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The Dynamics and Emergence of Shared Viruses in Honey Bees and Native Bees

By
Nina Sokolov

A dissertation submitted in partial satisfaction of the
requirements for the degree of
Doctor of Philosophy
in
Integrative Biology
in the
Graduate Division
of the
University of California, Berkeley

Committee in charge:
Professor Michael Boots, Chair
Professor of the Graduate School Wayne Sousa
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Abstract

The Dynamics and Emergence of Shared Viruses in Honey Bees and Native Bees

By

Nina Sokolov

Doctor of Philosophy in Integrative Biology

University of California, Berkeley

Professor Michael Boots, Chair

It is estimated that 40% of managed honey bee hives are lost each year in the United States. The little data we have on wild bees highlights significant declines in many species. Pathogens are implicated as one of the many important drivers behind losses. RNA viruses are of particular concern due to their high mutation rate and thus are considered the highest risk for spillover into a new species. Although these viruses are often described as ‘honey bee viruses’, their origins are almost entirely unknown, with many of these viruses also being found in wild bees. With the existing data we currently have, we cannot infer the direction of spillover, the main host reservoirs of the viruses, nor the ecological dynamics of these viruses. One way of addressing this issue is through longitudinal sampling in which different bee species are collected through time and their viruses are quantified to make a time series. By monitoring these epidemiological dynamics, we can see if epidemics begin in certain species before being transmitted to others and infer transmission dynamics. These data are lacking but necessary to accurately assess the risk of viruses being transmitted between managed and wild bees. The severity of viral infections has increased after the recent emergence and spread of a novel parasitic mite, *Varroa destructor*. Varroa mites arrived in the United States only in the 1980s and have subsequently made beekeeping a lot more challenging and unsustainable. This mite parasitizes honey bees by consuming their fat body, an organ essential for mounting immune responses, and acts as a vector for viruses. Before varroa mites, most bee viruses experienced direct transmission where infectious virions were primarily ingested by the bee host consuming contaminated floral products or interacting with conspecifics. With the arrival of varroa, this mite adds a novel transmission pathway in which the viruses are directly injected into the bee’s hemolymph, the circulatory fluid within invertebrates. This bypasses any of the viral defenses the bees have evolved in their gut lining; the virus passes directly into their blood and leads to higher viral loads in varroa-parasitized colonies. As a result, managed hives experience high overwintering losses. This has led to both increased use of pesticides against the mite (and subsequent evolution of resistance of the mite against the acaricides), and the pressure to breed honey bees that are resistant to varroa.

Notwithstanding these observations, studies of bee virus dynamics are lacking in California, even with a massive agricultural industry, and high native bee diversity. During the almond bloom in the Central Valley, over 1.7 million hives are shipped in on truck beds from across the country, potentially bringing novel pathogens to local sites. Subsequently, honey bees share floral resources at stopover sites with wild bees, which creates a prime zone for

interspecies disease transmission. This mass mixing event has the potential to select for more deadly pathogens as these RNA viruses are greeted with an enormous influx of novel susceptible hosts to spread through. According to disease ecology theory, increased spatial connectivity between host populations allows for the evolution of increased virulence, meaning the deadliness of a pathogen. This could be acting as a mass mixing event that has the capacity to evolve more deadly diseases for bees, thus there is a pressing need to track the viral dynamics occurring in association with the almond bloom.

These pollination events in conjunction with contemporary beekeeping management practices all have a part to play in the disease ecology emerging within the honey bees. But pollinators do not exist in a closed system, and the consequences of industrial agriculture on honey bees are also being felt in the greater pollinator community. After managed honey bees participate in crop pollination events, they share floral resources with native bees at stopover sites between pollination events, creating a risk for inter-species transmission. As it stands, we know little about viral dynamics in honey bees at the population level, nor about the effect of crop pollination events on disease emergence in honey and native bees.

This dissertation is first comprised of a literature review on the status of efforts to breed honey bees for varroa resistance but finds that most of these actions are in fact breeding for parasite tolerance. I describe the distinct epidemiological, evolutionary, and ecological consequences of tolerance for resistance to varroa. This is discussed for both the honey bees which are directly parasitized by varroa, and the indirect community-level consequences of that parasitism on wild bees through shared viruses. During this time, I have developed three independent field systems in Northern California through a connection of local landowners, crop growers, gardeners, ranchers, and community members, along with commercial, professional, and hobbyist beekeepers. This network allowed me to consistently collect honey and native bees for viral quantification through time, resulting in a frozen time series collection of bees from 2019 – 2024. Two of these systems will be described in this dissertation while the third will be the focus of my postdoctoral research. These field sites allowed for the regular longitudinal sampling of bee viruses through time starting in February at the almonds blooming in the Central Valley. After the almonds finished blooming, this beekeeping operation moved their hives to the Sierra foothills for the spring before being experimentally placed at high-elevation montane meadows in the Sierra for breeding projects and honey production. This time series data set allowed me to track honey bee viral dynamics in association with the almond bloom and then test the impact of commercially managed honey bees in a montane wildflower system.

Chapter 1: Avoiding the tragedies of parasite tolerance in Darwinian beekeeping.

Bee losses in the United States across the last several decades have been attributed, in part, to the parasitic virus vectoring mite *Varroa destructor*. In response, beekeepers have conducted breeding efforts to confer resistance to these invasive parasites. In this chapter, I review the current literature on honey bee breeding for *Varroa* and highlight the many instances these bees are tolerating rather than resisting mite parasitization, and by proxy, tolerating viral infections. Although both strategies lead to greater survivorship amongst those tolerant honey bees, I stress the critical difference between resistance versus tolerance from an ecological and pathogen spillover perspective. Existing literature shows that selecting for tolerance will not only lead to more spillover from honey bees but may also select for viruses that are more virulent in wild bees leading to the ‘Tragedies of Tolerance’. I argue the evolutionary ecology of pathogen

spillover must be considered in ecologically responsible honey bee management. By selecting for varroa resistance specifically, we can improve both honey bee and wild bee health.

Chapter 2: Changes in the honey bee virome during the almond bloom in California

Natural migrations of animals as well as managed transport of agricultural species can enhance the geographical spread of pathogens and increase interspecies transmission. According to disease theory, spatial structure can have impacts on the evolution of virulence, with increased connectivity leading to higher levels of pathogen virulence. In this chapter, I track viruses in honey bees throughout the almond bloom to quantify viral dynamics through a mass pollination event. I sampled honey bee hives in February 2021 across four time points: before the almonds bloom, twice during the bloom, and then at their next stopover site a month afterward. Almond hives were compared to “control” hives that did not participate in the almond bloom and instead stayed at their home yard in Grass Valley, CA. I sampled 32 hives through time and quantified their viruses through RNA sequencing. Overall, I detected 34 viral species through time, which included RNA viruses, DNA viruses, phages, and plant viruses. Almond-pollinating hives had higher viral species richness at all time points in comparison to the controls, except at the last time point when the almond-pollinating hives returned to the control’s location. There it appears that the viral species richness was transferred to the control hives, with the control hive’s viral richness significantly higher once the almond bees returned. Individual viral taxa experienced different temporal dynamics and associations with the almond bloom. Black queen cell virus was consistently lower in almond-pollinating bees, whereas the Lake Sinai Virus (LSV) complex was more prevalent in the almond hives. *Apis mellifera* filamentous virus showed viral dynamics that were independent of the bloom, and instead showed seasonal dynamics that were similar between locations. These results show that these mass migrations are impacting viral dynamics in honey bees, even in honey bees that did not participate in crop pollination. Additionally, certain viruses are more highly associated with the bloom than others which highlights focal viral species that should be monitored genetically. As these conditions are primed for the evolution of increased virulence, we must begin storing genetic data to act as possible repositories in the case of emergent new pathogen strains.

Chapter 3: Viral dynamics between managed honey bees and native wild bees.

Wildflower meadows in the Sierra Nevada Mountain range boast high levels of native bee and floral diversity along with low honey bee abundance. This provides a unique environment to quantify bee viral dynamics in a field system that is isolated from agriculture. By taking advantage of the low honey bee density of this area, this chapter reports the results around a 'natural experiment,' using longitudinal sampling of wild bee populations for viruses before and after the human-mediated migration of commercial honey bees onto private land within the Tahoe National Forest. In this system, we can ask whether viruses were in native bees before honey bee arrival, if honey bees brought viruses with them when they migrated, and whether there was evidence of spillover between species. Native bees including bumble bees (genus: *Bombus*), and mason bees (Genus: *Osmia*) were collected before and at three-time points after honey bee arrival across eight sites in the summer of 2021. Honey bee hives that had previously participated in almond crop pollination were sampled before migration. Then, honey bee hive and foraging honey bees were collected at three-time points after arrival in the Sierran meadows.

Sites ranged in their abundance of honey bees foraging on flowers and in the presence of an apiary. The effect of honey bee abundance and apiary presence was tested as a possible explanation behind any patterns of viral prevalence in native bees. Deformed wing virus strain B (DWV-B), Sacbrood virus (SBV), and Chronic bee paralysis virus (CBPV) were all already present in *Bombus* species before honey bee arrival, suggesting that bumble bees can independently maintain these viruses. Honey bees brought each of these viruses with them when they migrated. However, the temporal dynamics of each of these viruses were distinctly different. DWV-B increased in *Bombus* over time, regardless of honey bee abundance or apiary presence, indicating an epidemic within bumble bee populations. SBV was highly prevalent in *Bombus* early in the season and decreased over time in all species, while *Osmia* mason bees showed no SBV infection. Whereas honey bee foragers barely experienced any evidence of SBV, the hive samples exhibited high SBV prevalence which also declined through the season. As both *Bombus* and hive SBV prevalence decreased through time, this suggests a seasonality trend for this virus. CBPV exhibited high prevalence in honey bees but low prevalence in *Bombus*, suggesting honey bees as more competent hosts with minimal direct impact on bumble bees. *Osmia* mason bees had positive cases of both DWV-B and CBPV, but their prevalence levels were far lower than the honey bees and bumble bees. These insights emphasize the necessity for long-term temporal survey data to understand bee virus ecological dynamics and their potential impact on native bee health.

Dedication

*I would like to dedicate this dissertation
To every single bee
that had to be sacrificed for this research.
I am dedicated to protecting you,
Nurturing you into the future,
Helping you sip from the finest ambrosia.
Thank you for the wildflower meadows you cultivate.
Thank you for the food you provide.
Thank you for the opportunity to bear witness
to the parts of your microscopic communities
that plague you.
I dedicate this work,
And my life's work to
The Bees.*

Table of Contents

Abstract.....	1
Dedication	i
Table of Contents	ii
Acknowledgments	iv
Introduction: The role of viruses in regulating bee populations and the risk of inter-species transmission.....	vi
Chapter 1: Avoiding the tragedies of parasite tolerance in Darwinian beekeeping	1
1.1 Abstract:.....	1
1.2 Introduction:.....	2
1.2.1 Box 1: Varroa, viruses, and honey bees.....	4
1.3 The impact of honey bee viruses on wild bee populations:	5
1.4 Tolerance versus resistance as distinct host defense mechanisms:.....	7
1.4.1 Box 2: The conceptual basis of the evolution of host defenses to pathogens.	7
1.4.2 Box 3: Three Tragedies of Tolerance	8
1.5 Tolerance and resistance to viruses and mites in honey bees:	9
1.6 Conclusion:	13
1.7 Author’s contributions:	13
Chapter 2: Honey bee virome dynamics during a mass pollination event	13
2.1 Abstract:.....	13
2.2 Introduction:.....	14
2.3 Methods:	16
2.4 Data Analysis:.....	17
2.5 Statistical Analysis:.....	19
2.6 Results:.....	20
2.7 Discussion:	25
2.8 Conclusion:	28
2.9 Acknowledgements:.....	29
Chapter 3: Bee virus dynamics between managed honey bees and native wild bees in a Sierra Wildflower System	29
3.1 Abstract:.....	29
3.2 Introduction:.....	30
3.3 Methods:	33
3.4 Results:.....	37
3.5 Discussion:	47

3.6 Conclusion:	50
3.7 Acknowledgements:.....	50
Concluding Remarks	51
Conclusions:.....	51
Future research:.....	53
Bibliography	62
Appendices.....	80

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Introduction:

The role of viruses in regulating bee populations and the risk of inter-species transmission.

Over the last 50 years, demand for animal-pollinated crops has nearly tripled (Aizen & Harder, 2009). Bees in general act as key pollinators for over 70% of the major crops in our diet (Klein et al., 2007). Although total honey bee colony numbers in the US have remained relatively stable in the last two decades, each year they experience unsustainably high rates of loss. Across the last decade of surveys, national over-winter colony mortality rates oscillated from 21.1% - 37.7% (Bruckner et al., 2023). Losses of managed and native pollinators will negatively impact pollination services, threatening crop production and ultimately human food security (Potts et al., 2010), health, and welfare (Garibaldi et al., 2022). The economic impacts of these losses will be high, with honey bees and native pollinators together pollinating \$15 billion worth of crops and seeds in the United States alone (Calderone, 2012). Native bees, specifically, are critical pollinators of crops and are often more efficient pollinators than honey bees alone due to their specialist foraging tendencies (Garibaldi et al., 2013). We know little about the population dynamics of native bees, and this is for a number of reasons that are well described by Wagner et al., (2021). First, the species level of identification of bees is predicated on specialist taxonomists, and there are far more specimens that need to be identified than there are taxonomists. Second, to claim that a species is in decline requires us to know what their populations were in the past to have evidence that it has declined from a greater past abundance. Very few species have that level of resolution to be able to use historical data to claim decline. Insects in particular have highly cyclical population dynamics that make inferring general patterns of declines hard to decipher (Didham et al., 2020). The data we do have is primarily for the charismatic megafauna of bees, the bumble bees.

According to the International Union for the Conservation of Nature (IUCN), more than one-quarter of North American bumble bees are in a Threatened Category (Hatfield et al., 2015). In 2017, the rusty patch bumble bee (*Bombus affinis*) was the first bee to be listed as endangered in the continental United States under the federal Endangered Species Act (USFWS, 2017). In 2019, California determined four bumble bee species may be warranted to be listed as endangered under the California Endangered Species Act (CESA). After experiencing backlash in court, the state of California ruled in favor of insects being possible candidates for listing under the CESA in 2022. We are still on the road towards gaining official state protection for these species, but candidates receive the same legal protection afforded to listed species. This is all just for bumble bees, arguably the most highly studied of the native Californian bees. Of the remaining nearly 1,600 species of other native bees in the state, little is known about their population statuses, and they therefore remain unprotected. In California, the only solitary bee with enough data to be listed by the Xerces Society for Invertebrate Conservation is *Halictus harmonius*, a sweat bee with a very narrow range. We should be concerned about these unsustainable losses of both managed agricultural species and wild native pollinators from the perspective of our food systems and broader ecosystem health and function.

Infectious disease is recognized as a major determinant of population and community dynamics in wildlife as well as in managed and human populations (Anderson and May, 1978; Daszak, 2000; Hassell et al., 2017; Wiethoelter et al., 2015). Pathogens are implicated as one of

the many important drivers of declines in bees (Singh et al., 2010) alongside habitat loss, climate change, pesticides, and poor nutrition (Goulson, 2015). RNA viruses are of particular concern due to their high mutation rates, allowing them to infect many species (Tehel et al., 2016). Certain pathogens are nearly ubiquitously found in honey bees including the high prevalence of Deformed Wing Virus (DWV) and black queen cell virus (BQCV) (Budge et al., 2015a; Natsopoulou et al., 2017a). Honey bee hives provide crowded warm conditions and seasonal host confinement in the winters, which favor disease outbreaks (DeGrandi-Hoffman and Chen, 2015). Many viruses persist throughout hives as chronic relatively asymptomatic infections, with symptom severity increasing when the host becomes stressed, leading to high colony loss in the winter and early spring (Döke et al., 2015). This set of conditions sets the stage for high levels of potential interspecies transmission events (Manley et al., 2015a; McMahon et al., 2015a). However, the extent to which these viruses are causing active and symptomatic infections is still unclear.

Beekeeping became a lot more challenging after the emergence of the parasitic mite, *Varroa destructor*. A spillover event occurred in the early 1900s in Eastern Asia from the eastern honey bee (*Apis cerana*) and their parasitic mite (*Varroa jacobsoni*) to the western honey bee that we use in crop pollination (*Apis mellifera*) (Wilfert et al., 2016). This happened because of the co-occupation of both honey bee species in the same apiaries. When the varroa mite began infecting *A. mellifera* it found a host that had even larger colonies and no adaptations to dealing with this mite's parasitism. This led to rapid proliferation and subsequent speciation of this mite into, the aptly named, *Varroa destructor* (Anderson and Trueman, 2000). This mite has spread across the world through the globalized trade of honey bees and their products (Wilfert et al., 2016). This mite arrived in the United States in the late 80's and has spread across the country, devastating the beekeeping industry along the way (Morfin et al., 2023). These mites are large parasitic organisms that consume the bee's lipids and fat body, a tissue that is critical in immune function (Ramsey et al., 2019). The mite also vectors viruses, with the most clearly associated with Deformed Wing Virus (Wilfert et al., 2016). Parasitism is associated with higher viral prevalence and load in bees and is one of the drivers of the unsustainably high levels of overwintering mortality (Martin, 2001). The viruses the bees are dealing with may not be new, but all their evolved adaptations to mount an immune response were in association with oral transmission of pathogens from conspecifics or from their environment. Yañez et al., (2020) describes the various routes of viral transmission in bees. Specifically, they describe how immune defenses are located within the bee's gut lining to fight off orally consumed pathogens. The mite, however, bypasses all these immune adaptations and pierces directly into the bee's hemolymph. It is currently unclear whether the mite is acting as a mechanical vector, meaning the mite is facilitating disease transmission by spreading it between hosts, or a biological vector, where the viruses are actively replicating in the mite (Damayo et al., 2023). Regardless, vector-borne disease transmission has also been shown to be associated with more virulent infections than pathogens spread through direct transmission (Boots and Sasaki, 1999). This is hypothesized to be due to the vector's ability to spread diseases across larger spatial scales than the host alone.

Viruses thought to be honey bee specific are now being found in other bee species (Alger et al., 2019a; McMahon et al., 2015), and moreover, declines of native bees coinciding with the introduction of managed species carrying novel infectious pathogens have already been

documented (Montalva et al., 2011). Shared disease have the potential to play a central role in the population and community dynamics of natural systems, with spillover and spillback having the potential to be fundamental processes in natural communities (Guth et al., 2022a). Spillover is the transmission of a parasite or pathogen from a definitive maintenance host, into an incidental or non-maintenance species (Daszak et al., 2000). With the addition of new incidental or maintenance hosts, there can be an increase in disease transmission (Power & Mitchell, 2004). Furthermore, shared disease can play a central role in the population and community dynamics of natural systems, with spillover and spillback having the potential to be fundamental processes in natural communities (Guth et al., 2022). In a prominent example, the leading hypothesis behind the massive decline of the rusty patch bumble bee (*Bombus affinis*), the poster child of endangered bees, was due to the novel emergence of *Nosema bombii* that spilled over from commercial *Bombus* (Cameron et al., 2016a). Notably, *Nosema* parasite species were one of the first demonstrations of invasive novel honey bee pathogens spilling over and causing disease in wild bees (Graystock et al., 2013a). However, RNA viruses are of particular concern due to their high mutation rates. This allows for rapid adaptation to novel host immune systems, and thus RNA viruses are considered the highest risk for spillover between hosts (Holmes, 2009). We are only just beginning to understand the impact of viral emergence on bee populations (McMahon et al., 2015a). Although RNA viruses have the capacity to cause frequent spillover events due to their high mutation rates, most viruses do not evolve sustained transmission cycles within their novel host (Holmes, 2009). Determining the scenarios in which cross-species transmission is established and maintained in a novel host species rather than dying out from not being able to maintain the pathogen's basic reproductive number (R_0) is a cornerstone of understanding disease emergence in new hosts. It is therefore critical that we better understand the ecological dynamics of shared disease in natural communities.

Thus far, 36 honey bee-associated viruses have been described, most of which are positive-sense single-stranded RNA viruses. Many of these viruses have been implicated in causing hive mortality with symptoms of deformity, paralysis, decreased movement, and larvae failing to emerge (Benaets et al., 2017) while others appear asymptomatic (Grozinger and Flenniken, 2019). Although these viruses are described as honey bee viruses, their origins are almost entirely unknown, with eleven of these viruses also being found in wild bees so far (Tehel et al 2016). The first published instance showing spillover of a honey bee pathogen to another bee species was in 1964 (Bailey & Gibbs, 1964). Now with novel molecular techniques, there are over eighty-one studies demonstrating spillover between honey bees and other arthropod species (Nanetti et al., 2021). Some studies even claim that spillover between plants and bees is possible in the case of the Tobacco Ringspot Virus (Li et al., 2014), though additional evidence is necessary to confirm this claim. An increasing number of studies have shown that 'honey bee' viruses throughout nearly all of the major bee families including other members of Apidae (Dolezal et al., 2016a; Levitt et al., 2013a; McMahon et al., 2015a; Singh et al., 2010a; Tapia-González et al., 2019a; Tehel et al., 2016a), Halictidae (Dolezal et al., 2016a; Levitt et al., 2013a; Singh et al., 2010a), Andrenidae (Dolezal et al., 2016a; Ravoet et al., 2014; Singh et al., 2010a; Tehel et al., 2016a), and Megachilidae (Dolezal et al., 2016a; Ravoet et al., 2014; Tehel et al., 2016a), and Colletidae (Dolezal et al., 2016a; Tapia-González et al., 2019a). While not all these studies looked for evidence of active replication by testing for negative strands of these ss-RNA viruses, active replication in native bees has been found for acute bee paralysis virus (ABPV), DWV, black queen cell virus (BQCV), Israeli acute paralysis virus (IAPV), Kashmir bee virus

(KBV), Lake Sinai virus (LSV) and slow bee paralysis virus (SBPV). The lack of demonstrations of active replication in native bees is likely due to logistical challenges rather than biological reality (Levitt et al., 2013a). Phylogenetic relationships among the viruses show that viruses do not cluster by host bee species and instead cluster by geography, suggesting free dissemination of viruses between species (McMenamin and Flenniken, 2018a; Tehel et al., 2016a).

Intra-species transmission likely occurs via the oral-fecal route whereby an infected bee visits a flower and defecates, leaving infectious virions behind for another bee to contact when they visit the same flower. Environmental transmission has been shown experimentally with *Crithidia bombi* (Durrer & Schmid-Hempel, 1994a) and more recently with DWV on blooming clover flowers (Burnham et al., 2021). DWV is the most common virus found in honey bees (Martin and Brettell, 2019a) and the bee virus that has been most frequently found in other insects thus far (Tehel et al., 2016a), boasting the highest host breadth of any of the honey bee-associated viruses across its 65 arthropod ‘host’ species (8 insect orders, 3 Arachnida orders) (Martin and Brettell, 2019a). Crucially, DWV prevalence in these species is correlated with DWV load and prevalence in the overlapping honey bee populations (Manley et al., 2015a; Murray et al., 2019a; Santamaria, 2018). For example, Alger et al. (Alger et al., 2019a, 2019c) looked at the spillover of viruses between honey bees and bumble bees and found the highest viral prevalence in bumble bees associated with high viral load in honey bees. A direct demonstration of this principle intersecting with the effect of *Varroa* in managed honey bees was shown by (Manley et al., 2019a), who compared viral load and prevalence in honey bees and bumble bees on a series of replicate islands in the UK that existed with and without *Varroa*. They found that the presence of *Varroa* leads to higher viral loads and prevalence in honey bees. This in turn leads to higher prevalence and load in sympatric *Bombus*. Although viruses are being found in a range of other bee species, the virulence, or how deadly the pathogen is, remains largely unknown. Some estimates of the effect of viruses on non-*Apis* bees primarily come from experimental inoculation studies of *Bombus*. For example, the virulence of DWV in *Bombus* was shown through experimental inoculation leading to an increased host mortality rate (M A Fürst et al., 2014). However, it is well known that immune activation in bees is energetically expensive and that bees without *ad libitum* food are much more vulnerable to starvation when infected (Graystock et al., 2013a), a critical limit of laboratory studies. We therefore need to look to ‘natural experiments’ for more evidence of virulence in cases of bee pathogen spillover. Declines of native bees coinciding with the introduction of managed species have been documented, notably with *Bombus dalhombii*, the Chilean giant bumble bee. Although once one of the most abundant bee species found in Chile, it faced major declines after the introduction and successful invasion of commercial species *Bombus ruderatus* and *Bombus terrestris* (Montalva et al., 2011a). A dramatic reduction in species abundance includes a clear role of pathogen spillover, whereby the neo-gregarine parasite, *Apicystis bombi*, has been implicated as a cause of *B. dalhombii*’s precipitous population decline. This parasite was not found in the native *Bombus* species before the introduction of *Bombus terrestris* but was found at high prevalence thereafter (Arbetman et al., 2013a). However, demonstrating that insect populations are declining as well as finding the causal agent of these declines remains logistically challenging, especially for RNA viruses which require special handling. I argue that this necessitates the generation of long-term temporal survey data to determine disease dynamics’ association with population dynamics, and ultimately species declines.

The positive associations between honey bee viruses and native wild bee viruses are well known (Iwasaki and Hogendoorn, 2022). However, we cannot infer a number of important ecological factors, including the direction of spillover, meaning, which species is giving the disease to the other (Fürst et al., 2014) nor the main host reservoirs of the viruses. We also barely understand the ecological dynamics of the viruses resulting in ‘source-sink’ dynamics, or if there is ‘back-and-forth’ transmission between species (Tehel et al., 2016). Pathogen persistence can either depend on a primary host species, known as the maintenance host or can be maintained in a community (Haydon et al., 2002). Source-sink dynamics mean that one primary maintenance species is acting as a source of spillover to the rest of the interacting community (Roberts and Heesterbeek, 2020). This means that without interaction with the primary reservoir host, the pathogen would die out in the ‘sink’ community (Ng et al., 2023). Back-and-forth transmission indicates that all hosts are competent, and the pathogen epidemic can be maintained regardless of the presence of any one species.

To address the question of directionality, we need viral time series data, not just samples at one point in time. This is necessary to accurately assess the risk of disease transmission between managed and wild bees. The total host breadth, or how many species can be infected in any one ecosystem, has also yet to be established. Spillover of emergent infectious diseases has been modeled to occur even when the disease is rare in the host population (Otterstatter & Thomson, 2008). This could represent a major cause of mortality of wild bees wherever honey bees are maintained. We know little about viral dynamics in bees, nor the effect of crop pollination events on disease emergence. Studies of bee virus dynamics are specifically lacking in California, even with its massive agricultural industry, and high native bee diversity in the state. The state is home to over 500,000 honey bee hives, however, this is still not enough to meet demands. When the almonds bloom in the Central Valley, over 1.7 million hives are shipped to the orchards on semi-trucks, bringing their pathogens along for the ride (Glenny et al., 2017). Subsequently, honey bees share floral resources at stopover sites between pollination events with native bees which creates a prime zone for disease transmission between species (Fürst et al., 2014; Manley et al. 2015). Clear benefits of putting hives on natural lands between pollination stints exist, as diverse floral forage has been shown to lower pathogen loads and increase colony survival (Degrandi-Hoffman et al., 2016). However, this benefit must be weighed against the cost of exposing native bees to novel pathogens from the crop blooms. All these dynamics together could represent a major cause of mortality of wild bees wherever honey bees are maintained. Therefore, there is a need to understand the impact of shipping managed bees for crop pollination on potential spillover events in wild bees to help conserve these native species into the future.

This dissertation first reviews the literature on the status of honey bee breeding efforts for varroa resistance but to the contrary, finds that most of these programs are in fact breeding for parasite tolerance. I describe the distinct epidemiological, evolutionary, and ecological consequences of the distinction between tolerance and resistance to parasites both for the honey bees, and the wild bees that they interact with. Next, to understand the epidemiological and transmission dynamics of these viruses in the field, I used longitudinal monitoring of bee viruses in the field in both an agricultural system and a Sierran wildflower system. I tracked viral dynamics in honey bees before, during, and after participation in the almond bloom pollination to assess the effect of a mass migration event on viral species richness and individual viral

dynamics. After participating in almond pollination, the commercial pollinating honey bees were placed at seasonal stopover sites on private land before being moved to high-elevation meadows in the Sierra for wildflower honey production and breeding. There they interact with a high diversity of native bees in a rich floral landscape. Wildflowers provide both nourishment for honey bees that are often resource-limited, but also provide the opportunity for disease emergence in the native bees through sharing of floral resources. This means there is the potential for viruses associated with the almond bloom to impact bees far away from the bloom itself. This community also includes a candidate for the California Endangered Species Act, *Bombus occidentalis*. Therefore, determining the possible impact of honey bees in this area is even more pressing from a conservation perspective. Broadly, I am interested in (1) how honey bees resist varroa mites and the viruses they vector (2) how crop pollination events play a role in disease emergence (3) the seasonal dynamics of bee viruses, and (4) whether the transmission dynamics are due to recurring spillover events, resulting in ‘source-sink’ dynamics, or if there is ‘back-and-forth’ transmission between managed and native bees. I believe that through the knowledge and understanding of principles and theory underlying disease ecology and evolution, we can inform strategies that will help conserve these important beings into the future. I look forward to playing a role in the generation of that knowledge and using it to assist in pollinator conservation.

Chapter 1: Avoiding the tragedies of parasite tolerance in Darwinian beekeeping

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1.1 Abstract:

Declines in both managed and wild bees are of economic, ecological, and conservation concern. Losses of bees in the US have been attributed in part to the impacts of invasive or emerging parasite outbreaks. In honey bees, major losses are associated with the virus-vectoring mite *Varroa destructor*, and in response, beekeepers have focused breeding efforts on enhancing the resistance of bees to this key parasite. A popular approach includes survival-based beekeeping in which bees that can survive over the winter with varroa after no mite treatment are selected for breeding. We posit that survival-based beekeeping approaches will typically not lead to resistance but rather tolerance to the focal parasite. Parasite resistance involves host behavioral or immunological mechanisms that allow the host to either prevent infection in the first place or the intensity of infection low. Tolerance, on the other hand, involves the host minimizing the fitness costs associated with parasitism without affecting parasite abundance. There is a clear epidemiological distinction between resistance and tolerance to parasites that lead to very different ecological consequences. Moreover, honey bee disease dynamics impact other pollinators due to spillover of pathogens, especially viruses, into wild bee populations. Crucially, selecting for tolerance will not only lead to more spillover from honey bees but may also select for viruses that are more virulent in wild bees: leading to ‘Tragedies of Tolerance’ (Best et al., 2014). An alternative approach to survivor-stock breeding is “Darwinian beekeeping” in which a threshold for varroa is set, and bees that are unable to keep mites below that threshold are either culled or otherwise do not participate in the breeding programs. This actively selects for bees that can keep mite numbers low via resistance mechanisms, rather than surviving despite high parasitization through tolerance. We argue the evolutionary ecology of pathogen spillover must be considered in ecologically responsible honey bee management.



Figure 1. Graphical abstract illustrating the differences between tolerance and resistance to *Varroa* mites in honey bees, and the impact of spillover onto co-occurring wild bees. In the tolerance example (left) a honey bee is shown with high varroa mite infestation, shedding more viruses into the environment. This leads to an increased risk of subsequent infection for bumble bees that are co-foraging with honey bees. Alternatively, in the resistance example (right) honey bee is shown to be free of varroa mites, therefore shedding fewer viral particles into the environment and leading to decreased rates of infection in co-occurring bumble bees.

1.2 Introduction:

Bees, both managed and wild, have significant agro-economic and cultural value (Gallai et al., 2009; Potts et al., 2016)). However, managed bees in North America face high annual losses and wild bees have experienced substantial declines in parallel, leading to several species now being listed as threatened at both the state and federal levels (Goulson et al., 2015; Potts et al., 2010). Declines are due to a range of factors including habitat and forage loss, pesticide exposure, and parasite stressors (Goulson et al., 2015; Smith et al., 2013). These stressors compound via biological interactions whereby one stressor leads to further vulnerabilities to another. For example, bees exposed to pesticides may be immunosuppressed thereby increasing their vulnerability to parasites and pathogens (Annoscia et al., 2020; Grassl et al., 2018), and bees experiencing infection have higher energetic demands, putting further strain on already limited foraging resources (e.g. Dolezal et al., 2019). Solving this multifaceted problem requires action at multiple levels, including efforts to reduce parasite and pathogen burdens on bee populations.

The role of parasites in managed honey bee declines is widely documented, including recent re-emerging viral epidemics (Wilfert et al., 2016a) interacting with contemporary host-jump events by both micro- and macro-parasites (Genersch, 2010). Emphasis has been placed on the impact and putative vectoring of certain viruses by the invasive ectoparasitic mite *Varroa destructor* (Anderson and Trueman, 2000), which is the principal driver of honey bee loss in the USA after its introduction in the 1980s (Bruckner et al., 2023; Steinhauer et al., 2021; Wilfert et al., 2016a). Parasites and pathogens are spread through intraspecific transmission between honey

bee colonies either directly through the drift of individuals between colonies and hive robbing or indirectly through interactions on flowers (Peck et al., 2016). Simultaneously, efforts have highlighted parallel putative stressors in wild bees in the form of novel or outbreaking parasites (McMahon et al., 2018). While the interacting effects of poor nutrition and pesticide exposure are partly responsible for observations of parasite-driven declines, newly emerged parasites almost certainly play a part in underpinning wild bee health concerns and broadly negative population trends (Wilfert et al., 2021).

Pathogen spillover events are defined by a reservoir host population transmitting a disease to a novel host population. Many pathogen stressors of wild bees are the result of perpetual dead-end, or recent species-transfer, spillover events from either imported managed bees or managed bees that have acquired new parasites (Alger et al., 2019b; Manley et al., 2019b). Particularly in North America, this is a serious problem with migratory beekeeping for pollination leading to mass accumulation and transplantation of managed, non-native honey bees from geographically distant locations (Goodrich et al., 2019; Welch et al., 2009). Novel honey bee-associated pathogens are either regularly encountered by wild bees whilst out foraging (M. A. Fürst et al., 2014) or have been acquired recently before subsequent intraspecific transmission (Manley et al., 2015b) and exacerbated in severity by pesticide and nutritional stressors (Sánchez-Bayo et al., 2016). Evidence points towards the proximate role of infected managed honey bees in the spillover of these viruses (Graystock et al., 2020; Manley et al., 2019, McArt 2021). It is therefore clear that not only is honey bee health indirectly linked to wild bee health via shared stressors, but that the two are directly linked through the spillover of honey bee parasites into wild bee populations. Consequently, the ecological and evolutionary context of pathogen spillover must be included in responsible discussions of honey bee health.

To reduce unsustainable honey bee losses to *Varroa*, a focus has been placed on finding or breeding ‘better bees’ with higher survivorship has been the goal of both researchers and practitioners. Consequently, there is widespread academic and applied interest in ‘*Varroa*-resistant’ honey bee stock, seeking to overcome the challenges imposed on the beekeeping industry by this parasite (Le Conte et al., 2020; Noël et al., 2020, 2020; Spivak and Danka, 2021; Van Alphen and Fernhout, 2020) (Box 1). This is a worthy goal, often paired with concern over pesticides used in miticidal treatments (Bartlett, 2022; Toufalia et al., 2014, Delaplane et al. 2005). However, only recently has bee-breeding research been framed in the wider context of host-parasite systems in evolutionary ecology (O’Shea-Wheller et al., 2022). There has historically been a lack of differentiation between mechanisms of defense against parasites, including resistance versus tolerance. This distinction has, however, been discussed in the plant-herbivore literature (Rosenthal & Kotanen, 1994). As different strategies to improve honey bee health in the long term are embraced by practitioners and breeders at large, we must frame these exercises in directed evolution within broader paradigms of host-parasite evolution.

