IMPROVING RESISTANCE TO *VARROA* MITES IN HONEY BEE COLONIES VIA CULTURAL BROOD MIXING

by

John F. Menz

A dissertation submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Entomology

Winter 2024

IMPROVING RESISTANCE TO *VARROA* MITES

IN HONEY BEE COLONIES

VIA

CULTURAL BROOD MIXING

by

John F. Menz

Approved: _____

Jacob L. Bowman, Ph.D.
Chair of the Department of Entomology and Wildlife Ecology

Approved: _____

Calvin L. Keeler, Ph.D.
Interim Dean of the College of Agriculture and Natural Resources

Approved: _____

Louis F. Rossi, Ph.D.
Vice Provost for Graduate and Professional Education and
Dean of the Graduate College

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed: _____

Deborah A. Delaney, Ph.D.
Professor in charge of dissertation

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed: _____

Vincenzo A. Ellis, Ph.D.
Member of dissertation committee

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed: _____

Gregory W. Shriver, Ph.D.
Member of dissertation committee

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed: _____

Keith S. Delaplane, Ph.D.
Member of dissertation committee

ACKNOWLEDGMENTS

I would like to express profound gratitude to Dr. Deborah Delaney, Dr. Vincenzo Ellis, Dr. Gregory Shriver, and Dr. Keith Delaplane for helping me plan this project, acquire funding, execute the experiments, and overcome obstacles along the way. This work would not have been possible without dedicated assistance from Ben Sammarco, Jack Geraghty, Daniel Borkoski, Cameron Green, Jesse Baker, Tommy Cael, Ella Owen, and Ann Weaver. I am thankful for the support of my peers Tyler Hagerty, Lisa Elizondo, Ziyad Abdul Qadir, and Grace Savoy-Burke.

This research was funded by the UD Department of Entomology and Wildlife Ecology, by USDA-NIFA-AFRI grant award number 2021-67013-33563, and by USDA Hatch Funds DEL00774.

This dissertation is dedicated to Dr. Francesco Randi, whose support, patience, and encouragement made this work possible.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Honey bee, *Apis mellifera*, colonies are susceptible to failure due to the novel parasitic mite *Varroa destructor*. Genetic diversity has been shown to be vital to colony health, productivity, and resistance to mites. Genetic diversity can be augmented within a colony via artificial insemination, which mimics the derived polyandrous state of all members of the genus *Apis*. Alternatively, brood mixing is a manual method of transferring immature bees between colonies, thereby providing a non-technical method of increasing colony genetic diversity. To evaluate the effectiveness of brood mixing for improvement of colony strength and resistance to *Varroa* mites via augmented genetic diversity, I conducted two field experiments over two seasons. In the first season (2021), honey bee colonies were established with one of four types of queens from distinct geographic breeding regions in the continental US: Florida, Georgia, and 2 queen types from California. These queens did not have distinct selected resistance traits against *Varroa* mites and there were only minimal effects of brood mixing and queen source on colony productivity and mite levels. In the second field trial (2022), I investigated the mixing effect between only two types of colonies: those with the highly selected *Varroa*-sensitive hygienic (VSH) trait and wildtype colonies (WT) that lacked the trait. Mixed colonies were observed to investigate the possibility of trait sharing between colonies via repeated brood mixing and resulted in

colonies with intermediate adult bee populations and intermediate mite levels, with WT-control colonies having the highest mite levels and VSH-control colonies having the lowest mite levels. Finally, I conducted a thorough pathogen screen using relative quantification of honey bee viruses, and microsporidian and bacterial parasites using real-time qPCR at 3 time points in the 2021 field trial to assess the risk of pathogen and disease spread between brood mixed colonies. Brood mixing did not affect pathogen prevalence nor relative quantities, however, general increases in Deformed Wing Virus and decreases in Black Queen Cell Virus and Sacbrood Virus were observed over the season.

Chapter 1

INTRODUCTION

1.1 Issues in honey bee health

European honey bees, *Apis mellifera* L. 1758, provide substantial pollination services in natural ecosystems and are responsible for 13% of total global flower visits (Hung et al., 2018). They contribute even more to crop agro-systems where honey bees are the single most common visitor to the 35% of agricultural crops dependent on insect pollination (Garibaldi et al., 2013; Klein et al., 2007). Honey bees are the single most important managed pollinator in the United States and their services annually contribute $17 billion to the US agricultural economy (Calderone, 2012; Morse & Calderone, 2000). There has been concern that the increase in demand for commercial pollination will outstrip the available supply of honey bee colonies (Aizen & Harder, 2009) due to the increasingly difficult economic model for beekeepers that struggle with high colony loss rates that have hovered between 30% and 40% since 2006. These losses represent double the acceptable attrition rate as reported by beekeepers. Survey data from 2018-2019 show the highest overwintering loss rate (37.7%) in 12 years (Bruckner et al., 2019). Beekeepers have been able to maintain colony numbers from year to year via reproductive splitting of survivor colonies, however this current model will be unsustainable if colony losses continue to increase as observed in 2018-2019. Efforts to reduce colony losses are necessary to combat these concerning trends.

1.2 *Varroa destructor* and methods of control

Honey bees in the United States suffer from a variety of health pressures including pesticide exposure and lack of forage (Brosi et al., 2017). The primary global threat to honey bee survival has been the parasitic mite *Varroa destructor* (Anderson & Trueman, 2000; Dietemann et al., 2012; Genersch, 2010) which not only feeds directly on honey bee fat bodies (Ramsey et al., 2019) but also vectors and increases virulence of viral pathogens that devastate colonies within two years without intervention (Rosenkranz et al., 2010). General consensus suggests that the *Varroa* mite is the primary cause of diseased honey bee colonies and their eventual failure (Guzmán-Novoa et al., 2010; Kielmanowicz et al., 2015).

Beekeeper intervention is essential to control *Varroa* infestation levels and prevent colony death. *Varroa* mites reproduce inside the sealed brood cells of honey bees. Mite populations are relatively low in colonies after winter when there is little brood, or immature bees, present. However, as the season progresses, the ratio of mites to bees increases exponentially to its highest point in autumn without treatment or control (Traynor et al., 2016). Multiple avenues of control have been developed since the arrival of *Varroa* to the United States more than 30 years ago, but none are currently sustainable (Dietemann et al., 2012; Rosenkranz et al., 2010). Synthetic acaricides have been successful in suppressing colony mite levels, however, acaricide resistance has already been documented for several products (Elzen et al., 2000; Rinkevich, 2020; Rinkevich et al., 2017). The most effective and widespread acaricide compound in use, amitraz (Haber et al., 2019), has shown adverse interactions with agrochemicals that reduce colony health (Johnson et al., 2013), can reduce individual bees' ability to tolerate viral infections (O'Neal et al., 2017), and permits near constant exposure to both bees and mites because of accumulation in beeswax (Johnson et al.,

2009; Traynor et al., 2016). Within the first month of 2020 there were several authenticated reports of amitraz-resistant *Varroa* across the United States (Rinkevich, 2020). Calls for judiciousness in synthetic acaricide use stem from *Varroa*'s extreme propensity to evolve resistance due to its short generation times and nearly clonal reproductive cycle (Beaurepaire et al., 2017; Rinkevich, 2020; Rosenkranz et al., 2010). Organic (carbon-containing) acids have proven to be effective treatments that are unlikely to select for resistant mites but are highly temperature sensitive, which gives them a narrow therapeutic index and limits repeat use due to queen mortality risks (Dietemann et al., 2012). Breeding bees for resistance was regarded as the most sustainable route for controlling *Varroa* (Dietemann et al., 2012), however, use of selectively bred resistant honey bee stock has not been adopted *en masse* because the inbred lines must be maintained via artificial insemination, requiring frequent replacement of queens (Danka et al., 2012). Furthermore, the presence of basic resistance behavior does not preclude the need to chemically treat colonies with high levels of mite infestation (Spivak & Reuter, 2001).

However, preventative measures and breeding resistant bees can be exceptionally useful in suppressing mite population increase and thereby reducing the number of chemical treatments needed per season (Danka et al., 2012; Delaplane et al., 2005; Lodesani et al., 2019). Despite this being the most sustainable future for honey bee management and *Varroa* control, non-chemical methods are associated with higher rates of colony loss (Haber et al., 2019; Underwood et al., 2019), displaying the need for additional, effective control methods that can be integrated into current beekeeping operations without additional costs.

There has been renewed focus on the genetic diversity of honey bee breeding stock in the United States (Delaney et al., 2009) and the importance of intra-colonial diversity to overall honey bee health. Exploration of increasing genetic diversity for colony benefit is based upon the evolutionary history of *Apis* spp. bees, which all display extreme levels of polyandry, resulting in decreased relatedness among sterile worker sisters in a colony, which balances the high level of relatedness derived from sharing a diploid mother that mates with haploid males. An extensive body of research has demonstrated the importance of polyandry and genetic diversity to colony health in *Apis mellifera* and this aspect of honey bee natural history can be exploited to improve colony fitness in a managed agricultural setting.

1.3 Importance of natural polyandry and genetic diversity for colony health

Honey bee queens are highly polyandrous, meaning they mate with multiple males. The haploid nature of male drones creates distinct groups of highly related workers within a colony known as patrilines and the number of patrilines expressed in a colony is dependent upon the queen's mating number (Estoup et al., 1994). Theoretical models predict that mating with six drones provides 90% of the genetic variation observed in an average colony (Palmer & Oldroyd, 2000), however, natural mating numbers are usually between 12 and 20 (Tarpy et al., 2004). The genetic variance (GV) hypothesis is the leading rationale for the evolution of this seemingly excessive number of matings. It suggests that even a slight increase in genetic variation from extra matings provides colony benefits (Crozier & Fjerdingstad, 2001) in the form of more efficient genetic task specialization (Calderone & Page, 1988, 1992; J. Jones et al., 2004; Oldroyd & Fewell, 2007), increased parasite and disease resistance (Seeley & Tarpy, 2007; M. Simone-Finstrom et al., 2016; Tarpy, 2003),

greater productivity (Mattila et al., 2012; Mattila & Seeley, 2007) and even improved microbiome diversity and increased representation of beneficial gut bacteria (Mattila et al., 2012). From this body of research, it stands to reason that further benefits could be reaped from even higher levels of polyandry, but such levels may be impossible to achieve due to the physical limits of mating. This addition to the GV hypothesis was supported via a test of hyperpolyandrous queens (Delaplane et al., 2015).

1.4 Added benefits of artificial 'hyper'polyandry

Delaplane et al. (2015) tested the limits of the GV hypothesis by artificially inseminating queens with pooled semen from up to 60 drones, thereby creating hyperpolyandrous queens (mating number ≥30); this technique overcomes the physical limits to mating number. Their results showed that hyperpolyandrous queens produced colonies with reduced *Varroa* mite levels and increased brood rearing efficiency. Neither the queens nor the drones came from selected lines of bees (Delaplane et al., 2015). Hyperpolyandry exploits a caveat to the GV hypothesis that suggests that higher mating numbers increase the chances of capturing rare resistance alleles already present at low levels within a population and allow colonies to better cope with parasite and pathogen pressure (Fuchs & Moritz, 1998). For example, resistant individuals may remove infected brood, thereby preventing infections from spreading (Spivak & Gilliam, 1998). As shown via the positive results of the artificial insemination experiment and a separate direct test of the rare-allele hypothesis (Delaplane et al., 2021) the physical costs of natural mating likely prevent colonies from reaping the benefits of higher mating numbers and the increased representation of rare resistance alleles. Although breeding programs have been successful in elevating the expression of specific resistance alleles in selected lines of honey bees,

these resistant lines have not been widely adopted due to difficulty in maintaining expression of these traits at the colony level (Dietemann et al., 2012; Plate et al., 2019). Resistance alleles have already been found in different, unselected populations of honey bees in the United States (Harbo & Hoopingarner, 1997) and hyperpolyandry provides a key to increase diversity of resistance alleles at the colony level.

1.5 Improving social immunity: Balancing breeding and genetic diversity to improve resistance to parasites

Significant research has been conducted on the major behavioral and physiological mechanisms that can contribute to a colony that is functionally resistant to parasites such as *Varroa*, and to pathogens such as viruses, bacteria, and fungi (Biganski et al., 2018; Evans & Spivak, 2010; Kurze et al., 2016). Honey bee colonies are made up of thousands of individuals that respond to stimuli, stress, and their environment in different ways according to their genetics and their age. Within a colony there may be multiple mechanisms to either resist or tolerate a pathogen with great variation between individuals. Examples of well-studied resistance traits include hygienic behavior (Evans & Spivak, 2010) where bees can detect and remove diseased brood, and *Varroa* Sensitive Hygienic (VSH) behavior where bees can identify and remove brood that is specifically infested with *Varroa*, briefly interrupting its reproductive cycle (Villa et al., 2009). The great multiplicity of resistance tactics is the foundation of social immunity, a phenomenon which refers to the behavioral or physiological traits of individuals contributing to colony-level resistance to parasites or infectious disease (Cremer et al., 2007). Not all individuals need immunity to a disease or expression of a resistance trait for a colony to survive an infestation or infection (Kurze et al., 2016), however, the proportion individual bees with a

resistance phenotype can influence the colony level expression of the trait in that a certain threshold of individuals may need to be met for the resistance traits to have any observable effects (Arathi & Spivak, 2001; Delaplane et al., 2021).

Breeding programs have shown great potential to increase the expression of specific resistance traits like hygienic behavior and *Varroa*-sensitive hygienic (VSH) behavior (Danka et al., 2013), however there is concern for the effect of long-term breeding practices on overall genetic diversity and the potential loss of other, unidentified beneficial traits that might occur when selecting for single traits (Leclercq et al., 2017; Meixner et al., 2010). In Europe there has been extensive research on conservation of native honey bee genetic resources and populations of managed honey bees were found to have reduced genetic diversity compared to wild populations (Jaffé et al., 2010; Meixner et al., 2010; Moritz et al., 2007). Decreases in genetic diversity are especially relevant to US honey bee populations, which suffered an initial bottleneck due to a small European founder population and a second bottleneck as a result of current breeding practices whereby most queens bred in the US each year descend from as few as 600 mother queens (Delaney et al., 2009). This makes genetic diversity an area of concern that should receive high priority when considering honey bee health. Hyperpolyandry via artificial insemination attempts to incorporate all available resistance traits in a population into a single colony in a manner that could complement traditional breeding practices that target specific traits. Hyperpolyandry's major contribution to social immunity is that it provides a colony with greater opportunity to express a multiplicity of resistance traits that would be less likely under natural mating conditions. It is therefore an untapped method of improving honey bee health, and has basis in other insect models that studied virus transmission in systems

with varying host heterogeneity (Dwyer et al., 1997). Unfortunately, creating artificially inseminated (AI) hyperpolyandrous queens is financially and technologically implausible for most beekeepers. Furthermore, AI queens can suffer from reduced longevity (S. W. Cobey, 2007) making them impractical in the long term for implementing hyperpolyandry as an applied management strategy for honey bee health. Brood mixing, however, is a simple method of manually increasing colony genetic diversity that is feasible for all beekeepers.

1.6 Brood mixing: a practical method of simulating hyperpolyandry and improving social immunity

Brood mixing overcomes the obstacle of increasing patriline numbers without jeopardizing queen longevity or necessitating intensive technical training and costly equipment. Although it does not generate intrinsic hyperpolyandry, brood mixing simulates the effect of hyperpolyandry by temporarily increasing the number of patrilines through manual transfer of brood frames between sets of colonies (Fig. 1.1). From each colony within a set of four colonies, three brood frames are removed and distributed equally so that each colony now contains three donated frames of brood from its neighbors. If we assume an average mating number of 12 drones for each queen in a colony, which means that each colony on average would contain bees from 12 distinct patrilines (and one matriline), the result after brood frame transfer is 4x12 patrilines = 48 effective patrilines in each of the four mixed colonies, plus 3 additional matrilines (Fig. 1.1). This treatment can therefore be scaled depending on the number of colonies and brood frames available. For example, the brood mixing ‘dosage’ can be increased or decreased by sharing frames between a set of two colonies (2x12 patrilines = 24 patrilines), or a set of six colonies (6x12 patrilines = 72 patrilines). The

effect of increasing colony genetic diversity is heightened if a diverse queen population is used. As research accumulates to illuminate the multiple avenues by which honey bee social immunity limits *Varroa* infestation and increases disease resistance (Kurze et al., 2016), focusing on single traits for resistance to *Varroa* is not necessarily the most straightforward approach (Leclercq et al., 2017). Brood mixing can theoretically help a colony achieve resistance to *Varroa* by maximizing population genetic diversity, and plausibly increasing the diversity of resistance alleles, in multiple colonies simultaneously and with minimal cost to beekeepers.

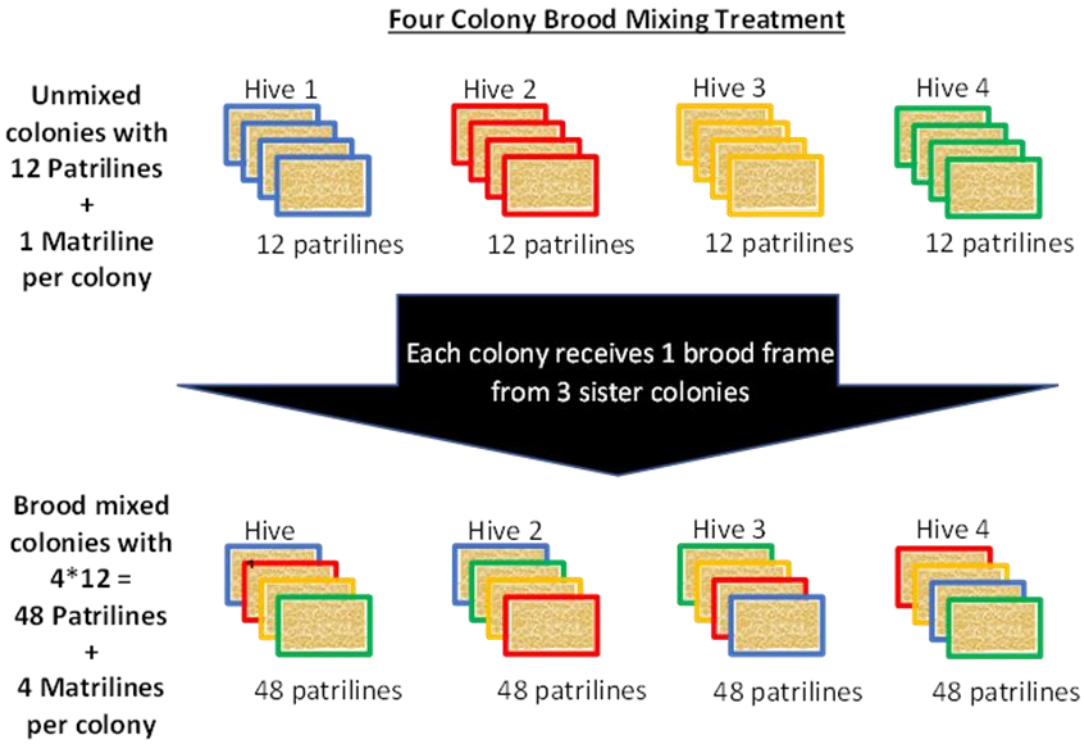


Figure 1.1: Representation of a 4-colony brood mixing treatment. Each colony receives one brood frame, represented by colored rectangles, from each sister colony within the quartet while retaining one of its own brood frames. The result is a temporary quadrupling in colony worker patriline number, thereby simulating an intrinsically hyperpolyandrous state with the added benefit of 3 extra matrilines and associated genetic recombination with their respective patrilines.

1.7 Pathogen transmission risks from brood mixing

Studies show drifting of adult bees and robbing of weak colonies by strong colonies are a source of movement of adult bees between colonies, especially in high density apiaries without visual and spatial differentiation between colonies (Dynes et al., 2019; Peck & Seeley, 2019). Viruses are abundant in honey bee colonies, and can spread through various means, such as these drifting or robbing events, infection with *Varroa* mites, or exposure during foraging at high activity floral sites (Grozinger &

Flenniken, 2019). It stands to reason that the frequent, prescribed movement of larval bees between colonies could be a source of transmission between colonies. Honey bee colonies also frequently suffer from bacterial and fungal infections (Budge et al., 2010; Jensen et al., 2013), making it prudent to include a thorough pathogen screening analysis for any field test of brood mixing.

1.8 Research objectives

To test the effectiveness of brood mixing for improvement of colony strength and resistance via augmented genetic diversity, I conducted two field experiments over two seasons.

In the first season (2021), honey bee colonies were established with one of four types of queens from distinct geographic breeding regions in the continental US: Florida, Georgia, and 2 queen types from California. These queens did not have distinct selected resistance traits against *Varroa* mites. These 4 queen types were subjected to a mixing group treatment and compared to control colonies.

In the second field trial (2022), I investigated the mixing effect between only two types of colonies: those with the highly selected *Varroa*-sensitive hygienic (VSH) trait and control colonies that lacked the trait. Mixed colonies in this field trial were observed to explore the possibility of trait sharing between colonies via repeated brood mixing.

Finally, I conducted a thorough pathogen screen using relative quantification of honey bee viruses, and microsporidian and bacterial parasites using real-time qPCR at 3 time points in the year 1 field trial to assess the risk of pathogen and disease spread between brood mixed colonies.

Chapter 2

BROOD MIXING BETWEEN COLONIES WITHOUT SELECTED *VARROA* RESISTANCE TRAITS

2.1 Objective

Objective: Demonstrate the effectiveness of repeat brood mixing in suppression of *Varroa* population growth and colony strength in the absence of selected resistance traits.

- Hypothesis 1: Mixed colonies will show lower *Varroa* mite population sizes than unmixed colonies at mid-season and end-season time points.
- Hypothesis 2: Mixed colonies will show improved results across all colony strength and social immunity metrics compared with unmixed colonies.

2.2 Methods

2.2.1 Experimental Design

The 2021 field experiment sought to create and evaluate mixing groups of 4 colonies with each colony headed by a queen of different genetic origin. All queen stocks were unselected for specific resistance traits to *Varroa*. Three apiaries were established: 2 apiaries at the University of Delaware Research Farm in Newark, DE, USA (39.66747247627695, -75.74645904580896) and one apiary 5 miles to the northeast in Fair Hill NRMA in Elkton, MD, USA (39.71601607608891, -75.82417514703252).

Each apiary contained 20 colonies arranged in an outward facing circle with a 25 meter diameter. The circles were divided into 5 segments each containing 4 colonies which were then randomly assigned one of the 4 queen types, so that each queen type was represented in each of the circle segments to avoid clumping of specific queen types in any one section of the circle. For each queen type, colonies were randomly allocated to either a control or brood mixing treatment, with two mixing treatments allowed per apiary. See Table 2.1 for sample size allocation at the apiary and full trial levels.

Table 2.1: Sample sizes of 2021 field experiment for individual apiaries and whole experiment totals.

Single apiary replicate			
		Mixing	
		Mixed	Control
Queen Source	A	2	3
	B	2	3
	C	2	3
	D	2	3
	Total	8	12
Full field trial (3 apiary replicates)			
		Mixing	
		Mixed	Control
Queen Source	A	6	9
	B	6	9
	C	6	9
	D	6	9
	Total	24	36

2.2.2 Colony Establishment and Mixing Treatments

Sixty colonies were established in standard 10 frame Langstroth deep boxes on April 16th, 2021 with 1.4 Kg packages of worker bees from a producer in Georgia, USA and were fed 1:1 w/v sugar solution and 50 g of supplemental pollen. Two of the 10 frames contained fully drawn wax comb to assist colony establishment, the rest of the frames contained only undrawn plastic comb foundation. An additional 12 colonies were established at the same time in a separate apiary and served as backup replacements for failed experimental colonies prior to the baseline data assessment. Packages came with a caged, open-mated Georgia queen. The GA queen was replaced on the day of installation for colonies assigned to Florida, California-1, or California-2 queen groups. Colonies were inspected on April 19th to ensure that all queens had been successfully released.

In mid-May colonies were equalized between the same queen source colonies so that each colony had 5 frames of brood from the same genetic stock to avoid a genetic mixing effect between queen sources. A second, deep 10 frame Langstroth deep box was added to all colonies before baseline assessment data was taken in the 1st week of June. Medium frame honey supers with undrawn plastic foundation were added as needed to colonies in June and July.

Three colony strength assessments and three brood mixing treatments were conducted over the course of the 2021 summer season. During each brood mixing treatment, frames were removed from mixing colonies and removed of adult bees by gently brushing the frames, which were then allocated to mixing group colonies according to Figure 1. To control for handling effects, control colonies also had three frames of brood removed, brushed of adult bees and returned to the same colony in a different order.

2.2.3 2021 Field Experiment Timeline

Figure 2.1 displays key timepoints in the 2021 field experiment. A baseline colony strength assessment was taken the week of May 31st, 2021, which was designated Week 1 in analyses. Starting at Week 1, weekly 48-hr *Varroa* mite fall was measured until Week 15. An initial brood mixing treatment was conducted for "mixed" colonies on June 4th, followed by a second mixing treatment 24 days later on June 28th (Week 5). A second colony strength assessment was conducted the week of July 19th (Week 8), once all brood that had been mixed among colonies at the second mixing treatment had emerged. This ensured that all brood assessed in a colony was laid by the colony's resident queen, and not the queen of a brood donor colony. A third mixing treatment was conducted on July 23rd (Week 8) and a final assessment was conducted the week of August 23rd (Week 13).

Colonies were treated for *Varroa destructor* on September 7th (Week 15) and weekly mite fall counts were taken to record an endpoint knockdown mite fall through Week 17.

All colonies received 2 gallons of 2:1 w/v sugar solution in October in preparation for overwintering. Additionally, 3.2 Kg of dry sucrose was given to each colony in December as additional overwintering feed.

Colonies were inspected on March 14th, 15th, and 18th in 2022 to determine overwintering survival, concluding the 2021 brood mixing field experiment.

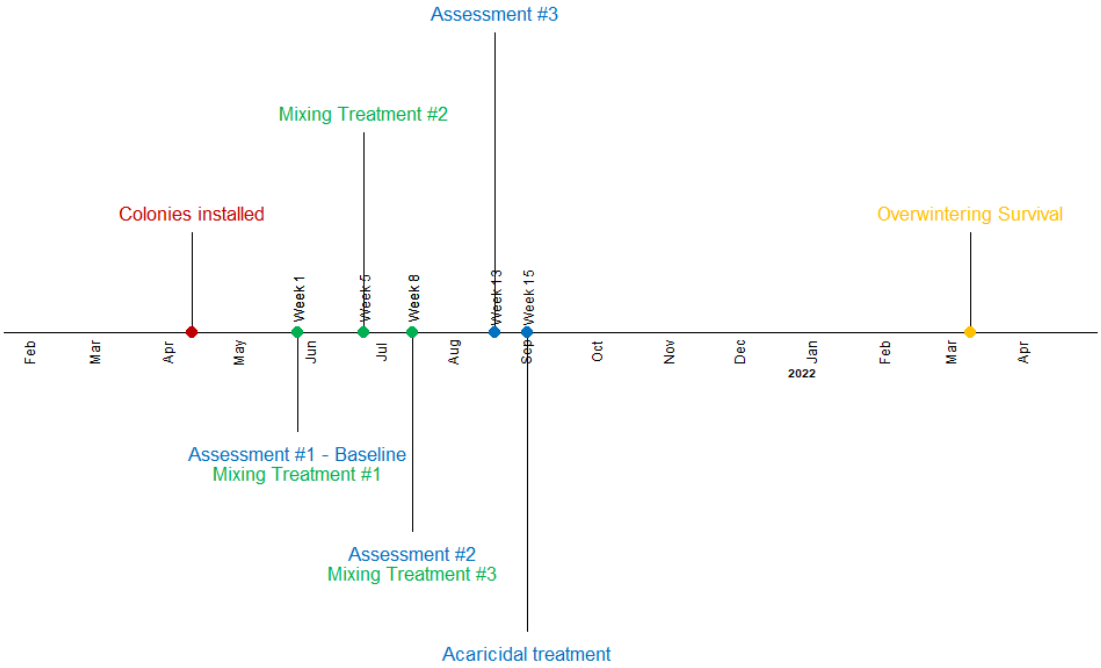


Figure 2.1: Timeline of 2021 brood mixing field experiment with 4 queen sources.

2.2.4 Data Collection Protocols

Colony Strength Assessments

The following assays were conducted during the 3 assessment timepoints and provided relative measures of a colony's population, brood rearing capacity, and productivity. Each metric was visually assessed as an area proportion of a frame averaged from 2 experienced assessors as described in (Delaplane et al., 2013). Coverage proportions were converted to total frame area per colony with measured areas of this experiment's deep Langstroth frames (860 cm^2) and medium Langstroth frames (559 cm^2) and then converted to count data based on bee density and cell density.

