Improving the health and survivorship of commercial honey bee colonies

A Dissertation
SUBMITTED TO THE FACULTY OF THE
UNIVERSITY OF MINNESOTA
BY

Kathleen Lee

IN PARTIAL FULFILLMENT OF THE REQUIERMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

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December 2018

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Chapter 1 has previously been published (citation: Lee, K., Steinhauer, N., Travis, D.A., Meixner, M.D. and Deen, J., and vanEngelsdorp, D. 2015. Honey bee surveillance: a tool for understanding and improving honey bee health. *Current Opinion in Insect Science*, 10: 37-44.) Permission for use here granted by first author Kathleen V. Lee and publisher Elsevier.

Chapter 2 has previously been published (citation: Lee, K.V., Steinhauer, N., Rennich, K., Wilson, M.E., Tarpy, D.R., Caron, D.M., Rose, R., Delaplane, K., Baylis, K., Lengerich, E., Pettis, P., Skinner, J., Wilkes, J.T., Sagili, R., and vanEngelsdorp, D. for the Bee Informed Partnership. 2015. A national survey of managed honey bee 2013–2014 annual colony losses in the USA. *Apidologie*, 46, 292–305. doi:10.1007/ s13592-015-0356-z) Permission for use here granted by first author Kathleen V. Lee and publisher Springer.

Chapter 4 has previously been published (citation: Lee, K.V., Goblirsch, M., McDermott, E., Tarpy, D.R., and Spivak, M. Is the Brood Pattern within a Honey Bee Colony a Reliable Indicator of Queen Quality? *Insects*, in press.) Permission for use here granted by first author Kathleen V. Lee and publisher MDPI.

Acknowledgements

Like all good research, my dissertation greatly benefited from the help of others. I would be nowhere without the support of many people.

Without the support of funding organizations, none of the research would have been possible: RARF, USDA-NIFA, North Dakota Department of Agriculture and North Dakota Beekeepers Association, Project Apis m, and the American Beekeeping Federation.

Thank you to my lab mates and Bee Squad for helping foster an atmosphere of curiosity and collaboration. Thank you to Mike Goblirsch for being my editor and sending me Simpsons videos. And especially thank you to Gary Reuter for teaching me how to keep bees and for being someone I have consistently been able to ask for advice on anything from the size of a syrup tank to how to fix a toilet.

Thank you to all those at the Bee Informed Partnership that helped make the Tech-Transfer Teams possible and run smoothly: the Tech-Transfer Team members, Heather Eversole and the sample processing lab at the University of Maryland, Michael Wilson at the University of Tennessee and the IT Team at Appalachian State, Nathalie Steinhauer, the Bee Informed Partnership board, and everyone that helped me collect filed data. In particular, thank you to Karen Rennich and Dennis vanEngelsdorp for all your work and encouragement.

I've had the privilege of knowing a group of super competent, bright, and fantastic women that are obsessed with honey bees and have been there for me when I needed support or a different perspective: Megan Mahoney, Ellen Topitzhofer, Kristy Allen, Elizabeth Frost, and Rebecca Masterman.

My favorite part of this dissertation was the field work that allowed me to travel and meet an incredible group of beekeepers. Thank you to all the beekeepers on both the California and Minnesota Tech-Transfer Teams that were willing to let me paw through their colonies. The work provided me with the opportunity to learn from this group of people that have so much knowledge to share about beekeeping and life in general. In particular, thank you to those of you I consider to be a part of my beekeeping family.

To my committee, thank you for your guidance throughout the process and for steering me and pushing me to learn more. The access to your collective and individual knowledge has made me a better scientist and has improved the content of my work.

Finally, thank you to Marla Spivak. It has been an absolute privilege being your student. I feel incredibly fortunate to have stumbled into your social insects class and then your lab. It has been an incredible journey and I couldn't have imagined or asked for a better advisor. I have learned so much and truly appreciate your patience, wisdom, and guidance.

Dedication

To my parents, Mark and Cathy Lee, for your support, and for fostering a love of the outdoors.

And to my best friend and partner in this crazy world, David Robertson.

Abstract

The health of honey bees (*Apis mellifera* L.) is essential for the health of U.S. agricultural and natural ecosystems. In particular, the sustainability of commercial beekeeper operations (beekeepers that manage >500 colonies) is of critical importance to agriculture as these operations manage most of colonies in the U.S. and fulfill the majority of crop pollination contracts. Identifying risk factors and best management practices for these beekeepers can improve colony health and survivorship, decreasing total U.S. colony mortality. My dissertation uses epidemiological methods to identify and quantify risk factors in real-world commercial beekeeping operations in four chapters.

Chapter 1 reviews recent publications that use epidemiological methods to study honey bee health. The reviewed research efforts used surveillance methods to quantify colony mortality, describe disease prevalence and incidence, and identify risk factors. Surveillance can be used to highlight potential areas of further research and measure improvements in honey bee health.

Chapter 2 quantifies the problem of colony mortality by reporting the results of the eighth year (2013 – 2014) of the Bee Informed Partnership's annual colony loss survey. The survey reports on summer, winter, and annual colony mortality of 7,425 voluntary respondents representing approximately 19% of U.S. colonies, and includes different beekeeper operation sizes, regions, and migratory statuses. Although only 1.4% of respondents were commercial beekeepers, they managed 89% of the colonies represented in this survey. Similar to previous surveys, commercial beekeepers had lower winter and annual loss than beekeepers that managed fewer colonies, with summer losses not different among beekeepers grouped by operation size. Contrary to public perception,

migratory operations did not have higher losses than those operations that did not move colonies. Commercial beekeepers identified *Varroa destructor* mites and queen failure as the top two contributors to colony mortality.

Chapter 3 is an observational study that identifies risk factors using real-world data from commercial beekeeper operations collected by the Bee Informed Partnership's Tech-Transfer Team. Colony inspection metrics that predicted colony health or survival included an estimation of the adult bee population, loads and signs of the parasitic mite *V. destructor*, spore loads of the microsporidian *Nosema* spp., and the continuous presence of a queen bee. Overall, this study provides a way to make management decisions based on the likelihood of a colony health outcome.

Chapter 4 determines if a colony phenotype can be attributed to a poor-quality queen. The failure of the queen bee was identified by the surveys and the Tech-Transfer Team data as a major contributor to colony mortality. One sign commonly attributed to the failure of a queen is a poor brood pattern – i.e. the continuity of cells on a comb containing sealed pupae. This study had two objectives: 1) determine if a poor brood pattern was reliably associated with other measures of queen quality (2016 and 2017); and 2) determine if brood pattern was influenced more by factors associated with the colony environment or by the queens' egg-laying capacity (2017). Although brood pattern was not associated with measures of queen quality, brood patterns improved after a queen with a poor brood pattern was introduced into a colony with a good brood pattern, suggesting that colony environment had a larger impact on brood pattern than the queens' egg laying capacity. The results imply that brood pattern is not a reliable indicator of queen quality and factors other than the queen can influence brood pattern.

Overall, these studies contribute to the understanding of commercial beekeeping and can inform management practices to increase colony health and survivorship.

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Introduction

Honey bees (*Apis mellifera* L.) have piqued human interest for centuries. In addition to their fascinating social behaviors, the honey bee's ability to make surplus honey, provide pollination services, and be kept in movable colonies have made them an integral part of both agriculture and human culture. Honey bees can be managed in a way that makes them similar to livestock, but make no mistake about it, they are wild. Although we keep them in boxes to collect their honey and move them across the country on trucks for pollination services, we have no control over where bees fly. Honey bees collect their own food and they can live and reproduce on their own without our interference, as demonstrated in New York's Arnot Forest (Seeley 2007). Successful beekeeping entails applying an understanding of honey bee biology to coax bees to behave a specific way while maintaining a colony's health. Beekeepers keep bees on different scales and are generally classified into three groups based on the number of colonies they manage: backyard beekeepers manage <50 colonies, sideline beekeepers manage 50-500 colonies, and commercial beekeepers manage > 500 colonies, with the largest operation managing >70,000 colonies. Commercial beekeepers make up the minority of the beekeepers in the U.S., but they are critical to our agricultural system as they manage the majority of the colonies that perform pollination services needed for fruit, vegetable, nut, and other seed crop production (Calderone 2012; Daberkow et al. 2009). While basic beekeeping practices remain the similar among the three beekeeper groups, the challenges and level of management are most intense in commercial operations.

Commercial operations are in many ways similar to farming livestock as they share parallel pressures, such as potentially high disease transmission, unpredictable weather, and maintaining healthy animals to derive income. Commercial beekeepers also have the lowest colony mortality of the three beekeeping groups according to the Bee Informed Partnership's annual survey on colony mortality. Commercial beekeeping operations suffer approximately a 34% annual total loss compared to the 51% total annual loss of backyard beekeepers (Kulhanek et al. 2017; Lee et. al 2015; Seitz et al. 2016; Spleen et al. 2013; Steinhauer et al. 2014; vanEngelsdorp et al. 2007; 2008; 2010; 2011; 2012a). Despite the difference, commercial beekeepers still experience considerable colony mortality, and the level of mortality varies among individual operations. Lowering colony mortality for this group of beekeepers has the largest potential for improving overall health of U.S. colonies as these beekeepers produce the majority of bees and queens used across the nation. My dissertation is on addressing commercial beekeeper colony health with the ultimate long-term goal of decreasing colony mortality and morbidity.

My main goal was to use epidemiological methods to identify risk factors that contribute to poor colony health through the use of survey and field data collected from commercial beekeeping operations. These methods are commonly used in livestock and the same principles can be applied to honey bees. Commercial beekeeping is multifaceted with operations following different migratory routes, and using different ways of deriving income from their bees (e.g. pollination, honey production, queen production, bee sales). Beekeepers in different regions can face different issues due to availability of forage, severity of pests, density of other beekeepers, and environmental influences (Giacobino

et al. 2018; Meixner et al. 2015). The variety of challenges in different locations makes it difficult to identify which management practices are the most effective in keeping colonies healthy. Epidemiological methods allow for this real-world complexity when identifying risk factors associated with health and survival.

Honey bees and commercial beekeeping have been a deep interest of mine since my master's thesis at the University of Minnesota Spivak Bee Lab on developing a standard sampling plan for the parasitic mite *Varroa destructor*. The interest increased with my work on the Tech-Transfer Teams (T^3) in California and Minnesota, both a part of the University of Maryland-based non-profit the Bee Informed Partnership (beeinformed.org). The T^3s work for commercial beekeepers to perform colony assessments using standardized metrics of colony health, sample for diseases and pests, and test for the genetic trait of hygienic behavior that confers disease resistance to colonies. Participating beekeepers pay for the service, and data are provided to them via a report in a timely manner to allow for management decisions based on the results. I established a T^3 at the University of Minnesota in May 2012 to work with commercial beekeepers that maintain their bee colonies in Minnesota and North Dakota over the honey production season. I began my PhD in Fall 2013 and simultaneously ran the T^3 full-time until January 2017, when I switched to completing my PhD. Using these data to provide results back to beekeepers was the reason I chose to pursue a PhD.

My dissertation addresses the following objectives in four chapters:

1. Review recent publications that used epidemiological methods to study honey bee health.

2. Quantify the problem of colony mortality by reporting the results of one of the Bee Informed Partnership's annual colony loss surveys.
3. Identify inspection metrics that predict colony health (loads of *V. destructor mites* and *Nosema* spp.) and survivorship using real-world data from commercial beekeeper operations.
4. Determine if a colony phenotype can be attributed to a poor-quality queen.

Chapter 1 is a published, invited, review paper on recent studies that applied epidemiological methods to honey bee populations to learn more about colony health (Lee et al. 2015a). These studies identify risk factors using observational data in complex and real-world situations often without manipulation of the environment. The paper discusses different types of surveillance methods used to describe diseases and pests and identify risk factors, including the level of investment needed for each type of data collection. This paper sets the stage for the following chapters as observational data were collected and analyzed in Chapters 2 and 3. Queen failure was identified as a risk factor in Chapters 2 and 3, and in Chapter 4 I attempted to find the putative cause of one of the conditions, poor brood pattern, associated with poor queens.

Chapter 2 contains the analysis of one survey in the series of annual beekeeper surveys on colony mortality that began in 2006-2007 and is now conducted by the Bee Informed Partnership (Kulhanek et al. 2017; Lee et. al 2015; Seitz et al. 2016; Spleen et al. 2013; Steinhauer et al. 2014; vanEngelsdorp et al. 2007; 2008; 2010; 2011; 2012). These surveys were a response to the identification of Colony Collapse Disorder and the reported abnormally high colony mortality in 2006 – 2007 (vanEngelsdorp et al. 2009).

However, yearly colony mortality had not been previously quantified, so it was unclear if the losses were abnormal. The USDA-NASS has produced annual reports that estimated the number of honey producing colonies since 1943 (vanEngelsdorp and Meixner 2010), but the estimations report only colony numbers and not colony mortality. Colony numbers do not necessarily reflect mortality as beekeepers are able to make up losses by spitting surviving colonies in two, thus the number of colonies they maintain can remain constant. The annual surveys have helped quantify colony mortality and establish a baseline of colony loss in the U.S. overall and for individual beekeeper groups and regions.

My survey publication (Lee et al. 2015b) was the eighth in the series and the second to estimate summer and annual losses, with the surveys prior to Steinhauer et al. (2014) estimating only winter loss. Along with the other surveys, this study highlighted differences in operational losses. Commercial beekeepers tended to lose the fewest colonies compared to sideline and backyard beekeepers, suggesting that the relatively intense management practices of commercial beekeepers can lower colony losses despite the increased potential stress on colonies due to transportation (Ahn et al. 2012; Simone-Finstrom et al. 2016) or increased disease exposure (DeGrandi-Hoffman et al. 2016; Frey and Rosenkranz 2014; Nolan and Delaplane 2017; Seeley and Smith 2015). Losses can vary widely among commercial operations, leading to the postulation that different management practices may be effective at lowering colony loss. The identification of practices that are most effective could help beekeepers decrease annual colony mortality; a topic addressed in Chapter 3.

In Chapter 3, I addressed the utility of simple metrics collected by the Minnesota T^3 in commercial beekeeper operations. The inspection metrics included colony population as estimated by frames of bees, queen status, clinical signs of *V. destructor* mites, and *V. destructor* and *Nosema* spp. loads. I examined if the metrics collected were predictive of colony health (*Nosema* spp. and *V. destructor* loads) and colony survivorship. I looked at four specific time periods that corresponded to sampling bouts based on beekeepers' production and management windows, and examined if the inspection metrics in one sampling period could predict a health or survivorship outcome in the following period. Overall, the metrics with the strongest association with colony health or survivorship were queen events, adult bee population size, clinical signs of *V. destructor*, *V. destructor* and *Nosema* spp. loads., and *V. destructor* treatment products and timing. This study provides beekeepers with information that they can use to make management decisions.

Chapter 4 is a study on one colony phenotype commonly attributed to the risk factor of queen failure: poor brood pattern, or the continuity cells on a comb containing sealed pupae. Over May and June in 2016 and 2017, I addressed two questions for this study: 1) Could a poor brood pattern be attributed to the quality of the queen; and 2) Was the brood pattern due to the queen or to the colony environment. For the first question, I worked with commercial beekeepers to identify colonies with poor and good brood patterns. Metrics of queens from colonies with poor and good brood pattern were compared (e.g. sperm count and viability) and contrary to my expectations, none of the metrics were significantly different between the two groups, indicating that brood pattern was not a reliable indicator of queen quality. In 2017 for the second question, colonies

were identified with poor and good brood patterns, then queens were exchanged from colonies with poor brood patterns into colonies with good brood patterns, and vice versa. I measured sealed brood patterns before and 21 days after the exchange and found that the brood pattern for the queens from poor brood colonies improved after the exchange and the patterns for queens from good brood colonies became worse. This result suggests that brood patterns were more due to the colony environment than to a factor associated with the queen. This study was a collaborative work between the University of Minnesota Spivak Bee Lab and Dr. David Tarpy's Honey Bee Lab at the Entomology Department, North Carolina State University.

My results use epidemiological methods to provide context for the level of honey bee colony mortality in the U.S. and to identify metrics predictive of colony health and survivorship. The complexity of commercial beekeeper operations provides a fascinating system in which to study the health of honey bees and one that I hope to continue to learn more about.

Chapter 1. Honey bee surveillance: a tool for understanding and improving honey bee health

Publication citation

Lee, K., Steinhauer, N., Travis, D.A., Meixner, M.D., Deen, J., and vanEngelsdorp, D. 2015. Honey bee surveillance: a tool for understanding and improving honey bee health. *Current Opinions in Insect Science* (10): 37–44.

1.1 Synopsis

Honey bee surveillance systems are increasingly used to characterize honey bee health and disease burdens of bees in different regions and/or over time. In addition to quantifying disease prevalence, surveillance systems can identify risk factors associated with colony morbidity and mortality. Surveillance systems are often observational, and prove particularly useful when searching for risk factors in real world complex systems. We review recent examples of surveillance systems with particular emphasis on how these efforts have helped increase our understanding of honey bee health.

1.2 Surveillance in honey bees

'Observation sets the problem; experiment solves it' Jean-Henri Fabre, (1823–1915)

Surveillance is an observation-based method of quantifying levels of 'disease' in a population. At their core, surveillance efforts quantify disease prevalence and incidence over space and time, which can help identify risk factors that contribute to disease

incidence when coupled with other data. Data from surveillance efforts can identify or confirm risk factors that predict disease outcomes, and can guide the development of experimental approaches to demonstrate causation. Further, identification of risk factors can inform disease mitigation practices that can improve health at the population level (vanEngelsdorp et al. 2013, 2014).

Health and/or disease surveillance systems exist for most human and production animal health programs. When implemented sustainably, they help mitigate and prevent important diseases in populations. Considering the importance of honey bees (*Apis mellifera*) for pollination of agricultural crops (Gallai et al 2009; Klein et al. 2007; vanEngelsdorp et al. 2013; Winfree et al. 2001), it is not surprising that many surveys have quantified health and disease burdens. Surveillance of non-apis species also exists, but is less developed compared to honey bees (Box 1.1). Surveillance system design is dictated by many factors, most importantly by the objectives of the study and availability of resources (Figure 1.1). Here we review examples of honey bee surveillance efforts, emphasizing their contribution toward understanding and improving honey bee health (summarized in Table 1.1).

Box 1.1. Non-apis bee surveillance

Non-apis bee species are major contributors to agricultural and natural pollination systems (Garibaldi et al. 2013; Hoehn et al. 2008; Isaacs et al. 2010; Klein et al. 2007;). These species are largely unmanaged and have multiple different life histories, thus requiring specialized surveillance techniques.

Recently there have been several efforts to standardize survey effort approaches that document the abundance and diversity of non-apis species (Banazak et al. 2010).

Application of standardized collection methods allows for ecological network analyses to help quantify the structure of bee-plant networks in various landscapes (Geroff et al. 2014; Gunnarsson and Federsel 2014; Yamamoto et al. 2014; Zotarelli et al. 2014). When standardization is not possible (such as in the case of comparing changes in abundance and diversity over time by using historical collections), statistical analyses can help elucidate important drivers of changing populations, including changes in agricultural policy and practice (Ollerton et al. 2014), ecological succession (Gardner and Spivak 2014), landscape (Viotti et al. 2013) and climate change (Ploquin et al. 2013).

Surveys of non-apis bee populations have been conducted to identify disease loads in populations (Cameron et al. 2011; Jones and Brown 2014; Ravoet et al. 2014; Reynaldi et al. 2014), although generally these studies have concentrated on possible disease spillover from honey bees. Further surveillance on non-apis bees and their diseases is much needed.

1.3 Detection, characterization, quantification of disease

Monitoring is a regular, repetitive and intermittent series of measurements designed to detect changes in the health status of a defined population (see Table 1.1 for examples). Apiary inspections are an example of monitoring as they have long been used to estimate disease in managed honey bee populations. These inspections quantify disease prevalence and range by sampling a number of ‘analytic units’ (individual bees, colonies, apiaries, or operations; vanEngelsdorp et al. 2013a) over a defined period of time and population. Traditionally, apiary surveillance was used to identify disease outbreaks in order to enforce regulations aimed at eliminating or containing disease spread. This

approach is largely credited for reducing the incidence of the bacterial disease American foulbrood (*Paenibacillus larvae*) in the US (vanEngelsdorp et al. 2014). More recently, disease surveys have expanded to include early detection of non-extant (or recently introduced) disease threats such as *Tropilaelaps clareae* mites in the US (Pettis et al. 2013; USDA-AHPIS 2014), small hive beetles (*Aethina tumida*) in Europe (Chauzat et al. 2014), or *Varroa destructor* mites (*Varroa*) in Australia (Plant Health Australia 2014). Determination of disease free status for particular pests has implications for trade of bees and bee products (USDA-AHPIS 2014). Early detection of a new organism can permit containment efforts, such as the Australian effort to contain *Apis cerana* (Austalian Government 2010). The utility of surveillance efforts in epidemiologic studies is dependent on numerous factors, including how samples are selected, number of analytic units sampled, specificity of the diagnostic test, and sample collection methodologies (vanEngelsdorp et al. 2013a); all of which are constrained by the pragmatic reality of limited resources.

A notable monitoring program quantified disease load and colony mortality by inspecting randomly selected apiaries in 17 different European countries (Chauzat et al. 2014). By randomly selecting colonies and implementing a standardized inspection approach, the resulting data avoided selection biases inherent with many survey efforts. The ability to randomly select colonies from a known population is a central tenant of good survey design, but in practice is problematic as random sampling requires a near-complete description and access to the honey bee population, which is often difficult to attain or create.

Modified apiary inspections can be used to perform more directed surveillance for the discovery and characterization of potential new diseases and/or pathogens, including virulence and distribution. Identifying new cases helps direct future monitoring or research to better understand if they contribute to colony mortality and morbidity. Several surveillance programs have utilized new molecular tools to discover new, possibly pathogenic, honey bee viruses, including Aphid Lethal Paralysis virus strain Brookings, Big Sioux River virus, four strains of the Lake Sinai virus, and the tobacco ringspot virus (Li et al. 2014; Ravoet et al. 2013; Runckel et al. 2011).

Surveillance of known pathogens can shed light on the etiology of disease and support a hypothesis that is difficult to test experimentally. Examining the relationship between disease and other variables of interest at a single time point in a defined population can be done using cross-sectional studies (Table 1.1). In a cross-sectional study of Hawaiian colonies, Deformed Wing Virus (DWV) prevalence was correlated with the number of years *Varroa* was present on the island (Martin et al. 2012). The observed DWV strain diversity was greatest in samples from Varroa free islands, while a single DWV strain replaced all others when *Varroa* was present for over three years. These findings imply that mite-mediated transmission of DWV favors certain, possibly more virulent, DWV strains (Martin et al. 2012). This hypothesis is supported by survey results that demonstrate *Varroa* dramatically changes the viral complex in infested honey bee populations (Mondet et al. 2014), and experimental research showing one virulent DWV strain benefits from the direct injection route mediated by *Varroa* (Ryabov et al. 2014).

Generally, understanding the dynamics of 'new' host– parasite/pathogen interactions does not lend itself well to direct hypothesis testing, but benefits from surveillance efforts. A survey in Kenya documented the prevalence of *Varroa*, *Nosema* spp., DWV, Black Queen Cell Virus (BQCV), and Acute Bee Paralysis Virus (ABPV) (Muli et al. 2014). The surveyed pathogens did not appear to affect colony strength suggesting the presence of more benign diseases in the region, a more resistant host, or a combination of both. Data from a Swedish effort that looked at viral levels in '*Varroa* tolerant' colonies seems to support the concept of host-based tolerance, as the study population has increased virus tolerance (Locke et al. 2014) and an ability to reduce mite fitness (Locke et al. 2012).

1.4 Identification of risk factors

Surveillance can be used to investigate putative causes of unexplained disease states. Analyses of a limited number of disease samples can be reported as a case study: a detailed description and analysis of the occurrence of a particular health problem, its development and its outcome (Table 1.1). Although widely used in human and other animal health fields, case study reporting is rare for honey bees. A recent exception described efforts to determine the putative cause of two collapsed colonies (Bekele et al. 2014), with the clinical disease symptoms ascribed to *Nosema ceranae*. On its own, a case study has limited utility in explaining population level health. However, if findings from other case studies make similar conclusions, the results can identify associated risks. In case–control studies, colonies are first selected based on whether or not they have the disease/health status of interest, and then their exposure histories are obtained and

compared to identify correlations between different risk factors (Table 1.1). One case–control study found apiaries with high rates of loss were less likely to have been treated for *Varroa* than apiaries with low rates of loss (Chauzat et al. 2010). Another found higher pathogen loads and a different gut microbe community in collapsing colonies as compared to apparently healthy colonies (Cornman et al. 2012).

Surveillance efforts can also help validate experimentally identified risk factors. An experiment that measured effects of temperature on spore viability of *Nosema apis* and *N. ceranae*, found *N. ceranae* spores were more tolerant of higher temperatures while *N. apis* spores were more tolerant of colder temperatures (Fenoy et al. 2009). This suggests *N. apis* should be more prevalent in colder locations, a finding documented by surveillance programs in Sweden, Germany, and Taiwan (Chen et al. 2012; Forsgren and Fries 2013; Gisder et al. 2010).

Surveillance efforts need not require field visits. Owner or caretaker observations can capture information about a population's health, and have been used extensively to document colony losses (vanEngelsdorp et al. 2013a; van der Zee et al. 2013). Repeated surveys have shown that losses are highly variable between regions and over time (Steinhauer et al. 2014; van der Zee et al. 2012, 2014), which may be explained by subgroups within a population. A consistent finding within US loss data is that beekeepers in northern states lose more colonies than those in southern states (Lee et al. 2015b; Spleen et al. 2013; Steinhauer et al. 2014; vanEngelsdorp et al. 2008, 2010, 2011, 2012a), suggesting winter temperature may explain some variability in loss rates. Indeed, winter loss rates in Pennsylvanian beekeeping operations were correlated to average winter temperature (vanEngelsdorp et al. 2008).

Although questionnaires are relatively easy to conduct, they are prone to biases: respondents may not be representative of the population, have poor recall, among others (van der Zee et al. 2013). Further, when comparing results among different questionnaire-based surveillance efforts, consideration of different methodologies and definitions is essential. For instance, the timeframe for 'winter' loss calculations can be a set date (Pirk et al. 2014; vanEngelsdorp et al. 2013a) or self-determined by the responding beekeeper (van der Zee et al. 2014), making direct comparisons of loss rates difficult to interpret.

Properly designed questionnaires increase the ability to identify management practices correlated with increased survivorship. A consistent finding in several loss and management surveys has linked application of *Varroa* treatments with increased winter survival (Clermont et al. 2014; van der Zee et al. 2014; vanEngelsdorp et al. 2012a). Despite the consistency of these findings across different surveys, this relationship remains correlative and should not be interpreted as causative unless data from experimental testing is considered (Giovenazzo and Dubreuil 2011; Gregorc and Planinc 2012; Semkiw et al. 2013). However, resource availability can limit the ability to conduct experimental studies. In these cases, a combination of questionnaires and field surveys can be informative. An Argentinian study paired monitoring of *Varroa* loads with retrospective management survey data to identify factors that were predictive of above threshold *Varroa* populations at the time of sampling (Giacobino et al. 2014). Another cross-sectional study, which also combined field sampling and questionnaires, found that acaricide treatments were 4.9 times more likely to fail when applied to colonies with *Varroa* infestations over a 3 mites per 100 bees threshold (Giacobino et al. 2015). Both these and other cases demonstrate a strong link between *Varroa* levels and colony

mortality (Genersch et al. 2010) and validate this method as a cost-effective surrogate approach to more intensive surveillance.

Longitudinal surveillance (repeated sampling of the same colonies over time) is a powerful tool for connecting 'risk factor' exposure with disease outcomes. This approach can quantify the association between a risk factor and likelihood that a disease outcome will result in the future. Cohort studies are a form of longitudinal monitoring that compares the incidence of a particular health outcome between subsets of defined populations selected for having experienced a common exposure status (Table 1.1). For example, a US cohort study followed colonies in different migratory beekeeping operations and identified a close association with the occurrence of a queen event or the presence of 'Idiopathic Brood Syndrome' with subsequent colony mortality (vanEngelsdorp et al. 2013b). Similar cohort studies have found relationships between *Varroa* (or its control), DWV, or a combination as being predictive of mortality (Büchler et al. 2014; Francis et al. 2013; Frey and Rosenkranz 2014; Lodesani et al. 2014).

Longitudinal studies can also identify and quantify nonbiotic factors that may predict disease outcomes. European researchers recently published a multi-year study conducted in 21 apiaries in 11 countries identifying effects of bee genotype and environmental factors on mortality and morbidity (Hatjina et al. 2014; Meixner et al. 2014). They demonstrated that location strongly influenced autumn mite loads and viral (ABPV and DWV) prevalence. Location effects had a more pronounced affect on disease outcome than host genotype, suggesting disease thresholds likely differ by geography (Meixner et al. 2014). Colonies in locations with a shorter active season (i.e. temperate regions) have longer lived winter bees compared to colonies with a longer active season

(Hatjina et al. 2014), suggesting colonies managed in temperate regions require more vigilant pathogen control. Longitudinal data has also been paired with landscape data to develop a model, EcoBEE, that predicts optimal apiary sites (Odoux et al. 2014). Multi-year longitudinal monitoring of colony health can identify region specific risk factors associated with colony mortality. Once identified, these factors can inform management and research priorities. Longitudinal trends in disease and/or risk factor prevalence may help predict future morbidity and mortality. Unfortunately, few of these long-term monitoring efforts exist, with some notable exceptions based in Europe (Genersch et al. 2010; Meixner et al. 2014; Topolska et al. 2009) and more recently in the US (vanEngelsdorp et al. 2012b).

1.5 Conclusions and further directions

Honey bee health surveillance efforts quantify disease and disease risk factors in managed honey bee populations. Over time, these efforts provide data that can identify emerging threats and place disease measures in context by establishing baseline metrics. As surveillance based studies are becoming more common, increased efforts to standardize approaches (BEEBOOK Volume II) would foster greater comparison among studies and increase potential benefits. To maximize benefits and allow for comparison of studies across time and regions, several challenges need to be addressed: designs that ensure representative data are obtained, better coordination among efforts, and standardization of approaches (Hendrikx et al. 2009). The value of continuing and initiating other long-term surveillance efforts cannot be over stated. Surveillance data can be used to guide disease intervention methods and policy, hypothesis driven research

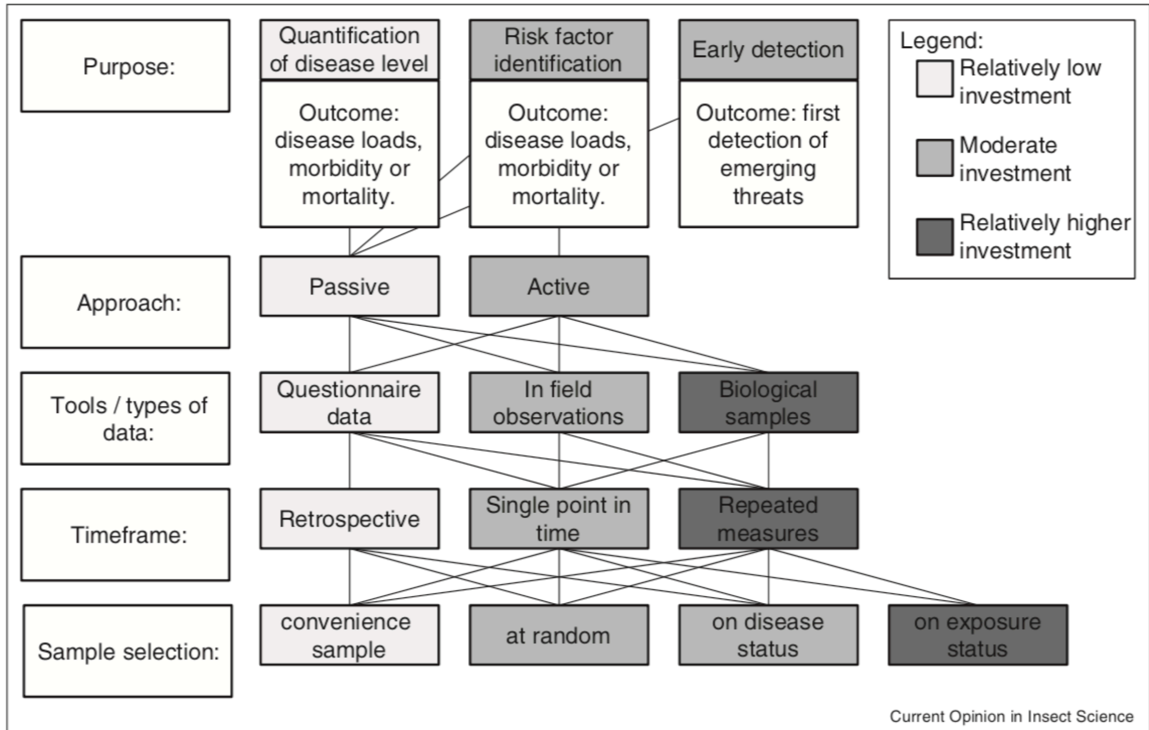
efforts focused on discovering causes of disease, and, most importantly, measure the impact the application of this knowledge has on improving bee health.

1.6 Acknowledgements

We thank Kirsten Traynor for her review of this paper.

1.7 Figures

Figure 1.1. Describes different approaches to honey bee surveillance and the corresponding relative degree of investment (time and monetary). Purpose: objective of the surveillance program. Outcome: measure of health under surveillance. Passive: approach: no intervention imposed on the regular management of the colonies under surveillance. Active: approach: implicates manipulation of the conditions experienced by (at least part of) the colonies under surveillance. Questionnaire data: interview or self-reported recollection from the stakeholder. In field observations: the overt symptoms expressed in the colonies under surveillance. Biological samples: clinical diagnostics from a physical sample collected from colonies under surveillance. Retrospective: collection of data regards exclusively past events. Single point in time: cross-sectional design where the collection of data (exposures and outcomes) are made at the same unique point in time. Repeated measures: the same colonies under surveillance are assessed repeatedly through time. Convenience sample: sample from the target population is only determined by the availability and willingness of the stakeholders. At random: selection of the sample from the target population is completely randomized, meaning all individuals from the target population have the same probability of being sampled. Selection on disease status: case–control studies comparing individuals classified as ‘diseased’ versus individuals classified as ‘disease-free’ for the disease of interest. Selection on exposure status: cohort studies comparing individuals classified as ‘exposed’ to individuals classified as ‘non-exposed’ for the risk factor of interest.


Purpose:
Quantification of disease level
Risk factor identification
Early detection
Outcome: disease loads, morbidity or mortality.
Outcome: disease loads, morbidity or mortality.
Outcome: first detection of emerging threats
Legend:
Relatively low investment
Moderate investment
Relatively higher investment
Approach:
Passive
Active
Tools / types of data:
Questionnaire data
In field observations
Biological samples
Timeframe:
Retrospective
Single point in time
Repeated measures
Sample selection:
convenience sample
at random
on disease status
on exposure status
Current Opinion in Insect Science

1.8 Tables

Table 1.1. Surveillance types. Without judging for the individual design's potential precision level, biases and confounding effects, we classified recent honey bee research according to the type of surveillance design to identify methodologies underrepresented in current publication trends.

Survey Design	**Samples (S) / Questionnaires (Q)**	**Transversal (T)/ Longitudinal (L)**	**References**	**Objectives**		
				Early warning surveillance	Monitoring (colony, disease prevalence)	Identification of risk factors
Monitoring	S/Q	L	Chauzat et al. 2014	X	X	X
	S	L	Runckel et al. 2011; Forsgren and Fries 2013; Chen et al. 2012; Topolska et al. 2009		X	
	S	T	Ravoet et al. 2013		X	X
	S	L	Li et al. 2014; Gisder et al. 2010		X	X
	S	T	Muli et al. 2014		X	
	Q	L	Clermont et al. 2014; Lee et al; 2015; Spleen et al. 2013; Steinhauer et al. 2014; van der Zee et al. 2014; vanEngelsdorp et al. 2008, 2010, 2011, 2012		X	X
	S/Q	L	Genersch et al 2010; Francis et al 2013		X	X
	S/Q	T	Giacobino et al. 2014, 2015		X	X
	S/Q	L	Odoux et al 2014		X	
Cross-sectional	S	T	Martin et al. 2012; Mondet et al. 2014		X	
Case studies	S	T	Bekele et al. 2014			X
Case-control	S/Q	T	Chauzat et al. 2010			X
	S	T	Cornman et al. 2012			X

Cohort	S/Q	L	vanEngelsdorp et al. 2013	X
	S	L	Büchler et al. 2014; Frey and Rosenkranz 2014; Lodesani et al. 2014; Hatjina et al. 2014; Meixner et al. 2014	X

T (transversal), Collected at one point in time

L (longitudinal), More than one observation per replicate over time

Chapter 2. A national survey of managed honey bee 2013–2014 annual colony losses in the USA

Publication citation

Lee, K.V., Steinhauer, N., Rennich, K., Wilson, M.E., Tarpy, D.R., Caron, D.M., Rose, R., Delaplane, K.S., Baylis, K., Lengerich, E.J., Pettis, J., Skinner, J.A., Wilkes, J.T., Sagili, R., and vanEngelsdorp, D., for the Bee Informed Partnership. 2015. A national survey of managed honey bee 2013–2014 annual colony losses in the USA. *Apidologie* 46 (3): 292–305.

2.1 Synopsis

Honey bee colony losses are a major concern in the USA and across the globe. Long-term data on losses are critical for putting yearly losses in context. US colony loss surveys have been conducted yearly since the winter of 2006–2007. Here, we report the results from the eighth annual survey on winter losses and the second annual survey of summer and annual losses. There were 7425 valid respondents (7123 backyard, 190 sideline, and 112 commercial beekeepers) managing 497,855 colonies, 19 % of the total US colonies. Total losses reported were 19.8 % [95 % CI 19.3–20.3 %] over the summer, 23.7 % [95 % CI 23.3–24.1 %] over the winter, and 34.1 % [95 % CI 33.6–34.6 %] for the whole year. Average losses were 15.1 % [95 % CI 14.5–15.7 %] over the summer, 44.8 % [95 % CI 43.9–45.7 %] over the winter, and 51.1 % [95 % CI 50.2–51.6 %] for

the whole year. While total winter loss was one of the lowest reported in 8 years, 66 % of all beekeepers had higher losses than they deemed acceptable.

2.2 Introduction

Honey bee (*Apis mellifera* L.) colony losses are a major concern worldwide. Mortality can be driven by a number of interacting factors, including the parasitic mite *Varroa destructor*, other parasites and diseases, nutrition, pesticides, and socioeconomic factors (Berthoud et al. 2010; Dainat et al. 2012a; Dainat et al. 2012b; Ellis et al. 2010; Le Conte et al. 2010; Potts et al. 2010a; vanEngelsdorp and Meixner 2010). From 1947 to 2008, the total US honey bee population has declined by 61 % (Ellis et al. 2010; vanEngelsdorp and Meixner 2010). In spite of this long-term trend and recent winter losses, the US Department of Agriculture National Agricultural Statistics Service (USDA-NASS) estimates that colony numbers increased from 2.39 million in 2006 (USDA-NASS 2007) to 2.64 million in 2013 (USDA-NASS 2014). Colony losses have not resulted in declines, as colony losses can be mitigated by beekeepers splitting colonies to recover or even exceed winter losses, a springtime activity that may mask the severity of a recent winter die-off (vanEngelsdorp and Meixner 2010). In addition, income from record high honey prices (USDA-NASS 2014) and increased compensation for almond pollination in California (vanEngelsdorp and Meixner 2010) provide beekeepers incentives to increase colony numbers. However, it is unclear if beekeepers will be able to sustain this level of annual loss and meet pollination demand while the acreage of pollinator-dependent crops continues to increase faster than the honey bee population (Aizen et al. 2008; Aizen and Harder 2009).

Documenting colony losses is critical for putting losses into context and identifying potential causes of mortality, especially in different regions. To better understand the distribution of colony losses, researchers have conducted surveys at national or regional scales (Aston 2010; Brodschneider et al. 2010; Charrière and Neumann 2010; Clermont et al. 2014; Dahle 2010; Gajger et al. 2010; Gray et al. 2010; Hatjina et al. 2010; Ivanova and Petrov 2010; Mutinelli et al. 2010; Neumann and Carreck 2010; Nguyyen et al. 2010; Pirk et al. 2014; Potts et al. 2010b, Soroker et al. 2010; Topolska et al. 2010; van der Zee 2010; van der Zee et al. 2012; van der Zee et al. 2013; van der Zee et al. 2014; Vejsnæs et al. 2010), including the United States (Spleen et al. 2013; Steinhauer et al. 2014; vanEngelsdorp et al. 2007, 2008, 2010, 2011, 2012). Multi-year records are especially important to understanding the variability of losses.

In the USA, surveys have been conducted since the winter of 2006–2007. The surveys asked beekeepers about numbers of living colonies at different points in the year, decreases and increases of colonies, the level of winter loss that they deemed acceptable, the state(s) the colonies were kept in, if the beekeepers moved colonies across state lines, if the colonies were used for almond pollination, and the perceived causes of those losses, including colony collapse disorder (CCD) (vanEngelsdorp et al. 2009). From the previous surveys, the total US winter losses were 32, 36, 29, 34, 30, 22, and 31 % for the winters of 2006–2007, 2007–2008, 2008–2009, 2009–2010, 2010–2011, 2011–2012, and 2012–2013, respectively (Spleen et al. 2013; Steinhauer et al. 2014; vanEngelsdorp et al. 2007, 2008, 2010, 2011, 2012). Total US summer and annual losses for 2012–2013 were reported as 25 and 45 %, respectively (Steinhauer et al. 2014). Acceptable losses of previous US surveys ranged from 13.2 to 17.6 % (vanEngelsdorp et al. 2010, 2011, 2012; Spleen et al. 2013; Steinhauer et al. 2014).

This study is based on the latest US colony mortality survey conducted by the Bee Informed Partnership (BIP, beeinformed.org). It addresses colony mortality from 1 April 2013 to 1 April 2014. It is the second survey in the series to include summer and annual losses along with winter losses. We include the level of acceptable losses reported by beekeepers and the percent of beekeepers that exceed the level of loss they deem acceptable. The results contain loss comparisons by operation type, losses by state, pollination of almonds, migratory status, and the self-reported causes of death, including the percent of colonies that died with the symptom “no dead bees in the hive or apiary.” As in previous surveys, responding beekeepers were categorized by operation type (backyard, sideline, or commercial) based on the number of colonies they managed, as backyard, sideline, and commercial beekeepers tend to have different management practices. Commercial beekeepers are more likely to be migratory, use their colonies to pollinate almonds, have more intensive management practices, and keep colonies in high-density locations that can affect disease transmission and virulence (Royce and Rossignol 1990). Backyard beekeepers tend to be stationary, have fewer colonies, and manage less rigorously. Sideline beekeepers tend to be between the other two groups. Beekeepers were also categorized by state, as reporting the state(s) in which the colonies were kept can help account for differences colony losses due to the climate or regional practices. In addition, as causes of mortality can be multifactorial and vary among operation types and colony location, asking beekeepers to report what they think is their primary cause(s) of death can lead to insights about the most influential factors of loss for beekeepers in different regions and demographics.

2.3 Methods

2.3.1 Survey

The survey to estimate colony losses of 2013 to 2014 was provided online through the Internet platform SelectSurvey.com. Beekeepers were invited to participate via email by distribution through lists maintained by two national beekeeping organizations (American Beekeeping Federation and American Honey Producer's Association), a beekeeping supply company (Brushy Mountain Bee Farm), two honey bee brokers, two beekeeping journals (American Bee Journal and Bee Culture), and two subscription listservs (Catch the Buzz and ABF Alert). An e-mail request to participate in the survey was sent to 8679 beekeepers that signed up to participate on beeinformed.org, responded to a previous BIP survey and indicated their willingness to participate in future surveys, or participated in the USDA Animal Plant Health Inspection Service National Honey Bee Disease Survey and provided their e-mail. All survey requests asked beekeepers to forward the survey on to other beekeepers. Requests to distribute letters were sent to the Apiary Inspectors of America, state extension apiculturists, industry leaders, and to a number of beekeeping clubs, including the Eastern Apicultural Society. To specifically encourage the participation of commercial beekeepers, we conducted surveys over the phone or mailed paper surveys (n =1200) either through BIP or a state apiarist. As our methods for soliciting responses depended on other organizations and requests to pass on the invitation, we are unable to calculate a total number of beekeepers contacted. The survey was not randomly conducted as described by van der Zee et al. 2013, which could lead to bias in the results. To compensate for the potential bias, we used a variety of other contact methods to reach a diverse group of beekeepers and contacted every registered commercial beekeeper.

At the request of several commercial beekeepers and due to the longer than typical winter weather in some states, we extended the survey to encompass the entire month of April. The survey was available online from 1 to 30 April 2014. Paper surveys were mailed on 26 March, and completed surveys returned by 9 May were included in the analyses.