This review capitalizes on the rich external body of theory and experimental work underpinning the evolution of host defenses against parasites. We argue that the impact on wild bees through the spillover of honey bee viruses will differ based on the selection approach used by beekeepers. Survival can arise from either tolerating high levels of the mite, likely through tolerating viral infections, or reducing mite survival or fecundity, leading to small mite populations and low viral loads (Figure 2). ‘Darwinian’ beekeeping approaches were initially popularized by Tom Seeley (Seeley 2019; Radcliffe and Seeley 2021); Seeley’s Darwinian beekeeping approach sets a *Varroa* mite threshold for a colony, with colonies above that

threshold culled or otherwise prevented from breeding This is different to, but often confused with, survivor-stock selection (Blacqui re et al., 2019; Neumann and Blacqui re, 2017). Survivor stocks are defined as honey bees that can survive overwintering with varroa mites without any chemical interventions, which then subsequently have offspring that are also able to survive with varroa mites over winter. The unit of selection is on colony survival by any underlying parasite defense mechanism, rather than breeding for specific resistant traits. We explain why tracking parasite populations is critical while breeding honey bees, as the mechanistic differences between tolerance and resistance to parasites have far-reaching ecological consequences. Additionally, we highlight how tolerance is the most likely outcome of survivor-stock selection and is actually the mechanism by which purportedly ‘resistant’ honey bee genetic lines survive. Although both mechanisms lead to honey bee survival, breeding methods that do not monitor parasites can lead to evolving a highly tolerant host and subsequent ‘tragedies of tolerance’ (Box 3), potentially devastating the greater community of wild bees.

1.2.1 Box 1: Varroa, viruses, and honey bees

Varroa mites are an ectoparasitic mite of *Apis* spp. Historically a host species jump occurred from the Asian honey bee *Apis cerana* to the Western honey bee *Apis mellifera*, following which *Varroa* have spread nearly worldwide (Wilfert et al., 2016a). The original honey bee species host, *Apis cerana*, has co-evolved with *Varroa jacobsoni* for long enough to develop complex behavioral adaptations to resist this parasite. *Apis mellifera*, on the other hand, has none of these adaptations. *Varroa* have caused widespread losses of honey bee colonies in parts of the world (Le Conte et al., 2010), principally through their apparent vectoring of honey bee viruses (McMahon et al., 2018; Wilfert et al., 2021). Viral transmission putatively occurs during the parasite’s reproductive stage whilst feeding on developing honey bee pupae (Martin and Brettell, 2019b). Although pupae survive parasitization, once they emerge as adults, they experience highly virulent viral infections. A notable example is the physical deformities of bees caused by vectored Deformed Wing Virus (DWV) (Martin and Brettell, 2019b). While the parasitic vector load can be controlled through human management and chemical pesticides (Delaplane et al., 2005; Traynor et al., 2020), unmanaged or uncontrolled *Varroa* infestations are associated with high virus titers, loss of colony productivity, and highly elevated colony mortality (No l et al., 2020; Woodford et al., 2022).

Differentiating outcomes around parasite tolerance and resistance mechanisms is a necessary next step in bee health efforts worldwide. Box 2 describes how tolerance is understood as a strategy to reduce susceptibility to parasites by mitigating the costs of infection without reducing parasite load. It is fundamentally different from other forms of disease defense which rely on avoidance (reducing the likelihood of becoming infected to avoid fitness loss), resistance through control (reducing parasite replication upon infection to reduce fitness loss), or recovery (rapidly clearing parasites to overcome infection and reducing duration to reduce fitness loss). The evolutionary and ecological role of tolerance is well differentiated from these alternative strategies. Originally characterized in the plant literature (Rosenthal and Kotanen, 1994; Simms and Triplett, 1994; Stowe et al., 2000; Strauss and Agrawal, 1999; Tiffin, 2000, 2000b) it has in recent decades also been explored in animal pathogen defense (Baucom and De Roode, 2011; Medzhitov et al., 2012; R berg, 2014; R berg et al., 2009; Schneider and Ayres, 2008). There is now a rich body of theoretical evolutionary ecology underpinning our understanding of tolerance strategies, including how they arise and their consequences (see Box 2).

In this review, we synthesize the tolerance-resistance evolutionary and ecological theory and apply it to current efforts in honey bee management and discuss the unintended consequences for wider pollinator health. In particular, we argue that there is substantial risk to wild bees associated with the outcomes of a new emphasis on black-box ‘Darwinian beekeeping’ which breeds for survivor stock bees that are able to survive varroa infestations over winter while withholding acaricide treatments (Blacqui re et al., 2019; Neumann and Blacqui re, 2017; Robinson, 2019, Radcliffe and Seeley 2021). However, we also highlight how current and proposed culling programs and selection efforts for specific phenotypic traits can avoid the risks posed by tolerant honey bees, ultimately leading to healthier honey bee stocks and reduced risk for wild bees. Honey bee management could stand to be a major success story of intentional guided evolution by avoiding multiple ‘tragedies of tolerance’ (Miller et al., 2006).

1.3 The impact of honey bee viruses on wild bee populations:

Interspecific pathogen spillover is important in the context of tolerance where the tolerant species increases the force of infection, or the transition rate between susceptible and infectious. The field of invasion ecology has frequently highlighted the ability of an invasive species to devastate and displace a native competitor via transmission of a pathogen to which it is tolerant but the competitor susceptible (Holmes, 1982; Prenter et al., 2004; Dunn et al., 2012; Strauss et al., 2012). One of the most studied systems demonstrating this phenomenon is that of invasive, squirrel pox-tolerant grey squirrels (*Sciurus carolinensis*) in the United Kingdom, where they have displaced the native, squirrel pox-susceptible red squirrels (*Sciurus vulgaris*) (Rushton et al., 2006; White et al., 2014).

Certain pathogens are nearly ubiquitously found in honey bees (Bartlett et al., 2019), exemplified by the extremely high prevalence of DWV (Budge et al., 2015b; Natsopoulou et al., 2017b; Stamets et al., 2018). These conditions set the stage for high levels of potential interspecies transmission events (Manley et al., 2015b; McMahon et al., 2015b). Native bee species are at risk of pathogen spillover pathogens through foraging on shared floral resources (M. A. F rst et al., 2014; Graystock et al., 2016, 2016, 2015; Pritchard et al., 2021). This was first shown experimentally with *Crithidia bombi* (Durrer & Schmid-Hempel, 1994b). Correspondingly, studies have found evidence of ‘honey bee’ viruses throughout nearly all the major bee families including other members of Apidae, Halictidae, Andrenidae, and Megachilidae (Dolezal et al., 2016b; Levitt et al., 2013b; McMahon et al., 2015b; Piot et al., 2022; Singh et al., 2010b; Tapia-Gonz lez et al., 2019b, 2019b; Tehel et al., 2016b). However, detection of a virus does not necessarily indicate infection, nor the competency of that host. Active replication can be quantified by using primers specific for the negative strands of these single-stranded-RNA viruses. Replication of viruses in native bees has been demonstrated for ABPV, DWV, BQCV, IAPV, KBV, LSV, & SPBV (McMahon et al., 2018), but there is still very little known about which species act as primary host reservoirs of any of these viruses. Examining the phylogenetic relationship among the viruses extracted from different bee species, viruses do not cluster by species and instead cluster by geography, suggesting free dissemination of viruses between species (McMenamin and Flenniken, 2018b; Tehel et al., 2016b). DWV is the most common virus found in honey bees (Martin and Brettell, 2019b) and the bee virus that has been most frequently found in other insects thus far (Tehel et al., 2016b). Crucially, DWV prevalence in these species is correlated with DWV load and prevalence in the overlapping honey bee populations (Jones et al., 2021; Manley et al., 2015b; Murray et al., 2019b; Santamaria et al., 2018). This virus is understood to be of honey bee origin (Wilfert et al.,

2016a), providing evidence that the recent emerging epidemic of DWV has led to radiation from a common source of honey bees and *Varroa* into many other species, in part due to its prevalence and severity. DWV remains, however, one of many honey bee pathogens that show evidence of spillover into wild bees.

Assessment of the effect of these pathogens on non-*Apis* bees primarily comes from experimental inoculation studies of *Bombus*. Virulence of DWV in *Bombus* was evaluated through experimental inoculation that led to an increased host mortality rate (M. A. Fürst et al., 2014; Streicher et al., 2023; Tehel et al., 2022). However, a critical limitation of laboratory studies is that immune activation in bees is energetically expensive and therefore bees without *ad libitum* food are much more vulnerable to starvation when infected (Graystock et al., 2013b). Slow bee paralysis virus does not affect orally inoculated *Bombus* under satiated conditions, however, once starved the mortality rate increased by 1.6 times the control (Manley et al., 2017). This is critical for understanding the differential virulence of bee pathogens between wild and managed bees, as honey bees inherently have a nutritional advantage by virtue of active management compared to wild bees. This management includes providing food sources in times of drought or seasonal lulls in foraging (Noordyke and Ellis, 2021) as well as direct management of parasites and pathogens either through acaricide treatments for varroa (Locke et al., 2012), and, more recently a bee vaccine against the bacterial pathogen, American Foul Brood (Dickel et al., 2022). Consequently, human-mediated management means honey bees are likely much more able to tolerate infections compared to their wild counterparts. This conditional tolerance provides another mechanism by which pathogens that have evolved a less virulent relationship to their tolerant honey bee host may be highly virulent when they spillover into wild bees.

Declines of native bees coinciding with the introduction of managed bee species have been documented, notably with *Bombus dalhombii*, the Chilean giant bumble bee. Although once among the most abundant bee species found in Chile, it faced major declines after the introduction and successful invasion of commercially managed species *Bombus ruderatus* and *Bombus terrestris* (Montalva et al., 2011b). After introduction, *Bombus terrestris* became the most abundant *Bombus* species in the area, while *Bombus dalhombii* populations shrank (Morales et al., 2013). The spillover of the parasite *Apicystis bombi* has been implicated as the cause of *B. dalhombii* population declines. It was not found in the native *Bombus* species before the introduction of *Bombus terrestris* but was found at high prevalence thereafter (Arbetman et al., 2013b). Additionally, the infection wavefront outpaced the expansion of *B. terrestris* itself, meaning that the disease spread through the native *B. dalhombii* faster than the invading host could arrive. This spread of a novel disease and subsequent declines in native populations have been proposed as one of the mechanisms behind *B. terrestris* successful invasion, with *Apicystis bombi* opening the niche that the native bumble bee once filled. In another example, the dramatic declines of the rusty patch bumble bee (*Bombus affinis*), emblematic of endangered bees in the USA, are hypothesized as due to the spillover of *Vairimorpha bombii* (Tokarev et al., 2020) from managed *Bombus* colonies (Cameron et al., 2016b). Notably, *Vairimorpha* parasite species were one of the first demonstrations of invasive novel honey bee pathogens spilling over and causing disease in wild bees (Graystock et al., 2013b).

While improving honey bee health will not eliminate the risk of spillover of bee pathogens, significantly reducing the prevalence and severity of infection may reduce infectious propagule pressure on wild bees coexisting with managed honey bees. We expect that pathogen load and transmission success are linked as inter-species infection is understood to occur via

environmental transmission. Specifically, through foraging on contaminated floral resources after an infected bee collects resources and sheds viral particles onto the flower (Durrer & Schmid-Hempel, 1994b; Singh et al., 2010b; Van Wyk et al., 2023). Manley et al. (2019) compared viral load and prevalence in honey bees and bumble bees across 12 sites which included islands in the United Kingdom that differed in their presence of *Varroa*. Four *Varroa*-free islands were compared with three *Varroa*-present islands and five *Varroa*-present mainland sites. They found that *Varroa* presence led to higher viral loads and viral prevalence in honey bees, and this was associated with higher prevalence and load of those viruses in sympatric *Bombus* populations when compared to islands that did not have *Varroa*. Similarly, Alger et al., (2019 a, b) also looked at spillover of viruses between honey bees and bumble bees and found that the highest viral prevalence in bumble bees was associated with higher viral load in honey bees. ‘*Varroa* tolerant’ honey bees experience higher viral loads and are likely exerting more severe propagule pressure on wild bees. This ultimately can lead to more spillover events and higher viral prevalence in co-foraging bumble bees.

1.4 Tolerance versus resistance as distinct host defense mechanisms:

While the study of resistance versus tolerance evolution began in plants (Box 2), there has been a recent integration of tolerance evolution, ecology, and immunology in animals (Baucom and De Roode, 2011) alongside resistance. The mechanisms underpinning tolerance and resistance evolution are not well understood, but advances have come concerning its integration with immunology (Graham et al., 2005; McKean et al., 2008; Råberg, 2014; Råberg et al., 2009; Soares et al., 2017), including in insects (Schneider and Ayres, 2008). Through synthesizing these studies and the rich body of theory outlined in Boxes 2 & 3, we can make important predictions on how resistance and tolerance are likely to manifest in an applied system and the subsequent ecological and evolutionary consequences. We focus on tragedies of tolerance: ecological and evolutionary phenomena associated with tolerance (but not resistance) which in applied contexts are broadly negative.

1.4.1 Box 2: The conceptual basis of the evolution of host defenses to pathogens.

Broadly, host defense can be divided into one of three categories: (1) avoidance, where hosts minimize their chance of either encountering or being infected by a parasite (infections are reduced); (2) control/clearance, where hosts limit parasite population growth to reduce the cost of being infected (infections are less severe/shortened); (3) tolerance, where hosts mitigate the costs of being parasitized without altering the infection course (infections may be common and parasite loads high, but fitness costs small to hosts). Of these, control/clearance are often mechanistically linked and are conceptually very similar, and avoidance/ control/clearance may be grouped as ‘resistance’ since they all reduce pathogen fitness while tolerance does not.

Tolerance is the most distinct of these from an evolutionary and ecological perspective as it is the only host strategy that reduces susceptibility without imposing a fitness cost on the parasite (Boots and Bowers, 1999; Vitale and Best, 2019). Moreover, the evolution of tolerance is self-reinforcing if it lengthens the infectious period of the host. As tolerance strategies become more common in a population, parasites become more prevalent, the force of infection (‘parasite pressure’) therefore increases, which strengthens the selection pressure on non-tolerant hosts to evolve to further increase their tolerance to the parasite. This is in stark contrast to the other classes of resistance introduced above. In cases of resistance evolving, costly resistance

mechanisms experience negative frequency-dependent selection, as a partly resistant population reduces the force of infection by reducing transmission, thus lowering the selection pressure on susceptible hosts to defend themselves from the parasite (Miller et al., 2005). The fixation of tolerance strategies is therefore much easier than other resistance strategies (Boots and Bowers, 1999; Vitale and Best, 2019). The difference between tolerance and resistance mechanisms is more profound where coevolution is considered, as parasites experience strong selection to overcome avoidance, clearance, or control strategies. Conversely, tolerance of a host does not reduce parasite fitness and so does not participate in ‘arms-race’ coevolutionary dynamics.

As in Box 3, the original ‘tragedy of tolerance’ was the observation that whilst individual infection-mortality rates will substantially decline as a host population evolves tolerance, the much higher infection rates associated with tolerant populations may mean that the total number of deaths from infections across a tolerant population is higher than in a susceptible population (Best et al., 2014; Miller et al., 2006). Managed honey bees live at such high densities as to always achieve a high prevalence of infection (Bartlett et al., 2019), and therefore this original single-population tragedy of tolerance has clear implications for spillover in the honey bee virus system whereby the force of infection experienced by non-*Apis* wild bees increases as tolerance in honey bees evolves. In addition, the evolution of pathogens in their tolerant host is likely to lead to higher virulence when infecting a different species (Long and Boots, 2011). These predictions are well established in the theory of tolerance evolution (Day et al., 2007; Miller et al., 2006), and have been investigated in specific cases in applied systems such as malaria (Long et al., 2008; Long and Graham, 2011) and more recently in bat viruses (Brook et al., 2020; Brook and Dobson, 2015; Guth et al., 2022) where tolerance of pathogens in bats, enabled by specific immuno-physiological adaptations, leads to elevated virulence of those pathogens when they spill over into other hosts. We explain the theoretical basis for these tragedies in Box 3 verbally and posit that the honey bee – wild bee virus spillover system is an ideal case to test these predictions about the nature of tolerance in underpinning virulent cases of disease spillover.

1.4.2 Box 3: Three Tragedies of Tolerance

Tolerance has been shown to be easy to evolve to fixation. With tolerant hosts, parasites do not experience any fitness loss from this strategy, thus co-evolutionary arms race dynamics that are often thought of in host-parasite evolution do not occur (Miller et al., 2006). However, tolerance is also associated with negative ecological outcomes, which have been dubbed ‘tragedies of tolerance’ (Best et al., 2014). These tragedies of tolerance are all relevant to the ecological outcomes of honey bee breeding efforts.

1. **Increased mortality at population level:** Miller et al. (2006) demonstrated that if host-parasite systems do not co-evolve toward full commensalism, then while tolerance strategies may reduce case mortality (likelihood of any given infection killing the host) they can increase the absolute parasite-driven mortality through increased total prevalence across the whole population. Tolerance can therefore represent an evolutionary end-state where more individuals are dying from a parasite than was the case when the population was fully susceptible to the pathogen.

2. **Increased prevalence and infection rate:** While this original ‘tragedy of tolerance’ focused exclusively on the focal host population, additional tragedies are apparent from the evolutionary response of parasites to a tolerant host. Miller et al. (2006) also detail that in response to host tolerance, parasites may evolve higher replication rates due to the removal of virulence costs of high replication rates under the widely accepted virulence-transmission trade-off framework. An example of this is seen in virulence modulated through immunopathology, whereby evolving tolerance reduces immune response to the pathogen even at high replication rates (Day et al., 2007; Long and Boots, 2011). Ultimately this leads to tolerant hosts being able to withstand higher infection rates, leading to increased prevalence of infected focal hosts.
3. **Higher spillover risk:** The final tragedy lies in the infectiousness of each infected host being higher due to elevated pathogen loads, therefore leading to the potential for increased spillover risk to other species. This spillover context also underpins a further evolutionary tragedy of tolerance. Higher parasite replication rates are typically associated with greater virulence of infection (Alizon et al., 2009; Anderson and May, 1982; Bull, 1994; De Roode et al., 2008) when transmitted to non-tolerant hosts. Consequently, parasites adapted to a tolerant focal host may experience much higher rates of viral replication when they find themselves infecting non-focal hosts, leading to more severe pathology (Best et al., 2014; Miller et al., 2006).

1.5 Tolerance and resistance to viruses and mites in honey bees:

A key consideration of the honey bee disease interaction is that in addition to viruses, honey bees are parasitized by mites that may also vector the viruses (Box 1). Honey bees can evolve tolerance and resistance mechanisms to both the varroa mites and the viruses they vector. In Figure 2, we outline the ways to breed honey bees for defense against *Varroa* by looking at the tolerance and resistance options for both *Varroa* and viruses. In this simplified example, honey bees are assumed to start off susceptible to both and then are selected to be either resistant or tolerant of viruses, and resistant or tolerant of *Varroa* (Fig. 2). This is an extension of current frameworks (Penn et al., 2022; Van Alphen and Fernhout, 2020) to avoid the pitfalls of ‘black-box’ survival-based selection (Blacquière et al., 2019). While each of these combinations is worth consideration, we will focus on the resistant/resistant or tolerant/tolerant outcomes as the most likely.

(1.) Virus resistant, *Varroa* tolerant.

There is circumstantial evidence from the Brazilian island Fernando de Noronha that honey bees successfully tolerate high mite infestation rates due to an apparent lack of replicating vectored virus in the population (Brettell and Martin, 2017). A similar example has been documented in Papua New Guinea, where the absence of DWV leads to a new low-virulence *Varroa* spillover into *Apis mellifera* (Roberts et al., 2020). These examples illustrate the fundamental concept of how an absence of significant viral replication (through avoidance, clearance, or control of viruses) allows for the tolerance of *Varroa*, as most of the virulence-associated with *Varroa* is through its interaction with viruses (Di Prisco et al., 2016; Traynor et al., 2020). However, the evolutionary stability of this state is doubtful. *Varroa*-associated viruses are rapidly evolving and re-emerging pathogens (Ryabov et al., 2019a; Wilfert et al., 2016a) and we would expect from evolutionary theory for there to be strong selective pressure on them to overcome resistance

mechanisms in the honey bee host, should adequate variants in the virus population arise. The putative immunosuppression of honey bees by *Varroa* also makes this outcome unlikely from a mechanistic standpoint (Yang and Cox-Foster, 2005).

(2.) Virus tolerant, *Varroa* tolerant.

Conceptually very similar to circumstance (1.), honey bees that experience little virulence from viral infection are tolerant of *Varroa* by extension. Theoretically, this is the expected / likely outcome of natural selection as we outline in Box 2, in part due to its fundamental evolutionary stability. However, this carries substantial risk for wild bees. Recent work from isolated wild populations of *Varroa* & virus parasitized honey bees demonstrate this evolutionary outcome in the ‘wild’ (Thaduri et al., 2019). They show that if their ‘mite-resistant’ Gotland bees are challenged with viral inoculum, they are infected by viruses at the same rate as mite-susceptible bees and show infections with the same viral titers as mite-susceptible bees, but do not suffer the same viral infection-associated mortality rates. As such, they tolerate *Varroa* infestations by not suffering pathology from prevalent high-titer viral infections. This is a demonstration of the evolution of parasite tolerance (Locke et al., 2014). Further evidence of this viral tolerance has been recently observed in feral ‘survivor / Darwinian’ bees in the U.S. (Bartlett et al., 2021; Hinshaw et al., 2021), where feral honey bee colonies may exhibit elevated viral titers despite predictions following the logic of resistance-evolution reasoning (Brosi et al., 2017). Even more recently, Penn et al. (2022) demonstrated this same principle by comparing different breeds of *Varroa*-resistant bees and building on the work of Khongphinitbunjong et al., (2015), whereby Pol-line bees bred to exert strong control on *Varroa* were highly susceptible to DWV infections, whereas Russian bees (originated initially as a ‘survivor stock’ line from eastern Russia, and since maintained as a breeding program) were tolerant of those same DWV infections, exhibiting the same viral loads but far less severe pathology. Overall, from both theoretical understanding and evidence from wild bees, we expect this outcome of tolerance from ‘survivor stock’ breeding programs (Blacqui re et al., 2019; Neumann and Blacqui re, 2017).

(3.) Virus tolerant, *Varroa* resistant.

This unlikely combination of viral tolerance and *Varroa* resistance is a highly speculative incident inspired by comments made in (Mondet et al., 2014). They posit that during the invasion of *Varroa* in New Zealand, *Varroa* populations declined because of mortality associated with the extremely high incidence of DWV. Parasitic arthropod vectors of pathogens are known to also be engaged in an evolutionary arms race with the pathogens they vector (Mellor, 2000; Oliveira et al., 2020), which could limit their populations if they are intolerant of infection. Hypothetically, honey bees tolerant of viruses that are virulent when infecting parasitic *Varroa* may be resistant to *Varroa* parasitization as a result; comparable observations have been made in other systems (Norris, 1999). While we consider the likelihood of this circumstance to be low, (Ryabov et al., 2022) recently showed that *Varroa* mites infected with and more competently vectoring DWV have significantly reduced lifespans compared to their counterparts.

(4.) Virus resistant, *Varroa* resistant

This double-resistant outcome is traditionally what bee breeding efforts are allegedly pursuing to minimize mite-associated losses. As it stands, beekeepers control *Varroa* through miticidal chemical applications to prevent vector-borne transmission of virulent viruses (Delaplane et al.,

2005; Traynor et al., 2020). It is intuitive to breed bees that control *Varroa* on their own and maintain low mite numbers without chemical treatment. If honey bees evolve adaptations to control *Varroa*, the resistance to the mite will by proxy grant resistance to the viruses via the avoidance mechanism (see Box 2). If achieved, it may hold promise rather than risk for wider bee health by decreasing viral prevalence and load in honey bees, thus decreasing the potential force of infection in the native pollinator community.

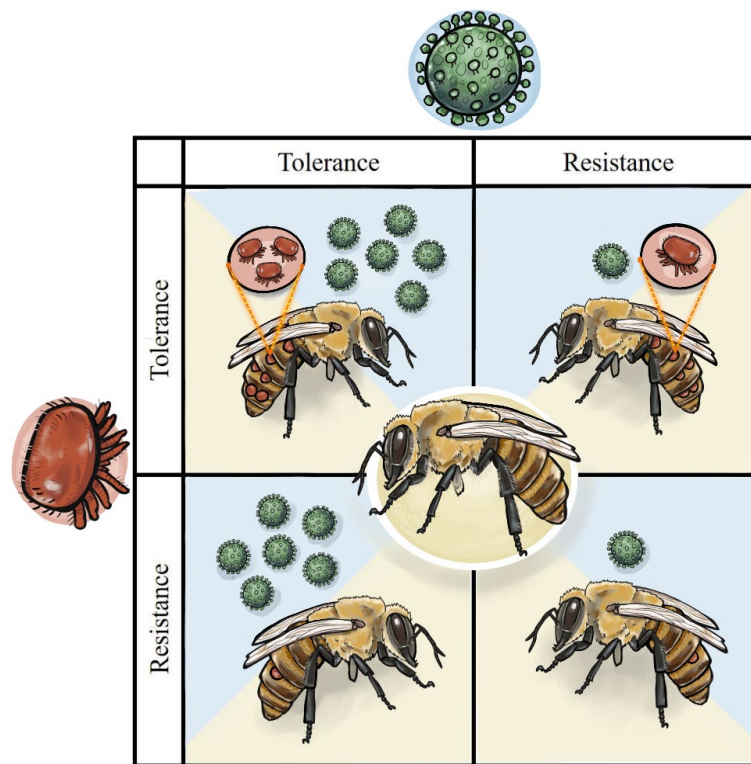


Figure 2. Graphical summary of the mechanistic combinations possible in resistance/tolerance to *Varroa*/viruses in honey bees.

Thaduri et al. (2019) and Penn et al. (2022) confirm the arguments and framework explained above through their examination of the mechanisms underlying honey bee survival of *Varroa* parasitization. They highlight the evolution of tolerance in current honey bee breeding regimes and detail that the Gotland honey bees survive viral infection at much higher rates despite the same infection intensity as susceptible honey bees. Further evidence has also shown honey bee tolerance of viral infection confers tolerance of *Varroa* infestation (Fig. 2) (Hinshaw et al. 2021; Bartlett et al., 2021). These studies, however, do not nest this issue in the broader theory underlying the evolution of tolerance versus resistance, nor do they examine the context of spillover consequences to native bees. The Gotland natural experiment exemplifies the outcome of ‘survivor stock’ breeding through undirected natural selection leading to the evolution of tolerance. This is in agreement with the core theory we have described so far and thus of serious concern to wild bees (Locke et al., 2014). Similarly, Penn et al. (2022) document how lineages of bees bred to survive *Varroa* parasitization do not differ in their resistance to DWV infection but do differ in their tolerance. Notably, the lineages they examine which were

selected specifically for low *Varroa* populations (Pol-line bees) show low tolerance to viruses, while lineages originated from what was initially an imported ‘survivor stock’ population (Russian bees) show undifferentiated viral titers, but much higher tolerance.

Whilst interest amongst beekeepers in survivor stock has been spurred on by a focus on an amorphously defined popular concept of ‘Darwinian beekeeping’, tolerance is not the only outcome of this process (Seeley 2019; Radcliffe and Seeley 2021). Seeley’s approach to Darwinian beekeeping does not choose or identify specific mechanisms, such as behavioral traits or physiological indicators, to selectively breed for (Seeley 2019). Instead, the phenotype being selected for is simply ‘low *Varroa* parasitism rates’, achieved by choosing a mite infestation threshold beyond which he deems a colony ‘doomed’ and culled, or not used in further breeding regimes. Seeley presents this as an ‘acceleration’ of Darwinian natural selection. Crucially, Seeley’s breeding paradigm does not act in the same way as natural selection via survivor-stock as described by other honey bee researchers (Neumann and Blacqui re 2017; Blacqui re et al. 2019), and the evolutionary outcomes will not be the same. By selecting for low mite parasitic load, Seeley’s culling approach cannot permit the evolution of tolerance of mites in honey bees, only resistance. Currently, Seeley’s efforts in popularizing selection approaches for better bees (Radcliffe and Seeley, 2021) have been lost to a broader survivor-stock understanding of Darwinian beekeeping. We predict these will have very different outcomes to Seeley’s specific mite-threshold-culling approach that will only select for resistance.

The mechanistic underpinnings of how Seeley’s breeding program achieves mite resistance remain open to the selection process. However, resistance can be bred by selecting specific behavioral-immunological phenotypes to control (resist) mites. Behavioral social-immunity mechanisms have been longstanding targets for selective breeding of honey bees in the face of *Varroa* (B chler et al., 2010; Rinderer et al., 2010). For example, *Varroa*-sensitive-hygiene includes a suite of behaviors in which the honey bees can detect infested brood, uncap infected cells, and remove parasitized brood (Panziera et al., 2017; Toufailia et al., 2014; Tsuruda et al., 2012). Additionally, ‘mite-biter’ bees can bite and damage the mites to the point of decreasing the mite’s fitness (Morfin et al., 2020). By breeding for these particular behaviors, beekeepers can actively select for resistance mechanisms which actively clear *Varroa* (see control, Box 2).

We must note that the mechanisms associated with defense against *Varroa* mites go against the current needs of commercial beekeepers. This includes smaller colony sizes and an increased propensity to swarm (Loftus et al., 2016; Mondet et al., 2020). Breeding bees that have characteristics of *Varroa* resistance and viability in the agricultural system poses a significant challenge. However, advances have been made, including the identification of genetic markers for some of these resistance traits (Sainsbury et al., 2022) that can be used as part of selective breeding regimes. This highlights the achievability of aiming for truly resistant bees. Again, these *Varroa*-control selective efforts should only lead to ecologically responsible resistance-resistance outcomes (Fig. 2). Generalizing these principles to other scenarios is conceptually simple (Van Alphen and Fernhout, 2020). As momentum builds amongst beekeepers and honey bee scientists in exploring this topic, our understanding of resistance traits in honey bees only grows. For example, heightened immunity amongst developing bees has been directly linked to reduced *Varroa* fecundity, thereby helping control mite levels (Annoscia et al., 2020; Erez et al., 2022; Scaramella et al., 2023; Von Virag et al., 2022). This high degree of understanding

provides multiple avenues for bee breeders to be purposeful and specific in their selection regime, and by virtue of that understanding and purpose, avoid the tragedy of tolerance.

1.6 Conclusion:

The evolution of parasite tolerance in honey bees risks three tragedies (1) For honey bees, tolerance represents an evolutionary end-state where more individuals are dying from a parasite than was the case when the population was fully susceptible. (2) Spillover incidence will increase to native bees in the community due to the greater number of infected honey bees in the landscape, each being more infectious due to higher pathogen replication rates and (3) When spillover occurs from honey bees to wild bees, the infection is more virulent in wild bees. Unfortunately, tolerance of *Varroa* and corresponding viruses is the likely outcome of current ‘survivor stock’ selection regimes. However, these risks to both managed and wild bees can be circumvented by the selection for resistance to *Varroa* in managed honey bees. Resistance is the expected outcome of programs selecting for specific bee phenotypes such as hygienic bees and mite-biters. The evolution of resistance is also the predicted outcome for ‘Darwinian beekeeping’ the method of culling colonies above a certain *Varroa* infestation threshold championed by Tom Seeley. While tolerance is dangerous, and, if left unaddressed, a very likely outcome in the honey bee system, it is not inevitable. Beekeeper education on why certain breeding programs and approaches are more ecologically responsible may allow this critical livestock industry to both avoid the tragedies of tolerance for wild bees and mitigate current honey bee losses. Rapid gains in mechanistic understanding of honey bee resistance phenotypes make intentional outcomes in selective breeding very achievable for the industry and provide a conceptually fruitful arena for future work examining in detail how these breeding choices impact the spillover of viruses.

1.7 Author’s contributions:

NAS and LJB conceived and coordinated this review. NAS drafted the manuscript and illustrated the figures. All authors contributed to the final writing and editing of the manuscript.

Chapter 2: Honey bee virome dynamics during a mass pollination event

2.1 Abstract:

Each year, nearly 1.8 million hives are shipped to California's Central Valley to pollinate nearly the entirety of the world’s supply of almonds. This human-mediated “migration” clearly poses a risk of spreading and mixing viral pathogens from across the United States during the bloom itself, and after the bloom finishes and honey bees are moved to their next destination. Longitudinal studies monitoring individual colonies during the actual almond bloom are rare but necessary to understand the spatiotemporal patterns of bee viruses. This study generated a time series of viral richness and individual viral species dynamics in hives participating in the almond bloom compared to hives that were maintained at a control site approximately 100 kilometers away from the almond groves. The findings indicate that viral richness was significantly higher in the almond-pollinating bees than in the control hives before, during, and after the almonds bloomed. However, when the pollinating hives returned to the same site as the control hives, the viral richness in the control hives increased significantly, surpassing that of the almond hives at that time. This suggests that the viral diversity experienced in the almonds was transmitted to the controls after the bloom finished and the hives reunited. Individual viral taxa exhibited unique

spatiotemporal dynamics, with some viruses being exclusively associated with the almond-pollinating bees and others with the control hives. Viruses also showed very different temporal dynamics during the bloom. This chapter highlights some of the potential impact of long-distance mass hive translocation on pathogen emergence in honey bees by tracking the temporal changes in the honey bee virome during the almond bloom in California.

2.2 Introduction:

Migrations have the potential to enhance the geographical spread of pathogens and increase interspecies transmission (Altizer et al., 2011), but surprisingly few examples of long-distance pathogen dispersal by migrating animals have been documented in the published literature (Altizer et al., 2011; Morgan et al., 2007). Whether the net effects of migration will increase or decrease prevalence depends in large part on the mode of parasite transmission and the level of host specificity, both of which will affect opportunities for cross-species transmission at stopover sites. Generalist pathogens especially will likely increase in prevalence if there are opportunities at stopover sites for cross-species transmission (Altizer et al., 2011).

Each season the majority of commercially managed honey bee colonies, nearly 1.8 million hives, are transported to the Central Valley to pollinate California's almonds (Ferrier et al., 2018; USDA-NASS, 2018). Each acre of almonds requires two honey bee hives for pollination (Carman, 2011). Honey bees will regularly fly over 3 km away from their hives to forage which means that bees in a large orchard could share forage and indirectly interact with bees from thousands of other colonies (Alger et al., 2018). The viral pathogens of interest are highly generalist, infect a wide range of insect taxa, and therefore pose high risks of transmission to other bee species at stopover sites (McMahon et al., 2015a). Although there is concern about what migratory practices, in general, do to honey bee health and exposure to novel pathogens and parasites, few studies show the negative impact of commercial hive movement on disease emergence (Alger et al., 2018). Longitudinal studies that monitor individual colonies throughout the almond bloom are rare (Faurot-Daniels et al., 2020), with most assessing health at the total apiary level looking at viruses across all hives rather than looking at individual hive dynamics. Other studies have shown that migratory bee colonies face heightened degrees of oxidative stress levels during migratory events which in turn could be negatively impacting the immune function of individual bees (Simone-Finstrom et al., 2016). Alger et al., (2018) conducted an experiment where "migratory" hives that pollinated the bloom were exposed to stationary hives that did not participate in the bloom. The exposed hives experienced higher deformed wing virus (DWV) load and prevalence one month after foraging next to migratory hives than the isolated hives that did not come into contact with almond bees. *Varroa destructor*, the parasitic mites that vector many of the major honey bee viruses (Wilfert et al., 2016b), was not explanatory of these differences, as parasite burden decreased in the migratory hives and were the same between isolated and exposed hives. One explanation is that the migratory honey bees returned with a more virulent strain of DWV that quickly spread through the new host population as the exposed group had higher *Varroa* numbers than the migratory group. To determine if this is true, the genetic variation between DWV strains needs to be quantified through targeted sequencing. Previous studies have described the viral genome of DWV as nearly clonal (Martin et al., 2012; Ryabov et al., 2014) making it hard to infer phylogenetic relationships from those variants. However, recent work has shown that DWV in the United States has a surprisingly high level of heterogeneity, suggesting a post-bottleneck expansion of genetic diversity (Ryabov et al., 2019b). It is important to look at strain diversity as differences in viral genotypes that coexist in a

single population could be due to diversification via selection by the host's immune system (Ryabov et al., 2019b). Additionally, recombination events between DWV strains A and B have been associated with increases in virulence levels as documented in the UK (Ryabov et al., 2014). Therefore, it is important to track the changes in genotypic diversity of the honey bee virome to monitor what managed bees are being exposed to in the bloom. Additionally, a newly discovered virus, Lake Sinai virus (LSV), is highly prevalent during these blooms, with unknown consequences on the honey bees or other bee species (Daughenbaugh et al., 2015). Some evidence points towards negative health consequences for infected hives (Faurot-Daniels et al., 2020) but overall there is no one stressor associated with poor hive performance.

