ABSTRACT

GIUFFRE, CARL JOHN. Technological Methods in the Health Assessment of Western Honey Bees (Apis mellifera). (Under the direction of Dr. David R. Tarpy and Dr. Sharon R. Lubkin).

The Western honey bee (Apis mellifera), one of the most important pollinators worldwide, has been faced with many recent health challenges. Important health issues, such as the invasive mite Varroa destructor, are well-documented, but significant scientific opportunities remain in describing their role as a vector for disease. Other pressing health issues, such as Colony Collapse Disorder, have yet to be fully described, due to the many behavioral and environmental variables at play in the honey bee system.

In this dissertation, we hope to exploit novel advances in computer algorithms and imaging technology, by presenting methods in three current topics of bee health: grooming, parasites, and nutrition. We developed methods in image analysis to describe time-continuous grooming behavior of known commercial honey bee stocks. We analyzed grooming videos using a differential equation model to parameterize bee grooming rates, then used statistics to draw comparisons between the stocks. We used video object tracking to describe the behavior of the important bee parasite, Varroa destructor in vitro. By corroborating mite phenotypical behavior with genotypical data, we proved that viral count nontrivially changes mite behavior. Finally, we used digital imaging, colorimetry, and machine learning to determine plant source for corbicular pollen, a very important component of bee nutrition. We provide some initial sensitivity analysis of the pollen imaging system, and describe future directions, opportunities, and applications as a framework for larger pollen studies.
Technological Methods in the Health Assessment of Western Honey Bees (*Apis mellifera*)

by

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DEDICATION

I dedicate this thesis to my family. To my loving parents, Alan and Patricia, and to my inspirational siblings, Mary, Jon, Tony, Carrie, and Laura. To Marley, and others on the way.

*Hamilton: We’re finally on the field, we’ve had quite a run.*

*Lafayette: Immigrants -

*Hamilton and Lafayette: - we get the job done!*

BIOGRAPHY

Carl John Giuffre was born on February 16, 1982 in the city of Oconomowoc, Wisconsin, a named derived from “Coo-no-mo-wauk”, the Potawatomi term for “waterfall”. His earliest interest in population ecology and invasive species stems from growing up on a lake where purple loosestrife (*Lythrum salicaria*) had invaded the surrounding marshland.

He completed his secondary education at Arrowhead High School in Hartland, Wisconsin. From there, he went to the University of Wisconsin-Milwaukee to pursue an undergraduate education in theatre. His creativity and passion for mathematics and biology quickly changed this pursuit, and eventually he changed his focus from theatre to mathematics. Carl became actively involved in digital image processing as an extension of his undergraduate research work at the Great Lake Freshwater Institute on the species *Paramecium tetraurelia*. Carl earned a Bachelor of Science in Mathematics degree in May 2011.

In Fall 2011, Carl began his doctorate work in the Department of Mathematics at North Carolina State University. He hopes to one day share the vision of Mark Zuckerberg, only with social insects, instead of human beings.
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CHAPTER 1.

Introduction

Honey bees (*Apis mellifera*) are indigenous to Europe and Africa, but they have been exported worldwide because of the important pollination services that they provide. Apiculture is estimated to contribute 162 billion USD to the global economy annually (Gallai *et al.*, 2009). The true economic estimate of bee services is difficult to measure, especially since a foraging bee can range up to 3 km or more from the nest site (Winston, 1987). Furthermore, unmanaged bee populations contribute to both local and exotic pollination, which cannot be accurately measured (Ricketts *et al.*, 2004). The honey, pollen, wax, textiles, and produce industries are all directly impacted by honey bee populations.

Unfortunately, honey bee populations are on the decline worldwide, and recently U.S. beekeepers have been losing roughly one third of their colonies over winter (Ellis *et al.*, 2010; Neumann and Carreck, 2010). Colony Collapse Disorder (CCD), although sensationalized by the media, has mysteriously facilitated these negative population trends; pesticides, microbes, viruses, parasites, and genetics have all been implicated for playing a role in its cause (Oldroyd, 2007; Evans *et al.*, 2009; Guzmán-Novoa *et al.*, 2010; Dainat *et al.*, 2012; Cox-Foster *et al.*, 2007). Regardless of the specific factors causing CCD, it is clear from diminished health and productivity that honey bee populations are under threat.

Recent advances in image processing have proven empirically relevant in behavioral sciences and industry. Object recognition and colorimetry have paved the way for high-throughput, observational studies, that objectively reduce human error. Studying bee behavior,
using image processing as a vehicle, would offer deeper perspectives on the larger issues in bee health sciences.

1.1 Levels of honey bee health and defense

Honey bee health is complicated to study, as there are five levels of defense relevant to honey bee health: individual, pairwise, colony, structural, and environmental (Evans and Spivak, 2010). **Individual** bees have their own immune systems, uniquely determined by their genetics. Since a queen mates multiply (and therefore her workers are sired by different males), individual innate-immunity in a single colony can be quite varied (Winston, 1987). Undertaking (removal of dead adults), hygienic behavior (removal of dead or diseased brood), and autogrooming (self-grooming) are all behavioral traits that preventatively protect a honey bee from pathogens (Evans and Spivak, 2010).

**Pairwise defense** is the result of honey bee social interactions, resulting in elevated immunity, also known as social immunity. Trophallaxis, although primarily a mechanism for food exchange and communication, also exchanges beneficial gut microbiota, which has been shown to be important in bee immunity (Martinson, 2012). Allogrooming (grooming of another individual) is such an important pairwise defense that honey bees evolved a specialized dance to initiate grooming from another bee (Land and Seeley, 2004).

Since healthy bee colonies are comprised of one reproductive female and ~50,000 of her offspring, immunological traits within the **colony** are quite interrelated and present a unique set of challenges (Evans and Schwarz, 2011). Thus, it is often advantageous to think in terms of colony health and colony immunity. Colony health, or the health of the
“superorganism,” is a complex combination of individual and pairwise defenses (Seeley, 1989; Winston, 1987). Stinging behavior is the most obvious form of an adaptive suicide-mechanism that benefits the health of the superorganism. In fact, immunologically compromised bees have been shown to altruistically remove themselves from the hive (Rueppell et al., 2010). Colony immunity, or the immune responses collectively pooled in a population, has been examined in several important studies using both behavioral (Wilson-Rich et al., 2009) and genetic (Evans and Pettis, 2005) approaches. Task allocation and age polyethism are behavioral manners by which individual bees perform individual tasks (Winston, 1987). These tasks are determined, in part, by the current health needs of the colony.

Evans and Spivak (2010) highlight a nuanced difference between structural and environmental defenses. Environmental defenses are methods that bees use to exploit their surroundings for protection. The hive entrance functions as a structural bottleneck for intruders, such as parasites or diseased bees (Seeley, 1989). Bees also sanitize the hive using propolis from tree resins to create an antiseptic “envelope” that protects the colony from foreign microbes (Simone-Finstrom and Spivak, 2009). Amino acids from various pollen sources reduce oxidative stress and enhance bee longevity (Herbert and Shimanuki, 1978; Huang, 2012).

1.2 The Vector Manipulation Hypothesis

Studies of disease vectors and vector behavior are equally important in describing the health of the host species they affect. Many traditional studies in parasitism explore the host-vector relationship as it relates to behavior, immunology, and prevention. Others study disease-
host interactions, usually at the microscopic or viral level, in efforts to interrupt key moments of the disease’s life cycle, or to determine candidate cures. Several studies have recently revealed that in the vector-disease relationship, vector behavior may be altered based on the viruses they harbor; this is known as the Vector Manipulation Hypothesis (Ingwell et al., 2012; Cator et al., 2012).

The Vector Manipulation Hypothesis contends that vectors for disease perform a host-preference shift from their infected hosts to their uninfected counterparts (Ingwell et al., 2012, Cator et al., 2012). This train of thought is quite important, since it suggests that diseases asymptomatically and facilitatively modify vector behavior to ensure reproductive success of the virus.

1.3 The problem with connecting bee phenotypes and genotypes

Since the mapping of the honey bee genome in 2006, many efforts have been made to integrate macro- and molecular-level characteristics of bee health. Molecular-level contributions have proven effective, and as technology has advanced, the price for these methods has decreased over the past 10 years, while the quality of analysis has increased (Everett et al., 2010). Genetics has revolutionized the scientific approach in studies of bee behavior, immunology, and nutrition (DeGrandi-Hoffman et al., 2010). Even though the price of genetic analysis has decreased, most methods are still quite costly and time-consuming (Everett et al., 2010). Genetic data are also limited by reducing the temporal complexities of bee society into a single snapshot and removing live individuals from future studies.
Genetics have been important in advancing our understanding of bee health, but behavioral studies of honey bees are equally complicated. Behavioral studies require hours of direct human observation, often *in vitro* or outside the context of a typical colony environment (Camazine, 1991; Robinson, 1987; Peng *et al.*, 1987). Behavioral studies are nonetheless important, as they can capture social dynamics that have the potential to correlate with the ever-growing body of genetic honey bee data. This creates an unfortunate gap between bee behavioral and genetics literature.

1.4 Computer vision and color space

Image processing is at the core of this thesis. The subtleties of colorimetry are especially important in Chapter 4. Pixel data from an $m \times n$ color image are stored on a computer in the form of an $m \times n \times 3$ matrix, with three color spaces typically used in their analysis: the RGB, HSV, and CIE-Lab color spaces. Coordinates for all three spaces depend on the software and language interpreting the image; numerical values are appropriately scaled in practice, and the values below are provided to assist with familiarity in understanding a critical component of this present work.

The RGB color space, which is standard for digital photography and displays, represents color on a cube by assigning integer values between 0 and 255 on red, green, and blue (RGB) axes. These numbers translate to saturation values on a computer monitor, with 0 representing unsaturated ([0 0 0] = white) and 255 completely saturated ([255 255 255] = black). This coordinate system is very straightforward to use, since the brain can easily visualize values on a cube. However, this ease-of-use does not translate well in analytical
studies; monitors, printers, and other imaging devices cannot display every RGB color combination (known as the device’s gamut), and images themselves are highly dependent upon how the imaging device stores pixel data numerically. At the data level, the three color channels are highly correlated with one another, making the measurement of differences in color difficult (Tkalcic and Tasic, 2003; Cheng et al., 2001; Ford and Roberts, 1998).

![Figure 1-1. Visualization of the RGB color space.](https://upload.wikimedia.org/wikipedia/commons/a/af/RGB_color_solid_cube.png)

HSV—or hue, saturation, and value—is the color space used widely in generating computer graphics. In this conic color space, hue represents the rotation about a central axis, saturation the normalized unit distance vector from the central axis, and value the unit vector from 0 (= black) to 1 (= white) vertically along the axis. Although the coordinate system is
harder to visualize, this color space is very useful for imaging techniques (such as image thresholding), since value can easily be employed to detect reflections or shadows in an image. HSV inherits the same color-correlation issues as RGB, since it is simply a nonlinear transformation of that space. The HSV space also suffers from gamut and device dependence issues, as a result.

