ABSTRACT

Title of Dissertation: Feeding behavior and distribution of *Varroa destructor* on adult bees of *Apis mellifera*

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Dissertation directed by: Dr. David J. Hawthorne, Department of Entomology

Varroa destructor is a competent vector of honey bee viruses and the leading cause of colony losses worldwide. Much about its feeding behavior and distribution on adult bees remains unknown. This work shows that *Varroa* are promiscuous feeders of adult bees, actively switching from one host to another. Laboratory trials showed there is a large heterogeneity in the host switching rate with some *Varroa* switching infrequently while others switched at high rates. The consequences of *Varroa* feeding on adult bees is largely unknown because adult feeding has largely been overlooked. This work shows that there is a high relative risk of death from *Varroa* feedings. Adult workers die quickly without developing high levels of infection after being fed upon by an infectious *Varroa*, and confer lower risk to their non-parasitized nestmates than counterparts which were nestmates to longer lived parasitized bees. Further experiments showed communicable routes of virus transmission may explain these findings. Trophallaxis between adult workers allowed for the movement of the

pathogen to naïve nestmates. These nestmates act as an infectious reservoir to naïve *Varroa* showing communicable transmission between hosts can influence the acquisition and subsequent vectoring of the same pathogen by the vector. Another social behavior, cannibalization, was shown to have the same influence on *Varroa* vectoring. *Varroa* were also shown to be susceptible to viral acquisition through shared feedings on adult bee and brood hosts. Naïve *Varroa* readily acquired and then transmitted deformed wing virus when sharing the same host with an infectious *Varroa*. Collectively this work exemplifies how host social behavior and *Varroa*-*Varroa* transmission routes can increase the risk of vectors becoming infectious. *Varroa* feedings and virus transmission on adult workers cannot describe one of the most glaring features of *Varroa* infestations. For a portion of the year *Varroa* aggregate predominantly on adult drones, largely ignoring the worker cohort. Parasite burden only shifts onto workers when drone production ceases.

FEEDING BEHAVIOR AND DISTRIBUTION OF VARROA DESTRUCTOR ON ADULT BEES OF APIS MELLIFERA

by

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Dedication

To Jacks, Stefanie, Nicole and Nitin

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General Introduction

Varroa destructor, deformed wing virus, and the eusocial honey bee *Apis mellifera* form a unique relationship rarely observed in other parasite-pathogen-host relationships. This triad represents both an emerging pathogen, an evolutionary host jump of a parasite, and a disease system which supports both communicable and vectored transmission[1,2].

The host jump from *A. cerana* to *A. mellifera* was a recent event in the long evolutionary history of the honey bee[3]. Once established on its new host, *Varroa* began the vectoring, or direct transmission, of deformed wing virus (DWV) to both brood and adult bees through its feeding[4]. For the first time we know of, DWV went from an obscure, communicably spread pathogen rarely observed in the millions of managed colonies across the globe, to a prevalent vector-borne disease detected wherever *Varroa* has become established[1]. On an evolutionary time scale this happened in a blink of the eye. DWV is now the most widely studied insect virus, and an emerging pathogen which has been detected in over 65 arthropod species[5].

As an infectious disease DWV has another rare characteristic that makes its transmission between bee and mite extremely unique. Most infectious diseases are communicable or vector-borne, but rarely both[6]. For example, malaria is vectored by biting mosquitos, but human hosts cannot communicably spread it from person to person[7]. Likewise, tuberculosis can readily be spread from human to human, but vectors such as mosquitos cannot acquire and transmit the pathogen when feeding on a sick person. There are only a handful of infectious diseases which are efficiently transmitted by both modes. They are the salmon isavirus of salmonoids, West Nile

virus of wild birds, Tembusu virus of farmed poultry, and the bacterial pathogen Yersinia pestis which causes the plague[8-11]. The disease transmission dynamics in these few infectious diseases are complex and interactive between host and vector transmission cycles.

The following body of work attempts to illuminate driving factors that may affect disease transmission in this relationship between *Varroa*, virus and bee. I first focus on the feeding behavior of *Varroa* on adult bees; namely if *Varroa* actively switch from host to host to feed. Disease transmission in vector-borne systems are extremely sensitive to the biting rate of arthropod vectors[7,12]. Small changes in the biting rate can cause non-linear (quadratic) changes in vectorial capacity[13]. To fully appreciate, and to mathematically describe DWV transmission both communicable and vectored routes must be described. I carried out a series of experiments to study host-host, host-vector and vector-vector transmission. Ultimately, I asked if social behaviors of honey bees could influence the acquisition and subsequent vectoring of DWV. After studying both behaviors of the vector and host, I asked which bees in natural colonies bear the majority of parasite burden. I noticed all experiments to date have excluded adult male bees in their experimental designs[14-18]. In a series of experiments, I studied *Varroa* parasitism on both drones and workers, and investigated if there would be a change in *Varroa* distribution once the seasonal male cohort was absent from colonies. In this manner I attempted to fill gaps in knowledge in the disease transmission epidemiology of *Varroa*, virus and bee.

Chapter 1: Promiscuous feeding across multiple honey bee hosts amplifies the vector capacity of *Varroa destructor*.

Abstract

Varroa destructor is a cosmopolitan pest and leading cause of colony loss of the European honey bee. Historically described as a competent vector of honey bee viruses, this arthropod vector is cause for the global pandemic of Deformed wing virus, now endemic in honeybee populations. Our work shows viral spread is driven by *Varroa* actively switching from one adult bee to another as they feed. Assays using fluorescent microspheres were used to show the movement of fluids in both directions between host and vector when *Varroa* feed. Therefore, *Varroa* could be in either an infectious or naïve state dependent upon the disease status of their host. I tested this and confirmed that the relative risk of a *Varroa* feeding was dependent on the infectiousness of their previous host. *Varroa* exhibit heterogeneity in this host switching behavior, with some *Varroa* switching infrequently while others switch at least daily. As a result, relatively few of the most active *Varroa* parasitize the majority of bees. This multiple feeding behavior has analogs in vectorial capacity models of other systems, where promiscuous feeding by individual vectors is a leading driver of vectorial capacity. I propose that the honeybee-*Varroa* relationship offers a unique opportunity to apply principles of vectorial capacity to a social organism, as virus transmission is both vectored and occurs through multiple host-to-host routes common to a crowded society.

Introduction

Varroa destructor is an ectoparasitic mite found on *A. mellifera,* with a nearly global distribution[1]. *Varroa* and associated viruses are suspects in large colony losses experienced in North America in the early 2000s[19]. Numerous studies have since linked *Varroa* and a pathogen it vectors, Deformed wing virus, as a driver of colony loss[20]. While female *Varroa* reproduce only on developing honey bee brood (late-stage larvae and pupae), they also feed on adult bees[21-23]. Little is known about the feeding dynamics of *Varroa* on adult bees as this stage of *Varroa*'s lifecycle has been understudied compared to its reproductive phase. *Varroa* are described as regularly leaving their original bee host after emergence from a brood cell, preferring nurse bees, and leaving that adult bee prior to its death[17,24,25]. In observational studies, *Varroa* have been described to leave hosts in wintering clusters, suggesting they may actively switch from one host to another[26]. To date there are no further descriptions of *Varroa* host-switching behavior on adult bees. This switching rate is critical for determining the vectorial capacity of *Varroa* with respect to honey bees.

Vectorial capacity describes the potential of a vector to transmit a pathogen arising from a single infectious case in a day, and is constructed using measures of the proportions of hosts and vectors, the frequency of infection, survivorship and the rates of biting or feeding on hosts by the vector.[27] The biting rate (host switching rate) is one of the most influential parameters influencing disease transmission, and heterogeneity in vector biting rates and heterogeneity in host exposure to bites overwhelmingly shape disease transmission[12,28]. The biting rate has not been measured for the honey bee *Varroa* relationship and obtaining an estimate of that

heterogeneity will improve estimates of vectorial capacity for modeling of DWV transmission in honey bee colonies. *Varroa* are described as regularly leaving their adult bee host after emergence from a brood cell, and preferring nurse bees[17,24,25]. That *Varroa* feed on adult bees has been confirmed through several studies, both through the visualization of bee material inside of *Varroa* and through the uptake of tagged material from experimental bees[21-23]. Applying the biting rate as described in vectorial capacity models of other systems may inform our understanding of the *Varroa* – and the honey bee - Deformed wing virus system.

Here I describe a series of experiments to measure the biting rate in the honey bee-*Varroa* relationship. First, I show that *Varroa* indeed feed when they enter known feeding positions of the adult honey bee and that the infectiveness of a mite depends on the viral state of the previous host. I used fluorescent microspheres to show material passes in both directions between the host and vector, suggesting that *Varroa* can both transmit and acquire viruses from their adult hosts. The consequences of a *Varroa* feeding event may be dependent on the infectiousness of the previously parasitized host, and not solely because of an inherent characteristic of the individual *Varroa*. To test this I followed *Varroa* in either infectious or naïve states and observed their direct feeding on individual adult bees where I found striking differences in virus levels and relative risk between treatment groups. differences between parasitized and non-parasitized nestmates. Finally, I measured the movement of *Varroa* among hosts to estimate the host switching rate. In this manner, I describe the relative risk of *Varroa* feeding on viral-mediated mortality, transmission of virus between vectors and hosts, among hosts, and variation in their host switching

behavior. I found remarkable promiscuity by feeding *Varroa*, with often daily switches from one bee to the next. These insights help clarify the roles played by *Varroa* in spreading disease as well as the roles played by honey bees as reservoirs for nestmates and subsequent parasite encounters.

Materials and Methods

Cage design

A cage design by Evans et al. (2009) was used in all experiments for this study[29]. I used a clear plastic 16 ounce tumbler (Uline Crystal Clear Plastic Cups 16oz, S-22276) covered with a *Varroa* proof mesh (noseum-netting) which also provided ventilation. A small insertion into the fabric lid was made with a razor blade and a 2 ml Eppendorf tube was pushed through this insertion to serve as feeders. The tubes were perforated with a brad nail or a 5/64 drill bit, filled with water or 40% sucrose solution by weight. Trap doors were cut from the lower portion (side approximately 1x1 inch) of each cage allowing for removal of dead samples during trials. These holes were sealed by creating a duct tape door. Duct tape was folded back onto itself to seal all sticky portions and then cut into squares slightly larger than the hole in the cup. A strip of lab tape was used to secure the door to the cup. By folding back a short section from one end of the lab tape onto itself, a handle was made which allowed for easy closure and opening of the trap door. To ensure no accidental escape could occur through the trap door, the whole cage was slid into another plastic cup. Cages constructed in this manner allowed for the containment of both *Varroa* and

bees. The cages were well ventilated, and collection of dead samples was easy without interrupting the live samples.

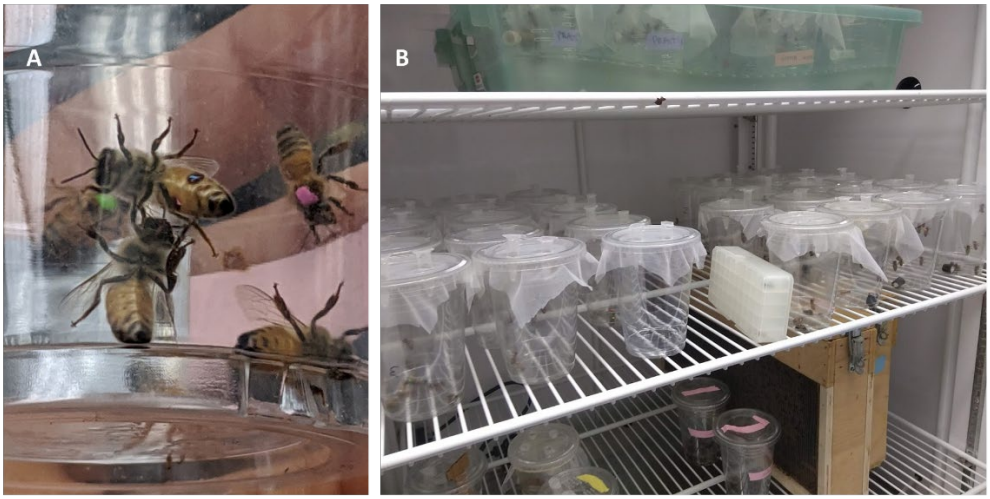

Figure 1.1 (A) Inside of an experimental cage. The ventral abdomen of a bee is depicted with *Varroa* visibly in feeding positions between the sternites. Bees are marked on their thorax for individual identification. **(B)** Numerous experimental cages are established and maintained inside an incubator. Noseum netting is visible and serves to hold the sucrose feeders in place, while providing ventilation and a mite proof barrier.

Experiments 1 and 2: Detections of feeding through microspheres

Fluorescent microspheres were used as a surrogate for bee tissue in order to test if *Varroa* were feeding on adult bees each time when they entered a known feeding position. Adult nurse bees were obtained and chilled for 10 minutes at 4 degrees Celsius. 3uL of 10^7 dapi microspheres (FluoSpheres 1.0um, blue [365/415], Invitrogen, Thermo Fisher Scientific) were injected into the hemocoel between the 5^{th} and 6^{th} tergite with a 31 guage Hamilton syringe (Hamilton Company, Reno, Nevada). Injections that showed visible dripping were rejected and not included in the study. The bees were returned to their cages and incubated for 4 hours so that the injection wounds could heal. A *Varroa* was passaged onto the bee host and left for 24

hours. After 24 hours *Varroa* were recollected from their adult bee hosts. Their position on the bee was recorded and these positions were described as feeding or not feeding positions. Using a #5 Dumont tweezer (Montignez, Switzerland), the honey bee host was secured, and using a Chinese grafting tool (HD390 ,Mann Lake, Hackensack, MN), the *Varroa* was gently scooped away from its bee host. *Varroa* were anesthetized on ice for microscopy. Using another set of tweezers, the dorsal carapace was removed exposing the interior of the *Varroa*. The internal tissues of the *Varroa* were then smeared onto a glass slide, 2 uL of PBS was added and then mounted with a cover slip. Samples were viewed under fluorescent microscopy. Positive detections were determined by visualization of dapi fluorescent spheres, and tallied to estimate the proportion of *Varroa* that acquired microspheres from their host.

Experiment 2: Passage of microspheres from *Varroa* to adult bee

In order to test if microspheres could be transferred from a *Varroa* to an adult bee via *Varroa* feeding, fluorescent microspheres were first introduced into *Varroa*. I accomplished this by having *Varroa* feed on pupae in which 3uL of $1x10^7$ DAPI microspheres in PBS buffer was injected (31 gauge needle, Micro4 microsyringe pump controller (World Precision Instruments, Sarasota, FL). A second group of pupae which served as a control were injected with PBS. The injected pupae were incubated at 34 ºC for 24 hours before being fed on by *Varroa*. Pupae showing onset of melanization were removed from the study. *Varroa* were placed onto the injected pupae and allowed to feed for 48 hours. *Varroa* were then removed and transferred to a cage of adult bees for 24 hours. Pupae were incubated at 34 ºC degrees in 00 gel

caps (Capsule Connection, Prescott, Arizona), adult bees were incubated at 34 ºC degrees in groups of approximately 40 bees in a common cage.

Bees with a *Varroa* in the feeding position were removed for dissection. Positive detections were determined by visualization of fluorescent spheres, and bees with and without microspheres were tallied to estimate the proportion of bees that acquired microspheres from a *Varroa*.

Detection of fluorescent microspheres

In order to train the researcher to visualize dapi fluorescent spheres under microscopy a positive of control of stock solution and injected pupae were prepared on slides and then viewed under fluorescent microscopy. Visual scanning, and dorsal and ventral z-stacks of *Varroa* samples were taken of *Varroa*. *Varroa* samples were then smeared on a glass slide after confirmation of microspheres were not present on the exterior of the *Varroa*.

Experiment 3: Observation and quantification of host switching

Observations of mites switching from adult bee host to host was observed across 4 trials in the laboratory. For all laboratory cage trials, a single frame of emerging bees was collected from healthy queen right colonies exhibiting no visible signs of disease. The frames were collected 48 hours prior to emergence and incubated at 34 ºC. Newly emerged bees were collected and given a color paint mark on their thorax. Cages were then made with 8 bees, individually distinguishable by their painted thorax. We utilized 7 different colors and one unmarked bee per cage. The cages were given a 40% sucrose solution and incubated for 3 days. At the beginning of day 4, a single *Varroa* was placed into each cage. Phoretic *Varroa* were captured from a single

colony, and then incubated on a purple eye worker pupae (~ 16 -17 days old) in a 00 gel cap for 48 hours prior to transferring to the cage of workers on day 4. The presence of the *Varroa* on a host bee, and which bee it was on was recorded 2 hours after introduction, and every 12 hours thereafter for 15 days. In this way the number of parasitized hosts and the frequency of host switching for each *Varroa* was recorded.

It was essential in this experiment to distinguish among *Varroa* in feeding and non-feeding positions. *Varroa* in feeding positions (left, right or distal) were on the abdomen partially covered by the sternites of the bee. Non-feeding positions include the thorax or abdomen when the entire *Varroa* was visible, without any part of the *Varroa* enveloped by the bees sternites. *Varroa* in non-feeding positions (on cage surface or in a non-feeding position on a bee) were recorded and their movement to new hosts also recorded. Parasitized bees were those in which a *Varroa* was observed in a feeding position. Daily bee and *Varroa* mortality was recorded.

Switching Rates

A switch was considered when a *Varroa* was observed on a different bee than its previously parasitized host. The first bee a *Varroa* was observed parasitizing did not count as a switch. Each new host subsequent to this one did. Observations were made every 12 hours during trials (+/- 2 hours)

Pupae and *Varroa*

Pupae (early pink eyed: ~ 14-15 days old) were obtained for injection by gently removing their cell capping and extracting pink eyed pupae with a pair of soft tip tweezers. Injections were performed with a 31 gauge needle using a WPI Micro4 MicroSyringe Pump.

Varroa were captured along with their host bees from an infested colony. Bees with *Varroa* were placed into a a cage and maintained at 34 ºC and 50% humidity. Pupae were removed from the comb and placed into 00 gel caps. The *Varroa* were removed individually from their honey bee host and placed in a 00 gel cap with a (early purple eyed) pupae host for 24 hours. In this way all *Varroa* collected for experiments were equalized by being on the same type of host prior to the start of the experiment.

Experiment 4: Relative risk of *Varroa* parasitism on adult workers

We used the same cage design described previously with 8 individually marked bees to carry out this study (Evans, 2009)[29]. The bees in each cage represented a fixed population of bees which were either unchallenged or challenged by one *Varroa*. Groups challenged with a *Varroa* were further divided into groups based on the infectious status of the *Varroa*: non-infectious control, +DWV or +VDV1 (Table 1.1 below). In this way 4 groups established the study. A single *Varroa* was used in each cage replicate (n = 10 cages per group, 40 total cages. A single *Varroa* was used per cage replicate to facilitate observation of *Varroa* amongst a small group of bees. A single *Varroa* was used to reduce confounding by introducing multiple vectors within a population.-The proportion of vector to host was

fixed with 1 vector to 8 hosts (12.5%), a realistic infestation rate observable in honeybee colonies[7].

Table 1.1 Explanation of experimental groups

Group name	Treatment	Names used in this text	Replicates in trial
unchallenged	Bees are not exposed to *Varroa* during trial	Unchallenged, negative control group	9
Challenged	Bees are exposed to a *Varroa* that fed on a pupae injected with PBS during trial	Challenged control group	10
Challenged + DWV	Bees are exposed to a *Varroa* that fed upon a pupae injected with DWV-A inoculum prior to start of trial	*Varroa* challenged + virus group, *Varroa* challenged + DWV group	10
Challenged + VDV1	Bees are exposed to a *Varroa* that fed upon a pupae injected with VDV1 inoculum prior to start of trial	*Varroa* challenged + virus group, *Varroa* challenged + VDV1 group	9

Table 1.1 Groups, description of treatments, naming and number of replicates in the trial. One replicate was removed from the unchallenged group and the challenged + VDV1

Introduction of viral inoculum

Viral inocula (supplied by Ryabov and Evans[30], 1 ul (10^7 GE per ul) of inoculum in 9 ul of PBS) were injected per pupae using Micro4 microsyringe pump controller (World Precision Instruments, Sarasota, FL). Pupae were incubated for 48 hours following injection and then *Varroa* were introduced to the pupae by enclosing both *Varroa* and pupae in a 00 cellulose gel cap for 72 hours. *Varroa* were then removed and placed individually into cages of 8 marked bees described previously. *Varroa* were considered non-infectious and acted as a control if they fed on the PBS injected pupae prior to the start of the experiment. *Varroa* were considered infectious if they fed upon a pupae injected first with viral inoculum. Because all *Varroa* in this experiment were collected from field colonies with unknown baseline levels of virus,

Varroa in this trial harbored an unknown viral load. To account for this, we collected and treated the *Varroa* the same for all groups. The only difference was the pupae they fed upon prior to the start of the trial.

Statistical analysis

Data was analyzed in Rstudio using BaseR and various imported packages. In experiments 1 and 2, the frequency of microsphere presence in the parasitic *Varroa* and the host bees were tallied, and no further analysis was performed. In experiment 3, the per-day switching rate of *Varroa* was calculated by dividing the number of host switches by the number of days a *Varroa* persisted in the trial. The total number of parasitized hosts included the first parasitized bee. Variation among *Varroa* in host switching rate was estimated using summary statistics but differences among *Varroa* in number of hosts was not tested. In order to assess the relationship between number of bees parasitized by each *Varroa* over the number of days in the trial we performed a weighted least squares by calculating fitted values from a regression and using weights of fitted values. Initial models resulted in residuals not meeting assumptions of normality. For this reason non-parametric tests were used. These included the Mann-Whitney-U Test, Kruskal-Wallis and weighted least squares regression.

In experiment 4 I assessed patterns of mortality of bees in the four treatment groups (unchallenged, challenged control, challenged + DWV-A, challenged + VDV1) using a Kaplan-Meier survivor analysis (estimated using the survival and survminer packages in R). A log-Rank test was used for comparison of survivorship amongst

treatments. A bee or *Varroa* was considered to survive the trial when it remained alive for the whole length of the trial, which was set to 15 days.

