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MORBIDITY AND MORTALITY IN THE BEE YARD

A Dissertation in

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by

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ABSTRACT

Managed honey bee (*Apis mellifera* L.) populations have steadily declined in the Unites States over the last 60 years (NRC 2006). The causes for these losses are multiple and complex (vanEngelsdorp and Meixner 2010; CHAPTER 2), and are of particular concern considering the importance of honey bees as pollinators of many agricultural crops. An estimated 35% of the western human diet benefits, directly or indirectly, from honey bee pollination (Klein et al., 2007).

Considering the honey bees' vital role in the commercial production of many crops, it is somewhat surprising that the steady decline in colony numbers was largely ignored until the unusually high losses reported in the US over the winter of 2006-2007 (vanEngelsdorp et al. 2007). Since then in the US, heavy overwintering losses have been documented every winter (vanEngelsdorp et al. 2008, vanEngelsdorp et al. 2010a, vanEngelsdorp et al. 2011; CHAPTER 3). Troublingly, the cause or causes for these apparent elevated rates of winter loss were and remain unclear.

Previous work on honey bee disease tends to concentrate on the etiology of individual diseases and stresses. This dissertation borrows from the well-established field of human epidemiology to look at the complexity and interrelationships of multiple disease determinate factors.

In the US at least, a portion of the honey bee colonies lost in the winter of 2006-2007 and every year thereafter died with a distinct set of symptoms: 1) no dead bees in the colonies or apiary, (2) adult populations rapidly declined leaving brood poorly or completely unattended, and (3) the absence of robbing or kleptoparasitism in collapsed colonies (Cox-Foster et al. 2007). A review of the historical bee literature suggests that large localized unexplained losses have occurred at least 20 times over the last 150 years, and many of those losses occurred with symptoms very similar to the losses of 2006-2007 (Underwood and vanEngelsdorp 2007). In the past these conditions had been given a variety of names including "Fall Dwindle Disease", "May disease", "disappearing disease", and "disappearing syndrome". However, none of these names seemed appropriate (the disease occurred between November and March – not exclusively the fall or May; the disease nor the syndrome did not disappear, the bees did). As a result, during a conference call by investigators who would eventually make of the core of the Colony Collapse Disorder working team, the term "Colony Collapse Disorder", or "CCD" was coined. This "word" has subsequently been included in the New Oxford American Dictionary, and was selected by dictionary's editors as the runner up "new word of 2007".

Efforts to find a cause for CCD were intense. Initial efforts identified Israeli Acute Paralysis Virus (IAPV) as highly associated with diseased colonies along with Kashmir bee virus, *Nosema apis* and *Nosema ceranae* (Cox-Foster et al. 2007). While IAPV is able to cause colony collapse (Maori et al. 2009), its potential role as the sole cause of CCD has not been substantiated (vanEngelsdorp et al. 2009; CHAPTER 4). In the most comprehensive study of the disorder to date, vanEngelsdorp and colleges (2009; CHAPTER 4) compared 61 different variables, including pathogen and pesticides prevalence and load, in bees collected from CCD and non-CCD colonies. While some single pathogen loads differed between affected and non-affected colonies, no single pathogen was consistently found associated with the condition.

Notably absent were differences in the *Nosema* spore counts and *Varroa* levels between CCD and control colonies and apiaries. *Varroa* mites, likely in association with the viruses they vector (Martin 2001), are known to cause colony mortality, although such collapses usually occur at the tail end of the nectar flow and are usually accompanied with large numbers of bees crawling in the affected apiary. *Nosema ceranae*, a more recently introduced pathogen of bees, has been implicated in large scale die-offs in southern Spain (Martín-Hernández et al. 2007), and studies have shown that the in advanced stages of collapse colonies die with symptoms similar to CCD (Higes et al. 2008). The study outlined in CHAPTER 4 did not support these two organisms as having a direct role in colony collapse, and as a result, an additional criterion was proposed for inclusion in CCD's case definition – "4) at the time of collapse *Varroa* and *Nosema* populations are below levels thought to cause economic injury or colony decline".

Although no evidence was found for a single causal agent, the descriptive epizoological study summarized in CHAPTER 4 did document evidence that pathogens played an important role in the condition. Colonies neighboring colonies affected by CCD were more likely to have the condition than chance would suggest, implying that the condition was either contagious or the result of exposure to a common risk factor. Pathogen prevalence rates in control and CCD populations were similar, suggesting that pathogen exposure was also similar for both groups. However, CCD colonies had higher pathogen loads, and were much more likely to be co-infected with more than three pathogen, suggesting some underlying factor or factors may affect a colony's ability to resist disease (Cox-Foster and vanEngelsdorp 2009).

Pesticides are commonly postulated as potentially explaining increased disease susceptibility in bees (Mullin et al. 2010). While pesticides almost certainly can have negative

effects on bee health, the study outlined in CHAPTER 4 found no evidence for impact of a single pesticide as being associated with CCD. In fact, of the 50 pesticides and metabolites found in samples tested, only two – Coumaphos and Esfenvalerate – were found at levels that differed between CCD and control populations. In both cases levels of these products were found at higher levels in non-CCD colonies. A classification and regression tree analysis (CART) preformed on the same data set (vanEngelsdorp et al. 2010b; CHAPTER 5) more starkly highlighted the ability of pesticide levels – and specifically coumaphos levels – ability to differentiate CCD from control populations. Colonies with high levels of coumaphos were healthier. As coumaphos is commonly used by beekeepers to control *Varroa* populations, this finding suggests that healthy colonies had mite populations that were more aggressively or persistently controlled. Although *Varroa* mite levels were not different between CCD and control populations at the time of sampling, it is possible that mite populations differed at some time before sample collection. CCD may therefore be a consequence of elevated levels of mites some time before CCD onset.

The need to monitor colonies over time is highlighted by the potential for a "legacy" effect from risk factor exposure occurring some time prior to sample collection. To this end, a longitudinal study was initiated that monitored colonies operated by three different east coast migratory operations (vanEngelsdorp et al. submitted; CHAPTER 6). In sum, 58% of the monitored colonies died over the 10 months they were observed; and while too few colonies died with symptoms that would allow for CCD diagnosis, several factors were identified that had measurable impacts on colony survivorship. Notably colonies diagnosed with the presence of brood suffering from symptoms indicative of Parasitic Mite Syndrome (PMS), with evidence of queen loss or replacement, or with poor brood pattern had an elevated risk of dying in the subsequent 50 days, when compared to colonies without any of these symptoms. This role of

queen-related issues in colony mortality substantiate claims by beekeepers that poor queens play an important role in high rates of winter mortality (as documented invanEngelsdorp et al. 2011; CHAPTER 3).

In summary, this thesis applied epidemiological techniques to describe mortality and morbidity in honey bee colonies. First, it attempted to place colony losses in a historical context and grossly identify those factors that may influence long-term trends in honey bee declines (CHAPTER 2). It then quantified overwinter colony loss more precisely over time, and placed these losses into geographic and operational context (CHAPTER 3). A study designed to specifically describe CCD (an emerging and poorly understood threat to honey bee colonies) was preformed. These studies compared an exhaustive list of colony health and risk factor measures (like wing symmetry, pathogens, and pesticides) thought to affect or to be an indirect measure of colony health (CHAPTER 4). To better understand the relative importance and interrelationships between these factors, a CART analysis was done on the same data set (CHAPTER 5). While these efforts failed to find a single cause for the condition, they did suggest that CCD may be the result of several risk factors working in synergy (Cox-Foster and vanEngelsdorp 2009). In particular, the potential legacy effect of past risk factor exposure was highlighted, calling for the need to conduct longitudinal colony health studies to identify and quantify risk. Partial results of such a study were summarized in CHAPTER 6 where risk factors that were self-identified by beekeepers as leading causes for high overwinter rate (CHAPTER 3) were substantiated. Like all epidemiological studies, the findings of these studies are not meant to be conclusive, but rather informative; they are intended to direct and focus future hypothesis-based investigations. Investigations aimed at elucidating the etiology of Parasitic Mite Syndrome, and the causes of queen failure and superscedure in managed colonies are highlighted as areas for future work aimed at mitigating unsustainable rates of managed colony mortality.

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PREFACE

A majority of the work presented in this dissertation was the result of collaborations with many individuals at many different institutions. Without these collaborators, this dissertation would not have been possible. Below my (DvE) specific contributions to the multi-authored work presented in this dissertation are summarized.

Chapter 2: DvE was responsible for the overall article and specifically focused on colating, discussing, and summarizing North American honey bee health data.

Chapter 3: DvE was responsible for survey design and delivery, data analysis, and lead article writing efforts.

Chapter 4: DvE was responsible for sample collection, sample distribution to collaborators, data management, analysis, and lead article writing efforts.

Chapter 5: DvE coordinated data sharing, lead efforts to interpret and present the results, and was chiefly responsible for writing the manuscript.

Chapter 6: DvE was primarily responsible for experimental design, data collection, analysis, and writing the manuscript.

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I find myself amazed that this dissertation is actually finished. Its completion was in no small part due to the encouragement, guidance, and nudging of the many colleagues and friends who played a role in making this happen.

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It has been a privilege and honor.

FRONTISPIECE

Glass plate of a swarm of bees. Circa 1920

Photographer unknown.

Chapter 1:

ADOPTION OF EPIDEMIOLOGICAL METHODS FOR THE STUDY OF MORBIDITY AND MORTALITY IN HONEY BEE COLONIES.

Managed honey bee (*Apis mellifera* L.) populations have steadily declined in the United States over the last 60 years (NRC 2006). These losses are of particular concern considering the importance of honey bees as pollinators of many agricultural crops. An estimated 35% of the western human diet benefits, directly or indirectly, from honey bee pollination (Klein et al., 2007).

Considering the honey bee's vital role in the commercial production of many crops, it is somewhat surprising that the steady decline in colony numbers was largely ignored until the unusually high losses reported in the US over the winter of 2006-2007 (vanEngelsdorp et al. 2007). Since then in the US, heavy overwintering losses have been documented every winter (vanEngelsdorp et al. 2008, 2010, 2011). As demonstrated in the web of causation schematic (Figure 1) the factors potentially influencing changes in the population of managed honey bees are multiple and interrelated. Indeed, while biological factors such as honey bee genetics and pathogen and parasite exposure play an important role in these changes, other factors, including socio-economic, political and environmental factors also influence both the rate at which colonies are lost and the rate at which lost colonies are replaced or populations grow.

By and large past efforts aimed at understanding honey bee diseases have focused on studying disease etiology at the individual bee or colony level. Indeed, as depicted in the model presented in Figures 1 and 2, the individual bee and the colony itself act as the core unit needed to

understand specifically colony health and more generally the changes in managed honey bee colony numbers. By borrowing and expanding the conceptual model used to explain how social inequalities influence human health (Dahlgren and Whitehead 1992), the complex systems and dynamic relationships between biotic and abiotic factors are more easily understood (Gunning-Schepers 1999). It is precisely the complexity and interrelated nature of the factors that influence colony health that makes the use of epidemiological methods an appropriate avenue for identifying and quantifying diseases and their determinants in honey bee colonies.

Epidemiology is the study of the distribution and determinants of disease within a human population (Woodward 2005). Ultimately, epidemiologists aim to help prevent disease and alleviate suffering (Koepsell and Weiss 2003). To accomplish this, epidemiological studies attempt to identify factors that may explain or contribute to disease outbreaks. Once identified, these factors not only inform future clinical etiological studies, but also, and perhaps more importantly, they inform disease prevention and control programs (Mausner and Kramer 1985).

The success of human epidemiologists in reducing disease occurrence over the last century is undeniable. Identifying factors that contribute to the occurrence of diseases like lung cancer (smoking), sexually transmitted diseases (unprotected sex), and cardiovascular disease (high blood pressure) have permitted targeted community health initiatives aimed at preventing or controlling risk factor exposure. These initiatives, in turn, have helped reduce the rate of disease in targeted populations (Mausner and Kramer 1985, Koepsell and Weiss 2003, Woodward 2005).

Considering the success of human epidemiologists, it is not surprising that epidemiological methods have been adopted by those wishing to understand and reduce disease outbreak in non-human populations, such as plants and non-human animals (Nutter 1999b).

Building on this tradition, this dissertation proposes to apply epidemiological approaches to understanding disease occurrence in managed honey bee colonies.

Nutter (1999) argued that the application of epidemiological methods to understand disease outbreaks in plant, human, and animal populations involves the implementation of six common steps: (1) Defining disease problems in quantitative terms; (2) Quantifying state and rate variables of the disease system's components; (3) Identifying the most effective management strategy(ies) to achieve effective disease control; (4) Developing and then quantifying the impacts of specific and integrated management tactics on disease dynamics as a means to evaluate a proposed disease management strategy(ies); (5) Integrating management tactics into management programs and reevaluating the epidemiologic impacts of whole disease management programs on disease dynamics, and finally; (6) Assessing the economic and environmental risks versus the actual benefits achieved by implementing integrated disease management programs. This holistic and comprehensive approach to disease mitigation is well beyond the scope of the work presented in this dissertation. Instead, this dissertation's work will focus on developing and applying methods to quantitatively describe the health outcomes in honey bee populations (Nutter's step 1) and identifying and quantifying some of the factors that contribute to rates of disease in honey bee populations (Nutter's step 2). It is hoped that future efforts will utilize and expand on the methods used here in order to develop, implement, and evaluate programs designed to reduce disease occurrence in honey bee populations (Nutter's Steps 3- 6).

To successfully develop tools which either quantify the rate of disease in a population or quantify the factors which may contribute to disease occurrence, the "disease" of interest must be clearly defined. Broadly speaking, disease is any departure from perfect health (Woodward 2005). When applied to specific studies, a precise definition - the case definition – must be developed which unambiguously allows subjects to be classified as a case or not. In the work

presented here, the case definition for the health outcomes of interest differed depending on the objective of the study preformed. When documenting the rate of winter loss in colonies, the case definition for the outcome of interest is dead colonies, while the efforts which attempted to elucidate the cause or causes of Colony Collapse Disorder (CCD) used a specific set of symptoms which characterized colonies lost to this condition. When all "cases" in a given population are identifiable, measures of disease rate can be calculated and compared (Koepsell and Weiss 2003).

Epidemiologists have developed several different ways to express and quantify disease within a population. Two common measures are the incidence rate and the prevalence rate. The calculation, and hence the definition, of both of these terms is not uniform across the branches of epidemiology. For instance, medical (human) epidemiologists define disease prevalence as the number of existing cases of disease at a given point in time, veterinarian epidemiologists calculate disease prevalence as the number of cases (both old and new) in a defined population at a given point in time, while a botanical epidemiologists define disease prevalence as the number of geographical sampling units (i.e. fields) where the disease in present divided by the total number of geographic units assessed (Nutter et al. 1991, Nutter 1999a, Woodward 2005). The differences in disease prevalence calculations reflect important differences in the availability of data, in the ease and economy by which disease data can be generated, and the reality of important differences in the management systems employed to control disease in the different systems. By and large, human medical records are much more complete than for other animal and plant systems. Further, while some animals do aggregate in herds, the unit of interest for the veterinarian epidemiologist is almost certainly individuals as the health status of each individual is easily obtained. This is not the case for botanical epidemiologist, who considers disease occurrence (and treatment strategies) in terms of groups of individuals (i.e. fields) rather than individuals.

Considering the subtle differences in the definition and calculation of basic epidemiological terms between medical, veterinarian, and botanical epidemiologists it seems reasonable to expect that the application and calculation of basic measures of disease occurrence in honey bee populations will need to be specifically adapted for epidemiological studies of this organism as well. This is especially true when one takes into account that honey bees are social insects. Contemporary thinkers suggest that honey bee colonies should be considered "super-organisms", for the colony acts as the vehicle by which honey bees propagate their genes (Seeley 1989, Moritz and Fuchs 1998). While, in theory honey bee "health" (or disease) can be studied by describing disease occurrence in individual bees within a colony, it seems more appropriate to consider individual colonies as the epidemiological unit of interest, for it is at that level that most disease conditions are diagnosed and disease mitigation strategies implemented.

Underpinning all epidemiological studies is an assumption that disease is not randomly distributed within a population. An epidemiological approach identifies factors which may explain or contribute to disease outbreak by characterizing differences in the frequency and/or types of disease found between groups of individuals within a population, or within the same group over time. This approach acknowledges that disease causation is not simply the result of exposure to a disease agent, but rather the result of several, potentially interrelated factors which contribute to a specific defined outcome (a disease case). These factors include both host (intrinsic) factors which govern the susceptibility of a host, and environmental (extrinsic) factors which influence the exposure and possibly susceptibility to disease agents. The complex nature of the relationship between factors which contribute to health outcomes are often described as webs of causation. Schematic representations of these webs of causation can provide a framework to more easily understand the relationships (either theoretical or evidence based) between environmental, socio-political, biological, and genetic determinates of disease.

Ultimately, the aim of epidemiological research as applied to honey bee colonies is to ensure adequate populations of honey bees for pollination of pollinator-dependent crops. Numerous factors have been theorized or demonstrated to affect changes in colony populations from year to year (Figure 1). The schematic representation of these factors and their potential interrelationships acts as a theoretical framework to guide epidemiological studies to areas which are lacking knowledge and suggest diseases mitigation strategies which could help increase colony numbers. Notably, the schematic highlights the potential importance of beekeeper profitability as a key component which indirectly drives colony numbers. It is profitability that dictates the amount and quality of inputs a beekeeper can implement to control and replace colony losses. This potential impact needs to be verified using retrospective analysis of colony numbers linked to some indication of beekeeper profitability. One such analysis, as well as a broad overview of the factors that affect managed honey bee populations in both North America and Europe are addressed in Chapter 2.

One key component which drives colony losses and replacement on a year-to-year basis which is not demonstrated in the web of causation model (Figure 1) is the degree to which various factors contribute to colony losses. Studies that attempt to quantify the risk and impact of the various direct causes of mortality would be especially informative in that they would not only elucidate our limited understanding of determinants of colony mortality, but potentially would highlight areas where beekeeping management could help negate disease determinants and ultimately colony losses. Such studies can be survey-based and have a broad scope – such as the winter mortality study presented in Chapter 3; can quantify the distribution of disease determinants between diseased and non-disease colonies, such as the investigations seeking to identify the cause or causes of CCD (Chapters 4 and 5); or can quantify the risk of mortality resulting from risk factor exposure over time (as was done in Chapter 6).

In summary, this dissertation proposes to implement epidemiological methods to highlight disease determinants in honey bee colonies. To fulfill this aim, I utilized historical records, conducted beekeeper surveys, preformed descriptive comparisons of diseased and non-diseased colonies, and performed longitudinal monitoring of colony health. The results of these efforts were summarized using epidemiological methods specifically adapted for application and use on the honey bee system.

Figure 1: Web of causation: Numerous interrelated factors likely influence the change in the number of managed honey bee colonies from year to year.

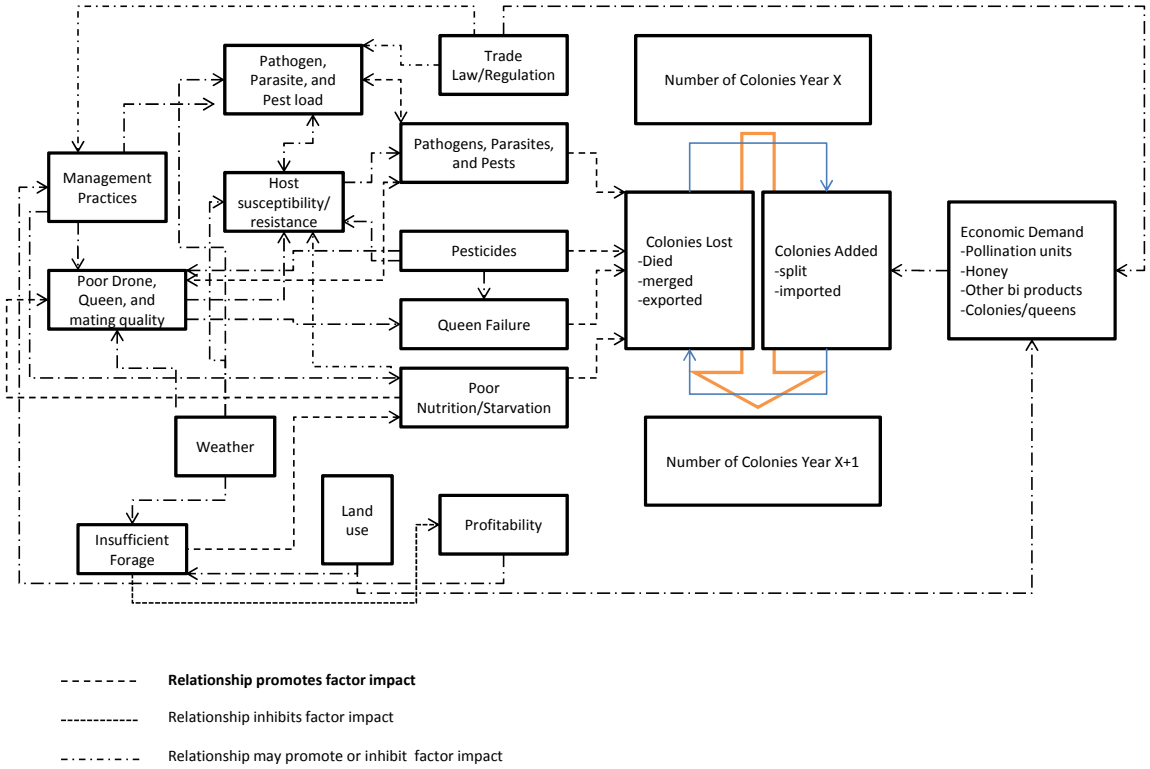
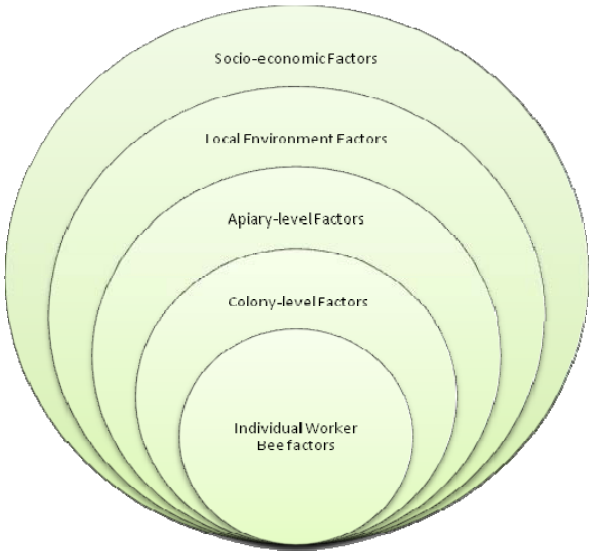


Figure 2: Determinants of health in honey bee colonies. The determinants of health in honey bee colonies are multiple and interrelated. At their core, they deal with individual worker bees whose nutritional status as a larva and young worker bee, as well as her genetics will influence her health. This health is also influenced by colony-level factors such as the genetic diversity of sister groups within the colony. Apiary and/or operational factors, such as production goals and resulting management (migratory vs. stationary), and operator management philosophy (chemical aversions) also potentially influence disease determinant factors. More broadly, the environments surrounding apiaries have many factors which potentially influence health, including resource abundance, pesticide exposure, and proximity to disease and pest agents. Finally, socio-economic factors governing the movement of bees, and ability to pay for management inputs also influence health outcomes for honey bee colonies.



References

Dahlgren, G., and M. Whitehead. 1992. Policies and strategies to promote social equity and health. World Health Organisation, Copenhagen.

Gunning-Schepers, L. J. 1999. Models: instruments for evidence based policy. J Epidemiol Community Health. 53: 263.

Koepsell, T. D., and N. S. Weiss. 2003. Epidemiologic methods: studying the occurrence of illness. Oxford University Press, New York.

Mausner, J. S., and S. Kramer. 1985. Epidemiology: an introductory text. W. B. Saunders Company, Philadelphia.

Moritz, R., F.A., and S. Fuchs. 1998. Organization of honeybee colonies: characteristics and consequences of a superorganism concept. Apidologie 29: 7-21.

NRC. 2006. Status of Pollinators in North America, pp. 317. National Academy of Sciences, Washington, D.C.

Nutter, F. W. 1999a. Epidemiological Concepts in Human, Veterinary, and Botanical Ecosystems: An Introduction to This Special Issue of Ecosystem Health. Ecosystem Health 5: 128-130.

Nutter, F. W., P. S. Teng, and F. M. Shokes. 1991. Disease assessment terms and concepts. Plant Disease 75: 1187-1188.

Nutter, F. W., Jr. 1999b. Understanding the interrelationships between botanical, human, and veterinary epidemiology: the Ys and Rs of it all. 5: 131-140.

Seeley, T. D. 1989. The honey bee colony as a superorganism. American Scientist 77: 546-551.

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

vanEngelsdorp, D., J. Hayes, Jr., R. M. Underwood, and J. Pettis. 2008. A survey of honey bee colony losses in the U.S., Fall 2007 to Spring 2008. PLoS ONE 3: e4071.

vanEngelsdorp, D., J. Hayes Jr, R. M. Underwood, and J. S. Pettis. 2010. A survey of honey bee colony losses in the United States, fall 2008 to spring 2009. Journal of Apicultural Research 49 7-14.

vanEngelsdorp, D., J. Hayes Jr, R. M. Underwood, D. Caron, and J. S. Pettis. 2011. A Survey of managed honey bee colony losses in the U.S., fall 2009 to winter 2010. Journal of Apicultural Research 50: 1-10.

Woodward, M. 2005. Epidemiology. Study Design and Data Analysis. Champman & Hall/CRC, New York.

Chapter 2

A HISTORICAL REVIEW OF MANAGED HONEY BEE POPULATIONS IN EUROPE AND THE UNITED STATES AND THE FACTORS THAT MAY AFFECT THEM.[1]

[1] **vanEngelsdorp, D., and M. D. Meixner**. 2010. A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. Journal of Invertebrate Pathology 103: S80-S95.Elisivier Publishers. Reprinted with kind permission.

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A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them

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ABSTRACT

Honey bees are a highly valued resource around the world. They are prized for their honey and wax production and depended upon for pollination of many important crops. While globally honey bee populations have been increasing, the rate of increase is not keeping pace with demand. Further, honey bee populations have not been increasing in all parts of the world, and have declined in many nations in Europe and in North America. Managed honey bee populations are influenced by many factors including diseases, parasites, pesticides, the environment, and socio-economic factors. These factors can act alone or in combination with each other. This review highlights the present day value of honey bees, followed by a detailed description of some of the historical and present day factors that influence honey bee populations, with particular emphasis on colony populations in Europe and the United States.

1. Introduction – the value of honey bees

The European honey bee, *Apis mellifera* L., is the most commonly managed bee in the world. A highly adaptable species, it has a native range that stretched from the southern parts of Scandinavia to Central Asia and throughout Africa (Seeley, 1985; Ruttner, 1988; Sheppard and Meixner, 2003). Since the 1600s, however, *A. mellifera's* range has expanded to nearly all habitable corners of the globe. Most of the European honey bee's range expansion has been the result of deliberate human transport (Crane, 1999). "Like the dog, the honeybee (*sic*) had accompanied man on most of his major migrations, and some of the early settlers in each part of the New World took hives of bees with them" (Crane, 1975). Unlike dogs however, honey bees were imported by settlers for their ability to make honey and bees wax. Honey was the only sweetener available to early African, Middle Eastern and European civilizations, and demand for the product no doubt lead to the domestication of bees by the Ancient Egyptians sometime before 2600 BCE. The practice of keeping bees was passed to the ancient Greeks by 650 BCE, who in turn passed the art to the Romans (by 150 BCE) who spread the art throughout what would become medieval Europe. It was the descendants of medieval European beekeepers who eventually spread both the practice of beekeeping and the bees themselves around the world (Ransome, 1937).

1.1. Honey

Honey was the only readily available sweetener to the peoples of Europe until methods were developed for refinement of sugar from sugar beets and sugar cane (Voorhies et al., 1933). Honey remains an important international commodity with global production estimated at 1.07 million metric ton in 2007, a 58% increase in production since 1961 (FAO, 2009). Using the average 2006 US price for honey, $1168 metric ton, the global value of honey production in 2007 had an estimated worth of US$1.25 billion.

1.2. Pollination

By far the most important contribution honey bees make to modern agriculture is the pollination services that they provide. Fifty-two of the 115 leading global food commodities depend on honey bee pollination for either fruit or seed set (Klein et al., 2007). Some (five) honey bee-dependant commodities would have $\geqslant$90% yield reduction without honey bees (Klein et al., 2007). In addition, yields in terms of fruit size, quality, or quantity would be greatly reduced (90–40%) in 16 commodities, modestly reduced (10–40%) in a further 19 commodities, and slightly reduced (<10%) in a further 13 commodities (Klein et al., 2007). In total, 22.6% of all agricultural production in the developing world, and 14.7% of agricultural production in the developed world is directly reliant on animal pollination to some extent (Aizen et al., 2008). However, when foods that indirectly benefit from pollination are included, 35% of the human diet is thought to benefit from pollination (Klein et al., 2007). Globally, the value of insect pollination has been esti-

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mated at US$ 212 billion (€153 billion), which represents about 9.5% of the total value of agricultural production. The value of insect pollination to agriculture is approximately the same for EU25 €14.2 billion (US$19.8 billion) and North American (excluding Mexico) nations (€14.4 billion; US$20.1 billion; (Gallai et al., 2009)).

Not all animal-dependent pollination is provided by honey bees, nor are honey bees the most efficient pollinators of most crops (NRC, 2006). However, they remain the most important pollinator for most crop monocultures worldwide (McGregor, 1976; Delaplane and Mayer, 2000). Managed honey bees are ideally suited for the pollination of large monocrop plantings for several reasons. Colonies of bees have a relatively large year round work force of 10,000–40,000 individuals, approximately one-third of which are foragers (Seeley, 1985). Beekeepers can stimulate the growth of these populations in preparation of a pollination event by feeding artificial diets of sucrose or high fructose corn syrup and artificial protein diets. Further, managed colonies are maintained in standardized equipment which facilitates the transport of colonies over large distances to pollination sites.

The biology of honey bees also makes them well suited as commercial pollinators. Honey bees are generalists, visiting a wide range of flower types, even those they are not well suited to pollinate, such as blueberries and alfalfa. Traveling an average of 4.5 km to forage (Seeley, 1985), honey bees are able to pollinate crops over an area of 6360 ha, allowing colonies to be placed in groups in the center of large orchards without affecting pollination in the orchards' periphery. Further, a bee's ability to communicate the location of floral resources to her nest mates makes honey bees particularly efficient pollinators (Seeley, 1985).

Crops not-dependent on animals for pollination represent the majority of caloric intake in human diets (Klein et al., 2007). While the total land area under cultivation has increased globally over the last 46 years, the proportion of land dedicated to the production of non-pollinator-dependent crops has shrunk when compared to land used to cultivate pollinator-dependent crops (Aizen et al., 2008). In part, this shift in land use is motivated by the fact that pollinator-dependent crops tend to have higher value than non-pollinator-dependent crops (Gallai et al., 2009). Between 1961 and 2006, agriculture industry's dependence on pollinators has increased by 50% and 62% in the developed and developing world, respectively (Aizen et al., 2009). This rate of increase surpasses that of global increases in the number of managed honey bee colonies, suggesting that pollinators may limit production of pollinator-dependent crops in the future (Aizen and Harder, in press).

The loss of all pollinators would reduce agricultural production by an estimated 8%. However, because many crops are not 100% reliant on insect pollination, some reduced production could be compensated for by increasing cultivated acreages. The loss of animal pollinators would require the developed and developing world to increase land cultivated in pollinator-dependent crops by 15% and 42%, respectively, to make up production deficits (Aizen et al., 2009). Pollinator declines and/or failure of pollinator populations to increase at the rate of pollinator-dependent crop expansion could have serious effects on world food security, just as the recent increased demand for corn for ethanol production has had significant effects on food prices (Elobeid, 2007).

2. Populations of managed honey bees

2.1. Worldwide

The total number of managed honey bee colonies worldwide was estimated at 72.6 million in 2007 (FAO, 2009). This represents a 64% increase in the total number of colonies managed since 1961 (Fig. 1). This crude approximation overestimates the change in managed bee populations because it does not account for changes in the number of nation states reporting colony numbers over the period. Aizen and Harder (in press) estimated that global stocks have increased by ~45%, after excluding all states that did not report colony numbers for the entire time series between 1961 and 2007.

While it is clear that global stocks of honey bees have increased over the last five decades, not all regions have experienced gains. Notably, in the period between 1961 and 2007, managed colonies decreased in both Europe (−26.5%) and North America (−49.5%), while large increases were recorded for Asia (426%), Africa (130%), South America (86%), and Oceania (39%) (FAO, 2009). Even within regions there was considerable variability in the honey bee colony population trends. For example, in North America, both the US and Mexico saw declines over the 46 year period, while Canada saw increases in colony numbers. In Europe, similar discrepancies in trends were apparent (Fig. 2: FAO, 2009).

2.2. United States

The number of honey-producing colonies in the US dropped 61% from their high of 5.9 million managed in 1947 to the low of 2.3 million reported in 2008 (Fig. 3). The number of honey-producing colonies has been tabulated by the USDA National Agricultural Statistics Service (NASS) for almost all years since 1943. Between 1982 and 1985 NASS discontinued its survey and colony numbers for those years were estimated by the Agricultural Stabilization and Conservation Service (Rodenberg, 1992) (Fig. 3). The annual census was designed to capture the number of honey-producing colonies in each state. As a result, the survey counts colonies that produce honey in more than one state multiple times, potentially inflating national figures (NRC, 2006). In addition, after 1985, NASS no longer counted beekeepers with five or fewer hives, potentially explaining some of the steep decline in colony numbers recorded between 1985 and 1986 (Fig. 3) (Rodenberg, 1992).

NASS also counts honey bee colonies as part of its agricultural census, an effort it conducted once every 5 years since 1982 (Fig. 3). The agricultural census (Ag Census) effort is meant to provide comprehensive information about US farms, including those with apicultural enterprises. It specifically inventories the number of honey bee colonies owned on farms on December 31 of survey years. This may underestimate the number of "production" colonies in the country as beekeepers may reduce colony numbers going into winter to avoid overwintering costs (Daberkow et al., 2009). The census survey also excludes beekeepers who do not produce or sell $1000 worth of produce per year (Hoppe et al., 2007). Total colonies inventoried by the Ag Census show a period of decline in managed colonies similar to that recorded by the Honey report between 1987 and 2002 (17% vs. 22% respectively), however, between 2002 and 2007 the number of colonies recorded by AG Census dramatically increased.

Standardized periodic surveys that quantify colony numbers provide a measure of total losses and/or gains over a period, but do not necessarily capture actual losses over that period. Beekeepers can quickly replace large losses (i.e. winter losses) by splitting surviving colonies and/or by purchasing and installing packages of bees (vanEngelsdorp et al., 2007). It is, therefore, possible for inventories of colonies reported by a given periodic survey to remain stable or even increase when substantial losses occurred between survey dates (Daberkow et al., 2009). This appears to have been the case in 2007 and 2008. After an estimated overwintering loss of 32% and 36% in the winters of 2006–2007 and 2007–2008, respectively (vanEngelsdorp et al., 2007, 2008), the total number of colonies recorded by the Honey report increased by 5% between

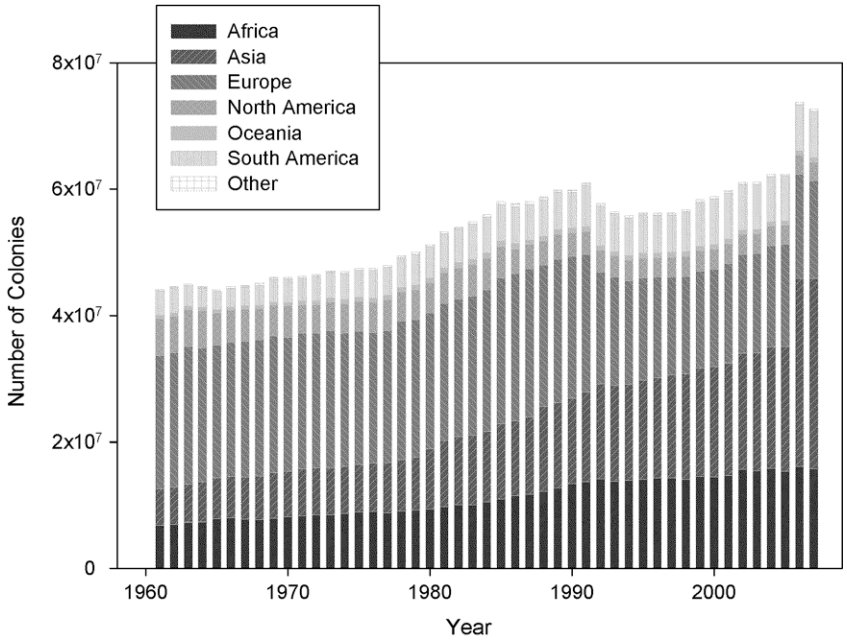

Fig. 1. Total global number of managed honey bee colonies between 1961 and 2007 (FAO, 2009). The large increase in Asian bee populations between 2005–2006 primarily results from countries reporting managed colonies to the FAO for the first time in 2006.

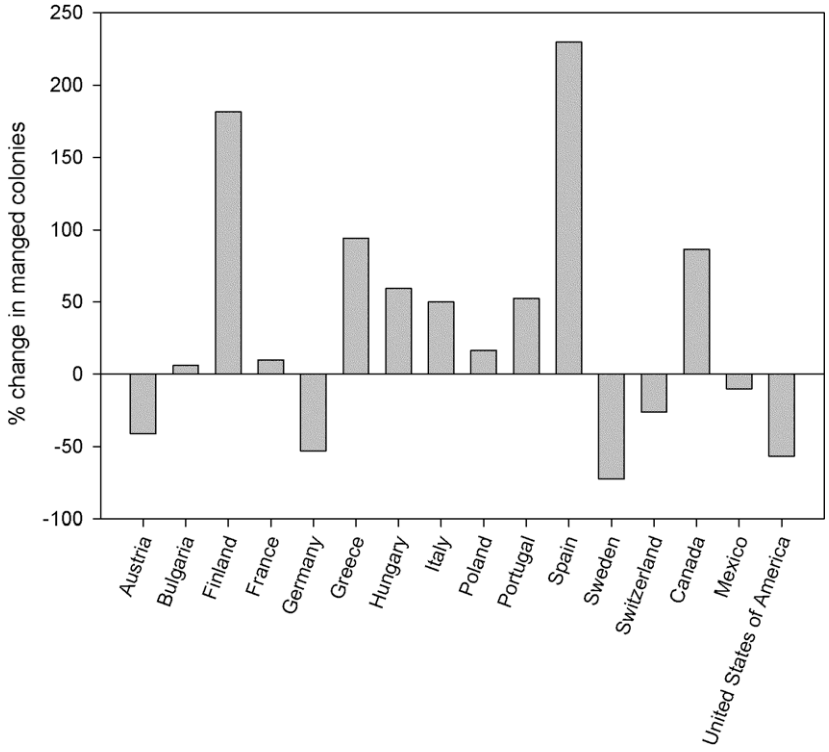

Fig. 2. Percent change in number of managed bee colonies between 1961 and 2006 in selected countries in Europe and North America (FAO, 2009).

2006 and 2007, and decreased by 14% between 2007 and 2008 (USDA-NASS, 2009a,b).

2.3. Europe

Colony numbers in Europe decreased from over 21 million in 1970 to about 15.5 million in 2007 (FAO, 2009). While this decrease was slow and gradual before 1990, a much steeper decline was observed thereafter. As there is no Europe-wide central annual census, comparable to the National Agricultural Statistics Service in the US, estimations of colony numbers and fluctuations over years are much harder to compile. Colony numbers for most countries are reflected in the FAO figures, but for several countries colony number data are either incomplete or do not exist at all. In addition, in some cases the FAO numbers are estimates made by the FAO or the reporting country.

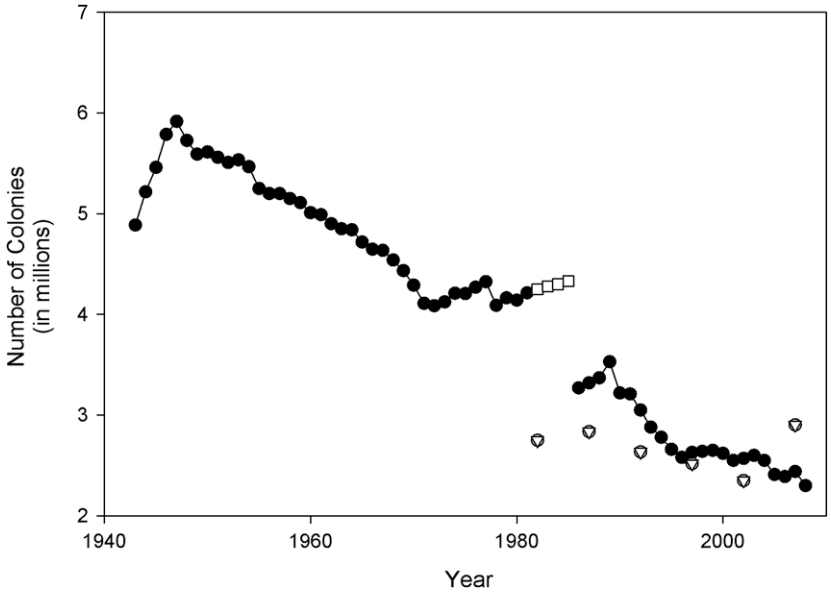

Fig. 3. Numbers of managed honey bee colonies in the United States of America 1944–2008. Annual estimates of the number of honey-producing colonies (solid circles) were obtained from the annual Honey reports with the expectation of the years 1982–1985 when the survey was discontinued. During these years estimates are provided by the USDA Agricultural Stabilization and Conservation Service (hollow squares). Estimates of the total number of colonies as inventoried by AG census are also provided (hollow triangles) (USDA-BAE, 1949; USDA-AMS, 1955; USDA-NASS, 1967, 1972, 1978, 1981; Rodenberg, 1992; USDA-NASS, 1999, 2004a,b, 2009a,b).

Over the last 48 years the change in the number of honey bee colonies managed in individual European states has been extremely variable (Fig. 2). Since 1961, colony numbers in several countries have increased; remarkably Finland and Spain have seen managed colonies increase by more than 50%. These numbers should, however, be viewed with caution as early data were based on FAO estimates. In contrast, Austria and Germany saw a decline in the number of managed bee colonies over the same period, while Sweden saw a drop of approximately 75%.

The changes in populations experienced by different nation states did not occur consistently over time. For instance, colony numbers in Germany have been increasing slowly since 2000, despite experiencing an absolute loss of more than 50% when compared to 1961 populations. In contrast, while the number of colonies managed in France is greater today when compared to 1961 populations, colony numbers have consistently decreased after reaching a peak in 2000.

2.4. Factors to consider when comparing variations in winter mortality between nations: Survey effort and reporting

Overwintering mortality can be extremely variable within a region, for instance in the US in the winter of 2007–2008 the average winter mortality in the US was 35.8%; however, the total loss in individual states ranged from 7.3% to 56.2% (vanEngelsdorp et al., 2008). Overwintering losses in Europe over the winter of 2008–2009 also showed similar variation (Fig. 4). Some caution, however, is required when comparing overwintering losses in different nations, as survey efforts from different countries are not the same. For instance, some results reported in Fig. 4 come from survey efforts that were conducted by beekeeper groups (e.g. Finland), others by regulatory and research officials (e.g. Canada and the US), and others by monitoring specific colonies (e.g. Germany). Typically, beekeepers responding to these surveys are not randomly selected potentially biasing results; for instance, if only beekeepers attending national meetings fill out surveys, non-attending beekeepers' losses, which could be quite different, are not tabulated. In other cases, such as France, only a randomly selected portion of the nation's largest beekeepers were surveyed. Equally variable is the presentation of survey results. In all cases total colony losses were reported, but this number is biased by larger operations, whose losses may differ from smaller operations. As increased attention is given to annual winter losses, uniform survey and reporting methods would be beneficial (see Fig. 4).

3. Factors affecting managed honey bee populations

Many factors may account for the declines of honey bees in the US and Europe. In all likelihood, no one factor on its own can account for all losses or gains over a given time period. Many factors can occur simultaneously and some influence one another. The remainder of this article is a general review of some important factors thought to impact colony numbers and a discussion of their likely impact on honey bee populations.

With few exceptions it is nearly impossible to determine the cause of a honey bee colony death after the fact. If a colony dies during winter, a considerable amount of time may pass before it is noticed by the beekeeper, and clues to the cause are usually lost. To definitively determine the cause or causes of mortality in colonies *a priori* sampling and analysis of a representative portion of colonies is needed. Such longitudinal studies enable causes of mortality to be inferred and the relative risk of risk factors (on their own or in combination) to be calculated.

Several national colony monitoring programs have been initiated. One of the first and most comprehensive of these programs was the German Honey Bee Monitoring Program (http://www.ag-bienenforschung.de), where about 1200 colonies are continuously followed over a period of several years. Colony strength and health status are regularly assessed, and samples are taken and checked for disease and parasite loads. Although laborious and cost-intensive, this project has proven useful, because it generates reliable data enabling relationships between risk factors and colony death to be determined.

3.1. Diseases and parasites

There are many honey bee diseases (bacterial, fungal, viral, microsporidial), parasites (mites), predators (bears, birds, humans),

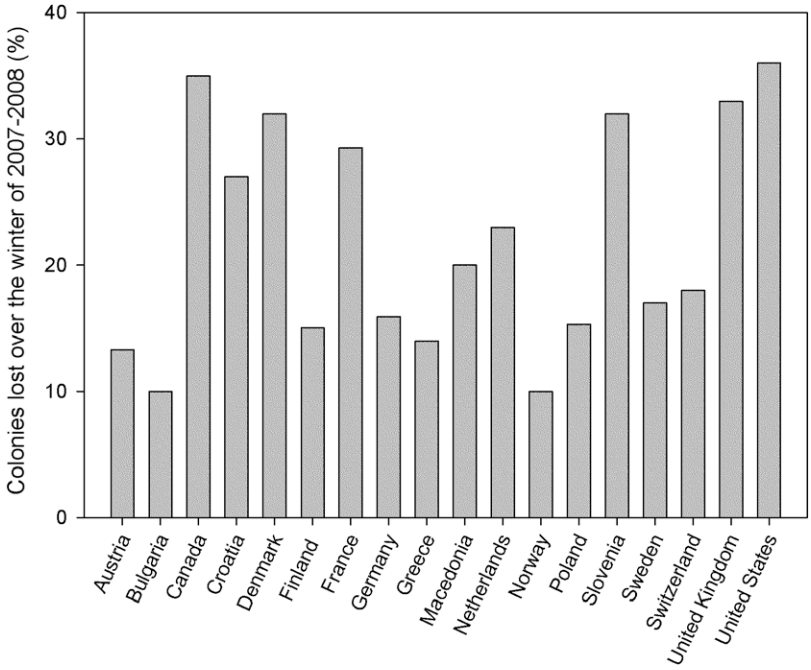

Fig. 4. Percentage of colony winter losses in 2007/2008 in several countries (Anonymous, 2008; Pernal, 2008; Coloss, 2009).

and pests (beetles, moths) that can adversely affect managed honey bee productivity and survival (Morse and Flottum, 1997). A comprehensive discussion of the most important diseases and parasites of bees is provided in subsequent chapters of this issue. Here, we provide a brief discussion of a few of the most significant diseases and parasites, specifically those that may have and/or continue to play a significant role in changing honey bee populations.