The survey consisted of two parts: the "loss survey" and the "management survey." After completion of the loss survey, beekeepers were given the option to continue to the management survey. Only the responses to the loss survey are addressed in this study. Figure S2.1 contains the loss survey questions and the corresponding definition for valid responses to each question. Loss questions were based on the survey designed by Prevention of honey bee COlony LOSSes (COLOSS), a research group that measures colony losses internationally (van der Zee et al. 2013). However, the definition of colony loss in this survey differs from the COLOSS survey. We consider a colony as "living" if it is "alive on that date, independent of future prospects," while the COLOSS survey takes the future prospects of the colony into account. Definitions for a "colony," "living" colonies, and "increases" are provided in Figure S2.1.

The 2013–2014 survey included the same core questions as the previous years' winter loss surveys and the same summer and annual loss questions as last year's loss survey (Steinhauer et al. 2014). As in the previous US surveys, summer was defined as the period from 1 April 2013 to 1 October 2013, winter from 1 October 2013 to 1 April 2014, and annual from 1 April 2013 to 1 April 2014 (Spleen et al. 2013; Steinhauer et al. 2014; vanEngelsdorp et al. 2007, 2008, 2010, 2011, 2012). New to the current survey were questions 11, 12, 20, 21, and 22 (Figure S2.1). Winter, summer, and annual are classified as a fixed time period as there is no definable winter in some states. We

account for colony increases and decreases during the fixed time periods in the current survey. A fixed winter definition is a deviation from the methods by van der Zee et al. 2013, but the same as the 2010 questionnaire used for countries without a definable winter (van der Zee et al. 2012).

The loss data were edited to remove invalid response (i.e., negative numbers, responses that exceeded 100,000 managed colonies). Duplicate entries were removed, as were entries from non-US respondents. The questionnaire included a multiple choice question with an open entry “other” category, where responses were sorted to either keep the entry as “other” if the cause of death written was effectively different from the listed categories or revised to one of the preexisting categories where appropriate. After the initial validation, three subsets of data based on the three time periods were created for the analyses: valid for winter loss, valid for summer loss, and valid for annual loss. These subsets were necessary because not all respondents answered the entire set of loss questions. To be valid in a time period, beekeepers needed to start that time period with at least one colony.

Each beekeeper’s set of managed colonies will be referred to as that beekeeper’s “operation.” To compare different operation sizes, beekeepers were classified into three groups based on the number of living colonies managed on 1 October 2013: “backyard beekeepers” managed 50 or fewer colonies, “sideline beekeepers” managed between 51 and 500 colonies and “commercial beekeepers” managed more than 500 colonies. These classifications are identical to those used in the previous surveys.

2.3.2 Statistics

Total and average colony losses for summer, winter, and the annual period were calculated for all operations based on vanEngelsdorp et al. (2013) and Steinhauer et al. (2014). First, percent colony loss for each time period for each respondent was calculated by dividing the number of colonies the beekeeper lost by the number of colonies at risk in summer, winter, and annual (Figure S2.1, questions 2–5, 5–8, and 2–8, respectively). The total % loss for each time period (summer, winter, and annual) were calculated by dividing the total number of colonies lost in that time period by the total number of colonies at risk in the same time period and multiplying by 100. Results from the total % loss calculations were applied to calculate the average % loss for each time (summer, winter, and annual). Average losses were calculated by summing all the individual % losses for that time period, then dividing by the number of respondents for that same time period. All equations can be found in Steinhauer et al. 2014. The 95 % confidence intervals (95 % CIs) for the total losses were calculated using a generalized linear model (quasibinomial distribution) (R Development Core Team, 2009; code provided by Y. Brostaux and B.K. Nguyen). The 95 % CI for average losses were calculated using the Wald's formula (see vanEngelsdorp et al. 2013 for details).

Total loss calculations count each individual colony equally, without regard to operation size. This means beekeepers with more colonies have greater influence in the total loss results. For comparison, in the average loss calculations, each beekeeper's operation is counted once, meaning each operation has the same weight whether it is backyard, sideline, or commercial. Total loss calculations are more representative of commercial operations as they manage significantly more colonies compared to backyard and sideline operations. Average loss calculations are more representative of backyard

beekeepers as there are more backyard than commercial or sideline operations. Total loss is more informative to compare losses among seasons and among states, and average loss is more informative to compare categories of respondents.

The winter loss data were used to compare operation types (backyard, sideline, commercial), losses by state, migratory status (beekeepers that moved colonies at least once during the year), beekeepers that use their colonies to pollinate almond trees in California, acceptable winter losses, and causes of colony death, including the percent of colonies that died with the symptom "no dead bees in the hive or apiary" (a characteristic associated with CCD). The Kruskal-Wallis rank sum test was used to compare average losses among groups, which, if significant, was followed with a Mann-Whitney U test (Wilcoxon rank-sum test) for a pairwise test to check for significant differences between groups and a Bonferroni correction for multiple comparisons. All statistics were performed using statistical program R (R version 3.1.0 (2014-04-10), and all tests used a significance level of $\alpha=0.05$. To report the state losses, we followed the USDA-NASS method of counting colonies of multistate beekeepers in each state in which the beekeeper reported having colonies (USDA-NASS 2014). Multistate beekeepers can be migratory or stationary. If a state had five or fewer respondents, the losses for that state were not reported to preserve the identity of the respondent(s).

2.4 Results

2.4.1 Average and total losses

There were a total of 7425 loss entries (7123 backyard, 190 sideline, and 112 commercial beekeepers) after the data were validated and duplicate responses removed. There were 5962 respondents with valid data in the summer loss data subset, 7189

respondents in the winter loss data subset, and 6105 respondents in the annual loss subset. The total number of colonies managed by the respondents on 1 October 2013 was 497,855 or approximately 18.9 % of the 2.64 million total colonies in the nation (USDA-NASS 2014). Of the 7189 valid winter loss respondents, 1994 beekeepers (27.7 % of all respondents) reported losing no colonies over winter (1984 backyard beekeepers, 7 sideline beekeepers, and 3 commercial beekeepers).

Table 2.1 provides a summary of the total number of colonies managed by the respondents at the start and end of each of the time periods, the total colony increases and decreases for each period, and the total and average losses of each period. Over the winter, total colony losses reported were 23.7 % [95 % CI 23.3–24.1 %] and the average winter losses were 44.8 % [95 % CI 43.9–45.7 %]. Total summer losses were 19.8 % [95 % CI 19.3– 20.3 %], and the summer average losses were 15.1 % [95 % CI 14.5–15.7 %]. Total annual losses were 34.1 % [95 % CI 33.6–34.6 %], and the average annual losses were 51.1 % [95 % CI 50.2–51.6 %]. Note that different pools of respondents were analyzed for each of the time periods.

2.4.2 State losses

States had dramatically different numbers of respondents, ranging from 1 in Puerto Rico to 1080 in Pennsylvania, with a large range of total and average losses. The range in total losses was from 2.3 to 71.1, 11.1 to 71.1, and 20.1 to 89.7 % for summer, winter, and annual, respectively. Average losses ranged from 4.2 to 24.2, 11.1 to 69.1, and 24.4 to 72.2 % for summer, winter, and annual, respectively. Figure S2.2 shows images of US maps with the total and average losses plotted for each state and the number of winter loss respondents. To indicate the distribution of multistate beekeepers,

we have included the percent of beekeepers that operate exclusively within the state (ranging from 3.2 to 100 %) and the percent of colonies that were kept exclusively within the state (ranging from 0.04 to 100 %). As stated in the methods, beekeepers that manage colonies in more than one state were counted in each state. Therefore, states with a small percentage of beekeepers operating exclusively inside the state require caution when interpreting the results. Table S2.1 summarizes the following for each state: number of respondents and colonies, number of respondents from each operation type in, percent colonies operating exclusively in that state, and the summer, winter and annual losses.

2.4.3 Losses by operation type

Response rates to the survey were different for the three operation types. For the winter loss data subset, 96.0 % of the total number of respondents were backyard beekeepers (n =6899), 2.6 % were sideline beekeepers (n=186), and 1.4 % were commercial beekeepers (n =104). The three different operation types managed very different numbers of colonies. Of the 497,855 colonies managed on 1 October 2013, the backyard beekeepers managed 39,188 colonies (7.9 % of the total number of colonies), sideline beekeepers managed 27,288 colonies (5.5 %), and commercial beekeepers managed 431,379 (86.6 %) (Table 2.2). There was a seasonal difference in the total losses for sideline and backyard beekeepers: more colonies died in the winter compared to the summer. Winter and summer losses for commercial beekeepers were not different. A visualization of the seasonal average losses for each operation type is provided in Figure 2.1.

There were significant differences in mortality among seasons and operation types (statistics summarized in Table S2.2). All beekeeper operation types had significantly

different average annual losses (Kruskal-Wallis χ^2=39.2306, all Mann-Whitney P <0.05), with backyard beekeepers having the highest losses and commercial beekeepers with the lowest losses (Table 2.2). Comparing the average losses across operation types for summer and winter, only the winter losses of sideline and backyard beekeepers were not different (Kruskal-Wallis χ^2=61.6678, Mann-Whitney P =0.064).

To compare average winter losses of migratory to non-migratory beekeepers and beekeepers that used colonies to pollinate almonds to those that do not pollinate almonds, we performed separate analyses for sideline and commercial beekeepers since the two operation types had significantly different winter losses (Kruskal-Wallis χ^2=21.6678, Mann-Whitney P <0.0001) (Table 2.3). This comparison differs from last year's survey that included both commercial and sideline beekeepers (Steinhauer et al. 2014). Backyard beekeepers were not included due to few being migratory or commercial almond pollinators (1.2 and 0.1 % of backyard beekeepers, respectively). The only significant difference found was migratory sideline beekeepers which had lower losses compared to non-migratory sideline beekeepers (Kruskal-Wallis χ^2=7.1623, Mann-Whitney P =0.007445). There was no difference between migratory and non-migratory in commercial groups, but the P value was close to 0.05 (P =0.065). Losses of sideline beekeepers using their colonies to pollinate almond was not different than non-pollinator losses, but the P value was again close to 0.05 (P =0.060).

2.4.4 Acceptable winter losses

On average, beekeepers reported that a 19.1 % (95 % CI 18.6–19.5 %) winter loss was acceptable. Separated by operation type, commercial beekeepers had the lowest self-

reported average acceptable winter loss of 16.8 % (95 % CI 14.5– 19.2 %), sideline beekeepers reported an average acceptable loss of 18.4 % (95 % CI 16.2–20.6 %), and backyard beekeepers had the highest level of 19.1 % (95 % CI 18.7–19.6 %). The self-reported acceptable winter loss ranged from 0 to 100 %. We compared each beekeeper's actual winter losses to the loss they reported as acceptable and found that 2447 beekeepers (34.0 % of respondents) had a winter loss that they considered to be acceptable, and 4742 beekeepers (66.0 % of respondents) exceeded the winter loss they considered acceptable. Beekeepers that were below their self-reported acceptable winter loss had an average winter loss of 2.3 % (95 % CI 2.1–2.5 %). Beekeepers that exceeded what they deemed an acceptable loss had an average winter loss of 66.7 % (95 % CI 65.9–67.5).

Beekeepers that reported that their winter losses compared to last year were lower, same, higher, no bees, or do not know lost had average losses of 19.6 % (95 % CI 18.4–20.7), 38.6 % (95 % CI 36.6–40.7), 66.5 % (95 % CI 65.3– 67.7), 44.0 % (95 % CI 41.4–46.5), or 29.5 % (95 % CI 24.5–34.4) of their colonies, respectively (Table 2.4). All loss level groups had significantly different loss averages except for the comparison between the "same loss" and "do not know" groups (Kruskal-Wallis χ^2=1543.264, df =4, P <0.0001; all Mann-Whitney P <0.05, except comparison between "same loss" and "do not know").

2.4.5 Self-reported causes of winter loss

In the winter loss data subset, a total of 4903 beekeepers (4635 backyard, 172 sideline, and 96 commercial beekeepers) had losses and reported at least one cause of death. The selected causes of death in order were poor wintering conditions (n =2237),

starvation (n =1774), weak in the fall (n=1610), queen failure (956), do not know (921), *Varroa destructor* (n =836), other (n=455), pesticides (n=325), CCD (n=324), *Nosema* spp. (n =261), small hive beetle (n =250), and disaster (n =100). Common causes of death written in the “other” category were wasps (n =59), ventilation/moisture (n =48), wax moth (n =46), swarming (n =41), and robbing (n =38). The relative frequency of responses was separated by operation type to show the relative frequency of the selection of each cause of death (Figure 2.2). For both backyard and sideline beekeepers, the top three self-reported causes of colony death in order were poor wintering conditions, starvation, and weak colonies. Commercial beekeepers chose queen failure, *V. destructor*, and pesticides. Beekeepers that reported losing colonies to poor wintering conditions, CCD, or did not know reported losing more bees than those who did not report those causes (Kruskal-Wallis χ^2=286.5315, 4.2501, and 31.2649, respectively, with all Mann-Whitney $P < 0.05$). Beekeepers that reported losing colonies to queen failure, *V. destructor*, weak in the fall, or “other” had fewer loses compared to beekeepers that did not report those causes (Kruskal-Wallis χ^2=151.9933, 26.234, 44.018, and 5.0879, respectively, with all Mann-Whitney $P < 0.05$). Results and statistics are summarized in Table S2.3.

For the question that asked for if colonies that died over the winter had the symptom “without dead bees in the hive or apiary,” there were a total of 4907 valid responses with 1455 beekeepers reporting having at least one colony that died with the symptom and 3452 reporting the absence of this symptom. We estimate that 46,765 colonies died with this symptom or 34.5 % of the total colonies that died over the winter. This number was estimated using the number of beekeepers reporting the symptom, the

percent at which they reported the symptom, and the number of colonies that died over the winter in those beekeepers' operations. Beekeepers that reported the symptom did not have higher losses than those that did not report the symptom (Kruskal-Wallis χ^2 =2.3436, Mann-Whitney P =0.1258). Commercial beekeepers were 2.9 times more likely to report the symptom compared to backyard beekeepers (Pearson's χ^2=197.3449, df =2, P <0.0001).

2.5 Discussion

This is the eighth in a series of surveys estimating annual US colony winter losses since 2006–2007 (Spleen et al. 2013; Steinhauer et al. 2014; vanEngelsdorp et al. 2007, 2008, 2010, 2011, 2012) and the second year to report annual and summer losses (Steinhauer et al. 2014). While the results showed a lower total winter loss, the average winter loss was among the highest of all the surveys, with a large difference in winter losses among operation types. This year, the survey respondents reported one of the highest acceptable winter loss levels; however, 66 % of beekeepers still exceeded their level of acceptable loss. Summer losses were considerable, emphasizing that surveys should measure annual losses to estimate colony mortality.

2.5.1 Average and total losses

This year's total winter loss of 23.7 % was similar to the lowest winter loss in the 8-year survey set of 22.5 % in 2011–2012 (Spleen et al. 2013). Even with this year of lower loss, the average total winter loss of all the US surveys is 29.4 %. This year's average winter loss of 44.8 % was the same as the highest average winter loss of the

previous seven surveys of 44.8 % that occurred in 2012– 2013 (Steinhauer et al. 2014). In other countries, beekeepers are also having high winter losses. In 2008–2009, losses ranged from 6.3 to 21.7 % for Austria, Belgium, Denmark, Germany, Ireland, Italy, Netherlands, Norway, Poland, Sweden, Switzerland, and the UK (van der Zee et al. 2012). The following year in 2009–2010, losses were higher for all the countries polled with a range from 8.0 to 37.8 % (van der Zee et al. 2012). Caution needs to be used when making this comparison as these losses do not all have a standard winter loss time frame. Instead, beekeepers were asked to define the winter period on their own, as compared to US survey that defined "winter" as the 6-month period between 1 October and 1 April. High winter losses (20–50 % total losses) have also been reported in other years in Italy (Mutinelli et al. 2010), Denmark (Vejsnæs et al. 2010), Austria and South Tyroll (Brodschneider et al. 2010), Scotland (Gray et al. 2010), England (Aston 2010), Israel (Soroker et al. 2010), Switzerland (Charrière and Neumann 2010), and South Africa (Pirk et al. 2014). Not all losses have been high. Moderate winter losses of about 10 % have been reported in Bulgaria (Topolska et al. 2010) and Norway (Dahle 2010). Low losses (under 5 %) have been reported in China and various other regions within countries (van der Zee et al. 2012).

As demonstrated by the 2012–2013 survey (Steinhauer et al. 2014), winter losses alone do not provide the full picture of yearly colony mortality. Many regions within the US lack a temperate winter, so losses that occur may not have to do with winter. In 2012–2013, the total winter losses were 30.6 %, with a 25.3 % total summer loss, and a 45.2 % annual total loss. If losses were not assessed over the full year, the winter losses would have grossly underestimated the total yearly mortality. Summer losses in other regions have been low (under 5 %) (Dahle 2010; Gray et al. 2010; Peterson et al. 2010; van der

Zee 2010), or higher and varied by region and year (Gray et al. 2010; Mutinelli et al. 2010).

2.5.2 State losses

The USA has a varied climate range that likely affects the loss rate in the different states, especially for stationary beekeepers. The winter in the Midwest in 2013–2014 was one of the coldest on record and could be reflected in the highest loss averages recorded in that region. However, the Midwest region tends to have a higher average winter loss in other years as well (Steinhauer et al. 2014), which may indicate the importance of preparing colonies for winter. Correlating losses with US weather data should be further investigated. Participation levels varied widely from state to state, which could lead to bias in loss calculations at the state level. This phenomenon is not unique to the USA, as other researchers have seen this high level of variation among regions within and among other countries (van der Zee et al. 2012).

2.5.3 Losses by operation type

While commercial beekeepers manage many colonies and move their bees, they do not appear to have higher losses than the other two beekeeper groups. In this survey, commercial beekeepers had lower winter and annual losses. In previous US surveys, commercial beekeepers either had the same level of winter loss (vanEngelsdorp et al. 2007, 2008, 2012; Spleen et al. 2013) or significantly lower losses (vanEngelsdorp et al. 2010, 2011; Steinhauer et al. 2014) than backyard beekeepers. Sideline beekeepers were not different from commercial or backyard beekeepers in the rest of the survey years, except for 2012–2013 when losses were higher than commercial beekeeper losses and

lower than backyard beekeeper's losses (Steinhauer et al. 2014) and in 2009–2010 when losses were higher than commercial beekeeper losses (vanEngelsdorp et al. 2011). This trend of lower losses for larger operation extends out of the USA. A survey of 19 mostly European countries also found that the larger operations (150 colonies or more) had significantly lower losses than the smaller operations (van der Zee et al. 2014).

The dramatic difference between the total winter loss of 23.7 % and the average winter loss of 44.8 % was largely due to the total loss being more reflective of the commercial losses and the average loss reflecting the backyard beekeeper losses. Commercial beekeepers have the majority of colonies and more influence over the total loss, and backyard beekeepers are the majority of the survey respondents and have more influence over the average loss calculations. Commercial beekeepers are generally migratory and keep their bees out of the temperate zones in winter. In addition, backyard beekeepers may be less willing to treat their bees for *V. destructor*, which could result in high winter losses (Dainat et al. 2012b; Le Conte et al. 2010; vanEngelsdorp and Meixner 2010).

In this survey and all previous US surveys, beekeepers that are migratory or use their colonies to pollinate almonds had equal or lower losses compared to beekeepers that do not do these practices (vanEngelsdorp et al. 2007, 2008, 2010, 2011, 2012; Spleen et al. 2013; Steinhauer et al. 2014). These results suggest that moving colonies or pollinating almonds does not increase the chance of mortality as some have suggested. In fact, there may be a benefit associated with pollinating almonds or the management practices employed by beekeepers that pollinate almonds better protect colonies. This may not be applicable to other countries, especially if the causes of mortality are

different, as a survey in South Africa found higher losses among the migratory beekeepers (Pirk et al. 2014).

2.5.4 Acceptable winter losses

Even though this year's total winter losses were lower than the previous 7-year average of 30 % total winter loss, 66 % of survey respondents still had losses higher than the average 19 % loss they reported as acceptable. This 19 % acceptable loss was the highest reported acceptable loss of all the past US surveys, although it was similar to the 17.6 % acceptable loss reported in 2008– 2009 (vanEngelsdorp et al. 2010). Otherwise, the previous acceptable loss range reported from 2009–2010 to 2012–2013 ranged from 13.2 to 14.6 % (Spleen et al. 2013; Steinhauer et al. 2014; vanEngelsdorp et al. 2011, 2012). This higher reported level of acceptable loss could be due in part to influence of the media focusing on high honey bee colony losses or to beekeepers becoming more accustomed to higher losses. For comparison, the acceptable average winter colony loss was reported to be 10 % in both Switzerland (Charrière and Neumann 2010) and Germany (Genersch et al. 2010), and 12 % for Denmark (Vejsnæs et al. 2010).

2.5.5 Self-reported causes of winter loss

Beekeepers were asked to choose the factors that had the greatest effect on their colony death over the winter. In previous US surveys, the most common causes of colony death reported by beekeepers were queen failure, *V. destructor*, starvation, weak in the fall, pesticides, poor wintering conditions, and CCD (vanEngelsdorp et al. 2007, 2008, 2010, 2011, 2012; Spleen et al. 2013; Steinhauer et al. 2014). In this survey, backyard and sideline beekeepers both chose poor wintering conditions, starvation and weak

colonies, in that order. Commercial beekeepers chose queen failure, *V. destructor*, pesticides, and CCD, in that order. The ranked list of causes of death for commercial beekeepers was identical to the ranked list of causes of death for commercial beekeepers in 2012–2013 (Steinhauer et al. 2014). In 2012– 2013, the backyard beekeepers chose weak, starvation, and that they did not know (Steinhauer et al. 2014). Poor winter was ranked sixth in 2012–2013. The high ranking of poor winter this year could point to a driver of high colony mortality in temperate zones; however, the backyard average winter loss in 2012–2013 (45.4 %) was very similar to the backyard average winter loss in 2013– 2014 (45.3 %). Further investigation into the regional differences in colony loss could shed light on this apparent discrepancy.

The survey question asking for a percentage of colonies that are “lost without dead bees in the hive or apiary” is a proxy question for CCD as it is one of the classically described symptoms (vanEngelsdorp et al. 2009). In previous US surveys, beekeepers reporting that at least one colony died with the CCD symptom lost significantly more colonies compared to beekeepers that did not report losing colonies with the symptom. However, this year beekeepers that reported the CCD symptom did not have higher losses. Interestingly, when asked directly if a cause of loss was CCD, beekeepers selecting CCD had higher losses compared to beekeepers that did not select CCD as a cause of death. This could be due to confusion of the definition of CCD, which may have been caused in part by the high media attention. In Europe, beekeepers that reported losing colonies with no dead bees present lost more colonies than those beekeepers that did not report the symptom (van der Zee et al. 2014).

2.5.6 Potential sources of bias

There are a few potential sources of bias that could affect the results of this survey. One source could be that the survey was not random as described by van der Zee et al. (2013), which may result in bias in the type of respondents. Beekeepers with access to a computer and those that are more Internet-literate may be a larger portion of our respondents. To help compensate for the potential bias, we mailed paper surveys to every registered commercial beekeeper in the USA and any beekeeper that requested a paper copy. We also widened our respondent pool by providing information in beekeeping journals, a beekeeping supply company, and at meetings.

Bias could be introduced through the location and type of the respondents. Some regions had a lower number of respondents, which could bias the results. In future surveys an emphasis should be made on recruiting more beekeeper participants from areas with low respondents, like Puerto Rico where there was only a single voice, to decrease the state-to-state response bias. Differences among this survey and previous surveys could be due to a difference in the respondent pool. Fewer commercial beekeepers participated in this year's survey (n=112, 1.5 % of respondents) compared to last year (n=135, 2.1 % of respondents) (Steinhauer et al. 2014). This could influence the estimation of total losses. There is a possibility that beekeepers with higher losses were more likely to take the time to do the survey. This year had one of the lowest total colony losses, meaning that it is unlikely that commercial beekeepers that had high losses were more likely to respond. However, this survey also had one of the highest average losses, which could mean that backyard beekeepers with higher losses were more likely to fill out the survey. This could result in bias in the reported average losses.

There is a possibility for bias as different respondents could interpret the survey differently or if they had poor recollection of the past. There was no definition for the potential cause of death “weak in the fall” or “poor winter,” which could lead to differences in interpretation. The survey was designed to ask questions about the number of living colonies a beekeeper has on a specific date and not about the number of colonies that died. The definition of a “living” was provided, but there may be beekeepers that interrupted the definition of “living” differently. For example, if a colony had a very small population or no queen, a beekeeper may consider it to be not living since the chances of survival are small. Results of the survey could also be altered by recall bias, as the survey asked beekeepers to remember the past.

2.6 Conclusions

This study highlights the benefits of performing multiyear surveys to better understand yearly trends. It also demonstrates the importance of considering the individual operation types separately and reporting annual and season-specific losses to best represent the colony losses of the beekeeping industry. Even in a relatively low winter loss year, beekeepers still lost 34 % of their colonies over the full year. Last year, beekeepers lost close to 45 % of their colonies over the full year (Steinhauer et al. 2014). Total winter losses were lower this year, but beekeepers are still experiencing unacceptably high losses.

2.7 Acknowledgements

We thank all respondents for their participation. We thank Brian Decker, Heather Eversole, Rachel Fahey, Andrew Garavito, and Meghan McConnell for entering the

paper surveys into the database. Our gratitude goes out to the many beekeeping organizations, industry leaders, and beekeeping clubs that forwarded our appeal for participation emails. A special thank you is owed to USDA APHIS, the Apiary Inspectors of America, Eastern Apiculture Society, the American Honey Producers Association, the American Beekeeping Federation, Brushy Mountain Bee Farm, Bee Culture magazine, and American Bee Journal for sending out participation requests to their online audiences. This project was funded by a Coordinated Agricultural Project (CAP) grant from US Department of Agriculture-National Institute of Food and Agriculture (USDA-NIFA): the Bee Informed Partnership and includes, in addition to several of the authors, Marla Spivak, Angela Spleen, Jerry Hayes, Robert Snyder, Ben Sallman, Liana Teigen, Ellen Topitzhofer, Dan Wyns, Danielle Downey, Lauren Rusert, Ed Levi, Shayne Madella, Grace Kunkel, Marjorie Gurganus, Karen Roccasecca, and Robyn Underwood, and the USDA-ARS Areawide Program on Bee Health. Thank you to the reviewers for the helpful comments.

2.8 Figures

Figure 2.1 A comparison of the average (%) summer (1 April 2013 to 1 October 2013), winter (1 October 2013 to 1 April 2014), and annual (1 April 2013 to 1 April 2014) losses (with 95 % CI) of the three beekeeping operation types (backyard, sideline, and commercial).

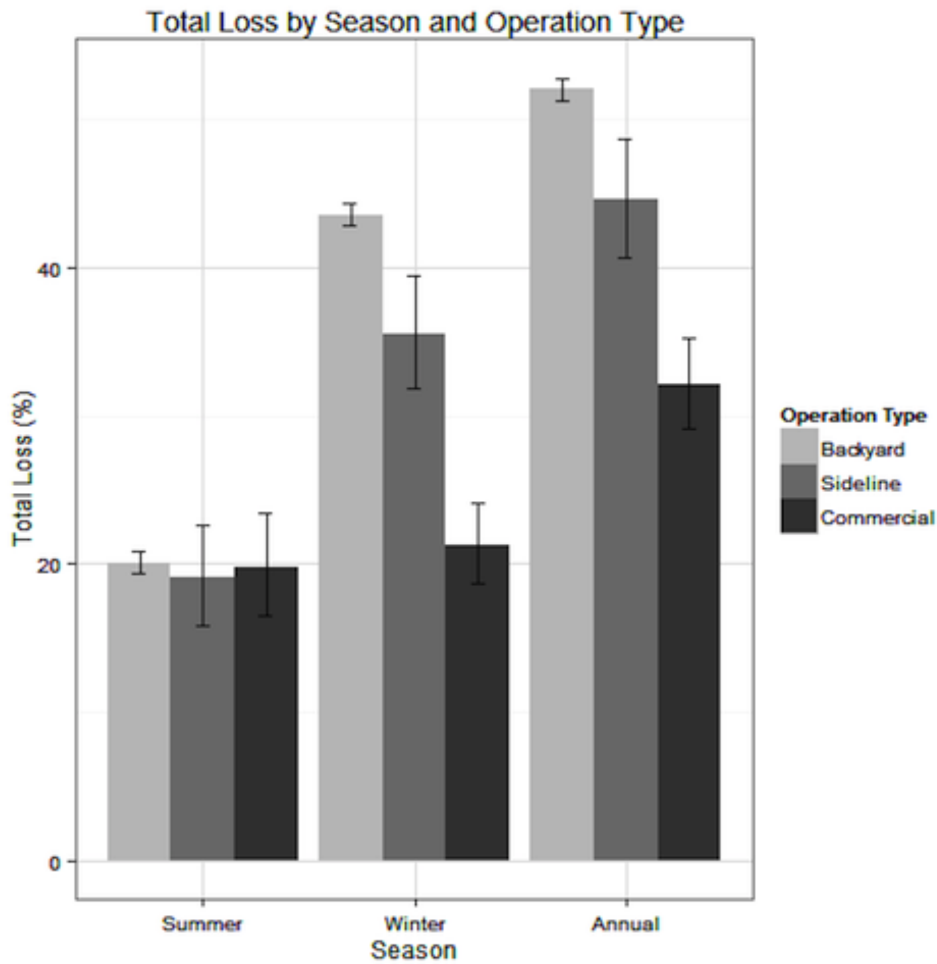


Figure 2.2 The relative frequency of the most prominent causes of colony winter mortality as chosen by the survey respondents and separated by operation type (backyard, sideline, and commercial). Respondents were able to choose more than one cause of death. SHB small hive beetle, CCD colony collapse disorder, DK do not know, Disaster natural disaster and alike (e.g. flood and bear).

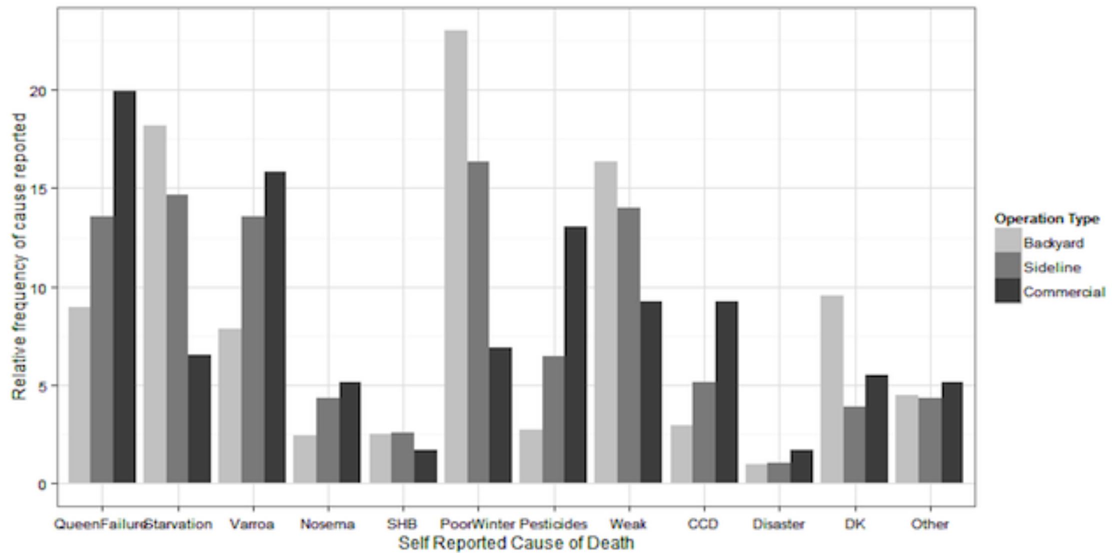


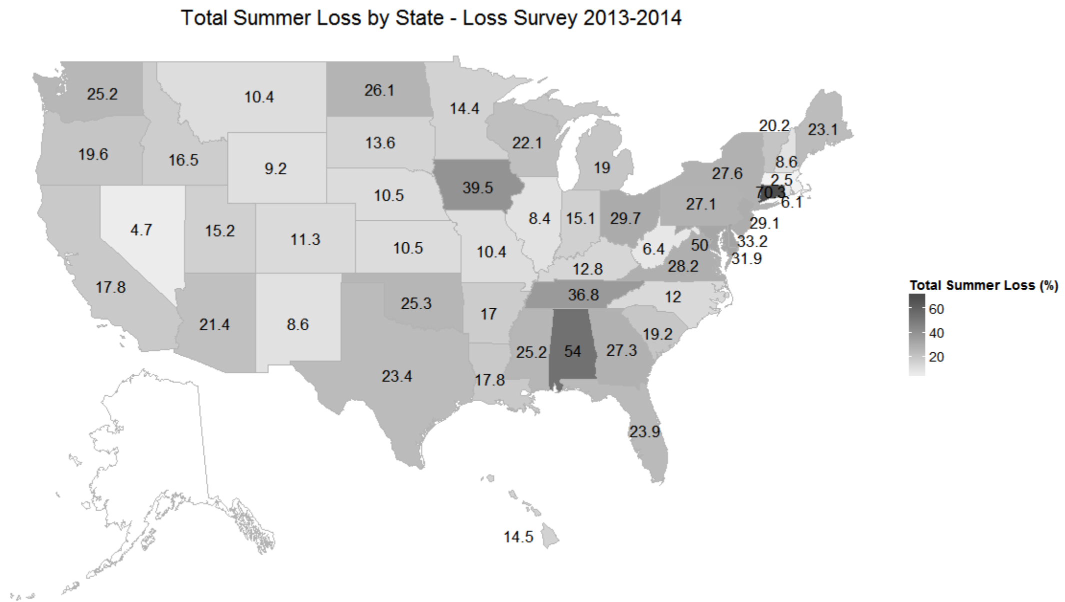
Figure S2.1 Survey questions used to determine the winter, summer and annual losses from 1 April 2013 to 1 April 2014. The allowed entries are included below each question. A required response is indicated by a "*." Definitions for a "colony," "living" colonies and "increases" were provided with the appropriate questions as the following: a colony is a queen right unit of bees that include full size colonies and queen right nucs (do not include mating nucs); "living" means alive on that date, independent of future prospects; and "increases" include successfully hived swarms and/or feral colonies.

1. In what state(s) did you keep your colonies in between April 2013 - April 2014?* Multiple choice with multiple selections allowed of all US states, or "Other" category with open entry
2. How many living colonies did you have last spring on April 1, 2013?* Numeric (integer) open entry
3. How many colonies, splits, and/or increases did you make / buy between April 1, 2013 and October 1, 2013?* How many colonies, splits, and/or increases did you sell or give away between April 1, 2013 and October 1, 2013?* Numeric (integer) open entry
4. How many colonies, splits, and/or increases did you sell or give away between April 1, 2013 and October 1, 2013?* Numeric (integer) open entry
5. How many living colonies did you have on October 1, 2013?* Numeric (integer) open entry
6. How many colonies, splits, and/or increases did you make / buy between October 1, 2013 and April 1, 2014?* Numeric (integer) open entry
7. How many colonies, splits, and/or increases did you sell / give away between October 1, 2013 and April 1, 2014?* Numeric (integer) open entry
8. How many total living colonies (overwinter surviving colonies plus purchase or splits) did you have on April 1, 2014?* Numeric (integer) open entry
9. What was the largest number of living colonies you owned between April 1, 2013 and April 1, 2014? Numeric (integer) open entry
10. What was the smallest number of living colonies you owned between April 1, 2013 and April 1, 2014? Numeric (integer) open entry
11. You indicated you had ###### colonies alive on April 1, 2013. How many of those specific colonies were still alive on October 1, 2013? Numeric (integer) open entry

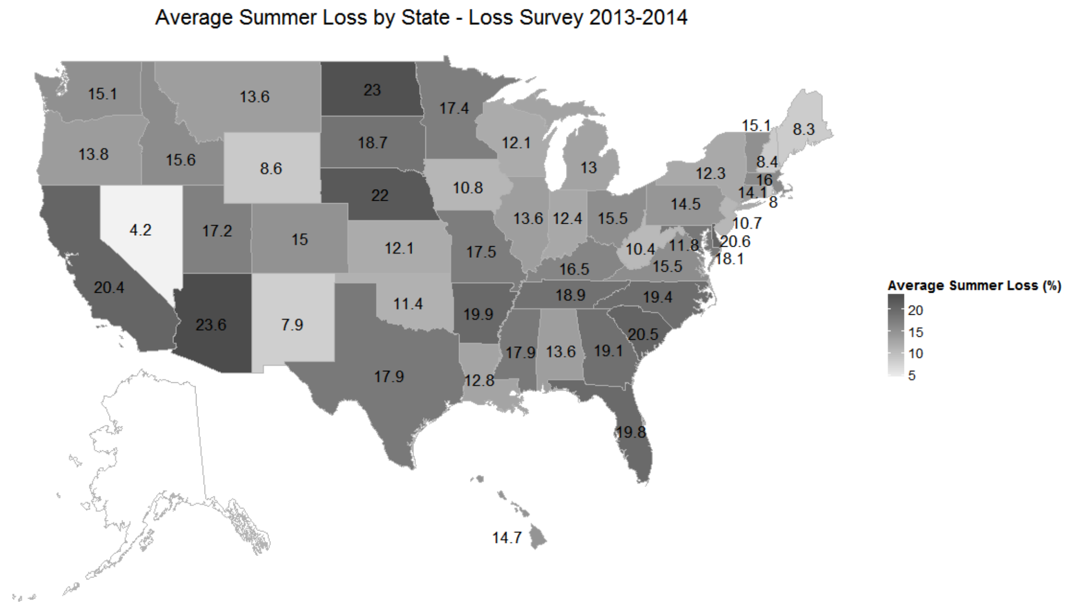
12. You indicated you had ###### colonies alive on October 1, 2013. How many of those specific colonies were still alive on April 1, 2014? Numeric (integer) open entry
13. What percentage of loss, over the winter, would you consider acceptable? Percentage with value between 0-100
14.Single choice entry with the following possible choices: Higher, Lower, Same, Unsure, Did not keep bees last year
15. What percentage of the colonies that died over the winter (between October 1, 2013 and April 1, 2014) were lost without dead bees in the hive or apiary? Percentage with value between 0-100
16. In your opinion, what factors were the most prominent cause (or causes) of colony death in your operation between October 1, 2013 and April 1, 2014? Multiple choice with multiple selections allowed of the following answers: I did not experience any winter loss, Queen failure, Starvation, *Varroa* mites, *Nosema* disease, Small Hive Beetles, Poor wintering conditions, Pesticides, Weak in the fall, Colony Collapse Disorder, Natural disaster and alike (ex: flood, bear, ...), Don't know, Other to specify (open entry)
17. Did you move any of your colonies last year (between April 1, 2013 and April 1, 2014) at least once across state lines?* Single choice of Yes or No
18. In what zip or postal code is your operation based? Numeric open entry
19. What percentage of your hives did you send to or move into California almond orchards for pollination in 2014? Percentage with value between 0-100
20. Approximately what percentage of your operation moved across state lines at least once between April 1, 2013 and April 1, 2014? Percentage with value between 0-100
21. Please indicate in which states you kept bees for the months listed. Multiple choice, with multiple selections allowed of the following: all states, all months
22. On December 31, 2013, please list the number of colonies you had in each state. Numeric (integer) entry, 1 per state

Figure S2.2 US maps of the total losses (%) and average losses (%) by state for: (a) total summer losses (%), (b) average summer losses (%) by state, (c) total winter losses (%), (d) average winter losses (%), (e) total annual losses (%), (f) average annual losses (%). The map of the number of winter loss respondents by state is shown in (g). Colonies owned by beekeepers operating in multiple states are counted in all states in which the beekeeper reported having colonies. Results from states with fewer than five respondents are not shown.

a.



b.



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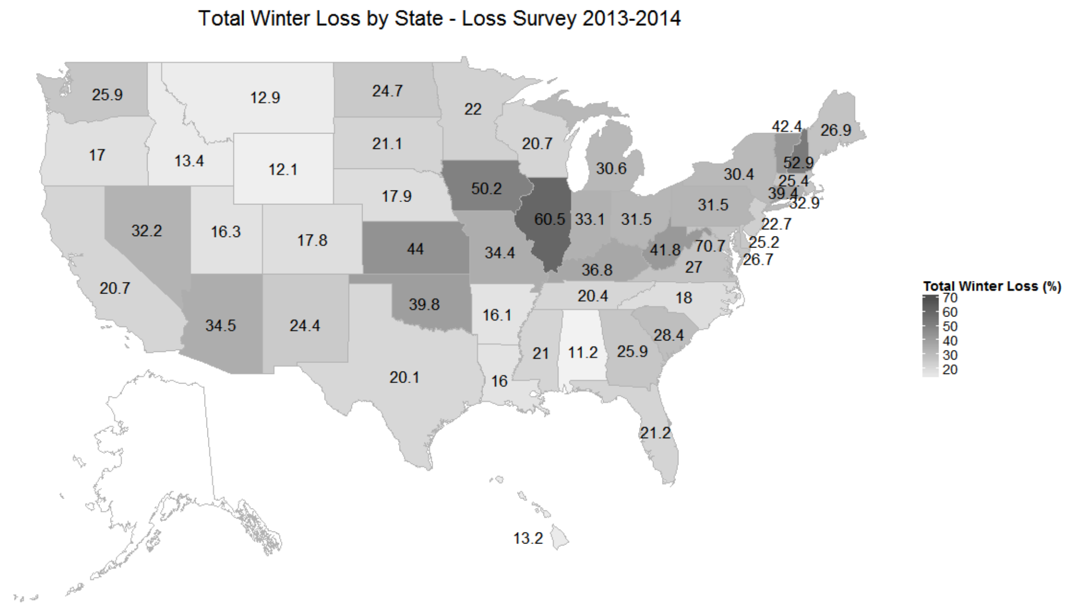
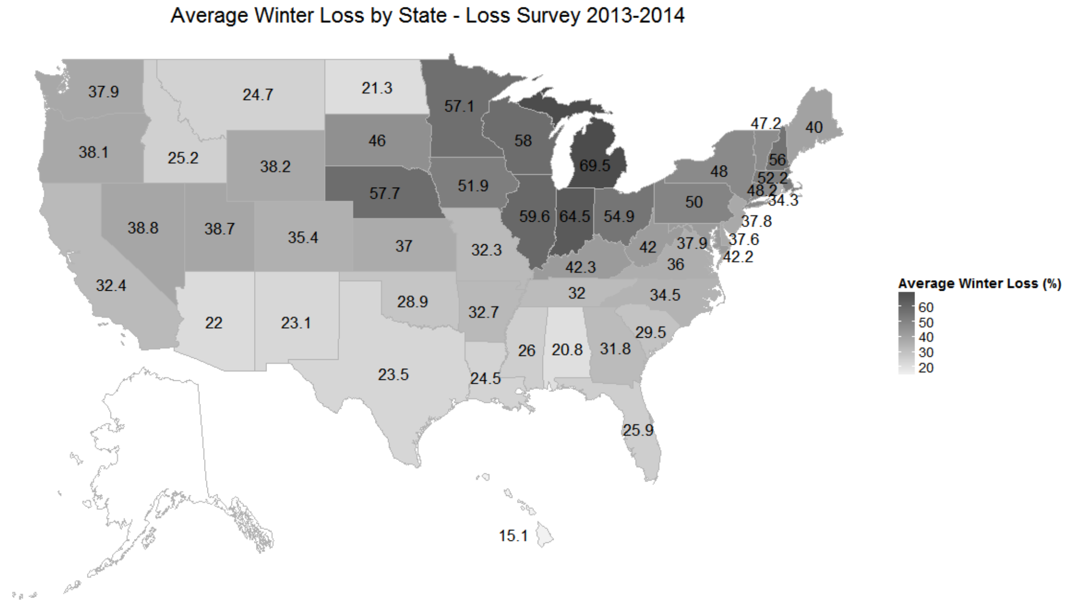
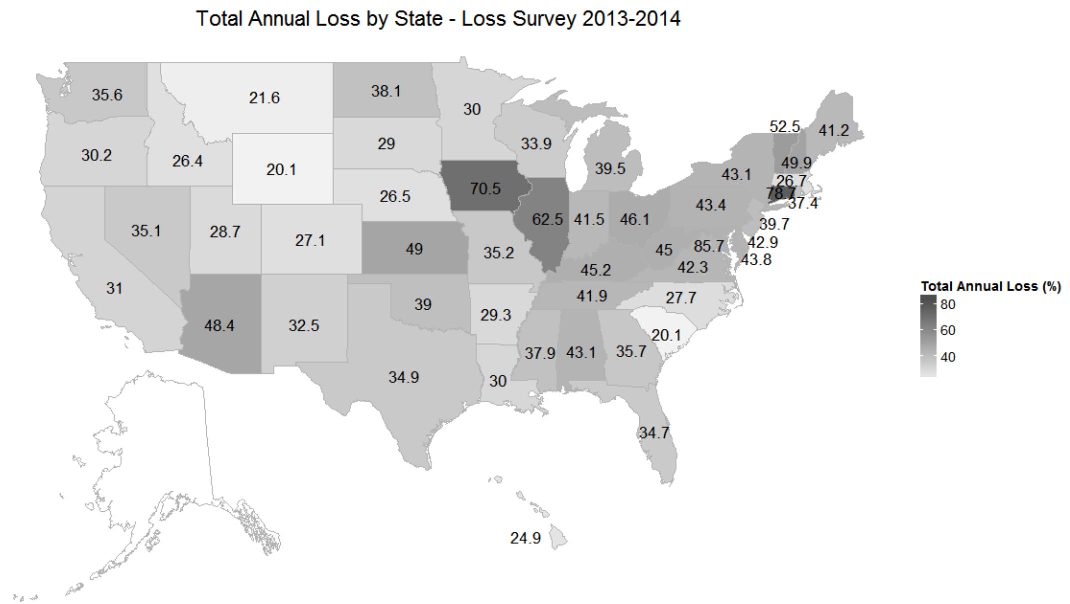


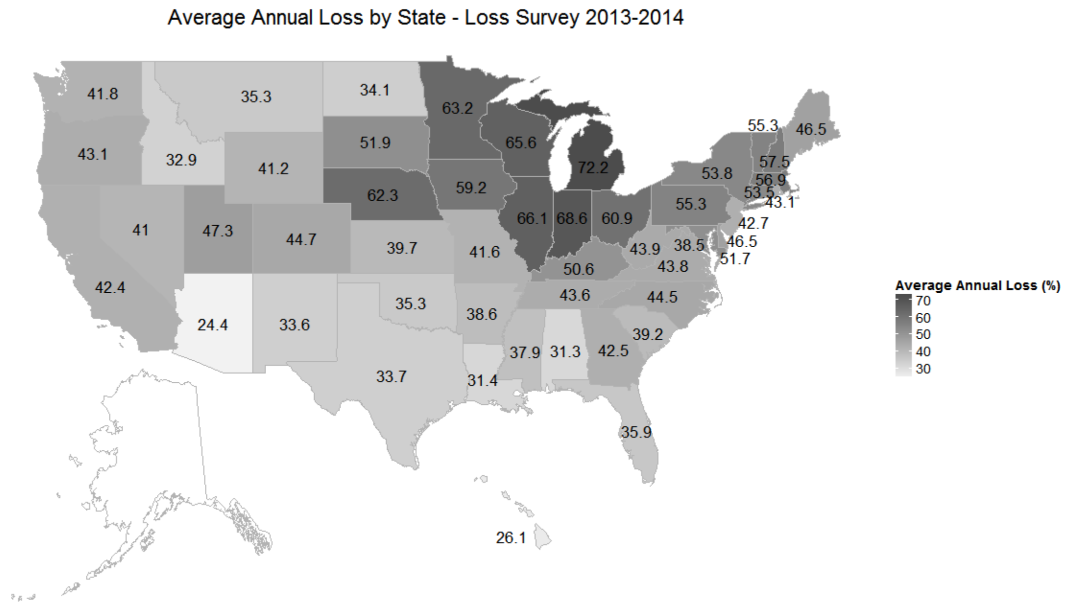
d.