![Figure 1-2. Visualization of the HSV color space. (source:](https://upload.wikimedia.org/wikipedia/commons/f/f1/HSV_cone.jpg)

In 1976, the Commision Internationale de l’Eclairage (CIE) proposed a color space where the Euclidean distance between two colors is strongly correlated to human perception of color, a term known as perceptual uniformity (Tkalcic and Tasic, 2003). The space's axes are called $L$, $a$, and $b$, so the space was named CIE-Lab. CIE-Lab colors are represented on a sphere, where $L$ ranges from 0 (= black) to 100 (= white), $a$ ranges along a -50 (= green) to 50 (= red) axis, and $b$ ranges along a -50 (= blue) to 50 (= yellow) axis. Unlike its RGB and HSV
counterparts, CIE-Lab is device independent, making it a useful tool in many current analytical studies on color and computer vision.

Figure 1-3. Visualization of the CIE-Lab color space. (source: https://openclipart.org/download/218708/joede--cie-color-space-coordinate-system-B3.svg)
1.5 Aim

The goal of this thesis is to address important behavioral components relevant to the more global problem of honey bee health, using digital imaging technology and behavioral modeling.

In this thesis, I bridge the gap between genotypic and phenotypic honey bee studies by alleviating the financial burdens of genetics studies and the time pressures of behavioral ones. This thesis outline methods and results relevant to the individual, colony, and environmental levels of bee defense and honey bee health.

Chapter 2 highlights honey bee autogrooming, an important behavioral defense mechanism in individual bee health. Chapter 3 approaches bee health indirectly, focusing on the behavior of varroa, and tests the Vector Manipulation Hypothesis. Chapter 4 combines machine vision and colorimetry to study honey bee pollen, important in honey bee nutrition and environmental defenses. All three chapters are important in advancing our understanding of specific components of honey bee health.
1.6 References


CHAPTER 2.

Automated assay and differential model of Western honey bee (*Apis mellifera*)

autogrooming using digital image processing
Automated assay and differential model of western honey bee (Apis mellifera) autogrooming using digital image processing

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A B S T R A C T

In animals, self-grooming is an important component of their overall hygiene because it reduces the risk of disease and parasites. The European honey bee (Apis mellifera) exhibits hygiene behavior, which refers to the ability of the members of a colony to remove diseased or dead brood from the hive. Individual grooming behavior, however, is when a bee grooms itself to remove parasites. While both behaviors are critical for the mitigation of disease, hygienic behavior is overwhelmingly more studied because, unlike grooming behavior, it has a simple bioassay to measure its phenotype. Here, we develop a novel bioassay to expedite data collection of grooming behavior by testing different honey bee genotypes (stocks). Individual worker bees from different commercial stocks were coated in baking floor, placed in an observation arena, and digitally recorded to automatically measure grooming rates. The videos were analyzed in MATLAB, and an exponential function was fit to the pixel data to calculate individual grooming rates. While bees from the different commercial stocks were not significantly different in their grooming rates, the automation of grooming measurements may facilitate future research and stock selection for this important mechanism of social immunity.

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1. Introduction

The European honey bee (Apis mellifera) belongs to the insect order Hymenoptera, which includes many of the highly eusocial insect societies (ants, bees, and wasps). As in all organisms, honey bees have their own individual immune defenses. However, social groups increase the complexity of their relationships with parasites and pathogens (Schmid-Hempel, 1998). On the one hand, pathogens can spread more readily when high concentrations of susceptible host are in close proximity and (usually) in stable environments. On the other hand, social insects may also exhibit so-called social immunity (Cremer et al., 2007), which is the collective ability of a society to deal with parasites either behaviorally or via innate immune responses. In honey bees, autogrooming (Evans and Spivak, 2010), resin collection (Simone et al., 2009), and the removal of diseased or dead brood (Spivak and Reuter, 2001) have all been identified as behavioral mechanisms of social immunity in honey bee colonies. Techniques for improving both individual and social immunity in honey bee stocks should, therefore, have strong economic implications for commercial breeding programs.

Commercial honey bee stocks exhibit a spectrum of traits across many important characteristics, such as honey production, winter hardness, disease resistance, hygienic behavior, and grooming behavior (Winston, 1987). Different commercial stocks of bees exist naturally and through artificial breeding (e.g., Minnesota Hygienic, Italian, Russian, Carniolan, and Varroa Sensitive Hygiene). In particular, the hygienic and grooming behaviors have been the topics of interest for many beekeepers because of what they imply for the longevity of the colony. Hygienic behavior in the strict sense refers to the colony’s ability to mitigate diseases by removing diseased or dead brood from the colony, varroa mites from infested larvae, or foreign objects from the hive (Spivak and Reuter, 2001). Grooming behavior, in contrast, refers to an individual bee grooming herself to remove parasites from the exterior of her body or that of other honey bees (Winston, 1987).

Modern assays to measure hygienic behavior have proven useful in breeding programs (Spivak and Reuter, 2001). One such assay focused on manually distinguishing classes of observable behavior of individual honey bees after being exposed to the external parasite Varroa destructor (Auneier, 2001; Gazman-Novoa et al., 2012).
Manual observation assays have an unfortunately laborious bottleneck, requiring repeated human observation that can be very time consuming. Other assays involve counting chewed mites dropped onto sticky-board placed beneath the hive (Arechavaleta-Velasco and Guzman-Novoa, 2001; Andino and Hunt, 2011). Exposure to external parasites elicits a very confined set of behaviors, specific to parasite response, which may not be indicative of grooming as a function of pathology resistance in general. More recent studies have explored grooming as a function of various control (Pritchard, 2016), although very little has been done to study the generalized act of self-grooming (also known as autogrooming) in the absence of parasites, which may play a key role in colony-level disease pathology.

The seminal work done by Tu et al. (2016) has established both the need for and the capability of technology in automated, behavioral studies of pollinators. More broadly, the overall objective of this study was to develop a novel bioassay that may alleviate the severe bottleneck and constraints of acquiring precise data on grooming behavior. By automating and accurately quantifying grooming rates of individual bees without direct human observation (which is both time consuming and subject to inter-individual measurement error), our aim is to address this important—but largely neglected—behavioral mechanism of social immunity in honey bees.

2. Materials and methods

2.1. Experimental colonies

Queens from commercial stocks of Italian, Russian, and Minnesota Hygienic honey bees were obtained through commercially available queen breeders. The hives were located at the Lake Wheeler Honey Bee Research Facility located in Raleigh, NC. Queen age-time was added to their respective colonies using conventional apicultural practices (Büchter et al., 2013). Since a full brood rearing cycle lasts approximately three to four weeks, the colonies were allowed 4 weeks to ensure that the emerging broods were from the appropriate genetic source.

Frames of newly emerging workers were gathered from 18 different colonies (6 frames from each commercial stock), and stored in an incubator at brood nest conditions (34 °C and ~50% RH) for approximately 12 h. Roughly 300 ‘callow’ honey bees from each frame were then marked on their thorax with colored paint (Tesors). The marks were color-coded so that they could be identified for both the age and stock of each individual. The bees were then introduced to separate, foreign colonies (“common gardens”), thereby controlling for the effects of colony environment and social dynamics on adult behavior. Approximately 3000 honey bees, in total, were marked in this manner. To control for seasonal factors, all cohorts were marked within a three-week window of time, spanning from late June through early July.

Bees that are one week old are typically identified as ‘nurse bees’ (Winston, 1987) as their behavior tends to focus on brood rearing, nursery maintenance, pathogen defense, and grooming. Thus, bees were gathered at approximately one week of age, when their grooming behavior would be most prevalent.

2.2. Setup and recording

On recording day, the desired bees were collected from their respective common gardens. Groups of five bees were collected using soft forceps, and placed into a specially modified test tube with multiple holes for ventilation. The tubes were kept in an incubator, set at 34 °C, until it was time for that group to be recorded. At that time, the group was taken from the incubator, and a small quantity of all-purpose wheat flour (Gold Medal™) was added. The bees were tossed in the flour for about one minute, or until fully coated. Once the bees were fully coated in flour, they were quickly transferred to a 125 mm petri dish using soft forceps. To facilitate, the bees were temporarily dumped onto a coffee filter, where they could more easily be gathered.

Honey bee grooming was then recorded. To expedite data collection, two cameras simultaneously recorded two experiments being run at the same time. All recordings took place in a dark room with Sony Handycams mounted approximately 10% inches (27 cm) above the petri dishes containing the experimental bees. Four utility light clamps (Coleman) were installed with 10 W (60 W-equivalent) LED light bulbs (Lighting Science) and arranged in a square on the perimeter of both experiments. This provided uniform lighting, which reduced both the reflective glare from the tops of the petri dish and shadows generated by the bees. Beneath the petri dishes was an even layer of the same flour in which they had been coated. As this required separate recording devices, it was important to color calibrate each video sequence, and an X-Rite Color Checker was also present in the recording area, which enabled pixel color calibration of the recordings (Fig. 1).

The bees were recorded for 10 min at 60 frames per second, with 2 min of buffering at the beginning of each video, for a total recording time of approximately 1.2 min. A total of 105 videos were recorded in a span of three weeks. Of these, 9 of the videos were discarded due to events such as defecation or bee death, leaving a total of 96 videos for analysis.

2.3. Image analysis

Once the videos were recorded, an algorithm written in-house in MATLAB (The MathWorks) was used to analyze the video data. Our algorithm follows three steps which require minimal user intervention: (1) Frame extraction, (2) Color correction, (3) Thresholding/pixel counting and pixel-to-metric conversion (Fig. 2).

One goal of this study was to provide an algorithm that could be performed across a variety of personal computers, recording devices, and lighting arenas.

2.3.1. Color correction

Our setup enables us to simultaneously record two experiments in the same twelve-minute timeframe. However, once the frames were extracted, it was important to color-correct across the multiple recording devices, to ensure accurate data analysis. Pixel data were compared to the expected color values of an X-Rite Color Checker using polynomial regression analysis (Hardeberg, 2001). This technique removes the device-dependence of the data, and enables data to be compared, even though separate recording devices were used. A third-degree polynomial was chosen, since reduction in standard error was significant compared to linear or quadratic fits.

2.3.2. Thresholding

Next, the corrected images were thresholded to obtain the binary pixel data. The images were examined using the HSV computer color spectrum and thresholded on the V-channel. As honey bees were recorded, they moved around the petri dish and cast shadows against the flour background. Since shadow-colored pixels are dark, and the goal of this assay was to measure dark pixels as a function of grooming, it was important to estimate the threshold based on the shadows being cast by the honey bees. Thresholding on V at a value of 0.6275 enabled us to conservatively distinguish between background flour, shadows, and the bees. This value was obtained using Imago (Schneider et al., 2012) on selected frames from the start and finish of prototypical experiments.
Fig. 1. Experimental setup. Five honey bees were placed in a petri dish with a diameter of 12.5 cm. Beneath this dish was an inverted petri dish lid with a small bed of flour (~0.5 mm deep). A camera was mounted 27 cm directly above the petri dish, and a color-checker was placed in the recording area. Four utility lamps were mounted in a square surrounding the recording area, angled to minimize glare off the petri dish and maximize illumination of the bees. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. Steps in the processing algorithms: (left) raw images, (middle) color adjusted images, and (right) binary images. The top and bottom rows are images characteristic of the starts and ends of our experiments, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
2.3.3. Scaling

The thresholded pixels were counted every 100 frames. These pixel counts were then scaled to square millimeters using the known measurements of the X-Rite Color Checker as a scale. This produced a time series $\mathbf{x}(t)$ for each experiment.