3.1.1. Varroa destructor

The parasitic mite, *V. destructor* (Anderson and Trueman, 2000; formerly known as *Varroa jacobsoni*), is the most detrimental honey bee parasite in the world today (Rosenkranz et al., 2010). This mite moved from its original host, the Asian bee *Apis cerana*, to *A. mellifera* colonies imported to Asia. On their new host, varroa mites have spread to nearly all continents where *A. mellifera* are kept. Today, it can safely be assumed that all honey bee colonies within the mite's range harbor varroa mites. As a consequence of mite infestation, dramatic colony losses have repeatedly occurred in affected countries (Finley et al., 1996; Martin et al., 1998; vanEngelsdorp et al., 2007).

Female varroa mites feed on adult bees, but depend on bee brood for reproduction. Both the adult female and her offspring feed on pupae, where they can cause damage by ingestion of hemolymph, resulting in severe nutritional deficits for the developing bee (Duay et al., 2003; Garedew et al., 2004). In addition, alteration of the bee's physiology and secondary infections contribute to the damage (Amdam et al., 2004).

The level of infestation of varroa mites that cause colony damage appears to have decreased over time. In the early 1980s, in Europe, a bee colony could harbor several thousand mites without dramatic symptoms (Boecking and Genersch, 2008). Today, however, a fall infestation rate of 10%, corresponding to about one thousand mites in a colony of 10,000 bees, is considered to be a critical threshold for winter survival of the colony (De Jong, 1997; Siede et al., 2008).

3.1.1.1. Interactions between viruses and mites. Colonies with varroa mite infestations that are not effectively controlled quickly develop disease symptoms and, if left untreated, inevitably will collapse. The damage is manifested by reduced colony development, the presence of malnourished, deformed, and underweight bees, or crawling bees that are unable to fly or have crippled wings (De Jong, 1997). Brood in infested colonies may also have a condition termed "parasitic mite syndrome (PMS)" (Shimanuki et al., 1994). Many of these symptoms are thought to be caused by viruses associated with varroa mite infestations (Hung et al., 1995, 1996). Varroa mites can vector several viruses, most of which were present in honey bees before varroa invasion (Bailey and Ball, 1991), but remained covert, symptomless infections (Bowen-Walker et al., 1999; Yue and Genersch, 2005).

For several of the about 18 known honey bee viruses (Chen and Siede, 2007) interactions with *V. destructor* are known, either through virus transmission by the mite, or through other means of action. For instance, pupae parasitized by varroa mites may suffer from an impaired immune system and seem to be more susceptible to virus infections (Yang and Cox-Foster, 2005). The distribution of many viruses appears to match the distribution of the varroa mite, but, for some viruses, there also appear to be regional differences (Ellis and Munn, 2005). Results from the German Bee Monitoring Program over 4 years indicate a clear and highly significant correlation between colony winter mortality, fall mite infestation rates, and both Deformed wing virus (DWV) and Acute bee paralysis virus (ABPV) loads. Colonies with a high mite load in October had both more viruses and a significantly higher risk of mortality in the winter (Anonymous, 2008).

Although DWV can be transmitted directly from bee to bee, expression of clinical symptoms, such as crippled wings or a shortened abdomen, only occurs after mite-to-pupa transmission of virus particles (Bowen-Walker et al., 1999; Yue and Genersch, 2005; Yue et al., 2006, 2007; Tentcheva et al., 2006). DWV has repeatedly been shown not only to be efficiently transmitted by the mite, but also to replicate in mite tissues (Bowen-Walker et al., 1999; Shen et al., 2005; Yue and Genersch, 2005; Tentcheva et al., 2006). Thus, the number of viral copies transmitted to the bee by the mite is dramatically increased, perhaps also accompanied by immunosuppression of the bee (Shen et al., 2005) or an

increase in virulence of the virus (Gisder et al., 2009). The biology of DWV and in particular the interactions between DWV and *V. destructor* have recently been described in detail (de Miranda and Genersch, 2010).

Like DWV, ABPV was known as a honey bee virus before the arrival of varroa mites, although it usually did not cause clinical symptoms or lead to colony death (Bailey and Gibbs, 1964). Nevertheless, the prevalence of ABPV in Europe was shown to increase after the arrival of the mite (Allen and Ball, 1996), which had been identified as an efficient transmission vector (Ball, 1983). While there is currently no experimental evidence for viral replication of ABPV in varroa mites, it has been confirmed that infections with this virus are more deadly in combination with the mites. A recent study found a strong correlation between high fall mite loads, viral loads and increased winter mortality (Siede et al., 2008). In contrast, all colonies with viral infections, but without detectable mite levels in the fall, survived (Siede et al., 2008).

The highly virulent Kashmir bee virus (KBV) has been found to be present in countries (e.g. Australia) still free of varroa mites (Bailey et al., 1979); however, interactions between the virus and the mite have been established. KBV can be transmitted by varroa mites, but there is still no proof of viral replication in mite tissues (Chen et al., 2004; Shen et al., 2005). The presence of mites clearly elevates viral titers in infected bee pupae suggesting that increased viral replication in the bee is correlated with parasitization although the exact mechanism remains elusive (Shen et al., 2005). It has been hypothesized that immunosuppression of the bee by protein components of the mite saliva facilitates virus replication (Shen et al., 2005). KBV has been shown to be prevalent in the U.S, but is unevenly distributed in Europe. It was found in France, but appears to be mostly absent in Germany (Siede and Büchler, 2004).

The Israeli acute paralysis virus (IAPV) has received considerable scientific interest as a potential causative agent for Colony Collapse Disorder (CCD), because its presence was correlated to an increased risk for colony collapse (Cox-Foster et al., 2007). Because IAPV has been detected in samples that predate CCD (Chen and Evans, 2007), its role in CCD is likely secondary (Cox-Foster and vanEngelsdorp, 2009). An interaction between IAPV and varroa mites has not been demonstrated to date.

However, recent data suggest that ABPV, KBV, and IAPV may not represent clearly separated, different species, but rather form a complex of closely related species. Due to their close genetic relationship, especially KBV and IAPV sequences have been frequently misclassified in the literature and the public sequence databases (de Miranda et al., 2010). The similarity of these three viruses has to be considered when evaluating their impact on colony health.

3.1.1.2. Impact of varroa mites on US bee populations. The negative impact of varroa mite parasitism on individual colonies is clear (Rosenkranz et al., 2010). However, its overall impact on managed bee populations may be less pronounced. According to the USDA (USDA-NASS, 1999, 2004a, 2009b,c), the number of managed colonies in the US dropped some 26% since the mite was introduced in 1987, a decline often linked to varroa-mediated mortality (NRC, 2006). However, the rate of decline after 1987 (1.09% per year) is barely different from the rate of decline recorded between 1947 and 1987 (1.11% per year) suggesting that varroa mites did not have a direct effect on the rate of colony loss, which began more than six decades ago. This is not to say that the mite has had no impact on the US apicultural industry. A majority of beekeepers in the US (~70%) are relatively small, managing less than 25 colonies. Between 1987 and 2002, there was a ~40% decline in the number of these small apicultural farms, but these operations accounted for only a small portion of the colonies managed in the nation (<10%) (Daberkow et al., 2009). It seems likely that many of those small operations leaving the apicultural industry over the period did so as a result of an inability to control varroa mites. Concurrent with a decrease in the number of small beekeepers was a 66% increase in the number of colonies managed by the remaining beekeeping operations (Daberkow et al., 2009). These larger operations are presumably better able to control mite populations and may have increased operational sizes in anticipation of increased annual losses (Burgett, 2004).

A simple comparison of colony numbers from year to year may mask fluctuations in colony numbers that can occur between survey dates. Prior to the introduction of varroa and tracheal mites, overwintering losses of 10% were normal (Voorhies et al., 1933). More recently, beekeepers reporting "normal" losses had an average loss of 21% (vanEngelsdorp et al., 2008). Since the introduction of mites, severe overwintering losses have been recorded. Some of these losses are almost certainly linked to mite infestation (NRC, 2006). For instance, over the winter of 1995–1996 Pennsylvania beekeepers recorded an average loss of 53%. Those beekeepers that reported treating colonies with Apistan (for varroa mite control) in the fall of 1995 reduced their overwintering loss by an average of 26% (Finley et al., 1996). In a more recent survey of winter losses in the US, beekeepers considered varroa mites to be the third most important contributor to mortality following queen failure and starvation (vanEngelsdorp et al., 2008).

While overwintering losses do not seem to have had a pronounced effect on the overall rate of declines in managed honey bees enumerated in the United States, they have almost certainly had a pronounced effect on beekeepers' bottom line (Kemp, 2000). Managing varroa mite populations has directly increased operational costs because of the costs associated with purchasing and applying control products (NRC, 2006). The costs of mite control applications may have been passed onto producers renting bees for pollination by way of increased fees (Burgett, 2004). Mites have also indirectly affected beekeeper profitability as colonies made or purchased to replace mite-killed colonies are smaller than full sized overwintered colonies, and hence, tend to be less productive (NRC, 2006).

3.1.2. Nosema spp.

Although not always resulting in evident disease symptoms, infections with microsporidia of the genus *Nosema* are regarded among the diseases that are most economically important for beekeepers (Fries, 1993, 1997, 2010). Infections with *Nosema* spp. are known to be correlated with reduced lifespan of individual bees, reduced performance of colonies, and increased winter mortality (Fries et al., 1984). The honey bee is host to two different species, *Nosema apis*, which has been known for a long time as a bee pathogen (Zander, 1909), and the recently described *Nosema ceranae* (Fries et al., 1996). *Nosema* infections are transmitted horizontally among bees, by ingestion of spores from the environment. For example, housecleaning bees, on removing nosema-infected bee feces deposited in the hive, ingest nosema spores, which then germinate in the ventriculus, causing inflammation of and damage to the gut epithelial cells.

N. ceranae originates from Asia and was originally described as a pathogen of the Asian cavity nesting bee *A. cerana* (Fries et al., 1996). It was later found to occur in colonies of *A. mellifera* in Taiwan (Huang et al., 2007) and reported from Spain (Higes et al., 2006). It has been suggested that *N. ceranae* may be more virulent than *N. apis* when infecting *A. mellifera*, and it has been reported to cause severe colony losses, especially in southern Europe (Higes et al., 2007, 2008). *N. ceranae* has been present in the US since at least 1995 (Chen et al., 2007) and in Europe (Finland) since 1998 (Paxton et al., 2007). While the time of the *N. ceranae* jump to *A. mellifera*, and the date of its arrival in Europe

and North America remain unknown, these are most likely recent events (Paxton et al., 2007).

3.1.3. Bacterial brood diseases

American foulbrood (AFB; *Paenibacillus larvae*) is the most serious bacterial disease of the honey bee (for a recent review see: Genersch, 2010). Early apiculturists did not distinguish this disease from European foulbrood (EFB; *Melissococcus plutonius*; for a recent review see: Forsgren, 2010), which is now mostly considered less virulent than AFB.

3.1.3.1. American foulbrood. American foulbrood is a bacterial disease of the bee brood, caused by the gram-positive bacterium *Paenibacillus larvae*. Due to its high contagiousness, easy and rapid spread within a colony, among colonies in an apiary, and between apiaries, American foulbrood is a notifiable disease in many countries where it is subject to strict regulations, enforced by veterinary authorities. Usually, colonies with active AFB have to be destroyed (burned) to prevent the disease from spreading further. While several countries, like the US, permit the prophylactic use of antibiotics to control AFB, many countries follow an opposite approach, interdicting any antibiotic treatment. Antibiotics are not effective in killing spores, and non-destructive control methods, such as the "shook swarm technique" (i.e., shaking bees onto new comb foundation and destroying the infected comb), are also available (Pernal, 2008). Common problems associated with antibiotic use are increased occurrence of resistant AFB strains and antibiotic residues in honey (Miyagi et al., 2000; Mussen, 2000; Kochansky et al., 2001a; Lodesani and Costa, 2005).

Several European countries are currently changing the focus of AFB control toward a more efficient prevention of clinical AFB outbreaks by prophylactic determination of *P. larvae* spores in honey samples. By recognizing infected although not yet diseased colonies, i.e. before clinical symptoms of the disease appear, sanitation measures can be taken at an early stage and outbreaks can be prevented.

3.1.3.2. European foulbrood. European foulbrood is a disease of bee larvae, caused by the gram-positive bacterium *Melissococcus plutonius* (formerly known as *Melissococcus pluton*) (Bailey and Ball, 1991; Shimanuki, 1997). In several countries, EFB is a notifiable disease and currently appears particularly prevalent and dramatically increasing in the UK (Wilkins et al., 2007; Tomkies et al., 2009) and Switzerland (Forsgren et al., 2005; Belloy et al., 2007; Roetschi et al., 2008).

3.1.3.3. Impact of American and European foulbrood on US managed honey bee populations. Of all diseases of the honey bee, AFB has had the greatest impact on the industry. In 2000, annual economic loss attributed to AFB infection in the US was estimated at US$5 million (Eischen et al., 2005). This estimate is likely only a fraction of the cost to the industry prior to the mid-1900s.

The earliest known documented shipment of bees to the Americas occurred from England in December of 1621 which likely arrived several months later (Oertel, 1976). By 1650 nearly all farms in New England are reported to have had a colony or two of bees. However, the number of bees managed by these colonists rapidly declined after 1670, presumably because of AFB (Pellett, 1938). Substantive documentation of AFB's presence in the new world, however, did not occur until more than a century later, by the late 1800s and early 1900s. Then, AFB and EFB were a "veritable scourge" in many parts of the country (Surface, 1916) resulting in the passage of many state bee laws and implementation of state apiary inspection programs (Phillips, 1920). These early apiary laws proposed to mitigate the spread of AFB by requiring the destruction of all infected colonies and the burning of infected bee equipment. This effort was not insignificant, for instance, in the state of Pennsylvania over 32,000 colonies were burned between 1930 and 1965, a number that is just shy of the total number of colonies in the state in 2007 according to the Ag Census (PA Dept of Ag, unpublished records; USDA-NASS, 2009c). Beekeepers that did not burn their colonies had their colonies burned by inspectors, and some particularly uncooperative individuals were fined $100 dollars (~US$1,173-2007 adjusted dollars) or jailed for non-compliance.

Early laws also required the mandatory transfer of colonies from box and gum hives (i.e. colonies established in crudely made boxes without frames, or in hollowed out tree trunks) into movable frame hives which permitted inspection of colonies for disease. The Rev. L. L. Langstroth's discovery of the bee space (that is the space of about 0.95 cm between hive components which bees will not fill with propolis nor fill with additional comb), and subsequent development of the movable frame hive in 1852 revolutionized the practice of keeping bees.

Pellett (1938) claims that prior to the widespread adoption of movable frame hives, honey bee diseases in America were not widely distributed. While he does not explicitly provide evidence for this claim, one can assume the basis of his claim relies on the fact that, except for wax moth, *Galleria mellonella*, there exists little discussion of disease in the robust bee literature of the time prior to the 1860s. Many factors, however, could have contributed to underreporting of disease. As Pellett himself states, before the adoption of the movable frame hive, little was known about the biology of the colony, and so diseases, if present, would have been permitted to run their course. Weakened colonies provide opportunity for wax moth larvae, which in the process of consuming pollen and cocoon castings in the comb, destroy them. Wax moth damage is distinct, and it is likely that many cases of colony death attributed to the moth were, in fact, caused by other factors such as a failing queen or a disease (Benton, 1899). As summarized by Miller (1901), blaming wax moth for colony death is the same as concluding that "maggots had killed a horse if (one) should find a horse filled with them a few weeks after it had been shoot."

Beekeeping practice prior to moveable frame hives may also have had an indirect role on disease mediation. Beekeepers killed both the heaviest and weakest fall hives to harvest honey The strongest colonies were chosen because they were heavy with honey, and the weakest were chosen because they were the most likely to die over the winter. The annual killing of weak (and possibly diseased) colonies and rendering their comb could have slowed the spread and multiplication of disease harbored in the comb similar to the way regular comb replacement reduces disease incidence (Fries, 1988).

Prior to 1943, US honey and bees wax production data were collected every 10 years by the National Census, however colony numbers were not consistently reported, and when they were reported were tabulated at different times of the year, making meaningful comparisons of colony numbers difficult. Thus, the impact of AFB/EFB on US colony numbers is difficult to assess. Using honey production figures as a rough estimate of colony numbers, Voorhies et al. (1933) noted a distinct increase in honey production between 1860 and 1890, followed by a two decade decline in production, and then an increase in production in 1920–1930. While many factors likely contributed to these gains and losses, including changing climate and forage availability (Voorhies et al., 1933), it is of note that decreased productivity between 1890 and 1910 was associated with notable outbreaks of both EFB and AFB (Surface, 1916; Voorhies et al., 1933). Increased production observed in the 1920s coincided with decreased disease incidents. The incidence of EFB, which reportedly killed many thousands of colonies, was dramatically reduced by changing the race of bees used. The EFB-susceptible German black bee (*A. mellifera mellifera*) was largely replaced with

the EFB-resistant Italian bee (*A. mellifera ligustica*) in the second decade of the 1900s (Voorhies et al., 1933). Early State efforts certainly reduced AFB incidence, however the widespread use of antibiotics (Sulfathiazole in the 1940s and 1950s, Oxytetracycline from the 1950s until the 1990s, and more recently Tylosin tartrate: Haseman and Childers, 1944; Turell, 1974; Elzen et al., 2002) has significantly (and arguably more dramatically) reduced the incidence of bacterial diseases. However, while prophylactic use of antibiotics may prevent disease outbreak, discontinuation of regular antibiotic application often results in disease reoccurrence (Alippi et al., 1999).

3.1.4. Unexplained or unresolved bee epidemics

The beekeeping literature is ripe with incidents of bee epidemics, localized or regional events typified by mass mortality of honey bee colonies (Underwood and vanEngelsdorp, 2007). Many of these losses remain unexplained, or their cause remains disputed. Perhaps the most infamous honey bee epidemic occurred on the Isle of Wight during the early 1900s. In three events between 1905 and 1919, 90% of the island's bees was lost (Bailey, 1964; Adam, 1968). The cause of the affliction remains disputed, with some arguing that the protozoan fungus, *N. apis* was the cause (Fantham and Porter, 1912), while others believe the honey bee tracheal mite *Acarapis woodi* was to blame (Adam, 1968). Both diseases of adult bees are known to have a pronounced negative effect on colony overwintering ability. In a survey of Pennsylvania beekeepers conducted just as *A. woodi* was first spreading in the state, beekeepers who overwintered colonies with *A. woodi* infestations lost an average of 31% of their colonies as compared to the 11% loss suffered by their non-infested neighbors (Frazier et al., 1994). More recently, large losses of honey bees were experienced by Spanish beekeepers and blamed on *N. ceranae* (Higes et al., 2008).

Colony Collapse Disorder (CCD) is a condition of colonies that first came to light in the United States in the fall of 2006. The condition is defined by a clear set of symptoms that distinguishes it from most other conditions. These include the total lack of dead bees in the colony or apiary, evidence that the loss of adult bees from dead or dying colonies was rapid, and a lack of kleptoparasitism in dead hives despite the presence of surplus honey and pollen stores (Cox-Foster and vanEngelsdorp, 2009). Outbreaks of colony mortality similar to CCD have occurred in the US before (Underwood and vanEngelsdorp, 2007), although not to the extent documented in the winters of 2006–2008 (vanEngelsdorp et al., 2007, 2008).

The cause of CCD remains unknown (Cox-Foster and vanEngelsdorp, 2009). It is likely that several "stress factors", acting alone or in combination, contribute to weakening the bees and allowing opportunistic pathogens to infect and eventually kill colonies (Cox-Foster and vanEngelsdorp, 2009). Over the winter of 2007–2008 operations suspected of suffering from CCD lost more than two times the number of colonies lost by operations not suffering from the condition (vanEngelsdorp et al., 2008).

The effect of high overwintering losses on total managed bee populations is not clear. As previously discussed, high overwintering losses do not necessarily translate to an overall reduction in colonies managed by beekeepers in the summer (vanEngelsdorp et al., 2008). In fact, the high losses experienced over the winter of 2006–2007 may explain the increase in colonies enumerated in December of 2007 by the Ag Census (Fig. 3). Since 2004, an increasing number of US commercial beekeepers have begun moving colonies from across the continent to pollinate almonds, motivated largely by the increased demand for pollinating units, which caused colony rental prices to increase from US$54 a unit in 2004 to US$136 in 2006 (Sumner and Boriss, 2006). The large losses experienced by beekeepers in the winter of 2006–2007 left several operations without enough bees to meet their contractual obligations. As a result, many migratory beekeepers may have increased their stocks the following winter in anticipation or fear of higher losses. The greatest increases in colony inventories occurred in California and in states in which large numbers of colonies are wintered (Florida, Georgia, and Texas) before moving to California tacitly supporting this hypothesis (USDA-NASS, 2004a, 2009c).

3.2. Non-disease factors influencing managed honey bee populations

3.2.1. Pesticides

Modern agriculture increasingly depends on the use of chemical substances to control weeds, fungi and arthropod pests to ensure high yields. Honey bees may frequently become exposed to environmental chemicals as a consequence of their foraging activities, and traditionally, the focus of pesticide regulations was more on protection of bees against direct poisoning (Croft, 1990; Thompson, 2003; Desneux et al., 2007). However, since the substances that are being used have changed, damage from acute toxicity is not the only threat to bees. Instead, sub-lethal effects such as paralysis, disorientation or behavioral changes, both from short-term and long-term exposure, increasingly come into focus.

3.2.1.1. Direct effects – poisoning. In most countries, a legal framework is in place to protect honey bees and other pollinator insects from the negative effects of pesticides and other agrochemicals. The relevant decrees are the European Council Directive 91/414 in Europe, and the Federal Insecticide Fungicide and Rodenticide Act in the US. To determine the effects of pesticide exposure on bees, the standard methods used are the calculation of the LD50 (median lethal dose) or LC50 (median lethal concentration) of a given substance with respect to adult bees or larvae. Another common measure is the hazard quotient which is based on the LD50. Based on the results of these assays, substances are then classified into different categories of risk to bees (e.g. in Germany B1–B4), and conditions and restrictions for application of substances in each category are defined.

As a consequence of the protection by laws and decrees, direct poisoning of honey bees by pesticides in the field is now a comparatively infrequent event in most countries of Europe and North America. For instance, the absolute number of samples with damaged bees sent to the Julius-Kühn-Institut (JKI) in Germany (the central institution for analysis of damage by poison), decreased from more than 400 in the 1970s to 67 in 2004 (www.jki.bund.de). However, the hazards of agricultural pesticides to honey bees have been most dramatically illustrated by a recent accident in southern Germany, where in the spring of 2008, more than 11,000 honey bee colonies were severely damaged by direct poisoning. The colonies were poisoned by toxic dust containing neonicotinoid insecticides that had become loosened from dressed corn seed due to incomplete incrustation during the dressing process. When the corn seed was sowed using pneumatic sowing machines, the dust became windborne and drifted across the fields onto colonies and other plants visited by honey bees at the time. Unfortunately, major nectar and pollen sources, such as dandelion, oilseed rape and fruit trees were blooming at the time so that millions of foraging bees were poisoned by the dust (http://www.jki.bund.de/presse).

The seed dressing with the neonicotinoid insecticide Clothianidin had been made compulsory by decree for broad areas in southern Germany to prevent outbreaks of the corn root worm *Diabrotica virgifera*. As a consequence of the accident, the registration of Clothianidin in Germany has been withdrawn, and the ingredient may currently not be sold or used (http://www.bvl.bund.de). Among EU countries, the legal situation concerning neonicotinoid insecticides is currently quite variable. For example, they are banned in France, but seed dressing of corn is still a compulsory measure against corn root worm in some other countries, including Austria.

3.2.1.2. Sub-lethal effects. In contrast to direct poisoning of bees that is apparent and easily observable, sub-lethal effects of pesticides on honey bees and other pollinators are much more difficult to demonstrate. However, they have received growing scientific interest (recently reviewed by Desneux et al. (2007)). Sub-lethal negative side effects of pesticides often may become apparent only after prolonged exposure. They may affect various life stages and organizational levels of honey bees, ranging from cell physiology or the immune system of the individual bee to consequences affecting the colony as a whole, such as effects on learning, behavior and communication (Desneux et al., 2007).

While data on effects of pesticides on singular aspects of honey bee life are emerging, very few datasets exist that describe the pesticide load within honey bee colonies, or indicate possible correlations between pesticide exposure and colony losses. In a recent survey conducted in the US, a considerable number of pesticides were detected in samples of pollen (108 samples) and beeswax (88 samples) (Frazier et al., 2008). In association with elevated pesticide levels, a new, albeit rare, condition in bee bread recently was described from honey bee colonies, and named "entombed pollen" (vanEngelsdorp et al., 2009). Compared to normal pollen, samples of entombed pollen contained significantly higher levels of pesticides, most prominently among them the miticides coumaphos and fluvalinate, and the fungicide chlorothalonil. While experimental feeding with entombed pollen did not lead to increased mortality of larvae or adult bees, colonies containing such pollen had a higher risk of mortality in the field (vanEngelsdorp et al., 2009).

In a recent field study in France, residues of several pesticides, including neonicotinoid insecticides and their metabolites, were detected in honey bee colonies. Although no direct correlations between colony mortality and residues could be observed, synergistic effects of pesticides with other factors affecting colony health could not be precluded (Chauzat et al., 2009).

Within the German Honey Bee Monitoring Program, possible effects of pesticide exposure in relation to winter losses have also been investigated using a sensitive method allowing detection and quantification of a total of 258 relevant pesticides (Anonymous, 2008). No direct correlations between single substances and colony death were found, although the analysis focused on samples with poor overwintering scores. The most unexpected result of this survey was the considerable number of agricultural pesticides found in the bee bread. Most samples contained more than one pesticide and only 24% of the samples were free of residues. The total number of single residues found is compiled in Fig. 5, broken down by substance class, but irrespective of residue level. Fungicides were the most frequent pesticides detected, but the number of both herbicides and insecticides/acaricides increased in 2007 compared to previous years. Among all contaminants, the miticide coumaphos, applied by beekeepers to control varroa mites, was the most frequently detected single substance (46 detections in 2005/06, 33 in 2007).

The comparison of the results from Germany and the US reveals striking differences in the residue levels between pollen samples collected in these two countries, although this observation has to be interpreted with caution in light of the small and unequal sample sizes (vanEngelsdorp et al., 2009; Anonymous, 2008). For example, residues of chlorothalonil (a fungicide), with mean levels of over 1300 ppb in entombed pollen from the US, were not found in Germany. Samples of entombed or capped pollen in the US study had mean coumaphos residue levels of about 800 ppb, while the highest single coumaphos residue detected in German bee bread samples was about 130 ppb. Likewise, the highest residue of the acaricide tau-fluvalinate in Germany was about 20 ppb, compared to 600 ppb in the US study. This can be explained by the fact that tau-fluvalinate is not registered for the control of varroa mites in Germany, but is registered for this use in the US.

3.2.1.3. Residues of varroacides. Residues of varroacides, substances used to kill varroa mites, increasingly appear to be of major importance in the discussion of sub-lethal pesticide loads in honey bee colonies. Varroacides, such as coumaphos or fluvalinate, commonly used for varroa mite control and registered for use in many formulations in several countries, have been frequently found in the honey bee environment in significant concentrations. In recent surveys from France and the US, 100% of all tested wax samples were contaminated with both substances (Martel et al., 2007; Frazier et al., 2008). No recent data on wax are available from Germany, where

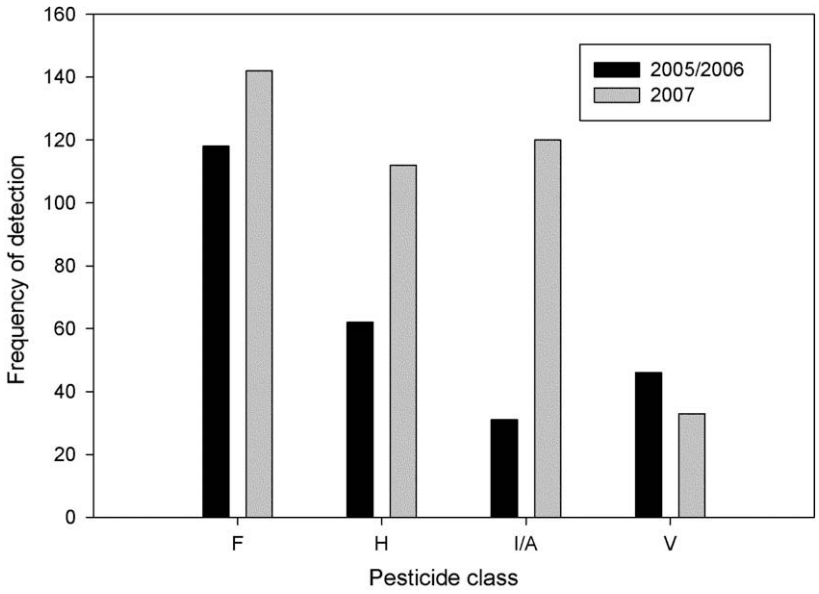

Fig. 5. Number of single pesticide residues in bee bread from Germany in the years 2005/06 (105 samples) and 2007 (110), broken down by substance class. *X*-axis: F = fungicides, H = herbicides, I/A = insecticides and acaricides, V = varroacides; *Y*-axis: number of times a substance from the respective classes was detected in the samples, irrespective of residue level. The figure also contains all instances where a substance was detected, but in concentrations too low to quantify (Anonymous, 2008).

in 1999 coumaphos was found in 28% of wax samples (Wallner, 1999). The situation is further exacerbated by the fact that mites have increasingly developed resistance against various treatments in different parts of the world (Milani, 1999; Pettis, 2004; Lodesani and Costa, 2005). In response to this problem, and driven by economic considerations, beekeepers may resort to the use of unauthorized products, often in excessive quantities (Martel et al., 2007; Chauzat et al., 2009).

While pesticide residues in honey, wax and other parts of the "bee environment" have been explored (Wallner, 1999; Kochansky et al., 2001b; Tremolada et al., 2004; Bogdanov, 2006), their effect on bee health is largely unknown (Martel et al., 2007; Frazier et al., 2008; Desneux et al., 2007). The consequences of long-term exposure to varroacide residues on larvae, pupae and adult bees remain unknown and, thus, future research is clearly needed.

3.2.2. Effect of pesticide poisoning on managed honey bee colony numbers

The adverse effect pesticide application has on colony numbers dates back to the early part of the 1900s, when arsenic spraying of fruit trees was listed as one of the top five reasons why colony numbers in California were declining (Voorhies et al., 1933). Agricultural sprays used widely in the 1960s and 1970s were particularly hard on bees, with a 48% drop in colony numbers experienced in Arizona between 1963 and 1977 that was blamed on pesticide-mediated bee kills. Between 1962 and 1972 California beekeepers were thought to have lost an average of 62,500 colonies a year (~11.5%) from pesticide poisoning. While many of these colonies were replaced, the cost of bee kills to the industry was not insignificant. A 1962 study of Washington beekeepers concluded that as a result of pesticide-mediated bee kills, they lost about 3.2% on their apicultural investments compared to an 11% gain they would have received in the absence of any such bee losses (Johansen and Mayer, 1990).

3.3. Genetically Modified Crops

Genetically Modified Organisms (GMOs) were developed, in part, to help prevent the potentially adverse effects of pesticides on pollinators (NRC, 2006). Initial concerns that GMO crops with insecticidal properties would have a negative, albeit sub-lethal, effect on bees (Malone and Pham-Delègue, 2001) have not been verified (Marvier et al., 2007; Duan et al., 2008). For example, worker bees and colonies fed pollen from genetically modified Bt corn did not have increased rates of mortality (Rose et al., 2007). Further, pollen from Bt corn did not affect the microflora in bee intestines (Babendreier et al., 2007) nor did it affect hypopharyngeal gland development (Malone et al., 2004). The Cry1Ab toxin expressed in Bt corn did not affect learning performance of the honey bee under natural conditions suggesting that consumption of Bt corn pollen expressing Cry1Ab is unlikely to have an effect on colony performance (Ramirez-Romero et al., 2008).

3.4. Genetic variability of honey bee colonies

The evolution of beekeeping as a cultural practice and as a profession (Crane, 1999) has resulted in the development and use of techniques that may ultimately reduce the vitality of honey bee colonies. Selective pressures on the bee population are routinely being influenced by management decisions like the regular use of medication to control parasites and diseases, the protection of hives against cold weather, and artificial feeding. Consequently, weak and susceptible colonies are kept alive and given a chance to participate in the reproductive process. Colonies that were treated against diseases or parasites may be selected over non-treated colonies that have been forced to cope with the pathogens, thereby lowering the natural resistance against diseases and other environmental selection factors in the honey bee population.

In addition, beekeeping has also favored the distribution of the preferred commercial subspecies outside of their native range, usually to the disadvantage of less productive subspecies or species of honey bees. Thus, genetic diversity can be lost rapidly as native populations are threatened by newly introduced parasites or replaced by imported stock. Large parts of the original *A. m. mellifera* areas in Western Europe are today occupied by introduced stock with more desirable apicultural traits.

As a consequence of professional beekeeping, specialists in queen breeding produce and distribute large numbers of progeny from few queen mothers, a process which inevitably reduces genetic variability in honey bee populations. To increase genetic diversity, several European countries, especially those with a tradition in the production of the commercially most desirable races (*A. m. ligustica* and *A. m. carnica*), have coordinated national breeding schemes. From these programs, thousands of queens are produced and exported across Europe and the world (Lodesani and Costa, 2003). In the US, honey bees are not native and were first imported in the 17th century (Sheppard, 1989a,b). Thus, the genetic variability of the US honey bee population is reduced compared to that of indigenous honey bee populations of Europe (Sheppard, 1988). In addition, it has been reported that as few as 500 breeder queens have been used to provide progeny for most of the commercial hives present in the US (Schiff and Sheppard, 1995, 1996; Delaney et al., 2009). Breeding, thus, can act as a bottleneck, significantly reducing genetic variability in honey bee populations. Genetic similarity among colonies in wide areas increases the chances of successful disease transmission, and therefore the risk of colony losses.

Sufficient genetic variability within the colony is also known to be important for disease resistance, homeostasis, thermoregulation and overall colony fitness (Tarpy, 2003; Jones et al., 2004; Graham et al., 2006; Mattila and Seeley, 2007). If genetic variability is important for immune response and defense against parasites, colonies with diminished genetic variability are left with a reduced capacity to ward off stressors.

3.5. Poor queens

Anecdotal reports of increased rates of queen failure, supersedure, and drone laying have persisted in the US since in the mid-1990s (Camazine et al., 1998). Over 4 years of monitoring, the Pennsylvania apiary inspection program found that, on average, 2.36% of all inspected colonies were queenless (PDA, unpublished data). US beekeepers ranked poor queens as the number one cause of winter mortality (vanEngelsdorp et al., 2008). The reason for poor queen quality is not understood, and could be related to several factors. *N. apis* and possibly other infections of queens may be responsible for increased rates of supersedure (Camazine et al., 1998; Loskotova et al., 1980). Rates of queen failure may be related to environmental factors such as placement under high power electrical lines (Greenberg et al., 1981). Colonies headed by queens that are being superseded are less productive, and there is a significant risk that supersedure will fail, leading to queenlessness (Camazine et al., 1998). Poor queens may also be the result of the presence of pesticides in wax comb. The use of synthetic miticides to control varroa mite populations is common in the US, and these lipophilic products can build up in the wax over time (Bogdanov, 2006). Coumaphos, a product almost universally found in wax from the brood nest (Frazier et al., 2008), is known to have a detrimental effect on queen rearing (Pettis et al., 2004). Thus, it is possible that colonies with high coumaphos loads in their wax are having difficulty replacing failing queens.

3.6. Bee forage

The availability of adequate bee pasture has impacts on both beekeeping profitability and bee health. The need for adequate forage was recognized early on by US beekeepers and motivated early migratory beekeeping (Anonymous, 1792). The amount and quality of bee pasture in the US has been declining consistently for over the last half a century, largely on account of changing agricultural practices. For example, the use of fertilizers has allowed for a reduction in the rotation of legumes into cropping systems and the extensive use of herbicides has reduced weeds both within crops and at crop edges (Bohan et al., 2005). In addition, reduced pasturing of cattle and the harvesting of alfalfa before bloom to maximize protein content, have all played a role in the reduction of available bee forage. The result has been a near stagnant colony productivity between 1945 and 1981 (19.4 and 20 kg/colony per year) (Ayers and Harman, 1992; Bohan et al., 2005).

Increased colony losses suffered by individual states between 1992 and 2003 have been linked with decreased ratios of open land to developed land. States with greater amounts of open space tended to have more productive colonies, presumably because they had more available forage (Naug, in press). Decreased productivity can have a dramatic effect on total colony numbers in several ways. Productive colonies are less likely to starve over the winter, and starvation has been identified as the second most important cause of winter mortality in the US (vanEngelsdorp et al., 2008). Malnourished colonies are more susceptible to disease outbreaks (Gilliam, 1986) and are less able to tolerate pesticide exposure (Wahl and Ulm, 1983). Finally, and perhaps most importantly, less productive colonies translate into decreased profitability for beekeepers. The near exponential increase in colony productivity that occurred in Canada between 1945 and 1982 has been linked to the increased acreages of superior nectar crops like canola, making beekeeping more profitable, which in turn played a role in the 70% increase in colony numbers during that period (Ayers and Harman, 1992).

Changing agricultural practices as well as increased urbanization and suburban sprawl have also decreased available apiary sites. The consequence of reduced apiary locations on total colony numbers is difficult to ascertain but is almost certainly detrimental. Reduction in apiary locations is of particular concern in areas where Africanized "killer" bees have become or are becoming established. Florida has recently been colonized with feral populations of Africanized honey bees. As a result, some private and public land owners, fearing litigation, have requested that colonies be removed from long-established apiary sites (Jerry Hayes, personal communication). Public concern over "killer" bees has also motivated the passage of local ordinances that forbid or restrict beekeeping – a practice that is counterproductive as the presence of managed European bees is thought to slow the establishment of Africanized bees (Jerry Hayes, personal communication). It should be noted that there is no evidence that Africanized honey bees have directly caused honey bee declines since their introduction into the United States in 1990 (NRC, 2006).

3.7. Weather and climate

Weather has a very real effect on colony welfare. Extended periods of cold, rainy, and hot weather have been blamed on severe, oft unexplained, colony mortality in the past (Anonymous, 1869; Kauffeld et al., 1976). Beekeepers identified severe winter weather as the fourth most important contributor to winter mortality in the US (vanEngelsdorp et al., 2008). Weather can have a direct effect on colony productivity. For example, higher ambient temperatures tend to increase colony productivity because of reduced metabolic demands on foragers (Harrison and Fewell, 2002), while long periods of rain and cool weather have a detrimental effect on productivity as bees remain in the hive.

Arguably, the more significant effects of weather on colony productivity, both positive and negative, are indirect. High temperatures and sufficient precipitation are both correlated to increased nectar production (Shuel, 1992), which in turn translates to increased colony productivity (Voorhies et al., 1933). Conversely, insufficient rain or rain at inopportune times can have a negative effect on colony productivity. Both prolonged summer drought and persistent fall rains have been blamed on poor overwintering in the northeastern US as they prevent fall plants, such as goldenrod and aster, from producing their usual amounts of pollen and nectar. Dwindling pollen reserves in the fall result in early cessation of brood rearing that triggers the premature development of long-lived winter bees (Mattila and Otis, 2007). Colonies containing winter bees that were reared early because of pollen scarcity are less likely to survive the winter than those colonies that rear winter bees later in the fall.

Weather can also have an effect on pathogen loads within colonies. For example, temperature and humidity have a direct effect on varroa mite population growth (Harris et al., 2003). Conversely, cool weather, especially when a colony's adult population is small (which is common in the spring), can result in chilled brood. While chilling can kill immature bees outright, brood chilling is required for some pathogens, such as chalkbrood, *Ascosphaera apis*, to become established (Bailey and Ball, 1991), and adult bees that were chilled when immature are more susceptible to *A. woodi* infestation (McMullan and Brown, 2005).

In tropical regions, where floral resources are available year round, brood rearing also occurs year round. As a consequence, populations of parasites that reproduce on immature bees, like the varroa mite, grow much more quickly than they would if brood rearing was interrupted (Calis et al., 1999).

3.8. Socio-political factors affecting managed colony populations

3.8.1. Trade

Over the last several decades the world has seen increased international trade. International trade agreements that facilitate trade liberalization provide ways for nations to prevent the import of bees or bee products if such imports pose a risk to domestic bee stocks (Matheson, 1995a). Risk assessments must be justified by sound technical evidence, such as the presence of a disease or parasite in the exporting country that is not present in the importing country. The recent relaxing of bans on importation of live bees from Australia to the US no doubt has helped offset some of the declines in bee populations over the last several years (Sumner and Boriss, 2006). Importation from Australia was facilitated by the fact that Australia harbored no bee pathogens or parasites not already established in the US. However, such risk assessments, as dictated by international law, do not account for possible introduction of different pathogen strains (Palacios et al., 2008) or parasite haplotypes (Solignac et al., 2005), and so, do not protect against the introduction of potentially more virulent varieties of established disease or parasites. By far the largest threat to the beekeeping industry, however, is the illegal importation of queens or bees. Bee smuggling is thought to be a major vehicle for the spread of bee diseases and parasites (Matheson, 1995b).

While liberalized trade can have both positive and negative impacts on managed populations, trade restrictions can also have an effect. The detection of *A. woodi* in Europe and its early linkage to the Isle of Wight disease resulted in the US federal government passing the Honeybee Act of 1922. This law initiated a long period of restricted bee imports into the US from all but a few countries. Considering that the law prevented the arrival of *A. woodi* for over 66 years, its passage seemed warranted, although the cost to

beekeepers is unknown (Mussen, 2001). The closure of the Canadian border to US imports had a more measurable effect. In 1987, the detection of *A. woodi* in California queen breeder operations coincided with the first detections of varroa mites in the US, and exports of bees into Canada were banned (Mussen, 2001). California breeders supplied 250,000 packages of bees to Canadian provinces each year before the ban. The closure of the Canadian border therefore, explains both the dramatic decrease in colony sales recorded by the AG census (1987 sales ~600,000 colonies vs. 2002 sales ~76,000 colonies) (Daberkow et al., 2009) and the 22% drop in colonies managed in California (compared to a national drop of 16%) over the subsequent decade (USDA-NASS, 1995, 1999).

Increased world trade in non-bee products can also inadvertently introduce new bee pests and diseases. For example, the small hive beetle, *Aethina tumida*, was thought to have been imported to the US in a shipment of citrus from South Africa (Hood, 2000; Le Conte, 2008). Not all introduced "pests", however, are detrimental to beekeeping. At least 66 different plants or genera of plants that have been introduced into the US and Canada provide bee forage (Ayers and Harman, 1992). Some, like purple loosestrife (*Lythrum salicaria*) and Japanese knotweed (*Fallopia japonica)*, are the principle honey source in some regions, and beekeepers often resist efforts to control these "noxious weeds".

3.8.2. Economics

The profitability of beekeeping operations likely has a major influence on managed colony populations (Sumner and Boriss, 2006). Colony declines in the US prior to mite introduction have been linked to stagnant honey production figures (honey produced per colony per year), while increased colony productivity (and, presumably, profitability) over the same period has been used to explain increasing numbers of managed colonies in Canada (Ayers and Harman, 1992). Annual records of the price of honey (Fig. 6) and colony numbers in the US (Fig. 3) provide data needed to crudely examine what, if any, effect the price of honey has on national colony numbers. The price (adjusted to 2008 US$) of honey in a given year and the percent change in national colony numbers between years are related ($F = 27.81$, df = 1, 58, $P < 0.0001$; Fig. 7). The resulting regression suggests that only when the price of honey exceeds US$ 1.45 per lb (2008 adjusted) do colony numbers increase nationally. This threshold has only been surpassed 16 times in the last 66 years (Fig. 6). Increased demand for honey, and thus, increased price during the First World War is cited as the underlying reason for the increase in the number of managed colonies during that period (Phillips, 1928).

In the US, beekeepers have derived income not only from hive products, but also from renting colonies for pollination. This practice is not new and dates back to the early 1900s (Voorhies et al., 1933). Colony rentals have become an important source of income for many beekeepers, especially to meet the demand created by the increasing number of almond bearing acres in California. Almond bearing acres have increased dramatically over the last decade, with some projections estimating that as many as 2 million colonies (some 86% of current US bee stock) will be needed by 2012 to meet demand (Sumner and Boriss, 2006). These estimates, however, do not take into account the current drought facing California, and the resulting water restriction that forced many almond producers to plow under mature groves in early 2009. The effect this drought will have on pollination demand remains to be determined. Nonetheless, the high price paid for colonies in California certainly has been an incentive for some operations to increase in size and, as previously discussed, may help to account for the increase in colony numbers recorded by the 2007 Ag Census.

Almonds, however, are not the only crop requiring pollination. East and west coast berry, stone, and pit fruits, and cucurbits all require pollination services. It is not uncommon for a beekeeper to travel 37,000–40,000 miles per year to pollinate four or more different crops (Rucker et al., 2001). The price received for rental of colonies varies by crop, with those crops that produce honey (e.g. apples) generally commanding a lower price than crops that do not produce honey (e.g. pumpkins) (NRC, 2006). Rental prices also tend to increase as honey prices increase (Rucker et al., 2001).

