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Travis Dynes Date

Genetics and human management in *Varroa destructor* parasitism

By

Travis Dynes
Doctor of Philosophy

Graduate Division of Biology and Biomedical Sciences
Population Biology, Ecology, and Evolution

_____
Berry Brosi, Ph.D.
Co-advisor

_____
Jacobus de Roode, Ph.D.
Co-advisor

_____
Keith Delaplane, Ph.D.
Committee Member

_____
Gonzalo Vazquez Prokopec, Ph.D.
Committee Member

_____
Lance Waller, Ph.D.
Committee Member

Accepted:

_____
Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____
Date

Genetics and human management in *Varroa destructor* parasitism

By

Travis Dynes
B.A. Luther College, 1998
M.S. Yale University, 2002

Advisor: Berry J. Brosi, Ph.D.
Advisor: Jacobus C. de Roode

An abstract of
A dissertation submitted to the Faculty of the
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Abstract

Genetics and human management in *Varroa destructor* parasitism
By Travis Dynes

European honey bee colonies have experienced widespread losses in the past decades, a particular concern due to the importance that honey bees play in agricultural services critical in both economic terms and to human health. In the first half of the 20th century the obligate ectoparasitic mite *Varroa destructor* made a sustained host switch from the Asian honey bee to the European honey bee. Since that time *V. destructor* has spread around the world and become the largest pathogenic threat currently facing the beekeeping industry.

When *Varroa* arrived in the United States in the 1980's beekeepers were able to effectively treat *Varroa* infections with miticides. However, the mites quickly developed resistance to these miticides. This was unusual considering *Varroa* is characterized by a lack of genetic diversity. Our research was able to show that there was more genetic diversity at fine scales than would have been predicted. This implies that there was also more transmission of mites between colonies than would have been predicted. Human management of honey bee colonies places colonies in densities that more than three orders of magnitude greater than would be found in feral colonies. This increased density has implications for parasite transmission, colony health and survival. We found that increased density leads to more potential for disease transmission, decreased colony health and productivity, and increased winter mortality.

According to the virulence-transmission theory, *Varroa* populations evolving under these different management intensities (from feral to heavily managed) may face different selection pressures for population growth and virulence. Our research was consistent with this hypothesis for population growth. However, our virulence results suggest that there are genotype by genotype interactions that are occurring.

Genetics and human management in *Varroa destructor* parasitism

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I would also like to thank my committee for their guidance both scientifically and professionally. I specifically, thank Keith Delaplane for his willingness read drafts of manuscripts and to open up his lab to me. Outside of my committee, I want to pay a special thanks to the University of Georgia Honey Bee Lab. The hard-working and fun crew there not only made the work enjoyable but without their endeavors my research would not be possible. Specifically, I want to single out Jennifer Berry, Brett Nolan, Nicholas Weaver, Ben Rouse, and Jack Garrison for their dedication and support of this research. I also want to thank the hard-working lab manager Emily Dobbs for her assistance in anything needed for my research.

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Chapter 4: Reduced density and visually complex apiaries reduce parasite load and promote overwintering survival in honey bees

CHAPTER 1

Introduction

The global decline of pollinators (National Research Council, 2007; Potts et al., 2016), which are vital to reproduction of 90% of the world's flowering plants, has been a cause for widespread concern (National Research Council, 2007; Ollerton, Winfree, & Tarrant, 2011). For example, the managed western honey bee (*Apis mellifera*) populations in the United States declined 61% from 1941 to 2008 (Dennis vanEngelsdorp & Meixner, 2010). This notable decline in the honey bee colonies in the past decades is particularly troubling due to the disproportionate importance that honey bees play in critical pollination services in the agricultural sector (J.S. Pettis & Delaplane, 2010). Animals contribute an estimated US $200 billion a year to the world economy through pollination (Gallai, Salles, Settele, & Vaissière, 2009). In addition, pollinated crops form approximately one third of the calories consumed by humans, contributing meaningfully to human health and nutrition (Klein et al., 2007). Interferences with the pollination services that honey bees provide could prove harmful to global agriculture and human health and nutrition.

While many factors, including land use change and pesticides, are implicated in threats confronting honey bees, parasites have emerged as one of the principal causes (Goulson, Nicholls, Botías, & Rotheray, 2015; Otto, Roth, Carlson, & Smart, 2016; Dennis vanEngelsdorp et al., 2009). Among these biotic threats, the obligate ectoparasitic mite *Varroa destructor* is the most prominent factor in colony losses worldwide (Guzmán-Novoa et al., 2010; Le Conte, Ellis, & Ritter, 2010; Rosenkranz, Aumeier, & Ziegelmann, 2010). *Varroa destructor* is also a vector for a number of important secondary viral infections that, when coinfected with *Varroa,* are

considered the foremost cause of these colony losses (Dainat & Neumann, 2013; Nazzi et al., 2012; Dennis vanEngelsdorp et al., 2009). *V. destructor* originated in Asia following a host switch from the Asian honey bee (*Apis cerana*) and has quickly spread wherever it has been introduced (D.L. Anderson & Trueman, 2000). Following the introduction of *Varroa* into North America in the 1980s infections were effectively treated using acaricides (Rosenkranz et al., 2010). However, the mites developed resistance to these chemicals relatively quickly and infected colonies that are left untreated typically do not survive for more than 2 years (Gracia-Salinas et al., 2006; Jeff S. Pettis, 2004).

Despite the extreme importance of *V. destructor* to honey bee health and the attention that has been paid to this parasite, we still have a limited understanding of the basic biology of the mite and the factors involved in the disease ecology of the *Varroa*-honey bee interactions. The means and level of parasite transmission is of particular importance for understanding the dynamics that govern disease dynamics (Agnew & Koella, 1997; R. M. Anderson, 1982; Grassly & Fraser, 2008), but transmission of *Varroa* remains poorly characterized. The research described in this thesis attempts to address this shortcoming in three fundamental ways.

First, we attempt to utilize genetic tools, specifically neutral microsatellite markers, to determine whether we are able to observe transmission (in the form of gene flow), between colonies. The use of genetic analyses in *V. destructor* is severely impaired by a distinctive lack of genetic diversity. This lack of diversity is due to the previously described host switch and multiple founding effects as *Varroa* was introduced around the world, as well as a significant amount of inbreeding in the population. Chapter 2 will present the results of an analysis of the fine scale

genetic population structure of mites to determine the partitioning of genetic variation within and between managed colonies (Dynes et al., 2017). This previously unstudied gap in the local genetic structure needs to be addressed to deal with important questions such as regional transmission, pathogenicity, and evolution in *Varroa destructor*.

Second, we address how the spatial arrangement and density of honey bee colonies affects the potential for *Varroa* transmission and population dynamics, as well as colony level health metrics and survival. Specifically, we look at the extent to which the potential for transmission through bee drifting (when bees enter non-natal colonies) is affected by colony density and visual cues. In the process of this research, we developed a novel method to monitor the movement of tagged individuals through video monitoring and utilizing a new video analysis pipeline (Chapter 3) (Rossetti, Dynes, Brosi, de Roode, & Kong, 2017). Theoretical and empirical studies have both found that host population density is a key factor driving disease and dynamics (Roy M. Anderson, May, & Anderson, 1992; Hudson, Rizzoli, Grenfell, Heesterbeek, & Dobson, 2002). The densities of honey bee colonies vary drastically between feral colonies and those managed by humans. Feral honey bee colonies typically range in density between 1 and 6 colonies per km^2 (Ratnieks, A. Piery, & Cuadriello, 1991; T. Seeley, 2007), while those in managed apiaries typically are closer than 1m apart and may have thousands of colonies in an area similar to that of one feral colony (T. D. Seeley & Smith, 2015). Chapter 4 presents the results of a large replicated two-year study using highly standardized colonies in two densities of apiaries where we actively cleared colonies of mites and then randomly inoculated colonies with a standard dose of mites. We monitored colony health, parasite burden, and bee drifting

behavior. The goal of this research was to examine the role of colony density in driving colony health, productivity, parasite dynamics and the potential for disease transmission.

Third, we examine how mites that have evolved under different honey bee management regimes, with drastically different transmission potentials, interact with bees from one management background. According to the virulence-transmission trade-off theory in disease ecology (Alizon, Hurford, Mideo, & Van Baalen, 2009a), industrial bee management is predicted to have favored increased population growth rates and virulence of mites. Chapter 5 presents a study in which we asked how mites from three bee management intensities (feral, lightly managed, and heavily managed) differentially affect standardized honey bee colonies from a common, lightly managed background. We used a large replicated virulence assay study with colonies initially actively cleared of *Varroa* mites and then inoculated with a standardized dose of mites from the different backgrounds. We measured mite levels, colony health and survival for the colonies for over two years of the experiment. The goal of this experiment was to determine whether mites evolved under these different honey bee management backgrounds showed differential population growth rates and/or virulence (colony health and survival) when interacting with bees from a single management background.

Taken together this research has the potential to add to our understanding of *Varroa destructor* biology and honey bee disease ecology. Specifically, we can learn how: 1) fine scale genetics can help understand the amount of diversity we can expect in an apiary setting and how *Varroa* quickly evolved resistance to miticides; 2) the density of honey bee colonies affects colony level health and survival, parasite burdens, and potential for disease transmission; and 3) how

evolution may have shaped the growth rate and virulence of mites from different management regimes. These advances in our understanding of genetics and human genetics in *Varroa destructor* parasitism can inform a better understanding of the role of transmission in the *Varroa*-honey bee disease dynamics.

CHAPTER 2

Fine scale genetic structure of *Varroa destructor*, an ectoparasitic mite of the honey bee (*Apis mellifera*)

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Introduction

There is widespread concern about the global decline of pollinators which are integral to the reproduction of nearly 90% of the world's flowering plant species (National Research Council, 2007; Ollerton et al., 2011). Specifically, the precipitous decline in honey bee (*Apis mellifera*) colonies over the past decades is of particular cause for alarm due to the disproportionate role honey bees play in critical agricultural pollination services (J.S. Pettis & Delaplane, 2010). Pollination services by animals contribute more than an estimated US $200 billion a year to the world economy (Gallai et al., 2009). In addition, to this large economic value, animal-pollinated crops contribute greatly to human health and nutrition. These crops form approximately one-third of the calories consumed by humans (Klein et al., 2007), and represent close to 10% of agricultural yields (Gallai et al., 2009). Disruptions in pollination services could thus pose a serious and destabilizing risk to the global agriculture enterprise as well as human health and nutrition.

Pathogens and parasites are implicated as one of the primary factors in current honey bee declines (Dennis vanEngelsdorp et al., 2009). Of these biotic threats, the ectoparasitic mite *Varroa destructor* is the most important explanatory factor in colony losses worldwide

(Rosenkranz et al., 2010). Originally from Asia, *V. destructor* has spread quickly wherever it has been introduced, and infested colonies typically do not survive for more than two years without treatment (Boecking & Genersch, 2008). *V. destructor* is also a significant vector for secondary viral infections (Kevan, Hannan, Ostiguy, & Guzman-Novoa, 2006) and may facilitate higher viral replication when coinfection occurs (Nazzi & Conte, 2016). Following its introduction to North America in the mid 1980s (Rosenkranz et al., 2010), *V. destructor* was effectively controlled with acaricides but has now developed resistance to most of these chemicals (Gracia-Salinas et al., 2006; Jeff S. Pettis, 2004). Despite the attention being given to management of *V. destructor*, we still have a surprisingly limited understanding of its basic biology, including the population structure within and among honey bee colonies and apiaries.

Our ability to apply population genetic tools to questions of basic *V. destructor* biology has been handicapped by a distinctive lack of genetic diversity. This low diversity limits the ability of analyses to resolve differences between populations at multiple levels. Three factors contribute to this low diversity: genetic bottleneck events, haplodiploid sex determination, and a unique life history resulting in a majority of matings occurring between siblings (Rosenkranz et al., 2010).

Neutral microsatellite markers have been developed and used to describe the genetic structure of *V. destructor* at a global scale by utilizing samples from geographically widespread populations (Evans, 2000; Solignac et al., 2003, 2005), but there is a dearth of in-depth information on the amount of variation found at a fine spatial scale between populations, within populations, and within colonies. How much genetic variation can be expected within a population or colony—or indeed whether we can expect any variation at all - remains unknown. Such questions are directly relevant to understanding parasitism dynamics in mites. This relevant

gap in the knowledge of local genetic structure for *V. destructor* needs to be addressed to deal with important questions such as regional transmission, pathogenicity and evolution.

To help fill this gap, we studied two aspects of fine scale genetic population structure of *V. destructor*. First, we investigated the partitioning of genetic variation within and between managed colonies. We also examined whether this variation changes over time and if variation is related to geographic spatial structure. Second, we assessed the ability of current microsatellite markers to inform us about mite transmission between honey bee colonies, including distinguishing between horizontal and vertical transmission.

Materials and Methods

In the spring and summer of 2011, we obtained mite samples from ten apiaries managed by the University of Georgia Honey Bee Lab around Athens, Georgia, USA. Each apiary consisted of 20 honey bee colonies housed in standard Langstroth hive boxes. We analyzed 589 adult female mites (from a total of 200 colonies and over three time periods: April, July, and September) for microsatellite genotypes. The colonies and apiaries were established at the start of the study. There was no exchange of bees or equipment between these apiaries and no additional bees were added to the study from other sources (details in Supplementary Material).

We extracted total genomic DNA from adult female mites using the DNeasy Blood and Tissue Kit from Qiagen (Valencia, CA, USA, details in supplement) and amplified ten polymorphic neutral microsatellite loci identified from two previous studies that were useful for population studies and amplify reliably: vj272, vj292, vj294, vj295 (Evans, 2000), and VD001, VD112,

VD114, VD119, VD126, VD163 (Solignac et al., 2003). We used Qiagen Type-It Microsatellite PCR Kits for the PCR reactions.

We analyzed microsatellite loci for the presence of null alleles, large allele dropout and scoring errors due to stutter peaks using MICRO-CHECKER v.2.2.3 (Van Oosterhout, Hutchinson, Wills, & Shipley, 2004). We assessed departure from Hardy-Weinberg equilibrium (HWE) using ARLEQUIN v.3.5.1.3 (Excoffier & Lischer, 2010) using Fisher's exact tests at each locus/population combination (100 within apiary locus by locus tests and 100 within colony locus by locus tests each employing 1,000,000 Markov chain steps) with a sequential Bonferroni correction (Rice, 1989). Most polymorphic pairwise loci/population combinations were not in HWE (Table 2.S1), as was expected based on the high level of inbreeding in *V. destructor*. This departure of HWE limited the types of population genetic analyses that we could perform. We could not, for example, use Bayesian clustering programs, such as STRUCTURE (Pritchard, Stephens, & Donnelly, 2000) due to assumptions of HWE and random mating populations.

We conducted baseline analyses to determine the number of alleles per locus, and allelic range and subsequent derivation of the Garza-Williamson Index. These were calculated in ARLEQUIN to empirically test for genetic evidence of past reduction in population size, or bottleneck events (Garza & Williamson, 2001). The index uses a generalized step-wise mutation model specifically designed for microsatellite data. We also employed BOTTLENECK program v.1.2.02 (Piry, Luikart, & Cornuet, 1999) to test for reductions in population size.

We calculated overall and pairwise estimates of R_{ST} and F_{ST}, as well as F_{IS} and F_{IT} in FSTAT and ARLEQUIN to determine genetic differentiation. R_{ST} is the preferred parameter when dealing with microsatellite data as it explicitly deals with the stepwise mutation nature of microsatellites (Slatkin, 1995). It has become common practice, however, to report both measures (Balloux &

Lugon-Moulin, 2002). Pairwise F_{ST} and R_{ST} values were calculated for loci found to be in HWE using GENEPOP v.4.2 (Rousset, 2008). The results were recalculated using adjusted allele frequencies calculated by MICRO-CHECKER.

To evaluate the partitioning of genetic variation within and between mite populations (in this case at the apiary, colony, and individual levels), we used one-way analysis of molecular variance (AMOVA) in ARLEQUIN, and determined locus-by-locus significance using 10,000 permutations. We examined changes in haplotype frequencies between the sampling dates using χ^2 tests. We used genetic assignment to assign mites to apiaries and to determine detection of first-generation migrants using GENECLASS2 (Piry et al., 1999).

We also examined changes in population structure over time and over geographic space. We ran the same AMOVA analyses over different time periods to assess temporal changes. To evaluate isolation by distance (IBD; i.e. population genetic changes in geographic space with individual mite data pooled by apiary), we used IBDWS v.3.23 (J. L. Jensen, Bohonak, & Kelley, 2005) to assess the correlation of Euclidean distance between populations and genetic differentiation with Mantel tests. We ran a Mantel test, for pairwise combinations of the genetic similarity (M) and the log transformation of geographic distance for each combination for those values. We ran 10,000 randomizations on F_{ST} and R_{ST} using both the unadjusted and adjusted allele frequencies (adjusted allele frequencies account for bias resulting from null alleles adjusting the frequencies of the amplified alleles) determined by MICRO-CHECKER.

To compare the allelic diversity we found with that of previously reported diversity (Solignac et al., 2005) at a global scale we used a rarefaction approach to perform a new analysis on the raw data of the global survey (kindly provided by Michel Solignac) and our current study to account for different sampling intensities. This new analysis used the nine microsatellite loci in common

between our study and the 2005 data (vj292, vj294, vj295, VD001, VD112, VD114, VD119, VD126 and VD163). Of note is that locus vj272 was not used in the 2005 study and this was the locus in our study that had the greatest allelic diversity. We used ADZE (Szpiech, Jakobsson, & Rosenberg, 2008) to perform a rarefaction analysis calculating the number of unique alleles that can be expected for a random sample of each population at different sample sizes. We compared our samples to the previously reported global survey at the continental level. Lastly, we used results from the previously described analyses related to population genetic differentiation and variation to determine if inferences can be made about parasite transmission, including distinction of horizontal vs. vertical transmission.

Results

We analyzed 589 *V. destructor* mites from 200 honey bee colonies arranged in 10 equal-size apiaries at each of 10 microsatellite loci. In our baseline analyses we identified 42 alleles. Numbers of alleles per locus ranged from 2 to 13. The observed heterozygosity for the population across all 10 loci was 0.09, a level consistent with the inbreeding-biased mating system of this mite. Microsatellite scoring analyses detected no evidence for large allele dropout. Seven (VD126, VD112, vj295, vj294, vj292, VD001, and vj272) of the ten loci showed indications of homozygote excess, which is to be expected for a population such as *V. destructor* that violates panmixia assumptions. However, it could also suggest that null alleles are present. Locus VD001 exhibited a number of alleles with one unit difference; these may be due to a PCR stuttering artifact that can result in scoring errors.

At the apiary level (i.e. pooling the allele frequencies across all colonies within apiaries), 78 out of 91 (86%) polymorphic locus/population combinations were not in HWE (Table 2.S1; $P < 0.05$; range from $P < 0.0001$ to $P = 0.048$). This is not unexpected considering the phylogenetic history and reproductive system of *V. destructor* and the assumptions built into Hardy-Weinberg equilibrium. At the colony level (i.e. pooling of allele frequencies across all individuals within one colony which was performed on one colony for each apiary), 42 out of 77 (55%) colony samples were not in HWE ($P < 0.05$; range from $P < 0.0001$ to $P = 0.042$). This decrease when looking at individual mites within colony values is most likely due to insufficient statistical power due to the lower mite sample size in individual colonies compared to apiaries (Tables 2.S1 and 2.S2).

We found a low, but statistically significant, level of population differentiation across all apiaries and loci (global $F_{ST} = 0.017$, $P < 0.0001$; global weighted $R_{ST} = 0.096$, $P < 0.0081$). The individual apiaries ranged in F_{IS} value from 0.384 to 0.659 with a statistically significant overall population F_{IS} value of 0.479 ($P < 0.0001$). Population pairwise F_{ST} values at the apiary level are shown in Table 2.1. The F_{IT} value that measures the deviation from HWE in the total population is 0.488 ($P < 0.0001$).

AMOVA calculations from ARLEQUIN are shown in Table 2.2 The percentage of variation that can be accounted for within individuals is 51.20%. Genetic assignment procedures using GENECLASS2 were only able to successfully assign 19.7% of the individuals (assignment threshold of 0.05) to the proper apiary and analysis of first generation migrants was therefore not useful, meaning we could not use microsatellites to determine inter-colony transmission. The apiary mean across all loci for the Garza-Williamson Index (GWI) had a fairly tight range over the 10 apiaries (0.36 to 0.41, Fig. 2.S1). A general rule of thumb for the GWI is that a value

below 0.7 indicates a population has gone through a bottleneck (Garza & Williamson, 2001). None of the loci in any of the apiaries had a GWI greater than 0.67 (Fig. 2.S1). The BOTTLENECK analysis similarly showed a reduction in past population size with 8 out of the 10 apiaries exhibiting heterozygous excess consistent with a recent bottleneck (two-tailed Wilcoxon sign-rank test using a two-phase mutation model for microsatellite data; significant *P* values ranged from 0.0019 to 0.027; while insignificant values were 0.20 and 0.25).