- **Adult worker population**: The number of adult bees was estimated from the total frame area covered by adult bees. Adult population count was calculated as proportion coverage*frame area*1.38 adult bees/cm^2 (Burgett & Burikam, 1985).
- **Brood production**: The total area (cm^2) of capped brood was calculated from averaged visual assessments. Cell count was obtained by multiplying proportion coverage*frame area*3.8cells/cm^2.
- **Comb construction:** The total area (cm^2) of drawn wax comb on plastic foundation was calculated from averaged visual assessments. Wax cell count was obtained by multiplying proportion coverage*frame area*3.8cells/cm^2.
- **Honey stores**: The amount of colony honey stores in both deep and medium colony boxes was estimated from the total area of capped honey (cm^2). Cell count was obtained by multiplying proportion coverage*frame area*3.8cells/cm^2.
- **Pollen stores**: The amount of colony pollen stores was estimated during the last two assessments from the total area of pollen cells on a frame (cm^2). Cell count was obtained by multiplying proportion coverage*frame area*3.8cells/cm^2.
- **Harvested honey**: Honey was harvested from medium supers at Week 14 after the 3rd assessment and end of season honey weight (Kg) was recorded.
- **Pollen foraging:** Anatomic front mount pollen traps (Betterbee® Greenwich, NY, USA) were placed on colonies for 24-hour periods in August during Week 10, Week 11, Week 12, and Week 14. Collected pollen was weighed (g) to determine foraging strength of colonies.

Foraging diversity

Pollen trapped during Week 12 (week of August 16th) was selected for further analysis by subsampling 100 pellets (clumped corbicular pollen from a bee's foraging

trip) from the full day's pollen sample. Pollen pellets subsamples representing each colony were initially sorted by color, mixed in deionized water to produce a slurry, and then prepared for photography on a glass slide with safranin dye and covered with a coverslip. Photographs of pollen samples were taken at 10X, 40X, or 100X magnification from 3 separate frames of view for each slide and 3 replicates of each frame were photographed at different focal depths to provide a full survey of the pollen diversity on each slide and the morphology of the pollen grains.

Pollen was categorized into 31 morphological types based on symmetry, shape, size, aperture number, and orientation. The number of morphotypes present in each colony's subsample was recorded to represent the level of pollen foraging diversity. Example images and size ranges of each morphotype are included in Appendix 1.

Varroa population quantification

Three methods of quantifying the level of *Varroa* infestation were used to provide an accurate estimate of mite population on immature (brood) and adult bees, and to quantify the expression of a *Varroa*-specific resistance trait.

- **Weekly colony mite fall**: Screened bottom boards underneath each colony allowed debris and mites to fall onto a sheet of corrugated plastic coated with vegetable oil, thereby trapping mites during a 48 hour period each week during the field season. The number of fallen mites was recorded and normalized for differences in colony size using the adult population metric (Dietemann et al., 2013).

- **Knockdown mite fall**: Mite fall was recorded and summed for Weeks 15, 16, and 17 after the acaricidal treatment to calculate an endpoint mite fall count for each colony.
- **Mites on adult bees**: 300 to 600 adult bees were rinsed in 70% ethanol for 1 minute and the total number of mites per bees was recorded for each of the 3 assessments (Dietemann et al., 2013).
- **Mites in capped brood**: 50 to 100 capped brood cells were uncapped and larvae were removed from brood frames using forceps. Both larva and empty cells were inspected for mites and the number of cells with mites was recorded.
- **Varroa sensitive hygienic (VSH) trait assay**: VSH behavior is a heritable, additive trait found in honey bee populations around the world whereby honey bees detect *Varroa* infested brood and remove it, thereby disrupting the mite's reproduction (Kirrane et al., 2015; Villa et al., 2009). Expression levels of the trait vary between worker patrilines and colonies, which will provide us with a quantitative metric of a *Varroa*-specific resistance trait. VSH bees specifically target fertile *Varroa* mites, leaving infertile foundress mites alone. Therefore, VSH was quantified by removing 100 pupae 7 to 11 days post-capping, during the "purple-eyed stage", and recording the number of cells with mites and whether the mites are reproductive or not. The VSH score of the colony will be determined by the proportion of non-reproductive mites in the 100-pupae sample (Harbo, 2020). This assay is only utilized when mite levels are high, therefore it was conducted only during the 3rd assessment. Although none of the queens used in this field experiment were selectively bred for the VSH trait, low level expression of the

trait may be expected to occur and could indicate unselected, underlying mechanisms of resistance to *Varroa*.

Propolis deposition

Propolis is an amalgamation of antimicrobial plant resins collected by foraging honey bees in the late summer and autumn. Propolis has been shown to reduce the severity of brood fungal infections and improve immune system regulation in individual bees. Therefore, the collection of propolis is a colony-level indicator of social immune function (Borba et al., 2015; M. Simone-Finstrom et al., 2017; M. D. Simone-Finstrom & Spivak, 2012). I evaluated brood mixing's effect on this quantifiable mechanism of social immunity by placing propolis traps (Mann Lake LTD, Wilkes-Barre, PA, USA) on each colony on the week of July 19th (Week 8). The propolis was collected and weighed (g) seven weeks after the final mixing treatment on Week 15.

Overwintering survival

Whether a colony can survive the winter season is an indicator of the intensity of autumn *Varroa* infection and the ultimate assessment of a colony's health from the previous season (Guzmán-Novoa et al., 2010). Colony survival was determined the week of March 14th, 2022.

2.2.5 Data Analysis Methods

Colonies that either failed or swarmed during the experimental period were removed from the study due to a break in the honey bee brood cycle, which interferes with the mite reproductive cycle. Because of this interruption, the mite levels of these

colonies could no longer be compared to other experimental colonies and were removed from their respective apiaries. Eight of the control colonies were therefore removed from final data analysis and one mixed colony was removed. The mixed colony failed after the third brood mixing treatment and before the last colony strength assessment, allowing the other three colonies in its mixing group to continue in the study and remain in the final data analysis.

Table 2.2: Final 2021 season colony sample size.

Final Analysis Sample Size 2021 Season				
		Mixing		
		Mixed	Control	Total
Queen Source	CA-1	6	7	13
	CA-2	6	7	13
	FL	5	8	13
	GA	6	6	12
	Total	23	28	51

All statistical analyses were conducted in R statistical software v.4.3.1 (R Core Team, 2023). The fixed effects of mixing and queen source (CA1, CA2, FL, or GA) were evaluated for the measured parameters. Response variables that were recorded at a single time point, such as harvested honey, were analyzed via generalized linear model (GLM) using R package 'MASS' (Venables & Ripley, 2002). For repeated measures responses, such as adult bee population, a mixed model (GLMM) with assessment included as a fixed effect and colony identification number as a random effect were used from either R package 'lme4' (Bates et al., 2015) or 'glmmTMB' (Brooks et al., 2017) when zero-inflation and overdispersion prevented model

convergence in 'lme4'. Model offsets, distributions, and transformations are specified for each response variable and model below. All model assumptions and residuals were confirmed using DHARMa package (Hartig, 2022). The 'car' package (Fox & Weisberg, 2019) was used to conduct model effect ANOVAs and estimated marginal means and *post hoc* comparisons were calculated using the 'emmeans' package (Lenth, 2023).

Colony Strength Assessments

Adult bee population and capped brood population were recorded for each colony at three separate assessment time points. Wax cell count and capped honey cell count were recorded at each of the three assessments and adjusted in their respective GLMMs by the corresponding adult bee population at each assessment point to control for variation in colony sizes. Harvested honey (Kg) was recorded after the third assessment and is offset by the adult bee population at the nearest assessment.

Table 2.3: Data analysis parameters for 2021 colony strength measures.

Response variable	**Fixed effects**	**Random effects**	**R package**	**Distribution**	**Offset**
Adult bee population (log)	Mixing*Assessment*Queen	(1|Colony)	lme4	Gaussian	-
Capped brood population	Mixing*Assessment*Queen	(1|Colony)	lme4	Gaussian	-
Wax cell count (sqrt)	Mixing*Assessment*Queen	(1|Colony)	lme4	Gaussian	sqrt(Adult bee population)
Honey cell count (log)	Mixing*Assessment*Queen	(1|Colony)	lme4	Gaussian	log(Adult bee

					population)
Honey harvest (Kg)	Mixing*Queen	-	MASS	Gaussian	Adult bee population at assessment 3

Pollen and propolis

Pollen store cell counts were recorded at assessment 2 and 3 and were adjusted by the corresponding assessment bee population to control for variation in colony size. Foraged pollen was collected from colonies at weeks 10, 11, 12, and 14 and an average mass (g) was analyzed for each colony adjusted by assessment 3 (week 13) adult bee population. Propolis mass (g) for each colony was adjusted by adult bee population at assessment 3 as well. The pollen morphotype counts for week 12 were analyzed without a bee population adjustment.

Table 2.4: Data analysis parameters for 2021 pollen and propolis data.

Response variable	**Fixed effects**	**Random effects**	**R package**	**Distribution**	**Offset**
Pollen stores cell count	Mixing*Assessment*Queen	(1|Colony)	lme4	Gaussian	log(Adult bee population)
Pollen foraging (g) average	Mixing*Queen	-	MASS	Gaussian	log(Adult bee population only at assessment 3)
Pollen morphotype richness	Mixing*Queen	-	MASS	Gaussian	-
Propolis (g)	Mixing*Queen	-	MASS	Gaussian	log(Adult bee

					population only at assessment 3)

Varroa mite population quantification

Alcohol wash mite counts (adult bee mites) were recorded at each of the three assessments and offset in the GLMM by the number of adult bees in the wash sample to control for sampling effort.

The total count of mites in brood cells was analyzed only during assessment 3 because counts from the first two assessments were too zero-inflated for the model to converge. The GLM was offset by the number of brood cells sampled. The GLM of the non-reproductive mite count was offset by the total number of brood mites recorded at assessment 3. The knockdown mite counts after the acaricidal treatments were summed and offset by assessment 3 adult bee population.

The weekly 48-hour mite fall count was recorded for each colony for weeks 1 through 13. In order to offset the mite count by bee population at each week, bee population was interpolated using each colony's population at weeks 1, 8, and 13 and the quadratic formula lm(bee_pop ~ I(week^2)+week). Figure 2.2 displays un-interpolated and interpolated bee population data. The mite fall GLMM retained week as a categorical variable in the analysis.

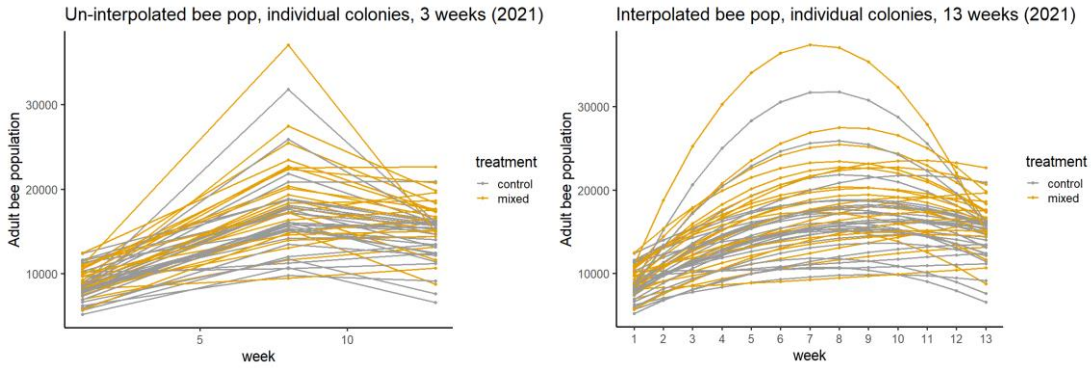


Figure 2.2: Representation of 2021 adult bee population for individual colonies in its un-interpolated (left) and interpolated (right) forms. Interpolating bee population for each week allowed for an offset term to be included for each week of mite fall data.

Table 2.5: Data analysis parameters for 2021 *Varroa* mite counts.

Response variable	**Fixed effects**	**Random effects**	**R package**	**Distribution**	**Offset**
Adult bee mites	Mixing*Assessment*Queen	(1|Colony)	glmmTMB	Neg binom 1	log(Number of bees washed)
Brood mites	Mixing*Queen	-	MASS	Negative Binomial	log(Total brood cells checked for mites)
Non-reproductive mites	Mixing*Queen	-	MASS	Poisson	log(Total number of brood mites)
Weekly mite fall	Mixing*Week*Queen	(1|Colony)	glmmTMB	Neg binom 2	log(Interpolated weekly bee population)
Knockdown mite fall	Mixing*Queen	-	MASS	Negative binomial	log(Assessment 3 bee population)

Overwintering survival

Overwintering survival was analyzed via a binomial GLM with "alive" colonies coded as 1 and "dead" colonies coded as 0. Three out of 5 of the FL-mixed group colonies failed between October and December 2021 and were therefore not included in overwintering survival analyses.

Table 2.6: Data analysis parameters for 2021 colony survival in spring 2022.

Response variable	**Fixed effects**	**Random effects**	**R package**	**Distribution**	**Offset**
Overwinter survival	Mixing*Queen	-	MASS	Binomial (1=alive, 0=dead)	-

2.3 Results

Colony Strength Assessments

Assessment time had a significant effect for all colony strength measures in which it was included, and a summary of all main and interaction effects are presented in Table 2.7. There were no significant interaction effects on adult bee population (Fig. 2.3); however, there was a significant effect of queen source, $\chi^2(3) = 10.2$, $p<0.05$. The FL queen source had significantly fewer bees when averaged over treatment and assessment periods (Fig. 2.4).

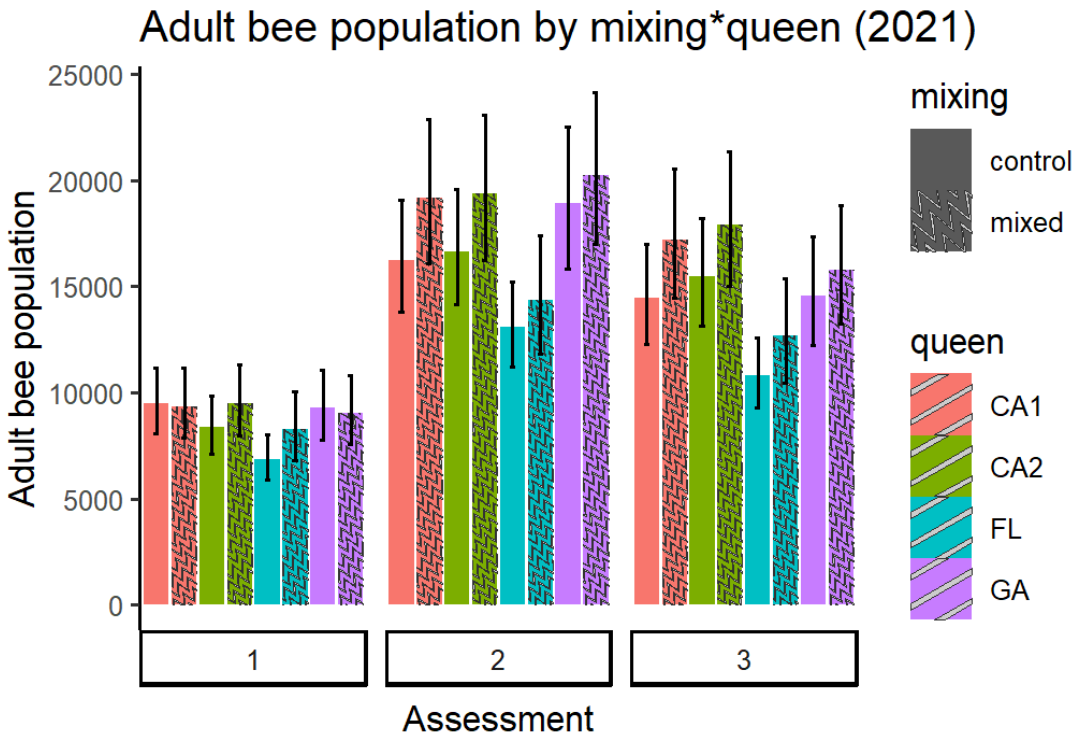


Figure 2.3: Adult bee population by mixing*queen over 3 assessment periods. Each bar represents marginal means with 95% CI presented in the response scale but Wald III tests were performed on the log scale. There was a significant effect of queen source ($\chi 2$ (1) = 10.2, p<0.05), but no significant interactions or main effects.

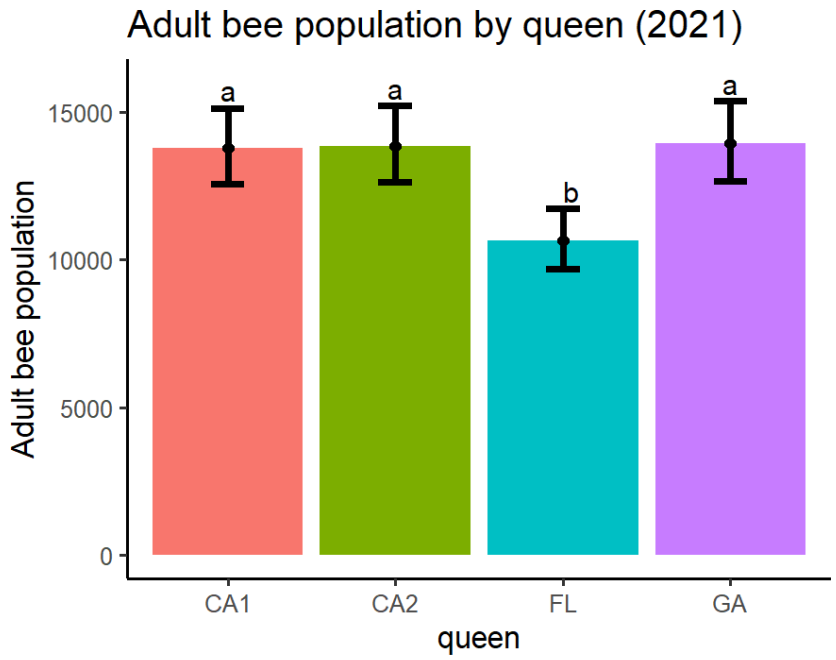


Figure 2.4: Effect of queen source averaged over mixing and assessment on adult bee population. Predicted means and 95% CIs are presented in the response scale but Wald III tests were performed on the log scale. Marginal means were separated by Tukey-Kramer test $\alpha \leq 0.05$.

There was a significant interaction between mixing and assessment time for brood population, χ^2 (2) = 6.6, p<0.05; however, *post hoc* analysis found only a marginally significant increase in mixed colony brood population at assessment 2 when control colonies had on average 1286 ± 769 fewer brood cells, t(111)=-1.672, p=0.097.

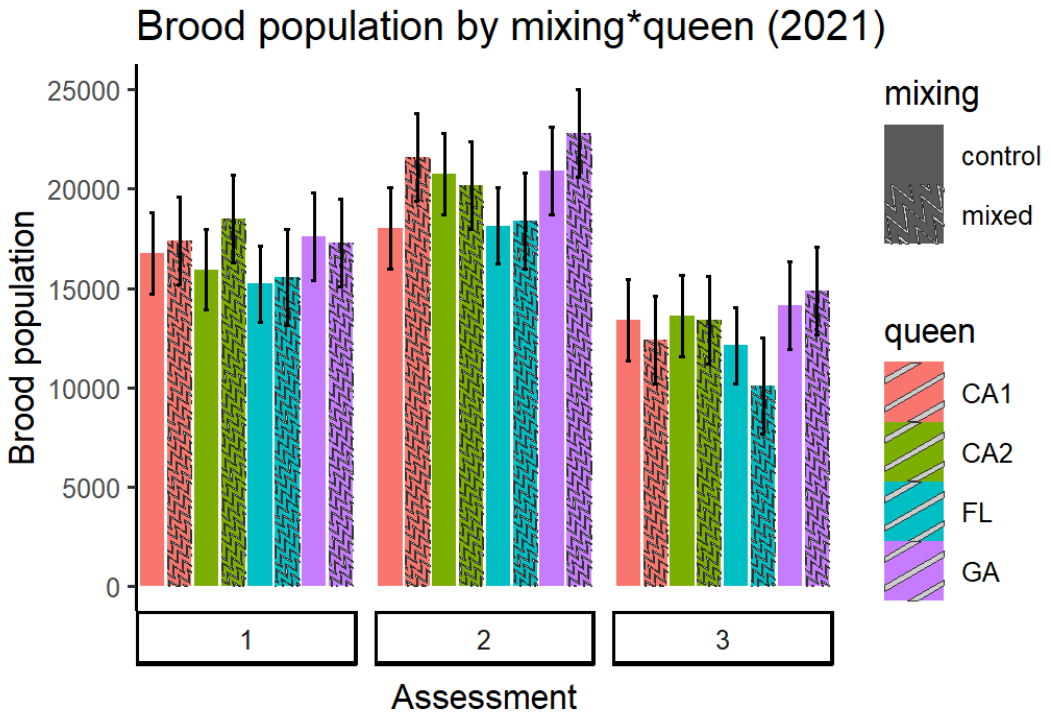


Figure 2.5: Adult brood population over 3 assessment periods grouped by mixing and queen source. Each bar represents marginal means with 95% CI. There was a significant effect of mixing*assessment, $\chi^2(2) = 6.6$, $p<0.05$. However, *post hoc* one-way ANOVAs found only a marginally significant increase in brood population at assessment 2, $t(111)=-1.672$, $p=0.097$.

There were no significant effects for wax nor honey cell counts, other than between assessment times. Harvested honey weight had no significant effects and was only measured once and therefore did not include assessment time in its GLM.

Table 2.7: 2021 Colony strength ANOVAs (Type III) of the main effects of mixing (M), time (T), and queen source (QS) and their interactions on colony-level measures of strength. Differences accepted at $\alpha \leq 0.05$.

Adult bee population	**Chisq**	**DF**	**Pr(>Chisq)**	*****
Mixing (M)	0.0126	1	0.9106	
Time (T)	38.3552	2	4.69E-09	***
Queen Source (QS)	10.2448	3	0.0166	*
M*T	2.5544	2	0.2788	
M*QS	2.1504	3	0.5418	
T*QS	4.8723	6	0.5603	
M*T*QS	2.5507	6	0.8628	
Capped brood population	**Chisq**	**DF**	**Pr(>Chisq)**	*****
Mixing (M)	0.1735	1	0.6770101	
Time (T)	14.8695	2	0.0005904	***
Queen Source (QS)	2.9346	3	0.4018241	
M*T	6.6198	2	0.0365205	*
M*QS	1.9602	3	0.5807061	
T*QS	4.707	6	0.5819055	
M*T*QS	8.8882	6	0.1799646	
Wax cell count	**Chisq**	**DF**	**Pr(>Chisq)**	*****
Mixing (M)	0.024	1	0.8768	
Time (T)	145.6397	2	<2e-16	***
Queen Source (QS)	1.8885	3	0.5959	
M*T	0.2613	2	0.8775	
M*QS	1.1403	3	0.7674	
T*QS	3.8167	6	0.7015	
M*T*QS	1.5662	6	0.955	
Honey cell count	**Chisq**	**DF**	**Pr(>Chisq)**	*****
Mixing (M)	1.062	1	0.30277	
Time (T)	93.7521	2	< 2e-16	***
Queen Source (QS)	5.6521	3	0.12982	
M*T	0.4838	2	0.78514	
M*QS	2.1215	3	0.54757	
T*QS	4.0753	6	0.66648	
M*T*QS	3.2946	6	0.77107	
Honey harvest (Kg)	**Chisq**	**DF**	**Pr(>Chisq)**	*****
Mixing (M)	0.63476	1	0.4256	
Queen Source (QS)	1.54671	3	0.6715	
M*QS	0.98365	3	0.8052	

Pollen and propolis

There were significant main effects of mixing, time, and queen source on pollen stores, but no interaction effects (see Table 2.8). Overall, mixed colonies had greater stores of pollen than their control counterparts, however, the effect amounted to only 589 ± 659 more pollen cells, χ^2 (1) = 7.09, p<0.01. FL colonies had overall greater pollen stores than GA colonies when averaged across assessment 2 and 3, χ^2 (3) = 9.3, p<0.05 with a much greater difference than the mixing effect. GA colonies had 2160 ± 946 fewer pollen cells than FL colonies. There was a significant decrease in pollen stores overall between assessment 2 and assessment 3 (t(43) = 8.489, p < .0001). The GLMs for pollen foraging (g), pollen morphotype richness, and propolis (g) found no significant main effects nor interactions (Table 2.8).

Table 2.8: 2021 Pollen and propolis ANOVAs (Type III) of the main effects of mixing (M), time (T), and queen source (QS) and their interactions on pollen and propolis measures. Differences accepted at α≤0.05.

Pollen stores cell count	**Chisq**	**DF**	**Pr(>Chisq)**	*****
Mixing (M)	7.0908	1	0.007748	**
Time (T)	5.9733	1	0.014524	*
Queen Source (QS)	9.346	3	0.025028	*
M*T	2.0629	1	0.150921	
M*QS	6.5088	3	0.089316	.
T*QS	4.3078	3	0.230089	
M*T*QS	2.3011	3	0.512316	
Pollen foraging (g) average	**Chisq**	**DF**	**Pr(>Chisq)**	*****
Mixing (M)	1.76434	1	0.1841	
Queen Source (QS)	0.29677	3	0.9606	
M*QS	1.73669	3	0.6288	
Pollen morphotype richness	**Chisq**	**DF**	**Pr(>Chisq)**	*****
Mixing (M)	0.1213	1	0.7276	
Queen Source (QS)	2.2813	3	0.5161	
M*QS	2.1416	3	0.5435	
Propolis (g)	**Chisq**	**DF**	**Pr(>Chisq)**	*****

Mixing (M)	0.00156	1	0.9685	
Queen Source (QS)	2.37256	3	0.4988	
M*QS	0.04485	3	0.9975	

Varroa mite population quantification

There were no significant effects (Table 2.9) for mites on adult bees or for weekly mite fall (Fig. 2.6) other than the main effects of assessment. No significant effects were observed for non-reproductive mites nor for knockdown mite fall counts.

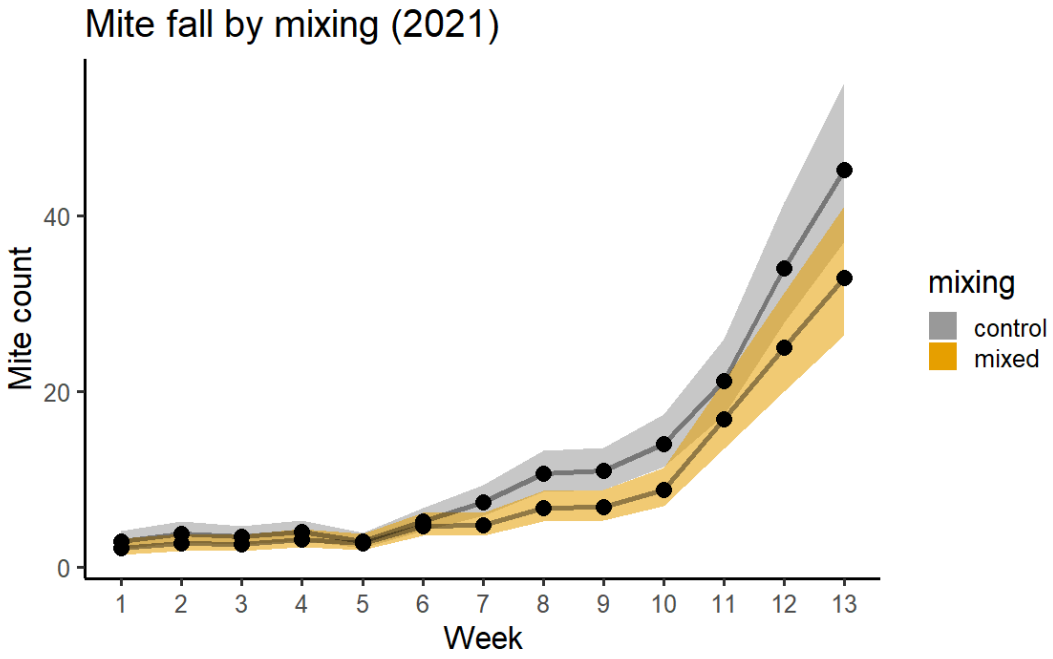


Figure 2.6: Effect of mixing averaged over queen source on 2021 weekly mite fall. Predicted means and 95% CIs for each week are presented in the response scale but Wald III tests were performed on the log scale and found no significant interaction between mixing and week, $\chi^2(12) = 7.7$, p=0.8. The GLMM included an offset term for adult bee population.