In Europe, a dramatic decline in the number of hives was observed during the early 1990s. It is safe to assume that this decline resulted from the political and economic disruption caused by the Soviet collapse, rather than from widespread ecological factors, because it largely disappeared when data for Soviet Bloc countries were excluded from statistical analysis (Aizen et al., 2009). The economic situation of beekeepers drastically changed with the

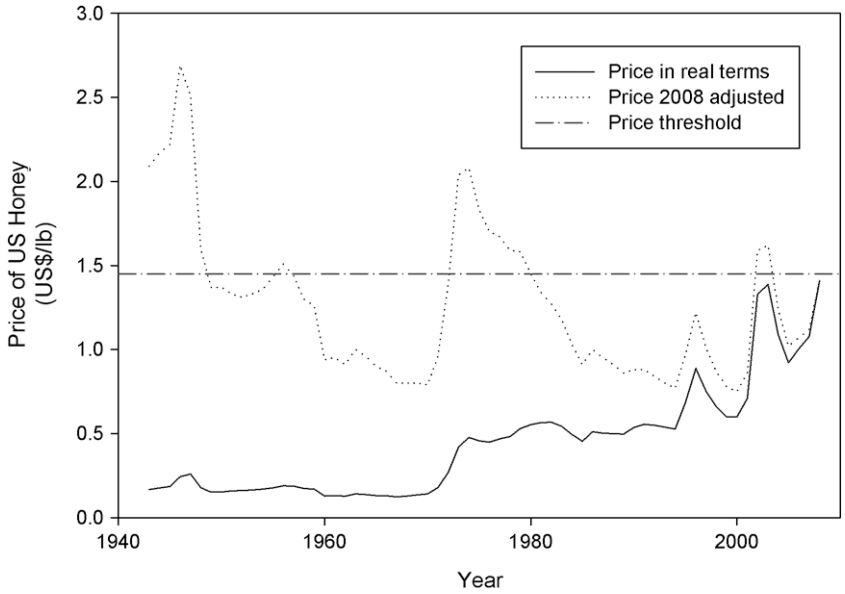

Fig. 6. Average price of honey ($/lb) in the US (Rodenberg, 1992), Prices are presented in actual (solid blue line) and dollars adjusted for inflation presented in US$2008 (dotted blue line) (Williamson, 2008). The horizontal red dashed line represents the theoretical threshold price: when prices exceed this threshold, colony numbers begin to increase in the United States (see text for details). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

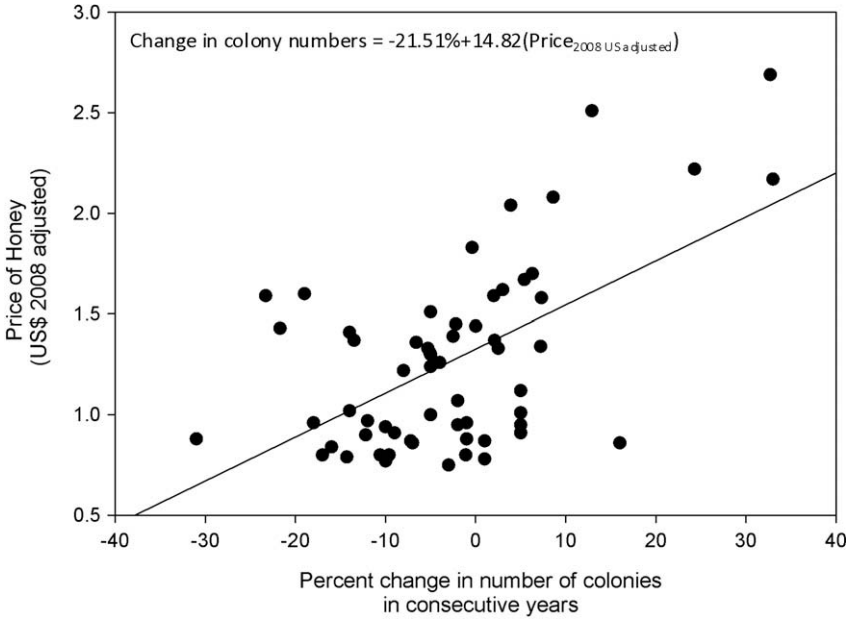

Fig. 7. The percent change in colony numbers in the US as compared to the average retail price of honey (in 2008 adjusted dollars/lb). A significant relationship occurs between the factors, with increases in colony numbers seen when the price of honey exceeds $1.43/lb (see text for details).

dissolution of the Soviet Union. Honey served as a second currency in many countries of the Soviet Bloc and, thus, many people were motivated to keep bees. Due to the political and economic upheaval in eastern Europe in the early 1990s, honey lost its relevance in those countries and the number of bee hives for instance in the former German Democratic Republic dropped by approximately 75% within a year's time (data from the German Bee Keeping Association), underlining the importance of economic factors in bee keeping.

4. Summary

Managed honey bees remain a critical resource for world agricultural and food security. While global honey bee populations have increased over the last 5 decades, this increase has not been universal. Notably, Europe and North America have suffered steep declines in managed populations. However, within these regions some nations have seen increases while others have seen decreases. Disease factors, such as the bacterial diseases AFB and EFB, have likely played an important role in honey bee colony declines in the US over a century ago; however, their role in current overall declines is likely minimal. Varroa mites, together with the virus complex associated with mite parasitism, are likely one of the major causes for considerable overwintering losses documented by many northern nations over the last several years. However, overwintering losses do not have a direct or measurable effect on total managed colony numbers as enumerated by national surveys in the US, likely because beekeepers are able to replace losses quickly. Pesticides, specifically those that directly affect colony health, had a pronounced effect on colony populations in the US. However, modern pesticides with reduced acute toxicity may have sub-lethal effects that are more difficult to quantify. Additional factors, such as reduced bee forage, climate, narrowing of the gene pool, poor queens, and socio-economic factors all have measurable effects on managed honey bee populations. Many of these factors influence the profitability of beekeeping which may have the most dramatic effect on managed populations of honey bees.

Conflicts of interest

There are no conflicts of interest to be declared.

References

Adam, B., 1968. "Isle of Wight" or acarine disease: its historical and practical perspectives. Bee World 49, 6–18.
Aizen, M.A., Harder, L.D., in press. The global stock of domesticated honey bees is growing slower than agricultural demand for pollination. Curr. Biol. doi: 10.1016/j.cub.2009.03.071
Aizen, M.A., Garibaldi, L.A., Cunningham, S.A., 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, 1572–1575.
Aizen, M.A., Garibaldi, L.A., Cunningham, S.A., Klein, A.M., 2009. How much does agriculture depend on pollinators? Lessons from long-term trends in crop production. Ann. Bot. 103, 1579–1588.
Allen, M.F., Ball, B.V., 1996. The incidence and world distribution of honey bee viruses. Bee World 77, 141–162.
Alippi, A.M., Albo, G.N., Leniz, I., Rivera, M., Zanelli, L., Roca, A.E., 1999. Comparative study of tylosin, erythromycin and oxytetracycline to control American foulbrood of honey bees. J. Apicult. Res. 38, 149–158.
Amdam, G.V., Hartfelder, K., Norberg, K., Hagen, A., Omholt, S.W., 2004. Altered physiology in worker honey bees (Hymenoptera: Apidae) infested with the mite *Varroa destructor* (Acari: Varroidae): a factor in colony loss during overwintering? J. Econ. Entomol. 97, 741–747.
Anderson, D.L., Trueman, J.W.H., 2000. *Varroa jacobsoni* (Acari: Varroidae) is more than one species. Exp. Appl. Acarol. 24, 165–189.
Anonymous, 1792. Observations on bees. Am. Mus. 12, 22.
Anonymous, 1869. Beekeeping Statistics. Report of the Commissioner of Agriculture for the Year 1868. Washington, DC, US Government Printing Office, pp. 272–281.
Anonymous, 2008. Monitoringprojekt Völkerverluste". Untersuchungsjahre 2004–2008. Zusammenfassung und vorläufige Beurteilung der Ergebnisse. http://www.ag-bienenforschung.de (in German).
Ayers, G.S., Harman, J.R., 1992. Bee forage of North America and the potential planting for bees. In: Graham, J.M. (Ed.), The Hive and the Honey Bee, Revised ed. Bookcrafters, Hamilton, IL, pp. 437–493.
Babendreier, D., Joller, D., Romeis, J., Bigler, F., Widmer, F., 2007. Bacterial community structures in honeybee intestines and their response to two insecticidal proteins. FEMS Microbiol. Ecol. 59, 600–610.
Bailey, L., 1964. The 'Isle of Wight disease': the origin and significance of the myth. Bee World 45, 32–37.
Bailey, L., Ball, B.V., 1991. Honey Bee Pathology. Academic Press, London.
Bailey, L., Gibbs, A.J., 1964. Acute infection of bees with paralysis virus. J. Ins. Pathol. 6, 395–407.
Bailey, L., Carpenter, J.M., Woods, R.D., 1979. Egypt bee virus and Australian isolates of Kashmir bee virus. J. Gen. Virol. 43, 641–647.

Ball, B.V., 1983. The association of *Varroa jacobsoni* with virus diseases of the honey bee. Exp. Appl. Acarol. 19, 607–613.

Belloy, L., Imdorf, A., Fries, I., Forsgren, E., Berthoud, H., Kuhn, R., Charrière, J.-D., 2007. Spatial distribution of *Melissococcus plutonius* in adult honey bees collected from apiaries and colonies with and without symptoms of European foulbrood. Apidologie 38, 136–140.

Benton, F. 1899. The Honey Bee: A Manual of Instruction in Apiculture. United States Department of Agriculture.

Boecking, O., Genersch, E., 2008. Varroosis – the ongoing crisis in bee keeping. J. Verbr. Lebensm. 3, 221–228.

Bogdanov, S., 2006. Contaminants of bee products. Apidologie 37, 1–18.

Bohan, D.A., Boffey, C.W.H., Brooks, D.R., Clark, S.J., Dewar, A.M., Firbank, L.G., Haughton, A.J., Hawes, C., Heard, M.S., May, M.J., Osborne, J.L., Perry, J.N., Rothery, P., Roy, D.B., Scott, R.J., Squire, G.R., Woiwod, I.P., Champion, G.T., 2005. Effects on weed and invertebrate abundance and diversity of herbicide management in genetically modified herbicide-tolerant winter-sown oilseed rape. Proc. Roy. Soc. Lond., B 272, 463–474.

Bowen-Walker, P.L., Martin, S.J., Gunn, A., 1999. The transmission of deformed wing virus between honeybees (*Apis mellifera*) by the ectoparasitic mite *Varroa jacobsoni* Oud. J. Invertebr. Pathol. 73, 11–106.

Burgett, M., 2004. Pacific Northwest Honey Bee Pollination Survey – 2003. National Honey Report XXIII.

Calis, J.N.M., Fries, I., Ryrie, S.C., 1999. Population modelling of *Varroa jacobsoni* Oud. Apidologie 30, 111–124.

Camazine, S., Çakmak, I., Cramp, K., Finley, J., Fisher, J., Frazier, M., Rozo, A., 1998. How healthy are commercially-produced US honey bee queens? Am. Bee J. 138, 677–680.

Chauzat, M.-P., Carpentier, P., Martel, A.-C., Bougeard, S., Cougoule, N., Porta, P., Lachaize, J., Madec, F., Aubert, M., Faucon, J.-P., 2009. Influence of pesticide residues on honey bee (Hymenoptera: Apidae) colony health in France. Environ. Entomol. 38, 514–523.

Chen, Y.-P., Evans, J.D., 2007. Historical presence of Israeli acute paralysis virus in the United States. Am. Bee J. 147, 1027–1028.

Chen, Y.-P., Siede, R., 2007. Honey bee viruses. Adv. Virus Res. 70, 33–80.

Chen, Y.-P., Evans, J.D., Smith, I.B., Pettis, J.S., 2007. *Nosema ceranae* is a long-present and wide-spread microsporidian infection of the European honey bee (*Apis mellifera*) in the United States. J. Invertebr. Pathol. 97, 186–188.

Chen, Y.-P., Pettis, J.S., Evans, J.D., Kramer, M., Feldlaufer, M.F., 2004. Transmission of Kashmir bee virus by the ectoparasitic mite *Varroa destructor*. Apidologie 35, 441–448.

COLOSS, 2009. In: Proceedings of the 4th COLOSS Conference, Zagreb. <http://www.coloss.org>.

Cox-Foster, D., vanEngelsdorp, D., 2009. Solving the mystery of the disappearing bees. Sci. Am. 2009 (April), 40–47.

Cox-Foster, D.L., Conlan, S., Holmes, E.C., Palacios, G., Evans, J.D., Moran, N.A., Quan, P.L., Briese, T., Hornig, M., Geiser, D.M., Martinson, V., vanEngelsdorp, D., Kalkstein, A.L., Drysdale, A., Hui, J., Zhai, J., Cui, L., Hutchinson, S.K., Simons, J.-F., Egolm, M., Pettis, J.S., Lipkin, W.I., 2007. A metagenomic survey of microbes in honey bee colony collapse disorder. Science 318, 283–287.

Crane, E., 1975. Honey: A Comprehensive Survey. Morrison and Gibb Ltd., London.

Crane, E., 1999. The World History of Beekeeping and Honey Hunting. Gerald Duckworth & Co. Ltd, London.

Croft, B.A., 1990. Arthropod Biological Control Agents and Pesticides. Wiley, New York.

Daberkow, S., Korb, P., Hoff, F., 2009. Structure of the US beekeeping Industry: 1982–2002. J. Econ. Entomol. 102, 868–886.

De Jong, D., 1997. Mites: Varroa and other parasites of brood. In: Morse, R.A., Flottum, K. (Eds.), Honey Bee Pests, Predators and Diseases. A.I. Root Company, Medina.

De Miranda, J., Cordoni, G., Budge, G., 2010. The acute bee paralysis virus – Kashmir bee virus – Israeli acute paralysis virus complex. J. Invertebr. Pathol. 103, S30–S47.

De Miranda, J., Genersch, E., 2010. Deformed wing virus. J. Invertebr. Pathol. 103, S48–S61.

Delaney, D.A., Meixner, M. D., Schiff, N.M., Sheppard, W.S., 2009. Genetic characterization of commercial honey bee (Hymenoptera: Apidae) populations in the United States by using mitochondrial and microsatellite markers. Ann. Entomol. Soc. Am. 102, 666–673.

Delaplane, K.S., Mayer, D.F., 2000. Crop Pollination by Bees. CABI Publishing, New York.

Desneux, N., Decourtye, A., Delpuech, J.-M., 2007. The sublethal effects of pesticides on beneficial arthropods. Annu. Rev. Entomol. 52, 81–106.

Duan, J.J., Marvier, M., Huesing, J., Dively, G., Huang, Z.Y., 2008. A meta-analysis of effects of Bt crops on honey bees (Hymenoptera: Apidae). PLoS ONE 3 (1), e1415.

Duay, P., De Jong, D., Engels, W., 2003. Weight loss in drone pupae (*Apis mellifera*) multiply infested by *Varroa destructor* mites. Apidologie 34, 61–65.

Eischen, F.A., Graham, R.H., Cox, R., 2005. Regional distribution of *Paenibacillus larvae* subspecies *larvae*, the causative organism of American foulbrood, in honey bee colonies of the Western United States. J. Econ. Entomol. 98, 1087–1093.

Ellis, J.D., Munn, P.A., 2005. The worldwide health status of honey bees. Bee World 86, 88–101.

Elobeid, A., 2007. Ethanol expansion in the food versus fuel debate: how will developing countries fare? J. Agric. Food Ind. Org. 5, 1–23.

Elzen, P.J., Westervelt, D., Causey, D., Ellis, J., Hepburn, H.R., Neumann, P., 2002. Method of application of tylosin, an antibiotic for American foulbrood control, with effects on small hive beetle (Coleoptera: Nitidulidae) populations. J. Econ. Entomol. 95, 1119–1122.

Fantham, H.B., Porter, A., 1912. The morphology and life history of *Nosema apis* and the significance of its various stages in the so-called 'Isle of Wight' disease in bees (Microsporidiosis). Ann. Trop. Med. Parasitol. 6, 163–195.

Food and Agriculture Organization of the United Nations (FAO), 2009. FAOSTAT. <http://faostat.fao.org>.

Finley, J., Camazine, S., Frazier, M., 1996. The epidemic of honey bee colony losses during the 1995–1996 season. Am. Bee J. 136, 805–808.

Forsgren, E., Cassel Lundhagen, A., Imdorf, A., Fries, I., 2005. Distribution of *Melissococcus plutonius* in honeybee colonies with and without symptoms of European foulbrood. Microbiol. Ecol. 50, 369–374.

Frazier, M.T., Finley, J., Collison, C.H., Rajotte, E., 1994. The incidence and impact of honey bee tracheal mites and nosema disease on colony mortality in Pennsylvania. Bee Sci. 3, 94–100.

Frazier, M., Mullin, C., Frazier, J., Ashcraft, S., 2008. What have pesticides got to do with it? Am. Bee J. 148, 521–523.

Fries, I., 1988. Comb replacement and nosema disease (*Nosema apis* Z.) in honey bee colonies. Apidologie 19, 343–354.

Fries, I., 1993. Nosema apis – a parasite in the honeybee colony. Bee World 74, 5–19.

Fries, I., 1997. Protozoa. In: Morse, R.A., Flottum, K. (Eds.), Honey Bee Pests, Predators and Diseases. A.I. Root Company, Medina.

Fries, I., 2010. Microsporidia. J. Invertebr. Pathol. 103, S73–S79.

Fries, I., Ekbohm, G., Villumstad, E., 1984. *Nosema apis*, sampling techniques and honey yield. J. Apicult. Res. 23, 102–105.

Fries, I., Feng, F., da Silva, A., Slemenda, S.B., Pieniazek, N.J., 1996. *Nosema ceranae* n. sp. (Microspora, Nosematidae), morphological and molecular characterization of a microsporidian parasite of the Asian honey bee *Apis cerana* (Hymenoptera, Apidae). Eur. Protistol. 32, 356–365.

Forsgren, E., 2010. European Foulbrood. J. Invertebr. 103, S5–S9.

Gallai, N., Salles, J.-M., Settele, J., Vaissière, B.E., 2009. Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. Ecol. Econ. 68, 810–821.

Garedew, A., Schmolz, E., Lamprecht, I., 2004. The energy and nutritional demand of the parasitic life of the mite *Varroa destructor*. Apidologie 35, 419–430.

Genersch, E., 2010. American Foulbrood in honeybees and its causative agent *Paenibacillus larvae*. J. Invertebr. Pathol 103, S10–S19.

Gilliam, M., 1986. Infectivity and survival of the chalkbrood pathogen *Ascosphaera apis* in colonies of honey bees *Apis mellifera*. Apidologie 17, 93–100.

Gisder, S., Aumeier, P., Genersch, E., 2009. Deformed wing virus: replication and viral load in mites. J. Gen. Virol. 90, 463–467.

Graham, S., Myerscough, M.R., Jones, J.C., Oldroyd, B.P., 2006. Modelling the role of intracolonial genetic diversity on regulation of brood temperature in honey bee (*Apis mellifera* L.) colonies. Ins. Soc. 53, 226–232.

Greenberg, B., Bindokas, V.P., Frazier, M.J., Gauger, J.R., 1981. Response of honey bees, *Apis mellifera* L., to high-voltage transmission lines. Environ. Entomol. 10, 600–610.

Harris, J.W., Harbo, J.R., Villa, J.D., Danka, R.G., 2003. Variable population growth of *Varroa destructor* (Mesostigmata: Varroidae) in colonies of honey bees (Hymenoptera: Apidae) during a 10-year period. Environ. Entomol. 32, 1305–1312.

Harrison, J.F., Fewell, J.H., 2002. Environmental and genetic influences on flight metabolic rate in the honey bee, *Apis mellifera*. Comp. Biochem. Physiol. A 133, 323–333.

Haseman, L., Childers, L.F., 1944. Controlling American foulbrood with sulfa drugs. Univ. Missouri Coll. Agric. Bull., 1–16.

Higes, M., Martin, R., Meana, A., 2006. *Nosema ceranae*, a new microsporidian parasite in honeybees in Europe. J. Invertebr. Pathol. 92, 93–95.

Higes, M., Garcia-Palencia, P., Martin-Hernandez, R., Meana, A., 2007. Experimental infection of *Apis mellifera* honeybees with *Nosema ceranae* (Microsporidia). J. Invertebr. Pathol. 94, 211–217.

Higes, M., Martín-Hernández, R., Botías, C., Bailón, E.G., González-Porto, A.V., Barrios, L., del Nozal, D.J., Bernal, J.L., Jiménez, J.J., Palencia, P.G., Meana, A., 2008. How natural infection by *Nosema ceranae* causes honeybee colony collapse. Environ. Microbiol. 10, 2659–2668.

Hood, W.M., 2000. Overview of the small hive beetle, *Aethina tumida*, in North America. Bee World 81, 129–137.

Hoppe, R., Korb, P., O'Donoghue, E., Banker, D., 2007. Structure and Finances of U.S. Farms: Family Farm Report, 2007 edition. US Department of Agriculture, Economic Research Service, Economic Information Bulletin Number EIB-24.

Huang, W.F., Jiang, J.-H., Chen, Y.-W., Wang, C.-H., 2007. A *Nosema ceranae* isolate from the honeybee *Apis mellifera*. Apidologie 38, 30–37.

Hung, A.C., Shimanuki, H., Knox, D.A., 1995. Bee parasitic mite syndrome: II. The role of Varroa mite and viruses. Am. Bee J. 135, 702.

Hung, A.C., Shimanuki, H., Knox, D.A., 1996. The role of viruses in bee parasitic mite syndrome. Am. Bee J. 136, 731–732.

Johansen, C.A., Mayer, D.F., 1990. Pollinator Protection. A Bee and Pesticide Handbook. Wicwas Press, Cheshire, CT.

Jones, J.C., Myerscough, M.R., Graham, S., Oldroyd, B.P., 2004. Honey bee nest thermoregulation: diversity promotes stability. Science 305, 402–404.

Kauffeld, N.M., Everitt, J.H., Taylor, E.A., 1976. Honey bee problems in the Rio Grande Valley of Texas. Am. Bee J. 116, 220. 222, 232.

Kemp, W.P., 2000. The future of crop pollination. Am. Bee J. 140, 851–853.

Klein, A.M., Vaissière, B.E., Cane, J.H., Steffan-Dewenter, I., Cunningham, S.A., Kremen, C., Tscharntke, T., 2007. Importance of pollinators in changing landscapes for world crops. Proc. Roy. Soc. Lond., B. 274, 303–313.
Kochansky, J., Knox, D.A., Feldlaufer, M., Pettis, J.S., 2001a. Screening alternative antibiotics against oxytetracycline-susceptible and -resistant *Paenibacillus larvae*. Apidologie 32, 215–222.
Kochansky, J., Wilzer, K., Feldlaufer, M., 2001b. Comparison of the transfer of coumaphos from beeswax into syrup and honey. Apidologie 32, 119–125.
Le Conte, Y., 2008. Climate change: impact on honey bee populations and diseases. Rev. Sci. Tech. 27, 499.
Lodesani, M., Costa, C., 2003. Bee breeding and genetics in Europe. Bee World 84, 69–85.
Lodesani, M., Costa, C., 2005. Limits of chemotherapy in beekeeping: development of resistance and the problem of residues. Bee World 86, 102–109.
Loskotova, J., Peroutka, M., Vesely, V., 1980. Nosema disease of honeybee queens (*Apis mellifica* L.). Apidologie 11, 153–161.
Malone, L.A., Pham-Delègue, M.H., 2001. Effects of transgene products on honey bees (*Apis mellifera*) and bumblebees (*Bombus* sp.). Apidologie 32, 287–304.
Malone, L.A., Todd, J.H., Burgess, E.P.J., Christeller, J.T., 2004. Development of hypopharyngeal glands in adult honey bees fed with a Bt toxin, a biotin-binding protein and a protease inhibitor. Apidologie 35, 655–664.
Martel, A.-C., Zeggane, S., Aurières, C., Drajnudel, P., Faucon, J.-P., Aubert, M., 2007. Acaricide residues in honey and wax after treatment of honey bee colonies with Apivar® or Asuntol®50. Apidologie 38, 534–544.
Martin, S.J., Hogarth, A., van Breda, J., Perrett, J., 1998. A scientific note on *Varroa jacobsoni* Oudemans and the collapse of *Apis mellifera* colonies in the United Kingdom. Apidologie 29, 369–370.
Marvier, M., McCreedy, C., Regetz, J., Kareiva, P., 2007. A meta-analysis of effects of Bt cotton and maize on nontarget invertebrates. Science 316, 1475–1477.
Matheson, A., 1995a. All change for global trade and the bee world. Bee World 76, 165–168.
Matheson, A., 1995b. World bee health update 1995. Bee World 76, 31–39.
Mattila, H.R., Otis, G.W., 2007. Dwindling pollen resources trigger the transition to broodless populations of long-lived honeybees each autumn. Ecol. Entomol. 32, 496–505.
Mattila, H.R., Seeley, T.D., 2007. Genetic diversity in honey bee colonies enhances productivity and fitness. Science 317, 362–364.
McGregor, S.E., 1976. Insect Pollination of Cultivated Crop Plants. US Department of Agriculture, Washington, DC.
McMullan, J.B., Brown, M.J.F., 2005. Brood pupation temperature affects the susceptibility of honeybees (*Apis mellifera*) to infestation by tracheal mites (*Acarapis woodi*). Apidologie 36, 97–105.
Milani, N., 1999. The resistance of *Varroa jacobsoni* Oud. to acaricides. Apidologie 30, 229–234.
Miller, C.C., 1901. Bee Culture. Dept. of Agriculture, Commonwealth of Pennsylvania. 104.
Miyagi, T., Peng, C.Y.S., Chuang, R.Y., Mussen, E.C., Spivak, M.S., Doi, R.H., 2000. Verification of oxytetracycline-resistant American foulbrood pathogen *Paenibacillus larvae* in the United States. J. Invertebr. Pathol. 75, 95–96.
Morse, R.A., Flottum, K. (Eds.), 1997. Honey Bee Pests, Predators, and Diseases. A.I. Root Company, Medina, Ohio, USA.
Mussen, E.C., 2000. Antibiotic-resistant American foulbrood. Am. Bee J. 140, 300–301.
Mussen, E.C., 2001. Introduction, spread and economic impact of tracheal mites in North America. In: Mites of the Honey Bee. Dadant and Sons, Inc, Hamilton, IL, pp. 43–56.
National Research Council (NRC), 2006. Status of Pollinators in North America. National Academy of Sciences, Washington, DC.
Naug, D., in press. Nutritional stress due to habitat loss may explain recent honeybee colony collapses. Biol. Conserv, doi:10.1016/j.biocon.2009.04.007.
Oertel, E., 1976. Bicentennial bees: early records of honey bees in the eastern United States. Am. Bee J. 116. 70–71, 114, 128, 156–157, 214–215, 260–261, 290.
Palacios, G., Hui, J., Quan, P.L., Kalkstein, A., Honkavuori, K.S., Bussetti, A.V., Conlan, S., Evans, J., Chen, Y.P., vanEngelsdorp, D., Efrat, H., Pettis, J., Cox-Foster, D., Holmes, E.C., Briese, T., Lipkin, W.I., 2008. Genetic analysis of Israel Acute Paralysis Virus: distinct clusters are circulating in the United States. J. Virol. 82, 6209–6217.
Paxton, R., Klee, J., Korpela, S., Fries, I., 2007. *Nosema ceranae* has infected *Apis mellifera* in Europe since at least 1998 and may be more virulent than *Nosema apis*. Apidologie 38, 558–565.
Pellett, F.C., 1938. History of American Beekeeping. Collegiate Press, Inc. Ames, Iowa.
Pernal, S. 2008. CAPA Statement on Honey Bees Losses in Canada (Spring 2008) – Final Revision, Canadian Association of Professional Apiculturists.
Pettis, J.S., 2004. A scientific note on *Varroa destructor* resistance to coumaphos in the United States. Apidologie 35, 91–92.
Pettis, J.S., Collins, A.M., Wilbanks, R., Feldlaufer, M.F., 2004. Effects of coumaphos on queen rearing in the honey bee, *Apis mellifera*. Apidologie 35, 605–610.
Phillips, E.F., 1920. Control of American Foulbrood. Farmers Bull. 1084, 1–16.
Phillips, E.F., 1928. Beekeeping. The Macmillan Company, New York.
Ramirez-Romero, R., Desneux, N., Decourtye, A., Chaffiol, A., Pham-Delegue, M.H., 2008. Does Cry1Ab protein affect learning performances of the honey bee *Apis mellifera* L. (Hymenoptera, Apidae)? Ecotoxicol. Environ. Safety 70, 327–333.
Ransome, H.M., 1937. The Sacred Bee. Bee Books New and Old, London.
Roetschi, A., Berthoud, H., Kuhn, R., Imdorf, A., 2008. Infection rate based on quantitative real-time PCR of *Melissococcus plutonius*, the causal agent of European foulbrood, in honeybee colonies before and after apiary sanitation. Apidologie 39, 362–371.
Rodenberg, H., 1992. Marketing the crop of commercial beekeepers. In: Graham, J.M. (Ed.), The Hive and the Honey Bee, Revised ed. Bookcrafters, Hamilton, IL.
Rose, R., Dively, G.P., Pettis, J.S., 2007. Effects of Bt corn pollen on honey bees: emphasis on protocol development. Apidologie 38, 368–377.
Rosenkranz, P., Aumeier, P., Ziegelmann, B., 2010. Biology and control of Varroa destructor. J. Invertebr. Pathol. 103, S96–S119.
Rucker, R.R., Thurman, W.N., Burgett, M., 2001. An Empirical Analysis of Honeybee Pollination Markets. American Agricultural Economics Association Annual Meeting., Chicago, IL.
Ruttner, F., 1988. Taxonomy and Biogeography of Honey Bees. Springer, Munich.
Seeley, T.D., 1985. Honeybee Ecology. Princeton University Press, Princeton, NJ.
Schiff, N.M., Sheppard, W.S., 1995. Genetic analysis of commercial honey bees (Hymenoptera: Apidae) from the southern United States. J. Econ. Entomol. 88, 1216–1220.
Schiff, N.M., Sheppard, W.S., 1996. Genetic differentiation in the queen breeding population of the western United States. Apidologie 27, 77–86.
Shen, M., Yang, X., Cox-Foster, D., Cui, L., 2005. The role of varroa mites in infections of Kashmir bee virus (KBV) and deformed wing virus (DWV) in honey bees. Virology 342, 141–149.
Sheppard, W.S., 1988. Comparative study of enzyme polymorphism in United States and European honey bee (Hymenoptera: Apidae) populations. Ann. Entomol. Soc. Am. 81, 886–889.
Sheppard, W.S., 1989a. A history of introduction of honey bee races into the United States. Part 1. Am. Bee J. 129, 617–619.
Sheppard, W.S., 1989b. A history of introduction of honey bee races into the United States. Part 2. Am. Bee J. 129, 664–667.
Sheppard, W.S., Meixner, M.D., 2003. *Apis mellifera pomonella*, a new honey bee subspecies from Central Asia. Apidologie 34, 367–375.
Shimanuki, H., 1997. Bacteria. In: Morse, R.A., Flottum, K. (Eds.), Honey Bee Pests, Predators, and Diseases. A.I. Root Company, Medina, Ohio, pp. 35–54.
Shimanuki, H., Calderone, N.W., Knox, D.A., 1994. Parasitic mite syndrome: the symptoms. Am. Bee J. 134, 827–828.
Shuel, R.W., 1992. The Production of Nectar and Pollen. In: Graham, J.M. (Ed.), The Hive and the Honey Bee, Revised ed. Bookcrafters, Hamilton, IL, pp. 401–433.
Siede, R., Büchler, R., 2004. First detection of Kashmir bee virus in Hessen. Berl. Münch Tierärztl. Wochenschr. 117, 12–15.
Siede, R., König, M., Büchler, R., Failing, K., Thiel, H.J., 2008. A real-time PCR based survey on acute bee paralysis virus in German bee colonies. Apidologie 39, 650–661.
Solignac, M., Cornuet, J.M., Vautrin, D., Le Conte, Y., Anderson, D., Evans, J., Cros-Arteil, S., Navajas, M., 2005. The invasive Korea and Japan types of *Varroa destructor* ectoparasitic mites of the Western honeybee (*Apis mellifera*) are two partly isolated clones. Proc. Roy. Soc. Lond., B. 272, 411–419.
Sumner, D.A., Boriss, H., 2006. Bee-economics and the leap in pollination fees. ARE update. Giannini Found. Agric. Econ. 9, 9–12.
Surface, H.A., 1916. Bee diseases in Pennsylvania. Zool. Bull. 6, 1–23.
Tarpy, D.R., 2003. Genetic diversity within honeybee colonies prevents severe infections and promotes colony growth. Proc. Roy. Soc. Lond., B 270, 99–103.
Tentcheva, D., Gauthier, L., Bagny, L., Flevet, J., Dainat, B., Cousserans, F., Colin, M.E., Bergoin, M., 2006. Comparative analysis of deformed wing virus (DWV) RNA in *Apis mellifera* and *Varroa destructor*. Apidologie 37, 41–50.
Thompson, H.M., 2003. Behavioural effects of pesticides in bees: their potential for use in risk assessment. Ecotoxicology 12, 317–330.
Tremolada, P., Bernardelli, I., Colombo, M., Spreafico, M., Vighi, M., 2004. Coumaphos distribution in the hive ecosystem: case study for modeling applications. Ecotoxicology 13, 589–601.
Tomkies, V., Flint, J., Johnson, G., Waite, R., Wilkins, S., Danks, C., Watkins, M., Cuthbertson, A.G.S., Carpana, E., Marris, G., Budge, G., Brown, M.A., 2009. Development and validation of a novel field test kit for European foulbrood. Apidologie 40, 63–72.
Turell, M., 1974. A history of the use of drugs in the prevention and cure of American foulbrood. Am. Bee J. 11, 13–14. 17.
Underwood, R., vanEngelsdorp, D., 2007. Colony collapse disorder: have we seen this before? Bee Culture 35, 13–18.
United States Department of Agriculture – Agricultural Marketing Service (USDA-AMS), 1955. U.S. Honey and Beeswax Production – 1954. US Department of Agriculture, Washington, DC.
United States Department of Agriculture – Bureau of Agricultural Economics (USDA-BAE), 1949. Honey and Beeswax Production – 1948. US Department of Agriculture, Washington, DC.
United States Department of Agriculture – National Agricultural Statistics Service (USDA-NASS), 1967. Honey and Beeswax by state 1955–64. US Department of Agriculture, Washington, DC.
USDA-NASS, 1972. Honey and Beeswax by state 1965–69. US Department of Agriculture, Washington, DC.
USDA-NASS, 1978. Honey Production Final Estimates 1970–75. US Department of Agriculture, Washington, DC.
USDA-NASS, 1981. Honey Production Final Estimates 1976–79. US Department of Agriculture, Washington, DC.
USDA-NASS, 1995. Honey Production Final Estimates 1987–1992. US Department of Agriculture, Washington, DC.
USDA-NASS, 1999. Honey: Final Estimates1993–1997. US Department of Agriculture, Washington, DC.
USDA-NASS, 2004a. 2002 Census of Agriculture. USDA–NASS, Washington, DC.

USDA-NASS, 2004b. Honey: Final Estimates1998–2002. US Department of Agriculture, Washington, DC.
USDA-NASS, 2009a. 2007 Census of Agriculture. US Department of Agriculture, Washington, DC.
USDA-NASS 2009b. Honey. US Department of Agriculture, Washington, DC.
USDA-NASS 2009c. Honey: Final Estimates 2003–2007. US Department of Agriculture, Washington, DC.
vanEngelsdorp, D., Evans, J.D., Donovall, L., Mullin, C., Frazier, M., Frazier, J., Tarpy, D.R., Hayes Jr., J., Pettis, J.S., 2009. "Entombed Pollen": a new condition in honey bee colonies associated with increased risk of mortality. J. Invertebr. Pathol. 101, 71–76.
vanEngelsdorp, D., Hayes Jr., J., Underwood, R.M., Pettis, J., 2008. A Survey of Honey Bee Colony Losses in the U.S., Fall 2007 to Spring 2008. PLoS ONE 3, e4071.
vanEngelsdorp, D., Underwood, R., CaronD, D., 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.
Voorhies, E.C., Todd, F.E., Galbraith, J.K., 1933. Economic aspects of the Bee industry. Univ. California Coll. Agric. Bull. 555, 1–117.
Wahl, O., Ulm, K., 1983. Influence of Pollen Feeding and Physiological Condition on Pesticide Sensitivity of the Honey Bee *Apis mellifera carnica*. Oecologia 59, 106–128.
Wallner, K., 1999. Varroacides and their residues in bee products. Apidologie 30, 235–248.
Wilkins, S., Brown, M.A., Cuthbertson, A.G.S., 2007. Perspective: The incidence of honey bee pests and diseases in England and Wales. Pest Manage. Sci. 63, 1062–1068.
Williamson, S. H., 2008. Six Ways to Compute the Relative Value of a U.S. Dollar Amount, 1790 to Present. Measuring Worth. <http://www.measuringworth.com/uscompare/>.
Yang, X.L., Cox-Foster, D.L., 2005. Impact of an ectoparasite on the immunity and pathology of an invertebrate: evidence for host immunosuppression and viral amplification. Proc. Natl. Acad. Sci. USA 102, 7470–7475.
Yue, C., Genersch, E., 2005. RT-RCR analysis of deformed wing virus in honeybees (*Apis mellifera*) and mites (*Varroa destructor*). J. Gen. Virol. 86, 3419–3424.
Yue, C., Schröder, M., Bienefeld, K., Genersch, E., 2006. Detection of viral sequences in semen of honeybees (*Apis mellifera*): evidence for vertical transmission through drones. J. Invertebr. Pathol. 92, 93–96.
Yue, C., Schröder, M., Gisder, S., Genersch, E., 2007. Vertical transmission routes for deformed wing virus of honeybees (*Apis mellifera*). J. Gen. Virol. 88, 2329–2336.
Zander, E., 1909. Tierische Parasiten als Krankheitserreger bei der Biene. Leipzig. Bienenztg 24, 147–150. 164–166.

Chapter 3

A SURVEY OF MANAGED HONEY BEE COLONY LOSSES IN THE U.S., FALL 2009 TO WINTER 2010.[2]

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ORIGINAL RESEARCH ARTICLE

A survey of managed honey bee colony losses in the USA, fall 2009 to winter 2010

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Summary

This study records the fourth consecutive year of high winter losses in managed honey bee (*Apis mellifera)* colonies in the USA. Over the winter of 2009-2010, US beekeepers responding to this survey lost an average of 42.2% of their colonies, for a total loss of 34.4%. Commercial beekeepers (those operating more than 500 colonies) experienced lower total losses as compared to sideline and backyard beekeepers. Similarly, operations that maintained colonies in more than one state and operations that pollinated almond orchards over the survey period had lower total losses than operations either managing colonies in one state exclusively or those not pollinating almonds. On average beekeepers consider acceptable losses to be 14.5%, and 65% of all responding beekeepers suffered losses in excess of what they considered acceptable. The proportion of operations that experienced losses and reported having no dead bees in their colonies or apiaries was comparable to that reported in the winter of 2008-2009. Manageable conditions, such as starvation and a weak condition in the fall were the leading self-identified causes of mortality as reported by all beekeepers. Commercial beekeepers were, however, less likely to list such manageable causes, instead listing poor queens, mites, and pesticides most frequently as the self-identified causes of mortality in their operations.

Una encuesta sobre la gestión de las pérdidas de colmenas de abejas en los EE.UU., entre el otoño de 2009 hasta el invierno de 2010

Resumen

Este estudio documenta el cuarto año consecutivo de altas pérdidas invernales en las colmenas de abejas manejadas (*Apis mellifera*) en los EE.UU. Durante el invierno de 2009-2010, los apicultores de EE.UU. que respondieron a este estudio, perdieron un promedio del 42,2% de sus colmenas, siendo la pérdida total de un 34,4%. Los apicultores comerciales (los que operan con más de 500 colmenas) experimentaron pérdidas totales menores en comparación a los apicultores aficionados y sin organización. Del mismo modo, las operaciones que mantienen a las colmenas en más de un estado y las operaciones de polinización de almendros en el período de muestreo tuvieron menos pérdidas totales que las operaciones que mantienen a las colmenas en un estado exclusivamente o a las que no polinizaron almendros. Como media los apicultores consideraron que una pérdida aceptable era del 14,5%, y el 65% de todos los apicultores respondieron haber sufrido pérdidas superiores a lo que consideraban como aceptable. La proporción de operaciones que experimentaron pérdidas y que informaron de no tener abejas muertas en sus colmenas o apiarios fue comparable a la documentada en el invierno de 2008-2009. Las condiciones de manejo, tales como la inanición y una condición débil en el descenso fueron las principales causas auto-identificadas de la mortalidad según lo informado por todos los apicultores. Los apicultores comerciales fueron, sin embargo, menos críticos al listar las causas de manejo, en su lugar, auto-identificaron a las reinas malas, a los ácaros y a los pesticidas como las causas más probables de la mortalidad en sus operaciones.

Keywords: Honey bees, overwinter, mortality, USA, 2009-2010

Introduction

Over the last few years, high rates of overwintering mortality have been reported in honey bee (*Apis mellifera*) colonies in many European and North American countries (vanEngelsdorp *et al.*, 2008, 2010; Currie *et al.*, 2010; Neumann and Carreck, 2010; Nguyen *et al.*, 2010). In the USA specifically, high overwintering losses (32%, 36% and 29% for the winters of 2006-2007, 2007-2008, and 2008-2009, respectively) have been reported (vanEngelsdorp *et al.*, 2007, 2008, 2010).

It is clear that these losses have not resulted in a pronounced decrease in the number of honey producing colonies managed by US beekeepers in the subsequent summers (USDA-NASS, 2009). In fact, the 2007 US Agricultural Census, a survey conducted once every five years, reported a dramatic increase in the number of colonies managed on 31 December 2007, as compared to the total number of honey producing colonies enumerated the preceding summer (USDA-NASS, 2009). This apparent discrepancy may be explained by beekeepers who, fearing heavy losses, overwintered larger numbers of colonies to better ensure that they would have enough colonies to meet spring's pollination demands (vanEngelsdorp and Meixner, 2010). Beekeepers can easily increase the number of colonies they manage by either purchasing package bees or splitting existing hives. A recent survey of Pacific Northwest beekeepers revealed that in both 2008 and 2009, beekeepers replaced more colonies than they lost in the preceding winter (Caron *et al.*, 2010).

The reason for the high level of losses is not completely understood. Whilst annual overwintering loss surveys are not designed to identify factors responsible for losses, each survey has asked beekeepers to self-identify the reasons why they believe they experienced high losses. Among the most mentioned factors have been queen failure, starvation and the parasitic mite *Varroa destructor* (vanEngelsdorp *et al.*, 2007, 2008, 2010). While not conclusive, these self-identified causes of mortality do suggest that a multitude of factors are contributing to colony mortality, and so suggest that efforts aimed to reduce losses will likely need to be as diverse as the causes.

In keeping with previous years' efforts, this survey's objective was to quantify the mortality of overwintered colonies in the USA over the winter of 2009-2010. Here, we compare the rate of loss by operation size and activity, and also quantify the suspected reasons for loss as reported by the surveyed beekeepers.

Materials and methods

An email soliciting responses to an online survey posted at Surveymonkey.com was sent to state apiarists, presidents of national and state beekeeping organizations, and to online beekeeping lists. This email encouraged beekeepers to forward the request to other beekeepers that they knew. In addition to the state apiarists, 43 different state and county beekeeping organizations were contacted, and 42 of these agreed to forward the survey request to their distribution lists. Because of the nature of this approach, the exact number of beekeepers contacted cannot be calculated but based on the subscription rates of electronic list serves such as BEE-L and Catch the Buzz, it can be assumed to be above 20,000 (Flottum 2010). In an attempt to compare the web-based survey results with past efforts, the USDA also contacted commercial beekeepers by phone and asked the same questions.

Some of the questions asked were established by a working group of the international COST (European Cooperation in Science and Technology) network of bee researchers with the acronym COLOSS (Prevention of honey bee COLony LOSSes). The following questions were asked: 1. in what state(s) did you keep your hives in 2009?; 2. how many living colonies did you have on 1 October 2009?; 3. how many living colonies did you have on 1 April 2010?; 4. how many splits, increases, and / or colonies did you make / buy between 1 October 2009 and 1 April 2010?; 5. how many splits, increases, and / or colonies did you sell between 1 October 2009 and 1 April 2010?; 6. what percentage of the colonies that died between 1 October and 1 April were lost without dead bees in the hive or apiary?; 7. what percentage of loss, over this time period, would you consider acceptable?; 8. to what do you attribute the cause of death for the colonies that died?; 9. what percentage of your hives did you send to California for almond pollination?; 10. how many times, on average, did you move your colonies last year?; and 11. how many years have you been keeping honey bees?

Beekeepers were given the option to provide their email address if they were interested in seeing the results of the survey effort. In addition to recording the survey responses, the web-based survey tool also recorded the IP address of respondents. In all cases, except for question 1, the survey called for beekeepers to type in their answers (i.e. possible answers were not provided). Thus, responses to question 8 were categorized into broad groups (e.g. lack of food = starvation) for analysis. Beekeepers were assigned to operational size groups by the following criteria: beekeepers managing 50 or fewer colonies were classified as "backyard beekeepers"; those managing between 51 and 500 colonies were classified as "sideline beekeepers"; and beekeepers managing 501 or more colonies were classified as "commercial beekeepers".

Calculations and statistical analysis

Total colony losses were calculated for each reporting operation, for the sum total of all respondents, and for various subgroup classifications. Total losses were calculated by first calculating the total number of monitored colonies at risk of dying over the period (colonies 1 October (Q2) + colonies split or purchased (Q4) – colonies

sold (Q5)). The total colonies that died over the period (total monitored colonies – total colonies 1 April (Q2)) was then divided by the total monitored colonies multiplied by 100%. To account for the nested nature of total loss data, the standard error of the intercept of a null General Linear Model with quasi-binomial family error distribution was used to calculate the confidence limits for total loss data (McCullagh and Nelder, 1989; R development Core Team, 2009 (code provided by Y Brostaux and B K Nguyen; pers. communication)). The mean of individual operation losses was calculated to determine the average loss among subgroups and 95% confidence intervals (CI) were also calculated (SAS, 2007).

Comparisons of total losses between different groups of operations were conducted using the chi-square test. When more than two groups were compared within a test, pair-wise comparisons between groups were conducted. When multiple comparisons were made, the α used to reject the null hypothesis was adjusted appropriately (Abdi, 2007). Comparisons of average operational losses were made using the Kruskal-Wallis rank sum test. Only significant results ($P < 0.05$) are reported.

The total number of colonies lost with the symptom of no dead bees in the colony was calculated for individual operations by multiplying the number of colonies lost in an operation by the reported percentage lost without dead bees. When calculating losses in individual states, colonies belonging to operations that managed colonies in more than one state were counted multiple times; once in each listed state. This same practice is used by the National Agricultural Statistics Service when calculating the number of honey-producing colonies in each state (USDA-NASS, 2009a).

Results

Average and total losses

National losses

The web-based survey recorded 4,284 responses, of which 4,262 provided all of the information needed to quantify overwintering losses. Of these, 34 respondents gave responses that suggested their losses were less than 0%, so these respondents were excluded. In all, 85 distinct IP addresses were used more than once to submit responses; of these, 24 responses were clearly duplicate data and were also excluded. The remaining 4,204 respondents managed a total of 296,507 living colonies on 1 October 2009, representing 12.0% of the 2.46 million honey-producing colonies estimated to have been managed in the US in 2009 (USDA-NASS, 2010). On average, the beekeepers in this group lost 42.4% (95% CI: 41.3-43.5%) of their colonies, while the total loss suffered was 32.2% (95% CI: 31.6-32.8%).

The USDA phone effort interviewed a total of 22 respondents. In total, this group reported managing 142,615 colonies on 1 October 2009; 5.8% of the total honey-producing colonies managed in the US in 2009. The average operational loss suffered by this group was 34.0% (95% CI: 23.9-44.0%), while the total loss suffered was 38.4% (95% CI 29.0–48.0%).

The average operational loss suffered by respondents in the two surveys did not differ ($\chi^2 = 0.20$, d.f. = 1, $P = 0.6472$), and so the databases were combined. The duplicate response provided by a beekeeper who answered both surveys was removed from the merged dataset. The combined dataset had a total of 4,225 respondents who collectively operated 436,802 colonies on 1 October 2009; 17.7% of the total colonies managed in the summer of 2009. These same beekeepers reported having 375,501 living colonies on 1 April 2010. When colonies made / bought (n = 143,973) or sold (n = 8,136) are considered in the calculation (see materials and methods) these beekeepers experienced an average operational loss of 42.2% (95% CI: 41.3-43.4%) and a total loss of 34.4% (95 % CI: 33.7-35.1%). Should these results be representative of national losses, between 829,020 and 863,460 of colonies were lost over the winter of 2009-2010.