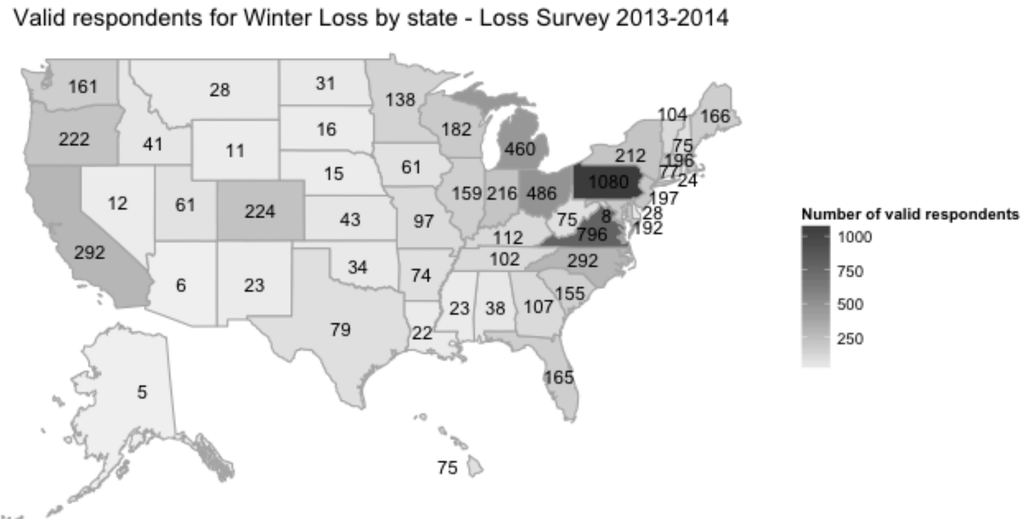
e.



f.



g.



2.9 Tables

Table 2.1 A summary of the three loss periods (summer, winter, and annual) of the self-reported colony loss data from 1 April 2013 to 1 April 2014, with the total number of respondents, the total number of colonies on each date, the total number of colonies increases (+) and decreases (−), and the total loss and average loss for each period (%) [95 % CI].

Period	Number	Total number of colonies managed on					Total loss (%)	Average loss (%)
		1 April 2013		1 October 2013		1 April 2014		
Summer loss	5962	397,611	(+186,361) (−18,509)	453,459		–	19.8 [19.3–20.3]	15.1 [14.5–15.7]
Winter loss	7189	–		497,855	(+86,220) (−11,716)	436,759	23.7 [23.3–24.1]	44.8 [43.9–45.7]
Annual loss	6105	435,662	(+197,549) (−23,270)	505,003	(+91,993) (−13,440)	453,525	34.1 [33.6–34.6]	51.1 [50.2–51.9]

Table 2.2 Average and total losses by operation type (total and average loss (%) [95 % CI]), including the number of operations included in each analysis, the number of colonies at the beginning of the specified analysis period, and the relative percent of colonies in the respondent pool run by each operation type.

Period	Operation type	Number	No. of colonies (start)	% Colonies (start)	Total loss (%) [95 % CI]	Average loss (%) [95 % CI]
Summer loss	Backyard	5695	26,903	6.8	20.1 [19.4–20.8]	15.1 [14.5–15.7]
	Sideline	164	18,357	4.6	19.1 [15.8–22.6]	12.5 [10.0–14.9]
	Commercial	103	352,351	88.6	19.8 [16.6–23.4]	18.7 [15.7–21.7]
Winter loss	Backyard	6899	39,188	7.9	43.6 [42.8–44.3]	45.3 [44.4–46.2]
	Sideline	186	27,288	5.5	35.5 [31.8–39.4]	38.9 [34.9–42.8]
	Commercial	104	431,379	86.6	21.3 [18.6–24.2]	22.7 [19.6–25.8]
Annual loss	Backyard	5815	27,738	6.4	52.0 [51.2–52.8]	51.6 [50.7–52.5]
	Sideline	180	19,470	4.5	44.6 [40.6–48.7]	44.5 [40.5–48.4]
	Commercial	110	388,454	89.2	32.1 [29.1–35.2]	32.6 [29.4–37.5]

Table 2.3 Results of a Kruskal-Wallis rank-sum test and followed-up by a Mann-Whitney test used to compare the average winter losses (%) [95 % CI] among commercial and sideline operations that do or do not take their colonies to pollinate almonds in California, and beekeepers that are migratory (moved their bees at least once during the past year) to those that are not.

Operation type	Factor	Selection	Number	Average winter loss (%) [95 % CI]	Kruskal-Wallis chi-squared	*P* value
Commercial	Almond pollination	No	22	27.6 [19.3–35.9]	1.5348	0.2154
		Yes	76	22.1 [18.7–25.4]		
	Migratory	No	22	29.5 [21.5–37.5]	3.3969	0.0653
		Yes	76	21.5 [18.2–24.9]		
Sideline	Almond pollination	No	151	40.2 [35.8–44.6]	3.5249	0.0605
		Yes	27	30.7 [20.5–40.9]		
	Migratory	No	135	41.7 [37.1–46.3]	7.1623	0.0075*
		Yes	43	29.5 [21.7–37.4]		

*$P<0.05$, significant

Table 2.4 Comparison of the responses to the survey question "Was your winter loss this year higher or lower than last year?"

Winter loss level	No. of backyard beekeepers	No. of sideline beekeepers	No. of commercial beekeepers	Average winter loss (%) [95 % CI]
Lower	1,604	64	34	19.6 [18.4–20.7]
Same	1,351	24	20	38.6 [36.6–40.7]
Higher	2,426	82	38	66.5 [65.3–67.7]
No bees	1,083	2	0	44.0 [41.4–46.5]
Do not know	142	5	5	29.5 [24.5–34.4]

The number of respondents in each operation type is provided, along with the overall average winter loss (%) [95 % CI] for each possible response

Table S2.1 US state estimates of total and average losses for summer, winter and annual (% [95% CI]). Included are the numbers of beekeeper operations reporting for each state (BK = beekeeper, N/A=not applicable). If a state had fewer than five respondents, the losses are not shown to protect the identity of the respondents. Estimates of the total number of colonies and total and average losses are calculated using the USDA-NASS method of counting colonies of multi-state beekeepers where colonies are counted multiple times, once for each state in which the beekeeper reported keeping bees during the monitoring period. Percent beekeepers and colonies operating exclusively within a state are included.

	Summer Loss				Winter Loss									Annual Loss		
	n (# of operations)	Total # of colonies (04/2013)	Total Loss mean [95%CI]	Average Loss mean [95%CI]	n (# of operations)	n Backyard BK	n Sideline BK	n Commercial BK	% BKs exclusively in state	Total # of colonies (10/2013)	% colonies exclusively in state	Total Loss mean [95%CI]	Average Loss mean [95%CI]	n (# of operations)	Total Loss mean [95%CI]	Average Loss mean [95%CI]
US	5,962	397,611	19.8 [19.3-20.3]	15.1 [14.5-15.7]	7,189	6,899	186	104	N/A	497,855	N/A	23.7 [23.3-24.1]	44.8 [43.9-45.7]	6,105	34.1 [33.7-34.6]	51.1 [50.2-52.0]
STATE:																
Alabama	35	599	54 [43.1-64.7]	13.6 [64.7-13.6]	38	37	1	0	94.7	345	74.5	11.2 [6.2-18]	20.8 [18-20.8]	35	43.1 [38.9-47.4]	31.3 [47.4-31.3]
Alaska	3	.	.	.	5	5	0	0	.	.	.	.	.	4	.	.
Arizona	6	204	21.4 [17.2-25.9]	23.6 [25.9-23.6]	6	5	1	0	83.3	362	98.3	34.5 [21.1-49.9]	22 [49.9-22]	6	48.4 [36-60.9]	24.4 [60.9-24.4]
Arkansas	61	6,713	17 [15.5-18.5]	19.9 [18.5-19.9]	74	69	3	2	94.6	8,676	7.8	16.1 [13.5-18.8]	32.7 [18.8-32.7]	64	29.3 [26.7-31.9]	38.6 [31.9-38.6]
California	269	294,045	17.8 [16.2-19.5]	20.4 [19.5-20.4]	292	187	28	77	68.2	391,481	8.9	20.7 [19.1-22.4]	32.4 [22.4-32.4]	275	31 [29.3-32.7]	42.4 [32.7-42.4]
Colorado	179	42,715	11.3 [10.9-11.7]	15 [11.7-15]	224	.	.	.	97.8	68,982	1.2	17.8 [17.3-18.3]	35.4 [18.3-35.4]	183	27.1 [26.5-27.6]	44.7 [27.6-44.7]
Connecticut	66	1,295	70.3 [59.2-80]	14.1 [80-14.1]	77	73	4	0	90.9	816	68.3	39.4 [33.6-45.5]	48.2 [45.5-48.2]	67	78.7 [71-85.2]	53.5 [85.2-53.5]
Delaware	22	12,097	33.2 [31.9-34.4]	20.6 [34.4-20.6]	28	.	.	.	78.6	10,160	0.7	25.2 [24.1-26.3]	37.6 [26.3-37.6]	23	42.9 [42.1-43.7]	46.5 [43.7-46.5]
District of Columbia	6	110	50 [41.3-58.7]	11.8 [58.7-11.8]	8	7	1	0	62.5	192	9.9	70.7 [54-84.3]	37.9 [84.3-37.9]	6	85.7 [68.7-95.7]	38.5 [95.7-38.5]
Florida	170	52,965	23.9 [21.4-26.4]	19.8 [26.4-19.8]	165	137	9	19	82.4	50,493	7.2	21.2 [19.2-23.4]	25.9 [23.4-25.9]	167	34.7 [32.2-37.3]	35.9 [37.3-35.9]
Georgia	102	14,949	27.3 [24.2-30.6]	19.1 [30.6-19.1]	107	95	6	6	87.9	12,352	12.4	25.9 [21.9-30.3]	31.8 [30.3-31.8]	101	35.7 [31.2-40.5]	42.5 [40.5-42.5]

	Summer Loss				Winter Loss									Annual Loss		
	n (# of operations)	Total # of colonies (04/2013)	Total Loss mean [95%CI]	Average Loss mean [95%CI]	n (# of operations)	n Backyard BK	n Sideline BK	n Commercial BK	% BKs exclusively in state	Total # of colonies (10/2013)	% colonies exclusively in state	Total Loss mean [95%CI]	Average Loss mean [95%CI]	n (# of operations)	Total Loss mean [95%CI]	Average Loss mean [95%CI]
Hawaii	67	11,804	14.5 [12.8-16.4]	14.7 [16.4-14.7]	75	.	.	.	98.7	13,511	48.2	13.2 [11.1-15.5]	15.1 [15.5-15.1]	68	24.9 [21.9-28.1]	26.1 [28.1-26.1]
Idaho	34	81,574	16.5 [12.9-20.6]	15.6 [20.6-15.6]	41	22	2	17	61.0	96,469	8.4	13.4 [11.6-15.2]	25.2 [15.2-25.2]	37	26.4 [22.6-30.4]	32.9 [30.4-32.9]
Illinois	127	973	8.4 [6.3-10.9]	13.6 [10.9-13.6]	159	155	3	1	96.2	2,438	47.5	60.5 [57.4-63.4]	59.6 [63.4-59.6]	135	62.5 [59.5-65.5]	66.1 [65.5-66.1]
Indiana	179	2,919	15.1 [13-17.5]	12.4 [17.5-12.4]	216	210	5	1	96.8	3,713	49.0	33.1 [28.6-37.9]	64.5 [37.9-64.5]	183	41.5 [36.9-46.2]	68.6 [46.2-68.6]
Iowa	55	818	39.5 [30.3-49.3]	10.8 [49.3-10.8]	61	57	4	0	96.7	1,257	83.2	50.2 [42.9-57.6]	51.9 [57.6-51.9]	55	70.5 [62.7-77.7]	59.2 [77.7-59.2]
Kansas	38	365	10.5 [6.7-15.5]	12.1 [15.5-12.1]	43	41	2	0	95.3	575	64.5	44 [35.3-53]	37 [53-37]	40	49 [40.2-57.8]	39.7 [57.8-39.7]
Kentucky	96	1,097	12.8 [9.4-16.8]	16.5 [16.8-16.5]	112	105	7	0	96.4	1,500	73.0	36.8 [32.4-41.4]	42.3 [41.4-42.3]	99	45.2 [40.5-49.9]	50.6 [49.9-50.6]
Louisiana	23	7,802	17.8 [17-18.7]	12.8 [18.7-12.8]	22	19	1	2	95.5	9,276	24.5	16 [13.7-18.5]	24.5 [18.5-24.5]	22	30 [27.6-32.4]	31.4 [32.4-31.4]
Maine	141	27,162	23.1 [20.3-26]	8.3 [26-8.3]	166	160	2	4	96.4	29,096	3.6	26.9 [25.5-28.4]	40 [28.4-40]	142	41.2 [38.8-43.6]	46.5 [43.6-46.5]
Maryland	158	13,252	31.9 [30.6-33.1]	18.1 [33.1-18.1]	192	187	3	2	93.2	11,871	13.4	26.7 [25.2-28.1]	42.2 [28.1-42.2]	162	43.8 [42.5-45.2]	51.7 [45.2-51.7]
Massachusetts	151	7,463	2.5 [1.4-4.1]	16 [4.1-16]	196	190	4	2	95.4	10,490	18.2	25.4 [23.1-27.8]	52.2 [27.8-52.2]	155	26.7 [24.2-29.3]	56.9 [29.3-56.9]
Michigan	339	22,706	19 [17.3-20.9]	13 [20.9-13]	460	439	12	9	97.0	24,523	19.7	30.6 [28.6-32.7]	69.5 [32.7-69.5]	357	39.5 [37.3-41.8]	72.2 [41.8-72.2]
Minnesota	95	69,640	14.4 [12.8-16.2]	17.4 [16.2-17.4]	138	123	6	9	91.3	95,626	2.7	22 [20.2-23.9]	57.1 [23.9-57.1]	99	30 [28-32.1]	63.2 [32.1-63.2]
Mississippi	26	70,611	25.2 [16.1-36.2]	17.9 [36.2-17.9]	23	15	2	6	65.2	85,643	0.3	21 [18.8-23.2]	26 [23.2-26]	24	37.9 [31.8-44.3]	37.9 [44.3-37.9]
Missouri	83	1,137	10.4 [8.1-13.1]	17.5 [13.1-17.5]	97	93	4	0	96.9	1,396	83.4	34.4 [29.7-39.4]	32.3 [39.4-32.3]	89	35.2 [30.1-40.6]	41.6 [40.6-41.6]
Montana	23	25,793	10.4 [9-11.9]	13.6 [11.9-13.6]	28	22	1	5	75.0	28,378	0.4	12.9 [10.1-16.2]	24.7 [16.2-24.7]	23	21.6 [18-25.5]	35.3 [25.5-35.3]
Nebraska	11	53,652	10.5 [8.4-12.9]	22 [12.9-22]	15	12	1	2	80.0	79,237	0.1	17.9 [16.2-19.6]	57.7 [19.6-57.7]	13	26.5 [23.9-29.2]	62.3 [29.2-62.3]

	Summer Loss				Winter Loss									Annual Loss		
	n (# of operations)	Total # of colonies (04/2013)	Total Loss mean [95%CI]	Average Loss mean [95%CI]	n (# of operations)	n Backyard BK	n Sideline BK	n Commercial BK	% BKs exclusively in state	Total # of colonies (10/2013)	% colonies exclusively in state	Total Loss mean [95%CI]	Average Loss mean [95%CI]	n (# of operations)	Total Loss mean [95%CI]	Average Loss mean [95%CI]
Nevada	12	424	4.7 [2.5-8]	4.2 [8-4.2]	12	10	2	0	75.0	625	11.7	32.2 [23.7-41.6]	38.8 [41.6-38.8]	12	35.1 [26.1-44.9]	41 [44.9-41]
New Hampshire	58	176	8.6 [5.1-13.3]	8.4 [13.3-8.4]	75	74	1	0	94.7	531	62.1	52.9 [46.2-59.5]	56 [59.5-56]	61	49.9 [42.1-57.7]	57.5 [57.7-57.5]
New Jersey	171	16,953	29.1 [27.9-30.4]	10.7 [30.4-10.7]	197	190	5	2	95.9	14,714	9.0	22.7 [21.1-24.4]	37.8 [24.4-37.8]	172	39.7 [38.3-41.2]	42.7 [41.2-42.7]
New Mexico	18	59	8.6 [3.8-16.1]	7.9 [16.1-7.9]	23	23	0	0	100.0	116	100.0	24.4 [14.2-37]	23.1 [37-23.1]	18	32.5 [20.1-46.8]	33.6 [46.8-33.6]
New York	165	22,029	27.6 [25.1-30.1]	12.3 [30.1-12.3]	212	193	14	5	94.3	20,608	18.3	30.4 [28.2-32.6]	48 [32.6-48]	170	43.1 [40.3-45.9]	53.8 [45.9-53.8]
North Carolina	260	45,056	12 [11.3-12.9]	19.4 [12.9-19.4]	292	285	5	2	94.5	71,168	2.9	18 [17.2-18.7]	34.5 [18.7-34.5]	260	27.7 [26.9-28.5]	44.5 [28.5-44.5]
North Dakota	26	128,819	26.1 [17.9-35.6]	23 [35.6-23]	31	2	3	26	3.2	150,882	0.0	24.7 [19.5-30.4]	21.3 [30.4-21.3]	28	38.1 [32.5-44]	34.1 [44-34.1]
Ohio	416	14,106	29.7 [28.6-30.8]	15.5 [30.8-15.5]	486	479	6	1	97.9	13,724	25.2	31.5 [29.8-33.1]	54.9 [33.1-54.9]	428	46.1 [44.9-47.4]	60.9 [47.4-60.9]
Oklahoma	26	4,718	25.3 [23.3-27.4]	11.4 [27.4-11.4]	34	32	0	2	91.2	6,551	3.7	39.8 [37.1-42.5]	28.9 [42.5-28.9]	29	39 [36.6-41.5]	35.3 [41.5-35.3]
Oregon	176	34,975	19.6 [17.4-21.9]	13.8 [21.9-13.8]	222	207	3	12	92.8	50,691	10.1	17 [15.3-18.8]	38.1 [18.8-38.1]	183	30.2 [27.8-32.6]	43.1 [32.6-43.1]
Pennsylvania	857	23,575	27.1 [25.9-28.2]	14.5 [28.2-14.5]	1080	1059	18	3	98.1	23,116	31.0	31.5 [30.4-32.6]	50 [32.6-50]	887	43.4 [42.1-44.7]	55.3 [44.7-55.3]
Puerto Rico	0	.	.	.	1	.	.	.	.	.	.	.	.	1	.	.
Rhode Island	22	119	6.1 [2.9-10.9]	8 [10.9-8]	24	23	1	0	70.8	205	38.0	32.9 [23.7-43]	34.3 [43-34.3]	22	37.4 [27.8-47.7]	43.1 [47.7-43.1]
South Carolina	159	4,350	19.2 [17.3-21.2]	20.5 [21.2-20.5]	155	149	5	1	96.1	2,313	57.6	28.4 [25.2-31.8]	29.5 [31.8-29.5]	156	20.1 [17.1-23.4]	39.2 [23.4-39.2]
South Dakota	15	58,410	13.6 [9.5-18.6]	18.7 [18.6-18.7]	16	11	1	4	56.3	76,028	0.0	21.1 [15.9-27]	46 [27-46]	15	29 [22.2-36.5]	51.9 [36.5-51.9]
Tennessee	94	1,020	36.8 [30.1-43.9]	18.9 [43.9-18.9]	102	97	5	0	95.1	1,066	81.6	20.4 [16-25.4]	32 [25.4-32]	96	41.9 [38-45.8]	43.6 [45.8-43.6]
Texas	80	117,086	23.4 [18.6-28.6]	17.9 [28.6-17.9]	79	59	7	13	79.7	119,507	2.1	20.1 [18.2-22.1]	23.5 [22.1-23.5]	81	34.9 [31-38.8]	33.7 [38.8-33.7]

	Summer Loss				Winter Loss									Annual Loss		
	n (# of operations)	Total # of colonies (04/2013)	Total Loss mean [95%CI]	Average Loss mean [95%CI]	n (# of operations)	n Backyard BK	n Sideline BK	n Commercial BK	% BKs exclusively in state	Total # of colonies (10/2013)	% colonies exclusively in state	Total Loss mean [95%CI]	Average Loss mean [95%CI]	n (# of operations)	Total Loss mean [95%CI]	Average Loss mean [95%CI]
Utah	55	4,409	15.2 [12.4-18.3]	17.2 [18.3-17.2]	61	52	5	4	83.6	9,250	2.6	16.3 [13.6-19.3]	38.7 [19.3-38.7]	57	28.7 [24.9-32.7]	47.3 [32.7-47.3]
Vermont	82	1,317	20.2 [18-22.4]	15.1 [22.4-15.1]	104	101	2	1	96.2	1,696	95.9	42.4 [39-45.8]	47.2 [45.8-47.2]	87	52.5 [49.1-55.9]	55.3 [55.9-55.3]
Virginia	662	16,043	28.2 [27.2-29.2]	15.5 [29.2-15.5]	796	781	14	1	98.2	15,726	35.1	27 [26-28]	36 [28-36]	671	42.3 [41.3-43.2]	43.8 [43.2-43.8]
Washington	123	44,946	25.2 [22.8-27.7]	15.1 [27.7-15.1]	161	141	9	11	90.1	74,378	1.7	25.9 [22.7-29.2]	37.9 [29.2-37.9]	125	35.6 [32.8-38.4]	41.8 [38.4-41.8]
West Virginia	65	867	6.4 [4.3-9.1]	10.4 [9.1-10.4]	75	70	5	0	92.0	1,125	67.9	41.8 [35-48.8]	42 [48.8-42]	70	45 [38.2-51.9]	43.9 [51.9-43.9]
Wisconsin	138	37,324	22.1 [20-24.3]	12.1 [24.3-12.1]	182	159	13	10	89.6	35,044	6.1	20.7 [18.4-23.2]	58 [23.2-58]	139	33.9 [31.4-36.4]	65.6 [36.4-65.6]
Wyoming	10	14,184	9.2 [4.2-16.7]	8.6 [16.7-8.6]	11	6	1	4	36.4	20,661	0.1	12.1 [9.6-14.8]	38.2 [14.8-38.2]	11	20.1 [14.8-26.2]	41.2 [26.2-41.2]

Table S2.2 Comparing the average losses among operation types for each time period using the Kruskal-Wallis rank sum test, followed by a Mann Whitney test. The number of operation types included in each analysis is provided. The associated "*" indicates significance where the Mann Whitney p-value is <0.05.

Period	Operation Type	n		Operation Type	n	Kruskal-Wallis χ^2	Mann Whitney p-value	
Summer Loss	Backyard	5,695	vs.	Sideline	164	61.6609	0.0011	*
	Backyard	5,695	vs.	Commercial	103		< 0.0001	*
	Sideline	164	vs.	Commercial	103		< 0.0001	*
Winter Loss	Backyard	6,899	vs.	Sideline	186	21.6678	0.0640	
	Backyard	6,899	vs.	Commercial	104		< 0.0001	*
	Sideline	186	vs.	Commercial	104		< 0.0001	*
Annual Loss	Backyard	5,815	vs.	Sideline	180	39.2306	0.0148	*
	Backyard	5,815	vs.	Commercial	110		< 0.0001	*
	Sideline	180	vs.	Commercial	110		0.0014	*

Table S2.3 Comparison of beekeeper self-reported winter causes of death of colonies and the associated average winter loss (%) [95% CI] for beekeepers that selected a factor and those that did not select that factor using the Kruskal-Wallis rank-sum and followed-up with a Mann Whitney test. P-values less than 0.05 are considered significant and indicated with a "*".

	Factor selected		Factor not selected				
Factor	**n**	**Average winter loss (%) [95%CI]**	**n**	**Average winter loss (%) [95%CI]**	**Kruskal-Wallis χ^2**	**Mann Whitney p-value**	
Queen failure	956	51.2 [49.3-53.2]	3,947	65.0 [64.0-65.9]	151.9933	< 0.0001	*
Starvation	1,774	62.3 [60.9-63.7]	3,129	62.3 [61.2-63.4]	0.0397	0.8420	
Varroa destructor	836	57.4 [55.3-59.4]	4,067	63.3 [62.3-64.2]	26.234	< 0.0001	*
Nosema spp.	261	59.3 [55.9-62.8]	4,642	62.4 [61.6-63.3]	2.6427	0.1040	
Small hive beetle	250	59.8 [56.0-63.6]	4,653	62.4 [61.5-63.3]	1.6903	0.1936	
Poor wintering conditions	2,237	70.5 [69.3-71.7]	2,666	55.4 [54.2-56.6]	286.5315	< 0.0001	*
Pesticides	325	63.6 [60.3-66.8]	4,578	62.2 [61.3-63.1]	0.3604	0.5483	
Weak in the fall	1,610	58.1 [56.6-59.6]	3,293	64.3 [63.3-65.4]	44.018	< 0.0001	*
CCD	324	65.9 [62.6-69.2]	4,579	62.0 [61.1-62.9]	4.2501	0.0393	*
Disaster	100	64.4 [58.8-70.1]	4,803	62.2 [61.4-63.1]	0.4277	0.5131	
Don't know	921	67.2 [65.3-69.2]	3,982	61.1 [60.2-62.1]	31.2649	< 0.0001	*
Other	455	59.3 [56.5-62.1]	4,448	62.6 [61.7-63.5]	5.0879	0.0241	*

Chapter 3. Utility of field measures in predicting honey bee colony health and viability in commercial beekeeping operations based in the Upper Midwest

3.1 Synopsis

Maintaining honey bee (*Apis mellifera* L) colony health in commercial beekeeping operations is critical to the health of U.S. agriculture. Factors affecting colony health are broad and multifaceted, and monitoring these factors, such as pesticide exposure, are prohibitively expensive for most beekeepers. Instead beekeepers use different colony metrics in order to assess colony health. Here, I evaluated the utility of easy standard colony health metrics to predict future colony health and survivorship. To do this, colonies owned by 23 commercial beekeepers that participated in Bee Informed Partnership's Upper Midwest surveillance services were assessed. I used generalized linear mixed models with a binomial distribution to examine inspection metrics that predicted colony health (loads of the microsporidian *Nosema* spp. and the parasitic mite *Varroa destructor* loads) and survivorship. The colony metrics that were most useful in predicting colony health or survivorship were an estimation of the adult bee population, loads and damage signs of *V. destructor*, *Nosema* spp. loads, and queen events. Overall, this study allows for beekeepers to implement management decisions based on the likelihood of a colony health outcome.

3.2 Introduction

High rates of honey bee (*Apis mellifera* L) colony mortality in commercial beekeeping operations are of concern as this category of beekeeper manages the majority of the 2.63 million colonies in the U.S. (USDA-NASS 2018) but in terms of number of

operations, are the smallest category of the U.S. apicultural industry (Daberkow et al. 2009). U.S. beekeeping operations are classified into three groups based on the number of colonies they manage: commercial beekeepers manage >500 colonies, sideliners manage 50-500 colonies, and backyard beekeepers <50 colonies. Commercial operations lose approximately 30% of their colonies each year, and, on average, have the lowest colony mortality compared to other beekeeper groups that manage fewer colonies, although the losses can vary widely among operations (Kulhanek et al. 2017; Lee et al. 2015b). High rates of colony mortality can threaten a commercial operation's finical viability since these beekeepers are dependent on the health and productivity of their colonies for income, and so, these beekeepers tend to implement intensive and expensive management practices (Caron 2009). To diversify their income, many commercial operations are migratory, meaning they transport colonies to different locations for honey production, raising queens and new colonies, and/or providing pollination services for different crops (Caron 2009). Currently, the average commercial beekeeping operation in the U.S. receives approximately 40% their revenue from pollination, and 82% of that income specifically from almond pollination (Ferrier et al. 2018). Colonies used for pollination are often graded for size, with more populous colonies receiving larger pollination fees (Sagilli and Burgett 2011), so providing populous colonies to almond growers in February is critical to many commercial operations' profitability.

Honey bees are having health problems and the causes are still not well understood. Following the large-scale losses of honey bee colonies reported in 2006-2007 that were the result of a specific set of conditions whose cause remains unresolved, there was a flurry of nationwide efforts to surveil colonies to better understand factors contributing to colony mortality and morbidity and to set colony loss baselines (reviewed

in Lee et al. 2015a). Research findings are revealing that there is no definitive cause of high annual colony death. Rather, a number of factors may contribute directly or through complex interactions to produce negative outcomes on honey bee health (Dainat et al. 2012a; Steinhauer et al. 2018).

Two of the most prevalent parasites of honey bees that can affect colony health are the parasitic mite *Varroa destructor* and the microsporidian *Nosema* spp. (Traynor et al. 2016a). *Varroa destructor*, or *Varroa* mites, are obligate ectoparasites that have been identified as a major cause of colony mortality (Genersch et al. 2010; Nazzi and Le Conte 2016; van Dooremalen et al. 2012). *Varroa* mites are ubiquitous across the U.S. (Traynor et al. 2016a) and reproduce on developing bees (Rosenkranz et al. 2010). Since these mites need developing bees to produce a new generation, *Varroa* mite levels grow as the bee population grows (Martin 1998). Canadian, European, and South American studies have clearly documented increased risk of colony mortality associated with mite levels. Measurable impacts of increased colony mortality occurred when mite densities surpassed 2 mites per 100 bees in spring, ≥3 mites per 100 bees in summer and fall, and ≥1 mite per 100 bees in winter (Currie and Gatien 2006; Genersch et al. 2010; Giacobino et al. 2016, 2017). Treatment efficacy can be variable, and beekeepers often need to apply several treatments to colonies over the course of a year. Windows of time that permit implementation of many *Varroa* mite management practices are limited in commercial operations as many treatments cannot be applied when colonies produce honey, or while colonies are being transported. An ability to predict when *Varroa* mite populations will surpass thresholds would be valuable for beekeepers.

The microsporidian *Nosema* spp. is a common honey bee parasite that has been associated with poor colony health, as it often becomes elevated when colonies are

exposed to other stressors like viruses, poor nutrition, or pesticides (reviewed in Martín-Hernández et al. 2018). There are two species of *Nosema* that infect honey bees: *N. apis* and *N. ceranae*. Currently, *N. apis* is relatively rare in the U.S., and *N. ceranae* is widespread and relatively recently found in the U.S. bee population (reviewed in Goblirsch 2017; Traver and Fell 2011; Traynor et al. 2016a). *Nosema* spp. infection leads to premature foraging, consequently disrupting the division of labor in the colony (Goblirsch et al. 2013). In contrast to *Varroa* levels, *Nosema* spp. levels in colonies tend to decrease over the summer (Traver and Fell 2011; Traynor et al. 2016a). The only registered treatment for *Nosema* spp. in the U.S. is the antibiotic fumagillin, but no treatment threshold has been established for *N. ceranae*.

Other metrics in addition to *Varroa* mite and *Nosema* spp. loads can indicate the health of a colony such as virus type and load, nutritional and immune biomarkers, or pesticide residues (Cornman et al. 2012; Ravoet et al. 2013; Runckel et a. 2011; Sanchez-Bayo and Goka 2014; Smart et al. 2016; Traynor et al. 2016a). However, in contrast to quantifying *Varroa* mite and *Nosema* spp. loads, these additional metrics can be expensive and require special handling and equipment for processing, as well as assistance with interpreting results. Therefore, the question posited for my study was whether simple and inexpensive colony assessments that are easily taken by commercial operations could be used to predict colony health outcomes far enough in the future so that beekeepers can implement management practices to reduce colony mortality.

To answer my research question, I used real-world data collected through the monitoring effort of the Bee Informed Partnership (BIP, beeinformed.org; vanEngelsdorp et al. 2012b) from 23 migratory commercial beekeeping operations that transport their colonies to the Upper Midwest for the summer months, and to California and/or the

southern U.S. for the winter months to pollinate almonds or produce new queens and bee colonies. Colony inspections were conducted at four different periods each year to record the following colony health metrics: adult bee population, queen status, and incidence of clinical diseases, parasites, pests, and atypical conditions. Adult bee samples were taken to quantify the colony loads of *Varroa* mites and *Nosema* spp. I used the colony health metrics to determine: 1) if visual signs of *Varroa* mites, observed by a beekeeper, could be used to estimate if a colony was above a *Varroa* mite threshold; 2) which metrics predicted *Varroa* mite load as quantified by taking a sample of adult bees at four time periods; 3) which metrics predicted *Nosema* spp. load at four time periods; and 4) which metrics predicted colony nonviability at two primary production and management windows. Nonviability is defined here as a colony being dead or having too small of an adult bee population to generate an income. The primary production and management windows were in fall before a beekeeper moved colonies to their wintering location, and in winter just before almond bloom and queen production. The results showed that: 1) any visual signs of *Varroa* mites indicated the colony was already above a mite threshold; 2) adult bee population and estimations of *Varroa* load in the previous sampling period, and treatment with a *Varroa* control product were good predictors of *Varroa* load; 3) *Nosema* spp. loads were predicted by frames of bees, *Nosema* spp. loads in the previous sampling period, and the time of year; and 4) the best predictors of colony nonviability were *Varroa* load, *Nosema* spp. load, the occurrence of a queen event, and the adult bee population size. These results can provide beekeepers with easy tools to predict colony health and viability and make more informed management decisions based on their individual risk tolerance, operation goals, and economics.

3.3 Methods

3.3.1 Data collection

3.3.1.1 Data collection in a bee-year

Tech-Transfer Teams (T^3) developed by BIP in 2011 conducted colony health inspections for commercial beekeeper operations. Specifically, this study used data from the T^3 at the University of Minnesota that collected information from colonies of commercial, migratory beekeeping operations based out of Minnesota and North Dakota during the summer for honey production, and that moved California for pollination and/or to the southern U.S. (hereinafter referred to the Deep South, i.e., Louisiana, Mississippi, and southeastern Texas) for honey production and to produce new queens and colonies. Colony inspections consisted of collecting metrics that were analyzed quickly to provide a rapid (<10 days) summary of results to each operation, which in turn allowed the beekeeper to make changes in management based on the results. Participating operations that requested T^3 services paid a fee-for-service; however, management recommendations were not provided.

Field data were collected from May 2012 to March 2017 by the BIP T^3, representing 5 "bee-years." A bee-year started and ended when new colonies were initiated in March through April, and often included replacing older queens and/or splitting and equalizing colonies. Sampling occurred at four distinct production and management windows over the bee-year:

1. Sampling period 1, SP-I (i.e., mid-May to late-June): Sampling occurred after old colonies were split and equalized and most queens were replaced, and generally after application of spring mite treatments but before the main honey flow.

2. Sampling period 2, SP-II (i.e., mid-July to late-August): Sampling occurred generally after the first round of honey production and before summer/fall mite treatments. Colonies were not sampled in early-July as this is a not a management window due to higher honey production.
3. Sampling period 3, SP-III (i.e., early-September to early-October): Sampling occurred after honey production and often during or after fall mite treatments. Colonies were not sampled from mid-October to early-January to reduce potential injury caused by opening colonies when ambient temperatures are low. Furthermore, colonies were inaccessible from mid-October through November, as they were being moved or held in wintering locations.
4. Sampling period 4, SP-IV (i.e., mid-January to early-March): Sampling occurred in holding yards, in almond orchards in California, or in production (queen or honey) apiaries in the Deep South. This was a major production window, especially for colonies leased for pollination of California almonds.

Colonies were not followed after March because most beekeepers replace the old queen with a new one, and/or add or remove significant amounts of adult or developing bees, essentially changing the identity of the colony.

3.3.1.2 Apiary metrics

Apiaries are groups of colonies placed at the same location. Colonies were placed on pallets, four to six colonies per pallet, to facilitate transportation by the beekeeper. Based on conversations with participating beekeepers, colonies within an apiary and apiaries within an operation were managed more alike than apiaries across different beekeeping operations. For this study, participating beekeepers chose the apiaries and

sometimes also the colonies within the apiary to be monitored. Beekeepers often chose the same apiary to be monitored for more than one bee-year. GPS coordinates and U.S. state were recorded for each apiary.

3.3.1.3 Colony inspection metrics

For each sampling period, inspected colonies from a given operation were sampled within a one-week period and were considered to be a part of a single sampling event. At the initial inspection, each sampled colony was given a unique number and operation code, and when possible, the same colonies within an operation were monitored over the entire bee-year. When monitoring the same set of colonies was not possible, a new set was chosen. To facilitate locating tagged colonies after they were moved, all colonies on a pallet were sampled when possible, which allowed for calculations of colony mortality from SP-III to SP-IV. Each unique number and code combination was retired following the death of the colony or at the start of a new bee-year. Each bee-year a new cohort of colonies was sampled from participating operations, with no colonies sampled across multiple bee-years.

Colony inspections were performed to record the following metrics (hereafter called inspection metrics): queen status; size of adult bee population; sealed brood pattern quality; incidence of clinical diseases, parasites, pests, and atypical conditions. Additionally, adult bee samples were collected to estimate colony loads of *Varroa* mites and *Nosema* spp. spores (see table 3.1).

3.3.1.4 Queen status

A colony was considered “queen-right” if the queen was seen or if eggs were observed in the brood nest. A colony was considered to have suffered a “queen event” if the colony had no queen present (i.e. laying worker bees indicated by multiple eggs per cell or the lack of eggs and larvae during a population growth period) or one of the following signs was observed: developing larval or pupal queens, a virgin queen, exclusively drone brood present, or the beekeeper noted the replacement of a queen in the period of time between sampling visits.

3.3.1.5 Size of adult bee population

Adult population size was estimated using a visual approximation of the number of frames completely covered by adult bees within all boxes containing the colony (Figure 3.1A) (Burgett and Burikam 1985; Delaplane et al. 2013; Nasr et al. 1990; Sagili and Burgett 2011; van Dooremalen et al. 2018). The number of frames covered by bees was determined by removing the hive cover and looking down between the frames. If the top view was not sufficient to make an accurate estimate, then the observer tipped up the box which provided a view from the bottom of the frames. When a colony was kept in more than one box, the population in each was estimated and summed. Not all boxes are the same depth, and so, when summing frame counts, a conversion factor was used to standardize the number of frames of bees: for hives boxes smaller than the standard 24.4cm size, a conversion factor of 0.67 for 16.8cm boxes and 0.5 for 13.7cm boxes was applied. Dead colonies were graded as having 0 frames of bees.

3.3.1.6 Quality of sealed brood pattern

Sealed brood pattern, or how contiguous the pattern of capped pupae is on a comb, can serve as a measure of the survival of immatures from egg to eclosion, and/or whether the queen is laying eggs in all cells. A poor pattern is discontinuous with a large number of empty cells surrounding a few sealed cells. This type of pattern can indicate the presence of a disease or a future queen event (vanEngelsdorp et al. 2013a). However, a poor brood pattern has not been linked to the quality of queen mating or queen pathogen measures (Lee et al. in press). Sealed brood patterns were rated by visually estimating the number of empty cells in an area of sealed brood on at least 3 frames in a colony. Patterns were scored on an ordinal scale of 1-5, with 1 being the poorest pattern with the most empty cells, and a score of 5 represented the most continuous pattern with the fewest empty cells, indicating consistent egg-laying by the queen and high brood survivorship (Lee et al. in press; modified from Guzmán-Novoa and Page 1999) (Figure 3.1B). Colonies with no sealed brood were given a value of 0, which often occurred during a queen event or if the queen stopped laying eggs during periods of cold or dearth (occasionally in October and January). Sealed brood patterns were not recorded in the first bee-year of 2012-2013.

3.3.1.7 Incidence of clinical diseases, parasites, pests, and atypical conditions

Visual signs of the following diseases, parasites, pests, and atypical conditions were recorded: the fungal pathogen chalkbrood (*Ascosphaera apis*); the bacterial brood diseases American foulbrood (*Paenibacillus larvae*) and European foulbrood (*Melissococcus plutonius*); Sacbrood virus; Deformed wing virus (DWV); idiopathic brood disease syndrome (vanEngelsdorp et al. in 2013a); parasitic mite syndrome (PMS,

described by Shimanuki et al. in 1994); *Varroa* mites on adult bees; chewed down brood (CDB) – hygienic response to a pupa infected by mites as indicated by the presence of white-colored mite frass in the cell; wax moth (*Galleria mellonella* and *Achroia grisella*) adults, larvae, pupae, or webbing; small hive beetle (*Aethina tumida*) adults or larvae; and entombed pollen – cells containing brick-red pollen underneath a wax or propolis cap (vanEngelsdorp et al. 2009b). Parasitic mite syndrome was distinguished from idiopathic brood disease syndrome by the presence of mites or mite frass in the cell. Signs of *Varroa* mite infestation – *Varroa* mites on adult bees, presence of DWV, PMS, or CDB (Figure 3.2) – were recorded as each being present/absent, and then the cumulative signs of *Varroa* mites score was assigned based on the number of signs present (0, 1, or >1).

3.3.1.8 Estimating *Varroa* mite and *Nosema* spp. loads

Approximately 300 adult bees were collected from a brood frame in each colony and preserved in either an alcohol or saturated sodium chloride solution with detergent before being processed for *Varroa* mite and *Nosema* spp. loads. The University of Maryland Bee Lab, Maryland USA, processed all samples. *Varroa* mites were dislodged from adult bees in alcohol by hand-agitation or a mechanized Burrell Scientific Wrist Action® Shaker for 35 min according to the protocol described in Dietemann et al. (2013) and Fries et al. (1991). A subsample of 100 bees was weighed and an estimate of the total number of bees in a sample was calculated by multiplying total bee mass by 100 and dividing by the mass of the subsample. Drone bees were excluded prior to weighing, counted separately, then added to the total bee count. *Varroa* mite load was calculated by dividing the number of *Varroa* mites dislodged in the alcohol wash by the total number

of bees based on sample weight and drone number, then multiplying by 100. *Varroa* mite levels are reported as mites per 100 bees.