Relative risk estimates were calculated for treatment groups parasitized and non-parasitized bees using an unconditional maximum likelihood estimation. In all relative risk assessments bees were compared with counterparts that had equal exposures. Confidence intervals for these groupings were calculated using normal approximation. The Epitools package was used to calculate the relative risk estimates. Time to death (TtD) was calculated by measuring the length of time between when a bee was first observed parasitized and when first observed dead. TtD was compared across groups using an ANOVA and Tukey post hoc analysis with Bonferroni adjustments. Viral loads (DWV-A and VDV1) of bees, estimated via rtPCR, were estimated and compared across treatments including comparing parasitized and non-parasitized bees using a non-parametric Kruskal-Wallis ANOVA with a post hoc Dunn test.

Results

Experiments 1 and 2: Detection of microspheres from adult bee host to *Varroa* and *Varroa* to bee

Most *Varroa* (93.75%, 16/17 *Varroa* observed) had observable fluorescent microspheres within their digestive tract after entering feeding positions on injected adult bees. While detection of fluorescent microspheres was reliable for the movement of microspheres from bee to *Varroa*, detection of the microspheres which moved from *Varroa* to bee was less so (1/17 *Varroa* observed). Microspheres were observed freely moving within the hemocoel of the honeybee under fluorescent

microscopy, from the outer abdominal wall inwards, while the *Varroa* was still in a feeding position between the 3rd and 4th sternites of sampled bees.

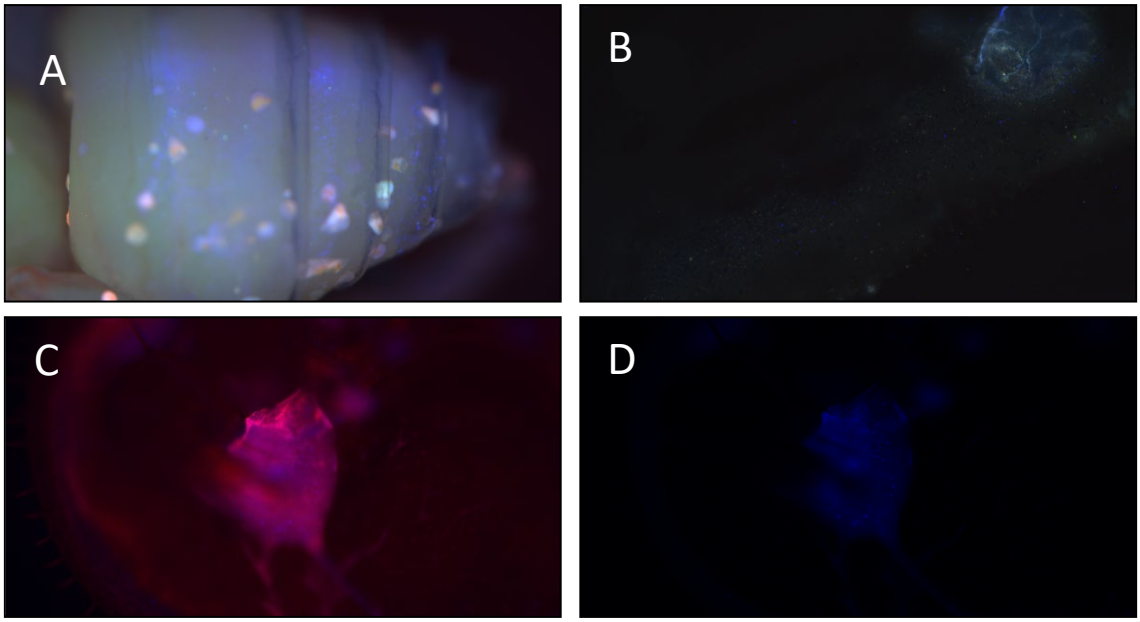

Figure 1.2: A. DAPI microspheres 1.0um, blue [365/415], Invitrogen, Thermo Fisher Scientific visualized under fluorescent microscopy through the cuticle of a worker pupae. **B.** DAPI FluoSpheres present in a *Varroa* which fed upon an injected adult bee. **C-D** The dorsal surface was partially removed to visualize the microspheres within the *Varroa*.

Experiment 3: Switching rates of *Varroa* destructor on adult worker bees

Varroa were observed across 4 trials (N = 70). *Varroa* switched hosts every 2.5 days on average (switching rate mean ± SD = 0.369 ± 0.21 hosts/day). Time was significant, but did not account for approximately half of the variability in the number of switches made by *Varroa* over the trials (WLS regression, $R^2 = 0.5514$, $F_{1, 68} = 83.58$, $p < 0.0001$). I accounted for longevity of the *Varroa* by dividing the number of switches a *Varroa* would make by the number of days that *Varroa* survived in the experiment (mean = 12.5 days ± SD = 3.5). The switching rate was not significantly different between trials (Kruskal-Wallis, $H^2 = 5.697$, DF(3), $p = 0.127$). Time did not

explain a majority of the variability in the number of bees parasitized within the trials (WLS regression, $R^2 = 0.571$, $F(1, 68) = 90.59$, $p < 0.0001$, Figure 1.3).

Varroa did not equally contribute to the number of parasitized bees in the trials (Table 1.1). Mites which were the lowest frequency switchers contributed to fewer bees on average than the highest frequency switchers, while on average surviving for equal times in the study (Table 1.2).

Table 1.2 Mite switches and contribution to parasitized bees

Number of *Varroa* which contributed to bites (percent of population)	Number of individual adult bees parasitized (mean ± SD)	Percent of bees parasitized of total population	Cumulative percent of bees parasitized	Cumulative time (days) these *Varroa* were alive in the trial (mean ± SD)
27 (38.6%)	82 (3 ± 1)	25%	25%	343 (12.5 ± 3.5)
17 (24.2%)	82 (4.8 ± 1.51)	25%	50%	207.5 (12 ± 3)
13 (18.6%)	82 (6.3 ± 1.60)	25%	75%	162.5 (12.5 ± 3.5)
13 (18.6%)	84 (6.5 ± 2.40)	25%	100%	160.5 (12 ± 4)

Table 1.2 Counts of *Varroa* in the trials and the number of bees which were parasitized by them. The percentage and cumulative percentage of parasitized bees are presented. The mean time of *Varroa* survivorship is presented here.

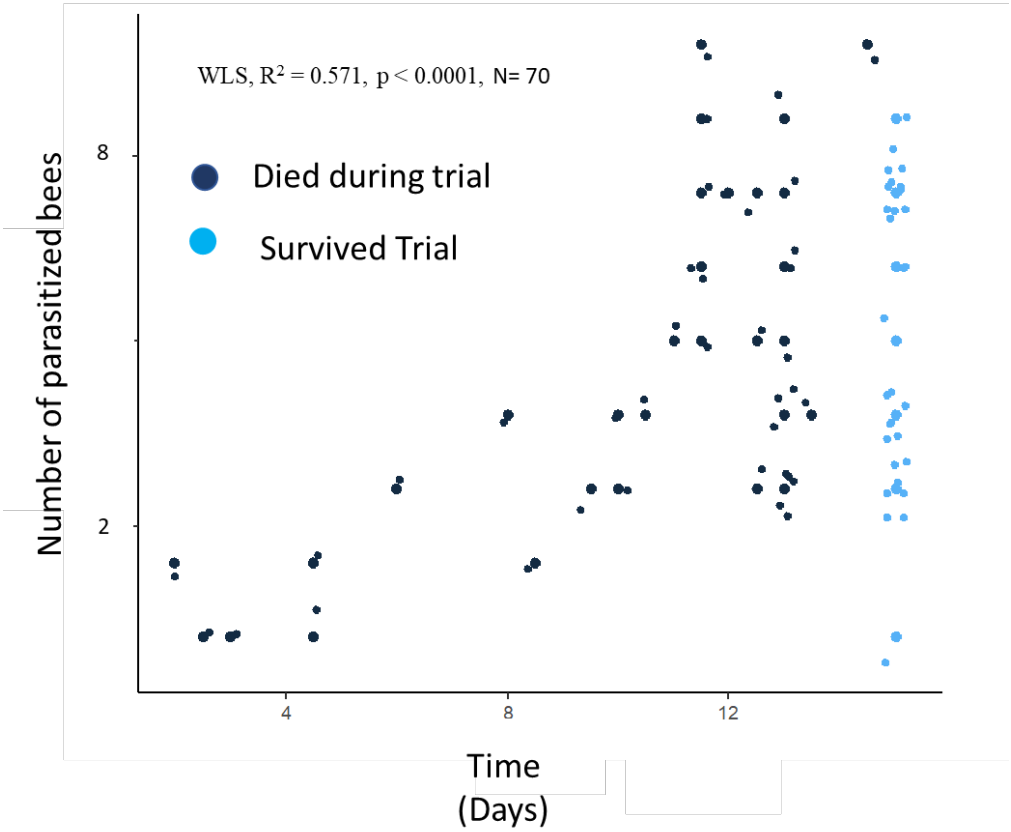

Figure 1.3 Number of bees parasitized by individual *Varroa* over time Each individual point is a *Varroa* observed over the course of a 15 day trial as it parasitized a small group of bees in the laboratory (8 bees per group, N = 70 replicates). Longevity was not a significant factor contributing to the number of parasitized hosts ($p = 0.124$, Mann-Whitney U Test, nor was time which weakly correlated. (WLS regression, $R^2 = 0.571$, $F(1, 68) = 90.59$, $p < 0.0001$)

Experiment 4: Relative risk of *Varroa* parasitism on adult workers

The presence of a *Varroa* among a group of worker bees was associated with increased bee mortality. Overall survivorship was highest in the unchallenged group, and significantly different from any of the *Varroa* challenged groups (Kaplan-Meier survival analysis, $p < 0.0001$, N = 303). Bees in the challenged groups died at faster and higher rates than bees within the unchallenged group (Kaplan-Meier survivor analysis, $p < 0.0001$, N = 303). However, there was no significant difference in

survivorship of bees did not differ between any of the *Varroa* challenged groups whether or not an added virus was present (i.e., whether *Varroa* had fed on artificially virus-infected pupae or on non-infected pupae prior to transferring to worker bees) (Pairwise Log-Rank post hoc test, $p = 0.33\text{-}0.6081$). However, bee survivorship within the *Varroa*-challenged groups was significantly influenced by parasitism and viral treatments. Bees parasitized by a *Varroa* died at faster rates than their non-parasitized counterparts only within the challenged +VDV1 and challenged + DWV groups (Kaplan-Meier survival analysis, $p < 0.0001$). There was no significant difference in survivorship between parasitized and non-parasitized individuals within the challenged control group. (Kaplan-Meier survival analysis, $p = 0.12$). The length of time from first observed *Varroa* feeding on an adult bee to death was longest in the challenged control group. Bees in this group lived for an average of 128 hours after first observed *Varroa* feeding (SD = 79 hours, n = 30). Time to death was shorter in the challenged + DWV group (96 hours) and shortest in the challenged + VDV group (87 hours). Differences were significant between the challenged control and challenged + VDV1 group, but not significantly different between the challenged control and challenged + DWV group. ($p = 0.027$, ANOVA, Tukey post hoc, $p =$ *0.0275* challenged + VDV1 and 0.082 challenged + DWV

Varroa mortality was recorded twice daily. More *Varroa* died in the challenged + virus groups but survivorship of the vector was not significantly different across any of the groups (Kaplan-Meier survivor analysis, $p = 0.43$, N = 29).

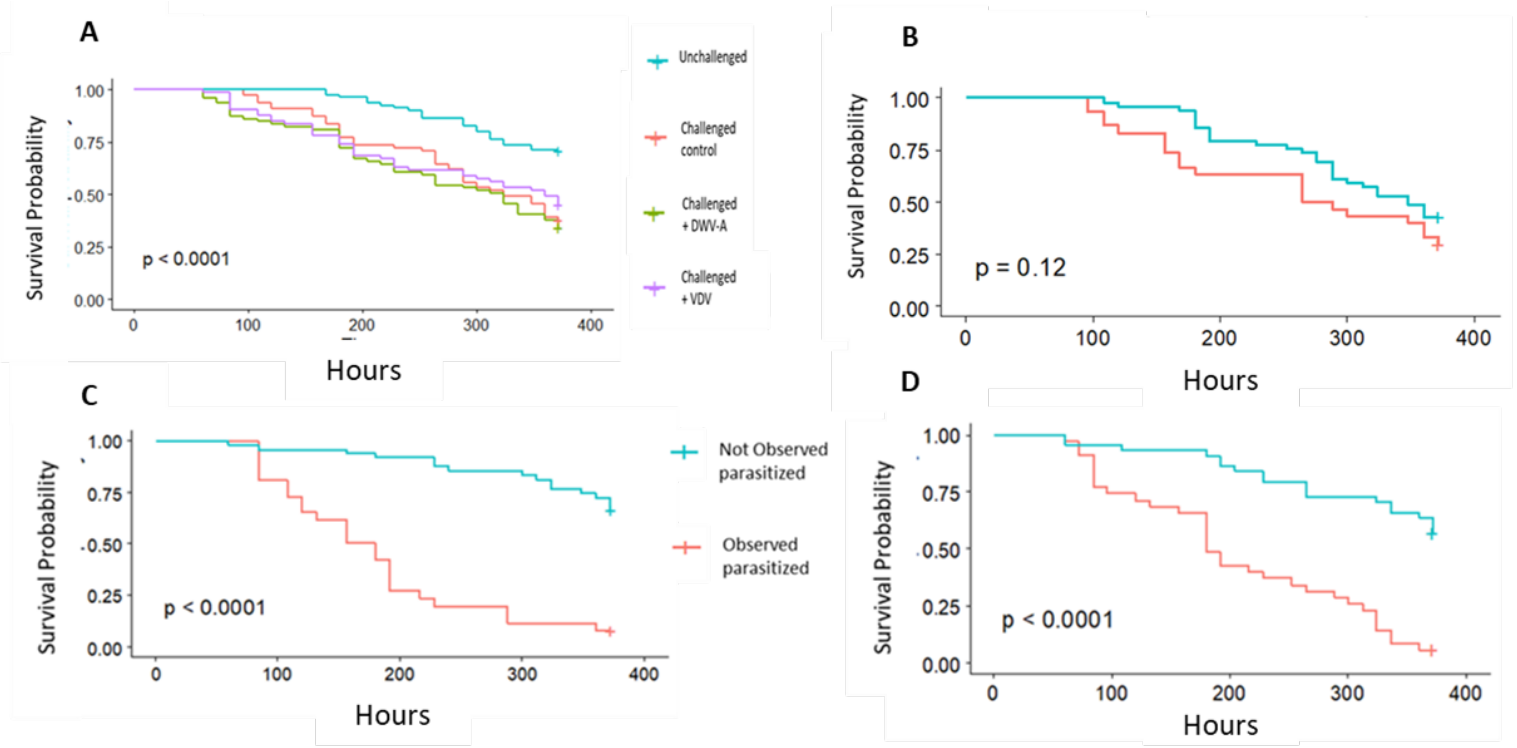

Figure 1.4 Survivorship of parasitized and non-parasitized bees: **A**. Survival analysis of bees from all treatment groups in the trial. **B, C, D** Survival analysis of parasitized and non-parasitized bees within each challenged group of the trial. **B.** Survivorship analysis of challenged control bees. **C**. Survivorship analysis of challenged + DWV bees. **D.** Survivorship analysis of challenged + VDV1 bees. There was a significant difference in survivorship between parasitized bees in the challenged + virus groups (p <0.0001), but not in the challenged control group (p = 0.12)

Bees in the unchallenged group had the highest survivorship, and incurred the least risk versus members of any other group (Table 1.3). The relative risk of death was higher and significantly different between every challenged group and the unchallenged group (2.16 challenged control, 1.91 challenged + VDV1, 2.29 challenged + DWV). Within groups, the relative risk of a *Varroa* feeding event was dependent upon the initial host source provided to the *Varroa* at the start of the experiment, and whether the bee was parasitized or not. Parasitized bees in the infectious groups had a high relative risk of death from a *Varroa* feeding and died shortly after being parasitized, while their non-parasitized nestmates experienced a relatively low risk of death (Tables 1.3). There was no significant difference in risk between the non-parasitized bees within the challenged + VDV1 or challenged + DWV groups compared to bees within the unchallenged group. However, relative risk was higher and significantly different between non-parasitized bees in the challenged control group compared with bees within the unchallenged group. In fact, relative risk was higher for non-parasitized bees in the challenged control group than non-parasitized counterparts in either of the challenged + virus groups (Table 1.3).

Group	Risk	Relative risk (95CI)	Relative risk of non-parasitized bees (95CI)	Relative risk of parasitized bees (95CI)	Within group relative risk between non-parasitized and parasitized bees (95CI)
Unchallenged	0.29	-	-	-	-
Challenged	0.62	2.16 (1.47, 3.17)	-	-	1.23 (0.87, 1.72)
Challenged + DWV	0.66	2.29 (1.57, 3.35)	0.76 (0.50, 1.15)	1.35 (1.05, 1.73)	2.18 (1.54, 3.09)
Challenged + VDV1	0.56	1.91 (1.27, 2.85)	0.60 (0.37, 0.95)	1.32 (1.02, 1.71)	2.71 (1.79, 4.10)

Table 1.3 Risk and relative risk table Risk reported for bees within their own group. Relative Risk estimates with confidence intervals reported for different bee cohorts within the study.

Survivorship after *Varroa* feeding events

There were significant differences in survivorship of bees after *Varroa* feeding events between the three challenged groups. The length of exposure of non-parasitized nestmates with parasitized nestmates was highest in the challenged control group, followed by the challenged + DWV-A and challenged + VDV1 groups. In both *Varroa* challenged + virus groups, challenged + DWV-A and challenged + VDV1, observable feeding resulted in the death of 94.3% and 92.9% of parasitized bees, respectively. High levels (above 8 $\log_{10}$) of DWV-A genome equivalents (GE) per bee never occurred among these individuals while 8 parasitized individuals in the challenged + VDV1 group had high levels of VDV1, all of which died during the 15 day trial. Non-parasitized individuals represented the majority of high VDV1 infections within this group with 14 of the 22 most infectious individuals in the challenged + VDV1 group being non-parasitized, of which only 2 died prior to the end of the trial. (Tables 1.4 and 1.5 for descriptive statistics and count data)

Table 1.4: Mean time to death after a Varroa feeding

Group	**Mean Time to death (hours) after *Varroa* feeding(SD)**	**Number of Parasitized bees (total bees in trial)**	**Mean percentage of non-parasitized to parasitized nestmates (±SD)**
Varroa Challenged	128 (79)	30 (72)	62.14% (0.184)
Challenged + DWV	96 (46)	35 (79)	55.36% (0.182)
Challenged + VDV1	87 (24)	26 (73)	64.35% (0.221)

Table 1.4 Mean time (hours) to death reported for bees within each challenged group. Count data is provided along with the average ratio of parasitized to non-parasitized bees in each group (±SD)

Table 1.5: Counts of parasitized and non-parasitized bees during experiment 4

Group	Non-Parasitized bees did not die during trial	Non-Parasitized bees that died during trial	Parasitized bees did not die during trial	Parasitized bees that died during trial
Unchallenged	-	-	-	-
Challenged	21	28	9	21
Challenged + DWV	25	19	2	33
Challenged + VDV1	31	16	2	24
Group	Non-parasitized bees (survived) with high levels DWV-A (VDV1)	Non-parasitized bees (died) with high levels DWV-A (VDV1)	Parasitized bees (survived) with high levels DWV-A (VDV1)	Parasitized bees (died) with high levels DWV-A (VDV1)
Unchallenged	-	-	-	-
Challenged	0 (0)	1 (0)	2 (0)	1 (0)
Challenged + DWV	1 (0)	4 (0)	0 (0)	0 (0)
Challenged + VDV1	1(12)	1(2)	0 (0)	0 (8)

Table 1.5: Count data provided for parasitized and non-parasitized bees in Experiment 4.

Viral loads across groups

Viral loads differed significantly across groups and between parasitized and non-parasitized bees within their own respective groups. There was a significant difference in viral loads across groups ($p < 0.0001$, Tables 1.7-1.8.) DWV-A levels were lowest in the unchallenged group, and significantly different between bees within all challenged groups (post-hoc Dunn test, $p < 0.0001$). DWV-A levels were highest in the challenged control group and were significantly higher than in the two other challenged groups + virus (post-hoc Dunn's test, $p < 0.0001$). DWV-A loads were not significantly different between the two challenged + virus groups. Parasitized bees in the challenged + virus groups failed to develop high levels of DWV-A infection (Table 1.6). VDV1 levels were highest in the challenged group + VDV1, and significantly higher than any other group ($p < 0.0001$, Kruskal-Wallace, df = 4, post-hoc Dunn's test, $p < 0.0001$). There was no significant difference between VDV1 levels and any other group in the trial.

Table 1.6: Mean Viral Loads DWV-A and VDV1 across experimental groups (log^{10} GE per bee)

Group	Mean DWV-A loads (SD)	Mean VDV1 loads (SD)	Mean DWV-A loads non-parasitized bees (SD)	Mean DWV-A loads parasitized bees (SD)	Mean VDV1 loads non-parasitized bees (SD)	Mean VDV1 loads parasitized bees (SD)
PreTrial Collection	3.74 (0.62)	6.45 (0.14)	-	-	-	-
Unchallenged	5.20 (1.68)	5.40 (1.42)	-	-	-	-
Challenged	4.47 (1.72)	5.41 (1.48)	4.91 (1.44)	5.70 (1.94)	5.44 (1.48)	5.32 (1.34)
Challenged + DWV	3.68 (0.44)	5.06 (1.45)	4.63 (2.09)	4.25 (0.97)	5.36 (1.53)	5.49 (1.43)
Challenged + VDV1	4.13 (1.22)	7.17 (1.64)	4.23 (1.46)	3.93 (0.52)	7.35 (1.53)	6.83 (1.81)

Table 1.6: Means ± SD for DWV-A and VDV1 viral loads provided in order from group, and within parasitized and non-parasitized cohorts. Analysis can be found on subsequent Tables 1.7 – 1.8

Table 1.7: Kruskal-Wallis ANOVA of DWV-A levels (log^{10} GE per bee) across groups

Group	*Unchallenged*	*Challenged*	*Challenged + DWV*	*Challenged + VDV1*
PreTrial Collection	0.96	0.027	0.40	0.48
Unchallenged	-	<0.0001	0.004	0.025
Challenged		-	0.0004	<0.0001
Challenged + DWV			-	0.61
Challenged + VDV1				-

Table 1.7: Comparison of DWV-A levels between bees across all groups. There was a significant difference in DWV-A levels across groups (Kruskal-Wallis $H^2 = 50.143$, df=4,$P < 0.0001$). Dunn post hoc analysis with Benjamini-Hochberg method of DWV-A levels across groups. reported in the above table.