Table 2.1. Population (apiary) pairwise F_{ST} values. Significant differences at the $P < 0.05$ level are indicated with asterisks. Numbers in parentheses indicate number of mites genotyped.

Apiary	1 (51)	2 (49)	3 (58)	4 (44)	5 (30)	6 (41)	7 (38)	8 (28)	9 (35)
2 (49)	0.032*								
3 (58)	0.026*	0.018*							
4 (44)	0.028*	0.014*	0.016*						
5 (30)	0.018*	0.270*	0.025*	0.014					
6 (41)	0.008	0.013	0.000	0.010	0.012				
7 (38)	0.026*	0.038*	0.024*	0.035*	0.022*	0.005			
8 (28)	0.040*	0.031*	0.021*	0.014	0.016*	0.015	0.020*		
9 (35)	0.006	0.025*	0.019*	0.023*	0.019*	0.001	0.016	0.028*	
10 (34)	0.021*	0.035*	0.036*	0.033*	0.030*	0.009	0.014	0.029*	0.019*

Table 2.2. Results of hierarchical analysis of molecular variance (AMOVA). Comparing *V. destructor* mites from 10 apiaries.

Source of Variation	d.f.	Sum of squares	Variance components	Percentage of variation	*P*-value
Among groups (between apiaries)	9	27.16	0.012	1.32	0.0020
Among populations within groups (between colonies)	164	226.04	0.022	2.55	0.040
Among individuals within populations (within colonies)	415	515.35	0.40	44.93	<0.00001
Within individuals	589	265.5	0.45	51.20	<0.00001
Total	**1177**	**1034.04**	**0.88**	**100.00**	

We compared the allelic diversity we found with previously reported *V. destructor* microsatellite data at the continental scale (Solignac et al., 2005) in a new analysis (Figure 2.1). As expected, the allelic diversity of *V. destructor* is highest in Asia ($N = 41$ unique alleles across the nine loci) where the initial host switches are thought to have occurred. However, the fine scale diversity found in this study is greater than what would be expected from the samples previously taken from North America. We found 27 unique alleles for the nine loci in our ten intensively sampled sites, while the 2005 study (Solignac et al., 2005) found 18 unique alleles in the six North American sites surveyed (three sites in the US and three in Mexico). We expected our samples to contain a subset of the previously reported diversity from North America, but instead we found substantially greater diversity. We also compared the allelic richness between the ten apiaries in this study (Figure 2.2). The diversity was mostly similar across apiaries (and comparable with the overall rarefaction analysis for the current study in Figure 2.1) while one apiary (referred to

as apiary PS) showed a reduced allelic richness compared with the other apiaries (more in line with the richness identified for North America in Figure 2.1).

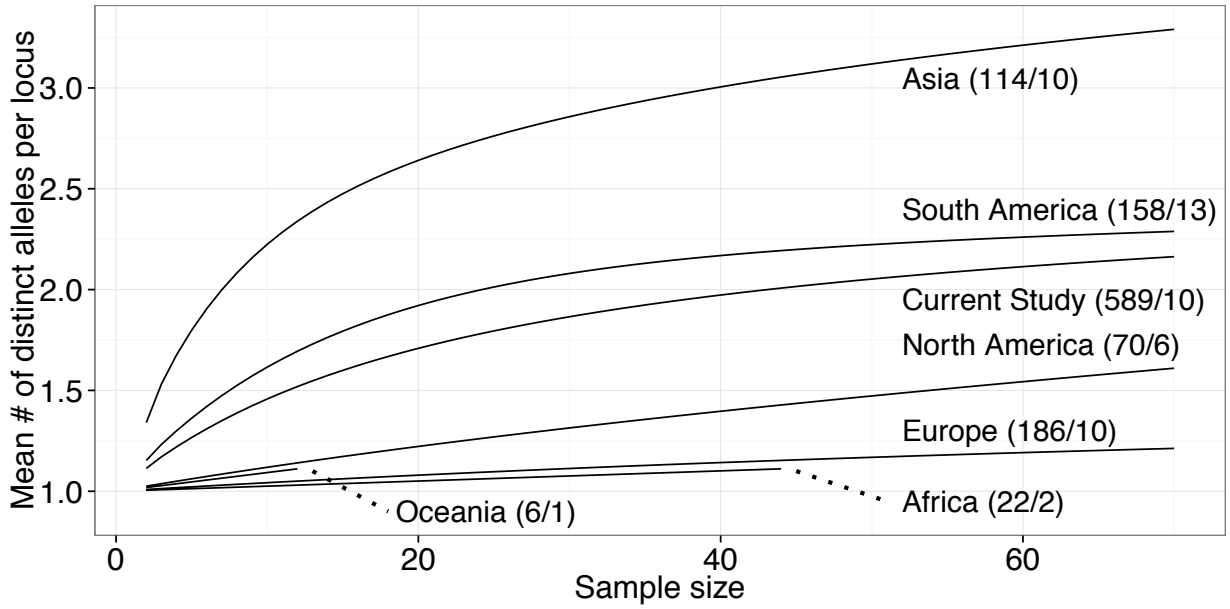

Figure 2.1. Allelic diversity. Fine scale allelic diversity (this study) compared to a new analysis of previously reported global allelic diversity (Evans, 2000; Solignac et al., 2003, 2005) using rarefaction to account for different sampling intensities. The estimated number of distinct alleles that can be expected for a random sample of each population is shown for different sample sizes. The number of individuals genotyped for the nine microsatellite loci and the number of sample sites are indicated in the parentheses. Note that Africa and Oceania did not have enough individuals genotyped to perform a full analysis.

Figure 2.3 shows the change in variation (AMOVA) over our three sample periods (all individual mite data pooled by time period). Due to the reduced number of samples when pooling time periods we can not analyze AMOVA between apiaries. This shows a modest increase in the amount of total variation accounted for within individuals over time. Finally, we assessed isolation by distance. The Mantel test for IBD showed no significant correlation between the

genetic similarity (*M*) and log-transformed (most appropriate for our two-dimensional spatial data; (Rousset, 1997) geographic distance between the apiaries (*M* vs. log dist. $P = 0.27$).

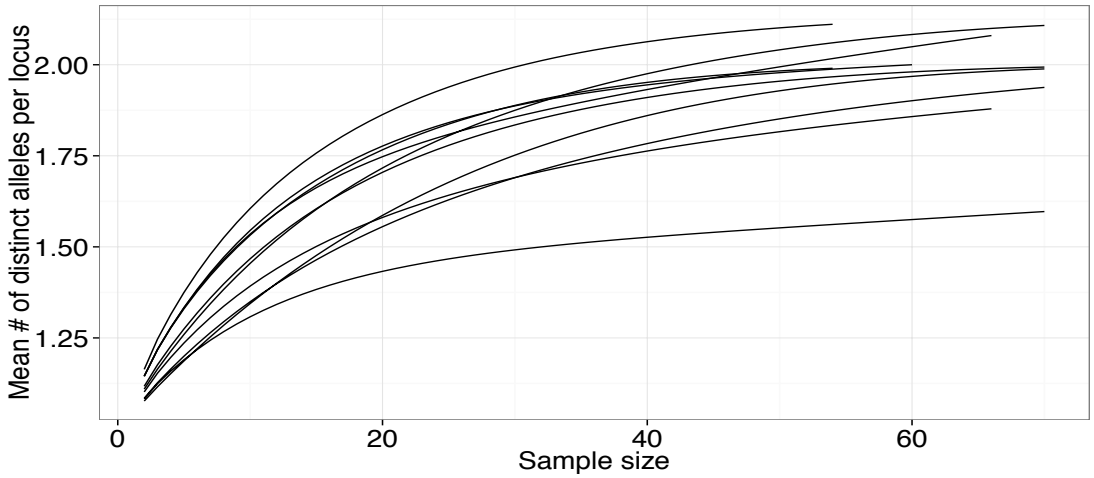

Figure 2.2. Allelic diversity in ten apiaries using a rarefaction approach. The estimated number of distinct alleles that can be expected for a random sample of each population is shown for different sample sizes. Each line refers to an individual apiary. The outlier with reduced allelic diversity is apiary PS.

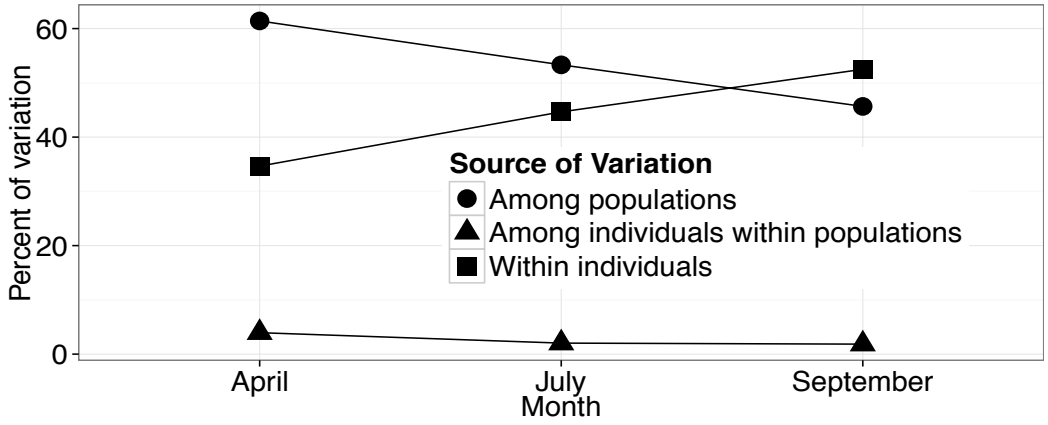

Figure 2.3. AMOVA change in source of population variation over time.

Discussion

In this first analysis of the fine scale population structure of *V. destructor* in a managed apiary setting, we found a surprising amount of genetic diversity given the mite's legacy of genetic bottlenecks and its inbred reproductive system. Despite these diversity-limiting factors, there is still detectable variation between and within apiaries and colonies, though we did not detect any relationship between geographic and genetic distance. We also documented a modest increase in amount of total variation accounted for within individuals over time (Figure 3.3), which could be due to *de novo* generation of diversity or more likely—because of the short time scales in this study—horizontal transmission of mites between colonies.

Genetic variation in Varroa destructor

In some ways, our findings are paradoxical: we found little genetic variation in *V. destructor*, but given its host-switching and biogeographical history and life cycle, the amount of assignable variation is surprisingly high. Since *Apis mellifera* is a social insect it is logical calculate variation at the four levels (including within- and between-colony levels) assigned in our AMOVA test (Table 3.2), which means that direct comparisons to *F*-statistics (F_{ST}, F_{IS}, and F_{IT}) are not appropriate. We found that the AMOVA (Table 3.2) percentage of variation within populations (colonies, 44.93%) and within individuals (51.20%) to make up the most significant part of the fine scale variation within *V. destructor*. This indicates a population structure with genetically distinct individuals and more sexual outcrossing than we would expect. The small amount of variation shown among groups (between apiaries, 1.32%) and among populations within groups (between colonies, 2.55%) indicates a genetic similarity between the groups and gene flow between them.

Three elements of *V. destructor* and honey bee biology and management may contribute to the genetic variation we observed: 1) within-colony mite genetic exchange; and between-colony mite transmission driven by 2) natural bee movement; and 3) human-induced movement. First, one potential explanation of this diversity is within-colony genetic exchange between mites. Most matings in *V. destructor* occur between siblings. If, however, circumstances encourage more than one foundress mite to enter the same brood cell to lay eggs, outcrossing can occur, enhancing within-colony genetic diversity. This scenario may occur more frequently at points in the annual cycle when the ratio of mites to bee brood is high, as in late summer. Such within-cell-outcrossings would particularly increase genetic diversity if there were mites from multiple sources within the same colony, i.e. if mite transmission between colonies had occurred.

Horizontal transmission of mites between colonies is another possible driver of higher-than-expected mite genetic diversity. Transmission is primarily driven by bee movement between colonies that can occur through two mechanisms: natural or human-mediated movement. Natural movement of bees between colonies occurs through robbing and drifting (Pfeiffer & Crailsheim, 1998). It is well known that honey bees will rob other (usually weaker) colonies, primarily for their stored honey. “Drifting” refers to movement of bees between colonies, for example if a worker honey bee becomes disoriented and enters an alien colony after foraging. Drifting is likely aggravated in human-managed apiaries with high colony densities. Unfortunately, rates of inter-colony movement via robbing and drifting remain poorly characterized. Recent research suggests that robbing and drifting can result in a large amount of mite transfer especially in apiaries that contain a high density of colonies (Frey & Rosenkranz, 2014). There remains, however, ambiguity in the published work linking bee drift to mite movement, with evidence that

bees from heavily infested colonies do (Kralj & Fuchs, 2006) or do not (Goodwin, Taylor, Mcbrydie, & Cox, 2006) drift more often than their mite-free counterparts.

Bee movement between colonies is also mediated by humans via beekeeping management. For example, movement of frames of brood (which can contain large mite numbers) and adult bees between colonies is a common beekeeping practice. Beekeepers may do this, for example, to bolster a struggling colony. At a commercial scale, many of the 36,000 US beekeeping operations and their 3 million colonies (USDA, 2014) are migratory. These large scale operations move hundreds of colonies at a time on trailers, often over thousands of kilometers a year (Rucker, Thurman, & Burgett, 2012). This movement is driven by crop pollination contracts, as with the almond industry that is wholly dependent on honey bee pollination and requires 1.4 million migratory colonies to descend on one half million acres in California for three weeks every year (National Research Council, 2007). Thus if two migratory beekeeping operations were to pollinate crops in adjoining farm properties, this could lead to interactions between bees from two different geographic provenances. This outcome is underscored by the rapidity of the spread of *V. destructor* in New Zealand, following its recent introduction there (Stevenson, Benard, Bolger, & Morris, 2005).

The intensity of human management also likely explains our inability to detect isolation by distance (IBD) or use genetic assignment criteria in *V. destructor*. In our study, bees and frames were explicitly not moved in order to control for this confounding factor and to assess the background level of mite transmission in an apicultural setting without human intervention. Because the colonies we used came from a single non-migratory beekeeping operation, prior to the study the typical beekeeping practices used in this operation likely led to a high potential for admixture. There was likely not sufficient time following establishment of our study to evolve

differences based on apiary isolation. An additional factor to consider is that IBD may occur in nature only over very large spatial scales in *V. destructor*, given that honey bee swarms can travel large distances (Schmidt, 1995). While beekeepers work to prevent swarming, a certain level of swarming is inevitable. Thus, even if our study had had sufficient time for evolutionary divergence the spatial scale may have been too small to pick up an IBD signal.

The comparison of allelic diversity to previously reported continental level diversity shows more diversity than might be expected ($N = 27$ unique alleles across nine loci in the current study compared to $N = 18$ found in the 2005 study for North America; Figure 2.1). We expected the diversity in our current study to be a subset of that found across the six sites previously surveyed in North America (three sites in the US and three in Mexico). One likely reason for this unexpected result is that the number of mite individuals from North America sampled previously ($N = 70$ from 6 sites) was too low to fully gauge the diversity of this population compared to the intensive fine scale sampling completed for the current study ($N = 589$ from 10 sites). Figure 2.1 shows the continental level rarefaction analysis. The North American population is increasing linearly rather than asymptotically indicating that the previously reported sampling had not exhausted the process of finding unique alleles. Thus, there may be more within-apiary and local allelic diversity than expected and potentially less diversity at larger scales. This is not inconsistent with the recent introduction and rapid spread of *V. destructor* where the diversity could be haphazardly scattered rather than systematically patterned. Future studies should consider the need to sample some smaller units intensively in order to assess hierarchical partitioning of genetic variability.

Finally, the level of within-colony variation that we found is consistent with horizontal transmission (between unrelated colonies) of *V. destructor*. If there were solely vertical

transmission we would not expect to see this level of diversity. Single transmission events of foundress mites, with subsequent sibling-only matings, would have resulted in much lower levels of variation within individuals. Indeed, the fact that we see such high levels of variation accounted for at the within-individual level lends credence to the idea that transmission between colonies is maintained by multiple sustained events rather than rare single transmission events.

Little is known empirically about the mechanisms and magnitudes of transmission of *V. destructor*, and this finding adds to anecdotal evidence supporting horizontal transmission (Frey, Schnell, & Rosenkranz, 2011). For example, *V. destructor* spread very quickly after it was introduced into North America (including to low-density feral populations) (Kraus & Page, 1995), implying that there is likely a mechanism beyond vertical transmission by which *V. destructor* infests new colonies. Unfortunately, the microsatellite markers that exist for *V.* destructor do not provide enough resolution to use these markers to track this transmission.

Conclusion

We found that existing microsatellite markers in *V. destructor* display unanticipated levels of diversity, in contrast to the limited diversity we would expect given historical bottlenecks, haplodiploidy, brother-sister matings, and previously reported diversity within North America. This variation may help explain how a species with such low genetic diversity can nevertheless evolve acaricide resistance and spread rapidly wherever it has been introduced. We still know little about the mechanisms and magnitude of *V. destructor* transmission, which is a key parameter underlying parasite dynamics. While new genetic markers with higher resolution (e.g. SNPs) could contribute to understanding transmission patterns, empirical work must also be

done on understanding the extent of bee movement between colonies, whether by human management or naturally via robbing and drifting.

Supplementary Materials

Sample collection

Colonies were originally obtained from a single beekeeping operation. Once the study began, we sought to minimize human-mediated mite transmission by refraining from typical beekeeping practices such as moving frames between colonies. Within each apiary, individual colonies were separated by 1.22 m in a linear arrangement. Each apiary was at least 3 km away from the others (range from 3.5 to 44.5 km). We sampled colonies for *V. destructor* at three different time periods (April, July, and September) in 2011 using the sticky board method (Calderone & Lin, 2003). This is a routine non-invasive beekeeping technique to sample and estimate population densities of mites by placing a thin piece of cardboard with a sticky surface beneath the colony to capture any mites that fall off their host bee. 51 out of the 200 initial colonies did not survive the entire sampling period, and thus we were not able to obtain all three samples from these colonies. In order to examine *Varroa destructor* genetic structure both within and between colonies we selected one colony from each apiary to analyze all of the mites collected at each sampling period (ranging from 7 to 15 mites, individually analyzed within a single sample period). In addition, one mite was selected for analysis from the remaining 19 colonies at each apiary for each sampling period.

DNA extraction and PCR

Each mite was ground with a sterile pestle in a microcentrifuge tube. Following the animal tissue spin-column protocol, we determined the DNA concentration using a NanoDrop 2000 (Thermo Scientific; Wilmington, DE, USA).

We followed standard kit protocol with the exception of lysing cells for 2 hours on an incubating shaker and eluting with 50µL elution buffer in order to maximize DNA yield and concentration. We ran 15µL multiplex reactions, each containing 0.2 µM of each primer and 20-50 ng of genomic DNA. We optimized the published PCR conditions for an Eppendorf Mastercycler ep gradient S thermocycler (Happauge, NY, USA).

Microsatellite genotyping and analyses

We analyzed amplification of PCR products via gel electrophoresis, and genotyped amplified DNA templates on an ABI 3100 genetic analyzer (Perkin Elmer, Applied Biosystems, Foster City, CA, USA) at the Emory Integrated Genomic Core (EIGC; Atlanta, GA, USA). We used GeneMarker version 4.0 software (SoftGenetics LLC, State College, PA, USA, 2010) to visualize and score the allele fragment sizes of each female diploid mite at all of the microsatellite loci. We scored the microsatellite loci in a semi-automated fashion, following the procedures recommended by Dewoody, Nason, and Hipkins (Dewoody, Nason, & Hipkins, 2006). In addition to using defaults on GeneMarker, we visually inspected each profile to prevent scoring errors introduced through automatic typing. We determined genotypes for the 589 mites for each of the ten microsatellite loci, taking mite samples from each colony in each apiary across the three separate sampling periods.