2.3.4. Efficiency

Performing mathematical operations on entire video sequences is computationally time-consuming on most personal computers. Therefore, every 100 frames were extracted from each video sequence. Initially, the algorithm was performed on the full 10 min of video footage. However, in most cases, the measurable grooming occurred within the first 5 min of the video. Therefore, as another efficiency measure, only the first five minutes of each video were used in analysis.

2.4. Mathematical model

The background of the recording area was the same color as the floor in which the bees were coated. Therefore, the recorded pixel data were treated as binary data where only non-flour colored pixels were counted. We were interested in formulating a model that captured the dynamics of the honey bees as they transition from flour-coated, or “dirty,” to clean.

The model needed to be characteristic of the observed behaviors. One behavior observed was that the bees engaged strictly in autogrooming, despite the presence of 4 other bees. Second, honey bees would rigorously groom themselves until they were mostly clean, and this process was quite rapid. Once the bees were mostly clean, the emphasis changed from autogrooming to other behaviors. Finally, since the heaviest grooming occurs at the start of the experiment, a model that parameterized how coated the bees actually were when the video recordings started was important. These assumptions and observations can be expressed simply as

\[
\frac{\text{rate of change of}}{\text{flour-covered area}} = \alpha \text{ proportional to } \frac{\text{flour-covered area}}{\text{area}}
\]

Writing $A$ as the (fixed) total area of the clean honey bees (mm$^2$), $a_0$ the initial cleanliness (mm$^2$), and $k$ as the grooming rate (mins$^{-1}$) converts the “word equation” into the differential equation

\[
\frac{\partial a}{\partial t} = -A (1 - a/A) + \alpha \text{ with } a(0) = a_0 \text{ and } \lim_{t \to \infty} a(t) = A.
\]

This has solution

\[
a(t) = A - (A - a_0) \exp(-kt)
\]

illustrated in Fig. 3a. In the context of our grooming assay, $\lim_{t \to \infty} a(t) = A$ represents the assumption that if an experiment was theoretically allowed to run forever, then eventually $a(t)$, the area of the clean honey bees at time $t$, will asymptotically approach the quantity $A$.

Since honey bees range from 50 mm to 150 mm in length, it was important to normalize by bee size. Defining $\phi(t) = a(t)/A$, the fraction of bee area which is clean, and $\phi_a = a_0/A$, the fraction clean at the start of the experiment, gives the resulting non-dimensional differential equation

\[
\frac{\partial \phi}{\partial t} = k \phi^{(1 - \phi_a) \exp(-kt)}
\]

with solution $\phi(t) = 1 - (1 - \phi_a) \exp(-kt)$, illustrated in Fig. 3b, where $k$, again, is the grooming rate (min s$^{-1}$). The condition

Fig. 3. Data and curve fitting of bee cleanliness over time. (a) Dimensional and (b) non-dimensional areas can be approximated by the expressions $\mathbf{x}(t) = A - (A - a_0) \exp(-kt)$ and $\phi(t) = 1 - (1 - \phi_a) \exp(-kt)$ respectively, where the parameter $A$ represents the long-term asymptotically clean area of fly honey bees (mm$^2$), $a_0$ represents the area of five honey bee areas that were clean at the start of the experiment, and $k$ is the grooming rate (time$^{-1}$). Parameters and curves in (a) and (b) are for illustration only. (c) Individual time series are highly stochastic due to bee motion and grooming behaviors, but the fit to model curves from (a) and (b) yields the individual grooming rate parameter $k$, quantifying the grooming process ($A = 26.8$ mm$^2$, $a_0 = 10.2$ mm$^2$, $k = 0.46$ min$^{-1}$). (d) Curve fits for different experiments may have very different grooming rates and starting cleanliness ratios. One curve appears to have decreasing cleanliness, but is treated as an outlier (see Section 4).
\[ \lim_{t \to \infty} \phi(t) = \phi_0 \]

is defined in a manner similar to the dimensional differential equation, using proportions of clean bee area, rather than total bee area.

3. Results

3.1. Time series

The time series \( \phi(t) \) of the data were then fitted to the dimensional model to obtain the parameters \( \phi_0, A, \) and \( k \) for each experiment. The global data were highly stochastic due to bee motion and other behaviors, so parameter fitting was done with the MATLAB package i nomin (The MathWorks). Fig. 3c shows a typical time series and resulting curve from nonlinear least-squares fit. These data were then converted to their non-dimensional counterparts, using the parameters from the dimensional fit.

3.2. Histogram and ANOVA of log grooming rates

Although the primary goal of this study is in assay development, we examined the grooming behavior of three commercial stocks of bees to calculate their grooming rates as a proof-of-concept. We chose to study Russian, Italian, and Minnesota Hygienic honey bees, since the Russian and Minnesota Hygienic bees are bred for their superior hygienic traits (Spivak and Reuter, 2001). We wanted to explore if these two stocks have statistically significant higher grooming rates than the Italian bees, which are known for their relatively high honey production and low defensiveness. The hygienic behaviors of the Russian and Minnesota Hygienic bees have previously been shown to be quantitatively greater than that for the Italian bees (Spivak and Reuter, 2001). If the grooming rates of the Russian and Minnesota Hygienic bees are equivalent to the rate obtained from the Italian bees, then this would suggest that hygienic behavior and grooming behavior are uncoupled at the individual and colony levels.

In order to explore the null hypothesis that different honey bee commercial stocks exhibit different grooming rates, here, denoted \( k \), analysis of the time until clean (1/\( k \)) and a means comparison test of log grooming rates was performed. Fig. 4a shows that approximately 94.5% of the 96 grooming experiments were complete in seven minutes or less. This percent changes to approximately 88% in five minutes or less. The null hypothesis that different stocks exhibit different grooming rates was tested with a one-way ANOVA, and was rejected with p-value 0.13 (Table 1). A swarm plot of the respective stock log grooming rates (Fig. 4b) gives visual confirmation of the overall equivalence of grooming rates between different stocks (plot routine from the MATLAB File Exchange package plotSpread; Jonas, 2016). However, it is possible that there are subtle differences, such as a markedly narrower distribution for the Russian stock. Although the mean \( \phi_0 \) was different for the three stocks (\( p = 0.3 \)), since the starting line coverage was parameterized by experiment, that experiment should uniquely define the resulting grooming rates. In this pilot study, there are no statistically significant differences in the grooming rates between stocks. Larger studies may find differences not evident in this present study.

4. Discussion

Dead or diseased honey bee brood are a critical factor in determining the overall health of a honey bee colony. The Minnesota Hygienic bees are a special commercial stock of honey bees, sele-
ertia bred for their ability to remove dead honey bee brood from the hive (Spiwak and Reuter, 2001). This behavior leads to a healthier colony, as harbingers of diseases and pathogens are quickly removed from the hive before they have the opportunity to spread.

Grooming is another important aspect of honey bee health. Honey bees that exhibit high levels of grooming should be less susceptible to diseases than bees that favor other behaviors (Arenchaveta-Velasco et al., 2012). One goal of this study was to test whether hygienic honey bees are any better at grooming than bees whose hygienic behaviors are unknown. Since this hypothesis was rejected, it implies that more work needs to be done on the study of honey bee grooming as a function of social immunity, independently from hygienic behavior. Our results demonstrate, however, that the underlying genetic mechanisms that govern hygienic behavior (Lapidge et al., 2002) are likely distinct and uncoupled from those underlying grooming behavior.

This assay is an important step towards studying honey bee behaviors that have been largely neglected due to the amount of labor needed to acquire even a basic understanding of bee ethology. Coating bees in baking flour is an extraordinarily cheap, quick, and safe way to instigate autogrooming behavior. Most scientists have access to recording equipment and personal computers, so this assay can be easily replicated in any lab. The most challenging aspect of this setup is the lighting; special care needs to be taken to ensure the recording area has proper illumination, without causing reflective glare from the petri dish or shadows cast by the honey bees.

There are multiple changes that can be made to our experimental protocol to ensure greater success in the future. Although much care was taken to ensure that bees were coated in flour and transferred to the recording arena immediately, it appeared that much of the grooming occurs within the first two to four minutes of the recording, and as such parameterization of initial bee cleanliness was necessary. The two-minute acclimation buffer may be unnecessary, and certainly could be shortened, but direct experimentation would be required. Moreover, across all of our experiments, it seems clear that 12 min of recording time was far more than necessary. All of our experimental timeframes could be shortened conservatively to ~6 min, making this method even more efficient than originally anticipated.

One possible explanation for the different mean values across stocks might be effects of temperature or humidity on the days each experiment was performed, but neither factor predicted parameter fits ($p > 0.3$). A different explanation might be differences in hair coverage between the stocks. The honey bee exoskeleton is covered with thousands of macro- and microscopic setae (or simply, hairs) that both provide sensory information from the environment function and facilitate pollen foraging (Winston, 1987). Bee hair was not measured, but it is plausible that there are consistent differences in hair between stocks.

Honey bees are eusocial insects, and prior to running the experiments, it seemed intuitive to film experiments as groups of five honey bees, in case both allo- and autogrooming events were observed. Unfortunately, it turned out that the observed behavior was quite different than originally hypothesized. Honey bees that were overwhelmed with external stimuli, in fact, exhibited extremely selfish behavior, and seemed to focus on autogrooming entirely. To this end, it would be beneficial to build on this experimental design by focusing on one bee at a time, rather than on groups of five. Since the first six minutes of this protocol are critical for examining honey bee grooming behavior, this change in protocol would facilitate a faster turnover between the time the bee has been coated in flour and the time the recording process begins. That said, pooling groups of five was intentionally done as a means of controlling for variability of individuals belonging to a significantly larger collective. Comparisons between individual versus pooled protocols need to be made. A modified experimental design that might capture both allo- and autogrooming behaviors might be adding a single bee coated in flour to a dish with one (or more) uncoated bees. This design might allow both grooming behaviors to be studied at the same time, more accurately capturing the overall grooming rate in social insects.

The chosen model has reasonable assumptions, and characterizes the typical forms of these time series. Although other models (not included in this study) were tested, they were rejected either due to their complexity, or because they were lacking a parameter describing how clean the bees were at the start of recording ($q_0$). One small issue with the selected mathematical model is that the data may start above the asymptotic value of a clean honey bee because of stochastic noise. For example, one curve started at a value of 120% (Fig. 3d). Since our model is based on a differential equation, the fit cannot pass below the asymptotic value of 100%.