All brood mite main effects and interactions were significant (Table 2.9). *Post hoc* analysis found that within queen groups, the CA1-mixed colonies had significantly fewer brood mites than CA1-control with 10.07 ± 3.97 fewer mites, z = 2.539, p = 0.011. (Fig. 2.7). The main effect of mixing on brood mites was significant, $\chi^2(1) = 7.7$, $p<0.01$, however the effect was minimal, with mixed colonies having an average of 1.6 ± 1.57 fewer mites than control colonies. The main effect of queen source on brood mites was significant, $\chi^2(3) = 14.6$, $p<0.01$, and indicated that GA colonies had 5.2 ± 2.3 more brood mites than FL colonies, however, *post hoc* analysis did not yield significant differences from CA1 or CA2 colonies (Fig. 2.8).

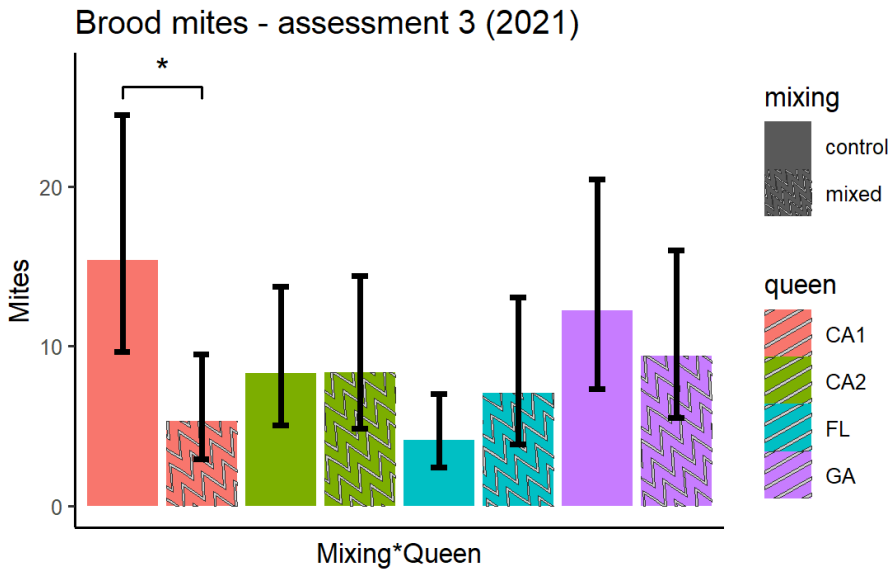


Figure 2.7: Effect of queen*mixing on brood mite counts at assessment 3. Predicted means and 95% CIs are presented in the response scale, but tests were performed in the log scale. The marginal means were separated by z-test with $\alpha \leq 0.05$ using nesting of mixing|queen.

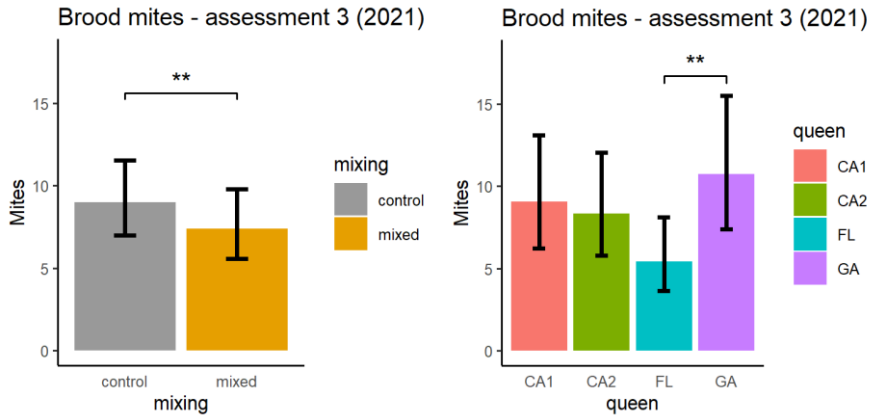


Figure 2.8: Main effects of mixing (left) and queen (right) on brood mite counts at assessment 3. Predicted means and 95% CIs are presented in the response scale, but tests were performed in the log scale. The marginal means were separated by Wald III Anova with $\alpha \leq 0.05$.

Table 2.9: 2021 *Varroa* mite count ANOVAs (Type III) of the main effects of mixing (M), time (T), and queen source (QS) and their interactions on mite count measurements. Differences accepted at $\alpha \leq 0.05$.

Adult bee mites	**Chisq**	**DF**	**Pr(>Chisq)**	*****
Mixing (M)	0.0342	1	0.8533	
Time (T)	46.2869	2	8.89E-11	***
Queen Source (QS)	0.3435	3	0.9517	
M*T	0.4361	2	0.8041	
M*QS	0.0395	3	0.9979	
T*QS	7.1119	6	0.3106	
M*T*QS	2.7813	6	0.8358	
Brood mites	**Chisq**	**DF**	**Pr(>Chisq)**	*****
Mixing (M)	7.7043	1	0.005509	**
Queen Source (QS)	14.6784	3	0.002113	**
M*QS	8.6528	3	0.034281	*
Non-reproductive mites	**Chisq**	**DF**	**Pr(>Chisq)**	*****
Mixing (M)	0.3348	1	0.5628	
Queen Source (QS)	5.1038	3	0.1644	
M*QS	0.1841	3	0.9801	
Weekly mite fall	**Chisq**	**DF**	**Pr(>Chisq)**	*****
Mixing (M)	1.5397	1	0.2147	
Time (T)	403.6121	12	<2e-16	***

Queen Source (QS)	3.7705	3	0.2873	
M*T	7.7739	12	0.8025	
M*QS	3.9088	3	0.2715	
T*QS	42.8752	36	0.2001	
M*T*QS	33.4295	36	0.5915	
Knockdown mite fall	**Chisq**	**DF**	**Pr(>Chisq)**	*****
Mixing (M)	1.1374	1	0.2862	
Queen Source (QS)	7.3585	3	0.06131	
M*QS	2.4313	3	0.48783	

Overwintering survival

No significant main effects or interactions were observed for overwintering survival of 2021 colonies (Table 2.10). Survival proportions by mixing*queen are presented in Fig. 2.9.

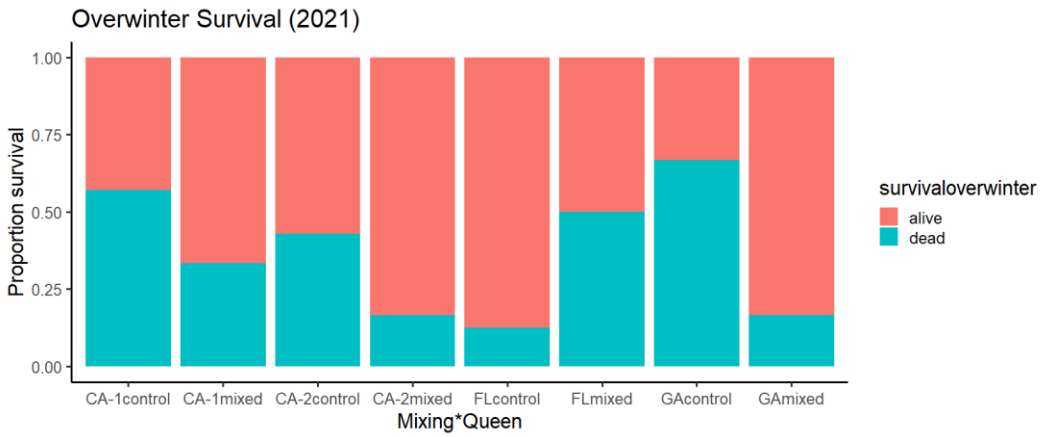


Figure 2.9: Proportions of alive and dead colonies for the overwinter survival of 2021 experimental colonies by mixing*queen. The binomial GLM found no significant effects (Table 2.10).

Table 2.10: Overwinter survival ANOVA (Type III) of the main effects of mixing (M), queen source (QS) and their interaction on 2021 colony survival in spring 2022. Differences accepted at $\alpha \leq 0.05$.

Overwinter survival	**Chisq**	**DF**	**Pr(>Chisq)**	*****
Mixing (M)	0.7459	1	0.3878	
Queen Source (QS)	5.4549	3	0.1414	
M*QS	3.6562	3	0.3011	

2.4 Discussion

Despite repeated brood mixing between 4 queen sources of distinct geographic origin, there were no observed effects of mixing for any response variable, except for a marginal decrease of 1.6 ± 1.57 brood mites for mixed colonies, which was not observed in any of the other 3 mite count measures, and a marginal increase of 589 ± 659 stored pollen cells. FL queen source colonies had a reduced adult bee population compared to the other three queen sources but did not significantly differ in measures of brood population, wax production, or honey production. There were no differences in mite levels within queen sources, except for the brood mite assay, where CA-1 mixed colonies had 10.07 ± 3.97 fewer mites than CA1-control colonies. Also, a significantly lower count of brood mites was observed in FL colonies (-5.2 ± 2.3) compared to GA colonies. These significant differences in mite levels were not observed in adult bee mite assays, mite fall, or the knockdown mite fall. No differences in pollen foraging, pollen morphotype diversity, or propolis foraging were observed for mixing or queen source groups.

Overall colony strength trends over time followed normal seasonal patterns of a peak in brood and adult bee populations in midsummer and an increase in the ratio of mites to bees.

This study did not find comparable results to Delaplane et al. (2015), which found increased brood rearing efficiency and lower mite levels in artificially inseminated queens with 30 to 60 patrilines per colony in the United Kingdom. In the 2021 field trial, brood mixed colonies theoretically had 12*4 = 48 patrilines per colony (Tarpy et al., 2004), with the added advantage of 3 more matrilines represented in their mixed populations, making their genetic diversity comparable to and arguably greater than the hyperpolyandrous colonies in Delaplane et al. (2015). Furthermore, I sourced queens from both the south-eastern US and western US breeding populations, which are known to have genetic distinctions in population analyses (Delaney et al., 2009), in order to obtain more diverse genetic profiles for the mixing treatments.

A plausible explanation for the lack of effect in the 2021 brood mixing trial compared to the UK artificial insemination experiment is the lack of diverse resistance traits and background population genetic diversity in the US breeding population compared to European populations. Multiple factors have contributed to the bottlenecking of US honey bee population genetics, beginning with the importation of only a few representative subspecies from the European continent. An importation ban in the 1920's due to the threat of tracheal mites, *Acarapis woodi*, effectively cut off gene flow between the old and new world populations of *A. mellifera* and was followed by an effective loss of US feral honey bee genetic resources after the arrival of *Varroa destructor* mites in the 1980's (Cobey et al., 2011; Sheppard, 1988). Yet another genetic bottleneck emerged as queen breeders increased production capacity,

selecting for highly productive colonies under the protective purview of synthetic acaracides, resulting in many queens from relatively few sources of breeder queen stock that has not allowed for natural selection for resistance to mites (Delaney et al., 2009; Schiff & Sheppard, 1995, 1996; van Alphen & Fernhout, 2020).

With these genetic limitations in mind, brood mixing among standard production queens in the US may not have an effect when the queen sources lack resistance traits to be shared in the first place. Mixing may have greater effect in regions such as Europe that have greater overall genetic variation (Sheppard, 1988), or where there is still gene flow between managed and feral populations, as feral populations have been found to have greater genetic diversity (Jaffé et al., 2010; Moritz et al., 2007).

Future studies could test brood mixing without specifically selected *Varroa*-resistance traits in a region with greater natural diversity in resistance traits, for example in far eastern Russia where *A. mellifera* has had the longest opportunity to co-evolve with *V. destructor* (van Alphen & Fernhout, 2020). In the US however, attempting to utilize stock with specific resistance traits, such as *Varroa*-Sensitive Hygienic trait, may be a pathway to sharing specific resistance traits between colonies using the simple methodology of brood mixing instead of relying completely on artificial insemination practices to maintain expensive inbred lines.

Comparing mite count methodologies

A key feature of this study was the use of multiple mite count methodologies to ensure that the counts of mites on adult bees, in brood, and overall mite fall have general consensus. High variability in counts that focus just on mites in their dispersal

stage on adult bees or only on reproductive mites in the brood may obscure the trends in mite growth between mixing groups, especially in field experiments with smaller sample size or variation in brood available for mites to infest. As the majority of mites may be found in honey bee brood (Frey & Rosenkranz, 2014), the brood assay is likely the best indicator of mite level during the brood rearing season. I found that the relative mite levels were consistent in each of the 8 mixing*queen groups in the 2021 season across 4 measures of mite counts. Figure 2.10 shows the endpoints of adult bee mites, brood mites, mite fall, and the knockdown mite fall. The concentrations of mites were highest for the brood assay and the knockdown mite fall assay. The knockdown mite fall assay allowed me to see the total colony mite population of adult bee mites and brood mites combined, as the acaricidal treatment kills mites as they emerge from brood cells over an extended period of time in addition to mites on adult bees.

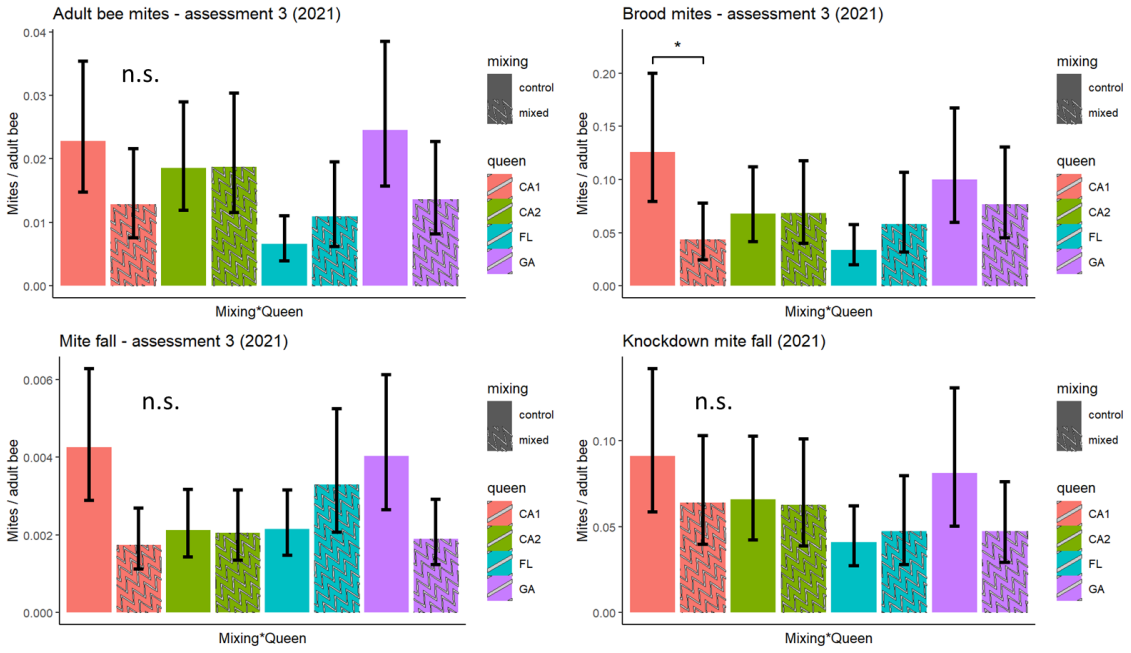


Figure 2.10: A comparison of 4 methods of counting *Varroa* mites at the endpoint of the 2021 field season. Scales have been adjusted to represent mites per unit bee or brood cell to better display relative mite levels between methods.

Chapter 3

BROOD MIXING BETWEEN COLONIES WITH A SELECTED RESISTANCE TRAIT: VARROA-SENSITIVE HYGIENIC TRAIT SHARING BETWEEN COLONIES

3.1 Objective

Objective: Demonstrate the effectiveness of repeat brood mixing in suppression of *Varroa* population growth and colony strength in the presence of selected resistance traits such as *Varroa*-sensitive hygienic (VSH) behavior.

- Hypothesis 1: Mixing between wild-type (WT) and VSH colonies will create an intermediate mite-level effect, with WT-mixed colonies showing significantly reduced mite levels when compared to WT-control colonies.
- Hypothesis 2: Mixed colonies will produce intermediate results across all colony strength and social immunity metrics compared with control colonies.

3.2 Methods

3.2.1 Experimental Design

The 2022 field experiment sought to create and evaluate mixing groups of 2 colonies with each colony headed by either a wild-type (WT) queen or a *Varroa*-sensitive hygienic (VSH) queen. Three apiaries were established: two apiaries at the University of Delaware Research Farm in Newark, DE, USA (39.66747247627695, -

75.74645904580896) and one apiary 5 miles to the northeast in Fair Hill NRMA in Elkton, MD, USA (39.71601607608891, -75.82417514703252).

Each apiary contained 20 colonies arranged in an outward facing circle with a 25 meter diameter. The circles were divided into 5 segments each containing 4 colonies which were then randomly assigned one of the 4 treatment groups WT control or mixed and VSH control or mixed, so that each treatment group was represented in each of the circle segments to avoid clumping in any one section of the circle. See Table 3.1 for sample size allocation at the apiary and full trial levels.

Table 3.1: Sample sizes of 2022 field experiment for individual apiaries and whole experiment totals.

Single apiary replicate				
		Mixing		
		Mixed	Control	Total
Queen Source	WT	5	5	10
	VSH	5	5	10
	Total	10	10	20

Full Field Trial (3 apiary replicates)				
		Mixing		
		Mixed	Control	Total
Queen Source	WT	15	15	30
	VSH	15	15	30
	Total	30	30	60

3.2.2 Colony Establishment and Mixing Treatments

Sixty colonies were established in standard 10 frame Langstroth deep boxes on March 31st, 2022 with 1.4 Kg packages of worker bees with WT queens from a

producer in Georgia, USA and were fed 1:1 w/v sugar solution and 50g of supplemental pollen. Four of the 10 frames contained fully drawn wax comb to assist colony establishment, the rest of the frames contained only undrawn plastic comb foundation. An additional 15 colonies were established at the same time in a separate apiary and served as backup replacements for failed experimental colonies prior to the baseline data assessment. Colonies assigned a VSH queen had the WT queens that came with the packages replaced on April 13th, 2022. All queens were caged prior to the VSH queen installations to control interrupted egg laying across queen source type. Colonies were inspected on April 15th to ensure that all queens had been successfully released.

On April 24th, colonies were equalized between the same queen source colonies so that each colony had 5 frames of brood from the same genetic stock to avoid a mixing effect. Between April 26th and May 17th, each colony was manually inoculated with a total of 12 live *Varroa* mites in increments of 2-4 mites at a time in order to ensure the starting presence of mites in all colonies. Mites were harvested from non-experimental colonies on the University of Delaware Farm that had not received acaricidal treatments during the prior year. For each inoculation, 5 nurse bees were placed in a petri dish with moistened filter paper. Live mites were pulled from capped brood cells and gently lifted with a paintbrush and tapped against a nurse bee. If the mite did not attach to the nurse bee, it was discarded to ensure that all colonies received viable adult female mites. The infested nurse bees were then placed in the feeding chamber of a colony with the petri dish open and the exit hole facing down into the brood nest with the colony entrance on the opposite end, forcing nurse bees to interact with the brood nest.

Six colony strength assessments and six brood mixing treatments were conducted over the course of the 2022 summer season, beginning on May 3rd, which ensured full population turnover of any WT worker brood remaining in colonies requeened with VSH queens. As the mixing groups consisted of only 2 colonies, colonies could be assessed and mixed simultaneously, thereby reducing handling stress on all colonies and controlling for handling effects between mixed and control colonies. Figure 3.1 demonstrates mixing brood frames between two queen type colonies.

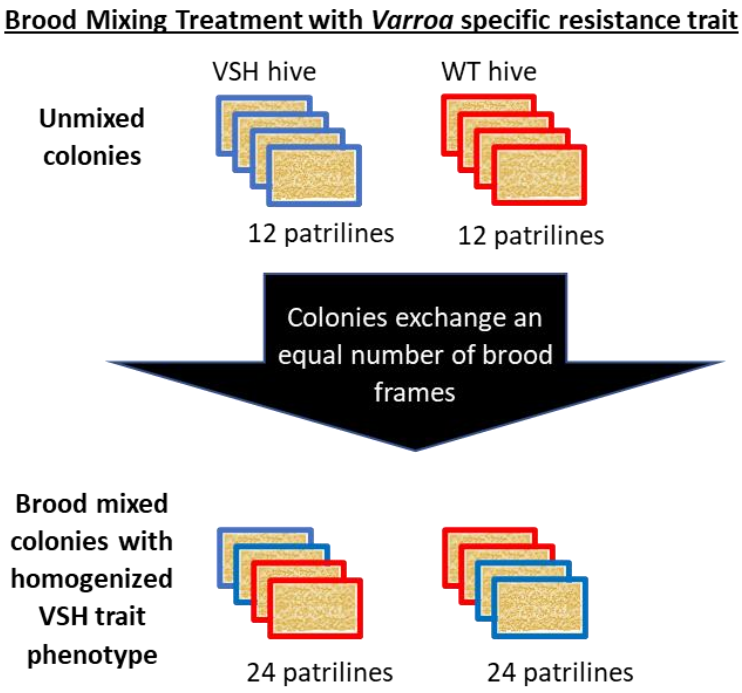


Figure 3.1: Representation of a 2-colony brood mixing treatment between a wild-type (WT) and *Varroa*-sensitive hygienic (VSH) colony.

3.2.3 2022 Field Experiment Timeline

Figure 3.2 displays key timepoints in the 2022 field experiment. A baseline colony strength assessment was taken the week of May 3rd, 2022, which was designated Week 1 in analyses. Starting at Week 3, weekly *Varroa* mite fall was measured until Week 19. Weekly mite fall was the sum of a 96-hour sticky board count from Thursdays to Mondays and a 72-hour sticky board count from Mondays to Thursdays. Assessments and mixing treatments were repeated every 3 weeks once all brood that had been mixed among colonies at the prior mixing treatment had emerged. This ensured that all brood assessed in a colony was laid by the colony's resident queen, and not the queen of a brood donor colony.

A sixth and final strength assessment and mixing treatment was completed on the week of August 15th (Week 16). Weekly mite fall counts continued until Week 19, when a VSH brood assay was completed for all colonies to determine the level of VSH trait in each colony. All colonies received an acaricidal treatment during Week 19 and sticky board knockdown mite fall counts were taken for three weeks.

All colonies received 2 gallons of 2:1 w/v sugar solution in October in preparation for overwintering. Additionally, 3.2 Kg of dry sucrose was given to each colony in December as additional overwintering feed.

Colonies were inspected on March 27th in 2023 to determine overwintering survival and record adult bee population and capped brood population. Colonies were again treated with acaricide, and the knockdown mite fall counts were recorded for 16 days.

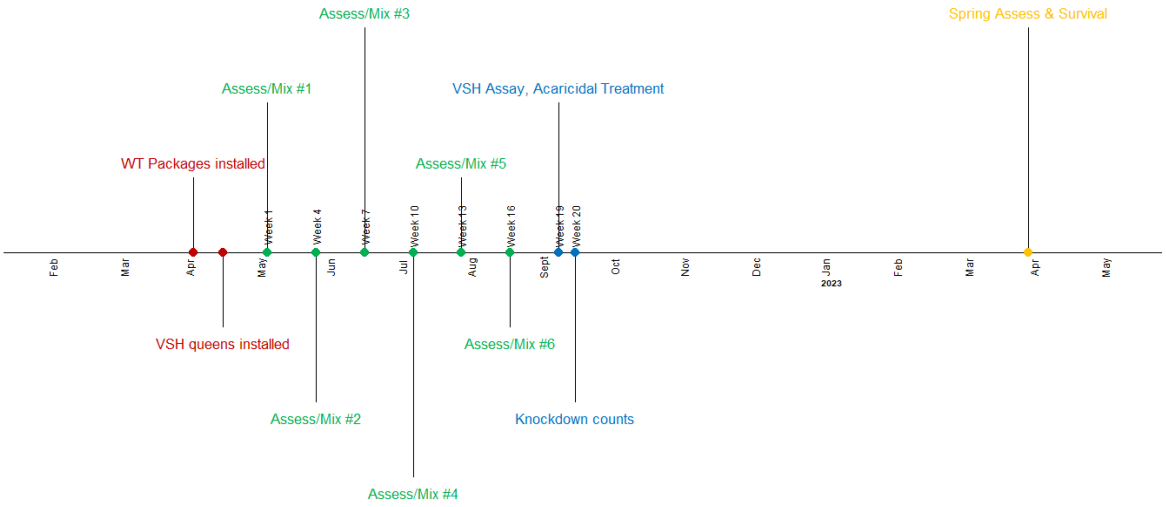


Figure 3.2: Timeline of 2022 brood mixing field experiment with VSH vs. WT queens.

3.2.4 Data Collection Protocols

Colony strength assessments and propolis

At each of the six colony strength assessments, adult bee population, capped brood population, and wax and honey cell count were recorded and calculated as described in section 2.2.4. Pollen stores, foraging, and pollen morphotype richness were not studied during the 2022 field season. Medium honey supers were harvested after the 6th assessment (Week 17). Propolis traps were placed on colonies during Week 17 and removed during Week 23.

Varroa population quantification

Three methods of quantifying the level of *Varroa* infestation were used to provide an accurate estimate of mite population on immature and adult bees and quantify the expression of the *Varroa*-sensitive hygiene trait.

- **Weekly colony mite fall**: Screened bottom boards placed underneath each colony allowed debris and mites to fall onto a sheet of corrugated plastic coated with vegetable oil, thereby trapping mites. Weekly mite fall for the 2022 field season was the sum of a 96-hour sticky board count from Thursdays to Mondays and a 72-hour sticky board count from Mondays to Thursdays. The number of fallen mites were recorded and normalized for differences in colony size using the adult population metric (Dietemann et al., 2013). Mite fall was recorded from Week 3 to week 19.
- **Knockdown mite fall**: Mite fall was recorded in 2 to 3 day increments and summed for Weeks 20, 21, and 22 after the acaricidal treatment to calculate an endpoint mite fall count for each colony.
- **Mites on adult bees**: 300 adult bees were rinsed in 70% ethanol for 1 minute and the total number of mites was recorded for assessments 3 through 6 and again during week 19 during the VSH trait assay (Dietemann et al., 2013).
- ***Varroa* sensitive hygienic (VSH) trait assay**: VSH behavior is a heritable, additive trait found in honey bee populations around the world whereby honey bees detect *Varroa* infested brood and remove it, thereby disrupting the mite's reproduction (Kirrane et al., 2015; Villa et al., 2009). Expression levels of the trait vary between worker patrilines and colonies, which will provide us with a quantitative metric of a *Varroa*-specific resistance trait. VSH bees specifically target fertile *Varroa* mites, leaving infertile foundress mites alone. Therefore, VSH trait was quantified by removing 200 pupae 7 to 11 days post-capping, during the "purple-eyed stage", and recording the number of cells with mites and whether the mites are reproductive or not. The VSH score of the colony will be determined by

the proportion of non-reproductive mites in the 200-pupae sample (Harbo, 2020). This assay is only utilized when mite levels are high, therefore it was conducted only at week 19, just before the acaricidal treatment was applied to obtain the knockdown mite fall count. This allowed me to 1) confirm the presence of VSH trait in the VSH colonies overall and 2) evaluate if the VSH trait was increased in WT mixed colonies compared to WT control colonies. Additionally, I was curious if VSH mixed colonies might suffer a dilution of their VSH trait levels compared to VSH control colonies. The VSH assay also served to record an endpoint value of the total mite infestation level in capped brood, which can be compared against mite alcohol washes and mite fall counts from the same colonies to look for general consensus between the mite levels of the 4 treatment groups (Mixing*Queen source).

Overwinter survival and spring 2023 assessment

Colonies were inspected for survival on March 27th, 2023. Additionally, a strength assessment for adult bee population and capped brood population was taken. An acaricidal treatment was applied the following day and knockdown mite fall was recorded in 2 to 3 day increments for 16 days.

3.2.5 Data Analysis Methods

Colonies that either failed or swarmed during the experimental period were removed from the study due to a break in the honey bee brood cycle, which interferes with the mite reproductive cycle. Because of this interruption, the mite levels of these colonies could no longer be compared to other experimental colonies and were removed from their respective apiaries. Six colonies failed in each of the WT-mixed,

VSH-mixed, and VSH-control groups. Four colonies failed in the WT-control group. Final analysis sample sizes are displayed in Table 3.2.

Table 3.2: Final 2022 season colony sample size.

Final Analysis Sample Size 2022 Season				
		Mixing		
		Mixed	Control	Total
Queen Source	WT	9	11	20
	VSH	9	9	18
	Total	18	20	38

All statistical analyses were conducted in R statistical software v.4.3.1 (R Core Team, 2023). The fixed effects of each mixing and queen source (WT or VSH) were evaluated for the measured parameters. Response variables that were recorded at a single time point, such as harvested honey, were analyzed via generalized linear model (GLM) using R package 'MASS' (Venables & Ripley, 2002). For repeated measures responses, such as adult bee population, a mixed model (GLMM) with assessment included as a fixed effect and colony identification number as a random effect were used from either R package 'lme4' (Bates et al., 2015) or 'glmmTMB' (Brooks et al., 2017) when zero-inflation and overdispersion prevented model convergence in 'lme4'. Model offsets, distributions, and transformations are specified for each response variable and model below. All model assumptions and residuals were confirmed using DHARMa package (Hartig, 2022). The 'car' package (Fox & Weisberg, 2019) was used to conduct model effect ANOVAs and estimated marginal means and *post hoc* comparisons were calculated using the 'emmeans' package (Lenth, 2023).