Losses by state

There was considerable variation in both the average (Table 1; Fig. 1) and total (Table 1; Fig. 2) loss suffered by beekeepers operating in different states. When generating these figures, operations managing colonies in more than one state had their colonies counted in all states in which the operation managed bees. This is in keeping with the practice of NASS when they annually quantify honey producing colonies. The percentage of colonies and operations per state that operated exclusively in a given state is summarized in Table 1. It should be noted that operations that report managing colonies in more than one state, do not necessarily move all their colonies into and out of a given state. For instance, the one beekeeper in Hawaii who reported having colonies in more than one state almost certainly did not move colonies between Hawaii and the mainland. Thus, some caution is needed when comparing state colony losses where a large proportion of the colonies are managed by operations managing bees in several states.

Losses by operation classification

Average losses suffered by commercial beekeepers tended to be lower than that suffered by sideline and backyard beekeepers, but this difference was not significant (Table 2). There was, however, a difference in the total losses suffered by these groups ($\chi^2 = 2,125$, d.f. = 2, $P < 0.0001$; Table 2). Pairwise chi-square comparisons of total loss data suffered by the sub-groups revealed that sideline beekeepers suffered the largest total loss as compared to all other groups, while the total losses suffered by commercial beekeepers was the lowest.

Fewer than 4% of survey respondents reported maintaining colonies in more than one state. There was no difference in the average loss

experienced by those beekeepers who maintained / did not maintain colonies in more than one state (P > 0.9). The two groups did differ, however, in the total losses reported (χ^2 = 731, d.f. = 1, P < 0.0001). Total losses experienced by beekeepers maintaining colonies in more than one state (33.6% (95% CI: 30.5–36.8%), *n* = 469,962) was lower than the total loss experienced by beekeepers maintaining colonies in only one state (38.3% (95% CI: 37.5–39.1%), *n* = 102,787).

Fewer than 2.5% of responding beekeepers reported moving at least some of their operations into almonds for pollination during the survey period. On average, beekeepers pollinating almonds moved 80.4 ± 2.94% (*n* = 460,607) of their colonies into the almond orchards. The average loss experienced by beekeepers who moved or did not move bees into almond orchards for pollination was not different (P > 0.2). Beekeepers who pollinated almonds experienced lower total losses than those not pollinating almonds (χ^2 = 6,332, d.f. = 1, P < 0.0001; Table 3).

On average all responding beekeepers moved their colonies 0.31 ± 0.02 times (*n* = 4,209). There was no correlation between the number of times beekeepers moved their colonies and total losses suffered by operations (P = 0.23). On average responding beekeepers reported keeping bees for 8.85 ± 0.85 years (*n* = 4,214). There was no correlation between years of beekeeper experience and total losses suffered by operations and the number of years beekeepers reported keeping bees (P = 0.56).

Table 1. The number of operations and colonies contributing to the average and total and losses by state (also summarized in Fig. 1 and Fig. 2) and the percentage of operations and colonies in each state that operated exclusively in that state. Operations reporting managing colonies in more than one state have their colonies counted in all states in which they report managing colonies. Results for states with fewer than six responders are not presented.

State	No. Operations	operations exclusively in state (%)	Total Colonies	colonies exclusively in state (%)	Average Loss (mean (95% CI))	Total Loss (mean (95% CI))
Alabama	46	95.7	1,441	93.5	35.7 (25.6-45.7)	26.6 (23.7-34.8)
Alaska	3					
Arizona	5					
Arkansas	50	96.0	460	23.5	26.8 (18.2-35.4)	23.3 (16.8-31.3)
California	166	38.0	445,639	5.2	39.4 (34.8-44.0)	31.7 (28.9-34.6)
Colorado	99	96.0	7,714	12.2	42.4 (35.2-49.7)	33.0 (30.3-35.9)
Connecticut	58	87.9	760	39.1	50.6 (40.3-61.0)	50.5 (43.5-57.5)
Delaware	15	93.3	104	93.3	54.8 (32.7-77.0)	54.8 (33.5-72.2)
Florida	155	89.7	56,508	9.4	28.6 (23.7-33.4)	53.4 (48.8-56.8)
Georgia	87	92.0	8,548	8.9	43.2 (36.0-50.4)	47.7 (43.2-52.1)
Hawaii	9	88.9	58	93.1	10.2 (0-22.1)	20.7 (11.9-33.6)
Idaho	27	92.6	27,034	0.8	43.8 (30.2-57.4)	27.3 (23.1-31.9)
Illinois	49	87.8	968	27.9	48.2 (37.6-58.9)	73.0 (66.2-78.9)
Indiana	85	95.3	4,574	17.3	47.5 (39.7-55.3)	57.1 (54.3-59.9)
Iowa	56	94.6	1,167	56.7	57.0 (47.9-66.3)	73.4 (67.6-78.4)
Kansas	10	70.0	5,753	1.4	41.0 (19.8-62.1)	39.6 (33.1-45.7)
Kentucky	25	88.0	790	69.9	38.1 (24.1-52.0)	48.8 (34.4-63.5)
Louisiana	3					
Maine	89	92.1	29,790	1.7	22.9 (15.7-30.1)	56.9 (52.4-61.4)
Maryland	171	94.2	4,763	81.8	36.3 (30.6-41.9)	38.7 (35.6-41.9)
Massachusetts	196	96.9	25,224	6.2	45.5 (39.8-51.1)	63.2 (61.1-65.3)
Michigan	231	96.5	13,446	31.9	49.9 (45.2-54.6)	44.9 (41.2-48.6)
Minnesota	37	75.7	158,846	0.6	50.0 (38.5-61.5)	31.8 (29.2-34.5)
Mississippi	14	85.7	17,454	2.3	38.4 (21.3-55.4)	45.8 (35.5-56.5)
Missouri	42	95.2	1,058	73.5	36.4 (25.9-46.9)	28.9 (23.0-35.7)
Montana	24	37.5	123,459	0.1	28.6 (18.5-39.0)	29.5 (20.0-39.8)
Nebraska	17	70.6	139,286	0.1	57.9 (39.5-76.3)	28.5 (24.0-33.5)
Nevada	5					
New Hampshire	76	93.4	821	79.9	37.2 (28.3-46.1)	26.2 (20.3-33.1)

Table 1. Cont.

State	No. Operations	operations exclusively in state (%)	Total Colonies	colonies exclusively in state (%)	Average Loss (mean (95% CI))	Total Loss (mean (95% CI))
New Jersey	31	77.4	3,966	3.4	34.0 (20.0-47.9)	10.4 (6.1-17.2)
New Mexico	9	100.0	3,248	100.0	58.2 (30.0-86.5)	31.8 (34.4-41.1)
New York	163	85.3	28,740	24.7	43.5 (35.6-48.4)	40.1 (36.9-43.4)
North Carolina	191	95.8	3,689	78.9	36.0 (31.1-40.8)	45.7 (41.3-50.0)
North Dakota	30	26.7	243,331	4.7	31.6 (22.2-41.0)	26.6 (4.8-32.0)
Ohio	203	97.0	5,330	33.4	44.0 (39.0-48.9)	52.4 (50.0-54.4)
Oklahoma	10	90.0	141	96.5	32.9 (19.0-46.8)	39.0 (29.4-49.6)
Oregon	49	89.8	30,927	3.5	53.2 (42.8-63.5)	29.7 (23.6-36.6)
Pennsylvania	546	96.9	10,619	50.4	46.3 (43.7-49.9)	42.6 (40.8-44.5)
Rhode Island	67	92.5	279	92.5	41.4 (31.9-50.9)	37.3 (29.5-45.8)
South Carolina	127	88.2	14,747	11.6	38.1 (32.3-43.9)	37.2 (33.1-41.6)
South Dakota	16	12.5	212,653	0.0	34.3 (21.0-47.6)	28.1 (20.8-36.8)
Tennessee	62	98.4	702	85.3	39.6 (30.1-40.1)	28.9 (22.0-36.9)
Texas	59	83.1	61,907	8.6	32.7 (25.4-40.0)	38.6 (35.0-42.4)
Utah	65	93.8	8,184	12.7	45.4 (36.3-54.5)	20.8 (17.0-25.2)
Vermont	68	94.1	3,189	58.1	40.0 (31.7-48.3)	29.0 (24.7-33.7)
Virginia	481	97.1	3,498	93.2	37.8 (34.6-41.0)	38.0 (35.3-40.7)
Washington	144	92.4	84,674	1.4	40.9 (34.8-47.1)	32.5 (29.6-35.5)
Washington, D.C.	2					
West Virginia	117	91.5	1,461	61.5	52.6 (46.2-59.1)	50.1 (44.4-55.8)
Wisconsin	155	92.3	12,119	25.5	59.2 (53.7-64.7)	33.2 (28.9-37.9)
Wyoming	4					

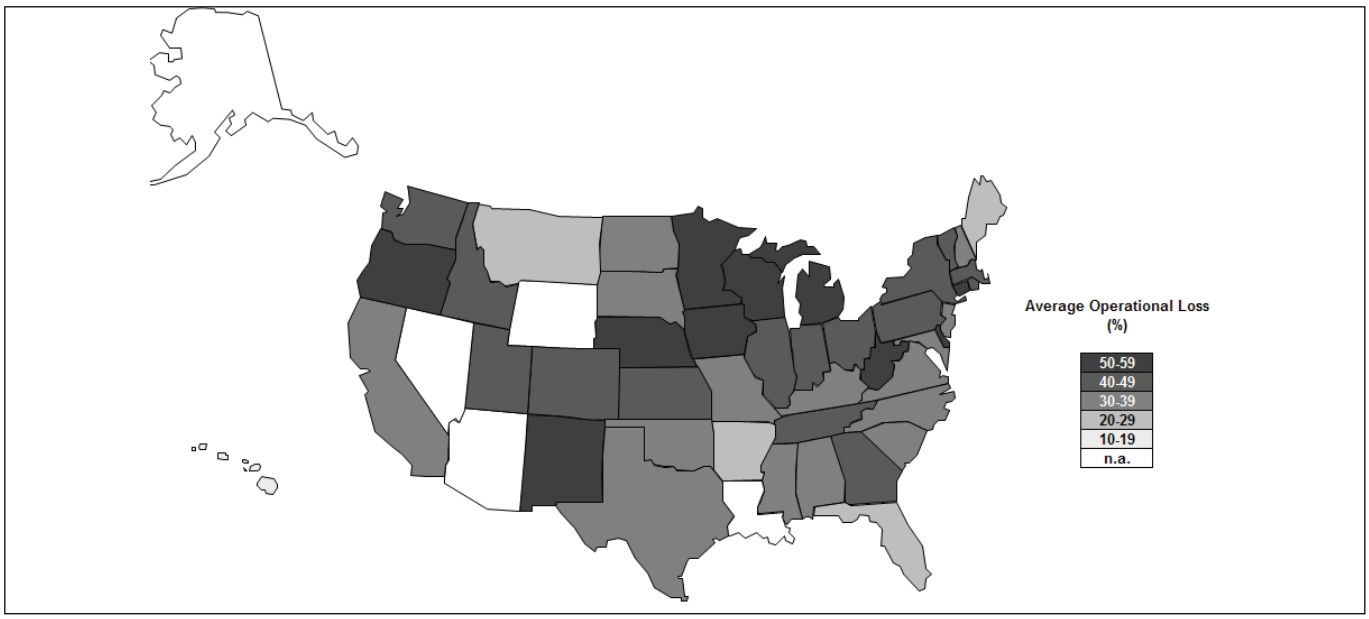

Fig. 1. Average operational loss by US state. Operations who reported managing colonies in more than one state had their losses included in all of the states in which they reported managing colonies (see Table 1). States which had fewer than six responders (n.a.) are not included.

Fig. 2. Total colony losses by state. Operations who reported managing colonies in more than one state had their losses included in all of the states in which they reported managing colonies (see Table 1). States which had fewer than six responders (n.a.) are not included.

Table 2. Average and total losses suffered by beekeepers grouped by the size of their operation. * indicates a significance difference between groups. Different letters in different rows indicate differences between groups in pair-wise chi-square comparisons (P < 0.0001).

Operation Type	Respondents	Average Loss Mean (95 % CI)	Total colonies	Total Losses* Mean (95 % CI)
Backyard	3,944	42.5 (41.3 – 43.6)	25,954	42.9 (41.9 – 43.8)[a]
Sideline	174	42.5 (38.5 - 46.4)	28,217	44.5 (40.6 – 48.4)[b]
Commercial	107	36.0 (31.7 – 40.4)	518,518	33.5 (29.7 - 37.4)[c]

Table 3. Comparison of average and total losses in operations that moved or did not move colonies into almonds for pollination. *indicates significant difference between groups.

	Respondents	Average Loss Mean (95 % CI)	Total colonies	Total Loss Mean (95 % CI)*
Moved into Almonds?				
No	4,063	42.5 (41.4 - 43.6)	112,082	44.6 (43.8 -45.3)
Yes	103	35.5 (30.2 - 40.0)	460,607	32.0 (28.2 – 35.9)

Table 4. Percentage of respondents reporting and the estimated percentage of colonies found dead with the condition of "no dead bees in the colony or apiary" by size of operation. The percentage of beekeepers reporting the condition differed between beekeepers when grouped by operation size (see text).

Operation Type	Respondents	% of respondents reporting condition	Total colonies lost by respondents	% of total colonies lost with condition
Backyard	2,535	25.3	10,261	13.1
Sideline	158	59.0	11,453	35.2
Commercial	88	80.0	135,367	57.3

Losses in operations reporting the symptom of "no dead bees in the hive or apiary"

One of the defining characteristics of Colony Collapse Disorder (CCD) is the complete absence of dead bees in the colony or apiary (vanEngelsdorp *et al.*, 2009), but this survey cannot differentiate between colonies lost to CCD and other conditions that may cause the same symptom. In all, 65% of surveyed beekeepers answered the question "What percentage of the colonies that died between 1 October and 1 April were lost without dead bees in the hive or apiary?"; of those 28.9% reported at least some of their colonies died with the absence of dead bees condition. Average losses were elevated in operations reporting this condition (62.2% (95% CI: 60.2-64.2%), n = 805) when compared to operations that did not report the condition (58.2% (95% CI: 57.0-59.6%), n = 1,976; χ^2 = 10.3, d.f.= 1, P = 0.0014). Beekeepers reporting the condition also experienced higher total losses (44.1% (95% CI: 42.8–45.5%), n = 287,234) as compared to those not reporting the condition (26.7% (95% CI: 25.7-27.7%), n = 113,703; χ^2 = 9,491, d.f. = 1, P < 0.0001).

Commercial beekeepers were 3.1 and 1.4 times more likely to report having some of their dead colonies die with an absence of dead beesthan were backyard and sideline beekeepers (χ^2 = 194, d.f. = 2, P < 0.001; Table 4). By multiplying the self reported proportion of colonies without dead bees by the number of colonies lost in operations reporting this condition we can therefore surmise that 42.1% of all colonies reported dead in this survey died with the "absence of dead bees" condition.

Table 5. Total loss experienced by different beekeeping operations groups classified by operation size and by self-identified leading cause or causes of mortality. *indicates total loss significantly different (Bonferroni-corrected P < 0.006) than total loss experienced by group; chi-square test.

Operation type	**Commercial**			**Sideline**			**Hobby**			**Total**		
No. Respondents	105			170			2,673			2,948		
No. Colonies	513,122			27,745			21,585			562,452		
Total Loss	33.5% (29.7-37.5)			45.0% (41.1-49.0)			49.9% (48.9-51.0)			34.7% (33.9-35.5)		
Factor	**Rank**	**% Resp**	**Total Loss**	**Rank**	**% Resp**	**Total Loss**	**Rank**	**% Resp**	**Total Loss**	**Rank**	**% Resp**	**Total Loss**
Starvation	5	18	18.3% (23.2-26.7) *	1	41	38.4% (32.5-44.6) *	1	31	44.5% (48.1-55.1) *	1	32	24.0% (22.7-25.4) *
Weather	7	7	43.6% (31.8-56.2) *	2	29	45.3% (37.6 49.6)	2	29	56.8% (55.1-58.5) *	2	29	45.1% (43.3-46.3) *
Weak in Fall	8	1	-	7	7	44.7% (27.9-62.8)	3	14	41.7% (39.1-42.3)	3	23	36.8% (29.4-49.6) *
Mites	2	22	40.1% (30.4-50.6) *	3	28	45.5% (32.8-52.8)	4	10	49.7% (46.5-53.0)	4	12	46.8% (38.2-43.4) *
Queen	1	35	27.2% (21.5-33.8) *	4	16	28.7% (20.9-38.4) *	5	9	35.7% (32.4-39.2) *	5	16	45.1% (43.8-46.3) *
CCD	4	20	36.4% (28.4-45.1) *	5	11	53.3% (44.7-61.6) *	6	3	65.6% (60.0-70.8) *	6	4	27.3% (25.2-29.5) *
Nosema	6	12	19.1% (12.1-28.7) *	5	11	45.1% (39.6-56.0)	6	3	45.0% (39.4-50.1)	6	4	37.5% (31.1-40.3) *
Pesticides	3	21	45.4% (37.7-53.7) *	8	4	59.7% (47.2-71.9) *	8	2	65.7% (58.5-73.2) *	8	3	45.6% (41.7-49.6) *

Table 6. Average losses reported by beekeepers who listed one or more factors as the leading cause of mortality in their beekeeping operation as compared to responding beekeepers not listing that particular cause as important.

	Responding			Not responding				
Factor	***N***	**Avg Loss % (95%CI)**		***n***	**Avg Loss % (95%CI)**		X^2	**P**
Starvation	930	54.2 (52.4 – 56.1)		657	62.5 (60.2 - 69.8)		28.6	<0.0001
Weather	825	65.8 (63.9-67.7)		790	56.8 (54.6-59.0)		38.3	<0.0001
Weak in fall	385	54.0 (50.9-57.0)		961	60.0 (58.1-61.9)		11.8	0.0006
Mites	339	55.7 (52.6-58.9)		1000	59.9 (58.1-61.8)		4.48	0.034
Queen	274	45.1 (41.6-48.7)		1045	60.4 (58.6-62.2)		56.2	<0.0001
CCD	124	64.5 (59.7-69.3)		1097	58.8 (57.0-60.6)		4.19	0.1212
Nosema	113	51.6 (46.4-56.8)		1093	59.3 (57.5-61.1)		7.51	0.0061
Pesticides	79	62.9 (56.8 - 69.0)		1,120	59.0 (57.3 - 60.8)		1.27	0.2598

Acceptable losses

Surveyed beekeepers were asked "What percentage of loss, over this time period, would you consider acceptable?" On average, responding beekeepers (n = 3,979) reported that a winter loss of 14.5% (95% CI: 13.9-15.1%) was considered acceptable. Sixty-five percent of responding beekeepers experienced losses higher than that which they considered acceptable. The average losses experienced by this group were higher than the average loss experienced by those who had losses below that which they considered to be acceptable (61.6% (95% CI: 60.6–62.5%) *vs.* 6.9% (95% CI: 5.6-8.3%) respectively; χ^2 = 2,301, d.f.= 1, P < 0.0001).

Perceived causes of losses

Seventy percent of responding beekeepers answered the question "To what do you attribute the cause of death for the colonies that died?" Of these, 413 responded that they did not know. Beekeepers listed eight different potential causes of winter mortality most frequently (Table 5). The frequency with which these causes were listed by beekeepers differed between beekeeper groups when classified by operational size. For instance, 31% of all beekeepers listed "starvation" as a leading cause of mortality. While starvation was the most frequently listed self identified cause reported by backyard and sideline beekeepers, only 18% of responding commercial beekeepers mentioned starvation as an important cause, ranking it below poor queens, mites, CCD, and pesticides for this sub-group of beekeepers. Total losses suffered by beekeepers reporting starvation as an important factor were lower than the total loss suffered by responding beekeepers overall (Table 5). Pesticides were considered an important cause of mortality by only 3% of all responding beekeepers, but 21% of responding commercial beekeepers listed pesticides as an important cause, ranking it as the third most frequently mentioned cause by this group. The total losses experienced by those listing pesticides as a cause of mortality was higher than the overall total losses reported by all responding beekeepers and subgroups of beekeepers (Table 5). The average loss experienced by all those listing pesticides as an important cause of mortality was no different than the average loss experienced by beekeepers not reporting pesticides as an important cause (Table 6). While average losses were also similar between those reporting CCD as a principle cause of loss and those not reporting CCD, for all other factors differences were noted. Beekeepers listing starvation, weak colonies in the fall, mites, queens, and nosema as a principal cause of mortality lost, on average, fewer colonies than those not reporting the condition. Only those reporting weather as a major contributor to their winter losses had higher average losses than those that did not (Table 6).

Discussion

This survey records the fourth consecutive year of overwintering colony losses well above the level US beekeepers consider acceptable. Survey respondents reported total colony losses at 34.4% and average operational losses at 42%. Should these survey results be representative of national losses, between 829,020 and 863,460 of colonies were lost in the US over the winter of 2009-2010, but caution should be used when interpreting this projection. This survey cannot be considered random, and the email solicitation of beekeeper respondents probably biased participation to the subgroup of beekeepers that are internet literate. As no comprehensive census of US beekeepers exists, we have no way to quantify and adjust for this potential bias. For a second consecutive year, beekeepers that used at least part of their operation for almond pollination had significantly lower total losses than their non-almond pollinating counterparts (Table 3). Furthermore, this survey found that operations that managed colonies in more than one state had lower losses than those that did not. While we were unable to find relationships between the

numbers of times colonies were moved the previous year and total or average colony losses, all told our data do not support the hypothesis that moving colonies causes increased mortality (Oldroyd, 2007). If transporting colonies does indeed have negative effects on colony health, these data suggests that these effects can be mitigated by beekeeper management.

While larger operations had lower total losses when compared to smaller sized operations (Table 2), larger operations were also more likely to report having some of the colonies in their operation die with colonies and apiaries absent of dead bees (Table 4). This symptom is one of the defining characteristics of CCD, and as in previous years, those losing some of their colonies to this condition experienced greater total losses than those not reporting the condition (Table 5).

Responding beekeepers most frequently self identified "manageable" conditions, such as starvation, poor weather, and weak in the fall, as the leading causes of mortality in their operations (Table 5), but there was a distinct difference in how beekeepers of different sized operations answered this question. Commercial beekeepers were much more likely to identify non-manageable conditions, such as poor queens and pesticides as leading causes of their losses. While *V. destructor* remained a concern for all beekeepers, it ranked as the second most frequently self-identified cause among commercial beekeepers, and total losses experienced by those identifying mites as a leading cause of mortality were elevated. These differences between groups suggest that extension efforts aimed at curbing high overwintering losses should not be uniform and should be tailored to specific apicultural subgroups.

In summary, this survey effort once again records high rates of mortality in overwintering colonies in the US. Losses suffered by smaller sized operations were higher than the losses suffered by larger operations, even though larger operations were more likely to report having some of their losses occur in the absence of dead bees in the colony or apiary, a defining symptom of CCD. While smaller operations were more likely to self-identify manageable conditions as the cause of mortality, larger operations were more likely to report non-manageable conditions such as queen failure and pesticides as the leading cause of mortality.

These results all point to the continuing need to describe colony losses on an annual basis. Concentrated efforts aimed at understanding the underlying causes of these losses are also needed.

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References

ABDI, H (2007) The Bonferonni and Šidák corrections for multiple comparisons. In N Salkind (Ed.) *Encyclopedia of Measurement and Statistics*. Thousand Oaks; Sage, CA, USA. pp. 103-107.

CARON, D M; BURGETT, M; RUCKER, R; THURMAN, W (2010) Honey bee colony mortality in the Pacific Northwest winter 2008/2009. *Americian Bee Journal* 150: 265-269.

CURRIE, R W; PERNAL, S F; GUZMÁN-NOVOA, D E (2010) Honey bee colony losses in Canada. *Journal of Apicultural Research* 49(1): 104-106. DOI: 10.3896/IBRA.1.49.1.18

DABERKOW, S; KORB, P; HOFF, F (2009) Structure of the US beekeeping industry: 1982-2002. *Journal of Economic Entomology* 103: 868-886. http://hdl.handle.net/10113/29783

FLOTTUM, K (2010) Inner cover. *Bee Culture* 138(6): 10.

MCCULLAGH, P; NELDER, J (1989) *Generalized Linear Models* (2 Ed.). Chapman and Hall / CRC. London

NEUMANN, P; CARRECK, N L (2010) Honey bee colony losses. *Journal of Apicultural Research* 49(1): 1-6. DOI: 10.3896/IBRA.1.49.1.01

NGUYEN, B K; MIGNON, J; LAGET, J; DE GRAAF, D C; JACOBS, F J; VANENGELSDORP, D; BROSTAUX, Y; SAEGERMAN, C; HAUBRUGE, E (2010) Honey bee colony losses in Belgium during the 2008-2009 winter. *Journal of Apicultural Research* 49(4): 333-339. DOI: 10.3896/IBRA.1.49.4.07

OLDROYD, B P (2007) What's killing American honey bees*? PLoS Biology* 5: e168. DOI:10.1371/journal.pbio.0050168

PAOLI, B; HAGGARD, L; SHAH, G; (2002) *Confidence intervals in public health.* Office of Public Health Assessment, Utah Department of Health, USA. 8 pp.

R DEVELOPMENT CORE TEAM. (2009) R: A language and environment for statistical computing. R Foundation for Statistical Computing; Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.

SAS (2007) JMP computer program. Cary, NC, USA.

UNITED STATES DEPARTMENT OF AGRICULTURE NATIONAL STATISTICS SERVICE (USDA-NASS) (2009) *2007 Census of Agriculture.* Department of Agriculture; Washington, D.C., USA. 6 pp.

UNITED STATES DEPARTMENT OF AGRICULTURE NATIONAL STATISTICS SERVICE (USDA-NASS) (2010) *Honey.* Department of Agriculture; Washington, D.C., USA. 6 pp.

VANENGELSDORP, D; 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. *Journal of Invertebrate Pathology* 103: S80-S95. DOI:10.1016/j.jip.2009.06.011

VANENGELSDORP, D; UNDERWOOD, R; CARON, D; HAYES, J Jr (2007) An estimate of managed colony losses in the winter of 2006-2007: a report commissioned by the Apiary Inspectors of America. *American Bee Journal* 147: 599-603.

VANENGELSDORP, D; HAYES, J Jr; UNDERWOOD, R M; PETTIS, J (2008) A survey of honey bee colony losses in the US, Fall 2007 to Spring 2008. *PLoS ONE* 3: e4071. DOI: 10.1371/journal.pone.0004071

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; PETTIS, J S (2009) Colony Collapse Disorder: A descriptive study. *PloS ONE* 4: e6481. DOI: 10.1371/journal.pone.0006481

VANENGELSDORP, D; HAYES, J Jr; UNDERWOOD, R M; PETTIS, J S (2010) A survey of honey bee colony losses in the United States, fall 2008 to spring 2009. *Journal of Apicultural Research* 49(1): 7-14. DOI: 10.3896/IBRA.1.49.1.03

Chapter 4

COLONY COLLAPSE DISORDER: A DESCRIPTIVE STUDY.[3]

[3] **vanEngelsdorp, D., J. D. Evans, C. Saegerman, C. Mullin, E. Haubruge, B. K. Nguyen, M. Frazier, J. Frazier, D. Cox-Foster, Y. Chen, R. Underwood, D. R. Tarpy, and J. S. Pettis.** 2009. Colony Collapse Disorder: A descriptive study. PloS ONE 4: e6481. Open Access.

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Colony Collapse Disorder: A Descriptive Study

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Abstract

Background: Over the last two winters, there have been large-scale, unexplained losses of managed honey bee (*Apis mellifera* L.) colonies in the United States. In the absence of a known cause, this syndrome was named Colony Collapse Disorder (CCD) because the main trait was a rapid loss of adult worker bees. We initiated a descriptive epizootiological study in order to better characterize CCD and compare risk factor exposure between populations afflicted by and not afflicted by CCD.

Methods and Principal Findings: Of 61 quantified variables (including adult bee physiology, pathogen loads, and pesticide levels), no single measure emerged as a most-likely cause of CCD. Bees in CCD colonies had higher pathogen loads and were co-infected with a greater number of pathogens than control populations, suggesting either an increased exposure to pathogens or a reduced resistance of bees toward pathogens. Levels of the synthetic acaricide coumaphos (used by beekeepers to control the parasitic mite *Varroa destructor*) were higher in control colonies than CCD-affected colonies.

Conclusions/Significance: This is the first comprehensive survey of CCD-affected bee populations that suggests CCD involves an interaction between pathogens and other stress factors. We present evidence that this condition is contagious or the result of exposure to a common risk factor. Potentially important areas for future hypothesis-driven research, including the possible legacy effect of mite parasitism and the role of honey bee resistance to pesticides, are highlighted.

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Introduction

The winter of 2006/2007 witnessed large-scale losses of managed honey bee (*Apis mellifera* L.) colonies in the United States [1]. Those losses continued into the winter of 2007/2008 [2]. In the U.S., a portion of the dead and dying colonies were characterized *post hoc* by a common set of specific symptoms: (1) the rapid loss of adult worker bees from affected colonies as evidenced by weak or dead colonies with excess brood populations relative to adult bee populations (Figure 1); (2) a noticeable lack of dead worker bees both within and surrounding the affected hives; and (3) the delayed invasion of hive pests (e.g., small hive beetles and wax moths) and kleptoparasitism from neighboring honey bee colonies [3]. Subsequently, this syndrome has been termed Colony Collapse Disorder, or CCD.

Large-scale losses are not new to the beekeeping industry; since 1869, there have been at least 18 discrete episodes of unusually high colony mortality documented internationally [4]. In some cases, the descriptions of colony losses were similar to those described above. For example, a condition named "May Disease" occurred in Colorado in 1891 and 1896, where large clusters of bees completely disappeared or significantly declined over a short period of time [5].

Numerous causes of CCD have been proposed, often with little or no supporting data [6]. In an attempt to identify the potential cause(s) of CCD, we conducted an epizootiological survey of CCD-affected and non-affected apiaries. In doing so, we set an operational case definition that we verified by taking measurements of colony populations (brood and adult bees) and collecting samples of adult bees, wax comb, beebread (stored and processed pollen), and brood to test for known honey bee parasites (i.e., varroa mites, *Varroa destructor*, and honey bee tracheal mites, *Acarapis woodi*), pathogens (i.e., bee viruses and *Nosema* spp.), pesticide residues, protein content, genetic lineage, and morphological measurements. The results of an initial metagenomic analysis of some of the samples collected from this effort have already been reported [3].

Broadly defined, epizootiological studies are the study of disease occurrence in animal (in this case honey bee) populations. A primary function of epizootiology is to provide clues as to the

Figure 1. Frames of brood with insufficient bee coverage, indicating the rapid loss of adult bees.
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etiology of disease [7] as defined in the broadest sense - a departure from perfect health [8]. Descriptive epizootiological studies attempt to elucidate the cause(s) of disease by comparing health and risk factors in "diseased" and "non-diseased" populations [8]. A hallmark of these studies is that they are performed without a specific hypothesis, but they require an ability to classify the surveyed population into "diseased" and "non-diseased" individuals (in this case, colonies) based on a case definition.

Case definitions, especially when little is known about the disease, are often inductive and based on shared readily observable clinical characteristics [9]. Clinical characteristics, such as those used to classify colonies as suffering from CCD, are based on readily available (albeit sometimes broad) characteristics easily identified by "clinicians", which are often referred to as operational case definitions [8]. The operational case definition of CCD, used in this study, may have a low level of specificity and, thus, runs the risk of misclassifying individual colonies, which in turn can bias results [10]. Some of the characteristics used to define CCD, such as the lack of kleptoparasitism or the rapid loss of adult bees, are not easily quantified yet are readily identified by experienced beekeepers. Such ambiguity often results in skeptics dismissing the described condition as too vague to warrant recognition. The human medical literature, however, is filled with examples of such broadly defined disease (e.g., Gulf war syndrome [11]). Studies based on initially broad operational definitions permit the refinement of the case definition as more knowledge is gained about the condition [8]. Thus, the use of a sensitive, potentially overly inclusive definition is typical when investigating conditions for which the inclusion of suspect cases cannot be validated (e.g., by using laboratory test) and is common when investigating apparently new disease events, particularly when that event may be a new outbreak or epidemic.

The current study aimed to (a) characterize the spatial distribution of strong, weak, and dead colonies in apiaries containing colonies with and without CCD symptoms; (b) quantify and compare measurements among populations suspected to be suffering from CCD with apparently healthy colonies; and (c) gain insight into the cause of CCD. By physically mapping dead and weak colonies within CCD-affected and non-affected apiaries, we determined whether colonies graded with the same "condition" were randomly distributed within apiaries. A non-random distribution (e.g., dead colonies tending to neighbor other dead colonies) would suggest that an infective agent or exposure to a common risk factor may underlie the disorder.

We recognized, up front, that our characterization of CCD is not without bias; many measures, such as quantifying the colony population, are confounded with the overt symptom of CCD (i.e., lack of adult bee population). Other confounding measures are those that quantify colony stress. For example, whole-bee protein levels can serve as an indirect measure of developmental stress [12]. Honey bee larvae require sufficient protein in their brood food to ensure proper development and to optimize their activities during the winter. Farrar [13] showed that the quantity of stored pollen within a colony in the fall is significantly correlated with its spring adult bee population. Measures of mass, total protein, and protein-mass ratio can therefore act as an indirect measure of colony nutrition [13–19], parasitism [20–23], or both. Differences in these measures may be a consequence (i.e., collapsing colonies are less able to acquire sufficient forage to maintain proper colony health and function) or a contributing cause of the syndrome (e.g., nutritionally stressed colonies are more susceptible to pathogen attack). Another indirect measure of developmental stress is fluctuating asymmetry (FA). FA is defined as random differences in the shape or size of a bilaterally symmetrical character [24], which can be an indicator of individual fitness [25] because organisms exposed to stress during early development show less symmetry than unstressed organisms [26–33].

Some factors quantified and compared in this study have known impacts on colony health. Elevated populations of varroa mites, *Nosema* spp., and honey bee tracheal mites (HBTM) are known to damage colonies and may contribute to CCD. Both the HBTM and the varroa mite were introduced into the U.S. in the 1980's and are now widespread. While the number of managed honey bee colonies has been in decline in the U.S. since the 1940's, these mites have been implicated in drastic losses of colonies since their introduction [34]. Similarly, two species of *Nosema* are now widespread across the continental U.S. Historically, nosema disease was thought to be caused by the gut parasite *Nosema apis*, which can be particularly problematic for overwintering colonies [35,36]. However, a recent survey of historical samples collected

from across the U.S. suggests that *N. apis* has been largely displaced by *N. ceranae* over the past decade [37]. While the etiology of *N. ceranae* is poorly understood, it has been implicated with recent large-scale losses experienced by Spanish beekeepers [38,39]. Other pathogens, including bacteria, fungi, trypanosomes, and viruses, can also significantly impact colony health. An extensive survey of declining and healthy honey bee populations, using metagenomics and targeted polymerase chain reaction (PCR), helped to identify several microbial associates of CCD colonies, the most informative of which was the discistrovirus Israeli acute paralysis virus (IAPV) [4]. In the current study, we assayed colonies for the presence of 12 organisms spanning these different groups using sensitive PCR-based techniques [3,40,41]. Moreover, using established protocols testing mitochondrial DNA markers [42], we were able to assign the sampled colonies as either European in origin (Eastern vs. Western) or as African in origin (Northern vs. Southern). If certain mitotypes are found to be more affected by CCD, it could pin-point specific genetic strains of interest for future analyses [43,44] as well as induce future explorations into unique host-pathogen interactions.

Pesticide exposure is also a risk factor that was quantified in this study. Honey bees can contact and collect pesticides when foraging on crops that have been treated to control pest insects, pathogens, or weeds. In addition, since the late 1980's, U.S. beekeepers have been using miticides within their beehives to control parasitic mites (primarily Varroa mites). A diverse range of pesticides, both grower- and beekeeper-applied, have been detected in hive matrices [45–47], and many of these products are known to adversely affect colony health [48–50]. Here, we compare both the prevalence and load of different pesticides in the wax, beebread, brood, and adult bees in a subset of CCD-affected and non-affected populations.

Materials and Methods

Apiary selection and CCD assessment

In January and February 2007, we selected colonies resident in Florida and California distributed across 13 apiaries owned by 11 different beekeepers. Apiaries were classified as (1) having no colonies with CCD symptoms ('control') or (2) having colonies with CCD symptoms ('CCD'). The operational case definition employed to classify CCD cases verses non-cases were qualitative and made in the field by researchers experienced in clinical bee disease diagnosis. This was as follows (1) the apparent rapid loss of adult worker bees from affected colonies as evidenced by weak or dead colonies with excess brood populations relative to adult bee populations; (2) the noticeable lack of dead worker bees both within and surrounding the hive; and (3) the delayed invasion of hive pests (e.g., small hive beetles and wax moths) and kleptoparasitism from neighboring honey bee colonies. In those CCD colonies where some adult bees remained, there were insufficient numbers of bees to cover the brood, the remaining worker bees appeared young (i.e., adult bees that are unable to fly), and the queen was present. Notably, both dead and weak colonies in CCD apiaries were neither being robbed by bees (despite the lack of available forage in the area as evidenced by the lack of nectar in the comb of strong colonies in the area and by conversations with managing beekeepers) nor were they being attacked by secondary pests (despite the presence of ample honey and beebread in the vacated equipment).

The physical locations of the hives in a subset of the visited apiaries (n = 9) were mapped. We classified these colonies as either 'alive' or 'dead' (i.e., no live bees) and we classified the living colonies as either 'weak' or 'acceptable' based on the number of frames of bees (with those having four or fewer frames of bees being considered 'weak').

Colony strength and sample collection

In all, 91 colonies were sampled and used in subsequent analyses. The populations of adult bees and brood were measured in living colonies (n = 79) through the estimation of the total area of comb covered by adult bees or brood [after 51].

At the time of sampling, the presence of overt brood infections (pathogens) was noted. The condition of the quality of the brood pattern was also noted with areas of capped brood containing less than 80% viable brood (as indicated by cells empty of brood) were considered "spotty" while those brood patterns that had less than 20% brood mortality were considered "solid".

Samples of adult bees (~150 bees) were removed from a central brood frame, placed into a 50 ml centrifuge tube, and temporarily stored on dry ice before being frozen at –80°C for future processing. A subset of these bees was used for pathogen, protein, and pesticide analyses. An additional sample of ~320 bees, collected from the same frame, was placed in 75% ethanol in a 125 ml sampling container and used for quantification of varroa mite mean abundance, HBTM prevalence, and *Nosema* spp. spore prevalence and load. Finally, all live and dead (n = 12) colonies had ~15 cm×15 cm sections of brood comb removed from them, which contained wax and often (but not always) bee brood and beebread. Sampled comb was stored on dry ice before long-term storage at −20°C.

Physiological and morphological measures

Body mass and protein analyses. We used BCA Protein Assay kits (Pierce Scientific, Rockford, IL) to quantify protein content from six separate adult worker honey bees from each of the sampled colonies containing live bees (n = 79). This process uses bicinchoninic acid for the colorimetric detection and quantification of soluble protein (Bradford assay), which indicates the developmental nutrition of bees within a colony during larval feeding [52].

We removed each bee from −80°C storage onto ice and separated its head, gaster (abdomen), and thorax with a razor blade. After the wings and legs were removed from the thorax (because, during shipping, many bees did not have a full complement of appendages), we weighed each body segment to the nearest 0.1 mg using a Metler digital scale. Immediately after weighing, each segment was placed into a separate 1.5 ml microcentrifuge tube on ice. We then added 150 µl, 600 µl, and 500 µl of extraction buffer (1×PBS+0.5% Triton X-100) to the head, abdomen, and thorax tubes, respectively. Each sample was homogenized using a clean plastic pestle, placed on ice for 30 min, and centrifuged at 14,000 g for 5 min. The supernatant was then transferred from each tube to a separate 0.5 ml microcentrifuge tube and frozen at −20°C until further analysis.

We performed the BCA tests by adding 18 µl of 1x phosphate-buffered saline, 2 µl thawed protein extract, and 100 µl BCA working reagent (Pierce Scientific, Rockford, IL) to individual PCR reaction tubes, vortexing and spinning the tubes to homogenize the reagents, and incubating them for 30 min at 37°C on a thermocycler. We then cooled the tubes on ice for 15 min and immediately read their absorbance using a Nanodrop® spectrophotometer. Following the Bradford assay, we calculated the final levels of soluble protein using a standard curve generated from known concentrations of Bovine Serum Albumen.

Morphometric measures. From each living colony from which adult bees were sampled into ethanol (n = 76), both forewings from 10 workers were removed and mounted on microscope slides

using transparent tape. The wings were then scanned at 600 dpi using a Hewlett Packard ScanJet ADF flatbed scanner. The centroid size of each wing was calculated by determining the relative position (landmark) of 12 vein intersections [after 53] and then calculating the square root of the sum of squared distances between each landmark and the centroid of each forewing [54]. The relative position of each landmark was determined using a script written for UTHSCSA Image Tool software (downloaded from http://ddsdx.uthscsa.edu/dig/itdesc.html) and the resulting data were imported into SAS [55] to automate the centroid-size calculation.

To distinguish between true measures of FA and measurement error, a randomly selected sub-sample of up to 10 bees from 24 colonies (n = 216) had their centroid sizes recalculated from the original scanned image. A two-way ANOVA (repeated measures) revealed that the mean square of the interaction between individual bees and wing side was significantly larger than the mean square of the error term ($F = 4.66$, df = 215, 432; $P<0.0001$), suggesting that measurement error was not a significant source of centroid size variation [56].

A simple linear regression was conducted [57] comparing centriod size and FA. As no association was found ($F = 0.085$, df = 1, 7, $P = 0.7714$), no correction for scale effect was warranted [56]. Consequently, FA_1 [58] measures were calculated by determining the absolute difference in centroid size between an individuals left and right wings.

Risk exploratory variables

Macro-parasite and pathogen quantification. The mean abundance of varroa mites (mites per bee, or mpb) was determined by separating mites from the entire sample of bees stored in ethanol by shaking them in soapy water and then counting both the number of mites and bees in the sample [59–61]. Thirty of these bees also had their abdomens removed to measure the mean abundance of *Nosema* spp. spores (spores per bee) following Cantwell [62]. Finally, using the methods outlined by Delfinado-Baker [63], the prevalence of honey bee tracheal mites (*Acarapis woodi*) was determined by examining thoracic slices of 16 bees per colony, which is the number suggested for differentiating highly infested colonies (prevalence >30%) and colonies with low infestation (prevalence<10%) [64]. For all of these tests, colonies were additionally classified as being affected or not affected by the parasite or pathogen, regardless of the load.

Pathogen analyses. We determined the prevalence (proportion of colonies affected) of several pathogens, including bacteria, trypanosomes, *Nosema* species, and numerous viruses: Acute Bee Paralysis Virus (ABPV), Black Queen Cell Virus (BQCV), Chronic Bee Paralysis Virus (CBPV), Deformed Wing Virus (DWV), Israeli Acute Paralysis Virus (IAPV), Kashmir Bee Virus (KBV), and Sacbrood Virus (SBV). Each pathogen was targeted with a single diagnostic primer [3, 40, 41; Table 1] except IAPV, for which we employed three distinct primer pairs as a means of capturing all members of this diverse lineage. For IAPV, we present relative transcript abundances based on each primer pair separately and an aggregate (arithmetic mean; $IAPV_{Avg}$) from all primer pairs. We extracted total RNA from pooled abdomens of eight worker bees from each colony (n = 76) by grinding abdomens in 1 ml guanidine thiocyanate lysis buffer, pelleting debris, and then extracting RNA from the supernatant using the RNAqueous procedure (Ambion). We then generated cDNA from approximately 500 ng of total RNA using a mixture of poly-dT primers [40] and Superscript II reverse transcriptase (Roche). We carried out quantitative PCR on individual samples and targets using the fluorescent intercalating dye SYBR Green and a Bio-Rad Icycler thermal cycler. We optimized primer pairs for each pathogen target (Table 1) and conducted all PCR reactions using a thermal profile of 3 min at 94°C, followed by 40 cycles of 94°C (30 s), 60°C (30 s), 72°C (30 s), and 78°C (20 s). The 78°C step was used to avoid background signals from potential primer-dimer artifacts. We normalized the estimates of pathogen transcript abundance by the ddC_T method [65], using the geometric mean C_T value of three honey bee housekeeping genes (actin, RPS5, and mGsT) as a reference for pathogen transcript abundance.

Pesticide analyses. Multi-residue pesticide analysis was conducted by the USDA-AMS-NSL at Gastonia, NC, using a modified QuEChERS method [66]. Of the 22 samples of brood comb that contained beebread, 7 had insufficient quantities (<3 g) to analyze on their own, so samples were pooled with other colonies within the same apiary having the same condition (CCD

Table 1. Quantitative-PCR primers for measuring transcript abundances of honey bee pathogens.

Locus	Forward Primer	Reverse Primer
ABPV	ACCGACAAAGGGTATGATGC	CTTGAGTTTGCGGTGTTCCT
BQCV	TTTAGAGCGAATTCGGAAACA	GGCGTACCGATAAAGATGGA
DWV	GAGATTGAAGCGCATGAACA	TGAATTCAGTGTCGCCCATA
KBV	TGAACGTCGACCTATTGAAAAA	TCGATTTTCCATCAAATGAGC
IAPV_B4SO427	CGAACTTGGTGACTTGAAGG	GCATCAGTCGTCTTCCAGGT
IAPV-F1a	GCGGAGAATATAAGGCTCAG	CTTGCAAGATAAGAAAGGGGG
IAPVpwF16	ACCCCCAACTGCTTTCAACAG	CTGGATATAGTACATTAATGTCCTGC
SBV	GGGTCGAGTGGTACTGGAAA	ACACAACACTCGTGGGTGAC
N. apis	CAATATTTTATTGTTCTGCGAGG	TATATTTATTGTATTGCGCGTGCT
N. ceranae	CAATATTTTATTATTTTGAGAGA	TATATTTATTGTATTGCGCGTGCA
Trypanosome	CTGAGCTCGCCTTAGGACAC	GTGCAGTTCCGGAGTCTTGT
Bact774	GTAGTCCACGCTGTAAACGATG	GACGGGCGGTGTGTRCA
RPS5	AATTATTTGGTCGCTGGAATTG	TAACGTCCAGCAGAATGTGGTA
Am actin	TTGTATGCCAACACTGTCCTTT	TGGCGCGATGATCTTAATTT
MGST	TTGCTCTGTAAGGTTGTTTTGC	TGTCTGGTTAACTACAAATCCTTCTG

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or control) (n = 18). Comb wax, beebread, brood, or adult bees (3 g) were extracted with 27 ml of 44% water, 55% acetonitrile, and 1% glacial acetic acid, after which 6 g of anhydrous magnesium sulfate and 1.5 g anhydrous sodium acetate were added. A 1–2 ml portion of the supernatant was then treated with primary secondary amine, anhydrous magnesium sulfate, and C18 (LC only) or graphitized carbon black (GC only). The resulting supernatant was analyzed by both high-performance liquid chromatography/tandem mass spectroscopy (LC/MS-MS) on a Thermo-Fisher TSQ triple quadrupole MS and gas-liquid chromatography/mass spectroscopy (GC/MS) on an Agilent 5975 triple quadrupole MS for up to171 pesticides and related metabolites [46]. Choices of insecticides, fungicides, and herbicides to analyze were based largely on their frequency of use where bees may be exposed (e.g., in-hive miticides, plant systemics), and their potential for bee toxicity. Limit of detections were in the low part per billion (ppb) range.