Although other studies have characterized honey bee viral prevalence and load before and after almond blooms, they did this at a coarser time scale (Alger et al., 2018; Glenney et al., 2017), and only two were sampled during the pollination event itself (Cavigli et al., 2016; Faurot-Daniels et al., 2020). Fine-scale temporal tracking of viruses throughout the almond bloom has not been done, nor have the contemporary phylogenetics of viral strain diversity. Overall, there are few studies monitoring individual commercial honey bee hive viruses at a transcriptomic scale through time. This resolution is necessary to monitor potentially novel and virulent viral strains associated with the mass movements of honey bee hives for crop pollination. (Runckel et al., 2011) found the dynamics to be sporadic cases of acute infections that hives were able to clear infections after brief periods of time, with BQCV being the only virus found to persist across time points as a chronic infection. (Alger et al., 2018) also saw BQCV persisting across their experiments. A study that sampled in February and then again in March showed that their hives had the lowest pathogen prevalence during the almond bloom itself, but then the total number of pathogens sharply increased after the bloom (Cavigli et al., 2016). Certain viruses have been shown to be prevalent before and throughout the bloom such as BQCV and LSV (Cavigli et al., 2016). Faurot-Daniels et al. (2020) followed a cohort of 50 individual hives before, during, and after the bloom across the span of a year. They found the highest total number of pathogens per colony a month after almond pollination. This correlated with seasonal buildups of hive population numbers and increased springtime foraging in the almond bloom and beyond. Although little is known about Lake Sinai Virus, they found a correlation between increased LSV2 abundance in weaker colonies, suggesting it is a pathogen of concern. DWV prevalence and load were highest in the fall, LSV was higher in the spring, and BQCV and SBV were both present throughout.

My study worked to understand the consequences of long-distance human-mediated mass movement of crop-pollinating hives on pathogen emergence in honey bees. This was done by quantifying the temporal changes in the honey bee virome throughout the almond bloom in comparison to hives that do not participate in crop pollination. A longitudinal survey was conducted following a cohort of 32 honey bee hives across four-time points in the Spring of 2021. Sampling occurred before, during, and after participation in almond bloom in California while in parallel 'control' samples were collected from hives that did not go to the bloom. A viral time series was generated from the resulting mRNA sequencing data. This allowed me to quantify community diversity metrics to investigate viral richness and evenness, and to quantify individual viral dynamics through their relative abundance and differential expression. I was interested in understanding what the impact of participating in the almond pollination event is on the viral dynamics of bees by comparing them to hives that did not go to bloom and 2) the seasonal dynamics of viral infections through time.

In this chapter, I tested the following hypotheses:

Hypothesis 1: Viral species richness changes through time, with increasing richness after the almond bloom in the almond-pollinating bees.

Hypothesis 2: Viral richness will show spatial heterogeneity, with almond-pollinating bees having higher species richness than controls due to the stressful act of being transported to the orchards and subsequent exposure to a high density of novel honey bees.

Hypothesis 3: The relative abundance of different viruses will show different dynamics through space and time, with some viruses being associated with the almond bloom and others not.

Alternatively, the evidence could show that the bloom is not associated with increases in viral diversity. This could occur as almond pollen is known to be nutritious and thus could bolster honey bee immune function through the bloom (Loper et al., 1980). On the other hand, a diversity of pollen in a honey bee diet is associated with increased immune competence (Di Pasquale et al., 2013) and evidence has been shown that honey bee health can be compromised due to the nutritional limitations of monocultures (Naug, 2009). Additionally, due to the high density of blooming flowers, there could be a dilution effect where the likelihood that any two bees visit the same flower and are exposed to novel virus strains is decreased (Piot et al., 2022).

According to disease theory, spatial structure and changing population connectivity can have impacts on the evolution of virulence or the deadliness of a pathogen. Increased connectivity between individuals can lead to higher levels of pathogen infectivity and virulence (Boots and Sasaki, 1999). Imagine this as a spatially structured population of susceptible hosts and a focal pathogen that can transmit within and between spatially clustered hosts. Lower connectivity means pathogens are spread locally, within a cluster, and have a low chance of infecting a separate host cluster. Higher connectivity associated with global transmission means there is a higher chance of the pathogen successfully jumping between host clusters. This increased connectivity has been hypothesized to allow for the evolution of increased virulence as local transmission limits how deadly a pathogen can be before running out of susceptible hosts within the local cluster and the pathogen dying out by proxy. Under global transmission, pathogens are not constrained by the limited local pool of susceptible hosts and therefore can evolve to be more virulent (Boots and Sasaki, 1999). The theory would suggest that this mass movement of hives has a chance of selecting for more virulent pathogens, many of which are generalist and found replicating in a wide range of species (Tehel et al., 2016). This would pose a considerable risk of interspecies transmission at honey bee stopover sites and justifies the need for monitoring through surveillance transcriptomic testing.

2.3 Methods:

Longitudinal sampling of honey bees was conducted in the almonds versus control hives in February of 2021. All the ‘treatment’ hives were in an orchard within Arbuckle, CA (Lat: 39.0135, Long: -122.0555) and sampled between: [02/09/21 – 04/20/21]. This resulted in samples across four time points, two weeks before the bloom (T1), during the bloom (T2), two weeks after the bloom (T3), and then a final time point when the almond and control hives were brought together three weeks after the bloom (T4). At the same time as this almond sampling, control hives located in Grass Valley, CA (site = 112 kilometers away from the focal almond

orchard) were sampled. These hives were maintained by the same beekeeping operation as the experimental almond orchard hives which is important as beekeeping operation and management practices have been shown to be a significant contributor to variation (Glenny et al., 2017; Martínez-López et al., 2022). Almond growers need approximately two bee colonies per acre to pollinate their crops. The spatial composition of the orchard consisted of approximately 25 hives per 500 meters in clustered “blocks”. All the treatment hives were within a 3 km radius of one another, as were all of the control hives. The control hives were in the foothills of the Sierra, isolated from direct interactions with monoculture crops. A total of 32 treatment hives were sampled through this time all from the same beekeeping operation across six blocks. Due to constraints with the focal beekeeping operation, the control hive sample size was low. In total, only four hives were able to be left behind from the bloom to meet the pollination contract. This number dropped to three after one hive died before the final sampling event.

Samples of foragers were collected by using a hand vacuum at the hive entrance, using a hive-blocking tool to minimize the aggression of guard bees. After approximately 50 foragers were collected, the vacuum was placed onto dry ice. From there, individuals were sampled into a 50 ml falcon tube, with a subset of five honey bees per hive placed into a single 2 ml cryovial tube for later viral quantification. Samples in falcon tubes were placed on dry ice during transport, while the cryovial samples were placed in a liquid nitrogen dewar before being stored at -80 degrees. Five foragers per hive were pooled together by homogenizing their tissue in bead-beating tubes containing a single steel ball and zirconia beads (4m/second; 30 seconds; 3 cycles; 20-second dwell) (Runckel et al., 2011). From there the RNA was extracted using Trizol protocols (Manley et al., 2020) and cleaned using an RNA clean and concentrator kit (Zymo). DNA was removed from the samples using TURBO DNA-free DNase Treatment (Thermo-Fisher). Sample RNA quantity and quality were quantified using a range of methods, including Nanodrop, Qubit, and Bioanalyzer, after which samples that passed quality control checks were sent off for mRNAseq on the Illumina NovaSeq PE150 machines through the company Novogene (Moore et al., 2011; Ryabov et al., 2019b). A poly-A tail enrichment step was done during library prep as a way to preferentially sequence RNA viruses (along with host mRNA) that often have long 3’poly(a) tails. In total 32 treatment hives and four control hives were sampled through time and a total of 131 mRNA libraries were generated.

2.4 Data Analysis:

CZID was used for initial bioinformatic quantification methods (Kalantar et al., 2020). From the processed data from CZID, combined sample taxon results were downloaded looking at the nucleotide total reads (NT r) as well as the reads per million (RPM) for all taxa in my samples. The data were filtered specifically for taxa that had >10 mapped reads present in each sample (Chen et al., 2023). From there, viruses were the specific focus for this chapter, but this RNAseq data possesses broad information on both the host transcriptomics as well as non-viral pathogens that could be of interest for future analysis. First, a taxonomy table was generated to describe the taxonomic classification of each of the focal viruses. This table focuses on qualitatively describing the taxonomic ranks which included Kingdom, Phylum, Class, Order, Family, Genus, and Species. Next, a viral OTU table was generated which quantified either the total read counts for each of the viral species, or the relative abundance (number of reads per million), for each hive sample at each time point. Taxonomic classification was carried out by assigning a rank to each ahead and then querying that dataset against a reference database to assign taxonomic identifiers (Kadlečková et al., 2022).

Viral species richness can be quantified through alpha diversity metrics which look at the diversity of species within a single sample, such as Shannon's diversity Index and Simpson's Diversity Index. Shannon diversity gives more weight to rare species. Simpson's diversity index weighs evenness more heavily in the calculations and is more sensitive to common species (Roswell et al., 2021). Additionally, beta diversity metrics allow for the comparison of microbial communities by measuring the degree of change in viral species composition between hive samples. Viral relative abundance is defined as the ratio of the number of reads in a sample that belongs to a specific viral OTU to the total number of reads in the sample. Total reads or the raw counts from RNAseq data were the primary data used for analysis. This also retains an absolute abundance of taxa in each sample. However, these counts are intrinsically biased by differences in library size and sequencing depth which would alter the total number of reads present between samples. Therefore, it is common practice to use a normalized metric, including reads RPM to normalize these differences. This is necessary for beta diversity metrics where relative abundance is compared between samples (Chen et al., 2023).

All data analysis was conducted in R Studio. BiocManager was used to install Bioconductor packages which specifically focus on bioinformatics (Morgan and Ramos, 2024). This package also ensures that the packages and their dependencies are correctly installed. With these data, I aimed to determine what the differences were in viral communities through time and across spatial scales. First, I calculated alpha diversity metrics (Shannon index, Simpson index) to estimate within-sample diversity and beta diversity metrics to estimate between-sample diversity. Alpha diversity metrics were calculated using 'phyloseq' for both Shannon and Simpson diversity to quantify the viral biodiversity of these hives. Phyloseq is a package that is specifically designed for high-throughput microbiome RNAseq data (McMurdie and Holmes, 2013). This was used to calculate diversity indices, transform read count data to relative abundances, calculate ordinations, and plot the results. These measures include species richness (number of viral species) and evenness (relative abundance of viral species). From there, alpha diversity metrics were plotted through space and time to visually assess differences using ggplot2 (Wickham, 2016). Beta diversity was calculated using normalized read per million data (RPM), the 'phyloseq' package, and the 'vegan' package (Oksanen et al., 2024). First, the data was transformed into relative abundance to calculate the Bray-Curtis dissimilarity matrix from the viral OTU matrix to get a dissimilarity value in comparisons between hives. Multivariate techniques were used to visualize the differences between samples in reduced dimensions using the 'vegan' package (Koch et al., 2018; Oksanen et al., 2024). A Principal Coordinates Analysis (PCoA) was conducted to visualize the dissimilarity matrix between samples using distance metrics. Non-metric Multidimensional Scaling (NMDS) was done to visualize the dissimilarity matrix using rank differences as another way of assessing viral community composition differences. To incorporate weights into the dimensionality reduction process, Weighted Classical Multidimensional Scaling (WCMDscale) was calculated on the Bray-Curtis dissimilarity matrix and plotted. To identify the clusters within this WCMD plot and to continue looking for any explanation for Beta diversity differences, hierarchical clustering was done using the base R stats package and Euclidean distance between the MDS coordinates. This hierarchical clustering was visualized through a dendrogram.

To begin assessing community composition differences between times and location, relative abundance was calculated for the RPM data and plotted for different taxa across samples (Koch et al., 2018). Relative abundance was graphed across a range of taxonomic groups using

‘ggplot2’ comparing spatiotemporal patterns. Next, a heat map with differential abundance comparisons between all samples was generated using ‘pheatmap’ (Kolde, 2018). The DESEQ2 package (Love et al., 2014) was used to better normalize the data and assess statistically significant differences amongst viral taxa between environments through time used as a differential abundance analysis method to highlight which taxa were changing significantly between environments. The package takes raw counts and normalizes them to account for variations in sequencing depth. The statistical model underlying the package controls for the high variation that is possible in microbiome count data by accounting for the mean-variance relationship in the model (Love et al., 2014). Only differentially expressed genes that exhibited a fold change of 2.0 or greater were used for downstream analysis. From the output, higher “baseMean” values indicate higher levels of expression. The “padj” is an adjusted p-value using the Benjamini-Hochberg procedure to control for false discovery rate from multiple tests. The ‘Ggplot2’ package was used to visualize each of the graphs. The “dplyr” package was used for data manipulation (Wickham et al., 2022)

2.5 Statistical Analysis:

Generalized linear mixed models were used to test for the effect of time and participation in the almond bloom, and the interaction between time and almonds, on the Shannon Diversity Index (Bolker et al., 2009). A Shapiro Wilk Test was used to plot the distribution of Shannon Diversity values to assess the distribution normality. A Levene’s Test for homogeneity of variances between groups was performed using the “car” package as this test does not require normal distributions (Zhou et al., 2023). From there, appropriate model selection was done to minimize any assumption violations. A generalized linear model with gamma distribution and log link function was parameterized with Shannon Diversity Index being the dependent variable and “Time” (Levels: T1 = Before bloom; T2 = During bloom, T3 = After bloom; T4 = All back together), “Location” (Levels: Almonds versus Control) as fixed effects and the interaction between the Time and Location being the interaction effect. Assumption and goodness of fit for the model were quantified and visualized as Q-Q, Residual vs Fitted plots, Scale-location, and residuals vs leverage plots. An ANOVA was conducted with a Chi-squared test to assess the significance of factors in the model and then a likelihood ratio test was conducted to see the significance of adding the interaction effect between time and location into the model. The model’s dispersion statistic was also quantified. DESEQ2 was used to assess the significance of the spatiotemporal factors on specific viral OTUs (Love et al., 2014). This package analyzes count data from sequencing experiments such as RNAseq. Using raw viral counts, this package identifies statistically significant differentially expressed genes between conditions/treatments through quantifying log fold changes between samples and their adjusted p-values. Using the ‘DESEQ2’ package statistical differences were quantified between differential gene expressions of each of the focal viruses. First, a DESeqDataSet object with normalized counts was created, low-count genes were filtered out normalization was re-calculated after filtration, and size factors were estimated using the geometric mean of positive counts. before DESeq analysis was performed. The significance of differential expression of viral OTUs was tested for the interaction between each of the “Time_point” between time point 1 and all other time points, as well as between “Location”.

2.6 Results:

Alpha Diversity:

For Shannon/Simpson diversity metrics, a higher value for each of these metrics is indicative of greater species richness or higher evenness. Shannon's Diversity metrics are reported here as the patterns were similar between Shannon's and Simpson's. The calculated Shannon diversity index was plotted through time comparing the almond location to the control sites (Figure 1). A boxplot shows a visual representation of the spread and variability of the data. As the boxplot whiskers indicate 1.5 times the box's interquartile range, any data points that could be considered outliers would be indicated as points outside the whiskers. No outliers were detected using this method. When testing for the homogeneity of variance between comparisons of location and time point, Levene's test ($p\text{-value} > 0.05$) indicates we failed to reject the null hypothesis, so we can assume equal variances across groups. Throughout the time series, the almond hives had higher values of the Shannon diversity index but at the final time point, control hives increased in viral richness when the hives came back together after the bloom finished.

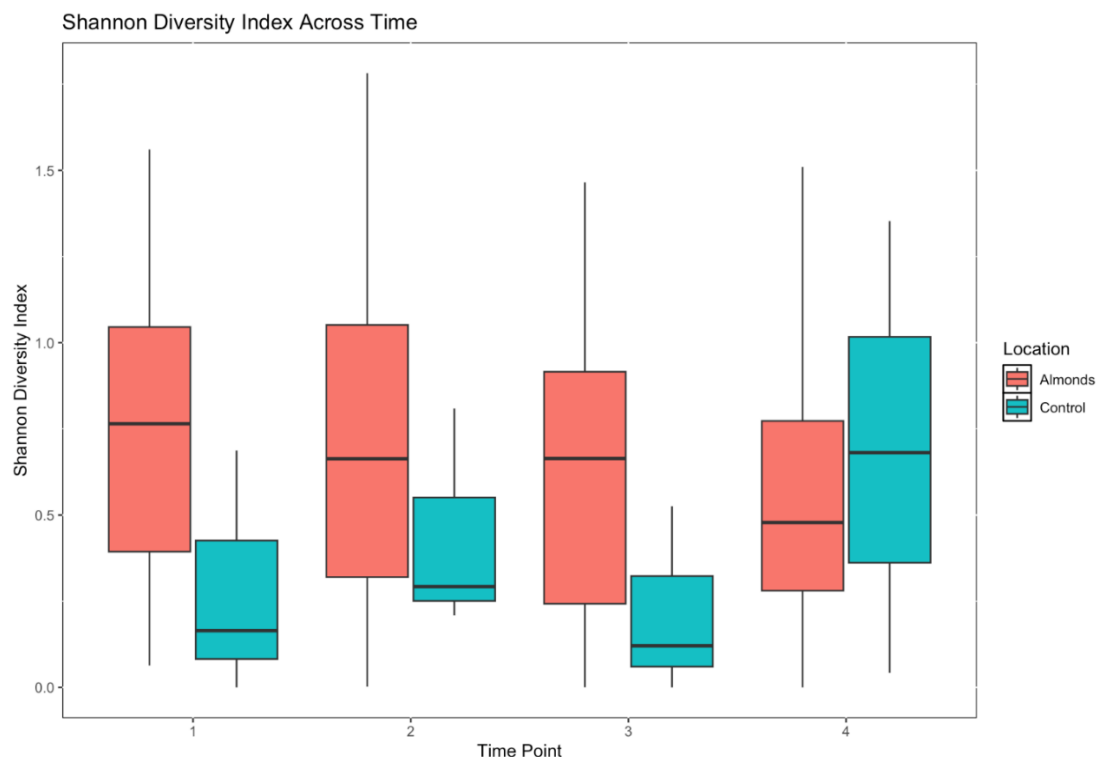


Figure 1. A boxplot showing Shannon diversity differences between time points, before, during, and after the bloom and then once both come together again, comparing the almond location (Arbuckle, CA) to the control (Grass Valley, CA). Boxplot whiskers are error bars that represent 1.5 times the interquartile range which is indicated as the box. The line in the middle of the box indicates the median or 50th percentile.

To better visualize the variation between samples, mean Shannon diversity was plotted through time between locations with standard error bars (Figure 1B). This metric shows the precision of the sample means estimating the actual population mean. The small sample size of the controls is indicated by the associated large standard error bars. In the first three time points,

the standard error bars do not overlap between locations substantially and therefore mean viral richness appears to be different between locations. The final time point shows a high degree of overlap between the almond and control estimates. This means we likely cannot infer the statistical significance of the effect of time and location on viral richness at that time point. Instead, we can test if there is a significant difference between the viral species richness between the first and final time points for the controls.

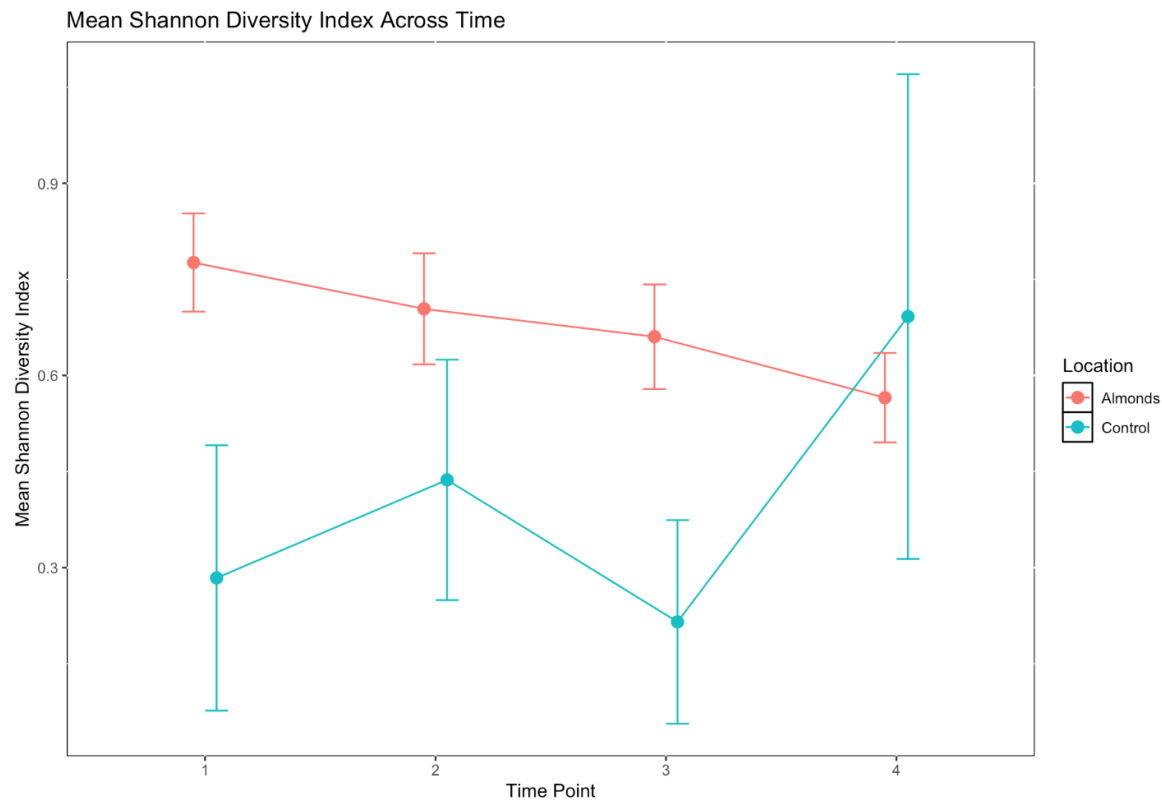


Figure 1B. Mean Shannon diversity index plotted through time between almond and control locations, with error bars representing $\pm$ one standard error.

Beta Diversity Metrics

Neither the PcoA clustered by time nor location showed any distinct clustering (Appendix 2.1, 2.2). WCMDScale was also performed to further investigate and did not show any distinct clustering based on the factors of interest (Appendix 2.3, 2.4). The Principal Components axes explained some amount of variation (PC1 = 17.12%, PC2 = 14.31%). The dendrogram that was generated from the MDS coordinates showed evidence of three potential clusters (Appendix 2.5). When the clustering of factors of interest (time, location) was compared with the clusters, no distinct patterns emerged (Appendix 2.6).

Prevalence

In total, 34 viral species were detected in samples over time, which included RNA viruses, DNA viruses, phages, and plant viruses. There were a total of 20 confirmed bee-infecting viruses and 13 plant-infecting viruses. To understand the broad landscape of viral incidence through time, prevalence was assessed for the almond hives and the control hives as the number of hives found infected with that focal virus over the total amount of hives. Black

Queen Cell Virus was prevalent throughout the almond hives (84%) and control hives (50%). Lake Sinai Virus showed high strain diversity in these samples, with six strains present in total. Lake Sinai Virus 3 is prevalent throughout the almond hives (59.7%), while the control sites had LSV-3 (25%). Lake Sinai Virus 2 was also at high prevalence in the almond bees (53.8%) and lower prevalence in the controls (25%). Lake Sinai Virus 1 was found to be at low prevalence in both almonds (26.9%) and control hives (25%). Lake Sinai Virus 4 was found at low prevalence in both locations as well with almonds (7%) and controls (8.3%). Sacbrood virus was found at low prevalence in general, but only in the almond bees and never in the controls. Deformed Wing Virus (DWV) appears prevalent at 42% through the almond pollinating bees, with certain hives having very high read counts whereas the prevalence in controls was lower (25%). Whereas DWV-B (also known as Varroa destructor virus-1) was found at lower prevalence in the almond bees (18.5%) and was not found in the controls. Prunus Ringspot Virus was found at high prevalence (48.7%) in the almond bees but was never found in the control bees. This virus was present in the almond bees before the major beginning of the bloom. However, early blooming varieties could have been flowering in areas away from the site but within flight distance of the bees.

Relative abundance

The relative abundance of species was assessed in pooled samples at each time point between locations (almonds versus control) (Figure 2, 3). These graphs indicated which viral species were found at high relative abundance across most samples. On a coarse level, we can see that the ‘almond’ hives have more colors indicating more viral species that comprise the samples than the controls (Figure 2). The Lake Sinai Virus complex (LSV) was highly abundant in the almond hives whereas the Black Queen Cell Virus comprised a majority of the represented abundance in the control hives through time.

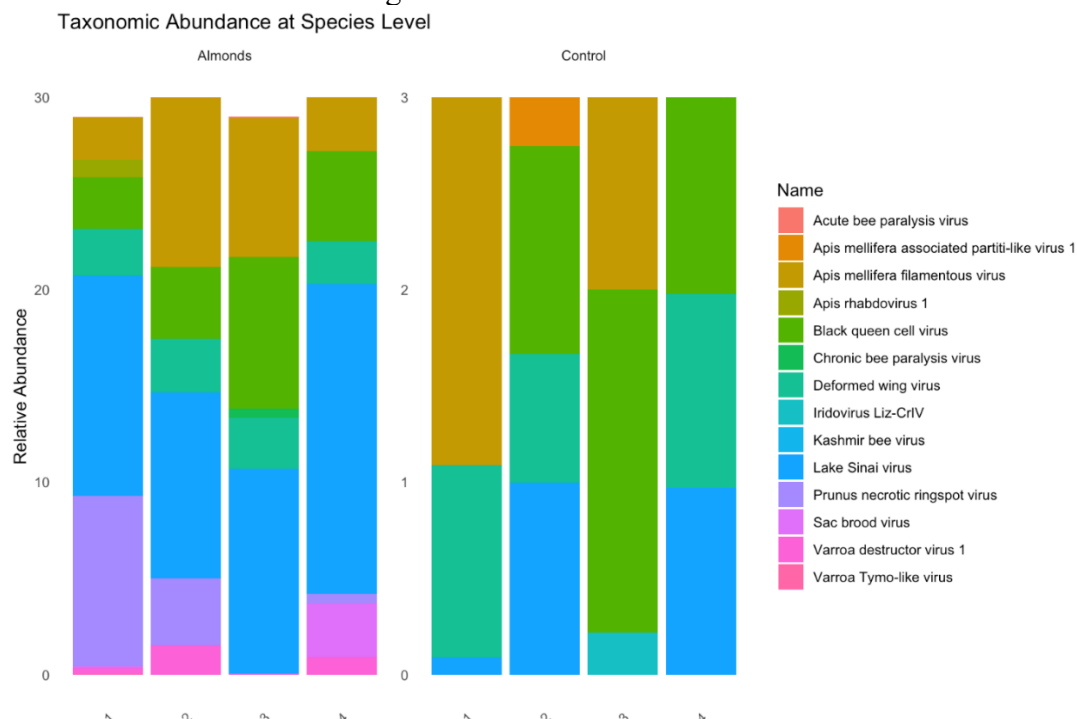


Figure 2. Relative abundance of viral species across all hive samples through time comparing almond hives (left) to control hives (right) plotted in a stacked bar plot.

Figure 3 shows the relative abundance of each of the focal viruses that had sufficient data through time and grouped by site. Acute Bee Paralysis Virus (ABPV), Sacbrood Virus (SBV), Prunus necrotic ringspot virus (PNRV), Varroa destructor virus 1 (VDV-1), Kashmir bee virus (KBV), Chronic bee paralysis virus (CBPV), and Apis rhabdovirus were all never found in the controls. PNRV was found in the almond hives even before most of the bloom began, but then dropped precipitously through the time series. CBPV was only seen in the almond hives after the almonds finished blooming. ABPV was found in the almond hives before, during, and after the bloom but was gone by the time the almond and controls came back together. Black Queen Cell Virus was found infecting both the almond and control hives throughout all time points, with higher relative abundance seen primarily in the controls. The Lake Sinai Virus complex was found infecting almond hives throughout time while the controls had sporadic evidence of infections. Some LSV variants were circulating in the controls even as the almond bees were in the middle of the bloom but then decreased at the subsequent time point before increasing again when the almond hives returned. *Apis mellifera* filamentous virus (AMFV) had a higher abundance in the controls initially in the study but then decreased through time while in the almonds it was present throughout. Deformed wing virus (DWV) showed consistently low but present relative abundance in the almond hives, while it was high in the control hives before the bloom and decreased in relative abundance after the bloom but increased in relative abundance once almond hives returned.

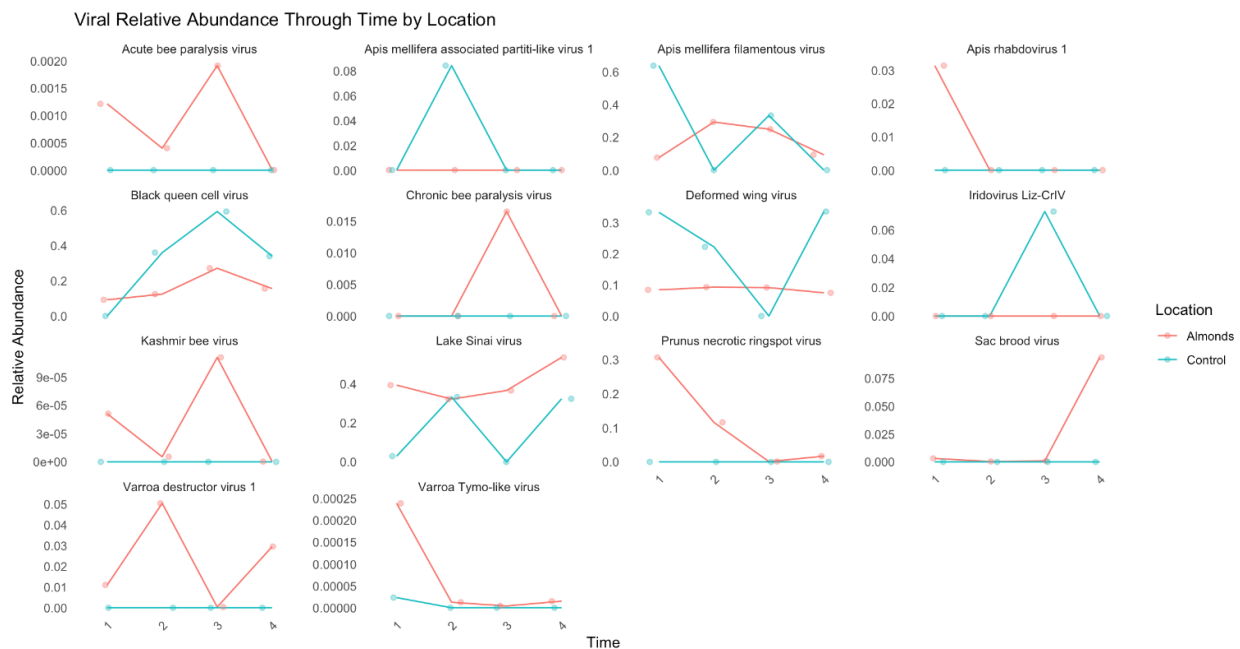


Figure 3. Line plot with the relative abundance (number of reads per million) of bee-infecting viruses showing the interaction of time and location per individual virus. The different colors indicate the location of the hives (Almonds; Controls).

To determine if there were any viral species with high relative abundance but low prevalence, clusters of high abundance were plotted in a heatmap for all samples (Figure 4). Certain hive samples had much higher relative abundance values of rarer viral OTUs that were at very low prevalence in the overall population. The following viruses were highly abundant in one to three hives at these time points and were contributing to overall viral richness metrics but

were not prevalent enough in the total samples to be statistically relevant: Apis rhabdovirus 1, Iridovirus Liz-CrIV, *Apis mellifera* associated partition-like virus 1, Chronic bee paralysis virus, Varroa Tymo-like virus, Acute bee paralysis virus, Kashmir bee virus, Varroa destructor virus 1. *Apis mellifera*-associated partition-like virus 1 and Iridovirus Liz-CrIV were highly abundant in the controls for one-time points. In the almond hives, Apis rhabdovirus 1 and Varroa Tymo virus were only present before the almonds bloomed, and SBV was only found in the almond hives at the final time point once all the hives came back together at the control location.

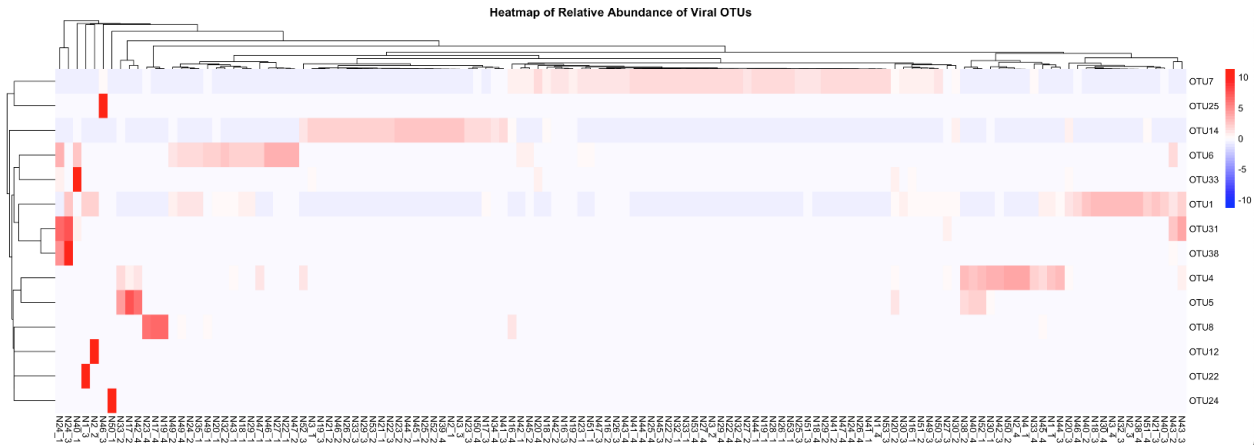


Figure 4. Heat map plot for relative abundance (number of reads per million) of each of the viral taxa (OTUs) between all hive samples. Red indicates higher relative abundance.

The previous figures highlighted differences across all the viral taxa focused on in the study. The viral dynamics of viruses in the genus Sinaivirus (Species: Lake Sinai Virus Complex) (Appendix 2.7, 2.8) and Iflavirus (Deformed Wing Virus (DWV), Sacbrood Virus, Varroa-Destructor Virus-1) were plotted across space and time (Appendix 2.9, 2.10). Focal viral species dynamics were plotted for DWV (Appendix 2.11).

Statistics:

First, the distribution of Shannon Diversity values was plotted, and it showed a positive skew to the right. A Shapiro Wilk Test was used to confirm the data was not normally distributed. Levene's test did not indicate a significant difference in variance between group comparisons ($p\text{-value} > 0.05$). Therefore, a GLM with gamma distribution was used to account for the right-skewed distribution. Assumptions of the model were tested by plotting Q-Q, Residual vs Fitted plots, Scale-location, and residuals vs leverage plots (Appendix 2.12). The following model was generated to test the significance of the effect of time, location, and the interaction between time and location on viral richness via the Shannon Diversity Index:

```
Model_1 <- (glm(formula = Shannon ~ Time_point * Location, family = Gamma(link = "log"), data = alpha_div).
```

To see the effect of adding the interaction between time and location into the GLM in comparison to a model without it, a likelihood ratio test was conducted and showed a marginal albeit still insignificant effect ($p\text{-value} = 0.079$). Due to the biological relevance of this comparison and the value being almost significant, the interaction was retained in the model. The

results from the ANOVA Chi-squared test looking at type II error showed that time alone did not explain a significant difference in Shannon Diversity. However, the effect of location was indeed significant (p -value = 0.029). Viral richness was assessed in pairwise comparisons of time. By looking at the model output, pairwise comparisons between the almond hives' first time point and the control hive's first time point were compared to each respective combination. Each time point and location was compared to the viral richness of the first time point at that location. The effect on time and location was significant for the final time point (p -value = 0.039), meaning that the viral richness of the control hives at the final time point was significantly higher than the viral richness in the control hives at the first time point. While at the other time points, the interaction was not significant. A dispersion statistic was calculated for this model which had a value of less than 1, indicating under dispersion of the model. This means that the variances of the residuals are smaller than expected under the assumed model and therefore some model refinement is still required. Modeling a linear mixed effects model that looked at this interaction while also integrating hive number identity as a random effect did not show evidence of explaining any more of the variation witnessed. Therefore, the random effect was excluded in favor of the simpler model explained above.