Table 1.8: Kruskal-Wallis ANOVA of VDV1 levels (log^{10} GE per bee) across groups

Group	*Unchallenged*	*Challenged*	*Challenged + DWV*	*Challenged + VDV1*
PreTrial Collection	0.09	0.19	0.2	0.36
Unchallenged	-	0.31	0.2	< 0.0001
Challenged		-	0.73	< 0.0001
Challenged + DWV			-	<0 .0001

Table 1.8: Comparison of VDV1 levels of bees across groups. There was a significant difference in VDV1 levels across groups (Kruskal-Wallis, $H^2 = 91.044$, df=4, $P < 0.0001$). Dunn post hoc analysis with Benjamini-Hochberg method of VDV1 levels across groups reported in the above table.

Viral loads in non-parasitized bees

Non-parasitized bees developed high levels of DWV-A infection (> 8 log_{10} GE per bee) in all of the *Varroa* challenged groups. There was a significant difference in DWV-A viral loads per bee across groups (Kruskal-Wallis $H^2 = 35.255$, df=3, $p < 0.0001$). Non-parasitized bees in the challenged control group and the challenged + DWV group had significantly higher levels of DWV-A than bees in the unchallenged group (Dunn post hoc test, $p < 0.0001$ and $p = 0.046$) There was no significant difference in DWV-A levels between non-parasitized bees in the challenged + VDV group and bees in the unchallenged group (Dunn post hoc test, $p = 0.053$). VDV1 levels were significantly different when compared across all groups (Kruskal-Wallis

H^2 = 71.774, df=4, $p < 0.0001$). VDV1 levels were highest in non-parasitized bees in the challenged + VDV1 group, and significantly different when compared to non-parasitized bees in all other groups (Dunn post hoc test, $p < 0.0001$). There were no significant differences between VDV1 levels and any other group (Table 1.9).

Table 1.9 Kruskal-Wallis ANOVA of DWV-A levels between non-parasitized bees across groups

Group	*Unchallenged*	*Challenged control*	*Challenged + DWV*	*Challenged + VDV1*
PreTrial Collection	0.99	0.045	0.51	0.45
Unchallenged	-	< 0.0001	0.046	0.053
Challenged control		-	0.01	0.015
Challenged + DWV			-	1.00

Table 1.9: Comparison of DWV-A viral levels between non-parasitized bees across all groups. (Kruskal-Wallis H^2 = 31.906, df=4, $p < 0.0001$) Dunn post hoc analysis with Benjamini-Hochberg method of DWV-A levels between non-parasitized bees across groups reported above.

Table 1.10 Kruskal-Wallis ANOVA of VDV1 levels between non-parasitized bees across groups

Group	*Unchallenged*	*Challenged control*	*Challenged + DWV*	*Challenged + VDV1*
PreTrial Collection	0.08	0.2	0.2	0.3
Unchallenged	-	0.23	0.33	< 0.0001
Challenged control			0.82	<0 .0001
Challenged + DWV			-	< 0.0001

Table 1.10: Comparison of VDV1 levels in non-parasitized bees across groups. VDV1 levels were significantly different for non-parasitized bees across all groups (Kruskal-Wallis H^2 = 71.774, df=4, $p < 0.0001$) Dunn post hoc analysis with Benjamini-Hochberg method of VDV1 levels between non-parasitized bees across groups reported above.

Viral loads in parasitized bees

Parasitized bees, bees in which a *Varroa* was observed in feeding position during at least one time during the trial, only developed high levels of DWV-A infection in the challenged control group, and not within the challenged + virus groups (Table 1.6). Parasitized bees with high levels of DWV-A represented a minority of all bees which developed high levels of infection: 3 out of 11 bees. DWV-

A levels were significantly higher for parasitized bees in the challenged control group than any other group (Figure 1.5). VDV1 levels were highest in the challenged + VDV1 group (Table 1.6). There was no significant difference in viral levels between parasitized or non-parasitized bees within the challenged + VDV1 group, although there were more observations of non-parasitized bees developing high levels of DWV-A infection levels than their parasitized counterparts. 8 parasitized bees developed high levels of which all died during the 15 day trial. Non-parasitized individuals still represented the majority of high VDV1 infections within this group with 14 of the 22 most infectious individuals in the challenged + VDV1 group being non-parasitized, of which only 2 died prior to the end of the trial.

Table 1.11 Kruskal-Wallis ANOVA of DWV-A levels between parasitized bees across groups

Group	*Unchallenged*	*Challenged*	*Challenged + DWV*	*Challenged + VDV1*
PreTrial Collection	0.98	0.02	0.30	0.50
Unchallenged	Na	< 0.0001	0.45	0.11
Challenged		Na	0.02	0.009
Challenged + DWV			Na	0.51

Table 1.11: Comparison of DWV-A viral levels between parasitized bees. DWV-A levels of parasitized bees were significantly different across groups (Kruskal-Wallis $H^2 = 35.852$, df=4, $p < 0.0001$) Dunn post hoc analysis with Benjamini-Hochberg method of DWV levels between parasitized bees across groups reported above.

Table 1.12 Kruskal-Wallis ANOVA VDV1 levels between parasitized bees across groups

Group	*Unchallenged*	*Challenged*	*Challenged + DWV*	*Challenged + VDV1*
PreTrial Collection	0.06	0.09	0.21	0.69
Unchallenged	Na	0.76	0.19	< .0001 ***
Challenged		Na	0.46	< .0001 ***
Challenged + DWV			Na	< .0001 ***

Table 1.12: Comparison of VDV1 levels in parasitized bees across groups. VDV1 levels were significantly different for parasitized bees across groups (Kruskal-Wallis $H^2 = 32.683$, df=4, $p < 0.0001$) Dunn post hoc analysis with Benjamini-Hochberg method of VDV1 levels between parasitized bees across groups reported above.

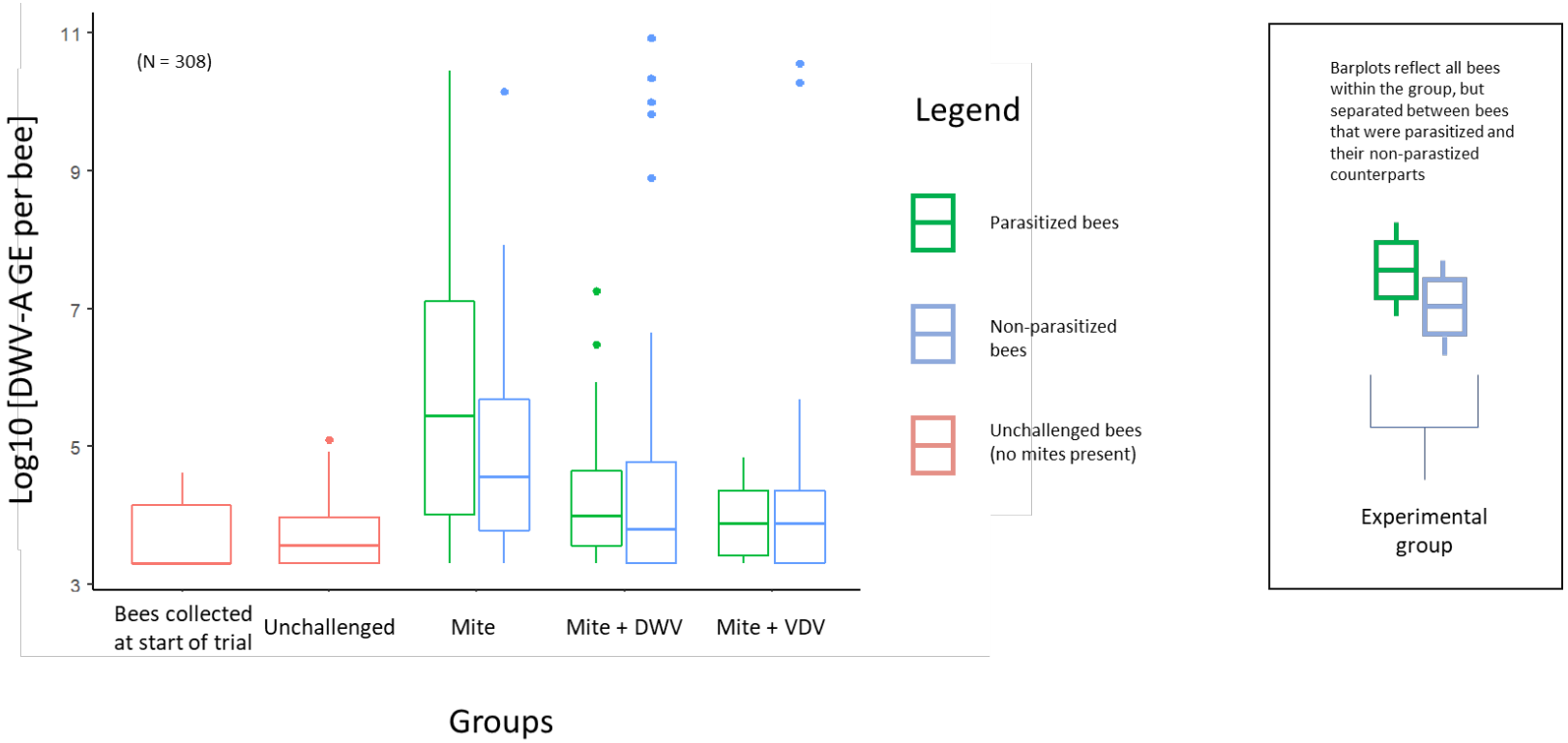

Figure 1.5: DWV-A genome equivalents (GE) per bee loads of 308 individual bees sampled from experiment 4. The four treatment groups are shown, as well as an additional group (furthest left) of bees collected at the start of the trial. For the 3 *Varroa* challenged groups, dual boxplots were used to display DWV-A GE per bee separately for parasitized and non-parasitized bees. The relative proportion of parasitized to non-parasitized bees can be found on **Table 1.5** and AOV analysis from **Tables 1.6-1.10**.

Discussion

Our experiments provide quantitative estimates for the host switching rates of *Varroa* mites from one adult bee to another, and the impacts of those switches on disease vectoring. The consistent acquisition of fluorescent microspheres by *Varroa* when feeding on adult bees shows that host switching is best seen as a pursuit of resources from adult bees, not simply a search for safe refuge. *Varroa* primarily feed on the fat body of adult honey bees, while incidentally ingesting hemolymph[21,31]. Regular uptake of the microspheres in our trials also confirmed ingestion of free-floating material, suggesting virus particles distributed throughout the hemocoel could be acquired independently of their presence in fat body. I observed fluorescent microspheres moving bidirectionally between vector and host, suggesting that *Varroa* can acquire infectious material from one host, and pass that material onto a subsequent host. Microspheres were more easily observable in a *Varroa* which fed on an injected adult bee. However, it was difficult to find and visualize microspheres inside of an adult bee on which a Varroa had fed. I could not determine in these samples if microspheres were indeed present and just not detected under fluorescent microscopy. Coupled with previous work, *Varroa* infectiousness may be partly dependent on the infectiousness of the host they most recently parasitized[32,33]. If so, *Varroa* which engage in the highest frequency switching are most likely to acquire and transmit DWV. A similar phenomenon is observed in mosquitos, i.e., high-frequency biters shape pathogen transmission[12].

Varroa are promiscuous feeders on adult bees, and expressed a great degree of heterogeneity in the host switching rate. *Varroa* which engaged in frequent switches were responsible for nearly three times as many parasitized hosts as their lower switching counterparts. For example, some *Varroa* switched 12-15 times in 15 days, returning to previously fed upon hosts because all non-parasitized bees had been exhausted. Meanwhile, slower switching counterparts switched only once in the same 15 day period, meaning that most bees in that cohort were bees not bitten. High-frequency switchers would be more likely to feed upon an already infectious adult bee than a low switching counterpart. After becoming infectious, these *Varroa* would be responsible for the most infected hosts, thus increasing the risk to all other *Varroa* which feed upon an infectious host, as well as the hosts themselves. The underlying mechanism driving heterogeneity in this behavior was not studied, but warrants future research. Behavioral heterogeneity could be explained by genetic differences in the *Varroa* population, whether *Varroa* had already produced or are callow daughters, or how long *Varroa* have been in the dispersal stage. Our studies attempted to limit the heterogeneity in the host population so that I could observe differences in *Varroa* behavior without confounders. In a true bee population of mixed ages, phenotypes, and sexes there would likely be an interaction between behaviors of the vector and availability or unavailability of ideal hosts. The host switching rate may also be affected by similar factors that influence the amount of time *Varroa* spend on adult bees such as host condition and brood availability, which have already been shown to affect the amount of time *Varroa* spend in their dispersal phase[16].

There are clear costs and benefits for estimating the host switching behavior and consequences of their feeding on adult bees in laboratory settings. Here, I used a fixed host population size since both basic reproductive rate (R_0 and vector capacity models utilize fixed populations in their estimates[7,34]. The use of artificial arenas reduced the number of confounders normally inherent in a honeybee colony as cage designs eliminate many key characteristics of a honeybee colony[29] while it also reduces the degree of field relevance[29]. This allowed us to study the relative risk of direct feeding on adult bees and the conferred harm to nestmates without confounders and survivorship bias inherent in colony settings. Because of social organization of a honeybee colony it's possible the conferred harm we observed in our trials to non-parasitized bees would not be observable in colony states where there are an ample number of newly emerged bees. A field study that tried to answer this question would be affronted by numerous confounding variables such as many *Varroa* with varying degrees of infectiousness, unobserved parasitism by *Varroa*, cannibalized pupae as a vector for honeybee viruses, and survivorship bias from death and removal by nest mates. .Observed feeding by *Varroa* was a significant predictor for bee mortality in our trials, but *Varroa* feeding could only partially explain bee deaths. The mean time to death was significantly shorter after a *Varroa* bite in the challenged plus virus groups compared to a *Varroa* bite in the control group feeding contact rates between non-parasitized and parasitized nestmates would have been longest in the challenged control group. Our results suggest long lived parasitized bees confer risk of death and viral transmission to non-parasitized nestmates. If true, trophallaxis or the oral exchange of food between nestmates may serve as a more impactful route for viral

transmission than currently appreciated. Relative risk was higher for non-parasitized bees in the challenged control group than non-parasitized counterparts in the viral challenged groups. This is likely possible because bees in this group lived for long periods after a *Varroa* feeding, giving more opportunities for contact and trophallactic interactions with non-parasitized nestmates. In fact, our data suggest parasitized bees which died quickly after *Varroa* feeding may be conferring protection to non-parasitized nestmates by limiting opportunities for host to host transmission, whereas long lived survivors may elevate risk to nestmates.

Continued research is warranted to understand how oral and contact transmission affects virus transmission dynamics in a honeybee colony. I suggest these asymptomatic, non-parasitized bees may be responsible for maintenance of the pathogen and potentially serve as a reservoir of infectious bees in a dense honeybee colony. I draw this hypothesis from the results of this experimental study and upon similar phenomena observed in other disease systems; namely emerging viruses that are both communicably spread between hosts and vectored borne. Just like social bees, birds which received West Nile virus or Tembusu virus through communicable routes developed high levels of infection and lived longer than parasitized or experimentally injected subjects[9,35,36]. A recent study confirmed this alarming trend. Older, asymptomatic ducks shed high levels of virus to flock mates, supporting the role "supershedders" may have in an epidemic.[37] In the honeybee colony the production of supershedders may be produced by the continual production of parasitized bees and susceptible individuals that trophallaxis with them. It is quite possible this circulation between *Varroa*-host and host-host transmission could

increase the risk of naïve *Varroa* acquiring infectious levels of DWV as they jump from bee to bee.

Continued research is needed to understand the impacts of this economically important pest on adult bees. *Varroa* switching from one adult bee to another to feed would jump trophallaxis networks which are carefully structured to maintain cohesion in the colony[38]. Not only could individual bees be connected due to a lineage of *Varroa* feedings, but entire social networks within the colony could be bridged[39]. These social networks, which naturally exhibit degrees of independence from each other[38], would be connected via promiscuous vectors. Prolific switching by vectors would also mean the infestation rate, often measured as a proportion of *Varroa* in a sample of bees[40], would not reflect the gross number of bees actually fed upon. In short, there could be more bees having been fed upon at any given time than the total number of *Varroa* present in the colony. Finally, *Varroa*, DWV and the honeybee offer a unique relationship in which to apply vectorial capacity principles as the relationship offers multiple communicable modes of transmission, not just vectored routes. Vectoring of DWV by *Varroa* is also an evolutionarily recent phenomenon, where mathematical analysis would help describe co-adaptation by vector, pathogen and host over time.

Chapter 2: Oversharing by honey bees and the spread of viruses

Abstract

Deformed wing virus (DWV) is an emerging insect pathogen efficiently transmitted through communicable and vector-borne routes with *Apis mellifera*. Continual transmission of DWV between hosts and vectors is required for maintenance of the pathogen within the population. This pathogen-vector-host system offers potentially unique interactions for transmission to be maintained in vectors as potentially both direct vector to vector transmission, host to vector and host to host to vector routes are possible. Can communicable transmission between hosts cause vectors to acquire and later transmit the pathogen? In a series of experiments, we study how vector-vector, host-host and host-vector transmission circulations maintain DWV in a honey bee population. We found co-infestations on shared hosts allowed for movement of DWV from mite-mite. Additionally, two social behaviors of the honeybee, trophallaxis and cannibalization of pupae, provide routes for communicable transmission from bee-bee. Communicable circulation of the virus solely amongst hosts was then shown to act as a reservoir of DWV for naïve *Varroa* to acquire and subsequently vector the pathogen.

Introduction

Continual transmission between infectious and susceptible individuals is essential for the persistence of a pathogen in a population[41]. Infectious individuals, aptly called maintenance hosts, form the host reservoir. Over the last 60 years, numerous theoretical models have been developed to describe the transmission

dynamics of infectious diseases[27,34], ultimately encompassing both host-parasite-vector systems and the threats posed by emerging diseases[10,42]. These models rely on complete and accurate estimates of routes and parameters of transmission, which may be lacking in emerging diseases. One such disease is the emerging pathogen Deformed wing virus (DWV). Once rarely observed in its honeybee host *Apis mellifera*, this virus has been detected in more than 65 arthropod species[5]. DWV is an RNA IIflaviridae family virus that is transmitted both communicably and as a vector-borne pathogen in global honey bee populations[1]. This virus was present, but had low prevalence in honeybee populations until the introduction of *Varroa destructor*, an ectoparasitic mite and competent vector of honeybee viruses that jumped hosts from *Apis cerana* to *Apis mellifera*[43]. Impacts of DWV garnered global attention when severe losses in managed and feral honeybee colonies were reported in 2006 in North America[44], leading to intensive research on DWV and its impacts[5].

Honeybees are the primary host of DWV, with other Hymenoptera serving as dead end hosts for this virus[1]. DWV is transmitted via multiple communicable modes among the thousands of members found in honeybee colonies[2]. Mockel *et al* showed that adult workers can develop covert (subclinical) infection after oral exposure to the virus[4]. Later, consumption of infectious pupae by adult bees followed by spread through trophallaxis between adult bees was shown to be an efficient route for transmission, which also resulted in covert infection[45]. Brood rearing is an essential function in a honeybee colony. Yue and Genersch detected DWV in larval food, suggesting brood rearing may be a route of infection for developing bees[46]. In addition, infection in developing brood has been shown to originate through the

queen via vertical transmission. Amiri *et al.* demonstrated experimentally 1/3 of colonies had eggs infected with DWV. The virus was deposited on the surface of the eggs indicating transovum transmission[47]. Direct infection to queens has been demonstrated experimentally through artificial insemination of virgin queens and in field studies through natural mating[48,49]. Importantly, covert infections arising from the queen and passing vertically to her thousands of offspring provide for sustained transmission within the colony, a key parameter in the maintenance of DWV. De Miranda and Fries postulated that infections through this route would influence *Varroa*-mediated and fecal-oral-cannibalization transmission routes by potentially infecting a large swath of bees[50].

Varroa as an active vector for honey bee viruses is a recent phenomenon. *Varroa* feed on bees both during their reproductive phase on a developing larva, and during a dispersal phase on adult bees[21,51]. Feeding bouts during both periods offer opportunities for vector-mediated transmission to hosts. Overt (clinical) infections are most evident in the form of crippled wings, shortened abdomens, and adults with motor-paralysis resulting from transmission during larval development[44,52]. Subclinical infections occur when bees are parasitized during development but do not develop physical deformities, and are often associated with lower viral loads[44,53]. *Varroa* can be infectious with a virus, or naïve, without a virus[54]. Bee to mite transmission occurs when *Varroa* feed on an already infected host which has developed a high level of infection prior to the *Varroa*'s feeding. Bowen-Walker and Gunn inferred this from the high levels of DWV in *Varroa* collected from infectious

bees with crippled wings[55]. Hosts harboring high levels of DWV will be especially infectious for naïve *Varroa* which feed upon them.

Mite-mite transmission has not yet been observed but could potentially occur during both the *Varroa*'s reproductive phase or during the dispersal phase when two or more *Varroa* share the same larval or adult bee host, simultaneously or concurrently. Multiple infestations are not as common as single infestations, but may be an important feature in the maintenance and spread of DWV in the *Varroa* population[56]. Similar features are important in the maintenance of pathogens in other parasites and vectors, most notably with cofeeding ticks.[57-59] Both Mite-mite and bee-mite transmission routes could be important in the maintenance of DWV in the *Varroa* population.

In a series of experiments, I ask if transmission occurs mite to mite on shared hosts, and through bee to mite routes. When examining bee to mite transmission routes I asked if communicable transmission of a virus among honeybees can influence acquisition of the virus by a *Varroa* and future vector transmission of the virus. In this way, I attempted to describe purported modes of transmission in a pathogen-vector-host relationship which is unlike most infectious disease systems studied to date. I show a surprising degree of interaction between communicable, and vector borne transmission modes; social behaviors of the host species aid in transmission, acquisition and future vectoring of the pathogen by the introduced parasite. In the most notable example, hygienic removal of diseased pupae by adult bees, a form of social defense in honeybees, aids in the spread and persistence of

DWV in hosts and *Varroa*. This study integrates these routes, showing that honey bees themselves can amplify the impacts of a new vector.