Genetic analyses. We extracted the DNA from three adult worker bees from each sampled colony (n = 73) using Puregene DNA extraction kits (Gentra systems, Inc.). We then employed an established mitotyping protocol as outlined in Nielsen et al. [42]. This procedure amplifies small (≈1 kb) sections of mitochondrial DNA from the COI and rRNA gene sequences and then subjects them to restriction enzyme digests using *HimfI*, *EcoRI*, and *HincII*. Splicing and banding patterns of the resultant amplified PCR product determined the maternal origin of the bees as either West European (subspecies including *Apis mellifera mellifera*), East European (subspecies including *A. m. ligustica*), North African (*A. m. lamarkii*), or South African (*A. m. scutellata*) after they were electrophoresed on 1.5% agarose gels and visualized with ethidium bromide.

Statistical analyses

Neighboring colony strength ratings. The colonies in all of the mapped CCD apiaries were managed on palletized systems, with either four or six colonies per pallet. Should CCD be caused by an infectious condition or exposure to a common risk factor, we would not expect that colonies in dead or weakened states to be randomly distributed within an apiary but rather be in closer proximity to one another. We tested this hypothesis by comparing the expected and observed frequencies of neighboring colonies (those sharing the same pallet and those with entrances facing in the same direction) with the same or different classifications (dead, weak, or acceptable). As is common in epizootiological studies (e.g. [67]), we examined possible relationships between apparently healthy and diseased colonies by comparing the expected (the number of categorized colonies expected to neighbor one another based on the overall frequency of that condition within an apiary) and observed frequencies of colonies sharing the same strength classification in mapped apiaries using a Chi-square test. The degree (or risk) associated with neighbouring weak or dead colonies in CCD-affected and non-affected apiaries was quantified by calculating odds ratio (95% confidence intervals (logarithmic approximation)). Each neighbor-to-neighbor rating is compared to the reference group as "Adequate – Adequate" neighbor pairings. A P value≤0.05 was considered significant.

CCD characterization. For statistical purposes, we used two methods to compare CCD and control populations. First, we grouped all colonies within an apiary, and thus compared apiary averages for a given measure in CCD vs. control apiaries. This approach averages the measurements from colonies regardless of whether any particular colony showed signs of collapse and so may include data from colonies not suffering from CCD. However, as sampled apiaries contained colonies that were actively collapsing, colonies graded as "adequately strong" or "control" in CCD apiaries could have been at an early, asymptomatic stage of collapse. Comparing CCD vs. control apiaries reduced the sample size and, consequently, the power of statistical analysis.

The second approach compared adequately strong colonies (control) with colonies that were obviously suffering from CCD (or had presumably died from CCD, such as those that had wax samples analyzed for pesticides; n = 11). While this approach increased the statistical power of analysis, it risked including colonies that were at the early stages of collapse in the control group. We performed and report both types of exploratory comparisons; CCD vs. control populations classified at the apiary- and individual-colony level.

Risk explanatory variables analyses. We compared individual- and colony-level measurements between CCD and control apiaries and colonies using Wilcoxon rank sum tests. Nonparametric tests were employed because the basal assumptions of parametric tests (i.e., normality and constant variance) were not satisfied [68]. We assumed that the observations in the two independent samples are representative of the populations of interest. We also compared the incidence (proportion of colonies affected) of the fungal disease chalkbrood (*Ascosphaera apis*), European foulbrood (*Melissococcus pluton*), and spotty brood patterns between the two groups using a Chi-square test or Fisher's exact test when the observed frequency in any cell was less than 5.

Unless otherwise noted, all statistical analyses were carried out using SAS JMP 9.0 [57] When risk factor prevalence data is presented, 95% confidence intervals on the point estimate were calculated by hand to adjust for incident rates based on 100 or fewer cases [8].

Results

Colony strength measurements

As the operational case definition for CCD was based, in part, by a clinical assessment that adult bee populations were in rapid decline, differences between non-affected and CCD-affected colony strength measures are not surprising (Table 2 and 3). These results verify that the application of the operational case definition was able to segregate the two populations in a discreet and non-random way.

Comparison of apiaries and ratings of neighboring colony strength

CCD-affected apiaries contained 3.5 times the number of dead colonies compared to control apiaries. Similarly, CCD apiaries contained 3.6 times more weak colonies compared to control apiaries (Table 4). In CCD apiaries, neighbouring colonies that were both of adequate strength ('acceptable') were 2.3 times less frequent than would have been expected, while neighboring colonies that were both 'weak' or both 'dead' were approximately 1.3 times more frequent than expected (Table 5). The opposite was true in control apiaries, where adequately strong colonies were 2.6 times more likely to neighbor other colonies of adequate strength. Moreover, the odds ratio demonstrated that in CCD apiaries there was an increased risk of colonies being weak or dead when they neighbored other weak or dead colonies (Table 5). This suggests that CCD is either a contagious condition or results from exposure to a common risk factor.

Comparison of protein and mass measurements

None of the measurements of soluble protein, mass, or protein-to-mass ratio were different when colonies from CCD apiaries were compared to colonies from control apiaries (Wilcoxon rank sum test; P>0.10; Table 2). Similarly, no measures of mass, soluble

Table 2. Strength and mean physiological and morphometric measurements of bees from colonies (N_t) located in CCD and control apiaries.

Variable		CCD Apiaries	Mean±S.E.	Median (25th & 75th percentiles)	Control Apiaries	Mean±S.E.	Median (25th & 75th percentiles)	Wilcoxon rank sum test
		N_t			N_t			*P*
Strength	Frames of brood	56	2.0±0.24	2.0 (0.3–3.0)	18	1.7±0.45	1.3 (0.8–1.9)	0.46
	Frames of bees	60	5.4±0.68	4.0 (2.0–8.0)	18	7.8±1.26	6.0 (4.0–9.8)	0.02*
	Ratio bees/brood	53	4.7±0.89	2.0 (1.0–4.0)	17	7.5±1.44	4.5 (4.0–10.0)	0.00*
Proteins#	Proteins in the head [A]	60	2.2±0.18	1.3 (1.1–3.4)	18	1.7±0.27	1.3 (1.1–1.7)	0.48
	Proteins in the abdomen [B]	61	12.7±0.82	10.2 (5.6–12.7)	18	10.0±0.98	10.2 (6.0–12.7)	0.21
	Proteins in the thorax [C]	61	4.1±0.87	4.2 (3.4–4.2)	18	4.4±0.18	4.3 (3.9–4.9)	0.19
	Total proteins [D]	60	16.4±0.82	15.4 (12.2–18.4)	18	14.8±1.21	15.4 (10.3–18.3)	0.71
	Mass of the head [E]	60	12.1±0.13	12.1 (11.3–13.1)	18	12.1±0.21	12.1 (11.4–12.9)	0.91
	Mass of the abdomen [F]	61	64.9±1.99	61.6 (55.2–72.3)	18	59.4±3.36	61.1 (47.8–67.4)	0.27
	Mass of the thorax [G]	61	33.5±0.33	33.8 (31.8–35.6)	18	34.1±0.44	34.3 (32.7–35.6)	0.46
	Total mass [H]	60	103.6±2.43	102.5 (92.3–113.4)	18	101.7±3.97	99.9 (91.5–113.2)	0.78
	Ratio [A]/[E]	60	0.10±0.003	0.10 (0.09–0.11)	18	0.11±0.01	0.11 (0.09–0.12)	0.11
	Ratio [B]/[F]	61	0.18±0.007	0.18 (0.15–0.22)	18	0.16±0.01	0.18 (0.15–0.22)	0.22
	Ratio [C]/[G]	61	0.12±0.003	0.12 (0.11–0.14)	18	0.13±0.01	0.13 (0.12–0.14)	0.41
	Ratio [D]/[H]	60	0.15±0.005	0.15 (0.12–0.17)	18	0.14±0.01	0.14 (0.12–0.17)	0.43
Morphological measures	Centroid size	58	59.7±0.79	58.8 (56.6–61.3)	18	60.9±0.73	60.7 (58.4–63.3)	0.08
	FA	58	1.7±0.116	1.48 (1.30–1.98)	18	1.9±0.11	1.9 (1.5–2.2)	0.04*

FA: Fluctuating asymmetry.
#A total of 6 heads or abdomens or thoraces from one colony were used.
*$P<0.05$.
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protein, or protein-to-mass ratio differed between the two types of colonies (Wilcoxon rank sum test; $P>0.06$; Table 3).

Comparison of morphometric measurements

The average forewing centroid size in bees from colonies sampled in CCD apiaries was no different than bees from colonies sampled in control apiaries ($P=0.08$). In contrast, a comparison of the absolute difference between the centroid size in right and left wings (FA_1) revealed that bees from colonies in CCD apiaries were more symmetrical than those in control apiaries (Wilcoxon rank sum test; $P=0.04$; Table 2).

Similarly, the average centroid size in bees sampled from CCD and control colonies was not different ($P=0.34$). Bees from CCD colonies, however, were more symmetrical than those in control colonies (Wilcoxon rank sum test; $P=0.01$; Table 3).

Comparison of overt signs of disease and brood pattern

Six percent of colonies from CCD apiaries had clinical infections of chalkbrood disease (CB) and 8% had clinical infections of European foulbrood (EFB; Table 6). While none of the colonies in control apiaries had clinical infections with these common brood diseases, the incidence of colonies affected did not differ significantly between apiary types (Fisher's exact test: $P>0.50$). Fifty-five percent of colonies from CCD apiaries had spotty brood patterns, which was not different than the 43% of colonies in control apiaries that had the same condition ($P=0.41$).

Colonies suffering from CCD did not have a higher incidence rate of either CB or EFB, nor did they have a greater incidence of poor brood patterns when compared to colonies not apparently suffering from CCD ($P>0.35$; Table 7).

It is of interest to note that EFB-infected larvae found in one apiary suffering from CCD were distinctly corn-yellow in appearance (Figure 2A) as opposed to the usual beige appearance of infected larvae (Figure 2B). Microscopic examination of smears from these samples revealed nearly pure cultures of EFB's causal agent *Melissococcus pluton*. This is unusual, as EFB smears usually reveal high levels of opportunistic bacteria such as *Paenibacilus alvei*, *Brevibacillus laterosporus*, and *Enterococcus faecalis* with little or no evidence of the causal agent *M. pluton* [60].

Comparison of macro-parasite and pathogen prevalence and load

Neither the proportion of colonies affected nor the mean abundance of varroa mites or *Nosema* spp. spores differed between CCD apiaries and control apiaries ($P>0.05$; Table 6). HBTM infection was more than three times as prevalent in control apiaries as compared to CCD apiaries (43% vs. 14% of colonies affected, respectively; $\chi^2=6.41$, $P=0.01$; Table 6). The mean prevalence of HBTM in bees from infected colonies was higher in control apiaries than CCD apiaries (8% vs. 1%, respectively; $\chi^2=7.71$, $P=0.01$; Table 6).

Neither the prevalence of colonies with varroa mites, *Nosema* spp. spores, or HBTM, nor the load of infection for these macro parasites/pathogens differed between CCD and control colonies ($P>0.05$; Table 7).

Comparison of pathogen prevalence

None of the screened pathogens showed higher prevalence or load in colonies from CCD apiaries when compared to colonies from control apiaries (Table 6).

Table 3. Strength and mean physiological and morphometric measurements of bees from colonies considered to be normal (control) or affected by CCD (N_t).

Variable		CCD Colonies	Mean±S.E.	Median (25th & 75th percentiles)	Control Colonies	Mean±S.E.	Median (25th & 75th percentiles)	Wilcoxon rank sum test
		N_t			N_t			*P*
Strength	Frames of brood	38	1.5±0.23	1.0 (0.3–3.0)	36	2.4±0.34	1.9 (0.6–3.5)	0.04*
	Frames of bees	39	3.6±0.64	2.0 (1.0–4.5)	39	8.3±0.86	8.0 (4.0–11.00)	0.00*
	Ratio bees/brood	35	4.9±1.15	2.0 (1.0–5.0)	35	6.0±1.00	4.0 (2.3–8.0)	0.05*
Proteins#	Proteins in the head [A]	39	2.2±0.24	1.3 (1.0–3.5)	39	1.9±0.19	1.3 (1.1–2.7)	0.96
	Proteins in the abdomen[B]	39	13.4±1.11	10.9 (9.6–16.6)	40	10.7±0.77	10.3 (6.7–13.4)	0.12
	Proteins in the thorax [C]	39	4.1±0.111	4.2 (3.5–4.6)	40	4.3±0.16	4.2 (3.7–4.8)	0.40
	Total proteins [D]	39	17.1±1.14	15.4 (12.8–18.4)	39	14.9±0.76	15.4 (10.3–18.4)	0.53
	Mass of the head [E]	39	12.1±0.18	11.9 (11.2–13.2)	39	12.2±0.13	12.1 (11.6–12.9)	0.48
	Mass of the abdomen [F]	39	67.2±2.58	63.9 (57.6–72.7)	40	60.2±2.19	58.9 (49.8–70.0)	0.06
	Mass of the thorax [G]	39	33.2±0.41	33.4 (31.7–35.5)	40	34.1±0.34	34.5 (33.0–35.7)	0.12
	Total mass [H]	39	105.6±3.31	102.7 (91.9–116.7)	39	100.8±2.46	101.5 (92.1–112.6)	0.38
	Ratio [A]/[E]	39	0.10±0.004	0.09 (0.08–0.11)	39	0.10±0.003	0.10 (0.09–0.11)	0.20
	Ratio [B]/[F]	39	0.19±0.008	0.18 (0.16–0.23)	40	0.17±0.008	0.18 (0.13–0.20)	0.16
	Ratio [C]/[G]	39	0.12±0.003	0.12 (0.11–0.14)	40	0.13±0.005	0.13 (0.11–0.14)	0.69
	Ratio [D]/[H]	39	0.16±0.006	0.15 (0.14–0.18)	39	0.14±0.005	0.15 (0.11–0.17)	0.22
Morphological measures	Centroid size	36	59.9±1.17	58.8 (56.5–61.1)	40	60.0±0.59	60.0 (56.9–62.4)	0.34
	FA	36	1.5±0.06	1.4 (1.3–1.8)	40	2.0±0.16	1.9 (1.4–2.2)	0.01*

FA: Fluctuating asymmetry.
#A total of 6 heads or abdomens or thoraces from one colony were used.
*$P<0.05$.
N_t: Number of colonies tested.
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Kashmir Bee Virus (KBV) was more prevalent in colonies suffering from CCD as compared to control colonies (42% vs. 8%, respectively; Fisher's exact test $P=0001$; Table 7). KBV virus titers were higher in CCD colonies when compared to control colonies ($P=0.01$; Table 7).

Table 4. Percentage of adequately strong, weak and dead colonies in apiaries containing colonies with symptoms of CCD and apparently healthy (control) apiaries.

Apiary	Location	N	Dead (%)	Weak (%)	Strong (%)
CCD	FL	66	18.1	39.4	42.2
	FL	88	30.6	69.3	0.0
	FL	200	41.0	47.0	12.0
	CA	76	7.9	42.1	50.0
	CA	28	25.0	57.1	17.9
	CA	48	20.8	35.4	43.8
Subtotal		506	28.4	48.6	22.9
Control	FL	64	0	0	100
	CA	34	23.4	38.2	38.2
	CA	88	7.9	13.6	78.4
Subtotal		186	8.1	13.4	78.5

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Overall, 55% of CCD colonies were infected with 3 or more viruses as compared to 28% of control colonies (Table 8: $\chi^2=5.4$, $P=0.02$). Both *Nosema* species were equally prevalent in CCD and control colonies (Table 7). However, 34% of CCD colonies were found to be co-infected with both *Nosema* species as compared to 13% of control colonies (Fisher's exact test, $P=0.05$).

CCD colonies were co-infected with a greater number of known pathogenic organisms (viruses and *Nosema* species) than control colonies (4.34±0.37 vs. 3.0±0.37, respectively; Wilcoxon rank sum test $P=0.026$).

Comparison of pesticide prevalence and residue levels

In all, 50 different pesticide residues and their metabolites were found in the 70 wax samples tested, 20 were found in the 18 pollen (beebread) samples tested, 5 in the 24 brood sampled tested, and 28 in the 16 adult bees tested.

There are some notable constraints with this pesticide data set. The number of beebread and adult-bee samples in control apiaries was low. This was largely a result of insufficient amounts of pollen collected from CCD-affected colonies (n = 7), leading to combining colony samples to obtain a sufficient quantity for analysis (n = 3). After adult bees had been distributed for protein and pathogen analysis, there was only one adult bee sample from a colony in a control apiary available for pesticide analysis. Another issue is that pesticides and metabolites were added to the screen as they became identified within samples. Because the beebread samples were analyzed earlier than the adult bee or brood samples, potentially important pesticides (such as chlorothalonil,

Table 5. Observed and expected frequencies of neighboring colonies with similar or different strength ratings in CCD and control apiaries.

Strength Rating		CCD (N = 6)		Control (N = 3)		OR (95% CI)#
Colony 1	Colony 2	Observed	Expected	Observed	Expected	
Adequate	Adequate	28	65	60	23	–
Adequate	Weak	26	26	9	9	5.98 (2.52–14.2)*
Adequate	Dead	15	14	4	5	7.38 (2.36–23.1)*
Weak	Weak	59	44	0	15	255 (15.2–4273)*
Weak	Dead	64	50	3	17	39.5 (12.3–126.5)*
Dead	Dead	25	18	0	7	109.3 (6.42–185.9)*

*$P<0.05$.
#OR: odds ratio; CI: confidence interval.
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amitraz metabolites, and the coumaphos metabolite, chlorferone) were left out of the former but not the latter analyses. Also, a majority of the wax samples were not analyzed for amitraz metabolites, the fungicides boscalid and iprodione, and the coumaphos metabolites chlorferone, coumaphos oxon, and potasan. Where only some of the samples in a given matrix were analyzed for coumaphos metabolites, only coumaphos (and not 'total coumaphos' levels - coumaphos plus metabolites) were compared. Lastly, a lack of detection of some chemicals does not necessarily rule out potential exposure. Chemicals that metabolize or break down quickly may have been removed from the various matrixes tested. Alternatively, some chemicals may have been consumed (in the case of beebread) before samples were collected.

There were no differences in the mean number of pesticides detected in the wax of colonies from CCD apiaries (5.96 ± 0.63) compared to colonies from control apiaries (4.87 ± 0.48; $\chi^2=0.125$, $P=0.72$). Similarly, there were no differences in the number of detections in beebread (CCD: 4.18 ± 0.62 vs. control: 7.50 ± 0.62; $\chi^2=1.83$, $P=0.175$) or brood (CCD: 2.15 ± 0.08 vs. control: 2.00 ± 0.00; $\chi^2=0.65$, $P=0.42$).

None of the pesticides detected in more than 20% of the samples in a given matrix was more prevalent in CCD apiaries than in control apiaries (Table 9). There were, however, higher levels of coumaphos in the wax of control apiaries than was detected in CCD apiaries (Wilcoxon rank sum test, $P=0.05$, Table 9).

There were neither differences in the mean number of pesticides detected in the wax of CCD-affected colonies (5.92 ± 0.84) compared to control colonies (5.67 ± 0.84; $\chi^2=0.001$, $P=0.97$) nor the number of detections in beebread (CCD: 5.09 ± 0.71 vs. control: 5.14 ± 1.14; $\chi^2=0.038$, $P=0.85$), brood (CCD: 2.18 ± 0.12 vs. control: 2.07 ± 0.07; $\chi^2=0.57$, $P=0.44$), or adult bees (CCD: 4.37 ± 1.73 vs. control: 9.00 ± 3.88; $\chi^2=0.89$, $P=0.34$).

Esfenvalerate was more prevalent in the wax of control colonies (32%) when compared to CCD colonies (5%) (Fisher's exact test, $P=0.001$; Table 10). Mean levels of this product were also higher in both the wax and adult bees from control colonies when compared to CCD colonies ($P=0.002$ and 0.04, respectively; Table 10). Coumaphos levels in wax, brood, and adult bees were higher in control colonies than in CCD colonies ($P=0.009$, 0.04, and 0.03, respectively; Table 10).

Comparison of mitotypes

Only one of the 98 colonies screened for mitotype was found to be Western European in matrilineal origin. The remaining colonies were all found to be of Eastern European origin. None were positively detected as being African in origin.

Discussion

This descriptive epidemiological study was initiated to better characterize CCD and compare risk-factor exposure between control and afflicted populations in hopes of identifying factors that cause or contribute to Colony Collapse Disorder. Of the more than 200 variables we quantified in this study, 61 were found with enough frequency to permit meaningful comparisons between populations. None of these measures on its own could distinguish CCD from control colonies. Moreover, no single risk factor was found consistently or sufficiently abundantly in CCD colonies to suggest a single causal agent. Nonetheless, our results help to elucidate this poorly understood affliction of the honey bee colonies and provide insight into the planning of hypothesis-driven research.

CCD apiaries contained more dead and weak colonies than did control apiaries and the distribution of dead and weak colonies in CCD apiaries was not random. Dead and weak colonies were more likely to neighbor each other in CCD apiaries as compared to control apiaries (Table 3), suggesting that an infectious agent or the exposure to a common risk factor may be involved in colony collapse.

While no single pathogen or parasite was found with sufficient frequency to conclude a single organism was involved in CCD, pathogens seem likely to play a critical (albeit secondary) role. CCD colonies generally had higher virus loads and were co-infected with a greater number of disease agents than control colonies. Elevated virus and *Nosema* spp. levels potentially explain the symptoms associated with CCD. One possible way honey bees regulate pathogen and parasite loads within a colony is for infected individuals to emigrate from their hive [69]. This behavior has been proposed to explain the rapid loss of adult populations in colonies collapsing from *N. ceranae* [39]. Whether infected individuals die away from the hive as the result of an evolved response (suicidal pathogen removal [69]) or from a sudden debilitating process by which forager bees cannot return to the hive [39] is irrelevant to understanding how colony collapse can unfold. Premature loss of worker bees does not preclude non-pathogenic causes; recent work has shown that worker bee longevity can be reduced when they are exposed to sub-lethal levels of coumaphos during the larval and pupal stages (Pettis, unpublished). The premature loss of forager bees, the older cohort in a colony, results in younger bees prematurely becoming forager bees [70]. If

Table 6. Parasite and pathogen loads of bees from colonies (N_t) located in CCD and control apiaries.

Variable		CCD Apiaries		Load		Control Apiaries		Load		Prevalence		Load (Wilcoxon rank sum test)
		N_t	Prevalence** (95% CI)	Mean±S.E.	Median (25th & 75th percentiles)	N_t	Prevalence** (95%CI)	Mean±S.E.	Median (25th & 75th percentiles)	χ^{2*}	*P*	*P*
Brood condition	Spotty Brood	49	55 (41–73)			14	43 (23–72)			0.66	0.41	
	Chalkbrood	51	6 (4–8)			17	0				0.56	
	European foulbrood	51	8 (6–11)			17	0				0.57	
Parasites	Varroa†	51	64 (48–84)	0.086±0.0284	0.007 (0–0.038)	17	53 (31–84)	0.020±0.014	0.003 (0–0.014)	0.74	0.39	0.24
	HBTM‡	51	14 (10–18)	1±0.6	0 (0–0)	17	43(25–69)	8±2.9	0 (0–16)	6.41	0.01*	0.01*
	Nosema♠	51	55 (41–72)	1.82±0.486	0.7 (0.0–1.80)	17	35 (20–56)	0.34±0.201	0.0 (0.0–0.15)	1.96	0.16	0.09
Pathogens♥	ABPV	58	45 (34–58)	4.4±0.86	0.0 (0.0–7.5)	18	33 (20–52)	2.3±0.90	0.0 (0.0–5.20)	0.75	0.38	0.29
	Bacteria	58	93 (70–100)	12.7±0.80	13.4 (9.1–18.0)	18	100 (59–100)	13.3±1.25	14.2 (7.7–18.0)		0.57	0.86
	BQCV	58	72 (54–93)	9.6±1.02	10.2 (0–13.8)	18	78 (46–100)	7.7±1.67	5.5 (1.1–15.4)		0.77	0.40
	CBPV	58	33 (25–43)	2.4±0.54	0.0 (0.0–3.8)	18	50 (30–79)	1.5±0.42	0.5 (0–3.27)	1.75	0.19	0.66
	DWV	58	44 (34–57)	5.7±0.99	0.0 (0.0–10.1)	18	66 (39–100)	5.6±1.21	5.50 (0–8.5)	2.62	0.11	0.56
	IAPVF1a	58	22 (17–28)	2.0±0.56	0.0 (0.0–0.0)	18	17 (10–27)	1.6±1.06	0.0 (0.0–0.0)		0.74	0.63
	IAPVpw1617	58	16 (12–21)	1.8±0.62	0.0 (0.0–0.0)	18	6 (3–9)	1.2±1.15	0.0 (0.0–0.0)		0.43	0.32
	IAPV_B4SO427	58	10 (8–13)	1.5±0.60	0.0 (0.0–0.0)	18	6 (3–9)	1.1±1.09	0.0 (0.0–0.0)		1	0.58
	IAPVAvg	58	28 (21–36)	1.8±0.52	0.0 (0.0–1.2)	18	17 (10–27)	1.3±1.03	0.0 (0.0–0.0)		0.53	0.36
	KBV	58	29 (22–37)	3.0±0.76	0.0 (0.0–4.8)	18	2 (1–3)	0.7±0.53	0.0 (0.0–0.0)		0.21	0.11
	SBV	58	16 (12–21)	0.8±0.29	0.0 (0.0–0.0)	18	28 (16–44)	2.0±0.84	0.0 (0.0–4.5)	1.37	0.24	0.21
	Nosema ceraneae	58	47 (36–61)	5.8±1.00	0.0 (0.0–12.9)	18	72 (42–100)	6.0±1.60	0.0 (0.0–14.6)	3.63	0.06	0.31
	Nosema apis	58	28 (21–36)	3.5±0.83	0.0 (0.0–6.47)	18	11 (7–17)	0.09±0.07	0.0 (0. 0–0.0)		0.21	0.09
	Trypanasomes	58	76 (58–98)	8.3±0.84	8.8 (0.0–13.6)	18	94 (56–100)	10.6±1.54	8.17 (5.5–17.7)		0.10	0.26

*Where no statistic is presented, Fisher's exact test was used as some cells had fewer than 5 responses.

**% of colonies infected with organism.

†Load = mean abundance (number of varroa mites per bee) in colonies.

‡Load = the percentage of bees infested with HBTM per colonies.

♠Load = mean abundance (number of spores per bee ($\times 10^6$)) in colonies.

♥♥All pathogen loads are scaled relative to the geometric mean of honey bee housekeeping genes RPS5, MGsT, and actin [37].

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Table 7. Parasite and pathogen loads of bees from colonies considered to normal (control) or affected by CCD (N_t).

Variable		CCD Colonies		Load		Control Colonies		Load		Prevalence		Load (Wilcoxon rank sum test)
		N_t	Prevalence **(95% CI)	Mean±S.E.	Median (25th & 75th percentiles)	N_t	Prevalence **(95% CI)	Mean±S.E.	Median (25th & 75th percentiles)	X^{2*}	*P*	*P*
Brood condition	Spotty Brood	35	60 (42–83)			27	52 (34–76)			0.04	0.85	
	Chalkbrood	38	5 (4–7)			39	3 (2–4)				0.62	
	European foulbrood	38	8 (6–11)			39	3 (2–4)				0.36	
Parasites	Varroa†	32	53 (36–75)	0.054±0.020	0.002 (0.0–0.037)	36	70 (49–97)	0.084±0.0372	0.007 (0.0–0.0 29)	1.91	0.17	0.37
	HBTM‡	32	19 (13–27)	0±0.0	0 (0–0)	36	22 (15–30)	10±2	0 (0–0)	0.13	0.72	0.44
	Nosema♠	32	63 (43–89)	1.9±0.59	0.1 (0.0–1.9)	36	39 (27–54)	1.0±0.47	0.0 (0.0–0.3)	3.79	0.05*	0.06
Pathogens♥	ABPV	38	47 (33–65)	5.1±1.23	0.0 (0.0–8.7)	38	37 (26–51)	2.6±0.62	0.0 (0.0–6.1)	0.86	0.35	0.22
	Bacteria	38	97 (69–100)	12.9±0.91	13.6 (9.2–17.6)	38	92 (65–100)	12.8±0.98	14.1 (8.4–18.0)		0.61	1.00
	BQCV	38	79 (56–100)	10.8±1.31	11.6 (3.5–14.6)	38	68 (48–93)	7.4±1.09	6.3 (0.0–13.7)	1.09	0.30	0.07
	CBPV	38	37 (26–51)	2.6±0.70	0.0 (0.0–3.8)	38	37 (26–51)	1.9±0.51	0.0 (0.0–3.3)	0.00	1.00	0.73
	DWV	38	61 (43–84)	7.7±1.3	6.5 (0.0–12.9)	38	40 (28–55)	3.6±0.86	0.0 (0.0–7.0)	3.37	0.07	0.02*
	IAPVF1a	38	24 (17–33)	2.1±0.69	0.0 (0.0–0.8)	38	18 (13–25)	1.7±0.71	0.0 (0.0–0.0)	0.32	0.57	0.57
	IAPVpw1617	38	16 (11–22)	2.0±0.82	0.0 (0.0–0.0)	38	11 (8–15)	1.3±0.71	0.0 (0.0–0.0)		0.74	0.48
	IAPV_B4SO427	38	11 (8–15)	1.6±0.80	0.0 (0.0–0.0)	38	8 (6–11)	1.2±0.69	0.0 (0.0–0.0)		1.00	0.67
	IAPVAvg	38	29 (21–40)	1.9±0.68	0.0 (0.0–1.5)	38	21 (15–29)	1.4±0.63	0.0 (0.0–0.0)	0.63	0.43	0.42
	KBV	38	42 (30–58)	4.4±1.10	0.0 (0.0–7.0)	38	8 (6–11)	0.5±0.29	0.0 (0.0–0)		0.00*	0.00*
	SBV	38	18 (13–25)	1.1±0.41	0.0 (0.0–0.0)	38	18 (13–25)	1.1±0.44	0.0 (0.0–0.0)	0.00	1.00	0.98
	Nosema ceranae	38	55 (39–76)	6.9±1.30	2.3 (0.0–13.8)	38	50 (35–67)	4.8±1.09	0.7 (0.0–9.8)	0.21	0.65	0.34
	Nosema apis	38	29 (21–40)	3.5±1.03	0.0 (0.0–6.5)	38	18 (13–25)	1.8±0.80	0.0 (0.0–0.0)	1.16	0.28	0.25
	Trypanosomes	38	82 (58–100)	9.6±1.00	9.8 (5.0–14.1)	38	79 (56–100)	8.2±1.09	7.0 (1.1–13.1)	0.08	0.77	0.31

*Where no statistic is presented, Fisher's exact test was used as some cells had fewer than 5 responses.

**% of colonies infected with organism.

†Load = mean abundance (number of varroa mites per bee) in colonies.

‡Load = the percentage of bees infested with HBTM per colonies.

♠Load = mean abundance (number of spores per bee ($\times 10^6$)) in colonies.

♥♥All pathogen loads are scaled relative to the geometric mean of honey bee housekeeping genes RPS5, MGsT, and actin [37].

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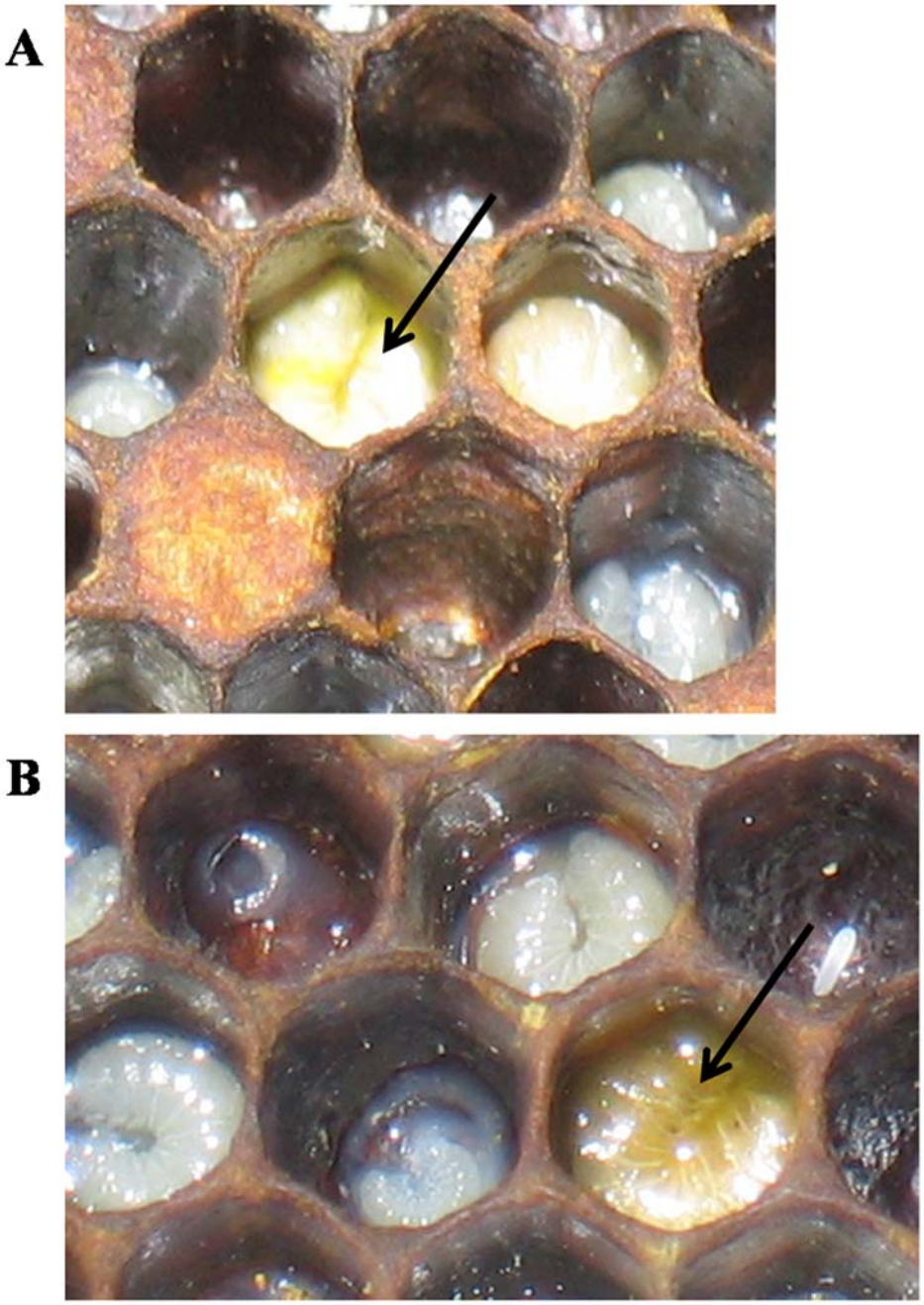

Figure 2. EFB-infected larvae (←) in some CCD-affected colonies were ''corn yellow'' (A) rather than the typical ''beige yellow'' (B).
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these replacement bees die at a rate that exceeds the colony's ability to replace them, the result would be rapid depopulation, a reduction in the bee-to-brood ratio, and eventually colony failure.

This study verified initial field observations [1] that there was a difference in the bee-to-brood ratio between CCD-affected populations when compared to controls. If the bees in colonies undergoing CCD collapse are young bees (as field observations suggest), we would expect to find indirect evidence of this in the measures of parasite loads with known associations to bee age. Tracheal mite loads increase as bees age [71], possibly explaining why HBTM incidence and prevalence were higher in control apiaries than in CCD-affected apiaries. Alternatively, HBTM levels may be lower in CCD colonies because infested individuals left the colony.

An unavoidable bias that results from sampling colonies in the midst of collapse is that only surviving bees are collected. These bees, arguably, are the least sick or most fit individuals. Asymmetry is expected to increase when stressful conditions disturb the normal development of insects [72]. In honey bees specifically, increased levels of symmetry correlates to increased fitness [53]. Bees from colonies suffering from CCD were consistently more symmetrical than those from control colonies. It is therefore reasonable to assume that bees surviving in CCD colonies, while young, were the fittest bees, surviving longer than their less-fit sisters. While this assumption needs to be verified experimentally, a comparison of the ranges of FA in populations of bees from CCD colonies versus control colonies provides tacit support to this hypothesis. The lower ranges of FA

Table 8. Percentage of Control and CCD colonies infected with Y or more viruses.

Colony classification	n	Percentage (%)				
	Y	1	2	3	4	5
Control		81.6	60.5	28.9	15.8	7.9
CCD		84.2	71.1	55.3	31.6	23.7
	X^2	0.09	0.94	5.4	2.6	Fisher's
	P	0.76	0.33	0.02	0.10	0.05

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measures were comparable between CCD and control populations (25^{th} percentile: 1.3 vs. 1.4 for CCD and control colonies, respectively), while the upper range of FA measures was notably higher in control colonies when compared to CCD colonies (75^{th} percentile: 2.2 vs. 1.8, respectively), suggesting that bees in CCD colonies under the most development stress (and with the greatest FA) had left or been removed from colonies before sampling.

Recently, *N. ceranae* was linked to colony losses in Spain [73], and a subsequent study documented how pathogen levels developed over time. In the final stages of collapse, the young bees remaining in the colony became heavily infected with this agent [39]. Our survey found only about half of the colonies sampled, both in CCD and control populations, were infected with *N. ceraneae*, and while some colonies had levels of infection that likely contributed to colony loss, this was not the case for the majority.

In a previous study using subsamples from the same colonies sampled here, IAPV was identified as highly correlated to CCD [3]. This expanded study did not replicate those results. The overall incidence of IAPV reported here was generally lower than found in the prior survey. This result might reflect decreased sensitivity of the assay used here, although prevalence of other viruses generally was comparable to prior results. Alternatively, the discrepancy in findings might reflect unappreciated genetic variation across lineages of IAPV, to the extent that primers poorly matched template cDNA. To minimize this risk, we estimated transcript levels using three published primer pairs for three regions of the genome, and we found broadly concordant results (Tables 6 and 7). As in [3], we treated products for any of the three used primer pairs as evidence for IAPV presence. Finally, the current survey included more colonies and covered a wider geographical range than the previous survey. IAPV shows strong geographical patterns (Evans JD et al., unpublished), and it is expected that surveys for this and other pathogenic viruses will differ across apiaries and regions [74].

The intrinsic bias associated with sampling only surviving (and presumably the least-sick) bees did not prevent us from establishing that workers in CCD colonies were more ill than those in control colonies. Co-infection with both *Nosema* species was 2.6 times greater in CCD colonies when compared to control colonies, and colonies co-infected with 4 or more viruses were 3.7 times more frequent in CCD colonies than in control colonies. While honey bee colonies are commonly infected with one or more pathogens, often without exhibiting overt signs of illness [75], the greater prevalence and abundance of infectious agents in CCD colonies does suggest that either they were exposed to a greater number of pathogens or their ability to fight infection had been compromised.

Several factors are known or suspected to be able to compromise the honey bee immune response. One proposed factor is poor nutrition. In this study, we measured protein content as a surrogate for evidence of poor nutrition in CCD colonies, and these results suggest that nutrition does not play a decisive factor. However, caution is needed in drawing strong inferences from these findings, as nutritional deficiencies may have much more subtle effects on bee development and immunity than can be detected with our methods.

Chronic or sub-lethal exposure to agricultural- or beekeeper-applied pesticides can weaken the honey bee immune system [48], hampering the ability of bees to fight off infection. This study found no evidence that the presence or amount of any individual pesticide occurred more frequently or abundantly in CCD-affected apiaries or colonies. In fact, the opposite was true; two products, esfenvalerate in wax, and coumaphos in wax, brood, and adult bees were found more frequently and at higher levels in control colonies than in CCD colonies.

Esfenvalerate or fenvalerate (racemic form), a pyrethroid insecticide, is considered to be highly toxic to bees [76], but its threat to honey bees is thought to be minimal as it tends to repel them. Exposed forager bees are thought to die in the field before returning to the hive [77], so detection of this product in wax is curious. Finding this product more frequently and at higher levels in control colonies may be spurious, however, similar residue levels in both CCD and control apiaries suggest uniform in-field exposure between populations.

Coumaphos is a product used by beekeepers to control varroa mites. Elevated levels of this product in control apiaries suggest that beekeepers managing those apiaries had more aggressively controlled for this parasitic mite than beekeepers managing CCD apiaries. In addition, control apiaries tended to have higher levels of fluvalinate ($P = 0.06$), another approved acaricide. Regardless of these differences in mite-control compounds, we were unable to detect differences in varroa mite levels in CCD- compared to control apiaries or colonies, suggesting that this mite was not the immediate cause of CCD. This does not necessarily mean that mite infestations have no role in collapse. It is possible that some of the sampled colonies had their mite populations controlled by miticides a few months prior to our sampling. Thus, while mite populations were comparable between the two groups at the time of sampling, there may have been a difference in the mite populations prior to mite treatment applications. Varroa mite parasitism is known to weaken the bees' immune system [78] and facilitate the transmission of viruses to brood and adult bees [79]. Further, high virus levels resulting from high populations of varroa mites are not always immediately suppressed by effective mite control [80]. The potential "legacy" effect of high mite populations in CCD-affected colonies should be the focus of future longitudinal epidemiological studies prior to the categorical dismissal of varroa mites as a causal or contributing agent in CCD.

Coumaphos, an organophosphate, is lipophilic, and so accumulates in wax. Increased levels of the compound in wax have been shown to decrease survivorship of developing queens [81,82]. Similar results with worker bees have also been recorded (Pettis, unpublished). A quick method to assess larval survival is to quantify the number of empty brood cells in an area of capped brood or, to

Table 9. The pesticide residue prevalence and load in wax, beebread, and brood from colonies (N_t) located in CCD and control apiaries.

Matrix	Chemical	CCD Apiaries		Load		Control Apiaries		Load		Prevalence (Fisher's exact test)	Load (Wilcoxon rank sum test)
		N_t	Prevalence (%)	Mean±S.E.	Median (25th & 75th percentiles)	N_t	Prevalence (%)	Mean±S.E.	Median (25th & 75th percentiles)	*P*	*P*
Wax	Boscalid	30	40 (27–57)	29.7±8.46	0.0 (0.0–0.0)	5	0			0.14	0.09
	Chlorothalonil	62	33 (25–42)	24.0±9.11	0.0 (0.0–7.75)	8	13(7–27)	4.9±1.72	0.0 (0.0–0.0)	0.42	0.25
	Chlorpyrifos	62	71 (54–91)	6.3±1.23	4.1 (0.0–7.5)	8	100 (43–100)	5.8±0.64	6.4 (4.1–7.4)	0.11	0.20
	Coumaphos	62	100 (77–100)	3373±552	1750 (719–4085)	8	100 (43–100)	6398±1815	6050 (2110–8992)	1.00	0.05*
	Dicofol	62	17 (13–22)	4.1±3.33	0.0 (0.0–0.0)	8	0			0.34	0.20
	Endosulfan	62	25 (19–32)	4.4±2.16	0.0 (0.0–0.33)	8	13(7–27)	0.6±0.55	0.0 (0.0–0.0)	0.67	0.42
	Esfenvalerate	62	13 (10–17)	1.7±0.97	0.0 (0.0–0.0)	8	38(16–75)	1.0±0.52	0.0 (0.0–2.10)	0.11	0.09
	Fluvalinate	62	100 (77–100)	12508±1718	8530 (2452–15608)	8	100 (43–100)	41737±23748	19800 (6510–42575)	1.00	0.06
	Iprodine	35	21 (15–30)	48.9±20.97	0.0 (0.0–0.0)	5	0			0.56	0.26
Beebread	Atrazine	16	32 (18–52)	8.4±16.61	0.0 (0.0–4.57)	2	0	0.0±0.0	0.0 (0.0–0.0)	1.00	0.37
	Chlorpyrifos	16	88 (50–100)	1.8±0.74	0.65 (0.32–1.75)	2	0	0.8±0.05	0.8 (0.7–0.8)	1.00	0.62
	Coumaphos	16	50(29–81)	18.5±7.7	2.1 (0.0–26.3)	2	50 (6–100)	3.6±3.6	0.0 (0.0–4.5)	1.00	0.89
	Dicofol	16	19 (11–31)	0.2±0.49	0.0 (0.0–0.0)	2	100 (12–100)	0.6±0.15	0.0 (0.0–0.7)	0.07	0.08
	Endosulfan1	16	32 (18–52)	0.2±0.38	0.0 (0.0–56)	2	50 (6–100)	0.4±0.4	0.4 (0.0–0.8)	1.00	0.50
	Fenpropathrin	16	50 (29–81)	1.0±0.30	0.4 (0.0–1.6)	2	100 (12–100)	1.5±0.70	1.5 (0.8–2.2)	0.47	0.30
	Fluvalinate	16	94 (54–100)	276±162.5	76 (8–270)	2	100 (12–100)	68±56	68 (12–124)	1.00	0.78
	Malathion	16	19 (11–31)	0.7±0.48	0.0 (0.0–0.0)	2	50 (6–100)	1.8±1.8	1.8 (0–3.6)	0.41	0.29
	Tebuthiuron	16	38 (22–62)	5.2±2.57	0.0 (0.0–2.85)	2	50 (6–100)	24±24	24 (0–48)	1.00	0.42
Brood	Coumaphos	20	100 (61–100)	51.8±12.83	27.5 (5.6–101)	4	100 (27–100)	92.4±32.2	114 (24–139)	1.00	0.33
	Fluvalinate	20	100 (61–100)	844±315	282 (149–930)	4	100 (27–100)	887±418	817 (127–1720)	1.00	0.53

Only pesticides found in 20% or more of samples are reported.

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Table 10. The pesticide residue prevalence and load in wax, beebread, brood and adult bees from colonies considered to be normal (control) or affected by CCD (N_t).