Colony Strength Assessments

Adult bee population and capped brood population were recorded for each colony at six separate assessment time points. Wax cell count and capped honey cell count were recorded at each of the six assessments and adjusted in their respective GLMMs by the corresponding adult bee population at each assessment point to control for variation in colony sizes. Harvested honey (Kg) was recorded after the sixth assessment and is offset by the adult bee population at assessment 6. Propolis mass (g) for each colony was adjusted by adult bee population at assessment 6 as well.

Table 3.3: Data analysis parameters for 2022 colony strength measures.

Response variable	**Fixed effects**	**Random effects**	**R package**	**Distribution**	**Offset**
Adult bee population (log)	Mixing*Assessment*Queen	(1|Colony)	lme4	Gaussian	-
Capped brood population	Mixing*Assessment*Queen	(1|Colony)	lme4	Gaussian	-
Wax cell count (sqrt)	Mixing*Assessment*Queen	(1|Colony)	lme4	Gaussian	log(Adult bee population)
Honey cell count (sqrt)	Mixing*Assessment*Queen	(1|Colony)	lme4	Gaussian	log(Adult bee population)
Honey harvest (Kg)	Mixing*Queen	-	MASS	Gaussian	log(Adult bee population assessment 6)
Propolis (g)	Mixing*Queen	-	MASS	MASS	log(Adult bee population assessment 6)

Varroa mite population quantification

Alcohol wash mite count (adult bee mites) was recorded at assessments 3 through 6 and additionally at week 19. GLMMs did not include an offset term by the number of adult bees in the wash sample because 300 bees were sampled each time.

The total count of mites in brood cells was sampled only during the VSH assay during week 19 and the GLM was not offset by the number of brood cells sampled because 200 cells were sampled for each colony. The GLM of the non-reproductive mite count was offset by the total number of brood mites recorded during the Week 19 VSH assay. The full offset term was log(total brood mites +1) because 3 of the VSH-control colonies had 0 brood mites. The knockdown mite counts after the acaricidal treatments were summed and offset by assessment 6 adult bee population.

The weekly total mite fall count was recorded for each colony for weeks 3 through 19. In order to offset the mite count by bee population at each week, bee population was interpolated piecewise between weeks with known population, which were Weeks 1, 4, 7, 10, 13, and 16. Weeks 17, 18, and 19 retain the same population as week 16, as there is not a reliable way of extrapolating population past the last known population measure. The resulting interpolation is presented in Fig. 3.3. The weekly mite fall GLMM retained week as a categorical variable in the analysis.

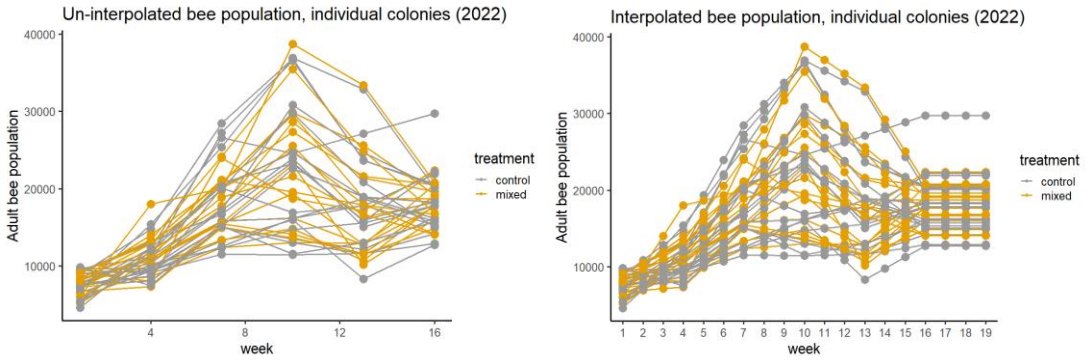


Figure 3.3: Representation of 2022 adult bee population for individual colonies in its un-interpolated (left) and piecewise interpolated (right) forms. Weeks 17, 18, and 19 are an extension of Week 16 population. Interpolating bee population for each week allowed for an offset term to be included for each week of mite fall data.

Table 3.4: Data analysis parameters for 2021 *Varroa* mite counts.

Response variable	**Fixed effects**	**Random effects**	**R package**	**Distribution**	**Offset**
Adult bee mites	Mixing*Assessment*Queen	(1|Colony)	glmmTMB	Neg binom 1	-
Brood mites	Mixing*Queen	-	MASS	Negative Binomial	-
Non-reproductive mites	Mixing*Queen	-	MASS	Poisson	log(Total number of brood mites + 1)
Weekly mite fall	Mixing*Week*Queen	(1|Colony)	glmmTMB	Neg binom 2	log(Interpolated weekly bee population)
Knockdown mite count (log)	Mixing*Queen	-	MASS	Gaussian	log(Assessment 6 bee population)

Overwintering survival and spring 2023 assessment

Overwintering survival was analyzed via a binomial GLM with "alive" colonies coded as 1 and "dead" colonies coded as 0. Spring 2023 adult bee population, brood population, and a knockdown mite fall count were analyzed separately from Summer 2022 data as the mixed colonies were no longer fully mixed, however, legacy effects from the prior season's treatments might be observed.

Table 3.5: Data analysis parameters for 2022 colony survival, strength, and mite measures in spring 2023.

Response variable	**Fixed effects**	**Random effects**	**R package**	**Distribution**	**Offset**
Overwintering survival	Mixing*Queen	-	MASS	Binomial (1=alive, 0=dead)	-
Adult bee population (log)	Mixing*Queen	-	MASS	Gaussian	-
Capped brood population	Mixing*Queen	-	MASS	Gaussian	-
Knockdown mite fall (log)	Mixing*Queen	-	MASS		log(Spring adult bee population)

3.3 Results

Colony Strength Assessments

Assessment time had a significant effect for all colony strength measures in which it was included, and overviews of all main and interaction effects are presented in Table 3.6. For adult bee population, there was a significant interaction between

mixing*queen, χ^2 (5) = 24.5, p<0.001, and a significant three-way interaction of mixing*assessment*queen, χ^2 (5) = 14.3, p<0.05. *Post hoc* results for each time point indicate that VSH queens had lower population from assessment 2 to 6 (Fig. 3.4) and that mixed colony populations were generally intermediate between the WT-control and the VSH-control populations (Fig. 3.5)

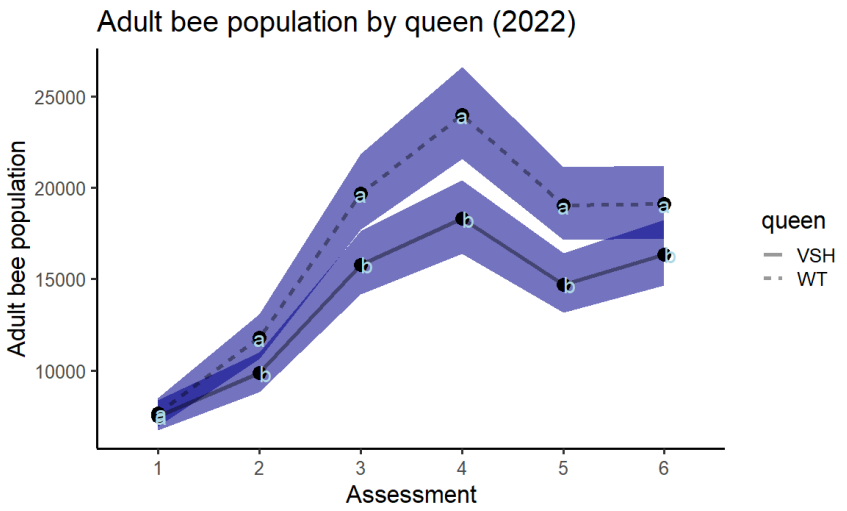


Figure 3.4: Effect of queen averaged over mixing on 2022 bee population. Predicted means and 95% CIs for each week are presented in the response scale but Wald III tests were performed on the log scale and found a significant interaction between mixing*assessment, χ^2 (5) = 24.5, p<0.001. Marginal means at each assessment were separated by t-test for queen|assessment with $\alpha \leq 0.05$.

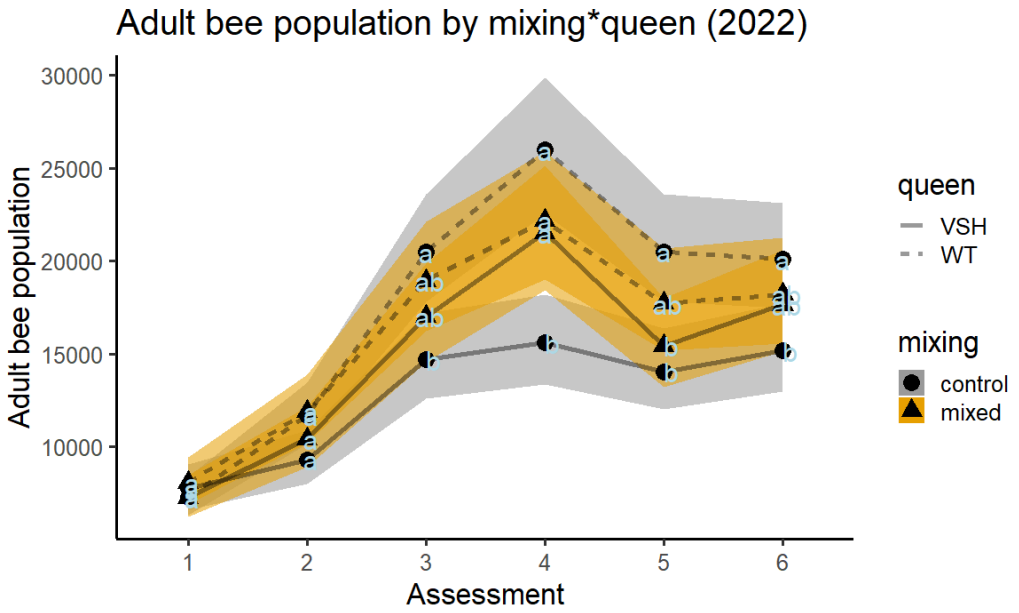


Figure 3.5: Effect of mixing*queen|assessment on 2022 bee population. Predicted means and 95% CIs for each week are presented in the response scale but Wald III tests were performed on the log scale and found a significant interaction between mixing*time*queen, χ^2 (5) = 14.3, p<0.05. Marginal means at each assessment were separated by Tukey-Kramer test $\alpha \leq 0.05$.

There were no significant effects other than assessment for brood population (Fig. 3.6) or honey cell count. Wax cell count had a significant interaction of queen*assessment, χ^2 (5) = 33.8, p<0.001, with increased wax production in WT colonies as the season progressed (Fig. 3.7).

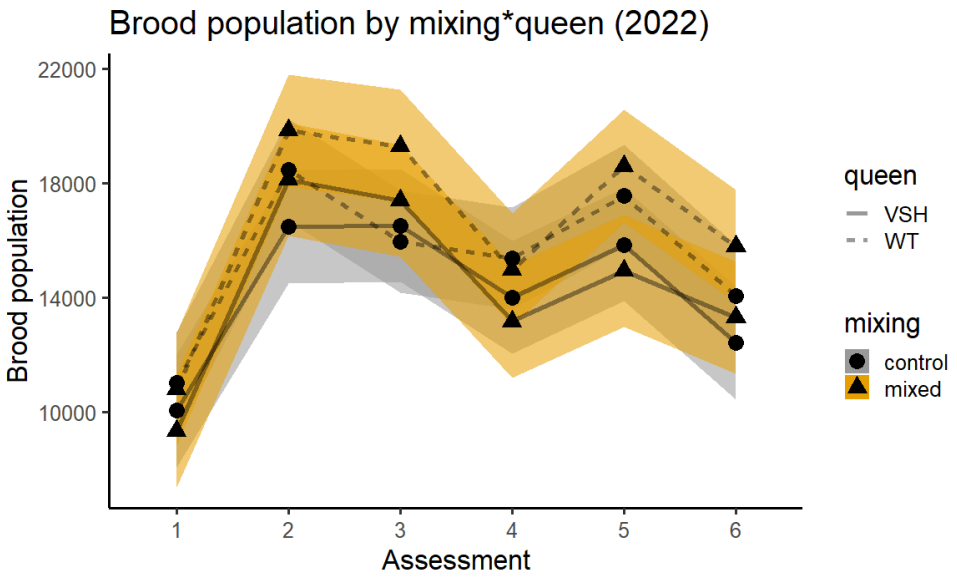


Figure 3.6: Effect of mixing*queen on 2022 brood population over 6 assessments. Predicted means and 95% CIs for each assessment are presented. Wald III tests were performed on the response scale and found no significant interaction between mixing*time*queen, $\chi^2(5) = 1.8$, $p=0.86$.

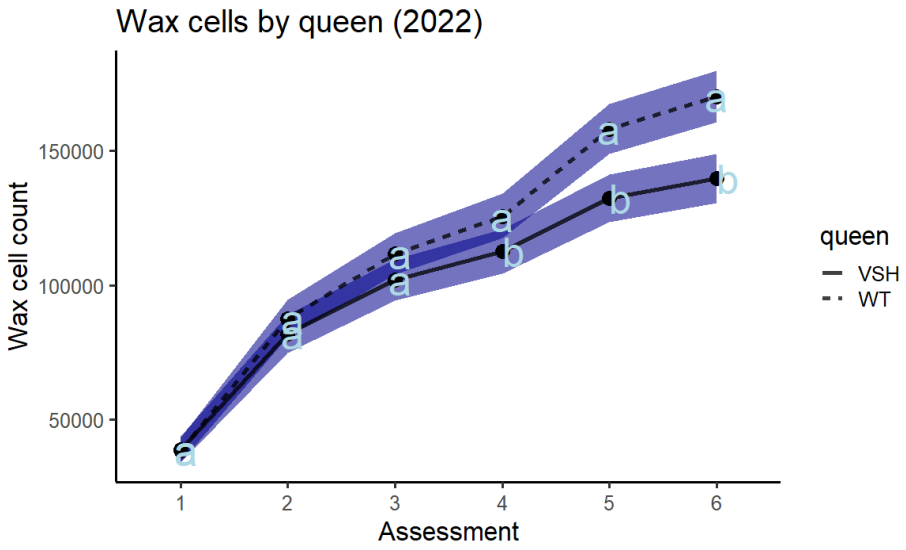


Figure 3.7: Effect of queen averaged over mixing on 2022 wax cell counts. Predicted means and 95% CIs for each week are presented in the response scale but Wald III tests were performed on the sqrt scale and found a significant interaction between queen*time, χ^2 (5) = 33.86, p<0.001. Marginal means at each assessment were separated by t-test for queen|assessment with $\alpha \leq 0.05$.

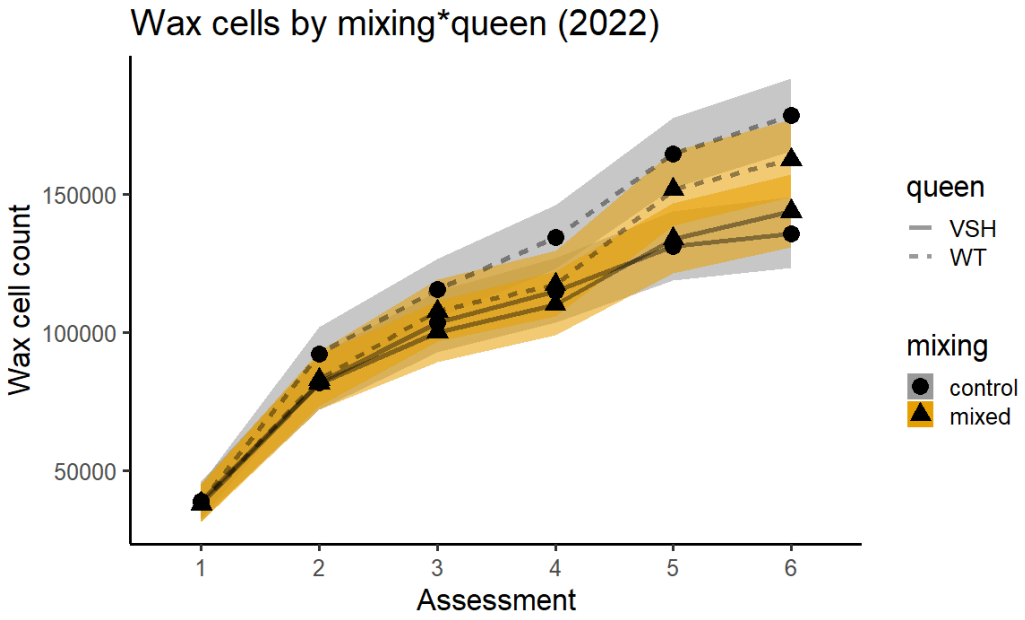


Figure 3.8: Effect of mixing*queen on 2022 wax cell count over 6 assessments. Predicted means and 95% CIs for each assessment are presented. Wald III tests were performed on the sqrt scale and found no significant interaction between mixing*time*queen, $\chi^2(5) = 4.7$, p=0.45.

Harvested honey (Kg) had a significant main effect of queen, $\chi^2(1) = 9.5$, p<0.01, but no mixing nor interaction effects (Table 3.6). There was a significant difference in harvested honey (Kg) by queen, with VSH queens having on average 8.7 ± 2.37 Kg less honey than WT queens (Fig. 3.9). No main effects or interactions were found for propolis (g) (Table 3.6).

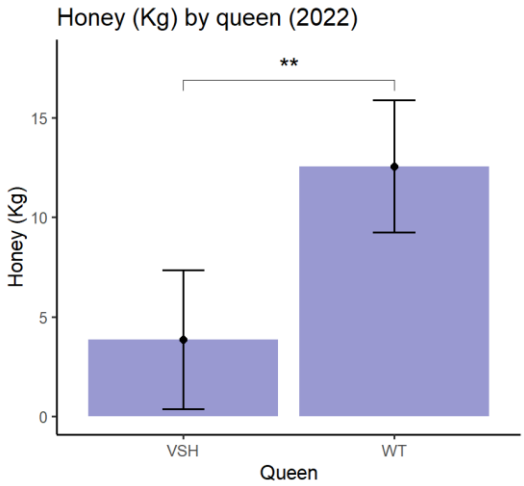


Figure 3.9: Effect of queen averaged over mixing on 2022 endpoint harvested honey (Kg). Predicted means and 95% CIs for each assessment are presented. Wald III tests were performed on the response scale and found a significant main effect of queen, $\chi^2(1) = 9.5$, $p<0.01$. VSH queens had on average 8.7 ± 2.37 Kg less honey than WT queens.

Table 3.6: 2022 Colony strength ANOVAs (Type III) of the main effects of mixing (M), time (T), and queen source (QS) and their interactions on colony-level measures of strength. Differences accepted at $\alpha \leq 0.05$.

Adult bee population	**Chisq**	**DF**	**Pr(>Chisq)**	*****
Mixing (M)	0.3187	1	0.5723904	
Time (T)	106.8896	5	< 2.2e-16	***
Queen Source (QS)	0.3288	1	0.5663476	
M*T	9.2483	5	0.0995596	.
M*QS	1.1651	1	0.2804175	
T*QS	24.5689	5	0.0001687	***
M*T*QS	14.3904	5	0.0133106	*
Capped Brood Population	**Chisq**	**DF**	**Pr(>Chisq)**	*****
Mixing (M)	0.2525	1	0.6153	
Time (T)	45.3928	5	1.21E-08	***
Queen Source (QS)	0.5307	1	0.4663	
M*T	4.1426	5	0.5291	
M*QS	0.0592	1	0.8078	

T*QS	3.1794	5	0.6724	
M*T*QS	1.8945	5	0.8635	
Wax cell count	**Chisq**	**DF**	**Pr(>Chisq)**	*****
Mixing (M)	0.0377	1	0.8461	
Time (T)	634.3613	5	< 2.2e-16	***
Queen Source (QS)	0.0078	1	0.9297	
M*T	3.3701	5	0.6431	
M*QS	0.0013	1	0.9713	
T*QS	33.8662	5	2.53E-06	***
M*T*QS	4.7034	5	0.4531	
Honey cell count	**Chisq**	**DF**	**Pr(>Chisq)**	*****
Mixing (M)	0.036	1	0.8495	
Time (T)	358.0947	5	2.20E-16	***
Queen Source (QS)	0.1223	1	0.7266	
M*T	8.6286	5	0.1248	
M*QS	0.1371	1	0.7112	
T*QS	3.2667	5	0.6589	
M*T*QS	2.5957	5	0.762	
Honey harvest (Kg)	**Chisq**	**DF**	**Pr(>Chisq)**	*****
Mixing (M)	0.0403	1	0.840944	
Queen Source (QS)	9.5094	1	0.002044	**
M*QS	0.3377	1	0.561157	
Propolis (g)	**Chisq**	**DF**	**Pr(>Chisq)**	*****
Mixing (M)	0.286217	1	0.5927	
Queen Source (QS)	0.085623	1	0.7698	
M*QS	0.191439	1	0.6617	

Varroa mite population quantification

There was a significant interaction between queen and week for alcohol washed mites from adult bees, $\chi^2(4) = 15.06$, $p<0.01$ (Table 3.7), with more mites found in WT colonies as the season progressed (Fig. 3.10). There was not a significant interaction between mixing*week*queen, $\chi^2(4) = 7.8$, $p=0.098$, for mites on adult bees (Fig. 3.11).

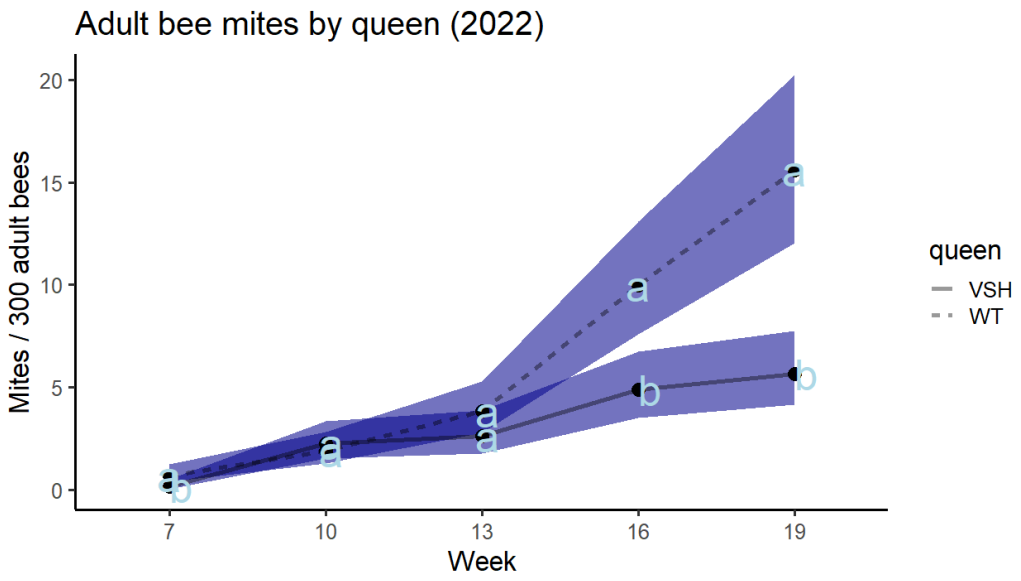


Figure 3.10: Effect of queen averaged over mixing on 2022 alcohol wash mites from adult bees. Predicted means and 95% CIs for each week are presented. Wald III tests were performed and found a significant interaction between queen*week, $\chi^2(4) = 15.06$, $p<0.01$. Marginal means at each week were separated by *t*-test with $\alpha \leq 0.05$.

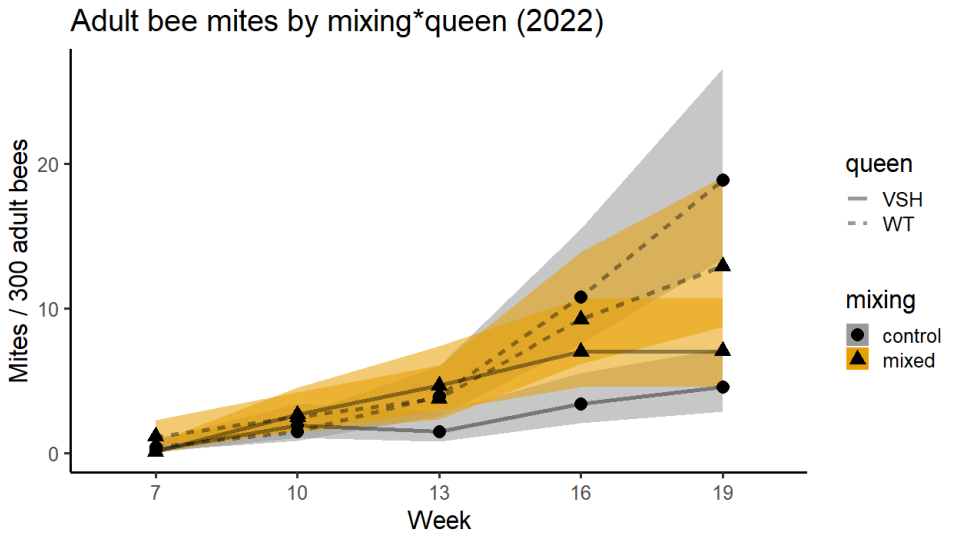


Figure 3.11: Effect of mixing*queen on 2022 alcohol wash mites from adult bees. Predicted means and 95% CIs for each week are presented. There was not a significant interaction between mixing*week*queen, $\chi^2(4) = 7.8$, $p=0.098$.

The endpoint measure of mites in brood had a significant interaction effect between mixing*queen, $\chi^2(1) = 7.5$, $p<0.01$. Of note, WT-control colonies had significantly more brood mites than the WT-mixed colonies (Fig. 3.12). WT-mixed colonies specifically had 15.23 ± 4.44 fewer brood mites than their WT-control counterparts, $t(34) = -3.431$, $p < 0.01$.

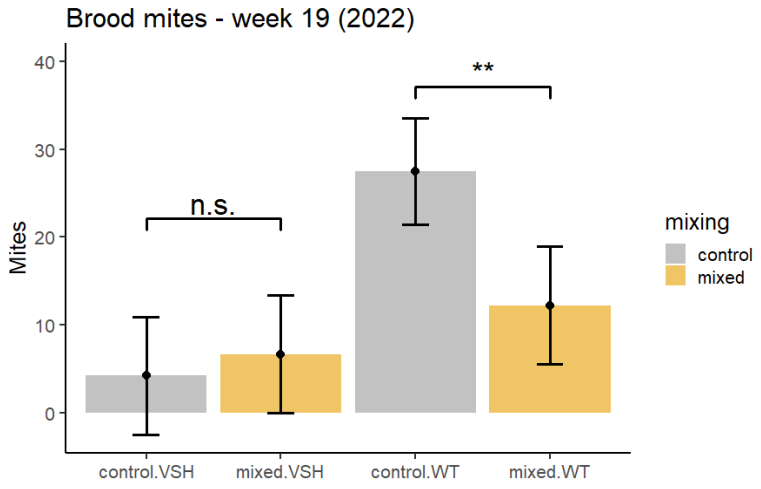


Figure 3.12: Effect of mixing*queen on 2022 brood mite count. Predicted means and 95% CIs for each assessment are presented. Wald III tests found a significant main effect of mixing*queen, $\chi^2(1) = 7.5$, $p<0.01$). Marginal means of mixing|queen were separated by Tukey-Kramer test $\alpha \leq 0.05$. There was also a significant difference between VSH and WT queens when averaged over mixing levels, $\chi^2(1) = 27.3$, $p<0.001$).

The proportion of brood mites that were non-reproductive (Fig. 3.13) was an important feature of the VSH trait assay and the main effect of queen was significant $\chi^2(1) = 10.5$, $p<0.01$. VSH queens had a greater proportion of nonreproductive mites. The interaction mixing*queen was not significant, $\chi^2(1) = 0.17$, $p<0.67$, but predicted means and confidence intervals are presented in Figure 3.14.