Materials and Methods

Construction of custom cages

Custom laboratory arenas were constructed from acrylic sheets so that bees could be physically isolated in adjoining, but divided chambers. The divider was a solid piece of acrylic with a 7/8 inch hole cut in the center. The hole was sealed with 1/8 inch hardware cloth. This served as a trophallaxis port in which bees could orally exchange food with li*Varroa*d physical contact. 1/8 fiberglass mesh screen was used for ventilation at the end of each chamber. The interior dimensions of each cage were 2.5” x 2.5” x 2.75”.

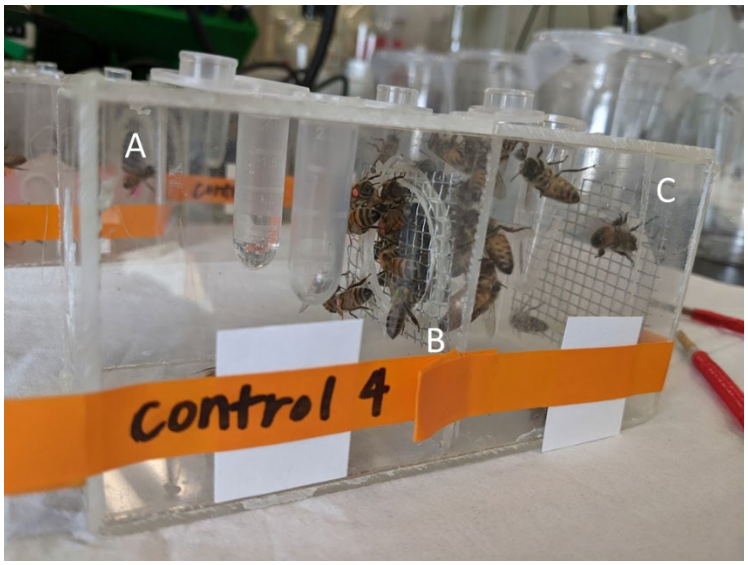

Figure 2.1 (A) Donor bee compartment. **(B)** Mesh screen divider. **(C)** Recipient bee compartment.

Viral inoculum used for studies

A clone derived variant of DWV-A (NLuc) was used throughout the trials[60]. This virus has two inserts in its nucleotide sequence which allow for reliable detection.One non-essential modification, the nucleotide sequence of NLuc reporter, is detectable with RTqPCR. A second modification involves an introduction of a rare cut restriction-enzyme site (Pac1) that can be verified by digestion of RT PCR product and standard gel imaging[60]. In this way, dual verification of the presence of the novel virus was achieved. For the rest of the document inoculum refers to this as the DWV-A NLuc virus. Stock samples were provided by Evans and Ryabov and maintained at the USDA-ARS Bee Research Laboratory in Beltsville, Maryland.

Molecular preparation and qPCR

All samples were extracted using standard techniques[61]. Whole bee specimens were extracted in trizol and then RNA was used to produce cDNA using single reaction reverse transcriptase according to manufacturer specifications (BioRad, IScript). Total viral cDNA was quantified using real time qPCR and a 10 fold dilution series of prepared standards exactly as described in Posada-Florez et al.[45]. Verification of 5′ region of DWV RNA (30–1266 nt) containing the PacI site was performed through digestion of the Pac1 and then visualization on an agarose gel[60].

Experiment 1: Can communicable transmission of DWV between honeybees amplify vectored disease?

Phase 1: Establishing infectious donor bees

White-eyed pupae were injected with 1 ul of either viral inoculum (10^7 GE) or PBS and incubated for 12 hours at 34 ºC and 40% humidity. *Varroa* were sampled

from adult bees collected from a moderately infested colony not exhibiting any signs of overt varroosis or brood disease. *Varroa* were serially passaged for five days on pupae procured from a healthy colony that did not yield any *Varroa* detections (5 mites per pupa host). *Varroa* were randomly assigned to a control (PBS injected) or experimental (viral injected) pupae for their last passage. At 12 hours after injection, pupae were transferred into a 00-sized gel cap. *Varroa* (4 to 7) were then placed inside the gel cap with the pupae, and returned to the incubator to feed for 24 hours (36 hours since injection).

After feeding for 24 hours on injected pupae, *Varroa* were transferred to 3 day old adult bees. Adult bees were procured from a frame of emerging brood sourced from a healthy colony exhibiting no clinical signs of disease that yielded no *Varroa* detections via an alcohol wash. Bees and *Varroa* were incubated at 34ºC at 40% humidity, and checked twice daily for *Varroa* feeding. The bee was removed from the cage with a pair of soft tweezers when a *Varroa* was observed in a known feeding position. The bee was held upside down by the wings or thorax with one hand, while the researcher agitated the *Varroa* from the feeding position with a Chinese grafting tool (Mann Lake, Hackensack, MN). Once removed, the *Varroa* was scooped from the bee and stored in a -80 ºC freezer. The bee was thoroughly inspected for additional *Varroa*, and then transferred to one compartment of the custom arenas. This side of the compartment was labelled “donor”. Bees were added in this manner to the arenas over the course of 4 days and incubated for a total of 7 days (from first addition). Bees were checked twice daily for the presence of *Varroa*, and to record bee mortality.

Phase 2: Addition and maintenance of recipient bees

At day 8 of the trial, recipient bees were added to the second compartment of the custom arenas. Recipient bees were newly emerged and procured in the same way as the donor bees. Recipient bees were thoroughly inspected for *Varroa* at emergence before introduction into the custom arenas, and inspected twice daily each day thereafter. Beginning on the second day after introduction of recipient bees trophallaxis was encouraged by removing sucrose feeders from the recipient's side. Sucrose feeders were removed and the arenas were returned to the incubator for four hours, after which bees were checked for *Varroa*, and the sucrose feeders returned. This continued for 8 more days (15 total days since first addition of donor bees). *Varroa* that were not experimentally inserted were not observed in any of the replicates during the trial.

Phase 3: Removal of donor bees and addition of *Varroa*

On day 16, the recipient bees were removed from the arenas and stored in a -80 ºC freezer. Recipient bees were removed by lightly chilling the whole arena in a walk in incubator (4ºC) for 10 minutes. The lid of the recipient compartment was removed, and the bees were knocked quickly into a wide lip plastic cup. A fine mesh net was promptly sealed over the bees to prevent escaping. The cages now with only recipient bees were returned to the incubator.

Recipient bees were thoroughly inspected for *Varroa* one final time (no *Varroa* were found in any of the arenas). *Varroa* were then added to each cage of recipient bees. *Varroa* were procured and serially passaged as previously described on non-injected pupae prior to their introduction onto the adult recipient bees. *Varroa*

were allowed to feed on adult recipient bees for 4 days after which *Varroa* were removed as previously described and placed onto a white eyed pupae (non-infested, procured from a healthy colony) in 00 gel caps. *Varroa* and pupae were incubated for 6 days and then preserved in a -80 ºC freezer for later RNA extraction.

Experiment 2: Do *Varroa* acquire virus when feeding upon adult bees which previously cannibalized pupae?

Pupae were injected with the viral inoculum as previously described or PBS and then incubated as previously described. *Varroa* were collected and introduced to the pupae as previously described. After 24 hours of feeding on pupae, *Varroa* were removed from the pupae and passaged onto a new pupa. *Varroa* were incubated on their new pupae for 5 days, and then frozen in a -80 ºC freezer. These pupae served as the experimental or control pupae for the experiment.

Cages of 30 adult bees were made by collecting worker bees from a healthy colony which did not yield detections of *Varroa* in an alcohol wash. Cages were constructed in exactly the same way and with the same materials as in Chapter 1[62]. Cages were randomly assigned to one of three treatment groups: no pupae, pupae with virus, pupae without virus. One pupa was removed from the -80ºC freezer for each experimental cage, thawed and immediately fed to the bees. This was repeated on the following day, after which bees were incubated for an additional 9 days (11 days total since first introduction).

A new series of *Varroa* were procured and passaged as previously described, and then introduced into the experimental cages. *Varroa* were allowed to feed upon

these adult bees for 4 days, after which *Varroa* were individually removed and introduced into a 00 gel cap with a white eyed pupa (healthy, non-injected). *Varroa* were allowed to feed on pupae for 48 hours, at which point the *Varroa* were removed and stored at -80 ºC. Pupae were incubated for 4 more days and then stored at -80 ºC.

Experiment 3 -4: Do *Varroa* transmit virus via shared honeybee hosts?

Collection and marking of *Varroa*

Varroa destructor were sourced and hand collected from a heavily infested colony not showing overt signs of *Varroa* parasitism. Adult bees were inspected and those with *Varroa* in their feeding positions were stored in a ventilated cage[62]. Bees and *Varroa* were returned to the laboratory and stored in an incubator at 34 ºC and 40% humidity.

Varroa were marked in these trials with a small amount of paint applied dorsal-distally directly onto the *Varroa*'s carapace. Paint was applied with a 0000 fine tip paintbrush (Javis: 4/0 nylon, England) with a small downward strike moving posteriorly from the dorsal tip of the *Varroa*. Paint from fine-tipped oil-based permanent markers were used. Paint was procured by ejecting the tip of the marker onto a plastic surface until a pool of paint formed. The tip of the brush was then dabbed against the surface of the puddle. In this way, a small amount of paint could be applied to the surface of a *Varroa*. Colors were used to identify *Varroa* by group. For example, *Varroa* painted blue were part of the viral group, clearly identifying them differently than the *Varroa* from the control group which were painted orange. The researcher could observe, track and recollect multiple *Varroa* co-housed on the same group of bees.

Maintenance

Varroa were serially passaged on pupae procured from healthy (no observed *Varroa*) colonies as a means of reducing potential viral loads carried by these *Varroa*[63]. *Varroa* were enclosed with pupal hosts in a 00 gel cap and pupae were changed every 2 days, a method shown to ultimately cleanse *Varroa* of DWV-A. *Varroa* were passaged 3 times prior to the start of the trial. Next, experimental-phase *Varroa* were prepared by passaging on pupae that had been injected with either 10ul of PBS or PBS with viral inoculum 12 hours prior to *Varroa* introduction. These *Varroa* were incubated in 00 gel caps with their respective pupa for 24 hours (total 36 hours post injection), prior to their use in the below trials.

Experiment 3- Examining potential *Varroa* to *Varroa* transmission on shared pupal hosts

A frame of recently capped worker brood was removed from a healthy *Varroa* free colony and brought to the laboratory. The corner of a cell capping was lifted with a razor blade so that *Varroa* could be introduced into the recently capped larval cell. *Varroa* were introduced by picking one up with a Chinese grafting tool, and then tipping the tip of the grafting tool with the *Varroa* into the opening of the torn cell. *Varroa* were coaxed into the cell by prodding them with an additional grafting tool held in the other hand. A second *Varroa* was immediately introduced in the same manner. Cell cappings were resealed by pressing the torn capping back down, and using another cell capping as a "bandage". This was done by removing an entire cell capping with a pair of tweezers, flipping it over, and firmly pressing the waxed side

(silk side up) against the "wound" of the experimental cell. If a cell was damaged too greatly to be re-sealed in this way, then it could be capped with a #6 gel cap.

Two *Varroa* were introduced into each cell. Introductions were made by pairings: control x control, or control x virus-exposed (viral). Experimental cells were numbered in chronological order of insertion, with *Varroa* insertion, and the source pupa of each respective *Varroa*. White paint markers were placed to provide a geographical reference point on the surface of the frame, to locate experimental cells. *Varroa* and pupal hosts were recovered 9 days after insertion. Experimental cell locations were confirmed by references cells and the laboratory log. Once confirmed, cell cappings were removed with a pair of tweezers. The pupae were removed and saved at -80ºC. *Varroa* were collected from the cells using a Chinese grafting tool, and then inserted with a pink-eyed pupal host in a 00 gel cap. Each gel cap was marked with a sharpie marker recording its origin. Pupae and *Varroa* were incubated for 48 hours, at which time *Varroa* were removed and stored at -80ºC until RNA extraction. Pupae were returned to the incubator for another 4 days, after which samples were preserved at -80ºC.

Examining mite to mite transmission on shared adult hosts Experiment 4

Bee cages

For experiment 4, cages of 8 individually marked bees were used, and established in the same manner in Material and methods, Chapter 1. Marked *Varroa* were placed into prepared cages of marked 3-day-old adult bees. *Varroa* locations were checked 2 hours after introduction and then every 12 hours thereafter for 10 continuous days. Daily *Varroa* and host mortality was recorded. All samples were

saved at -80ºC until extraction. The location of each *Varroa* was recorded every 12 hours, recording whether the *Varroa* was feeding or not on the host bee, and specifically which host bee. Recordings were made in a way to differentiate each *Varroa* from the other, and to track them independently.

Statistical Analysis

All statistical analysis were performed in RStudio using BaseR and associated packages. For all trials, count data were used to report the observed number of infectious bees or **Varroa** over the number of non-infectious bees or **Varroa** in a trial. In order to test viral load differences in exposed and non-exposed Varroa and bees to the NLuc virus an ANOVA was performed to see if there were significant differences between the groups. The residuals of data were visualized and then tested using the Shapiro-Wilk test in order to test for normality. DWV-A titers were compared across groups using an ANOVA. When the assumptions of normality were not met with NLuc titers a non-parametric Kruskal-Wallis ANOVA was used. Statistical significance was set at $P < 0.05$. Results

Experiment 1 Can *Varroa* acquire virus following communicable transmission between honeybees?

Using tagged virus clones, I showed that *Varroa* acquired DWV by feeding on adult bees which had become infected via food delivered to them by infected colony-mates. A group of 12 *Varroa* exposed to infectious (experimental) bees and 7 *Varroa* exposed to non-infectious (control) bees were individually sampled for presence of the tagged virus. Of the *Varroa* exposed to adult bees in the experimental group, 50% were positive for the novel virus (6/12 bees), while none of the controls reported

detections (0/7 bees). Of the *Varroa* which reported detections, qPCR results showed low levels of the tagged virus (mean ± SD = $4.61\log_{10} \pm 0.17$, n = 6). Natural DWV-A levels were not significantly different between exposed and unexposed *Varroa* in this study (AOV, $F_{1,17} = 1.38$, $p = 0.26$). 100% of *Varroa* in the experimental group had detections of DWV-A while 71.4% of *Varroa* which fed upon the unexposed bees had detections. (Table 2.1) After their removal from their adult bee hosts 9 of the 12 *Varroa* in the exposed group were transferred to feed upon pupae. (The other three *Varroa* were removed from their adult bee hosts and saved directly at -80ºC until RNA extraction. These *Varroa* did not have a paired pupal host). Of the 9 *Varroa* with a paired pupal host, 5 of the *Varroa* had detectable levels of the Nluc RNA, while only one paired *Varroa*-pupal host also had detectable levels. These samples were screened for the tagged virus via the *Pac*1 restriction site and gel electrophoresis and detection was verified in both the *Varroa* and pupal host. 2 other positive detections of the NLuc RNA were observed in pupae not paired with *Varroa* samples. Overall observed transmission from *Varroa* to pupal hosts was 17.6% N = 17 (Appendix 1. . There was a general trend for less prevalence of the DWV pathogen after transmission cycles further away from the index host or index vector.

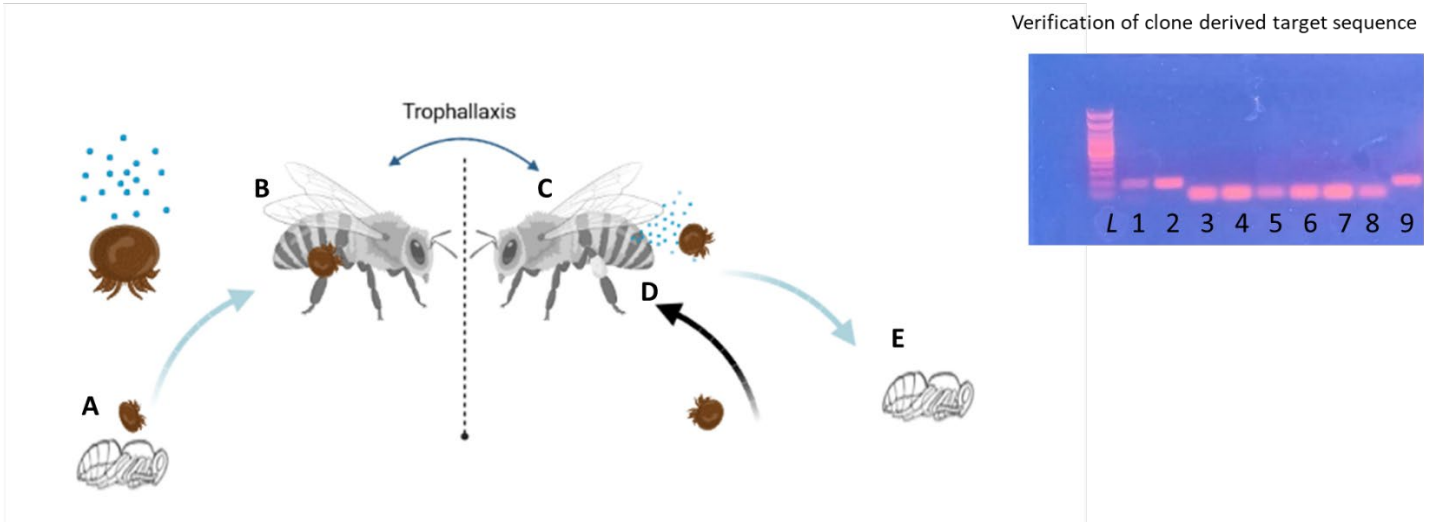

Figure 2.2 Transmission of DWV-A: (A)DWV-A (with NLuc reporter) was introduced into our system via infectious *Varroa*. (**B)** *Varroa* successfully vectored the pathogen to adult bee hosts who subsequently transmitted the pathogen through trophallaxis to naïve recipient bees **(C)** (unexposed to original *Varroa*). **(D)**New *Varroa* were introduced into the system which acquired the pathogen from the recipient bees, and **(E)** then vectored it to new pupal hosts. Detections of the tagged virus was accomplished through restrictive primers using qPCR and gel electrophoresis (**Right)***L: ladder.* **1:** *Varroa* with wildtype and (faint) Pac1 sequennce. **2.** *Varroa* with wildtype DWV sequence. **3-4** paired samples: infectious *Varroa* (3) shared adult bee host with naïve *Varroa*(4); which both fed upon the same adult bee samples. **5** Pupae(**E)** which acquired tagged virus from (**1)** Samples **6-7** were *Varroa* which shared the same pupal host. Sample 6 was originally naïve and sample 7 infectious. **8.** Is wild type DWV positive control. **9.** Is Pac1 site positive control. Images produced and licenses through BioRender.

Experiment 2: Do Varroa acquire virus when feeding upon adult bees which previously cannibalized pupae?

Naïve *Varroa* transmitted the novel tagged virus to a pupal host after feeding upon adult bees which had cannibalized infectious pupae. Of 21 staged *Varroa*, 17 (80.9%) successfully transmitted the acquired tagged virus to a naïve bee host. No detections of the novel nLuc insert were observed in the control groups (n = 23). There was no significant difference in wild type DWV levels between experimentally exposed and control *Varroa* (AOV, $F_{1,43} = 1.51$, $p = 0.29$, Table 2.1)

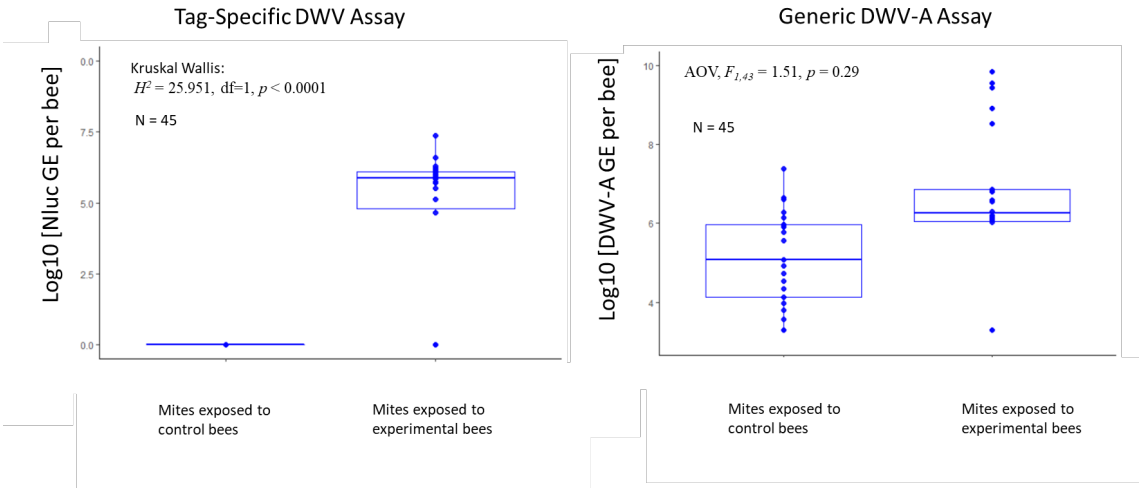

Figure 2.3: (Left) Detections of NLuc nucleotide sequence in pupae which mites fed upon after removal from their adult bee host. *Varroa* were exposed to either control or experimental adult bees. *Varroa* which fed on adult bees that cannibalized infectious pupae were highly likely to later transmit the tagged virus acquired through adult feeding (80.9%, N = 20). **(Right)** Detections of DWV-A in pupae which *Varroa* fed upon after removal from their adult bee host. There was no significant difference in natural DWV-A loads between control and experimental groups (AOV, $F_{1,43} = 1.51$, $p = 0.29$).

DWV levels in pupae *Varroa* fed upon after feeding on adult bees (all values in log10 GE per bee)						
Trial	**Group**	**State**	**Primer**	**Mean**	**Median**	**SD**
Cana	Experimental	Naive	DWV	6.34	6.25	2.08
Cana	Control	Naive	DWV	5.85	5.77	2.23
Cana	NegControl	Naive	DWV	5.50	4.83	2.06
NLuc levels in pupae *Varroa* fed upon after feeding on adult bees (all values in log10 GE per bee)						
Trial	**Group**	**State**	**Primer**	**Mean**	**Median**	**SD**
Cana	Experimental	Naive	DWV	4.60	5.86	2.60
Cana	Control	Naive	DWV	0	0	0
Cana	NegControl	Naive	DWV	0	0	0

Table 2.1 Mean, median and SD of DWV-A and Nluc levels between experimentally exposed or control *Varroa*.