Matrix	Chemical	CCD Colonies		Load		Control Colonies		Load		Prevalence (Fisher's exact test)	Load (Wilcoxon rank sum test)
		N_t	Prevalence (%) (95%CI)	Mean±S.E.	Median (25th & 75th percentiles)	N_t	Prevalence (%) (95%CI)	Mean±S.E.	Median (25th & 75th percentiles)	*P*	*P*
Wax	Boscalid	20	40 (24–62)	35.9±11.96	0.0 (0.0–66.0)	15	27 (15–45)	11.59±5.62	0.0 (0.0–0.0)	0.48	0.27
	Chlorothalonil	42	36 (26–49)	22.5±7.53	0.0 (0.0–16.15)	28	21 (14–30)	19.88±17.04	0.0 (0.0–0.0)	0.28	0.16
	Chlorpyrifos	42	73 (53–99)	6.5±1.62	4.1 (0.0–7.52)	28	75 (50–100)	5.83±1.25	5.6 (0.25–7.4)	1	0.68
	Coumaphos	42	100 (72–100)	2645±500	1335 (524–3320)	28	100 (67–100)	5330±1053	4090 (1435–6270)	1	0.0*
	Dicofol	42	19 (14–26)	5.3±4.92	0.0 (0.0–0.0)	28	11 (7–16)	0.9±0.58	0.0 (0.0–0.0)	0.51	0.43
	Endosulfan	42	21 (15–28)	2.0±0.94	0.0 (0.0–0.0)	28	25 (17–36)	6.88±4.57	0.0 (0.0–1.2)	0.77	0.67
	Esfenvalerate	42	5 (4–7)	1.4±1.33	0.0 (0.0–0.0)	28	32 (21–46)	1.98±0.84	0.0 (0.0–1.6)	0.00*	0.00*
	Fluvalinate	42	100 (72–100)	11825±1906	9420 (3067–15275)	28	100 (67–100)	21844±7350	9565 (2570–29675)	1	0.53
	Iprodine	24	21 (13–31)	32.5±15.83	0.0 (0.0–0.0)	14	14 (8–24)	59.5±42.6	0.0 (0.0–0.0)	1	0.75
Beebread	Atrazine	11	36 (18–64)	8.8±5.31	0 (0–4.7)	7	16 (6–33)	5.42±5.42	0.0 (0.0–0.0)	0.60	0.32
	Chlorpyrifos	11	91 (45–100)	2.14±1.03	0.7 (0.3–1.8)	7	86 (35–100)	1.0±0.47	0.7 (0.4–0.8)	1	0.82
	Coumaphos	11	45 (22–81)	24.02±10.73	0 (0–67)	7	57 (23–100)	5.4±2.87	4.2 (0.0–7.2)	1	0.81
	Dicofol	11	18 (9–32)	0.22±0.16	0 (0–0)	7	42 (17–87)	0.29±0.15	0.0 (0.0–0.7)	0.32	0.39
	Endosulfan1	11	27 (12–48)	0.18±0.10	0 (0–0.5)	7	43 (17–89)	0.37±0.18	0.0 (0.0–0.8)	0.63	0.39
	Fenpropathrin	11	54 (27–48)	1.45±0.41	0.8 (0–1.6)	7	57 (23–100)	0.83±0.36	0.8 (0.0–2.0)	1	0.85
	Fluvalinate	11	100 (50–100)	351±235	94 (7.4–339)	7	86 (35–100)	99±42.1	59 (12–193)	0.38	0.75
	Malathion	11	27 (13–48)	0.96±0.70	0 (0–0.9)	7	14 (6–29)	0.51±0.51	0 (0–0)	1	0.57
	Tebuthiuron	11	27 (13–48)	4.69±3.07	0 (0–1.6)	7	57 (23–100)	11.42±7.12	0 (0–27)	0.33	0.19
Brood	Coumaphos	13	100 (53–100)	30.76±11.88	21 (3.9–40.5)	11	100 (50–100)	91.39±18.26	111 (17–137)	1	0.04*
	Fluvalinate	13	100 (53–100)	1044±479	279 (135–1043)	11	100 (50–100)	623±197	364 (149–1270)	1	0.67
Adults	Chlorothalonil	9	33 (15–63)	4.56±4.07	0.0 (0.0–2.0)	7	29 (12–60)	0.81±0.53	0.0 (0.0–2.5)	1	0.89
	Chlorpyrifos	9	33 (15–63)	0.37±0.19	0.0 (0.0–1.0)	7	29 (12–60)	0.34±0.22	0.0 (0.0–1.0)	1	0.94
	Coumaphos	9	78 (36–100)	48.3±40.12	7.4 (0.5–22.5)	7	100 (40–100)	57.2±15.53	65 (11–100)	0.48	0.03*
	Esfenvalerate	9	43 (20–82)	0±0	0.0 (0.0–0.0)	7	11 (4–23)	3.75±1.77	0.0 (0.0–8.5)	0.26	0.04*
	Endosulfan (total)	9	0	0.9±0.9	0.0 (0.0–0.0)	7	29 (12–60)	0.8±0.8	0.0 (0.0–0.0)	0.18	0.92
	Fluvalinate	9	100 (46–100)	1769±814.6	238 (88.5–4540)	7	100 (40–100)	333±215.0	142 (91–185)	1	0.22

Only pesticides found in 20% or more of samples are reported.

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use the beekeeper colloquial term, brood "spottiness". We found no evidence that bees from control colonies had a greater frequency of spotty brood than CCD colonies despite the elevated levels of coumaphos in wax in the control colonies. This suggests that bees in control colonies had developed a tolerance to coumaphos exposure. Coumaphos-tolerant bees may be afforded protection through several routes. First, by living on wax comb with elevated miticide levels, varroa mite populations may remain lower than they would in colonies with lower levels of coumaphos residues in their brood nest. However, as coumaphos-resistant mites are widespread in the U.S. [81], this explanation seems unlikely unless coumaphos-resistant mites are less fit than non-resistant mites. Even a small reduction in the reproductive fitness of varroa mites could have a pronounced effect on their population growth and thus their effect on colony health [83]. Second, coumaphos (and/or fluvalinate) tolerance in bees provides cross-resistance to pesticide exposures from other organophosphates and pyrethroids [84] which may be affecting CCD-afflicted bees at sub-lethal doses. Honey bees, as compared to other insects, are notably lacking in detoxification enzymes which provide moderate levels of cross-resistance to pesticides [85]. Any enhancement in these enzyme levels may greatly improve the ability of bees to tolerate the numerous pesticides they encounter in-hive or while foraging.

When unexplained disease outbreaks occur, epidemiologists use descriptive studies to help identify possible cause(s). By definition, descriptive studies are non-hypothesis driven but rather highlight differences between diseased and non-diseased populations in an effort to inform future research.

This descriptive study looked for differences in colony strength, morphometrics, and risk factors in CCD and control colonies. Like all descriptive studies, we cannot make any definitive statement concerning which factors do or do not contribute to or cause CCD. However, our results permit some valuable inferences to be drawn, as the distribution of CCD-infected colonies was not random in infected apiaries and thus the underlying factor is likely contagious or caused by exposure to a common risk factor(s). As no one disease agent was found in all CCD colonies, and because bees derived from CCD colonies were infected with more pathogens then their control colony counterparts, we suspect that while pathogen infection may cause the symptoms of collapse, these infections are secondary and are the result of some other factor or combination of factors that reduce the bees' ability to mitigate infection. As mentioned throughout the text, these inferences must be considered in concert with the limitations and assumptions that are intrinsic to epidemiological studies.

For practical reasons, quantifying most factors in honey bee colonies (e.g., parasite loads, physiological measures, pesticide and pathogen loads) involves testing a sub-sample of colonies in a population. While increasing sample size would obviously result in increased test specificity, this was not always logistically possible. Moreover, our approach assumes that the factor(s) responsible for CCD would occur with high frequency in the affected population. Should this not be the case, our efforts may not have been resolute enough to detect it. Our study also assumes that the factor(s) responsible for CCD were present in the colonies at the time of sample collection, which also may not have been the case. For example, if pollen contaminated with a pesticide were responsible for CCD, contaminated pollen would have been consumed prior to sample collection and thus would not have been detected in the samples collected. Similarly, bees infected with the causative disease agent could have died away from the colony and thus not collected. Finally, Varroa mites or other parasites could have differed among populations prior to sampling, but effective control measures masked these differences at the time of sample collection.

Descriptive studies rely on operational case definitions. The case definition used in this study was applied by experienced bee clinicians using easily observable characteristics [9]. While the application of the case definition may have misdiagnosed colonies, our finding that colony strength measures differed between CCD and control colonies suggests the classification of colonies into affected and non-affected groups was not random. As with other descriptive studies based on case definitions, our findings enable us to propose refining the operational case definition of CCD [8]. In addition to the characteristics of CCD colonies previously described—(1) no dead bees in the colonies or apiary, (2) adult populations rapidly declined leaving brood poorly or completely unattended, and (3) the absence of robbing or kleptoparasitism in collapsed colonies—we now propose that the operational case definition for CCD include (4) at the time of collapse, varroa mite and nosema populations are not at levels known to cause economic injury or population decline. This additional characteristic should assist in distinguishing diminishing populations associated with elevated levels of varroa mites (and virus) [86] and *N. ceranae* [39] from collapsing populations associated with CCD.

The primary aim of descriptive studies is to help narrow future efforts that attempt to identify the cause of disease. This study suggests that future, longitudinal studies should focus on monitoring parasite (varroa mite), pathogen, and pesticide loads while quantifying pesticide tolerance in study populations. More specific studies that investigate potential interactions among pesticides and pathogen loads are also warranted.

This is the first descriptive epizootiological survey of honey bee colonies that provides evidence that the condition known as CCD is consistent with a contagious condition or reflective of common risk factors within apiaries Of the 61 variables quantified (including adult bee physiology, pathogen loads, and pesticide levels), no single factor was found with enough consistency to suggest one causal agent. Bees in CCD colonies had higher pathogen loads and were co-infected with more pathogens than control populations, suggesting either greater pathogen exposure or reduced defenses in CCD bees. Levels of the miticide coumaphos were higher in control populations than CCD-affected populations. Potentially important areas for future hypothesis-driven research, including the possible legacy effect of mite parasitism and role of honey bee resistance to pesticides, are highlighted.

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Author Contributions

Conceived and designed the experiments: Dv JSP. Performed the experiments: Dv JDE CM MF JF DRT. Analyzed the data: Dv CS EH BKN RMU. Contributed reagents/materials/analysis tools: JDE CM MF JF DLCF YPC DRT. Wrote the paper: Dv JDE CS CM EH BKN RMU DRT JSP.

References

1. vanEngelsdorp D, Underwood R, Caron D, Hayes J Jr (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.

2. vanEngelsdorp D, Hayes J Jr, Underwood RM, Pettis J (2008) A survey of honey bee colony losses in the U.S., fall 2007 to spring 2008. PLoS ONE 3: e4071.

3. Cox-Foster DL, Conlan S, Holmes EC, Palacios G, Evans JD, et al. (2007) A metagenomic survey of microbes in honey bee colony collapse disorder. Science (Washington) 318: 283–286.
4. Underwood R, vanEngelsdorp D (2007) Colony Collapse Disorder: have we seen this before? Bee Cult 35: 13–18.
5. Aikin RC (1897) Bees evaporated: a new malady. Glngs Bee Cult 25: 479–480.
6. Oldroyd BP (2007) What's killing American honey bees? PLoS Biol 5: e168.
7. Friedman GD (1987) Primer of epidemiology. New York: McGraw Hill, Inc. pp 401.
8. Koepsell TD, Weiss NS (2003) Epidemiologic methods: Studying the occurrence of illness. New York: Oxford University Press. 528p.
9. Goodman RA, Buehler JW (2002) Field epidemiology defined. In: Gregg M, ed (2002) Field epidemiology. New York: Oxford University Press. pp 3–7.
10. Dicker RC (2002) Designing studies in the field. In: Gregg M, ed (2002) Field epidemiology. New York: Oxford University Press. pp 117–131.
11. Hymans KC (1998) Developing case definitions for symptom-based conditions: the problem of specificity. Epidemiol Rev 20: 148–156.
12. Scharlaken B, deGraaf DC, Memmi S, Devreese B, vanBeeumen J, Jacobs FJ (2007) Differential protein expression in the honey bee head after bacterial challenge. Arch Insect Biochem Physiol 65: 223–237.
13. Farrar DL (1936) Influence of pollen reserves on the surviving populations of over-wintered colonies. Am Bee J 76: 452–454.
14. Somerville DC (2001) Nutritional value of bee collected pollens. Barton: NSW Agriculture, Rural Industries Research and Development Corporation 176.
15. Sagili RR, Pankiw T, Zhu-Salzman K (2005) Effects of soybean trypsin inhibitor on hypopharyngeal gland protein content, total midgut protease activity and survival of the honey bee (Apis mellifera L.). J Insect Physiol 51: 953–957.
16. Mattila HR, Otis GW (2006) The effects of pollen availability during larval development on the behaviour and physiology of spring-reared honey bee workers. Apidologie 37: 533–546.
17. Mattila HR, Otis GW (2006) Influence of pollen diet in spring on development of honey bee (Hymenoptera: Apidae) colonies. J Econ Entomol 99: 604–613.
18. Mattila HR, Otis GW (2006) Effects of pollen availability and *Nosema* infection during the spring on division of labor and survival of worker honey bees (Hymenoptera: Apidae). Environ Entomol 35: 708–717.
19. Sagili RR, Pankiw T (2007) Effects of protein-constrained brood food on honey bee (Apis mellifera L.) pollen foraging and colony growth. Behav Ecol Socio 61: 1471–1478.
20. Bowen-Walker PL, Gunn A (2001) The effect of the ectoparasitic mite, *Varroa destructor* on adult worker honeybee (*Apis mellifera*) emergence weights, water, protein, carbohydrate, and lipid levels. Entomol Exp Appl 101: 207–217.
21. Amdam GV, Hartfelder K, Norberg K, Hagen A, Omholt SW (2004) Altered physiology in worker honey bees (Hymenoptera: Apidae) infested with the mite *Varroa destructor* (Acari: Varroidae): a factor in colony loss during overwintering? J Econ Entomol 97: 741–747.
22. Contzen C, Garedew A, Lamprecht I, Schmolz E (2004) Calorimetrical and biochemical investigations on the influence of the parasitic mite *Varroa destructor* on the development of honeybee brood. Thermo Acta 415: 115–121.
23. Gregory PG, Evans JD, Rinderer T, deGuzman L (2005) Conditional immune-gene suppression of honeybees parasitized by varroa mites. J Insect Sci 5: 7.
24. Palmer AR, Strobeck C (1986) Fluctuating asymmetry: measurement, analysis, patterns. Annu Rev Ecol Syst 17: 391–421.
25. vanValen L (1962) A study of fluctuating asymmetry. Evolution 16: 125–142.
26. Leary RF, Allendorf FW (1989) Fluctuating asymmetry as an indicator of stress: implications for conservation biology. TREE 4: 214–217.
27. Clarke GM (1992) Fluctuating asymmetry: a technique for measuring developmental stress of genetic and environmental origin. Acta Zool Fenn 191: 31–35.
28. Parsons PA (1992) Fluctuating asymmetry: a biological monitor of environmental and genomic stress. Heredity 68: 361–364.
29. Markow TA (1995) Evolutionary ecology and developmental instability. Annu Rev Entomol 40: 105–120.
30. Leung B, Forbes MR (1997) Modeling fluctuating asymmetry in relation to stress and fitness. Oikos 78: 397–405.
31. Hendrickx F, Maelfait J-P, Lens L (2003) Relationship between fluctuating asymmetry and fitness within and between stressed and unstressed populations of the wolf spider *Pirata piraticus*. J Evol Biol 16: 1270–1279.
32. Møller AP, Manning J (2003) Growth and developmental instability. Vet J 166: 19–27.
33. Tuyttens FAM (2003) Measures of developmental instability as integrated, *a posteriori* indicators of farm animal welfare: a review. Animal Welfare 12: 535–540.
34. Committee on the Status of Pollinators in North America, National Research Council (2006) Status of Pollinators in North America. Washington, DC: National Academy of Sciences. 317p.
35. Zander E (1909) Tierische Parasiten als Krankenheitserreger bei der Biene. Münchener Bienenztg 31: 196–204.
36. Ellis JD, Munn PA (2005) The worldwide health status of honey bees. Bee World 86: 88–101.
37. Chen Y, Evans JD, Smith IB, Pettis JS (2008) *Nosema ceranae* is a long-present and wide-spread microsporidian infection of the European honey bee (*Apis mellifera*) in the United States. J Invert Pathol 97: 186–188.
38. Higes M, Martín-Hernández R, Meana A (2006) *Nosema* ceranae, a new microsporidian parasite in honeybees in Europe. J Invert Pathol 92: 93–95.
39. Higes M, Martín-Hernández R, Botías C, Bailón EG, González-Porto AV, et al. (2008) How natural infection by *Nosema ceranae* causes honeybee colony collapse. Environ Microbiol 10: 2659.
40. Evans JD (2006) Beepath: an ordered quantitative-PCR array for exploring honey bee immunity and disease. J Invert Pathol 93: 135–139.
41. Chen Y, Evans JD, Smith IB, Pettis JS (2007) *Nosema ceranae* is a long-present and wide-spread microsporidian infection of the European honey bee (*Apis mellifera*) in the United States. J Invert Pathol 97: 186–188.
42. Nielsen DI, Ebert PR, Page RE Jr, Hunt GJ, Guzman-Novoa E (2000) Improved polymerase chain reaction-based mitochondrial genotype assay for identification of the Africanized honey bee (Hymenoptera: Apidae). Ann Entomol Soc Am 93: 1–6.
43. Franck P, Garnery L, Celebrano G, Solignac M, Cornuet J-M (2000) Hybrid origins of honeybees from Italy (*Apis mellifera ligustica*) and Sicily (*A. m. sicula*). Molec Ecol 9: 907–921.
44. Clarke KE, Oldroyd BP, Javier J, Quezada-Euán G, Rinderer TE (2001) Origin of honeybees (*Apis mellifera* L.) from the Yucatan peninsula inferred from mitochondrial DNA analysis. Molec Ecol 10: 1347–1355.
45. Martel AC, Zeggane S, Aurieres C, Drajnudel P, Faucon JP, Aubert M (2007) Acaricide residues in honey and wax after treatment of honey bee colonies with Apivar® or Asuntol®50. Apidologie 38: 534–544.
46. Mullin CA, Frazier M, Frazier JL, Ashcraft S, Simonds R, et al. (2009) Pesticides and honey bee health: High levels of acaricides and crop protection chemicals in US apiaries. In preparation.
47. Nguyen BK, Saegerman C, Pirard C, Mignon J, Widart J, et al. (2009) Does imidacloprid seed-treated maize have an impact on honey bee mortality? J Econ Entomol 102: 616–623.
48. Desneux N, Decourtye A, Delpuech J-M (2007) The sublethal effects of pesticides on beneficial arthropods. Annu Rev Entomol 52: 81–106.
49. Pettis JS, Kochansmky J, Feldlaufer MF (2004) Larval *Apis mellifera* L. (Hymenoptera: Apidae) mortality after topical application of antibiotics and dusts. J Econ Entomol 97: 171–176.
50. Collins AM, Pettis JS, Wilbanks R, Feldlaufer MF (2004) Performance of honey bee (*Apis mellifera*) queens reared in beeswax cells impregnated with coumaphos. J Apic Res 43: 128–134.
51. DeGrandi-Hoffman G, Wardell G, Ahumada-Segura F, Rinderer T, Danka R, Pettis J (2008) Comparisons of pollen substitute diets for honey bees: consumption rates by colonies and effects on brood and adult populations. J Apic Res 4: 126–130.
52. Keller I, Fluri P, Imdorf A (2005) Pollen nutrition and colony development in honey bees - Part II. Bee World 86: 27–34.
53. Schneider SS, Leamy LJ, Lewis LA, deGrandi-Hoffman G (2003) The influence of hybridization between African and European honey bees, *Apis mellifera*, on asymmetries in wing size and shape. Evolution 57: 2350–2364.
54. Dryden IL, Mardia KV (1998) Statistical Shape Analysis. Chichester: John Wiley & Sons Ltd. 376p.
55. SAS Institute Inc (2002) SAS computer program, version 9.1. Cary: SAS Institute, Inc.
56. Palmer AR, Strobeck C (2003) Fluctuating asymmetry analyses revisited. In: Polak M, ed (2003) Developmental Instability: Causes and Consequences. New York: Oxford University Press. pp 279–319.
57. SAS Institute Inc (2007) JMP computer program, version 7.0. Cary: SAS Institute, Inc.
58. Palmer AR (1994) Fluctuating asymmetry analyses: a primer. In: Markow TA, ed (1994) Developmental instability: its origins and evolutionary implications. Dordrecht: Kluwer. pp 335–364.
59. Margolis L, Esch GW, Holmes JC, Kuris AM, Schad GA (1982) The use of ecological terms in parasitology (report of an ad hoc committee of the American Society of Parasitologists). J Parasitol 68: 131–133.
60. Shimanuki H, Knox DA (2000) Diagnosis of honey bee diseases. U.S. Department of Agriculture Agricultural Research Service. 57p.
61. Rinderer T, deGuzman L, Sylvester HA (2004) Re-examination of the accuracy of a detergent solution for varroa mite detection. Am Bee J 144: 560–562.
62. Cantwell GE (1970) Standard methods for counting nosema spores. Am Bee J 110: 222–223.
63. Delfinado-Baker, M (1984) *Acarapis woodi* in the United States. Am Bee J 124: 805–806.
64. Frazier M, Finley J, Harkness W, Rajotte ER (2000) A sequential sampling scheme for detecting the presence of tracheal mites (*Acarapis woodi*) infestations in honey bee (*Apis mellifera* L.) colonies. J Econ Entomol 93: 551–558.
65. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, et al. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol:RESEARCH0034.
66. Lehotay SJ, Mastovska K, Lightfield AR (2005) Use of buffering and other means to improve results of problematic pesticides in a fast and easy method for residue analysis of fruits and vegetables. J AOAC Int 88: 615–629.
67. Saegerman C, Speybroeck N, Roels S, Vanopdenbosch E, Thiry E, et al. (2004) Decision support tools for clinical diagnosis of disease in cows with suspected bovine spongiform encephalopathy. J Clin Microbiol 42: 172–178.
68. Petrie A, Watson P (2006) Statistics for Veterinary and Animal Science, Second Edition. Ames: Blackwell Publishing. 312p.
69. Kralj J, Fuchs S (2006) Parasitic mites influence flight duration and homing ability of infested *Apis mellifera* foragers. Apidologie 37: 577–587.

70. Robinson GE (1992) Regulation of division of labor in insect societies. Annu Rev Entomol 37: 637–665.
71. Bailey L (1958) The epidemiology of the infestation of the honeybee, *Apis mellifera* L., by the mite *Acarapis woodi* Rennie and the mortality of infested bees. Parasitol 48: 493–506.
72. Trotta V, Calboli FCF, Garoia F, Grifoni D, Cavicchi S (2005) Fluctuating asymmetry as a measure of ecological stress in *Drosophila melanogaster* (Diptera: Drosophilidae). Euro J Entomol 102: 195–200.
73. Martín-Hernández R, Meana A, Prieto L, Martínez Salvador A, Garrido-Bailón E, Higes M (2007) Outcome of colonization of *Apis mellifera* by *Nosema ceranae*. Appl Environ Microbiol 73: 6331–6338.
74. Palacios G, Hui J, Quan PL, Kalkstein A, Honkavuori KS, et al. (2008) Genetic analysis of Israel acute paralysis virus: distinct clusters are circulating in the United States. J Virol 82: 6209–6217.
75. Chen YP, Zhao Y, Hammond J, Hsu HT, Evans J, Feldlaufer M (2004) Multiple virus infections in the honey bee and genome divergence of honey bee viruses. J Invert Pathol 87: 84–93.
76. Stoner A, Wilson WT, Harvey J (1985) Honey bee exposure to beeswax foundation impregnated with fenvalerate or carbaryl. Am Bee J 125: 513–516.
77. Extension Toxicology Network (1994) Esfenvalerate. Ithaca: Cornell University. Available: http://pmep.cce.cornell.edu/profiles/extoxnet/dienochlor-glyphosate/esfenvalerate-ext.html. Accessed 4 February 2009.
78. Yang XL, Cox-Foster DL (2005) Impact of an ectoparasite on the immunity and pathology of an invertebrate: evidence for host immunosuppression and viral amplification. Proc Natl Acad Sci USA 102: 7470–7475.
79. Chen YP, Pettis JS, Evans JD, Kramer M, Feldlaufer MF (2004) Molecular evidence for transmission of Kashmir bee virus in honey bee colonies by ectoparasitic mite, *Varroa destructor*. Apidologie 35: 441–448.
80. vanEngelsdorp D, Underwood RM, Cox-Foster DL (2008) Short-term fumigation of honey bee (Hymenoptera: Apidae) colonies with formic and acetic acids for the control of *Varroa destructor* (Acari: Varroidae). J Econ Entomol 101: 256–264.
81. Pettis JS (2004) A scientific note on *Varroa destructor* resistance to coumaphos in the United States. Apidologie 35: 91–92.
82. Pettis JS, Collins AM, Wilbanks R, Feldlaufer MF (2004) Effects of coumaphos on queen rearing in the honey bee, *Apis mellifera* L. Apidologie 35: 605–610.
83. Milani N (1999) The resistance of *Varroa jacobsoni* Oud. to acaricides. Apidologie 30: 229–234.
84. Johnson RM, Wen Z, Schuler MA, Berenbaum M (2006) Mediation of pyrethroid insecticide toxicity to honey bees (Hymenoptera: Apidae) by cytochrome P450 monooxygenases. J Econ Entomol 99: 1046–1050.
85. Claudianos C, Ranson H, Johnson RM, Biswas S, Schuler MA, et al. (2006) A deficit of detoxification enzymes: pesticide sensitivity and environmental response in the honeybee. Insect Mol Biol 15: 615–636.
86. Martin SJ (2001) The role of Varroa and viral pathogens in the collapse of honeybee colonies: a modelling approach. J Appl Ecol 38: 1082–1093.

Chapter 5

WEIGHING RISK FACTORS ASSOCIATED WITH BEE COLONY COLLAPSE DISORDER BY CLASSIFICATION AND REGRESSION TREE ANALYSIS.[4]

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Weighing Risk Factors Associated With Bee Colony Collapse Disorder by Classification and Regression Tree Analysis

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ABSTRACT Colony collapse disorder (CCD), a syndrome whose defining trait is the rapid loss of adult worker honey bees, *Apis mellifera* L., is thought to be responsible for a minority of the large overwintering losses experienced by U.S. beekeepers since the winter 2006–2007. Using the same data set developed to perform a monofactorial analysis (PloS ONE 4: e6481, 2009), we conducted a classification and regression tree (CART) analysis in an attempt to better understand the relative importance and interrelations among different risk variables in explaining CCD. Fifty-five exploratory variables were used to construct two CART models: one model with and one model without a cost of misclassifying a CCD-diagnosed colony as a non-CCD colony. The resulting model tree that permitted for misclassification had a sensitivity and specificity of 85 and 74%, respectively. Although factors measuring colony stress (e.g., adult bee physiological measures, such as fluctuating asymmetry or mass of head) were important discriminating values, six of the 19 variables having the greatest discriminatory value were pesticide levels in different hive matrices. Notably, coumaphos levels in brood (a miticide commonly used by beekeepers) had the highest discriminatory value and were highest in control (healthy) colonies. Our CART analysis provides evidence that CCD is probably the result of several factors acting in concert, making afflicted colonies more susceptible to disease. This analysis highlights several areas that warrant further attention, including the effect of sublethal pesticide exposure on pathogen prevalence and the role of variability in bee tolerance to pesticides on colony survivorship.

KEY WORDS colony collapse disorder, epidemiology, classification and regression tree analysis, pathogens, *Apis mellifera*

Large-scale losses of managed honey bees, *Apis mellifera* L., have been reported globally (Haubruge et al. 2006, vanEngelsdorp and Meixner 2010). In the United States, a portion of the dead and dying colonies were characterized by a common set of specific symptoms: 1) the rapid loss of adult worker bees from affected beehives, resulting in weak or dead colonies with excess brood present relative to adult bees; 2) a noticeable lack of dead worker bees both within and surrounding the hive; and 3) the delayed invasion of hive pests (e.g., small hive beetles and wax moths) and kleptoparasitism from neighboring honey bee colonies (Cox-Foster et al. 2007). Subsequently, this syndrome has been termed colony collapse disorder (CCD), and its case definition has been revised to include 4) the absence of varroa and nosema loads at levels thought to cause economic damage (vanEngelsdorp et al. 2009).

In an attempt to better characterize CCD, an initial descriptive epizootiological study was conducted (vanEngelsdorp et al. 2009). This monofactorial study focused on identifying and quantifying direct and indirect measures of risk in affected populations and comparing these measures with apparently healthy populations. Some measures of risk differed between apparently healthy and unhealthy populations, although no one factor clearly separated the two groups. Generally, CCD-affected colonies had higher pathogen incidence and pathogen loads, but no pathogen on its own was found in all CCD colonies. This finding

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suggests that some underlying risk factor or combination of risk factors compromises the immunity of bees and thus decreases a colony's ability to fight pathogenic infection (vanEngelsdorp et al. 2009). A recent effort found broad changes in gene expression between bees from healthy and collapsed colonies, along with elevated pathogen levels in CCD colonies, but no systematic differences in RNA transcripts for genes implicated in honey bee immunity (Johnson et al. 2009b).

A classification and regression tree (CART) analysis is a useful nonparametric data-mining technique. This analysis is particularly helpful when attempting to investigate which direct and indirect measures of risk are predictive of a newly emerging or complex disease (Saegerman et al. 2004). Contrary to classical regression that uses linear combinations, CART does not require the data to be linear or additive. Furthermore, CART analysis does not require possible interactions between factors to be prespecified (Breiman et al. 1984). In essence, the classification trees resulting from a CART analysis accommodate more flexible relationships among variables, missing covariate values, multicolinearity, and outliers in an intuitive manner (Speybroeck et al. 2004). When values for some predictive factors are missing, they can be estimated using other predictor ("surrogate") variables, permitting the use of incomplete data sets when generating regression trees. Another advantage of a CART analysis (compared with a classical multivariate regression analysis) is that it allows for the calculation of the overall discriminatory power, or relative importance, of each explanatory variable.

The monofactorial study by vanEngelsdorp et al. (2009) investigated >200 variables, but only 61 occurred with enough frequency to make meaningful comparisons between diseased (CCD) and apparently healthy populations. Included in this list were six variables that were directly linked with either the operational or refined definition of CCD: frames of bees, ratio of bees to brood, presence of varroa mites, *Varroa destructor* (Anderson & Trueman), spore loads and presence of *Nosema ceranae*, *Nosema apis*, or both (see case definition discussion above). Although the inclusion of these variables either validated the application of the operational case definition (or justified the revision of the original case definition of CCD), the use of these "case defining" variables in a multifactorial analysis could skew results as these variables are inherently not independent. In the current study, we preformed a CART analysis to help identify those variables that, independently or in combination, best discriminate CCD from non-CCD populations. However, to avoid creating a circular argument, we included only truly independent variables ($n = 55$) and discarded those ($n = 6$) that were intrinsic to CCD's case definition. This study is the first to apply a CART analysis to honey bee pathology in an attempt to advance the understanding of the underlying causes of CCD.

Materials and Methods

Study Apiaries and Colonies. As outlined in vanEngelsdorp et al. (2009), 91 colonies from 13 apiaries resident in either Florida or California during January and February 2007 had adult bees, brood, wax, beebread (pollen provisions), or a combination, and were sampled for further analysis.

Case Definition. Select colonies were classified in the field as either 1) not having CCD symptoms (39 "control" colonies) or 2) having CCD symptoms (52 "CCD" colonies). Colonies were considered to have CCD symptoms when adult bee populations were in obvious rapid decline leaving brood poorly attended or were dead in an apiary having clear symptoms of CCD. In those CCD colonies in which bees remained, there were insufficient number of bees to cover the brood, the remaining worker bees seemed young (i.e., adults bees that were unable to fly), and the queen was present. Notably, both dead and weak colonies in CCD apiaries were not being robbed by other bees despite the lack of bloom in the area, neither were they being attacked by secondary pests despite the presence of honey and beebread in the vacated equipment (vanEngelsdorp et al. 2009).

Explanatory Variables. After elimination of six variables inherently linked to defining CCD colonies (vanEngelsdorp et al., 2009; see above), the remaining variables were either indirect measures of colony stress (e.g., adult bee physiological and morphological measures) or direct measures of risk that are thought to directly and adversely affect colony health (e.g., parasite, pathogen, and pesticide loads).

Classification and Regression Tree Analysis. A CART analysis was conducted on the data set, where colony status (CCD or control) was used as the dependent variable and the 55 direct/indirect measures of risk were used as independent or predictor variables. A CART analysis is a nonlinear and nonparametric model that is fitted by binary recursive partitioning of multidimensional covariate space. Using CART 6.0 software (Salford Systems, San Diego, CA), the analysis successively splits the data set into increasingly homogeneous subsets until it is stratified meet specified criteria (Saegerman et al. 2004, Thang et al. 2008). The Gini index was used as the splitting method, and 10-fold cross-validation was used to test the predictive capacity of the obtained trees. CART performs cross validation by growing maximal trees on subsets of data then calculating error rates based on unused portions of the data set. To accomplish this, CART divides the data set into 10 randomly selected and roughly equal "parts," with each part containing a similar distribution of data from the populations of interest (i.e., CCD versus control). CART then uses the first nine parts of the data, constructs the largest possible tree, and uses the remaining 1/10 of the data to obtain initial estimates of the error rate of the selected subtree. The process is repeated using different combinations of the remaining nine subsets of data and a different 1/10 data subset to test the resulting tree. This process is repeated until each 1/10

Table 1. Ranking of CCD colony risk factors by overall discriminatory power without a cost of misclassifying a CCD-diagnosed colony as a non-CCD colony

Variable	Power
Coumaphos in brood	100.00
Fluctuating asymmetry	50.15
Esfenvalerate in wax	33.91
Coumaphos in wax	29.42
Iprodione in wax	17.65
Dicofol in breebread	7.65
Chronic bee paralysis virus	6.77
Centriod size	5.74
Chlorothalonil in wax	5.03
Protein in the abdomen	4.49
Acute bee paralysis virus	3.58
Endosulfan in beebread	2.89

subset of the data has been used as to test a tree that was grown using a 9/10 data subset. The results of the 10 minitests are then combined to calculate error rates for trees of each possible size; these error rates are applied to prune the tree grown using the entire data set. The consequence of this complex process is a set of fairly reliable estimates of the independent predictive accuracy of the tree, even when some of the data for independent variables are incomplete, specific events are either rare or overwhelmingly frequent, or both.

For each node in a CART generated tree, the "primary splitter" is the variable that best splits the node, maximizing the purity of the resulting nodes. When the primary splitting variable is missing for an individual observation, that observation is not discarded but, instead, a surrogate splitting variable is sought. A surrogate splitter is a variable whose pattern within the data set, relative to the outcome variable, is similar to the primary splitter. Thus, the program uses the best available information in the face of missing values. In data sets of reasonable quality, this allows all observations to be used. This is a significant advantage of this methodology over more traditional multivariate regression modeling, in which observations which are missing any of the predictor variables are often discarded.

In this study, two classification and regression tree models were constructed: one without and one with a cost of misclassifying a CCD-diagnosed (positive) colony as an apparently healthy (negative) colony. For the second tree, several possibilities were tested, but the tree generated allowing for a misclassification cost of two resulted in the smallest number of misclassified colonies while minimizing the size (complexity) of the resulting tree (cf. Suman et al. 2010 for details). The cost (penalty) is a measure of the likelihood of misclassifying a CCD-diagnosed (positive) colony as an apparently healthy (negative) colony. This classification enabled us to make a distinction between groups of colonies containing at least one colony with CCD from groups of colonies without any CCD-diagnosed colonies. The discriminatory power of each variable included in the analysis also was calculated.

Results

Classification and Regression Trees Analysis Without a Misclassification Cost. The CART analysis without a misclassification cost showed that coumaphos load in brood (p: 100.00) and the fluctuating asymmetry (p: 50.15) were the two predictor variables with the strongest overall discriminating power (Table 1; Fig. 1). Generally, CCD colonies had lower levels of coumaphos in brood and their adult bees were more symmetrical compared with samples taken from apparently healthy colonies. As indicated by having a discriminatory power of >15%, three additional variables, i.e., variables that did not act as nodes on the regression tree (Fig. 1) also had significant discriminating power: loads of esfenvalerate (p: 33.91), coumaphos (p: 29.42), and iprodione (p: 17.65) in the wax (Table 1). Overall, the resulting tree (Fig. 1) had a sensitivity of 65% and a specificity of 87%.

Classification and Regression Trees Analysis With a Cost of Misclassification. When conducting the CART analysis with a misclassification cost of 2, at least five variables distinguished themselves as most important: coumaphos in brood (p: 100.00), coumaphos in bee-

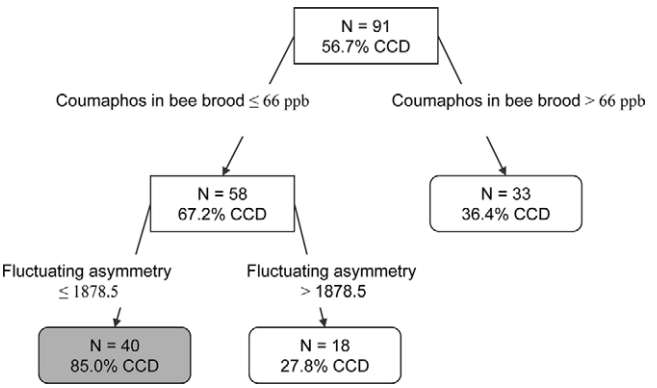

Fig. 1. Classification tree of the risk factors for CCD colonies without a cost of misclassifying a CCD-diagnosed colony as a non-CCD colony.

Table 2. Ranking of CCD colony risk factors by overall discriminatory power with a cost of 2 for misclassifying a CCD-diagnosed colony as a non-CCD colony

Variable	Power
Coumaphos in brood	100.00
Coumaphos in beebread	81.11
Fluctuating asymmetry	42.48
Mass of the head	36.07
Coumaphos in wax	27.39
Proteins in the thorax	12.71
Proteins in the abdomen	9.66
Acute bee paralysis virus	8.76
Dicofol in beebread	7.54
Proteins in the head	6.16
Centriod size	5.57
Total proteins	4.75
Chlorothalonil in wax	4.31
Mass of the abdomen	3.75
Endosulfan in beebread	2.71
Ratio proteins in the thorax/mass of the thorax	2.57
Ratio proteins in the abdomen/mass of the abdomen	1.91
Frames of brood	1.64
Ratio total proteins/total mass	1.04

bread (p: 81.11), fluctuating asymmetry (p: 42.48), mass of the head (p: 36.07), coumaphos in wax (p: 27.39), and proteins in the thorax (p: 12.71; Table 2). Some of these variables did not act as splitting nodes in the regression tree (Fig. 2). As with the first model, the tree permitting misclassification first segregated the study population based on coumaphos loads in bee brood. A majority of healthy colonies had coumaphos loads in bee brood >66 ppb. Both of the resulting branches were further split by three other variables (Fig. 2) and resulted in five terminal nodes, including one node that contained only CCD colonies. Generally, this model revealed that when compared with CCD colonies, control colonies are best characterized as having higher levels of coumaphos in brood, the adult bees were more asymmetrical and had heads with a greater mass. This entire tree had a sensitivity of 85% and a specificity of 74%.

Discussion

In the United States, overwintering losses of honey bee colonies have averaged 30% or more over the winters 2006–2007, 2007–2008, and 2008–2009 (vanEngelsdorp et al. 2007, 2008, 2010). Although most operations identify known threats as the cause of mortality (e.g., poor queens, colony starvation, and varroa mite parasitism), some of these losses shared symptoms associated with CCD (specifically, no dead bees in affected colonies). Previous attempts to find the cause of CCD failed to identify a single factor that explained all cases of CCD (Cox-Foster et al. 2007, Johnson et al. 2009b, vanEngelsdorp et al. 2009). In an attempt to better characterize CCD after an initial descriptive (and monofactorial) study, we present here the results of a multifactorial CART analysis.

The use of CART analysis in epidemiological studies permits the identification of risk factors that are useful in disease diagnosis (Saegerman et al. 2004) as well as those that may play an important role in disease occurrence (Thang et al. 2008). CART analysis is a valuable tool in epidemiological studies because it generates a nonlinear and nonparametric model. In addition, this approach is particularly useful when, as in this case, the data set includes missing values, because the CART model generates surrogate data points based on relationships identified within the existing data (Saegerman et al. 2004, Thang et al. 2008).

Among 55 variables used in our CART analysis, one variable stood out as the most important when differentiating CCD from control colonies: coumaphos levels in brood. In both the tree with and without a

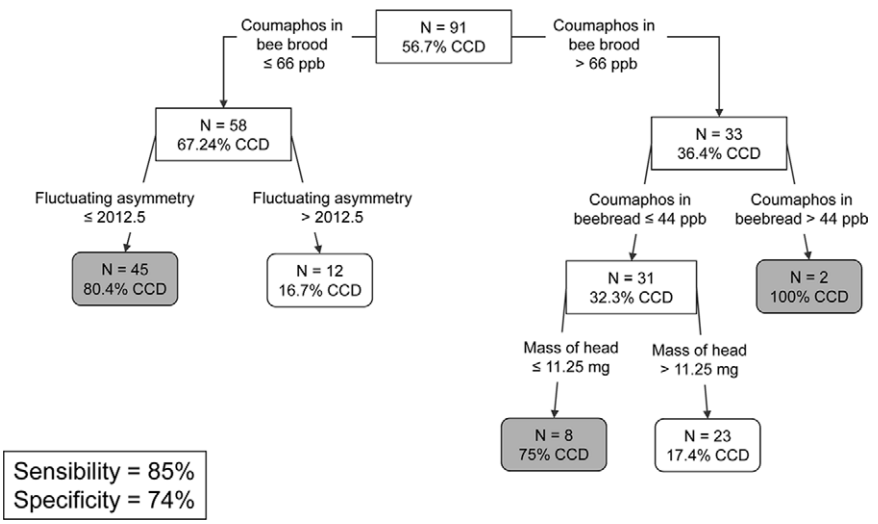

Fig. 2. Classification and regression tree of the risk factors for CCD colonies with a cost of 1.8 points for misclassifying a CCD-diagnosed colony as a non-CCD colony.

misclassification cost, colonies from control colonies had the highest level of coumaphos in brood.

The presence of some pesticide products found in hives is not surprising (Bogdanov et al. 1998, Tremolada et al. 2004, Martel et al. 2007). Coumaphos is the active ingredient found in varroa mite control products widely used by U.S. beekeepers. This lipophilic product is known to accumulate in wax. It is therefore not surprising that this product is found extensively in beekeeping operations both in the United States and Europe (Mullin et al. 2010, vanEngelsdorp and Meixner 2010). Even one treatment of the organophosphorus miticide coumaphos, marketed as CheckMite+ (Bayer), can elevate coumaphos levels in broodchamber honey stores to 60 and 111 ppb (Karazafiris et al. 2008). The discriminatory value of coumaphos in brood suggests that healthy colonies had mite populations that were more aggressively or persistently controlled by the beekeepers. Although varroa mite levels were not different between CCD and control populations at the time of sampling (vanEngelsdorp et al. 2009), it is possible that mite populations differed at some time before sample collection. CCD may therefore be a consequence of elevated levels of mites—relative to mite levels in control colonies—some time before sampling. Clearly, longitudinal studies that monitor the mite levels before the onset of CCD are needed to quantify the effect of mite levels before colony collapse.

Coumaphos was initially selected as a mite control agent because of its relative low toxicity to honey bees. Despite this low toxicity, chronic sublethal exposure to this product can have detrimental effects on colony health (Pettis et al. 2004). Furthermore, the low toxicity of this product also relies, at least in part, on the rapid detoxification of these miticides by the exposed bees (Johnson et al. 2009a). Honey bees, compared with other insects, have relatively few insecticide detoxifying genes (Claudianos et al. 2006), which may in part explain why honey bees are relatively sensitive to pesticide exposure (Atkins 1992). One gene family in particular, cytochrome P450 monooxygenase enzymes (P450) is used by honey bees to detoxify coumaphos (Johnson et al. 2006, 2009a). As a result, exposure to both products (e.g., coumaphos and fluvalinate) simultaneously has a synergistic effect on toxicity toward bees (Johnson et al. 2009a). Although unproven, it does stand to reason that certain populations of honey bees can vary in their tolerance of pesticide exposure as a result of differences in the expression of detoxifying genes. Should this be the case, differences in pesticide resistance could explain the relative importance of some pesticide loads in distinguishing CCD populations from control populations. In the monofactorial analysis, coumaphos and esfenvalerate in wax were consistently found at higher concentrations in the control colonies (vanEngelsdorp et al. 2009). Pathogenic attack, specifically viral attack, may arrest translation of proteins that mediate pesticide detoxification (Johnson et al. 2009b). Alternatively, because sublethal pesticide exposure can increase susceptibility to pathogen attack (Bendahou et al. 1997), it is possible that colonies afflicted with CCD are less tolerant to environmental pesticide exposure and consequently are more susceptible to pathogen attack, which leads to collapse.

Although higher levels of coumaphos may benefit colonies by controlling mite populations (vanEngelsdorp et al. 2009), this hypothesis does not explain completely why pesticides not used in beekeeping are important discriminating variables when distinguishing control colonies from CCD colonies. As determined by the CART analysis (Tables 1 and 2), the pesticides that are important distinguishing variables come from diverse classes such as coumaphos (an organophosphate), esfenvalerate (a pyrethroid), dicofol (an organochlorine), iprodione and chlorthalonil (two fungicides), and endosulfan (a cyclodiene). More work is needed to explain why some exogenous chemicals are positively associated with CCD but others are negatively associated.

As in the current study, fluctuating asymmetry (FA) was found to discriminate between CCD and non-CCD colonies in our earlier monofactorial comparisons (vanEngelsdorp et al. 2009). In this current effort, FA was an important discriminating factor in both CART models (without a misclassification cost: second most predictive variable, $P = 50.15$; with a misclassification cost: third most predictive variable, $P = 42.48$). FA, defined as random differences in the shape or size of a bilaterally symmetrical character (Palmer and Strobeck 1986), can be an indicator of individual fitness (VanValen 1962) because organisms exposed to stress during their development show less symmetry than unstressed organisms (Tuyttens 2003). Average FA scores of worker bees have been suggested previously as a measure of colony level fitness (Schneider et al. 2003). Although measuring fluctuating asymmetry is a less sensitive test when it comes to differentiating control colonies from CCD colonies compared with other variables, it is a more practical test than expensive and time-consuming pesticide analyses needed to determine coumaphos levels in brood and beebread. It is not, however, as easily measured as some other discriminating variables (such as head mass). The value of FA as a measure to predict colony health in general and CCD in particular, warrants further investigation.

Head masses between of bees from CCD and non-CCD populations were not significantly different overall (vanEngelsdorp et al. 2009). However, as a discriminating risk factor in CART model with a cost of misclassification, head mass seems to be important. For example, of the 31 individual colonies that had low coumaphos levels in beebread ($\leq$44 ppb), those from control colonies had heavier heads (Fig. 2). The heads of winter bees are $\approx$15% lighter than the heads of summer bees (Meyer-Rochow and Vakkuri 2002), which may be the result of reduced hypopharyngeal gland size in winter bees (Fluri et al. 1982) or because summer bees have larger brains (Meyer-Rochow and Vakkuri 2002). The volume of certain brain regions, and presumably the mass of the total bee brain, also changes as summer bees age, with antennal lobes in

forager bees being larger than those of 4-d-old house bees (Brown et al. 2002). As bees age, the size of their hypopharyngeal glands increases for 1 wk and then decreases (Crailsheim and Stolberg 1989). It is therefore possible that the increased head mass in healthy colonies reflects the overall age profile of the bees sampled, because bees remaining in CCD colonies are thought to be young (vanEngelsdorp et al. 2009).

The ability of individual pathogen loads to distinguish CCD and non-CCD colonies was minimal. This confirms previous findings that none of the pathogens quantified by this effort can be implicated as the sole "cause" of CCD. This is not to say, however, that disease agents play no role in CCD, because they clearly do (Cox-Foster et al. 2007, Johnson et al. 2009b, vanEngelsdorp et al. 2009). The use of CART analysis in epidemiological studies permits the identification of risk factors that are useful in disease diagnosis (Saegerman et al. 2004) as well as those that may play an important role in disease occurrence (Thang et al. 2008). This study is the first to apply this analytical tool to bee pathology in general and CCD in particular. It is important to note that this study, being an epizootiological study, did not set out to test a specific hypothesis (Koepsell and Weiss 2003) and so did not intend to identify the cause or causes of CCD. Rather, the results of this analysis are intended to act as a guide for further epidemiological- and hypothesis-driven research. To that end, the CART analysis presented here highlights several areas that warrant further attention, including the effect that sublethal pesticide exposure may have on pathogen prevalence, and the potential effect that tolerance to pesticides has on colony survivorship. This analysis also provides further evidence that CCD is probably the result of several factors, acting in concert, which together decrease colony fitness and make affected colonies more susceptible to disease.