Supplementary population genetic analyses

We used the web based software SMOGD to calculate genetic diversity indices G'_{ST} (Hedrick, 2005) and D (Crawford, 2010; Jost, 2008). These parameters were developed as an alternative to traditional parameters such as F_{ST}, because F_{ST} and similar estimators can approach zero when heterozygosity is high, regardless of the true genetic differences among populations (Heller & Siegismund, 2009; Jost, 2008, p. 200).

Supplementary results

The estimated alternative measures of genetic differentiation G'_{ST} and D ranged from 0.0061 to 0.041 and 0.0008 to 0.0046 respectively for 9 of the loci. The highly polymorphic locus vj272 was an outlier in each case with values of 0.136 and 0.101 respectively (Table S3).

Table 2.S1. Observed and expected heterozygosity levels within apiaries. *P*-values in bold indicate loci out of Hardy-Weinberg equilibrium ($P < 0.05$). Two letter apiary name designation in parenthesis following apiary number.

	Apiary 1 (PS)				Apiary 2 (MA)				Apiary 3 (DG)				Apiary 4 (DC)				Apiary 5 (BA)			
Locus	N	H_O	H_E	*P* value	N	H_O	H_E	*P* value	N	H_O	H_E	*P* value	N	H_O	H_E	*P* value	N	H_O	H_E	*P* value
VD126	51	0.042	0.144	**0.000**	49	Monomorphic			58	0.028	0.054	**0.042**	44	0.016	0.134	**0.000**	30	0	0.081	**0.000**
VD112	51	0.07	0.145	**0.001**	49	0.03	0.058	**0.045**	58	0.042	0.118	**0.000**	44	0.063	0.147	**0.001**	30	0.042	0.189	**0.000**
VD114	51	0.014	0.014	1.000	49	0.03	0.086	**0.003**	58	0.028	0.028	1.000	44	0.048	0.161	**0.000**	30	0.021	0.021	1.000
VD119	51	0	0.028	**0.007**	49	0.015	0.1	**0.000**	58	0	0.054	**0.000**	44	Monomorphic			30	Monomorphic		
vj295	51	0	0.028	**0.007**	49	0.015	0.155	**0.000**	58	0.014	0.144	**0.000**	44	0.048	0.107	**0.001**	30	0.042	0.118	**0.005**
vj294	51	Monomorphic			49	0.03	0.058	**0.045**	58	0.014	0.041	**0.021**	44	0.032	0.062	**0.048**	30	0.625	0.137	**0.012**
vj292	51	0.014	0.042	**0.022**	49	0.03	0.086	**0.002**	58	Monomorphic			44	0.032	0.091	**0.003**	30	0.208	0.101	**0.000**
VD001	51	0.085	0.264	**0.000**	49	0.015	0.1	**0.000**	58	0.014	0.118	**0.000**	44	0.063	0.12	**0.013**	30	0.083	0.252	**0.000**
VD163	51	9.056	0.179	**0.000**	49	0.015	0.072	**0.001**	58	0.153	0.231	**0.015**	44	0.179	0.235	**0.000**	30	0.146	0.205	0.096
vj272	51	0.69	0.767	**0.019**	49	0.493	0.723	**0.000**	58	0.653	0.77	**0.005**	44	0.508	0.675	**0.002**	30	0.625	0.754	0.312
Mean		0.108	0.179			0.075	0.16			0.105	0.173			0.11	0.192			0.199	0.206	
	Apiary 6 (GR)				Apiary 7 (FA)				Apiary 8 (WR)				Apiary 9 (SO)				Apiary 10 (RE)			
Locus	N	H_O	H_E	*P* value	N	H_O	H_E	*P* value	N	H_O	H_E	*P* value	N	H_O	H_E	*P* value	N	H_O	H_E	*P* value
VD126	36	0.018	0.052	0.263	36	0.041	0.151	**0.000**	27	0.116	0.111	1.000	35	0.058	0.092	0.096	34	0.036	0.035	1.000
VD112	36	0	0.101	**0.000**	36	0.082	0.247	**0.000**	27	0.07	0.226	**0.000**	35	0.038	0.176	**0.000**	34	0.089	0.284	**0.000**
VD114	36	0.035	0.068	**0.053**	36	Monomorphic			27	0.023	0.023	1.000	35	0.019	0.092	**0.002**	34	Monomorphic		
VD119	36	0.035	0.101	**0.004**	36	0.041	0.116	**0.005**	27	0	0.09	**0.000**	35	Monomorphic			34	0.018	0.179	**0.000**
vj295	36	0.018	0.052	**0.009**	36	0.02	0.099	**0.000**	27	0.023	0.069	**0.012**	35	0.038	0.075	0.058	34	0.036	0.134	**0.000**
vj294	41	0.065	0.123	**0.013**	38	0.075	0.174	**0.001**	28	0.045	0.271	**0.000**	35	Monomorphic			34	0.018	0.118	**0.000**
vj292	41	0.016	0.107	**0.000**	38	0.057	0.056	1.000	28	0.023	0.11	**0.000**	35	0.038	0.129	**0.000**	34	0.018	0.182	**0.000**
VD001	36	0.053	0.116	**0.009**	37	0.039	0.239	**0.000**	27	0	0.131	**0.000**	35	0.058	0.16	**0.001**	34	0	0.221	**0.000**
VD163	36	0.069	0.187	**0.000**	37	0.137	0.253	**0.006**	27	0.093	0.208	**0.005**	35	0.058	0.191	**0.000**	34	0.089	0.307	**0.000**
vj272	36	0.667	0.802	**0.013**	38	0.596	0.836	**0.000**	27	0.512	0.728	**0.001**	35	0.654	0.841	**0.001**	34	0.446	0.724	**0.000**
Mean		0.097	0.171			0.121	0.241			0.091	0.197			0.12	0.22			0.083	0.243	

Table 2.S2. Observed and expected heterozygosity levels within colonies. *P* values in bold indicate loci out of Hardy-Weinberg equilibrium ($P < 0.05$).

	Colony PS7				Colony MA29				Colony DG57				Colony DC80				Colony BA92			
Locus	N	H_O	H_E	*P* value	N	H_O	H_E	*P* value	N	H_O	H_E	*P* value	N	H_O	H_E	*P* value	N	H_O	H_E	*P* value
VD126	23	0.043	0.125	0.066	21	Monomorphic			17	0	0.114	**0.031**	22	0	0.089	**0.023**	21	Monomorphic		
VD112	23	0.043	0.125	0.067	21	0.048	0.048	1.000	17	0	0.114	**0.03**	22	0.091	0.089	1.000	21	0.05	0.224	**0.011**
VD114	23	0.043	0.043	1	21	0.095	0.093	1.000	17	Monomorphic			22	0.045	0.273	**0.002**	21	Monomorphic		
VD119	23	0	0.085	**0.022**	21	0	0.177	**0.002**	17	Monomorphic			22	Monomorphic			21	Monomorphic		
vj295	23	0	0.085	**0.022**	21	0.048	0.136	0.073	17	0.059	0.269	**0.003**	22	0.136	0.13	1.000	21	0	0.097	**0.026**
vj294	23	Monomorphic			21	0.048	0.048	1.000	17	Monomorphic			22	0.045	0.045	1.000	21	0.05	0.142	0.077
vj292	23	0.043	0.125	0.066	21	0.048	0.136	0.073	17	Monomorphic			22	0.091	0.089	1.000	21	0	0.097	**0.026**
VD001	23	0.087	0.294	**0.005**	21	0.048	0.215	**0.009**	17	0	0.214	**0.003**	22	0	0.089	**0.023**	21	0.15	0.224	0.245
VD163	23	0.13	0.198	0.214	21	0	0.093	**0.024**	17	0.176	0.258	0.288	22	0	0.304	**0.000**	21	0.15	0.142	1.000
vj272	23	0.783	0.748	**0.036**	21	0.476	0.696	**0.004**	17	0.882	0.711	0.641	22	0.5	0.615	0.224	21	0.5	0.647	0.070
Mean		0.13	0.203			0.09	0.182			0.186	0.28			0.101	0.192			0.129	0.225	
	Colony GR114				Colony FA130				Colony WR142				Colony SD180				Colony RE197			
Locus	N	H_O	H_E	*P* value	N	H_O	H_E	*P* value	N	H_O	H_E	*P* value	N	H_O	H_E	*P* value	N	H_O	H_E	*P* value
VD126	23	0.043	0.043	1.000	17	Monomorphic			19	0.056	0.056	1.000	17	0.053	0.149	0.081	25	Monomorphic		
VD112	23	0	0.162	**0.002**	17	0.059	0.258	**0.014**	19	0	0.108	**0.029**	17	0	0.102	**0.027**	25	0.04	0.393	**0.000**
VD114	23	0.043	0.043	1.000	17	Monomorphic			19	Monomorphic			17	Monomorphic			25	Monomorphic		
VD119	23	Monomorphic			17	0	0.114	**0.030**	19	Monomorphic			17	Monomorphic			25	0	0.327	**0.000**
vj295	23	0	0.085	**0.022**	17	0	0.214	**0.003**	19	Monomorphic			17	0.053	0,152	**0.028**	25	0	0.15	**0.001**
vj294	23	0.087	0.165	0.130	17	0.059	0.258	**0.015**	19	0.056	0.322	**0.003**	17	Monomorphic			25	0.04	0.115	0.060
vj292	23	0	0.085	**0.022**	17	0.059	0.057	1.000	19	Monomorphic			17	0.053	0.243	**0.002**	25	0	0.274	**0.000**
VD001	23	0.13	0.198	0.214	17	0.059	0.337	**0.004**	19	Monomorphic			17	0.158	0.371	**0.028**	25	0	0.372	**0.000**
VD163	23	0.087	0.162	0.131	17	0.118	0.371	**0.013**	19	0.167	0.322	0.085	17	0.053	0.149	0.081	25	0.04	0.184	**0.007**
vj272	23	0.783	0.817	**0.042**	17	0.706	0.749	0.696	19	0.611	0.702	0.072	17	0.632	0.679	0.252	25	0.2	0.647	**0.000**
Mean		0.13	0.196			0.132	0.295			0.178	0.302			0.143	0.264			0.04	0.308	

Table 2.S3. SMOGD alternative estimates of population differentiation.

Basic Parameters

Locus	n	H_S	H_T	D_{ST}	G_{ST}	H_{ST}	Δ_{ST}	D	H_S/H_T	Δ_S/Δ_T
VD126	10	0.085	0.086	0.001	0.016	0.001	1.001	0.002	0.984	0.999
VD112	10	0.168	0.171	0.003	0.02	0.004	1.004	0.004	0.98	0.996
VD114	10	0.049	0.05	0.001	0.028	0.001	1.001	0.002	0.972	0.999
VD119	10	0.066	0.068	0.002	0.028	0.002	1.002	0.002	0.972	0.998
vj295	10	0.097	0.099	0.001	0.014	0.002	1.002	0.002	0.986	0.998
vj294	10	0.097	0.101	0.004	0.044	0.005	1.005	0.006	0.956	0.995
vj292	10	0.091	0.093	0.001	0.016	0.002	1.002	0.002	0.984	0.998
VD001	10	0.171	0.174	0.003	0.017	0.004	1.004	0.004	0.983	0.996
VD163	10	0.205	0.207	0.003	0.013	0.003	1.003	0.004	0.987	0.997
vj272	10	0.756	0.785	0.03	0.038	0.121	1.138	0.134	0.962	0.879

Estimated Parameters

Locus	$\tilde{N}$	H_{S_est}	H_{T_est}	G_{ST_est}	G'_{ST_est}	D_{est}
VD126	56.21	0.085	0.086	0.008	0.008	0.001
VD112	56.21	0.169	0.171	0.012	0.014	0.003
VD114	56.21	0.049	0.05	0.02	0.021	0.001
VD119	56.21	0.067	0.068	0.02	0.022	0.002
vj295	56.21	0.098	0.099	0.006	0.007	0.001
vj294	56.21	0.098	0.102	0.037	0.041	0.005
vj292	56.21	0.092	0.093	0.008	0.008	0.001
VD001	56.21	0.173	0.174	0.009	0.011	0.002
VD163	56.21	0.207	0.208	0.005	0.006	0.001
vj272	56.21	0.762	0.786	0.03	0.136	0.11

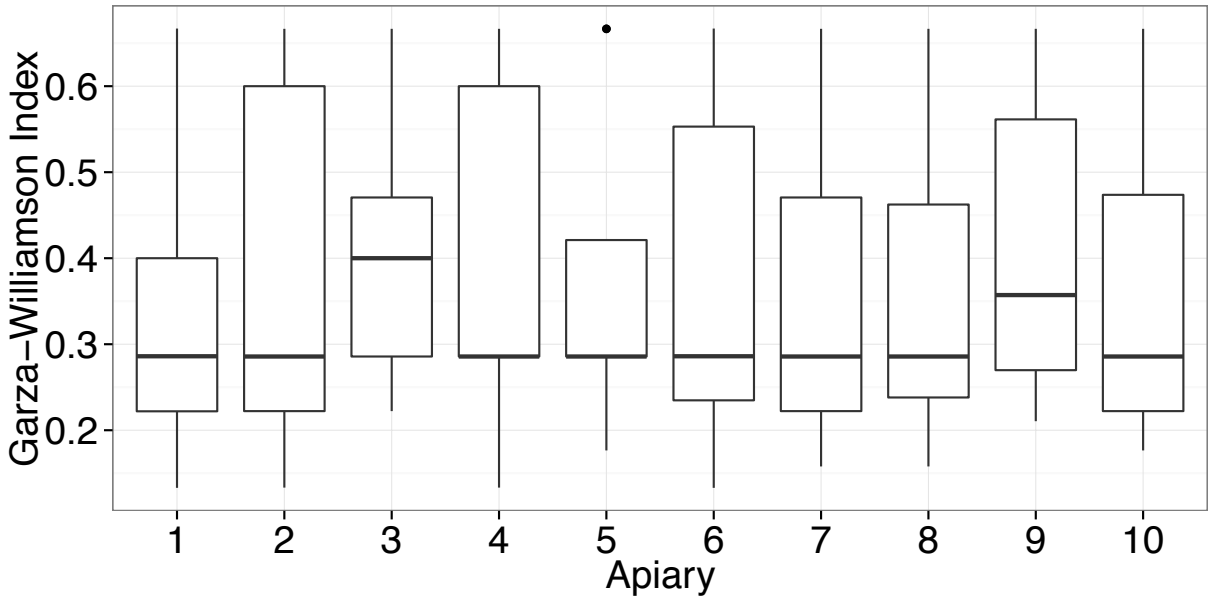

Figure 2.S1. Boxplot of Garza-Williamson Index values. Values for 10 loci at each of the 10 apiaries (The centerline of the boxplot indicates the median for each apiary while the extent of the box indicates the 25th and 75th percentiles and the whiskers indicate 1.5 times the interquartile range). The Index provides an indication of past reduction in population size. Generally, a population with an Index value lower than 0.7 indicates that a population has gone through a bottleneck.

CHAPTER 3

GRAPHITE: A graphical environment for scalable *in situ* video tracking of moving insects

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Introduction

Measurement of animal movement is key to understand ecological and evolutionary processes, such as dispersal, population and metapopulation dynamics, disease transmission, and gene flow, among others (Turchin, 1998). Many of these studies are highly relevant to conservation efforts (Fahrig, 2007). Despite this central prominence, we have a limited understanding of animal movement in most applications. For example, there are very few diseases for which we have a well-characterized empirical understanding of spatial transmission dynamics driven by host movement. This is true even for diseases important to public health (Riley, 2007; Wang, Wang, Zhang, & Li, 2013).

Existing categories of methods for animal movement measure include: (1) direct human observation, involving marking individuals (Ricketts, 2001) or not (Gómez, 2003); (2) trace-based methods with visible trail markers such as powdered dyes (Adler & Irwin, 2006); (3) active and passive electronic tags, including radio tracking (Aebischer, Robertson, & Kenward, 1993), harmonic radar (Osborne J.L. et al., 1999), GPS tags (Recio, Mathieu, Denys, Sirguey, & Seddon, 2011), and RFID (Kissling, Pattemore, & Hagen, 2014); (4) biomarkers including stable

isotopes (Rubenstein & Hobson, 2004); and (5) image-based methods including camera traps, video tracking (Dell et al., 2014), and fingerprinting methods (Kühl & Burghardt, 2013; Pérez-Escudero, Vicente-Page, Hinz, Arganda, & de Polavieja, 2014). These methods balance trade-offs between screening time, cost, accuracy, reliability, tracking area, continuity between tracking stations, ability to distinguish individuals, number of simultaneously tracked animals, a priori information and behavior-altering impediments.

However, few methods for measuring movement are available for small organisms including insects because they require the use of small components that are susceptible to false negative detection. Within the existing repertoire, even fewer methods offering consistent individual-level resolution are affordable, scalable and operable in the field (Campbell, Mummert, & Sukthankar, 2008; Crall, Gravish, Mountcastle, & Combes, 2015; Kimura, Ohashi, Okada, & Ikeno, 2011; Mersch, Crespi, & Keller, 2013; Tu, Hansen, Kryger, & Ahrendt, 2016). Our method combines automated image capture and a graphical interface to quantify motion dynamics of insects from discrete locations by video analysis of inexpensive ($\ll$\$0.01 per tag) and lightweight tags attached to individual insects. We have deployed consumer-grade digital cameras for video capture (Steen, 2016) with simple weatherproofed enclosures, keeping the cost of the entire system low. The key developmental component is a video analysis and graphical editing software that identifies potential tags in video frames, assembles these discrete tags into "tracks" of the same insect moving through scenes, infers the tag identifier by digit recognition, and provides a user-friendly graphical environment for editing tracking data. The goal is to reduce the time a research must spend screening video data while also minimizing the false negative tag detection rate.

This system is designed for use in field settings, in contrast to other image-based methods that are typically used in laboratory studies with predefined tracking areas (Noldus, Spink, & Tegelenbosch, 2001). Compared with typical camera traps (Rowcliffe & Carbone, 2008), this system distinguishes individuals via human-readable tags with unique numeric identifiers. Moreover, our new image-based method overcomes key limitations of existing RFID technology. Most notably, it enables easier access to location-based data by monitoring more colonies at a fraction of the cost of comparable RFID systems.

We show proof-of-concept of this method by tracking honey bees (*Apis mellifera*) at the entrance of beehives. However, this method can be generally deployed to track uniquely marked insects (*c*. 3 mm and larger) *in situ*. In particular, our method is most readily applied to a range of central place foragers with small nest, colony, or roost entrances relative to animal body size, allowing consistent tag detection within the camera's visual field. It would also be straightforward to deploy our method in studies with bait stations and/or feeders, such as artificial flowers and pollinator feeding stations for honey bees (Gould, 1975), and social stingless bees (Hubbell & Johnson, 1978), among others. Although free-ranging animal movements could also be tracked, this would be more challenging than the present study. Limitations to this method are two-fold. First, organisms need to be tagged. This necessitates prior capture as well as knowledge about which individuals are expected to be seen at a camera location. Second, the system is not expected to be as effective with solitary animals. Given the low cost, convenience, individual-level resolution, scalability, extensibility, and user-friendly graphical analysis and editing software, our system has the potential to contribute to a spectrum of insect movement studies.

Experimental Setup

Tagging

Tags were designed to be durable in the outdoor environment, easily visible, lightweight, and low-cost. Each tag consisted of a unique three-digit number that was inkjet-printed on white card stock (Neenah Exact Index, Item# 40508) with UV-resistant ink (PrintPayLess Black UV-Resistant Dye Ink). An inverted color scheme can be used for white insects. Tags were punched from the card stock and trimmed to a final size of 2.5 × 6 mm. The tags were then sprayed with a UV-resistant coating (Krylon UV-Resistant Clear Acrylic Coating Spray, Item# 1305) and a waterproof coating (Scotchgard Outdoor Water Shield, Item# 5019-6).

We recorded bee movement for a total of ninety colonies at six apiaries managed by the University of Georgia. To ensure that bees were correctly tagged with their respective colony and queen, brood frames were moved to an enclosed environment one day prior to tagging. We tagged newly emerged worker bees, which have the advantages of not being able to fly and having reduced stinging ability. A unique tag was secured to the thorax of newly emerged bees, using waterproof glue (Titebond III, Item# 1411). The glue was allowed to become tacky then applied to the bees using a wood toothpick. The tag was then affixed and held briefly to set. All tags were oriented with the rightmost number towards the head of the bee (see supplemental). Ethical considerations must be given to the tagging of sensitive or threatened species and the impact of tagging on the tracked animals.