This type of curve can occur when bees clump together at the start of the experiment, resulting in the illusion that the bees are becoming dirtier. This event only occurred once, however, and was treated as an outlier.

5. Conclusion

Image thresholding could be applied in any system whereby an organism can be recorded against a sufficiently contrasting solid background. Grooming can be measured as a function of time. While more research is required to directly measure honey bee grooming, it is clearly an important yet understudied aspect of social immunity. Many current assays only measure grooming as a function of parasite removal. Hopefully, the novel assay described here will lead to additional empirical studies on this topic, or successful breeding programs. Future work could be expanded to other insects such as ants, wasps, and other bee species.

Conflict of interest

No conflict of interest.

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CHAPTER 3

Viral load alters behavior of bee parasite *Varroa destructor*
Abstract

The invasive mite Varroa destructor has negatively impacted global apiculture, by being a vector for many viruses of the honey bee (Apis mellifera). Until now, most studies have been limited to varroa-honey bee or virus-honey bee interactions. The aim of this study is to bridge the important research gap of varroa-virus interactions by correlating varroa behavior with viral load. Ten-minute video recordings of 200 varroa mites were analyzed, and average speeds of the mites were compared to individual qPCR viral loads for deformed wing virus (DWV) and sacbrood virus (SBV). Statistical models reveal that colony, DWV, and SBV all play a significant role in mite behavior, suggesting that the varroa-virus interaction needs to be an integral part of future studies on honey bee pathogens.
3.1 Introduction

The invasive mite *Varroa destructor* has negatively impacted apiculture worldwide (Oldroyd, 2007). Varroa experienced an evolutionary host-shift from the Asian honey bee (*Apis cerana*) to the European honey bee (*Apis mellifera*) as early as 1960, and has been strongly implicated for playing a role in Colony Collapse Disorder and reduced health of bees in general (Oldroyd, 2007; Winston, 1987). These ectoparasites go through two major phases in their life cycle—the reproductive and phoretic stages. During the reproductive stage, a single varroa female infests the cell of an immature honey bee (pupa), feeding on the hemolymph of the developing bee. In doing so, the parasite can directly vector several viruses within honey bee colonies (Rosenkranz *et al.*, 2010; de Miranda *et al.*, 2013). During the phoretic stage, varroa mites emerge with the enclosed bee and continue to feed on adult honey bee hemolymph for sustenance (Rosenkranz *et al.*, 2010) and continue to spread viral pathogens horizontally among nestmates (Chen *et al.*, 2006). Honey bee colonies can exceed 50,000 bees, with only one female reproductive (the queen) (Winston, 1987). When a queen is infected with viral pathogens, the health of the entire colony can be compromised as she then has the potential to vertically transmit virus to her offspring through oviposition (Chen *et al.*, 2006).

The Vector Manipulation Hypothesis suggests that pathogens can modify the motility behavior or host preference for vector organisms, aiding in the spread of pathogens to the target hosts (Ingwell *et al.*, 2012). There are many known examples in arthropods of viruses or parasites altering vector behavior (Ingwell *et al.*, 2012; Cator *et al.*, 2012). The Vector Manipulation Hypothesis supports the possibility that infected mite vectors could exhibit higher motility than uninfected individuals. Such behavioral modifications could determine the
success or failure for certain viruses to spread within and among honey bee colonies, and it could have significant consequences for global bee health and the means to mitigate disease.

The study of varroa is important for the future success of honey bee management. The introduction of varroa to the United States has heavily impacted colony health, honey bee economics, and integrated pest management (Oldroyd, 2007; Spreafico et al., 2001; Pettis, 2004). The mode of action for many pesticides is similar for both insects and arachnids, both belonging to the same phylum, Arthropoda (Thrasyvoulou et al., 1988). Thus, treating for varroa can have negative implications on honey bee colony health, even leading to mortality within or of the colony (Thrasyvoulou et al., 1988). On the other hand, mites themselves impact colony health by vectoring pathogens (Tentcheva et al., 2004). This tradeoff forces beekeepers to make difficult decisions, and optimal varroa treatment strategy is not always clear.

As a further complication, varroa have developed resistance to common pesticide treatments (Spreafico et al., 2001; Pettis, 2004), which has influenced modern studies emphasizing behavioral treatments over chemical ones. Since the introduction of varroa to honey bees has presumably modified bee behavior, most previous studies focus on how honey bees behave toward varroa or bee grooming (Ibrahim and Spivak, 2006; Harris, 2007; Giuffre et al., 2017). Most of these assays examine some indirect artifact of honey bee behavior, such as the freeze-brood and sticky-board assays. One assay more directly measured the ability for a honey bee to bite and damage the exoskeleton or legs of varroa, rendering them dead or useless (Hunt et al., 2016).

More than 18 honey bee viruses have been identified, with six major viruses at the center of global scientific interest: deformed wing virus, sacbrood virus, black queen cell virus,
Kashmir bee virus, acute bee paralysis virus, and chronic bee paralysis virus (Chen and Siede, 2007; Genersch and Aubert, 2010; de Miranda et al., 2013). Due to the bees available for the present study, focus is narrowed onto two major players in honey bee virology: deformed wing virus (DWV) and sacbrood virus (SBV). DWV, in particular, has been of scientific interest because of its connection with varroa transmission. Although DWV can be spread to larvae by vectored mites, physiological differences in infected individuals are not apparent until the adult stage of the honey bee (Chen and Siede, 2007; Genersch and Aubert, 2010; Gisder et al., 2009). The host bee pupates and often develops with deformed wings, rendering adults unable to contribute to foraging duties in the colony (Chen and Siede, 2007). SBV targets the brood cycle of honey bees, preventing a brood from pupating and thus resulting in larval death. Both these viruses have been found in varroa, though it appears varroa are only vectors for DWV and not SBV (Chen and Siede, 2007; de Miranda et al., 2013). The low mortality and virulence of DWV benefits both virus prevalence and varroa dispersal, whereas SBV increases the chance of varroa mortality alongside the dead honey bee brood (Mondet et al., 2014). The aim of this study is to test the Vector Manipulation Hypothesis by correlating mite behavioral phenotypes to their viral status, identifying the role this interaction plays in the entire honey bee system.

3.2 Methods

3.2.1 Mite collection

Mites were collected at the Lake Wheeler Honey Bee Research facility located in Raleigh, NC. Once varroa infestations were identified in a colony, mites were gathered using the sugar shaking method, a process that safely dislodges live mites off their honey bee hosts.
The mites were subsequently gently rinsed in phosphate-buffered solution, removing excess sugar from their exoskeleton (Dietemann et al., 2013). Mites were then placed in a 60 mm-diameter petri dish, creating a small arena for the mite to explore over the course of the experiment. Fifty mites were gathered from each of four unrelated colonies, yielding 200 experimental subjects.

**Figure 3-1. Experimental setup.** A camera was mounted 27 cm directly above a collection of 10 petri dishes, with one varroa mite per dish. Two utility lamps were mounted in a square surrounding the recording area, angled to minimize glare off the dish and maximize illumination of the recording area (figure not to scale). Once the footage was recorded, mites were individually transferred to micropipette tubes and placed in a -80 °C freezer for subsequent analysis of viral loads.
All recordings were taken in a dark room with one Sony Handycam mounted approximately 10⅝ inches (27 centimeters) above the dishes containing the mites (Fig 3-1). Two utility light clamps (Coleman) were installed with 10W (60W-equivalent) LED light bulbs (Lighting Science) and placed on the perimeter of the recording arena to provide uniform lighting. Beneath the dishes was a sheet of white paper. Individual mites were recorded for 10 minutes at 30 frames per second with 1 minute of buffering at the beginning of each video, for a total recording time of 11 minutes. A total of 40 videos were recorded over the span of three days, with ten dishes per recording.

3.2.2 Quantitative PCR

RNA was extracted from individual varroa mites (de Miranda et al., 2013) using the BioBasic EZ-10 Spin Column Total RNA Mini-Preps Kit, resuspended in water, and tested for Vbeta-actin and Vdes-28s as reference genes (Evans, 2006) and for the same viral targets via real-time PCR on the same machine using KAPA SYBR FAST One-Step qRT-PCR ABIPrism Kit. The reaction mix contained 2.5 µL of SYBR, 0.25 µL of primer, 0.2 µL of KAPA RT Mix, 1.25 µL of water and 1µL of sample for a final reaction volume of 5.2 µL. The qPCR program ran at 42 °C for 5 min, 52 °C for 3 min, 95 °C for 3 min, then cycled 40 times through 95 °C for 3 sec, 58 °C for 25 sec, and 72 °C for 1 sec, then performed a melt curve step. Results were verified by melt curve temperature and normalized (Pfaffl, 2001) via GeNorm.
3.2.3 MATLAB

Videos were processed using a custom algorithm written in MATLAB R2015b (The Math Works Inc., Natick, Massachusetts, United States). This algorithm follows four steps, requiring minimal user intervention only on the first step: (1) Dish partitioning, (2) Frame extraction and thresholding, (3) Centroid calculation, and (4) Metric calculation. Steps 1-3 of the algorithm are visualized in Figure 3-2.

Because multiple dishes were filmed simultaneously, individual dishes had to be identified and segmented from the videos. The manual partitioning step forces the user to define boundaries on each dish using the first frame from the video and the MATLAB R2015b Image Processing Toolbox command `imcircle` (The Math Works Inc., Natick, Massachusetts, United States). This step serves a dual purpose in the context of this experiment. First, it enables the centroids calculated in step 3 to be joined and identified as belonging to the same varroa mite, then centered at the origin, irrespective of where the dish was placed in the recording area. Second, it allows for quick conversion between pixel and metric data, using the diameter of the petri dish as a scale.

Video frames were converted to grayscale. ImageJ (National Institutes of Health, Bethesda MD – USA) was used to estimate the proper binary thresholding value of (65 of 255 or $\approx 0.26$, 255 = white) for the mites, which was applied uniformly across every image sequence. It was important to ensure that the thresholding value was low enough to distinguish the mite from the background, but high enough to avoid tracking shadows cast by mites or dish edges. The result was a discrete sequence of images containing an isolated cluster of pixels, representative of a single varroa mite. On occasion, pixel values were misinterpreted in the
thresholding step. The MATLAB command `regionprops` (The Math Works Inc., Natick, Massachusetts, United States) computed the convex hull for candidate clusters of pixels that may have been the mite. If the cluster size was lower than the expected pixel area of a mite, it was eliminated as background noise. The centroid of the remaining pixel cluster was then interpreted as the Euclidean coordinate location of the mite, which was used to determine behavioral parameters that might be of interest.

![Figure 3-2. Visualization of the steps for the mite detection algorithm.](image)

For each video frame, (A) Petri dish edges were determined then (B) the images were converted to grayscale and binary images were produced using a threshold value of 0.255 on a 0-1 scale. Finally, (C) the convex hull of the mite was used to compute a centroid in the Cartesian plane.