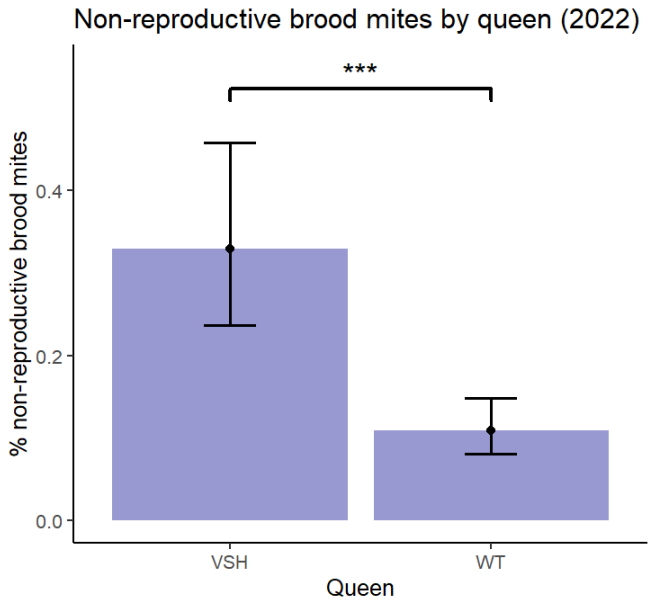


Figure 3.13: Effect of queen averaged over mixing on proportion of 2022 non-reproductive brood mites. Predicted means and 95% CIs are presented as proportions. Wald III tests were performed on the response scale and found a significant main effect of queen, $\chi 2(1) = 10.5$, $p<0.01$, with VSH queens having a greater proportion of nonreproductive mites.

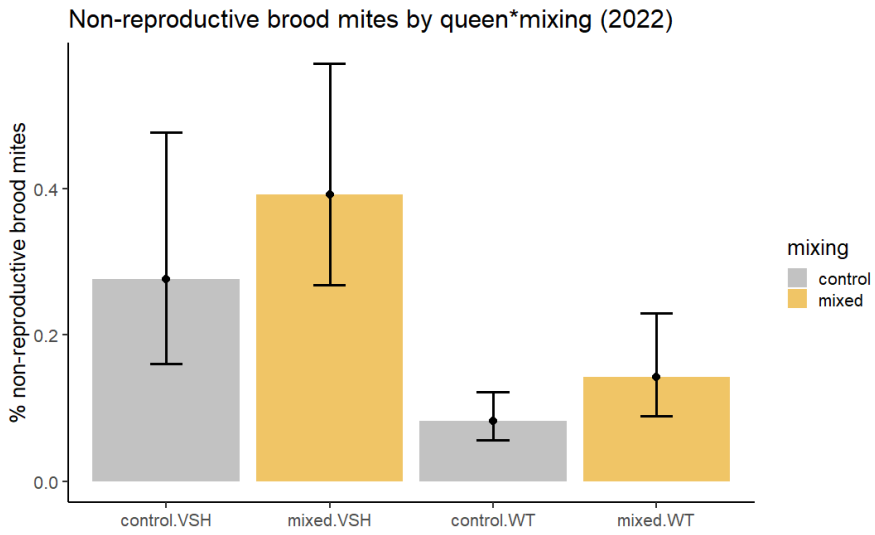


Figure 3.14: Effect of mixing*queen on 2022 non-reproductive brood mites. Predicted means and 95% CIs for each assessment are presented as proportions. Wald III tests performed on the two-way interaction were not significant, $\chi^2(1) = 0.17$, $p<0.67$.

There were significant two-way and three-way interactions for weekly mite fall (Table 3.7), with significant differences for mixed colonies within queen groups as the season progressed, most notably after week 13 (Fig. 3.15).

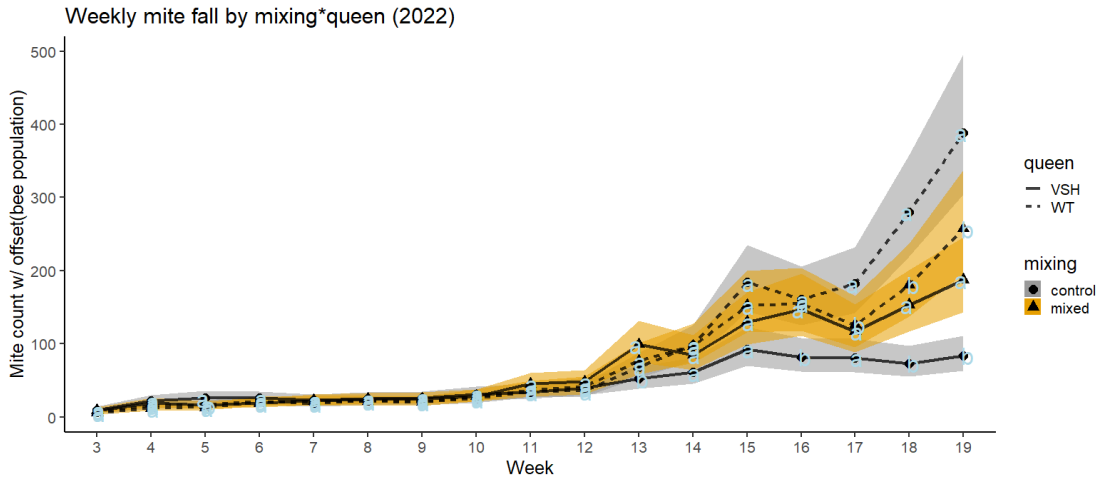


Figure 3.15: Effect of mixing*queen on 2022 weekly mite fall. Predicted means and 95% CIs for each week are presented and Wald III tests found a significant interaction between mixing*week*queen, χ^2 (16) = 63.9, p<0.001. The GLMM included an offset term for adult bee population. Marginal means for mixing|queen within each week were separated by Tukey-Kramer test $\alpha \leq 0.05$. **Letters refer to significant differences within queen type within each week.**

There were significant main effects of queen and mixing on the endpoint of knockdown mite fall and the interaction was also significant, $\chi^2(1) = 14.7$, p<0.001 (Fig. 3.16).

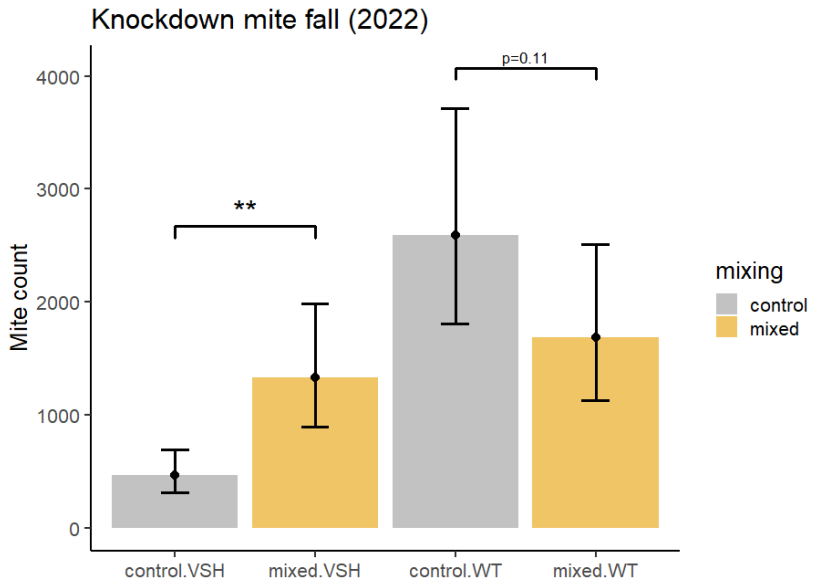


Figure 3.16: Effect of mixing*queen on 2022 knockdown mite fall adjusted for bee population using week 16 population data. Marginal means and 95% CIs are presented. Wald III tests performed on the two-way interaction were significant, $\chi^2(1) = 14.7$, $p<0.001$. VSH-control colonies had significantly fewer knockdown mites (-862 ± 277) than the VSH-mixed colonies, t(34) = -3.116, $p<0.01$.

Table 3.7: 2022 *Varroa* mite count ANOVAs (Type III) of the main effects of mixing (M), time (T), and queen source (QS) and their interactions on mite count measurements. Differences accepted at $\alpha \leq 0.05$.

Adult bee mites	**Chisq**	**DF**	**Pr(>Chisq)**	*****
Mixing (M)	0.3304	1	0.565399	
Time (T)	26.6869	4	2.30E-05	***
Queen Source (QS)	0.6495	1	0.420304	
M*T	4.7088	4	0.318499	
M*QS	1.5168	1	0.2181	
T*QS	15.061	4	0.004576	**
M*T*QS	7.8178	4	0.098484	.
Brood mites	**Chisq**	**DF**	**Pr(>Chisq)**	*****
Mixing (M)	0.2755	1	0.599634	
Queen Source (QS)	27.3788	1	1.67E-07	***
M*QS	7.5477	1	0.006009	**

Non-reproductive mites	**Chisq**	**DF**	**Pr(>Chisq)**	*****
Mixing (M)	1.0951	1	0.295334	
Queen Source (QS)	10.5616	1	0.001155	**
M*QS	0.1787	1	0.672471	
Weekly mite fall	**Chisq**	**DF**	**Pr(>Chisq)**	*****
Mixing (M)	0.0662	1	0.7969	
Time (T)	362.898	16	< 2.2e-16	***
Queen Source (QS)	1.4357	1	0.2308	
M*T	72.169	16	4.15E-09	***
M*QS	0.0335	1	0.8548	
T*QS	221.9269	16	< 2.2e-16	***
M*T*QS	63.9351	16	1.12E-07	***
Knockdown mite fall	**Chisq**	**DF**	**Pr(>Chisq)**	*****
Mixing (M)	14.171	1	0.0001669	***
Queen Source (QS)	41.848	1	9.87E-11	***
M*QS	14.793	1	0.0001199	***

Overwintering survival and spring 2023 assessment

No significant effects were found in GLMs for overwinter survival, spring adult bee population, or spring brood population (Table 3.8). There was a significant main effect of queen on the spring knockdown mite fall, $\chi^2(1) = 4.9$, $p<0.05$ and VSH colonies had significantly fewer mites than WT colonies.

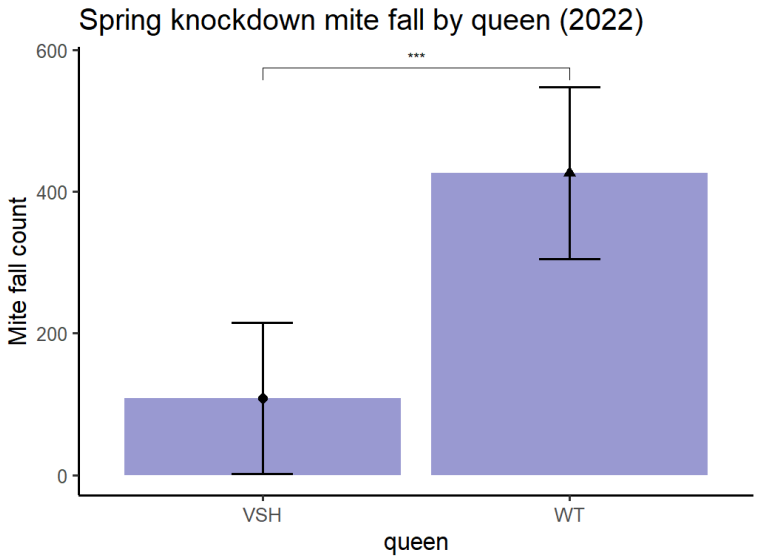


Figure 3.17: Effect of queen averaged over mixing on the 2022 field experiment's spring 2023 knockdown mite counts. Predicted means and 95% CIs are presented. Wald III tests were performed on the response scale and found a significant main effect of queen, $\chi^2(1) = 4.9$, $p<0.05$.

Table 3.8: Overwinter survival, colony strength, and mite fall ANOVAs (Type III) of the main effects of Mixing (M), queen source (QS) and their interaction on 2022 colony survival in spring 2023 and strength measures. Differences accepted at $\alpha \leq 0.05$.

Overwinter survival	**Chisq**	**DF**	**Pr(>Chisq)**	*****
Mixing (M)	1.33319	1	0.2482	
Queen Source (QS)	0.08636	1	0.7689	
M*QS	2.91601	1	0.0877	.
Spring Adult bee population	**Chisq**	**DF**	**Pr(>Chisq)**	*****
Mixing (M)	0.209546	1	0.6471	
Queen Source (QS)	0.058625	1	0.8087	
M*QS	0.079389	1	0.7781	
Spring brood population	**Chisq**	**DF**	**Pr(>Chisq)**	*****
Mixing (M)	0.084608	1	0.7711	
Queen Source (QS)	0.133128	1	0.7152	
M*QS	0.039701	1	0.8421	
Spring knockdown mite fall	**Chisq**	**DF**	**Pr(>Chisq)**	*****
Mixing (M)	0.0116	1	0.9143	

Queen Source (QS)	4.9696	1	0.0258	*
M*QS	1.2814	1	0.2576	

3.4 Discussion

In contrast to the 2021 field trial, I observed significant and consistent differences in colony population, productivity, and mite levels between the WT and VSH queen sources used in the 2022 season. The effect of the mixing treatment was more pronounced than in 2021 yet requires nuanced interpretation.

The growth of adult bee population followed a typical seasonal trend, with a peak in midsummer while brood production decreased slightly at the same time point, likely as a response to a midsummer dearth in floral resources. The GLMM for brood population found no significant effects, however, WT colonies tended to have higher brood production. The GLMM for adult bee population had significant three-way interaction effects. VSH-control colonies had significantly fewer adult bees than WT-control colonies for the last 4 out of 6 assessments. During the same period, mixed colonies had remarkably intermediate adult bee populations that were not significantly different from either control group.

VSH colonies had a significantly higher proportion of non-reproductive mites in brood cells, confirming the overall presence of VSH trait in the queens sourced for this study. VSH-mixed colonies had a slightly higher proportion of non-reproductive mites compared to the VSH-control colonies, likely due to their overall higher mite levels increasing detection probabilities and because 2 of the VSH-control colonies had 0 total brood mites. WT-mixed colonies had only a marginally higher proportion of non-reproductive mites compared to WT-control colonies, which may be caused by

VSH-trait bees aging out of nurse bee roles when the VSH assay was conducted 21 days after the final mixing treatment.

WT colonies were significantly more productive than VSH colonies in terms of honey and wax. There were no mixing effects for these measures of productivity, with mixed colonies trending towards their respective control counterpart groups. At the endpoint honey harvest, VSH queens had on average 8.7 ± 2.37 Kg less honey than WT queens. There were no significant model effects for propolis deposition, indicating that the selected VSH line had not suffered loss of propolis foraging traits compared to the WT colonies used in this study.

The alcohol mite wash for adult bees found significantly fewer mites in VSH colonies than WT colonies, however, the three-way interaction including mixing groups was not significant. At the endpoint measure of adult bee mites, WT-control had the highest mean mites, followed by WT-mixed, VSH-mixed, and finally VSH-control, suggesting an intermediate effect of mixing that was similar to that for adult bee population.

The endpoint (Week 19) measure of brood mites found a significantly greater number of mites in WT-control compared to WT-mixed colonies and no difference between mixing groups of VSH colonies (Fig. 3.12). WT-mixed colonies had an average of 15.23 ± 4.44 fewer brood mites than their WT-control counterparts, indicating a potentially strong effect of VSH trait sharing via brood mixing, without harming VSH-mixed colonies that would have had a dilution of the proportion of their workers with VSH phenotype.

Week 19 mite fall was again intermediate for mixed colonies and WT-mixed colonies benefited by having lower mite levels than WT-control colonies. However, in

this mite measure, VSH-mixed colonies had significantly greater mite fall than the VSH-control group, which may be cause for concern as VSH stock is, by definition, selected for *resistance* to the increase in *Varroa* populations, and not necessarily *tolerance* of higher mite levels and the accompanying increase in viral loads of Deformed Wing Virus (Grozinger & Flenniken, 2019).

Knockdown mite fall results further cloud interpretation of overall mite levels and mixing effects, as the relative mite counts among colonies followed the observed trend of WT-control>WT-mixed>VSH-mixed>VSH-control. *Post hoc* tests (Fig. 3.16) found that VSH-mixed colonies had significantly more mites than VSH-control, but the WT-control and WT-mixed colonies were not significantly different (p=0.11), essentially contradicting the significance results of the brood mite assay. This could indicate the fleeting nature of brood mixing's effect on trait sharing between colonies, with repeated mixing every 3 weeks being essential to maintaining the phenotypic effects. Figure 3.18 compares the 4 endpoint mite counts on a per bee scale.

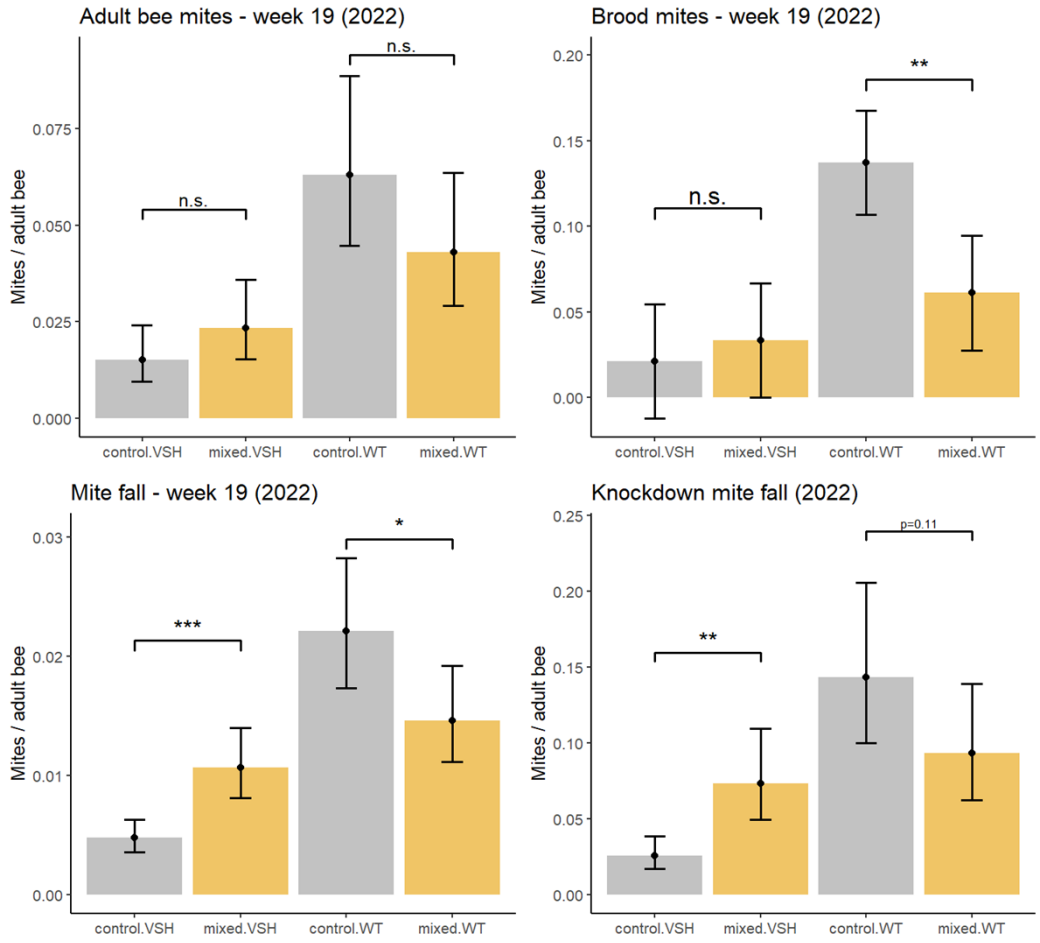


Figure 3.18: A comparison of 4 methods of counting *Varroa* mites at the endpoint of the 2022 field season. Scales have been adjusted to represent mites per unit bee or brood cell to better display relative mite levels between methods.

The 2022 mite results confirm that differential selected *Varroa*-resistance traits must be present within a mixing group in order to observe improvements in mite levels. The results also illustrate key tradeoffs when selecting livestock for specific resistance traits, notably, the loss of productivity in VSH queens. Leclerq et al. (2017) reviewed the benefits and drawbacks of hygienic behavior, which is distinct from

VSH, and found that colonies selected for high levels of removal of diseased brood did not suffer from loss of productivity or any other measurable traits. In contrast, experimental colonies selected for increased pollen hoarding behavior in the US found many tradeoffs when seeking to increase pollen foraging, such as longer maturation times for immature bees, lower body mass, and reduced productivity due to crowding of the brood nest (Page et al., 2012; Page & Fondrk, 1995). A separate line of pollen hoarding bees also suffered from decreased colony fitness in Poland (Wilde et al., 2011).

Maximizing specific traits can lead to unintended consequences from increased inbreeding. Of concern in the context of the 2022 field study are the increased mite levels in the VSH-mixed colonies, and whether the VSH line could be unprepared for increases in viral load when they are bred to resist mite population increase, but not necessarily tolerate high levels of mites and Deformed Wing Virus. Penn et al. (2022) found that Pol-Line bees, an outcrossed line of VSH bees, had the lowest tolerance of DWV, with more severe viral symptoms compared to Carniolan, Italian, Russian, and Saskatraz honey bee stocks. In some cases, the increased vulnerability of some selected stock may be adaptive, as described by Ihle et al. (2021) in Russian honey bee stock with high levels of social apoptosis, whereby brood with increased susceptibility to lower viral loads encourages workers to remove diseased or dying brood before the rest of the colony is exposed.

Many interacting factors can influence the social immunity of a colony, and for brood mixing to prove a scalable tool for *Varroa* management, future studies should investigate the benefits of phenotypic mixing of multiple *Varroa*-resistance traits. Grooming behavior would be a logical complementary resistance trait to VSH, as bees

selected for high levels of grooming (Morfin et al., 2020) might be able to actively remove mites from adult bees while bees selected for high VSH behavior could focus on disrupting mite reproduction within the brood. This would also ensure the activity of resistance behavior outside of the brood production season if a final mixing of winter bees was conducted. Additionally, combining VSH and grooming traits via brood mixing would overcome the inbreeding hurdles that would result from selecting for 2 unrelated resistance traits within a single line of selected bee stock. Special attention should be given to how the proportion of workers with certain resistance phenotypes might affect individual worker resistance behavior. Studies of hygienic, pollen hoarding, and VSH traits have found that varying proportions of workers with resistance phenotypes can encourage or discourage individual behavioral expression and, in some cases, a certain threshold of resistant individuals must be met for the resistance trait to have any colony-level effect (Arathi & Spivak, 2001; Calderone & Page, 1992; Delaplane et al., 2021).

Another issue to address prior to the adoption of brood mixing as a *Varroa* management technique is the high level of mites needed in order to see a benefit from brood mixing. Most beekeepers treat for *Varroa* when mite levels reach 3 mites per 100 bees (Jack & Ellis, 2021), however, we did not see differentiation between WT-control and WT-mixed colonies until WT-mite levels were closer to 5 mites per 100 bees on adult bees. Seasonal studies of VSH behavior have found that VSH activity is lower during the spring buildup when floral resources are abundant (Tison et al., 2021), which could further reduce the efficacy of sharing the VSH trait via brood mixing in an effort to reduce reliance on synthetic acaricides. The 2022 brood mixing experiment did not see the effect of VSH mixing until mite levels were higher than

most beekeepers would tolerate and is especially problematic when combined with the lower productivity of VSH colonies. These management factors further necessitate the exploration of combining VSH trait with grooming trait via brood mixing in future studies to explore resistance trait synergy.

Chapter 4

PATHOGEN DYNAMICS IN A BROOD MIXING SYSTEM

4.1 Background and Objectives

Objective: Investigate the effect of routine brood frame sharing on pathogen prevalence (presence or absence) and load (quantity of pathogen) over a full season.

- Hypothesis 1: The brood mixed treatment group will display either equal or higher prevalence of various pathogens as the season progresses.
- Hypothesis 2: The mixed treatment group will have similar pathogen loads to the unmixed groups as the season progresses.

To test these hypotheses, I conducted real-time quantitative PCR to determine the presence and relative normalized quantities of 6 pathogen targets in the 2021 brood mixing field experiment. The 2021 field season is described in full detail in Chapter 2 and entailed mixing brood between groups of 4 colonies with a reproductive queen sourced from either GA, FL, CA-1, or CA-2. Three pathogen samples were taken from each colony at the baseline (assessment 1), mid-season (assessment 2), and endpoint (assessment 3).

The viral targets include 3 ubiquitous viruses (Deformed Wing Virus, Black Queen Cell Virus, and Sacbrood Virus) and one uncommon virus (Israeli Acute Paralysis Virus). Additionally, I screened for the common microsporidian parasite *Nosema* spp., now genus *Vairimorpha* (Tokarev et al., 2020), and for the Gram-

positive bacteria *Melissococcus plutonius*, which is best known as the causative agent of the disease European Foulbrood.

4.2 Methods

4.2.1 Virus and Microsporidian pathogens

RNA extraction and reverse transcription

A sample of 50 live adult bees was taken from the brood nest of each colony at each of the 3 assessment points in the 2021 field season and frozen at -80 C. Bees were homogenized at -80 C in a 30 mL homogenization tube (OMNI Intl, SKU #19-6358Z) at 1500 strokes per minute for 2 min on a Spex Geno/Grinder™ 2000 (Cole-Parmer, Metuchen, NJ).

2.5 mL Monarch® DNA/RNA Protection Reagent (New England BioLabs, Inc., T2011L) and 2.5 mL water were added to the homogenate and then vortexed thoroughly. RNA was extracted following instructions from a Monarch® Total RNA Miniprep Kit (New England BioLabs, Inc., T2010S) with a final elution volume of 40 µl. RNA extract concentration and quality were analyzed with a Thermo Scientific™ NanoDrop™ One^{C}.

A cDNA template was created using 4 µg of RNA and random primers from a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CAT: 4368814).

Real-time qPCR

Using a CFX384 Touch Real-Time PCR Detection System (Bio-Rad), 10 µl reactions were carried out in triplicate for each sample using 40 ng of cDNA with 0.25 µl of each 10 µM forward and reverse primers and 5 µl of Luna Universal qPCR Master Mix (New England Biolabs). Each 384-well plate contained, in triplicate wells, an inter-plate positive control, a negative control with water in place of cDNA, and a standard dilution of the target to determine primer efficiency. Thermocycling conditions for all 5 pathogen targets and 1 honey bee endogenous reference (Table 4.1) were set to 95 C for 1 min, 40 cycles of 95 C for 15 sec and 60 C for 30 sec, and finally a melting curve analysis from 60 C to 95 C at a 0.5 C step to control for amplification product specificity.

Table 4.1: Primer sequences and sources of pathogen target genes and honey bee endogenous reference gene for real time qPCR.

Gene name	**Forward (5'-3')**	**Reverse (5'-3')**	**Category**	**Source**	**Observed efficiency**
Elongation factor 1-alpha	GGAGATGC TGCCATCG TTAT	CAGCAGCG TCCTTGAA AGTT	Reference gene	Lourenço et al., (2008)	94.1%
Deformed Wing Virus (DWV)	GTTTGTATG AGGTTATA CTTCAAGG AG	GCCATGCA ATCCTTCA GTACCAGC	Virus	Ryabov et al., (2014)	88.7%
Black Queen Cell Virus (BQCV)	GGAGTCGC AGAGTTCC AAATA	GAGATGCG TGAATACA GGGC	Virus	(Choi et al., 2015)	91.1%
Sacbrood Virus (SBV)	AACGTCCA CTACACCG AAATGTC	ACACTGCG CGTCTAAC ATTCC	Virus	(Blanchard et al., 2014)	91.2%
Israeli Acute Paralysis Virus (IAPV)	GTTGGATG ATAGGTCC ACCCC	TCAAGTGT CGGTTTTCG GTC	Virus	Jones et al., (2021)	94.3%

Nosema spp.	AGCAGCCG CGGTAATA CTTGTTC	GTTCGTCC AGTCAGGG TCGT	Microsporidian	Alburaki et al., (2018)	93.2%

Relative normalized quantification

CFX Maestro 1.1 v. 4.1.2433.1219 (Bio-Rad, 2017) software was used to calculate the normalized relative quantity of each pathogen target. The reference gene *ef1-alpha* was used to normalize RNA extraction efficiency among samples. The calculations that were used followed the Pfaffl method, whereby the observed primer efficiencies (Table 4.2.1) of the gene of interest and the reference gene are incorporated in calculations when efficiency is not 100% (Pfaffl, 2001). Relative normalized quantity was scaled to the sample with the lowest relative quantity level (highest ΔCt value between reference gene and target gene).