Camera and lighting

A camera housing was temporarily mounted to the entrance of each colony for monitoring tagged bees that exited and entered the apiary (Fig. 3.1). Each camera housing was 10 × 14 × 15.5 cm with a lower landing that extended 8.5 cm from the front face. Bees could pass through a 100 × 8 mm opening at the front of the camera housing. The camera compartment was separated from the passage by a 3 mm thick OPTIX acrylic sheet. The camera-facing-side of the acrylic sheet above the entrance was painted black except for an 18 mm viewport strip for video recording. The entrance-facing-side of the acrylic sheet was treated with a lubricant (3-IN-ONE Dry Lubricant, Item# 3IO-DL-00) to inhibit bees from walking in an orientation that obscured the tags. Lighting was provided by a 1.5 W battery-powered LED (LouisaStore Portable Pocket LED Card Light, Item# BOOPIU26TO) located within the camera compartment. Modifications to the camera housing can be made to accommodate alternative experiments and organisms provided the camera retains clear *en face* view of tags.

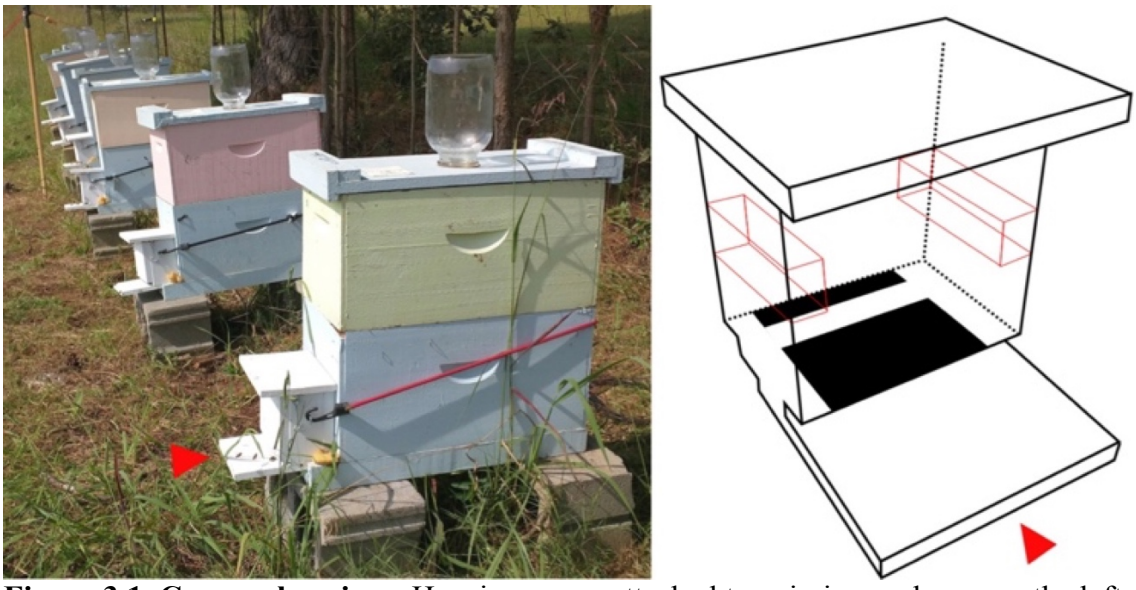

Figure 3.1. Camera housings. Housings were attached to apiaries as shown on the left. A diagram of the camera housing components (i.e. lower landing, viewport, and camera shelf) is shown on the right. The red arrows point to the lower landing.

Videos were recorded on Canon PowerShot SD1100 IS model cameras (30 fps; 640 × 480 px; automatic white balance; macro mode). Video duration ranged from 45 min to 1 hr. depending on the battery. The camera was mounted in the camera compartment on a wooden shelf 106 mm above the acrylic sheet. Frame-by-frame tracking was restricted to the viewport area; however, integration of data from multiple camera housings allowed low-resolution tracking of tagged insects across sites.

Modules and Editor

We have developed an analysis pipeline and graphical editor, called the GRAPHical Insect Tracking Environment (GRAPHITE), for end-to-end processing of video data. GRAPHITE is a modular set of functions with a user-friendly graphical interface written in MATLAB R2016a (url https://www.mathworks.com/). The software consists of a video preprocessor, tag detector, digit reader, and track assembler as well as a processing interface and graphical editor (Figure 3.2). Each module logs and accesses information within a central annotation MATLAB file. The user can choose to initiate the entire set of analysis routines as a single pipeline or access each module independently for a tailored analysis of a particular video (see supplemental). Batch processing is performed in parallel based on the number of cores available to MATLAB.

Video preprocessing module

The first pipeline module is a video preprocessor that prepares each video for tracking (Figure 3.2a). The module allows users to crop videos in the temporal and spatial dimensions. The user

can specify trimming times to remove frames from the beginning and/or end of each video. The spatial dimensions can also be cropped to remove areas that fall outside the apiary viewport as described in experimental setup and retain only the active region (Figure 3.3). Enabling active region cropping can reduce the searching space in subsequent modules for faster processing.

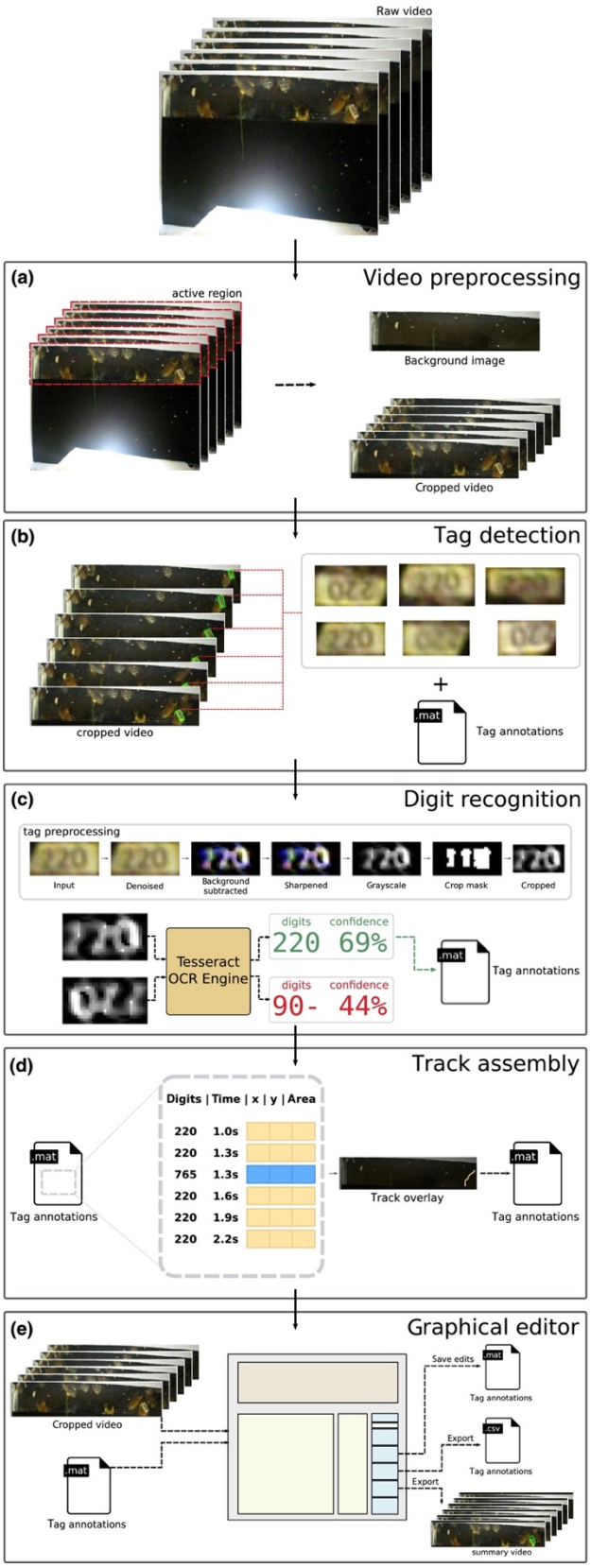

Figure 3.2. (right). Analysis pipeline. The analysis pipeline consists of five main modules: video preprocessing, tag detection, digit recognition, track assembly and a graphical editor**. (a)** The video preprocessor accepts a raw video as input and generates a background image and, optionally, a cropped video file as output. The cropped video file only includes the active regions detected within the raw video, and decreases the searching space for the follow-up tag analysis. **(b)** Each frame of the cropped video is searched for tag regions, and the resulting individual tag images are extracted, saved, and logged in an annotation file**. (c)** Tag images are preprocessed and provided to the Tesseract OCR engine for digit recognition. The orientation with the highest average confidence is chosen as the correct orientation, and digit recognition results are appended to the annotation file. **(d)** Tag data from different frames are linked as tracks based on their spatial locations and sizes**. (e)** The graphical editor can ease individual and global changes to tag data stored in the annotation file. The graphical editor also allows user to export the annotation data as either CSV or XLS files, in addition to a video file.

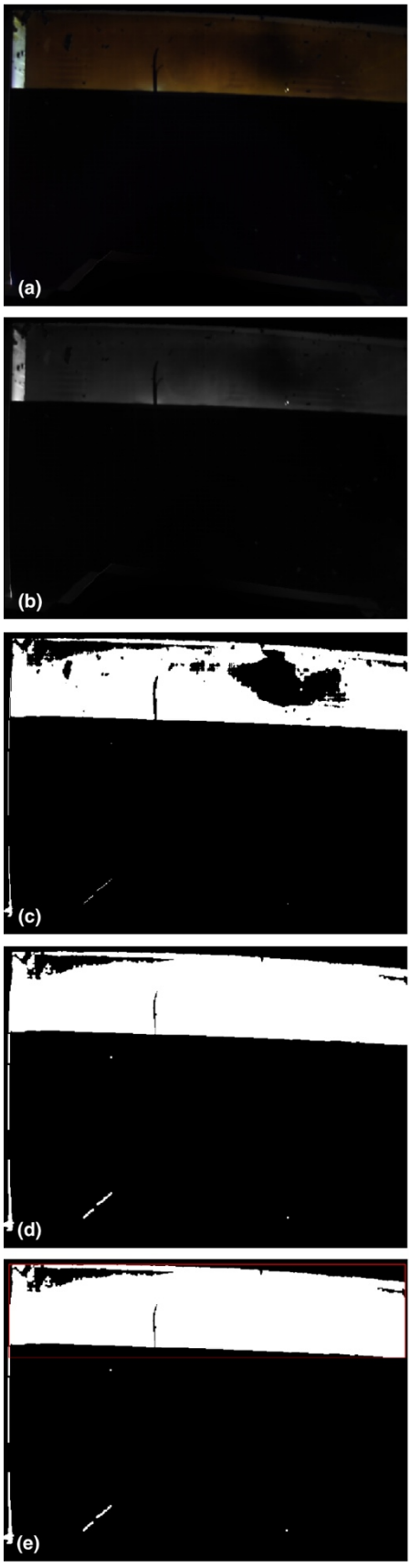

Figure 3.3. (right). **Active region.** Active region cropped from a raw video file. **(a)** A map of the active areas is determined by the pixel-wise variance over all frames. **(b)** The active region map is converted into grayscale where higher intensity values represent a greater deviation from the background, indicating motion events. **(c)** Thresholding is used to separate active (white) from static (black) regions in the grayscale activity map. **(d)** The binarized image is cleaned by morphological dilation and filling holes in the active regions. **(e)** The bounding box is determined for the largest active region (outlined in red), and the associated coordinates are used to crop the raw video and background image.

The active region is determined by the pixel-wise variance across the duration of the trimmed video sequence as pixels with more motion events have larger variance. The resulting variance matrix provides a map of activity that is segmented via Otsu thresholding into active and static regions. Otsu thresholding is a histogram-based method that generates a binary image by finding the optimal pixel value that separates bright foreground (active) regions from dark background (static) regions. The binary map is cleaned by a series of morphological operations to define the active region within the viewport. The bounding box coordinates of the active region are used to crop the video.

The video preprocessor also generates the static background image for the tag detection module. The grayscale background image is calculated as the mean pixel-wise

intensity over all frames of the videos sequence. This method leverages the a priori knowledge of a fixed field-of-view to produce background images regardless of moving object densities and motion speeds.

Tag detection module

Each frame in the preprocessed video sequence is searched for tags (Figure 3.2b). Moving objects fall into two categories: tagged and untagged objects. To differentiate these categories, we use color filtering to discard prominent non-tag colors (i.e. the color of the insect) with a user-determined RGB triplet. The frame and triplet are first converted to Hue-Saturation-Value color space, and the values of all pixels with a hue within ±15° of the specified color are set to zero. Finally, the filtered image is converted to grayscale for subsequent processing.

Next, the background image generated during video preprocessing is subtracted from the color-filtered frame to isolate pixels with motion events. The background subtracted frame is then passed to a Maximally Stable Extremal Region (MSER) feature detector to identify contiguous areas of stable pixel intensities. MSERs represent those appropriately colored moving objects with size ranging from 300 to 3,000 pixels (Figure 3.4a-e).

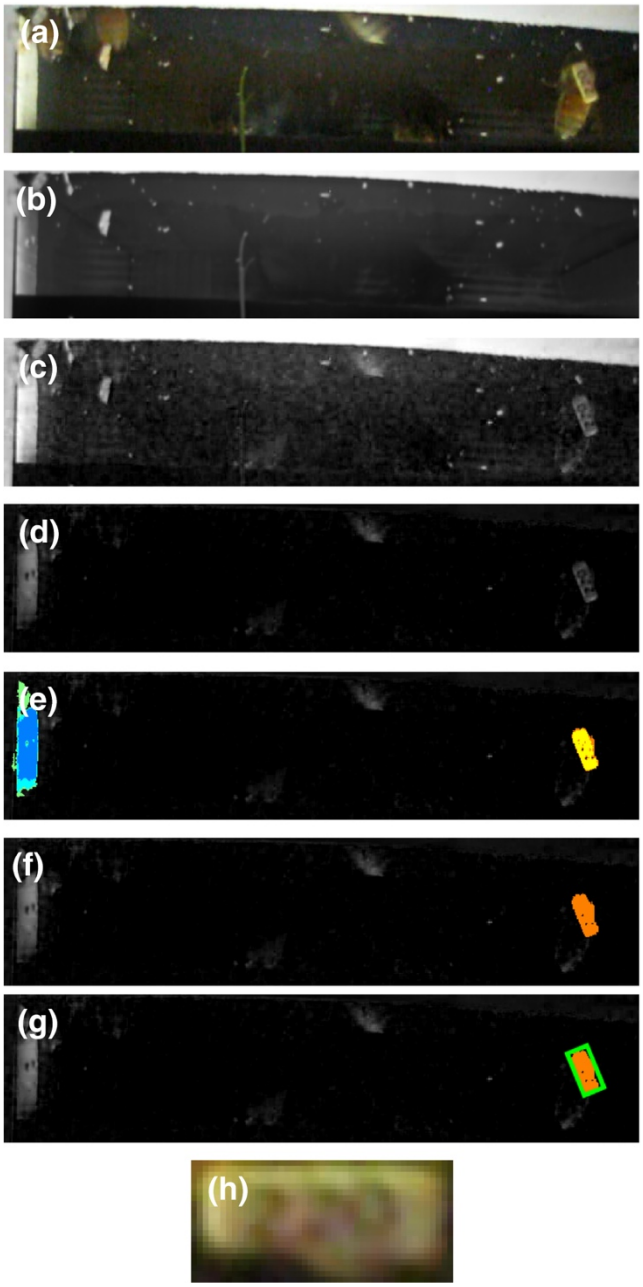

Figure 3.4. (right). Tag detection from a single video frame. (a) A full-color video frame and **(b)** a grayscale background image are passed to the tag detection module. **(c)** The yellow bee abdomens are removed by color filtering. The background image **(b)** is subtracted from the color filtered image **(c)** to produce **(d)** an image highlighting moving objects. **(e)** The MSER feature detector identifies five contiguous areas with stable intensity (labelled in orange, yellow, green, cyan, and blue). **(f)** MSERs are filtered by solidity, aspect ratio, and eccentricity to remove non-tag regions and retain tag regions (orange). **(g)** Remaining tag regions are fitted by MBRs. **(h)** The MBR coordinates are used to extract and rotate the tag region.

To this point, all steps focus on positive tag detection across video frames. We proceed with filtering out non-tag regions, or false positives. As the tag physical dimensions are known (tagging section in experimental setup), we use shape measurements to screen out non-tag regions. MSER detected regions are conservatively filtered by solidity, aspect ratio, and eccentricity. Solidity refers to the ratio of the region area to the convex hull area; aspect ratio is the ratio of the minor-axis length to the major-axis length of the region's fitted ellipse; and eccentricity is the distance between the fitted ellipse foci and the major-axis. When the MSER feature detector finds overlapping and duplicate regions, we only retain the smallest overlapping region by area (Figure 3.4f).

Although filtering by physical attributes removes most non-tag regions, additional steps are required to further reduce the number of false positives. In our solution, each potential tag region is fitted with a minimum-area bounding rectangle (MBR). The MBR coordinates are used to rotate and crop the region from the full-color video frame. Each cropped region is resized to 60 × 30 px and represented by a Histogram of Oriented Gradients (HOG) feature vector. A HOG feature vector is a series of one-dimensional histograms describing the edge orientations within each 4 × 4 px image patch. By encapsulating the shape components found within the image, we classify cropped regions as "tag" or "non-tag." The classification is performed by a two-class support vector machine (SVM) trained on HOG features from 4,093 false tag images and 880 positive tag images. Finally, remaining tag regions are classified as "tag" and processed for digit recognition.

Digit recognition module

Digit recognition from natural images has been an area of intensive research (Goodfellow, Bulatov, Ibarz, Arnoud, & Shet, 2013; Zhu, Yao, & Bai, 2016). This module uses the Tesseract optical character recognition (OCR) engine to identify digits in tag images (Figure 3.2c) (Smith, 2007). Each potential tag image is preprocessed to enhance the contrast of digit characters. Tag image preprocessing begins with channel-wise wavelet denoising and a rolling-ball background subtraction (Sternberg, 1983). Wavelet denoising uses a discrete stationary wavelet transform to remove noise in the image frequency domain without excessive edge blurring. The uneven white and black borders of tag images are removed with an estimated background from the denoised image. The rolling-ball background is generated by a

morphological open operation on each color channel with a 5 px radius spherical structuring element. Each channel is then normalized and sharpened by intensity before conversion to grayscale.

As any remaining marks within border regions can result in incorrect digit recognition, we limit analysis to the digit containing region. We multiply the column sum and row sum of the tag image and produce a map for the digit region. This map is binarized by Otsu thresholding and the bounding box coordinates for the foreground digit region are recorded. If a digit region is not found, the tag is marked as a false positive and removed from the following analysis.

Preprocessed tag images are next passed to the Tesseract OCR engine for digit prediction. The Tesseract OCR engine is trained with over 100 examples for each preprocessed digit. As tag images can be in two possible orientations (right-side up and upside down), digit predictions are made for both orientations. Digits with the highest three confidence levels are retained as the predictions for each orientation, and the highest average confidence level is used to indicate the correct orientation.

Track assembly module

Each video frame is analyzed independently up to this step. Relating frame-wise tag data into tracks is necessary to achieve an interpretation of bee activity (Figure 3.2d). For this purpose, the motion path of each tagged bee is assembled by linking tag data from frames based on centroid (*x*-*y*) location and tag size (area). The *x*-*y*-area feature vectors for sequential tag images are compared with a nearest neighbor algorithm. Euclidean distances between feature vectors are used to match a tag in one frame to a single tag in an adjacent frame. The track assembly

algorithm tolerates gaps between matches of 0.5 s to account for momentary occlusions. Matched tags are linked together into tracks with unique track identification numbers to represent tagged bee motion paths (Figure 3.5).

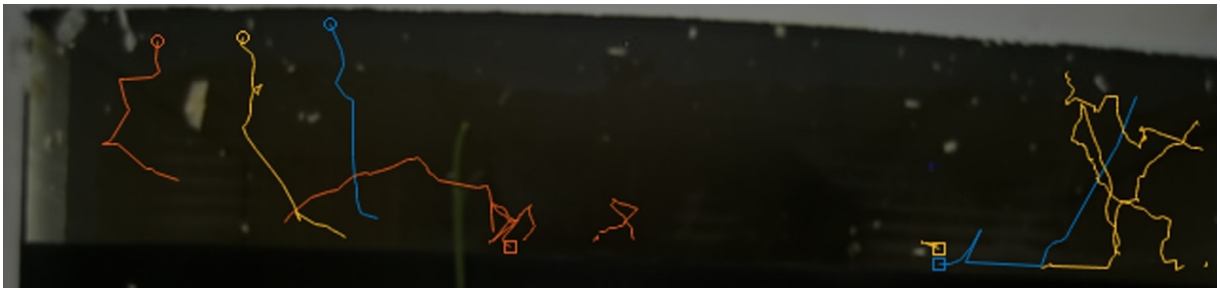

Figure 3.5. Tag tracks. The background image is overlaid with tracks of three tagged bees detected in a 50 min video. Each color (yellow, orange, and blue) represents the path of one tagged bee. Squares indicate the points of first observation, and circles denote the points of last observation. Gaps in the tracks indicate an occlusion of at least 1 s.