Experiments 3-4: Does *Varroa* to *Varroa* transmission occur on shared hosts?

Transmission occurred between *Varroa* that were concurrently feeding on pupal or adult bee hosts. Naïve *Varroa* acquired and subsequently transmitted a tagged virus while sharing a pupal host with another infectious *Varroa* (n = 20, 11/20 confirmed cases). There was no significant difference in wildtype or clone-derived virus loads between *Varroa* sharing a pupal host within the experimental group. (AOV, $F_{1,37} = 0.042$, $p = 0.84$ and $F_{1,37} = 1.70$, $p = 0.2$). Nor was there a significant difference in wildtype DWV-A levels between originally infectious and originally naïve *Varroa* within the experimental group which shared a pupal host (AOV, $F_{1,37} = 0.042$, $p = 0.84$). DWV-A loads in these *Varroa* averaged 8.09 $\log_{10}$ and 9.93 $\log_{10}$ GE per bee in the two trials, while their non-*Varroa* exposed counterparts averaged 4.47 $\log_{10}$ and 3.54 $\log_{10}$ GE suggesting *Varroa* infestations were causing covert infections (AOV, $F_{1,136} = 17.55$, $p < 0.0001$). Of the 11 *Varroa*, 4 acquired tagged virus (<>7 $\log_{10}$ GE/*Varroa*). *Varroa* which shared adult bee hosts (Experiment 4) were also shown to acquire the tagged virus (66.7% n = 6). All *Varroa* that originally were exposed to the tagged virus through pupal feedings had detectable levels of the

NLuc reporter sequence, with a modest (but non-significant, AOV, $F_{1,10} = 14.373$, $p = 0.10$) reduction in virus load for the naïve *Varroa* in mixed pairs. All *Varroa* in this trial had detectable levels of natural DWV-A (N = 18).

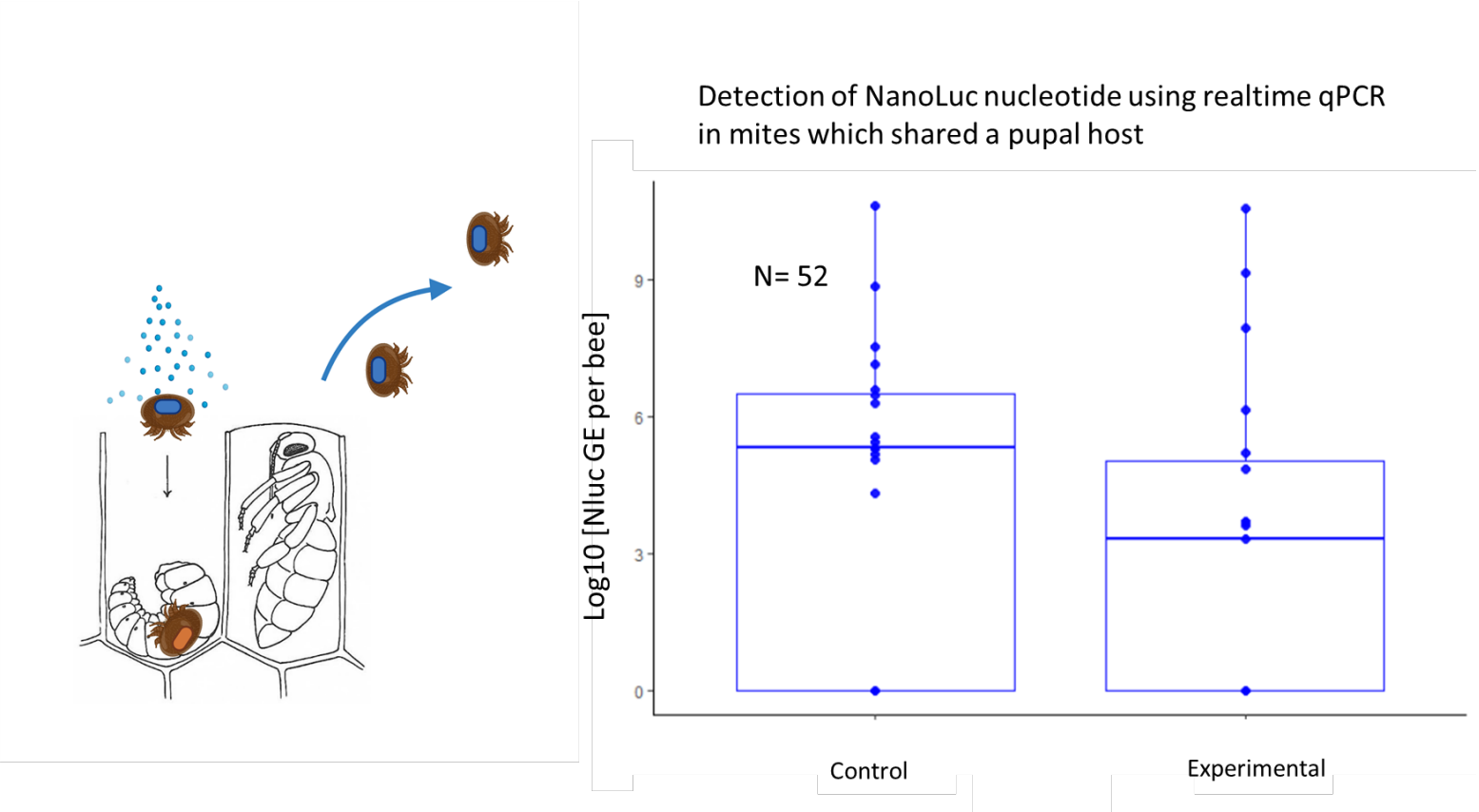

Figure 2.4 (**A**) A schematic describing the conceptual framework of the study. Naïve *Varroa* may acquire infectious DWV-A when sharing the same pupal host with an infectious *Varroa*. *Varroa* which were originally infectious did not have significantly higher levels of DWV-NLuc compared to the originally naïve *Varroa* which shared a host. Fewer naïve *Varroa* transmitted the tagged virus than originally infectious *Varroa* which maintained the virus. (10/18 (55.56%) naïve successfully trasnmitted versus 14/21 (66.67%) combined from both trials. (Pupal image credit: D G Mackean www.biology-resources.com)

Discussion

Deformed wing virus, *Varroa Varroa* and the honey bee offer a complex pathogen-vector-host relationship where the pathogen moves among hosts via multiple routes[2,4]. Here we provide experimental evidence confirming several of these routes in honey bee colonies and showing their relative importance for the maintenance of DWV in both *Varroa* and bee hosts. These insights lead to a predictive and testable theoretical framework for assessing how interactive effects determine communicable and vectored transmission dynamics.

Most importantly, while multiple *Varroa* are rarely found on the same bee at the same time, they exhibit serial parasitism, shifting from host to host. Shared hosts offer rich opportunities for bidirectional material exchange between host and vector. Indeed, we show that naïve *Varroa* acquire DWV when they feed on either adult or pupal bees following an infectious *Varroa*. This indirect *Varroa*-to-*Varroa* transmission confirms that *Varroa* are "hypodermic needles", adeptly moving pathogens from host to host thanks to their feeding mode and promiscuity. Vector-host exchanges of this sort are readily observed across arthropod vectors, most notably in ticks where co-feeding allows for non-systemic transmission between vectors[57,58]. To add another layer that is unique to social groups, *Varroa* also acquired DWV from non-parasitized bees that had themselves acquired this virus from nestmates via social transmission. This host to host transmission occurs through trophallaxis between adult bees, and our results strengthen and quantify previous observations that infectious hosts transmit DWV communicably to susceptible nestmates[45]. Our system of using tagged viruses shows that the original source of virus in workers was an infectious *Varroa*

which fed upon an adult bee (Figure 2.1). This virus was then traced from parasitized bees to nestmates and ultimately to a new *Varroa* vector, fulfilling the parameters of Koch's postulate[64]. While I show tagged viruses at each stage, I had anticipated greater levels of DWV amplification in bees which acquired the novel virus through trophallaxis (Appendix A). The experimental design could have impeded this result. The mesh screens in our design may have reduced trophallactic interactions. Alternatively, highly infectious bees might have died prior to phase two of the trial, impeding their ability to act as a vector of disease to susceptible nestmates. Such premature death of reservoir hosts is key for diminishing pathogen maintenance in a population and should be assessed in field trials[65].

In experiment two I asked if cannibalization, a social behavior common in honeybees as a route of removing infected nestmates or in times of nutritional stress, could provide a communicable route for transmission of DWV. I have previously shown that cannibalization of infectious pupae, coupled with oral transmission among nestmates through trophallaxis, could lead to viral transmission[45]. Here, I show that bees which cannibalize infectious pupae act as infectious hosts to naïve *Varroa* and that these *Varroa* subsequently vectored the novel DWV. These findings suggest that pupal cannibalization, which has been bred into honeybee colonies as a form of behavioral hygiene[45], presents a possible risk factor. Such behavioral traits provide benefits by reducing *Varroa* reproduction and survival[66], while likely increasing transmission of DWV to other nestmates. It is critical to assess, at the colony scale, the impacts of these opposing forces on colony health.

Although it was not within the scope of this study, there was an overall trend across experiments for the percentage of infectious vectors or hosts to be fewer after transmission cycles in the groups that did not originate with an index case. For example, at the end of the study there were more *Varroa* with detectable levels of DWV-A in experiment 1 within the *Varroa* + virus group than either of the control groups (Appendix A). The *Varroa* + virus group began the experiment with an infectious vector, whereas the other groups did not. It is likely transmission efficiencies are not 100% effective. This would mean after subsequent *Varroa*-bee feedings or bee-bee trophallaxis cycles we would would expect fewer infectious cases. Because I did not originally seek to answer this question my experimental design did not include a large enough number of observations to answer this question (Appendix A for count data on trials 1-4). The mechanisms behind this observation was outside the scope of this study, but warrants continued research.

DWV was present, but not prevalent, in honeybee populations before *Varroa destructor* jumped from *A. cerana* to *A. mellifera*[43,67,68]. Host-to-host transmission must have occurred to maintain DWV in honeybee populations prior to vectored transmission by *Varroa*. Host-to-host transmission has been attributed to vertical transmission of DWV from queen to progeny[47]. My results indicate that worker-to-worker routes of transmission rival those from queen to offspring. Since workers are targeted by *Varroa* more often than queens[69], I would argue that worker-worker transmission is the predominant means of maintaining DWV in colonies and puts these colonies highly vulnerable when the vector arrives. Continued dispersal by these *Varroa* supports this, i.e., when *Varroa* arrive in an area, DWV levels in worker

honey bees tend to increase exponentially and covary with *Varroa* infestation levels. For example, when *Varroa* were introduced to the Hawaiian islands, viral loads in individual worker bees increased by a million fold, establishing *Varroa*'s culpability in the emergence of DWV as a global pathogen[43]. The interplay of vector-host and host-host transmission for disease is rare in nature. In fact, the majority of infectious diseases are communicable, or vector borne, but very rarely both. Curiously, a select handful of emerging, RNA viruses, Zika, West Nile, Tembusu, salmon isavirus and DWV, are the exceptions to this rule[2,8-10,70]. As our results show, understanding this interplay can offer fundamental insights into routes of transmission and disease, and can point to management routes that might break transmission, improving the health of a key pollinator.

Chapter 3: Varroa aggregate on adult drones

Abstract

As an almost universal rule, parasites form aggregated distributions where a minority of hosts are responsible for harboring a majority of the parasite burden in any given population. For 30 years *Varroa* destructor has been described as preferring nurse bees of its host *Apis mellifera*, while largely leaving *Varroa*'s distribution undescribed. As a result disease transmission dynamics, sampling methodology and research focus has largely been a worker centric. For the first time we describe the intracolony dispersal patterns of *Varroa* on its western honey bee host. *Varroa* overwhelmingly distribute by sex and age cohorts, preferring young adult drones over any other cohort. We show *Varroa* form highly aggregated distributions on the drone

cohort early in the season when infestation levels are low, and distribute broadly onto the worker cohort later in the season when infestation levels are high. In 1978 Anderson and May first proposed the theoretical framework that parasites would have little destabilizing effect on the host population until the degree of aggregation was low, or approached a Poisson distribution. The distribution patterns of *Varroa* are in alignment with their theoretical work, and maybe the underlying mechanism responsible for historical losses of honey bee colonies observed over the last decade.

Introduction

Heterogeneities in host populations can give rise to the aggregation of parasites[71]. As a general rule, parasites form aggregated distributions[72]. Consequently, very few hosts are responsible for harboring the majority of macroparasites within the host population[71,73,74]. Heterogeneities in individual characteristics such as sex and age influence mean parasite burden. This may arise because of spatial temporal proximities (i.e. older hosts have more contact and exposure events with parasites) or from host susceptibility: specifically genetic differences in host susceptibility to infection[71]. Sex can be a driving difference amongst hosts in the attraction of parasites. Parasites show a bias in mammals towards males, with mean parasite burden and parasite induced mortality showing heavy sexual size dimorphism for larger males[75]. The differences across individuals in a host population will influence the degree of aggregation. When there are large differences between individuals, parasites will form highly aggregated distributions, while the degree of aggregation is reduced as a population becomes more homogeneous.

The degree to which macroparasites aggregate is central to the regulatory effect parasitism will have on the host population. In Anderson's theoretical model, regulation of the host population is limited when parasites form highly overdispersed distributions (very few hosts harbor all the parasites)[74]. Instead, when highly aggregated, parasites impact the fitness and mortality of just a select few individuals; these few individual hosts have an exaggerated influence on the fitness and survival of their parasites. Infestations that cause morbidity or shorten the lifespan of individual hosts will in turn reduce the reproductive fitness of the parasite. As a result, highly aggregated distributions impact hosts and macroparasites at the individual level while yielding few regulatory and destabilizing effects on the population level. Regulation of the host population, and not just individuals, occurs as the degree of aggregation becomes less severe so that more of the host population bears the infestation. As a result dispersal patterns influence stabilization of the host population[74].

Parasite dispersal patterns typically fit the negative binomial distribution[71], which is described by two parameters: the mean, or average parasite burden, and k, an aggregation parameter, which has an inverse relationship with the degree at which parasites are overdispersed[76]. When k is low, parasites are said to show a high degree of aggregation (few hosts harbor parasites while the majority are without infection). When k is large the mean approaches a Poisson distribution, at which point the probability of each host being parasitized becomes equal[76]. Anderson modeled the degree of aggregation as the degree of departure from a Poisson distribution[77] and both Anderson and May concluded that when distribution became less overdispersed

and approached randomness parasites would have regulatory and destabilizing effects on the host population[74,78]. Stability in the host population arises when parasite burden is limited by concentrating the effect on few hosts, and destabilizing effects arise when the parasite burden is less concentrated.

The destabilizing effects of parasitism largely arise from two factors: when parasite burden induces a reduction in host reproduction, and when the degree of aggregation becomes less severe so that parasite-burden falls across a larger proportion of the host population[79,80]. This phenomenon is observed across parasite-host relationships. For example, seasonal destabilization of several species of North American Drosophila is caused by a nematode which induces sterility and broadly parasitizes the host population[81]. This pattern is observable across numerous host-parasite relationships such as in an ant-hoverfly population, on North American moose and ticks[82], and numerously in mouse and helminths[83,84]. Cyclical population fluctuations in bird species, most notably quail and grouse populations, correlate with high density burden of helminth and nematode infections, and a reduction in reproductive fertility[85,86]. Here, I considered this common phenomena of parasites, aggregation, and applied it to the honeybee-*Varroa* relationship.

Varroa destructor (*Varroa*) is a parasite and an arthropod vector of honeybee viruses[1]. In the last 50 years *Varroa* has established near cosmopolitan distribution across the globe after it jumped hosts from the eastern honey bee, *Apis cerana*, to the western honeybee, *Apis mellifera*,[1,44,87]. The destabilizing effects of this macroparasite on honeybee populations is well documented, and *Varroa* is now considered the largest driver of colony loss[1]. The mechanisms by which this occurs

are partly described, interactive and not conclusive. The parasite's effects on individual hosts and the host population are more well described. *Varroa* shorten the lives of the bees they parasitize during development and adulthood[88,89]. Bees which are parasitized exhibit a reduction in their brood rearing capabilities, possibly extending harm to subsequent generations of bees[90]. Parasitized bees also begin foraging earlier than their non-parasitized counterparts, and are observed to perform more poorly at resource acquisition[91,92]. These effects alone can be quite damaging to honey bee colonies, though perhaps the most devastating effects of *Varroa* relate to their ability to vector disease in honey bee hosts. *Varroa* are associated with higher viral loads in honeybee colonies and are important in the amplification of deformed wing virus (DWV)levels[43]. This is likely because transmission of DWV and its variants amongst bees may be dependent upon the density of the *Varroa* parasite burden that befalls the worker cohort.

Varroa are described as preferring younger (nurse) bees over all other worker bee cohorts[14,15,17,18,93]. However, all studies which arrived at this conclusion excluded drone bees in choice tests. It is well established that *Varroa* prefer drone brood (developing bees enclosed in honeycomb cells) over worker brood when present in the colony[94], but no published work describes *Varroa* preferences between the sexes of adult bees. Such a gap in knowledge obscures our understanding of this important pest. Drones are seasonally present in a honeybee colony, and disappear as nectar availability diminishes[95]. Even if adult drones were to bear a small amount of parasite burden while they were seasonally present in a colony this could impact DWV transmission dynamics, direct impacts on the worker cohort, and sampling

methodologies which have been designed to collect nurse bees and largely exclude adult drones[96].

Here, I carried out a series of experiments to study host preference and *Varroa* distribution on age and sex cohorts. I first tested host preference in laboratory arenas, allowing *Varroa* to select either male or female bees within a small, controlled space. I then experimentally observed drones and workers from emergence through the first nine days of their adult life in natural colonies. In this way I could identify the mean parasite burden and the distribution of *Varroa* by sex and age cohorts. Because I tracked individual bees each day for the presence of *Varroa*, I could make inferences on intracolony dispersal: movement of *Varroa* amongst adult bees within a colony. The degree of aggregation of a parasite on its host is fundamentally important for regulation of the host population. I repeated these trials on the same colonies after seasonal drone production ceased to see if the degree of aggregation on the worker class became less severe. Finally, I tracked *Varroa* burden across sex and age cohorts in 8 different apiaries for four months to confirm that *Varroa* burden observed in our experimental studies was strong enough to be observed through random sampling of field colonies.

Material and Methods

Experiment 1: Laboratory Assays for host preference of *Varroa* destructor

In May and June of 2020, *Varroa* were hand collected from adult bees from a single colony. The colony was moderately infested, but was not exhibiting signs of varroosis[97]. *Varroa* were delicately transferred from their adult bee host to an

enclosure of a 00 gel capsule with a pink-eyed worker pupa and incubated for 48 hours at 34c and 40% humidity to simulate a sealed wax cell.

Worker and drone bees were marked at emergence from a colony unrelated to the *Varroa* source colony. This colony was healthy, exhibiting no signs of disease or *Varroa* infestation(confirmed with negative results from alcohol washes and manual inspection of drone brood). Workers and drones were continually marked at emergence over 4 days, and returned to the donor colony. The donor colony was a 5-frame nucleus colony made from the parent colony and requeened. The use of a nucleus colony allowed for easy re-collection of marked bees. Bees were checked for *Varroa* and then marked on the center of their thorax with a fine tip oil-based *Sharpie* marker. A different color was used to mark bees on each day to establish age groups.

At the start of the trial (Day 1) *Varroa* were incubated on pupae for a total of 48 hours and bees were marked as follows to establish different age cohorts: unmarked newly emerged, green 1 day old, white 2 days old, yellow 3 days old, and blue 4 days old.

Bees were hand collected from the donor colony, re-checked for *Varroa* and then distributed equally by *Age x Sex* cohorts to experimental cages (6 drones and 6 workers per arena). Experimental cages consisted of a modified petri dish[98] with the side wall of the arena covered with a partial sheet of wax foundation, and honey feeder. The arenas were individually labelled with a laboratory identification number and incubated at 34°C and 40% humidity until the start of the experiment. A seal around the edges of the arena was made with laboratory tape. This allowed for the visual recollection of *Varroa* that attempted to quest out of the arena.

At the start of the experiment 10 *Varroa* were introduced into the individual arenas (*total of 100 Varroa across 10 arenas). Varroa* were introduced by manually picking up *Varroa* with a Chinese grafting tool and inserting them into the arena. *Varroa* were inserted into a neutral part of the arena, never directly onto or near a host. The plastic rim of the arena and the wax foundational wall were both considered neutral space. If a potential host was near that space then the researcher lightly blew into the feeder hole which elicited the host to change direction. A total of 100 *Varroa* were introduced across 10 arenas over 45 minutes, during which time arenas were actively returned to the incubator after completion. During the inspection at 48 hours drones and workers were manually removed, counted for *Varroa*, and then that count was confirmed by dipping the individual 70% isopropyl alcohol.

Experiment 2: Distribution of *Varroa* destructor on sex and age cohorts in colonies

The distribution of Varroa on age x sex cohorts in honeybee colonies was studied. Candidate colonies were identified as healthy colonies without the overt presence of brood diseases, varroosis or queen issues, while still having an observable *Varroa* infestation. The size of a candidate colony was restricted with upper and lower bounds. For upper bounds, colonies over 60 frames (6 boxes) were excluded as these were deemed too large to appropriately carry out the study. For the lower bounds, colonies under 30 frames were excluded as these were considered unrepresentative of a typical colony in the experimental location at that time of the

year. Additionally, colonies smaller than 30 medium frames would not reflect the size of typical colonies used for commercial pollination.

In total, 5 colonies were used in 10 trials for this study. The 5 colonies used in the study were sampled once when drones were being produced and once when drones were no longer being produced within the colony. As a result, each colony was sampled twice, resulting in a total of 10 trials. In year 1 of the study, two colonies, were sampled over four trials, while three colonies, were sampled in year 2 for the remaining six trials. Because marked bees regularly drift from one colony to another, colonies were temporally or spatially isolated from one another to eliminate identification errors of marked bees. In year one, colonies were in separate yards and trials were run non-concurrently. In year two, colonies were in the same yards, spaced about 30 feet from each other. Trials were run non-concurrently to minimize erroneous assignment of marked bees.