Nosema spp. spore load was estimated using standard methods (Cantwell 1970). Briefly, bees from the subsample collected above (n = 100) were homogenized in 100 mL of water. A droplet of the homogenate was loaded into a hemocytometer and spores were visualized and counted under 400X magnification. Counts are reported as millions of *Nosema* spp. spores per bee (10^6 spores/bee). *Nosema* spp. spores cannot be confidently differentiated to species using this method; however, as *N. apis* is relatively rare in the U.S., if spores were present in a sample, they were assumed to likely be *N. ceranae* (Traver and Fell 2011; Traynor et al. 2016a).

3.3.1.9 Management practices

Information was recorded for each apiary or colony depending on if management practices were applied to the entire apiary or individual colonies within an apiary. Movement of colonies between sampling events was also recorded. Beekeepers frequently used different migratory routes for different sets of colonies within their operations. Except for two operations that wintered colonies in the Deep South, all monitored operations moved colonies to California by January or February for almond pollination. The various migratory routes for operations that sent colonies to California included: transporting colonies directly to California where they were kept in large apiaries called holding yards; a stop in the Deep South before transporting them to California; or overwintering colonies in temperature and CO_2 controlled wintering sheds prior to transporting them to California. Some beekeepers sent the majority of their colonies to California but kept a minority of their colonies in the Deep South for queen

production. Colonies were inspected in SP-IV either in California from mid-January to mid-February (n = 12 operations), or in the Deep South from mid-February to early-March (n = 9 operations). After almond pollination, some beekeepers moved their colonies to the Deep South, while others stayed in the western part of the U.S. to make new colonies and/or pollinate additional crops in California, Idaho, Oregon, and/or Washington. Colonies returned to Minnesota or North Dakota either from the Deep South (n = 12 operations) or the western U.S (n = 12 operations). A timeline of management practices conducted during a bee-year for three different operations is depicted in Figure 3.3.

Beekeeper applied *Varroa* and *Nosema* spp. treatments were recorded, including the product used, the application method, dosage, and approximate or exact treatment start date. All operations treated at least once per year to control *Varroa* mites. Products used included: Apivar ® (active ingredient amitraz), other amitraz-based products, formic acid-based products (Mite Away Quick Strips®, FormicPro™, or 65% formic acid), HopGuard® (active ingredient hops beta acids), oxalic acid (liquid or volatilized), Apiguard® (active ingredient thymol), ApiLifeVar® (active ingredients thymol, camphor, menthol and eucalyptol oil), or other thymol-based products.

3.3.2 Database management

Field and processed sample data were entered into the BIP's database managed at Appalachian State University, North Carolina USA. There, each change in the database was tracked and associated with the programmer, and the database was backed up nightly in multiple locations (personal communication, M. Wilson, University of Tennessee). A public interface is available at bip2.beeinformed.org.

Data were checked for different errors: biologically impossible or improbable outliers, conflicting entries, misclassified entries, duplicate entries, missing data, and incorrect calculations. Potential errors were flagged, and the original datasheets or lab records were examined. The error was either changed based on the original data or marked as invalid and removed from analysis.

Data were removed from all analyses if they were obtained from colonies considered to be a special case: colonies used for rearing queens and colonies headed by instrumentally inseminated queens. Colonies were occasionally sampled twice within one sampling period if the T^3 was testing the efficacy of a treatment product for the beekeeper; in these cases, the data from the last inspection period were used.

3.3.3 Statistical Analysis

3.3.3.1 Data description

The statistical program R was used for all analyses (R Core Team 2018). Seasonal trends in the inspection metrics of adult bee populations, brood patterns, and loads of *Varroa* mites and *Nosema* spp. of sampled colonies are shown using descriptive statistics. Kruskal-Wallis rank sum tests were used to examine seasonal differences of the inspection metrics, followed by a post-hoc Dunn test for comparisons among multiple levels with R package FSA (Ogle 2018). Odds ratios (ORs) were used to compare the odds of an event occurring in one group to the odds of it occurring in a different group. An OR >1 suggests a metric is positively associated with the response variable and an OR <1 suggests a negative association with the response variable. ORs are presented with their corresponding 95% confidence intervals.

3.3.3.2 Using visual signs of *Varroa* mites to estimate colony *Varroa* mite load

An OR was used to predict whether colonies with visual signs of *Varroa* mites, as observed in the field, were more likely to surpass the *Varroa* mite damage threshold of ≥3 mites per 100 bees (Genersch et al. 2010; Giacobino et al. 2016), as estimated in the laboratory, compared to colonies without signs of *Varroa* mites. A Kruskal-Wallis rank sum test with a post-hoc Dunn test in this case was used to examine if the colonies with different numbers of signs of mites observed in the field (0, 1, or >1) had different *Varroa* mite loads as determined by laboratory testing.

3.3.3.3 Metrics that predicted colony health and nonviability

Generalized linear mixed models (GLMM) with a binomial distribution were used to identify colony metrics that predicted *Nosema* spp. or *Varroa* mite loads for SP-I, SP-II, SPIII, and SP-IV. GLMMs with a binomial distribution were also used to identify inspection metrics that predicted colony nonviability in SP-III and SP-IV. The random effects variables of bee-year and apiary were nested within operation were included in each GLMM. The fixed effect variables were chosen for each model based on *a priori* knowledge of which inspection metrics might predict each response variable (Table 3.2 summarizes all variables used in the analyses). Assumptions of logistic regressions were checked by examining fixed effects variables for collinearity using a Spearman coefficient of ≥0.4 and removing one variable if collinearity was detected. Brood pattern was not used in any of the statistical models due to the high number of missing values. After variable selection, a full model was run followed by subsequent models with single variable deletions from the full model. Results for all models were generated using the glmer function in the lme4 package (Bates et al. 2015). The best fit model was selected

by choosing the most parsimonious model and an AIC value less than Δ2 using the MuMIn package (Bartoń 2018). Odds ratios and corresponding 95% confidence intervals were estimated using the confint function with the parameter set as beta using the lme4 package (Bates et al. 2015). The estimates, standard errors, and ORs with 95% confidence intervals are presented for the best fit model in summary tables. To allow for the interpretation of the sample sizes, each model output table includes the number of operations and colonies represented in each level of each fixed effect variable, and the percent of colonies in the response variable level that indicated poorer health (higher *Varroa* mite or *Nosema* spp. load) or colony nonviability. For categorical fixed effects variables, the level with an estimate of 0 is the reference to which the other levels are compared.

3.3.3.3.1 Metrics that predicted *Varroa* mite loads

For each of the four sampling periods, GLMMs with a binomial distribution were used to identify inspection metrics that predicted if a colony was above a seasonally appropriate *Varroa* threshold (mites per 100 bees, as estimated in the laboratory). The *Varroa* thresholds were based on previous publications: SP-I had a threshold of ≥2 mites per 100 bees (Currie and Gatien 2006; Giacobino et al. 2016), SP-II and SP-III had a threshold of ≥3 mites per 100 bees (Genersch et al. 2010; Giacobino et al. 2016), and SP-IV had a threshold of ≥1 mite per 100 bees (Giacobino et al. 2016). Valid datasets with no missing values were generated for each analysis. The following inspection metrics were included as fixed effects explanatory variables in the full model: queen status, number of frames of bees, *Varroa* mite and *Nosema* spp. load, use of a *Varroa* treatment

product, visual signs of *Varroa* mites (only in SP-I), and migratory route or wintering location. All inspection metrics were from the previous sampling period, except for the SP-I analysis where the metrics were recorded in SP-I. All GLMM steps were followed in the manner described above.

3.3.3.3.2 Metrics that predicted *Nosema* spp. loads

GLMMs with a binomial distribution were used to identify inspection metrics that predicted a colony was above a *Nosema* spp. threshold of 10^6 spores/bee (used in Traynor et al. 2016a). Due to the heavily left-skewed distribution of *Nosema* spp. loads and because there has not been a treatment threshold developed for *N. ceranae*, the binary threshold was used. An alternative could have been to use presence/absence of *Nosema* spp., but the 10^6 spores/bee threshold is more resistant to changes in the proportion of infected individuals within the colony as individual bee infection levels can differ by several magnitudes (Traver and Fell 2011). Analyses were performed for all four sampling periods. The following inspection metrics were included as fixed effects explanatory variables in the full model: queen status, frames of bees, *Varroa* mite and *Nosema* spp. load in the previous sampling period, use of the *Nosema* spp. treatment product fumagillin, the month of inspection, and migratory route or wintering location (summarized in Table 3.2). Frames of bees was used from the same sampling period as the response variable because the change in *Nosema* spp. abundance is expected decrease over the season. Fumagillin was the only treatment product used in the analysis as it is the only product registered to treat *Nosema* spp. in the U.S. All GLMM steps were followed in the manner described above.

3.3.3.3.3 Metrics that predicted colony nonviability

GLMMs with a binomial distribution were used to identify inspection metrics that predicted nonviable colonies (nonviable includes dead colonies) in SP-III and SP-IV, as these are critical production and management windows. In SP-III, the binary response variable cut-off for the GLMM analyses was assigned using the results of sensitivity analyses examining the likelihood a colony would be dead or nonviable in SP-IV based on the frame count in SP-III (methods following vanEngelsdorp et al. 2013). Of the colonies with ≤4 frames of bees in SP-III, 87.5% were nonviable by SP-IV (Table 3.3). As such, a colony was classified as viable if it had >4 frames of bees in SP-III and nonviable if it had ≤4 frames of bees in SP-III. For the binary response variable used in the SP-IV analysis, a colony was classified as viable if it had >4 frames of bees in SP-IV, and nonviable if it had ≤4 frames of bees in SP-IV. This cut-off was based on the minimum frame size deemed acceptable for orchard pollination of 4.5 frames of bees (Sagilli and Burgett 2011), as colonies with <4.5 frames can result in an economic loss when leasing colonies for almonds pollination.

Due to logistical complications of following and locating colonies from sampling SP-III to SP-IV when moved into orchards or other locations, the SP-IV dataset only included colonies from operations where colonies were inspected in SP-I, SP-II, and SP-IV. If colonies were not found in SP-IV, they were considered to be dead unless the beekeeper indicated that missing colonies could be in a different location. The following inspection metrics were included as fixed effects explanatory variables: queen status, frames of bees in the previous sampling period, *Varroa* mite and *Nosema* spp. load in the previous sampling period, use of the *Nosema* spp. treatment product fumagillin, treatment

with a *Varroa* product, and wintering location (summarized in Table 3.2). All GLMM steps were followed in the manner described above.

3.4 Results

3.4.1 Data description

3.4.1.1 Beekeeper operations

Twenty-three operations were sampled from May 2012 – March 2017. Participating operations managed ~ 900 to >20,000 colonies per operation. Twelve operations brought colonies to Minnesota or North Dakota from the western U.S. generally in April or May, and 12 brought colonies from Deep South in May or June. Colonies in a total of 470 different apiaries were sampled over the course of this study. Each operation had an average of 4.3 ± 2.2 (± SD, range: 1 – 32) apiaries inspected per visit with an average of 11.5 ± 10.3 (± SD, range: 1 – 103) colonies per apiary inspected, and 46.9 ± 26.5 (± SD, range: 7 – 187) colonies inspected per operation per sampling event. Over all bee-years, 2.3% and 7.4% of colonies originally sampled in SP-I were dead by SP-II and SP-III, respectively. Of the colonies followed from SP-I to SP-IV, 15.4% were dead by SP-IV.

3.4.1.2 Seasonal trends in colony inspection metrics

The number of frames of bees was significantly different among all four distinct sampling periods (Kruskal-Wallis chi-squared = 2507.2, df = 3, $p < 0.001$). Colonies were largest in SP-II (Dunn test $p < 0.05$). The average number of frames of bees was 9.9 ±

0.07 (± SE, n = 2,633), 13.5 ± 0.08 (± SE, n = 2,628), 10.1 ± 0.06 (± SE, n = 2,472), and 7.6 ± 0.07 (± SE, n = 1,845) for SP-I, SP-II, SP-III, and SP-IV, respectively (Figure 3.4).

Corresponding to frames of bees, brood patterns scores were significantly different among the four sampling periods (Kruskal-Wallis chi-squared = 274.08, df = 3, $p < 0.01$). Colonies in SP-II had the best brood patterns (Dunn test $p <0.01$). The average brood pattern was 3.77 ± 0.02 (± SE, n = 2,131), 3.82 ± 0.02 (± SE, n = 2,230), 3.47 ± 0.02 (± SE, n = 1,536), and 3.57 ± 0.02 (± SE, n = 1,034) for SP-I, SP-II, SP-III, and SP-IV, respectively (Figure 3.5).

3.4.1.3 Seasonal trends in *Varroa* mite and *Nosema* spp. loads

Varroa mite loads were significantly different between the following sampling periods: SP-I and SP-II, SP-I and SP-III, SP-II and SP-IV, and SP-III and SP-IV (Kruskal-Wallis chi-squared = 1215.6, df = 3, $p < 0.001$; Dunn test $p <0.001$) (Table 3.3). Mite levels were not different between SP-II and SP-III, with a combined average of 3.4 ± 0.08 (± SE, Dunn test $p = 0.99$), and between sampling periods SP-I and SP-IV, with a combined average of 0.8 ± 0.03 (± SE, Dunn test $p = 0.93$) (Figure 3.6). The average mite level across all sampling periods, for all bee-years was 2.2 ± 0.05 (± SE) mites per 100 bees with a median of 0.4 mites per 100 bees. The maximum level found in a colony was 66.4 mites per 100 bees in SP-II, and 42.3% of all samples had no mites detected (Figure 3.7).

Nosema spp. loads were significantly different among all four sampling periods (Kruskal-Wallis chi-squared = 1562.2, df = 3, $p <0.001$; Dunn test all $p <0.001$) (Table 3.3, Figure 3.8). *Nosema* spp. was the most prevalent in SP-I with 84.2% of sampled

colonies with a positive *Nosema* spp. detection, followed by 68.0%, 67.2%, and 43.3% for SP-II, SP-IV, and SP-III, respectively (Figure 3.9). The average spore load per colony was 0.606 ± 0.013 x 10^6 (± SE) spores per bee, with a median of 0.15 x 10^6 spores per bee. The maximum spore load estimated was 29.4 x 10^6 spores per bee in SP-IV, and spores were not detected in 34.8% of colonies inspected.

3.4.2 Metrics that predicted *Varroa* mite loads

3.4.2.1 Using visual signs of *Varroa* mites to estimate colony *Varroa* mite load

Observable signs of mites in the field – *Varroa* mites, DWV, PMS, and/or CDB – was a useful predictor of the overall *Varroa* infestation level in a colony determined from laboratory analysis of samples. If signs of mites were observed, it was likely the colony had a *Varroa* infestation of ≥3 mites per 100 bees. Fifty-one percent of all colonies with at least one sign observed (n = 1,631 observations) had *Varroa* levels ≥3 mites per 100 bees. *Varroa* levels were significantly different among colonies with 0, 1, and >1 observable signs of *Varroa* mites (Kruskal-Wallis chi-squared = 1590.7, df = 2, $p < 0.001$; Dunn test all $p < 0.001$) (Figures 3.10 and 3.11). Colonies with no observed signs had a mean of 1.3 ± 0.03 mites per 100 bees (± SE, n = 8,778); colonies with one sign (e.g., *Varroa,* DWV, PMS, or CDB) had a mean of 4.5 ± 0.19 mites per 100 bees (± SE, n = 1,075 observations); and colonies with >1 sign observed had a mean of 8.4 ± 0.38 mites per 100 bees (± SE, n = 547 observations). Colonies with at least one sign of *Varroa* mites had 7.4 (6.6 – 8.3, 95% CI; n = 10,400) higher odds of having ≥3 mites per 100 bees compared to colonies with no *Varroa* mites signs observed. For colonies without signs of mites (n = 8,778 observations), 12% had *Varroa* levels ≥3 mites per 100 bees.

3.4.2.2 Metrics that predicted *Varroa* mite loads in SP-I

Inspection metrics that predicted a colony would have ≥2 mites per 100 bees in SP-I were the observation of signs of *Varroa* mites and the type of *Varroa* treatment product used prior to SP-I or no treatment used (Table 3.5). Colonies with signs of mites in SP-I had 5.7 (3.6 – 9.1, 95% CI; $p < 0.001$) higher odds of having ≥2 mites per 100 bees when compared to colonies without any *Varroa* mite signs observed. Untreated colonies had 8.6 (3.4 – 24.3, 95% CI; $p < 0.001$) higher odds of having ≥2 mites per 100 bees compared to colonies treated with amitraz. Colonies treated with a product in the "other" category (HopGuard®, oxalic acid, Apiguard®, ApiLifeVar®, and other thymol-based products; Table 3.2) had 3.2 (0.99 – 10.42, 95% CI; $p < 0.05$) higher odds of having ≥2 mites per 100 bees compared to colonies treated with amitraz. Colonies treated with Apivar® or formic acid did not have significantly different odds of being above or below the 2 mites per 100 bees level as compared to colonies treated with amitraz ($p = 0.11$ and $p = 0.52$, respectively), suggesting that the amitraz-based and formic acid-based products were equally as effective in controlling mite populations in SP-I. Only 10.6% of all colonies had ≥2 mites per 100 bees in SP-I.

3.4.2.3 Metrics that predicted *Varroa* mite loads in SP-II

Inspection metrics that predicted a colony would have ≥3 mites per 100 bees in SP-II were the *Varroa* mite load and frames of bees in SP-I, and the type of *Varroa* treatment product used prior to SP-II or no treatment used (Table 3.5). Colonies with 1-2 mites per 100 bees and ≥2 mites per 100 bees in SP-I had 3.5 (2.1 – 5.9, 95% CI; p

<0.001) and 6.5 (3.6 – 12.2, 95% CI; p <0.001) higher odds of having ≥3 mites per 100 bees in SP-II compared to colonies with <1 mite per 100 bees. Larger colonies in SP-I were more likely to have ≥3 mites per 100 bees in SP-II, with the odds increasing by 19% (p <0.001) for each one-unit increase in the number of frames of bees. Compared to colonies treated with amitraz before SP-II, the odds a colony had ≥3 mites per 100 bees was 2.9 (1.2 – 6.9, 95% CI; p <0.05) higher for colonies treated with a product other than amitraz or formic acid (e.g., HopGuard®, oxalic acid, Apiguard®, ApiLifeVar®, and other thymol-based products; Table 3.2). Compared to colonies treated with amitraz before SP-II, colonies treated with Apivar® or formic acid were equally likely to have ≥3 mites per 100 bees (p = 0.12 and p = 0.98, respectively). Untreated colonies had 16.7 (5.9 – 50.6, 95% CI; p <0.001) higher odds of having ≥3 mites per 100 bees as compared to colonies treated with amitraz. The variable of a second treatment product (the product used on colonies treated more than once or no product) was positively correlated with the primary treatment product and not used in the models.

3.4.2.4 Metrics that predicted *Varroa* mite loads in SP-III

Inspection metrics that predicted a colony would have ≥3 mites per 100 bees in SP-III were the *Varroa* mite load and frames of bees in SP-II, and if a colony received an application of a *Varroa* treatment product prior to the SP-III inspection (Table 3.5). Compared to colonies with 0-1 mites per 100 bees in SP-II, colonies with 1-3 mites per 100 bees in had 7.2 (4.6 – 11.7, 95% CI; p <0.001) higher odds of having ≥3 mites per 100 bees in SP-III, and colonies with ≥3 mites per 100 bees in SP-II had 10.2 (6.0 – 17.8, 95% CI; p <0.001) higher odds of having ≥3 mites per 100 bees in SP-III. Larger colonies

in SP-II were more likely to have ≥3 mites per 100 bees in SP-III, with the odds increasing by 10% ($p < 0.001$) for each one-unit increase in the number of frames of bees. Colonies not treated with a *Varroa* product by the time of SP-III inspection had 37.9 (15.2 – 107.7, 95% CI; $p < 0.001$) higher odds of having ≥3 mites per 100 bees compared to treated colonies, and colonies that had a treatment ongoing during the SP-III inspection had 3.0 (1.5 – 6.3, 95% CI; $p < 0.01$) higher odds of having ≥3 mites per 100 bees. The only *Varroa* treatments used in August, September or October by operations represented in this dataset were amitraz, Apivar®, and formic acid-based products. The majority of all treatments were applied in September. The only product used by monitored beekeepers in October was amitraz-based.

3.4.2.5 Metrics that predicted *Varroa* mite loads in SP-IV

Inspection metrics that predicted a colony had ≥1 mite per 100 bees in SP-IV were *Varroa* mite load in SP-III and if the colony had a *Varroa* treatment product applied closer to the time of SP-IV (Table 3.5). Compared to colonies with 0-1 mites per 100 bees in SP-III, colonies with 1-3 mites per 100 bees or ≥3 mites per 100 bees had 2.8 (1.7 – 4.6, 95% CI; $p<0.001$) and 1.8 (1.1 – 3.2, 95% CI; $p < 0.05$) higher odds of having ≥1 mite per 100 bees in SP-IV, respectively. The discrepancy between colonies lower mite levels of 1-3 mites per 100 bees in SP-III having higher odds of being over the SP-IV *Varroa* threshold compared to colonies with ≥3 mites per 100 bees in SP-III was likely due to variations in the timing and type of treatments used or colonies with high mite levels already being dead. Indeed, the date a *Varroa* mite treatment product was applied had a significant effect on the odds a colony had ≥1 mite per 100 bees in SP-IV, with

0.29 (0.13 – 0.64, 95% CI; p <0.001) lower odds if a treatment was applied between December and February compared to August through November. The treatment applied in between December and February was an additional *Varroa* mite treatment after the primary fall *Varroa* mite treatment. All monitored beekeepers used a *Varroa* mite treatment between August and November. The *Varroa* treatment product and date the colony was treated were positively correlated due to operations using the "other" treatment product of oxalic acid between December and February, and, consequently, the factor of treatment product was removed from the analysis. The date a *Varroa* treatment product was applied was also positively correlated with wintering location due to the operations in California applying treatments between December and February, so wintering location was removed from the analysis.

3.4.3 Metrics that predicted *Nosema* spp. loads

3.4.3.1 Metrics that predicted *Nosema* spp. loads in SP-I

Inspection metrics that predicted a colony had $\geq 10^6$ spores/bee in SP-I were frames of bees in SP-I, migratory route, and if a colony was inspected in May instead of June (Table 3.6). Larger colonies were less likely to have $\geq 10^6$ spores/bee, with the odds decreasing by 5% (p <0.001) for each one-unit increase in the number of frames of bees. Compared to colonies from the western U.S., colonies in the Deep South prior to the SP-I inspection had 0.2 (0.1 – 0.4, 95% CI; p <0.001) lower odds of having $\geq 10^6$ spores/bee. Colonies inspected in June had 0.4 (0.2 – 0.6, 95% CI; p <0.001) lower odds of having $\geq 10^6$ spores/bee compared to colonies inspected in May. The interaction between migratory route and month inspected was not significant.

3.4.3.2 Metrics that predicted *Nosema* spp. loads in SP-II

Only the queen status inspection metric predicted if a colony would have $\geq 10^6$ spores/bee in SP-II. A colony that had a queen event during or prior to the SP-II inspection had 1.8 (1.1 – 2.9, 95% CI; $p < 0.05$) higher odds of having $\geq 10^6$ spores/bee compared to a colony that was queen-right (Table 3.6).

3.4.3.3 Metrics that predicted *Nosema* spp. loads in SP-III

Inspection metrics that predicted if a colony had $\geq 10^6$ spores/bee in SP-III were frames of bees in SP-III and if the colony had $\geq 10^6$ spores/bee in SP-II (Table 3.6). Larger colonies were less likely to have $\geq 10^6$ spores/bee, with the odds decreasing by 15% ($p < 0.01$) for each one-unit increase in the number of frames of bees. A colony with *Nosema* spp. $\geq 10^6$ spores/bee in SP-II had 2.6 (1.1 – 5.7, 95% CI; $p < 0.05$) higher odds of having $\geq 10^6$ spores/bee in SP-III.

3.4.3.4 Metrics that predicted *Nosema* spp. loads in SP-IV

The inspection metric that predicted a colony had $\geq 10^6$ spores/bee in SP-IV was $\geq 10^6$ spores/bee in SP-III, specifically 3.7 (1.5 – 8.9, 95% CI; $p < 0.001$) higher odds (Table 3.6). No other metrics were significant.

3.4.4. Metrics that predicted colony nonviability

3.4.4.1 Metrics that predicted colony nonviability in SP-III

Inspection metrics that predicted if a colony was nonviable in SP-III were frames of bees in SP-II, a queen event at any point from SP-I to SP-III, and *Varroa* mite load in SP-II (Table 3.7). Larger colonies in SP-II were less likely to be nonviable in SP-III, with the odds decreasing by 18% ($p < 0.001$) for each one-unit increase in the number of frames of bees. Colonies that underwent a queen event had 3.6 (2.2 – 5.8, 95% CI; $p < 0.001$) higher odds of being nonviable in SP-III compared colonies that were queen-right. Colonies with 1-3 mites per 100 bees in SP-II were not more likely to be nonviable in SP-III compared to colonies with <1 mite per 100 bees ($p = 0.49$). However, colonies with ≥3 mites per 100 bees in SP-II had 5.5 (3.1 – 9.9, 95% CI; $p < 0.001$) higher odds of being nonviable in SP-III compared to colonies with <1 mite per 100 bees. The number of treatments applied from SP-I to SP-III was not significant. Migratory route was not included in the analysis as it was positively correlated with the number of treatments used.

3.4.4.2 Metrics that predicted colony nonviability in SP-IV

Inspection metrics that predicted if a colony was nonviable in SP-IV were frames of bees in SP-III, a queen event at any point from SP-I to SP-IV, *Nosema* spp. and *Varroa* mite loads in SP-III, and if a colony was treated for *Varroa* mites by SP-III (Table 3.7). Larger colonies in SP-III were less likely to be nonviable in SP-IV, with the odds decreasing by 20% ($p < 0.001$) for each one-unit increase in the number of frames of bees. Colonies with a queen event had 2.5 (1.7 – 3.8, 95% CI; $p < 0.001$) higher odds of being nonviable by SP-IV compared to queen-right colonies. Colonies that had ≥10^6 *Nosema*

spp. spores per bee in SP-III had 2.9 (1.3 – 6.3, 95% CI; $p < 0.01$) higher odds of being nonviable in SP-IV compared to colonies with $<10^6$ spores per bee. Colonies with *Varroa* mite levels of 1-3 mites per 100 bees in SP-III had 1.6 (1.0 – 2.8, 95% CI, $p < 0.05$) higher odds of being nonviable in SP-IV compared to colonies with 0-1 mites per 100 bees. In addition, colonies with ≥3 mites per 100 bees in SP-III had 2.1 (1.3 – 3.4, 95% CI, $p < 0.001$) higher odds of being nonviable by SP-IV compared to colonies with *Varroa* mite levels of 0-1 mites per 100 bees. The average SP-III *Varroa* mite loads were 1.6 ± 0.14 mites per 100 bees (± SE, range: 0 – 58.6) for colonies that were viable in SP-IV, and 3.7 ± 0.45 mites per 100 bees (± SE, range 0 – 55.7) for colonies that were nonviable in SP-IV. These means were significantly different (Kruskal-Wallis chi-squared = 18.01, df = 2, $p < 0.001$). Colonies that had no *Varroa* mite treatment on by the time of the SP-III inspection had 3.1 (1.6 – 6.3, 95% CI, $p < 0.001$) higher odds of being nonviable in SP-IV compared to colonies that had been treated by SP-III. Colonies that had not been treated by the time of sampling had significantly higher *Varroa* mite levels compared to colonies that had been treated and had a treatment ongoing (Kruskal-Wallis chi-squared = 13.273, df = 2, $p < 0.001$; Dunn test $p < 0.001$, respectively), with means of 4.8 ± 0.04 (± SE) mites per 100 bees for the no treatment group, 1.5 ± 0.02 (± SE) mites per 100 bees for treatment ongoing, and 1.5 ± 0.02 (± SE) mites per 100 bees for treatment finished.

3.5 Discussion

This observational study was novel in that it used real-world data collected from a large number of commercial beekeeping operations to test whether simple and

inexpensive colony health assessments were predictive of colony health and viability over four sampling periods (SP-I to SP-IV) corresponding with four production and management windows. Based on statistical analyses, the inspection metrics that were useful in predicting binary colony health and viability outcomes were frames of bees, queen event occurrence, *Varroa* mite loads, and *Nosema* spp. loads. Colonies that had the highest odds of surviving from SP-I to SP-IV, irrespective of migratory route, had low levels of mites in SP-I and SP-II (<1 and <3 mites per 100 bees, respectively), were treated with an amitraz-based or formic-acid based treatment by SP-II, had less than one million spores per bee of *Nosema* spp. in SP-III, and were queen-right throughout the bee-year.

3.5.1 Metrics that predicted *Varroa* mite loads

Inspection metrics that predicted a colony would surpass a seasonal *Varroa* mite threshold were: *Varroa* mite level in the previous sampling period; a larger adult bee population in SP-I and SP-II; the type and timing of *Varroa* treatment products; and if any visual signs of *Varroa* mites were observed. Higher *Varroa* mite levels early in the bee-year led to higher mite levels later, which predicted that a colony would be nonviable in SP-III and SP-IV. These results suggest that colony survivorship is linked with early season *Varroa* mite loads, and controlling *Varroa* mites early in the year can increase beekeeper success.

There is an apparent trade-off between keeping larger colonies and controlling *Varroa* mites. While colonies with a larger adult bee population are at higher risk of having detrimental mite levels, they also tend to produce more honey (Graham 1992). Previous research has suggested keeping colony sizes smaller to produce fewer mites

(Seeley and Smith 2015), which may be a viable option if honey production is not a primary source of income. However, as honey production is a goal of most commercial operations, it is important to start out prior to the main honey production period with as low mite populations as possible, particularly if colonies have large bee populations at that time of year (>7 frames bees, following Figure 3.4). These findings suggest a potential management strategy of monitoring groups of colonies for *Varroa* levels within an operation that are more likely to have larger adult bee populations than others early in the bee-year. For example, colonies that were not divided into multiple smaller colonies, or colonies divided earlier in the bee-year may have higher *Varroa* levels, and may need to be treated first in fall.

The results show that amitraz- and formic acid-based treatments were effective at lowering mite levels, irrespective of migratory route. Treatments classified as “other” (HopGuard®, oxalic acid, Apiguard®, ApiLifeVar®, or other thymol-base products) tended to be less effective at keeping mite levels <3 mites per 100 bees in SP-I and SP-II. However, there was a large amount of variation in efficacy within the “other” group (Honey Bee Health Coalition 2018), which could be due to differences in application methods, the diversity of products, or the mite population prior to treatment as high mite levels are harder to effectively treat than low mite levels.

Monitoring colonies by sampling 300 adult bees and removing the mites using alcohol washes (Fries et al. 1991) or powdered sugar rolls (Macedo et al. 2002) to assess the mite load are currently the best ways to estimate the mite population (Lee et al. 2010). The absence of *Varroa* mites in a sample only means that no mites were detected, and it is likely that most, if not all, live colonies had a non-zero population of mites due to sampling error (Human et al. 2013). A more accurate estimation of colony *Varroa* mite

load would be to include the amount of brood in the colony (Lee et al. 2010); however, this is a time-intensive metric that is disruptive to the colony. An easier estimation of mite load would be the observable signs of mites in the field. However, this study indicates that if one or more *Varroa* mite signs are observed in a colony, then that colony likely already has ≥3 mites per 100 bees and a greater probability of becoming nonviable.

3.5.2 Metrics that predicted *Nosema* spp. loads

Inspection metrics that predicted higher *Nosema* spp. loads (≥10^6 spores/bee) were higher *Nosema* spp. loads in the previous inspection, smaller adult bee populations in SP-I and SP-III, the location of colonies prior to being move to the Upper Midwest, and a queen event by SP-II. A higher *Nosema* spp. load in SP-II was not predictive of colony nonviability in SP-III, but a higher *Nosema* spp. load in SP-III did predict a colony would be nonviable in SP-IV. However, only 39 of 970 colonies (4%) followed from SP-III to SP-IV had *Nosema* spp. loads ≥10^6 spores/bee in SP-III.

Large adult bee populations may dilute the prevalence of bees infected with *Nosema* spp., especially during bee growth seasons in the brood nest where the age of bees tends to be lower. In contrast, stressed colonies may have fewer bees and higher *Nosema* spp. loads. Colonies may be more likely to have a higher *Nosema* spp. load if the age cohort in a colony was disrupted earlier in the season (Khoury et al. 2011). Queen events often result in a break in colony brood rearing, which eventually results in a distribution of older adult bees in the colony than would have occurred if there was no brood interruption. Since older bees tend to have higher loads of *Nosema* spp. (Smart and Shepard 2011), this may explain the elevated loads seen. Overall, higher *Nosema* spp.

loads later in season (e.g., SP-III) may be symptomatic of other problems (reviewed in Martín-Hernández et al. 2018). A minority of colonies had *Nosema* spp. loads $\geq 10^6$ spores/bee in the SP-III dataset (90 of 1,472 or 6.1%), and 29% of those colonies with $\geq 10^6$ spores/bee in the belonged to a single beekeeper in bee-year 2015-16, where 64% (23 of 36 colonies) of that beekeeper's inspected colonies had $\geq 10^6$ spores/bee. The beekeeper noted that the set of colonies were not thriving over the summer and had low honey production, although this was not measured. However, the clustering of colonies with $\geq 10^6$ spores/bee and the suggestion by the beekeeper those colonies were unhealthy implies that those colonies may have already been exposed to a stressor that increased *Nosema* spp. loads prior to SP-III.

The description of *Nosema* spp. loads over bee-years and inspection periods suggest a seasonal pattern of high *Nosema* spp. loads earlier in the bee-year, that decrease through fall and increase again after the wintering period. This same seasonal pattern has been found in other studies (Gisder et al. 2010; Mortensen et al. 2018; Traver and Fell 2011; Traynor et al. 2016a) and was supported by the finding in this study that colonies sampled in June had lower odds having $\geq 10^6$ spores/bee compared to colonies sampled in May. *Nosema* spp. loads in this study followed a seasonal pattern irrespective of the application of fumagillin. Treatment with fumagillin did not predict if a colony would be below the *Nosema* spp. threshold at any of the inspection points, nor did it change the odds a colony would be viable in SP-III or SP-IV. In general, the data indicate that fumagillin treatments are not warranted since treatments did not change colony survival; however, any effect on honey production was not investigated. In addition, the timing of application was not consistent among operations, with the number of days the treatment

occurred prior to sampling ranging from 4 to 32 days, and the methods of applying fumagillin (in syrup, in a pollen patty, or as a drench) were not differentiated due to limited sample sizes. These factors could all affect the efficacy of the product. Despite these differences in application, our findings are supported by previous studies where *N. ceranae* was not reliably tied to colony mortality in field studies (Dainat et al. 2012a, 2012b; Desai and Currie 2016; Gisder et al. 2010; Smart et al. 2016; Williams et al. 2011; but see Botías et al. 2013).

An interesting trend from this study was that colonies that were located in the Deep South prior to movement to the Upper Midwest had lower *Nosema* spp. loads in SP-I compared to colonies that were located in the western U.S. This difference could be explained if colonies kept in the Deep South had better access to abundance and diversity of natural pollen since the quality of nutrition can impact the effects of *Nosema* spp. on honey bees (reviewed in Huang 2012). However, as *Nosema* spp. load decreased from SP-I to SP-III, even when colonies were untreated, higher levels in SP-I found in colonies from the western U.S. may not be of consequence as *Nosema* spp. loads in SP-I did not predict future colony health outcomes.

Overall, the results from this study suggests that it may not be worth the time it takes to sample for *Nosema* spp. Even if a colony has higher *Nosema* spp. loads in the SP-III, which was predictive of overwinter nonviability, the *Nosema* spp. loads may be indicative of another issue and an investment in a treatment with fumagillin may not change the odds of survival.

3.5.3 Metrics that predicted colony nonviability

Inspection metrics that predicted that a colony would be nonviable or dead in SP-III and SP-IV were fewer frames of bees in the previous sampling period, the occurrence of a queen event, and higher *Varroa* mite loads in the previous sampling period. Additional metrics that predicted a colony would be nonviable or dead in SP-IV were the *Varroa* treatment timing in SP-III and higher *Nosema* spp. loads in SP-III. Colonies that had not been treated by the time of SP-III had higher odd of being nonviable in SP-IV. In conjunction with the overall increased odds of nonviability when *Varroa* loads are high, these results suggest that if mite levels are high in SP-III, then a treatment applied earlier in SP-III could increase colony survival. The finding that colonies with queen events were significantly more likely to be weak or dead in sampling SP-III and SP-IV was similar to other studies (vanEngelsdorp et al. 2013a).

3.5.4 Comparing questionnaire surveys to colony inspections

Colony mortality was lower in this study compared to the surveys that quantify colony loss with a voluntary questionnaire (Kulhanek et al. 2017; Lee et al. 2015b; Seitz et al. 2016; Spleen et al. 2013; Steinhauer et al. 2014; vanEngelsdorp et al., 2007, 2008, 2010, 2011, 2012). Total summer loss (April 1 – October 1) for commercial beekeeper in the BIP surveys was approximately 20% averaged over all survey years, and approximately 7.4% in this study from SP-I (May-June) to SP-III (September-October). Including nonviable colonies (≤4 frames of bees) brought the estimate to 9.6% total summer loss in this study. Differences between the estimations of "summer" loss in the BIP surveys and this study may in part be explained by the non-random selection of queen-right colonies in this study in SP-I. Also, this study did not estimate colony

mortality that happened between the April 1 date used in the survey and the SP-I inspection.

Total winter loss (October 1 – April 1) for commercial beekeepers in the surveys was estimated at 21% as averaged over all survey years, and 8.8% in this study from SP-III (September-October) to SP-IV (January-February). Including nonviable colonies in the total winter loss calculation in this study brought the estimate to 25.9% nonviable, which is closer to the BIP estimation of total winter loss. Discrepancies in winter colony mortality may again be due to the different time periods over which losses were calculated, as this study does not account for colonies that died between SP-IV and the April 1 date used in the survey. Winter mortality may be overestimated in this study as colonies that were not found in SP-IV were assumed to be dead. Both the BIP surveys and this study had the potential for error to be introduced into the calculations due to bias in participants. BIP surveys had the potential for respondent bias as beekeepers volunteered to answer the survey, and the participants in the T^3 study may be a biased sample of beekeepers that tend to be proactive in their management and willing to participate in research.

3.5.5 Beekeeping strategies

Varroa mite loads and larger colonies were both predictive of higher *Varroa* mite loads in subsequent months and colony nonviability. Based on this result, beekeepers could use estimates of frames of bees and *Varroa* mite levels to decide which apiaries, or groups of colonies, to monitor more closely for detrimental *Varroa* mite loads, and perhaps reduce colony size or anticipate colony size earlier in the bee-year and treat with an effective *Varroa* control product before honey production. Observing signs of mites is

not useful as a preventive measure, but can be used to indicate a problem as even one sign of mites within a colony is a predictor that the colony already has ≥3 mites per 100 bees. The occurrence of a queen event is predictive that a colony will be nonviable later, which provides beekeepers an opportunity to manage those colonies (e.g., combine weak colonies), or decide to take losses in the fall.

Smaller colonies in SP-III had increased odds of being nonviable in SP-IV. However, there is a chance that these colonies could become viable by SP-IV based on the management. Some operations bolster smaller colonies by replacing queens in an overwintering location with the expectation the colonies would raise a new generation of bees. However, if there is no opportunity for the colony to raise new bees in winter, then it may be more cost-effective to avoid spending resources on the colony if it is small, has high *Varroa* mite or *Nosema* spp. levels in SP-III, and especially if it had a queen event. For example, operations that use wintering sheds often take their losses in the fall by combining weak colonies together or culling colonies, because a poor colony put into a shed is will remain a poor colony. In addition, sick colonies may transmit pathogens to neighboring colonies and may not be worth the risk to try to save. If an operation uses a two migration routes, they may send their best colonies to sheds and rebuild the smaller colonies in a warmer climate. The cost of transportation and management needs to be factored into whether a colony is worth saving. With the exception of very small colonies with a current or past queen event that may not survive until another inspection, many operations try to save every colony possible and take their losses in January or February because the current price per colony for almonds pollination contracts is at an all-time high (Ferrier et al. 2018).

3.5.6 Additional measures

The measures taken by the T^3, while informative, did not cover all potential indicators of colony health nor explain all of the colony death. This variability may be due to exposure to other risk factors that T^3 colony assessments do not routinely quantify. Monitoring pesticide exposure and virus levels are expensive, and so are probably only justified in operations that hive high rates of mortality without evidence of excessive *Varroa* mite populations. Even when colonies are exposed to high pressure from elevated *Varroa* mite loads, the impacts of this exposure are not consistent. For instance, one apiary had a mean of 21.5 ± 16.0 mites per 100 bees in September, with 15 of 19 colonies sampled having *Varroa* levels in excess of 10 mites per 100 bees. Of those 19 colonies, 8 were nonviable in January, with one death likely due to a queen event. This variation may be explained by viruses. *Varroa* mites spread detrimental viruses within and among colonies. In particular, DWV can have detrimental effects on colonies with the virulence differing among the three known strains of DWV (Martin et al. 2012; Mordecai et al. 2015, 2016). In addition, poor nutrition can lead to increased bee susceptibly to both viruses and pesticides (reviewed in Huang 2012). Indicators of the quality of nutrition in a colony, such as the quantification of lipids and the storage protein vitellogenin, have been identified as possible indicators of overwintering mortality (Dolezal et al. 2016; Smart et al. 2016), and may be associated with *Varroa* levels (Ramsey et al. in press). Operations experiencing excessive numbers of queen events may consider investing in additional metrics as exposure to high pesticide levels have been linked to queen events (Traynor et al. 2016b) and pathogens can have detrimental effects on queens (Alaux et al. 2011; reviewed in Amiri et al. 2017; Chaimanee et al. 2014; Gauthier et al. 2011).

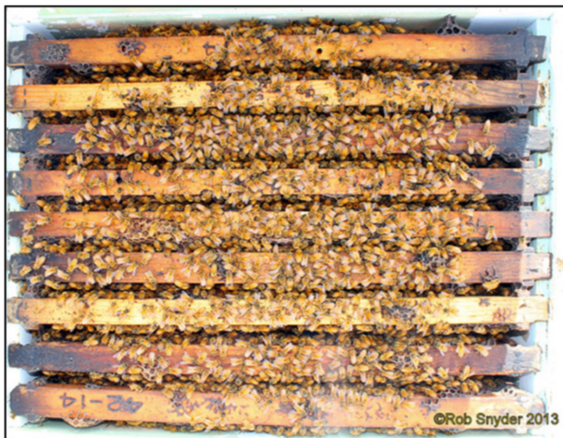
3.6 Conclusions

Simple metrics can be useful to inform management decisions of operations that spend summer months in the Upper Midwest. Many beekeepers already use these metrics to assess their colonies, but the value of the T^3 metrics is that they are standardized and used across operations, which allow for identifying and quantifying their utility in predicating health outcomes in a broad range of real-world colonies. These results allow for a more informed understanding of the probability of colony health and survival. Beekeepers can use these data to make decisions on management strategies to lower the number of nonviable colonies and decrease the amount of time and effort put into colonies that are likely to fail.

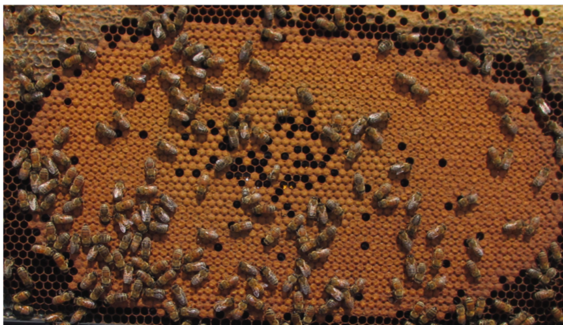
3.7 Figures

Figure 3.1 Images of frames of bees and brood patterns: A) different frames of bees counts with the left image a top view perspective of 8 frames of bees and the right image a bottom view perspective with 1.5 frames of bees (both out of 9 possible frames of bees) (photos by Rob Snyder); and B) the brood pattern assessment performed by ranking brood patterns by the amount of uninterrupted sealed pupal cells on a scale of 1-5, with 5 being the most solid, with the left image a brood pattern with a score of 5 and the right image of a brood pattern with a score of 2.

A.



B.



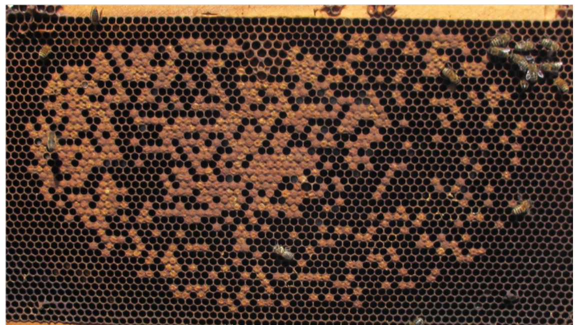


Figure 3.2 Signs of mites observed in a colony: A) an adult mite on a worker bee's thorax; B) chewed down pupae, or pupae being removed by hygienic bees responding to mite infestation; C) white frass left by a mite on the ceiling of a pupal cell; D) an adult bee with signs of deformed wing virus; and E) parasitic mite syndrome or dead and dying pupae with mites or mite fecal patches in cells. Photos A. and E. by Rob Snyder, the Bee Informed Partnership.