The DESEQ2 analysis showed the differential abundance for the top most significantly differentially expressed viral OTUs across each of the three possible interaction effects (Time point 2: Location; Time point 3: Location, Time point 4: Location). Black Queen Cell Virus (BQCV) and *Apis mellifera* filamentous virus (AMFV) show significant differential abundance between the almonds and control at the second time point once almonds began blooming, with BQCV being significantly lower (p adj < 0.001) and AMFV (p adj < 0.001) significantly higher in the almonds. Next, BQCV (p adj < 0.001), Lake Sinai Virus (LSV) (p adj < 0.001), Lake Sinai Virus 2 (LSV-2) (p adj < 0.001), Lake Sinai Virus 3 (LSV-3) (p adj < 0.001) show significant differential abundance between the two locations at time point 3. BQCV was significantly lower in the almonds while all the LSV strains were significantly higher in the almonds. BQCV (p adj < 0.001) and Sacbrood Virus (p adj = 0.01) show significant differential abundance between the two groups of locations when the hives returned together at the final time point. BQCV continued to be significantly lower in the almond pollinating bees and SBV was significantly higher in the almonds.

2.7 Discussion:

A time series of viral diversity was generated from a longitudinal RNAseq study of honey bee hives that were shipped to the Central Valley to participate in almond pollination in comparison to hives that did not participate in pollination. The results included a high diversity of viruses, including 20 confirmed bee-infecting RNA and DNA viruses. Our first key result is that viral richness measured through alpha diversity was higher in the almond bees than in the control bees before, during, and immediately after the almond bloom. This was the case even from the first time point, even though the controls and treatment hives had only been separated less than a week before. This suggests that the act of being moved into the almonds is an intrinsically stressful immunological event, leading to higher viral proliferation in the moved bees than in the controls. Alternatively, the hives that were left behind were in some way different or weaker from those selected to go to crop pollination. However, the lower species richness in the control hives overall would suggest that from an immune perspective, these hives were not dealing with as many different viruses as the almond bees. In future studies, it should

be prioritized to sample the almond-pollinating bees and control bees even before the pollinating hives are moved to the orchards to control for these variables.

When the hives were brought back together at the location of the controls at the end of the study, the increased viral diversity the almond hives were experiencing appeared to have been transferred to the controls. The controls had significantly higher viral richness at the end of the study when almond bees returned in comparison to the beginning. This time point is where the control hives' viral richness jumped to the highest levels they had been in the study thus far. This means that there could be a risk of honey bees transmitting the viral diversity they were exposed to back to their conspecifics that were not involved in the bloom upon returning from the bloom. To confirm this more definitively, however, would require a phylogenetic approach. This is a similar result to Alger et al., (2018) which showed that stationary hives that did not participate in crop pollination were impacted by the migratory honey bees that returned from pollination through an increase in viral load and prevalence.

The beta diversity metrics did not indicate any statistically distinct clustering, which means that the overall viral community composition was similar between locations and through time. This is consistent with the idea that a lot of variation in bee health outcomes comes from variations in beekeeping operation (Glenny et al., 2017). Since these hives were all within the same operation, they had consistent management practices that could decrease some of the total community variation. Having only one apiary reduces confounding variables (Faurot-Daniels et al., 2020), which makes the impact of location and time more accurate to pull out as effects in our models. This is because we have reduced the impact of unaccounted for variation inducing factors such as differences in varroa mite management treatment or supplementary feeding (Glenny et al., 2017).

The control hives sample size was low, with only four hives to start, dropping to three after one hive died before the end of the study. Coming out of the winter period is when beekeepers experience the highest loss of their bees, with mortality rates reaching nearly 50% over winter (Steinhauer et al, 2023). This coincides with the almonds blooming soon after in February when the most amount of honey bee hives are needed at any one time in the United States. This tension means that beekeepers are forced to send as many of their bees as possible to fulfill contracts. After 2020, this beekeeping operation experienced large losses and therefore had to ship most of their bees to the almonds, with only these four hives remaining back in Grass Valley, CA. Therefore, the comparisons between locations have intrinsically different sample sizes. Future research in this space should prioritize working with beekeepers and specifically paying them to leave hives behind, rather than requiring them to take on the financial burden of not getting paid for pollination services from a hive to serve as a control.

As the current statistical model for viral richness has evidence of under dispersion, continued refinement on model fitting will be conducted on these data to further tease apart the significant effects that location, time, and the interaction between both have on the explanation of viral richness differences. Specifically, we will use packages such as 'nlme' that are tailored for longitudinal data to parameterize a generalized least squares model which considers temporal autocorrelation (Pinheiro et al., 2021). From there we can test the model's dispersion to see if this is a better fit and is not violating any of the models' assumptions. Currently, the sample sizes between locations are so different that we may not have the statistical power to detect if the

locations are significantly different due to location or due to chance. Although GLMs can handle unequal sample sizes (Warton et al., 2016), we will also investigate alternative statistical comparisons that adjust for this difference. To account for differences in sample size we can employ stratified sampling to randomly select a sub-sample of the almond hives at each time point to match the number of controls (Qin et al., 2012). This can be done iteratively for the almond hives to calculate an average viral richness metric before running comparative statistics between the almond sub-samples and the control hives. This may solve some of the model assumption violations that occur when comparing data sets with different sample sizes.

When looking at individual viral taxa rather than total viral richness, different patterns emerge. When assessing viral relative abundance, the following viruses were all uniquely found in the almond pollinating bees. Acute Bee Paralysis Virus (ABPV), Sacbrood Virus (SBV), Prunus necrotic ringspot virus (PNRV), Varroa destructor virus 1 (VDV-1), Kashmir bee virus (KBV), Chronic bee paralysis virus (CBPV), and Apis rhabdovirus. In the almond hives, Apis rhabdovirus 1 and Varroa Tymo-like virus were only present before the almonds bloomed whereas CBPV was only seen in the almond hives after the almonds finished blooming. ABPV was found in the almond hives before, during, and after the bloom but was gone by the time the almond and controls came back together. Deformed wing virus (DWV) showed consistently low but present relative abundance in the almond hives, while it was high in the control hives before the bloom and decreased in relative abundance after the bloom but increased in relative abundance once almond hives returned, which also recapitulates Alger et al. (2018)'s observations of DWV increasing in hives that interacted with almond pollinating bees returning from pollination.

By looking at the differential expression of the viruses we could see which viruses were highly expressed when looking at the interaction of time and almond pollination. BQCV was significantly lower in the almond pollinating bees during the entirety of the study. Usually, this is a highly prevalent virus in commercial hives but exhibits overall low virulence. AMFV, on the other hand, was significantly higher upon the beginning of the almond bloom in the almond bees and then there was no difference between the almonds and controls nearly two weeks after that and remained the same when brought back together. This suggests this virus dynamics are just seasonal and do not necessarily have to do with the almonds. It appears with these data that for certain viruses the almond bloom matters, while for others, there are more seasonal dynamics that occur regardless of participating in the bloom. Some of the Lake Sinai Virus Complex (LSV, LSV2, LSV3) all showed to have significantly higher differential expression in the almonds which is in line with other studies that find LSV associated with almonds (Cavigli et al., 2016; Alger et al., 2018). Lastly, SBV was not present in any hives or time points until the end when some almond hives became infected and showed high relative and differential abundance in the almond hives.

A few viruses exhibited the pattern of having high relative abundance but exhibiting very low prevalence in the overall population. They are rare “one-offs”, only infecting between one to three hives in total across the duration of the study. Although we are not able to infer any dynamics, reporting the association of these viruses with the almonds and having genetic data is useful for broadening our understanding of the ecology of these viruses. To study low prevalence viruses such as these more in-depth, we would need to increase the total hive sample size. The viruses found at low prevalence and only one-time point included Apis rhabdovirus, Iridovirus

Liz-CrIV, *Apis mellifera* associated partition-like virus 1, Chronic bee paralysis virus (CBPV), Varroa Tymo-like virus. Acute bee paralysis virus (ABPV), Kashmir bee virus (KBV), and Varroa destructor virus 1 (VDV-1) were at low prevalence but found across multiple time points. Some of these viruses are known to be highly virulent, and thus their low prevalence could be attributed to a honey bee's mortality before sampling. While others are newly discovered, and the symptoms are relatively unknown due to the lower measurable pathogenicity. Overall, these members of the viral community contribute to increasing the viral diversity parameters but do not show significant spatiotemporal patterns in relative abundance or differential expression.

Finally, although not a virus, *Nosema ceranae*, a microsporidian parasite known to be pathogenic in honey bees, was found at very high prevalence and read count across all time points. This chapter exclusively focuses on viruses, but this data set broadly includes microbial mRNA transcripts as well as host mRNA transcripts and can warrant further investigation into non-viral microbial dynamics and host gene expression. Additionally, for some of these viruses, there is sufficient coverage to be able to build phylogenetic trees and even reconstruct entire viral consensus genomes. To gain a better understanding of a pathogen's origin, we must analyze the genetic information in a phylogenetic framework. In future studies, phylogenetic inference through phylodynamic methods (Grenfell et al., 2004) can detect patterns of transmission to understand the sharing of strains across geography (Murray et al., 2019). This will add another layer of information on whether the almonds are acting as a source of viral diversity for honey bees.

2.8 Conclusion:

Pollination events have the capacity to act as mass mixing events for honey bees that participate in crop pollination. This longitudinal study of viruses found in honey bees showcases the impact of participating in the almond bloom on the honey bee virome. Viral richness was higher in the almond pollinating honey bees throughout the study, even before the almonds bloomed. This suggests that the act of being transported to the bloom is intrinsically stressful. But when the almond pollinating bees returned to the same location as the controls, we saw a significant increase in viral richness in the controls. This diversity of viruses that the almond bees were experiencing appeared to be transferred to the control hives just three weeks after the hives returned together after the bloom. This shows some evidence that participating in the almonds impacts viral richness, and pollinating honey bees can have impacts on other honey bees that are not participating in pollination events. Additionally, this highlights that different viruses are experiencing unique spatiotemporal dynamics about the almond bloom itself. Some of the Lake Sinai Virus Complex (LSV, LSV2, LSV3) all showed to have significantly higher differential expression in the almonds, whereas BQCV was significantly lower in the almond pollinating bees during the entirety of the study. The AMFV, on the other hand, suggests that these dynamics are just seasonal and do not necessarily have to do with the almonds. It appears with these data that for certain viruses the almond bloom matters, while for others, these are more seasonal dynamics that occur regardless of participating in the bloom. Evolutionary disease theory suggests that when we go towards more global transmission dynamics that the virulence of pathogens can evolve to be more deadly. With greater than half of the entire commercial honey bee population arriving in a singular place in the Central Valley of California on an annual basis to pollinate most of the world's almonds before migrating across the entire country subsequently, this is an extreme example of more global transmission dynamics. We must maintain surveys of honey bee viral dynamics throughout the almond bloom specifically, as this

pollination event acts as a mixing pot that provides the ripe conditions necessary for the evolution of more highly virulent bee pathogens.

2.9 Acknowledgements:

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Chapter 3: Bee virus dynamics between managed honey bees and native wild bees in a Sierra Wildflower System

3.1 Abstract:

We know little about the ecological dynamics of the suite of RNA viruses found infecting bees. These viruses have a broad host range and evidence of viral infections has been documented across all the major families of bees. However, the competency of these other bee species is not well understood. PCR-based evidence of a virus on a novel bee species alone does not mean that there is an active infection, that the host would experience any pathology, nor that pathogens can successfully circulate in that species. Therefore, is still unclear how much other species of bees play a role in maintaining these viral epidemics. Honey bees are not native to North America and were brought by European colonizers in the 17th century for crop pollination and honey/wax production. Today they are considered ‘livestock’ purported to be a source of these viral infections to other species due to their role in industrial agriculture. These management stressors lead to higher disease incidence in livestock species due to crowded and stressed conditions of genetically homogenous hosts. Determining the role of honey bees in seeding epidemics into native bees, as well as the ability of native bees to maintain epidemics without honey bees are critical pieces of information to develop disease management strategies.

Due to the ubiquitous nature and range of honey bees, both managed by humans and living feral in the wild, it is challenging to find areas devoid of their pressures to assess the viral community of native bees alone. To begin understanding what native bee viral dynamics are through time with and without the direct influence of bees, I developed a field system in the Tahoe National Forest in the Sierra Nevada Mountains. These high elevation sub-alpine sites boast high bee and wildflower diversity and experience low honey bee density due to the harsh winters and lower rates of beekeeping. Commercial honey bee hives were brought into the Sierra for part of the summer season in 2021 which allowed for a ‘natural experiment’ where wild bee populations can be sampled for their viruses before and after the artificial migration of honey bees into the system. The study tested the following hypotheses: (1) whether honey bees bring viruses with them when they migrate, (2) if viral prevalence is highest near honey bee apiaries and lower at isolated sites, or if viruses are maintained in native bee populations independently of honey bee presence, (3) whether different viruses show distinct interactions with native bee populations, and (4) if there is any evidence of spillover between species. A longitudinal survey was conducted on the viral community of managed honey bees and native wild bees (Genus: *Bombus*, *Osmia*) before and after honey bee arrival across four-time points in the summer of 2021. Deformed Wing Virus-B (DWV-B), Sac Brood Virus (SBV), and Chronic Bee Paralysis Virus (CBPV) were the focal viruses of this study. Each of these viruses exhibited different

dynamics through time between different species of bees. DWV-B was already circulating in *Bombus* species before the arrival of honey bees. Its prevalence increased over time in *Bombus*, regardless of the abundance of honey bees in the environment, or the proximity of an apiary. Together this suggests an epidemic within bumble bee populations independent of honey bee presence and gives credence to the idea that *Bombus* are competent hosts for DWV. Different bee species experienced different seasonal dynamics, with *Bombus vandykei* showing high DWV-B prevalence later in the season, whereas *Osmia* mason bees consistently exhibited the lowest prevalence across all monitored bee species. SBV was highly prevalent in several *Bombus* species early in the season, even before the arrival of honey bees; following their arrival, SBV was highly prevalent in hives but minimally present in foragers. SBV prevalence decreased over time in both *Apis* and *Bombus*, which alludes to the seasonality of this virus rather than an example of spillover. *Osmia* mason bees showed no evidence of SBV infection throughout the time series. CBPV was also found in *Bombus* before honey bee arrival, with *Bombus vosnesenskii* having the highest prevalence. Honey bees consistently exhibited high CBPV prevalence, while *Bombus* showed generally low prevalence throughout time, suggesting that honey bees are more competent hosts, and there was not much evidence of direct impact on co-foraging bumble bees. These results show the importance of understanding the ecological dynamics of bee viruses and allude to seasonal viral dynamics in native wild bees in areas that are isolated from the direct impact of agriculture, but still are experiencing pathogen pressures. Furthermore, it highlights the necessity for long-term temporal survey data to inform the context-dependent risk and pressure of honey bee presence on native bee disease dynamics. In this study, we show that viruses are circulating in native bees, even before the arrival of honey bees. Additionally, the viral prevalence of DWV-B in *Bombus* increased throughout the season, regardless of honey bee abundance or apiary presence, and gives evidence that these native bee species can maintain epidemics of DWV, SBV, and CBPV. Only through regular temporal sampling of these important pollinator populations can we begin understanding questions of viral ecology and the effect of honey bees on wild bee population health

3.2 Introduction:

High elevation montane meadows in the Sierra boast high levels of both flower and native bee diversity. At the same time, non-native honey bees are unable to survive the harsh Sierran winters on their own, and consequently, feral honey bee hives are rare. There are some beekeeping operations in the cities and towns of this region, but beekeeping is inherently challenging due to black bear predation. This means meadows in the Sierra can at times be found free of honey bees, providing a system that allowed me to sample native bee communities before and after experimental honey bee arrival to test various spillover hypotheses. Bee viruses have not previously been comprehensively monitored in this area. I collaborated with commercial beekeepers who previously participated in the Central Valley almond pollination and subsequently brought a subset of their hives to these high elevation meadows located on private land in the Tahoe National Forest to produce wildflower honey and to conduct specific breeding programs in this relatively honey bee-free area. Although these activities took place on private property, honey bees can fly well beyond the borders of these lands and into the national forest to forage. Therefore, the benefits of breeding and honey production need to be weighed against the cost of potential competition with native bees for floral resources and disease transmission to these species.

This system allowed me to monitor viruses in native bee communities that are isolated from honey bees and subsequently ‘experimentally expose’ them to honey bee hives and track viral dynamics thereafter. Spillover of honey bee viruses could be happening due to an overlap in floral resource preferences between native and honey bees. Flowers have long been implicated as the mode of horizontal transmission of pathogens between bee species (Durrer & Schmid-Hempel, 1994), though until recently, few studies have unequivocally shown this to be the case (Burnham et al., 2021). Viruses that were once thought to be honey bee specific, are now being found in other bee species (Alger et al., 2019a; McMahon et al., 2015). Native bee species are at risk of spillover from any of the infectious diseases of honeybees through foraging on shared contaminated floral resources (Bailes et al., 2020; Fürst et al., 2014; Graystock et al., 2016). Moreover, declines of native bees coinciding with the introduction of managed species carrying novel infectious pathogens have already been documented (Montalva et al., 2011, Cameron et al., 2016, Graystock et al., 2013). An increasing number of studies have shown that ‘honey bee’ viruses infect nearly all of the major bee families, including other members of Apidae, Halictidae, Andrenidae, Megachilidae and Colletidae (Dolezal et al., 2016; Levitt et al., 2013; McMahon et al., 2015; Singh et al., 2010; Tapia-González et al., 2019; Tehel et al., 2016), Halictidae (Dolezal et al., 2016; Levitt et al., 2013; Singh et al., 2010). RNA viruses are of particular concern due to their high mutation rate which allows for rapid adaptation to novel host immune systems, but we are only just beginning to understand the impact of viral emergence on bee populations (McMahon et al., 2015). DWV is the most common virus found in honey bees (Martin and Brettell, 2019) and the bee virus that has been most frequently found in other insects (Tehel et al., 2016), boasting the highest host breadth of any of the honey bee associated viruses across its 65 arthropod ‘host’ species (8 insect orders, 3 Arachnida orders) (Martin and Brettell, 2019). Crucially, DWV prevalence in these species is correlated with DWV load and prevalence in the overlapping honey bee populations (Manley et al., 2015; Murray et al., 2019; Santamaria, 2018).

Most bee virus studies sample only one time point (Martin et al., 2019). Instead, I sampled at multiple time points across a season to quantify epidemiological dynamics. This system allowed me to survey the viral communities in bees before honey bees arrive and then after honey bees arrive, in comparison to “control” sites that are sufficiently removed from interactions with honey bees. This way we can see if sites that are isolated from honey bees can act as a refuge from viral spillover for native bees and determine the association between honey bee abundance and apiary presence and the prevalence of viruses in the native bees. Long-term control of disease in this system is predicated on identifying species acting as pathogen maintenance hosts to determine which species are sustaining epidemics. If honey bees are indeed sources of viral infection for native bees, we must test to see if spatial isolation from honey bees is enough to protect native bees from disease risks. Alternatively, if native bees are just as competent of maintenance hosts as the honey bees, then epidemics can be sustained even without the presence of honey bees. Therefore, it is critical to determine viral dynamics in sites with and without honey bees to infer host competency in the native bees. This knowledge can help inform policy surrounding the allowance of non-native honey bees on private land adjacent to wild areas.

A diversity of bumble bee species were sampled concurrently with honey bee sampling across sites. Bumble bees (genus: *Bombus*) are closely related to honey bees (Family: Apidae) and are also social and possess high species diversity in this region, with over 17 species of

Bombus found in the region (Hatfield and LeBuhn, 2007). This includes a candidate species for the California Endangered Species Act (CESA), *Bombus occidentalis* which will be the focus of my future research. *Bombus* are primitively eusocial ground-nesting bees with generalist foraging patterns, and annual colonies (Carnell et al., 2020). This exhibits a distinctly different life history strategy from honey bees, which survive as a perennial colony all year, whereas in bumble bees all individuals die each year except for the new queens. Additionally, a honey bee queen will never be found foraging on flowers. Bumble bee queens on the other hand have a distinctly solitary portion of their lifecycle, where the new queens produced from the previous season overwinter in a burrow after mating and diapause through the winter. Then in the spring queens emerge and begin foraging and searching for a new nest to begin their colony. The queen is in this solitary stage for a few weeks as she begins laying eggs and foraging for herself and her future colony while incubating her eggs. Once the eggs hatch and grow into adults, the workers are then the ones responsible for foraging while the queen remains in the nest and attends the growth part of her colony's life cycle. This means that queens are directly interacting with pathogens in the environment as they forage initially for themselves and their colony (Goulson et al., 2008). This could suggest an increased risk of vertical transmission for bumble bees in comparison to honey bees which have social immunological behaviors developed to minimize the direct exposure of the queen to pathogens. Additionally, male bumble bees (drones) also could exhibit unique viral patterns as the males interact with far fewer flowers than the workers as they are not responsible for gathering food for the colony. Thus, the prevalence of drones could be lower than workers due to less exposure to contaminated floral products (Van Der Steen, 2008).

In addition to bumble bees, *Osmia* mason bees were also collected. *Osmia* are solitary bees with annual life history dynamics and are a more distantly related lineage of bees (Family: Megachilidae) and possess a high species diversity at these sites. Considering threats to commercial honey bees, many farms have turned to supplementing honey bee pollination with mason bees, including the species *O. bicornis*, *O. cornifrons*, *O. cornuta*, and *O. lignaria* (Osterman et al. 2021). Mason bees are solitary cavity-nesting bees that construct brood chambers in the nest using mud. Due to their distinct life histories, it is largely unknown how the virus dynamics of mason bees relate to honey bees. Some viruses have been shown to spillover from honey bees and actively replicate, such as BQCV in *O. bicornis* (Radzevičiūtė et al. 2017). DWV, however, has been shown to not replicate in the same species, despite being present (Schauer et al. 2023). Other honey bee viruses, like SBV, Israeli acute paralysis virus (IAPV), Chronic Bee Paralysis Virus (CBPV), and Kashmir bee virus (KBV) have been detected in non-*Osmia* bee taxa and other non-bee insects (Levitt et al. 2013) but are still being explored in *Osmia*. As research expands more mason bee viruses are being discovered. For example, *Osmia*-associated bee chuvirus (OABV) was recently discovered in *O. taurus* bees from Fukushima, Japan (Takemae et al. 2023). To address conservation concerns, it is important to establish a baseline understanding of mason bee viruses by studying wild populations.

A longitudinal survey was conducted across four-time points during the summer of 2021. In this way, a viral prevalence time series was generated for Deformed Wing Virus-B (DWV-B), Sacbrood Virus (SBV), and Chronic Bee Paralysis Virus (CBPV) for honey bees (*Apis mellifera*), bumble bees (*Bombus spp*), and solitary mason bees (*Osmia spp*). Sampling through time, and specifically sampling native bees before honey bee arrival, allowed me to see whether honey bees bring viruses with them when they migrate, what the viral prevalence is in native

bees before honey bee arrival, and if areas absent of honey bees are free of these viruses. Sites with and without honey bees were compared, and the impact of honey bee abundance and proximity to the apiary was assessed on the impact on viral prevalence in co-interacting native bees.

In this chapter, I tested the following hypotheses:

Hypothesis 1: Viral prevalence will be highest in native bees living closest to honey bee apiaries and will be lower at sites with honey bee presence but without apiaries within a honey bee flight radius, and no bee viruses of any kind will be found in wild bees that are isolated from honey bees completely. Sites with the highest honey bee abundance foraging on flowers will also have the highest prevalence of native bees at the same site and time point.

Hypothesis 2 (Alternative): Viruses are being maintained in native bee populations even before honey bee arrival, suggesting either historical interactions with honey bees or that these are more general bee viruses and not necessarily honey bees in origin. Viral prevalence will be similar in native bees across sites, regardless of apiary presence or honey bee abundance.

Hypothesis 3: Different viruses will show different patterns, with viral dynamics for viruses that are ‘honey bee’ in origin, such as DWV, driving dynamics in native bees, while other viruses do not show evidence of correlation.

3.3 Methods: Sampling

Three treatment sites each had 25 managed honey bee hives placed on private land within the Tahoe National Forest. A subset of the honey bee hives were tracked since the beginning of the year as they had participated in the almond bloom (Chapter 2). By sampling hives before during and after crop pollination as well as throughout this study will produce a viral time series for these hives from February – August of 2021. Future work can analyze these hives specifically to see if there is a connection between viral dynamics in the almonds and these montane environments to assess the impact of crop pollination, even far away from the event itself.

Each of the apiary sites had pairs of associated “control” sites which were at increasing distances away from the apiary. Two control sites were sampled to maximize the chance that honey bees would not arrive at some point in the sampling season. Site selection occurred at a distance greater than 6 kilometers (km) away from the closest hives. This was the average maximum distance that a honey bee would fly during foraging (Figure 1) (Couvillon et al., 2015). Control sites were not established at any further distances beyond these sites due to attempts to control elevation and floral community as much as possible to minimize additional variation. Treatment versus control sites are indicated by either the presence or absence of an apiary, respectively. Honey bees have been shown to fly upwards of 10 km when necessary in times of low food resources (Couvillon et al., 2015). In anticipation that this might occur with 2021 being a drought year in California, we also quantified honey bee abundance at each site and time point to show (1) which sites remained ‘true controls’ where no honey bees were ever recorded at the site and (2) if there was any association between honey bee abundance and viral prevalence in native bees, regardless of apiary presence. At the beginning of each sampling event, honey bee along with bumble bee abundances were estimated by counting the number of individuals along a 100 meter (m) long, 5 m wide belt transect, walking at a consistent pace

through the densest patch of flowers. Sampling abundance was quantified as the number of bees per 500 m² (Gunnarsson and Federsel, 2014). The impact of both apiary presence and honey bee abundance was investigated as possible explanatory factors behind viral prevalence in native bees.

Native bees were collected from experimental and control sites before honey bee arrival in the meadows. Three apiaries were then moved from Grass Valley, CA for both breeding efforts and wildflower honey production. This beekeeping operation specifically installed ‘breeding’ hives which were undergoing selection for varroa mite resistance traits. Each apiary was kept enclosed in an electric fence to protect against bears. Honey bees and native bees were collected across all sites for three additional time points following the sampling scheme in Figure 2. At each time point for each site, there was a collection of reference material for flower, pollen, and bee species identification. This provided a reference collection to aid in identifying the species represented at each site. This list of species was the reference for the identification of the frozen samples for viral quantification. For diversity metrics at each site and time point, bees were net collected for 20 person-minutes into a jar of ethyl acetate. Sampling consisted of 20 person-minutes of collecting into kill jars with ethyl acetate. From the insect collection, the two most abundant native bee species were selected as the focus for pollen identification and viral detection. The collected bees were stored on wet ice before being pinned for later identification using discoverlife.org and relevant dichotomous keys (Discoverlife.org; Michener et al., 1994). The bees that were the focal species were then verified by a taxonomist. This identified collection will also be available as reference material for any future DNA barcoding projects.

Honey and native bees were collected using hand nets for virus quantification as they foraged on flowers. The species of flower the bee was foraging on was recorded for each specimen when possible. Nets were sterilized between sites in a diluted bleach solution before being sprayed with 75% ethanol. The most abundant species of native bees were collected for a total sample size of 10. If the sample size could not be reached for that species, other native bee species were collected ad libitum to reach the minimum sample size. Foraging male bumble bees (drones) were also collected and a subset was processed. Honey bees that were foraging on the flowers were also collected whenever possible to determine the viral loads of foragers in the environment with a maximum of 10 foragers collected per site per time point. To track individual hive dynamics and to assess the differences in viral composition between hives and foragers, honey bee samples were taken directly from the hives that I have monitored since the almond bloom (Chapter 2). Foragers were collected as they returned to the hive entrance and placed into tubes using soft forceps. At least 5 individuals per hive were collected, preferentially catching those carrying pollen back to the hive.

Viral quantification

Upon collection, bees were frozen on dry ice before being transferred into a dry shipper of liquid nitrogen and later stored at -80°C for further analysis of their RNA viruses. Bees were identified to the species level while on dry ice before dissection. In total, 730 individual bees were collected for viral analysis. Then the bee was dissected in half dorso-laterally from which half of the sample was homogenized and the other stored as a backup tissue sample. From there the RNA was extracted using Trizol protocols (Manley et al., 2020) and quantified with a Nanodrop. RT-PCR was performed to synthesize cDNA from the total RNA sample using cDNA

SuperScript iii (Invitrogen). For each sample, 2 µl of RNA was added to nuclease-free water along with random primers before being heated at 70 degrees Celsius for 5 minutes to melt secondary structures and bind the random primers to the template. Then the sample was placed directly onto ice before each sample was mixed with a mixture of buffer, dNTPs, and the Superscript iii enzyme before being incubated again at 37 degrees Celsius for 60 minutes. To quantify viral prevalence for the viruses of interest, GoTaq green master mix (Promega) was mixed with 2 µl of template cDNA, 12.5 µl of master mix, 9.5 µl of nuclease-free water, and 1 µl total of forward and reverse viral primers. BeeCox1 gene primers have been developed as specific DNA barcodes for bees and were used as positive controls to indicate successful cDNA synthesis. Viral-specific primers were used for Deformed wing virus B, Sacbrood virus, and Chronic bee paralysis virus. Gel electrophoresis was performed to identify the presence of viruses in individual bee samples. Agarose gels were made at a 1.5% concentration and stained with ethidium bromide. Gels were run at 120 V for 45 minutes before imaging using a Gel Doc Imager. Individual bees were scored as either 0 for no amplification or 1 if there was a fluorescent band that had the correct number of base pairs associated with each viral primer when compared to a 1 kb ladder. A score of 1 indicated evidence that the bee had the focal virus of interest. For honey bee samples with confirmed DWV-B pathogen presence, viral load was assessed using droplet digital PCR (Hindson et al., 2012) and custom made DWV-A and DWV-B probes. In total, 648 samples were screened for the focal viruses of interest. A table of this study's metadata and prevalence results can be found here (Appendix 3.1B).

Mason bee (*Osmia*) meta transcriptomics

One of my previous undergraduate students, Ricky Pankratz, did his honors senior thesis where we worked together on quantifying the viruses of wild *Osmia* through time in this study. To determine which focal viruses to screen for in the collected *Osmia* samples, stratified random individual samples were selected across sites and time points for processing and subsequent meta-transcriptomic sequencing. A pooled sample was generated from 10 clean *Osmia* RNA samples. Contaminant DNA was removed using the Turbo DNase Treatment kit (Thermo Fisher Scientific). Buffer and the Turbo DNase enzyme were added to the samples and incubated at 37 degrees for 30 minutes. Next, the DNase inactivation reagent was added and incubated at room temperature before being centrifuged and removed to generate a sample of RNA that was free from contaminant DNA. Then, samples were cleaned using the RNA Clean & Concentrator kit (Zymo). This cleaned and pooled RNA sample was sent to Novogene Inc. (Sacramento, California, USA) for library prep and subsequent RNA meta-transcriptomic sequencing to quantify the entirety of the microbial assemblage found in these pooled samples. These results were used to determine which viruses to test the *Osmia* for in downstream analysis. Novogene conducted the bioinformatic analysis for these samples.

Statistical Analysis

All data analysis was carried out using R Studio. The package “dplyr” was used for general data manipulation (Wickham et al., 2022). Viral prevalence through time and across all bee species was graphed for DWV-B, SBV, and CBPV. Each time point had a viral prevalence metric per species per site. A smooth line was fitted through the data for each species using a generalized additive mixed model (GAMM). GAMMs are appropriate models for longitudinal

studies as the model includes random effects to adjust for temporal auto-correlation (McCormick et al., 2023). Additionally, the viral prevalence of all *Bombus* through time was graphed using boxplots to understand the spread and variation of the data and the overall patterns observed in this genus. The effect of apiary presence, honey bee abundance, and the interaction between the two factors on viral prevalence in native bees were investigated and visualized using the package “ggplot2” (Wickham, 2016). Prevalence was calculated from count data and grouped by “Site”, “Time”, and “Species”. Honey bee abundance was log-transformed before further data analysis. To quantify the impact of honey bee abundance or apiary presence on viral prevalence in native bees, generalized linear models were fit to the data with prevalence being the response variable, and log abundance, apiary, site, and time were all investigated as possible significant fixed effects. Generalized linear models were fitted using the “lme4” package (Bates et al., 2015). From there, ANOVA with Chi-Squared tests was done on each of the resulting models to quantify the significance of the fixed effects on explaining the variation in native bee viral prevalence. A data table outlining the sample sizes and scale of replication across time points, sites, and species for this study is outlined in Appendix 3.1.B.

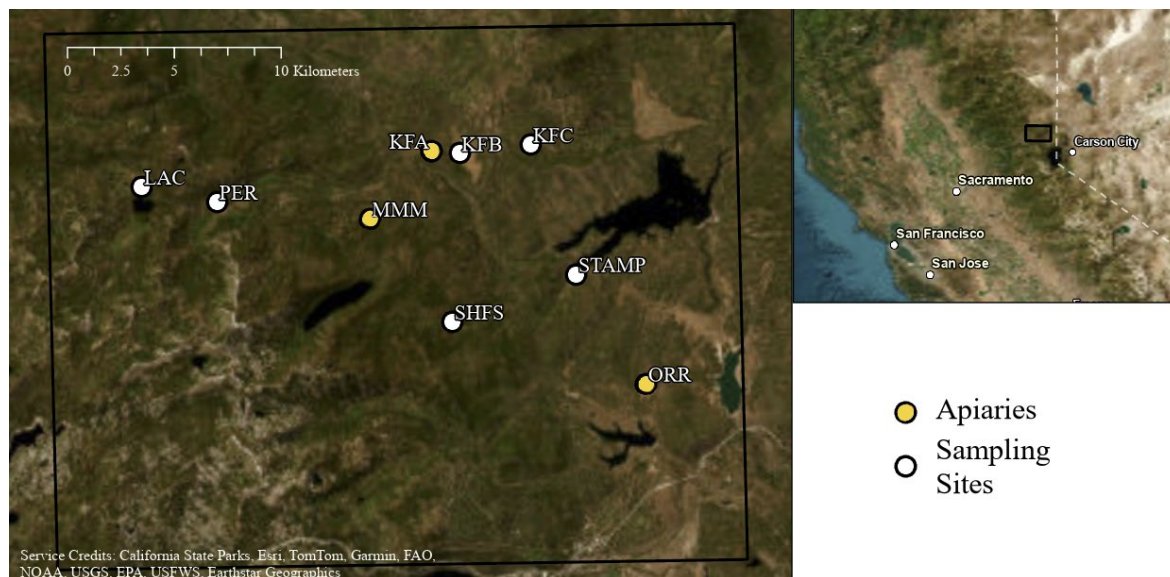


Figure 1. (Left) Distribution of sampling sites (white dots) and sites with experimental apiaries (yellow dots) in the Tahoe National Forest. (Right) Site location in reference to the San Francisco Bay Area. The map was generated using ArcGIS Pro version 3.2.1.