Graphical editor

The video analysis pipeline was designed to favor Type I errors in order to reduce manual screening time without missing tagged bees. Therefore, a full-featured graphical editor is provided to allow users to remove false positives and correct any errors in the automatically generated tag data (Figure 3.2e). The editor is designed to provide users with easy access to critical tag data, including tag digits, track identifiers, and false positive status (see supplemental).

The editor presents users with two tabular windows (Figure 3.6). The first window allows users to select one or more tracks. Once tracks are selected, the second window displays all tags included in those tracks. Selecting a tag will display tag-related video frames with a green

bounding box around the tag of interest. All other detected tags within frames are bounded in yellow. Edits can be made for individual tags, tracks, or groups of tracks for efficient bulk edits.

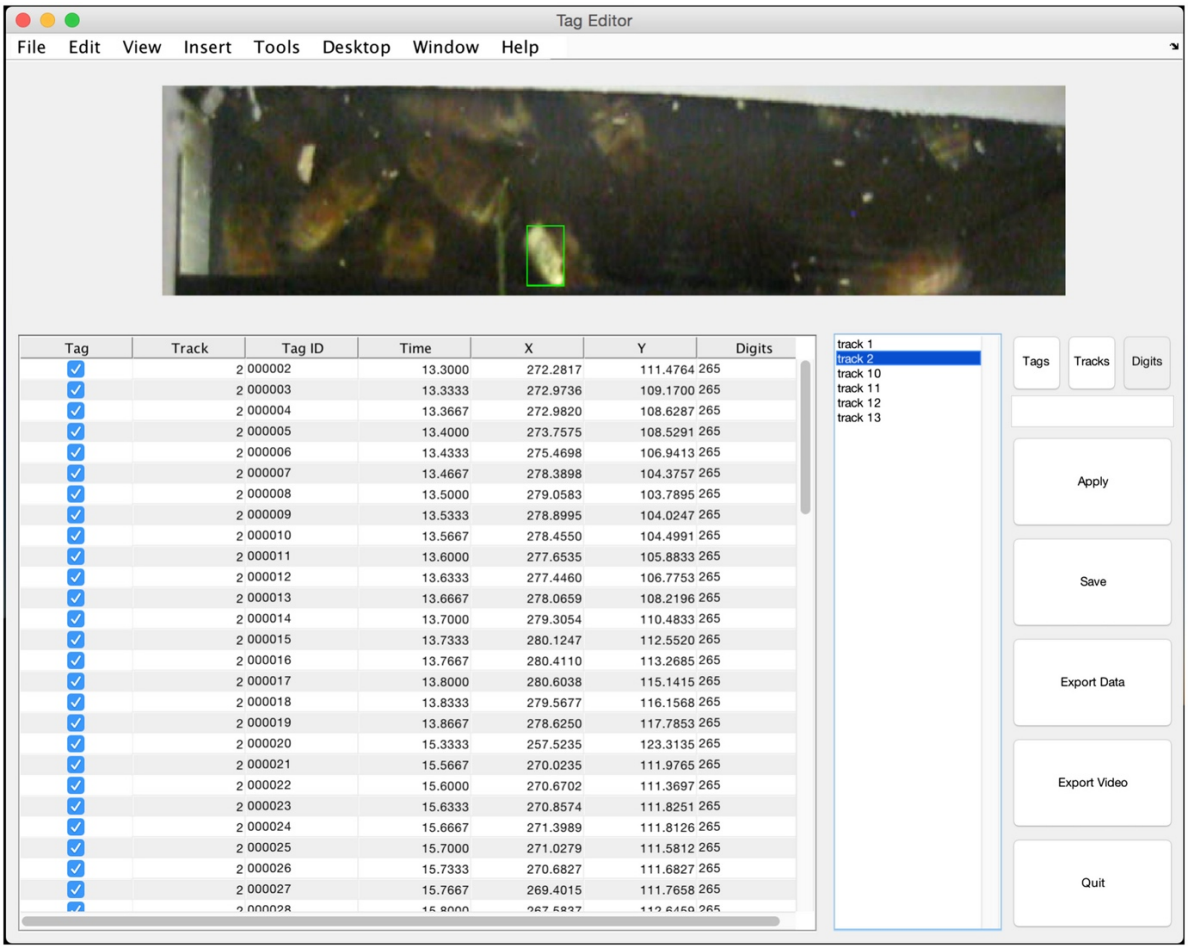

Figure 3.6. Graphical user interface. The graphical user interface for editing tag data automatically generated by the developed video analysis method.

After edits are made, users can export the annotations as either an Excel or CSV file. For an intuitive overview of the data, users can export a summary video that contains annotated video

segments of each tag track (see supplemental). Each track is represented by an MBR in a unique color and with tag digits displayed.

Evaluation

GRAPHITE has a human-in-the-loop design in which a user screens potential tracks that are automatically generated by the video analysis pipeline that preemptively minimizes the false negative detection rate. Monitoring was performed for ninety colonies at six apiaries resulting in 1,339 video files with a cumulative duration of *c*. 12,000 hr. Each video was processed in parallel using a 2.60 GHz Intel Xeon CPU (E5-2697 v3) at an average rate of 40 ms/frame. The false negative rate was determined by manually reviewing a random sample of 600 1-min videos segments. A set of 362 segments were randomly sampled from the 181 videos containing detected tags and 238 segments were randomly sampled from the remaining 1,157 videos. On average, a 1-min segment was reviewed for *c*. 20% of the videos where a tag was not detected. This review resulted in a false negative rate of 0%.

GRAPHITE detected 1160145 potential tag regions in 181 of these videos. Potential tag regions were manually reviewed with the graphical editor. 6,766 tags were identified (representing 450 tracks from 229 bees) resulting in a false positive rate of 99.4%. Despite the expectedly high false positive rate, the pipeline reduced the manual screening time by over 1,000× from *c*. 12,000 to *c*. 11 hr. without missing any tagged bees. In addition, false positives were mostly grouped into a small number of tracks that were quickly reviewed and removed in bulk.

Conclusions and Future Directions

GRAPHITE offers a low-cost, end-to-end animal movement tracking environment with a user-friendly graphical interface. We demonstrate the efficacy of the developed software with specific application to tracking tagged bees. The accessible and minimal hardware requirements along with the highly automated and flexible processing modules allow for many different experimental setups with various model organisms. This flexibility allows capabilities beyond video tracking software with no means to identify individuals traversing different tracking stations (Kimura et al., 2011; Tu et al., 2016).

A major advantage of this method is its ability to track individual insect movements in a low-cost field setting, as opposed to average movement rates that routine techniques such as powdered dye provide. Individual variation in movement can have large consequences for the ecology and evolution of species (Bolnick et al., 2003, 2011). For example, in infectious disease studies, certain individuals may be more likely to move and thereby have greater contact rates than other individuals. Highly mobile and connected individuals could thereby have major impacts on disease transmission, and in some cases act as super-spreaders (Lloyd-Smith et al., 2005).

In future work, the GRAPHITE digit reading module can be upgraded to other learning engines that allow corrections made via the editor to be fed back into the model for improved accuracy of digit recognition. The SVM classifier used to remove non-tag regions during tag detection could also benefit from the same feedback mechanism.

Supplementary Material

Figure 3.S1. Tagged bees.

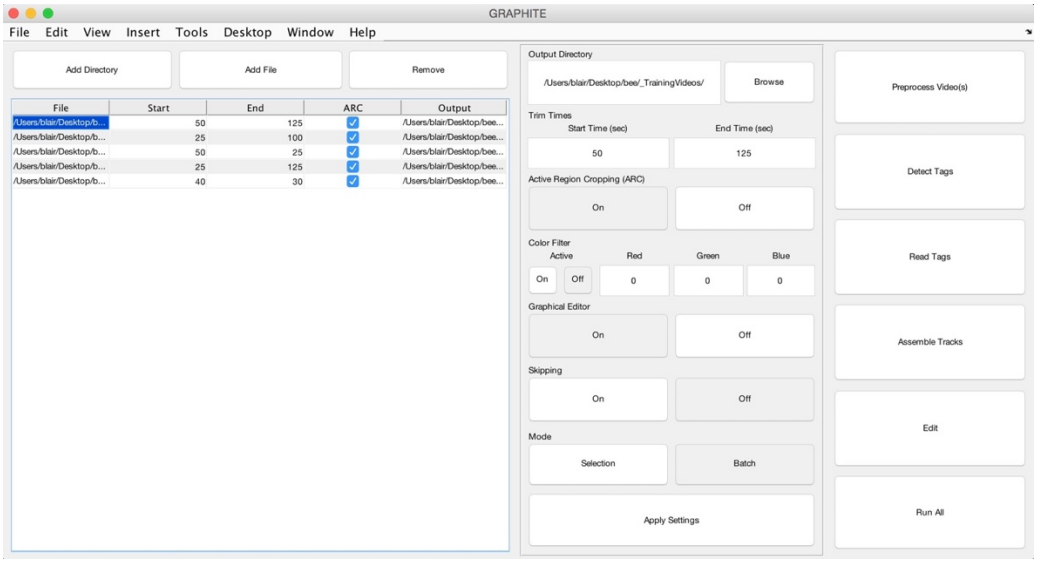

Figure 3.S2. Editor

CHAPTER 4

Reduced density and visually complex apiaries reduce parasite load and promote overwintering survival in honey bees

Introduction

There is broad concern over the sharp decline in managed honey bee colonies worldwide. For example, estimates indicate a decline of 61% in the number of managed colonies in the US from 1941 to 2008 (Dennis vanEngelsdorp & Meixner, 2010). Managed colonies are typically kept at a proximity and density that are many orders of magnitude higher than their feral or wild counterparts, which commonly range in density from 1-6 colonies per km^2 (Ratnieks et al., 1991; T. Seeley, 2007). Typical managed apiaries, in contrast, have colonies spaced ≤1m apart (T. D. Seeley & Smith, 2015) and may have thousands of colonies in densely arranged apiaries. Such dramatic altering of densities may have serious implications for colony health and survival, disease transmission, and drifting behavior (when bees enter a non-natal colony).

Population density has been studied as a key factor in ecological relationships going back to Malthus (1798), who first described density-dependent mortality and fecundity relationships. Density is known as an important driver of population dynamics across many taxa including insects (Stiling, 1988), fish (Lizaso et al., 2000), plants (Grace, 1999), and mammals (Fowler, 1987). Density can also be an important modulator of other ecological factors including landscape patterns such as patch size (Bowers & Matter, 1997) and ecological interactions such as the well-studied effects of prey density on predator consumption rates (Oaten & Murdoch, 1975). Studies have shown in other social insect species that competition for foraging space is

indicated in how close ant colonies are distributed (Gordon & Kulig, 1996) and worker ants in crowded colonies expend more energy which may impact colony performance and fitness (Cao & Dornhaus, 2008).

In agricultural systems high densities are common, and crowding can have negative consequences on animal performance. For example, high stocking densities can increase the regulation of stress genes and down-regulate immune genes in fish (Yarahmadi, Miandare, Fayaz, & Caipang, 2016), while cows in high-density management settings decrease the amount of time they spend feeding (Huzzey, DeVries, Valois, & von Keyserlingk, 2006). In honey bees, intracolony crowding can have detrimental effects on colony productivity and bee lifespan (Harbo, 1993; Rueppell, Kaftanouglu, & Page, 2009). Crowded foraging conditions can also initiate signals to stop foraging or decrease the recruitment of new foragers, thus reducing the foraging efficiency of the colony (Lau & Nieh, 2010; Thom, 2003). Further, intercolony crowding could have a detrimental effect on homing errors in drone bees and increase parasite loads (T. D. Seeley & Smith, 2015). In contrast, low-density apiaries could have a negative impact on the frequency in mating for polyandrous honey bee queens (Neumann, Moritz, & van Praagh, 1999).

Both theoretical and empirical ecological studies show that population density is also a key factor in driving disease ecology and dynamics (Roy M. Anderson et al., 1992; Hudson et al., 2002). Disease ecology predicts that higher host density and greater mixing of host populations will result in greater disease transmission and disease burdens (Ramsey et al., 2002) and can lead to the evolution of increased parasite virulence (Lively, 2006).

In many managed honey bee systems, there are high densities of honey bee colonies, crowding of foragers, and substantial levels of mixing of these colonies, either intentionally (transferring frames to equalize colony strength), or unintentionally through higher rates of bee drifting. Given that all of these factors are consistent with negative effects of density, we hypothesized that standard beekeeping management practices will increase exploitive competition between colonies, resulting in greater disease burdens and transmission, and overall negative effects on colony health, productivity, and survival. We carried out a two-year study in which we experimentally controlled colony density and monitored colony health, parasite burden, and bee drifting following controlled inoculations with the obligate ectoparasitic mite *Varroa destructor*. We chose this parasite because it is widely believed to be the greatest biotic threat facing honey bees (Rosenkranz et al., 2010).

The purpose of our study was to experimentally test the role of colony density driving colony health, productivity, and parasite burdens. Specifically, we set up six experimental apiaries, each consisting of eight honey bee colonies. In three apiaries, we placed colonies in a linear array, spaced 1m apart (high-density apiaries). In the other three, we placed colonies 10m apart in a circle, with hive entrances facing outwards. In order to further discourage drift in these low-density apiaries, colonies were painted different colors, placed at different heights, and marked with different symbols. We initially cleared all colonies of *V. destructor* and subsequently inoculated two randomly chosen colonies per apiary with 200 adult mites to reflect naturally occurring mite infestations. We then quantified the effect of varying colony density and arrangement, experimentally crossed with *V. destructor* infection on four aspects of honey bee

health: 1) colony strength (measured by adult bee population, brood population, and honey production); 2) colony survival; 3) *V. destructor* reproduction and spread; and 4) worker-bee drifting behavior. We predicted that *V. destructor* levels in high-density settings will increase faster and stay at a higher level than in low-density apiaries. Further, we expected colonies in the high-density apiaries to have lower colony strength and survival, as well as greater mite burdens, mite transmission and worker bee drifting. Finally, we expected to observe higher drifting rates in the high-density apiary and more drift in experimentally infected colonies.

Materials and Methods

Overview

In order to determine the effect of honey bee colony spacing and arrangement on colony health and disease spread, we compared a linear high-density colony arrangement with a circular low-density arrangement. We established three high-density apiaries and three low-density apiaries in June 2015 around Athens, Georgia, USA, maintained by the University of Georgia Honey Bee Lab. Each apiary consisted of eight colonies housed in standard five-frame Langstroth nucleus hive boxes, for a total of 48 colonies. We arranged high-density colonies in a straight line with 1m between colonies and with all entrances facing in the same direction (Fig. 4.1A). High-density colonies were all painted white and placed at a consistent height (200 cm) above the ground. This arrangement is typical of many beekeeping apiaries. We chose a circular layout for the colonies in the low-density apiaries with 10m between colonies and all entrances facing outwards from the center of the circle (Fig. 4.1B). To maximize bees' ability to visually distinguish between colonies, we painted the low-density colonies different colors, painted

different symbols at their entrances, and placed the colonies at three different heights above the ground (0, 200, 400 cm, with the spatially closest colonies at different heights).

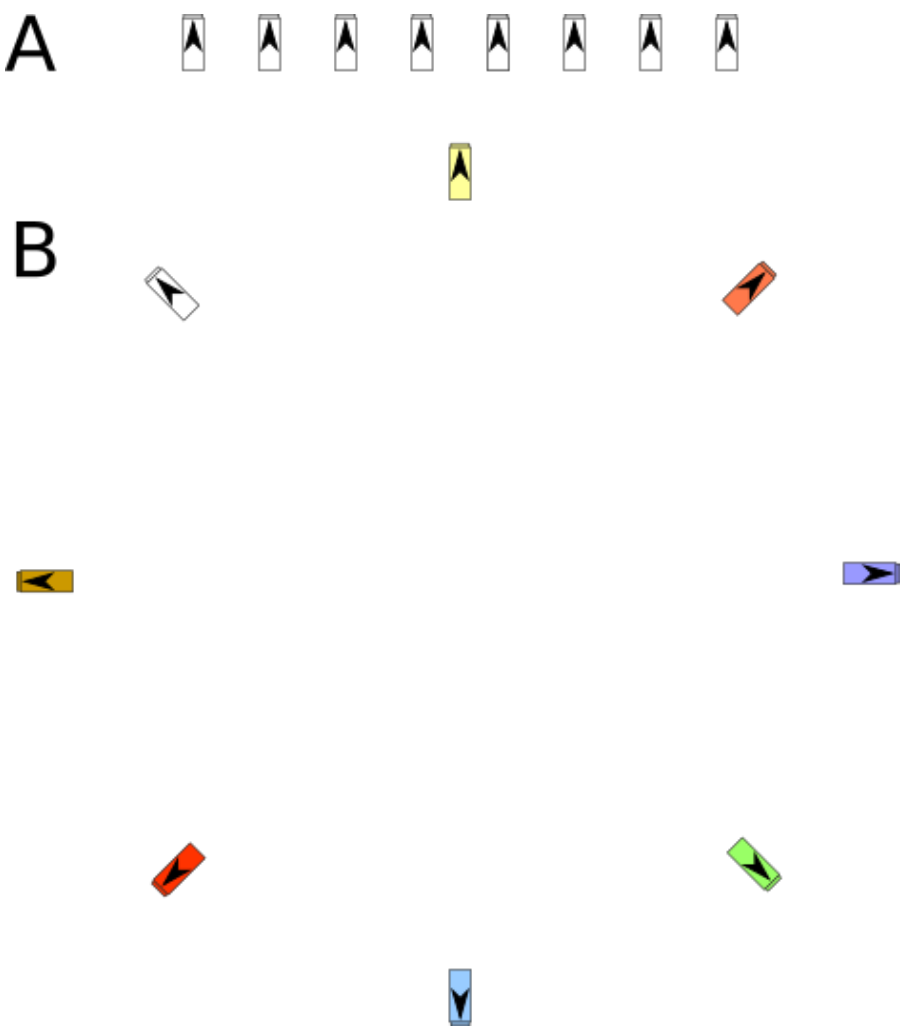

Figure 4.3. Scale representation of apiary arrangements from above. **(A)** shows a high-density apiary and **(B)** shows a low-density apiary. Each arrangement was replicated 3 times. The direction of the arrow indicates the colony entrance.

To minimize variation, we worked with highly standardized colonies. We established each colony with a mated queen and 1.1 kg (2.5 lb.) of adult bees, shaken into a package. The queens

were all from the same queen breeder in southern Georgia, USA and the adult bees were all from common-source backgrounds. The packages were treated for *V. destructor* using two separate methods to ensure maximum possible mite clearance. We first used the powder sugar method (Aliano & Ellis, 2005) to encourage bee grooming and mite dislodgement. The packages were then placed in a dark room overnight at 16.6°C (62°F), and sprayed with sugar water one hour prior to the application of 30mL of an oxalic acid solution (Milani, 2001). Each package was installed three days later in its randomly-assigned apiary. To investigate the effect of both apiary density and colony arrangement on parasite dynamics we randomly selected two colonies in each apiary and inoculated these colonies with 200 adult *V. destructor* mites. Mites were collected from source colonies outside of the experiment by sifting powdered sugar over the colony and collecting dislodged mites at the bottom of the colony. We used small natural fibered paintbrushes to place mites on damp coffee filters. We kept mites in an incubator set at 35°C (95°F) until all mites were collected. We then transferred all mites evenly to an open brood frame and waited one minute to ensure mites had attached to the wax cells in a colony. We emphasize that inoculated colonies are closer to naturally occurring conditions in typical beekeeping practices than the near-complete clearance treatment colonies in our experiment. To maintain our focus on these original colonies (and their queens), we enacted swarm control on colonies likely to swarm by splitting those colonies. We standardized swarm control in this way to ensure small colonies were not jeopardized by the procedure. A total of 38 out of the 48 colonies were split to prevent swarming, all occurring in March 2016. We employed a Fisher's exact test to determine that there was not a significant difference ($P = 0.29$) in the number of splits between our treatment groups. During the experiment, we did not conduct any control measures against *V. destructor*. We fed colonies a syrup solution, a common practice in

beekeeping management, that we standardized by giving equal volumes across all colonies regardless of need. We continued the experiment from June 2015 through May 2017, at which point only 12 of the original 48 colonies were still surviving.