Trial duration, and setup

Trials were considered to start on the first day when emerging bees were marked, and then ended on the final day of collection. Trials were restricted to a total of 9 days, which falls within (but does not entirely encompass) the typical range of nurse bee development[99]. Trial length was determined by this development window for nurses as well as feasibility (determined from 2 preliminary trials). Main considerations for feasibility were weather, workload and onset of foraging, and defensiveness of older bees.

The setup for these trials required additional hive bodies, lids, covers and bottom boards, a nucleus colony that acted as a temporary recipient colony, queen excluder, and an observation stand. An observation stand consisted of a frame holder at chest level that allowed the researcher to pick-up and observe bees with ease. The queen excluder was used to isolate emerged drones from older drones (explained in the subsequent section: *Marking bees*). An EZ-BZ plastic nucleus box was used as the temporary nucleus colony. An entrance was cut into the lid into which bees could be placed after inspection. A wooden block was used as a temporary "door" that prevented bees from escaping in between observations. The frames and bees within the nucleus box were sourced from the experimental colony, always included 3 frames, 2 of which were brood, and unmarked bees. Temporary housing was similar to that of the parent colony to reduce influence of *Varroa* dispersal, while functionally allowing for the inspection of bees each day without the chance of double sampling. The additional hive components housed frames after inspection. A schematic of the experimental design and workflow can be found in figure 3.1

Marking bees

Bees were marked each day at emergence with unique paint marks reflecting age and parasitism history. This was done by combining a fixed color pallet with geographical locations on the bees' body. By marking bees by *color x pattern* the researcher could quickly identify a bee unique from the rest of the bees within the colony. Bees were continually marked at emergence over the course of 5 days, and

subsequently followed for the remainder of the trial. Marking of emerging bees stopped after day 5, after which, bees were monitored.

One color was used to identify the bee by age, and that mark was placed geographically on the same spot of all bees: center thorax of workers or a longitudinal stripe along the center abdomen of the drones. 5 different colors were used to identify age in a non-repeating pattern. Blue was used for day one, yellow for 2, white for 3, green for 4 and finally pink for day 5. All bees collected at emergence without a *Varroa* on them were marked in this manner. Bees not parasitized during development were followed as a group as one worker with a blue thorax could not be differentiated from another worker with a blue thorax.

Markings for parasitism were given in such a way that would retain the information about the bees age while also uniquely setting it apart from every other bee in the colony. Pre-trials showed that diligent marking patterns had to be adopted for the repeatable identification of uniquely marked bees. As a result, a strict factorial marking pattern of *color x location* was followed. For example, worker bees observed with a *Varroa* at emergence were marked in the center of their thorax to denote age, and then marked in another location, potentially with another color, to denote parasitism. A factorial usage of colors prevented an age marking to be misinterpreted as a marking for parasitism. Bees which gained a *Varroa* during adulthood were re-marked to uniquely identify them from any other bee within the colony. As a result, bees which gained a *Varroa* were removed from the *never parasitized group* and subsequently would be followed as individuals and not as a group for the rest of the trial. There was no need to re-mark bees in the parasitized groups after the initial

marking. Subsequent gain or loss of *Varroa* was recorded in the field log. Errors, such as duplicate marked bees, were noted. In the case of such an error, an additional marking was given to the bee, ending its history as a duplicate bee. After marking or checking for *Varroa*, bees were introduced into the temporary nucleus colony until all bees were marked for the day, after which bees were returned to the parent colony.

Bees were handled briskly during markings and inspections with enough time to determine presence of *Varroa* and their feeding or non-feeding location, but briefly enough to prevent the researcher from disturbing the *Varroa*. In doing so I attempted to inspect for parasitism in a way without disturbing the *Varroa* if present. Bees were picked up by their wings, and turned over to expose their abdomens. Care was taken so that worker bees could not curl their abdomens and sting the researcher. If a worker stung the researcher or if drones inverted during an inspection this was recorded. *Varroa*, which were regularly seen moving along the adult body of adult drones in the laboratory trials, were also seen moving along handled drones in the field trials.

Figure 3.1 Schematic describing workflow: The following schematic describes the workflow during sampling events.

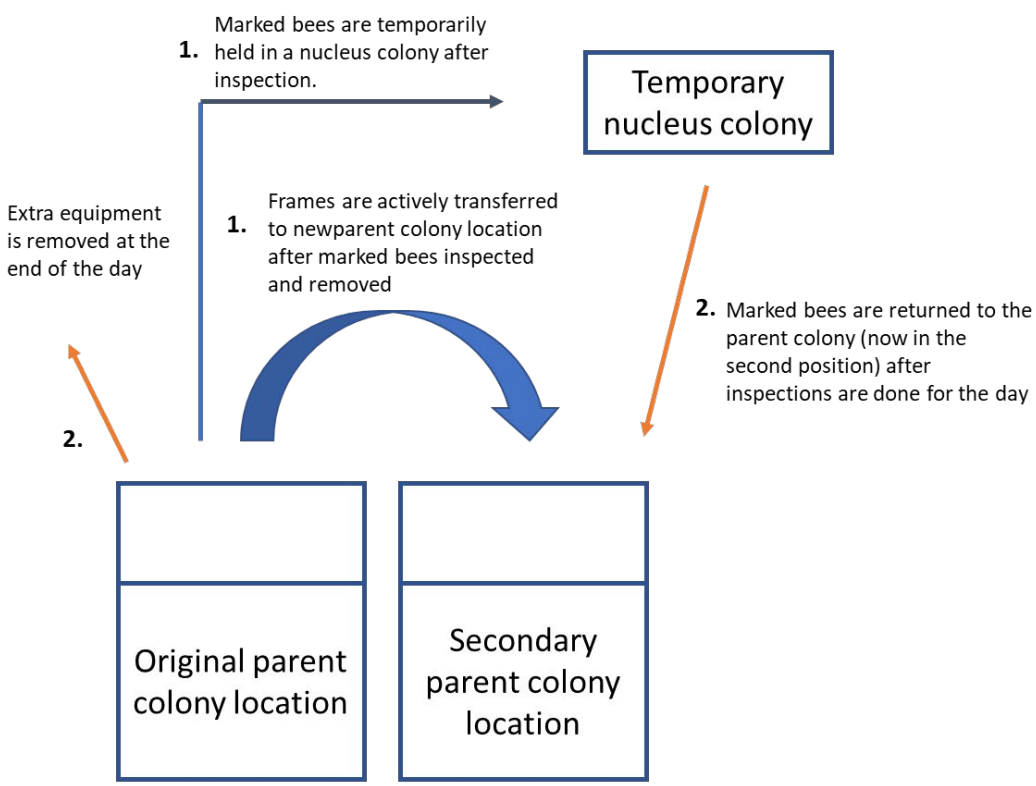

Figure 3.1 Schematic of workflow: **1.** During sampling marked bees are housed in a temporary nucleus colony so that resampling of the same bee is not possible. Frames which are fully inspected are transferred to a new parent hive setup. When a sufficient number of frames are inspected (subjective) the original parent colony maybe turned backwards to promote orientation to the secondary location (not shown). **2.** At the end of the day the temporary nucleus colony is united with the parent colony. Unoccupied hive components are removed from the yard to reduce orientation confusion. (*Right)* Parent colony ready to be transferred to secondary position during a trial. Temporary nucleus colony not depicted

Recording *Varroa* locations and bee activity

A field log was used to record observations over the course of the 9-day trial. *Non-parasitized bees* were observed and recorded as a group if they remained non-parasitized. *Parasitized bees* were recorded individually whether they retained, lost or gained a *Varroa*.

Varroa locations were recorded as either feeding or non-feeding. I determined feeding locations as a *Varroa* between the sternites of a bee's abdomen, and non-feeding as anywhere else on the bee. Non-feeding locations were not uniquely recorded except in year two when *Varroa* were found between the head and thorax of drones.

Bee activity was not followed directly during this study. However, overtly observable events were recorded. For example, I did not study onset of foraging during this trial or emigration of bees between colonies. However, observations were noted such as when a marked bee was observed returning to the colony with pollen or if marked bees were found in neighboring colonies.

Distinctions between bees parasitized during development and adulthood

Detection of a *Varroa* on an emerging worker or drone was considered indicative of being parasitized during larval development, while detection of a marked adult bee >24 hours post emergence was indicative of being parasitized during adulthood. I tested if this underlying assumption was true by inspecting bees immediately after emergence for the presence of a *Varroa*, and then inspecting their larval cell immediately for evidence of *Varroa*. Detections were in agreement between emerging bees and evidence of *Varroa* in their brood cell, except for one cell

which had a *Varroa* dead in the cell and no *Varroa* on the emerging bee. (N = 100, 99/100 in agreement). Detections of *Varroa* on newly emerged bees was considered a good proxy for parasitism during development, albeit deriving an infestation rate using this method does not account for uncapped or completely cannibalized *Varroa* infested brood. As a result, this proxy accounts for the infestation rate of asymptomatic brood and emerging workers, and not the infestation of the brood population as a whole.

Experiment 3: Targeted Sampling of *Varroa* in commercial apiaries

Colonies in two commercial operations across 8 different apiaries were sampled for Varroa monthly from May through August in Vermont, USA. A new sampling method was performed alongside an established and currently accepted sampling method. The purpose was to establish if future Varroa infestations could be predicted targeting small groups of adult drones.

Beekeeper and apiary selection

Colonies in this study were sampled from commercial apiaries in two different operations (Beekeepers A and B). Beekeepers were selected for this study based upon a previous relationship between the beekeepers and the researcher. Apiary selection was determined through availability and agreement between the beekeeper and researcher.

Beekeepers were approached by the researcher for several reasons. Both beekeepers maintained colonies with similar management strategies. Both were non-migratory operations located in geographically similar areas. Both beekeepers

produced their own queens for use within their apiaries instead of relying upon purchased stock. Neither beekeeper purchased bees in the form of packages or nucleus colonies. This reduced the confounder of importing colonies with artificially high *Varroa* loads. Neither beekeeper prophylactically treated their colonies with antibiotics for brood diseases, nor was either beekeeper known for having brood diseases prevalent in their operations. Each beekeeper kept brood configurations similarly and allowed unrestricted egg laying of queens. Neither beekeeper was expected to remove any portion of the colonies during this study in the form of splitting colonies. Finally, each beekeeper was in the practice of keeping approximately the same number of colonies per yard. As a result, *Varroa* infestation could be analyzed across apiaries and operations with li*Varroa*d confounders.

Beekeeper A treated their colonies with an acaracide a month prior to the start of the trial. Beekeeper B did not treat their colonies prior to the trial, but did request permission to treat colonies after they reached the economic threshold of 3 *Varroa* per 100 bees. This agreement was established, and the researcher informed the beekeeper when colonies reached threshold. Beekeeper B spot treated (treated colonies at threshold on a case-by-case basis) with formic acid (Formic Pro) and informed the researcher the exact date of application. The researcher continued to sample these colonies throughout the trial while noting the treatment confounder.

Colony selection criteria

In total, 48 colonies across 8 apiaries were included in this study. Both beekeepers maintained colonies in similar configurations. Beekeeper A overwintered

colonies in a 2 deep and one medium hive configuration (Langstroth universal dimensions). This configuration served as the brood area from spring through fall. Honey supers were placed directly atop the brood area prior to the start of the trial, and additional honey supers were added by the beekeeper as space was needed during the trial. Beekeeper B overwintered colonies in a 2 deep hive configuration. This area also served as the brood area from spring through fall. Honey supers were added prior to the trial, and the beekeeper likewise continued to add supers on an as needed basis.

There were no upper bounds for colony size during this trial. Lower bounds were set by the researcher at a minimum of 2 deeps in brood area at least 1 super to be candidates in the trial. Colonies having already swarmed, with mature queen cells or in a queenless state were excluded as candidates during the selection process. No colonies were observed with overt brood disease during the colony selection process.

After exclusion 5-8 colonies per yard were selected for the study. Selection was made from the remaining colonies by coin toss.

Sampling

Two sampling methods were performed once a month, over the course of 4 months on each colony in the trial. An alcohol wash was performed using standard methods of collecting ½ cup of bees from the center of the brood nest and placing them into alcohol. For consistency across the trial, bees were sampled from a "typical" frame from the brood nest. This frame had worker brood of multiple stages (open and capped). Frames which had emerging brood were avoided. Bees were collected by quickly shaking the adult bees from the frame into a steel pan (large hotel pan), knocking the bees quickly to one corner of the pan, and then scooping ½ cup directly

into alcohol. To facilitate collection and consistency across colonies, apiaries and operations, two researchers carried out this task jointly.

For an independent measure of *Varroa* levels, a brood frame adjacent to the one removed for the alcohol wash was removed from the brood nest. This frame was set horizontally across a spare telescopic cover that served as a table in the shade. Researchers proceeded to hand collect bees individually from the surface of the frame, inspect the bees for *Varroa* and then, after recording the observation, toss the bee into alcohol. *Varroa* were recorded as the number of *Varroa* on an individual host.

Worker bees were collected systematically across both surfaces of the frame. To reduce spatial site bias, researchers systematically collected bees in zones across the whole frame. To reduce unconscious bias from the researcher, the researcher was instructed to look away from the frame and then quickly look back at the frame and point at a bee. Whichever bee came into view was selected. Deviations from this methodology were allowed as follows: Newly emerged worker bees were collected (when available) and recorded as a separate worker cohort. This was deemed acceptable because statistical analysis could exclude this group easily, and an age cohort within worker bees could prove valuable.

Drones were also targeted for collection. Drones were hand collected from the frame. Drones were divided into three groups: emerged, non-buzzy and buzzy. Newly emerged drones cannot always be identified by visual cues alone. However, newly emerged drones have soft abdomens, an off white and weakly opaque endophallus when forced to invert, and do not buzz. Such a drone was recorded as *newly emerged*.

Drone abdomen muscles strengthen after emergence. As a result their abdomens feel slightly firmer than a newly emerged drone. If these drones did not buzz then they were assumed < 24 hours, but less than 2 days. These were recorded as *non-buzzy*. Drones begin to buzz when disturbed from 2 days onward. A slight vibration could be felt in drones picked up by their thorax. These were labelled as *buzzy*.

A maximum of 100 workers, 30 newly emerged workers, and a total of 75 drones were sampled from each colony. This sample was procured from the two adjacent frames in the brood nest next to the frame used for the alcohol wash. There was no lower end for newly emerged workers or drones for this sampling technique. To account for variability in drone presence across colonies, a grading scale was used to describe colonies. Grading used a subjective scale to describe the availability of adult drones and drone brood within the colony. A similar grading scale was used to describe the strength of the colony. Key features such as brood cannibalization, queen status, queen cell formation, and presence of brood disease were recorded.

Statistical Analysis

Statistical analysis was done in RStudio using baseR and various imported packages.

Differences in *Varroa* occurrence on drones or workers was tested using an ANOVA. In experiment 1 residuals of the data did not meet the assumptions of normality. A non-parametric Kruskal-Wallis ANOVA was used to compare differences between the groups. Each trial was analyzed separately to see if there was

an effect between trials and because bees in Trial 3 were collected slightly differently than bees in Trials 1 and 2.

For experiment 2, absolute parasite burden was calculated by tabulating the number of drones and workers observed at least once with a *Varroa* and dividing it by the total number of bees in the sample. Relative parasite burden was calculated as the number of *Varroa* collected on a small volume of bees (1/2 cup) via an alcohol wash[96].. Bees were sampled daily for the presence of a *Varroa*. Each individual bee was assigned to a sex x age cohort, and for each day of the experiment the detections on each sex x age cohort were summed. The number of *Varroa* detected on each sex x age cohort was fit to a negative binomial and analyzed with a GLMM negative binomial with a logit link function (using the MASS package). In order to see if age as a factor was an important contributor to the distribution of *Varroa* age was used as a categorical variable in all of our analysis. This determination was made because the experimental design and description of the results used age categorically and not as a continuous variable. The degree of aggregation was determined by implementation of Gini coefficients[77]. Gini Coefficients were calculated using the DescTools package and bootstrapping to 5000 for each sample. In order to test for overdispersion in the distribution of *Varroa* in our data set the data was fit to a GLMM set to a Poisson distribution. A Pearson's Chi-Squared statistic test was used to estimate dispersion, followed by subsequent analysis using the AER and DHARMa packages to estimate overdispersion via a nonparametric dispersion test by simulated-refitting of the fitted mean deviance residuals. The MASS package was employed for multiple analyses including Fisher T test, Wilcoxon-Ranked Test, and Chi-Square tests. The use of non-

parametric tests was implemented throughout analysis after visualization of the data or residuals including the Kruskal-Wallis ANOVA as a non-parametric alternative to compare groups.

Experiment 3: Targeted sampling of *Varroa* on adult drones and workers

In order to estimate the distribution of *Varroa* on adult drones and workers over the course of a season, adult bees were individually sampled for *Varroa* once a month for four continuous months. To determine if detections by sex were different by month this data was fit to a GLMM using a quasi-poisson distribution[100]. In order to tell if detections via an alcohol wash were different by month we fit detections from alcohol washes to a GLMM using a quasi-poisson distribution. From these observations prevalence was calculated as the number of adult drones or worker bees with 1 or more *Varroa* observed on them over the total number of sampled bees. The number and proportion of co-infested adult bees was also calculated, as was the proportion of infested adult drones compared to adult workers. To test for differences in proportion of workers and drones infested by *Varroa* a Fishers Exact Test was performed on the prevalence data for each month. .

In order to quantify the degree of aggregation of *Varroa* on adult bee hosts Gini coefficients were used. I used the DescTools package to construct Gini coefficients and 95% confidence intervals. Gini coefficients were constructed using detection data on the colony level (irregardless of sex of the host sampled) to compare the distribution of *Varroa* between operations.

Results

Experiment 1: Host selection by *Varroa* destructor in laboratory arenas

I compared host selection by *Varroa* in three separate trials. In trials 1 and 2 *Varroa* were exposed to drones and workers. Drones were a mix of 2-3 days old, and workers were a mix of 3-4 days old in these trials. *Varroa* aggregated on the drone bees over worker bees in trials 1 and 2. (Table 3.1). A third, non-concurrent trial was run in which same aged workers and drones were exposed to *Varroa* in laboratory arenas. There was no significant difference in the distribution of *Varroa* across same age sexes in this trial (Table 3.1).

Table 3.1 Experiment 1: Laboratory Choice Assays

Trial	Comparison	Total *Varroa* on Drones (percent of all hosts infested)	*Varroa* on Workers (percent of all hosts infested)	Chi-Squared Test statistic (Df)		pValue
Trial 1	Drones and workers of mixed ages (Kruskal-Wallis)	48(32%)	23(21.6%)	6.3309(1)		0.011
Trial 2	Drones and workers of mixed ages (Kruskal-Wallis)	62(36.4%)	(24.3%)	9.1298(1)		0.003
Trial 3	Drones and workers of same ages (Kruskal-Wallis)	33(22.5%)	40(23.4%)	0.03851(1)		0.84

Table 3.1 Count data from laboratory trials testing host selection of *Varroa* destructor. Kruskal-Wallis non-parametric analysis provided along with test statistics and degree of freedom.

Distribution of Varroa destructor on sex and age cohorts in colonies

Varroa formed an aggregated distribution. *Varroa* dispersed by sex and age cohorts (negative binomial, $k = 1.33$, $p < 0.0001$, Figure 3.2). *Varroa* were observed on drones in greater numbers and also by proportion of the host population (Figure 3.3). More *Varroa* were observed on 1-3 day old bees than any other age group by both a proportion of the host population or in total numbers (Table xx).

The degree of aggregation was determined by calculating Gini coefficients for each sex and age group within each trial (Appendix B). The degree of aggregation in workers was high in early season trials, and decreased in later season trials. Mean burden on workers approached or became random in every colony in the later part of the season (Table 3.2). This observation paralleled the relative and absolute number of detections of *Varroa* on workers: low early in the season, high later in the season.

Varroa prevalence was highest on drones across all early season trials (25.7% - 96.2%) compared to same aged worker bees sampled within the same colonies (5.2% - 42.8%). Incidence of *Varroa* on workers increased significantly in 4 of 5 colonies in the study between early season and late season trials (Fisher Exact T-Test, $p < 0.0001$, except for colony B, $p = 0.075$). The proportion of workers parasitized during larval development was significantly lower than the proportion of bees observed parasitized during adulthood (Table 3.3). This trend was strongly observed earlier in the season, and significant in 4 out of 5 of colonies later in the season. The ratio of workers parasitized during development versus workers parasitized during adulthood was most severe in early season trials when drones were available, and less severe later in the season when drones were absent.

3.2 Prevalence Table

	Early Season			Late Season		
Colony	Male	Female	Alcohol Wash	Male	Female	Alcohol Wash
House	65.43%	14.29%	5.1%	96.61%	59.95%	14.6%
Tower	25.70%	5.25%	2%	ND	20.52%	4.3%
A	78.20%	23.72%	1.1%	ND	38.94%	9.9%
B	96.30%	42.80%	2.5%	ND	50.49%	14.5%
C	77.51%	14.86%	1.3%	ND	45.10%	12%

Table 3.2 – Absolute parasite of drones and workers, and relative parasite burden (alcohol wash) for early and late season trials. Parasite burden on workers in late season trials was significantly higher than burden on workers in early season trials in 4 out of 5 colonies. (Fisher Exact T-Test, $p < 0.0001$, except for colony B, $p = 0.075$) ND = no detection.

Table 3.3 Is there a significant difference in the proportion of bees parasitized between early and late season inspections?

Is there a significant difference in the proportion of worker bees parasitized during **development** between early and late season?

Colony	Percentage of worker bees parasitized during development early season	Percentage of worker bees parasitized during development late season	Difference in incidence between trials Fisher Exact Test pvalue
House	3.52%	18.09%	<0.0001
Tower	0.71%	10.07%	<0.0001
A	5.87%	15.04%	<0.0001
B	11.28%	19.22%	= 0.0075
C	2.2%	14.01%	<0.0001

Is there a significant difference in the proportion of worker bees parasitized during **adulthood** between early and late season?

Colony	Percentage of worker bees parasitized during adulthood early season	Percentage of worker bees parasitized during adulthood late season	Difference in incidence between trials Fisher Exact Test pvalue
House	10.76%	23.93%	<0.0001
Tower	4.45%	12.97%	<0.0001
A	17.85%	23.89%	= 0.014
B	31.52%	31.27%	= 0.507
C	12.53%	31.09%	<0.0001

Is there a significant difference in the proportion of **worker bees parasitized** between early and late season?