4.2.2 Bacterial Pathogen - *Melissococcus plutonius*

DNA extraction and reverse transcription

A sample of 50 capped larval bees was taken from each colony at each of the 3 assessment points in the 2021 field season and frozen at -80C. Larvae were homogenized in 25 mL of nuclease-free water at 4 C in a 30 mL homogenization tube (OMNI Intl, SKU #19-6358Z) at 1500 strokes per minute for 2 min on a Spex Geno/Grinder™ 2000 (Cole-Parmer, Metuchen, NJ). Samples were then centrifuged at 1,200 x *g* for 14 min and then at 2,000 x *g* for 1 min to pellet debris. The supernatant was transferred to a 50 mL falcon tube and water was added for a total volume of 45 mL. Samples were pelleted by centrifuging for 25 min at 4,000 x *g.*

DNA was extracted from pelleted samples using a Monarch® Genomic DNA Purification Kit (New England BioLabs, CAT: T3010L) by following the protocol for Gram-positive bacteria. 80 µl of cold PBS was added to pellets with 40 µl of 25 mg/ml lysozyme solution (Millipore-Sigma) and 200 µl of Tissue Lysis Buffer. The solution was incubated and agitated at 1000 rpm at 37 C for 20 min. 20 µl of proteinase K was then added and samples were incubated and agitated at 1000 rpm at 56 C for 1 hour. Debris was pelleted by centrifuging for 2 min at 13,000 x *g*. A final incubation with 3 µl RNase A was done for 5 min at 56 C with agitation of 1000 rpm. The gDNA extraction was then completed following the kit manufacturer's protocol with a final elution volume of 60 µl.

Real time qPCR

Fluorogenic probes were used for the honey bee reference gene and the *M. plutonius* target gene and included double quenching chemistry with ZEN™ and 3' Iowa Black® (Integrated DNA Technologies). The reference gene was labeled with SUN™ and the *M. plutonius* target gene was labeled with FAM (Table 4.2).

Samples were analyzed with a QuantStudio 3 Real-Time PCR system (Applied Biosystems). 25 µl simplex reactions were run with 12.5 µl PrimeTime Gene Expression Master Mix (Integrated DNA Technologies, CAT: 1055772), 0.75 µl of the forward and reverse primers, 0.25 µl of the corresponding probe/quencher, and 10 µl of DNA extract. Primer and probe concentrations were 10 µM. Samples were run in duplicate on 95-well plates with an interplate positive control and a negative control with water in triplicate. Standard curves were included on each plate in duplicate.

Thermocycling conditions were set to 95 C for 3 min, 40 cycles of 95 C for 15 sec followed by 1 min annealing temperature of 55 C for *M. plutonius* 16s rRNA and 60 C for honey bee 18s rRNA.

Table 4.2: Sequences and sources of *M. plutonius* target gene and honey bee endogenous reference gene for real time qPCR.

Target	**Forward (5'-3')**	**Reverse (5'-3')**	**Probe**	**Source**	**Observed efficiency**
18s rRNA – *Apis mellifera*	TGTTTTCCC TGGCCGAAA G	CCCCAATCC CTAGCACGA A	5SUN/ **CCC GGG TAA**/ Int ZEN™/ **CCC GCT GAA CCT C** /3' Iowa Black®FQ/	Ward et al., (2007)	97.6%
16s rRNA – *Melissococcus plutonius*	TGTTGTTAG AGAAGAAT AGGGGAA	CGTGGCTTT CTGGTTAGA	56-FAM/ **AGA GTA ACT** /Int ZEN™/ **GTT TTC CTC GTG ACG GT** /3' Iowa Black®FQ/	Budge et al., (2010)	92.9%

Relative normalized quantification

Mean equivalent Ct values, whereby the original Ct values are projected to 100% target primer efficiency, and interplate standardization for each target gene was calculated using Relative Quantification Analysis software v1.1 (Applied Biosystems Cloud Connect App). Relative normalized quantification was then calculated separately via the $2^{-(\Delta\Delta Ct)}$ method (Livak & Schmittgen, n.d.) using the 18s rRNA gene

from *Apis mellifera* to normalize for DNA extraction efficiency among samples. Relative quantity of *M. plutonius* 16s rRNA was scaled to the sample with the lowest relative quantity (highest ΔCt value between reference gene and target gene).

4.2.3 Data analysis

The final sample sizes for each mixing*queen source group are identical to the data analysis for 2021 colony strength measures in Chapter 2 and are displayed again in Table 4.3.

Table 4.3: Final 2021 season colony sample size for pathogen screen.

Final Analysis Sample Size 2021 Season				
		Mixing		
		Mixed	Control	Total
Queen Source	CA-1	6	7	13
	CA-2	6	7	13
	FL	5	8	13
	GA	6	6	12
	Total	23	28	51

All statistical analyses were conducted in R statistical software v.4.3.1 (R Core Team, 2023). The fixed effects of each mixing treatment (control or brood mixed) and queen type (CA1, CA2, FL, or GA) were evaluated for the measured parameters. The response variable prevalence was analyzed via generalized linear model (GLM) with binomial distribution using R package 'MASS' (Venables & Ripley, 2002). For repeated measures responses, such as relative normalized quantity, a mixed model (GLMM) with assessment included as a fixed effect and colony identification number as a random effect was used from either R package 'lme4' (Bates et al., 2015) or

'glmmTMB' (Brooks et al., 2017) when zero-inflation and overdispersion prevented model convergence in 'lme4'. Model offsets, distributions, and transformations are specified for each response variable and model below. All model assumptions and residuals were confirmed using DHARMa package (Hartig, 2022). The 'car' package (Fox & Weisberg, 2019) was used to conduct model effect ANOVAs and estimated marginal means and *post hoc* comparisons were calculated using the 'emmeans' package (Lenth, 2023).

Viral targets

Baseline, mid-season, and endpoint relative normalized quantity of each viral target were analyzed in GLMMs with colony ID number as a random effect. DWV, BQCV, and SBV had 100% prevalence in all colonies at all timepoints. No model could be run for IAPV, however, because it had low prevalence at all time points: 5.8% in the 1st assessment, 15.6% in the 2nd assessment, and 3.9% in the 3rd assessment.

Table 4.4: Data analysis parameters for 2021 virus quantification.

Response variable	**Fixed effects**	**Random effects**	**R package**	**Distribution**
DWV – Relative normalized quantity (log2)	Mixing*Assessment*Queen	(1|Colony)	lme4	Gaussian
BQCV – Relative normalized quantity (log2)	Mixing*Assessment*Queen	(1|Colony)	lme4	Gaussian
SBV –	Mixing*Assessment*Queen	(1|Colony)	lme4	Gaussian

Relative normalized quantity (log2)				
IAPV – N/A	Prevalence too low for model analysis	-	-	-

Nosema

Prevalence of *Nosema* varied between groups and timepoints but was high enough to run a binomial GLM to compare prevalence between groups over time. Any samples with a Ct value greater than 30 were considered negative for *Nosema*.

In the GLMM of *Nosema* quantification, a transformation of log2(x+1) was applied to the relative normalized quantification for *Nosema* to account for the 0 values of colonies that were negative for *Nosema*. This model had a significant deviation from normal, which was resolved by removing the colony random effect, however, as ANOVA results were identical, it was decided to retain the colony random effect despite the slight deviation.

Table 4.5: Data analysis parameters for 2021 *Nosema* spp. prevalence and quantification.

Response variable	**Fixed effects**	**Random effects**	**R package**	**Distribution**
***Nosema* – Prevalence**	Mixing*Assessment*Queen	-	MASS	Binomial (1=present, 0=absent)
***Nosema* – Relative normalized quantity log2(x+1)**	Mixing*Assessment*Queen	(1|Colony)	glmmTMB	Gaussian

Melissococcus plutonius

The prevalence of *M. plutonius* varied between groups and timepoints but was high enough to run a binomial GLM to compare prevalence between groups over time. Any samples with a Ct value greater than 39 were considered negative for *M. plutonius*. Seven samples had amplification in one of two wells on an initial run and again in a second run, leading these undetermined samples to be excluded in prevalence and quantification models. The excluded samples included 2 samples from the 1st assessment, 2 from the 2nd assessment and 3 samples from the 3rd assessment.

In the GLMM of *M. plutonius* quantification, a transformation of log2(x+1) was applied to the relative normalized quantification for *M. plutonius* to account for the 0 values of colonies that were negative for *M. plutonius*.

Table 4.6: Data analysis parameters for 2021 *M. plutonius* prevalence and quantification.

Response variable	**Fixed effects**	**Random effects**	**R package**	**Distribution**
***M. plutonius* – Prevalence**	Mixing*Assessment*Queen	-	MASS	Binomial (1=present, 0=absent)
***M. plutonius* – Relative normalized quantity log2(x+1)**	Mixing*Assessment*Queen	(1|Colony)	lme4	Gaussian

4.3 Results

Deformed wing virus

Assessment had a significant effect on DWV levels, χ^2 (2) = 7.18, p<0.05, as shown in Figure 4.1. There was a significant difference between baseline and endpoint DWV levels, with a predicted fold increase of 2.6 in log2 scale, or 6x increase in DWV from the beginning of the season to the end when averaged over mixing and queen. There were no other significant effects and ANOVA results are summarized in Table 4.7. Predicted means of the three-way interaction are presented in Figure 4.2. DWV had 100% prevalence in all colonies and assessments.

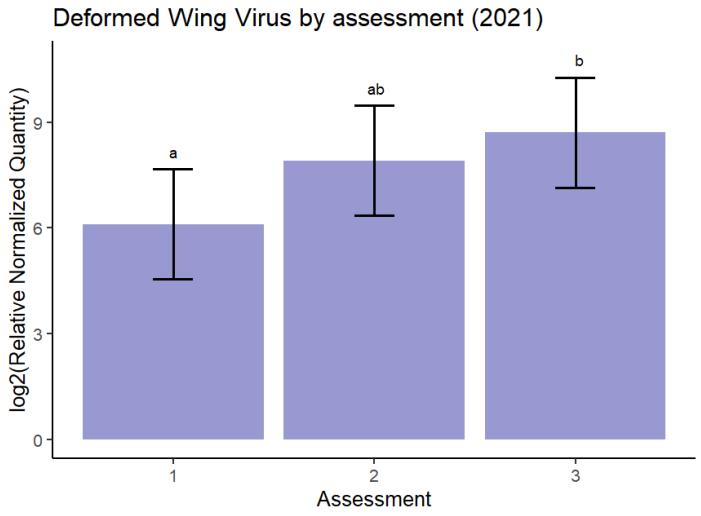


Figure 4.1: Effect of assessment time averaged over mixing and queen on 2021 DWV levels. Predicted means and 95% CIs for each assessment are presented. Wald III tests were performed on the log2 scale and found a significant main effect of assessment, χ^2 (2) = 7.18, p<0.05.

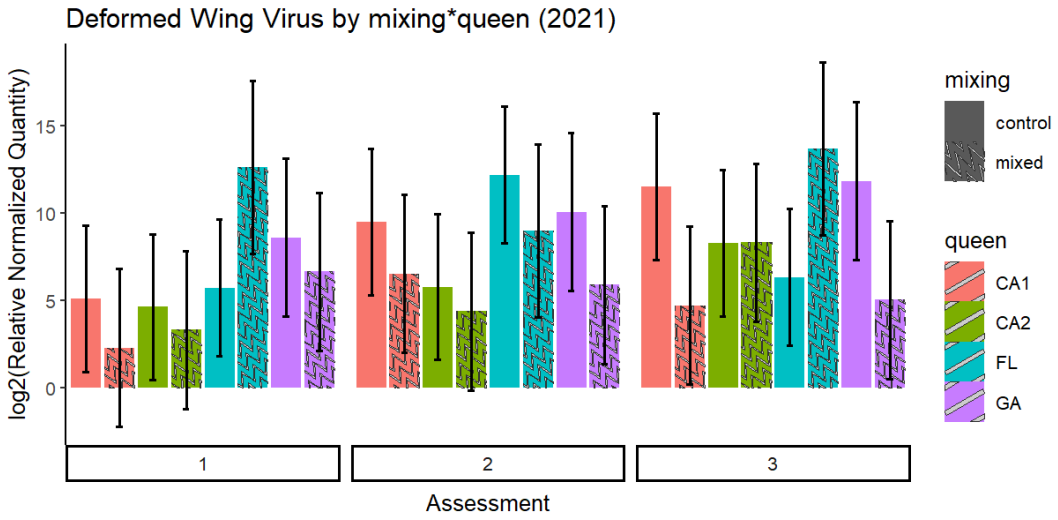


Figure 4.2: DWV levels over 3 assessment periods grouped by mixing and queen source. Each bar represents marginal means with 95% CI. There were no significant effects other than the main effect of assessment (Table 4.7).

Table 4.7: 2021 DWV ANOVAs (Type III) of the main effects of mixing (M), time (T), and queen source (QS) and their interactions on relative normalized quantities of DWV. Differences accepted at $\alpha \leq 0.05$.

Deformed Wing Virus	**Chisq**	**DF**	**Pr(>Chisq)**	*****
Mixing (M)	0.8142	1	0.36689	
Time (T)	7.181	2	0.02758	*
Queen Source (QS)	1.9144	3	0.59037	
M*T	1.5661	2	0.457	
M*QS	6.0578	3	0.10883	
T*QS	9.9653	6	0.12612	
M*T*QS	10.9943	6	0.08855	.

Black Queen Cell Virus

Assessment had a significant effect on BQCV levels, $\chi^2(2) = 38.9$, $p<0.001$, as shown in Figure 4.3. There was a significant difference between baseline, midseason, and endpoint BQCV levels. In contrast with DWV, there was a seasonal decrease in

BQCV, with a predicted fold decrease of -10.75 or 1722x less BQCV from the beginning of the season to the end when averaged over mixing and queen. There was a significant main effect of queen, χ^2 (3) = 8.7, p<0.05, indicating that GA queens had a higher overall level of BQCV compared to FL queens.

There were no other significant effects and ANOVA results are summarized in Table 4.8. Predicted means of the three-way interaction are presented in Figure 4.4. BQCV had 100% prevalence in all colonies and assessments.

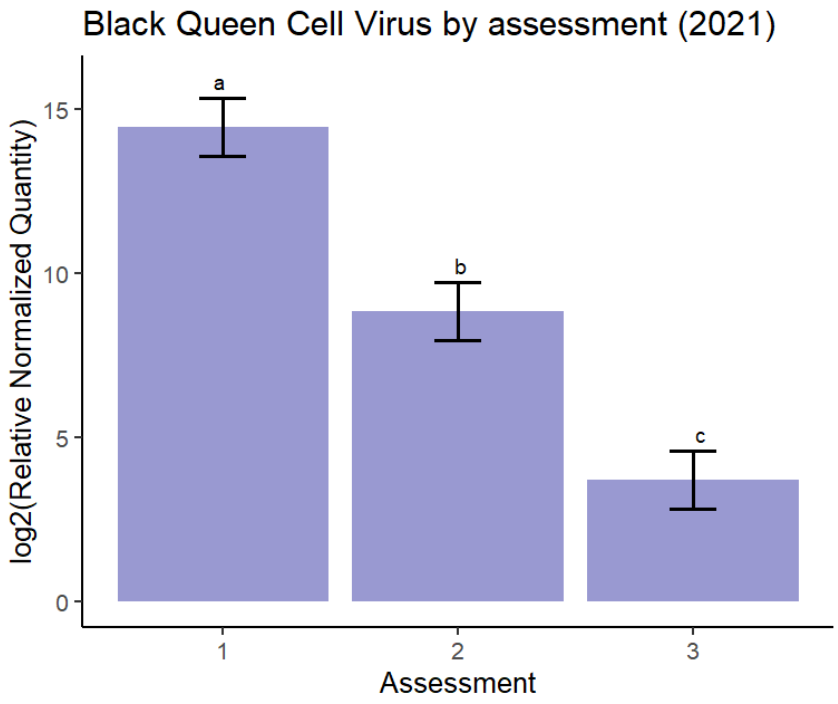


Figure 4.3: Effect of assessment time averaged over mixing and queen on 2021 BQCV levels. Predicted means and 95% CIs for each assessment are presented. Wald III tests were performed on the log2 scale and found a significant main effect of assessment, χ^2 (2) = 38.9, p<0.001.

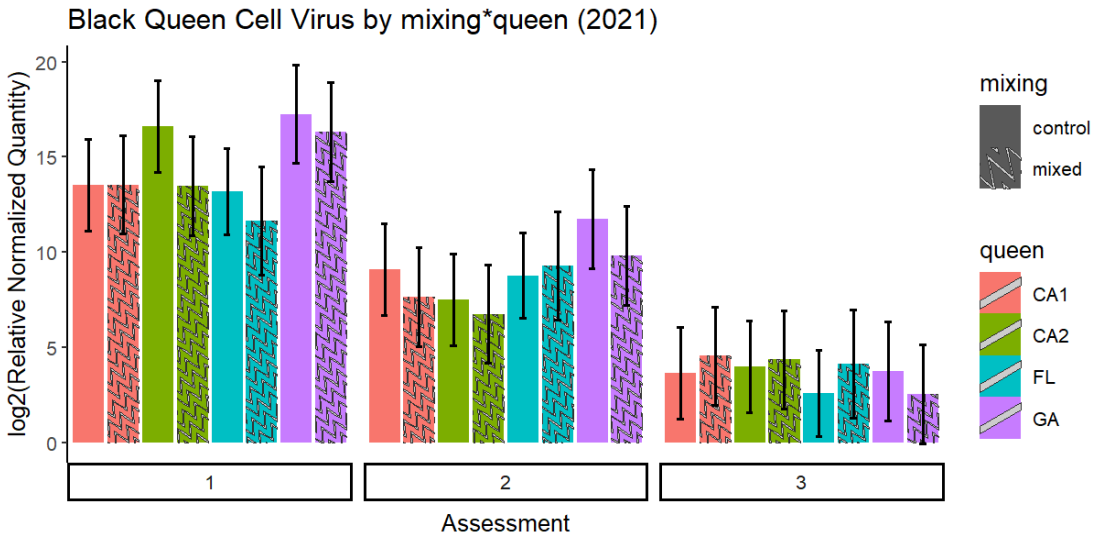


Figure 4.4: BQCV levels over 3 assessment periods grouped by mixing and queen source. Each bar represents marginal means with 95% CI. There were no significant effects other than the main effects of assessment and queen (Table 4.8). GA queens had higher overall BQCV levels compared to FL queens, χ^2 (3) = 8.7, p<0.05.

Table 4.8: 2021 BQCV ANOVAs (Type III) of the main effects of mixing (M), time (T), and queen source (QS) and their interactions on relative normalized quantities of BQCV. Differences accepted at $\alpha \leq 0.05$.

Black Queen Cell Virus	**Chisq**	**DF**	**Pr(>Chisq)**	*****
Mixing (M)	0.0001	1	0.99216	
Time (T)	38.9184	2	3.54E-09	***
Queen Source (QS)	8.7552	3	0.03273	*
M*T	1.0054	2	0.60489	
M*QS	1.6461	3	0.64898	
T*QS	8.7808	6	0.18628	
M*T*QS	2.7828	6	0.83557	

Sacbrood Virus

Assessment had a significant effect on SBV levels, χ^2 (2) = 24.3, p<0.001, as shown in Figure 4.5. There was a significant difference between baseline, midseason, and endpoint SBV levels. Similar to BQCV, there was a seasonal decrease in SBV, with a predicted fold decrease of -3.42 or 10.7x less SBV from the beginning of the season to the end when averaged over mixing and queen.

There were no other significant effects and ANOVA results are summarized in Table 4.9. Predicted SBV levels of the three-way interaction are presented in Figure 4.6. SBV had 100% prevalence in all colonies and assessments.

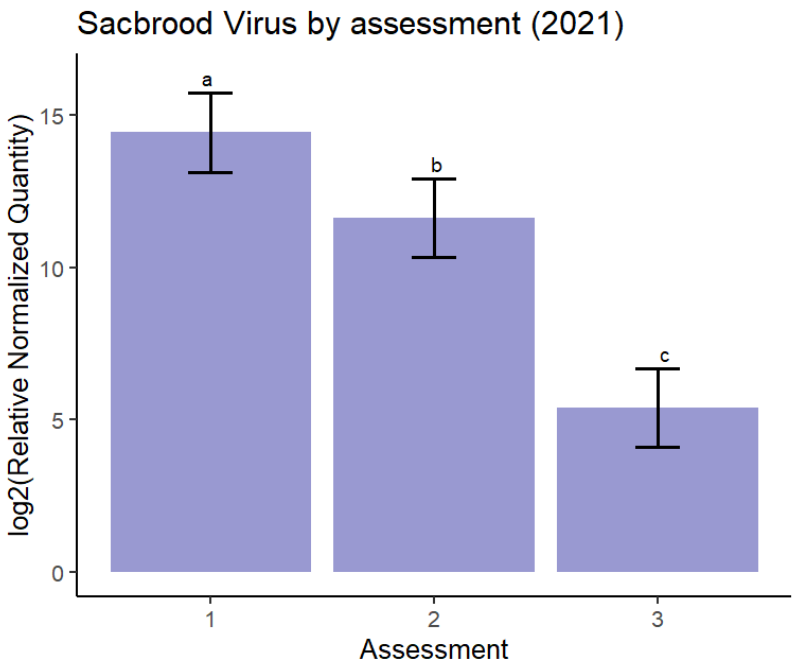


Figure 4.5: Effect of assessment time averaged over mixing and queen on 2021 SBV levels. Predicted means and 95% CIs for each assessment are presented. Wald III tests were performed on the log2 scale and found a significant main effect of assessment, χ^2 (2) = 24.3, p<0.001.

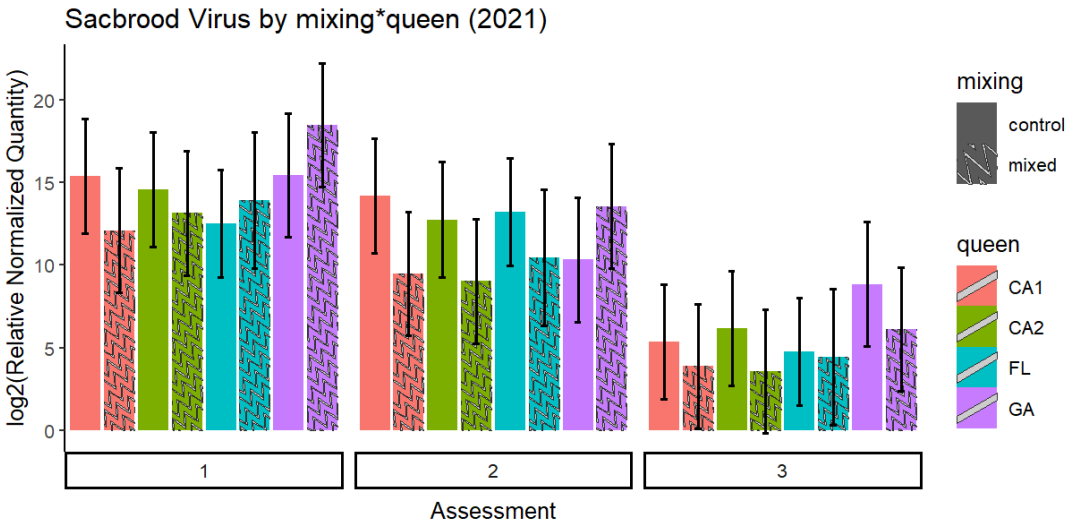


Figure 4.6: SBV levels over 3 assessment periods grouped by mixing and queen source. Each bar represents marginal means with 95% CI. There were no significant effects other than the main effect of assessment (Table 4.9).

Table 4.9: 2021 SBV ANOVAs (Type III) of the main effects of mixing (M), time (T), and queen source (QS) and their interactions on relative normalized quantities of SBV. Differences accepted at $\alpha \leq 0.05$.

Sacbrood Virus	**Chisq**	**DF**	**Pr(>Chisq)**	*
Mixing (M)	1.5997	1	0.206	
Time (T)	24.3834	2	5.07E-06	***
Queen Source (QS)	1.9501	3	0.5828	
M*T	0.9877	2	0.6103	
M*QS	3.492	3	0.3218	
T*QS	7.3704	6	0.2879	
M*T*QS	5.4875	6	0.483	

Israeli Acute Paralysis Virus

IAPV was uncommon at all assessment periods with a slight increase in prevalence during assessment 2 (Fig. 4.7). There were never more than 2 colonies in each mixing*assessment*queen grouping that tested positive for IAPV. Only a single

colony tested positive for IAPV more than once: colony #9 was a CA-2 control colony that tested positive at assessments 2 and 3. The only groups to never test positive for IAPV were control group CA-2 mixed and both FL groups.

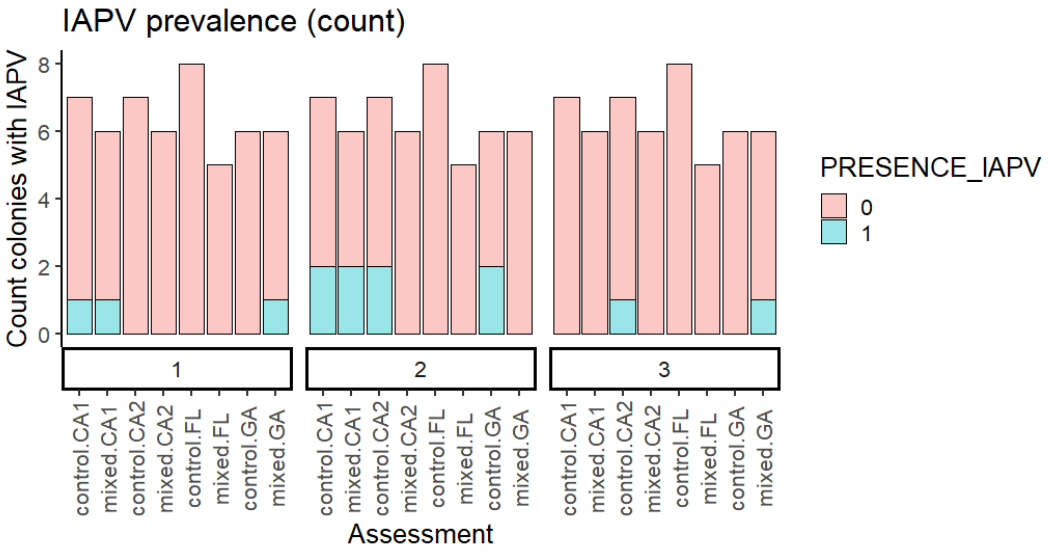


Figure 4.7: Prevalence by colony count of IAPV.

Nosema

There were no significant effects in the GLM for *Nosema* prevalence (Table 4.10). Three-way interactions of prevalence are displayed in Figure 4.8. Relative normalized quantity of *Nosema* also did not have significant effects (Table 4.11) and the three-way interactions of *Nosema* levels are displayed in Figure 4.9.

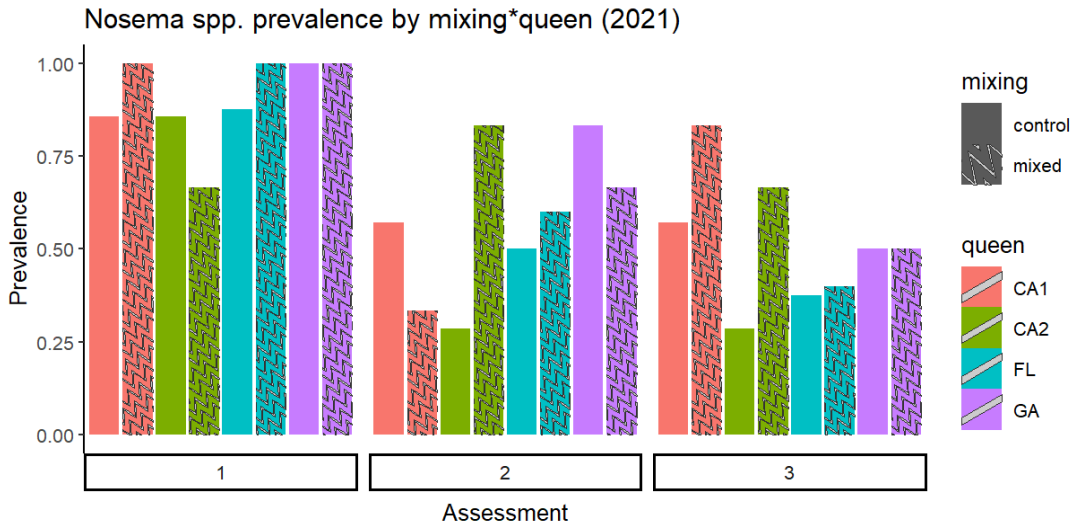


Figure 4.8: Prevalence of *Nosema* by proportion. There were no significant effects in the binomial GLM (Table 4.10).

Table 4.10: 2021 *Nosema* prevalence ANOVAs (Type III) of the main effects of mixing (M), time (T), and queen source (QS) and their interactions on prevalence of *Nosema*. Differences accepted at $\alpha \leq 0.05$.