Data Collection

Colony strength assessments

To determine the effect of apiary density and arrangement on colony health we took periodic health measurements throughout the experiment. We followed the colony strength assessment guidelines described in Delaplane et al. (2013) to measure the adult bee population, amount of brood, and amount of honey stored for each colony. We performed these colony assessments seven times over the two years of the experiment. We also recorded the date each colony was found to be dead and last known date it was alive for survival analyses.

Measuring V. destructor infestation

We measured *V. destructor* infestation levels in three different ways. First, we used an alcohol wash method described by Fries et al. (1991). This method involves destructively sampling 300 bees from a colony in alcohol and counting bees and mites (which detach from the bees allowing easier counting) to get a relative mite level on the adult bee population. We took nine alcohol wash samples throughout the experiment. Second, we used sticky boards (Branco, Kidd, & Pickard, 2006), a standard method to evaluate *V. destructor* levels in a colony by collecting mites that fall and become entrapped on a board placed at the bottom of a colony. We measured mite levels with sticky boards seven times through the first year of the experiment including one immediately following package installation to confirm colonies were *V. destructor* free. Sticky

board sampling was not continued in the second year of the experiment for logistical reasons. Third, we measured the mite population in brood cells by opening 100 covered brood cells in each colony and counting the number of mites. We measured mite levels in brood cells six times throughout the experiment.

Drifting behavior

To quantify potential effects of apiary layout on drifting behavior, we tagged individual bees with uniquely numbered tags and used video cameras located above each colony entrance to record bees entering and leaving (Rossetti et al., 2017).We performed the tagging and video capture in September 2015. We tagged newly emerged workers, ensuring tagged bees originated from that colony. Tagging was split into three consecutive weeks. Each week we tagged up to 100 bees in all colonies from one high-density apiary and one low-density apiary. On days 24 and 25 post-tagging, we recorded five to seven hours of video in one-hour segments at each colony. With computer science collaborators, we developed a Matlab video analysis pipeline called GRAPHITE to examine each frame of video and extract frames containing tags (Rossetti et al., 2017). Using the pipeline, we identified tags in the videos and determined: which colony the tagged bee was from; whether the bee was entering or exiting; and whether the bee was carrying a pollen load.

Statistical Analysis

Overview

We explored the effect of two main explanatory variables on several metrics of honey bee health, described above in the data collection section. The explanatory variables are: (1) density (high

versus low: the composite measure of apiary density, colony arrangement and colony color and symbol); (2) parasite treatment (cleared versus inoculated: whether the colony was inoculated with a standardized *V. destructor* infection at the start of the experiment). We conducted three general classes of analyses, based on the following response variables: 1) colony-level mite infection and colony health parameters; 2) colony-level survival; and 3) drifting behavior.

Colony strength and mite infection

Longitudinal repeated measures and nested designs, used in our experiment, can result in temporal and within-subject autocorrelation which violates the assumptions of independence for parametric and linear regression methods. Therefore, we used generalized estimation equations (GEE) to account for repeated measures including autocorrelation. We used the 'geeglm' function in the 'geepack' package v.1.2-1 (Højsgaard, Halekoh, & Yan, 2006) in R v.3.4.2 (R Core Team, 2017) to specify and evaluate the GEE models in particular because it allows for longitudinal data with missing observations. We blocked the data by apiary and colony and utilized an autoregressive (AR1) autocorrelation structure. Each initial model was specified using the two explanatory variables and their interaction. In cases where the GEE was unable to converge, a Wilcoxon signed-rank test was applied to each sample date and a Benjamini-Hochberg procedure was completed to adjust the false discovery rate of testing multiple comparisons.

Survival analysis

We performed survival analysis on the colonies using both explanatory variables and their interaction, apiary density and colony inoculation status. We also completed a separate winter

survival analysis since *V. destructor* infection is implicated in reduced winter survival (Dainat, Evans, Chen, Gauthier, & Neumann, 2012a; van Dooremalen et al., 2012). Colonies were inspected periodically throughout the experiment and exact timing of colony death could not be determined. Therefore, we used an interval of date of observed colony death and date of last known colony viability. Given this data structure, we analyzed survival with Cox proportional hazard models with interval censoring via the 'frailtypack' package (Rondeau, Mazroui, & Gonzalez, 2012) in R. Since the winter survival data consisted solely of binomial data at a single time point (i.e., survived vs. did not survive through the winter), we employed a separate binomial-errors generalized linear mixed model (GLMM) using the lme4 package (Bates, Maechler, Bolker, & Walker, 2015) in R with random effects for colony identity, nested within apiary.

Drifting data analysis

In order to understand how an inoculated colony status and apiary density/arrangement affect bee drifting behavior, we ran GLMMs using the lme4 package, with counts of drifting events modeled with Poisson errors. Drifting may also be correlated with proximally located neighbors. We employed a Mantel correlogram test to determine the relationship between the amount of drift and inter-colony distance.

Results

Overview

We collected extensive data on the strength of the colonies, mite levels, and the movement of individual bees throughout the experiment. The colony health assessments resulted in 202

measurements each of: the adult bee population, brood coverage, and honey storage. In order to evaluate *V. destructor* levels throughout the experiment, we collected 316 sticky boards, 279 alcohol washes (each containing approximately 300 worker bees), and 208 counts of mites in the brood (each including 100 brood cells). We recorded and used the GRAPHITE pipeline to process approximately 290 hours of video tracking individually tagged bees. We observed 120 uniquely tagged individual bees at 242 separate times.

Colony strength

The GEE model of honey storage did not converge, likely due to the very strong seasonal pattern in honey production. A Wilcoxon signed-rank test was completed for each of the sample dates for honey stores and after the Benjamini-Hochberg procedure for multiple comparisons was applied, low-density colonies had significantly more honey stores on four of five sample dates (P = 0.020, 0.010, 0.040; Fig. 4.2), with the only non-significant sample date being the first sample. Neither of the GEE models for the adult bee population and amount of brood had any significant terms.

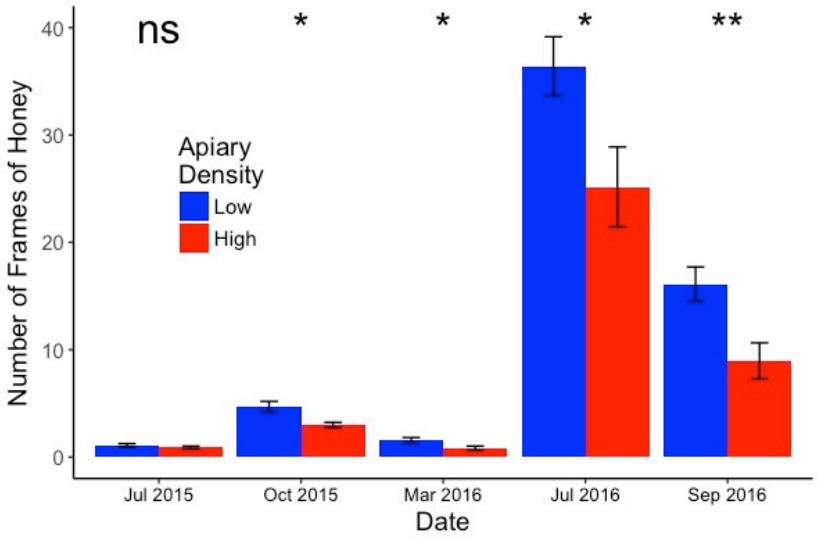

Figure 4.4. Honey production over time grouped by apiary density. A Wilcoxon test was applied to the density treatment comparison in each month. The Benjamini-Hochberg procedure was completed to confirm the significance of the multiple comparisons. Error bars represent standard error of the mean. *: P<0.05; **: P<0.01; ns: not significant.

Mite infection

A GEE model of the sticky board data showed a significantly ($P = 0.0188$) positive effect of inoculation on mite levels, but no effect of apiary density (Fig. 4.3). The GEE model did not show a significant relationship between apiary density or inoculation on mites per 100 brood cells. However, there was a significant interaction ($P = 0.0176$) between inoculation status and apiary density. The interaction indicates that that there were significantly more mites in brood when colonies were both inoculated and located in a high-density apiary (Fig. 4.4). The GEE for the mite levels as assessed by alcohol washes did not have any significant terms.

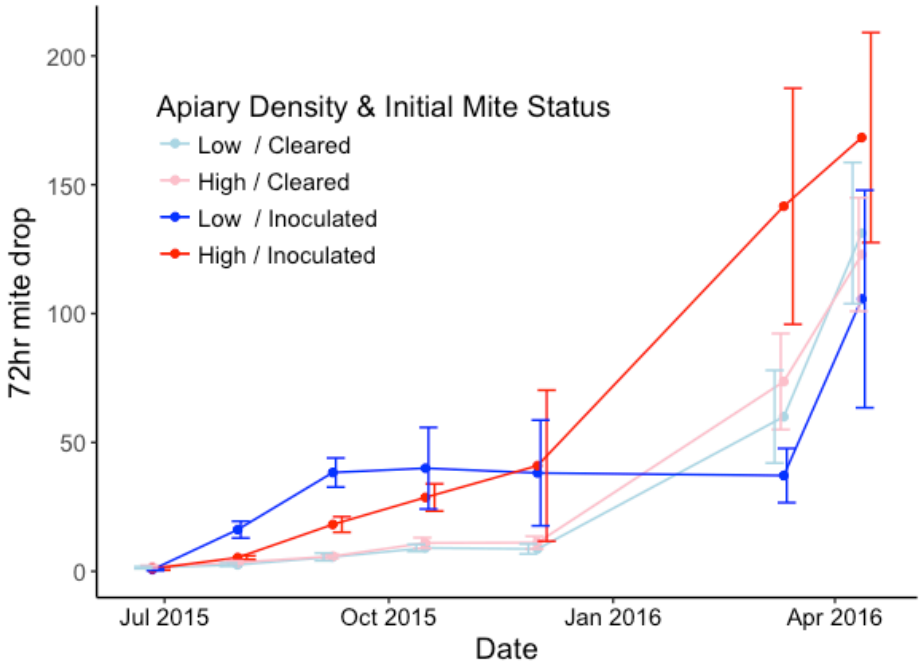

Figure 4.5. Mite count by sticky board separated by treatment and inoculation over time. Note the cleared colonies in both density treatments stayed at low levels throughout the first

winter while inoculated colonies had a steady increase. By the end of the first year however, the cleared colonies had reached the same infection levels as the inoculated colonies. Error bars represent the standard error of the mean. A GEE found a significant positive effect for inoculation on mite numbers ($P = 0.0188$).

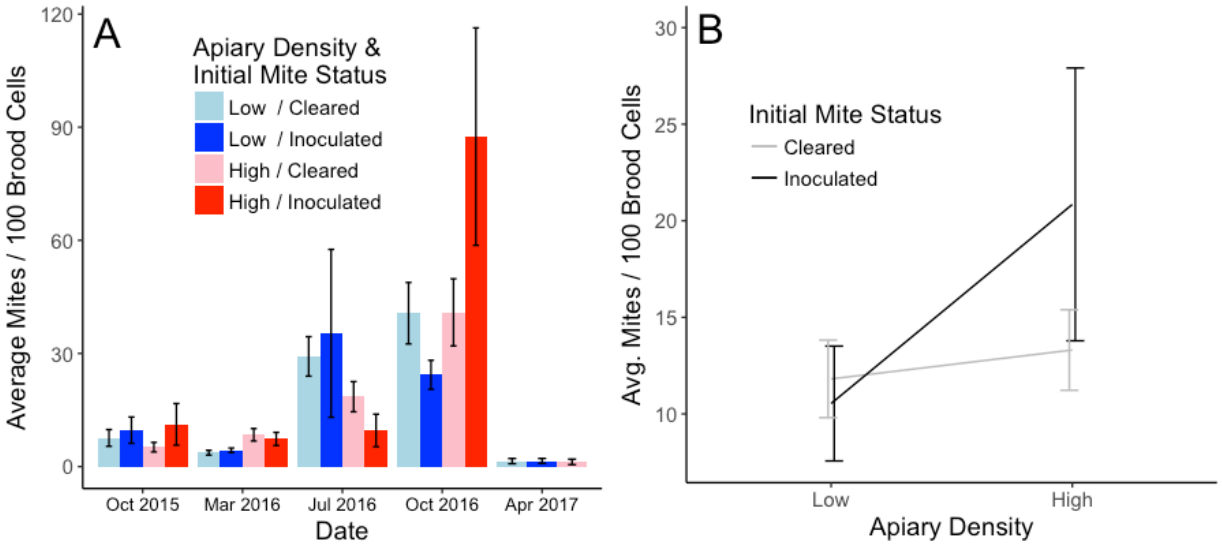

Figure 4.6. Mites levels in brood. (A) Mites in 100 brood cells by inoculation status and apiary density. **(B)** A GEE found a significant interaction ($P = 0.0176$) between inoculation status and density. Error bars in both plots represent the standard error of the mean.

Survival analyses

The Cox survival analyses did not show any significant relationship between colony density and inoculation status or their interaction (Fig. 4.5A). However, the binomial-errors GLMM showed that apiary density was significantly related to winter survival ($P = 0.037$), with colonies in the lower-density apiaries more likely to survive winter (Fig. 4.5B).

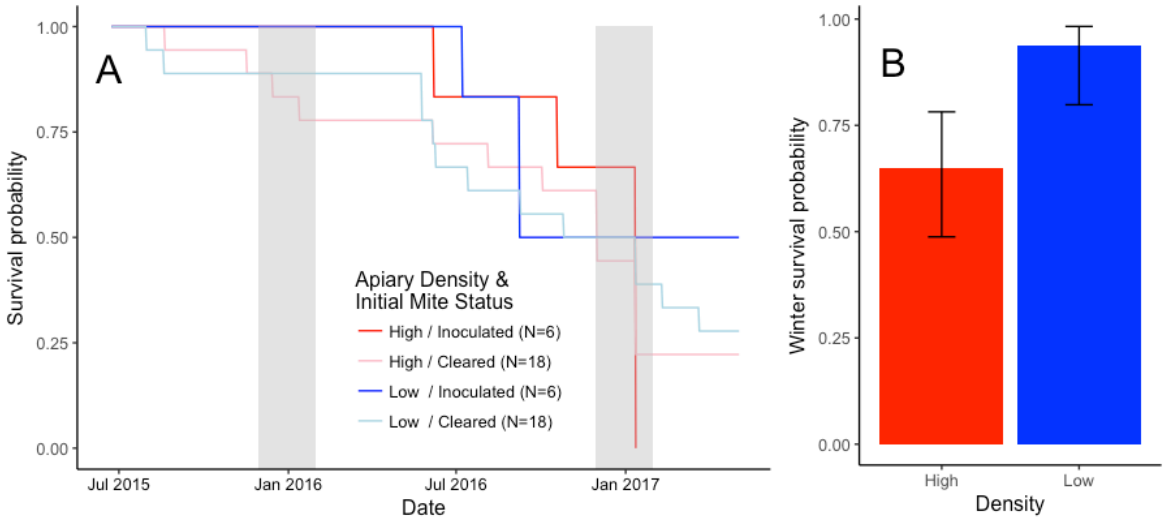

Figure 4.7. Survival curves and winter survival. **(A)** Survival curves by apiary density and inoculation status. Gray bars show the winter months of December and January. Note the large drop in survival over the second winter in the high-density apiaries. **(B)** Effect of density on winter survival (Note: a colony surviving until the start of the second winter is counted twice in this figure). A logistic model of winter survival did find that colonies in low density apiaries were significantly more likely to survive the winter ($P = 0.037$). Error bars in B show binomial confidence intervals.

Drifting analyses

Bees in high-density apiaries were significantly ($P = 0.048$) more likely to drift based on a mixed effects model (Fig. 4.6). We observed that 25.0% of all tagged bees in the high-density apiary drifted while 7.5% in the low-density apiary drifted. On four occasions we observed a drifting bee entering the same (non-natal) colony multiple times, and once we detected a bee that had drifted, return to its natal colony. Nearly all drifting in the high-density apiaries was to the nearest neighbor (1m) to the focal bee's natal colony (24 of 26 drifting events), with one drifting event each to the second and sixth-nearest neighbor. In the low-density apiaries, all three observations of drift occurred at the nearest-neighbor colonies (10m distant). While nearest colony neighbors were significantly associated with drifting ($P = 0.047$, Mantel correlogram),

other colony positions were not. Inoculation status was not significantly related to drifting behavior.

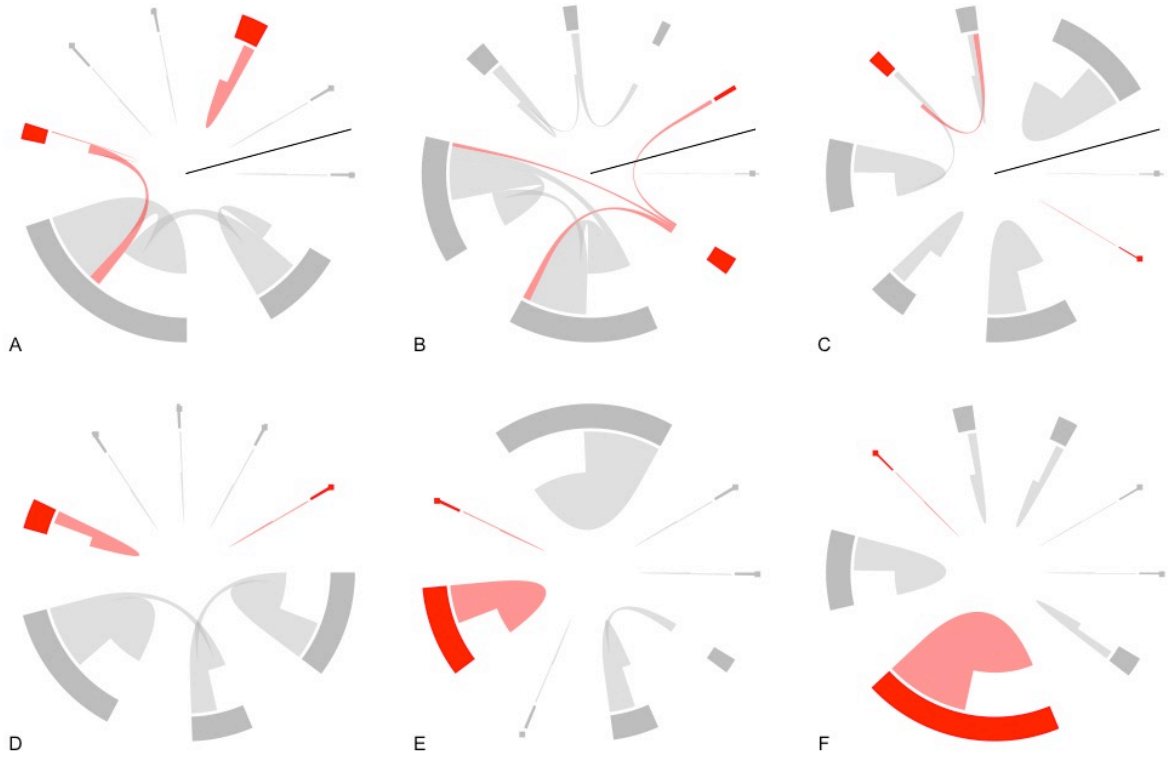

Figure 4.8. Representation of drift within each of the apiaries. Each panel shows eight colonies as subsections of the circle's circumference (the width of these subsections indicates number of unique bees observed at colony) and bee movements (individual bees are indicated with thin lines, so that large numbers of observed movement result in large "wedge-like" shapes). Direction of drift is indicated by how close an individual bee movement line is to the colony representation on the circle's circumference: a bee drifts to a colony where the line is close to the colony and came from a colony where there is a large gap between the line and the colony. Red indicates inoculated colonies while gray indicates cleared colonies. Panels **(A-C)** represent the high-density apiaries; these are represented in a circular format here for comparison to the low-density apiaries; the black lines in **(A-C)** represent where linear arrangement of them would be split to be put back in a straight line. Panels **(D-F)** represent drift in the low-density apiaries. All drift in **(D-F)** is to the nearest neighbor. In contrast, there is increased drift in **(A-C)** and there are two instances of drift beyond the nearest neighbor (both in **B**). The inoculation status of a colony was not found to have a significant effect on drift.