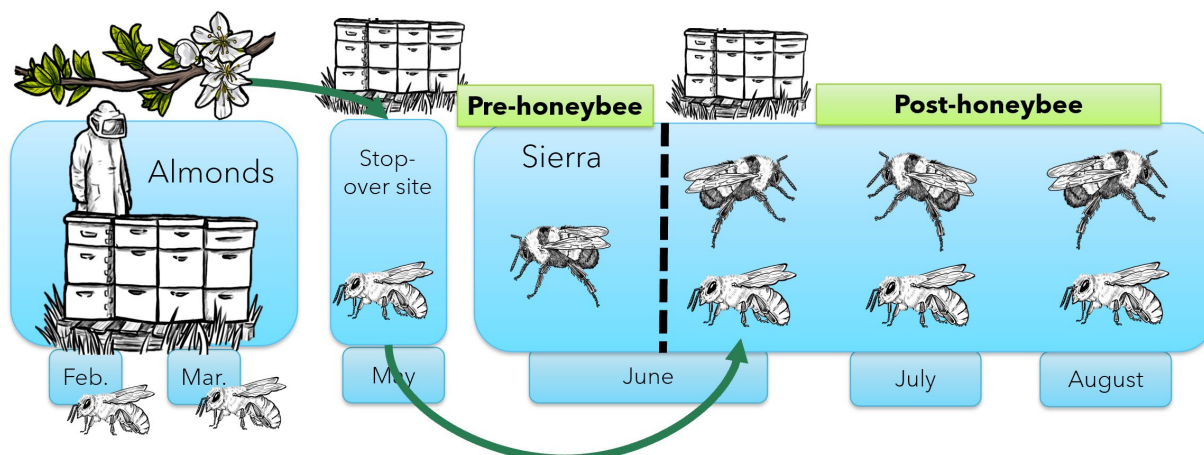


Figure 2. A flowchart schematic illustrating the sampling scheme for the Sierra 2021. Sampling began in the almonds in Arbuckle, CA, after which they were migrated to a stopover site in the Grass Valley, CA. From there I sampled bumble bees before honey bee arrival and after arrival across three time points in the summer. Each bee indicates a sampling event.

3.4 Results:

Eight sites were sampled in 2021 that were sampled across the entire season, with three treatment sites, each receiving an apiary consisting of 25 honey bee hives. These sites varied throughout the season in their abundance of honey bees on flowers. Two sites were completely free of honey bees throughout the entire season (Appendix 1). The final time point had impacts on the sample sizes collected as wildfires had begun in the area. Bees were sampled across the major bee families, which included bumble bees (genus: *Bombus*), mining bees (genus: *Osmia*), andrenid bees (Genus: *Andrena*), and sweat bees (genus: *Halictus*, *Lasioglossum*). Over 1,000 individual bee specimens were collected, pinned, and curated for a collection that will eventually be donated to the Essig Entomology Museum. Most of these specimens have been identified as species, and if not, all have been identified as genus. In 2021 four primary species of *Bombus* were sampled across the sites through time: *Bombus vosnesenskii*, *Bombus vancouverensis nearcticus*, *Bombus vandykei*, and *Bombus flavifrons*. In total, nine species of *Bombus* were collected during diversity surveys across these sites (Table 1). *Osmia* bees present a very challenging taxonomic conundrum, with species sharing overlapping characteristics, and diagnostic characters often being hard to access (mandibles, genitalia). Therefore, taxonomic expertise was employed to identify this group of species. Dr. John Ascher (University of Singapore) and taxonomic experts at the USDA Logan Bee Lab, including Dr. Terry Griswold and Skyler Burrows identified the 187 pinned *Osmia* individuals. In total, 33 species of mason bees were identified, including 3 unidentified species that may be undescribed (Table 3). As the viruses that could be infecting *Osmia* are generally not known, a pooled sample was first sequenced to determine focal viruses of interest. The resulting transcriptomic library was of high quality with over 99% clean reads (Appendix 3.2). The relative abundance of transcripts across taxonomic groups was plotted (Appendix 3.3) Three bee viruses were found through the transcriptomic analysis: DWV-B, CBPV, and Andrena-Associated Bee Virus-1 (AnBV-1) (Appendix 3.4) and therefore DWV and CBPV were the focal viruses examined for this chapter, along with SBV, which showed high prevalence in *Bombus* during a preliminary screening.

Table 1. *Bombus* species were identified by Nina Sokolov, Dr. John Ascher, and Anna Hatzakis in the total species diversity collection.

Genus	Species
<i>Bombus</i>	vancouverensis
<i>Bombus</i>	nearcticus
<i>Bombus</i>	flavifrons
<i>Bombus</i>	vosnesenskii
<i>Bombus</i>	vandykei
<i>Bombus</i>	mixtus
<i>Bombus</i>	californicus
<i>Bombus</i>	occidentalis
<i>Bombus</i>	insularis
<i>Bombus</i>	appositus

Table 2. *Osmia* species were identified from the pinned diversity collection by experts Dr. Terry Griswold, Skyler Burrows, & Dr. John Ascher.

Genus	Species	Genus	Species
<i>Osmia</i>	" <i>Acanthosmioides</i> " <i>sp.</i>	<i>Osmia</i>	<i>lanei</i>
<i>Osmia</i>	<i>albolateralis</i>	<i>Osmia</i>	<i>lignaria</i> <i>propinqua</i>
<i>Osmia</i>	<i>atrocyanea</i>	<i>Osmia</i>	<i>malina</i>
<i>Osmia</i>	<i>bella</i>	<i>Osmia</i>	<i>melanopleura</i>
<i>Osmia</i>	<i>brevis</i>	<i>Osmia</i>	<i>montana</i> <i>quadriceps</i>
<i>Osmia</i>	<i>californica</i>	<i>Osmia</i>	<i>nigrifrons</i> <i>nigrifrons</i>
<i>Osmia</i>	<i>cobaltina</i>	<i>Osmia</i>	<i>odontogaster</i>
<i>Osmia</i>	<i>coloradensis</i>	<i>Osmia</i>	<i>paradisica</i>
<i>Osmia</i>	<i>cyanella</i>	<i>Osmia</i>	<i>penstimonis</i>
<i>Osmia</i>	<i>densa</i>	<i>Osmia</i>	<i>proxima</i>
<i>Osmia</i>	<i>enixa</i>	<i>Osmia</i>	<i>pusilla</i>
<i>Osmia</i>	<i>exigua</i>	<i>Osmia</i>	<i>subaustralis</i>
<i>Osmia</i>	<i>indepressa</i>	<i>Osmia</i>	<i>trevoris</i>
<i>Osmia</i>	<i>juxta</i>	<i>Osmia</i>	<i>tristella</i>
<i>Osmia</i>	<i>laeta</i>	<i>Osmia</i>	<i>visenda</i>

Bee viruses:

When honey bees arrived at the wildflower meadows, both foragers and hive samples had evidence of infection for all five of the initially screened viruses. All five have been shown to spillover into other bee species (Tehel et al., 2016), including Deformed Wing Virus (DWV), Sacbrood Virus (SBV), Chronic Bee Paralysis Virus (CBPV), Black Queen Cell Virus (BQCV), and Lake Sinai Virus (LSV). Foraging honey bees displayed a 39% positivity rate for DWV-B, a 43% positivity rate for CBPV, and only a 2% SBV positivity rate across all time points. The honey bee hives on the other hand exhibited 48% positivity for DWV-B, 85% positivity for CBPV, and 47% positivity for SBV. These dynamics, however, shifted across sites and time and differed from the native bee's viral dynamics. The mean prevalence of DWV-B, SBV, and CBPV were grouped by site, plotted through time, and differentiated host species (Figures 3A, 4A, 5A). Individual honey bee foragers (*Apis mellifera*) are distinct from pooled hive samples (Hive). The first time point is before honey bees arrived with the second time point being one week after honey bee hives migrated to the Sierra. The honey bee "Hive" data points at the first time point indicate a sampling event of the hives before migration to the Sierra.

It is important to note that bee viruses were already circulating in bumble bees even before honey bees' arrival. By grouping all of the different *Bombus* species into a singular group, we can see a general pattern that DWV-B is increasing through time (Figure 3B), that SBV started at very high prevalence and then rapidly decreased through time (Figure 4B) and that CBPV is at low prevalence throughout, with a slight increase at the second time point once honey bees have arrived (Figure 5B). When *Bombus* species identity is overlaid with these data we can see that the viral prevalence dynamics of DWV-B, SBV, and CBPV showed variation between species of *Bombus* (Figures 3A, 4A, 5A). All male bumble bees were grouped into (Drones) as males are more taxonomically challenging to identify. Viral prevalence was compared between *Bombus* female workers and *Bombus* male drones to test if there was a higher prevalence among the workers. All female workers were compared to all male drone's viral prevalence, with prevalence grouped by site and time. Plots of viral prevalence showing the impact of sex on viral prevalence of DWV-B, CBPV, and SBV are highlighted respectively in (Appendix 3.5 – 3.7). There did not appear to be significant differences between worker and drone viral prevalence for any of the viruses.

Osmia mason bees were also collected and processed, but due to taxonomic constraints, only identified to genus before processing for viruses. There are now taxonomically verified *Osmia* species in the pinned reference collection generated for the diversity metrics (Table 3) which allows for future DNA barcoding to identify *Osmia* samples of interest. In an analysis of the 133 wild foraging *Osmia*, they had overall low prevalence with a total of nine individuals being found positive for DWV-B (Figure 3A). AnBV-1 was not able to be successfully amplified through PCR, although high read counts in the meta-transcriptomics reports indicate that this was the most abundant viral transcript in the pooled samples. CBPV was found at even lower rates, with only six positive cases across all samples (Figure 4A). DWV-B was present at five sites, whereas CBPV was present at four sites. Three sites had no viruses present at all. For CBPV, three out of the four sites it was found at were not near hives, whereas two out of three of the apiary sites lacked this virus completely. The sites that lacked DWV-B were all near Kyburz Flat Meadows. No *Osmia* were found to be positive for SBV at any time point.

Comparative temporal viral dynamics:

Deformed Wing Virus-B:

Once honey bees arrived at the sites the foragers (labeled as “*Apis mellifera*” in the resulting graphs) immediately had a high prevalence of DWV-B. Figure 3A shows the dynamics for DWV-B through time, where honey bee hives had high viral prevalence even before migration. When they arrived, their viral prevalence decreased before increasing again at the third and fourth time points. Foragers experienced the highest viral prevalence upon arrival but stayed at relatively the same prevalence levels throughout the season. DWV-B dynamics varied by *Bombus* species, but this graph shows that this virus was already circulating in bumble bees even before honey bee arrival. *B. vandykei* and *B. vosnesenskii* had the highest DWV prevalence at the first time point and both appear to decrease slightly through time before increasing in viral prevalence at the final time point. *B. vancouverensis* steadily increases in prevalence throughout the survey, as does *B. flavifrons*, before dropping in prevalence at the final time point. Overall, DWV-B prevalence increased in all the *Bombus* samples, going from a median of 0% prevalence at the beginning of the season to 50% prevalence by the end of the season across all species (Figure 3B) *Osmia* had exceedingly low viral prevalence before honey bee arrival and continued to be low during the second time point but increased more dramatically for the third time point (Figure 3A). No *Osmia* was sampled at the fourth time point.

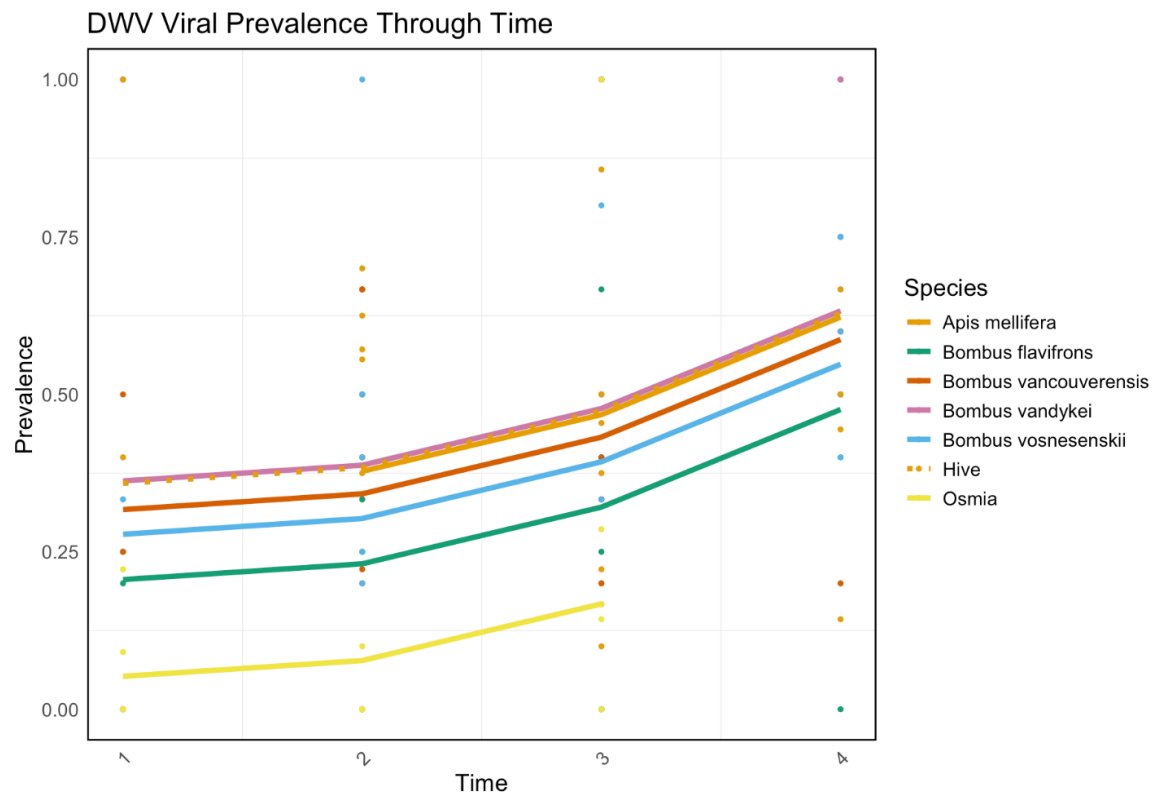


Figure 3A. Viral prevalence of DWV-B pooled across sites through time across all species. Each point indicates a data point of the viral prevalence of that species at a particular site. Lines indicate fitting from a generalized additive model.

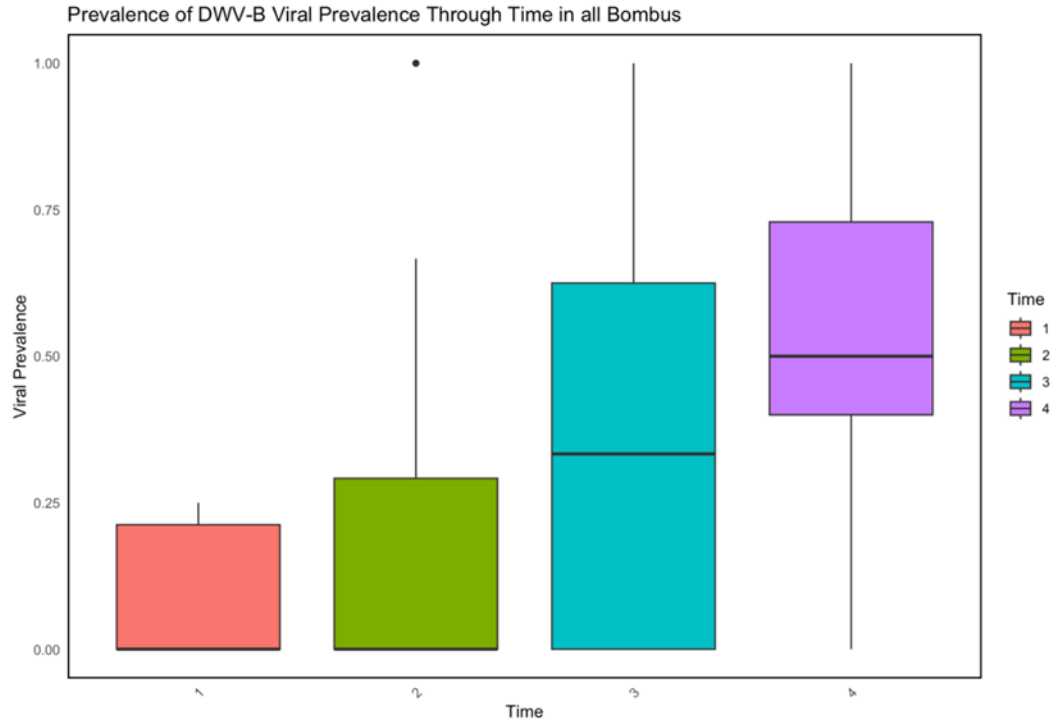


Figure 3B. Boxplots showing DWV-B viral prevalence of all *Bombus* samples through time. Dots outside of the box indicate outliers.

Chronic Bee Paralysis Virus:

Figure 4A highlights the dynamics of CBPV, where honey bee hives were found infected before being moved to these sites and experienced exceedingly high viral prevalence throughout the season. The prevalence was high in foraging honey bees once they arrived at the sites and remained so for the duration of the season. *Bombus flavifrons* and *B. vosnesenskii* both had positive cases of CBPV even before honey bees arrived. Overall, across all species, bumble bee prevalence for CBPV was low throughout the season and did not increase in any noticeable way after honey bees arrived (Figure 4B). *Osmia* had one positive case of CBPV before honey bee arrival, but otherwise had very low prevalence in the first two time points before increasing in prevalence slightly in the third time point, a month after honey bee arrival (Figure 4A). No *Osmia* were collected at the final time point.

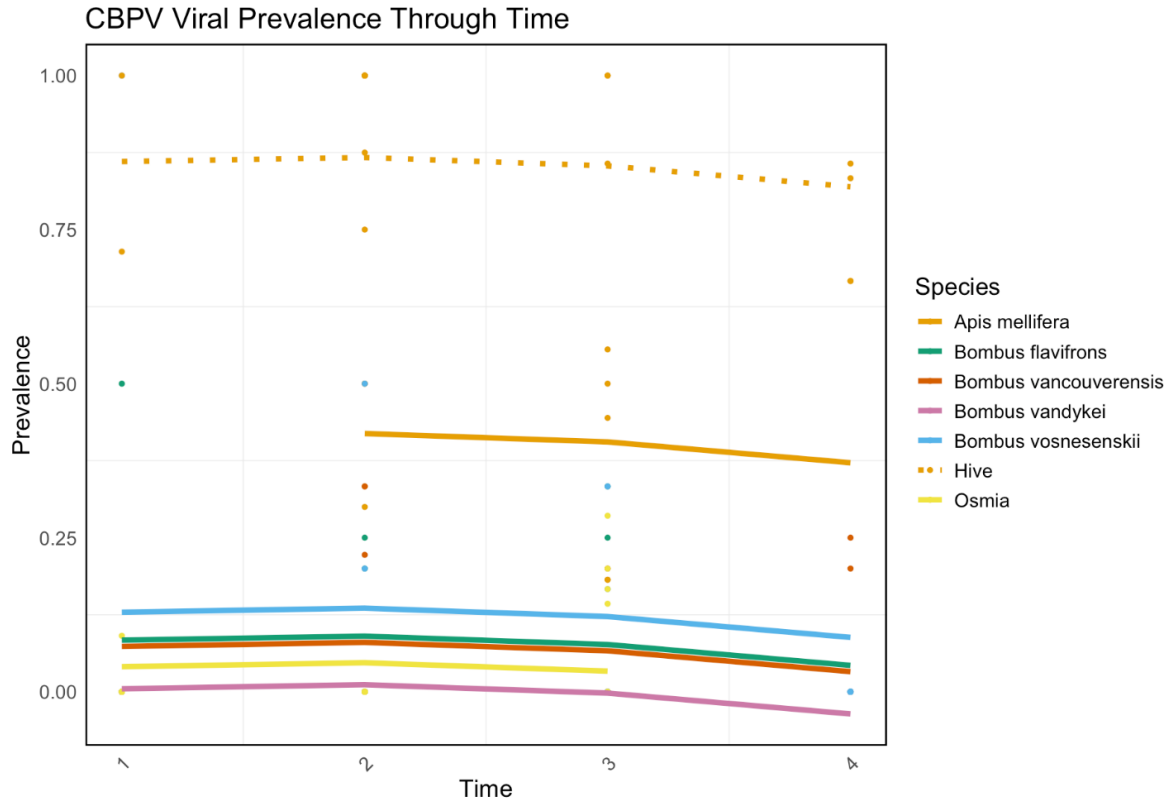


Figure 4A. Viral prevalence of CBPV grouped by sites through time across all species. Lines indicate fitting from a generalized additive model.

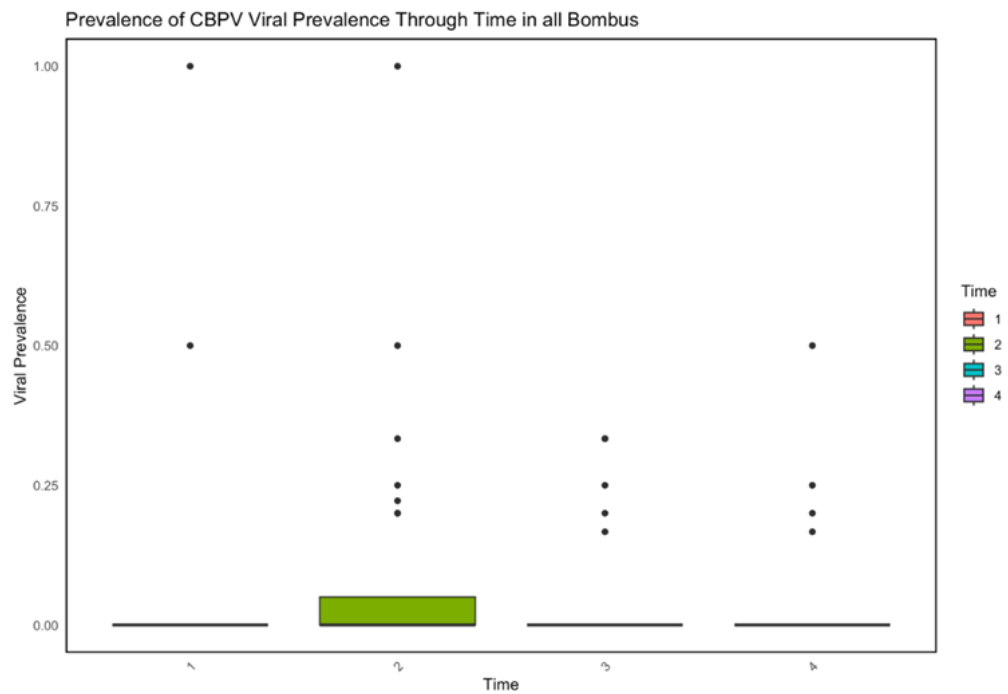


Figure 4B. Boxplots show CBPV viral prevalence of all *Bombus* samples over time. Dots outside of the box indicate outliers.

Sac Brood Virus:

Figure 5A shows SBV viral dynamics through time, where honey bee hives were highly infected with SBV before migrating to these sites but dropped in prevalence through the season. In contrast, honey bee foragers were not found highly infected upon arrival. SBV decreased precipitously through time for all species. SBV was highly prevalent in *B. vancouverensis*, *B. flavifrons*, *B. vandykei*, and *B. vosnesenskii* even before honey bees arrived. SBV prevalence dropped dramatically throughout the season for all the monitored species and was never detected in *Osmia* mason bees. SBV appears to be on a steady downward seasonal trend in all the bumble bees, with the median dropping from 75% prevalence at the start of the season to 0% by the final time point (Figure 5B).

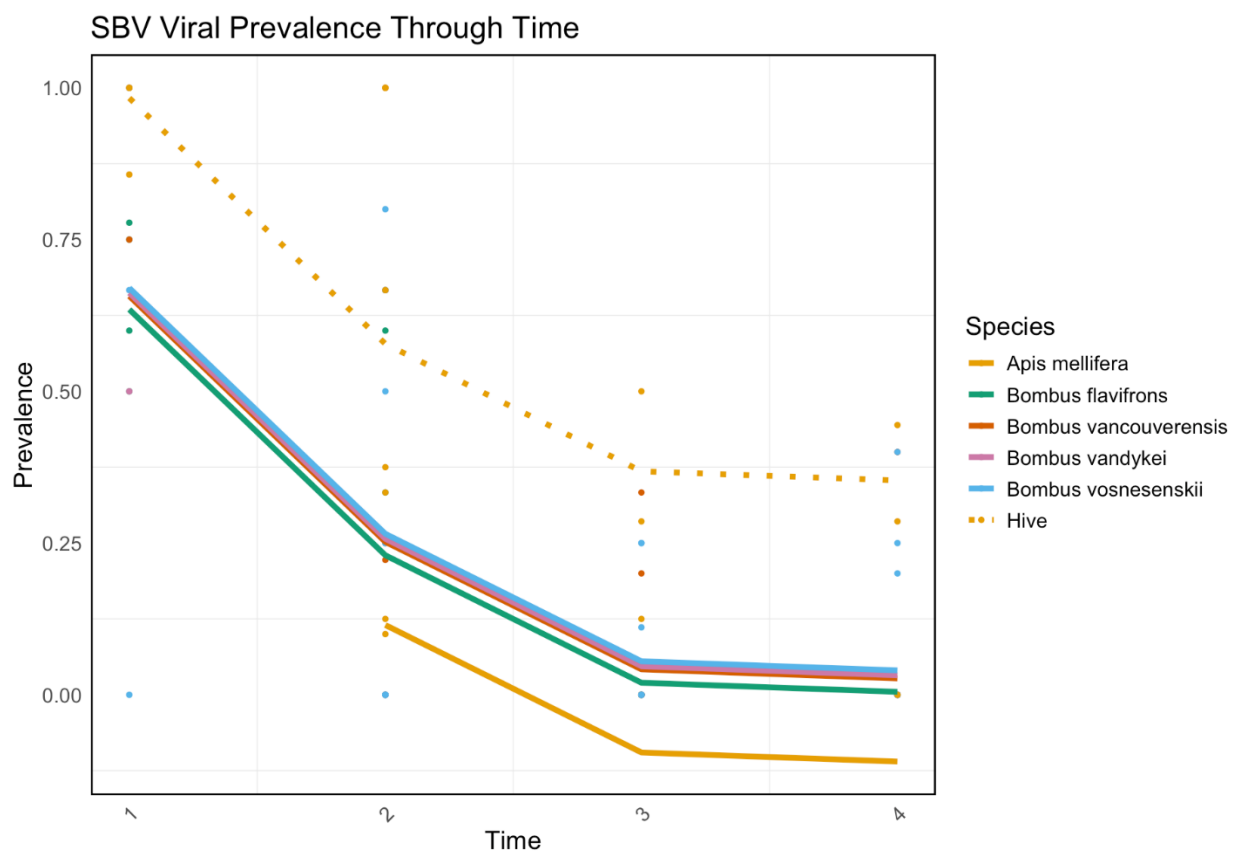


Figure 5A. Viral prevalence of SBV grouped by sites through time across all species. Lines indicate fitting from a generalized additive model.

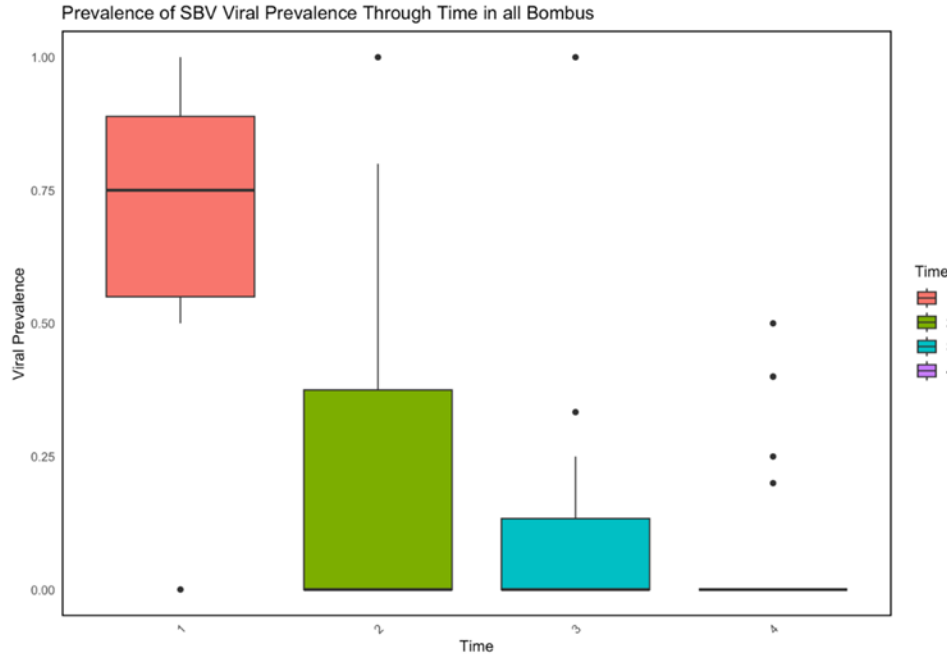


Figure 5B. Boxplots show the SBV viral prevalence of all *Bombus* samples over time. Dots outside of the box indicate outliers.

Effect of abundance and apiary:

Sites varied in their abundance of honey bees, with two control sites never having honey bees present, while the remaining sites had between (1-50) honey bees per 500 m² at least during one point in time (Appendix 3.1). Once honey bees arrived at the second time point, four of the 'control' sites did not have any honey bees recorded (site codes: KFC, PER, LAC, SHFS). However, STA had evidence of foraging honey bees already at this time point, but at a far lower abundance than the sites close in proximity to the apiaries. At the third time point two sites continued to lack honey bees completely (KFC, SHFS) whereas honey bees were found foraging at (PER). By the end of the study, KFC and SHFS were the only two sites where honey bees were never recorded.

The viral prevalence of DWV-B in native bees was plotted against honey bee abundance as well as apiary presence to assess the impact of honey bees in this environment. First looking at the relationship with DWV-B viral prevalence in native bees between sites with and without apiaries, we do not see any initial trends, as both areas with and without apiaries had highly variable viral prevalence (Figure 9). Honey bee abundance from (Appendix 3.1) was log-transformed and plotted against viral prevalence in native bees (Figures 10,11). Initially, the relationship between prevalence and abundance appears to be rather flat without showing much signal. When overlaid with apiary presence (Figure 10) we can see that abundance does not change prevalence in native bees when there is an apiary present, but when there is not an apiary present, there appears to be a slight positive relationship. To test the significance of this possible pattern, the following generalized linear model was parameterized:

```
Model_2 <- glm.nb(Prevalence ~ Log_Abundance * Apiary, data =
BB_DWV_B_prevalence)
```

Modeling the interaction between log abundance and the presence of apiary on viral prevalence in bees did not show a significant effect of abundance, apiary, nor the interaction between the two.

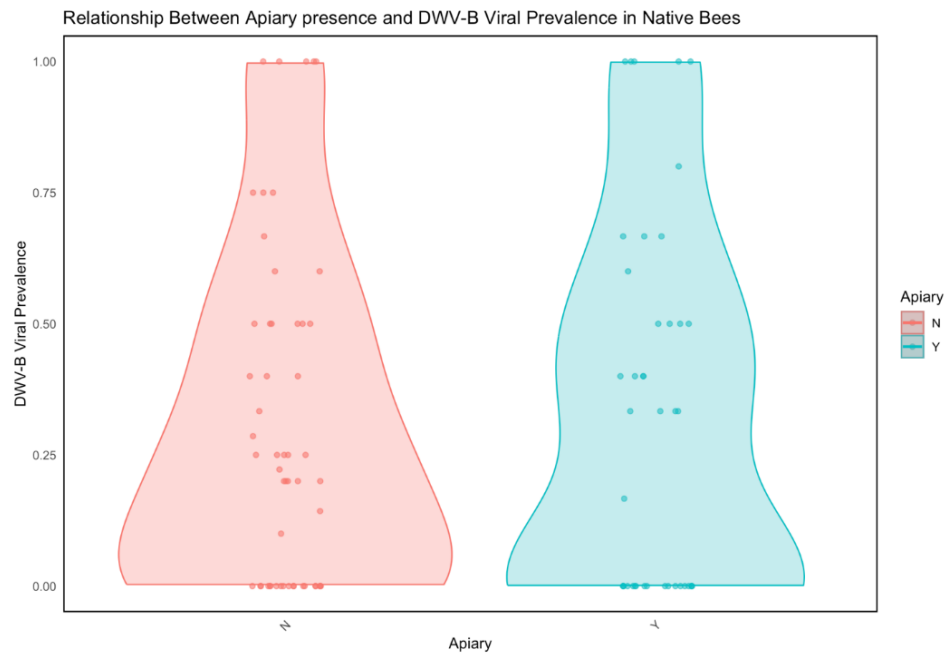


Figure 9. Violin plots show the difference in viral prevalence in native bees based on the presence of apiary (Y) or not (N).

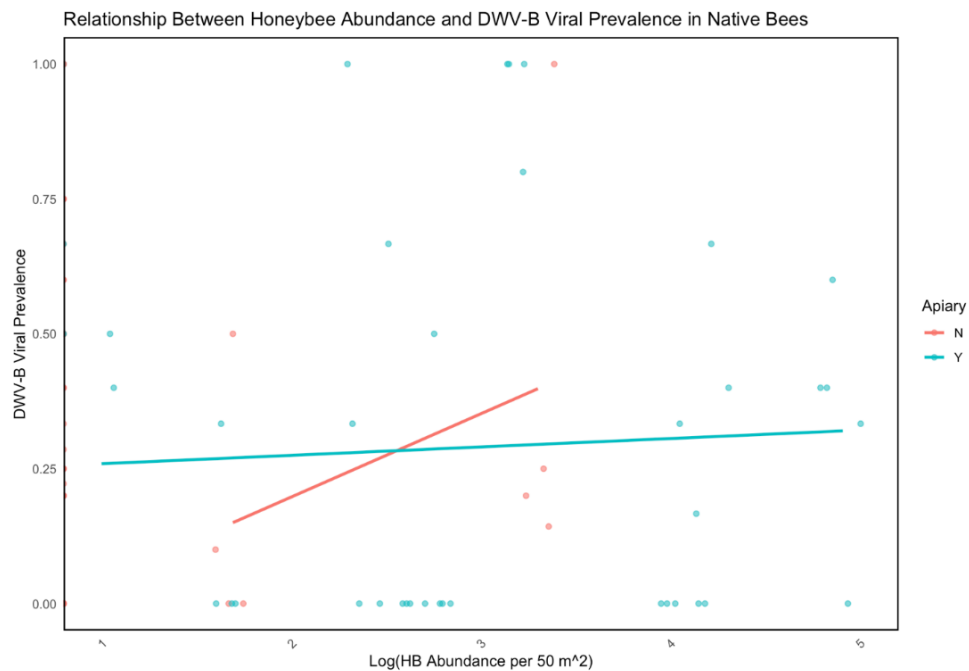


Figure 10. Relationship between log honey bee abundance and viral prevalence in native bees, in the presence or absence of an introduced apiary.

DWV-B prevalence appears to generally be increasing through time in native bees (Figure 3), regardless of the presence of apiary or not (Figure 11). The effect of the interaction between time and apiary presence on DWV-B viral prevalence in native bees was modeled using a generalized linear model with a negative binomial distribution and link: log function:

```
Model_3 <- glm.nb(formula = Prevalence ~ Time + Apiary, data = BB_DWV_B_prev,
  link = log)
```

An ANOVA with a Chi-Squared test was conducted on the resulting model. While the effect of “Apiary” was not significant, the effect of “Time” was approaching significance (p-value = 0.05931).