Standard measurements such as velocity and average speed are useful in analyzing complete, continuous, and smooth data. Within each mite’s movement track, there are isolated instances where a mite may have disappeared due to the mite crawling on the side of the petri dish. Varroa are extremely flat organisms, and therefore they appear as an ellipse when viewed
dorsally but only as a sliver when viewed laterally. Since any mite can be hard to detect if it
turns edge-on, careful consideration had to be made in regards to how behavioral metrics were
calculated in the presence of discontinuities in mite tracks. Furthermore, centroid calculation
can introduce small perturbations in coordinate data. These perturbations improperly skewed
typical rate-of-change calculations such as instantaneous speed and velocity.

To compensate, a surrogate metric for average speed was calculated in step 4 of the
processing algorithm. Because the mites are approximately 1 mm in width, dividing the total
track area covered by a mite per unit time by the mite width yields a reasonable estimate of the
mite average speed. Each dish was gridded with 1 mm$^2$ squares and treated as a binary 60 by
60 “visitation matrix” (Figure 3-3). If a mite centroid was found in any given grid square, it
was assigned a value of one, otherwise zero. The total area traveled, in square millimeters,
could then be divided by the amount of time the mite was successfully tracked, resulting in the
following estimate of average speed $S$:

$$ S (mm/min) = \frac{\text{(number of 1 mm}^2 \text{ squares)}}{\text{(total time tracked in mins)}} \times \frac{1}{1 mm} $$
3.3 Results

The behavioral and qPCR data were statistically analyzed using JMP Pro 11.0 (SAS Institute, Cary, NC, USA). Mites were tested for a suite of 8 common honey bee viruses, however the collected mites only tested positive for DWV and SBV. Of the 200 total mites, 194 remained for statistical analysis after accounting for various technical errors or small representation in categories (Table 3-1). Of those 194, whose tracks are displayed in Figure 3-4, 120 tested negative for infection (Figure 3-4A). Colonies 1 and 2 were primarily uninfected, with only two mites from each colony testing positive for both DWV and SBV. In contrast, colonies 3 and 4 displayed a variety of infection patterns: uninfected (Fig 3-4A), infected with DWV only (Figure 3-4B), and infected with both DWV and SBV (Figure 3-4C).
Table 3-1. Breakdown of processed data set and exclusions.

Of 200 mites, 6 were not included in statistical analysis: 1 mite was only infected by SBV, 3 mites were not analyzed by qPCR, and 2 mites could not be tracked for any portion of the ten-minute video. Infection types for the remaining 194 mites (uninfected/DWV-only/both) are also provided.

<table>
<thead>
<tr>
<th>colony</th>
<th>analyzed</th>
<th>not analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>uninfected</td>
<td>infected</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DWV</td>
</tr>
<tr>
<td>1</td>
<td>48</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>46</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>19</td>
<td>18</td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>20</td>
</tr>
<tr>
<td>totals</td>
<td>120</td>
<td>38</td>
</tr>
</tbody>
</table>
Figure 3-4. Mite tracks grouped by colony and viral status. (a) Uninfected mites grouped by colony and sorted by average speed. (b) Mites only infected by DWV, grouped by colony and sorted by average speed. (c) Mites infected with both DWV and SBV, sorted by SBV viron count.
Table 3.2. Statistical models. Models were combinatorially tested across multiple levels. The leading rows of the table provide quick identification for the model and parameters of interest for that model (i.e., Model CD uses parameters $\alpha$, $\beta$, and $\gamma$). Poor parameter estimates are highlighted (light red). AICc and BIC metrics were normalized according to the expression $\Delta IC = IC - \min(IC)$, then color-coded on a scale from 0 (green, strong information criterion model selection) to $\max(\Delta IC)$ (red, unfavorable information criterion model selection) so that model selection could be quickly verified. Models with $IC = 0$ are boldfaced. Additional rejected models ($p > .05$) not shown.

<table>
<thead>
<tr>
<th>model</th>
<th>terms</th>
<th>parameter estimates</th>
<th>model evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$\alpha$ (intercept) + $\beta$*colony + $\gamma$<em>kDWV + $\delta$<em>SBV + $\theta$</em>(kDWV-D0)</em>(SBV-S0) = average speed (mm/min)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\alpha$ mm/min</td>
<td>$\beta$ mm/min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$p$</td>
<td>$p$</td>
</tr>
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<td></td>
<td></td>
<td>est</td>
<td>SE</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td>37.4</td>
<td>2.1</td>
</tr>
<tr>
<td>D</td>
<td></td>
<td>37.5</td>
<td>2.2</td>
</tr>
<tr>
<td>S</td>
<td></td>
<td>37.3</td>
<td>2.2</td>
</tr>
<tr>
<td>CD</td>
<td></td>
<td>37.9</td>
<td>2.2</td>
</tr>
<tr>
<td>CS</td>
<td></td>
<td>37.6</td>
<td>2.2</td>
</tr>
<tr>
<td>CDS</td>
<td></td>
<td>38.1</td>
<td>2.2</td>
</tr>
<tr>
<td>DSN</td>
<td></td>
<td>36.2</td>
<td>2.3</td>
</tr>
<tr>
<td>CSN</td>
<td></td>
<td>36.4</td>
<td>2.2</td>
</tr>
<tr>
<td>CDSN</td>
<td></td>
<td>37.0</td>
<td>2.2</td>
</tr>
</tbody>
</table>
The statistical model

\[
\text{Average Speed (S)} = \alpha \text{ (intercept)} + \beta \ast \text{Colony} + \gamma \ast k\text{DWV} + \delta \ast \text{SBV} \\
+ \theta \ast (k\text{DWV} - D0) \ast (\text{SBV} - S0)
\]

includes terms for intercept, colony (C), DWV (D), SBV (S), and a nonlinear interaction (N) between viruses. We combinatorially tested models containing or omitting each of these terms, e.g. Model CD contains only the intercept, colony, and DWV dependence. Additionally, we tested models with nonlinear dependence on DWV or SBV. Fits for parameters are shown in Table 3-2. Since the order of magnitude of DWV virons was significantly larger than of SBV virons, we use kilo-DWV virons (kDWV) as a unit of DWV viral count. Fits of statistical models were tested, with average speed (mm/min) as a response variable, and colony, kilo-DWV virons, and SBV virons as the effect variables. In general, models were rejected at a level of \(\alpha = 0.05\); additional rejected models are not shown.

Colony effects are not the major effects of interest in this study. However, it was important to establish whether colony-level effects were present before building up more complicated models. Model C was accepted with a p-value < 0.01 (numerator d.f. = 3), indicating that the colony of origin influences average speed of the mite.

The main effects of viral loads (kDWV, SBV) on average speed were explored in Models D and S, ignoring colony-level effects. Model D (\(p = 0.66\), d.f. = 1) and Model S (\(p = 0.82\), d.f. = 1) were both rejected. However, addition of the nonlinear viral interaction yielded Model DSN, which was accepted with a p-value of 0.04 (d.f. = 3). Finally, Model CDS (\(p = 0.02\), d.f. = 5) and Model CDSN (\(p < 0.01\), d.f. = 6) were both accepted.
Selection of appropriate and parsimonious statistical models was done using Bayesian information criterion (BIC) and corrected Akaike information criterion (AICc), two philosophically different approaches for model selection (Table 3-2). Both metrics attempt to maximize goodness-of-fit, while minimizing the number of parameters used to avoid overfitting the data. BIC and AICc are reported in Table 3-2.

3.4 Discussion

It is not the aim of this paper to philosophically discuss merits of BIC versus AICc, as other contributions can be found discussing this issue (Burnham and Anderson, 2004; Link and Barker, 2006). Instead, model selection will be supported using biological arguments, and emphasize the need for future work to be done using similar assays.

BIC favors selection of Model C (smallest BIC) which suggests that mite viral load seems to be unrelated to mite behavior. At the colony level, phoretic mites may modify their behavior based on environmental conditions, such as bee population, brood availability, hive temperature, or even bee grooming. Although none of these variables were measured in the current study, environmental conditions clearly play a direct role in virus’ ability to spread by impacting vector motility. It could be argued that colonies have different viral profiles responsible for these global effects. Unfortunately, four colonies were not enough to determine such effects.

AICc selects Model CSN, which also has the lowest p-value. The estimates of $\gamma$ (mm/min/kDWV) have high variability between models, are nearly centered at zero, and have high p-values, bringing to question the kind of impact DWV has on mite behavior. However,
the selection of statistical models that include cross-terms without also including the related linear terms has been a topic of debate in the statistical community (Nelder, 1998; Rindskopf, 1990).

The most complicated Model CDSN also performs very well, but its AICc is higher than Model CSN's AICc. Nonetheless, Model CDSN is of interest, because while DWV is known to replicate in varroa, SBV is not (Chen and Siede, 2007; de Miranda et al., 2013). SBV should not be modifying varroa RNA, and therefore, should have no interactive relationship with their behavior (de Miranda et al., 2013). One possible explanation for this might be that SBV plays a more important role in colony-level effects, which indirectly changes their behavior, however the Model CS(C*S) with colony and SBV crossed was rejected at level $\alpha = 0.05$. Recent studies show that viruses can interact with one another in nontrivial ways, including altered host susceptibility due to a breakdown in physical barriers in both plants and animals, which may be true for vectors, as well (DaPalma et al., 2010; Syller, 2012; Celum et al., 2004). Although SBV does not modify varroa RNA, it could be that SBV-bearing mites are more susceptible to effects from DWV, or that SBV replication occurs in the presence of DWV, but this possibility will require further study. Our data indicate that the DWV-SBV interaction generally causes mites to move more slowly. SBV encourages faster mite movement, except in Models S, CS and CDS, where the p-values for $\delta$ (mm/min/SBV) estimation are high (Table 3-2).

Many questions remain, such as how or why DWV and SBV interact. This study had mite selection and tracking performed blind to the types and levels of infection: it was possible that none of the mites were infected, or that they had a suite of viruses with even more
complicated levels of cross-infection. The limited results of this study suggest the need for a more controlled experiment, where varroa are inoculated with specific virus levels and types, or where varroa are gathered from more than four colonies over a longer timeframe, with greater knowledge of colony phenotypes or environments.

This assay is a novel attempt at exploring varroa behavior, in addition to tying results to the Vector Manipulation Hypothesis. The AICc values for all models suggest that viral loads play a key role in how varroa explore their surroundings, supporting this hypothesis and akin to findings in other areas of entomology (Ingwell et al., 2012; Cator et al., 2012). Varroa behavior should be studied more for the role that it plays in the host-vector-disease interaction, and this study provides a framework for such future studies.

The bioassay developed in this paper attempts to facilitate data gathering by making it high-throughput and easy-to-track. It could be modified, e.g. by using particle image velocimetry, or restricting the mite to the upper or lower surface of the dish.