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References Cited

Atkins, E. 1992. Injury to honey bees by poisoning, pp. 1155–1203. *In* J. M. Graham [ed.], The hive and the honey bee, revised edition. Bookcrafters, Hamilton, IL.

Bendahou, N., M. Bounias, and C. Fleche. 1997. Acute toxicity of cypermethrin and fenitrothion on honeybees (*Apis mellifera mellifera*) according to age, formulations and (chronic paralysis virus)/insecticide interaction. J. Environ. Biol. 18: 55–65.

Bogdanov, S., V. Kilchenmann, and A. Imdorf. 1998. Acaricide residues in some bee products. J. Apicult. Res. 37: 57–67.

Breiman, I., J. H. Friedman, R. A. Olsen, and C. J. Stone. 1984. Classification and regression trees. Wadsworth, Pacific Grove, CA.

Brown, S., R. Napper, C. Thompson, and A. Mercer. 2002. Stereological analysis reveals striking differences in the structural plasticity of two readily identifiable glomeruli in the antennal lobes of the adult worker honeybee. J. Neurosci. 22: 8514–8522.

Claudianos, C., H. Ranson, R. M. Johnson, S. Biswas, M. A. Schuler, M. R. Berenbaum, R. Feyereisen, and J. G. Oakeshott. 2006. A deficit of detoxification enzymes: pesticide sensitivity and environmental response in the honeybee. Insect Mol. Biol. 15: 615–636.

Cox-Foster, D. L., S. Conlan, E. C. Holmes, G. Palacios, J. D. Evans, N. A. Moran, P. L. Quan, T. Briese, M. Hornig, D. M. Geiser, et al. 2007. A metagenomic survey of microbes in honey bee colony collapse disorder. Science 318: 283–286.

Crailsheim, K., and E. Stolberg. 1989. Influence of diet, age and colony condition upon intestinal proteolytic activity and size of the hypopharyngeal glands in the honeybee (*Apis mellifera* L.). J. Insect Physiol. 35: 595–602.

Fluri, P., M. Luscher, H. Wille, and L. Gerig. 1982. Changes in weight of pharyngeal gland and haemolymph titres of juvenile hormone, protein and vitellogenin in worker honey bees. J. Insect Physiol. 28: 61–68.

Haubruge, E., B. K. Nguyen, J. Widart, J.-P. Thomé, P. Fickers, and E. Depauw. 2006. Le dépérissement de l'abeille domestique, *Apis mellifera* L., 1758 (Hymenoptera: Apidae): faits et causes probables. Notes Fauniques Gembloux 56: 3–21.

Johnson, R. M., Z. Wen, M. A. Schuler, and M. R. Berenbaum. 2006. Mediation of pyrethroid insecticide toxicity to honey bees (Hymenoptera: Apidae) by cytochrome P450 monooxygenases. J. Econ. Entomol. 99: 1046–1050.

Johnson, R. M., H. S. Pollock, and M. R. Berenbaum. 2009a. Synergistic interactions between in-hive miticides in *Apis mellifera.* J. Econ. Entomol. 102: 474–479.

Johnson, R. M., J. D. Evans, G. E. Robinson, and M. R. Berenbaum. 2009b. Changes in transcript abundance relating to colony collapse disorder in honey bees (*Apis mellifera*). Proc. Natl. Acad. Sci. 106: 14790–14795.

Karazafiris, E., C. Tananaki, U. Menkissoglu-Spiroudi, and A. Thrasyvoulou. 2008. Residue distribution of the acaricide coumaphos in honey following application of a new slow-release formulation. Pest Manage. Sci. 64: 165–171.

Koepsell, T. D., and N. S. Weiss. 2003. Epidemiologic methods: studying the occurrence of illness. Oxford University Press, New York.

Martel, A. C., S. Zeggane, C. Aurières, P. Drajnudel, J. P. Faucon, and M. Aubert. 2007. Acaricide residues in honey and wax after treatment of honey bee colonies with Apivar(R) or Asuntol(R)50. Apidologie 38: 534–544.

Meyer-Rochow, V. B., and O. Vakkuri. 2002. Honeybee heads weigh less in winter than in summer: a possible explanation. Ethol. Ecol. Evol. 14: 69–71.

Mullin, C. A., M. Frazier, J. L. Frazier, S. Ashcraft, R. Simonds, D. vanEngelsdorp, and J. S. Pettis. 2010. High levels of miticides and agrochemicals in North American apiaries: implications for honey bee health. PloS ONE 5: e9754.

Palmer, A. R., and C. Strobeck. 1986. Fluctuating asymmetry: measurement, analysis, patterns; Augmented title: review. Annu. Rev. Ecol. Syst. 17: 391–421.

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

Saegerman, C., N. Speybroeck, S. Roels, E. Vanopdenbosch, E. Thiry, and D. Berkvens. 2004. Decision support tools for clinical diagnosis of disease in cows with suspected bovine spongiform encephalopathy. J. Clin. Microbiol. 42: 172–178.

Schneider, S. S., L. J. Leamy, L. A. Lewis, and G. DeGrandi-Hoffman. 2003. The influence of hybridization between African and European honey bees, *Apis mellifera,* on asymmetries in wing size and shape. Evolution 57: 2350–2364.

Speybroeck, N., D. Berkvens, A. Mfoukou-Ntsakala, M. Aerts, N. Hens, G. v. Huylenbroeck, and E. Thys. 2004. Classification trees versus multinomial models in the analysis of urban farming systems in central Africa. Agric. Syst. 80: 133–149.

Suman, P., E. Thys, A. Mfoukou-Ntsakala, L. Ali, M. Ouedraogo, P. Van den Bossche, G. Van Huylenbroeck, D. Berkvens, and N. Speybroeck. 2010. Methodology for assessing determinants of manure use in urban areas of Africa. Waste Manage. Res. (doi: 10.1177/0734242X09356016).

Thang, N. D., A. Erhart, N. Speybroeck, L. Hung, T. Le Khanh, H. Cong Trinh, K. Pham Van, M. Coosemans, and U. D'Alessandro. 2008. Malaria in central Vietnam: analysis of risk factors by multivariate analysis and classification tree models. Malar. J. 7: 28. (doi:10.1186/1475-2875-7-28).

Tremolada, P., I. Bernardinelli, M. Colombo, M. Spreafico, and M. Vighi. 2004. Coumaphos distribution in the hive ecosystem: case study for modeling applications. Ecotoxicology 13: 589–601.

Tuyttens, F.A.M. 2003. Measures of developmental instability as integrated, a posteriori indicators of farm animal welfare: a review. Anim. Welf. 12: 535–540.

vanEngelsdorp, D., and M. D. Meixner. 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., R. Underwood, D. Caron, and J. Hayes, Jr. 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., J. Hayes, Jr., R. M. Underwood, and J. Pettis. 2008. A survey of honey bee colony losses in the U.S., fall 2007 to spring 2008. PLoS ONE 3: e4071.

vanEngelsdorp, D., J. D. Evans, C. Saegerman, C. Mullin, E. Haubruge, B. K. Nguyen, M. Frazier, J. Frazier, D. Cox-Foster, Y. Chen, et al. 2009. Colony collapse disorder: a descriptive study. PloS ONE 4: e6481.

vanEngelsdorp, D., J. Hayes, Jr., R. Underwood, and J. S. Pettis. 2010. A survey of honey bee colony losses in the U.S., fall 2008 to spring 2009. J. Apicult. Res. 49: 7–14.

VanValen, L. 1962. A study of fluctuating asymmetry. Evolution 16: 125–142.

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Chapter 6

COLONY MORTALITY AND MORBIDITY IN MIGRATORY BEEKEEPING OPERATIONS IN THE EASTERN UNITED STATES: A LONGITUDINAL DESCRIPTIVE STUDY BASED ON RATES OF RISK FACTOR EXPOSURE.[5]

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[5] vanEngelsdorp, D., D. R. Tarpy, E. J. Lengerich, and J. Pettis. in revision**.** Colony mortality and morbidity in migratory beekeeping operations in the Eastern United States: A longitudinal descriptive study based on rates of risk factor exposure. Apidologie.

Abstract:

Using standard epidemiological methods, this study set out to quantify the risk associated with exposure to easily diagnosed risk factors on colony mortality and morbidity in three migratory beekeeping operations. Fifty-six percent of all colonies monitored during the 10-month period died. The relative risk (RR) that a colony would die over the short term (~50 days) was appreciably increased in colonies diagnosed with a "queen event" (evidence of queen replacement or failure; RR=3.1) and with Idiopathic Brood Disease Syndrome (IBDS, the brood condition associated with parasitic mite syndrome; RR =3.2). We also found that several risk factors, including the incidence poor brood pattern, chalkbood (CB), deformed wing virus (DWV), sacbrood virus (SBV), and exceeding varroa threshold of 5 mites per 100 bees were differentially expressed in different beekeeping operations. Further we found that a diagnosis of several risk factors increased and/or decreased the risk that colonies would be re-diagnosed with the same (poor brood pattern (RR=2.5), CB (RR=3.7), DWV (RR =6.6) and Queen events (RR=3.3)) or another risk factor (*e.g.* poor brood patterns and CB (RR=4.9), SBV (RR=5.8), IBDS (RR=3.6) and exceeding varroa threshold (RR=0.5)) during the next inspection. These results confirm the growing consensus that the causes of colony mortality are multi-factorial and complex.

Key Words: honey bee/epidemiology/mortality/relative risk

1. **Introduction**

Honey bees (*Apis mellifera* L.) play a vital role in modern agriculture. An estimated 35% of the western human diet benefits, directly or indirectly, from honey bee pollination (Klein et al. 2007). While colony numbers have increased globally over the last 60 years (Aizen et al. 2008), this increase has not kept pace with increased acreages planted with pollinator-dependent crops (Aizen and Harder 2009).Additionally, increases in colony numbers have not been consistent across all regions, with long-term losses documented in the US and European nations(Potts et al. 2010, vanEngelsdorp and Meixner 2010). These trends have raised fears that demand for pollinating units will outstrip supplies in the future (NRC 2006). While some have questioned the basis of these fears (Ghazoul 2005)researchers agree that there is a need for consistent and reliable enumeration of pollinator populations and focused research investigating the cause or causes of losses (Neumann and Carreck 2010, Nguyen et al. 2010) .

In recent years, there has been increased attention to documenting overwintering honey bee colony losses in North America (vanEngelsdorp et al. 2008, Currie et al. 2010, vanEngelsdorp et al. 2010a, vanEngelsdorp et al. 2011)and Europe (Brodschneider et al. 2010, Potts et al. 2010, Nguyen et al. in press). While these efforts have not attempted to directly ascertain the cause of mortality, most have accepted self-reported reasons from beekeepers about which factors they believe most likely contributed to colony mortality in their particular operation. (vanEngelsdorp et al. 2008, Brodschneider et al. 2010, vanEngelsdorp et al. 2010a, vanEngelsdorp et al. in press). Some factors self identified by survey respondents as leading causes for increased mortality, such as varroa mite parasitism, have been collaborated by more systematic surveys s(Haubruge et al. 2006, Chauzat et al. 2010a, Guzmán-Novoa et al. 2010). While varroa mites clearly contribute to colony mortality, other factors—including pesticide exposure, other bee parasites and pathogens, foraging conditions in the fall, and beekeeper management—may also negatively affect colony survival

(vanEngelsdorp and Meixner 2010). There seems little doubt that different factors can interact with one another. In Denmark, for instance, elevated losses were compounded when weather conditions in the fall prevented effective mite treatments, facilitating higher mite loads on bees that may not have had optimal pollen stores (Vejsnæs et al. 2010). The objective of this study was to identify and quantify risk factors associated with colony mortality in migratory beekeeping operations in the eastern U.S. Specifically we monitored risk factors that are readily identified by beekeepers during colony inspection or quantified by beekeepers by taking easily obtained samples and using basic diagnostic techniques. We used basic epidemiological methods to calculate and compare the relative risk associated with exposure to these easily quantified putative risk factors. This risk-factor approach is commonly used in human studies to inform future hypothesis-driven analytical studies designed to elucidate causes of disease and mortality (Koepsell and Weiss 2003). Just as in human studies, we intend for the results of this study to highlight areas for future research intended understand and mitigate colony losses.

2. Materials and Methods:

2.1 Colony selection

The study design employed was a longitudinal study of colonies in three migratory beekeeping operations (OP1, n=20; OP2, n=24; OP3, n=18). The study was conducted during a 10-month period (mean = 300 days) between March 2007 and January 2008. The selected operations were considered representative of east coast migratory operations, as the beekeepers transported honey bee colonies north and south within the eastern United States to pollinate and/or produce honey on a diverse variety crops and natural vegetation (Figure 1). All colonies contained within an apiary operated by the co-operating beekeepers were tagged with individually numbered

cattle ear tags. Upon first inspection, each colony's queen was located, marked, and had one of her wings clipped to help monitor queen replacement. Surviving colonies were inspected at intervals that varied depending on the frequency that the colonies were moved (Figure 1).

2.2 Colony measurements

During each inspection, the condition of monitored colonies was first noted. Colonies were considered dead when they were found completely depopulated of adult bees. The strength of surviving colonies was then assessed by estimating the number of frames covered with adult bees and containing capped brood (DeGrandi-Hoffman et al. 2008)The quality of brood was also assessed by averaging the number of empty brood cells in four randomly-selected patches of contiguous capped brood (100 brood cells per patch). When an average of ≥ 20% of the cells were found empty , the brood pattern was considered 'poor'.

During inspection, clinical symptoms of disease were noted, including Chalkbrood (CB), *Ascosphaera apis*; European foulbrood (EFB), *Melissococcus pluton*; American foulbrood (AFB), *Paenibacillus larvae*; Sacbrood virus (SBV); and Deformed wing virus (DWV). Also clinical symptoms of Idiopathic Brood Disease Syndrome (IBDS) were noted. Our exposure definition for IBDS was based on Shimanuki et al.'s (1994) work on Parasitic Mite Syndrome (PMS); specifically, the presence of brood at different ages that appear molten to the bottom of cells or may have other symptoms reminiscent of infection with AFB, EFB, or SBV. Unlike PMS however, we diagnosed IBDS based on brood symptoms alone and excluded adult bee symptoms, such as crawling bees, high levels of varroa mites, a rapid decline in adult populations, and increased supersedure rates which are required for the diagnosis of PMS(Shimanuki et al., 1994).

During each inspection the condition of a colony's queen was also assessed. Attempts were always made to find the original marked and clipped queen. In cases where the marked queen was

not found, it was assumed that she was present if eggs were found in the brood nest. A colony was diagnosed as having experienced a "queen "event" if the colony was found to have emergency or superscedure queen cells, a virgin or replacement (unmarked) queen, or was apparently queenless.

At each sampling point, colonies had samples of adult bees removed from a frame of brood and stored in 70% ethanol (~320 bees). . These bees l were used to determine the prevalence of varroa mites in adult bees (Rinderer et al. 2004). Moreover, a sample of 30 worker bees were macerated in 30 ml of water to determine the *Nosema* spp. spore loads (after Cantwell 1970).

2.3 Analysis

Colony survivorship was calculated for each group of colonies at the time of each inspection. To calculate the rate of mortality (% of colonies dying per month), colonies found dead during an inspection were assumed to have died midway between the last inspection (when they were found alive) and the inspection in which they were found dead.

In this study, we sought to quantify the risk of colony mortality associated with exposure to different easily diagnosed risk factors using standard epidemiologic methods (Koepsell and Weiss, 2003). We defined a "case", a basic epidemiological measure, as the death of a colony. We expressed the frequency of cases, or outcomes, as the mortality rate (%), that is the total number of colonies that died over the observation period divided by the total number of colonies monitored over the observation period and multiplied by 100. Similarly, we measured the presence of risk factors, which, unlike mortality can change over time *(e.g*. a colony can have clinical infection with CB during one inspection and not during the next). To account for this variability, we calculated the incident rates (IR) of outcomes for each of the various exposures measured by dividing the number of colonies with a recognized "exposure" by the total number "at risk" over time. For the

purposes of this study, time was represented by the number of inspections. On average, inspections included in this study were conducted at 50-day intervals (see Figure 1).

Comparing incidence rates by risk factor exposure is only meaningful if it is differentially expressed in a population. When some level of exposure to a risk factor is present ubiquitously (or nearly so), a more meaningful measure of exposure is the level of a given risk factor in a population (e.g., varroa mite prevalence or nosema spore load). We considered colonies to have been exposed to varroa mites and nosema spores only when these parasites surpassed a predetermined threshold. We defined this threshold for varroa mites to be greater than 5 mites per 100 adult bees (Genersch et al. 2010) and we considered a colony to have been exposed to nosema when the average spore load surpassed 1 million spores per bee (E. Mussen, personal communication).

A common method to quantify risk from exposure is by calculating the relative risk (RR) of mortality. To standardize the time of exposure we used only data from inspection periods in April-May; June-July; September–October; November-December; and January; see Figure 1).

2.4 Statistics

All statistical analyses were performed using JMP statistical package (SAS, 2007). We used chi-square test to compare total mortality between the different operations and mortality rates. Differences in incidence of mortality by operations were examined by ANOVA. For all other risk factors, we calculated the relative risk of colony mortality during the interval between the occurrence of a risk factor and the subsequent inspection. The statistical significance ($p<0.05$) of relative risk was determined using the Chi-square test, unless fewer than 5 expected or observed cases were noted, in which case Fisher's exact test was used.

3. Results:

3.1 Colony mortality

Fifty-six percent of the colonies died during the 10 months of this study (n = 62). This represents an average mortality rate of 14.9 ± 4.28% (mean ± SE) per 2-month period. This rate was not constant, with the rate of mortality increasing as the study progressed (Figure 2; $\chi^2 = 12.01$, df = 4, P =0.017). Total mortality did not differ among the three operations ($\chi^2 = 1.73$, df = 2, P = 0.42).

3.2 Colony size and parasite loads

Both the number of frames of bees ($F_{5,253} = 18.90$, $P < 0.0001$) and frames of brood ($F_{5,237} = 14.78$, $P < 0.0001$) changed over the course of study. Generally, colonies were largest during the first half of the study and were smallest in November and December (Figure 3). Average varroa prevalence and Nosema spore counts populations also changed over the course of study ($F_{5,256} = 26.26$, $P < 0.0001$ and $F_{5,256} = 3.20$, P =0.008 respectively). While varroa loads reached a peak in the September/October inspection period (Figure 4), Nosema spore loads peaked during the May/June inspection period (Figure 5).

3.3 Incidence Rates and Risk Factor Exposure

Clinical signs of two brood diseases, AFB and EFB, were detected only once over the course of study. AFB was found in one colony during the initial inspection period while EFB was found in another colony during the last inspection in January 2008. Due to their low incidence, these diseases were not considered for further analysis.

Non-surviving colonies had over twice (21.2 vs. 7.9 cases per 100 colonies inspected) the number of queen events when compared to surviving colonies ($F_{1,60} = 7.85$, P =0.007 0.05; Table

1). Overt DWV infection occurred at a higher incidence rate in surviving colonies (10.1 cases per 100 colonies per inspection) than in non-surviving colonies (1.1 cases per 100 colonies per inspection; $F_{1,60}$=11.7, P = 0.001; Table 1). Surviving colonies exceeded the varroa mite threshold of 5% (9.4 cases per 100 inspections) more often than non-surviving colonies (1.1 cases per 100 inspections; $F_{1,60}$=24.3 , P<0.0001). Non-surviving colonies had close to 4 times the number of cases of clinical SBV infections (22.2 cases per 100 inspections) as compared to surviving colonies (5.7 cases per 100 inspections; $F_{1,60}$=10.7 , P=0.0018)The incidence rate was not different for all other risk factors measured (all P > 0.05).

The incidence rate for all risk factors differed among operations except for queen events (15.4 per 100 colonies per inspection; 95% CI = 10.5-20.4), IBD (5.7 per 100 colonies per inspection; 95% CI = 2.7-8.7), and exceeding nosema spore threshold levels (3.5 per 100 colonies per inspection; 95% CI 29.8-40.6; Table 2). The colonies in OP1 had the highest incidence rate of poor brood patterns ($F_{2,59}$ = 21.0, P<0.0001), CB ($F_{2,59}$ = 7.58, P=0.0012), and SBV ($F_{2,59}$ = 12.76, P<0.0001); OP2 had the highest incidence rate for both DWV ($F_{2,59}$ = 4.94, P =0.01) cases and for exceeding the varroa mite infestation threshold ($F_{2,59}$ = 11.0, P < 0.0001; Table 2).

3.4 Relative Risk (RR) of mortality after risk factor exposure

Colonies diagnosed with IBDS were more than 3.8 times (95%CI 1.5-7.0) as likely to die by the next inspection period (50 days later, on average) when compared to colonies without the condition (χ^2 = 6.17, df = 1, P =0.013; Table 1). Colonies diagnosed with a queen event were more than three times (RR=3.1) as likely to die by the next inspection when compared to those without evidence of a queen event (χ^2 = 9.81, df = 1, P = 0.0017;Table 1).

3.5 Relative Risk of remaining or becoming diagnosed with a risk factor after exposure in the previous inspection period

Diagnosis of a poor brood pattern significantly elevated the risk that the colony would have a poor brood pattern (RR = 3.6; $\chi^2 = 23.45$, df = 1, $P < 0.001$), CB (RR=4.9; $\chi^2 = 22.50$, df = 1 $P < 0.0001$), SBV (RR=5.8; Fisher's exact test P = 0.02, and IBDS (RR = 3.6; $\chi^2 = 6.98$, df = 1, P =0.008) at the subsequent inspection. A colony diagnosed with a poor brood pattern had a decreased chance of being found to have varroa mite levels above threshold levels (RR=0.5; $\chi^2 = 6.14$, df = 1, P =0.003) during the next inspection period (Table 3).

Colonies showing clinical signs of CB disease had increased risk of being diagnosed with a poor brood pattern (RR = 2.1; $\chi^2 = 9.87$, df = 1, P = 0.0017), and CB (RR = 3.6; $\chi^2 = 16.99$, df = 1, $P < 0.0001$) during the next inspection (Table 3).

While colonies diagnosis with IBDS were more likely to be diagnosed with CB during the next inspection (RR = 2.8; Fisher's exact test, P=0.031) they were less likely to have nosema spore counts in excess of one million spores per bee during the next inspection (RR = 0.2; Fisher's exact test, P =0.038).

Colonies with varroa mite populations in excess of 5 mites per 100 bees were more likely to be diagnosed with clinical signs of DWV (RR = 5.6, $\chi^2 = 15.5$, df = 1, $P < 0.0001$) during the next inspection period. Conversely, colonies with average nosema spore counts above one million spores per bee were less likely to be diagnosed with DWV during the next inspection (RR = 0.3, Fisher's exact test, P =0.038).

In addition to indicating increased risk that colonies would be dead at the next inspection, a colony diagnosed with a queen event had 3.3 times (95% CI 1.6-6.5) the chance of being re-diagnosed with a queen event in the subsequent inspection ($\chi^2 = 8.64$, df = 1, P =0.003; Table 3).Colonies in which clinical signs of DWV were observed were more likely to have DWV re-

diagnosed (RR=6.6; $\chi^2 = 12.8$, df = 1, P = 0.003) when next inspected. Conversely, no colonies observed to have symptomatic DWV infections had varroa mite population exceeding threshold levels when next inspected (RR= 0; Fisher's exact test, P < 0.001; Table 3).

Colonies with average Nosema spore loads above 1 million spores per bee had decreased incidence of DWV (Fisher's exact test, P < 0.01) in the subsequent inspection period (Table 3).

4. Discussion

This study set out to quantify the impact that easily defined measures of risk on colony mortality and subsequent and persistent morbidity in migratory honey bee colonies. To that end, we found that, over the short term (~50 days), the presence of IBDS and evidence of a queen event increased the risk that a colony would die by 3.8 and 3.1 times respectively. We also found that exposure to several different factors increased the risk that a colony would be diagnosed with another risk factor or re-diagnosed with the same risk factor in the subsequent inspection (see Table 3). For instance, a colony with a poor brood pattern had an increased risk of being re-diagnosed with a poor brood pattern (RR=2.5), of being diagnosed with CB (RR=4.9), SBV (RR=5.8), and IBDS (RR=3.6), while having a decreased risk of being diagnosed with Varroa levels above threshold (RR=0.5) during the next inspection.

The complexity of the picture that emerges supports a growing consensus that causes of honey bee colony mortality and morbidity are multiple and interrelated (Genersch and Evans 2010, Genersch et al. 2010, Neumann and Carreck 2010, vanEngelsdorp et al. 2010b). However, this study is the first that quantifies the mortality and subsequent and persistent morbidity risk associated with exposure to certain risk factors using epidemiological methods.

Colonies diagnosed with IBDS were nearly 4 times more likely to die by the next inspection period compared to colonies without this condition. This was the most pronounced

measure of mortality risk recorded in this study. As outlined in the methods section, our case definition for determining "exposure" to this condition were based on the work of Shimanuki et al. (1994); specifically, the presence of brood at different ages that appear molten to the bottom of cells or may have other symptoms reminiscent of infection with AFB, EFB, or SBV while ignoring those symptoms of PMS that included mite levels or symptoms in adult bee population. As the name for PMS suggests, mites (specifically varroa mites) are thought to play a direct role in this condition. Our findings, as it relates to the symptoms in brood (called here IBDS), do not support this association; suggesting that cause of the characteristic "snotty brood" associated with PMS is a distinct condition in itself. Indeed, even the reports that initially described PMS suggested that the role of mites was likely secondary to the symptoms described (Shimanuki et al. 1994, Hung et al. 1996). Mites may play a role in creating PMS brood symptoms (IBDS) by acting as a vector for a causative agent or because mite feeding somehow activates asymptomatic infections (Hung et al. 1996). Attempts to isolate a single causative agent in symptomatic brood removed from PMS suffering colonies have failed, although viruses such as acute bee paralysis virus (ABPV) and Kashmir bee virus (KBV) have been implicated (Hung et al. 1996). If IBDS is indeed a symptom of viral infection, once established, its persistence in colonies with low varroa mite pressure is not surprising considering that viruses are able to persist in colonies even when mite levels remain low or after they have been controlled with chemical treatments (Highfield et al. 2009, Martin et al. 2010).

This study identified "queen events" as a leading factor in colony mortality. Not only was the relative risk of mortality increased in colonies diagnosed with a queen event significant, the incidence rate of queen events was nearly twice as high in colonies that died by the end of the study when compared to those colonies that had not died (Table 1). Beekeepers themselves have consistently self identified queen failure as a leading cause of winter mortality in recent winter loss

surveys (Brodschneider et al., 2010; vanEngelsdorp et al., 2010), and this study corroborates beekeeper suspicions. Additional studies are necessary to elucidate the underlying causes of queen events and the mechanism that governs its apparent association with increased colony mortality.

As previously mentioned, poor brood pattern (e.g., more than 20% of capped brood missing) indicated that colonies were of increased risk to be subsequently diagnosed with CB, SBV, IBDS, of being re-diagnosed with a poor brood pattern, and of having a decreased risk of being diagnosed with varroa levels above threshold (Table 3). There are many potential causes of a spotty brood pattern, including cannibalism of diploid drone larvae because of homozygosity at the *csd* locus (see Tarpy and Page 2000), larval death due to pesticide poisoning (Pettis et al. Submitted), and worker bee hygienic behavior which removes diseased or dead larvae (Gilliam et al. 1983, Boecking and Spivak 1999). It maybe that colonies diagnosed with a poor brood pattern are successfully removing diseased larvae infected with the agents causing disease (e.g. CB and SBV), and so, in some cases poor brood patterns may simply indicate that colonies are able to keep infections below levels needed for clinical infections to be diagnosed. Further, hygienic behavior is also known to reduce varroa populations as bees remove varroa when removing parasitized brood (Spivak and Reuter 2001). This may explain why colonies with poor patterns were half as likely to have varroa level surpassing threshold levels in the subsequent inspection period as measured in this study.

Comparing the incident rates of risk factors between different groupings of colonies also proved insightful. First, the difference in incidence rates in the three different beekeeping operations (Table 2) was notable. Many causes for these differences are possible, including differences in management practices, colony genetics, risk factor exposure, and the environment. While our study was not designed to identify the causes for these differences, this finding does support the growing consensus that many factors contribute to colony mortality and that studies to

understand these causes may need to be equally diverse (Williams et al. 2010, vanEngelsdorp et al. in press).

Also notable were the significant difference in incident rates of queen events, SBV, DWV and colonies exceeding threshold levels for varroa mites between surviving and non-surviving colonies. That queen events occurred 2.6 times more frequently in non-surviving colonies is not surprising considering the significant relative risk associated with this condition and the relative frequency of its diagnosis. The potential causes for queen failure are multiple, and include pathogen load (Loskotova et al. 1980, Camazine et al. 1998), pesticide exposure (Pettis et al. 2004) and mating number (Tarpy et al. unpublished data; see also Richard et al. 2007). Considering the pronounced effect queen events have on colony survivorship, studies specifically designed to measure and address possible interactions between risk factors and queen replacement are needed.

Over the long term, non-surviving colonies had nearly 4 times the incident rate of SBV when compared to surviving colonies. Unlike queen events, however, we did not observe increased risk of mortality in colonies over the short term (as indicated by a non-significant RR; Table 1). This suggests that the effects of SBV on colony health may be sub-lethal or that clinical infection with SBV maybe a consequence of some other sub-lethal factor which compromise colony health such as pesticide exposure or poor nutrition, both factors which are known to contribute to a compromised bee immune system (Alaux et al. 2010b, Alaux et al. 2010a). It is important to note that this study was not designed to look at sub-lethal effects (e.g. decreased productivity) of SBV or other risk factors measured. Therefore, our inability to detect negative consequences of exposure to certain risk factors is not equivocal to saying these risk factors had no effect on colony health.

The incident rate of DWV infection occurred at over 9 times the rate in surviving colonies as compared to non-surviving colonies while varroa exceeded threshold levels at over 8 times the

rate in surviving colonies as compared to non-surviving colonies. At first glance these findings are counter intuitive, as they could be interpreted to imply that DWV and high mite levels were beneficial to colony health. This is unlikely to be the case, as there is broad and compelling evidence that both varroa and DWV have negative effects on both individual bee and colony health (Korpela et al. 1992, Highfield et al. 2009, Chauzat et al. 2010a, Chauzat et al. 2010b, Genersch et al. 2010, Guzmán-Novoa et al. 2010, le Conte et al. 2010, Martin et al. 2010, Schäfer et al. 2010). A more probable explanation for higher incidence of these two factors in surviving colonies can be inferred after considering the population dynamics of varroa in observed colonies (Figure 4). Average varroa mite populations were highest in the September/October sampling period, and by this time 40% of the colonies that would die in the study had already died. Thus, many non-surviving colonies simply had not survived long enough to have mite levels surpass threshold levels. The same explanation potentially explains increased DWV rates as well. Both this and other studies have shown a linkage between high varroa mite levels and DWV infection (Table 3,(Bowen-Walker et al. 1999, Highfield et al. 2009). Indeed in this study we diagnosed 27 cases of colonies with clinical infections of DWV, however only 5 of these cases were diagnosed before the September-October inspection period (data unpublished). Again, only 60% of those colonies that would eventually die were left in the study population during this period. Had this study been larger, or had it continued for a longer period the negative effects of these factors may have been detected.

To conclude, this study used epidemiological methods to quantify the risk of exposure from several easily diagnosed factors on colony mortality and morbidity. Systematic epidemiologic methods have long been used to quantify risk for human and domestic animal mortality. As demonstrated by this study these methods hold promise for understanding the risk of bee populations as well. Specifically, this study identified two risk factors that were predictive of

colony mortality over the short term: queen events, and IBDS. The ease at which these conditions can be accurately diagnosed in colonies makes them excellent monitoring tools for beekeepers attempting to assess the health of their operations. Unfortunately, the underlying causes of these conditions are poorly understood and our findings suggest that previous assumptions—such as the putative role of varroa mites in IBDS—may be incorrect. These results add to the growing body of work that suggests that the causes of colony mortality and morbidity are multiple and complex. While further epidemiological studies are needed to help verify these findings, hypothesis-driven research specifically aimed at trying to understand the causes of queen failure and IBDS should be prioritized.

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General Summary:

Using standard epidemiological methods, this study set out to quantify the risk associated with exposure to easily diagnosed risk factors on colony mortality and morbidity in three migratory beekeeping operations. Fifty-six percent of all colonies monitored during the 10-month period died. The relative risk (RR) that a colony would die over the short term (~50 days) was appreciably increased in colonies diagnosed with a “queen event” (evidence of queen replacement or failure; RR=3.1) and with Idiopathic Brood Disease Syndrome (IBDS, the brood condition associated with

parasitic mite syndrome; RR =3.2). We also found that several risk factors, including the incidence poor brood pattern, chalkbood (CB), deformed wing virus (DWV), sacbrood virus (SBV), and exceeding varroa threshold of 5 mites per 100 bees were differentially expressed in different beekeeping operations. Further we found that a diagnosis of several risk factors increased and/or decreased the risk that colonies would be re-diagnosed with the same (poor brood pattern (RR=2.5), CB (RR=3.7), DWV (RR =6.6) and Queen events (RR=3.3)) or another risk factor (*e.g.* poor brood patterns and CB (RR=4.9), SBV (RR=5.8), IBDS (RR=3.6) and exceeding varroa threshold (RR=0.5)) during the next inspection. These results confirm the growing consensus that the causes of colony mortality are multi-factorial and complex.

Figure 1: Timeline of colony inspection over the course of the study. Placement of colonies on various floral sources is indicated (boxes), as are the times colonies were inspected (starbursts). Data from inspections collected at times indicated by solid star bursts were considered for calculating all case incidence rates, however, in an attempt to equalize "exposure time", only data from inspection periods indicated by large starburst were included for calculating relative risk variables and for comparing colony measures and disease prevalence over time. The period of time between these inspections is indicated within the starbursts and represent days between inspections (see text for details).

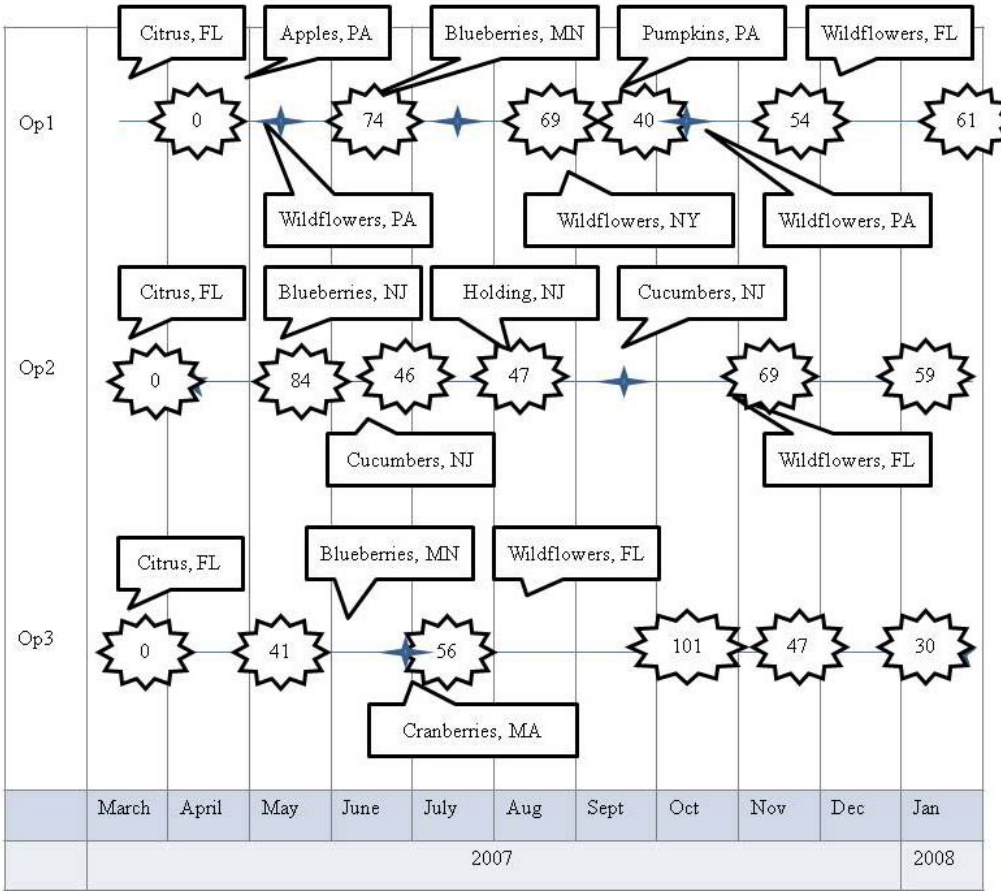

Figure 2: Rate of colony mortality over the course of the study was not equal.

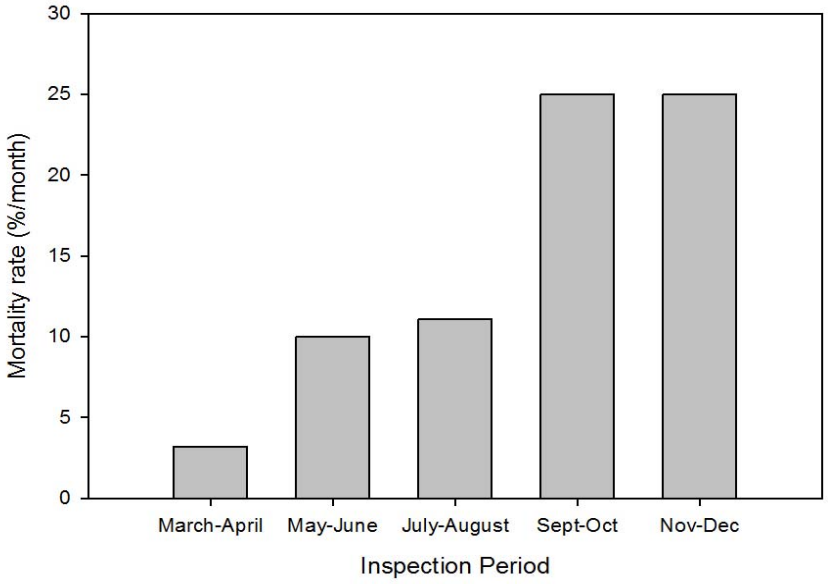

Figure 3: Mean number of frames of bees and brood in surviving colonies over the course of study.

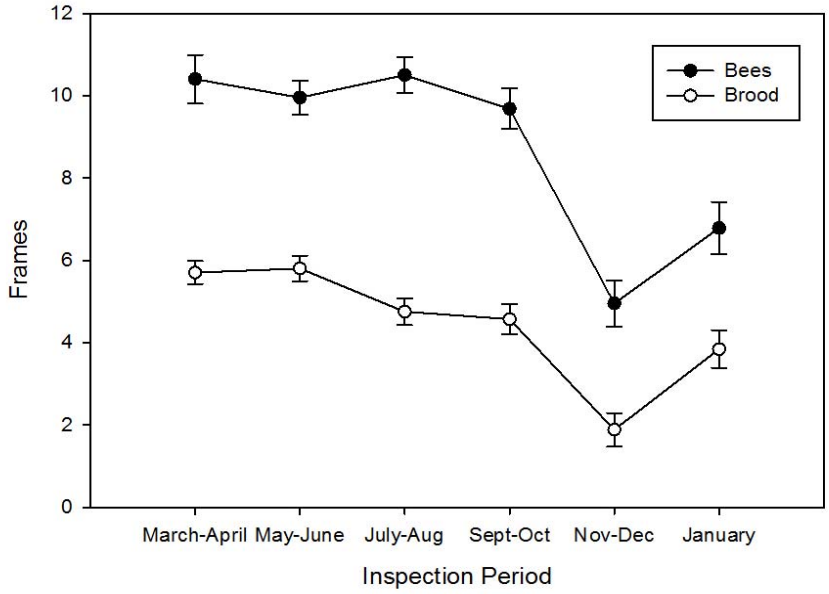

Figure 4: Average mite infestation in monitored colonies over the course of study.

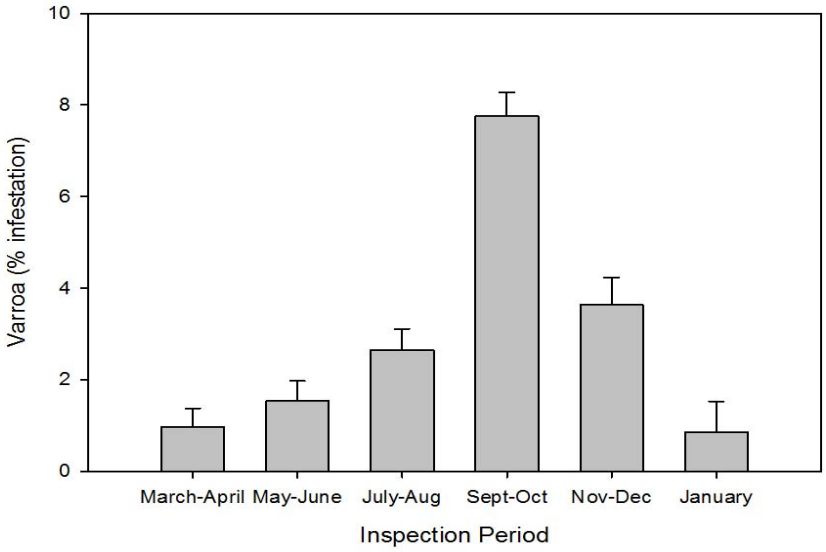

Figure 5: Average Nosema sp. spore load in colonies over the course of study.

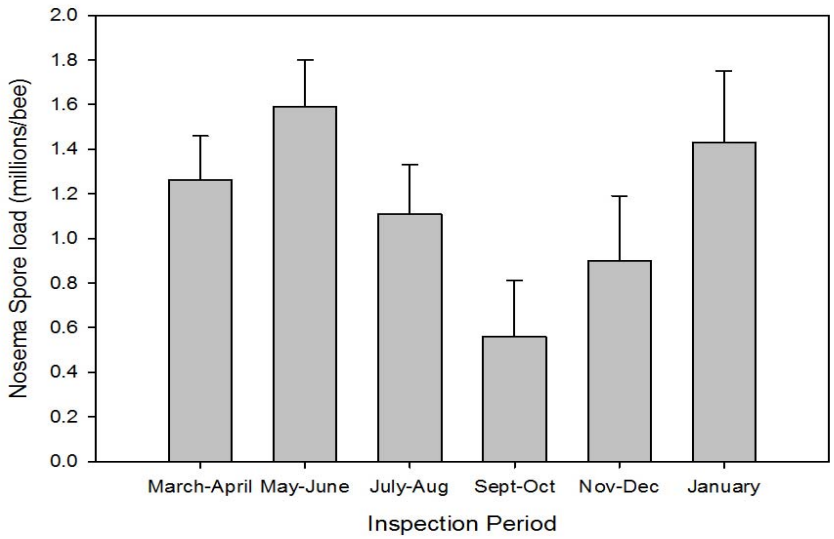

Table 1: A comparison of the Incidence Rates in surviving and non-surviving colonies and the Relative Risk of mortality after a diagnosis with a certain risk factor during the previous inspection. Differences in incident rates are indicated by different letters in the same row ($P<0.05$).

	Incidence Rates X (95%CI) cases per 100 colonies inspected		Relative Risk
	Surviving Colonies	Non-Surviving Colonies	
n	27	35	
Risk Factor			
Brood Condition/Disease			
Pattern†	31.4 (4.9-21.5)	26.8 (4.3-18.1)	1.6 (0.77-3.23)
Chalkbrood	11.9 (4.3-19.7)	8.4 (1.7-15.1)	1.1 (0.41-2.97)
Sacbrood	5.7 (0.0-13.30)b	22.2 (15.5-29.0)a	0.9 (0.85-0.92)
IBDS	3.9 (0.0-8.40)	7.1 (0.3-11.1)	3.2 (1.15-6.97)*
Adult Bee Disease			
DWV	10.1 (0.06-14.1)a	1.1 (0.0-4.7)b	0.6 (0.08-3.95)
Varroa‡	9.4 (6.8-11.9)a	1.1 (0.0-3.2)b	1.0 (0.42-2.19)
Nosema spore$^{\beta}$	35.2 (26.9-43.5)	35.1 (27.9-42.4)	1.4 (0.73-2.56)
Queen			
Queen event	7.9 (0.1-15.1)b	21.2 (15.0-27.5)a	3.1 (1.67-5.80)*

†Poor brood pattern as indicated by ≥20% of capped cells missing
‡Varroa mite threshold of 5 mites per adult bee exceeded
β Nosema spore load of 1 million spores per bee
For IR rates, differences between surviving and non-surviving colonies are indicated by different letters.
*Indicates significant RR ($P<0.05$).

Table 2: Incidence rates for risk factors which differed (P<0.05) between operations. Differences are indicated by different letters within the same rows.

	Incidence Rate X(95% CI) cases 100 colonies per inspection		
Factor	OP1	OP2	OP3
n	20	24	18
Pattern†	52.3 (43.4-61.3)a	20.0 (11.9-28.1)b	14.4 (5.0-23.8)b
CB	21.1 (13.9-29.1)a	0.0 (0-7.3)b	10.8(0.2-19.3)ab
DWV	0.0 (0.0-4.7)b	10.6 (5.7-14.4)a	4.1 (0.0-9.1)ab
SBV	29.6 (21.5-37.7)a	1.9 (0.0-9.3)c	2.2 (0-5.4)b
Varroa‡	0.8 (0-3.7)b	9.7 (7.1-12.3)a	2.2 (0-5.2)b

†Poor brood pattern as indicated by ≥20% of capped cells missing

‡Varroa mite threshold of 5 mites per adult bee exceeded

Table 3: Relative risk of being diagnosed or being re-diagnosed with a specific risk factor during the next inspection period after being diagnosed with a risk factor during an inspection.

	Relative Risk (RR (95% CI))of Risk Factor Diagnosis during next inspection							
	Risk Factor							
Risk Factor Exposure	Pattern†	Chalkbrood	Sacbrood	IBDS	DWV	Varroa‡	Nosemaβ	Queen Event
Brood Condition/Disease								
Pattern†	2.5 (1.7-3.5)*	4.9 (2.4-9.8)*	5.8(1.2-2.8)*	3.6(1.4-9.6)*	0.9(0.37-2.4)	0.5 (0.3-0.9)*	0.9 (0.6-1.3)	0.7 (0.3-1.7)
Chalkbrood	4.9 (2.4-9.8)*	3.7 (2.0-6.7)*	2.4(0.5-11.8)	2.7(0.9-7.9)	0.4(0.1-3.1)	0.4 (0.1-1.3)	1.0 (0.7-1.7)	1.0 (0.3-3.0)
Sacbrood	0.5 (0.1-3.1)	1.8 (0.5-6.4)	0	2.6 (0.4-16.8)	0	0.5 (0.1-3.6)	0.2 (0.0-2.4)	0
IBDS	0.5 (0.1-1.7)	2.8 (1.3-6.2)*	1.1(0.28-4.56)	1.2 (0.2-8.3)	1.1 (0.51-7.6)	0.3 (0.04-2.0)	0.2 (0.03-0.4)*	0.7 (0.1-4.6)
Adult Bee Disease								
DWV	1.0 (0.5-2.2)	0	0	1.0(0.1-7.4)	6.6 (2.9-14.7)*	0*	0.7 (0.3-1.8)	0
Varroa‡	1.2 (0.7-1.8)	0.8 (0.3-1.9)	0.6(0.1-4.5)	0.3 (0.04-2.1)	5.6(2.5-17.7)*	1.2 (0.6-2.1)	1.2 (0.8-1.8)	1.0 (0.4-2.4)
Nosema sporeβ	1.0 (0.7-1.5)	1.2 (0.7-2.3)	0.3(0.09-1.0)*	1.3 (0.5-3.2)	0.8(0.27-2.3)	0.7 (0.4-1.2)	1.1 (0.8-1.6)	1.6 (0.8-3.1)
Queen								
Queen event	1.0 (0.5-1.8)	1.9 (0.9-4.1)	0	0.5 (0.1-3.0)	0	0.7 (0.23-1.8)	1.2 (0.8-2.0)	3.3 (1.6-6.5)*

*indicates significant ($P<0.05$) relative risk

†Poor brood pattern as indicated by ≥20% of capped cells missing

‡Varroa mite threshold of 5 mites per adult bee exceeded

β Nosema spore load of 1 million spores per bee

References

Aizen, M. A., and L. D. Harder. 2009. The Global Stock of Domesticated Honey Bees is Growing Slower then Agricultural Demand for Pollination. Current Biology 19: 915-918.