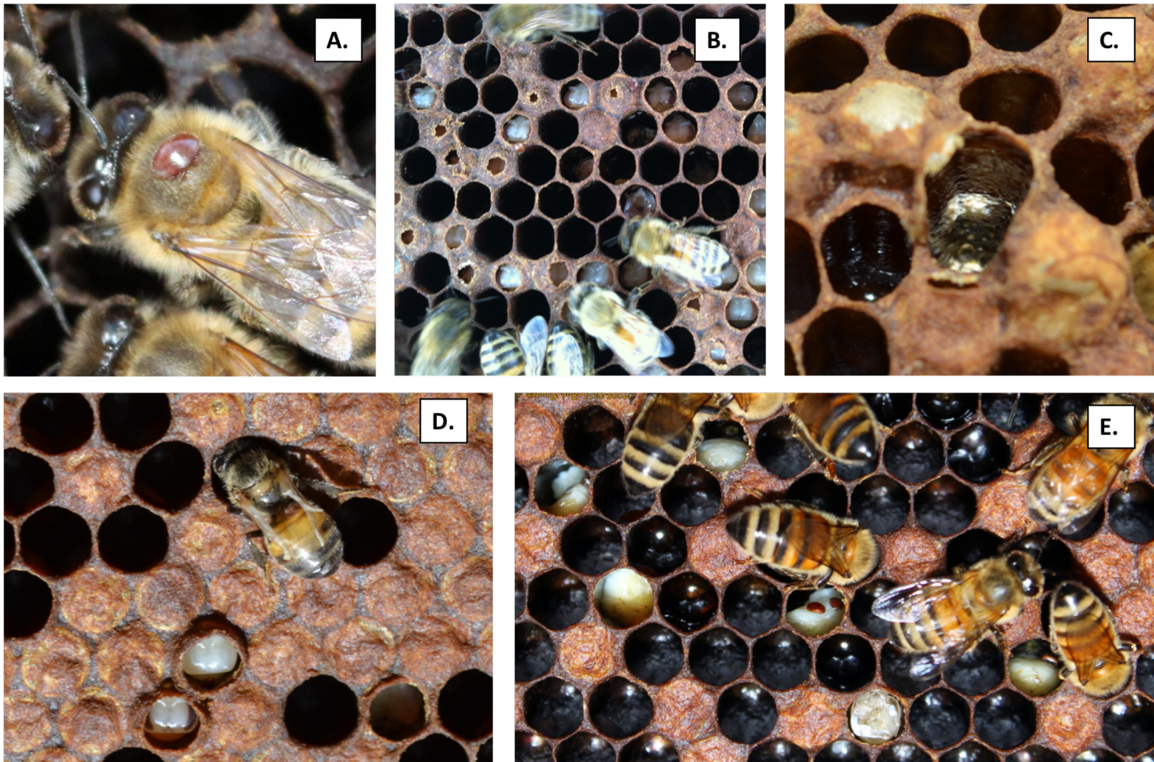


Figure 3.3 An example of a bee-year for three commercial beekeeping operations with different migratory paths, management, and *Varroa* mite and *Nosema* spp. treatment times.

<table>
<tr><td>Bee-year</td><td></td><td colspan="2">April</td><td colspan="2">May</td><td colspan="2">June</td><td colspan="2">July</td><td colspan="2">August</td><td colspan="2">September</td><td colspan="2">October</td><td colspan="2">November</td><td colspan="2">December</td><td colspan="2">January</td><td colspan="2">February</td><td colspan="2">March</td></tr>
<tr><td>Sample times</td><td></td><td></td><td></td><td></td><td colspan="3">SP-I</td><td></td><td colspan="3">SP-II</td><td colspan="3">SP-III</td><td></td><td></td><td></td><td></td><td></td><td></td><td colspan="4">SP-IV</td><td></td></tr>
<tr><td rowspan="4">Operation 1</td><td>Location</td><td colspan="3">California</td><td colspan="11">North Dakota</td><td colspan="10">California</td></tr>
<tr><td>Management</td><td colspan="3">Requeen, make new colonies</td><td></td><td></td><td colspan="6">Honey production</td><td colspan="3">Winter preperation: feed, equalize</td><td></td><td></td><td></td><td></td><td></td><td colspan="3">Almond pollination</td><td colspan="2">Make new colonies</td></tr>
<tr><td>Nosema spp. treatment</td><td></td><td></td><td></td><td>X</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td>X</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>Varroa treatment</td><td></td><td></td><td></td><td>X</td><td></td><td></td><td></td><td></td><td></td><td></td><td>X</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td>X</td><td></td><td></td><td></td><td></td></tr>
<tr><td rowspan="4">Operation 2</td><td>Location</td><td colspan="3">California</td><td colspan="11">North Dakota</td><td colspan="5">Wintering shed</td><td colspan="5">California</td></tr>
<tr><td>Management</td><td colspan="3">Requeen, make new colonies</td><td></td><td></td><td colspan="6">Honey production</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td colspan="3">Almond pollination</td><td colspan="2">Pollination contracts</td></tr>
<tr><td>Nosema spp. treatment</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td>X</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>Varroa treatment</td><td></td><td></td><td></td><td></td><td>X</td><td></td><td></td><td></td><td></td><td></td><td>X</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td>X</td><td></td><td></td><td></td><td></td></tr>
<tr><td rowspan="4">Operation 3</td><td>Location</td><td colspan="5">Texas</td><td colspan="9">Minnesota</td><td colspan="5">Texas</td><td colspan="3">California</td><td colspan="2">Texas</td></tr>
<tr><td>Management</td><td colspan="5">Requeen, make new colonies, honey production</td><td colspan="6">Honey production</td><td colspan="3">Winter preperation: feed, equalize</td><td></td><td></td><td></td><td></td><td></td><td colspan="3">Almond pollination</td><td colspan="2">Queen production</td></tr>
<tr><td>Nosema spp. treatment</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>Varroa treatment</td><td></td><td>X</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td>X</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
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Figure 3.4 Mean frames of bees counts (± SE) by bee-year and sampling periods.

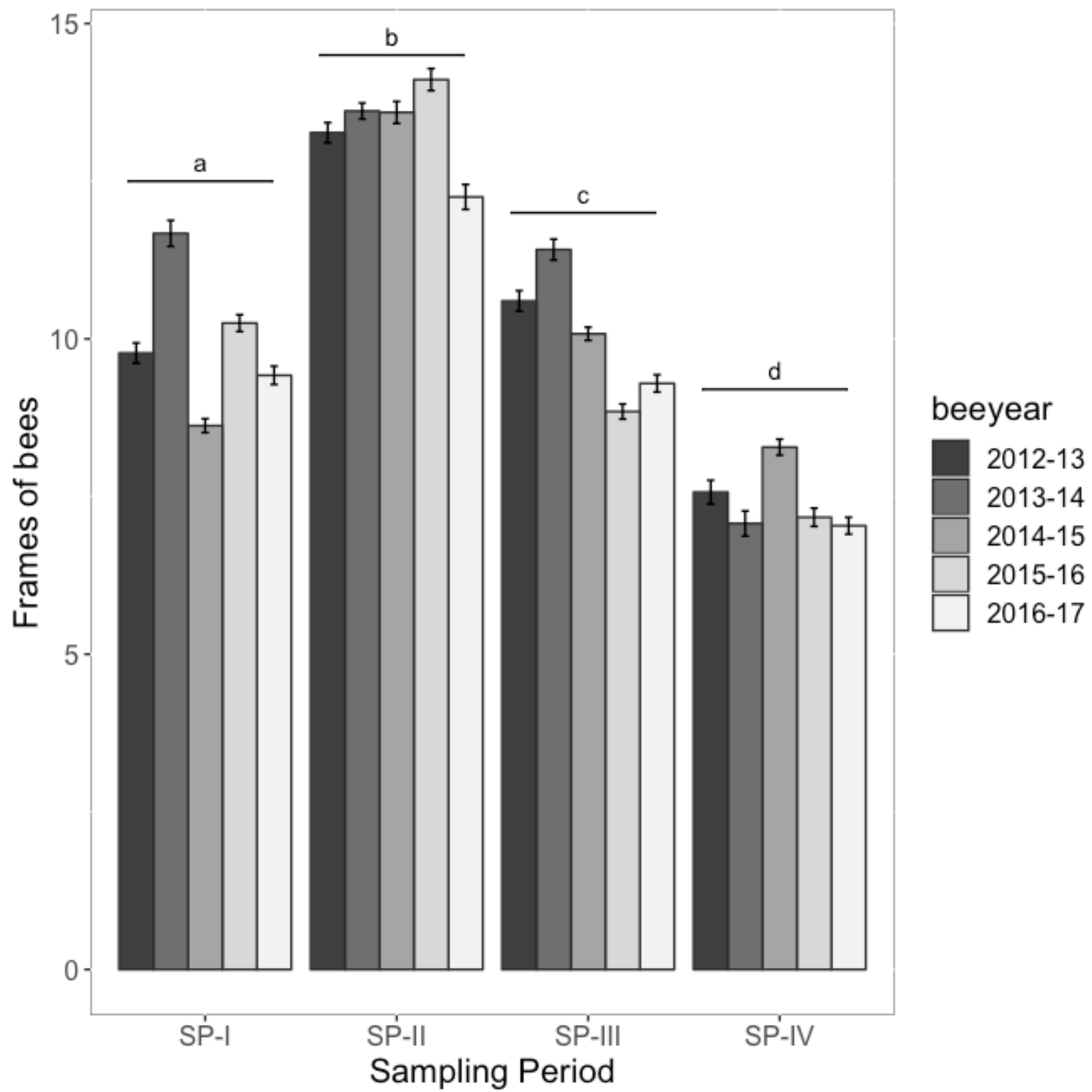


Figure 3.5 Mean brood pattern scores (± SE) by bee-year and sampling periods.

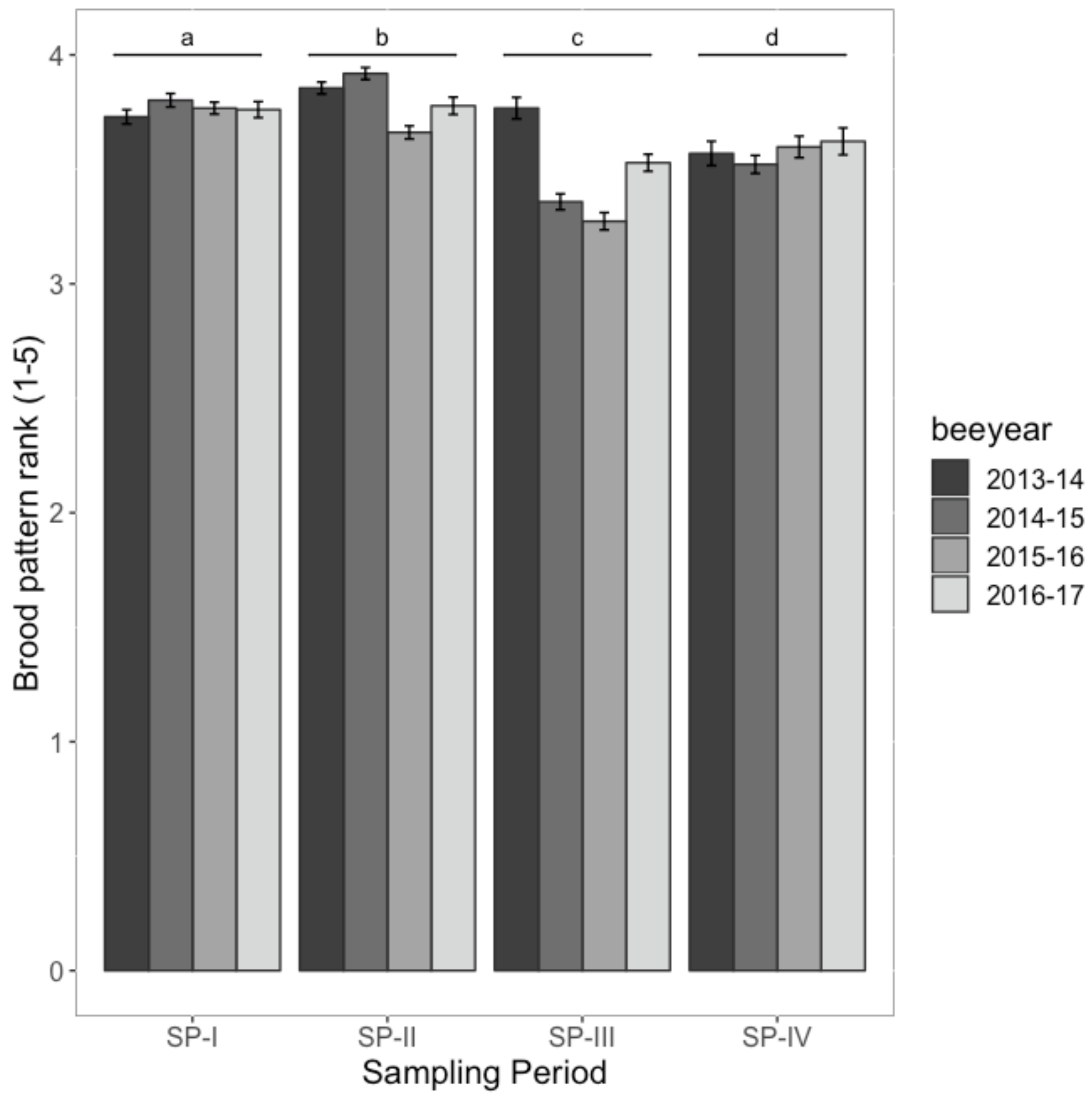


Figure 3.6 Mean *Varroa* mite loads (± SE) by bee-year and sampling periods.

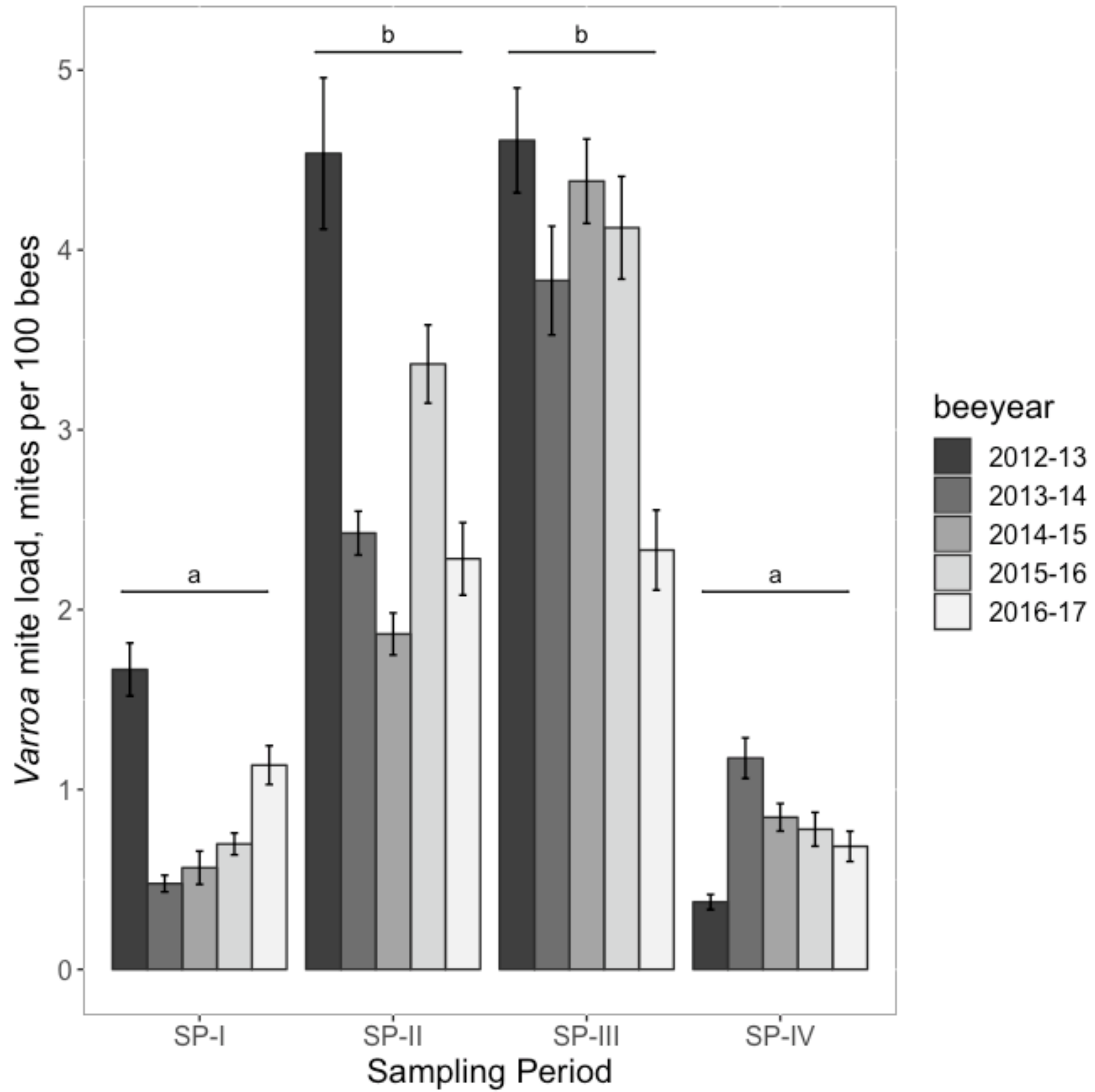


Figure 3.7 The proportion of samples with different levels of *Varroa* mites (mites per 100 bees) by sampling period.

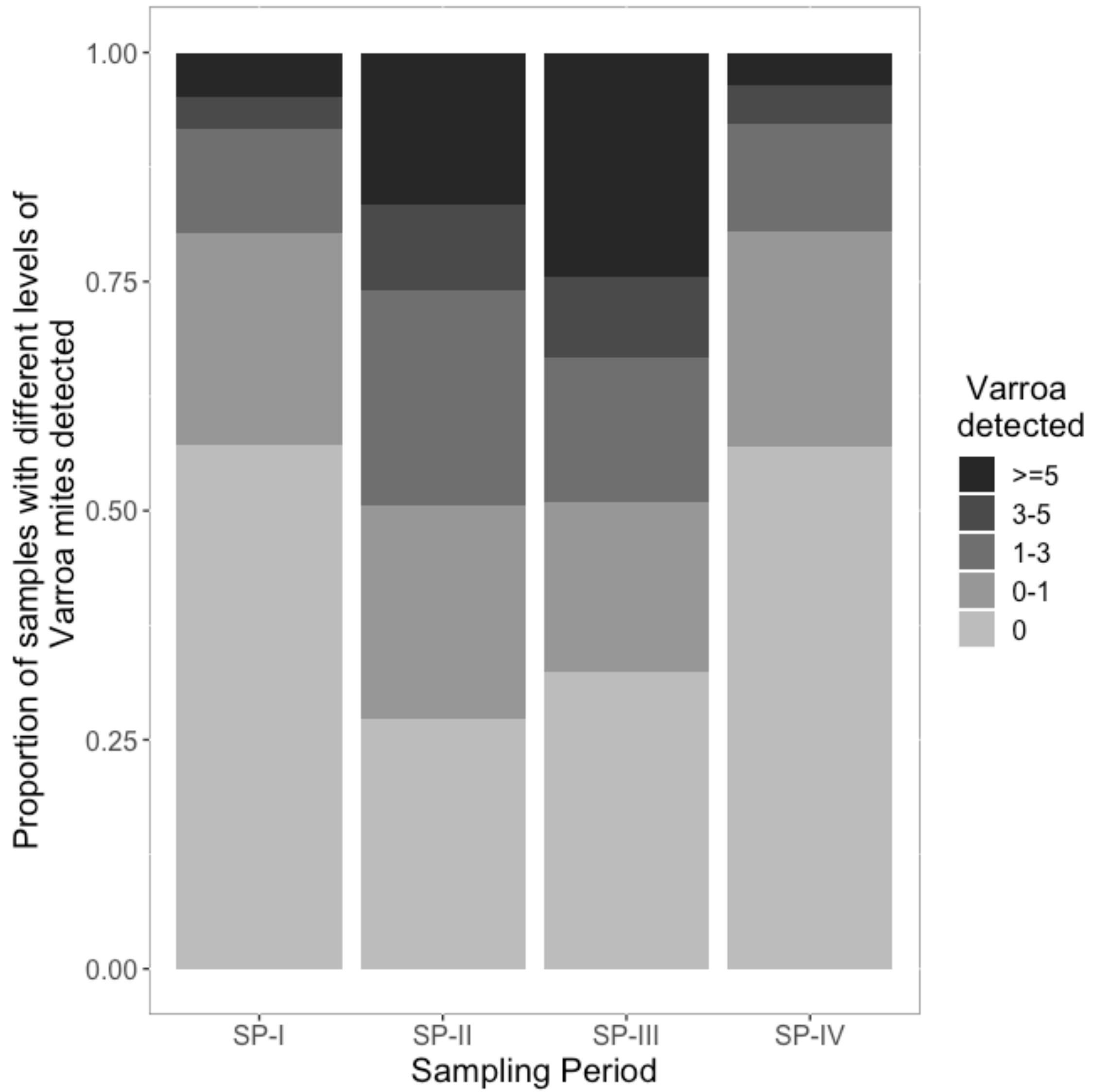


Figure 3.8 *Nosema* spp. spore load averages (± SE) by bee-year and sampling periods.

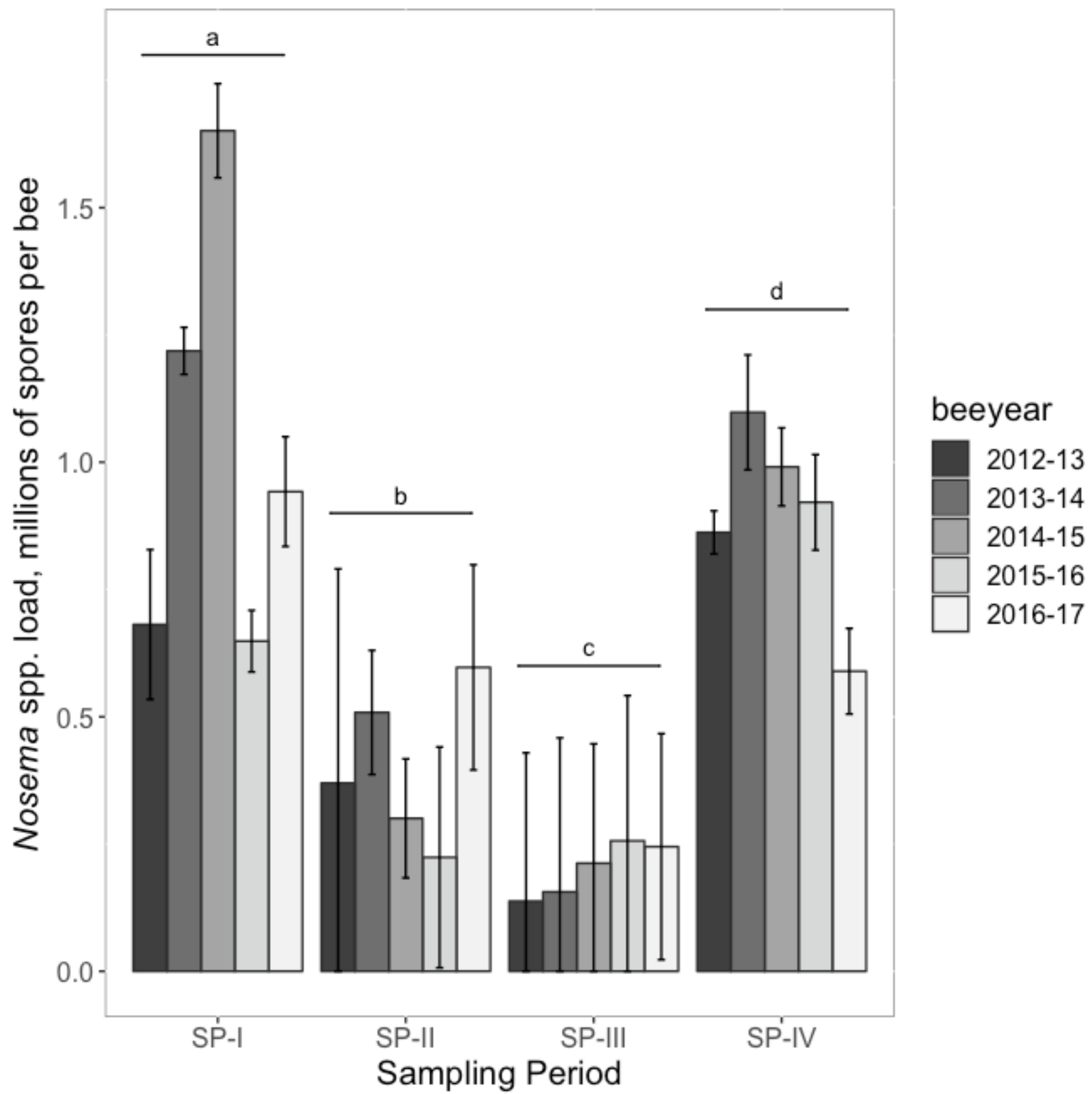


Figure 3.9 The proportion of samples with no *Nosema* spp. spores detected, 0-1 x 10^6 spores, or >1 x 10^6 spores per bee detected by sampling period.

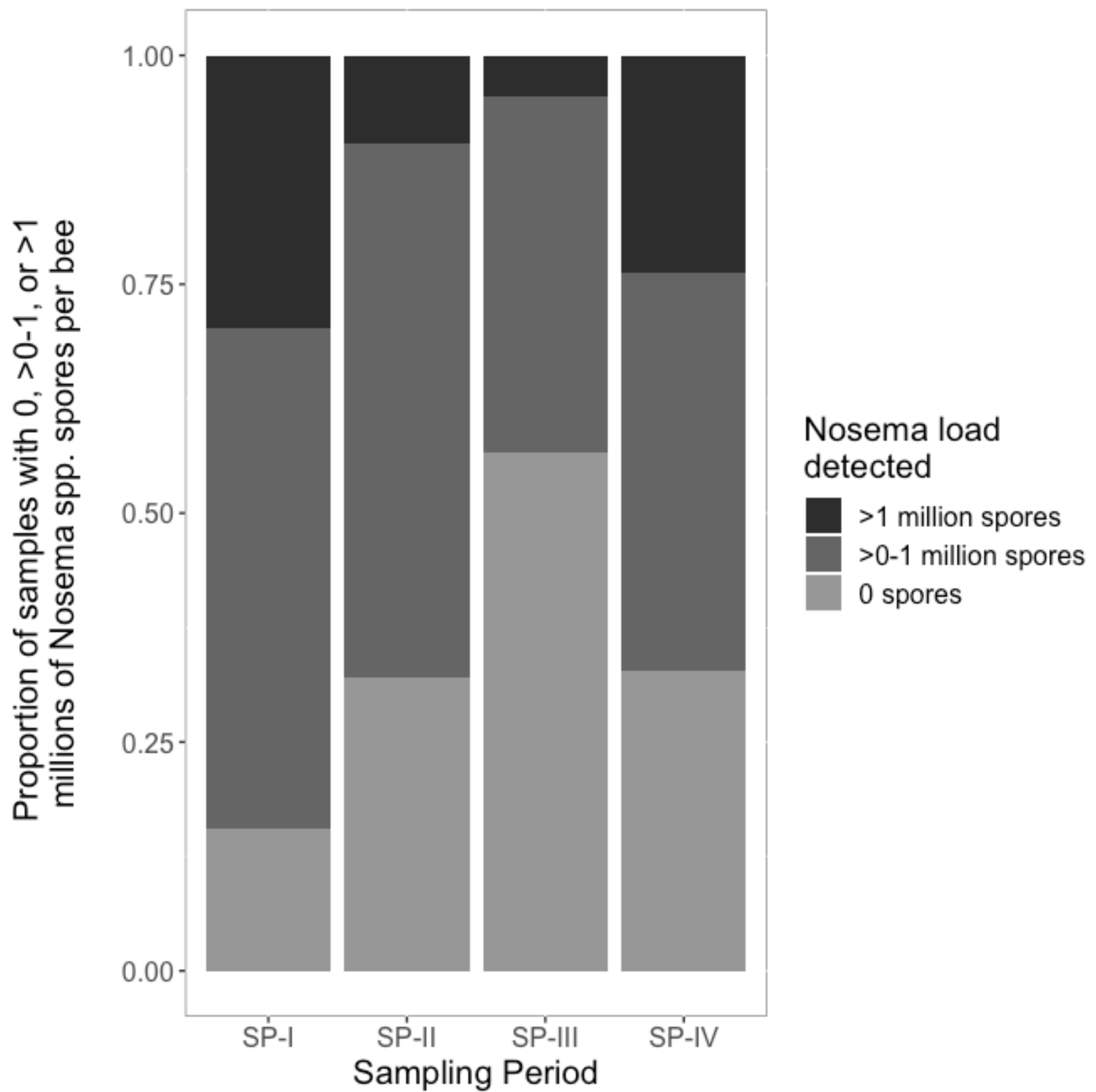


Figure 3.10 Mean *Varroa* mite load (± SE) as determined in the laboratory by the number of signs of mites visually observed in a colony. Means with different letters are significantly different (Kruskal-Wallis chi-squared = 1590.7, df = 2, p < 0.001; Dunn post hoc test; p <0.001).

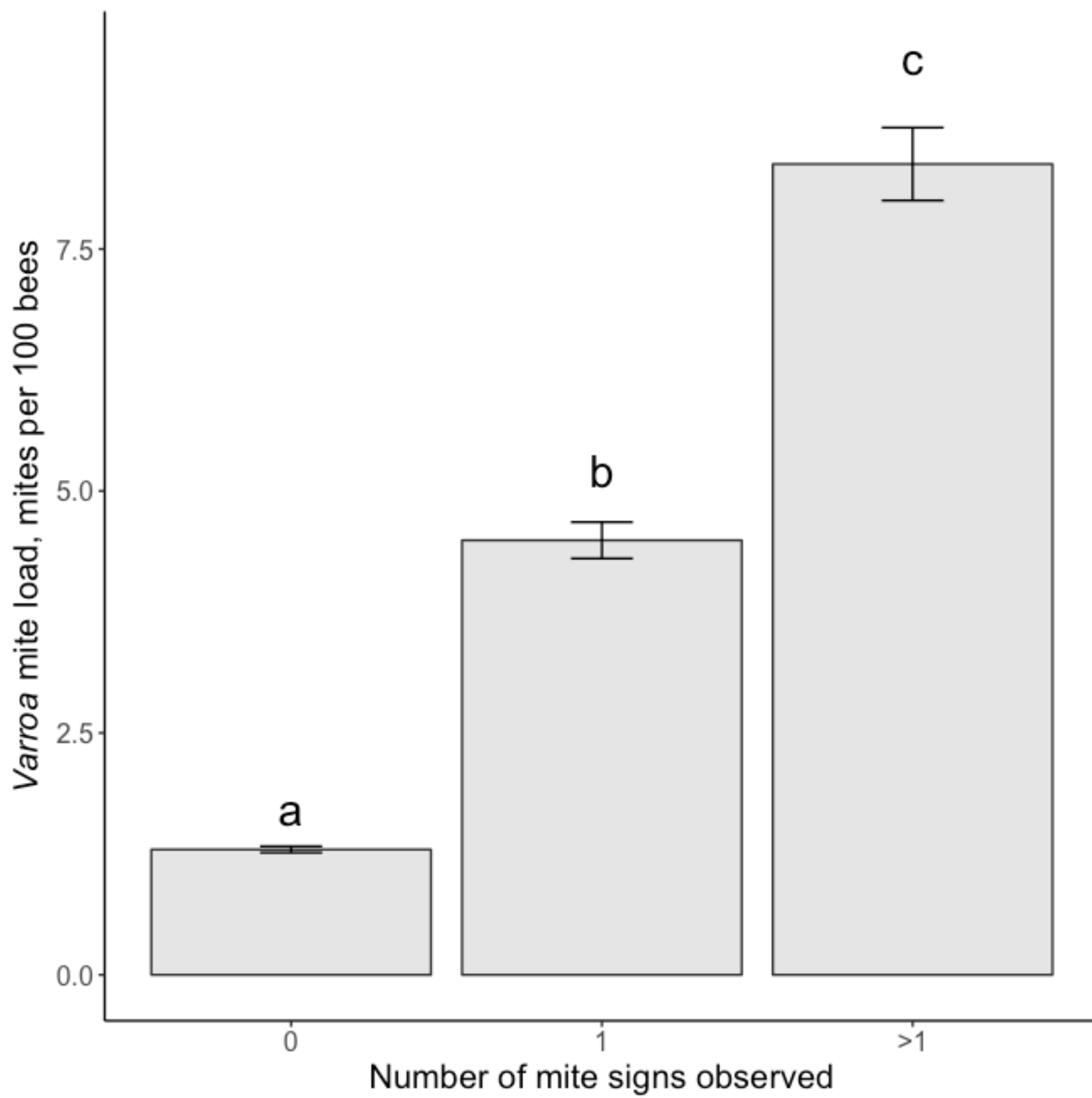
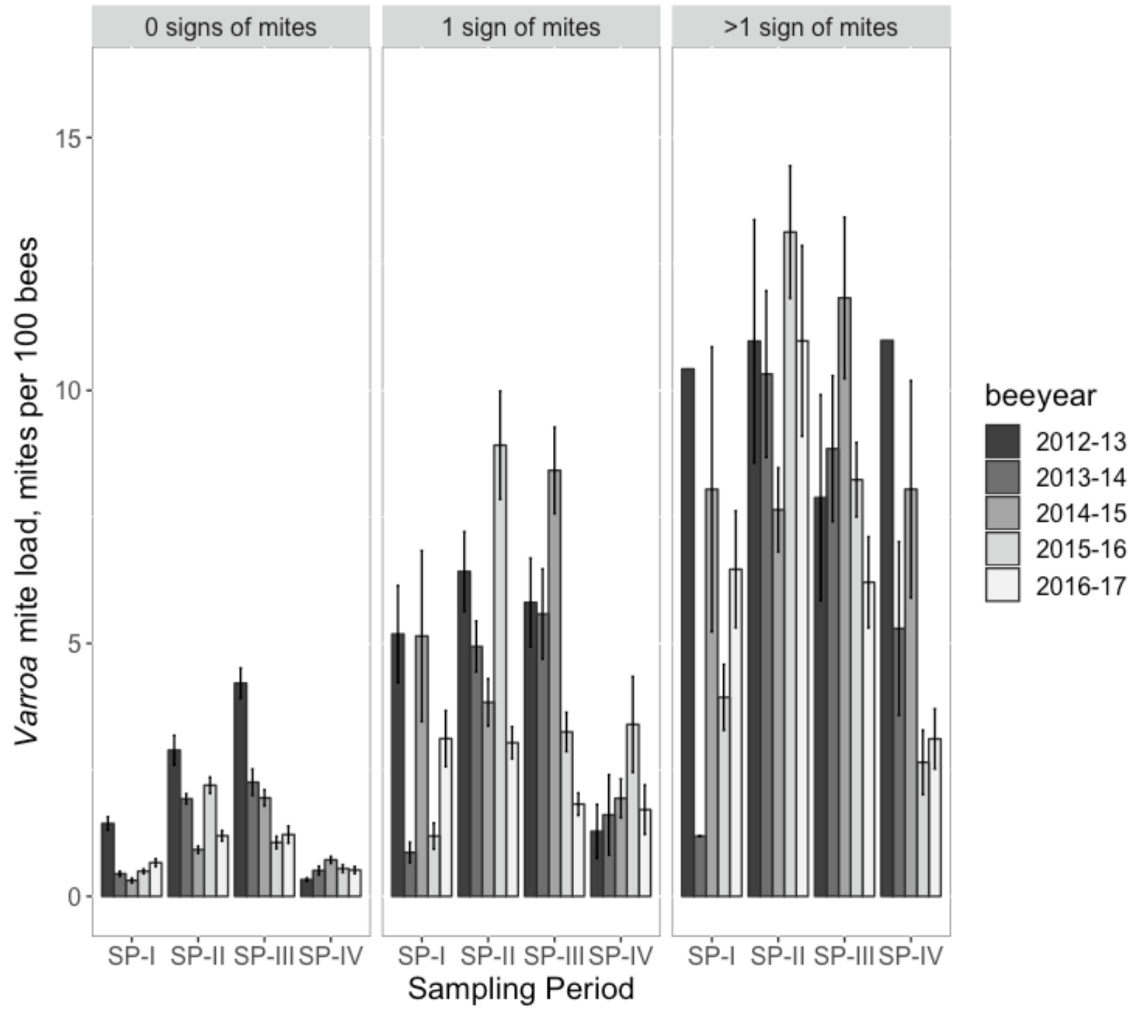


Figure 3.11 Mean *Varroa* mite loads (± SE) by the number of signs of mites (0, 1, or >1) by bee-year and sampling periods.



3.8 Tables

Table 3.1 Summary of the number of participating commercial beekeeper operations, colonies inspected, and inspection metrics by sampling period within bee-year. As some operations and colonies were inspected in more than one sampling period within a bee-year, the number of unique operations and colonies is included. Colonies were not inspected for more than one bee-year.

Bee-year	Sampling period	Beekeeper operations, n	Colonies inspected, n	*Varroa* mite samples, n	*Nosema* spp. samples, n	Frames of bees assessed, n	Brood pattern assessed, n
2012-13	SP-I	12	490	488	488	473	0
	SP-II	8	284	279	279	279	0
	SP-III	14	635	618	618	461	0
	SP-IV	13	502	445	445	384	0
	Unique number of colonies inspected: 1168; Unique number of operations: 14						
2013-14	SP-I	11	510	504	505	492	489
	SP-II	15	826	693	693	823	778
	SP-III	11	489	466	466	459	372
	SP-IV	13	603	418	418	393	283
	Unique number of colonies inspected: 1151; Unique number of operations: 16						
2014-15	SP-I	10	569	569	569	567	566
	SP-II	15	823	797	797	692	681
	SP-III	16	1143	1012	1012	767	715
	SP-IV	15	863	701	701	607	529
	Unique number of colonies inspected: 1623; Unique number of operations: 17						
2015-16	SP-I	14	731	682	683	573	568
	SP-II	14	679	634	635	515	500
	SP-III	14	629	546	546	509	398
	SP-IV	13	515	339	339	334	205
	Unique number of colonies inspected: 1222; Unique number of operations: 18						
2016-17	SP-I	16	549	549	549	529	525
	SP-II	12	459	441	441	362	347
	SP-III	11	418	384	384	410	384
	SP-IV	13	428	237	237	311	197
	Unique number of colonies inspected: 1176; Unique number of operations: 19						
Total	Unique number of colonies inspected: 6340; Unique number of operations: 23						

Table 3.2 Definitions of response, random effects, and fixed effects variables used in the GLMMs with a binomial distribution.

Response variables	Description
Varroa mite load, SP-I threshold	Binary: *Varroa* mite load above or below 2 mites per 100 bees (Currie and Gatien 2006; Giacobino et al. 2016).
Varroa mite load, SP-II and SP-III threshold	Binary: *Varroa* mite load above or below 3 mites per 100 bees (Genersch et al. 2010; Giacobino et al. 2016).
Varroa mite load, SP-IV threshold	Binary: *Varroa* mite load above or below 1 mite per 100 bees (Giacobino et al. 2016)
Nonviable colonies	Binary: viable or nonviable. Classified as viable if a colony had >4 frames of bees, and nonviable if a colony was dead or had ≤4 frames of bees.
Nosema spp.	Binary: *Nosema* spp. load above or below 10^6 spores/bee.

Random effects variables	Description
Bee-year	Categorical: 2012-13, 2013-14, 2014-15, 2015-16, or 2016-17. The seasonal cycle beginning in April and ending in April of the following calendar year in which a colony was inspected.
Beekeeping operation	Categorical: coded individual beekeeper operation that owned and managed an inspected colony.
Apiary	Categorical: coded location in which colony was inspected. Nested within beekeeping operation.

Fixed effects variables	Description
Frames of bees	Continuous: approximation of adult bee population. Estimated by counting the number of frames in the colony completely covered by bees.
Queen Status	Binary: "queen-right" or "queen event." Colony with a "queen event" had no queen, a virgin queen, drone laying queen, developing queen cells, or queen was replaced by the beekeeper. A "queen-right" colony had a queen present and lacked any symptoms of a queen event.
Migratory route	Categorical: Deep South (Texas, Mississippi, Louisiana) or West (California, Idaho, Oregon, Washington). Based on U.S. region where a colony was located prior to SP-I.
Wintering location	Categorical: wintering sheds, holding yards (California), or the Deep South (Louisiana, Mississippi, or Texas). Based on the location of a colony during or immediately prior to inspection.
Nosema spp. load	Binary: *Nosema* spp. load above or below 10^6 spores/bee.
Number of *Varroa* treatments	Categorical: number of *Varroa* mite treatments applied to the colony prior to the specified sampling period.
Varroa mite treatment product	Categorical: Apivar ®, amitraz-based, formic acid-based (Mite Away Quick Strips ® or 65% formic acid), other (Apiguard®, ApiLifeVar®, HopGuard®, oxalic acid, or other thymol-based products), or no treatment. If multiple treatments were used, the treatment product with the highest efficacy was chosen based on the Honey Bee Health Coalition's Tools for Varroa Management (2018).

Treatment before SP-III	Categorical: post-treatment, treatment present, and pre-treatment. Status of application of the primary product for *Varroa* mite treatment at the time of inspection.
Second treatment	Binary: treated or not treated. If a second *Varroa* mite treatment was applied to a colony.
Varroa mite level, SP-I threshold	Categorical: *Varroa* mite load binned at 0-1, 1-2, or ≥2 mites per 100 bees.
Varroa mite level, SP-II and SP-III threshold	Categorical: *Varroa* mite load binned at 0-1, 1-3, or ≥3 mites per 100 bees.
Date of *Varroa* mite treatment	Binary: August-November or December-February. Based on the timing of the last *Varroa* mite treatment prior to inspection.
Signs of mites	Binary: presence/absence of signs of mites. Signs of mites includes mites on adult bees, brood cells with mite frass, parasitic mite syndrome, and/or bees with deformed wings.
Nosema spp. treatment before SP-I	Categorical: untreated, treated in winter (January-March), or treated in spring (April-June).
Nosema spp. treatment before SP-III	Binary: untreated or treated with fumagillin by SP-III, or before SP-IV.
Month of inspection	Binary: May or June.

Table 3.3 Results of a sensitivity analysis that examined the number of colonies that were dead or nonviable (≤4 frames of bees, including dead colonies) by SP-IV relative to the number of frames of bees in colonies in SP-III. The numbers and percentages of dead or nonviable colonies in SP-IV are shown.

	Dead colonies in SP-IV				Nonviable colonies in SP-IV			
Frames of bees in SP-III	Dead, n	Alive, n	Total, n	Dead colonies, %	Nonviable, n	Viable, n	Total, n	Nonviable colonies, %
1	0	0	0		0	0	0	
2	1	0	1	100	1	0	1	100
3	3	4	7	42.9	6	1	7	85.7
4	12	12	24	50.0	21	3	24	87.5
5	18	25	43	41.9	34	9	43	79.1
6	24	56	80	30.0	47	33	80	58.8
7	29	155	184	15.8	76	108	184	41.3%

Table 3.4 Summary statistics for colonies tested over 5 bee-years for *Varroa* mites and *Nosema* spp. over four sampling periods. Means with different letters are significantly different.

	Varroa mites, mites per 100 bees						*Nosema* spp., 10^6 spores/bee					
Sampling period	n	Median	Mean	SE	Range	Samples with detectable *Varroa* mites, %	n	Median	Mean	SE	Range	Samples with detectable *Nosema* spp., %
SP-I	2792	0	0.89 a	0.04	0 - 29.9	42.9	2792	0.450	1.020 a	0.450	0 - 19.6	84.6
SP-II	2844	0.97	2.66 b	0.08	0 - 66.4	72.6	2845	0.100	0.387 b	0.100	0 - 10.6	68.0
SP-III	3026	0.95	4.04 b	0.12	0 - 58.6	67.6	3026	0	0.200 c	0	0 - 9.5	43.3
SP-IV	2140	0	0.78 a	0.39	0 - 23.1	42.9	2140	0.200	0.930 d	0.200	0 - 29.4	67.2

Table 3.5 Results from four GLMMs with a binomial distribution showing significant metrics that affected the odds a colony would be above a *Varroa* mite threshold of ≥2 mites per 100 bees in SP-I, ≥3 mites per 100 bees in SP-II and SP-III, and ≥1 mite per 100 bees in SP-IV. The metrics removed from the full model using AIC selection are listed as "not significant." For categorical fixed effects variables, the level with an estimate of 0 is the reference to which the other levels are compared. The number of colonies, the percent of colonies above the threshold, and the number of operations at each level within a category is noted.