Finally, this study opens the door for a host-preference study on varroa. Honey bee pheromones are notably vital for communication in the dark environment of a hive (Winston, 1987). Using a similar experimental design, one could test viral effects on host preference for *A. mellifera* drones, versus workers, versus *A. cerana*. Perhaps mite viral ethology could better explain this evolutionary jump, or further support it. Testing and measuring mite behavior in vitro may could help untangle the more complicated vector-disease dynamics of the hive.
3.5 References


18. Nelder JA. The selection of terms in response-surface models—how strong is the weak-


CHAPTER 4

A classification algorithm for honey bee (*Apis mellifera*) corbicular pollen using digital colorimetry
Abstract

Pollination is an important ecosystem service for agriculture, and pollen is the main protein source for bees that promotes their health and productivity. Understanding the source(s) of pollen collected by foraging bees is thus important for understanding foraging ecology and bee health. Many modern techniques for identifying pollen loads brought back to colonies of Western honey bee (*Apis mellifera*) are either time-consuming, expensive, or both. We present a naïve Bayes classification algorithm that uses pollen pellet color as the main identifying feature, and we develop a cheap, fast method for identifying species of honey bee pollen loads using a flatbed scanning device for data acquisition. This method could be combined with additional information such as time of year, geographical location, or other pollen databases to further expand our knowledge of pollinator services. We identify procedures to standardize the construction of a common color-library that links pollen type, plant source, and other information (e.g., genetic sequence, chemical analysis) to facilitate pollen identification in a rapid, objective manner.
4.1 Introduction

Pollen is the male gamete of flowering plants that is necessary for sexual reproduction. Angiosperms often require animal vectors for cross-pollination, and the Western honey bee (Apis mellifera) has historically been the most important managed pollinator in natural and agro-ecosystems (Winston, 1987). Angiosperm flowers from Europe and Asia have developed a wide array of colors, patterns, and scents to advertise resource abundance, and are known to have coevolved with honey bees as their primary pollinator (Menzel, 1985; Hill et al., 1997; Raguso, 2008). As such, since bee services are so critical, beekeepers from across the United States transport their colonies to various crops to perform pollination services (Almond Almanac, 2015), including citrus, squash, and almonds (Winston, 1987; Aizen and Harder, 2009; Morse and Calderone, 2000; Gallai et al., 2009). One recent study evaluated global pollinator services at $162 billion USD annually (Gallai et al., 2009), a significant portion of which is provided by honey bees.

Pollen is the primary source of protein for developing larvae and young adult honey bees less than four days old (Winston, 1987; Crailsheim et al., 1992). Honey bees are opportunistic and generalist foragers, but an individual forager collects food from only one plant source at a time (floral constancy) (Winston, 1987). This behavioral fidelity results in pollen grains on the surface of the visiting forager being deposited on the female structures of subsequent flowers, facilitating cross pollination and therefore the reproductive success of the plant (Winston, 1987; Dai and Law, 1995). At the colony level, however, different foragers can collect pollen from many different plant sources, often bypassing species that could potentially be more rewarding (Winston, 1987). Excluding abundance and availability, factors
such as pollen grain size, shape (Nicholls and Hempel de Ibarra, 2016), and olfactory cues (Arenas and Farina, 2012) all seem to influence the selection of certain plants over others, though some studies debate the specifics of their relative importance (Pernal and Currie, 2002).

Honey bees do not consume raw pollen brought back to the hive directly. When honey bees return to the hive with pollen in their corbiculae (or pollen-baskets), they seek out the areas within the nest, directly adjacent to the developing brood, where pollen is stored, then directly deposit their pollen load into a cell. Subsequently, middle-aged food-processor bees tightly pack the pollen into the cell, which over time may contain pollen from a variety of different plants. Honey, nectar, and glandular secretions are added by the processor bee, which facilitates beneficial microbes (mostly yeasts and fungi) fermenting the packed pollen into what is known as “bee bread” (Herbert and Simanuki, 1978). Bee bread is the final product consumed by developing brood and young adult bees, and it provides not only the main protein source but also sugar, starch, lipids, fiber, and pectin (Herbert and Simanuki, 1978).

Despite the nutritional importance of honey bee pollen, there has been a surprising lack of emphasis on quickly and accurately identifying a given pollen’s source. Most scientific studies either assume plant sources based on pollen grains found in honey (González-Miret et al., 2005) or were done before personal computers became widely accessible (Hodges, 1974; Kirk, 1994). A major reason for this scientific gap is that manual identification of a relatively small sample (< 1000) of pollen pellets can take many hours, requiring a high level of skill or equipment. To complicate matters further, only a handful of individuals have written honey bee pollen color keys, likely due to the labor required to produce a thorough and accurate pollen key (Hodges, 1974; Kirk, 1994; Pollen Color Chart, 2014; Sheffield Beekeepers'
Recent studies on pollen have used financially or time-expensive techniques, such as chemical composition (Almeida-Muradian et al., 2005), spectroscopy (Pappas et al., 2003), and microimaging (France et al., 1997).

Many algorithms for digital color imaging have proven successful in several industries. In textiles, for example, quality control for color in fabrics has been digitized, reducing the need for human intervention on defects (Anagnostopoulos et al., 2001). In agriculture, colorimetry has been used to test produce for freshness and pests (Brosnan and Sun, 2004). With interest in pollinator health in general and honey bee nutrition in particular, there is a great opportunity to incorporate colorimetry into pollen identification based on established technologies available in other industries. The goal of this study is to develop a high-throughput system of pollen identification based on color to more thoroughly process and describe the foraging efforts of individual honey bee colonies.

4.2 Materials and methods

4.2.1 Pollen collection

Pollen samples were gathered from individual colonies using pollen traps on during the summer of 2014, in Newark, Delaware, then stored in bags in a -80 °C freezer until further processing. Pollen pellets gathered in this manner average approximately 3 mm in diameter. Pollen samples were manually sorted by genera into piles and characterized based on their colors. Sorting was verified microscopically using other pellets from the same pile. In all, eight different pollen species were manually determined as an accurate overall-majority of the pollen gathered by the colony that day. Fifty individual pellets were randomly subsampled from each
of the eight species. Each subsample was scanned using a Canon LiDE 120 scanner at 600 dpi, then saved in uncompressed .tif image format. These eight images are defined in this paper as the “pollen library images.”

Two types of “pollen data images” were then fashioned similarly as described above: non-blind, where the pollen pellets were manually sorted then scanned; and blind, where pollen pellets from that day were randomly spread out and scanned, without any manual sorting performed. In the blind scans, one image was characterized as “wet” pollen, or pollen scanned directly from the -80 °C freezer, and one image was characterized as “dry” pollen, the same pollen from the wet sample, but heated at 30 °C for two hours.

4.2.2 Image analysis

First, all scanned images were color-corrected in the RGB color-space using an X-Rite Color Checker® with polynomial regression analysis (Figure 4-1a) (Hardeberg, 2001). Then, pollen pellet edges were detected using a thresholding-and-clustering algorithm (Figure 4-1b). In the process of arranging pollen pellets to be scanned, small flecks of pollen can break off and be seen in the resulting image. At a resolution of 300 dpi, the mean pixel area for the pollen pellets was approximately 867 pixels (≈ 2.8 mm diameter) with a standard deviation of 311 pixels. All clusters smaller than 100 pixels in area were interpreted as noise, and thrown out (Figure 4-1c). Many studies in colorimetry have established the device dependence, correlation of color channel components, and poor performance of mathematical operations in the RGB and HSV color spaces (Tkalcic and Tasic, 2003; Cheng et al., 2001; Ford and Roberts, 1998). In 1976, the Commission Internationale de l’Eclairage (CIE) proposed a color space
Figure 4-1. Steps in image processing. (a) Images were cropped and colors calibrated to color checker. Pollen pellets were segmented by thresholding. Segmented objects with area < 100 pixels were discarded. (b) Segmented pellets for each known species form pellet image libraries. (c) For each pellet, RGB color values are converted to CIE-Lab values. (d) Pixels from each pellet are averaged. (e) Each known species is represented by 50 pellets’ individual color measurements.
where the Euclidean distance between two colors is strongly correlated to human perception of color, also known as perceptual uniformity (Tkalcic and Tasic, 2003). The CIE-Lab (or, more simply, Lab) color space is the industry standard in many current applications of color science. Since the goal of this study was to actively compare the observed pixel colors between the library and data images, all pixel data were numerically converted from the RGB to the Lab color space.

4.2.3 Naïve Bayes classification

The naïve Bayes (NB) classification algorithm has many applications in image recognition, email spam filtering, and document classification (Mitchell, 1997). One reason for its popularity is that it can decouple multivariable input distributions and independently estimate each variable as a one-dimensional distribution. Since images are represented in three dimensions (or channels, i.e., Lab color space has variables $L$, $a$, and $b$), NB interprets each channel independently.

There are two core components in a NB classification scheme: the training model and the prediction step. Rigorous proof and details of this process can be found in (Mitchell, 1997). Presented here are generalized steps (modified from Mitchell, 1997) in the context of image analysis and pollen.

Given an unknown pollen sample’s Lab color values

$$X = (L, a, b)$$ (or, without loss of generality, $X = (x_1, x_2, x_3)$),

and a known discrete set of pollen classifications, by genera,

$$C = \text{(clover, sumac, corn, ...)} = (C_1, C_2, ..., C_k), \ k = \text{number of known pollen sources},$$
we wish to classify the unknown pollen $X$ as $y$ into one of our known sources, or equivalently, find the maximum probability that a pollen sample belongs to a class, given its Lab values, $p(C_k|X)$. Applying Bayes’ theorem, we have
\[
p(C_k|X) = \frac{p(C_k)p(X|C_k)}{p(X)}.\]
The term $p(X)$ represents the probability that pixel values belong to a pollen sample, which assumed to be true, and therefore, is equivalent to 1. The term $p(C_k)$ represents the underlying distribution of the pollen classes; ideally, this would be the true distribution of pollen samples that bees bring back to the hive. This distribution is sensitive to many external factors such as weather or time of year. For simplicity, we assume that $p(C_k)$ is the uniform distribution ($p(C_k) = 1/k$).

Assuming the three color channels are conditionally independent, the underlying distributions of the Lab values, given their class, can be decomposed as a product:
\[
p(X|C_k) = p(x_1, x_2, x_3|C_k) = p(x_1|C_k)p(x_2|C_k)p(x_3|C_k) = \prod_i p(x_i|C_k).
\]
Replacing this result and the underlying assumptions into the equation from Bayes’ theorem, we have,
\[
p(C_k|X) = \frac{1}{k} \prod_i p(x_i|C_k).
\]
This equation is also known as the NB training model. Since we wish to maximize the probability $p(C_k|X)$ over the classes $C_k$, the final classification scheme for $y$ is
\[
y = p(C_k|X) = \frac{1}{k} \arg\max_{C_k} \prod_i p(x_i|C_k).
\]
This equation is also known as the NB prediction step.

4.2.4 Pollen lookup table and data analysis

Images of 50 manually sorted pollen pellets from 8 plant species were scanned at the above specifications (Figure 4-1). Pollen color was measured as the mean tristimulus Lab value of all pixels within the boundary of a single pellet, then stored as a 1x3 vector in a pollen lookup table, or “pollen library”. Thus, the resulting library was a 400x3 matrix of mean Lab values. The MATLAB (The Math Works Inc., Natick, Massachusetts, United States) routine fitcnb was used on the library to train the NB model. Pollen color for both sorted and unsorted data images were similarly converted into \( N \times 3 \) matrices. The routine predict compared these data to the library model.