Colony	Percentage of parasitized in the worker population early season	Percentage of parasitized in the worker population late season	Difference in incidence between trials Fisher Exact Test p value
House	10.76%	23.93%	<0.0001
Tower	5.16%	13.14%	<0.0001
A	23.72%	39.38%	<0.0001
B	42.8%	50.49%	= 0.075
C	14.73%	45.48%	<0.0001
All colonies	14.4%	41.43%	<0.0001

Table 3.3 Tables depict the percentage of workers parasitized as adults or during development and whether there was statistical significant between the proportion of bees in early versus late season trials. Analysis was done using a Fisher's Exact T-test.

Table 3.4: Is there a significant difference in the ratio of bees parasitized during development versus adulthood?

	Early Season		Late Season	
Colony	**Ratio of workers parasitized during development to workers during adulthood**	**Chi-Square Goodness fit test: p value, df=1(X^2)**	**Ratio of workers parasitized during development to workers during adulthood**	**Chi-Square Goodness fit test: p value, df=1(X^2)**
House	19:58	<0.0001 (19.753)	59:76	= 0.143(2.141)
Tower	4:25	<0.0001 (15.207)	106:150	=0.0059(7.563)
A	24:73	<0.0001 (24.753)	34:54	=0.033(4.546)
B	29:81	<0.0001 (24.582)	59:96	=0.0029(8.832)
C	10:57	<0.0001 (32.97)	50:111	<0.0001 (23.112)

Table 3.4 Count data statistical analysis of the ratio of workers parasitized during development or parasitized during adulthood in early season or late season trials.

Varroa appeared to distribute more broadly over female age cohorts (less aggregated) in later season trials than in earlier trials. This visual trend was confirmed by calculating the degree of aggregation. Gini coefficient estimates and confidence intervals were calculated from detection data from all trials. (Appendix b) and used as a method to measure aggregation[77]. Gini coefficients in female workers was significantly lower (less aggregated) in later season trials than in early season trials (mean ± SD = 0.964, mean late season 0.914, Welch two sample t-test, $t(77) = 4.21$, $p < 0.0001$).

Figure 3.2: *Varroa* distribute by age and sex cohorts. The distribution was fit to a negative binomial model. **In early season** trials both sex and age were used as predictor variables, and the incidence of *Varroa* on worker bees was compared against the incidence of newly emerged bees ($p < 0.0001$ for both variables, negative binomial, df(age) = 8, df(sex) = 1) **In late season trials** drones were not well repreented (n = 59) and as a result were excluded from analysis (due to too small of a sample[101]). There was no significant difference between prevalence on newly emerged workers or 2 day old workers in late season trials ($p = 0.086$, df = 8).

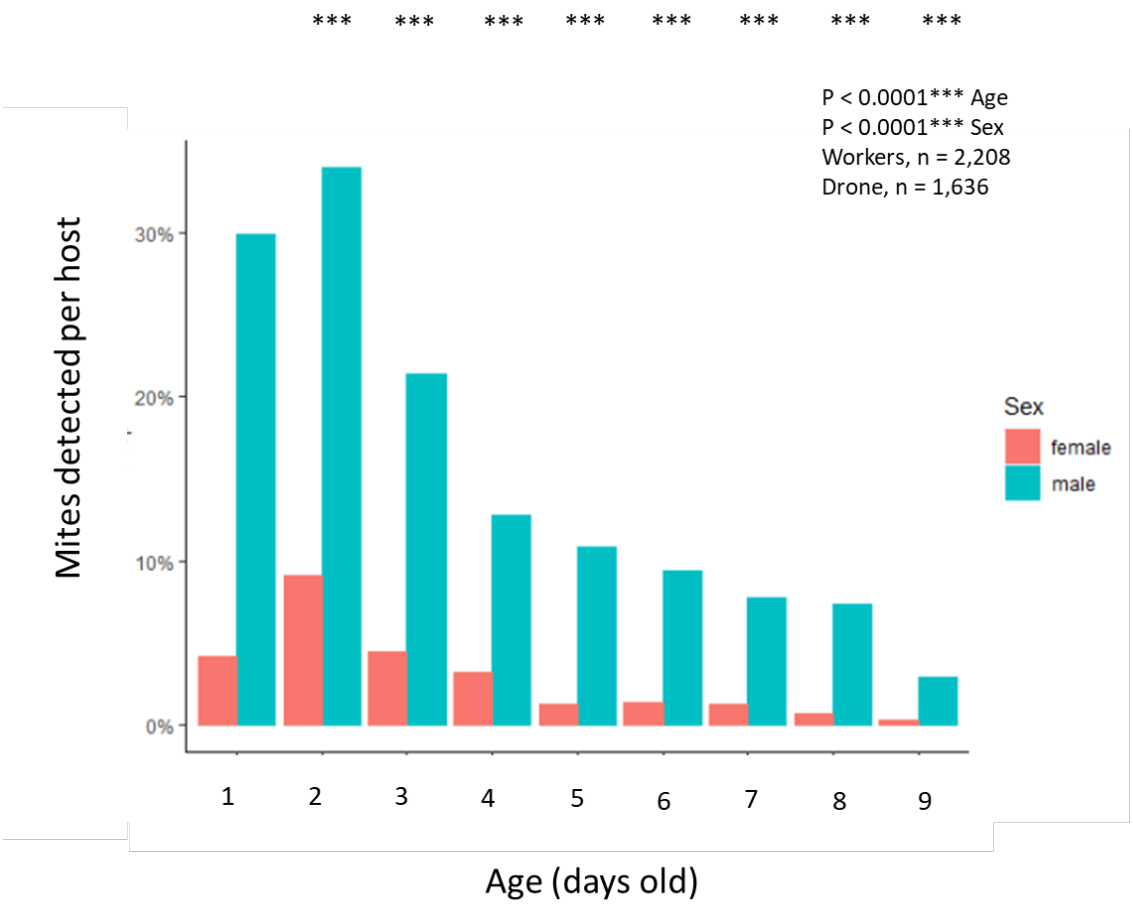

Figure 3.3: Drones by a proportion of the population were more infested than worker bees (GLMM, Negative binomial, df(1,8) $p < 0.0001$ of data combined from 5 trials)

Targeted Sampling of *Varroa* in commercial apiaries

(a) *Early season detections of Varroa destructor*

Detections of *Varroa* were equally low with both alcohol washes and hand sampling methods in May detecting *Varroa* in 58.6% and 63% of colonies respectively. A detection was considered as a single *Varroa* collected from either the alcohol wash or observed while hand sampling. *Varroa* were detected in the lowest abundance in May compared to any other month. Data from hand sampling revealed

74 *Varroa* were detected from a total of 6,377 bees. Of these infested bees, co-infestation with 2 or more *Varroa* was a rarity. Four bees were found to each harbor 2 *Varroa*, while another 2 bees were both found to harbor 3 *Varroa*. All co-infestations were found on males, none on female bees in the month of May. Of the 60 bees infested with a single *Varroa* 43 were found on males, 17 on females.

(b) Prevalence and co-infestations by sex cohorts

Prevalence, as measured by the proportion of infested individuals over the total bees in the sample, was highest in drones: 3.96% of drones had *Varroa* in May compared to 0.33% of workers. This trend was observed despite fewer drones (N = 1,237) were sampled in May compared to workers (N = 5,140). In addition to prevalence being higher on drones, a greater number of *Varroa* were detected on a smaller number of drones than workers. Prevalence patterns in June and July shared the same pattern as May: a minority of drones harbored a majority of *Varroa* detections. A total of 2,314 drones and 5,599 workers were sampled in June. The majority of *Varroa* were detected on drones where, 152 *Varroa* were detected on 139 of the drones compared to 49 *Varroa* on 49 workers. Co-infestations were more common, but still rare in June. 10 bees harbored 2 *Varroa* and 1 bee harbored 4 *Varroa*; all co-infested bees were observed on drones. Prevalence remained higher in drones (6.0%) compared to workers (0.88%). Prevalence estimates increased by 51.5% in drones and 167% in workers from May to June sampling events.

Co-infestations were still an uncommon occurrence but continued to increase in drones in July over the previous sampling periods, and co-infestations were detected on workers for the first time. In July, 41 drones harbored 2 *Varroa* each,

while 9 harbored 3 *Varroa* apiece. One drone was observed with 4 *Varroa*. Co-infestations were less common in workers with 5 workers harboring 2 *Varroa* each, while another 4 harbored 3 *Varroa*. As a percentage of infested bees, co-infestations had been a rarity in the early season sampling and non-observant in workers until July. I calculated the prevalence of co-infested bees (co-infested bees / infested bees). Co-infestations in July rose to 25.0% in drones from 7.9% in June. First time observations of co-infestations of workers in July represented 16.4% of all infested workers detected in July. Prevalence increased and mirrored similar trends in July as the previous months. A total of 266 *Varroa* were observed on drones and 68 on workers: 204 of the total 2,446 drones sampled in July were infested (8.3%) while 46 of the 4,958 workers were infested (1.1%). Prevalence estimates increased from June to July for both drones and workers (increase of 38.3% in drones and 25.0% in workers). More *Varroa* were still found on drones over the worker cohort even though the total number of drones sampled was less than half of workers.

Prevalence of *Varroa* on workers increased in August with 121 of the 4,218 workers infested (2.9%). Of those, 17 workers were co-infested with more than one *Varroa*. (11 workers with 2 *Varroa*, and 6 workers with 3 *Varroa*). Prevalence estimates increased by 109% in August from the previous month of July and co-infestations comprised 19.0% of all detections in workers. Drone abundance was the lowest in August with only 1,033 drones sampled in August. In August, 129 of those drones were infested with a single *Varroa*. I observed a greater variance in the number of *Varroa* co-infestating drones in August. In August, 16 drones were observed with 2 *Varroa*, while 7 drones were co-infested with 3 *Varroa*, 7 drones

were co-infested with 4 *Varroa*, and one drone harbored 5 *Varroa*. Co-infested *Varroa* represented 24.0% of all infested drones in August.

Month	Sex	Number of bees with one *Varroa*	Number of bees with 2 *Varroa*	Number of bees with 3 *Varroa*	Number of bees s > 4 *Varroa*	Total infeste d bees	Total bees sampled
May	female	17 (100%)	0	0	0	17	5,140
June	female	49 (100%)	0	0	0	49	5,599
July	female	39 (84.8%)	3 (6.5%)	4 (8.7%)	0	46	4,958
August	female	105 (86.8%)	11 (9.1%)	5 (4.1%)	0	121	4,218
May	male	43 (87.8%)	4 (8.2%)	2 (4.1%)	0	49	1,237
June	male	128 (92.1%)	10 (7.2%)	0	1 (0.7%)	139	2,314
July	male	150 (74.6%)	41 (20.4%)	9 (4.5%)	1 (0.5%)	201	2,400
August	male	86 (73.5%)	16 (13.7%)	7 (6%)	8 (6.8%	117	869

Table 3.5: Detections of *Varroa* on adult bees. Detections were organized by the number of *Varroa* observed on each individual bee, and then presented with the relative proportion they represented to all infested bees within their cohort for that month.

Month	Infested Drones	All Drones Sampled	Infested Workers	All Workers Sampled	Percent Infested Drones	Percent Infested Workers	Fisher Exact Test *P* (95CI)
May	49	1,237	17	5,140	3.96%	0.33%	<0.0001 (0.04, 0.15)
June	139	2,314	49	5,599	6.01%	0.88%	<0.0001 (0.10, 0.20)
July	201	2,400	46	4,958	8.38%	0.93%	<0.0001 (0.08, 0.15)
August	117	869	121	4,218	13.46%	2.87%	<0.0001 (0.16, 0.28)

Table 3.6: Sum of all detections of *Varroa* on adult drone and worker bees in 8 apiaries over the course of 4 months in similar regions Vermont and New Hampshire. A fisher exact t test was performed comparing the proportion of infested drones to workers in each month with 95% confidence intervals.

(c) *Gini coefficient to describe Varroa infestations*

I used the Gini index to describe unequal distributions of *Varroa* across colonies in the two operations in this study. Operation A was located in northern Vermont, while Operation B was located in the Upper Valley of New Hampshire and Vermont. Both operations kept bees in areas historically known as commercially successful

locations. I calculated a Gini co-efficient for each operation for each month by using data from the alcohol wash sampling (Table 3.7). Gini coefficients approaching one represent a highly aggregated distribution while values which were lower represented less aggregated. A zero value would represent an even distribution.

	Honeybee Operation A			Honeybee Operation B		
Month	Gini	LowerQ	UpperQ	Gini	LowerQ	UpperQ
May	0.91	0.80	0.98	0.53	0.39	0.79
June	0.89	0.78	0.96	0.56	0.44	0.81
July	0.82	0.71	0.91	0.55	0.40	0.70
August	0.74	0.64	0.84	0.63	0.46	0.87

Table 3.7: Gini co-efficients for each month for both operations in the study are reported here along with Lower and Upper Quartile ranges (set to 95% confidence intervals). A Gini coefficient was used as a reliable substitution for parameter k[77].

Detections by the alcohol wash method increased in number as the season progressed. The trend was similar to detections observed via the hand collection method (**Figures 3.4 and 3.5**)

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Figure 3.4

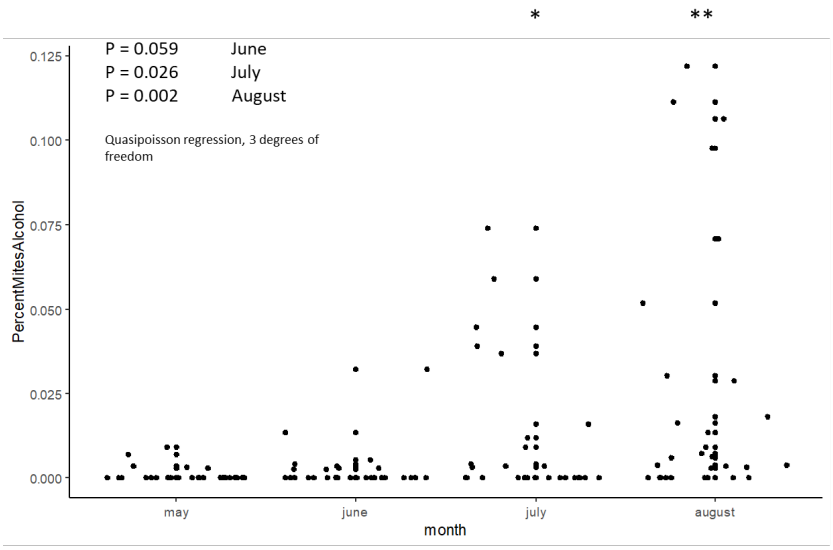

Figure 3.4: Detections from alcohol wash sampling over the four months of the trial are depicted. The number of *Varroa* counted in each sample was converted to a ratio and presented here as *Varroa*/100 bees. *P* represents statistical significance in detections compared to the month of May. *Data points were staggered to prevent overlapping and masking of data entries. All samples were collected at the same time each month

Figure 3.5

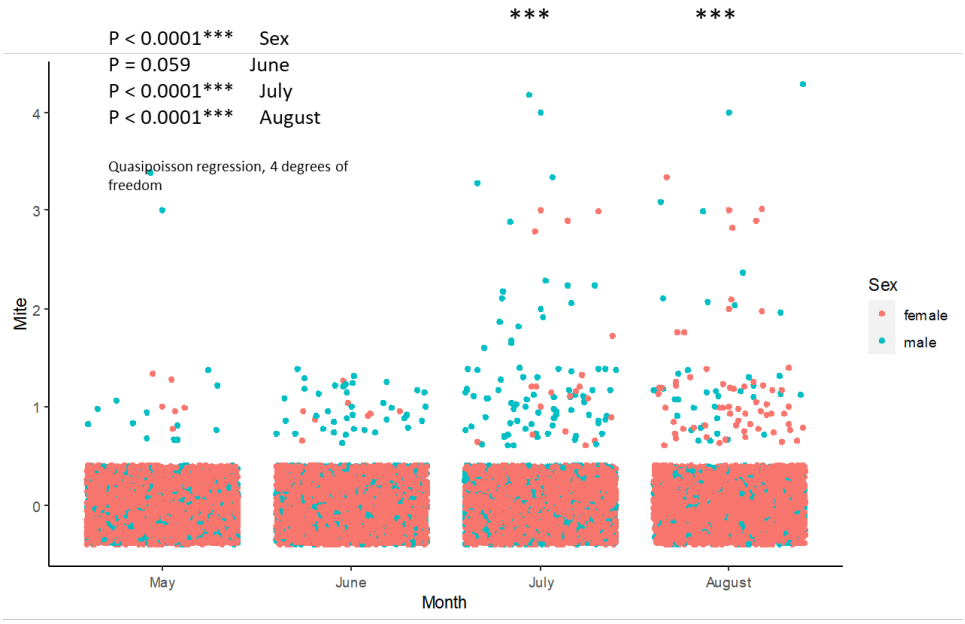

Figure 3.5*:* Detections from hand sampling data over four months are depicted in Figure 3.5. *Varroa* destructor was sampled from individual bees from 48 colonies over 4 months in 2021. *P* represents statistical significance in detections compared to the month of May. *Data points were staggered to prevent overlapping and masking of data entries.

Discussion

Drones bear a disproportionate parasite burden early in the season when mean parasite burden is low and the degree of aggregation is highly distributed onto relatively few individuals. This burden shifts onto the worker cohort later in the season when production of drones cease and mean parasite burden is high. In these trials, *Varroa* burden on developing workers accelerated concurrently with the disappearance of drones; where infestation on developing worker bees went from an infrequent occurrence early in the season, to a regular occurrence later in the season. Simultaneously, pressure on adult workers accelerated, becoming a predominate feature of late season *Varroa* infestations. A majority of worker bees in all trials were parasitized as adults and infested prior to their nursing stage (2-3 days old). My results repeatedly show *Varroa* infestations precede the onset of brood rearing. This is in disagreement with current consensus on *Varroa* feeding. Current literature state that the *Varroa* burden falls upon workers during their nursing stage (4-13 days old)[14,17,24]. The consequences of *Varroa* feeding on adult bees is largely unstudied, and the impacts on nursing behavior and transmission dynamics to the next generation of brood is currently unknown and warrants further study. I bring attention to this gap in our current understanding because *Varroa* feedings on adult bees maybe the most obvious feature of *Varroa* infestations. In highly infested colonies, the majority of worker bees can be parasitized in as little as 9 days. Absolute parasite burden surpassed 50% with upwards of 60% of worker bees observed to be parasitized. The majority of these parasitized worker bees are parasitized during adulthood, not during development. In all late season trials the absolute parasite

burden, measured as the sum of all parasitized bees over the total number of bees in the trial, exceeded the relative parasite burden which was calculated from standard sampling methods. There is a gross disconnect between infestation rates derived from alcohol washes and the rampant feeding occurring on worker bees within the colony.

Two events simultaneously occur at the end of the summer season: the host population becomes more homogeneous (the disappearance of one of the sexes) and *Varroa* infestations increase due to natural *Varroa* reproduction. The first condition meets current consensus that gives rise to a less aggregated distribution of parasites. The latter condition confounds our ability to assess the effect of the former independent of the confounders which naturally arise when parasite density increases. We do not know the relative influence homogeneity in the host population versus natural *Varroa* reproduction has on our repeated observations that later in the season the degree of aggregation is less on worker bees. I showed the degree of aggregation decreases on worker bees by calculating Gini coefficients on each age group of worker bees in all trials and then compared Gini coefficients between early and late season trials.

Other interactions may influence the change in *Varroa* distribution on worker bees later in the season. I did not ask if the age composition of worker bees in our colonies changed between early and late season trials, but a compositional change in the age structure of the worker bee population may have an impact on *Varroa* distribution within the population. We have some evidence from published works that seasonal changes could create a right skewed (older) worker bee population. The amount of brood area in a colony typically decreases later in the season[102]. This

would naturally reduce the emergence and availability of young workers. The occurrence of cannibalized worker pupae typically increases during this time as well. Cannibalization of pupae could further reduce the availability of young workers entering the worker population. A reduction in the successful egg to emergence rate occurs when cold weather sets in and pollen sources diminish[103]. This would also result in fewer young emerging bees entering the adult bee population in a colony. As a result a worker population could become right-skewed (proportionally older) leaving fewer young bees within the population.

Interactions between these three variables, loss of drones, increase in infestation levels, and a proportional age shift within the worker population, are three confounding variables I am suggesting that may drive the change in distribution in a colony later in the season.

Although we observed *Varroa* preferring drones over workers in multiple trials, we do not have an explanation for why *Varroa* were routinely attracted to drones. *Varroa* feeding on adult bees has been shown to increase their fecundity[17]. It is possible feeding on adult drones provide more nutrition and enhances a *Varroa*'s fecundity versus feeding on a worker. It is also possible feeding on adult drones is an evolved strategy from their time spent on *A. cerana*[104]. Other possibilities may include a lack of alogrooming on drones which is largely unstudied or feeding sites maybe easier to access on drones over workers[105]. However, even if we ignored drones during early season trials, we would still observe *Varroa* aggregated on select ages within the worker cohort. It is possible there are shared qualities in workers and drones of the same age that make them highly attractive to *Varroa*.

The impacts of *Varroa* parasitism may be cushioned by the seasonal presence of drones. Drones bear the majority of parasite burden while they simultaneously do not engage in colony maintenance tasks[106]. It is well established that parasitism by *Varroa* on worker bees shortens their lifespans, interrupts social interactions, foraging and the ability for workers to rear the next generation of brood[90,91,107-109]. This work also establishes *Varroa* feeding on adult bees with or without the presence of virus reduces their lifespan while conferring harm to non-parasitized nestmates[62]. The worker cohort is spared all of these debilitating effects of parasitism while *Varroa* continue to feed upon drone brood and adults early in the season. However, during this time worker bees are supporting the production and maintenance of drones within their colony *Varroa* prefer and reproduce more effectively on drone brood; inadvertently the relatively non-parasitized worker bee population is building reservoir of *Varroa* that will flood onto worker bees when seasonal drone production ceases at the onset of the summer dearth. The consequences of this finding are currently unknown and warrant further research. However I conjecture that the sheer number of parasitized workers may make it impossible for non-infected nestmates to avoid interacting with infested conspecifics while still being a cooperative organization.