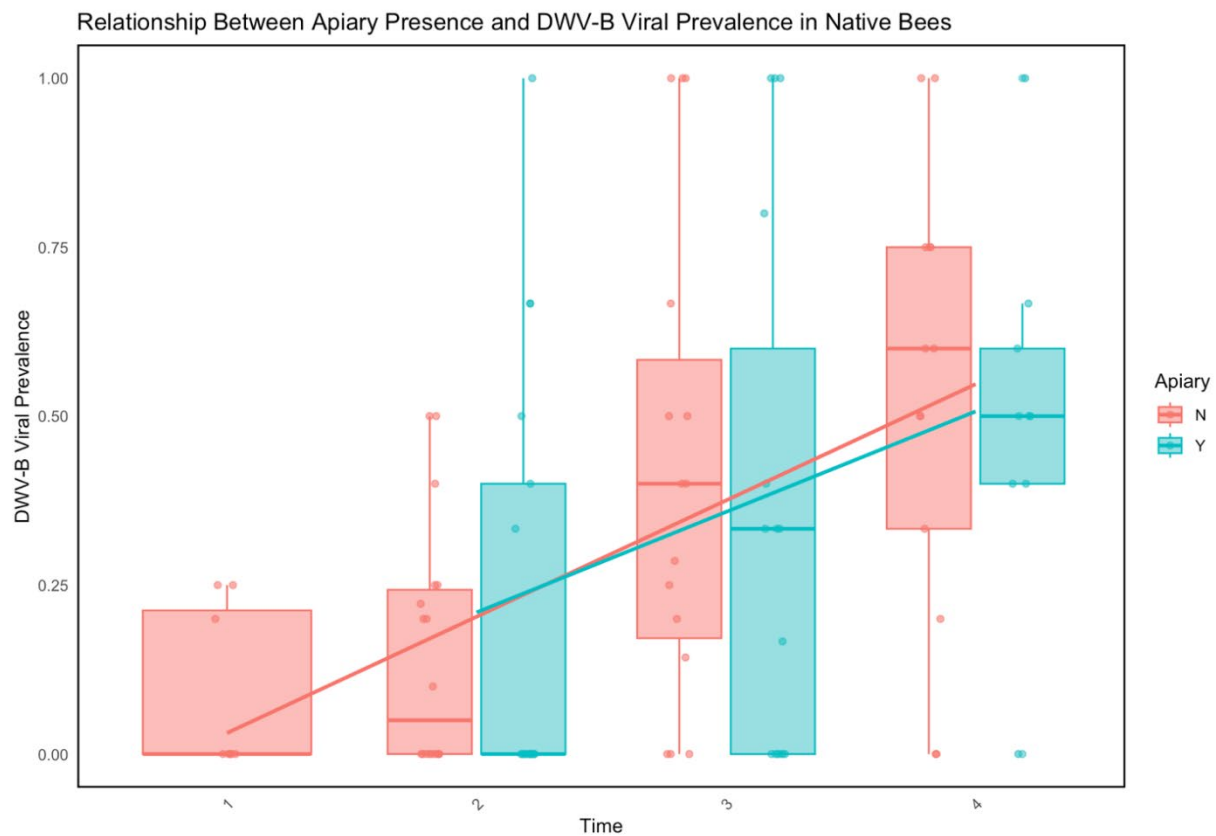


Figure 11. DWV-B Viral prevalence in native bees through time, grouped by whether or not there was an apiary present at the site.

Honey bee DWV-B viral load did not appear to change through time, nor were there differences between the viral load of foragers (*Apis mellifera*) versus Hives (Appendix 3.8). There were some site-specific differences, with some sites experiencing higher viral load on average than others (Appendix 3.9). When looking at the effect of DWV-B load acting as a predictor of DWV-B virus prevalence in native bees, we did not see an effect. DWV-B load alone does not seem sufficient to explain patterns. Future statistical modeling will investigate the interaction effect between viral load and honey bee abundance on native bee DWV-B prevalence.

3.5 Discussion:

Deformed wing virus (DWV) is known to be the most prevalent of honey bee viruses and is the one viral species found in the greatest number of other bee species (Tehel et al., 2016a). Many insect viruses have the propensity to be asymptomatic with covert infections (Britton and Jane White, 2021) but experience condition-dependent virulence where the more stressed the host is, the higher the virulence (Brown et al., 2000; Manley et al., 2017). DWV is characterized in its most symptomatic form as adults emerging with deformed wings, as the name implies. A bee that cannot fly is not capable of surviving long, and therefore its lifespan is cut short significantly. This can scale up to overall colony declines (McMahon et al., 2016). There are three master variants of DWV circulating the globe, with the recently emerged DWV-B being associated with varroa mites and increased virulence (Natsopoulou et al., 2017b). Preliminary work showed a low prevalence of DWV-A in this system, and therefore we focused on studying DWV-B. We found that DWV-B was already circulating in *Bombus*, even before honey bees arrived. Additionally, we saw that honey bees bring DWV-B with them when they migrate and the prevalences were similar between foragers and hive sampled individuals. Amongst the native bees, the dynamics were the most variable with *Bombus vandykei* having some of the highest prevalence later in the season and *Osmia* mason bees having the lowest prevalence of the native bees. When looking broadly across all species of *Bombus*, we can see that DWV-B prevalence is increasing over time, regardless of honey bee abundance or apiary presence. This suggests that this virus could be circulating as an epidemic within this population of bumble bees, with or without honey bees present, and hints towards the competency of bumble bees as a host for DWV. Overall, the temporal dynamics here indicate that DWV-B is increasing throughout the summer for both honey and native bees. Anecdotally, these hives were brought into this system specifically to breed for varroa mite resistance. Breeder hives were identified when the bees were able to keep varroa numbers below a specified threshold. With the current evidence, it does not appear that honey bee DWV dynamics impacted the native bee community. Future research could test this directly by comparing ‘varroa-resistant breeder’ hives to varroa susceptible hives and the bumble bees they are interacting with to see if there is indeed evidence of decreased spillover risk of DWV in varroa-resistant lines of honey bees.

Chronic bee paralysis virus (CBPV) was also found in *Bombus* before honey bee arrival, with *Bombus vosnesenskii* experiencing the highest viral prevalence, followed by *B. flavifrons*. Honey bees brought CBPV with them and experienced high prevalence levels in the hives for the entirety of the study, and relatively high viral prevalence in the foragers. In honey bees, CBPV is characterized visually by bees that are hairless with a greasy appearance and trembling which eventually ends in paralysis before death (Budge et al., 2020). Normally, this is a pathogen with severe symptomology, and therefore the high prevalence in the hives would be concerning. However, during the duration of this study, none of the colonies were lost. There were noticeable differences in prevalence between the forager honey bees and the hive samples, with the hives exhibiting high prevalence throughout the study, whereas foragers were at lower but consistent prevalence throughout the study. This could be explained by the honey bee age structure, where the foragers are the oldest members of the hives (Vance et al., 2009) and therefore could have cleared any remnants of infection by the time they aged up into the forager role. Bees collected from the nest entrance of the hive could have been guard bees which are younger than foragers and could still be experiencing covert or symptomatic infections. Since CBPV is known to have high pathology, individual honey bees that are infected with it as younger bees could experience increased symptoms when aging up into the foraging class. The job of a forager is a stressful

one, having to fly kilometers around their hive looking for resources for the colony. Once they begin foraging, they only live a couple more weeks. Therefore, if a bee is already sick with CBPV the stressful act of foraging may increase the virulence of this pathogen, leading to mortality before there is a chance to sample them. In opposition, *Bombus* had a low prevalence of this pathogen, with a general negative association between prevalence and time. This suggests honey bees are a more competent host for CBPV, but overall, we do not see evidence that honey bee CBPV dynamics seem to be directly impacting the bumble bees.

Sacbrood virus (SBV) was found at exceedingly high prevalence in *Bombus vosnesenskii*, *B. vancouverensis*, *B. vandykei*, and *B. flavifrons* at the first time point even before honey bee arrival. Before the honey bees were moved, their hives also had an exceedingly high prevalence of SBV. In honey bees, SBV is a larval disease and is characterized by discolored larvae that turn a brown color and die before emergence. Although this pathogen can weaken a colony, it is directly associated with high honey bee losses (Li et al., 2019). Once the honey bees were moved to the montane sites, the hives still had high prevalence whereas the foragers exhibited minimal evidence of SBV infection. Again, this could be explained by the age structure of honey bees. I would predict that for larval diseases such as SBV, the prevalence would be highest amongst the youngest age classes of bees. Therefore, by the time a honey bee ages into the forager class, it could have recovered from any larval diseases, thus leading to lower prevalence in foragers. Unlike CBPV, this virus is not known for its high virulence, so I would not predict that there is some heightened disease pressure that has infected bees die off before or at the beginning, of aging into the forager behavioral caste. All species experienced decreased SBV prevalence over time and thus suggests that these viral dynamics decrease seasonally through the summer for both *Apis* and *Bombus*. This makes some intuitive sense for *Bombus* as there becomes a point in the season where the queens transition from a growth phase of their life cycle to a reproductive phase to produce queens for the following year. I would posit that larval diseases such as SBV would be highest during the growth phase of a *Bombus* colony. *Osmia* was never found with positive cases of SBV. However, the lack of evidence of viral presence in any pathogen survey can mean that either the pathogen is not a problem at all, or it is highly virulent and experiencing mortality before the surveyor can sample them (White et al., 2014). Therefore, a lack of field survey pathogen data cannot be used to claim that a species does not get impacted by that pathogen. Increased sampling is required for these cases to increase the probability of capturing an individual before they succumb to the infection. But overall, this fundamental issue is what makes observing virulent pathogens in the field particularly challenging.

When comparing the viral prevalence of the female worker bumble bees to the male drones, we did not see any difference. This is surprising as the behaviors of the two sexes are very different with the workers having to forage for food for the entire colony, while the males just find floral resources for themselves. This would suggest that workers would be experiencing more contaminated floral resources and have higher viral prevalence. This is not seen here and could be due to the smaller sample size of drones than the sample size of workers, which could be attributed to this pattern. Alternatively, this could suggest that vertical transmission is playing a role in the viral dynamics of drones where the queen bumble bee could have been exposed to that virus during her solitary phase.

A balance had to be struck with the ‘control’ sites in this study, as we wanted the sites to be far enough away from the apiaries such that honey bees were less likely to fly that distance, but without being too far away such that the floral community or elevation changed significantly.

Throughout the season, honey bees did find some of the control sites. This is indicated as the honey bee abundance for the ‘control’ sites listed in Appendix 3.1. However, two sites did stay honey bee-free for the entirety of the study. In 2021 there was a drought in California, and this manifested in lesser snowpack in the Sierra. The runoff from the snowpack melting is essentially the only water that enters into the system at the beginning of the summer. The plant phenology runs off that initial influx of resources but quickly dries up. This year was the first year one of the landowners recalled that their water well had gone completely dry. This led to decreased floral forage and therefore it makes sense that honey bees were willing to fly farther to get their necessary nutrients.

Neither honey bee abundance, viral load, nor apiary presence could significantly explain any of the DWV-B prevalence patterns in native bees. Abundance can also be investigated as a possible explanatory factor behind SBV and CBPV. However, those viruses appear to be even less associated with honey bee dynamics and therefore I do not expect to find a pattern. Alternatively, the lack of pattern could be explained by the way that native bee viral prevalence was quantified. Due to differences in species sample sizes, there could be skews of prevalence being reported as being excessively high or low just based on low sample size. Further investigation of this pattern would require a subset of the native bee samples that have at least five individuals comprising the prevalence metric. The various models testing the effect of honey bee abundance, viral load, and apiary presence can be rerun on that subset of data to see if small sample sizes were skewing the results. If there continues to be no relationship between honey bee abundance and native bee viral prevalence, this gives further credence to the idea that these viruses can circulate in native bees, regardless of the presence of honey bees.

The fact that viruses are found in montane bumble bees before interacting with honey bees suggests that these viruses are successfully overwintering in these populations of *Bombus* unless there is an unknown reservoir species. This has implications for the understanding of these viruses’ ecological dynamics as it is still unknown whether these viruses are experiencing ‘source-sink’ dynamics, where honey bees are acting as the source of the viruses such that when honey bees are not present the epidemics should die out. Alternatively, they could be experiencing ‘back and forth’ transmission where all the species are competent hosts and can maintain epidemics, regardless of honey bee presence. Overall, for the viruses that have been screened thus far, there is minimal evidence of spillover from honey bees to native bees in this season. One explanation for this lack of spillover effects is the high floral diversity and abundance typical of meadows in the Sierra Nevada region. This diversity could act as protection against viral spillover due to the differential specialization of bee species on distinct sets of floral resources. If honey bees and bumble bees are never foraging on the same flowers, then they will be protected from inter-genera transmission, but bumble bees could still have their dynamics driven by other species in the genus *Bombus*. Future development of this work will aim to integrate floral preference data into the model to quantify if floral preferences are different between groups, and if those overlapping preferences are associated with increased viral sharing between species. Additionally, the work thus far has just been a vignette of three bee viruses. This work can be expanded, and other important viruses screened for to assess a broader range of viral diversity’s seasonal dynamics and to see if any other virus could have evidence of spilling over between species.

Although these viruses are being found in a range of bee species, the virulence and subsequent population impact are largely unknown. Therefore, it is still hard to say what the

impact of these viruses is on wild bee health. Some estimates of the effect of viruses on non-*Apis* bees come primarily from experimental inoculation studies of *Bombus*. For example, the virulence of DWV in *Bombus* was shown through experimental inoculation leading to an increased host mortality rate (Fürst et al., 2014). However, immune activation in bees is energetically expensive and bees without *ad libitum* food are much more vulnerable to starvation when infected (Graystock et al., 2013), which is a critical limit of laboratory studies. However, trade-off theory suggests that resources that an organism is devoting towards mounting an immune response cannot therefore go to other important life history traits such as growth, movement, or reproduction. Ultimately, there are likely sub-lethal effects that are challenging to measure with current techniques but could still have large impacts when applied to entire populations experiencing epidemics. Demonstrating that insect populations are declining as well as finding the causal agent of these declines remains logistically challenging, especially for RNA viruses which require special handling. To achieve this goal, we must continue to gather long-term temporal survey data to determine disease dynamics associated with host population dynamics and potential species declines.

3.6 Conclusion:

This chapter highlights the complex viral dynamics between managed honey bees and native wild bees in the high-elevation montane wildflower meadows of the Sierra Nevada Mountain range and is the first to characterize bee viruses in this region. By taking advantage of the low honey bee density of this area, this study conducted a 'natural experiment,' using longitudinal sampling through time of wild bee populations for viruses before and after the transplantation of honey bees into the system. We found that DWV-B, SBV, and CBPV were all already present in *Bombus* species before honey bee arrival, suggesting that bumble bees can independently maintain this virus. Additionally, we saw that honey bees brought each of these viruses with them when they migrated. However, the temporal dynamics of each of these viruses were distinctly different. The overall increasing prevalence of DWV-B in *Bombus* over time, regardless of honey bee abundance or apiary presence, indicates an epidemic within bumble bee populations. But different *Bombus* species did experience unique DWV dynamics, suggesting certain species are more impacted by this virus than others. SBV was prevalent in *Bombus* early in the season and decreased over time in all species, while *Osmia* mason bees showed no SBV infection. CBPV exhibited high prevalence in honey bees but low prevalence in *Bombus*, suggesting honey bees as more competent hosts with minimal direct impact on bumble bees. *Osmia* mason bees had positive cases of both DWV-B and CBPV but their prevalence levels were far lower than the honey bees and bumble bees. These insights emphasize the necessity for long-term temporal survey data to understand bee virus ecological dynamics and their potential impact on native bee health.

3.7 Acknowledgements:

The authors would like to thank the beekeeping operation that brought their hives as experimental apiaries for this research. Additionally, I would like to thank Pam, Fred, Mike, and Lauren for hosting the honey bee apiaries and allowing the sampling to occur on their land. Thank you to Dr. Neil Williams and Dr. Maureen Page for connecting me to these field sites and beekeepers. I would like to acknowledge the funding sources from the National Science Foundation (NSF) Graduate Research Fellowship program and the Carol Baird Graduate Student Award for Field Research. Thank you to Carol Baird herself for supporting field biology research and cultivating community amongst graduate student researchers. Lastly, I would like to thank the

staff at Sagehen Creek Field Station for their hospitality and support throughout the years that this research has been occurring.

Concluding Remarks:

Conclusions:

My dissertation research focused on assessing the risk of viruses in both honey and native bees, and how their struggles may be connected. First, I assessed this connection through a review of the parasitic mite, *Varroa destructor*. While this mite exclusively parasitizes honey bees, indirect effects are felt by native bees through their shared viruses. Varroa mites are a new stressor, having been accidentally imported into the United States in the late 80's and subsequently spreading across the country. Since the emergence of varroa, honey bees have been experiencing higher over-wintering mortality in association with increases in viral titers through this novel vector-borne transmission. Native bee health is intrinsically linked to honey bee health due to the sharing of floral resources. These viruses can be environmentally transmitted, meaning bees can pick up infectious virions from the flowers they pollinate. The higher the viral titers, the more viral particles an infected honey bee can shed into the environment. This can lead to an increased risk of successful environmental transmission to co-foraging native bees. In response to the unsustainable over-wintering losses associated with varroa mites and the evolution of resistance to acaricides by the mite, beekeepers have been attempting to breed for varroa mite resistance.

The existing honey bee breeding literature was reviewed in Chapter 1 to determine the mechanisms by which honey bees are surviving despite varroa mite presence and no mite treatments. We found that most instances of honey bee breeds that were touting varroa mite 'resistance' were 'tolerance' mechanisms. Resistance is defined as immunological defenses that prevent or reduce parasite burden. Tolerance, on the other hand, is a strategy that works to minimize the pathological damage from the infection, without reducing parasite burden. While on the outside this strategy manifests in less honey bee mortality, tolerance suffers from three tragedies: (1) For honey bees, tolerance represents an evolutionary end-state where more individuals are dying from a parasite than was the case when the population was fully susceptible. (2) Spillover incidence will increase to native bees in the community due to the greater number of infected honey bees in the landscape, each being more infectious due to higher pathogen replication rates and (3) When spillover occurs from honey bees to wild bees, the infection is more virulent in wild bees. Therefore, I argue that selectively breeding for varroa mite resistance through methods employed by Darwinian beekeeping in which colonies that are not able to maintain varroa mite counts below a certain threshold do not get to be a part of breeding programs. By doing mite counts and selecting bees that can keep mite numbers low to non-existent, resistance is actively selected as opposed to tolerance. more ecologically responsible may allow this critical livestock industry to both avoid the tragedies of tolerance for wild bees and mitigate current honey bee losses.

Each year pollination of nearly the world's supply of almonds requires close to two million honey bee hives to accomplish the task. With hives coming from all corners of the country, this pollination event has the risk of acting as a mass mixing event for disease. In Chapter 2 a time series of viral diversity was generated from a longitudinal RNAseq study of honey bee hives that were shipped to the Central Valley to participate in almond pollination in

comparison to hives that did not participate in pollination. We found that viral richness was higher in the almond hives than in the controls before, during, and after the almonds had bloomed. Viral richness was higher in the almond hives almost immediately upon arrival, as this sampling event occurred only a few days after the hives were moved to the orchards. This hints towards the idea that being moved is an intrinsically stressful event for the bees, thus leading to increased viral proliferation. To say that more definitively we would have needed to sample the hives before they were moved to the almonds. Interestingly, time alone did not appear to have an impact on viral richness metrics in the almonds. Once all the hives reunited in their home yard, viral richness in the controls increased significantly. This suggests that the effect of almond pollination on viral diversity can be experienced by bees that did not even participate in the event. When looking at individual viral taxa, the dynamics varied between almond and control hives. The Lake Sinai Virus Complex all showed to have significantly higher differential expression in the almonds. It appears from this data that for certain viruses the almond bloom matters, while for others, these are more seasonal dynamics that occur regardless of participating in the bloom. To investigate these patterns further, we must take a phylogenetic approach. Initially, I hypothesized that viral richness would be the metric that changes significantly throughout the bloom. Now I am interested in understanding what is happening to strain diversity during the bloom and what is occurring to genetic variability within a viral species through the bloom itself. Future studies working on this data set can integrate phylogenetic inferences based on viral sequence data that have sufficient coverage to see how viral strains are proliferating during the almonds.

After participating in the almond pollination, the beekeeping operation from Chapter 2 returned to the Sierra foothills for the spring before they experimentally moved a subset of their breeding hives to montane wildflower meadows at higher elevations in the Sierra Nevada. Chapter 3 focuses on the viral dynamics between managed honey bees and native wild bees at these sites in the Tahoe National Forest and is the first to characterize bee viruses in this region. The native bee virome was characterized before honey bee arrival. Both honey and native bees were collected through time across sites that varied in their presence of an apiary and their honey bee abundance. All of the initial viruses we screened for, DWV-B, SBV, and CBPV, were already present in *Bombus* species before honey bee arrival. This hints towards bumble bees having the host competency necessary to independently maintain this virus. Alternatively, there could be other unaccounted for species in the environment that are driving these dynamics. This beekeeping operation had set up their apiaries at these sites the previous year as well. I hypothesize that interactions between honey bees and future bumble bee queens in the previous season could have seeded the viral dynamics into the native bees, even without honey bees present. Honey bees also were shown to bring viruses with them when they were moved. However, the temporal dynamics of each of these viruses were distinctly different. DWV-B increased in time overall through the season. When assessing the relationship between honey bee abundance or apiary presence with native bee DWV-B prevalence, no statistically significant pattern emerged. This means that regardless of honey bee presence or density co-foraging, the prevalence of DWV-B was consistent across bumble bees. This provides another piece of evidence that DWV-B can circulate in *Bombus*, regardless of honey bee dynamics. SBV on the other hand showed high prevalence in honey bee hives and bumble bees broadly at the beginning of the season and then a precipitous drop through time. This suggests that SBV dynamics witnessed here are seasonal rather than one species influencing the other. Finally, CBPV

exhibited a high prevalence in honey bees but a low prevalence in *Bombus*, suggesting honey bees as more competent hosts. *Osmia* mason bees had positive cases of both DWV-B and CBPV, but their prevalence levels were far lower than the honey bees and bumble bees. These insights emphasize the necessity for long-term temporal survey data to understand bee virus ecological dynamics and their potential impact on native bee health.

During my dissertation, I developed three independent field systems across Northern California to determine the impact of crop pollination events on the viral community of honey and native bees. Fieldwork across these systems has been conducted over the last five years with the aim of surveying for viruses. This has generated a significant frozen time series of bees which can act as a repository of data. We lack so much knowledge about RNA viruses due to the delicate nature of RNA needing to be stored at ultracold conditions and with sterile materials as to not degrade the sample. Although this dissertation's chapters only highlight two of my systems in 2021, thousands more bee samples can be processed to have their viral dynamics quantified in the same way as I outlined here. I now possess the training and technical abilities to process this repository of data. My long-term research goal is to understand transmission dynamics between managed and native/wild bees to infer which species are maintaining these various viral epidemics. I aim to accomplish this by collecting bees for viral quantification, therefore generating a long-term epidemiological time series data set that highlights viral dynamics between species of bees across years. With this knowledge, I aim to design ecologically minded disease management strategies to help conserve bees into the future.

Future research:

I aim to continue in academia with a focus on disease ecology, insect viruses, and conservation. After completing my dissertation, I will continue as a postdoctoral researcher in the Boots' lab where I will expand the various bee virus systems I have developed. This will manifest a variety of ways, including in the following two projects outlined below:

Project 1: The role of honey bee management on viral dynamics in honey and bumble bees

In addition to developing the almond field system in chapter 2, and the Sierra wildflower system in chapter 3, during my dissertation I also developed a local field system centered in Marin County. There I was interested in understanding bee viral dynamics and the role honey bee management plays on disease both in honey bees and the bumble bees they interact with. This will form the basis for the postdoctoral research position that I will begin in the Boots' lab and is the foundation of an in-progress National Science Foundation grant.

The role of management:

In addition to the disease risks associated with moving commercial honey bees to the almond bloom, modern honey bee management may also impact disease prevalence. With intensive management practices, there is an increased usage of pesticides and miticides that can increase the survivorship of hives. Viruses associated with the Varroa mite increase through the season as mite populations increase into the fall, and this effect is stronger in hives with lower levels of management (Underwood et al., 2023). Alternatively, we might expect high-management sites to increase in pathogen prevalence through the season due to the larger population size of commercial apiaries (Bartlett et al., 2019). The stressful conditions in a dense population of honey bees could ultimately lead to greater spillover risks into native bee communities. We

propose to monitor viral dynamics and infer which stressors associated with management practices in the system could be leading to a higher pathogen burden. This project investigates the impact of management and honey bee migrations for crop pollination on the emergence of viruses, both within California honey bees and native wild bees. This will help protect pollinator services and increase understanding about the emergence of bee diseases in general.

The three specific objectives of this work are:

Objective 1: Determine the impact of different honey bee management regimes – feral, static, and migratory beekeeping – on viral dynamics within honey bees.

Objective 2: Determine the impact of the three different honey bee management regimes and environmental drivers on the subsequent spillover and community dynamics of honey bee viruses within the native bee communities: by determining the dynamics and directionality of viral spillover.

Objective 3: To build virus pollinator community models to examine the implications of multi-host disease dynamics and explore management scenarios.

This will be done through long-term longitudinal sampling and analysis of honey bee viruses emerging into native bees in the field. We aim to identify the major host reservoirs of the viruses and infer transmission dynamics between species. We propose to address the question of directionality in potential viral spillover, by documenting the viruses by sampling multiple times in a season across a time series. In addition, we will take advantage of a field perturbation “experiment” by comparing viral emergence across different pathogen risk settings created by honey bees with different management practices associated with the Almond Bloom in California. A graphical abstract of the sampling scheme is illustrated in Figure 1.

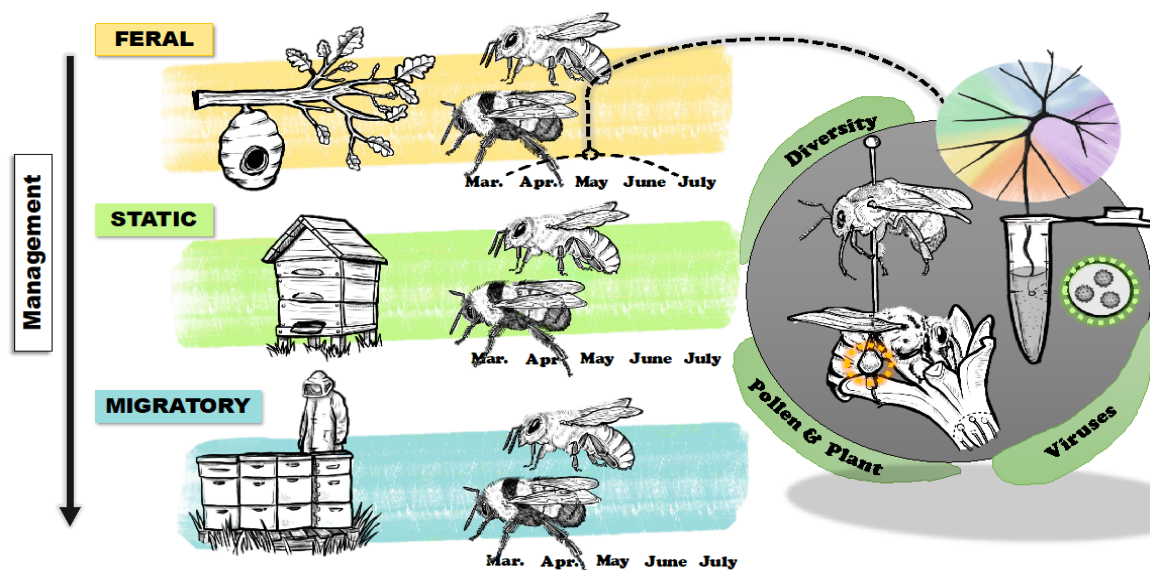


Figure 1. The sampling scheme for this project compares the effect of honey bee management styles on viral dynamics in honey and native bees through time.

Field system:

It is becoming increasingly popular to place commercial hives on large sprawling cattle ranches in Marin County. Here they interact with the native bees, sharing floral resources and potentially exposing the native bees to pathogens to which the honey bees could have been exposed during the pollination events. Additionally, hobbyist beekeeping is a common practice in the county, leading to a substantial population of static, non-migratory hives. Lastly, when honey bee hives get too big, they show swarming behavior, splitting their colony in half and taking the old queen to establish a new hive. These hives can be established in the wild in tree holes and are considered feral. For this work, we define feral hives as those that have survived at least one winter on their own without human-mediated management. We predict that the migratory, static, and feral colonies would be experiencing unique disease dynamics based on the variation in management techniques. In this environment, the sites quickly become barren of flowers as summer progresses. That means native bees are at risk of spillover from any of the diseases that the honey bees have been exposed to in an increasingly resource-limited environment. Both migratory and stationary honey bee hives have active management and treatment for parasites and pathogens and supplementation of food resources in times of scarcity. In contrast, feral populations experience lower densities, however, have none of human-mediated management to treat parasites and pathogens. Currently, it is unknown which management style would lead to more or less spillover of viruses to native bees. In Marin County we can track all three groups and the native bees they interact with to examine the impacts of honey bees on native bees through a natural perturbation ‘experiment’ by comparing communities with; (1) migratory crop-pollinating honey bees, (2) managed but static honey bees that are not used in crop pollination and (3) feral honey bees. We hypothesize that the different management regimes lead to changes in honey bee viromes with subsequent impacts on native pollinator communities.

Field work will occur in Marin County, which is near Berkeley, allowing for practical long-term repeated temporal sampling throughout the year. Commercial beekeeping operations participate in the almond blooms and then place hundreds of hives on local ranches. Additionally, there are multiple beekeepers and honey producers with stationary hives alongside high native bee diversity. Furthermore, due to the high population of managed bees in Marin County, there are established feral colonies of honey bee hives that survive in the wild without any human intervention. We have established connections and have received approval for our research from landowners and beekeepers supporting this work across each of the three management regimes with respective sites outlined on the map drawn in Figure 2.

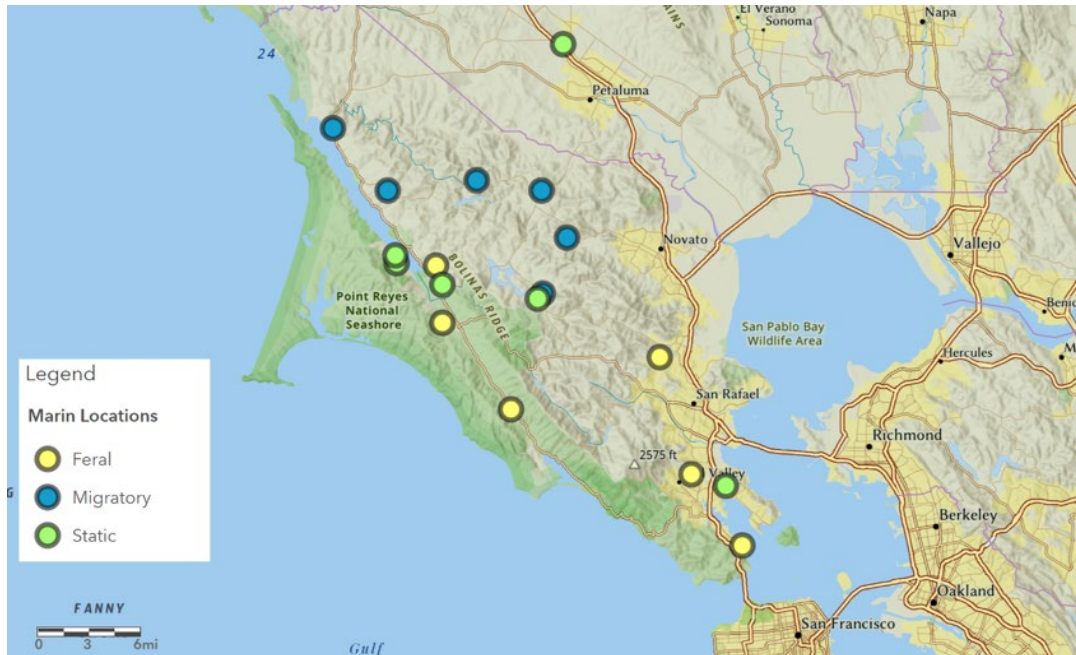


Figure 2. Map of Marin County detailing the established sites of Feral, Static, and Migratory honey bee hives.

Preliminary Results

In 2021 we carried out a preliminary examination of the impact of management practice by comparing feral honey bee hive virus dynamics to managed migratory ones across 12 sites for four key honey bee viruses across three time points in the spring. These are the two extremes of the honey bee management spectrum. We measured honey bee viruses and spillover into pollinator communities in four common species of bumble bees (Fisher et al., 2022) (*Bombus vosnesenskii*, *Bombus melanopygus*, *Bombus mixtus*, *Bombus californicus*) by collecting honey bees and bumble bees across 12 sites and three-time points in the spring. The most consistently prevalent virus found in both managed and feral honey bees was the Black Queen Cell Virus (BQCV), a pathogen that shows little virulence throughout the year, but can lead to colony failure by preventing the development and emergence of a new queen to replace dead queens (Murray et al., 2019) (Figure 3). The virulence of this pathogen on other eusocial bees such as bumble bees is unknown, however active replication through the detection of the negative strand of the BQCV has been shown (Peng et al., 2011).

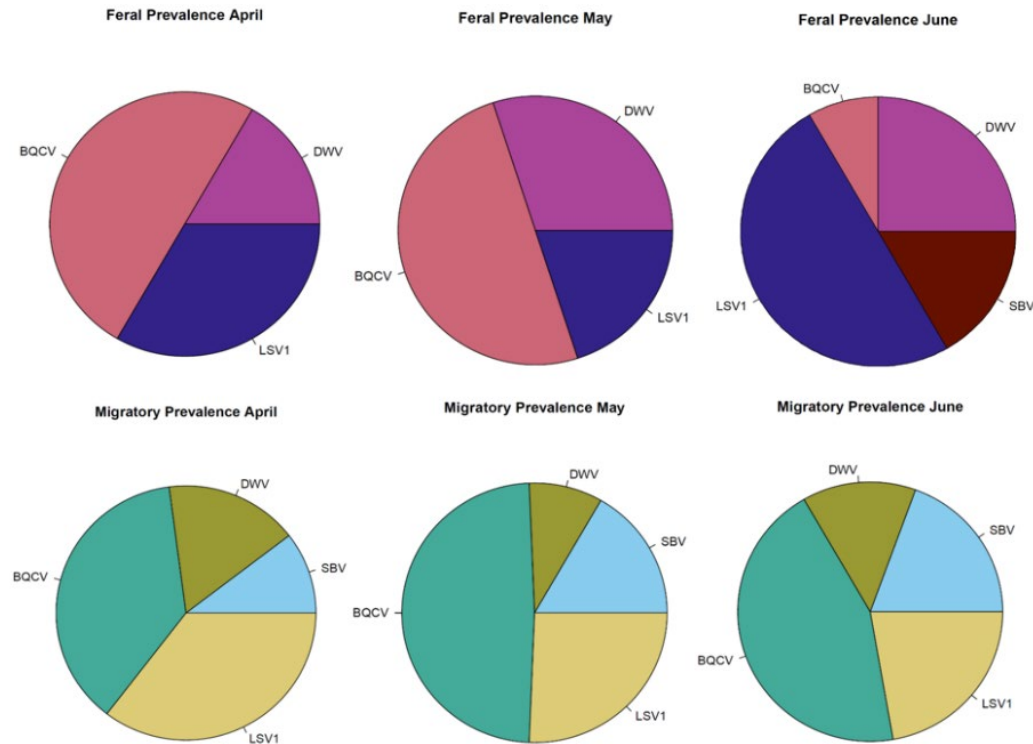


Figure 3. Pie charts indicate the differences in viral prevalence between “Feral” and “Migratory” operations of honey bees.