Nosema - Prevalence	**Chisq**	**DF**	**Pr(>Chisq)**	*****
Mixing (M)	1.3093	1	0.2525	
Time (T)	1.8705	2	0.3925	
Queen Source (QS)	1.5564	3	0.6693	
M*T	2.967	2	0.2268	
M*QS	2.9449	3	0.4002	
T*QS	2.1742	6	0.903	
M*T*QS	7.3542	6	0.2893	

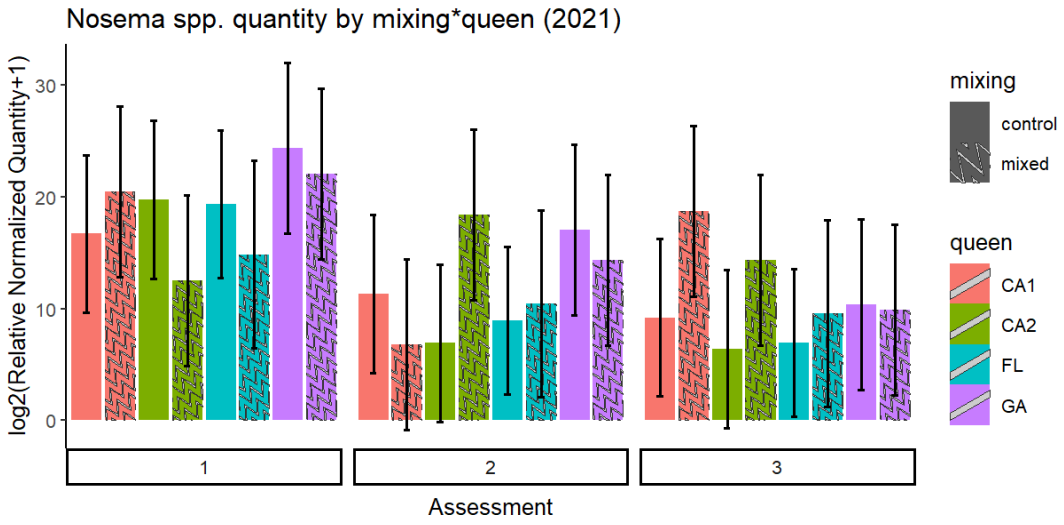


Figure 4.9: *Nosema* levels over 3 assessment periods grouped by mixing and queen source. Each bar represents marginal means with 95% CI. There were no significant effects (Table 4.11).

Table 4.11: 2021 *Nosema* ANOVAs (Type III) of the main effects of mixing (M), time (T), and queen source (QS) and their interactions on relative normalized quantities of *Nosema*. Differences accepted at $\alpha \leq 0.05$.

Nosema – Relative normalized quantity	**Chisq**	**DF**	**Pr(>Chisq)**	*****
Mixing (M)	0.5078	1	0.4761	
Time (T)	2.3325	2	0.3115	
Queen Source (QS)	2.1827	3	0.5354	
M*T	3.6273	2	0.1631	
M*QS	2.3612	3	0.5009	
T*QS	2.0224	6	0.9176	
M*T*QS	7.8185	6	0.2517	

Melissococcus plutonius

There were no significant effects in the GLM for *M. plutonius* prevalence (Table 4.12). Three-way interactions of prevalence are displayed in Figure 4.10.

Relative normalized quantity of *M. plutonius* had a significant effect of assessment (Fig. 4.11), χ^2 (2) = 7.6, p<0.05. There was a -3.69 fold change (12.9x decrease) in *M. plutonius* levels between the 1st and 3rd assessments. There were no other significant effects and ANOVA results are summarized in Table 4.13. Predicted means of the three-way interaction are presented in Figure 4.12.

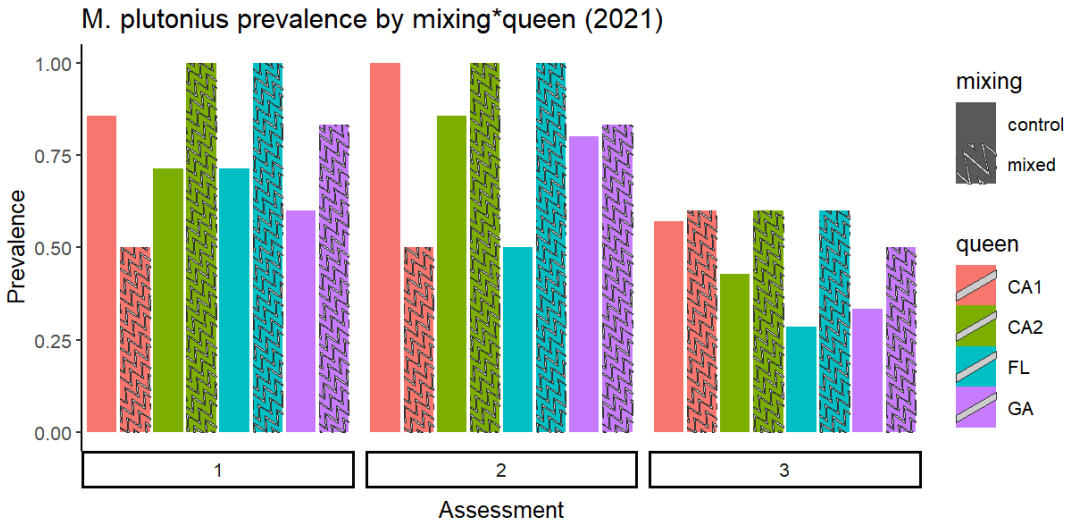


Figure 4.10: Prevalence of *M. plutonius* by proportion. There were no significant effects in the binomial GLM (Table 4.12).

Table 4.12: 2021 *M. plutonius* prevalence ANOVAs (Type III) of the main effects of mixing (M), time (T), and queen source (QS) and their interactions on prevalence of *M. plutonius*. Differences accepted at α≤0.05.

MP – Prevalence	**Chisq**	**DF**	**Pr(>Chisq)**	*****
Mixing (M)	1.9889	1	0.15846	
Time (T)	4.7138	2	0.09472	.
Queen Source (QS)	1.0663	3	0.78521	
M*T	3.7465	2	0.15362	
M*QS	7.21	3	0.0655	.
T*QS	3.0774	6	0.79907	

M*T*QS	7.175	6	0.30497	

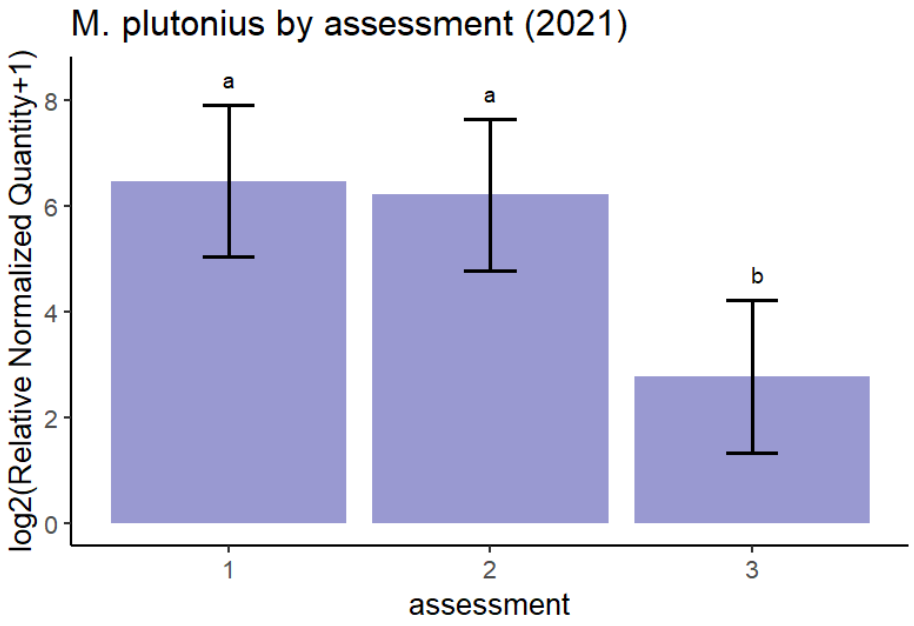


Figure 4.11: Effect of assessment time averaged over mixing and queen on 2021 *M. plutonius* levels. Predicted means and 95% CIs for each assessment are presented. Wald III tests were performed on the log2(x+1) scale and found a significant main effect of assessment, χ^2 (2) = 7.6, p<0.05.

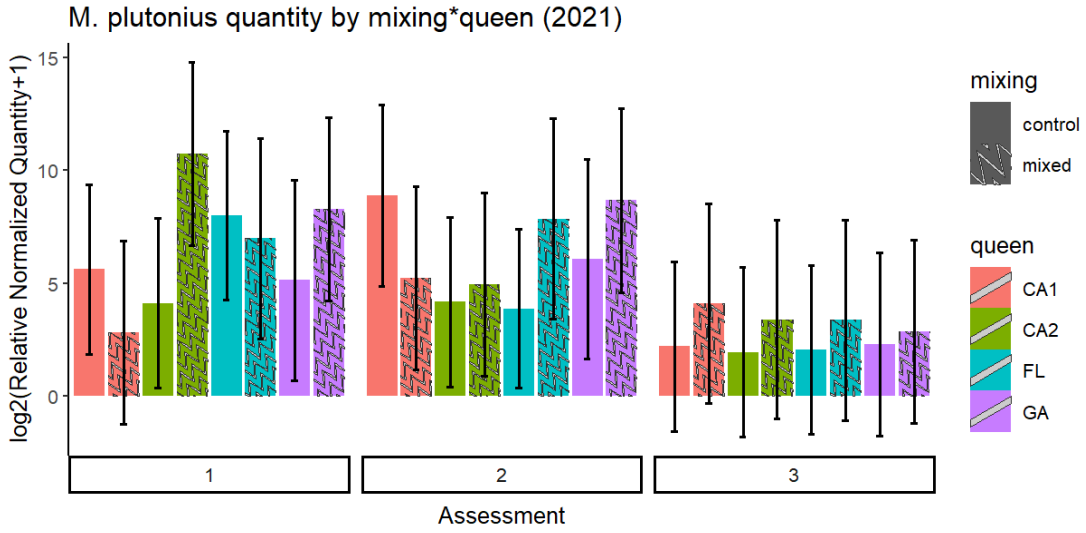


Figure 4.12: *M. plutonius* levels over 3 assessment periods grouped by mixing and queen source. Each bar represents marginal means with 95% CI. There were no significant effects other than the main effect of assessment (Table 4.13).

Table 4.13: 2021 *M. plutonius* ANOVAs (Type III) of the main effects of mixing (M), time (T), and queen source (QS)and their interactions on relative normalized quantities of *M. plutonius*. Differences accepted at $\alpha \leq 0.05$.

MP – Relative normalized quantity	**Chisq**	**DF**	**Pr(>Chisq)**	*****
Mixing (M)	0.9931	1	0.318986	
Time (T)	7.6352	2	0.02198	*
Queen Source (QS)	2.2487	3	0.522417	
M*T	2.7835	2	0.248635	
M*QS	6.7563	3	0.080084	.
T*QS	6.2079	6	0.400307	
M*T*QS	8.7227	6	0.189782	

4.4 Discussion

Brood mixing did not significantly affect prevalence or quantity of 6 honey bee pathogens. This observation is supported by studies that show drifting of adult bees and robbing behavior by strong colonies to be a known source of movement of adult bees between colonies, especially in high density apiaries without visual and spatial differentiation between colonies (Dynes et al., 2019; Peck & Seeley, 2019). Viruses can spread between colonies through various means, such as exposure during foraging at high activity floral sites (Grozinger & Flenniken, 2019) and brood mixing does not seem to remediate or exacerbate these typical trends in pathogen dynamics.

The overall seasonal trends in virus levels observed in 2021 are supported by literature. DWV quantity in colonies increased as the season progressed in tandem with mite levels. This is a well-documented phenomenon correlating mite loads with increased titers of DWV (Grozinger & Flenniken, 2019). In contrast to DWV, levels of BQCV, SBV, and *M. plutonius* decreased over the 13 weeks between June and August. D'Alvise et al. (2019) found a similar decrease in BQCV titers between June and September and suggested that the increased life span of winter bees may contribute to higher pathogen levels in the spring while shortened bee lifespan and high population turnover during the summer may contribute to declining levels for pathogens not directly associated with mite level increases. We found no seasonal variation in *Nosema* levels, which mirrors results from D'Alvise et al. (2019) in Germany and Hinshaw et al., (2021) in Pennsylvania, USA.

IAPV was scarce in the 153 pathogen samples, with a slight uptick in the number of colonies testing positive, 8 out of 51 colonies, during the 2nd assessment in July. Six of the 8 positive colonies were control colonies.

Melissococcus plutonius was found at all assessment points, with slightly decreasing prevalence and relative quantity at the end of the season. I was surprised by the number of colonies testing positive for the causative agent of European Foulbrood disease, as no colony displayed the symptomatic brown and twisted larvae at any assessment point. This high prevalence of asymptomatic *M. plutonius* infections is similar to findings by Budge et al. (2010) in the United Kingdom and to D'Alvise et al. (2019) in Germany.

Most pathogens are ubiquitous, especially at the transition period from spring to summer and neither brood mixing nor queen source significantly affected prevalence or relative quantity of any of the 6 pathogens tested. This is not a suggestion to disregard the potential spread of more serious diseases like American Foulbrood, which would be a greater concern if brood mixing were to occur between apiaries, instead of within closely monitored apiaries that are further subdivided by mixing groups of 2 to 4 colonies. Maintaining closed mixing groups and practicing careful monitoring would reduce the likelihood of spreading the causative agent of American Foulbrood, the spore-forming bacteria *Paenibacillus larvae*.

This study suggests that the regular beekeeping practice of moving frames of brood between colonies for equalization in a closed apiary setting is not a major source of pathogen transmission.

REFERENCES

Aizen, M. A., & Harder, L. D. (2009). The Global Stock of Domesticated Honey Bees Is Growing Slower Than Agricultural Demand for Pollination. *Current Biology*, *19*(11), 915–918. https://doi.org/10.1016/j.cub.2009.03.071

Alburaki, M., Chen, D., Skinner, J., Meikle, W., Tarpy, D., Adamczyk, J., & Stewart, S. (2018). Honey Bee Survival and Pathogen Prevalence: From the Perspective of Landscape and Exposure to Pesticides. *Insects*, *9*(2), 65. https://doi.org/10.3390/insects9020065

Anderson, D. L., & Trueman, J. W. H. (2000). Varroa jacobsoni (Acari: Varroidae) is more than one species. *Experimental & Applied Acarology*, *24*(3), 165–189. https://doi.org/10.1023/A:1006456720416

Arathi, H. S., & Spivak, M. (2001). Influence of colony genotypic composition on the performance of hygienic behaviour in the honeybee, Apis mellifera L. *Animal Behaviour*, *62*(1), 57–66. https://doi.org/10.1006/anbe.2000.1731

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software*, *67*, 1–48. https://doi.org/10.18637/jss.v067.i01

Beaurepaire, A. L., Krieger, K. J., & Moritz, R. F. A. (2017). Seasonal cycle of inbreeding and recombination of the parasitic mite Varroa destructor in

honeybee colonies and its implications for the selection of acaricide resistance. *Infection, Genetics and Evolution*, *50*, 49–54. https://doi.org/10.1016/j.meegid.2017.02.011

Biganski, S., Kurze, C., Müller, M. Y., & Moritz, R. F. A. (2018). Social response of healthy honeybees towards Nosema ceranae-infected workers: Care or kill? *Apidologie*, *49*(3), 325–334. https://doi.org/10.1007/s13592-017-0557-8

Blanchard, P., Guillot, S., Antùnez, K., Köglberger, H., Kryger, P., de Miranda, J. R., Franco, S., Chauzat, M.-P., Thiéry, R., & Ribière, M. (2014). Development and validation of a real-time two-step RT-qPCR TaqMan(®) assay for quantitation of Sacbrood virus (SBV) and its application to a field survey of symptomatic honey bee colonies. *Journal of Virological Methods*, *197*, 7–13. https://doi.org/10.1016/j.jviromet.2013.09.012

Borba, R. S., Klyczek, K. K., Mogen, K. L., & Spivak, M. (2015). Seasonal benefits of a natural propolis envelope to honey bee immunity and colony health. *Journal of Experimental Biology*, *218*(22), 3689–3699. https://doi.org/10.1242/jeb.127324

Brooks, M., E., Kristensen, K., Benthem, K., J. ,van, Magnusson, A., Berg, C., W., Nielsen, A., Skaug, H., J., Mächler, M., & Bolker, B., M. (2017). glmmTMB Balances Speed and Flexibility Among Packages for Zero-inflated Generalized Linear Mixed Modeling. *The R Journal*, *9*(2), 378. https://doi.org/10.32614/RJ-2017-066

Brosi, B. J., Delaplane, K. S., Boots, M., & de Roode, J. C. (2017). Ecological and evolutionary approaches to managing honeybee disease. *Nature Ecology & Evolution*, *1*(9), 1250–1262. https://doi.org/10.1038/s41559-017-0246-z

Bruckner, S., Steinhauer, N., Aurell, S. D., Caron, D., Ellis, J., Fauvel, A. M., Kulhanek, K., McArt, S. H., Mullen, E., Rangel, J., Sagili, R., Tsuruda, J., Willkes, J. T., Wilson, M. E., Wyns, D., Rennich, K., vanEngelsdorp, D., & Williams, G. (2019). 2018-2019 Honey Bee Colony Losses in the United States: Preliminary Results. *Bee Informed Partnership*. https://beeinformed.org/wp-content/uploads/2020/06/BIP_2019_2020_Losses_Abstract.pdf

Budge, G. E., Barrett, B., Jones, B., Pietravalle, S., Marris, G., Chantawannakul, P., Thwaites, R., Hall, J., Cuthbertson, A. G. S., & Brown, M. A. (2010). The occurrence of Melissococcus plutonius in healthy colonies of Apis mellifera and the efficacy of European foulbrood control measures. *Journal of Invertebrate Pathology*, *105*(2), 164–170. https://doi.org/10.1016/j.jip.2010.06.004

Burgett, M., & Burikam, I. (1985). Number of Adult Honey Bees (Hymenoptera: Apidae) Occupying a Comb: A Standard for Estimating Colony Populations. *Journal of Economic Entomology*, *78*(5), 1154–1156. https://doi.org/10.1093/jee/78.5.1154

Calderone, N. W. (2012). Insect Pollinated Crops, Insect Pollinators and US Agriculture: Trend Analysis of Aggregate Data for the Period 1992–2009. *PLoS ONE*, *7*(5), e37235. https://doi.org/10.1371/journal.pone.0037235

Calderone, N. W., & Page, R. E. (1988). Genotypic variability in age polyethism and task specialization in the honey bee, Apis mellifera (Hymenoptera: Apidae). *Behavioral Ecology and Sociobiology*, *22*(1), 17–25. https://doi.org/10.1007/BF00395694

Calderone, N. W., & Page, R. E. (1992). Effects of interactions among genotypically diverse nestmates on task specialization by foraging honey bees (Apis mellifera). *Behavioral Ecology and Sociobiology*, *30*(3), 219–226. https://doi.org/10.1007/BF00166706

Choi, N. R., Jung, C., & Lee, D.-W. (2015). Optimization of detection of black queen cell virus from Bombus terrestris via real-time PCR. *Journal of Asia-Pacific Entomology*, *18*(1), 9–12. https://doi.org/10.1016/j.aspen.2014.10.010

Cobey, S., Sheppard, W., & Tarpy, D. (2011). Status of breeding practices and genetic diversity in domestic U.S. honey bees. *Honey Bee Colony Health: Challenges and Sustainable Solutions*, 39–49.

Cobey, S. W. (2007). Comparison studies of instrumentally inseminated and naturally mated honey bee queens and factors affecting their performance. *Apidologie*, *38*(4), 390–410. https://doi.org/10.1051/apido:2007029

Cremer, S., Armitage, S. A. O., & Schmid-Hempel, P. (2007). Social Immunity. *Current Biology*, *17*(16), R693–R702. https://doi.org/10.1016/j.cub.2007.06.008

Crozier, R. H., & Fjerdingstad, E. J. (2001). Polyandry in social Hymenoptera—Disunity in diversity? *Annales Zoologici Fennici*, *38*(3/4), 267–285.

D'Alvise, P., Seeburger, V., Gihring, K., Kieboom, M., & Hasselmann, M. (2019). Seasonal dynamics and co-occurrence patterns of honey bee pathogens revealed by high-throughput RT-qPCR analysis. *Ecology and Evolution*, *9*(18), 10241–10252. https://doi.org/10.1002/ece3.5544

Danka, R. G., De Guzman, L. I., Rinderer, T. E., Sylvester, H. A., Wagener, C. M., Bourgeois, A. L., Harris, J. W., & Villa, J. D. (2012). Functionality of Varroa-Resistant Honey Bees (Hymenoptera: Apidae) When Used in Migratory Beekeeping for Crop Pollination. *Journal of Economic Entomology*, *105*(2), 313–321. https://doi.org/10.1603/EC11286

Danka, R. G., Rinderer, T. E., Spivak, M., & Kefuss, J. (2013). Comments on: " *Varroa destructor* , research avenues towards sustainable control." *Journal of Apicultural Research*, *52*(2), 69–71. https://doi.org/10.3896/IBRA.1.52.2.14

Delaney, D. A., Meixner, M. D., Schiff, N. M., & Sheppard, W. S. (2009). Genetic Characterization of Commercial Honey Bee (Hymenoptera: Apidae) Populations in the United States by Using Mitochondrial and Microsatellite Markers. *Annals of the Entomological Society of America*, *102*(4), 666–673. https://doi.org/10.1603/008.102.0411

Delaplane, K. S., Berry, J. A., Skinner, J. A., Parkman, J. P., & Hood, W. M. (2005). Integrated pest management against Varroa destructor reduces colony mite levels and delays treatment threshold. *Journal of Apicultural Research*, *44*(4), 157–162. https://doi.org/10.1080/00218839.2005.11101171

Delaplane, K. S., Given, J. K., Menz, J., & Delaney, D. A. (2021). Colony fitness increases in the honey bee at queen mating frequencies higher than genetic diversity asymptote. *Behavioral Ecology and Sociobiology*, *75*(9), 126. https://doi.org/10.1007/s00265-021-03065-6

Delaplane, K. S., Pietravalle, S., Brown, M. A., & Budge, G. E. (2015). Honey Bee Colonies Headed by Hyperpolyandrous Queens Have Improved Brood Rearing Efficiency and Lower Infestation Rates of Parasitic Varroa Mites. *PLOS ONE*, *10*(12), e0142985. https://doi.org/10.1371/journal.pone.0142985

Delaplane, K. S., van der Steen, J., & Guzman-Novoa, E. (2013). Standard methods for estimating strength parameters of Apis mellifera colonies. *Journal of Apicultural Research*, *52*(1). https://doi.org/10.3896/IBRA/1.52.1.03

Dietemann, V., Nazzi, F., Martin, S. J., Anderson, D. L., Locke, B., Delaplane, K. S., Wauquiez, Q., Tannahill, C., Frey, E., Ziegelmann, B., Rosenkranz, P., & Ellis, J. D. (2013). Standard methods for varroa research. *Journal of Apicultural Research*, *52*(1), 1–54. https://doi.org/10.3896/IBRA.1.52.1.09

Dietemann, V., Pflugfelder, J., Anderson, D., Charrière, J.-D., Chejanovsky, N., Dainat, B., de Miranda, J., Delaplane, K., Dillier, F.-X., Fuch, S., Gallmann, P., Gauthier, L., Imdorf, A., Koeniger, N., Kralj, J., Meikle, W., Pettis, J.,

Rosenkranz, P., Sammataro, D., … Neumann, P. (2012). Varroa destructor: Research avenues towards sustainable control. *Journal of Apicultural Research*, *51*(1), 125–132. https://doi.org/10.3896/IBRA.1.51.1.15

Dwyer, G., Elkinton, J. S., & Buonaccorsi, J. P. (1997). Host Heterogeneity in Susceptibility and Disease Dynamics: Tests of a Mathematical Model. *The American Naturalist*, *150*(6), 685–707. https://doi.org/10.1086/286089

Dynes, T. L., Berry, J. A., Delaplane, K. S., Brosi, B. J., & Roode, J. C. de. (2019). Reduced density and visually complex apiaries reduce parasite load and promote honey production and overwintering survival in honey bees. *PLOS ONE*, *14*(5), e0216286. https://doi.org/10.1371/journal.pone.0216286

Elzen, P. J., Baxter, J. R., Spivak, M., & Wilson, W. T. (2000). Control of Varroa jacobsoni Oud. Resistant to fluvalinate and amitraz using coumaphos. *Apidologie*, *31*(3), 437–441. https://doi.org/10.1051/apido:2000134

Estoup, A., Solignac, M., & Cornuet, J. (1994). Precise assessment of the number of patrilines and of genetic relatedness in honeybee colonies. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *258*(1351), 1–7. https://doi.org/10.1098/rspb.1994.0133

Evans, J. D., & Spivak, M. (2010). Socialized medicine: Individual and communal disease barriers in honey bees. *Journal of Invertebrate Pathology*, *103*, S62–S72. https://doi.org/10.1016/j.jip.2009.06.019

Fox, J., & Weisberg, S. (2019). *An R Companion to Applied Regression* (3rd Edition.). Sage. https://socialsciences.mcmaster.ca/jfox/Books/Companion/

Frey, E., & Rosenkranz, P. (2014). Autumn Invasion Rates of Varroa destructor (Mesostigmata: Varroidae) Into Honey Bee (Hymenoptera: Apidae) Colonies and the Resulting Increase in Mite Populations. *Journal of Economic Entomology*, *107*(2), 508–515. https://doi.org/10.1603/EC13381

Fuchs, S., & Moritz, R. F. A. (1998). Evolution of extreme polyandry in the honeybee Apis mellifera L. *Behavioral Ecology and Sociobiology*, *45*(3), 269–275. https://doi.org/10.1007/s002650050561

Garibaldi, L. A., Steffan-Dewenter, I., Winfree, R., Aizen, M. A., Bommarco, R., Cunningham, S. A., Kremen, C., Carvalheiro, L. G., Harder, L. D., Afik, O., Bartomeus, I., Benjamin, F., Boreux, V., Cariveau, D., Chacoff, N. P., Dudenhöffer, J. H., Freitas, B. M., Ghazoul, J., Greenleaf, S., … Klein, A. M. (2013). Wild pollinators enhance fruit set of crops regardless of honey bee abundance. *Science (New York, N.Y.)*, *339*(6127), 1608–1611. https://doi.org/10.1126/science.1230200

Genersch, E. (2010). Honey bee pathology: Current threats to honey bees and beekeeping. *Applied Microbiology and Biotechnology*, *87*(1), 87–97. https://doi.org/10.1007/s00253-010-2573-8

Grozinger, C. M., & Flenniken, M. L. (2019). Bee Viruses: Ecology, Pathogenicity, and Impacts. *Annual Review of Entomology*, *64*(1), 205–226. https://doi.org/10.1146/annurev-ento-011118-111942

Guzmán-Novoa, E., Eccles, L., Calvete, Y., Mcgowan, J., Kelly, P. G., & Correa-Benítez, A. (2010). Varroa destructor is the main culprit for the death and

reduced populations of overwintered honey bee (Apis mellifera) colonies in Ontario, Canada. *Apidologie*, *41*(4), 443–450. https://doi.org/10.1051/apido/2009076

Haber, A. I., Steinhauer, N. A., & vanEngelsdorp, D. (2019). Use of Chemical and Nonchemical Methods for the Control of Varroa destructor (Acari: Varroidae) and Associated Winter Colony Losses in U.S. Beekeeping Operations. *Journal of Economic Entomology*, *112*(4), 1509–1525. https://doi.org/10.1093/jee/toz088

Harbo, J. R. (2020). *Measuring Varroa Sensitive Hygiene*. Harbo Bee Company. https://static1.1.sqspcdn.com/static/f/1461081/28382514/1607875045023/measuring+vsh+rev+Dec2020.pdf?token=fDPRXvrucuVZKfIhC0KTtRP%2BQdQ%3D

Harbo, J. R., & Hoopingarner, R. A. (1997). Honey Bees (Hyntenoptera: Apidae) in the United States That Express Resistance to Varroa jacobsoni (Mesostigmata: Varroidae). *Journal of Economic Entomology*, *90*(4), 893–898. https://doi.org/10.1093/jee/90.4.893

Hartig, F. (2022). *DHARMa: Residual Diagnostics for Hierarchical (multi-Level / Mixed) Regression Models.* (R package version 0.4.6) [Computer software]. https://CRAN.R-project.org/package=DHARMa

Hinshaw, C., Evans, K. C., Rosa, C., & López-Uribe, M. M. (2021). The Role of Pathogen Dynamics and Immune Gene Expression in the Survival of Feral

Honey Bees. *Frontiers in Ecology and Evolution*, *8*. https://www.frontiersin.org/articles/10.3389/fevo.2020.594263

Hung, K.-L. J., Kingston, J. M., Albrecht, M., Holway, D. A., & Kohn, J. R. (2018). The worldwide importance of honey bees as pollinators in natural habitats. *Proceedings of the Royal Society B: Biological Sciences*, *285*(1870), 20172140. https://doi.org/10.1098/rspb.2017.2140