SAMPLING PERIOD 1 (SP-I)

Not significant: frames of bees in SP-I, *Nosema* spp. load in SP-I, and migratory route.

Random variables (variance ± SD): Apiary nested in operation (3.423 ± 1.85); Operation (1.096 ± 1.047); Bee-year (0.201 ± 0.449)

Variable	Level	Colonies, n	Colonies with ≥2 mites per 100 bees, %	Operations, n	Estimate	SE	Odds ratio	95% CI	P-value
Intercept					-4.1421	0.489			
Signs of mites present in SP-I[a]	False	2480	7.7	19	0				
	True	212	44.8	16	1.742	0.235	5.71	(3.61 - 9.07)	<0.001***
Varroa mite treatment product	Amitraz	960	7.0	14	0				
	Apivar®	510	1.2	8	-1.125	0.708	0.32	(0.07 - 1.23)	0.11
	Formic	328	6.1	4	-0.496	0.775	0.61	(0.12 - 2.70)	0.52
	Other[b]	398	12.6	6	1.162	0.591	3.20	(0.99 - 10.42)	<0.05*
	No treatment	496	28.8	5	2.153	0.501	8.61	(3.36 - 24.33)	<0.001**

SAMPLING PERIOD 2 (SP-II)

Not significant: queen status, *Nosema* spp. load in SP-I, and migratory route.

Random variables (variance ± SD): Apiary nested in operation (1.918 ± 1.385); Operation (1.977 ± 1.406); Bee-year (1.319 ± 1.148)

Variable	Level	Colonies, n	Colonies with ≥3 mites per 100 bees, %	Operations, n	Estimate	SE	Odds ratio	CI (95%)	P-value
Intercept					-4.351	0.786			
Frames of bees in SP-I	Continuous				0.171	0.029	1.19	(1.12 - 1.26)	<0.001***
Varroa mite level in SP-I, mites per 100 bees	0-1	1391	17.4	13	0				
	1-2	118	48.3	14	1.245	0.262	3.47	(2.06 - 5.90)	<0.001***
	≥2	151	62.9	12	1.874	0.304	6.52	(3.59 - 12.18)	<0.001***
Varroa mite treatment product	Amitraz	785	23.8	10	0				
	Apivar®	384	13.3	8	-0.556	0.356	0.57	(0.27 - 1.17)	0.12
	Formic acid	289	9.0	4	0.417	0.696	1.52	(0.35 - 6.19)	0.54
	Other	158	41.8	4	1.060	0.428	2.89	(1.24 - 6.92)	<0.05*
	No treatment	80	80.0	1	2.816	0.541	16.71	(5.86 - 50.59)	<0.001***

SAMPLING PERIOD 3 (SP-III)

Not significant: queen status and *Nosema* spp. load in SP-II.

Random variables (variance ± SD): Apiary nested in operation (1.683 ± 1.297); Operation (2.986 ± 1.728); Bee-year (0.179 ± 0.423)

Variable	Level	Colonies, n	Colonies with ≥3 mites per 100 bees, %	Operations, n	Estimate	SE	Odds ratio	CI (95%)	P-value
Intercept					-5.418	0.721			
Frames of bees in SP-II	Continuous				0.100	0.025	1.10	(1.05 - 1.16)	<0.001***
Varroa mite level in SP-II, mites per 100 bees	0-1	825	9.0	16	0				
	1-3	355	30.1	16	1.980	0.241	7.24	(4.55 - 11.72)	<0.001***
	≥3	365	34.8	15	2.319	0.278	10.16	(5.96 - 17.75)	<0.001***
Varroa mite treatment before inspection	Post-treatment	702	15.0	12	0				
	Treatment present	632	16.9	10	1.082	0.361	2.95	(1.53 - 6.27)	<0.01**

	Pre-treatment	211	60.2	7	3.636	0.500	37.94	(15.2 - 107.72)	<0.001***

SAMPLING PERIOD 4 (SP-IV)

Not significant: queen status, frames of bees in SP-III, and *Nosema* spp. load in SP-III.

Random variables (variance ± SD): Apiary nested in operation (1.442 ± 1.201); Operation (1.704 ± 1.305); Bee-year (0.231 ± 0.48)

Variable	**Level**	**Colonies, n**	**Colonies with ≥1 mite per 100 bees, %**	**Operations, n**	**Estimate**	**SE**	**Odds ratio**	**CI (95%)**	**P-value**
Intercept					-2.727	0.476			
Varroa mite level in SP-III, mites per 100 bees	0-1	655	9.2	16	0				
	1-3	194	26.3	14	1.013	0.261	2.8	(1.65 - 4.59)	<0.001***
	≥3	218	20.6	14	0.610	0.277	1.8	(1.07 - 3.17)	<0.05*
Date of last *Varroa* mite treatment	Aug-Nov	703	16.9	14	0				
	Dec-Feb	364	10.2	6	-1.253	0.411	0.29	(0.13 - 0.64)	<0.01**

[a] Signs of mites are *Varroa* mite on a bee, chewed down brood, parasitic mite syndrome, and/or deformed wings.

[b] Other treatment products were Hopguard®, Apiguard®, other thymol-based products, and oxalic acid.

Table 3.6 Results from four GLMMs with a binomial distribution showing significant metrics that affected the odds a colony was above or below a threshold of 10^6 *Nosema* spp. spores per bee in SP-I, SP-II, SP-III, and SP-IV. The metrics removed from the full model using AIC selection are listed as "not significant." For categorical fixed effects explanatory variables, the level with an estimate of 0 is the reference to which the other levels are compared. The number of colonies, the percent of colonies above the threshold, and number of operations in each category is noted.

SAMPLING PERIOD 1 (SP-I)

Not significant: *Varroa* mite load in SP-II and treatment with fumagillin in winter or spring.

Random variables (variance ± SD): Apiary nested in operation (1.209 ± 1.100); Operation (0.762 ± 0.873); Bee-year (0.552 ± 0.743)

Variable	Level	Colonies, n	Colonies with ≥10^6 spores/bee, %	Operation, n	Estimate	SE	Odds ratio	CI (95%)	P-value
Intercept					0.742	0.551			
Frames of bees in SP-I	Continuous	2678		18	-0.056	0.020	0.95	(0.91 - 0.98)	<0.01**
Migratory route	West	1457	45.7	10	0				
	Deep South	1171	12.0	9	-1.827	0.443	0.16	(0.07 - 0.41)	<0.001***
Month of inspection	May	522	48.3	7	0				
	June	2106	26.4	18	-0.950	0.247	0.39	(0.23 - 0.63)	<0.001***

SAMPLING PERIOD 2 (SP-II)

Not significant: frames of bees in SP-II, *Varroa* mite load in SP-I, migratory route, and treatment with fumagillin in winter or spring.

Random variables (variance ± SD): Apiary nested in operation (1.527 ± 1.236); Operation (2.557 ± 1.599); Bee-year: (0.516 ± 0.718)

Variable	Level	Colonies, n	Colonies with ≥10^6 spores/bee, %	Operations, n	Estimate	SE	Odds ratio	CI (95%)	P-value

Intercept					-3.347	0.571			
Queen status	Queen-right	1589	8.2	16	0				
	Queen event	227	15.4	14	0.574	0.237	1.77	(1.08 – 2.88)	<0.05*

SAMPLING PERIOD 3 (SP-III)

Not significant: queen status, *Varroa* mite load in SP-III, and treatment with fumagillin by SP-III.

Random variables (variance ± SD): Apiary nested in operation (1.332 ± 1.154); Operation (3.825 ± 1.956); Bee-year: (0.090 ± 0.299)

Variable	Level	Colonies, n	Colonies with $\geq 10^6$ spores/bee, %	Operations, n	Estimate	SE	Odds ratio	CI (95%)	P-value
Intercept					-3.828	0.975			
Frames of bees in SP-III	Continuous	1562		15	-0.163	0.055	0.85	(0.76 - 0.95)	<0.01**
Nosema spp. load in SP-II	$<10^6$ spores/bee	1419	5.0	15	0				
	$\geq 10^6$ spores/bee	143	13.3	8	0.944	0.411	2.57	(1.12 - 5.67)	<0.05*

SAMPLING PERIOD 4 (SP-IV)

Not significant: queen status, frames of bees in SP-IV, *Varroa* mite load in SP-III, wintering location, and treatment with fumagillin by SP-II.

Random variables (variance ± SD): Apiary nested in operation (1.963 ± 1.401); Operation (2.459 ± 1.568); Bee-year (0.133 ± 0.364)

Variable	Level	Colonies, n	Colonies with $\geq 10^6$ spores/bee, %	Operations, n	Estimate	SE	Odds ratio	CI (95%)	P-value
Intercept					-1.487	0.478			
Nosema spp. load in SP-III	$<10^6$ spores/bee	1055	23.0	17	0				
	$\geq 10^6$ spores/bee	41	51.2	9	1.299	0.432	3.67	(1.54 - 8.86)	<0.001**

Table 3.7 Results from two GLMMs with a binomial distribution showing significant metrics that affected the odds a colony would be nonviable in SP-III and SP-IV. The metrics removed from the full model using AIC selection are listed as "not significant." For categorical fixed effects explanatory variables, the level with an estimate of 0 is the reference to which the other levels are compared. The number of colonies, the percent of colonies above the threshold, and number of operations in each category is noted.

SAMPLING PERIOD 3 (SP-III)

Not significant: *Nosema* spp. load in SP-II and the number of *Varroa* mite treatment products used.

Random variables (variance ± SD): Apiary nested in operation (0.723 ± 0.850); Operation (0.033 ± 0.181); Bee-year (0.049 ± 0.221)

Variable	Level	Colonies, n	Nonviable colonies, %	Operations, n	Estimate	SE	Odds ratio	95% CI	P-value
Intercept					-1.547	0.490			
Frames of bees in SP-II	Continuous	1637			-0.193	0.032	0.82	(0.77 - 0.88)	<0.001***
Queen status	Queen-right	1344	5.1	14	0				
	Queen event	293	15.7	14	1.280	0.240	3.60	(2.24 - 5.77)	<0.01**
Varroa mite level in SP-II, mites per 100 bees	0-1	834	3.2	14	0				
	1-3	404	6.4	14	0.700	0.314	2.01	(1.09 - 3.74)	0.49
	≥3	399	15.5	14	1.705	0.293	5.50	(3.11 - 9.87)	<0.001***

SAMPLING PERIOD 4 (SP-IV)

Not significant: *Nosema* spp. treatment by SP-III and wintering location (sheds, California holding yard, or the Deep South).

Random variables (variance ± SD): Apiary nested in operation (0.372 ± 0.610); Operation: (0.009 ± 0.095); Bee-year: (0.37 ± 0.608)

Variable	Level	Colonies, n	Nonviable colonies, %	Operations, n	Estimate	SE	Odds ratio	CI (95%)	P-value
Intercept					0.086	0.511			

Frames of bees in SP-III	Continuous	970		9	-0.220	0.039	0.80	(0.74 - 0.86)	<0.001***
Queen event	Queen-right	752	20.7	10	0				
	Queen event	218	39.4	9	0.930	0.199	2.53	(1.71 - 3.76)	<0.001***
Varroa level in SP-III, mites per 100 bees	0-1	609	20.5	10	0				
	1-3	187	27.3	9	0.491	0.229	1.63	(1.04 - 2.57)	<0.05*
	>=3	174	37.9	9	0.751	0.232	2.12	(1.34 - 3.35)	<0.01**
Varroa treatment before inspection	Post-treatment	291	18.2	7	0				
	Treatment present	482	22.6	8	0.094	0.276	1.10	(0.63 - 1.91)	0.73
	Pre-treatment	197	40.6	6	1.13682	0.345	3.12	(1.57 - 6.31)	<0.001***
Nosema spp. load in SP-III	<10^6 spores/bee	931	24.0	10	0				
	≥10^6 spores/bee	39	48.7	6	1.056	0.396	2.87	(1.31 - 6.30)	<0.01**

Chapter 4. Is the Brood Pattern within a Honey Bee Colony a Reliable Indicator of Queen Quality?

Publication citation

Lee, K.V., Goblirsch, McDermott, E., Tarpy, D.R., and Spivak, M. Is the Brood Pattern within a Honey Bee Colony a Reliable Indicator of Queen Quality? *Insects*, in press.

4.1 Synopsis

Failure of the queen is often identified as a leading cause of honey bee colony mortality. However, the factors that can contribute to "queen failure" are poorly defined and often misunderstood. We studied one specific sign attributed to queen failure: poor brood pattern. In 2016 and 2017, we identified pairs of colonies with "good" and "poor" brood patterns in commercial beekeeping operations and used standard metrics to assess queen and colony health. We found no queen quality measures reliably associated with poor-brood colonies. In the second year (2017), we exchanged queens between colony pairs (n = 21): a queen from a poor-brood colony was introduced into a good-brood colony and vice versa. We observed that brood patterns of queens originally from poor-brood colonies significantly improved after placement into a good-brood colony after 21 days, suggesting factors other than the queen contributed to brood pattern. Our study challenges the notion that brood pattern alone is sufficient to judge queen quality. Our results emphasize the challenges in determining the root source for problems related to the queen when assessing honey bee colony health.

4.2 Introduction

The queen is arguably the most important member of a honey bee colony. She is tasked with the production of daughter workers that forage for resources and care for the brood—eggs, larvae, and pupae—and sons that support genetic diversity among colonies through mating with virgin queens from other colonies. The demand by the colony placed on the queen for a sustained, high-reproductive output underscores the importance of her well-being to a colony's success. Beekeepers are appreciative of queen health, as healthy queens ultimately lead to greater revenue generated from the sale of surplus bees, hive products, and pollination services. Beekeepers rely on various metrics associated with a queen's reproductive output when surveying their colonies to establish the health status of their queens. They then use this information to make management decisions based on whether a queen is judged to be "good" or "failing". However, are the signs and symptoms used to discern a good queen from a failing queen sufficient to inform management decisions? Finding an answer to this question is needed as queen health is a current issue in the beekeeping industry. Beekeepers repeatedly identify queen failure as a significant contributor to colony mortality in their responses on annual colony loss surveys, with commercial beekeepers—beekeepers that manage >500 colonies—ranking it as the first or second contributing factor (Kulhanek et al. 2017; Lee et al. 2015b).

Queens generally live one to three years. As a queen's age increases, so does the likelihood of a supersedure event occurring—when the bees raise a new queen to replace the old queen—or the queen dying (Szabo 1993). To avoid an interruption in brood production associated with aging queens, it is common practice for beekeepers to replace older queens annually. However, in recent years, beekeepers have reported queen failures

after introducing young, newly mated queens into colonies. Causes for why young queens fail are not well understood. One explanation for failure observed in young queens may be a breakdown in the linkage between sperm stored in the spermatheca and successful fertilization of worker-destined eggs (Baer et al. 2016). Approximately 10 days after emergence as an adult, queens acquire and store all of the sperm they will use throughout their lifetime from a single bout of mating with 10 to 20 drones that occurs over one or two days (Koeniger et al. 2014). Inadequate sperm quantity or quality, either because of too few matings or inviable sperm in the ejaculate of the drones, could lead to elevated rates of fertilization failure, to which the colony may respond by supersedure (Baer et al. 2016). However, sperm viability appears to be of greater concern than sperm load, as queens are often adequately mated—stored sperm counts within the queen are high (Tarpy et al. 2012)—but the viability of stored sperm diminishes over time (Lodesani et al. 2004; Tarpy and Olivarez 2014). Low viability can be an issue as the queen may have smaller brood patches (Collins 2000) or can become a "drone layer"—laying only unfertilized eggs (Delaney et al. 2011). Queens inbred to related drones produce inviable worker eggs (Koeniger et al. 2014), although this is uncommon in commercial queen production (Collins et al. 2004; Tarpy et al. 2012).

Additional factors that may play a causal role in queen failure are queen infection with pathogens or exposure to pesticides. There is evidence for negative effects of pathogens on queen physiology (reviewed previously in Amiri et al. 2017). Infection with deformed wing virus has been associated with ovarian degeneration (Gauthier et al. 2011), and an infection of the fungal pathogen *Nosema ceranae* may lead to relatively higher vitellogenin levels (Alaux et al. 2011) and upregulation of immune genes (Chaimanee et al. 2014). Physiological changes observed in queens exposed to different

pesticides include lower queen weight (Haarmann et al. 2002; Pettis et al. 2004) and fewer ovarioles (Haarmann et al. 2002). Moreover, pesticides have been shown to affect sperm viability in queens, including in-hive chemicals used by beekeepers to control the parasitic mite *Varroa destructor* (Burley et al. 2008; Chaimanee et al. 2016; Rangel and Tarpy 2016) and agricultural chemicals (Chaimanee et al. 2016). Pesticide exposure has also been linked to higher rates of queen supersedure (Sandrock et al. 2014; Tsvetkov et al. 2017; Traynor et al. 2016a) and decreased survival in combination with a *N. ceranae* infection (Dussaubat et al. 2016).

One sign commonly attributed to failing queens is a poor brood pattern. Brood pattern refers to wax-capped cells containing pupae, also called sealed brood. A brood pattern is considered to be poor if ≥20% of the cells within an area of sealed brood are empty (vanEngelsdorp et al. 2013a) and indicates that either the queen is not laying eggs well or the developing bees are not surviving to eclosion. In addition to queen quality measures, colony environment may influence brood pattern. Poor brood patterns have been associated with the fungal pathogen chalkbrood, sacbrood virus, and *Nosema* spp. infections of >1 million spores per bee (vanEngelsdorp et al. 2013a). Pesticide exposure in the comb (Wu et al. 2011) or lack of adequate nutrition may impact brood health (Brodschneider and Crailsheim 2010), leading to decreased brood viability. In contrast, there also are heritable traits where worker bees remove diseased or *V. destructor* infested brood resulting in a worse brood pattern but a healthier colony (Harbo and Harris 2009; Spivak and Reuter 1998).

The overall objective of this study was to examine if young, failing queens could be a major causal factor of poor sealed-brood patterns. In the first year of the study, we used standard metrics to assess queen and colony health in colonies with good-brood and

poor-brood patterns. In the second year, we included additional measures of colony environment to begin to untangle the effects of the queen and the colony. Our specific objectives were to (1) determine if brood pattern is a reliable indicator of queen quality, (2) identify colony-level measures associated with poor brood pattern colonies, and (3) examine the change in brood patterns after queens were exchanged into a colony with the opposite brood pattern classification.

4.3 Materials and Methods

4.3.1 Colony Selection

In 2016 and 2017, we identified colonies with poor sealed brood patterns and good sealed brood patterns in May and June. For each poor-brood colony, we identified a good-brood colony with the same management history within a commercial beekeeper operation based in North Dakota, Minnesota, or Texas. All colonies were headed by queens <6 months old and all queens were produced and mated in Texas or California. Data were collected from 34 colonies and queens from five operations in 2016, and 42 different colonies and queens from four operations in 2017 (Figure 4.1).

Sealed brood patterns were rated using an ordinal scale from 1 (poor) to 5 (excellent) (Figure 4.2) by two field technicians with extensive experience in using the rating system (modified from Guzmán-Novoa and Page 1999). A score of <3 was considered to be poor. In 2017, sealed brood pattern was also measured by quantifying the percent of sealed brood cells by placing a parallelogram large enough to occupy 100 cells over a section of sealed brood, then counting the number of empty cells—cells without sealed brood—within the parallelogram (described previously in Delaplane et al. 2013; vanEngelsdorp et al. 2013a). The number of empty cells was subtracted from 100,

and the average was taken from three separate frames containing the fewest empty cells. A brood pattern with <80% sealed brood was considered to be poor (Pettis et al. 2016; vanEngelsdorp et al. 2013a). The parallelogram method was also used to quantify the queen's egg-laying pattern by identifying the area of comb that had the most continuous patch of eggs in each colony, counting the number of empty cells, and subtracting the number of empty cells from 100.

In 2017, we used a partial reciprocal transplant design to quantify the change in brood patterns for queens placed into different colony environments (Figure 4.1b). Pairs of colonies with poor-brood and good-brood patterns were identified from the same apiary or a nearby apiary with the same management history. Queens were removed and marked with a paint pen for later identification, and then placed in queen cages provisioned with food. Queens were then exchanged between colony pairs, such that a queen previously identified from a poor-brood colony was introduced into a good-brood colony and a queen from a good-brood colony was introduced into its poor-brood colony pair. Caged queens were released manually approximately 3 days after introduction into their new colony. Brood pattern measurements were recorded before the reciprocal exchange and approximately 21 days after the queen's release to allow the queens to complete one full worker brood cycle in their new colony.

4.3.2 Queen Mating Quality and Morphometric Measurements

In 2016, queens were removed from their colonies and caged individually the same day colony metrics were recorded (Figure 4.1a). Cages were provisioned with food and seven worker bees from the colony where the queen was removed served as attendants. In 2017, only queens still alive after the exchange were collected. Within two

days of being sampled, all queens were shipped live overnight to the North Carolina State University Queen & Disease Clinic (NCSU-QDC). At the NCSU-QDC, queens were immobilized by carbon dioxide narcosis and external morphometrics were measured: head width (mm), thorax width (mm), and wet mass (mg). The spermatheca of each queen was then extracted and the sperm within was suspended in buffer and differentially (live-dead) dyed in accordance with the procedure that accompanies the Invitrogen Live-Dead Sperm viability kit (Invitrogen L7011). Twenty microliters of the sample was then transferred to a cell counting chamber and visualized on the Nexcelom Vision® System (Simone-Finstrom and Tarpy 2018). The total number of live and dead sperm were counted, and sperm viability was defined as the percent of live sperm out of the total sperm.

4.3.3 Colony Measurements

Adult bee populations were estimated by counting the number of frames in the colony that were fully covered by adult bees (described previously in Delaplane et al. 2013; Genersch et al. 2010). Presence of queen cells—which are indicators of swarming, supersedure, or queen loss—were noted along with any visual signs of disease or pests, including American foulbrood (*Paenibacillus larvae*), European foulbrood (*Melissococcus plutonius*), chalkbrood (*Ascosphaera apis*), sacbrood virus, hive beetles (*Aethina tumida*), wax moth (*Gallaria melonella*), *V. destructor* mites, deformed wings, and parasitic mite syndrome (Shimanuki et al. 2014). Entombed pollen (vanEngelsdorp et al. 2009) was noted if found.

From a brood frame, approximately 300 worker bees were collected into a 4oz bottle containing 70% ethanol from each colony to quantify the adult bee infestation

levels of *V. destructor* and *Nosema* spp. *Varroa destructor* levels were quantified using an alcohol wash to dislodge the mites from the adult bees in the sample (De Jong et al. 1982), then the mites and bees were counted and reported as mites per 100 bees. *Nosema* spp. levels were quantified by counting spores found in a composite sample of 100 bees (Cantwell 1970). The method of Cantwell (1970) does not differentiate between *N. apis* and *N. ceranae*, the two species known to cause infection in US honey bees. If a sample was found to be positive for *Nosema* spp. infection, it was assumed to be *N. ceranae* due to findings from a recent US survey on honey bee diseases (Traynor et al. 2016b).

A sample of empty wax comb (>3 g) was collected into a 50 mL conical tube and stored at −80 °C before shipment on ice to the USDA-AMS lab in Gastonia, North Carolina for pesticide residue analysis. Wax samples were screened for 175 and 202 pesticides and their metabolites in 2016 and 2017, respectively (analysis methods described previously (Mullin et al. 2010) (see Supplementary Material Dataset 1: S1b. Pesticides 2016 and S1d. Pesticides 2017). Not all pesticide samples were processed due to cost. Hazard quotients (HQs) and the total number of pesticides detected were used to establish the pesticide risk in each colony. HQs were calculated by dividing the amount of the pesticide found (ppb) in the wax sample by the adult bee contact LD_{50} reported for adult honey bees (methods described previously in Stoner and Eitzer 2013; Traynor et al. 2016a). The LD_{50} for each pesticide was obtained primarily by using US EPA Ecotox Database (US EPA Ecotox Database). Additional resources (Hertfordshire Pesticide Properties Database; Sanchez-Bayo and Goka 2014; Traynor et al. 2016a) were used when the adult bee contact LD_{50} was not available through the US EPA Ecotox Database (see Table S4.1). Wax HQs were considered elevated if they exceeded a value of 5000 (Traynor et al. 2016a). The total number of pesticide residues in the wax sample was

calculated by adding the number of unique pesticides detected for each colony. The HQ and total number of pesticides detected offer an approximate measure for pesticide exposure in the colony; however, they do not account for synergistic or sublethal effects, larval toxicity, or adult oral toxicity, but both have previously been associated with queen failure (Traynor et al. 2016a).

4.3.4 Molecular Analysis

Total RNA was extracted from the remaining queen tissues (after dissection of the spermathecae). Queens were homogenized in individual microcentrifuge tubes with a plastic pestle in an appropriate volume of Trizol (Thermo Fisher Scientific, HQ in Waltham, MA, USA) and extraction was performed by standard phenol-chloroform protocol. Samples were then tested on the NanoDrop for quality and concentration. RNA concentration was diluted to a normalized 200 ng/uL before cDNA (Biobasic Inc. in Markham, ON, Canada) was synthesized with the BioBasic Reverse Transcriptase Mix. Reverse transcription quantitative PCR (rt-qPCR) was performed following a previously described method (Alburaki et al. 2018) for detection of the following pathogens: *Nosema* spp. (universal primer), trypanosome spp. (universal primer), acute bee paralysis virus (ABPV), black queen cell virus (BQCV), chronic bee paralysis virus (CBPV), deformed wing virus type A (DWV-A) and type B (DWV-B), Israeli acute paralysis virus (IAPV), and Lake Sinai virus (LSV). qPCR was performed in triplicate with Power-Up SYBRGreen Mastermix (Thermo Fisher Scientific, HQ in Waltham, MA, USA) on a 384-well QuantStudio Flex 6 (Thermo Fisher Scientific, HQ in Waltham, MA, USA) and analyzed in the associated software. Cycling conditions were adapted from the Power-Up SYBR Green protocols. The standard curve for copy number quantification was

determined by running a dilution series of known plasmid standard on each plate. Results were normalized via GeNorm (reference) to the reference genes Actin, Apo28s, and GapDH, are reported as presence or absence of the pathogen.

In 2017 before queens were exchanged between pairs of colonies, >50 adult bees were collected from a brood frame into a 50 mL conical tube from each colony. Samples were frozen immediately using dry ice or liquid nitrogen, stored at −80 °C, and then shipped on dry ice to the NCSU-QDC. Samples were analyzed by rt-qPCR for pathogens (see above), the storage protein vitellogenin (*Vg*), heat shock protein HSP70 ab-like, and the immune peptides defensin and hymenoptacin. For each colony-level sample, 5 g (approximately 50 bees) were extracted. The entire 5 g sample was homogenized in an appropriate volume of Trizol and extracted by standard phenol-chloroform extraction. The rest of the extraction was performed as above. Expression levels of the immune genes, HSP70 ab-like and *Vg* were determined via ΔΔCt analysis as compared to the reference gene Actin, not by standard curve quantitation. These genes were tested as they can indicate the health of the bees: *Vg* can influence the lifespan and decrease the oxidative stress of worker bees (Amdam and Omholt 2002; Corona et al. 2007; Nelson et al. 2007; Seehuus et al. 2006), relatively higher values for the immune genes suggest an upregulated immune system (Evans and Pettis 2005; Simone et al. 2009), and the upregulation of heat shock proteins suggests a response to stressors resulting in denatured proteins (Even et al. 2012). Upregulation indicates that the immune system is more active—potentially in response to a pathogen or other stressor—and is costly to the individual bee.

4.3.5 Statistical Analysis

We used the statistical program R for all analyses (R Core Team 2017). All statistical assumptions were visually checked, and if violated an appropriate test was used—the nonparametric Kruskal–Wallis test or the Welch's *t*-test for unequal variances—or the data were transformed. Summary data are reported as means ± SD unless otherwise noted. Statistical comparisons were considered significant if $\alpha < 0.05$.

To ensure brood patterns were different between poor-brood and good-brood colonies, we compared the brood pattern scores—rating in 2016 and percent sealed in 2017—between the two groups using a Kruskal–Wallis test for the 2016 data and a Welch's *t*-test for the 2017 data. For the 2017 data, a simple linear regression was used to examine the relationship between the two methods of measuring sealed brood patterns.

For objectives 1 and 2, we used odds ratios (±95% confidence intervals) to compare the odds of a pathogen occurring in a poor-brood queen or colony compared to a good-brood queen or colony (vanEngelsdorp et al. 2013b). An odds ratio value significantly >1 indicates a positive association, and an odds ratio value significantly <1 indicates a negative association. To calculate the odds ratio in cases where no pathogen was detected, the Haldane–Anscombe correction was used (Anscombe 1956; Haldane 1940). We used *lme4* in R (Bates et al. 2015) to perform analyses using linear mixed effects models to compare the relationships between queen or colony measures and the binary brood pattern classification of good-brood or poor-brood. The brood pattern classification was used as a fixed effect, and beekeeper as a random factor with random slopes for the effect of the brood pattern classification. *p*-values were obtained by using likelihood ratio tests comparing the full model with the brood classification as a factor to

the model without the brood pattern classification. The effect levels are reported as the estimate ± standard errors.

For objective 3, we compared the sealed brood pattern of each queen in her original colony to her sealed brood pattern approximately 21 days after being released into her new colony. We predicted that if colony environment had an effect on brood pattern, then the pattern should either improve when a queen from a poor-brood colony was placed into a good-brood colony or worsen when a queen from a good-brood colony was placed into a poor-brood colony: the change in brood pattern (after minus before the exchange) would be significantly different than zero using a t-test. In addition, we examined the relationship between the brood pattern before the exchange, and the change in brood pattern (after minus before the exchange) using a simple linear regression. We predicted that the queens with best or worst brood patterns before the exchange would have the largest change in brood pattern after they the queens were transferred to their reciprocal colonies. We also compared queen egg patterns before and after the exchange using a t-test and a simple linear regression. Data were excluded from these analyses if the queen was not found after the exchange.

4.4 Results

4.4.1 Brood Pattern Classifications

Brood patterns were significantly different between good-brood and poor-brood pattern colonies in 2016 based on the brood rating scale ($H = 25.6$, df = 1, $p < 0.01$) and 2017 based on the percent of cells sealed ($t_{23.93} = 10.01$, $p < 0.01$), confirming that the poor-brood and good-brood classifications were different. In 2016, the mean brood rating was for 4.0 ± 0.4 good-brood colonies ($n = 17$) and 1.9 ± 0.5 for poor-brood pattern

colonies (n = 17). In 2017, the mean percent sealed brood was 93.0 ± 2.9% for good-brood colonies (n = 21) and 72.1 ± 9.1% for poor-brood colonies (n = 21). The brood rating scale was highly correlated to the percent brood measure in 2017 (R^2 = 0.90, $F_{1,79}$ = 731.2, p < 0.01), suggesting that the rating method sufficiently and accurately categorized brood patterns.

4.4.2 Measures Associated with Queens

Sperm number and sperm viability assessed from the queen spermathecae and queen morphometrics are summarized in Table 4.1. Data obtained from queens judged to be on average "high quality" from US commercial queen producers (Delaney et al. 2011; Tarpy et al. 2012) are included for comparison. In general, the queen morphometrics, and number and viability of sperm in the spermathecae of the queens from our study were similar or higher than the previous studies. In 2017, three queens did not survive until the second sampling: one queen from a good-brood colony and two queens from poor-brood colonies. One queen from a poor-brood colony in Operation 1 in 2017 had a sperm viability of 1.0%, which was examined as a possible error as it was more than 2 standard deviations from the mean. This queen continued to lay fertilized worker bee eggs, which is contrary to what would be expected from queens with similar levels of sperm viability (Collins 2000). Due to the biological improbability of the results, the data for this queen was removed from sperm viability analyses. The percent sperm viability was not different between the two brood pattern classification groups in either 2016 (χ^2 = 2.5, df = 1, p = 0.11) or 2017 (χ^2 = 0.02, df = 1, p = 0.90). In 2016, queens from poor-brood colonies tended to have fewer sperm than good-brood colonies, but the difference was not significant (χ^2 = 3.3, df = 1, p = 0.07). There was no difference in sperm count between

brood pattern groups in 2017 (χ^2 = 0.27, df = 1, p = 0.61). For both years, the average sperm count for both queen groups was over the 3 million sperm count threshold to be considered adequately mated (Woyke 1962) (Table 4.1). None of the queen mating or morphometric measures could be reliably associated with queens from poor-brood colonies.

None of the pathogens tested had significantly higher odds of being associated with queens from poor-brood pattern colonies (Table 4.2). The 2016 data for Operation 1 were not included in the PCR results because those samples were lost. Twenty-three percent of queens from 2016 and 78% of queens from 2017 had no pathogens detected from the panel of common honey bee pathogens used for screening. Moreover, ABPV, CBPV, trypanosomes spp., and *Nosema* spp. were not detected in any queens from 2016 or 2017. In both years, DWV-B was the most prevalent virus found in queen bees, followed by DWV-A. BQCV, LSV, and IAPV had low prevalence as they were found in only one or two queens in either 2016 or 2017.

4.4.3 Measures Associated with Colony Environment

4.4.3.1 Adult Bee Pathogens

None of the pathogens tested had significantly higher odds of being associated with a poor-brood pattern colony (Table 4.2). *Varroa destructor* levels were not different between good-brood and poor-brood colonies for either year, and overall levels were low with few colonies having a mite load higher than a treatment threshold of 3 mites per 100 bees (Genersch et al. 2010; Giacobino et al. 2015). Worker bees from poor-brood colonies were not more likely to be over the threshold of >1 *Nosema* spp. million spores per bee as quantified by microscopy (Traynor et al. 2016b), nor be more likely to test

positive for *Nosema* spp. as determined by PCR. In 2017, all worker bee samples tested positive for LSV and 35 samples also tested positive for *Nosema* spp. However, no 2017 queen tested positive for LSV or *Nosema* spp., suggesting that the queen was not vertically transmitting these pathogens and the workers did not transmit them to her.

4.4.3.2 Brood Pathogens

It was not always possible to choose poor-brood colonies with no clinical signs of disease. Due to the near ubiquity of chalkbrood in 2017, we chose five good-brood and six poor-brood colonies with chalkbrood before the exchange that had ≤5 cells presenting symptoms of infection. After the exchange, 52% of good-brood and 76% of poor-brood colonies had chalkbrood symptoms. However, chalkbrood was not more likely to be found in poor-brood pattern colonies (Table 4.2). For comparison, only one good-brood colony had chalkbrood in 2016. No other brood diseases were found in either year.

4.4.3.3 Worker Bee Vitellogenin, Immune Genes, and Heat Shock Protein

Vg levels in worker bees from poor-brood colonies were 0.90 ± 0.36 (standard error) higher than *Vg* levels in workers bees from good-brood colonies. This difference was significant ($\chi^2 = 13.1$, df = 1, $p < 0.01$), but may not be biologically relevant as it was under one ct cycle. We found no differences between the worker bees from good-brood and poor-brood colonies for defensin ($\chi^2 = 1.3$, df = 1, $p = 0.26$), hymenoptacin ($\chi^2 = 0.7$, df = 1, $p = 0.39$), or Hsp70ab-like ($\chi^2 = 1.9$, df = 1, $p = 0.16$). However, the levels of these genes were all significantly higher in Operation 1's worker bees from poor-brood colonies ($n = 6$) compared to the worker bees from good-brood colonies ($n = 6$): defensin ($H = 7.4$, $p < 0.01$), hymenoptacin ($H = 8.3$, $p < 0.01$), and Hsp70ab-like ($H = 5.0$, $p <$

0.05) (Figure 4.3). *Vg* was not different between good-brood and poor-brood colonies for Operation 1 ($H = 0.8$, $p = 0.38$). No other significant differences were found for the immune genes or heat shock protein genes. These results suggest that the worker bee immune systems in Operation 1's poor-brood colonies were upregulated.

4.4.3.4 Colony Pesticide Levels

Twenty-eight beeswax samples were processed for pesticides in 2016 and 24 samples in 2017 (results summarized in Table S4.1). The pesticide data are not directly comparable between years as there were different chemicals tested each year. In 2016, there was a range of 5–16 pesticides detected per sample, and a range of 9–31 pesticides detected per sample in 2017. In 2016, the most common pesticide class found was varroacides—pesticides used to control *V. destructor*—with 44% of pesticides found belonging to this class (Figure S4.1). Fungicides were most common in 2017 with 45% of pesticides found belonging to that class, followed by varroacides at 24%.

Overall HQ levels were low. Excluding Operation 5, the mean HQ in 2016 was 38 ± 71 ($n = 22$). For Operation 5, there was a high incidence of cyfluthrin (pyrethroid insecticide) in both the good-brood and poor-brood colonies resulting in higher HQs: 2093 ± 1940 ($n = 6$). One good-brood colony had an HQ >5000. In 2017, the mean HQ for good-brood colonies was 677 ± 801 ($n = 12$) and 1160 ± 894 ($n = 12$) for poor-brood colonies. All HQs in 2017 were <5000. The log transformed HQs were not significantly different between good-brood and poor-brood colonies in 2016 ($\chi^2 = 0.03$, df = 1, $n = 28$, $p = 0.86$) nor in 2017 ($\chi^2 = 1.97$, df = 1, $n = 24$, $p = 0.16$). However, the total number of pesticide residues in 2016 was significantly higher in poor-brood compared to good-brood colonies ($\chi^2 = 5.00$, df = 1, $n = 24$, $p < 0.05$), with poor-brood colonies having 1.9

± 0.7 (standard errors) more pesticides detected. In 2017, there was a trend toward more pesticides in poor-brood colonies, but this result was not significant ($\chi^2 = 3.8$, df = 1, n = 28, $p = 0.051$).

4.4.4. Brood Pattern Change

The change in sealed brood patterns for queens from poor-brood colonies exchanged into good-brood colonies was significantly different than zero with a mean increase of 11.6 ± 9.9 more sealed cells ($t_{17} = 5.0$, $p < 0.01$) (Figure 4.4a), indicating better patterns after the exchange. The brood patterns for queens from good-brood colonies were also significantly different after the exchange into poor-brood colonies with a mean of 8.0 ± 10.9 fewer sealed cells ($t_{18} = 3.2$, $p < 0.01$), indicating worse patterns after the exchange. The linear regression of the starting brood pattern against the change in brood pattern was significant ($R^2 = 0.50$, $F_{1,35} = 36.38$, $p < 0.01$), suggesting that queens with initially poor patterns tended to have improved patterns after the exchange and queens with initially better brood patterns tended to have worse patterns after the exchange (Figure 4.4b). This result implies that colony environment impacted the sealed brood pattern. To account for the potential effect of chalkbrood on sealed brood patterns, we removed the colonies with signs of chalkbrood after the exchange from the dataset and re-examined the relationship between the starting sealed brood pattern and the change in brood pattern. The relationship was still significant ($R^2 = 0.48$, $F_{1,14} = 14.99$, $p < 0.01$), suggesting that the change in brood patterns was not only due to chalkbrood.

Queens from poor-brood colonies had significantly worse egg patterns compared to queens from good-brood colonies before the exchange, with an average of 84.7 ±

16.0% sealed for poor-brood colonies compared to an average of 94.9 ± 4.7% sealed for good brood colonies ($t_{19.7} = 2.6$, $p < 0.05$). When the same <80% cut-off for a poor sealed brood pattern was used for the egg patterns, one queen from a good-brood colony and four queens from poor-brood colonies had "poor" egg patterns before the queen exchange. Queens from good-brood colonies transferred into poor-brood colonies had a mean egg pattern of 95.8 ± 3.4% after the exchange, and queens from poor-brood colonies transferred to good-brood colonies had a mean egg pattern of 90.8 ± 6.1%. While the difference in egg pattern was still significantly different between groups ($t_{26.41} = 3.1$, $p < 0.01$), only one queen, originally from a poor-brood colony, had a "poor" egg pattern of <80% after the exchange.

The change in egg pattern after queens were reciprocally transferred was not different than zero for queens from either good-brood ($t18 = 0.6$, $p = 0.58$) or poor-brood colonies ($t17 = 1.5$, $p = 0.16$) (Figure 4.5a), suggesting that egg patterns did not change after the queen exchange based on the binary sealed brood classification. Queens from good-brood colonies had good patterns before and after they were exchanged into a potentially worse colony environment. Egg patterns for queens from poor-brood colonies did not improve on average after the exchange and the variability in egg pattern change was higher for these queens. While there was no difference in the egg pattern change when classified by the binary good or poor sealed brood classification, the queens that initially had the worst egg patterns had better patterns after being exchanged, and the queens with good egg patterns had similar or worse patterns after the exchange ($R2 = 0.87$, $F1,16 = 115.5$, $p < 0.01$) (Figure 4.5b). This result suggests that colony environment may have influenced the egg patterns for queens with initially the worst egg patterns as those patterns improved after the exchange. However, it is unclear why some of the good

egg patterns for queens from poor-brood colonies were worse after the exchange as their egg laying potential was high.

4.5 Discussion

The results of this study suggest that a poor sealed brood pattern is not a reliable indicator of queen quality and is not necessarily a sign of queen failure. Queens from both good-brood and poor-brood colonies had sperm counts, sperm viability, body sizes, and weights that were comparable to queens considered to be of high quality in other studies (Delaney et al. 2011; Tarpy et al. 2012). Queens from poor-brood colonies were not more likely to have <3 million sperm in their spermathecae, which has been considered the threshold for being poorly mated (Woyke 1962). There were no differences in pathogen detections between the sets of queens, including viruses, *Nosema* spp., and trypanosomes.

The partial reciprocal transplant of queens in 2017 revealed that the sealed brood patterns of queens from poor-brood colonies improved significantly after they were placed into colonies with good patterns, suggesting an influence of colony environment on the sealed brood pattern rather than solely the queens' egg-laying capacity. None of the worker bee pathogen or immune gene measures were reliably associated with poor patterns. Levels of HQs in wax combs did not differ between brood pattern classifications. More specifically, Operation 5 reported issues with queens not being accepted by colonies in the spring of 2016; we found the highest HQs in those colonies. However, queen acceptance problems and high HQs were not found in other operations in this study. The total number of pesticides detected in wax combs was significantly higher in colonies with poorer patterns in 2016 and trended that way in 2017. Pesticide

exposure may have influenced brood survivorship and thus brood pattern, but this warrants further investigation.

In this study, we differentiated between queen and colony measures as possible causes of poor sealed brood patterns, but the queen and her colony are not mutually exclusive. Every colony phenotype is a result of both environment and genetics: how a queen's offspring interacts with the environment, which includes nutrition, pesticides, pathogens, and beekeeper management practices. After the queen exchange in 2017, we allowed queens to lay for 21 days before removing her from the colony for sampling. It is possible that if we had left the queen in the colony and sampled after 6 weeks—when the worker bees would have been progeny of the transferred queen—that we would have been able to see if the designation of poor or good brood patterns held with the new work force. Replacing the queen could result in a better brood pattern if the colony environment remained the same and the new workers were better able to thrive in that environment.

For practical purposes, the questions important to beekeepers are action-based: under what conditions will the colony improve if the queen is replaced? Further studies on brood pattern could help elucidate the cause(s) and indicate management steps to take. A full reciprocal transplant—exchanging queens between two good-brood colonies, between two poor-brood colonies, and the same queen exchanges performed in this study—could help tease out colony vs. queen effects on brood pattern by controlling for the influence of transferring queens and the changes in environmental conditions that occur as the season progresses. Further studies could investigate colony effects on egg laying patterns by caging the queen on a frame, noting the egg pattern, then following the brood viability over time. Collecting longitudinal data on pathogens and immune genes

could help determine if the brood pattern changes as these factors change. Additional measures could be included to more thoroughly judge queen quality, including the number of patrilines (Tarpy et al. 2013) and queen pheromone profile (Kocher and Grozinger 2011; Niño et al. 2013). To make the study more robust, it could be done at different times of year and with different ages of queens.

An important lesson from this study was that it was difficult to find queens with poor brood patterns without signs of brood disease. If queen failure is a leading cause of colony loss, then other symptoms besides poor brood patterns are likely to be more relevant. Beekeepers report multiple symptoms associated with younger queens failing, including stunted colony growth, relatively low brood production, irregular egg laying pattern, supersedure of apparently healthy queens, or queen death without replacement. These different symptoms may be attributed to different causes, so defining the specific symptoms and measures used to identify "failing" queens is critical to make progress in mitigating queen failures. Specifying details like queen age can make a difference in interpretations of measures like sperm viability that can decrease as queens age (Lodesani et al. 2004; Tarpy and Olivarez 2014). Quantifying the prevalence of different definitions of queen failure could help research target issues, and a specific definition would allow for the work to be repeatable.

Operation 1 serves as an example of why a specific definition of queen failure matters. Operation 1 selected colonies for us to sample that matched a different definition of "queen failure": colonies were selected based on relatively small amounts of brood—19 of approximately 800 inspected colonies—and we sampled those colonies with the worst brood patterns. In these preselected poor-brood colonies the immune systems of the worker bees were upregulated, making it appear that colony environment influenced

sealed brood pattern. Because sealed brood pattern was not the primary symptom used to identify the colonies, in effect we were examining a different type of failure. The definition of “failing” used by Operation 1 may be more relevant to beekeepers, although it again may not reliably be tied to queen quality.