Discussion

Overview

Managed honey bee colonies are kept at densities that far exceed naturally occurring densities in feral colonies. Current beekeeping practices which favor these high densities for logistical reasons are predicted to have detrimental effects on disease spread and colony survival (Brosi, Delaplane, Boots, & de Roode, 2017). Our present work generated four main findings related to how density affected honey bee health. First, increased colony density significantly decreased honey production. Second, increased density resulted in significantly higher levels of *V. destructor* infestation of honey bee brood. Third, increased apiary density had a significant detrimental effect on a colony's winter survival. Finally, drift was significantly increased in high-density apiaries.

Colony strength

We found that honey production was decreased in high density apiaries (Fig. 4.2). Colony honey hoarding is positively associated with adult bee population (Farrar, 1937), but as we found no concomitant reductions in either adult bee or brood populations, our present findings are better interpreted as direct effects of either 1) apiary density conditions or 2) increasing numbers of *V. destructor* mites.

First, honey bee colonies may operate less efficiently in a high-density setting because of confusion or mixed signaling, leading to lower forager efficiency. For example, one study found that forager crowding prompts foragers to signal to other bees to stop foraging (Lau & Nieh, 2010).

Second, increasing mite infestations may negatively impact colony honey stores. However, (Murilhas, 2002) could not detect progressively negative effects of mites on brood, adult populations, or honey stores until mite populations reached extreme highs. Moreover, he could detect no direct effects of colony mite level on honey stored per bee per day and concluded that putative effects of mites on colony honey stores are an indirect effect of crashing adult bee populations. Our data indicated increased mite burdens in the brood of inoculated colonies in high-density apiaries, but, like Murilhas, this occurred late in the experiment. However, in contrast to Murilhas, honey stores in our inoculated, high-density colonies were consistently lower throughout most of the experiment. In short, the effects of *V. destructor* on honey hoarding, at either the individual or colony level, remain ambiguous. However, it is well-known that *V. destructor* mites, in addition to causing pathology themselves, transmit pathogenic viruses (Dainat et al., 2012a; Nazzi et al., 2012), and it is possible that the observed reduction in honey stores was partly driven by these viruses. Further experiments are necessary to explore this possibility.

Mite infection

Our finding of a significant interaction between inoculation status and apiary density in brood mite levels (Fig. 4.4B) indicates that greatest mite levels occurred in inoculated colonies in high-density apiaries. Because our inoculations mimicked natural *Varroa* infestations, this result importantly demonstrates that the intensification of bee keeping increases the risk of high mite burdens. This high mite burden effect was most notable late during the experiment, which is also the time when colonies in high-density apiaries showed increased winter mortality, suggesting

that high mite burdens contributed to winter mortality. Our experiment also showed that one-time clearing of mites at the beginning of the season using a relatively simple method (oxalic acid spraying) can significantly suppress mite growth. This was most apparent during the first five months of the experiment, when mite levels measured by sticky boards in the cleared colonies stayed relatively low. During this same time, mite levels steadily increased in the inoculated colonies. Ten months into the experiment, mite levels in all colonies reached similar levels (Fig. 4.3), suggesting that mite transmission was low during the initial months and increased dramatically after the mite populations reached a certain threshold in the inoculated colonies. This hypothesized transmission between colonies is consistent with other studies. For example, one study found increased invasion rates and population levels of *V. destructor* in colonies that were surrounded by higher densities of colonies (Frey & Rosenkranz, 2014). In addition, colonies placed as far apart as 100m exhibited equalized mite numbers over time (Nolan & Delaplane, 2017) and genetic analyses are consistent with substantial horizontal transmission of mites between colonies (Dynes et al., 2017).

Survival analysis

Interestingly, survival analysis over the course of the entire experiment did not indicate greater mortality for inoculated than cleared colonies or differences between high- and low-density apiaries. This may have been partly due to other factors. For example, background mortality as driven by failing queens or other factors may have masked infection and density effects on mortality during the early stages of the experiment. In addition, as described above, mite dynamics suggested between-colony transmission of mites, thereby making cleared and inoculated colonies more equal in mite numbers.

However, when survival analyses were restricted to overwintering mortality, we did find a significant effect of apiary density. Indeed, while exactly 50 percent of inoculated colonies survived through two winters in low-density apiaries, not a single inoculated colony survived through two winters in the high-density apiaries (Fig. 4.5A). Our determination that winter survival was significantly greater in lower density apiaries is an important finding for beekeepers. Winter mortality is currently one of the greatest challenges facing beekeepers in moderate to cold temperate regions of the globe (Dainat, Evans, Chen, Gauthier, & Neumann, 2012b; van Dooremalen et al., 2012; D. vanEngelsdorp, Hayes, Underwood, & Pettis, 2010), and our experiment suggests that this mortality can be significantly reduced by managing colonies at lower densities. There are at least two factors that could have contributed to this increased survival in our experiment. First, low-density apiaries had higher honey production. Since colonies in temperate regions need adequate honey supplies to survive the winter (Free & Racey, 1968) it follows that the increased honey production could play a role in increased survival. While the colonies in our study were fed supplemental sugar syrup (standardized across colonies), as is standard practice in beekeeping management, there could have been population-based variation among colonies in their ability to convert this to honey stores (Farrar, 1937), along with variation in flower foraging rates, as well as differences in consumption rates which we did not measure. Second, the significantly higher mite levels in capped brood for inoculated colonies in high-density apiaries in the sample date before the second winter (Fig. 4.4A) may have contributed to the 100 percent mortality of the remaining colonies that winter.

Our results are consistent with another study, in which overwinter survival was also found to be significantly lower in a crowded apiary compared to a group of dispersed colonies (T. D. Seeley

& Smith, 2015). However, in that study, bee swarming was not suppressed, making that work less applicable to common beekeeping practices.

Drifting

It has long been known that distance, entrance direction, and apiary layout can affect drifting behavior in honey bees (Jay, 1966). We used these insights to minimize drifting in our low-density apiaries by placing colonies 10m apart in a circle, at different heights and with colonies facing outwards and being painted different colors and marked with different symbols. These measures were effective, reducing drift from 25 percent in the high-density to 7.5 percent in the low-density apiaries. This quantification is important for managers considering how to slow down disease transmission and for disease modelers working to parameterize the effect of space on disease spread. These observed drifting rates fall within the large ranges in the proportion of drifting individuals (0-89%) found in other studies (Free, 1958; Neumann, Moritz, & Mautz, 2000). Our observation of four bees staying in non-natal colonies after drifting suggested that drifting bees could permanently switch colonies after drifting rather than go back and forth, though these are very small sample sizes. Our experiment also showed that the majority of drifting occurred between nearest neighbors. Together these findings suggest relatively lower amounts of disease transmission, compared to the alternatives of bees drifting back and forth between natal and non-natal colonies, or bees randomly drifting to any colony which causes mites to be spread greater distances from the original inoculation. Although our experiment was not able to directly quantify between-colony transmission of mites through drift, it is highly likely that increased rates of drift increase disease transmission. The video-tracking of drifting

bees we used here (Rossetti et al., 2017) provides a fruitful method to further study the role of drifting in disease transmission.

Conclusion

Current apiary management utilizes colonies in high density arrangements for practical and logistical reasons. However, our experiment, using replicated high- and low-density apiaries, shows that high-density management can be detrimental to colony-level health and productivity. Our results suggest that by lowering the apiary density and making colonies visually distinctive, beekeepers can increase colony productivity, reduce overwinter mortality, and potentially reduce the spread of diseases within the apiary through reduced drift. These steps are relatively modest and should be possible to implement in many beekeeping operations.

CHAPTER 5:

Assessing virulence of *Varroa destructor* mites from different honey bee management regimes

Introduction

European honey bee (*Apis mellifera* L.) colonies have experienced widespread losses in the past decades in the US and Europe, which is a particular concern due to the importance that honey bees play in agricultural pollination services critical to both the economy and human health (National Research Council 2007; Pettis and Delaplane 2010). While honey bees are facing numerous challenges, from pesticides to land use changes, parasites have emerged as a significant factor in these losses (Potts et al. 2016). In the first half of the 20th century the obligate ectoparasitic mite *Varroa destructor* (Acari: Mesostigmata: Varroidae) made a sustained host switch from the Asian honey bee (Apis cerana) to the European honey bee (Rosenkranz et al. 2010). Since that time *V. destructor* has spread around the world and become the largest pathogenic threat, termed "varroosis", currently facing the beekeeping industry (Sammataro et al. 2000; Rosenkranz et al. 2010). In addition, *V. destructor* is a vector for a range of economically important viruses and the interaction between these viruses and *V. destructor* is considered the single most important factor in honey bee colony losses worldwide (Boecking and Genersch 2008; Wegener et al. 2016).

In the honey bee system, the dynamics by which *V. destructor* mites interact with honey bee colonies can vary drastically. Feral honey bee colonies, those colonies that are unmanaged by humans, typically occur at a density of around one per square kilometer in the USA (Seeley 2007). In these isolated settings, bees and mites are not likely to interact with individuals from

other honey bee colonies on a regular basis. In contrast, industrial beekeeping operations can manage thousands of colonies in a much smaller area. Virulence-transmission trade-off theory (Boots and Sasaki 1999; Boots et al. 2004; Alizon et al. 2009; Lion and Boots 2010; Webb et al. 2013) suggests that the higher colony densities, coupled with great rates of between-colony mixing, found in managed honey bee operations could favor *V. destructor* mites with increased reproduction and virulence. According to trade-off theory, natural selection can favor virulent parasites that cause reductions in host fitness by selecting for between-host parasite transmission (Levin and Pimentel 1981; Anderson and May 1982; Ewald 1983; Bremermann and Pickering 1983; Antia et al. 1994; Bull 1994; Levin 1996; Boots and Mealor 2007). This theory is based on the assumption that both between-host transmission and virulence (usually defined as parasite-induced host mortality) increase with increasing within-host parasite reproduction, an assumption that has found empirical support in a wide range of systems (Messenger et al. 1999; Mackinnon and Read 1999, 2004; Jensen et al. 2006; De Roode et al. 2008; Hawley et al. 2013). As a result, parasites are generally expected to evolve an intermediate level of within-host growth and consequently virulence: parasites with too low growth are selected against because of low between-host transmission, while parasites with too high growth are selected against by killing the host before transmission can occur (Levin and Pimentel 1981; Lenski and May 1994). The expected level of optimal virulence, however, depends strongly on the density of susceptible host individuals, as well as the spatial structure of the population (Kamo and Boots 2006; Boots and Mealor 2007). In well-mixed high-density host populations, transmission opportunities are ample and the cost of high virulence in terms of killing hosts before transmitting is low. This type of environment is common in agricultural settings and according to theory can favor the evolution of higher virulence (Kennedy et al. 2015). In contrast, in highly structured low-density

host populations, transmission opportunities are rare and costs of virulence are high. As a result, evolutionary theory predicts selection for greater virulence in highly dense and well-mixed populations than in low density population with high spatial structure. Evidence for such increased virulence evolution due to greater host density remains lacking, but it is now clear that agricultural the new selection practices imposed by agriculture can select for more deadly parasites, as has been demonstrated, for example, in the increased virulence of the virus causing Marek's disease due to vaccination of chickens (Atkins et al. 2013; Read et al. 2015).

The contrasting transmission conditions driven by density and population mixing are crucial to honey bees, where industrial beekeeping practices have shifted the host-parasite interaction from low densities with high spatial structure in feral bees to highly dense and well-mixed populations in industrially managed bees. Thus, based on virulence-transmission trade-off theory, we would expect greater selection for parasite growth and virulence in managed honey bee colonies than in feral colonies (Brosi et al. 2017). By promoting increased transmission opportunities, management practices, such as moving frames of brood to boost struggling colonies (a common beekeeping practice), and the high rates of mixing of managed bees due to migratory beekeeping could contribute to *Varroa destructor* virulence evolution and may be responsible for maintaining virulent *Varroa destructor* genotypes in managed honey bee colonies (Fries and Camazine 2001; Calderón et al. 2010; Guzmán-Novoa et al. 2010; Brosi et al. 2017).

Our current understanding of these relationships in the honey bee system is limited, but there is a small amount of research that is consistent with the virulence-transmission trade-off hypothesis. Based on a comparison of bee colonies infected with mites from different backgrounds, Seeley (2007) proposed that avirulent mite strains may explain feral colonies surviving *V. destructor* better than feral bee resistance to the mites. Migratory beekeepers have reported more colony

mortality than small scale beekeepers (Dahle 2010). More *V. destructor* transmission was observed in higher-density (compared to lower-density) honey bee colonies (Nolan and Delaplane, 2017, Dynes unpubl. data). Furthermore, studies indicate a genetic basis for variation in mite virulence, confirming that virulence could be acted upon by natural selection (De Jong and Soares 1997; Anderson 2000; Corrêa-Marques et al. 2002, 2003).

To understand if mites from different management regimes have evolved contrasting virulence, we completed a large and replicated study at the apiary level to examine varroosis using a highly standardized approach which to our knowledge has not been previously attempted. Specifically, we examined how mites evolved under different honey bee management intensities (feral, lightly managed, and heavily managed) differentially reproduced and affected no fewer than 88 honey bee colonies from a common, lightly managed background. We measured both mite burdens and effects on colony health over more than two years. The strength of our approach lies in our colony and queen standardization, mite clearance, standardized inoculations, and replication at the apiary level.

Materials and Methods

Overview

We performed a virulence assay on *V. destructor* mites collected from different honey bee management backgrounds on bees obtained from a similar lightly managed background such as you would find with backyard beekeepers. Our purpose was to determine whether management conditions have selected for mites with differential growth rate and/or virulence and whether the colony response differs between these backgrounds. We established eight apiaries, each consisting of 11 colonies, for a total of 88 colonies, in June 2015 around Athens, GA, USA,

maintained by the University of Georgia Honey Bee Lab. Colonies were initially cleared of mites and subsequently inoculated with mites (N = 100 in multiple doses over the course of two weeks). We had 7 mite donor colonies for each management background type (e.g. feral colonies). In order to ensure a sufficient quantity of mite inoculations for each experimental colony mites were pooled from between 1 and 3 of the 7 possible donor colonies (Table 1). Colonies in two apiaries each were inoculated with: mites from feral, lightly managed, or heavily managed backgrounds, while two apiaries were established as negative controls and were not inoculated with mites.

Table 1: Mite inoculation sources within each apiary

Apiary	Mite Background	Number of colonies (Mite source)
1	Negative Control	NA
2	Heavily Managed	5 (HM7), 2 (HM1/6), 1 (HM8/13), 1 (HM10/12), 1 (HM6/10/12)
3	Lightly Managed	3 (LM1/8), 2 (LM2), 2 (LM3), 2 (LM6/29), 1 (LM5)
4	Feral	4 (F7/13), 2 (F1), 2(F3/10), 1 (F6), 1 (F2/14), 1 (F6/13)
5	Lightly Managed	3 (LM5), 2(LM2), 2(LM3), 2(LM6/Farm9), 1 (LM1/8), 1 (LM1/2/8)
6	Heavily Managed	5 (HM7), 2 (HM1/6), 2(HM10/12), 1(HM2/27), 1(HM8/13)
7	Negative Control	NA
8	Feral	5 (F7/13), 3 (F6), 1 (F1/2), 1 (F2/14), 1 (F3/F10)

Mites from feral backgrounds were obtained from honeybee colonies that originated from swarm traps placed in a forest, while colonies from a typical backyard beekeeper management system provided mites for our lightly managed inoculations. We acquired mites from a migratory beekeeper that typically manages thousands of colonies for our heavily managed inoculation

treatments. Colonies were housed in standard five-frame Langstroth nucleus hive boxes and we attempted to minimize drift by arranging colonies in a circular layout with all entrances facing outwards from the center of the circle, with 1m between the colonies. We further attempted to minimize drift by maximizing bees' ability to visually distinguish between colonies (Dynes, unpubl. data). The colonies were painted different colors, placed at different heights above the ground (0, 200 and 400cm), with different symbols painted at the hive entrance.

We started with highly standardized colonies to minimize variation. We obtained mated queens from a single queen breeder in southern Georgia, USA and added 1.1kg (2.5 lb.) adult bees from a common-genetic background into a package. The packages were placed in a dark room overnight at 16.6ºC (62ºF), and sprayed with sugar water one hour prior to the application of 30mL of an oxalic acid solution (Milani 2001). Each package was installed three days later into a randomly-assigned apiary. Mites were collected from source colonies outside of the experiment by sifting powdered sugar over the colony and collecting dislodged mites at the bottom of the colony. We used small natural fibered paintbrushes to place mites on damp coffee filters. We kept mites in an incubator set at 35ºC (95ºF) until all mites were collected for each dose. We then transferred all mites evenly to an open brood frame and waited to ensure mites had attached to the wax cells in a colony before returning the frame to the colony. To maintain our focus on these original colonies (and their queens), we enacted swarm control on colonies likely to swarm by splitting those colonies. We standardized swarm control in this manner to ensure small colonies were not jeopardized by the procedure. A total of 33 out of the 72 colonies that remained alive were split in March and April of 2016. We employed a Fisher's exact test to determine that there was not a statistically significant difference ($P = 0.09198$) in frequency of splitting between our treatment groups. During the experiment, we did not conduct any control

measures against *V. destructor*. We continued the experiment from June 2015 through December 2017, at which point only 12 of the original 88 colonies were still surviving.

Data collection

Measuring V. destructor infestation

We measured *V. destructor* infestation levels using three different methods. First, we used an alcohol wash method described by Fries et al. (1991). This method involves destructively sampling approximately 300 bees from a colony in alcohol and counting bees and mites (which detach from the bees allowing easier counting) to get a relative mite level on the adult bee population. We took eight alcohol wash samples throughout the experiment. Second, we used sticky boards (Branco et al. 2006), a standard method to evaluate *V. destructor* levels in a colony by collecting mites that fall and become entrapped on a board placed at the bottom of a colony. We measured mite levels with sticky boards six times throughout the experiment including one measurement immediately following package installation to confirm colonies were *V. destructor* free. Third, we measured the mite population in brood cells by opening 100 covered brood cells in each colony and counting the number of mites. We measured mite levels in brood cells five times throughout the experiment.

Colony strength assessments

We took periodic health assessments throughout the experiment in order to evaluate the effect of mite background on colony health. We followed the assessment guidelines outlined in Delaplane et al. (2013) to measure colony strength in terms of: adult bee population, amount of brood, and amount of honey stored for each colony. We performed these colony assessments five times over

the two years of the experiment. We also recorded the date each colony was found to be dead and last known date it was alive for survival analyses.

Statistical analysis

Overview

We explored how our treatment levels (mites from feral, lightly managed, and heavily managed backgrounds) affected the mite burdens and health response outcomes at the colony level. We also assessed the effects of mites from our different mite donor colonies within each treatment level to determine whether variation exists within the treatment levels. We conducted analyses based on three classes of response variables: 1) colony-level mite infection levels; 2) colony health parameters; and 3) colony-level survival.

Mite infection levels and colony strength

Our experiment used longitudinal repeated measures and nested random effects which can result in temporal and within-subject autocorrelation and violates the assumptions of independence for parametric and linear regression methods. Therefore, we used generalized estimation equations (GEE) to account for repeated measures including autocorrelation. GEE models are similar to the more common generalized linear mixed models (GLMM), but handle within-group correlation as a marginal model rather than as a conditional model found in GLMM's. We used the 'geeglm' function in the 'geepack' package v1.2-1 (Højsgaard et al. 2006) in R v.3.4.2 (R Core Team 2017) to specify and evaluate the GEE models in particular because it allows for longitudinal data with missing observations. We blocked the data by apiary and colony and utilized an autoregressive (AR1) autocorrelation structure to compare treatment levels with negative control

colonies. We used the 'lsmeans' package v. 2.27 in R to conduct post hoc pairwise comparisons of response variables of mites from different donor colonies using Tukey's method for multiple comparisons (Lenth 2016). We used the 'missMDA' package v.1.12 in R (Josse and Husson 2016) to impute missing values ($N = 917$ out of a total of 1,869 values) for mite measurements that did not occur in the same months and then created a composite index combining the three methods of mite measure using a unity-based normalization index (Dodge et al. 2006). This index takes each method of mite measurement and scales the measurement to a value between 0 and 1 by comparing the measurement to the minimum and maximum value for that method. The normalized value for each method of measurement is then added to the other methods for that particular sample for a composite index value. We employed a GEE model to evaluate this composite index in addition to each of the individual mite measures. We similarly assessed colony strength measures (adult bee population, brood production, and honey stores) using GEE models to compare treatment levels to negative control colonies.