4.3 Results

4.3.1 Presorted pollen image

One image of presorted pollen was used as verification for the classification scheme (Figure 4-2). In this image, 12 pollen clusters were sampled and compared to the NB model. Altogether, this image had pollen from 11 plant species, with 8 of the 11 stored in the library matrix, and 3 from species not in the library. The last of the 12 clusters was used as a blind test to ensure there was no human confirmation bias in development of the classification algorithm.
Figure 4-2. Performance of naïve Bayes classification on a presorted data set. Pollen samples were presorted and compared against the known library (L1-8) of pollen species by color. U1-3 were three unknown pollen samples not present in the known library. Resulting classifications, Type-I and II errors, and error rates (green <= 10% incorrect, yellow <= 30%, red > 30%) are highlighted.
<table>
<thead>
<tr>
<th>Species</th>
<th>T2E</th>
<th>T1E</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1 clover</td>
<td>5/26 (19%)</td>
<td></td>
</tr>
<tr>
<td>L2 sumac</td>
<td>1/16 (6%)</td>
<td></td>
</tr>
<tr>
<td>L3 corn</td>
<td>2/17 (12%)</td>
<td></td>
</tr>
<tr>
<td>L4 willow</td>
<td>0/16 (0%)</td>
<td></td>
</tr>
<tr>
<td>L5 blind-type A</td>
<td>0/26 (0%)</td>
<td></td>
</tr>
<tr>
<td>L6 dandelion</td>
<td>3/21 (14%)</td>
<td></td>
</tr>
<tr>
<td>L7 blind-type B</td>
<td>2/30 (7%)</td>
<td></td>
</tr>
<tr>
<td>L8 blind-type C</td>
<td>0/20 (0%)</td>
<td></td>
</tr>
<tr>
<td>U1</td>
<td>28/28 (100%)</td>
<td></td>
</tr>
<tr>
<td>U2</td>
<td>29/29 (100%)</td>
<td></td>
</tr>
<tr>
<td>U3</td>
<td>19/19 (100%)</td>
<td></td>
</tr>
<tr>
<td>U4 sumac</td>
<td>5/20 (25%)</td>
<td></td>
</tr>
</tbody>
</table>
We define a Type I error (false positive) as the incorrect classification of a pollen sample that is not part of the pollen library. Similarly, we define a Type II error (false negative) as the classification of a pollen sample as one species, when it belongs to another. Overall, the NB predictor had a within-species Type-II error rate of 25% or less. Across the whole known pollen data set, approximately 9.4% (results not shown) of the pollen was misclassified. Since NB is a closed-set classification algorithm, data for pollen not already in the library results in a 100% Type-I error rate. Although the highest Type-II error rate happened to occur within our blind cluster sample, it should be noted that this was consistent with pollen 2 being improperly classified as pollen 6 (Figure 4-2). Principal component analysis (PCA) on the pollen library also revealed that the 95% confidence ellipses for pollens 2 and 6 have substantial overlap (Figure 4-3).
Figure 4-3. Pollen libraries against principal components and confidence ellipses. Principal component analysis was performed on the 8 pollen libraries. The resulting 95% confidence intervals on the principal component coordinates are indicated with ellipses.

For NB models, there is an important assumption of independence for the variables used to train the model. The angles between vectors on the PCA biplot (Figure 4-4, Table 4-1) indicate correlation between the $L$, $a$, and $b$, color channels in the pollen library. Although these correlation values indicate a violation of the independence assumption, empirical work has been done to show that often this violation does not matter, in practice (Domingos and Pazzani, 1997; Zhang, 2004).
Figure 4-4. Biplot for the principal components. The original Lab axes are projected onto the first two principal component axes, using the eigenvectors from the first two components. The angles between the three vectors ($\theta$) determine the correlation levels between the $L$, $a$, and $b$ channels according to the relationship $r = \cos(\theta)$. Correlations are provided in Table 4-1.
Table 4-1. Lab tristimulus channel correlation values. Angle measurements between the \( L, a, \) and \( b \) vectors from Figure 4-4. These angle measurements are used to calculate the correlation between the original tristimulus image channels. Since the \( L-a \) and \( a-b \) channels are 60% correlated, the NB independence assumption (Section 4.2.3) is violated. Since \( L \) and \( b \) are the least correlated of the three variables, pollen comparisons should be made on these two channels when PCA is unavailable on an unknown data set.

<table>
<thead>
<tr>
<th></th>
<th>( \theta = \text{angle (radians)} )</th>
<th>correlation = ( \cos(\theta) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( L )</td>
<td>( x )</td>
<td>2.22</td>
</tr>
<tr>
<td>( a )</td>
<td>( x )</td>
<td>0.93</td>
</tr>
</tbody>
</table>

4.3.2 Unsorted pollen images

Several unsorted pollen images were also scanned for analysis. However, constructing a complete pollen library for hundreds of geographically and seasonally relevant plant species is an onerous task. As such, one important underlying assumption in the analysis of these data images is that the pollen library is complete.

Water content of the nectar a forager mixes with pollen grains in the corbicula mostly determines pollen moisture (Winston, 1987; Reiter, 1947). Other factors, such as time in a pollen trap, exposure to heat, and evaporation/condensation in the freezer, can change pollen moisture, resulting in nontrivial color changes on the surface of the pellet. Therefore, it was important to characterize the color changes that might be observed in a data set. Approximately 650 pollen samples were taken directly from the freezer, spread out on a piece of paper, and imaged. Then, the paper was transferred to a hot plate, heated at 30 °C for two hours, transferred back to the scanner, and imaged again.

In our library, \( L \) and \( b \) are the least correlated color channels (Table 4-1), suggesting that \( L-b \) plane may be the best \( \mathbb{R}^2 \)-projection for visualization of the data samples. \( L-b \) coordinates for wet versus dry are visualized in Figure 4-5, and changes in the NB prediction
are quantified in Table 4-2. Pollen types 5 and 8 experienced the largest net change in their classifications, with approximately 170 pellets directly transferring from one class to the other. Since the color change because of drying out is inconsistent, a constant color shift for moisture would not correct the discrepancies. Therefore, this pilot comparison highlights the necessity to control pellet moisture, in both library and data image acquisition.
Figure 4-5. *L*-*b* scatterplot of wet and dehydrated pollen pellets. Wet pollen pellets were spread out, scanned, and classified using the Bayes model trained by the library (red). The same pellets were dehydrated and reclassified (blue). Shifts in the *L*-*b* plane are indicated by black arrows. Moisture level affects classification in some pollen data (8 to 5), but not others. The average norm for above data shifts was ~2.9 units, with a standard deviation of ~1.7 units. For visualization purposes, this figure contains only a random subsample (155 pellets) of the original image (648 pellets). Full data set is outlined in Table 4-2.
Table 4-2. Wet versus dehydrated pollen classification. The trained NB model predictions for a wet and dehydrated pollen data image. Predicted class counts are colored from low (blue) to high (yellow), and Δcount is colored from net loss (red) to net gain (green). One pollen sample was lost when transferring between the hot plate and the scanner.

<table>
<thead>
<tr>
<th>Predicted class</th>
<th>Wet counts</th>
<th>Dehydrated count</th>
<th>Δcount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8</td>
<td>5</td>
<td>-3</td>
</tr>
<tr>
<td>2</td>
<td>132</td>
<td>147</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>38</td>
<td>46</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>0</td>
<td>-2</td>
</tr>
<tr>
<td>5</td>
<td>66</td>
<td>242</td>
<td>176</td>
</tr>
<tr>
<td>6</td>
<td>31</td>
<td>12</td>
<td>-19</td>
</tr>
<tr>
<td>7</td>
<td>201</td>
<td>188</td>
<td>-13</td>
</tr>
<tr>
<td>8</td>
<td>170</td>
<td>7</td>
<td>-163</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>648</td>
<td>647</td>
<td></td>
</tr>
</tbody>
</table>
4.4 Discussion

Pollen identification is difficult to study, as the process can be onerous, expensive, and subjective rather than empirical. Nonetheless, there are many important reasons to empirically quantify pollen loads for their plant of origin. For example, growers contract honey bee colonies for their pollination services to improve yield, but verifying that the bees are actively foraging on the target crop is often anecdotal (Waller, 1980). One suggested method is quantifying the number of bees per 100 flowers (Waller, 1980), even though abiotic factors such as weather or time of day may skew these data. It would be much easier and statistically reliable to trap, image, and interpret pixel data from pollen loads to quickly confirm the percentage of pollen deriving from target versus non-target plant species. This method, therefore, has the potential to be developed into a simple and convenient app on a mobile phone that can quickly ascertain such information in the field and in real time.

4.4.1 Limitations

NB is a closed-set classification scheme; all data must be cast into an existing classification, even when it results in Type-I errors. Type-I errors are very difficult to deal with, since catching them requires manual identification, which fundamentally conflicts with the purpose of this study. Open-set recognition techniques, such as the 1-class SVM, 1-vs-set-machine algorithms, and the nearest non-outlier algorithm, attempt to address this problem by allowing the rejection of samples from a class, but current applications are limited to many spatial features in an entire image, not tristimulus pixel color (Wilber et al., 2013; Bendale and Boult, 2015). Unfortunately, open-set algorithms are very computationally expensive.
compared to closed-set techniques such as NB, and require a substantially-sized training set to succeed (Wilber et al., 2013; Bendale and Boult, 2015; Joshi et al., 2009). So-called “active learning” or “deep learning” models are relatively new, and beyond the scope of this study (Joshi et al., 2009; Rakotomamonjy et al., 2007). NB performs very well with a relatively small training set, and despite the independence assumption violation (common in image analysis), has become the modern standard in many face recognition and classification studies (Wold et al., 1987; Turk and Pentland, 1991; Kim et al., 2002; McCann and Lowe, 2012). Variables such as time of year, location, or weather might further improve pollen classification precision within the NB framework, and storing these informational variables is financially cheaper than storing the pollen itself.

Of course, the quality of pollen libraries is just as important as the data analysis. Such databases are only as strong as the level of manual verification that goes into constructing them, a labor-intensive process that would benefit from the contributions of many individuals, across multiple disciplines. This study has established the need for broader pollen color databases and the standardization of color by first dehydrating pollen samples.

Finally, much more work needs to go into the colorimetry of pollen, particularly sensitivity analysis of the Lab color space in distinguishing commonly encountered pollen colors, such as yellows and oranges.
4.4.2 Future work

Honey bees are known to fly up to 3 km from the hive in their foraging efforts. Time of year, resource abundance, and local ecology all play important roles in the plant species that a pollen forager might encounter, and therefore, the quality of the pollen library. To capture some of these complicated dynamics, a set of 4 images, spanning 4 days and 2 geographical locations, were also classified using the NB model. Unfortunately, due to the small data set, no strong connections between time of year and geographical location to pollen collection can be drawn in this study, but may be of interest to the reader (Figures 4-6 and 4-7), and is left for future work.