Importantly in the context of our proposal, the three other viruses (Lake Sinai Virus 1 (LSV1), Deformed Wing Virus (DWV), and Sac Brood Virus (SBV)) show clear differences in prevalence between the feral and the managed migratory honey bees. Furthermore, there is clear temporal variation through the season (Figure 2). Specifically, Sac Brood Virus is present in the migratory honey bees throughout the entire season, while it is absent in the feral honey bees until the final time point. From initial processing of the co-occurring *Bombus* specimens, SBV is found circulating in both *B. melanopygus* and *B. mixtus* from the very first time point at the feral sites. This suggests that the feral honey bees could have either been infected from *Bombus*, or the migratory honey bee operations nearby. The nearest migratory site is 5.6 kilometers away from the feral sites which is within the foraging radius honey bees are known to travel, however, this requires flying over the Tomales Bay which could act as a dispersal barrier. With the aid of this grant, we would be able to sequence the SBV strains from the feral and migratory hives and phylogenetically compare them to the *Bombus* strains to infer the most likely route of transmission. This preliminary data shows that management practice impacts the viral dynamics in honey bees and demonstrates the need to examine these differences across a time series. In this preliminary data, we are only comparing migratory managed and feral honey bees and therefore need to also examine the dynamics of managed static honey bees to control for human management and understand the impact of migrating honey bees to the almond bloom itself. In 2022 and 2023 we collected honey bee and bumble bee samples, this time comparing migratory to stationary hive dynamics along with the interacting native bees. Our preliminary data shows that these differences in the honey bees may also lead to knock-on impacts on the viral dynamics in the broader pollinator community. My future research will involve continuing to process the

data from sampling in 2022 and 2023 as well as leading field teams for continued longitudinal sampling and viral monitoring across this unique field system.

Project 2: The imperiled western bumble bee (*Bombus occidentalis*):

Bumble bees in the genus *Bombus*, specifically, have experienced dramatic reductions in their abundance and range (McFrederick and Lebuhn, 2006). Pathogens have been implicated as one of the many important drivers of declines in both managed and native bees (Singh et al., 2010). Notably, *Nosema* parasite species were one of the first demonstrations of invasive novel honey bee pathogens spilling over and causing disease in wild bees (Graystock et al. 2013). *Bombus occidentalis*, also known as the western bumble bee, was once the most common bumble bee species on the west coast of North America. But starting in the 1990's it began declining in range and abundance (Cameron et al, 2011). In 2019, California determined four bumble bee species may be warranted to be listed as endangered under the California Endangered Species Act (CESA), and in 2022 the state ruled in favor of insects being possible candidates for listing. One of those species is the western bumble bee (*Bombus occidentalis*). A leading hypothesis behind these declines includes the spillover and subsequent spread of an introduced pathogen, *Nosema bombi*, an intracellular microsporidian parasite found commonly in bumble bees throughout Europe (Thorp and Shephard, 2005). *Nosema* has been associated with the decline of *Bombus* in the US (Koch et al., 2012) and has been found at higher prevalence levels in *Bombus occidentalis* than in other, non-declining bumble bee species (Cordes et al., 2012; Cameron et al., 2016).

RNA viruses are also emerging as another potential threat and driver of bumble bee decline, as viruses that were once thought to be honey bee specific, are now being found in many other bumble bee species (McMahon et al., 2015). However, the consequences of these viral infections are generally unknown (Alger et al., 2019). Trade-off theory suggests that whenever a host has to mount an immune response, this takes resources away from life history traits such as growth and reproduction and has to put them towards defending the host against a pathogen. Therefore, hosting an infection will, by definition, be costly to the host and therefore is one of the many stressors leading to declines. Currently, there are no papers that discuss the virus prevalence found in *Bombus occidentalis*. Therefore, this project sheds light on how viruses could be interacting with this declining bee species.

This proposed research is critical to understanding how to protect this population of threatened bumble bees because very little work has been done at these sites in the first place at all. To my knowledge, the only study that has found *Bombus occidentalis* at these sites was conducted by Hatfield & Lebuhn (2006) which quantified *Bombus* diversity across a range of sites in the Tahoe National Forest. Subsequently, no known surveys have been conducted to assess their populations, their floral preferences, or their pathogen prevalence. Viral research has not been conducted on this species to date to my knowledge and therefore would make this a landmark study.

After obtaining the proper permitting from the California Department of Fish and Wildlife (CDFW) a field season was conducted in 2023 to survey for *B. occidentalis* abundance across the sites that I had established in Chapter 3. Abundance surveys highlighted which sites had *B. occidentalis* present and informed us how many specimens of *B. occidentalis* we could lethally sample for this study according to the permit. This imperiled species of bumble bee was collected alongside the other species of bumble bees that were found co-foraging at the same

sites and time points. Samples were collected using standard viral methods explained in this dissertation. *B. occidentalis* was focused on as an initial priority. RNA was extracted, cleaned, and sent to the company Novogene for meta-transcriptomic sequencing.

Preliminary Results

B. occidentalis was only found on private property in 2022 (Site: MMM) but then found on 3 separate sites in 2023: Sagehen Creek Field Station (SHFS), Perazzo Meadows (PER), and Stampede Reservoir (STAMP). Two out of three of these sites were new for the detection of this species. The distance between each of the sites extends beyond the 1.7 km a typical bumble bee is known to travel, suggesting the presence of at least three distinct colonies (Figure 4).

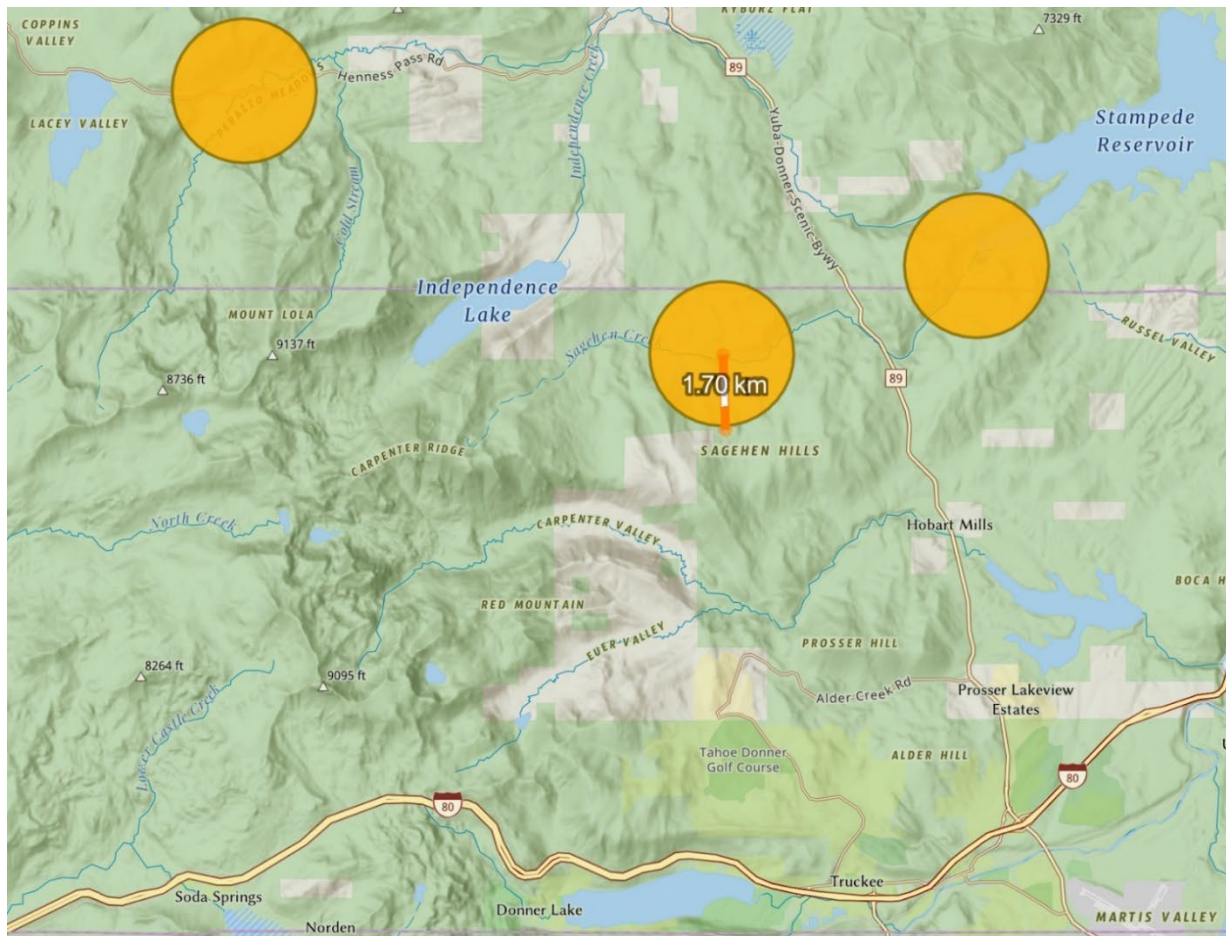


Figure 4: Sites in Tahoe National Forest where individuals of *Bombus occidentalis* were found.

We conducted abundance surveys and lethally collected a subset of the bumble bees based on the estimated density counts. Reports of the location and abundance information were communicated to the CDFW to inform the current work for re-listing this imperiled species. The density of *B. occidentalis* was greatest at STAMP, followed by SHFS, and finally PER. Notably, one of the individuals collected at STAMP was a drone, indicating a shift from the growth phase to the reproductive phase of that colony's life cycle. The meta-transcriptomic results for the four pooled samples quality control results showed successful sequencing of high-quality libraries. Figure 5 showed variation between sites and individuals in their microbial community

composition with the individual from the PER site having the highest relative abundance of a suite of microbes.

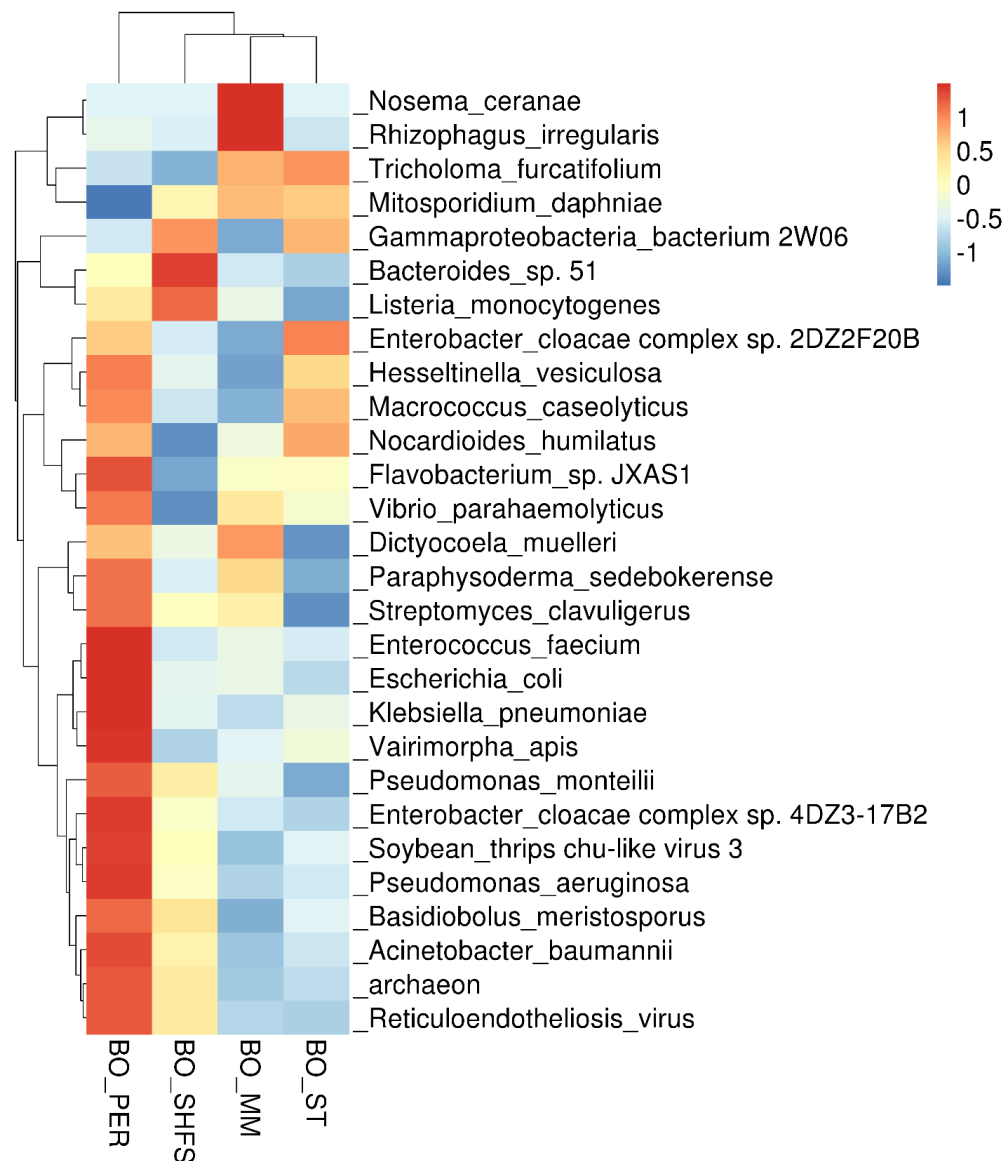


Figure 5. Cluster of species. The Bray-Curtis distance clustering tree structure is on the left side; the relative abundance distribution map of each sample is shown.

This was the first meta-transcriptomic study to investigate pathogen community composition in *Bombus occidentalis*. From the meta-transcriptomic sequencing results, a heatmap was generated to compare the relative abundance of each of the microbes present between samples. I found evidence of *Nosema ceranae* infection at the MMM site and *Vairimorpha apis* infection at the PER site. Interestingly, none of the four populations had any honey bee-associated viruses at all. This brings into question whether these bees are managing to resist any possible viruses they are encountering, and that is why they can survive here.

Currently, I am not able to address this question, as this is confounded with 2023 being the first year that local landowners did not allow commercial honey bee operations to place their hives on the private land neighboring these meadows. Therefore, this year could also just be a year free of honey bee viruses.

For my future research, I will address this question as I have other species of bumble bees collected at the same time and sites sitting in the freezer which I have already identified to species. I will conduct meta-transcriptomic sequencing on the other species of co-occurring bumble bees as well to see if they are also free of honey bee viruses this year, thus suggesting the benefit of not allowing honey bees to be placed in these Sierran meadows. Alternatively, if *B. occidentalis* is uniquely free of honey bee viruses, while the other species are found infected with them, this suggests that this area is possibly providing a refuge for this species to survive despite pathogen pressures. A possible explanation for that pattern could be that *B. occidentalis* have unique floral foraging preferences due to their short tongues. My former undergraduate student, Anna Hatzakis, focused her senior honor's project on this exact idea. Anna quantified the pollen preferences of this species from the pollen that was also collected from the bees that were sequenced. Through identification of pollen granules by light microscopy, she found that most of the pollen sample was composed of pollen from the Apiaceae family, driven by the pollen from Parish's Yampah (*Perideridia parishii*) (Figure 6), Anecdotaly, I have never seen other bumble bees foraging on this flower which is often associated with drier environments. If this pattern continues to hold, further investigation of the unique floral preferences in comparison to the other bumble bees of this area will be warranted.

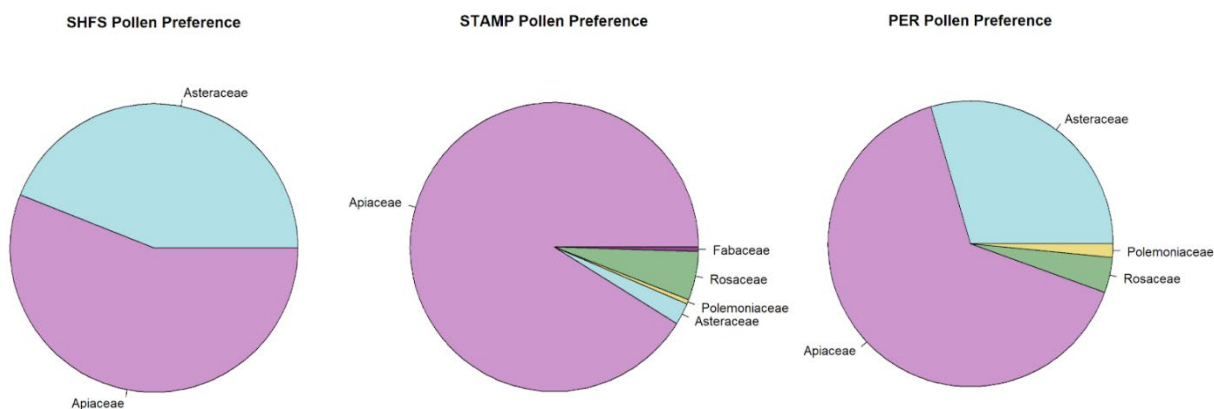


Figure 6. Floral family composition of pollen across sites (SHFS, STAMP, and PER).

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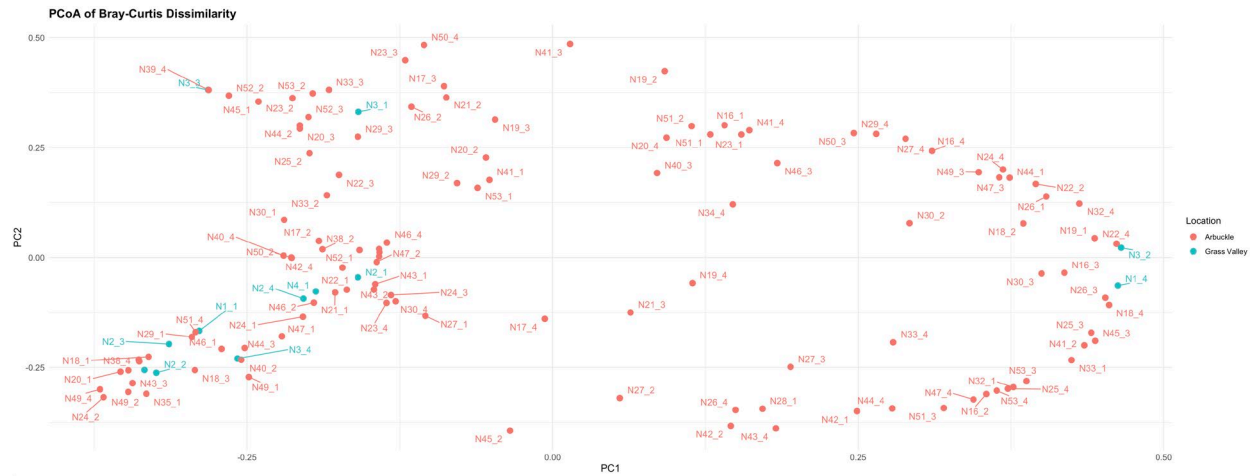
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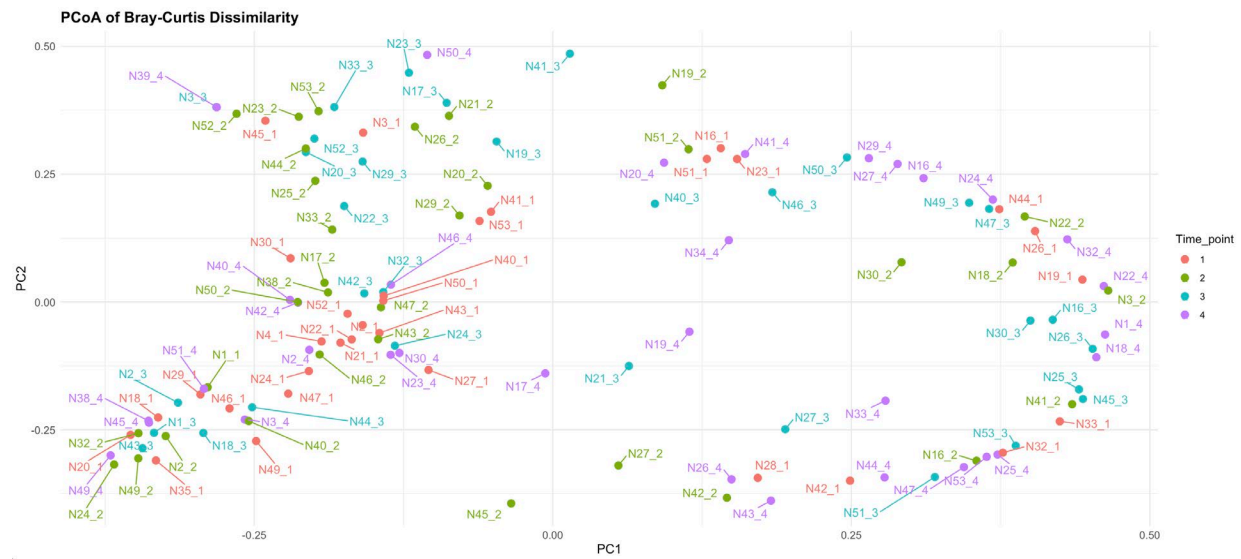
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Appendices:

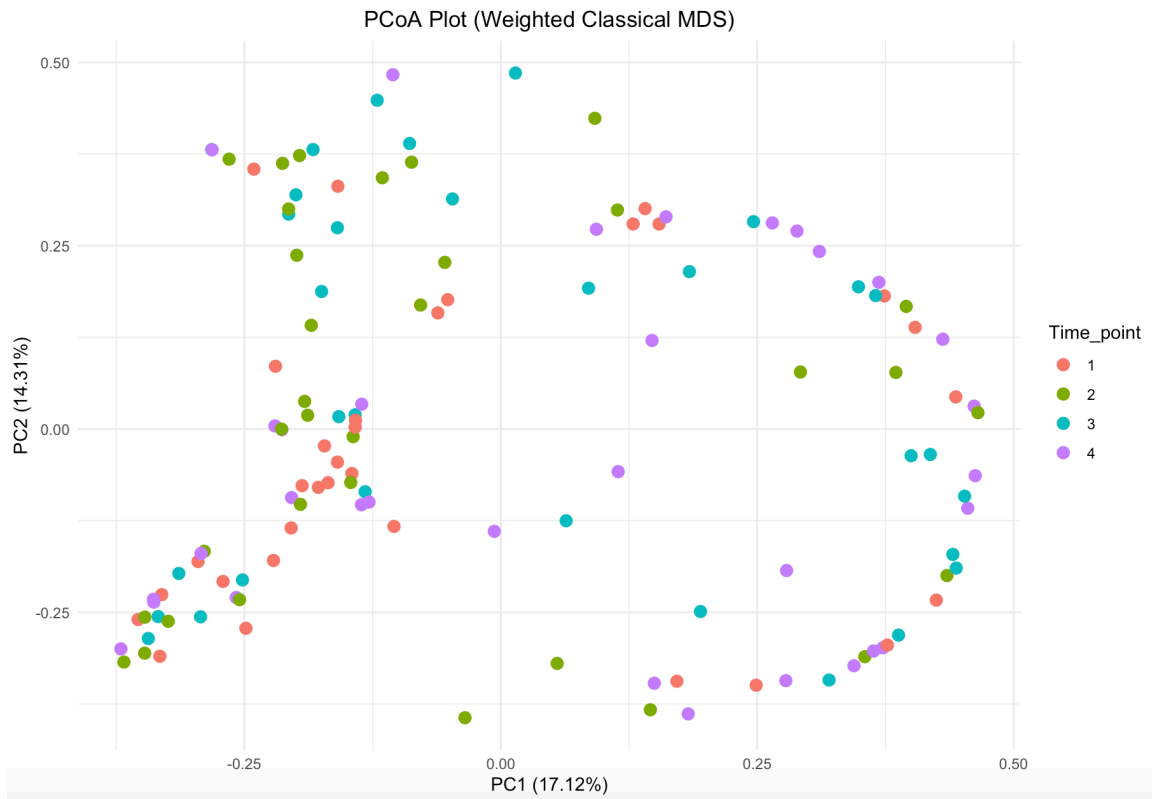
Chapter 2: Supplementary Figures



Appendix 2.1. Principal Coordinates Analysis plot of Bray-Curtis Dissimilarity metric across all hive samples, grouped by Location.



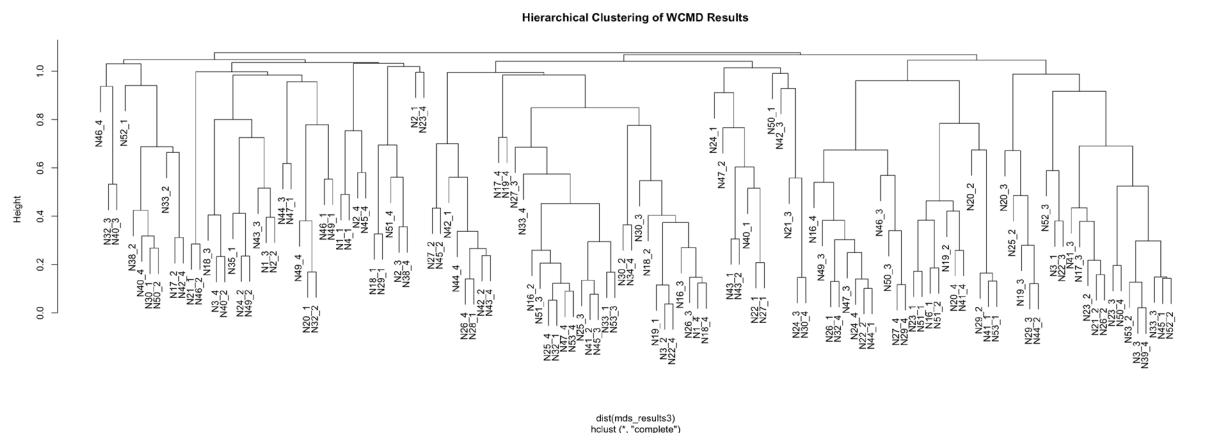
Appendix 2.2. Principal Coordinates Analysis plot of Bray-Curtis Dissimilarity metric across all hive samples, grouped by Time.



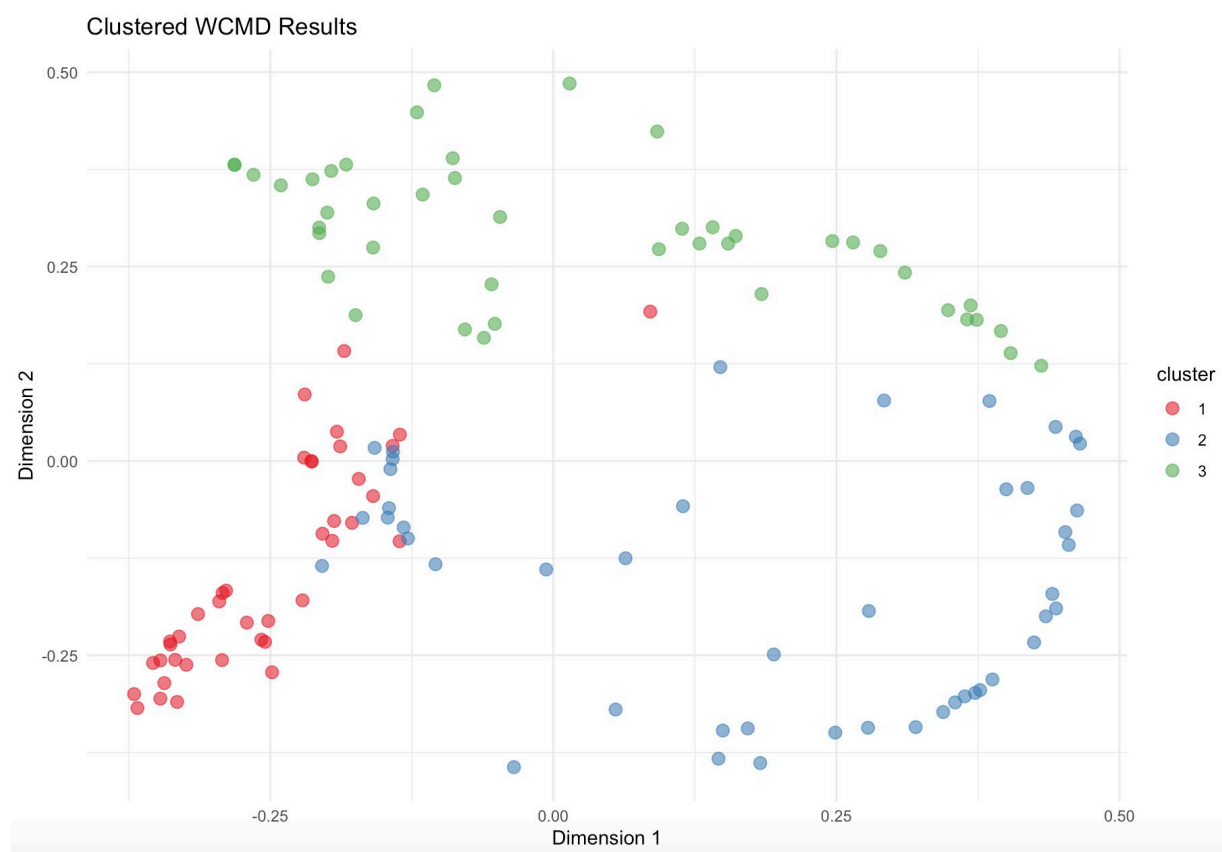
Appendix 2.3. Weighted Classical Multidimensional Scaling plot grouped by Time.



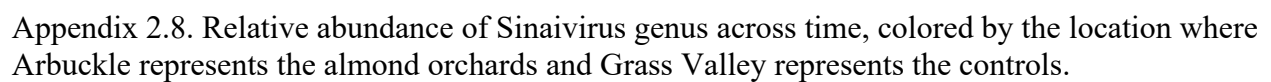
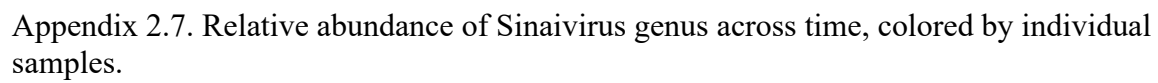
Appendix 2.4 Weighted Classical Multidimensional Scaling plot grouped by Location.

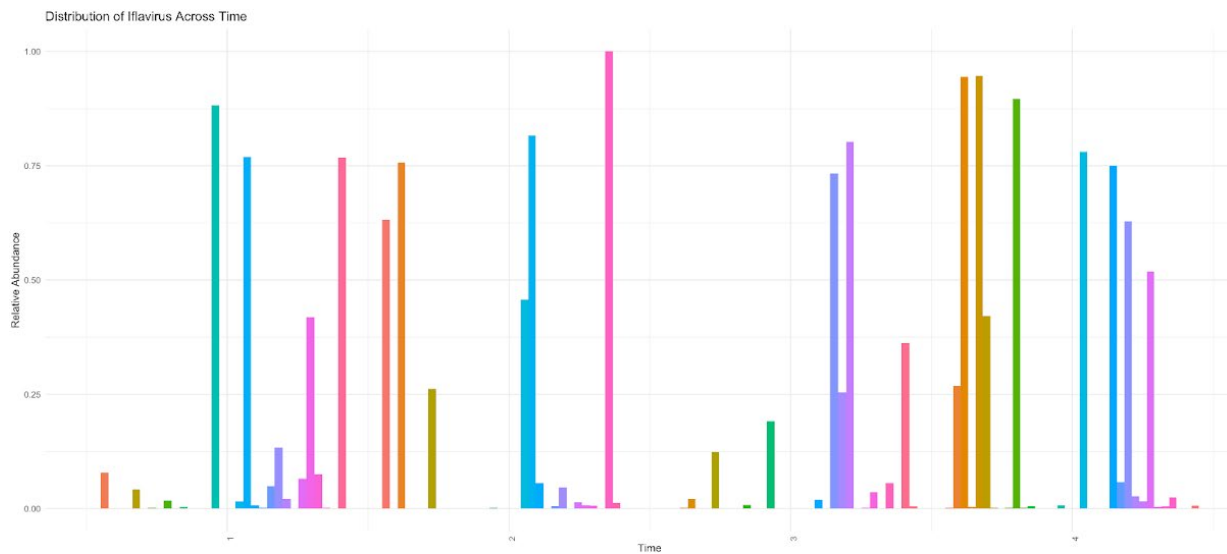


Appendix 2.5. Dendrogram of hierarchical clustering of WCMD Results.

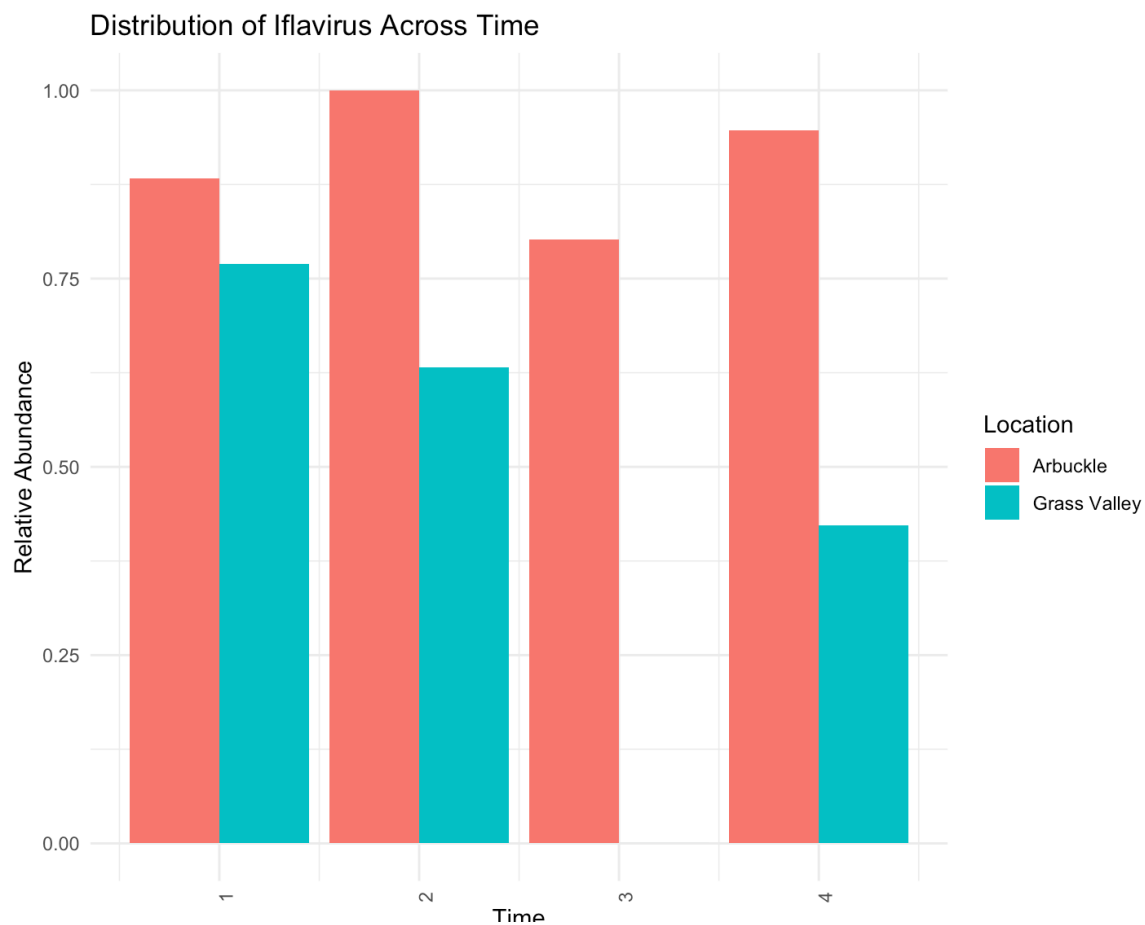


Appendix 2.6. A scatter plot of the Weighted Classical Multidimensional Scaling coordinates with the points colored according to their identified clusters.