4.6 Conclusions

Brood pattern alone was an insufficient proxy of queen quality. In future studies, it is important to define the specific symptoms of queen failure being studied in order to address issues in queen health.

4.7 Acknowledgments

We would like to thank Dennis vanEngelsdorp for his suggestions to improve the manuscript, Megan Mahoney for help in collecting queens, Deniz Chen for processing queen samples, the anonymous reviewers for their suggestions that strengthened the manuscript, and the beekeepers for their participation, support, and ideas.

4.8 Figures

Figure 4.1 Experimental design. (**a**) In 2016, 17 poor-brood pattern and 17 good-brood pattern colonies were identified, colony metrics recorded, and queens collected and shipped live for analysis. (**b**) In 2017, 21 poor-brood pattern and good-brood pattern colony pairs were identified (42 total colonies), colony metrics recorded, and samples taken. On the same day, queens were exchanged between poor-brood pattern and good-brood pattern colony pairs. In approximately 24 days, colony metrics were again recorded, and queens collected and shipped live for analysis.

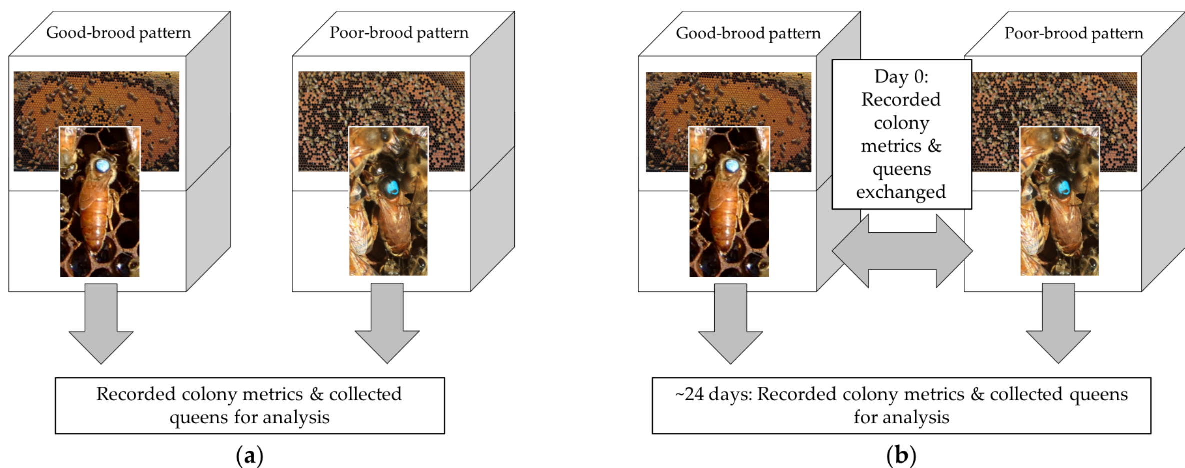


Figure 4.2 Sealed brood patterns rated a 1 (**left**), 3 (**middle**), and 5 (**right**). Photo credit: Rob Snyder.

Figure 4.3 The transcription levels (means ± 95% CI)) relative to the reference gene actin for the two immune gene peptides defensin (**a**) and hymenoptacin (**b**), and the heat shock protein HSP70ab-like (**c**). The significance asterisks indicate that the only significant comparisons were between the worker bees from good-brood colonies (light grey) compared poor-brood colonies (dark grey) within Operation 1.

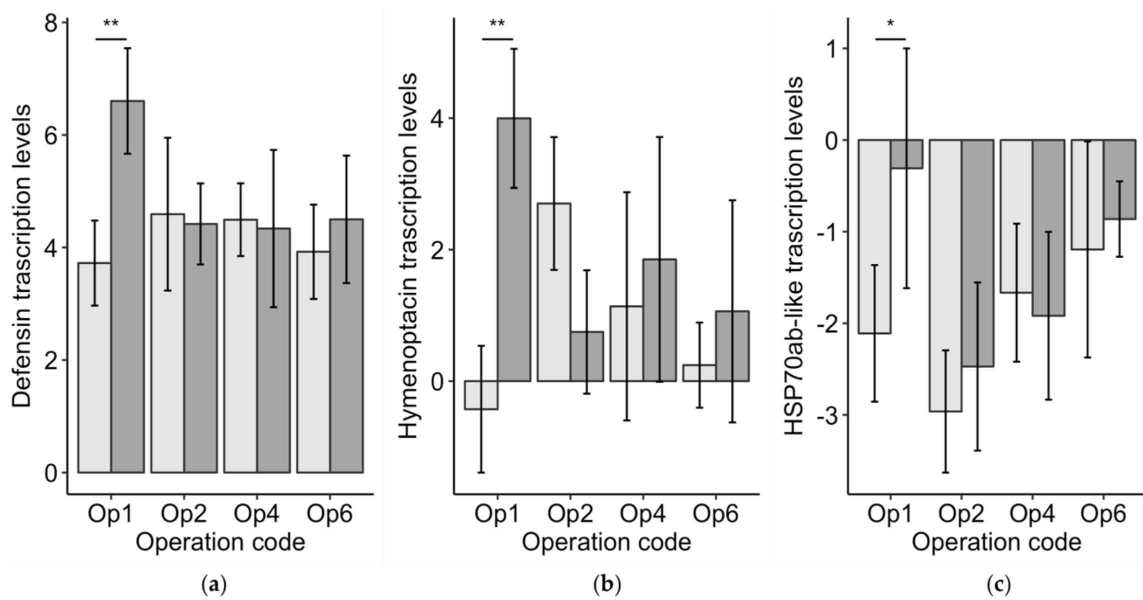
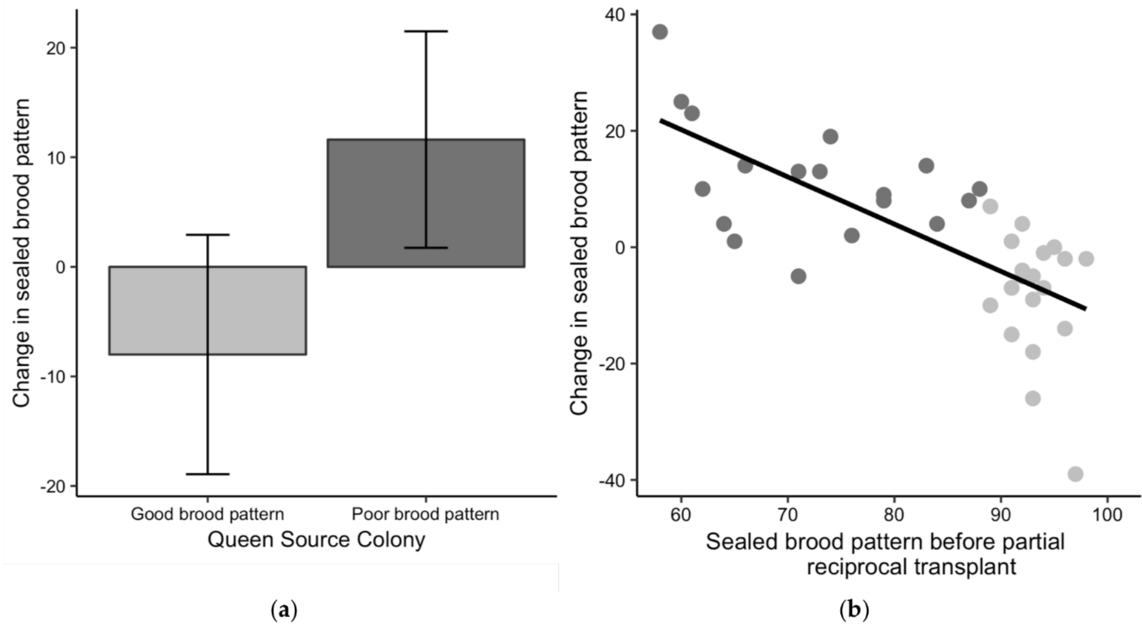


Figure 4.4 Changes in sealed brood pattern for the partial reciprocal transplant experiment in 2017. (**a**) Comparison of the change in the sealed brood pattern—a queen's percent of sealed brood cells after the exchange minus her percent of sealed brood cells before the exchange—to the initial brood pattern classification of good (light grey) or poor (dark grey). Positive values indicate an improved brood pattern after the exchange and negative values indicate the pattern was worse after the exchange. (**b**) The potential for change in brood pattern based on the variability in the starting brood patterns.



(**a**) (**b**)

Figure 4.5 Changes in egg pattern for the partial reciprocal transplant experiment in 2017. (**a**) Comparison of the change in the egg pattern—a queen's egg pattern after the exchange minus her egg pattern before the exchange—to the initial sealed brood pattern classification of good (light grey) or poor (dark grey). Positive values for the change in egg pattern indicate that the brood pattern improved after the exchange and negative values indicate the pattern was worse after the exchange. (**b**) The potential for change in egg pattern based on variability in the starting egg patterns.

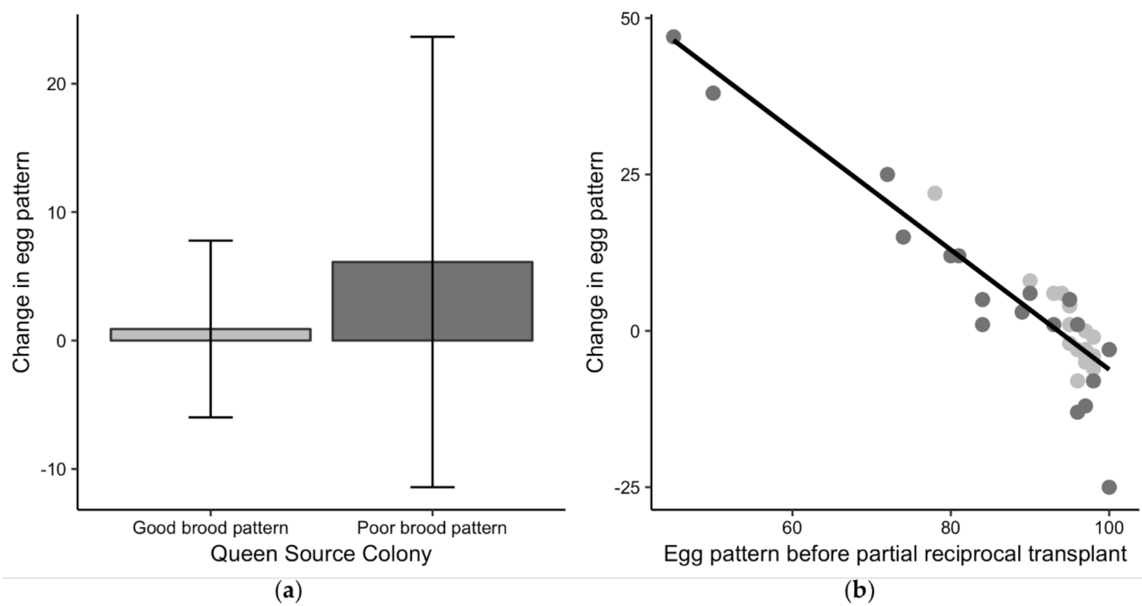
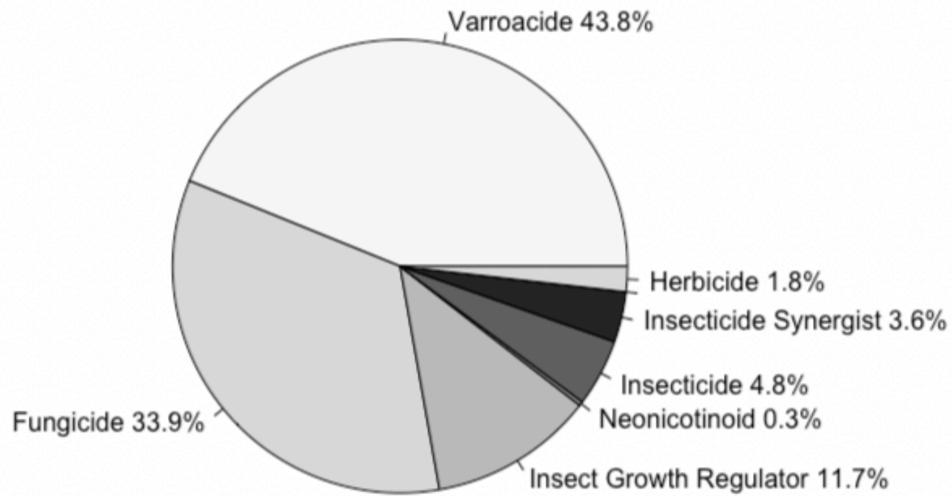
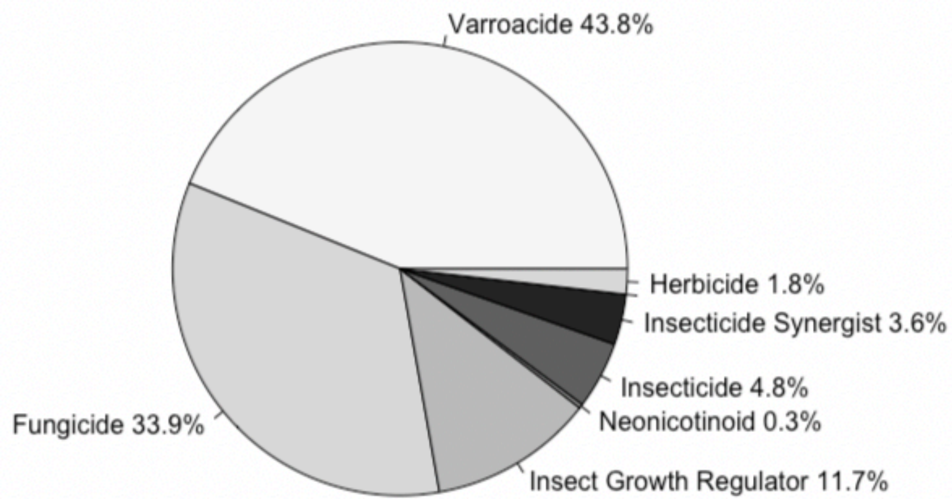


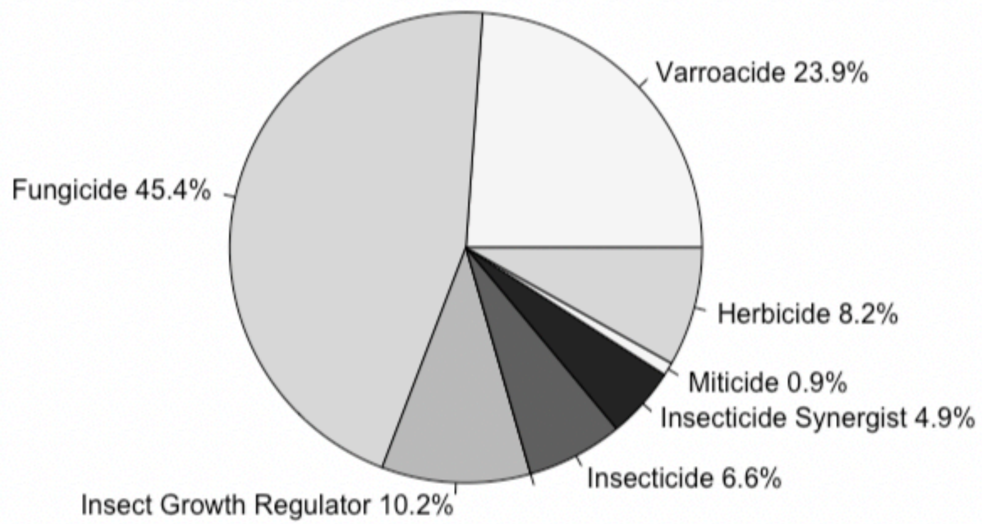
Figure S4.1. The relative percent of pesticide classes found in beeswax samples from a) 2016 and b) 2017.

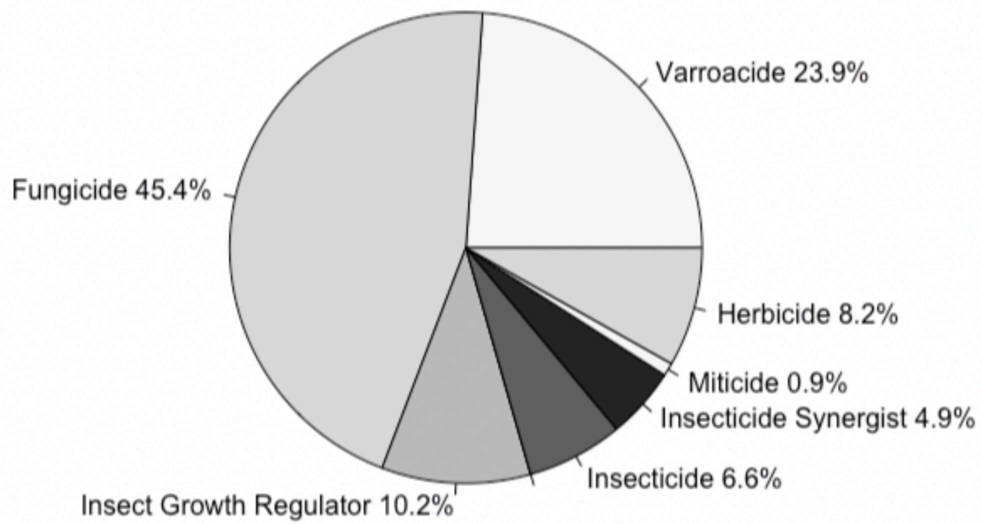
a.





b.





4.9 Tables

Table 4.1 The current study's summary of queen quality results compared to the results from previous studies (Delaney et al. 2011; Tarpy et al. 2012), including the number of queens tested (*n*) and the mean (±SD) values of morphometric and mating quality measures. Queens from this study are compared between brood pattern groups, with the queens from 2017 classified by their source colony status (before the exchange) of good-brood or poor-brood.

Paper (year)	Brood Pattern	*n*	Sperm Count, Millions (range)	Poorly Mated [1]	Sperm Viability (%)	Weight (mg)	Thorax Width (mm)	Head Width (mm)
This study (2016)	Good-brood	17	6.74 ± 1.95 (2.55–9.37)	6%	83.7 ± 6.3	223.9 ± 17.1	4.89 ± 0.15	3.79 ± 0.14
	Poor-brood	17	5.07 ± 2.51 (0.52–8.09)	24%	78.3 ± 11.2	216.5 ± 23.0	4.85 ± 0.18	3.8 ± 0.12
This study (2017)	Good-brood	19	5.69 ± 1.82 (1.39–8.4)	11%	78.0 ± 6.4	223.6 ± 27.9	4.93 ± 0.19	3.83 ± 0.10
	Poor-brood	18	5.88 ± 1.57 (2.8–8.1)	6%	78.3 ± 10.9 [2]	231.8 ± 23.0	4.94 ± 0.19	3.83 ± 0.09
Delaney et al. (2011)	NA	114	3.99 ± 1.50 (0.2–9.0)	18.6%	NA	184.8 ± 21.7	4.35 ± 0.19	3.62 ± 0.12
Tarpy et al. (2012)	NA	61	4.37 ± 1.45	13.6%, & 1 virgin queen	83.7 ± 3.3	218.7 ± 20.7	4.34 ± 0.23	3.45 ± 0.23

[1] Percent of queens tested that had <3 million sperm in their spermatheca (Woyke 1962).
[2] Queen with 1% sperm viability removed from summary.

Table 4.2 Summary of the odds ratios (95% CI range) and the percent of positive pathogen detections using PCR for worker bee samples (5 g composite sample) in 2017 and queen samples in 2016 and 2017 from colonies with good-brood or poor-brood patterns. Only pathogens with positive detections are included; chronic bee paralysis virus was not found in any samples. Also included are the comparisons between poor-brood and good-brood colonies with symptoms of the brood disease chalkbrood, and worker bee samples with *Varroa destructor* mite levels >3 mites per 100 bees, and *Nosema* spp. levels >1 million spores per bee as determined by microscopy. No pathogen had significantly higher odds of being in a poor-brood pattern colony or queen.

		% of Samples with Positive Detections		
Sample Type (year)	**Factor**	**Good-Brood**	**Poor-Brood**	**Odds Ratio (95% CI)**
Queens (2016)	No pathogens detected	33	13	0.31 (0.05–1.93)
	Black Queen Cell Virus	7	0	0.31 (0.01–8.29)
	Deformed Wing Virus type A	40	60	2.25 (0.52–9.70)
	Deformed Wing Virus type B	53	73	2.41 (0.52–11.1)
	Lake Sinai Virus	13	0	0.17 (0.01–3.96)
Queens (2017) [1]	No pathogens detected	79	78	0.93 (0.2–4.47)
	Deformed Wing Virus type A	5	11	2.25 (0.19–27.22)
	Deformed Wing Virus type B	16	17	1.07 (0.19–6.13)
	Israeli Acute Paralysis Virus	5	0	0.33 (0.01–8.73)
Worker bees (2016)	>3 *Varroa* mites per bee	6	6	1.00 (0.06–17.41)
	>1 million *Nosema* spores per bee, by microscopy	12	18	1.61 (0.23–11.09)
Worker bees (2017) [2]	>3 *Varroa* mites per bee	0	5	3.15 (0.12–81.74)
	>1 million *Nosema* spores per bee, by microscopy	33	48	1.82 (0.52–6.33)
	Acute Bee Paralysis Virus	10	5	0.48 (0.04–5.68)
	Black Queen Cell Virus	38	19	0.38 (0.09–1.55)
	Deformed Wing Virus type A	5	14	3.33 (0.32–34.99)
	Deformed Wing Virus type B	24	43	2.40 (0.64–9.03)
	Israeli Acute Paralysis Virus	19	5	0.21 (0.02–2.09)
	Lake Sinai Virus	100	100	1.00 (0.02–52.74)
	Trypanosomes	10	19	2.24 (0.36–13.78)
	Nosema spp., by PCR	90	86	0.63 (0.09–4.23)
Brood disease (2016)	Chalkbrood	6	0	0.31 (0.01–8.27)
Brood disease (2017)	Chalkbrood [3]	52	76	2.91 (0.78–10.89)

[1] Queens in 2017 were sampled after the queen exchange but classified by their source colony status (before the exchange) of good-brood or poor-brood.
[2] Sampled before the queen exchange.
[3] Accounts for chalkbrood found before and/or after queen exchange.

Table S4.1. Summary of each pesticide found in wax samples from colonies with good sealed brood patterns (2016: n = 14; 2017: n = 12) and poor sealed brood patterns (2016: n = 14; 2017: n = 12), including the mean ppb, mean hazard quotient (HQ), limit of detection (LOD) for 2016 and 2017, pesticide class, adult bee contact LD50, and the percent of positive detections. The adult bee contact LD50 was determined using the US EPA Ecotox Database (US EPA Ecotox Database) unless otherwise noted.

				2016						2017					
				Good-brood colony			Poor-brood colony			Good-brood colony			Poor-brood colony		
Pesticide	LOD (ppb), 2016/2017 (NT = not tested)	Class[1]	LD_{50}	Mean ppb	Mean HQ	% pos. detections	Mean ppb	Mean HQ	% pos. detections	Mean ppb	Mean HQ	% pos. detections	Mean ppb	Mean HQ	% pos. detections
2,4 Dimethylphenyl formamide (DMPF)	5/1.5	VARRO	75[3]	60.4	0.8	100	61.1	0.8	100	326.1	4.0	100	452.7	6.0	100
Acetochlor	15/100	HERB	1715							16.7	0.0	17	16.7	0.0	17
Atrazine	4/4	HERB	97	0.2	0.0	6	0.0	0.0	0			0			0
Azoxystrobin	5/1	FUNG	200							2.9	0.0	50	6.1	0.0	75
Bentazon	NT/30	HERB	100							0.0	0.0	0	2.5	0.0	8
Boscalid	10/5	FUNG	200	4.2	0.0	35	5.9	0.0	41	10.8	0.1	92	26.0	0.1	83
Buprofezin	60/2	IGR	200							1.3	0.0	33	31.9	0.2	67
Captan	50/250	FUNG	215	0.0	0.0	0	8.8	0.0	6			0			0
Carbaryl	2/5	INSECT	1.1	2.4	2.1	18	0.3	0.3	12	0.0	0.0	0	11.0	10.0	17
Carbendazim MBC	5/2	FUNG	50	1.0	0.0	18	6.6	0.1	41	18.4	0.3	50	6.5	0.1	67
Chlorothalonil	100/250	FUNG	181.3							41.7	0.2	17	62.5	0.3	25
Chlorpropham CIPC	10/15	HERB	36.6							6.1	0.2	25	100.3	2.7	25
Chlorpyrifos	5/5	INSECT	0.01							5.9	546.2	67	9.2	916.7	83
Chlorthal-dimethyl DCPA	NT/2	HERB	100[4]							0.0	0.0	0	0.6	0.0	17
Coumaphos	3/4	VARRO	20[5]	42.2	2.1	94	48.9	2.4	88	226.1	10.4	83	617.8	30.9	100
Coumaphos oxon	2/0.5	VARRO	20[5]	2.4	0.1	71	4.6	0.2	71	10.6	0.5	75	20.6	1.0	83
Cyfluthrin total	10/25	INSECT	0.037	19.4	523.1	24	8.9	240.1	18						
Cyhalothrin total	5/100	INSECT	0.022	1.2	53.5	6	0.7	32.1	6			0			0
Cyprodinil	10/2	FUNG	784	10.5	0.0	29	87.1	0.1	71	30.5	0.0	83	42.5	0.1	92
DDE, p,p'	5/2	INSECT	6.4[3]							0.3	0.0	17	0.3	0.1	17
Difenoconazole	10/4	FUNG	101	1.2	0.0	12	1.4	0.0	6						
Diflubenzuron	5/2	IGR	114	7.1	0.1	35	17.4	0.2	65	6.3	0.1	42	13.0	0.1	75

Diuron	NT/1	HERB	145							0.1	0.0	8	0.4	0.0	25
Endosulfan II	10/10	INSECT	7.1							0.4	0.1	8	0.0	0.0	0
Ethion	15/25	INSECT	11[5]							0.0	0.0	0	2.1	0.2	8
Fenamidone	30/1	FUNG	47.1							0.2	0.0	17	0.2	0.0	17
Fenbuconazole	15/3	FUNG	292	1.6	0.0	12	1.8	0.0	12	0.9	0.0	25	11.3	0.0	50
Fenpyroximate	4/3	VARRO	40	64.9	1.6	82	140.6	3.5	65	215.7	5.0	83	200.1	5.0	67
Fluopyram	5/1	FUNG	100	0.0	0.0	0	1.5	0.0	12	4.0	0.0	58	2.2	0.0	83
Fluvalinate	5/25	VARRO	8.1	132.4	16.3	71	189.5	23.4	65	117.5	13.4	100	446.8	55.2	100
Fluxapyroxad	5/2	FUNG	370.4							5.8	0.0	67	4.8	0.0	83
Hexythiazox	15/2	MITI	200							0.5	0.0	25	0.0	0.0	0
Imidacloprid	6/5	NEONIC	0.036	0.4	9.8	6	0.0	0.0	0						
Indoxacarb	30/7	INSECT	0.18	0.0	0.0	0	2.8	15.4	6			0			0
Iprodione	15/100	FUNG	200							442.9	2.0	83	571.3	2.9	100
Malathion	10/25	INSECT	0.64	0.9	1.4	6	0.0	0.0	0						
Mandipropamide	NT/2	FUNG	200							0.0	0.0	0	0.2	0.0	8
Metalaxyl	5/1	FUNG	100	0.0	0.0	0	0.3	0.0	6						
Metconazole	10/5	FUNG	100[4]	0.6	0.0	6	8.4	0.1	18	2.1	0.0	25	1.8	0.0	33
Methoxyfenozide	5/1	IGR	100	3.7	0.0	24	14.6	0.1	71	8.9	0.1	92	20.3	0.2	100
Metolachlor	5/25	HERB	110							6.3	0.1	25	8.3	0.1	33
MGK 264[2]	25/7	INS SYN								1.1	0.0	8	4.3	0.0	17
Myclobutanil	15/7	FUNG	33.9[4]							2.3	0.1	8	0.0	0.0	0
Novaluron	NT/5	IGR	100							0.0	0.0	0	1.0	0.0	17
Oxyfluorfen	5/100	HERB	100	1.4	0.0	18	1.1	0.0	12	0.0	0.0	0	10.8	0.1	8
Pendimethalin	15/50	HERB	100[4]							31.4	0.3	67	43.1	0.4	92
Penthiopyrad	NT/1	FUNG	500							1.0	0.0	17	7.3	0.0	67
Piperonyl butoxide	11/12	INS SYN	11	175.3	15.9	47	35.3	3.2	24	58.1	4.9	100	80.2	7.3	100
Prometon	NT/1	HERB	36							0.1	0.0	8	0.0	0.0	0
Propargite	15/2	MITI	15							1.4	0.1	17	0.0	0.0	0
Propiconazole	15/2	FUNG	25	2.6	0.1	18	22.9	0.9	41	29.1	1.1	92	13.3	0.5	92
Pyraclostrobin	5/2	FUNG	100	10.6	0.1	82	17.0	0.2	88	18.2	0.2	83	36.5	0.4	92
Pyridaben	5/2	INSECT	0.024							0.0	0.0	0	0.1	3.5	8

Pyrimethanil	100/5	FUNG	100	1.5	0.0	6	6.2	0.1	18	5.8	0.1	42	12.0	0.1	42
Pyriproxyfen	5/1	IGR	74	1.2	0.0	24	0.6	0.0	12	0.2	0.0	8	2.6	0.0	33
Spinetoram	NT/15	INSECT	0.024[4]							0.0	0.0	0	1.3	52.1	8
Spinosad	15/7	INSECT	0.047							1.8	34.4	25	2.9	62.1	42
Tebuconazole	15/5	FUNG	200[4]	2.6	0.0	18	7.6	0.0	47	3.8	0.0	42	8.7	0.0	50
Tetraconazole	15/2	FUNG	63[4]	1.8	0.0	12	0.9	0.0	6						
Thymol	50/2	VARRO	1273[4]	284.1	0.2	29	532.4	0.4	24	1582.0	1.1	100	1574.4	1.2	100
Trifloxystrobin	10/1	FUNG	200	0.0	0.0	0	2.2	0.0	6	6.0	0.0	58	7.1	0.0	83
Triflumizole	40/1	FUNG	160							0.2	0.0	17	0.1	0.0	8

[1] HERB = herbicide; FUNG = funigicide, IGR = Insect Growth Regulator; INSECT = insecticide; INS SYN = insecticide synergist; MITI = miticide; NEONIC = neonicotinoid insecticide; VARRO: varroacides applied by beekeepers for control of *V. destructor*.
[2] Not included in HQ calculations as it has no reported honey bee contact LD_{50}.
[3] LD_{50} determined using [23].
[4] LD_{50} determined using [43].
[5] LD_{50} determined using [44].

Summary of Thesis

This dissertation frames honey bee health in commercial beekeeper operations by describing colony mortality and identifying factors that increase the probability of colony mortality or morbidity. Chapter 1 serves as an introduction to epidemiological methods used to study honey bees and demonstrates their advantage in analyzing real-world data. Chapter 2 puts colony losses into context and identifies colony mortality risk factors using the results of a survey. Chapter 3 is an observational study that identifies colony health inspection metrics that predict colony health or survivorship, thereby providing data-based rationales for beekeepers to adjust management plans to prevent losses. Chapter 4 examines a colony phenotype often attributed to a failing queen, a risk factor identified in Chapters 2 and 3.

Chapter 1 reviews research efforts that used surveillance to quantify colony mortality, describe disease prevalence and incidence, and identify risk factors. These efforts can be used to highlight potential areas of further research and measure the improvements of honey bee health.

Chapter 2 and the other annual loss surveys demonstrate that commercial beekeeper colony mortality is lower on average compared to other beekeeper groups, but there is variability in the level of loss. This result suggests that there are practices that commercial beekeepers can implement in their operations to reduce colony death. Indeed, Chapter 3 identifies a number inspection metrics, including metrics that are influenced by management, that were predictive of colony health or survivability. For commercial operations based in Upper Midwest for honey production, monitoring *Varroa destructor*

mite populations and keeping levels below 1 mite per 100 bees before honey production and below 3 mites per 100 bees during honey production can decrease the probability a colony will be nonviable before winter months. Colonies with a larger population of adult bees before and during honey production had a higher probability of having higher mite loads and being nonviable after honey production, so it is especially important that larger colonies have low mite levels before honey supers are added. *Varroa* mite treatments of amitraz- or formic acid-based products kept mite levels lower, but other treatments could be used if the efficacy post-treatment is checked and adequate (<1 mites per 100 bees in spring), or potentially if colonies are kept small. Treatment with fumagillin for *Nosema* spp. did not predict colony viability. *Nosema* spp. levels above 10^6 spores per bee in fall were more likely to be nonviable in winter, but the prevalence of colonies above the threshold was low and appeared to be associated with other health metrics. Finally, a colony with a gap in a having a queen was more likely to be nonviable at the next sampling period, suggesting that if a colony has a queen event it may be less expensive to take the colony loss in fall than rather than transporting the colony to a wintering location.

Results from Chapter 4 indicate that poor brood pattern was not a reliable indicator of queen quality. While specific colony environmental measures were not identified as causing poor patterns, the one operation that chose colonies based on relative adult bee population size had higher levels of heat shock proteins and immune genes, suggesting that the immune systems of those bees were more active. This result highlights the importance of defining queen failure as different symptoms can be attributed to different causes. Other definitions of queen failure may be more relevant to

colony mortality as indicated by Chapters 2 and 3. Further research should address different types of queen failure.

In summary, these studies have added to the body of research on beekeeping and improved the understanding of the problems that beekeepers face. These results can be translated into practical recommendations for commercial beekeepers in the Upper Midwest and result in improved colony health and survivorship.

Bibliography

Ahn, K., Xie, X., Riddle, J., Pettis, J. and Huang, Z.Y. 2012. Effects of Long Distance Transportation on Honey Bee Physiology. *Psyche: J Entomol* 2012: 1–9.

Aizen, M., Garibaldi, L., Cunningham, S., and Klein, A.M. 2008. Long-term global trends in crop yield and production reveal no current pollination shortage but increasing pollinator dependency. *Curr Biol* 18 (20): 1572–1575.

Aizen, M., and Harder, L.D. 2009. The global stock of domesticated honey bees is growing slower than agricultural demand for pollination. *Curr Biol* 19(11): 915–918.

Alaux, C., Folschweiller, M., McDonnell, C., Beslay, D., Cousin, M., Dussaubat, C., Brunet, J.-L. and Conte, Y.L. 2011. Pathological effects of the microsporidium *Nosema ceranae* on honey bee queen physiology (*Apis mellifera*). *J Invertebr Pathol* 106 (3): 380–385.

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

Amdam, G.V. and Omholt, S.W. 2002. The Regulatory Anatomy of Honeybee Lifespan. *J Theor Biol* 216 (2): 209–228.

Amiri, E., Strand, M., Rueppell, O. and Tarpy, D. 2017. Queen Quality and the Impact of Honey Bee Diseases on Queen Health: Potential for Interactions between Two Major Threats to Colony Health. *Insects* 8 (2): 48.

Anscombe, F.J. 1956. On estimating binomial response relations. *Biometrika 4*: 461–464.

Aston, D. 2010. Honey bee winter loss survey for England, 2007–8. *J Apic Res* 49 (1): 111–112.

Australian Government, Department of Agriculture Fisheries and Forestry: Communique: Response to Asian Honeybees. 2010. Accessed at: http://www.agriculture.gov.au/SiteCollectionDocuments/about/media-centre/communique-asian-honeybee.pdf (on 2015.01.28)

Baer, B., Collins, J., Maalaps, K. and den Boer, S.P.A. 2016. Sperm use economy of honeybee (*Apis mellifera*) queens. *Ecol Evol* 6 (9): 2877–2885.

Banaszak, J., Banaszak-Cibicka, W., and Szefer, P. 2014. Guidelines on sampling intensity of bees (Hymenoptera: Apoidea: Apiformes). *J Insect Conserv* (18):651-656.

Bartoń, Kamil. 2018. MuMIn: Multi-Model Inference. R package version 1.42.1. https://CRAN.R-project.org/package=MuMIn.

Bates, D., Maechler, M., Bolker, B., and Walker, S. 2015. Fitting Linear Mixed-Effects Models Using lme4. *J Stat Softw* 67(1): 1-48.

Bekele, A.Z., Mor, S.K., Phelps, N.B.D., Goyal, S.M. and Armién, A.G. 2015. A case report of *Nosema ceranae* infection in honey bees in Minnesota, USA. *Vet Quart* 35 (1): 48–50.

Berthoud, H., Imdorf, A., Haueter, M., Radloff, S., and Neumann, P. 2010. Virus infections and winter losses of honey bee colonies (*Apis mellifera*). *J Apic Res* (49): 60–65.

Botías, C., Martín-Hernández, R., Barrios, L., Meana, A. and Higes, M. 2013. *Nosema* spp. infection and its negative effects on honey bees (*Apis mellifera* iberiensis) at the colony level. *Vet Res* 44 (1): 25.

Brodschneider, R. and Crailsheim, K. 2010. Nutrition and health in honey bees. *Apidologie* 41 (3): 278–294.

Brodschneider, R., Moosbeckhofer, R., and Crailsheim, K. 2010. Surveys as a tool to record winter losses of honey bee colonies: a two year case study in Austria and South Tyrol. *J Apic Res* 49 (1): 23–30.

Büchler, R., Costa, C., Hatjina, F., Andonov, S., Meixner, M.D., Conte, Y.L., Uzunov, A., Berg, S., Bienkowska, M., Bouga, M., Drazic, M., Dyrba, W., Kryger, P., Panasiuk, B., Pechhacker, H., Petrov, P., Kezić, N., Korpela, S. and Wilde, J. 2014. The influence of genetic origin and its interaction with environmental effects on the survival of *Apis mellifera L.* colonies in Europe. *J Apic Res* 53 (2): 205–214.

Burgett, M. and Burikam, I. 1985. Number of adult honey bees (Hymenoptera: Apidae) occupying a comb: a standard for estimating colony populations. *J Econ Entomol*, *78*(5):1154-1156.

Burley, L.M., Fell, R.D. and Saacke, R.G. 2008. Survival of Honey Bee (Hymenoptera: Apidae) Spermatozoa Incubated at Room Temperature from Drones Exposed to Miticides. *J Econ Entomol* 101 (4): 7.

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

Cameron, S.A., Lozier, J.D., Strange, J.P., Koch, J.B., Cordes, N., Solter, L.F., and Griswold, T.L. 2011. Patterns of widespread decline in North American bumble bees. *P Natl Acad Sci* 108:662-667

Cantwell, G.E. 1970. Standard methods for counting *Nosema* spores. Amer Bee J 110: 222–223.

Caron, D.M. 2009. *Honey bee biology and beekeeping*. Wicwas Press, Cheshire, CT, USA.

Chaimanee, V., Chantawannakul, P., Chen, Y., Evans, J.D. and Pettis, J.S. 2014. Effects of host age on susceptibility to infection and immune gene expression in honey bee queens (*Apis mellifera*) inoculated with *Nosema ceranae*. *Apidologie* 45 (4): 451–463.

Chaimanee, V., Evans, J.D., Chen, Y., Jackson, C. and Pettis, J.S. 2016. Sperm viability and gene expression in honey bee queens (*Apis mellifera*) following exposure to the neonicotinoid insecticide imidacloprid and the organophosphate acaricide coumaphos. *J Insect Physiol* 89: 1–8.

Charrière, J.-D., and Neumann, P. 2010. Surveys to estimate colony losses in Switzerland. *J Apic Res* 49 (1): 132–133.

Chauzat, M.-P., Martel, A.-C., Zeggane, S., Drajnudel, P., Schurr, F., Clément, M.-C., Ribière-Chabert, M., Aubert, M. and Faucon, J.-P. 2010. A case control study and a survey on mortalities of honey bee colonies (*Apis mellifera*) in France during the winter of 2005–6. *J Apic Res* 49 (1): 40–51.

Chauzat M.-P., Laurent, M., Riviere, M.-P., Saugeon, C., Hendrikx, P., Ribiere-Chabert, M.: Honeybee Pathology Unit. 2014. A pan-European Epidemiological Study on Honeybee Colony Losses 2012–2013. European Union Reference Laboratory for honeybee health (EURL).

Chen, Y.-W., Chung, W.-P., Wang, C.-H., Solter, L.F., and Huang, W.-F. 2012. *Nosema ceranae* infection intensity highly correlates with temperature. *J Invertebr Pathol* (111):264-267.

Clermont, A., Eickermann, M., Kraus, F., Georges, C., Hoffmann, L., and Beyer, M. 2014. A survey on some factors potentially affecting losses of managed honey bee colonies in Luxembourg over the winters 2010/2011 and 2011/2012. *J Apic Res* (53):43-56.

Collins, A.M. 2000. Relationship between semen quality and performance of instrumentally inseminated honey bee queens. *Apidologie* 31 (3): 421–429.

Collins, A.M., Williams, V. and Evans, J.D. 2004. Sperm storage and antioxidative enzyme expression in the honey bee, *Apis mellifera*. *Insect Molecular Biology* 13 (2): 141–146.

Cornman, R.S., Tarpy, D.R., Chen, Y., Jeffreys, L., Lopez, D., Pettis, J.S., vanEngelsdorp, D. and Evans, J.D. 2012. Pathogen Webs in Collapsing Honey Bee Colonies. *PLoS ONE* 7 (8): e43562.

Corona, M., Velarde, R.A., Remolina, S., Moran-Lauter, A., Wang, Y., Hughes, K.A. and Robinson, G.E. 2007. Vitellogenin, juvenile hormone, insulin signaling, and queen honey bee longevity. *P Natl A Sci* 104 (17): 7128–7133.

Currie, R.W. and Gatien, P. 2006. Timing acaricide treatments to prevent *Varroa destructor* (Acari: Varroidae) from causing economic damage to honey bee colonies. *The Can Entomol 138*(2): 238-252.

Daberkow, S., Korb, P. and Hoff, F. 2009. Structure of the U.S. Beekeeping Industry: 1982–2002. *J Econ Entomol* 102 (3): 868–886.

Dahle, B. 2010. The role of *Varroa destructor* for honey bee colony losses in Norway. *J Apic Res* 49 (1): 124–125.

Dainat, B., Evans, J.D., Chen, Y.P., Gauthier, L. and Neumann, P. 2012. Predictive markers of honey bee colony collapse. *PLoS one* 7(2): p.e32151.

Dainat, B., Evans, J.D., Chen, Y.P., Gauthier, L. and Neumann, P. 2012. Dead or alive: deformed wing virus and *Varroa destructor* reduce the life span of winter honeybees. *Appl Environ Microb 78*(4): 981-987.

De Jong, D., De Andrea Roma, D. and GonçAlves, L.S. 1982. A comparative analysis of shaking solutions for the detection of *Varroa jacobsoni* on adult honeybees. *Apidologie* 13 (3): 297–306.

DeGrandi-Hoffman, G., Ahumada, F., Zazueta, V., Chambers, M., Hidalgo, G. and Watkins deJong, E. 2016. Population growth of *Varroa destructor* (Acari: Varroidae) in honey bee colonies is affected by the number of foragers with mites. *Exp Appl Acarol* 69(1): 21-34.

Delaney, D.A., Keller, J.J., Caren, J.R. and Tarpy, D.R. 2011. The physical, insemination, and reproductive quality of honey bee queens (*Apis mellifera* L.). *Apidologie* 42 (1): 1–13.

Delaplane, K.S., van der Steen, J. and Guzman-Novoa, E. 2013. Standard methods for estimating strength parameters of *Apis mellifera* colonies. *J Apic Res* 52 (1): 1-12.

Desai, S.D. and Currie, R.W. 2016. Effects of Wintering Environment and Parasite–Pathogen Interactions on Honey Bee Colony Loss in North Temperate Regions. *PLOS ONE* 11 (7): e0159615.

Dietemann, V., Nazzi, F., Martin, S.J., Anderson, D.L., Locke, B., Delaplane, K.S., Wauquiez, Q., Tannahill, C., Frey, E., Ziegelmann, B. and Rosenkranz, P. 2013. Standard methods for varroa research. *J Apic Res 52*(1): 1-54.

Dolezal, A.G., Carrillo-Tripp, J., Miller, W.A., Bonning, B.C. and Toth, A.L. 2016. Intensively cultivated landscape and varroa mite infestation are associated with reduced honey bee nutritional state. *PloS one* 11(4): p.e0153531.

Dussaubat, C., Maisonnasse, A., Crauser, D., Tchamitchian, S., Bonnet, M., Cousin, M., Kretzschmar, A., Brunet, J.-L. and Le Conte, Y. 2016. Combined neonicotinoid pesticide and parasite stress alter honeybee queens' physiology and survival. *Sci Rep* 6 (1).

Ellis, J.D., Evans, J.D., and Pettis, J. 2010. Colony losses, managed colony population decline, and Colony Collapse Disorder in the United States. *J Apic Res* 49 (1): 134–136.