Survival analysis

We performed survival analyses to determine whether there was a difference in colony survival based on mite background. Colonies were inspected periodically throughout the experiment and exact timing of colony death could not be determined. Therefore, we used an interval of date of observed colony death and date of last known colony viability. Given this data structure, we analyzed survival with Cox proportional hazard models, which are mixed-effects survival (frailty) models, with interval censoring via the 'frailtypack' package (Rondeau et al. 2012) in R.

Results

Overview

We collected extensive data on mite levels and colony health parameters for each colony. The colony health assessments resulted in 231 measurements each of: the adult bee population, brood coverage, and honey storage. In order to evaluate *V. destructor* levels throughout the experiment, we collected 413 sticky boards, 353 alcohol washes (each containing approximately 300 worker bees), and 189 counts of mites in the brood (each including 100 brood cells).

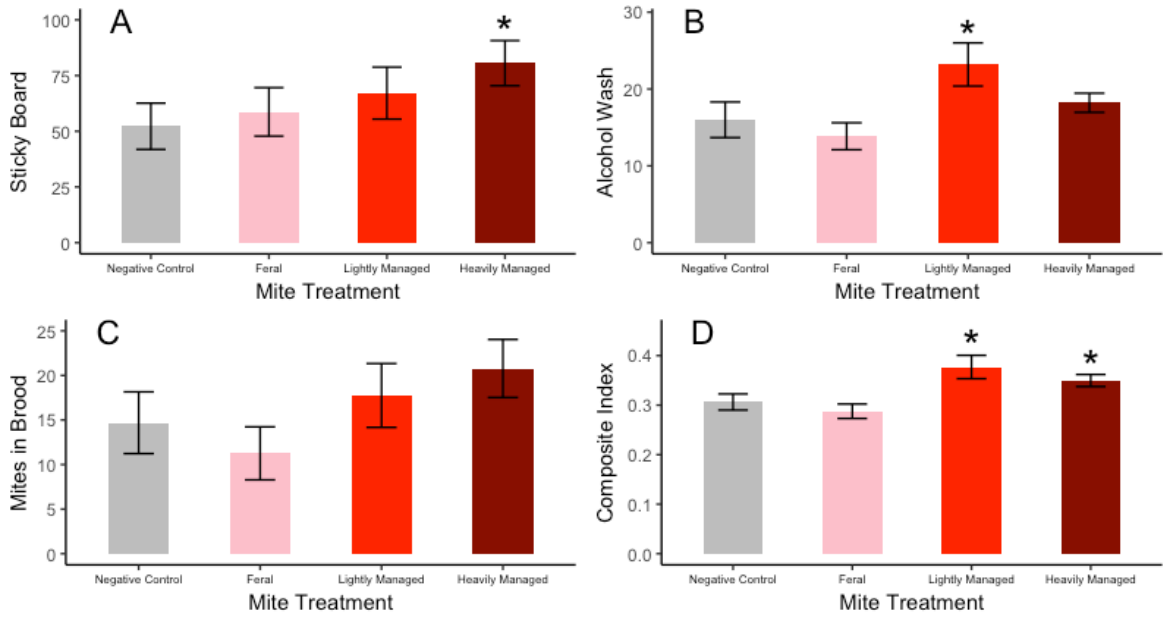

Figure 1: Measures of mite abundance by the treatment over the course of the experiment. (A) Sticky Board, (B) Alcohol Wash, (C) Mites in Brood, and (D) Composite index of all 3 measurements. GEE models were employed for data in each panel to determine significant differences from the negative controls. More mites were found in colonies with mites from heavily managed backgrounds (A; $P = 0.044$) and lightly managed backgrounds (B; $P = 0.047$). Note that while significance was not always found in each mite measurement (A-C) the trend in each is consistent with our hypothesis. A unity-based normalization index was used in panel D to combine all 3 mite measurements. This reduced the measurement variation and showed a significant difference between mites from the lightly managed ($P = 0.014$) and heavily managed ($P = 0.033$) backgrounds from the negative controls which is consistent with our hypothesis. Error bars represent S.E.M.

Mite infection levels

The GEE model for mite levels as assessed by sticky boards showed that colonies inoculated with mites from heavily managed backgrounds had significantly ($P = 0.044$) higher mite levels over the course of the experiment than the negative control colonies (Fig. 1A). The model for the alcohol wash data showed that colonies inoculated with mites from lightly managed backgrounds had significantly ($P = 0.047$) higher mite levels (Fig. 1B). The mites in brood measurement did not show any treatment level significantly different from negative controls (Fig. 1C). However, the trend in this measurement is consistent with the other two measures with colonies inoculated with feral mites tending to have the lowest mite levels and the treatment groups from managed backgrounds having the most mites. The GEE for the composite index, which combines the three measurements of mite level, indicated that colonies inoculated with mites from both lightly and heavily managed backgrounds had significantly ($P = 0.014$ and 0.033 respectively) higher mite levels than the negative controls. We did not find significant within treatment group differences based mite donor colonies for mite levels.

Colony strength and survival analysis

The GEE model for the amount of brood showed that colonies inoculated with mites from feral backgrounds to have significantly ($P = 0.0040$) lower levels of brood production (Fig. 2). The models for adult bee population and honey stores did not show any significant differences between the treatment groups and the negative control colonies. The feral and heavily managed treatments showed pairwise within treatment differences for adult bees based on mite donor colonies. The feral treatments had 3 significantly different pairwise comparisons ($P = 0.0000092$ to 0.042). The heavily managed treatments had 5 significantly different pairwise comparisons (P

= 0.00015 to 0.048). Eighty-six percent (76 of 88) of the colonies died over the two-year experiment. The Cox survival analysis did not show a significant difference in survival between the different treatment groups (Fig. 3).

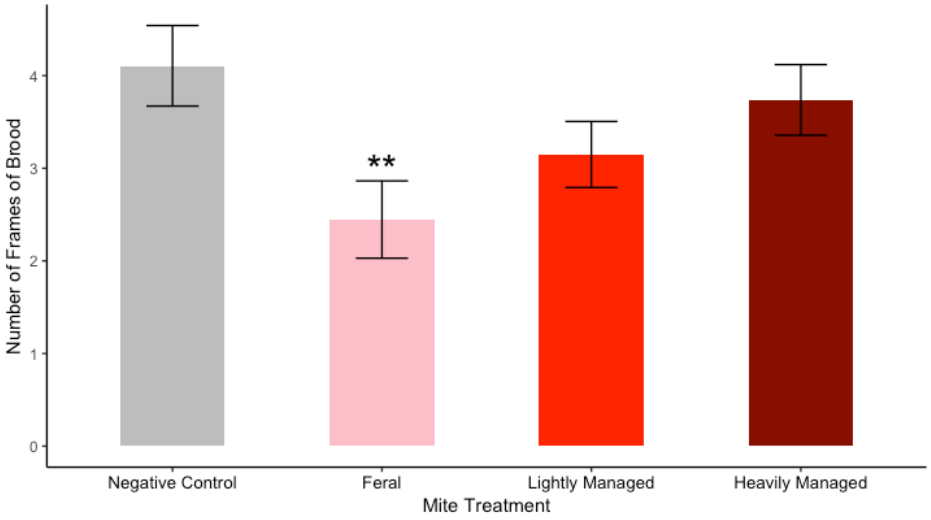

Figure 2: Number of frames of brood by treatment over the course of the experiment. A GEE model found significantly ($P = 0.004$) fewer frames of brood in the colonies inoculated with mites from a feral background. Note that the trend in the experimental treatment groups is opposite to what we predicted. Error bars represent S.E.M.

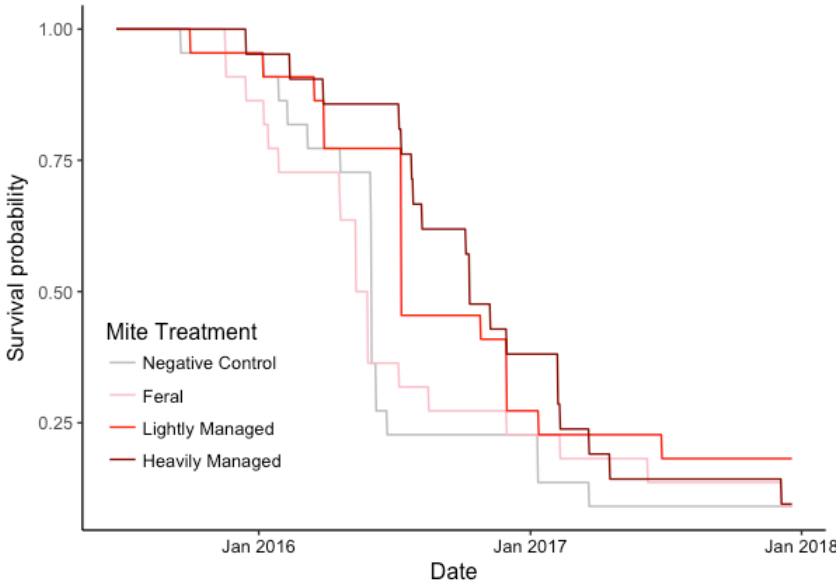

Figure 3: Survival curves by mite treatment. A Cox proportional hazard model with interval censoring did not find a significant difference between the groups.

Discussion

Overview

The conditions for *V. destructor* are vastly different in managed bee colonies versus feral bee colonies (Seeley 2007). The colony densities found in managed colonies far exceed those found in feral populations and may facilitate disease transmission (Seeley and Smith 2015). According to theory increased transmission between honey bee colonies may alter selection pressure that favors increased replication and virulence (Brosi et al. 2017). We performed a large replicated study assessing how mites from different management backgrounds interacted with honey bees from a single background. We were able to replicate varroosis by standardizing bee background, clearing mites, and inoculating with controlled doses of mites in a large replicated study, which has not been documented before. Our work provides evidence consistent with theory that densities in managed colonies have favored *Varroa destructor* strains with increased growth rates. Specifically, we found increased levels of mites in colonies inoculated with mites taken from managed honey bee populations. However, we did not find the negative consequences we expected for colony health and survival based on increased mite levels. In fact, for one response variable (brood production) we found that colonies inoculated with mites from feral backgrounds had a negative colony strength outcome relative to bees inoculated with mites from managed backgrounds.

Mite infection

Our finding of increased levels of *V. destructor* mites in colonies inoculated with mites from managed backgrounds (Fig. 1) suggests that honey bee management conditions have favored increased mite reproductive rates. While these levels were not always significantly different from

negative controls for each mite measure (Fig. 1A-C) the trend was always consistent with our predictions, with colonies inoculated with mites from feral backgrounds exhibiting the lowest mite levels and mites from managed backgrounds showing increased mite burdens. The composite index of all three mite measures (Fig. 1D) reduced within-group variation and showed colonies inoculated with mites from managed backgrounds had increased levels of infestation. This is consistent with the idea that mites from feral vs managed backgrounds are under different selection pressures with potential differences in mite growth and/or virulence (Corrêa-Marques et al. 2002, 2003).

Colony strength and survival analysis

We found significant within-treatment differences based on mite donor colony for the adult bee population, for both apiaries inoculated with mites from feral and heavily managed bees. This indicates genetic variation in mites among feral and heavily managed bee populations, such has been in other studies (Dynes et al. 2017). While we did not find significant differences in adult bee population or honey stores across our treatment groups, we found that bees inoculated with feral-background mites produced less brood than bees inoculated with mites from managed backgrounds (Fig. 2). This was surprising because we expected the opposite outcome: that higher levels of mites would lead to negative colony health outcomes. There are five potential explanations for this pattern that we consider here.

First, the bees we used could be adapted to the mite strain that they coevolved with Predicting the outcome of host-parasite interactions, such as in the honey bee - *V. destructor* system, can be more complicated when considering how different genotypes of hosts and parasites interact. Genotype-by-genotype (G × G) interactions result from interactions between different host and

parasite genotypes, such that some parasite strains are more successful against some host strains, and some hosts are less susceptible to certain parasite strains (Lambrechts et al. 2006). When G × G interactions occur, no single parasite strain optimally infects all host strains, while no single host strain is optimally defended against all parasite strains (Carius et al. 2001; Lambrechts et al. 2006; de Roode and Altizer 2010). Both theory and empirical studies indicate that coevolution can lead to increased host tolerance; as a consequence, a novel parasite strain from another evolutionary background could lead to more virulence than a coevolved parasite (Greischar and Koskella 2007; Miller et al. 2007; Read et al. 2008; Hawley et al. 2013; Gibson A. K. et al. 2015). If this is the case, the observed patterns of mite growth and colony strength may be due to a genetic mismatch between lightly managed bees and mites from feral colonies, with lightly managed bees resisting, but not tolerating, mites from feral colonies. This means that the bees are able to keep parasite population levels in check (resistance) but are unable to cope with the damage caused by these lower levels of parasites (tolerance) (Restif and Koella 2003; Best et al. 2009). Thus, while we would predict that the higher transmission opportunities in managed honey bees select for greater mite virulence, we may also predict greater selection for host resistance and tolerance, and the existence of mismatches in coevolved mite and honey bee strains may make virulence outcomes more difficult to predict. A full cross-infection experiment using bees from different backgrounds (in addition to mites of different backgrounds, as we assessed here) is needed to follow up and explore this hypothesis.

Second, honey bee queens may adjust their egg laying frequency based on mite-induced bee mortality. This pattern of increased brood production as a potential means of compensation for higher brood parasitism in *V. destructor*-infested colonies was noted by Delaplane and Hood (1999). Third, our negative controls, which were initially cleared of mites and not inoculated,

had greater mite levels than we expected. This suggests that horizontal transmission of mites from outside the experiment could have occurred (Nolan and Delaplane, 2017). We isolated our experimental apiaries from all known colonies by at least 5km to minimize this potential, but we cannot completely discount this as a possibility. Fourth, our mite clearance protocol may not have been as successful as we anticipated, and residual mite populations could have overtaken the inoculated population. However, our first sticky board samples taken after clearance and before inoculation showed most colonies having zero mites and an overall very low average of 2.29 mites detected in the 72hr sample per colony. Thus, our inoculation of 100 mites should have overwhelmed any residual mite population. Finally, it is well known that the negative consequences of *Varroa destructor* infestation are both due to the mites themselves and the viruses they transmit, and differences in viral virulence are now well established (Anderson 2000; Vojvodic et al. 2011; McMahon et al. 2016). As such it is possible that feral mites harbor different populations of viruses than those circulating in managed colonies and these feral viruses could have differential virulence and/or differential G × G interactions, leading to distinct health outcomes relative to mite infections on their own in the absence of viruses.

Colony level mortality was a key measurement in our assessment of virulence of *Varroa destructor* on the honey bee colonies. The level of colony mortality (86%) across two years by the simple addition of mites indicates just how virulent *V. destructor* mites are for honey bee colonies. These findings are in line with another study that determined *V. destructor* was responsible for >85% of the colony mortalities (Guzmán-Novoa et al. 2010). However, we did not find an effect of mite background on colony survival (Fig. 3). We had expected that the higher mite levels in colonies inoculated with mites from managed backgrounds would translate into worse health outcomes and reduced colony survival in these colonies. That we did not see

these results may suggest that there are other factors such as queen health (Amiri et al. 2017) or viral infections that may play a more important role than mite infestation. Additionally, the finding that our negative controls had similar survival outcomes as our treatment groups desmonstrates that a single treatment for *Varroa destructor* infestations is ineffective, even when that treatment clears all or nearly all mites from a colony. One study found that while a single treatment of oxalic acid caused 97.6% mortality in *V. destructor* mites, an additional treatment resulted in 99.6% mortality leaving the possibility that a small population of mites could reestablish after a single treatment (Al Toufailia et al. 2018).

Future research

While our study provided insights into how mites from different backgrounds interact with bee colonies of a similar background, our results also indicate that a cross-infection study with bees from different backgrounds could help us further understand the trade-offs that may occur in this system. Specifically, we suggest that future studies explore how human management may contribute to a possible virulence-transmission trade-off by measuring transmission and virulence of mites introduced into mite-free apiaries such as Hawley et al. performed with a bird disease (2013). Additionally, we need to determine the conditions under which mite levels are dissociated from colony harm. Future work also needs to focus on the role that viruses play in the *Varroa destructor*-honey bee system. This three-way system could interact in potentially unexpected ways including mechanisms that confound our present understanding.

Conclusion

Host population densities in managed honey bee apiaries are vastly different than what *Varroa destructor* experiences in feral honey bee populations. We provide evidence consistent with the idea that selection pressures on mites in these managed conditions may favor increased reproductive rates. This could act to increase the transmission rate in these managed environments. However, we did not find negative health outcomes and survival that we would expect with these higher mite burdens. Mites from feral backgrounds may have caused negative health outcomes due to a mismatch in coevolved bee and mite strains. Future research needs to determine the conditions under which mite levels are dissociated from virulence and whether human management of bee colonies is driving selection for more damaging mites.

CHAPTER 6:

CONCLUSION

The overarching goal of our research was to better understand the role of *Varroa destructor* transmission in the *Varroa*-honey bee interaction.

In Chapter 2, we documented more genetic diversity than expected based on previous research and a background characterized by limited diversity. This finding suggests more transmission of *Varroa* between colonies than expected. This higher than expected genetic diversity also suggests that *Varroa* has more evolutionary potential than previously suggested, thereby potentially explaining how *Varroa* quickly evolved resistance to miticides.

Using the bee tracking method described in Chapter 3, we measured bee drifting in apiaries with different density (Chapter 4). This revealed that bee drifting is higher in apiaries with higher colony density, increasing the transmission potential of *Varroa*. We also found that a higher density of honey bee colonies promotes increased overwinter mortality, reduced storage of honey and increased mite levels in the brood. This suggests that the high densities found in most beekeeping operations can have negative consequences for colony health and survival and disease spread. Importantly, the mechanisms by which we realized lower bee drifting and greater colony health are straightforward for implementation by keepers. Thus, this work has the potential to significantly mitigate the negative consequences of *Varroa* for beekeepers.

The experiment described in Chapter 5 indicates that mites that have evolved under human management regimes, which favor increased transmission potential, have increased population growth rates. This suggests that human management may increase selection pressure for increased fecundity of parasites. Surprisingly, we found that mites from a feral background caused reduced brood production in bees from a lightly managed background. This may indicate that queens are compensating for increased parasite induced bee loss by increasing egg laying. Alternatively, there may be a genotype by genotype interaction in feral mite strains interacting with lightly managed bees. This interaction may signify that lightly managed bees are more resistant (able to keep mite populations in check) but less tolerant (able to cope with the damage caused) of these lower mite levels. Alternatively, it is possible that the viruses circulating in feral bees are more virulent to bees from lightly managed backgrounds.

While the research we have presented here has advanced our understanding of *Varroa destructor* biology and the disease dynamics involved in its parasitism of honey bees, it also raises further questions that should be addressed by future research. We found, in chapter 4, that colonies in low density apiaries had significantly more honey stores. However, since we were simultaneously testing *Varroa* infections along with apiary density and visually distinctive colonies we can not determine definitively the causal factor affecting honey stores. Since this is an important finding for beekeeping management future research should examine these factors independently. Additionally, the unexpected result in chapter 5 that mites from a feral background were associated with decreased brood production suggests a possible genotype by genotype interaction. However, our study was not explicitly set up to disentangle these types of relationships. A full factorial cross-infection study that crosses mites from our three

management backgrounds with bees from the three management backgrounds would help us understand whether genotype by genotype interactions are actually occurring in the *Varroa*-honey bee system. A further factor that may play a role in this system that our study was not established to investigate is how viruses may confound the understanding of *Varroa*-honey bee interactions. Mites from different management may have different viral populations with distinctive virulence and effects on the two dynamic of mites and honey bees. Tracking mite and bee viral populations in our study described in chapter 5 or the proposed cross infection study could help resolve whether mite populations differ in their viral populations and whether they interact with *Varroa* infections to cause differential virulence. Lastly, a study that fully tests the virulence-transmission trade off that we explored in chapter 5 would be of interest to both beekeepers and evolutionary disease ecologists. By carefully tracking transmission rates and virulence of mite strains expected to be of high and low virulence would help understand the trade-offs shaping virulence evolution in the *Varroa*-honey bee system.

In addition to providing an overall better characterization of *Varroa*-honey bee disease dynamics our research reveals an enhanced understanding of *Varroa* biology and its transmission. Specifically, we found that *V. destructor* transmission is more common than expected and potential transmission is affected by apiary density and colony distinctiveness. Lastly, our finding of increased population growth rate in mites from managed bee populations suggests that these mites may compete better than other strains in a high transmission setting.

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