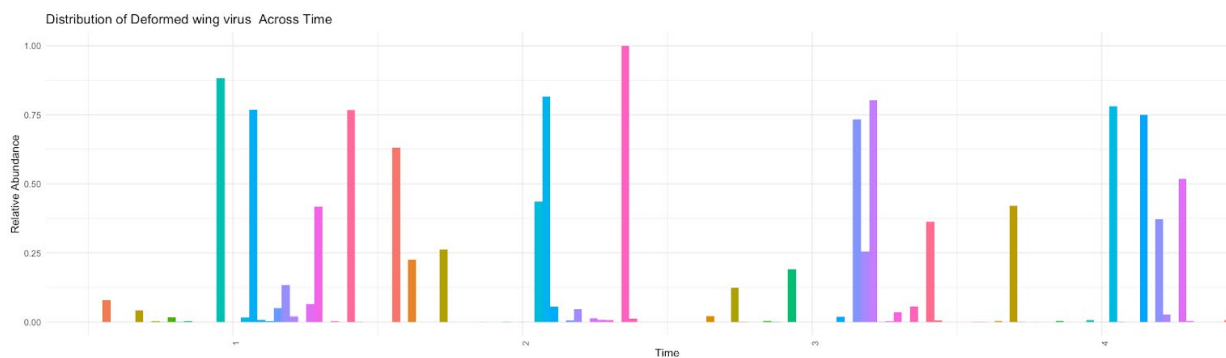




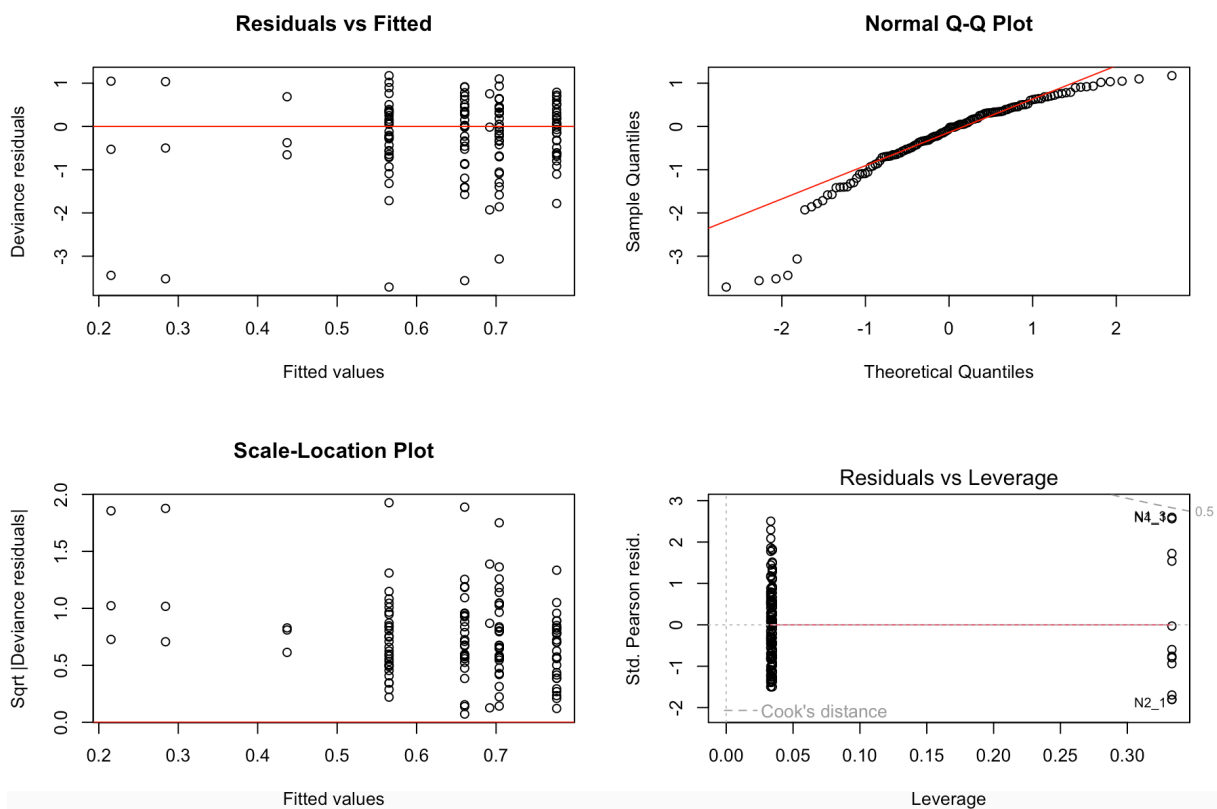
Appendix 2.9. Relative abundance of Iflavirus across time, with each bar representing a hive sample.



Appendix 2.10. Relative abundance of Iflavirus across time, filled by the location where Arbuckle represents the almond orchards and Grass Valley represents the controls.



Appendix 2.11. Relative abundance of Deformed wing virus with each bar representing a hive sample through time.



Appendix 2.12. Diagnostic plots for checking assumptions for the generalized linear model (GLM) with gamma distribution.

Chapter 3 Appendix:

Appendix 3.1: Density of honey bees (HB) and bumble bees per 500 m² across sites and time points in 2021 in order of ascending honey bee abundance. Sites with the presence of an apiary within 3 km are indicated as a Y (yes) and if further than 3 km as N (no). At the first time point, there were no honey bees present at any of the reported sites.

Time	Site	HB Density	BB Density	Apiary ?	Time	Site	HB Density	BB Density	Apiary ?
2	KFC	0	12	N	4	ORR	1	1	Y
2	PER	0	7	N	2	STA	2	20	N
2	LAC	0	20	N	3	KFA	2	6	Y
2	SHFS	0	13	N	2	ORR	4	3	Y
3	KFC	0	7	N	3	ORR	9	9	Y
3	SHFS	0	70	N	3	PER	10	8	N
3	STA	0	8	N	2	KFA	15	5	Y
4	KFA	0	3	Y	3	MM	21	2	Y
4	STA	0	20	N	2	MM	25	8	Y
4	PER	0	7	N	2	KFB	28	6	Y
4	SHFS	0	58	N	4	MM	49	1	Y
4	KFC	0	15	N	3	KFB	50	15	Y

Appendix 3.1.B: Data frame outlining the viral prevalence of each species per site per time point for DWV, CBPV, and SBV. Whether or not the site had an apiary is indicated as Yes (Y) or No (N). Count information shows the number of individuals screened for viruses.

#	Time	Species	Apiary	Site	DWV_Prev	CBPV_Prev	SBV_Prev	Count
1	1	<i>Bombus flavifrons</i>	Y	KFA	0	0	0.6	5
2	1	<i>Bombus flavifrons</i>	N	KFC	0.25	0	0.75	4
3	1	<i>Bombus flavifrons</i>	N	SHFS	0	0.5	0.5	4
4	1	<i>Bombus flavifrons</i>	N	STAM P	0.2	0	0.777777 8	10
5	1	<i>Bombus vancouverensis</i>	Y	KFB	0.5	0	1	2
6	1	<i>Bombus vancouverensis</i>	N	KFC	0	0	0	1
7	1	<i>Bombus vancouverensis</i>	N	PER	0.25	0	0.75	4
8	1	<i>Bombus vandykei</i>	Y	KFA	0	0	0.5	4
9	1	<i>Bombus vandykei</i>	Y	KFB	1	0	1	1

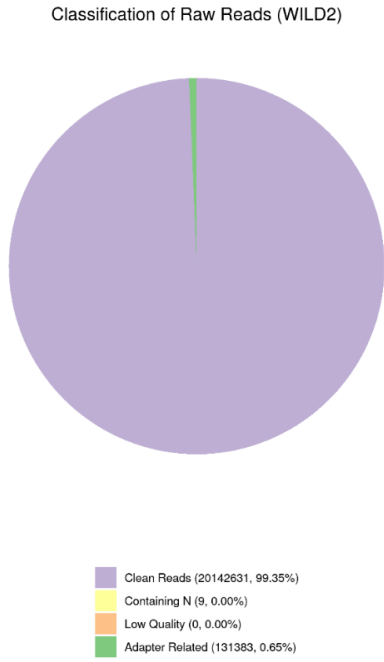
10	1	<i>Bombus vosnesenskii</i>	Y	KFA	1	1	0	1
11	1	<i>Bombus vosnesenskii</i>	Y	KFB	0.3333333	0.6666666		
12	1	<i>Bombus vosnesenskii</i>	N	PER	0	0	1	1
13	1	<i>Hive</i>	Y	GV	0.4375	0.6875	0.875	16
14	1	<i>Osmia</i>	Y	KFA	0	0	0	4
15	1	<i>Osmia</i>	Y	KFB	0	0	0	4
16	1	<i>Osmia</i>	N	KFC	0	0	0	10
17	1	<i>Osmia</i>	Y	MMM	0.0909090	0.0909090		
18	1	<i>Osmia</i>	Y	ORR	0.2222222			
19	1	<i>Osmia</i>	N	SHFS	2	0	0	9
20	2	<i>Apis mellifera</i>	Y	KFA	0	0	0	10
21	2	<i>Apis mellifera</i>	Y	KFB	0.7	0.5	0.1	10
22	2	<i>Apis mellifera</i>	Y	MMM	0.6666666			
23	2	<i>Apis mellifera</i>	Y	ORR	7	0.3	0	9
24	2	<i>Apis mellifera</i>	Y	MMM	0.4	0.5	0	10
25	2	<i>Apis mellifera</i>	Y	ORR	0.625	0.75	0.125	8
26	2	<i>Apis mellifera</i>	N	STAM P	0.3333333	0.3333333		
27	2	<i>Bombus flavifrons</i>	Y	KFA	3	3	0	3
28	2	<i>Bombus flavifrons</i>	Y	KFB	0	0	0.2	5
29	2	<i>Bombus flavifrons</i>	Y	KFB	0.5	0	0	2
30	2	<i>Bombus flavifrons</i>	N	KFC	0.25	0.25	0	4
31	2	<i>Bombus flavifrons</i>	Y	MMM			0.6666666	
32	2	<i>Bombus flavifrons</i>	Y	ORR	0	0	7	3
33	2	<i>Bombus flavifrons</i>	Y	ORR	0.3333333	0.3333333		
34	2	<i>Bombus flavifrons</i>	N	STAM P	3	0	3	3
35	2	<i>Bombus mixtus</i>	N	KFC	0	0.2	0.6	5
36	2	<i>Bombus mixtus</i>	N	KFC	0	0	0	2
37	2	<i>Bombus vancouverensis</i>	Y	KFA	0.6666666	0.3333333		
38	2	<i>Bombus vancouverensis</i>	Y	KFB	7	3	0	3
39	2	<i>Bombus vancouverensis</i>	Y	KFB	0	0	0	3
40	2	<i>Bombus vancouverensis</i>	N	KFC	0	0	0	3
41	2	<i>Bombus vancouverensis</i>	N	LAC	0.2222222	0.2222222	0.2222222	
42	2	<i>Bombus vancouverensis</i>	N	LAC	2	2	2	9
43	2	<i>Bombus vancouverensis</i>	Y	MMM	0.6666666		0.6666666	
44	2	<i>Bombus vancouverensis</i>	Y	MMM	7	0	7	3
45	2	<i>Bombus vancouverensis</i>	N	PER	0.2	0	0	5
46	2	<i>Bombus vancouverensis</i>	N	SHFS	0.4	0	0	5
47	2	<i>Bombus vandykei</i>	Y	KFA	0	0	0	1

40	2	<i>Bombus vandykei</i>	N	KFC	0.5	0	0	2
41	2	<i>Bombus vandykei</i>	Y	ORR	0	0	0	3
42	2	<i>Bombus vandykei</i>	N	STAM P	0	0	1	1
43	2	<i>Bombus vosnesenskii</i>	Y	KFA	0	1	0	1
44	2	<i>Bombus vosnesenskii</i>	Y	KFB	0	0	0.2	5
45	2	<i>Bombus vosnesenskii</i>	N	LAC	0	0	0	4
46	2	<i>Bombus vosnesenskii</i>	Y	MMM	0.4	0	0.8	5
47	2	<i>Bombus vosnesenskii</i>	Y	ORR	1	0	0.666666 7	3
48	2	<i>Bombus vosnesenskii</i>	N	PER	0.25	0	0	4
49	2	<i>Bombus vosnesenskii</i>	N	SHFS	0.2	0.2	0	5
50	2	<i>Bombus vosnesenskii</i>	N	STAM P	0.5	0.5	0.5	4
51	2	<i>Hive</i>	Y	GV	0	1	1	1
52	2	<i>Hive</i>	Y	KF	0.625	1	0.5	8
53	2	<i>Hive</i>	Y	MMM	0.555555 6	1	0.666666 7	9
54	2	<i>Hive</i>	Y	ORR	0.375	0.875	0.375	8
55	2	<i>Osmia</i>	Y	KFA	0	0	0	5
56	2	<i>Osmia</i>	Y	KFB	0	0	0	3
57	2	<i>Osmia</i>	N	KFC	0	0	0	7
58	2	<i>Osmia</i>	N	LAC	0	0	0	4
59	2	<i>Osmia</i>	Y	MMM	0	0	0	5
60	2	<i>Osmia</i>	Y	ORR	0	0	0	3
61	2	<i>Osmia</i>	N	PER	0	0	0	10
62	2	<i>Osmia</i>	N	SHFS	0	0	0	10
63	2	<i>Osmia</i>	N	STAM P	0.1	0	0	10
64	3	<i>Apis mellifera</i>	Y	KFA	0.222222 2	0.5	0	9
65	3	<i>Apis mellifera</i>	Y	KFB	0.2	0.444444 4	0	10
66	3	<i>Apis mellifera</i>	N	KFC	1	0	0	1
67	3	<i>Apis mellifera</i>	Y	MMM	0.166666 7	0.181818 8	0	12
68	3	<i>Apis mellifera</i>	Y	ORR	0.1	0.555555 6	0	10
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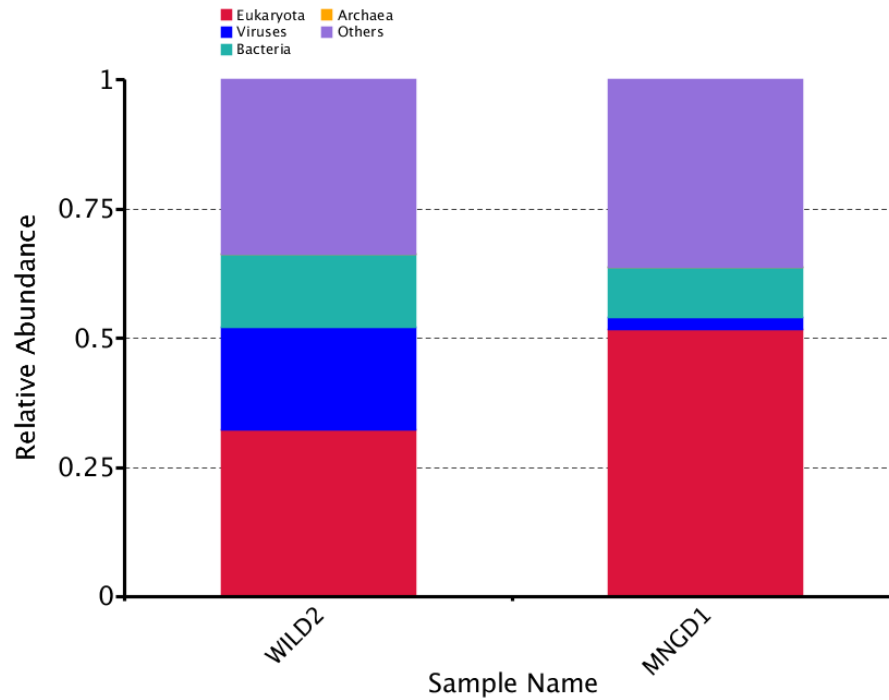
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72	3	<i>Bombus flavifrons</i>	N	KFC	0.6666666	7	0	0	6
73	3	<i>Bombus flavifrons</i>	Y	ORR	1		0	0	3
74	3	<i>Bombus flavifrons</i>	N	PER	0.3333333	0.25	3	0	4
75	3	<i>Bombus flavifrons</i>	N	STAM P	0.4		0.25	0	5
76	3	<i>Bombus mixtus</i>	Y	MMM	0		0	0	1
77	3	<i>Bombus vancouverensis</i>	N	LAC	1		0	0	1
78	3	<i>Bombus vancouverensis</i>	Y	MMM	0.3333333	3	0	0.3333333	3
79	3	<i>Bombus vancouverensis</i>	N	PER	0.2		0.2	0.2	5
80	3	<i>Bombus vancouverensis</i>	N	SHFS	0.4		0	0	5
81	3	<i>Bombus vandykei</i>	Y	KFA	0		0	0	1
82	3	<i>Bombus vandykei</i>	N	KFC	1		0	0	1
83	3	<i>Bombus vosnesenskii</i>	Y	KFA	0		0	0.25	4
84	3	<i>Bombus vosnesenskii</i>	Y	KFB	0.3333333	3	0.3333333	0.1111111	9
85	3	<i>Bombus vosnesenskii</i>	N	KFC	0		0	0	2
86	3	<i>Bombus vosnesenskii</i>	Y	MMM	0		0	0	3
87	3	<i>Bombus vosnesenskii</i>	Y	ORR	0.8		0	0	5
88	3	<i>Bombus vosnesenskii</i>	N	SHFS	0.5		0	0.25	4
89	3	<i>Bombus vosnesenskii</i>	N	STAM P	0.1666666	0.5	7	0	6
90	3	<i>Drone</i>	Y	MMM	0		0	0.25	2
91	3	<i>Drone</i>	Y	ORR	1		0	0	1
92	3	<i>Drone</i>	N	PER	1		0	1	1
93	3	<i>Drone</i>	N	SHFS	0		0	0	1
94	3	<i>Hive</i>	Y	KF	0.8571428	6	0.8571428	0.285714	7
95	3	<i>Hive</i>	Y	MMM	0.5		1	0.5	6
96	3	<i>Hive</i>	Y	ORR	0.375		1	0.125	8
97	3	<i>Osmia</i>	Y	KFA	0		0	0	2
98	3	<i>Osmia</i>	N	KFC	0		0.2	0	5
99	3	<i>Osmia</i>	Y	MMM	0.1666666	7	0.1666666	0	6

100	3	<i>Osmia</i>	Y	ORR	1	0	0	1
101	3	<i>Osmia</i>	N	PER	0.1428571 4	0.2857142 9	0	7
102	3	<i>Osmia</i>	N	SHFS	0.2857142 9	0.1428571 4	0	7
103	4	<i>Apis mellifera</i>	Y	MMM	0.4444444 4	0.6666666 7	0	9
104	4	<i>Bombus flavifrons</i>	Y	MMM	0	0	0	1
105	4	<i>Bombus flavifrons</i>	N	PER	0.5	0	0	2
106	4	<i>Bombus vancouverensis</i>	N	KFC	0.75	0	0	4
107	4	<i>Bombus vancouverensis</i>	Y	MMM	0.6	0.2	0.4	5
108	4	<i>Bombus vancouverensis</i>	N	PER	0.2	0.25	0	5
109	4	<i>Bombus vancouverensis</i>	N	SHFS	0.6	0	0	5
110	4	<i>Bombus vancouverensis</i>	N	STAM P	1	0	0	2
111	4	<i>Bombus vandykei</i>	Y	KFB	1	0	0	1
112	4	<i>Bombus vandykei</i>	N	STAM P	1	0	0	1
113	4	<i>Bombus vosnesenskii</i>	Y	KFA	0.5	0	0	2
114	4	<i>Bombus vosnesenskii</i>	Y	KFB	0.6666666 7	0	0	3
115	4	<i>Bombus vosnesenskii</i>	N	KFC	0.75	0	0.25	4
116	4	<i>Bombus vosnesenskii</i>	Y	MMM	0.4	0	0.2	5
117	4	<i>Bombus vosnesenskii</i>	Y	ORR	0.4	0	0.4	5
118	4	<i>Bombus vosnesenskii</i>	N	PER	0.5	0	0	4
119	4	<i>Bombus vosnesenskii</i>	N	SHFS	0.75	0	0	4
120	4	<i>Bombus vosnesenskii</i>	N	STAM P	0.6	0	0	5
121	4	<i>Drone</i>	Y	KFB	0.5	0.5	0	2
122	4	<i>Drone</i>	N	KFC	0.1666666 0	0.1666666 7	0	6
123	4	<i>Drone</i>	Y	ORR	0.5	0	0.5	2
124	4	<i>Drone</i>	N	SHFS	0	0	0	1
125	4	<i>Drone</i>	N	STAM P	0.3333333 3	0	0	6
126	4	<i>Hive</i>	Y	KF	0.8333333 0.5	0.8333333 3	0	6
127	4	<i>Hive</i>	Y	MMM	0.6666666 7	0.6666666 7	0.444444 4	9

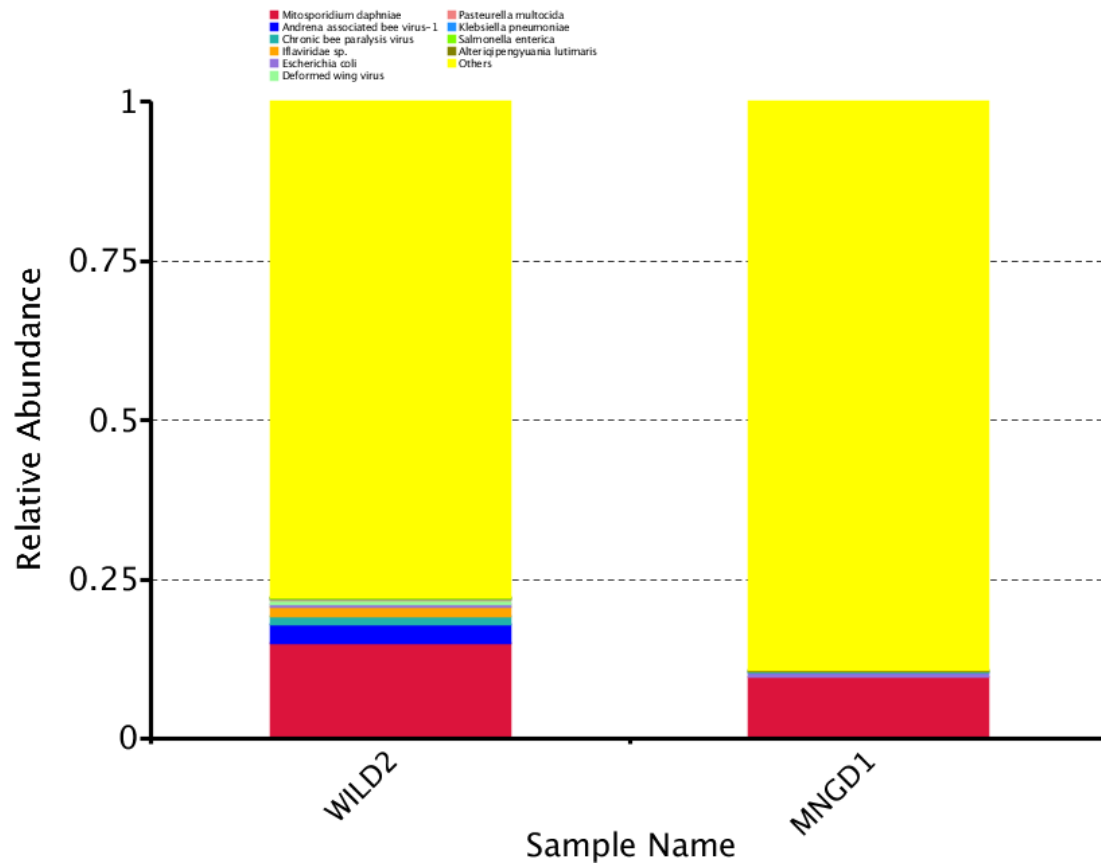
128	4	<i>Hive</i>	Y	ORR	0.1428571	0.8571428	0.285714	
					4	6	3	7



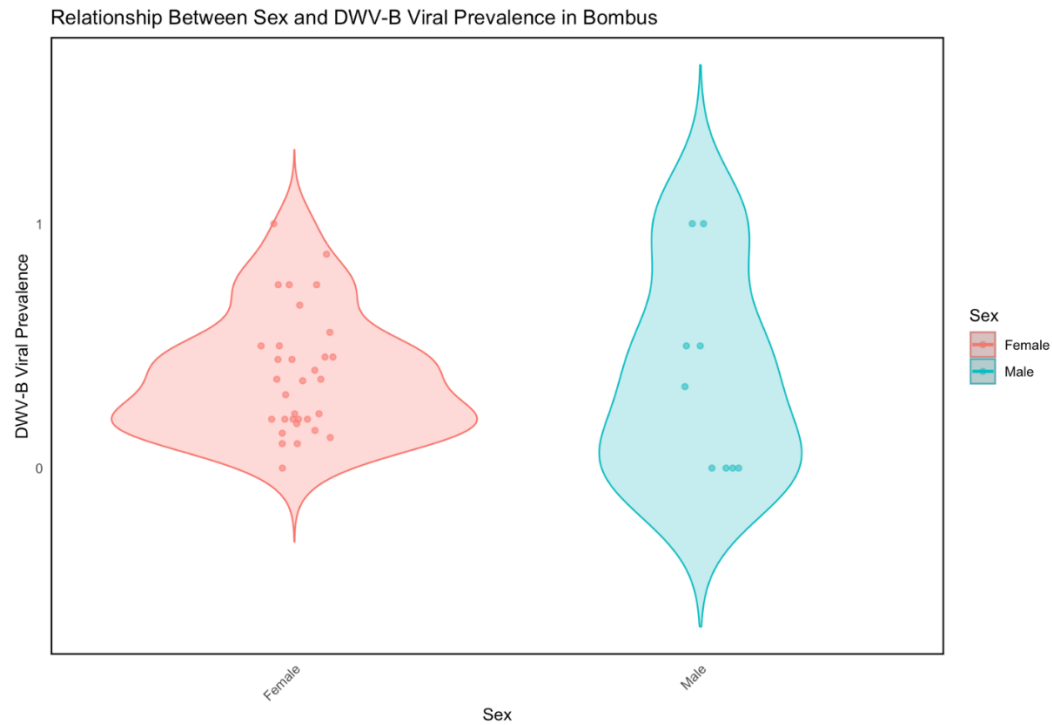
Appendix 3.2. Classification of raw reads from meta transcriptomic sequencing of a pooled *Osmia* sample showing read quality in a pie chart indicating a high-quality library.



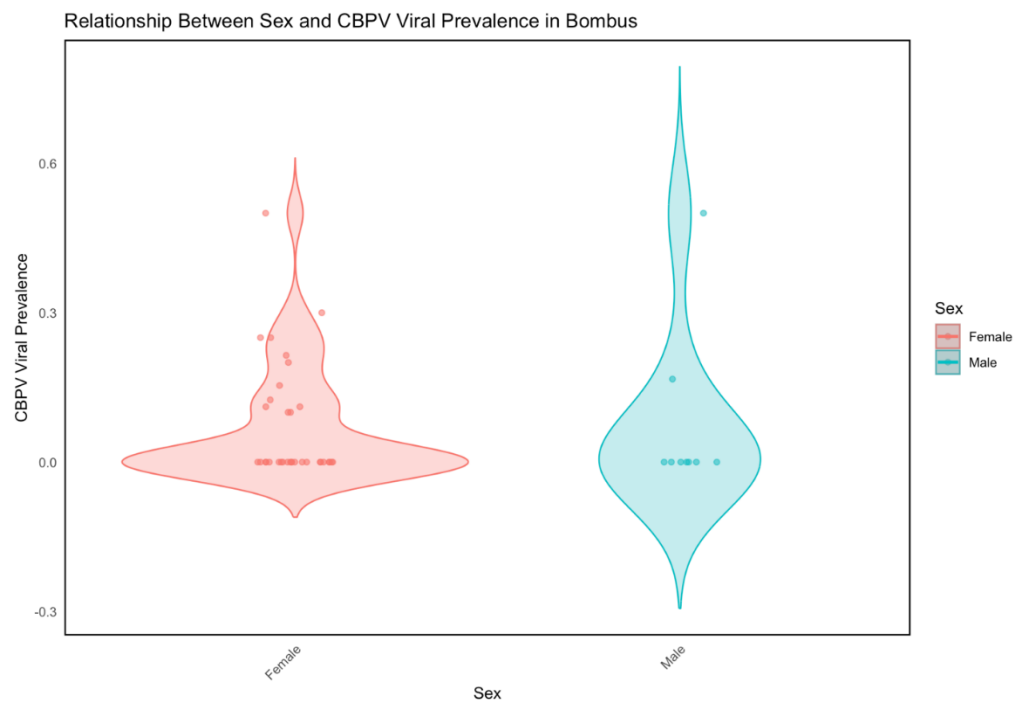
Appendix 3.3. Relative abundance of transcripts grouped by kingdom between wild *Osmia* foragers from the Sierra and managed *Osmia*. This comparison was to estimate the microbial differences between Sierran *Osmia* bees that were collected foraging in the wild versus *Osmia* bees that were reared from Sierran populations for use in managed pollination.



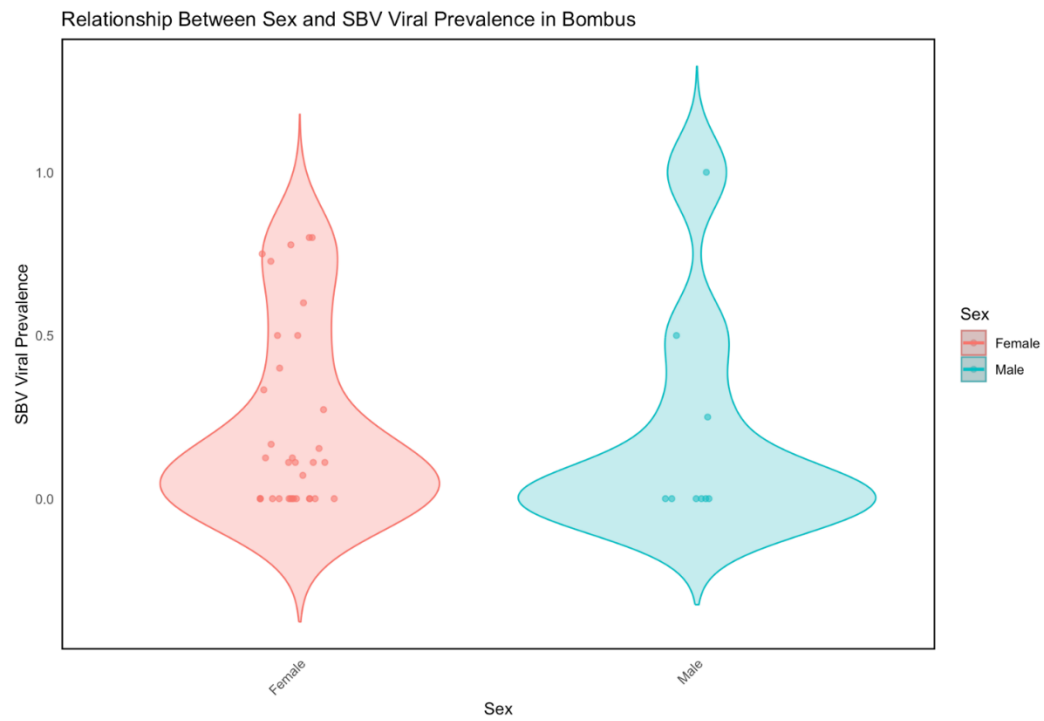
Appendix 3.4. Relative abundance of transcripts grouped by species between wild *Osmia* foragers from the Sierra and managed *Osmia* purchased from Foothill Bee Ranch. This comparison was to estimate the microbial differences between Sierran *Osmia* bees that were collected foraging in the wild versus *Osmia* bees that were reared from Sierran populations for use in managed pollination.



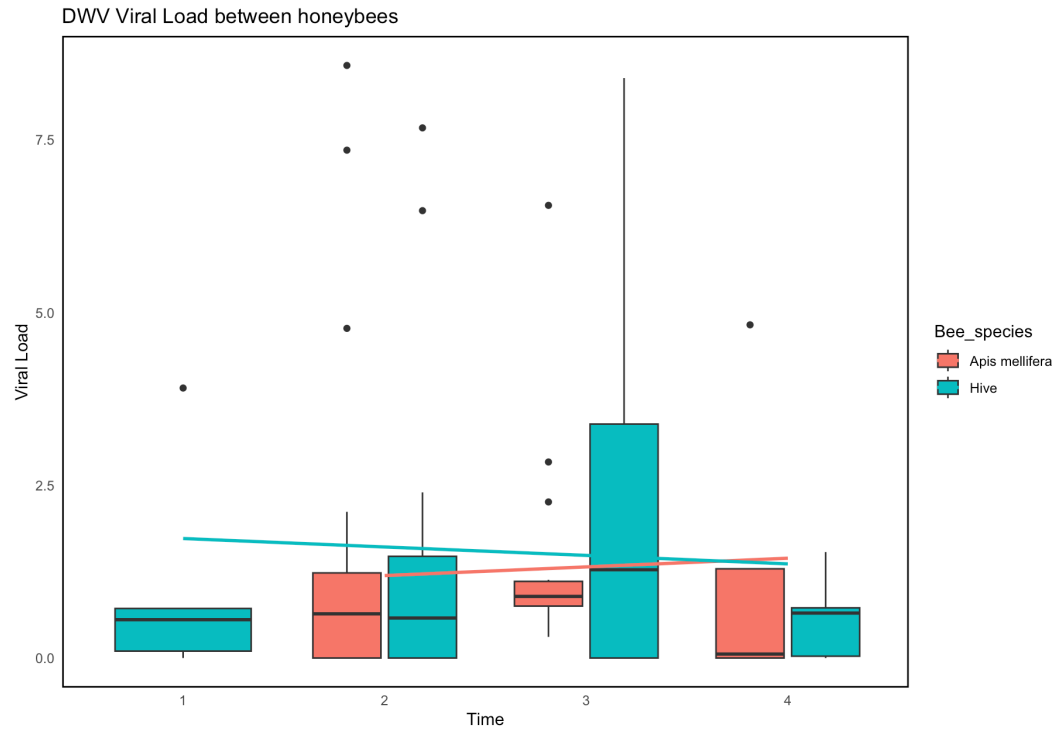
Appendix 3.5. Violin plots show the relationship between DWV-B viral prevalence and sex (female versus male) in all *Bombus* individuals across the surveys.



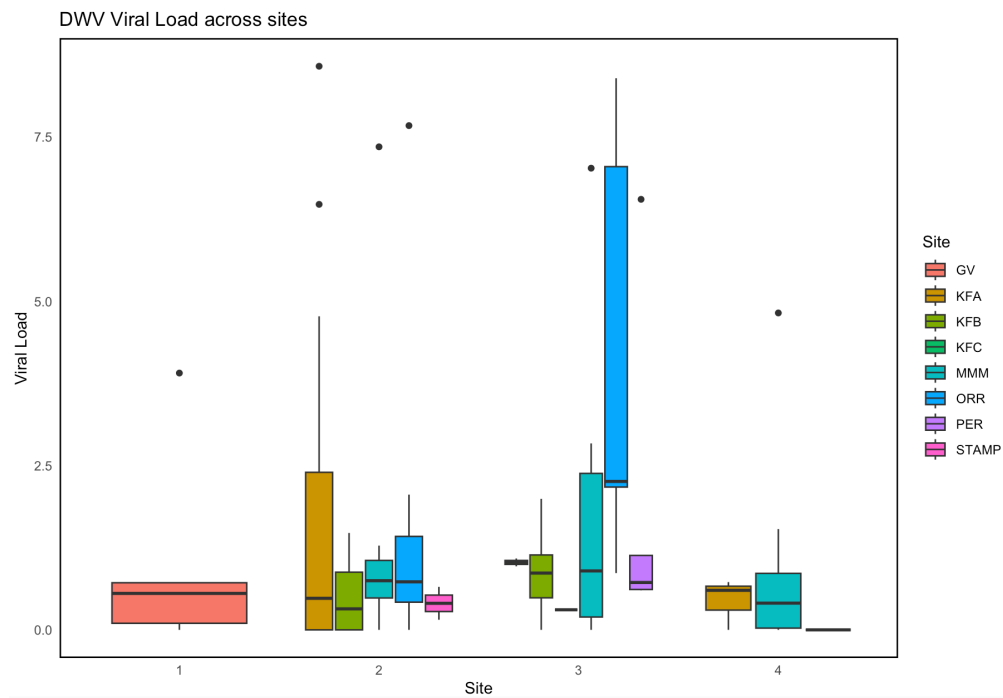
Appendix 3.6. Violin plots show the relationship between CBPV viral prevalence and sex (female versus male) in all *Bombus* individuals across the surveys.



Appendix 3.7. Violin plots show the relationship between SBV viral prevalence and sex (female versus male) in all *Bombus* individuals across the surveys.



Appendix 3.8. Box plots indicating DWV-B viral load through time and showing the differences between sample types being either *Apis mellifera* individual honey bee foragers, or hive samples that consisted of five individual honey bees pooled into one sample to be indicative of that hive.



Appendix 3.9. Bar plots indicating viral load data for DWV-B positive honey bees through time and across sites.