Ihle, K. E., de Guzman, L. I., & Danka, R. G. (2021). Social Apoptosis in Varroa Mite Resistant Western Honey Bees (Apis mellifera). *Journal of Insect Science*, *22*(1), 13. https://doi.org/10.1093/jisesa/ieab087

Jack, C. J., & Ellis, J. D. (2021). Integrated Pest Management Control of Varroa destructor (Acari: Varroidae), the Most Damaging Pest of (Apis mellifera L. (Hymenoptera: Apidae)) Colonies. *Journal of Insect Science*, *21*(5), 6. https://doi.org/10.1093/jisesa/ieab058

Jaffé, R., Dietemann, V., Allsopp, M. H., Costa, C., Crewe, R. M., Dall'Olio, R., De La Rúa, P., El-Niweiri, M. A. A., Fries, I., Kezic, N., Meusel, M. S., Paxton, R. J., Shaibi, T., Stolle, E., & Moritz, R. F. A. (2010). Estimating the Density of Honeybee Colonies across Their Natural Range to Fill the Gap in Pollinator Decline Censuses. *Conservation Biology*, *24*(2), 583–593. https://doi.org/10.1111/j.1523-1739.2009.01331.x

Jensen, A. B., Aronstein, K., Flores, J. M., Vojvodic, S., Palacio, M. A., & Spivak, M. (2013). Standard methods for fungal brood disease research. *Journal of*

Apicultural Research, *52*(1), 10.3896/IBRA.1.52.1.13. https://doi.org/10.3896/IBRA.1.52.1.13

Johnson, R. M., Dahlgren, L., Siegfried, B. D., & Ellis, M. D. (2013). Acaricide, Fungicide and Drug Interactions in Honey Bees (Apis mellifera). *PLOS ONE*, *8*(1), e54092. https://doi.org/10.1371/journal.pone.0054092

Johnson, R. M., Pollock, H. S., & Berenbaum, M. R. (2009). Synergistic Interactions Between In-Hive Miticides in Apis mellifera. *Journal of Economic Entomology*, *102*(2), 474–479. https://doi.org/10.1603/029.102.0202

Jones, J., Myerscough, M., Graham, S., & Oldroyd, B. (2004). Honey Bee Nest Thermoregulation: Diversity Promotes Stability. *Science (New York, N.Y.)*, *305*, 402–404. https://doi.org/10.1126/science.1096340

Jones, L. J., Ford, R. P., Schilder, R. J., & López-Uribe, M. M. (2021). Honey bee viruses are highly prevalent but at low intensities in wild pollinators of cucurbit agroecosystems. *Journal of Invertebrate Pathology*, *185*, 107667. https://doi.org/10.1016/j.jip.2021.107667

Kielmanowicz, M. G., Inberg, A., Lerner, I. M., Golani, Y., Brown, N., Turner, C. L., Hayes, G. J. R., & Ballam, J. M. (2015). Prospective Large-Scale Field Study Generates Predictive Model Identifying Major Contributors to Colony Losses. *PLOS Pathogens*, *11*(4), e1004816. https://doi.org/10.1371/journal.ppat.1004816

Kirrane, M. J., de Guzman, L. I., Holloway, B., Frake, A. M., Rinderer, T. E., & Whelan, P. M. (2015). Phenotypic and Genetic Analyses of the Varroa

Sensitive Hygienic Trait in Russian Honey Bee (Hymenoptera: Apidae) Colonies. *PLOS ONE*, *10*(4), e0116672. https://doi.org/10.1371/journal.pone.0116672

Klein, A.-M., Vaissière, B. E., Cane, J. H., Steffan-Dewenter, I., Cunningham, S. A., Kremen, C., & Tscharntke, T. (2007). Importance of pollinators in changing landscapes for world crops. *Proceedings of the Royal Society B: Biological Sciences*, *274*(1608), 303–313. https://doi.org/10.1098/rspb.2006.3721

Kurze, C., Routtu, J., & Moritz, R. F. A. (2016). Parasite resistance and tolerance in honeybees at the individual and social level. *Zoology*, *119*(4), 290–297. https://doi.org/10.1016/j.zool.2016.03.007

Leclercq, G., Pannebakker, B., Gengler, N., Nguyen, B. K., & Francis, F. (2017). Drawbacks and benefits of hygienic behavior in honey bees (Apis mellifera L.): A review. *Journal of Apicultural Research*, *56*(4), 366–375. https://doi.org/10.1080/00218839.2017.1327938

Lenth, R. (2023). *emmeans: Estimated Marginal Means, aka Least-Squares Means.* (R package version 1.8.7) [Computer software]. https://CRAN.R-project.org/package=emmeans

Livak, K. J., & Schmittgen, T. D. (n.d.). *Analysis of Relative Gene Expression Data Using Real- Tim e Quantitative PCR and the 2X□ □CT Method*.

Lodesani, M., Franceschetti, S., & Dall'Ollio, R. (2019). Evaluation of early spring bio-technical management techniques to control varroosis in Apis mellifera. *Apidologie*, *50*(2), 131–140. https://doi.org/10.1007/s13592-018-0621-z

Lourenço, A. P., Mackert, A., dos Santos Cristino, A., & Simões, Z. L. P. (2008). Validation of reference genes for gene expression studies in the honey bee, *Apis mellifera* , by quantitative real-time RT-PCR. *Apidologie*, *39*(3), 372–385. https://doi.org/10.1051/apido:2008015

Mattila, H. R., Reeve, H. K., & Smith, M. L. (2012). Promiscuous honey bee queens increase colony productivity by suppressing worker selfishness. *Current Biology: CB*, *22*(21), 2027–2031. https://doi.org/10.1016/j.cub.2012.08.021

Mattila, H. R., & Seeley, T. D. (2007). Genetic Diversity in Honey Bee Colonies Enhances Productivity and Fitness. *Science*, *317*(5836), 362–364.

Meixner, M. D., Costa, C., Kryger, P., Hatjina, F., Bouga, M., Ivanova, E., & Büchler, R. (2010). Conserving diversity and vitality for honey bee breeding. *Journal of Apicultural Research*, *49*(1), 85–92. https://doi.org/10.3896/IBRA.1.49.1.12

Morfin, N., Given, K., Evans, M., Guzman-Novoa, E., & Hunt, G. J. (2020). Grooming behavior and gene expression of the Indiana “mite-biter” honey bee stock. *Apidologie*, *51*(2), 267–275. https://doi.org/10.1007/s13592-019-00710-y

Moritz, R. F. A., Kraus, F. B., Kryger, P., & Crewe, R. M. (2007). The size of wild honeybee populations (Apis mellifera) and its implications for the conservation of honeybees. *Journal of Insect Conservation*, *11*(4), 391–397. https://doi.org/10.1007/s10841-006-9054-5

Morse, R. A., & Calderone, N. W. (2000). The Value of Honey Bees As Pollinators of U.S. Crops in 2000. *Bee Culture*, 2–15.

Oldroyd, B. P., & Fewell, J. H. (2007). Genetic diversity promotes homeostasis in insect colonies. *Trends in Ecology & Evolution*, *22*(8), 408–413. https://doi.org/10.1016/j.tree.2007.06.001

O'Neal, S. T., Brewster, C. C., Bloomquist, J. R., & Anderson, T. D. (2017). Amitraz and its metabolite modulate honey bee cardiac function and tolerance to viral infection. *Journal of Invertebrate Pathology*, *149*, 119–126. https://doi.org/10.1016/j.jip.2017.08.005

Page, R. E., & Fondrk, M. K. (1995). The effects of colony-level selection on the social organization of honey bee (Apis mellifera L.) colonies: Colony-level components of pollen hoarding. *Behavioral Ecology and Sociobiology*, *36*(2), 135–144. https://doi.org/10.1007/BF00170718

Page, R. E., Fondrk, M. K., & Rueppell, O. (2012). Complex pleiotropy characterizes the pollen hoarding syndrome in honey bees (Apis mellifera L.). *Behavioral Ecology and Sociobiology*, *66*(11), 1459–1466. https://doi.org/10.1007/s00265-012-1400-x

Palmer, K. A., & Oldroyd, B. P. (2000). Evolution of multiple mating in the genus Apis. *Apidologie*, *31*(2), 235–248. https://doi.org/10.1051/apido:2000119

Peck, D. T., & Seeley, T. D. (2019). Mite bombs or robber lures? The roles of drifting and robbing in Varroa destructor transmission from collapsing honey bee colonies to their neighbors. *PLOS ONE*, *14*(6), e0218392. https://doi.org/10.1371/journal.pone.0218392

Penn, H. J., Simone-Finstrom, M. D., Chen, Y., & Healy, K. B. (2022). Honey Bee Genetic Stock Determines Deformed Wing Virus Symptom Severity but not Viral Load or Dissemination Following Pupal Exposure. *Frontiers in Genetics*, *13*. https://www.frontiersin.org/articles/10.3389/fgene.2022.909392

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Research*, *29*(9), e45.

Plate, M., Bernstein, R., Hoppe, A., & Bienefeld, K. (2019). The importance of controlled mating in honeybee breeding. *Genetics Selection Evolution*, *51*(1), 74. https://doi.org/10.1186/s12711-019-0518-y

R Core Team. (2023). *R: A language and environment for statistical computing.* https://www.R-project.org/

Ramsey, S. D., Ochoa, R., Bauchan, G., Gulbronson, C., Mowery, J. D., Cohen, A., Lim, D., Joklik, J., Cicero, J. M., Ellis, J. D., Hawthorne, D., & vanEngelsdorp, D. (2019). Varroa destructor feeds primarily on honey bee fat body tissue and not hemolymph. *Proceedings of the National Academy of Sciences*, *116*(5), 1792–1801. https://doi.org/10.1073/pnas.1818371116

Rinkevich, F. D. (2020). Detection of amitraz resistance and reduced treatment efficacy in the Varroa Mite, Varroa destructor, within commercial beekeeping operations. *PLOS ONE*, *15*(1), e0227264. https://doi.org/10.1371/journal.pone.0227264

Rinkevich, F. D., Danka, R. G., & Healy, K. B. (2017). Influence of Varroa Mite (Varroa destructor) Management Practices on Insecticide Sensitivity in the

Honey Bee (Apis mellifera). *Insects*, *8*(1), Article 1. https://doi.org/10.3390/insects8010009

Rosenkranz, P., Aumeier, P., & Ziegelmann, B. (2010). Biology and control of Varroa destructor. *Journal of Invertebrate Pathology*, *103*, S96–S119. https://doi.org/10.1016/j.jip.2009.07.016

Ryabov, E. V., Wood, G. R., Fannon, J. M., Moore, J. D., Bull, J. C., Chandler, D., Mead, A., Burroughs, N., & Evans, D. J. (2014). A Virulent Strain of Deformed Wing Virus (DWV) of Honeybees (Apis mellifera) Prevails after Varroa destructor-Mediated, or In Vitro, Transmission. *PLoS Pathogens*, *10*(6), e1004230. https://doi.org/10.1371/journal.ppat.1004230

Schiff, N. M., & Sheppard, W. S. (1995). Genetic Analysis of Commercial Honey Bees (Hymenoptera: Apidae) from the Southeastern United States. *Journal of Economic Entomology*, *88*(5), 1216–1220. https://doi.org/10.1093/jee/88.5.1216

Schiff, N. M., & Sheppard, W. S. (1996). Genetic differentiation in the queen breeding population of the western United States. *Apidologie*, *27*(2), 77–86. https://doi.org/10.1051/apido:19960202

Seeley, T. D., & Tarpy, D. R. (2007). Queen promiscuity lowers disease within honeybee colonies. *Proceedings of the Royal Society B: Biological Sciences*, *274*(1606), 67–72. https://doi.org/10.1098/rspb.2006.3702

Sheppard, W. S. (1988). Comparative Study of Enzyme Polymorphism in United States and European Honey Bee (Hymenoptera: Apidae) Populations. *Annals*

of the Entomological Society of America, *81*(6), 886–889. https://doi.org/10.1093/aesa/81.6.886

Simone-Finstrom, M., Borba, R., Wilson, M., & Spivak, M. (2017). Propolis Counteracts Some Threats to Honey Bee Health. *Insects*, *8*(2), 46. https://doi.org/10.3390/insects8020046

Simone-Finstrom, M. D., & Spivak, M. (2012). Increased Resin Collection after Parasite Challenge: A Case of Self-Medication in Honey Bees? *PLOS ONE*, *7*(3), e34601. https://doi.org/10.1371/journal.pone.0034601

Simone-Finstrom, M., Walz, M., & Tarpy, D. R. (2016). Genetic diversity confers colony-level benefits due to individual immunity. *Biology Letters*, *12*(3), 20151007. https://doi.org/10.1098/rsbl.2015.1007

Spivak, M., & Gilliam, M. (1998). Hygienic behaviour of honey bees and its application for control of brood diseases and varroa. *Bee World*, *79*(4), 169–186. https://doi.org/10.1080/0005772X.1998.11099408

Spivak, M., & Reuter, G. S. (2001). Varroa destructor Infestation in Untreated Honey Bee (Hymenoptera: Apidae) Colonies Selected for Hygienic Behavior. *Journal of Economic Entomology*, *94*(2), 326–331. https://doi.org/10.1603/0022-0493-94.2.326

Tarpy, D. R. (2003). Genetic diversity within honeybee colonies prevents severe infections and promotes colony growth. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *270*(1510), 99–103. https://doi.org/10.1098/rspb.2002.2199

Tarpy, D. R., Nielsen, R., & Nielsen, D. I. (2004). A scientific note on the revised estimates of effective paternity frequency in Apis. *Insectes Sociaux*, *51*(2), 203–204. https://doi.org/10.1007/s00040-004-0734-4

Tison, L., Riva, C., Maisonnasse, A., Kretzschmar, A., Hervé, M., Le Conte, Y., & Mondet, F. (2021). Seasonal and environmental variations influencing the Varroa Sensitive Hygiene trait in the honey bee. *Entomologia Generalis*, *42*, 1–10. https://doi.org/10.1127/entomologia/2021/1280

Tokarev, Y. S., Huang, W.-F., Solter, L. F., Malysh, J. M., Becnel, J. J., & Vossbrinck, C. R. (2020). A formal redefinition of the genera Nosema and Vairimorpha (Microsporidia: Nosematidae) and reassignment of species based on molecular phylogenetics. *Journal of Invertebrate Pathology*, *169*, 107279. https://doi.org/10.1016/j.jip.2019.107279

Traynor, K. S., Rennich, K., Forsgren, E., Rose, R., Pettis, J., Kunkel, G., Madella, S., Evans, J., Lopez, D., & vanEngelsdorp, D. (2016). Multiyear survey targeting disease incidence in US honey bees. *Apidologie*, *47*(3), 325–347. https://doi.org/10.1007/s13592-016-0431-0

Underwood, R. M., Traver, B. E., & López-Uribe, M. M. (2019). Beekeeping Management Practices Are Associated with Operation Size and Beekeepers' Philosophy towards in-Hive Chemicals. *Insects*, *10*(1), Article 1. https://doi.org/10.3390/insects10010010

van Alphen, J. J. M., & Fernhout, B. J. (2020). Natural selection, selective breeding, and the evolution of resistance of honeybees (Apis mellifera) against Varroa. *Zoological Letters*, *6*(1), 6. https://doi.org/10.1186/s40851-020-00158-4

Venables, W. N., & Ripley, B. D. (2002). *Modern Applied Statistics with S*. https://cran.r-project.org/web/packages/MASS/citation.html

Villa, J. D., Danka, R. G., & Harris, J. W. (2009). Simplified methods of evaluating colonies for levels of Varroa Sensitive Hygiene (VSH). *Journal of Apicultural Research*, *48*(3), 162–167. https://doi.org/10.3896/IBRA.1.48.3.03

Ward, L., Waite, R., Boonham, N., Fisher, T., Pescod, K., Thompson, H., Chantawannakul, P., & Brown, M. (2007). First detection of Kashmir bee virus in the UK using real-time PCR. *Apidologie*, *38*(2), 181–190. https://doi.org/10.1051/apido:2006072

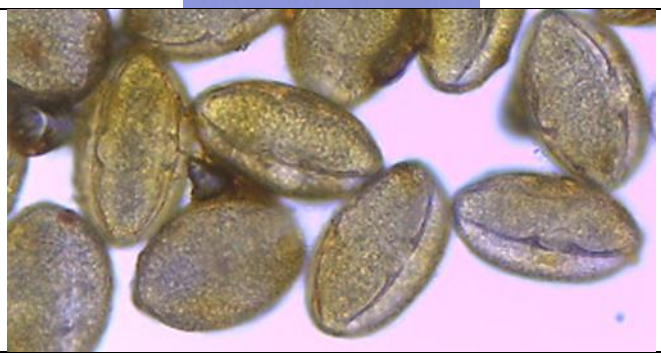
Wilde, J., Paleolog, J., Grabowski, P., Siuda, M., & Bratkowski, J. (2011). Correlated and direct responses to selection for high and low pollen yield in a small, open population of *Apis mellifera carnica*. *Journal of Apicultural Research*, *50*(3), 181–189. https://doi.org/10.3896/IBRA.1.50.3.01

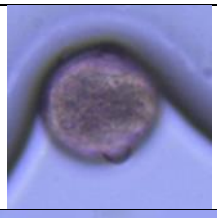
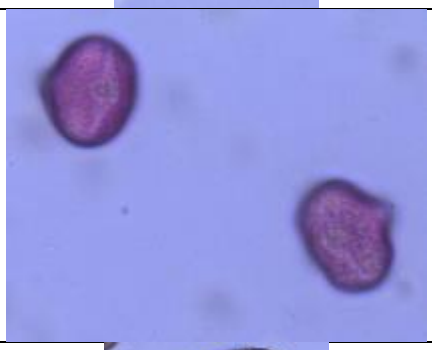
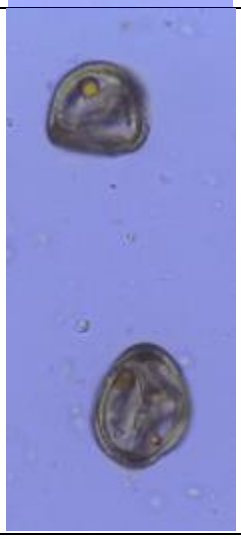
Appendix A

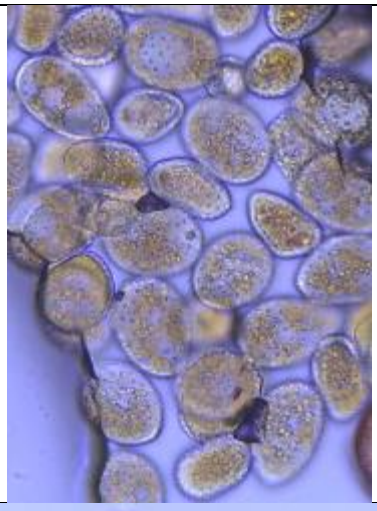
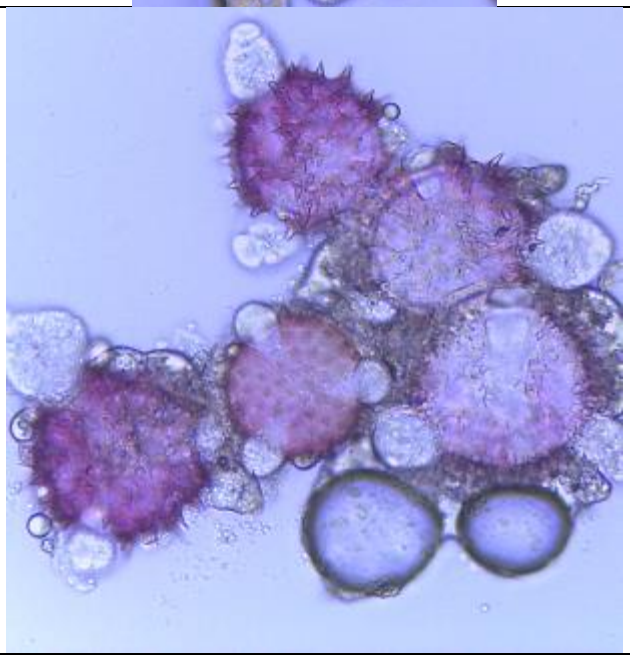
POLLEN MORPHOTYPES

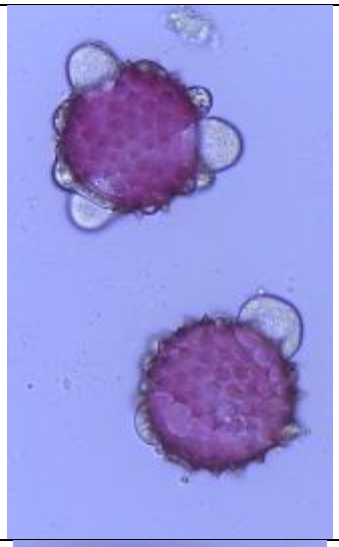
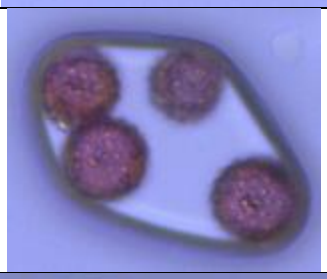
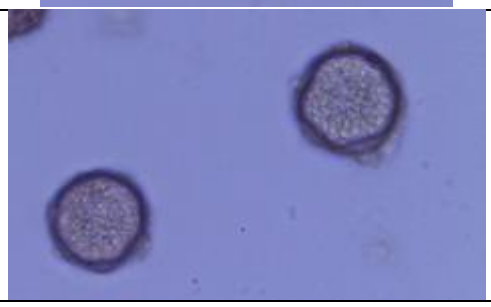
Table A.1 displays the 31 morphotype groups for pollen collected from experimental colonies the week of August 16th, 2021 and their size ranges.

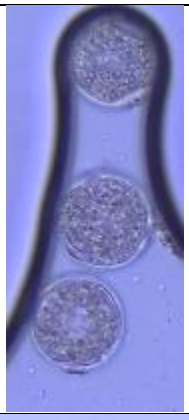
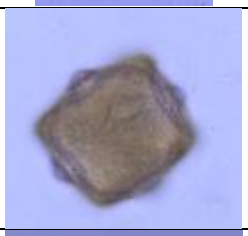
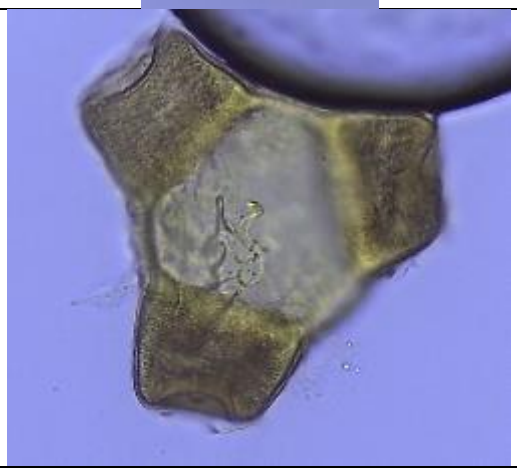
Table A.1: Pollen diversity morphotypes with representative microscopy image and size range (µM).

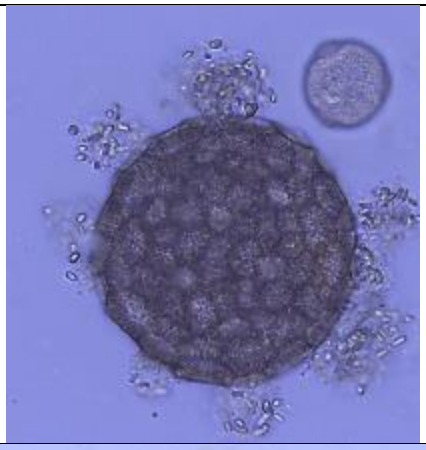
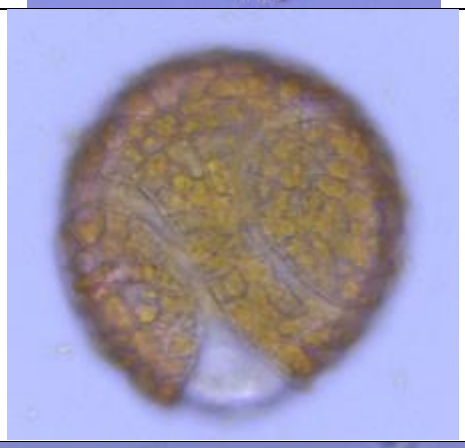
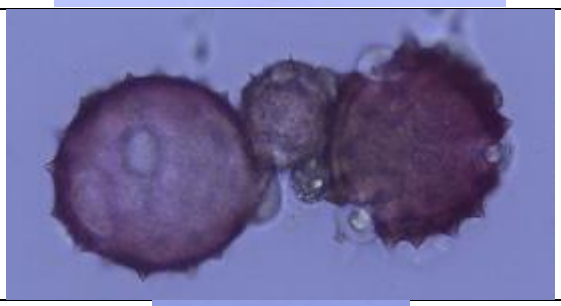
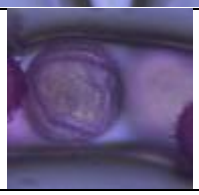
Pollen Morphotype Group	Photo	Size range (µM)
A		20.0 - 26.0
B		Long: 29.2-31.5 Short:18.0-22.2

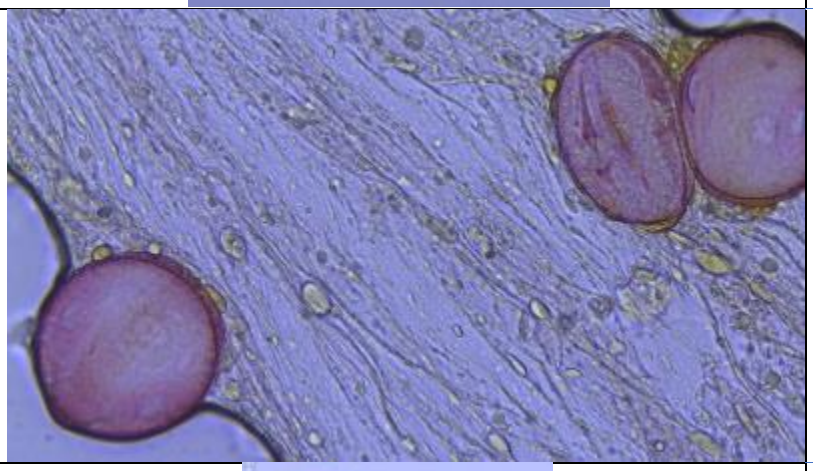
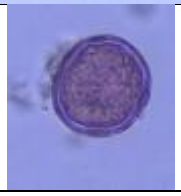
C		20.0 - 27.2
D		Long:16.0 - 20.0 Short: 10.0 – 16.0
E		Long: 20.0 - 30.0 Short: 15.0-20.0
F		Long:14.0 - 19.0 Short: 10.0 – 15.0

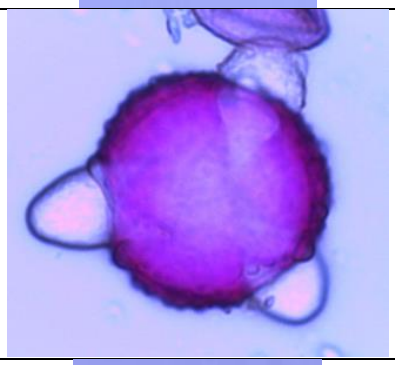
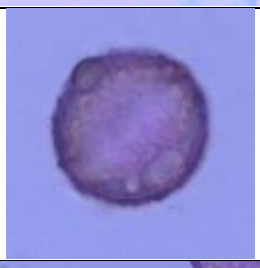
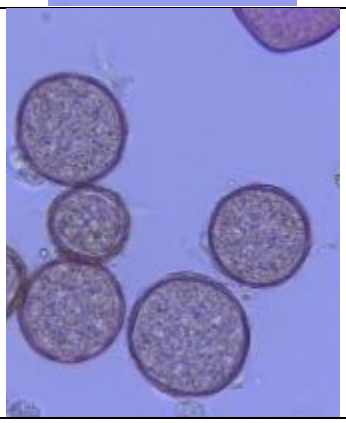
G		11.0 - 19.0
H		21.0 - 31.0

I		20.0 - 32.0
J		11.0 - 20.0
L		11.0 - 20.0
M		12.0 - 22.0

N		13.7 - 20.7
O		15.1 - 28.0
P		19.0 - 27.0
Q		55.7 - 84.3

R		32.4 - 42.4
S		48.0 - 77.0
T		20.0 - 29.0
U		20.0 - 25.2
V		15.0 - 16.0

W		24.0 - 35.0
X		37.0 - 48.4
Y		10.0 - 16.0
Z		19.0 - 22.4
A1		12.0 - 15.5

A2		17.1 - 23.6
A3		15.0 - 25.0
A4		20.0 - 25.0
B1		17.0 - 22.0
D1		18.0 - 21.0