Evans, J.D. and Pettis, J.S. 2005. Colony-level impacts of immune responsiveness in honey bees, *Apis mellifera*. *Evolution* 59 (10): 2270–2274.

Evans, J.D., Saegerman, C., Mullin, C., Haubruge, E., Nguyen, B.K., Frazier, M., Frazier, J., Cox-Foster, D., Chen, Y., Underwood, R. and Tarpy, D.R. 2009. Colony collapse disorder: a descriptive study. *PloS one* 4(8): p.e6481.

Even, N., Devaud, J.-M. and Barron, A. 2012. General Stress Responses in the Honey Bee. *Insects* 3 (4): 1271–1298.

Fenoy, S., Rueda, C., Higes, M., Martin-Hernandez, R., and del Aguila, C. 2009 High-level resistance of *Nosema ceranae*, a parasite of the honeybee, to temperature and desiccation. *Appl Environ Microbiol* 75:6886-6889.

Ferrier, P.M., Rucker, R.R., Thurman, W.N. and Burgett, M. 2018. Economic Effects and Responses to Changes in Honey Bee Health. USDA Economic Research Report Number 246.

Forsgren, E., and Fries, I. 2013. Temporal study of *Nosema* spp. in a cold climate. *Environ Microbiol Rep* (5):78-82.

Francis, R.M., Nielsen, S.L., and Kryger, P. 2013. *Varroa*–virus interaction in collapsing honey bee colonies. *PloS One* 8:e57540.

Frey, E. and Rosenkranz, P. 2014. Autumn Invasion Rates of *Varroa destructor* (Mesostigmata: Varroidae) Into Honey Bee (Hymenoptera: Apidae) Colonies and the Resulting Increase in Mite Populations. *J Econ Entomology* 107 (2): 508–515.

Fries, I., Aarhus, A., Hansen, H. and Korpela, S. 1991. Comparison of diagnostic methods for detection of low infestation levels of *Varroa jacobsoni* in honey-bee (*Apis mellifera*) colonies. *Exp Appl Acarol 10*(3-4): 279-287.

Gajger, I.T., Tomljanovic, Z., and Petrinec, Z. 2010. Monitoring health status of Croatian honey bee colonies and possible reasons for winter losses. *J Apic Res* 49 (1): 107–108.

Gallai, N., Salles, J.-M., Settele, J., and Vaissiere, B.E. 2009 Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. *Ecol Econ* (68):810-821.

Gardner, J.D., and Spivak, M. 2014 A survey and historical comparison of the megachilidae (Hymenoptera: Apoidea) of Itasca State Park, Minnesota. *Annals Entomol Soc Am* (107):983-993.

Garibaldi, L.A., Steffan-Dewenter, I., Winfree, R., Aizen, M.A., Bommarco, R., Cunningham, S.A., Kremen, C., Carvalheiro, L.G., Harder, L.D., Afik, O., Bartomeus, I., Benjamin, F., Boreux, V., Cariveau, D., Chacoff, N.P., Dudenhoffer, J.H., Freitas, B.M., Ghazoul, J., Greenleaf, S., Hipolito, J., Holzschuh, A., Howlett, B., Isaacs, R., Javorek, S.K., Kennedy, C.M., Krewenka, K.M., Krishnan, S., Mandelik, Y., Mayfield, M.M., Motzke, I., Munyuli, T., Nault, B.A., Otieno, M., Petersen, J., Pisanty, G., Potts, S.G., Rader, R., Ricketts, T.H., Rundlof, M., Seymour, C.L., Schuepp, C., Szentgyorgyi, H., Taki, H., Tscharntke, T., Vergara, C.H., Viana, B.F., Wanger, T.C., Westphal, C., Williams, N. and Klein, A.M. 2013. Wild Pollinators Enhance Fruit Set of Crops Regardless of Honey Bee Abundance. *Science* 339 (6127): 1608–1611.

Gauthier, L., Ravallec, M., Tournaire, M., Cousserans, F., Bergoin, M., Dainat, B. and de Miranda, J.R. 2011. Viruses Associated with Ovarian Degeneration in *Apis mellifera* L. Queens. *PLoS ONE* 6 (1): e16217.

Genersch, E., von der Ohe, W., Kaatz, H., Schroeder, A., Otten, C., Büchler, R., Berg, S., Ritter, W., Mühlen, W., Gisder, S., Meixner, M., Liebig, G. and Rosenkranz, P. 2010. The German bee monitoring project: a long term study to understand periodically high winter losses of honey bee colonies. *Apidologie* 41 (3): 332–352.

Geroff, R.K., Gibbs, J., and McCravy, K.W. 2014. Assessing bee (Hymenoptera: Apoidea) diversity of an Illinois restored tallgrass prairie: methodology and conservation considerations. *J Insect Conserv* 18:951-964.

Giacobino, A., Cagnolo, N.B., Merke, J., Orellano, E., Bertozzi, E., Masciangelo, G., Pietronave, H., Salto, C. and Signorini, M. 2014. Risk factors associated with the presence of *Varroa destructor* in honey bee colonies from east-central Argentina. *Prev Vet Med* 115 (3–4): 280–287.

Giacobino, A., Molineri, A., Cagnolo, N.B., Merke, J., Orellano, E., Bertozzi, E., Masciángelo, G., Pietronave, H., Pacini, A., Salto, C. and Signorini, M. 2015. Risk factors associated with failures of *Varroa* treatments in honey bee colonies without broodless period. *Apidologie* 46 (5): 573–582.

Giacobino, A., Molineri, A., Bulacio Cagnolo, N., Merke, J., Orellano, E., Bertozzi, E., Masciangelo, G., Pietronave, H., Pacini, A., Salto, C. and Signorini, M. 2016. Key management practices to prevent high infestation levels of *Varroa destructor* in honey bee colonies at the beginning of the honey yield season. *Prev Vet Med* 131: 95–102.

Giacobino, A., Pacini, A., Molineri, A., Bulacio Cagnolo, N., Merke, J., Orellano, E., Bertozzi, E., Masciangelo, G., Pietronave, H. and Signorini, M. 2017. Environment or beekeeping management: What explains better the prevalence of honey bee colonies with high levels of *Varroa destructor*? *Res Vet Sci* 112: 1–6.

Giacobino, A., Pacini, A., Molineri, A., Rodriguez, G., Crisanti, P., Cagnolo, N.B., Merke, J., Orellano, E., Bertozzi, E., Pietronave, H. and Signorini, M. 2018. Potential associations between the mite Varroa destructor and other stressors in honeybee colonies (Apis mellifera L.) in temperate and subtropical climate from Argentina. *Preventive veterinary medicine*, 159:143-152.

Giovenazzo, P., and Dubreuil P. 2011. Evaluation of spring organic treatments against *Varroa destructor* (Acari: Varroidae) in honey bee *Apis mellifera* (Hymenoptera: Apidae) colonies in eastern Canada. *Exp Appl Acarol* (55):65-76.

Gisder, S., Hedtke, K., Mockel, N., Frielitz, M.C., Linde, A. and Genersch, E. 2010. Five-Year Cohort Study of *Nosema* spp. in Germany: Does Climate Shape Virulence and Assertiveness of *Nosema ceranae*? *Applied and Environmental Microbiology* 76 (9): 3032–3038.

Gisder, S., Hedtke, K., Mockel, N., Frielitz, M.C., Linde, A. and Genersch, E. 2010. Five-Year Cohort Study of *Nosema* spp. in Germany: Does Climate Shape Virulence and Assertiveness of *Nosema ceranae*? *Appl and Environ Microb* 76 (9): 3032–3038.

Glenny, W., Cavigli, I., Daughenbaugh, K.F., Radford, R., Kegley, S.E. and Flenniken, M.L. 2017. Honey bee (*Apis mellifera*) colony health and pathogen composition in migratory beekeeping operations involved in California almond pollination. *PLOS ONE* 12 (8): e0182814.

Goblirsch, M., Huang, Z.Y. and Spivak, M. 2013. Physiological and Behavioral Changes in Honey Bees (*Apis mellifera*) Induced by *Nosema ceranae* Infection. *PLoS ONE* 8 (3): e58165.

Goblirsch, M. 2018. *Nosema ceranae* disease of the honey bee (*Apis mellifera*). *Apidologie* 49 (1): 131–150.

Graham, J.M. 1992. *The hive and the honey bee*. Dadant. Hamilton, Illinois.

Gray, A., Peterson, M., and Teale, A. 2010. An update on recent colony losses in Scotland from a sample survey covering 2006–2008. *J Apic Res* 49 (1): 129–131.

Gregorc, A., and Planinc, I. 2012 Use of thymol formulations, amitraz, and oxalic acid for the control of the *Varroa* mite in honey bee (*Apis mellifera* carnica) colonies. *J Apic Sci* (56):61-69.

Gunnarsson. B., and Federsel, L.M. 2014. Bumblebees in the city: abundance, species richness and diversity in two urban habitats. *J Insect Conserv* (18):1185-1191.

Guzmán-Novoa, E. and Page Jr, R.E., 1999. Selective breeding of honey bees (Hymenoptera: Apidae) in Africanized areas. *J Econ Entomol 92*(3): 521-525.

Haarmann, T., Spivak, M., Weaver, D., Weaver, B. and Glenn, T. 2002. Effects of Fluvalinate and Coumaphos on Queen Honey Bees (Hymenoptera: Apidae) in Two Commercial Queen Rearing Operations. *J Econ Entomol* 95 (1): 28–35.

Haldane, J.B.S. 1940. The mean and variance of the moments of chi-squaredwhen used as a test of homogeneity, when expectations are small. *Biometrika* 29: 133–134.

Harbo, J.R. and Harris, J.W. 2009. Responses to *Varroa* by honey bees with different levels of *Varroa* Sensitive Hygiene. *J Apic Res* 48 (3): 156–161.

Hatjina, F., Bouga, M., Karatasou, A., Kontothanasi, A., Charistos, L., Emmanouil, C., Emmanouil, N. and Maistros, A.-D. 2010. Data on honey bee losses in Greece: a preliminary note. *J. Apic. Res.* 49 (1): 116–118.

Hatjina, F., Costa, C., Büchler, R., Uzunov, A., Drazic, M., Filipi, J., Charistos, L., Ruottinen, L., Andonov, S., Meixner, M.D., Bienkowska, M., Dariusz, G., Panasiuk, B., Conte, Y.L., Wilde, J., Berg, S., Bouga, M., Dyrba, W., Kiprijanovska, H., Korpela, S., Kryger, P., Lodesani, M., Pechhacker, H., Petrov, P. and Kezic, N. 2014. Population dynamics of European honey bee genotypes under different environmental conditions. *J Apic Res* 53 (2): 233–247.

Hendrikx, P., Chauzat, M., Debin, M., Neuman, P., Fries, I., Ritter, W., Brown, M., Mutinelli, F., Le Conte, Y. and Gregorc, A. 2009. Bee Mortality and Bee Surveillance in Europe. EFSA Supporting Publications 6 (9).

Hertfordshire Pesticide Properties Database. Available online: http://sitem.herts.ac.uk/aeru/ppdb/en/ index.htm (accessed on 26 May 2018).

Hoehn, P., Tscharntke, T., Tylianakis, J.M., and Steffan-Dewenter, I. 2008. Functional group diversity of bee pollinators increases crop yield. *Proc R Soc Lond B Biol Sci* (275):2283-2291.

Honey Bee Health Coalition. 2018. Honey Bee Health Coalition's Tools for *Varroa* Management. The Keystone Policy Center.

Huang, Z. 2012. Pollen nutrition affects honey bee stress resistance. *Terr Arthropod Rev* 5 (2): 175–189.

Human, H., Brodschneider, R., Dietemann, V., Dively, G., Ellis, J.D., Forsgren, E., Fries, I., Hatjina, F., Hu, F.-L., Jaffé, R., Jensen, A.B., Köhler, A., Magyar, J.P., Özkýrým, A., Pirk, C.W.W., Rose, R., Strauss, U., Tanner, G., Tarpy, D.R., van der Steen, J.J.M., Vaudo, A., Vejsnæs, F., Wilde, J., Williams, G.R. and Zheng, H.-Q. 2013. Miscellaneous standard methods for *Apis mellifera* research. *J Apic Res* 52 (4): 1–53.

Isaacs, R., and Kirk, A.K. 2010. Pollination services provided to small and large highbush blueberry fields by wild and managed bees. *J Appl Ecol* (47):841-849.
Ivanova, E.N., and Petrov, P.P. 2010. Regional differences in honey bee winter losses in Bulgaria during the period 2006–9. *J Apic Res* 49 (1): 102–103.
Johnson, R.M. 2015. Honey bee toxicology. *Annu Rev Entomol* 60(1): 415-434.
Jones, C.M., and Brown, M.J.F. 2014. Parasites and genetic diversity in an invasive bumblebee. *J Anim Ecol* (83):1428-1440.
Kevill, J., Highfield, A., Mordecai, G., Martin, S. and Schroeder, D. 2017. ABC Assay: Method Development and Application to Quantify the Role of Three DWV Master Variants in Overwinter Colony Losses of European Honey Bees. *Viruses* 9 (11): 314.
Khoury, D.S., Myerscough, M.R. and Barron, A.B. 2011. A quantitative model of honey bee colony population dynamics. *PloS one 6*(4): p.e18491.
Klein, A.-M., Vaissiere, B.E., Cane, J.H., Steffan-Dewenter, I., Cunningham, S.A., Kremen, C. and Tscharntke, T. 2007. Importance of pollinators in changing landscapes for world crops. *P R Soc B-Biol Sci* 274 (1608): 303–313.
Kocher, S.D. and Grozinger, C.M. 2011. Cooperation, Conflict, and the Evolution of Queen Pheromones. *J Chem Ecol* 37: 1263–1275.
Koeniger, G., Koeniger, N., Ellis, J.D., and Connor, L. Mating Biology of Honey Bees (*Apis mellifera*). Wicwas Press LLC: Kalamazoo, MI, USA, 2014; ISBN 978-1-878075-38-3.
Kulhanek, K., Steinhauer, N., Rennich, K., Caron, D.M., Sagili, R.R., Pettis, J.S., Ellis, J.D., Wilson, M.E., Wilkes, J.T., Tarpy, D.R., Rose, R., Lee, K., Rangel, J. and vanEngelsdorp, D. 2017. A national survey of managed honey bee 2015–2016 annual colony losses in the USA. *J Apic Res* 56 (4): 328–340.
Le Conte, Y., Ellis, M., and Ritter, W. 2010. *Varroa* mites and honey bee health: can *Varroa* explain part of the colony losses? Apidologie 41 (3): 353–363
Lee, K.V., Moon, R.D., Burkness, E.C., Hutchison, W.D. and Spivak, M. 2010. Practical Sampling Plans for *Varroa destructor* (Acari: Varroidae) in *Apis mellifera* (Hymenoptera: Apidae) Colonies and Apiaries. *J Econ Entomol* 103 (4): 1039–1050.
Lee, K., Steinhauer, N., Travis, D.A., Meixner, M.D., Deen, J. and vanEngelsdorp, D. 2015. Honey bee surveillance: a tool for understanding and improving honey bee health. *Curr Opin Insect Sci* 10: 37–44.
Lee, K.V., Steinhauer, N., Rennich, K., Wilson, M.E., Tarpy, D.R., Caron, D.M., Rose, R., Delaplane, K.S., Baylis, K., Lengerich, E.J., Pettis, J., Skinner, J.A., Wilkes, J.T., Sagili, R., vanEngelsdorp, D., for the Bee Informed Partnership. 2015. A national survey of managed honey bee 2013–2014 annual colony losses in the USA. *Apidologie* 46 (3): 292–305.
Lee, K.V., Goblirsch, McDermott, E., Tarpy, D.R., and Spivak, M. In press. Is the Brood Pattern within a Honey Bee Colony a Reliable Indicator of Queen Quality? *Insects*, in press.
Li, J.L., Cornman, R.S., Evans, J.D., Pettis, J.S., Zhao, Y., Murphy, C., Peng, W.J., Wu, J., Hamilton, M., Boncristiani, H.F., Zhou, L., Hammond, J. and Chen, Y.P. 2014. Systemic Spread and Propagation of a Plant-Pathogenic Virus in European Honeybees, *Apis mellifera*. *MBio* 5 (1).
Locke, B., Conte, Y.L., Crauser, D. and Fries, I. 2012. Host adaptations reduce the reproductive success of *Varroa destructor* in two distinct European honey bee populations: Host Adaptations in *Varroa* -Resistant Honey Bees. *Ecology and Evolution* 2 (6): 1144–1150.

Locke, B., Forsgren, E., and de Miranda, J.R. 2014. Increased tolerance and resistance to virus infections: a possible factor in the survival of *Varroa destructor*-resistant honey bees (*Apis mellifera*). *PLoS ONE* (9):e99998.

Lodesani, M., Balduzzi, D. and Galli, A. 2004. A study on spermatozoa viability over time in honey bee (*Apis mellifera* ligustica) queen spermathecae. *J Apic Res* 43 (1): 27–28.

Lodesani, M., Costa, C., Besana, A., Dall'Olio, R., Franceschetti, S., Tesoriero, D. and Giacomo, D. 2014. Impact of control strategies for *Varroa destructor* on colony survival and health in northern and central regions of Italy. *Journal of Apicultural Research* 53 (1): 155–164.

Macedo, P.A., Wu, J. and Ellis, M.D. 2002. Using inert dusts to detect and assess varroa infestations in honey bee colonies. *J Apicul Res* 41(1): 3-7.

Martín-Hernández, R., Bartolomé, C., Chejanovsky, N., Le Conte, Y., Dalmon, A., Dussaubat, C., García-Palencia, P., Meana, A., Pinto, M.A., Soroker, V. and Higes, M. 2018. *Nosema ceranae* in *Apis mellifera*: a 12 years postdetection *perspective*: *Nosema ceranae* in *Apis mellifera*. *Environ Microb* 20 (4): 1302–1329.

Martin, S. 1998. A population model for the ectoparasitic mite *Varroa jacobsoni* in honey bee (*Apis mellifera*) colonies. *Ecological Modelling*, 109(3): 267-281.

Martin, S.J., Highfield, A.C., Brettell, L., Villalobos, E.M., Budge, G.E., Powell, M., Nikaido, S. and Schroeder, D.C. 2012. Global Honey Bee Viral Landscape Altered by a Parasitic Mite. *Science* 336 (6086): 1304–1306.

Meixner, M.D., Francis, R.M., Gajda, A., Kryger, P., Andonov, S., Uzunov, A., Topolska, G., Costa, C., Amiri, E., Berg, S., Bienkowska, M., Bouga, M., Büchler, R., Dyrba, W., Gurgulova, K., Hatjina, F., Ivanova, E., Janes, M., Kezic, N., Korpela, S., Conte, Y.L., Panasiuk, B., Pechhacker, H., Tsoktouridis, G., Vaccari, G. and Wilde, J. 2014. Occurrence of parasites and pathogens in honey bee colonies used in a European genotype-environment interactions experiment. *J Apic Res* 53 (2): 215–229.

Meixner, M.D., Kryger, P. and Costa, C. 2015. Effects of genotype, environment, and their interactions on honey bee health in Europe. *Current Opinion in Insect Science*, 10: 177-184.

Mondet, F., de Miranda, J.R., Kretzschmar, A., Le Conte, Y., and Mercer, A.R. 2014. On the front line: quantitative virus dynamics in honeybee (*Apis mellifera* L.) colonies along a new expansion front of the parasite *Varroa destructor*. *PLoS Pathog* (10):e1004323.

Mordecai, G.J., Brettell, L.E., Martin, S.J., Dixon, D., Jones, I.M. and Schroeder, D.C. 2016. Superinfection exclusion and the long-term survival of honey bees in *Varroa*-infested colonies. *The ISME Journal* 10 (5): 1182–1191.

Mordecai, G.J., Wilfert, L., Martin, S.J., Jones, I.M. and Schroeder, D.C. 2015. Diversity in a honey bee pathogen: first report of a third master variant of the Deformed Wing Virus quasispecies. *The ISME Journal* 10 (5): 1264–1273.

Mortensen, A.N., Jack, C.J., Bustamante, T.A., Schmehl, D.R. and Ellis, J.D. 2018. Effects of Supplemental Pollen Feeding on Honey Bee (Hymenoptera: Apidae) Colony Strength and *Nosema* spp. Infection. *J Econ Entom*: 1-7.

Muli, E., Patch, H., Frazier, M., Frazier, J., Torto, B., Baumgarten, T., Kilonzo, J., Kimani, J.N., Mumoki, F., Masiga, D., Tumlinson, J. and Grozinger, C. 2014. Evaluation of the Distribution and Impacts of Parasites, Pathogens, and Pesticides on Honey Bee (*Apis mellifera*) Populations in East Africa. *PLoS ONE* 9 (4): e94459.

Mullin, C.A., Frazier, M., Frazier, J.L., Ashcraft, S., Simonds, R., vanEngelsdorp, D. and Pettis, J.S. 2010. High Levels of Miticides and Agrochemicals in North American Apiaries: Implications for Honey Bee Health. *PLoS ONE* 5 (3): e9754.

Mutinelli, F., Costa, C., Lodesani, M., Baggio, A., Medrzycki, P., Formato, G. and Porrini, C. 2010. Honey bee colony losses in Italy. *J Apic Res* 49 (1): 119–120.

Nasr, M.E., Thorp, R.W., Tyler, T.L. and Briggs, D.L. 1990. Estimating honey bee (Hymenoptera: Apidae) colony strength by a simple method: measuring cluster size. *J Econ Entomol 83*(3): 748-754.

Nazzi, F. and Le Conte, Y. 2016. Ecology of *Varroa destructor*, the major ectoparasite of the western honey bee, *Apis mellifera*. *Annu Rev Entomol* 61: 417-432.

Nelson, C.M., Ihle, K.E., Fondrk, M.K., Page, R.E. and Amdam, G.V. 2007. The Gene vitellogenin Has Multiple Coordinating Effects on Social Organization. *PLoS Biology* 5 (3): e62.

Neumann, P., and Carreck, N.L. 2010. Honey bee colony losses. *J Apic Res* 49 (1): 1–6.

Nguyen, B.K., Mignon, J., Laget, D., de Graaf, D.C., Jacobs, F.J., vanEngelsdorp, D., Brostaux, Y., Saegerman, C. and Haubruge, E. 2010. Honey bee colony losses in Belgium during the 2008–9 winter. *J Apic Res* 49 (4): 337–339.

Niño, E.L., Malka, O., Hefetz, A., Tarpy, D.R. and Grozinger, C.M. 2013. Chemical Profiles of Two Pheromone Glands Are Differentially Regulated by Distinct Mating Factors in Honey Bee Queens (*Apis mellifera* L.). *PLoS ONE* 8 (11): e78637.

Nolan, M.P. and Delaplane, K.S. 2017. Distance between honey bee *Apis mellifera* colonies regulates populations of *Varroa destructor* at a landscape scale. *Apidologie* 48(1): 8-16.

Odoux, J.-F., Aupinel, P., Gateff, S., Requier, F., Henry, M. and Bretagnolle, V. 2014. ECOBEE: a tool for long-term honey bee colony monitoring at the landscape scale in West European intensive agroecosystems. *J Apic Res* 53 (1): 57–66.

Ogle, D.H. 2018. FSA: Fisheries Stock Analysis. R package version 0.8.20.

Ollerton, J., Erenler, H., Edwards, M., and Crockett, R. 2014. Extinctions of aculeate pollinators in Britain and the role of large-scale agricultural changes. *Science* (346):1360-1362.

Peterson, M., Gray, A., and Teale, A. 2010. Colony losses in Scotland in 2004–2006 from a sample survey. *J Apic Res* 48 (2): 145–146

Pettis, J.S., Collins, A.M., Wilbanks, R. and Feldlaufer, M.F. 2004. Effects of coumaphos on queen rearing in the honey bee, *Apis mellifera*. *Apidologie* 35 (6): 605–610.

Pettis, J.S., Rose, R., Lichtenberg, E.M., Chantawannakul, P., Buawangpong, N., Somana, W., Sukumalanand, P., and vanEngelsdorp, D. 2013. A rapid survey technique for *Tropilaelaps* mite (Mesostigmata: Laelapidae) detection. *J Econ Entomol* (106):1535-1544.

Pettis, J.S., Rice, N., Joselow, K., vanEngelsdorp, D. and Chaimanee, V. 2016. Colony Failure Linked to Low Sperm Viability in Honey Bee (*Apis mellifera*) Queens and an Exploration of Potential Causative Factors. *PLOS ONE* 11 (2): e0147220.

Pirk, C.W.W., Human, H., Crewe, R.M., and vanEngelsdorp, D. 2014. A survey of managed honey bee colony losses in the Republic of South Africa—2009 to 2011. *J Apic Res* 53 (1): 35–42.

Plant Health Australia: National Bee Pest Surveillance Program Operations Manual – Version 1.0. 2014. Accessed at: http:// www.planthealthaustralia.com.au/national-programs/ national-bee-pest-surveillance-program/ (on 2015.01.28).

Ploquin, E.F., Herrera, J.M., and Obeso, J.R. 2013. Bumblebee community homogenization after uphill shifts in montane areas of northern Spain. *Oecologia* (173):1649-1660.

Porta, M., Greenland, S., Hernán, M., Silva, I.D.S., and Last J.M. (Eds): A Dictionary of Epidemiology. Oxford University Press; 2014.

Potts, S.G., Biesmeijer, J.C., Kremen, C., Neumann, P., Schweiger, O., Kunin, and W.E. 2010. Global pollinator declines: trends, impacts and drivers. *Trends Ecol Evol* 25 (6): 345–353.

Potts, S.G., Roberts, S.P.M., Dean, R., Marris, G., Brown, M.A., Jones, R., Neumann, P. and Settele, J. 2010. Declines of managed honey bees and beekeepers in Europe. *J Apic Res* 49 (1): 15–22.

R Core Team. 2017. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing: Vienna, Austria. Available online: https://www.R-project.org/ (accessed on 26 May 2018).

R Core Team. 2018. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org.

Ramsey, S.D., Ochoa, R., Bauchan, G., Gulbronson, C., Mowery, J., Joklik, J., Cicero, J., Ellis, J., Hawthorne, D., and vanEngelsdorp, D. *Varroa* destructor feeds primarily on honey bee fat body tissue and not hemolymph. *Proc Natl A Sci*, in press.

Rangel, J. and Tarpy, D.R. 2016. In-Hive Miticides and their Effect on Queen Supersedure and Colony Growth in the Honey Bee (*Apis mellifera*). *J Environ Anal Toxicol* 06 (03).

Ravoet, J., De Smet, L., Meeus, I., Smagghe, G., Wenseleers, T., and de Graaf, D.C. 2014. Widespread occurrence of honey bee pathogens in solitary bees. *J Invertebr Pathol* (122):55-58.

Ravoet, J., Maharramov, J., Meeus, I., De Smet, L., Wenseleers, T., Smagghe, G. and De Graaf, D.C. 2013. Comprehensive bee pathogen screening in Belgium reveals *Crithidia mellificae* as a new contributory factor to winter mortality. *PLoS One* 8(8): p.e72443.

Reynaldi, F.J., Sguazza, G.H., Albicoro, F.J., Pecoraro, M.R., and Galosi, C.M. 2014. First molecular detection of co-infection of honey bee viruses in asymptomatic *Bombus atratus* in South America. *Braz J Biol* (73):797-800.

Rosenkranz, P., Aumeier, P. and Ziegelmann, B. 2010. Biology and control of *Varroa destructor*. *J Invertebr path* 103: S96-S119.

Royce, L.A., and Rossignol, P.A. 1990. Epidemiology of honey bee parasites. *Parasitol Today* 6 (11): 348–353.

Rueppell, O., Bachelier, C., Fondrk, M.K. and Page Jr, R.E. 2007. Regulation of life history determines lifespan of worker honey bees (*Apis mellifera* L.). *Exp Gerontol 42*(10): 1020-1032.

Runckel, C., Flenniken, M.L., Engel, J.C., Ruby, J.G., Ganem, D., Andino, R. and DeRisi, J.L. 2011. Temporal analysis of the honey bee microbiome reveals four novel viruses and seasonal prevalence of known viruses, *Nosema*, and Crithidia. *PloS one* 6(6): p.e20656.

Ryabov, E.V., Wood, G.R., Fannon, J.M., Moore, J.D., Bull, J.C., Chandler, D., Mead, A., Burroughs, N., and Evans, D.J. 2014. A virulent strain of deformed wing virus (DWV) of honeybees (*Apis mellifera*) prevails after *Varroa destructor*-mediated, or in vitro, transmission. *PLoS Pathogens* (10):e1004230.

Sagili, R., Burgett, D. 2011. Evaluating honey bee colonies for pollination. Pacific Northwest Extension Publications.

Sanchez-Bayo, F. and Goka, K. 2014. Pesticide Residues and Bees – A Risk Assessment. *PLoS ONE* 9 (4): e94482.

Sandrock, C., Tanadini, M., Tanadini, L.G., Fauser-Misslin, A., Potts, S.G. and Neumann, P. 2014. Impact of Chronic Neonicotinoid Exposure on Honeybee Colony Performance and Queen Supersedure. *PLoS ONE* 9 (8): e103592.

Seehuus, S.-C., Norberg, K., Gimsa, U., Krekling, T. and Amdam, G.V. 2006. Reproductive protein protects functionally sterile honey bee workers from oxidative stress. *Proc Natl A Sci* 103 (4): 962–967.

Seeley, T.D. 2007. Honey bees of the Arnot Forest: a population of feral colonies persisting with *Varroa destructor* in the northeastern United States. *Apidologie*, *38*(1): 19-29.

Seeley, T.D. and Smith, M.L. 2015. Crowding honeybee colonies in apiaries can increase their vulnerability to the deadly ectoparasite *Varroa destructor*. *Apidologie* 46(6): 716-727.

Seitz, N., Traynor, K.S., Steinhauer, N., Rennich, K., Wilson, M.E., Ellis, J.D., Rose, R., Tarpy, D.R., Sagili, R.R., Caron, D.M., Delaplane, K.S., Rangel, J., Lee, K., Baylis, K., Wilkes, J.T., Skinner, J.A., Pettis, J.S. and vanEngelsdorp, D. 2015. A national survey of managed honey bee 2014–2015 annual colony losses in the USA. *J Apicul Res* 54 (4): 292–304.

Semkiw, P., Skubida, P., and Pohorecka, K. 2013. The amitraz strips efficacy in control of *Varroa destructor* after many years application of amitraz in apiaries. *J Apic Res* (57):107-121.

Shimanuki, H., Calderone, N.W. and Knox, D.A., 1994. Parasitic mite syndrome: the symptoms. *Am Bee J* 134: 827–828.

Simone, M., Evans, J.D. and Spivak, M. 2009. Resin collection and social immunity in honey bees. *Evolution* 63 (11): 3016–3022.

Simone-Finstrom, M., Li-Byarlay, H., Huang, M.H., Strand, M.K., Rueppell, O. and Tarpy, D.R. 2016. Migratory management and environmental conditions affect lifespan and oxidative stress in honey bees. *Sci Rep* 6: 32023.

Simone-Finstrom, M. and Tarpy, D.R. 2018. Honey Bee Queens Do Not Count Mates to Assess their Mating Success. *J Insect Behav* 31 (2): 200–209.

Smart, M.D. and Sheppard, W.S. 2012. *Nosema ceranae* in age cohorts of the western honey bee (*Apis mellifera*). *J Invertebr Path* 109 (1): 148–151.

Smart, M., Pettis, J., Rice, N., Browning, Z. and Spivak, M. 2016. Linking Measures of Colony and Individual Honey Bee Health to Survival among Apiaries Exposed to Varying Agricultural Land Use. *PLOS ONE* 11 (3): e0152685.

Soroker, V., Hetzroni, A., Yakobson, B., David, D., David, A., Voet, H., Slabezki, Y., Efrat, H., Levski, S., Kamer, Y., Klinberg, E., Zioni, N., Inbar, S. and Chejanovsky, N. 2011. Evaluation of colony losses in Israel in relation to the incidence of pathogens and pests. *Apidologie* 42 (2): 192–199.

Spivak, M. and Reuter, G.S. 1998. Performance of hygienic honey bee colonies in a commercial apiary. *Apidologie* 29 (3): 291–302.

Spleen, A.M., Lengerich, E.J., Rennich, K., Caron, D., Rose, R., Pettis, J.S., Henson, M., Wilkes, J.T., Wilson, M., Stitzinger, J., Lee, K., Andree, M., Snyder, R., vanEngelsdorp, D. and for the Bee Informed Partnership. 2013. A national survey of managed honey bee 2011–12 winter colony losses in the United States: results from the Bee Informed Partnership. *J Apic Res* 52 (2): 44–53.

Steinhauer, N.A., Rennich, K., Wilson, M.E., Caron, D.M., Lengerich, E.J., Pettis, J.S., Rose, R., Skinner, J.A., Tarpy, D.R., Wilkes, J.T., vanEngelsdorp, D. and for the Bee Informed Partnership. 2014. A national survey of managed honey bee 2012–2013 annual colony losses in the USA: results from the Bee Informed Partnership. *J Apic Res* 53 (1): 1–18.

Steinhauer, N., Kulhanek, K., Antúnez, K., Human, H., Chantawannakul, P., Chauzat, M.-P. and vanEngelsdorp, D. 2018. Drivers of colony losses. *Curr Opin Insect Sci* 26: 142–148.

Stoner, K.A. and Eitzer, B.D. 2013. Using a Hazard Quotient to Evaluate Pesticide Residues Detected in Pollen Trapped from Honey Bees (*Apis mellifera*) in Connecticut. *PLoS ONE* 8 (10): e77550.

Szabo, T.I. 1993. Length of Life of Queens in Honey Bee Colonies. *Am Bee J* 133 (20): 723–724.

Tarpy, D.R., Keller, J.J., Caren, J.R. and Delaney, D.A. 2012. Assessing the Mating 'Health' of Commercial Honey Bee Queens. *J Econ Entomol* 105 (1): 20–25.

Tarpy, D.R., vanEngelsdorp, D. and Pettis, J.S. 2013. Genetic diversity affects colony survivorship in commercial honey bee colonies. *Naturwissenschaften* 100 (8): 723–728.

Tarpy, D.R. and Olivarez, R. 2014. Measuring sperm viability over time in honey bee queens to determine patterns in stored-sperm and queen longevity. *J Apic Res* 53 (4): 493–495.

Topolska, G., Gajda, A., Pohorecka, K., Bober, A., Kasprzak, S., Skubida, M. and Semkiw, P. 2010. Winter colony losses in Poland. *J. Apic. Res.* 49 (1): 126–128.

Topolska, G., Krzyzanska, K., Hartwig, A., and Gajda, A. The investigation of bee virus infections in Poland. 2009. Comparison of Two Methods of Monitoring Honeybee Infestation with *Varroa destructor* Mite. BEEBOOK Volume II: Standard methods for *Apis mellifera* pest and pathogen research — COLOSS, 33:125-133. Accessed at: http://www.coloss.org/ beebook/II (on 2015.01.28).

Traver, B.E. and Fell, R.D. 2011. Prevalence and infection intensity of *Nosema* in honey bee (*Apis mellifera* L.) colonies in Virginia. *J Invertebr Path* 107 (1): 43–49.

Traynor, K.S., Pettis, J.S., Tarpy, D.R., Mullin, C.A., Frazier, J.L., Frazier, M. and vanEngelsdorp, D. 2016. In-hive Pesticide Exposome: Assessing risks to migratory honey bees from in-hive pesticide contamination in the Eastern United States. *Sci Rep* 6 (1).

Traynor, K.S., Rennich, K., Forsgren, E., Rose, R., Pettis, J., Kunkel, G., Madella, S., Evans, J., Lopez, D. and vanEngelsdorp, D. 2016. Multiyear survey targeting disease incidence in US honey bees. *Apidologie* 47 (3): 325–347.

Tsvetkov, N., Samson-Robert, O., Sood, K., Patel, H.S., Malena, D.A., Gajiwala, P.H., Maciukiewicz, P., Fournier, V. and Zayed, A. 2017. Chronic exposure to neonicotinoids reduces honey bee health near corn crops. *Science* 356 (6345): 1395–1397.

United States Department of Agriculture – National Agriculture Statistics Service (USDA-NASS). 2007. Honey. Department of Agriculture; Washington DC, USA. 4 pp.

United States Department of Agriculture – National Agriculture Statistics Service (USDA-NASS). 2014. Honey. Department of Agriculture; Washington DC, USA. 6 pp.

United States Department of Agriculture – National Agriculture Statistics Service (USDA-NASS). 2018. Honey bee colonies. Washington, DC: Department of Agriculture.

United States Department of Agriculture – Animal and Plant Health Inspection Service (USDA APHIS): USDA Honey Bee Pests and Diseases Survey Project Plan for 2014. 2014. Accessed at:

http://www.aphis.usda.gov/wps/portal/aphis/ourfocus/planthealth/sa_domestic_pests_and_diseases/sa_pests_and_diseases/sa_non_regulated/sa_honey_bees/ (on 2015.01.28).

US EPA Ecotox Database. Available online: http://cfpub.epa.gov/ecotox/ (accessed on 26 May 2018).

van der Zee, R. 2010. Colony losses in the Netherlands. *J Apic Res* 49 (1): 121–123

van der Zee, R., Brodschneider, R., Brusbardis, V., Charrière, J.-D., Chlebo, R., Coffey, M.F., Dahle, B., Drazic, M.M., Kauko, L., Kretavicius, J., Kristiansen, P., Mutinelli, F., Otten, C., Peterson, M., Raudmets, A., Santrac, V., Seppälä, A., Soroker, V., Topolska, G., Vejsnæs, F. and Gray, A. 2014. Results of international standardised beekeeper surveys of colony losses for winter 2012–2013: analysis of winter loss rates and mixed effects modelling of risk factors for winter loss. *J Apic Res* 53 (1): 19–34.

van der Zee, R., Gray, A., Holzmann, C., Pisa, L., Brodschneider, R., Chlebo, R., Coffey, M.F., Kence, A., Kristiansen, P., Mutinelli, F., Nguyen, B.K., Noureddine, A., Peterson, M., Soroker, V., Topolska, G., Vejsnæs, F. and Wilkins, S. 2013. Standard survey methods for estimating colony losses and explanatory risk factors in *Apis mellifera*. *J Apic Res* 52 (4): 1–36.

van der Zee, R., Pisa, L., Andonov, S., Brodschneider, R., Charrière, J.-D., Chlebo, R., Coffey, M.F., Crailsheim, K., Dahle, B., Gajda, A., Gray, A., Drazic, M.M., Higes, M., Kauko, L., Kence, A., Kence, M., Kezic, N., Kiprijanovska, H., Kralj, J., Kristiansen, P., Hernandez, R.M., Mutinelli, F., Nguyen, B.K., Otten, C., Özkırım, A., Pernal, S.F., Peterson, M., Ramsay, G., Santrac, V., Soroker, V., Topolska, G., Uzunov, A., Vejsnæs, F., Wei, S. and Wilkins, S. 2012. Managed honey bee colony losses in Canada, China, Europe, Israel and Turkey, for the winters of 2008–9 and 2009–10. *J Apic Res* 51 (1): 100–114.

van Dooremalen, C., Gerritsen, L., Cornelissen, B., van der Steen, J.J.M., van Langevelde, F. and Blacquière, T. 2012. Winter Survival of Individual Honey Bees and Honey Bee Colonies Depends on Level of *Varroa destructor* Infestation. *PLoS ONE* 7 (4): e36285.

van Dooremalen, C., Cornelissen, B., Poleij-Hok-Ahin, C. and Blacquière, T. 2018. Single and interactive effects of *Varroa destructor*, *Nosema* spp., and imidacloprid on honey bee colonies (Apis mellifera). *Ecosphere*, *9*(8): p.e02378.

vanEngelsdorp, D., Underwood, R., Caron, D., and Hayes Jr., J. 2007. An estimate of managed colony losses in the winter of 2006–2007: a report commissioned by the Apiary Inspectors of America. *Am Bee J* (147): 599– 603.

vanEngelsdorp, D., Hayes, J., Underwood, R.M. and Pettis, J. 2008. A Survey of Honey Bee Colony Losses in the U.S., Fall 2007 to Spring 2008. *PLoS ONE* 3 (12): e4071.

vanEngelsdorp, D., Evans, J.D., Donovall, L., Mullin, C., Frazier, M., Frazier, J., Tarpy, D.R., Hayes, J. and Pettis, J.S. 2009. "Entombed Pollen": A new condition in honey bee colonies associated with increased risk of colony mortality. *J Inverteb Path* 101 (2): 147–149.

vanEngelsdorp, D., Evans, J.D., Saegerman, C., Mullin, C., Haubruge, E., Nguyen, B.K., Frazier, M., Frazier, J., Cox-Foster, D., Chen, Y., Underwood, R., Tarpy, D.R. and Pettis, J.S. 2009. Colony Collapse Disorder: A Descriptive Study. *PLoS ONE* 4 (8): e6481.

vanEngelsdorp, D., and Meixner, M.D. 2010. A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. *J Invertebr Pathol* 103: S80–S95.

vanEngelsdorp, D., Hayes, J., Underwood, R., and Pettis, J. 2010. A survey of honey bee colony losses in the United States, fall 2008 to spring 2009. *J Apic Res* 49 (1): 7–14.
vanEngelsdorp, D., Hayes, J., Underwood, R.M., Caron, D. and Pettis, J. 2011. A survey of managed honey bee colony losses in the USA, fall 2009 to winter 2010. *J Apic Res* 50 (1): 1–10.
vanEngelsdorp, D., Caron, D., Hayes, J., Underwood, R., Henson, M., Rennich, K., Spleen, A., Andree, M., Snyder, R., Lee, K., Roccasecca, K., Wilson, M., Wilkes, J., Lengerich, E., Pettis, J. and for the Bee Informed Partnership. 2012. A national survey of managed honey bee 2010–11 winter colony losses in the USA: results from the Bee Informed Partnership. *J Apic Res* 51 (1): 115–124.
vanEngelsdorp, D., Tarpy, D.R., Baylis, K., Spivak, M., Caron, D.M., Connell, J., Delaplane, K.S., Donohue, S., Esaias, W., Gross, B., Hayes, J., Lengerich, E.J., Pettis, J., Rennich, K., Underwood, R., Rose, R., Skinner, J. and Wilkes, J. 2012. The Bee Informed Partnership: Using Beekeeper's Real-World Experience to Solve Beekeepers' Real-World Problems. *Am Entomol* 58 (2): 116–118.
vanEngelsdorp, D., Lengerich, E., Spleen, A., Dainat, B., Cresswell, J., Baylis, K., Nguyen, B.K., Soroker, V., Underwood, R., Human, H., Le Conte, Y. and Saegerman, C. 2013. Standard epidemiological methods to understand and improve *Apis mellifera* health. *J Apic Res* 52 (1): 1–16.
vanEngelsdorp, D., Tarpy, D.R., Lengerich, E.J. and Pettis, J.S. 2013b. Idiopathic brood disease syndrome and queen events as precursors of colony mortality in migratory beekeeping operations in the eastern United States. *Prev Vet Med* 108 (2–3): 225–233.
vanEngelsdorp, D., Saegerman, C., Nguyen, B.K., and Pettis, J.S. 2014. Honey bee health surveillance. In Bee health and veterinarians. Edited by Ritter, W. OIE World Organisation for Animal Health: 215-222.
Vejsnæs, F., Nielsen, S.L., and Kryger, P. 2010. Factors involved in the recent increase in colony losses in Denmark. *J Apic Res* 49 (1): 109–110
Viotti, M.A., Moura, F.R., and Lourenco, A.P. 2013. Species diversity and temporal variation of the orchid-bee fauna (Hymenoptera, Apidae) in a conservation gradient of a rocky field area in the Espinhaco Range, State of Minas Gerais, Southeastern Brazil. *Neotrop Entomol* (42):565-575.
Williams, G.R., Shutler, D., Little, C.M., Burgher-Maclellan, K.L. and Rogers, R.E.L. 2011. The microsporidian *Nosema ceranae*, the antibiotic Fumagilin-B®, and western honey bee (*Apis mellifera*) colony strength. *Apidologie* 42 (1): 15–22.
Winfree, R., Gross, B.J., and Kremen, C. 2011. Valuing pollination services to agriculture. *Ecol Econ* (71):80-88.
Woyke, J. 1962. Natural and artificial insemination of queen honeybees. *Bee World* 43 (1): 21–22.
Wu, J.Y., Anelli, C.M., and Sheppard, W.S. 2011. Sub-Lethal Effects of Pesticide Residues in Brood Comb on Worker Honey Bee (*Apis mellifera*) Development and Longevity. *PLoS ONE 6*: e14720.
Yamamoto, M., Junqueira, C.N., Almeida Barbosa, A.A., Augusto, S.C., and Oliveira, P.E. 2014. Estimating crop pollinator population using mark-recapture method. *Apidologie* (45):205-214.

Zotarelli, H.G.S., Evans, D.M., Bego, L.R., and Sofia, S.H. 2014. A comparison of social bee–plant networks between two urban areas. *Neotrop Entomol* (43):399-408.