Figure 4-6. NB classification for four pollen images by hive and date. Each data sample came from 2 hives, across 4 unique images taken on different dates.
Another important application for rapid pollen identification could help mitigate the negative effects of environmental contaminants and therefore improve bee health. Previous studies have demonstrated that pollen contains numerous pesticides and other chemical toxins from the environment (e.g., Traynor et al., 2016; Mullin et al., 2010). Because corbicular pollen can contain up to ~5.5% lipids (Herbert and Simanuki, 1978), many lipophilic classes of pesticides and herbicides are inadvertently brought back to the colony and sequestered in the wax comb (Škerl et al., 2009; Chauzat et al., 2006; Johnson et al., 2010; Mullin et al., 2010). It would be beneficial to beekeepers if they could scan pollen loads to quickly diagnose what types of plants the bees are visiting, particularly when a colony dies suddenly or exhibits symptoms of an acute pesticide exposure.

Along with pesticide residues, it has been shown that pollen can be used as a bioindicator for pollution levels in the surrounding environment, which may have a subtle effect on bee health via oxidative stress and negatively influence average age in a colony (Kevan, 1999; Metcalfe and Alonso-Alvarez, 2010). The effects of urbanization on biodiversity have recently come forward as an important area of research in understanding the environment, particularly in applied entomology (Pompeu et al., 2005; McKinney, 2002; Pečarević et al., 2010; Meineke et al., 2013). Bees are an excellent system for studying plant biodiversity in any environmental setting, as they bring information about local plant diversity to a centralized location. If the current bottlenecks in pollen identification can be overcome, it would broaden the types of urbanization studies that could be done, and thus our approach may facilitate this process.
Other studies have suggested pollen choice may involve nutritional difference in protein (Di Pasquale et al., 2013; Herbert and Shimanuki, 1978) or amino acid (Di Pasquale et al., 2013; Cook et al., 2003) content of the host plant. One known aspect of pollen nutrition is that honey bee colonies sourcing pollen from monocultures perform worse than colonies with multiple pollen sources (Di Pasquale et al., 2013; Goulson et al., 2015; Vaudo et al., 2015; Bretagnolle and Gaba, 2015). This is likely because different pollen sources vary in their amino acid content, which bolsters the immune response towards oxidative stress in honey bees (Di Pasquale et al., 2013; Huang, 2012).
4.5 Conclusion

We have established a framework to study the color of pollen in the CIE-Lab color space using a closed-set NB training model. Our method demonstrated the ability to classify a known pollen data set (~ 9.4% Type-II error rate) with a minimal training set (50 pellets per species in the pollen library). We emphasize a need for data standardization procedures, particularly with the moisture levels during image capture. An open-set classification algorithm might prove more useful with the overwhelming number of plant species on which honey bees forage, but NB is the standard in most classification studies. Therefore, it remains crucial that colorimetric data are correlated with other variables, such as time of year or geographic location, and corroborated with other, more expensive, and time-consuming pollen databases. By developing a common, vetted database of pollen sources, our technique may alleviate a severe bottleneck in data processing and pollen identification that could have profound implications to a wide variety of applications.
4.6 References


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CHAPTER 5

Conclusion

How can we bridge the gap between behavioral and genetic studies with respect to honey bee health? Computer vision is a fast-growing field of research, and it features inexpensive, high-throughput techniques that remove certain limitations inherent to the latter two fields of study. Although the studies presented in this thesis have their own limitations, every assay is novel, and with further refinement they will continue to enhance the way we connect the behavioral phenotype to genotype and bee health.

5.1 Summary

We automated the analysis of approximately 100 10-minute videos, which would take an observer roughly 17 hours to simply watch. In a scientific study, accurate observation of a data set this large would take much longer. Since we were controlling for age, time-of-year, and colony-level effects, gathering data was the most severe bottleneck in our design. We studied bee autogrooming as a continuous variable using time series pixel data, offering a strong alternative to the manual monitoring of discrete grooming events. We have established how differential models can be used in parameterizing bee grooming rates, and presented a comparative analysis of four commercial stocks. Although the stock comparison concluded that there were no differences in grooming rates between stocks, we saw different grooming rates among the different experiments.

This thesis offers one of the first comprehensive studies of the varroa-virus relationship by testing the Vector Manipulation Hypothesis with a digital tracking assay. We correlated
mite speed with blind qPCR viral data and discovered that varroa speed varies based on viral load and colony-level effects. Many of the statistical models we examined offered a unique perspective on the interplay between colony, deformed wing virus (DWV), and sacbrood virus (SBV). Using corrected Akaike’s information (AICc) and Bayesian information criterion (BIC), we were able to select three biologically and statistically relevant models, all of which open the door for future work. With the exception of DWV, model coefficient values were quite robust, signifying that their contributions were meaningful beyond a simple p-value. SBV and colony level effects were universally apparent across the models of interest.

Pollen is an important component of bee health, since both nutrition and environment are critical to a colony’s success. We constructed pollen library images using 400 pollen pellets spanning 8 plant genera, measured their color, and constructed a naïve Bayes (NB) model for these color features in the CIE-Lab color space. The NB model was able to successfully classify ~90.6% of pollen from a known data set (~9.4% Type-II error rate). Color features of wet and dry pollen appear to change nonlinearly, and depend on the pollen species. Finally, principal component analysis revealed that the L and b channels have the weakest correlation, so future analysis and visualization methods should rely on these two channels to capture differences between data samples.

5.2 Limitations

The grooming assay started as a binary before-and-after concept, but this left too many mathematical questions that would be resolved with a time series. We did not adequately prepare for the statistical noise from a time-series approach to pixel analysis. We controlled
for certain factors by fine-tuning lighting conditions and the camera angle, yet some of the resulting data was still noisy. We pooled groups of five honey bees together with the expectation that we would broadly capture auto- and allogrooming dynamics. Our experimental design specifically instigated autogrooming behavior; allogrooming behavior was almost completely absent in our experiments. This created a somewhat skewed perspective of grooming, as pairwise defenses are important when addressing bee health. Pooling groups of honey bees made the pixel analysis more complicated than necessary, so we sacrificed data analysis in favor of unobserved behaviors. Finally, honey bee color varied significantly, ranging from darker black-browns to brighter orange-yellows. It would have been beneficial to calibrate our pixel data by prerecording a short 2-minute video of the bees before we coated them with flour. Although we marked thousands of bees to control for age, we experienced significant mortality over 2 weeks and the final number dwindled to hundreds.

Many advances in tracking technology have emerged in the past 5 years since this research began. Tracking work conducted on the fruit fly (*Drosophila melanogaster*) could have been applied to the varroa system (Branson *et al.*, 2009). Scientific discoveries are sometimes accomplished in parallel; current ant- and bee-tracking research projects (Fletcher *et al.*, 2011; Fasciano *et al.*, 2013) compliment the current tracking studies, and may inform other disciplines of entomology and biology.

Within the confines of the mite tracking algorithm we designed and used, there were other logistical hurdles. Measuring speed and velocity in an assay where mites were allowed to climb in three-dimensional space did not accurately represent our data set. Therefore, we had to conceptualize a surrogate metric for speed. Although this proved very useful for the data
we were trying to analyze, it limited our further exploration of differential models that may have contributed to our work. Since we conducted a blind study of varroa viral loads, we had no sense of the type of the data. This study would have benefitted greatly from a collection of varroa infected with only SBV or other single viruses, which would have simplified some of the interpretations in our results. Finally, model selection for AICc and BIC did not align, leaving some open statistical questions in our study.

Pollen research is very sensitive to the issue of scope. We described a simplified pollen library, completely uncharacteristic of the true diversity nature provides. We hope that our methods can be expanded and elaborated upon with a wider variety of pollen, but such a study would require the collaborative efforts of many scientific disciplines and individuals; thousands of hours of ground-truthing, trial, and error, are yet required to construct a pollen library that is representative of nature. We believe our method will perform quite well in practice, based on analysis of the principal components from our pollen library. Subtle yellow-orange-brown differences may not resolve in a NB model, but the precision and performance of NB on our data set has shown promise for future pollen research. Many studies of color rely on psychological experiments that match perceived color with numerical color, which is beyond the scope of this study. We anticipate that this research will be expanded or correlated with other (i.e., microscopic and pollen genetics) databases.

5.3 Future work

This thesis has established a foundation for future bee breeding programs where honey bees could be selectively bred for phenotypical grooming traits in the same manner Spivak and
Reuter (2001) established that bees selected for hygienic behavior showed improved resistance to disease. The scope of the grooming study (Chapter 2) is not limited to honey bees, and can more broadly be applied to other insects or pollinators, where grooming serves as a measurement of health, fitness, or immunology. There also is the pending question of the role grooming behavior plays in pollinator efficacy. For example, one study of five bumble bee species revealed no differences between grooming behavior and pollination (Asada and Ono, 1996), yet comprehensive studies on the physiology of the subfamily Apinae suggest that both specialized and generalized anatomical adaptations play key roles in pollination services (Thorp, 1979; Waser et al. 1996). The methods in this grooming chapter could be used to comparatively study the behavior, anatomy, and efficacy of many pollinator species.

The results from the mite assay (Chapter 3) conclusively supported the Vector Manipulation Hypothesis. None of the findings in this study were simple. They opened the door to many exciting questions about the varroa-virus relationship, which requires further exploration. Why does SBV, which is not known to replicate in varroa, modify varroa behavior? What sort of facilitative interaction is there between DWV and SBV, since this study at least hints that these viruses interplay on the behavioral level? Can we pinpoint the colony-level effects modifying mite behavior, by exploring other factors that this thesis work could not explain? Pheromones are known to play a role in signaling that brood are ready to be capped, and in turn, pheromone tells a phoretic varroa mite where it needs to go for reproduction. Future work with this assay could explore mite spatial distributions in a bee pheromone landscape as a Poisson point process (Giuffre et al., 2011). There also is an
opportunity to control the viral loads that mites are exposed to, furthering our understanding of the dynamics of mite behavior in a three-dimensional environment.

Future applications of the pollen study (Chapter 4) are quite broad. The extensive variability of pollen foraging patterns, resulting from factors such as climate, geospatial location, bee subspecies, crop management practices, and genetics, would make this an excellent citizen science project. Pollen color databases could be linked with existing microscopic, genetic, and spectrographic databases, thereby increasing the variable domain for deeper Bayesian models. Rigorous pesticide, bee nutrition, and urbanization studies could gain traction without the need for manual pollen sorting or expensive, genetic analysis. This proposed study would also be an excellent opportunity for development of a mobile phone application, making the technology available to any beekeeper who simply wants to know the pollen loads their honey bees are bringing back to the hive.

We have established several useful methods and applications of image science in the assessment of honey bee health. We analyzed bee health across many levels, ranging from individual bees up through the colony and the surrounding environment. We developed and interpreted bee observational data with mathematical and statistical models, making connections to genotypical data, when available. This thesis illustrates what important links can be made between computer vision and a wide range of bee studies.
5.4 References