Our findings suggest support to the theoretical framework first proposed by Anderson and May in 1978 that parasites regulate their host population when their distribution approaches random and when that burden reduces host reproduction. The distribution of *Varroa* in honeybee colonies followed a pattern of highly aggregated to less aggregated as the season progressed. As a result *Varroa* distribution heavily

impacts select drones early in the season, likely causing individual harm without conferring destabilizing effects on the population level. Whereas in the late season trials parasite burden broadly affected the worker cohort while coinciding with higher infestation levels. Workers are responsible for all colony maintenance tasks, including the successful rearing of progeny worker bees. Impeding worker's ability to rear brood would be equivalent of suppressing intra-colony reproduction. When parasite distribution was highly aggregated early in the season, workers were spared from infestations. However, when distributions became less aggregated *Varroa* predominately parasitized worker bees likely impeding their ability to successfully rear progeny. The similarities I observed in *Varroa* distribution in honeybee colonies are strikingly similar to the theoretic framework proposed by Anderson in parasites regulation of the host population[74].

My work fills gaps in knowledge about the biology and behavior of *Varroa* destructor that has eluded research over the last 40 years. Further work is needed to understand the implications of my findings. *Varroa* selectively target young adult bees, but we do not know where these *Varroa* are coming from. These could be *Varroa* actively switching from one adult bee to another or arising from emerging bees. *Varroa* actively switching amongst the same group of bees provide a rich opportunity for *Varroa*-*Varroa* transmission of DWV which I previously have shown[62]. An increase in the infectiousness of the *Varroa* population increases the relative risk of death for parasitized bees; parasitized bees would be expected to die quickly after a feeding[62]. Parasitized individuals that survive a *Varroa* feeding are prospective supershedders, capable of communicably spread DWV to many nestmates, and subsequently back to

Varroa. The full consequences of these dynamic transmission circulations is currently unknown, and warrants further study.

General Conclusion

For over 40 years the *Varroa* and honeybee relationship has been modeled as a brood and worker centric relationship. It was believed worker bees, especially nurse bees, were the preferred host for *Varroa* destructor, and researchers overwhelmingly studied bees parasitized during development, not during adulthood[14,17,24]. This understanding established the framework broadly influencing sampling methodologies, modeling approaches and the epidemiology of the complex relationship between bee, *Varroa*, and virus[96]. Our body of work here corrects important information regarding the biology, distribution and behavior of *Varroa* destructor. There is no study to date which confirms *Varroa* destructor prefers to feed on worker brood over adult bees, yet this has become the consensus in honey bee research[110]. We can only speculate how this underlying assumption took root and influenced research for over 40 years. However, our work shows *Varroa* actively switch from one adult bee to another to feed leaving bees in the adult form far more parasitized than during pupal development. Our work corrects the long held belief that Varroa prefer nurse bees. Instead *Varroa* aggregate on adult drones, and parasite burden shifts onto the worker cohort when seasonal drone production ceases. Because of this work we now have to question whether seasonal spikes in infestation levels are partly an artifact of a sampling method which is designed to select nurse bees and inadvertently excludes drones[96].

A worker centric model cannot accurately describe the interactive effects between this pathogen-vector-host triad as the parasite burden overwhelmingly befalls the drone cohort for much of the year, and only transitions to worker bees

once seasonal production of drones ceases. This shift in parasite burden likely means the many horizontal transmission routes of DWV between worker bees and mites described in this work is largely seasonal as well. Drones do not donate food in trophallaxis nor do they engage in resource acquisition and colony maintenance tasks[106]. Because of this I conjecture *Varroa* can reproduce at high rates and feed on the drone cohort for much of the year with little direct impact on the host colony.

Further research is needed to understand the effects from this new finding, but it may be safe to say that drones provide a seasonal cushion from *Varroa* infestations. For much of the year they bear by proportion a majority of the bites inflicted upon the colony during both development and adulthood. They bear the burden, while simultaneously supporting an ever-increasing reservoir of *Varroa* which will flood onto the worker population later in the summer. When this occurs the worker population is quickly overrun. *Varroa* are promiscuous feeders, switching from one adult bee to another in search of a good meal. Highly infectious *Varroa* kill the adult hosts they feed on quickly, while non-infectious *Varroa* pose less risk of immediate death[62]. These long lived, asymptomatic bees, however, are more likely to develop high levels of DWV, and confer higher risk to non-parasitized nestmates through contact or oral transmission of the virus. Social behaviors such as trophallaxis and cannibalization of infectious brood aid in the persistent transmission of honeybee viruses[45].

Varroa are the largest driver of honey bee colony health and continue to be implicated in historically high colony failures[20,111]. It would appear *Varroa* destructor has not reached equilibrium with its new host the western honeybee, and is making a

grave mistake fervently feeding on the adult bee population. Although this appears to be the case, I argue the opposite maybe true. I hypothesize *Varroa* maybe benefiting from cycles of rampant reproduction and subsequent colony failure and that arriving at equilibrium with its host species maybe at a fitness cost to the parasite. Honeybees are social insects which regularly drift into one another's colonies, which includes the act of robbing[112,113]. It is documented that failing colonies act as a source of *Varroa* through the robbing behavior of nearby colonies[112]. I hypothesize the intergenerational reproductive number of an original foundress would be highest with rampant early season reproduction and feeding on drones, and sudden colony failure during the summer dearth when temperatures are warm enough to sustain robbing and transfer young *Varroa* to new host colonies.

Theory suggests parasites should take a "prudent" approach to transmission in local populations to prevent killing their hosts[114], but there is also evidence that higher transmissibility and virulence is successful in spatially structured populations[115]. I hypothesize the latter is better for the reproductive potential of *Varroa.* If a *Varroa* suppresses her own reproduction to the benefit of not killing her host then she risks extinction from competition from other *Varroa* within her host colony that do fervently reproduce. There would also be a balancing act of suppressing reproduction enough not to debilitate the host before winter, and to produce enough offspring to have surviving daughters after the long winter. Winter is coming. However, if overwintered *Varroa* collectively engage in a reproductive "arms race" from the beginning of the spring then they can create numerous reproductive daughters. The original overwintered *Varroa* may pass away after a few

reproductive cycles on drone brood[116], but their potential daughters and their subsequent daughters could approach the hundreds from a single *Varroa* within a season. A colony actively failing and being robbed out while the weather is warm could potentiate sending those daughters to numerous, hopefully uninfested colonies[112].

Appendices

A. Count data for chapter 2. Listed in order of occurrence.

Experiment 1

Mean and standard deviation of recipient bees per group that died during the trial

Group	Total Recipients Added	Mortality	Average(STDEV)	AOV Mortality P (f)
Varroa + Virus	150	13	2.6 (2.70)	P = .015 (2.223)
Varroa	150	15	3 (2.92)	-
No *Varroa*	150	30	6 (2.74)	-

Number of *Varroa* individually sampled for presence of NanoLuc

Group	Total # *Varroa*	Number detected	Number not detected	Percent of samples w/ detection
Varroa + Virus	12	6	6	50%
Varroa	3	0	3	0%
No *Varroa*	4	0	4	0%

Number of *Varroa* individually sampled for presence of DWV-A

Group	Total # *Varroa*	Number detected	Number not detected	Percent of samples w/ detection
Varroa + Virus	12	12	12	100%
Varroa	3	2	1	67%
No *Varroa*	4	3	1	75%

Number of pupae individually sampled for presence of NanoLuc

These are pupae a Varroa fed upon in the final stage of the experiment

Group	Total # *Varroa*	Number detected	Number not detected	Percent of samples w/ detection
Varroa + Virus	17	3	14	17.6%
Varroa	14	0	14	0%
No *Varroa*	6	0	6	0%

Number of pupae individually sampled for presence of DWV-A

These are pupae a Varroa fed upon in the final stage of the experiment

Group	Total # *Varroa*	Number detected	Number not detected	Percent of samples w/ detection
Varroa + Virus	17	16	1	94.1%
Varroa	14	11	3	78.6%
No *Varroa*	6	4	2	66.7%

Experiment 1 (continued)

Mean and standard deviation of donor bees per treatment (replicates, n = 5)

Treatment	Total Donors Added	Mortality	Average(STDEV)	AOV Mortality P(F)
Varroa + Virus	74	20	4 (1)	P = .08 (3.206)
Varroa	64	15	3 (1.87)	-
No *Varroa*	75	8	1.8 (1.41)	-

Count data for viral detections in naïve *Varroa* which fed on recipient bees

Group	Target	Sample Type	Total Samples	Number detected	Number not detected	Percent of samples w/ detection
Varroa + Virus	NanoLuc	*Varroa*	12	6	6	50%
Varroa	NanoLuc	*Varroa*	3	0	3	0%
No *Varroa*	NanoLuc	*Varroa*	4	0	4	0%
Experimental	DWV-A	*Varroa*	12	12	12	100%
Varroa + Virus	DWV-A	*Varroa*	3	1	2	67%
Varroa	DWV-A	*Varroa*	4	3	1	75%
Varroa + Virus	NanoLuc	Pupa	17	3	14	17.6%
Varroa	NanoLuc	Pupa	14	0	14	0%
No *Varroa*	NanoLuc	Pupa	6	0	6	0%
Experimental	DWV-A	Pupa	17	16	1	94.1%
Varroa + Virus	DWV-A	Pupa	14	11	3	78.6%
Varroa	DWV-A	Pupa	6	4	2	66.7%

Experiment 3: Co-Feeding on brood

In This trial, Cofeeding on brood, CF (June, 2022) 19 hosts housed 38 *Varroa*. *Varroa* were divided into two groups (experimental (n = 25) and control (n = 6)

Recollection of *Varroa* and detection of molecular targets in pupae they fed upon after recollection

Group	Target	Total # recollected *Varroa*	Number pupae detected with NanoLuc	Number pupae without detection	Percent of samples w/ detection
Experimental-Naïve	NanoLuc	11	6	5	54.5%%
Experimental Infectious	NanoLuc	14	8	6	57.1%
Control	NanoLuc	6	0	6	0%
Experimental-Naïve	DWV	11	10	1	90.9%%
Experimental Infectious	DWV	14	13	1	92.9%
Control	DWV	6	5	1	83.3%

Recollection of *Varroa* and detection of DWV-A in pupae they fed upon after recollection

Group	Total # recollected *Varroa*	Number pupae detected with DWV-A	Number pupae without detection	Percent of samples w/ detection
Experimental-Naïve	11	10	1	90.9%%
Experimental Infectious	14	13	1	92.9%
Control	6	5	1	83.3%

Experiment 3: Co-Feeding on brood (continued)

Viral loads in CF-(Cofeeding-Brood Trial, June 2022)

Trial	Group	State	Primer	Mean	Median	SD
CF	Experimental	All Samples	DWV	8.090696	7.951268	2.393663
CF	Control	All Samples	DWV	6.125059	5.278225	2.750206
CF	Experimental	Infectious	DWV	8.004581	7.891846	2.36842
CF	Experimental	Naive	DWV	8.200296	7.951268	2.536781
CF	Control	All	LUC	No detection	No detection	Nodetection
CF	Experimental	All	LUC	3.783416	3.707195	3.811959
CF	Experimental	Infectious	LUC	4.314553	5.350022	3.836407
CF	Experimental	Naive	LUC	3.208017	1.845109	3.866971
CF	Background Control Pupae: Start		DWV	5.869487	5.896935	0.3925235
CF	Background Control Pupae: Middle		DWV	4.455652	4.295666	1.283693
CF	Background Control Pupae: End		DWV	4.471733	4.424042	1.284144

These include only samples with a positive detection of LUC and excludes all zeros (No detects)

Trial	Group	State	Primer	Mean	Median	SD
CF	Experimental	Infectious	LUC	7.011149	6.396124	1.903618
CF	Experimental	Naive	LUC	6.416034	5.689485	2.863271

Trial: Co-feeding on brood (CFA, August, 2021)
In This trial, Cofeeding on brood, CF (July, 2022) 24 hosts housed 48 *Varroa*. *Varroa* were divided into two groups (experimental (n = 24) and control (n = 24).
Recollection of *Varroa* and detection of NLuc in pupae they fed upon after recollection

Group	Total # recollected *Varroa*	Number pupae detected with NanoLuc	Number pupae without detection	Percent of samples w/ detection
Experimental- Naïve	7	4	3	57.1%%
Experimental Infectious	7	6	1	85.7%
Control	14	0	6	0%

Recollection of *Varroa* and detection of DWV-A in pupae they fed upon after recollection

Group	Total # recollected *Varroa*	Number pupae detected with DWV-A	Number pupae without detection	Percent of samples w/ detection
Experimental- Naïve	7	7	0	100%
Experimental Infectious	7	7	0	100%
Control	14	0	0	100%

Experiment 3: Co-Feeding on brood (continued)

Viral loads in CFA-(Cofeeding-Brood Trial, August 2022)

Trial	Group	State	Primer	Mean	Median	SD
CFA	Experimental	All Samples	DWV	9.926413	10.7184	1.436755
CFA	Control	All Samples	DWV	9.180214	9.169762	1.566407
CFA	Experimental	Infectious	DWV	10.03198	10.74724	1.336651
CFA	Experimental	Naive	DWV	9.820845	10.30884	1.630931
CFA	Experimental	All Samples	LUC	3.827413	4.596895	2.799905
CFA	Control	All Samples	LUC	No detection	No detection	Nodetection
CFA	Experimental	Infectious	LUC	4.827161	5.17646	2.334959
CFA	Experimental	Naive	LUC	2.827665	3.345055	3.033367
CFA	Background Control Pupae: Start		DWV	4.247459	4.243642	0.3959113
CFA	Background Control Pupae: Middle		DWV	4.236862	4.183093	0.2607861
CFA	Background Control Pupae: End		DWV	3.538158	3.547232	0.528503

These include only samples with a positive detection of LUC and excludes all zeros (No detects)

Trial	Group	State	Primer	Mean	Median	SD
CFA	Experimental	Infectious	LUC	5.631688	5.310558	1.051393
CFA	Experimental	Naive	LUC	4.948414	4.25416	2.100042

(continued on next page)

Experiment 4: Co-feeding on adult bees

The number of *Varroa* collected and individually extracted for NLuc

Group	Total # *Varroa*	Number detected	Number not detected	Percent of samples w/ detection
Experimental	12	12	12	100%
Control	6	5	2	41.7%
Experimental Naive	6	4	2	66.7%
Experimental Infectious	6	6	0	100%

The number of *Varroa* collected and individually extracted for DWV-A

Group	Total # *Varroa*	Number detected	Number not detected	Percent of samples w/ detection
Experimental	12	12	12	100%
Control	6	6	0	100%
Experimental Naive	6	4	2	100%
Experimental Infectious	6	6	0	100%

Appendix B: Experiment 2, Chapter 3

Gini Coefficient estimates and 95% confidence intervals calculated from detection data on induvial drones and workers by age over 10 trials

Colony	Order	Sex	Age	Gini	LQ	UQ
House	first	male	Age1	0.741338	0.706464	0.776529
House	first	male	Age2	0.739621	0.699494	0.778097
House	first	male	Age3	0.825865	0.788232	0.858362
House	first	male	Age4	0.920567	0.889532	0.945653
House	first	male	Age5	0.931925	0.897667	0.957277
House	first	male	Age6	0.939236	0.898148	0.965812
House	first	male	Age7	0.927673	0.866815	0.968554
House	first	male	Age8	0.928571	0.803571	0.964286
House	first	male	Age9	0.961539	0.692308	1
House	first	female	Age1	0.971392	0.955907	0.983271
House	first	female	Age2	0.924032	0.896887	0.94358
House	first	female	Age3	0.953157	0.92668	0.967413
House	first	female	Age4	0.975169	0.952596	0.986456
House	first	female	Age5	0.983133	0.963855	0.992771
House	first	female	Age6	0.985465	0.962209	0.994186
House	first	female	Age7	0.991968	0.963855	1
House	first	female	Age9	1	0.913793	1
House	second	male	Age1	0.374822	0.316523	0.458074
House	second	male	Age2	0.393939	0.325367	0.497966
House	second	male	Age3	0.686765	0.565707	0.813955
House	second	male	Age4	0.664615	0.527149	0.82026
House	second	male	Age5	0.846154	0.538462	0.978022
House	second	male	Age6	0.9	0.466667	1
House	second	male	Age7	0.666667	0	1
House	second	female	Age1	0.820884	0.788792	0.851478
House	second	female	Age2	0.78979	0.749413	0.826453
House	second	female	Age3	0.844344	0.802711	0.877717
House	second	female	Age4	0.879873	0.84	0.910794
House	second	female	Age5	0.883457	0.843974	0.915832
House	second	female	Age6	0.924603	0.883065	0.953571
House	second	female	Age7	0.842857	0.784105	0.886878
House	second	female	Age8	0.903509	0.824561	0.938597
House	second	female	Age9	0.947368	0.789474	1
Tower	first	male	Age1	0.886842	0.85728	0.911134
Tower	first	male	Age2	0.895827	0.865188	0.920497
Tower	first	male	Age3	0.963597	0.940043	0.976445

Tower	first	male	Age4	0.975169	0.952596	0.986456
Tower	first	male	Age5	0.987342	0.967089	0.994937
Tower	first	male	Age6	0.98855	0.962899	0.996183
Tower	first	female	Age1	0.994555	0.981704	0.998185
Tower	first	female	Age2	0.964618	0.944134	0.975791
Tower	first	female	Age3	0.996226	0.983019	1
Tower	first	female	Age4	0.998047	0.984375	1
Tower	first	female	Age6	0.994898	0.977041	1
Tower	second	female	Age1	0.923097	0.903327	0.941362
Tower	second	female	Age2	0.913219	0.886272	0.933377
Tower	second	female	Age3	0.958118	0.937328	0.972575
Tower	second	female	Age4	0.969524	0.948571	0.980952
Tower	second	female	Age5	0.978516	0.958984	0.988281
Tower	second	female	Age6	0.994709	0.976191	1
Tower	second	female	Age7	0.972222	0.940476	0.988095
Tower	second	female	Age9	0.988235	0.905882	1
A	first	male	Age1	0.744194	0.692756	0.79248
A	first	male	Age2	0.658844	0.600635	0.720136
A	first	male	Age3	0.770036	0.71233	0.821497
A	first	male	Age4	0.824405	0.760395	0.876984
A	first	male	Age5	0.862222	0.798333	0.909841
A	first	male	Age6	0.84153	0.766276	0.897782
A	first	male	Age7	0.909091	0.806818	0.954546
A	first	male	Age8	0.9375	0.823494	0.96875
A	first	female	Age1	0.954617	0.932997	0.971814
A	first	female	Age2	0.893504	0.860594	0.921447
A	first	female	Age3	0.928851	0.900298	0.952094
A	first	female	Age4	0.980822	0.958174	0.991781
A	first	female	Age5	0.991379	0.971264	0.997126
A	first	female	Age6	0.989247	0.960574	0.996416
A	first	female	Age7	0.984925	0.948849	0.994975
A	second	female	Age1	0.886714	0.843026	0.921996
A	second	female	Age2	0.775518	0.714953	0.826869
A	second	female	Age3	0.936585	0.892683	0.960976
A	second	female	Age4	0.949749	0.904523	0.969849
A	second	female	Age5	0.967593	0.931641	0.9875
A	second	female	Age6	0.96124	0.906977	0.984496
A	second	female	Age7	0.988372	0.918605	1
A	second	female	Age8	1	0.916667	1
A	second	female	Age9	1	0.761905	1
B	first	male	Age1	0.596929	0.528562	0.672862
B	first	male	Age2	0.431399	0.371603	0.508026

B	first	male	Age3	0.502701	0.431709	0.585705
B	first	male	Age4	0.611404	0.524286	0.707317
B	first	male	Age5	0.560234	0.467513	0.660528
B	first	male	Age6	0.651351	0.543704	0.754762
B	first	male	Age7	0.690925	0.54717	0.801258
B	first	male	Age8	0.752345	0.582927	0.853659
B	first	female	Age1	0.900635	0.858613	0.93375
B	first	female	Age2	0.789583	0.739298	0.832149
B	first	female	Age3	0.881805	0.833848	0.917031
B	first	female	Age4	0.892857	0.839286	0.924107
B	first	female	Age5	0.98	0.94	0.995
B	first	female	Age6	0.981367	0.937888	0.993789
B	first	female	Age7	0.990196	0.931373	1
B	first	female	Age8	1	0.898305	1
B	second	female	Age1	0.85628	0.819189	0.886572
B	second	female	Age2	0.726191	0.672532	0.774074
B	second	female	Age3	0.884066	0.841597	0.917582
B	second	female	Age4	0.91357	0.872457	0.942322
B	second	female	Age5	0.928477	0.889328	0.955957
B	second	female	Age6	0.932451	0.888585	0.958778
B	second	female	Age7	0.949181	0.900621	0.975155
B	second	female	Age8	0.902913	0.825243	0.941748
B	second	female	Age9	0.881356	0.745763	0.932203
C	first	male	Age1	0.668802	0.622255	0.715387
C	first	male	Age2	0.549608	0.499724	0.601149
C	first	male	Age3	0.679269	0.61841	0.73986
C	first	male	Age4	0.828447	0.779307	0.870147
C	first	male	Age5	0.880237	0.832669	0.916003
C	first	male	Age6	0.943036	0.901017	0.967136
C	first	male	Age7	0.979592	0.931973	0.993197
C	first	female	Age1	0.971154	0.949451	0.98315
C	first	female	Age2	0.943052	0.917995	0.961276
C	first	female	Age3	0.98818	0.966903	0.995272
C	first	female	Age4	0.967269	0.945137	0.982045
C	first	female	Age5	0.989637	0.968912	0.994819
C	first	female	Age6	0.993421	0.970395	1
C	first	female	Age7	0.991228	0.961087	1
C	first	female	Age8	0.993631	0.955414	1
C	second	female	Age1	0.883864	0.847545	0.913358
C	second	female	Age2	0.838264	0.795927	0.872566
C	second	female	Age3	0.889413	0.846626	0.920034
C	second	female	Age4	0.920794	0.885835	0.946395
C	second	female	Age5	0.917492	0.877888	0.940594

C	second	female	Age6	0.943529	0.904	0.967273
C	second	female	Age7	0.946705	0.901163	0.972301
C	second	female	Age8	0.940678	0.872881	0.974576
C	second	female	Age9	0.96	0.82	1

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