Aizen, M. A., L. A. Garibald, S. A. Cunningham, and A. M. Klein. 2008. Long-term global trends in crop yield and production reveal no current pollination shortage but increasing pollinator dependency. Current Biology 18: 1572.

Alaux, C., François Ducloz, Didier Crauser, and Y. L. Conte. 2010a. Diet effects on honeybee immunocompetence. Environmental Microbiology 12: 774-782.

Alaux, C., J. L. Brunet, C. Dussaubat, F. Mondet, S. Tchamitchan, M. Cousin, J. Brillard, A. Baldy, L. P. Belzunces, and Y. Le Conte. 2010b. Interactions between Nosema microspores and a neonicotinoid weaken honeybees (Apis mellifera). Environmental Microbiology 12: 774-782.

Boecking, O., and M. Spivak. 1999. Behavioral defenses of honey bees against *Varroa jacobsoni* Oud. Apidologie 30: 141-158.

Bowen-Walker, P. L., S. J. Martin, and A. Gunn. 1999. The transmission of deformed wing virus between honeybees (Apis mellifera L.) by the ectoparasitic mite Varroa jacobsoni Oud. Journal of Invertebrate Pathology 73: 101-106.

Brodschneider, R., R. Moosbeckhofer, and K. Crailsheim. 2010. Surveys as a tool to record winter losses of honey bee colonies: a two year case study in Austria and South Tyrol. Journal of Apicultural Research 49: 23-30.

Camazine, S., I. Çakmak, K. Cramp, J. Finley, J. Fisher, M. Frazier, and A. Rozo. 1998. How healthy are commercially-produced US honey bee queens? American Bee Journal 138: 677-680.

Cantwell, G. E. 1970. Standard methods for counting nosema spores. American Bee Journal 110: 222.

Chauzat, M., A. Martell, S. Zeggane, P. Drajnudell, F. Schurrl, M. Clementi, M. Ribiere-Chabertl, M Aubert, and J. Fauconl. 2010a. A case control study and a survey on mortalities of honey bee colonies (Apis mellifera) in France duringthe winter of 2005-6. Journal of Apicultural Research 49: 40-51.

Chauzat, M., P. Carpentier, F. Madec, S. Bougeard, N. Cougoule, P. Drajnudel, M. Clément, M. Aubert, and J. Faucon. 2010b. The role of infectious agents and parasites in the health of honey bee colonies in France. Journal of Apicultural Research 49: 31-39.

Currie, R. W., S. F. Pernal, and E. Guzmán-Novoa. 2010. Honey bee colony losses in Canada. Journal of Apicultural Research 49: 104-106.

DeGrandi-Hoffman, G., Gordon Wardell, Fabiana Ahumada-Segura, Thomas Rinderer, Robert Danka, and J. Pettis. 2008. Comparisons of pollen substitute diets for honey bees: consumption rates by colonies and effects on brood and adult populations. Journal of Apicultural Research 4: 126-130.

Genersch, E., and J. Evans. 2010. Honey bee disease overview. Journal of Invertebrate Pathology 103: S2-S3.

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

Ghazoul, J. 2005. Buzziness as usual? Questioning the global pollination crisis. Trends in Ecology & Evolution 20: 367-373.

Gilliam, M., S. Taber, III, and G. V. Richardson. 1983. Hygienic Behavior of Honey Bees Apis-Mellifera in Relation to Chalkbrood Disease. Apidologie 14: 29-40.

Guzmán-Novoa, E., L. Eccles, Y. Calvete, J. McGowan, P. G. Kelly, and A. Correa-Benítez. 2010. Varroa destructor is the main culprit for the death and reduced populations of overwintered honey bee (Apis mellifera) colonies in Ontario, Canada. Apidologie 41: 443-450.

Haubruge, E., B. K. Nguyen, J. Widart, J.-P. Thomé, P. Fickers, and E. Depauw. 2006. Le dépérissement de l'abeille domestique, *Apis mellifera* L., 1758 (Hymenoptera : Apidae) : faits et causes probables. Notes fauniques de Gembloux 56: 3-21.

Highfield, A. C., A. El-Nagar, L. C. M. MacKinder, L. M. L. J. NoÃ«l, M. J. Hall, S. J. Martin, and D. C. Schroeder. 2009. Deformed wing virus implicated in overwintering honeybee colony losses. Applied and Environmental Microbiology 75: 7212-7220.

Hung, A. C. F., H. Shimanuki, and D. A. Knox. 1996. The role of viruses in bee parasitic mite syndrome. American bee journal 136: 731-732.

Klein, A. M., B. E. Vaissière, J. H. Cane, I. Steffan-Dewenter, S. A. Cunningham, C. Kremen, and T. Tscharntke. 2007. Importance of pollinators in changing landscapes for world crops. Proceedings of the Royal Society of London. Series B, Biological Sciences 274: 303-313.

Koepsell, T. D., and N. S. Weiss. 2003. Epidemiologic methods: studying the occurrence of illness. Oxford University Press, New York.

Korpela, S., A. Aarhus, I. Fries, and H. Hansen. 1992. *Varroa jacobsoni* Oud. in cold climates: population growth, winter mortality and influence on the survival of honey bee colonies. Journal of Apicultural Research 31: 157-164.

le Conte, Y., M. Ellis, and W. Ritter. 2010. Varroa mites and honey bee health: can Varroa explain part of the colony losses? Apidologie 41: 353-363.

Loskotova, J., M. Peroutka, and V. Vesely. 1980. Nosema disease of honeybee queens (Apis mellifica L.). Apidologie 11: 153-161.

Martin, S. J., B. V. Ball, and N. L. Carreck. 2010. Prevalence and persistence of deformed wing virus (DWV) in untreated or acaricide-treated Varroa destructor infested honey bee (Apis mellifera) colonies. Journal of Apicultural Research 49: 72-79.

Neumann, P., and N. L. Carreck. 2010. Honey bee colony losses. Journal of Apicultural Research 49: 1-6.

Nguyen, B. K., R. Van der Zee, F. Vejsnæs, S. Wilkins, Y. Le Conte, and W. Ritter. 2010. COLOSS Working Group 1: monitoring and diagnosis. Journal of Apicultural Research 49: 97-99.

Nguyen, B. K., J. Mignon, D. Laget, D.C. de Graaf, F. J. Jacobs, D. vanEngelsdorp, Y. Brostaux, C. Saegerman, and E. Haubruge. in press. Honey bee colony losses in Belgium during the 2008-2009 winter. Journal of Apicultural Research.

NRC. 2006. Status of Pollinators in North America, pp. 317. National Academy of Sciences, Washington, D.C.

Pettis, J., K. Wilzer, and M. Feldlaufer. Submitted. The effects of coumaphos on worker longevity in the honey bee.

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

Potts, S. G., S. P. M. Roberts, R. Dean, G. Marris, M. A. Brown, R. Jones, P. Neumann, and J. Settele. 2010. Declines of managed honey bees and beekeepers in Europe. Journal of Apicultural Research 49: 15-22.

Richard, F.-J., D. R. Tarpy, and C. M. Grozinger. 2007. Effects of Insemination Quantity on Honey Bee Queen Physiology. PloS ONE 2: e980.

Rinderer, T., L. De Guzman, and H. A. Sylvester. 2004. Re -examination of the accuracy of a detergent solution for varroa mite detection. American Bee Journal 144: 560-562.

Schäfer, M. O., W. Ritter, J. S. Pettis, and P. Neumann. 2010. Winter losses of honeybee colonies (Hymenoptera: Apidae): the role of infestations with Aethina tumida (Coleoptera: Nitidulidae) and Varroa destructor (Parasitiformes: Varroidae). Journal of Economic Entomology 103: 10-16.

Shimanuki, H., N. W. Calderone, and D. A. Knox. 1994. Parasitic mite syndrome: the symptoms. American Bee Journal 134: 827-828.

Spivak, M., and G. S. Reuter. 2001. Varroa destructor infestation in untreated honey bee (Hymenoptera: Apidae) colonies selected for hygienic behavior. Journal of Economic Entomology 94: 326-331.

Tarpy, D. R., and R. E. J. Page. 2000. No behavioral control over mating frequency in queen honey bees (Apis mellifera L.): Implications for the evolution of extreme polyandry. American Naturalist 155: 820-827.

vanEngelsdorp, D., and M. D. Meixner. 2010. A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. Journal of Invertebrate Pathology 103: S80-S95.

vanEngelsdorp, D., J. Hayes, Jr., R. M. Underwood, and J. Pettis. 2008. A survey of honey bee colony losses in the U.S., Fall 2007 to Spring 2008. PLoS ONE 3: e4071.

vanEngelsdorp, D., J. Hayes Jr, R. M. Underwood, and J. S. Pettis. 2010a. A survey of honey bee colony losses in the United States, fall 2008 to spring 2009. Journal of Apicultural Research 49 7-14.

vanEngelsdorp, D., J. Hayes Jr, R. M. Underwood, D. Caron, and J. S. Pettis. 2011. A Survey of managed honey bee colony losses in the U.S., fall 2009 to winter 2010. Journal of Apicultural Research 50: 1-10.

vanEngelsdorp, D., J. Hayes Jr, R. M. Underwood, D. Caron, and J. S. Pettis. in press. A Survey of managed honey bee colony losses in the U.S., fall 2009 to winter 2010. Journal of Apicultural Research.

vanEngelsdorp, D., N. Speybroeck, J. Evans, B. K. Nguyen, C. Mullin, M. Frazier, J. Frazier, D. Cox-Foster, Y. Chen, D. R. Tarpy, E. Haubruge, J. S. Pettis, and C. Saegerman. 2010b. Weighing risk factors associated with bee Colony Collapse Disorder by classification and regression tree analysis. Journal of Economic Entomology 103: 1517-1523.

Vejsnæs, F., S. L. Nielsen, and P. Kryger. 2010. Factors involved in the recent increase in colony losses in Denmark. Journal of Apicultural Research 49: 109-110.

Williams, G. R., D. R. Tarpy, D. vanEngelsdorp, M. P. Chauzat, D. L. Cox-Foster, K. S. Delaplane, P. Neumann, J. S. Pettis, R. E. L. Rogers, and D. Shutler. 2010. Colony Collapse Disorder in context. BioEssays 32: 845-846.

Chapter 7:

THE USE OF EPIDEMIOLOGICAL METHODS TO DESCRIBE AND HELP ELUCIDATE THE CAUSES OF MORBIDITY AND MORTALITY IN HONEY BEE COLONIES.

Introduction:

Managed honey bee (*Apis mellifera* L.) populations have steadily declined in the Unites States over the last 60 years (NRC 2006). The causes for these losses are multiple and complex (vanEngelsdorp and Meixner 2010; CHAPTER 2), and are of particular concern considering the importance of honey bees as pollinators of many agricultural crops. An estimated 35% of the western human diet benefits, directly or indirectly, from honey bee pollination (Klein et al., 2007).

Considering the honey bees' vital role in the commercial production of many crops, it is somewhat surprising that the steady decline in colony numbers was largely ignored until the unusually high losses reported in the US over the winter of 2006-2007 (vanEngelsdorp et al. 2007). Since then in the US, heavy overwintering losses have been documented every winter (vanEngelsdorp et al. 2008, vanEngelsdorp et al. 2010a, vanEngelsdorp et al. 2011; CHAPTER 3). The causes of these elevated rates of winter loss are almost certainly multiple and are likely interrelated. Notably too, not only did the magnitude of losses differ between different segments of the apicultural industry (e.g. those pollinating almonds verses those who did not), but the self-identified causes for winter losses also differed (e.g. large commercial operations verses back yard beekeepers) (vanEngelsdorp et al. 2011). It is precisely the apparent complex nature of

colony losses that makes the use of epidemiological methods a valuable tool when trying to elucidate causes of morbidity and mortality in honey bee colonies.

Epidemiology, strictly speaking, is the study of the distribution of disease in human populations (Morton et al. 1990); however, the term has been widely adopted by those who study disease occurrence in animal and plant populations too (Nutter 1999). Regardless of the population of interest, the basic assumption that underlies all epidemiological studies is that disease is not randomly distributed within a population. Therefore, by characterizing differences in the frequency and/or types of disease found between groups of individuals within a population, or within the same group over time, an epidemiological approach aims to identify factors which may explain or contribute to disease outbreak. Once identified, these factors can guide clinical etiological studies, and also lay the foundation for disease prevention and control programs (Mausner and Kramer 1985). It is important to note that from the point of view of an epidemiologist "disease" is defined broadly: it is any departure from perfect health (Woodward 2005).

At their core epidemiological studies describe the distribution of disease within a population, and epidemiologists have developed many standardized ways to express rates of disease. The most basic of these rates, mortality rate, summarizes the proportion in a given population that have succumbed to the most serious departure from health possible – death. A basic epidemiological study would calculate mortality rates and the corresponding 95% Confidence Interval (95% CI) for different groups within a population in hopes of discovering informative differences. This basic approach can also be used when describing mortality rates in managed honey bee colonies, however, several features of the apicultural industry make calculating and summarizing mortality rates in managed honey bee colonies uniquely challenging.

Calculating colony losses:

Since the reporting of unusually high losses in the US and Europe in the winter of 2006-2007, there have been sustained efforts to tabulate the level of loss that occurs over the winter months. It is important to note that these losses do not necessarily translate into a drop in the total colonies managed in a country as determined by national census efforts. Beekeepers can replace winter losses by either buying bees (packages) or colonies from other producers, or by splitting their surviving colonies to make replacement colonies. Splitting is a process by which beekeepers take brood and bees from surviving colonies and adds a queen. Beekeepers are able to make one or more replacement colonies from one surviving colony. It is not unreasonable to expect a beekeeper that lost 30% or more of his or her colonies to have replaced all the dead colonies within one season. The activity of replacing lost colonies is not without cost. Splitting or buying colonies has real and significant direct (the price of queens or packaged bees) and indirect (lost productivity) costs. Attempts to gauge the health of the apicultural industry must not be limited to simply counting colonies at a given time each year (vanEngelsdorp and Meixner 2010), but need to include monitor winter losses. These efforts, in turn, may help us understand the factors underlying the cause or causes of losses (Nguyen et al. 2010).

Quantifying colony losses is a two part process. First, a survey must be designed, distributed, and responses collected. Secondly, the results must be tabulated and reported in standardized, transparent, and accurate ways. Both the questions asked and selection of survey respondents has not been uniform among the various recent national winter loss survey efforts. These differences make comparing results between efforts difficult. While a detailed analysis of these various approaches is beyond the intent of this discussion, it is clear that a more standardized approach would be of benefit. To this end, a working group of an international bee researchers with the acronym COLOSS (Prevention of Honeybee COLony LOSSes) has recently

released a "model" winter loss survey which includes a set of essential standardized winter loss questions. The international adoption of these questions should help make colony loss results more comparable. Furthermore, the standardization of winter loss survey questions permits the establishment of standardized winter loss calculations and reporting. Providing guidelines on how loss numbers should be calculated and reported should facilitate transparent and easy comparisons between different winter loss survey efforts.

When reporting colony loss figures, there are two different ways that losses should be reported; total colony losses, which are an aggregate of all losses suffered by all respondents, and average loss, which is the mean of the total losses suffered by each responding beekeeper. The utility of these two figures differ, in that both are intrinsically and unavoidably biased. This bias is the result of the demographics of the apicultural industry, where a relatively few members (commercial beekeepers) own the vast majority of colonies while members who make up the vast majority of the industry own a small percentage of the total colonies (Daberkow et al. 2009). As explained in detail below, total loss figures are more heavily influenced by the losses experienced by the few large operations, while average losses are more representative of the many small operations.

Calculating Total Colony Losses:

Total colony loss figures are the percent of all colonies lost in a defined group over a defined period of time. Total colony losses are calculated using the same equation used by epidemiologists to calculate mortality; e.g. Equation 1, where TL = total losses over a period, td = total colonies that died over a given period, and tc = the total number of monitored colonies that are at risk of dying over the period (Koepsell and Weiss 2003).

Equation 1:

$$TL = \left(\frac{td}{tc}\right) X\ 100\%$$

In colony loss surveys conducted using the COLOSS standardized questionnaire in 2010 (e.g.vanEngelsdorp et al. 2011), the data needed to make this calculation are derived, in part, from survey responses to two questions: 1) how many colonies did you have on October 1, 2009, and 2) how many colonies did you have on April 1, 2010. To account for the beekeeper practice of either selling, giving away, buying or making additional colonies over the winter period, calculating the *tc* must be adjusted using information derived from two additional essential questions 3) how many colonies did you sell or give away over the period between Oct 1 and April 1, and 4) how many colonies did you make or buy over the period? Thus, the total number of monitored colonies at risk of dying over the period (tc) is given by equation 2, where the total colonies at the beginning of the survey period (tcb) is the number of colonies alive on Oct 1 minus any colonies sold or given away over the period (tcs) plus total colony number increases (tci), e.g. colonies bought or made through splitting over the period.

Equation 2:

$$tc = tcb - tcs + tci$$

The total colonies that died over the period (*td*; equation 3), is then calculated by determining the difference between the total number of monitored colonies at risk of dying over the period (*tc*) and the total number of living colonies at the end of the period (*te*), e.g. April 1st.

Equation 3:

$$td = tc - te$$

In some previous reports, colony losses were not adjusted for changes in the colony counts that result from selling, buying, or increasing colony numbers by splitting (Brodschneider et al. 2010). Such an approach may have minimal effect on total loss calculations in regions where changes in colony numbers through management or commerce are not commonly practiced over the winter months (tcs << tc and tci << tc) or in regions where the total colonies sold is almost equal to the total colony numbers increases (tcs ≈ tci). For instance in Belgium, 14.3% (n=238) of responding beekeepers reported having sold or bought colonies over the 2009-2010 survey period (Nguyen et al. in press). The total number of colonies these beekeepers bought and sold make up a relatively small portion of the total colonies monitored (8.2%). Further, the difference in the number of colony decreases (colonies sold or given away) and the number of colony increases (splits or purchases) is relatively small (n = 53, or 1.7% of tc). Should the total loss calculations disregard the number of colonies bought or sold, the effect on total loss calculations would be minimal, changing the national total loss from 27.8% to 26.6 %. However, disregarding the number of colonies bought, given, sold, or increases made by beekeepers in regions where these practices are common can have pronounced effects on total loss calculations. In the 2010 US winter loss survey (vanEngelsdorp et al. 2011), a minority of responding operations (16.5%, n = 4,227) reported having sold, bought, or made increases in their operations during the survey period. However, while the number of colonies sold or given away in the period was minimal (n = 8,086 colonies or 1.4% of tc) the number of colonies bought or made during the period was substantial, totaling 135,837 colonies, or 23.7% of the total colonies

monitored (tc). Disregarding this information would have changed the total colony loss calculations from 34.7% to 14.0%, thus significantly under reporting losses.

Another approach to dealing with colony increase and decrease data is to remove all data obtained from responding beekeepers who reported buying, giving, or selling colonies, or making increases; this approach, however, risks biasing survey results. Of course the degree of potential bias varies depending on the degree to which beekeepers split, sell or buy colonies over the winter in different regions. As previously mentioned, only 14.3% of beekeepers in Belgium reported reducing or increasing their colony numbers through management or commerce for the 2009 – 2010 winter. Excluding these beekeepers from the calculation would have changed total loss figures for the country from 27.8 to 26.0%. In the US, however, excluding beekeepers who bought, gave, sold, or made colony increases over the survey period would bias results by disproportionally excluding larger operations. Sixty four percent of commercial beekeepers (operating more than 500 colonies) reported splitting, selling, or buying colonies which compares to only 27.0% of sideline beekeepers (51 – 500) and 9 % of backyard beekeepers (1-50 colonies) who engaged in one or more of these practices. The outright exclusion of those beekeepers would reduce the number of surveyed colonies (tc) by 76% (n = 572,641), and would change the total colony loss figures from 34.7% to 31.6% for the period. Therefore, in the same way that national mortality figures include persons born in the study period and exclude persons no longer residing in a region during a study period, calculating total colony losses for a defined period (Oct 1 thru April 1), should exclude colonies removed from monitoring (colonies sold or given) and include increases in the number of colonies (made or bought) during the period.

Calculating 95% CI for Total Colony Losses:

Confidence intervals (CI) are used to express the reliability of an estimate. CI's are defined by two numbers, or confidence limits, which straddle a mean (Zar 1996). In principle, calculating the confidence limits for total losses uses the same approach as is used to calculate the confidence limits for any proportion (Equation 4).

Equation 4

$$\text{confidence limits for } \widehat{TL} = \widehat{TL} \pm Z_{\alpha} \times \text{s.e.}(\widehat{TL})$$

where

$$\text{s.e.}(\widehat{TL}) = \sqrt{\frac{\widehat{TL}(1-\widehat{TL})}{n}}$$

To calculate a two-sided 95% CI, we would use Equation 4 setting Z_{α} ($\alpha = 0.05/2$) to 1.96. The resulting confidence limits would encompass the surveyed population's true TL with 95% certainty. In other words, the mean TL resulting from other survey efforts would fall between the confidence limits 95% of the time. Calculating confidence limits using equation 4 assumes a normal approximation of the binomial distribution, which is a safe assumption provided that td and tc-td (used to calculate TL in Equation 1) are greater or equal to 10 (Koepsell and Weiss 2003). While it is hard to envision a total colony loss calculation where this would not be the case, should td<10 or tc-td<10, then confidence limits would be more accurate if based on the binomial distribution. Several alternative methods to calculate confidence limits using binomial distributions are available (Koepsell and Weiss 2003, p. 85).

Another consideration that must be taken into account when using equation 4 is the value to use for "n" when calculating s.e. for $\overline{TL}$. Simply making n=tc clearly underestimates the real 95% CI (see table 1; (Brown et al. 2001, 2002)). To avoid this problem, previous efforts which calculate 95% CI for total losses have opted to set n as the number of survey respondents (vanEngelsdorp et al. 2008, Brodschneider et al. 2010, vanEngelsdorp et al. 2010a, Nguyen et al. in press). While expedient, this approach is also faulty, in that the resulting CI is overly pessimistic (see Table 1; (Brown et al. 2001, 2002)).

Underpinning the confidence limits calculation as presented in Equation 4 is an assumption that the data used to derive $\overline{TL}$ is independent. That is, all colonies were just as likely to die over the period as all others. However, colony losses are not independent, as there is a notable difference in the total loss experienced within different beekeeping operations. To accurately calculate 95% CI for colony loss data we must take the nested nature of colony loss data into account (see Table 1). This approach requires calculating and using the s.e. of the Generalized Linear Model, where TL is set as the dependent variable and the total colonies alive and dead at the end of the survey period are the independent variables. The inv.logit command in the statistical program R (Hornik 2010) is capable of such calculations .

TABLE 1: A comparison of 95% CI of total colony loss figures calculated using various methods. Belgian and US national loss figures for 2009-2010 are presented.

	95% CI for National Colony Losses 2009-2010		
Method to calculate s.e using Equation 3	Belgium	US	Result
N = tc	26.3 – 29.4	34.3 – 34.5	Conservative - underestimates true range
N = total respondents	22.1 – 33.5	32.9 – 35.9	Liberal - overestimates true range
s.e. of the Generalized Linear Model	23.9 – 32.2	33.7 – 35.1	Accurate

The importance of properly calculating 95% CI is self evident when extrapolating national loss numbers from the survey results. Caution must always be used when making such extrapolations as sampling bias may significantly over- or under-represent beekeepers in a given region or operation type. However, if we assume that the survey results are representative of losses nationally, and national honey bee colony numbers are known, we are able to use the 95% CI to extrapolate how many colonies died nationally over the period. In the US, the number of honey-producing colonies in 2009 was estimated to be 2.462 million (USDA-NASS 2010). Using "accurate" 95% CI calculated for US losses (table 1), and assuming that that survey was representative of the national losses, we can infer that between 829,694 and 861,720 colonies were lost over the winter of 2009-2010. Should we have set n=tc, then the resulting range would have underestimated the true range by 84.6%, while if n been set to equal the number of respondents, the range would have overestimated the true range by 231%.

***Calculating Average Colony Losses*:**

Average losses are the mean of the total colony losses experienced by all responding beekeepers in a defined group. The information needed to calculate average losses (AL; Equation 5) is identical to that needed to calculate TL, however, the TL for each responding operation (TL_O) is calculated and the number of responding beekeepers (nr) whose TL_O was calculated is required.

Equation 5:

$$AL = \frac{\sum TL_O}{nr}$$

Calculating 95% CI for Average Colony Losses

Calculating the 95% CI for average losses uses the same equation as presented in Equation 4, except that TL is replaced with AL, and,

$$S.E. = \frac{\sigma TLO}{\sqrt{nr}}$$

This approach assumes that the number of respondents used to calculate the AL was larger than 60, which permits us to assume that the data are normally distributed. In cases where nr is equal to or less than 60, a normal distribution of the data cannot be assumed. In such cases the Z_α used in Equation 4 needs to be replaced with a value derived from a t-distribution, where the t-distribution chosen is based on the data's number of degrees of freedom (nr-1). T-distribution values are easily obtained through the utilization of readily available statistical tables (Paoli et al. 2002).

Purpose of the epidemiological approach

As previously mentioned epidemiological studies describe the distribution of disease within a population. However, most studies also attempt to uncover relationships between "disease" and determinants of that disease. Disease determinants are those factors which precipitate or predict disease. Disease determinants are often referred to as risk factors. Risk factors can be measures of agents believed to be directly involved in the etiology of disease, such as *Nosema ceranae* spore load in Nosemosis type C collapse (Higes et al. 2010), as well as factors that may be indicative of an other factors or agents involvement, for example, entombed pollen is indicative of exposure to the fungicide chlorothalonil which may be involved in increased colony mortality (Woodward 2005, vanEngelsdorp et al. 2009a). Both the determination of "disease" (a dependent variable) and risk factors (independent variables) can be measured qualitatively or quantitatively (Friedman 1987). As in all disciplines, when it comes to honey bee epidemiology, the measure used to quantify the dependent variable is determined by the question being asked. A study intending to look at factors which may help explain colony mortality would use colony survivorship as the dependent variable (e.g. (vanEngelsdorp et al. in revision; CHAPTER 6)), while a study looking to identify possible causes for an apparently new disease would use the disease status as the dependent variable (e.g. presence or absence of the symptoms of Colony Collapse Disorder (vanEngelsdorp et al. 2009b; CHAPTERS 4 and 5)).

Epidemiological approach used to investigate CCD

In the US, at least, a portion of the honey bee colonies lost in the winter of 2006-2007 and every year thereafter died with a distinct set of symptoms: (1) no dead bees in the colonies or apiary, (2) adult populations rapidly declined leaving brood poorly or completely unattended, and (3) the absence of robbing or kleptoparasitism in collapsed colonies (Cox-Foster et al. 2007). A

review of the historical bee literature suggests that large localized unexplained losses have occurred at least 20 times over the last 150 years, and many of those losses occurred with symptoms very similar to the losses of 2006-2007 (Underwood and vanEngelsdorp 2007). In the past theses conditions had been given a variety of names including "Fall Dwindle Disease", "May disease", "disappearing disease", and "disappearing syndrome". However, none of these names seemed appropriate (i.e. the disease occurred between November and March – not exclusively the fall or May). As a result, during a conference call meeting of investigators who would eventually make of the core of the Colony Collapse Disorder working team, the term "Colony Collapse Disorder", or "CCD" was coined. This "word" has subsequently been included in the New Oxford American Dictionary, and was selected by dictionary's editors as the runner up "new word of 2007".

Efforts to find a cause for CCD were intense. Initial efforts identified Israeli Acute Paralysis Virus (IAPV) as highly associated with diseased colonies along with Kashmir bee virus, *Nosema apis* and *Nosema ceranae* (Cox-Foster et al. 2007). While IAPV is able to cause colony collapse (Maori et al. 2009), its potential role as the sole cause of CCD has not been substantiated (vanEngelsdorp et al. 2009b; CHAPTER 4). In the most comprehensive study of the disorder to date, vanEngelsdorp and colleagues (2009; CHAPTER 4) compared 61 different variables (potential risk factors), including pathogen and pesticides prevalence and load, in bees collected from CCD and non-CCD colonies. While some single pathogen loads differed between affected and non-affected colonies, no single pathogen or agent was consistently found associated with the condition.

Notably absent were differences in the *Nosema* spore counts and *Varroa* levels between CCD and control colonies and apiaries. *Varroa* mites, likely in association with the viruses they vector (Martin 2001), are known to cause colony mortality, although such collapses usually occur

at the tail end of the nectar flow and are usually accompanied with large numbers of bees crawling in the affected apiary. *Nosema ceranae*, a more recently introduced pathogen of bees, has been implicated in large scale die-offs in southern Spain (Martín-Hernández et al. 2007), and studies have shown that the in advanced stages of collapse colonies die with symptoms similar to CCD (Higes et al. 2008). The study outlined in CHAPTER 3 did not find that the these two organisms were differentially expressed in diseased verses non-diseased population, and as a result, an additional criterion was proposed for inclusion in CCD's case definition – "4) at the time of collapse *Varroa* and *Nosema* populations are below levels thought to cause economic injury or colony decline".

Although no evidence was found for a single causal agent, the descriptive epidemiological study summarized in CHAPTER 4 did document evidence that pathogens played an important role in the condition. Colonies neighboring colonies affected by CCD were more likely to have the condition than chance would suggest, implying that the condition was either contagious or the result of exposure to a common risk factor. Pathogen prevalence rates in control and CCD populations were similar, suggesting that pathogen exposure was also similar for both groups. However, CCD colonies had higher pathogen loads, and were much more likely to be co-infected with more than three pathogens, suggesting some underlying factor or factors may affected a colony's ability to resist disease (Cox-Foster and vanEngelsdorp 2009).

The population "unit" used in epidemiological approaches in apiculture

The work summarized in CHAPTER 3 does highlight an important consideration when it comes to applying epidemiological methods to honey bees – the importance of clearly defining the unit by which the dependent variable (the population) is defined. Honey bees are social insects. The proverb *Una apis, nulla apis* – one bee is no bee – succinctly and accurately

summarizes the reality that living as a collective is essential for honey bee survival (Preston 2006). Contemporary thinkers suggest that one way to look at honey bee colonies is as "superorganisms", for it is the colony which is the vehicle by which honey bees propagate their genes (Seeley 1989, Moritz and Fuchs 1998). Honey bee "health" (or disease) can therefore be studied by describing populations of individual bee or populations of superorganisms (colonies). Indeed, depending on the question one attempts to answer, either approach is appropriate. Take for instance the growing evidence that honey bee viruses are transmitted and/or latent infections of viruses are activated by *Varroa* parasitism. The studies used to test this hypothesis were largely done at the individual bee level – where the ratio of newly emerged individual bees infected with a virus were compared between populations who had or had not been parasitized by *Varroa* mites while pupating (Shen et al. 2005, Gisder et al. 2009). Studies aimed at understanding (or breeding) honey bees that are able to resist disease can also be performed at the individual bee level; however, often these studies more appropriately measure colony health (disease or parasite load) at the colony level while comparing (and in the case of breeding efforts selecting for) variables that may explain differences in health within a population of colonies. The plethora of efforts over the last 60 years aimed to understand and breed honey bees resistant to the highly virulent and persistent brood disease caused by *Paenibacillus larvae larvae* (American Foulbrood; AFB) is illustrative of the value in studying bee health on both the individual bee and colony level. Indeed individual bees can be more or less prone to infection (Rothenbuhler and Thompson 1956). Resistance to disease, however, can also be conferred to a colony at the superorganismal level. For instance, some resistant lines of honey bees contain individuals that produce brood food that inhibit AFB spore germination (Rose and Briggs 1969). This type of resistance was tested by comparing brood food extracts from individual bees from resistant and non-resistant populations. Other colony level resistance strategies are more complex, requiring more than one individual bee's participation for the behavior which confers resistance

to be realized. Take for instance hygienic behavior, the ability of a colony of bees to identify, uncap, and remove diseased individuals (Rothenbuhler 1964, Spivak and Downey 1998). This behavior is afforded a colony when it contains individuals who can identify dead or dying brood contained under capped cells and remove the capping from those cells, as well as individuals who can identify and remove the remains of dead larvae which have been exposed after their cells have been uncapped. Commonly, hygienic colonies are not composed of individuals who are proficient at both uncapping and removing behavior, rather they contain families of sister bees (sharing the same mother but not necessarily the same father) who are proficient at one behavior while also containing families of sister bees that are efficient at performing the other behavior (Lapidge et al. 2002, Oxley et al. 2010). The collective advantage resulting from membership in a "society" of semi-related individuals, who express behaviors differently, is arguable the driving force behind the evolution of castes, multiple mating, and ultimately social behavior (Mattila and Seeley 2007, Seeley and Tarpy 2007) .

Naturally, European honey bee colonies are not found in aggregations. However, honey bees are managed, and so are artificially placed in aggregations called apiaries. When colonies neighbor each other, as they do in apiaries, disease agents are easily moved between colonies as a result of bee drift, robbing behavior, exchange of colony equipment by beekeeper management practices, and through the migration of bees from dying colonies into stronger surviving colonies (Bailey 1953, Jay 1966, Pfeiffer and Crailsheim 1998, Goodwin et al. 2006). The relative free exchange of bees and/or disease agents between colonies within the same apiary highlights the necessity of considering apiaries as a level of interest in some epidemiological investigations. This certainly was the case for the efforts attempting to find the cause of CCD. Investigators recognized upfront that a distinction had to be made between "healthy" colonies in apiaries containing CCD infected colonies verses those healthy colonies that were sampled in apiaries

where there was no indication of CCD. This was necessary because the possibility existed that healthy colonies in CCD affected apiaries could harbor agents responsible for collapse but had not yet collapsed themselves. In actuality, no agent was found more frequently in colonies sampled in CCD affected apiaries when compared to colonies found in control apiaries. However, some viral pathogens were found at greater frequency (and in some cases higher levels) in CCD colonies as compared to healthy colonies. This finding suggests that exposure to pathogens was equal among colonies in CCD and healthy apiaries and that the actual symptoms of collapse were the result of pathogen load (vanEngelsdorp et al. 2009b). Left unanswered by this finding was what underlying factor or factors made CCD colonies more susceptible to pathogen attack.

Longitudinal studies

Pesticides are commonly postulated as potentially explaining increased disease susceptibility in bees (Mullin et al. 2010). While pesticides almost certainly can have negative effects on bee health, the study outlined in CHAPTER 4 found no evidence for impact of a single pesticide as being associated with CCD. In fact, of the 50 pesticides and metabolites found in samples tested, only two – coumaphos and esfenvalerate – were found at levels that differed between CCD and control colonies. In both cases levels of these products were found at higher levels in CCD colonies. A classification and regression tree analysis (CART) preformed on the same data set (vanEngelsdorp et al. 2010b; Chapter 5) more starkly highlighted pesticide levels – and specifically coumaphos levels – ability to differentiate CCD from control populations. Colonies with high levels of coumaphos were healthier. As coumaphos is commonly used by beekeepers to control *Varroa* populations, this finding suggests that healthy colonies had mite populations that were more aggressively or persistently controlled. Although *Varroa* mite levels were not different between CCD and control populations at the time of sampling, it is possible

that mite populations differed at some time before sample collection. CCD may therefore be a consequence of elevated levels of mites some time before CCD onset.

The potential "legacy" effect of risk factor exposure some time prior to sample collection does highlight the need to monitor colonies over time. To this end a longitudinal study was initiated which monitored colonies operated by three different East Coast migratory operations (vanEngelsdorp et al. submitted; CHAPTER 6). In all, 56% of the monitored colonies died over the 10 months observation peroid; and while too few colonies died with symptoms that would allow for CCD diagnosis, several factors were identified that had measurable impacts on colony survivorship. Notably colonies diagnosed either with Idiopathic Brood Disease Syndrome (IBDS; the brood condition associated with Parasitic Mite Syndrome (PMS)) or showed evidence of a "queen event" (evidence that the queen had been or was being replaced) had an elevated risk of dying in the subsequent 50 days when compared to colonies without any of these symptoms (Relative Risk (RR) = 3.2 and 3.1 respectively). The role of queen issues in colony mortality substantiate claims by beekeepers, especially commercial beekeepers, that poor queens influence high rates of winter mortality (vanEngelsdorp et al. 2011; CHAPTER 3).

In summary, this dissertation, in recognition of the complex and multiple factors that influence colony health, set out to apply epidemiological techniques to 1) define disease problems in honey bee colonies quantitative terms and 2) Quantifying the degree and magnitude different risk factor exposures had on colony health. These were the first 2 of the 6 steps proposed by Nutter (1999) as essential to the adoption of epidemiological techniques to non-human organism systems. As anticipated, when compared to the use of epidemiological methods in other systems (medical, veterinarian or botanical), the adoption of epidemiological terms required some adjustments to be made in both the means by which health outcomes in honey bee colonies are calculated and expressed (see discussion above regarding calculating colony losses). The efforts

described here to both define and quantify disease and disease determinates in honey bee populations are by no means exhaustive. As a review of the model web of causation schematics presented in the introductory chapter of this dissertation highlights (Figure 1.1) sets about a tentative frame work of factors which may influence colony health. Considering the overarching affect that environmental, socio-political and economic factors have on disease determinate factors (see Figure 1.2), it is important to note that even in those cases where the work presented in this dissertation confirms a relationship between colony health and a determining factor, there is value in having these efforts replicated and demonstrated in other environments. Importantly, future work should also focus on designing, implementing, and evaluating disease mitigation systems that are specifically informed by this and other epidemiological efforts.

References

Bailey, L. 1953. The Transmission of Nosema Disease. Bee World 34: 171-172.

Brodschneider, R., R. Moosbeckhofer, and K. Crailsheim. 2010. Surveys as a tool to record winter losses of honey bee colonies: a two year case study in Austria and South Tyrol. Journal of Apicultural Research 49: 23-30.

Brown, L. D., T. T. Cai, and A. DasGupta. 2001. Interval Estimation for a Binomial Proportion. Statistical Science 16: 101-117.

Brown, L. D., T. T. Cai, and A. DasGupta. 2002. Confidence Intervals for a Binomial Proportion and Asymptotic Expansions. The Annals of Statistics 30: 160-201.

Cox-Foster, D., and D. vanEngelsdorp. 2009. Solving the mystery of the disapearing bees. Scientific American 300: 40-47.

Cox-Foster, D. L., S. Conlan, E. C. Holmes, G. Palacios, J. D. Evans, N. A. Moran, P. L. Quan, T. Briese, M. Hornig, D. M. Geiser, V. Martinson, D. vanEngelsdorp, A. L. Kalkstein, A. Drysdale, J. Hui, J. H. Zhai, L. W. Cui, S. K. Hutchison, J. F. Simons, M. Egholm, J. S. Pettis, and W. I. Lipkin. 2007. A metagenomic survey of microbes in honey bee colony collapse disorder. Science (Washington) 318: 283-286.

Daberkow, S., P. Korb, and F. Hoff. 2009. Structure of the US beekeeping industry: 1982-2002. Journal of Economic Entomology 103: 19.

Friedman, G. D. 1987. Primer of Epidemiology. McGraw Hill Inc, New York.

Gisder, S., P. Aumeier, and E. Genersch. 2009. Deformed wing virus: replication and viral load in mites (Varroa destructor). Journal of General Virology 90: 463-467.

Goodwin, R. M., M. A. Taylor, H. M. McBrydie, and H. M. Cox. 2006. Drift of *Varroa destructor*-infested worker honey bees to neighbouring colonies. Journal of Apicultural Research 45: 155-156.

Higes, M., R. Martin-Hernandez, and A. Meana. 2010. Nosema ceranae in Europe: an emergent type C nosemosis. Apidologie 41: 375-392.

Higes, M., Raquel Martín-Hernández, Cristina Botías, Encarna Garrido Bailón, Amelia V. González-Porto, Laura Barrios, M. Jesús del Nozal, José L. Bernal, Juan J. Jiménez, Pilar García Palencia, and A. Meana6. 2008. How natural infection by *Nosema ceranae* causes honeybee colony collapse. Environmental microbiology 10: 2659.

Hornik, K. 2010. The R FAQ.

Jay, S. C. 1966. Drifting of Honeybees in Commercial Apiaries. II. Effect of Various Factors When Hives Are Arranged in Rows. Journal of Apicultural Research 5: 103-112.

Koepsell, T. D., and N. S. Weiss. 2003. Epidemiologic methods: studying the occurrence of illness. Oxford University Press, New York.

Lapidge, K. L., B. P. Oldroyd, and M. Spivak. 2002. Seven suggestive quantitative trait loci influence hygienic behavior of honey bees. Naturwissenschaften 89: 565-568.

Maori, E., N. Paldi, S. Shafir, H. Kalev, E. Tsur, E. Glick, and I. Sela. 2009. IAPV, a bee-affecting virus associated with Colony Collapse Disorder can be silenced by dsRNA ingestion. Insect Molecular Biology 18: 55-60.

Martín-Hernández, R., A. Meana, L. Prieto, A. Martínez Salvador, E. Garrido-Bailón, and M. Higes. 2007. Outcome of colonization of *Apis mellifera* by *Nosema ceranae*. Applied and Environmental Microbiology 73: 6331-6338.

Martin, S. J. 2001. The role of *Varroa* and viral pathogens in the collapse of honeybee colonies: a modelling approach. The Journal of Applied Ecology 38: 1082-1093.

Mattila, H. R., and T. D. Seeley. 2007. Genetic diversity in honey bee colonies enhances productivity and fitness. Science (Washington) 317: 362-364.

Moritz, R., F.A., and S. Fuchs. 1998. Organization of honeybee colonies: characteristics and consequences of a superorganism concept. Apidologie 29: 7-21.

Morton, R. F., J. R. Hebel, and R. J. McCarter. 1990. A study guide to epidemiology and biostatistics. Third Edition. Aspen Publishers Inc., Gaithersburg, MD.

Mullin, C. A., M. Frazier, J. L. Frazier, S. Ashcraft, R. Simonds, D. vanEngelsdorp, and J. S. Pettis. 2010. High levels of miticides and agrochemicals in North American apiaries: Implications for honey bee health. PLoS ONE 5: e9754.

Nguyen, B. K., R. Van der Zee, F. Vejsnæs, S. Wilkins, Y. Le Conte, and W. Ritter. 2010. COLOSS Working Group 1: monitoring and diagnosis. Journal of Apicultural Research 49: 97-99.

Nguyen, B. K., J. Mignon, D. Laget, D.C. de Graaf, F. J. Jacobs, D. vanEngelsdorp, Y. Brostaux, C. Saegerman, and E. Haubruge. in press. Honey bee colony losses in Belgium during the 2008-2009 winter. Journal of Apicultural Research.

NRC. 2006. Status of Pollinators in North America, pp. 317. National Academy of Sciences, Washington, D.C.

Nutter, F. W., Jr. 1999. Understanding the interrelationships between botanical, human, and veterinary epidemiology: the Ys and Rs of it all. 5: 131-140.

Oxley, P. R., M. Spivak, and B. P. Oldroyd. 2010. Six quantitative trait loci influence task thresholds for hygienic behaviour in honeybees (Apis mellifera). Molecular Ecology 19: 1452-1461.

Paoli, B., L. Haggard, and G. Shah. 2002. Confidence intervals in public health, pp. 8. Office of Public Health Assessment, Utah Department of Health.

Pfeiffer, K. J., and K. Crailsheim. 1998. Drifting of honeybees. Insectes Sociaux 45: 151-167.

Preston, C. 2006. Bee. Reakton Books Ltd., London.

Rose, R. I., and J. D. Briggs. 1969. Resistance to American foulbrood in honey bees. Effects of honey-bee larval food on the growth and viability of Bacillus larvae. Journal of invertebrate Pathology 13: 74-80.

Rothenbuhler, W. C. 1964. Behavior Genetics of Nest Cleaning in Honey Bees. IV. Responses of F1 and Backcross Generations to Disease-Killed Brood. American Zoologist 4: 111-123.

Rothenbuhler, W. C., and V. C. Thompson. 1956. Resistance To American Foulbrood in Honey Bees. I. Differential Survival of Larvae of Different Genetic Lines. Journal of Economic Entomology 49: 470-475.

Seeley, T. D. 1989. The honey bee colony as a superorganism. American Scientist 77: 546-551.

Seeley, T. D., and D. R. Tarpy. 2007. Queen promiscuity lowers disease within honeybee colonies. Proceedings of the Royal Society of London. Series B, Biological Sciences 274: 67-72.

Shen, M. Q., X. L. Yang, D. Cox-Foster, and L. W. Cui. 2005. The role of varroa mites in infections of Kashmir bee virus (KBV) and deformed wing virus (DWV) in honey bees. Virology 342: 141-149.

Spivak, M., and D. L. Downey. 1998. Field assays for hygienic behavior in honey bees (Hymenoptera: Apidae). Journal of Economic Entomology 91: 64-70.

Underwood, R., and D. vanEngelsdorp. 2007. Colony Collapse Disorder: Have we seen this before? Bee Culture 35: 13-18.

USDA-NASS. 2010. Honey, pp. 6. Department of Agriculture., Washington DC.

vanEngelsdorp, D., and M. D. Meixner. 2010. A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. Journal of Invertebrate Pathology 103: S80-S95.

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

vanEngelsdorp, D., J. Hayes, Jr., R. M. Underwood, and J. Pettis. 2008. A survey of honey bee colony losses in the U.S., Fall 2007 to Spring 2008. PLoS ONE 3: e4071.

vanEngelsdorp, D., J. Hayes Jr, R. M. Underwood, and J. S. Pettis. 2010a. A survey of honey bee colony losses in the United States, fall 2008 to spring 2009. Journal of Apicultural Research 49 7-14.

vanEngelsdorp, D., D. R. Tarpy, E. J. Lengerich, and J. Pettis. in revision. Colony mortality and morbidity in migratory beekeeping operations in the Eastern United States: A longitudinal descriptive study based on rates of risk factor exposure. Apidologie.

vanEngelsdorp, D., D. R. Tarpy, E. J. Lengerich, and J. Pettis. submitted. Colony mortality and morbidity in migratory beekeeping operations in the Eastern United States: A longitudinal descriptive study based on rates of risk factor exposure. Apidologie.

vanEngelsdorp, D., J. Hayes Jr, R. M. Underwood, D. Caron, and J. S. Pettis. 2011. A Survey of managed honey bee colony losses in the U.S., fall 2009 to winter 2010. Journal of Apicultural Research 50: 1-10.

vanEngelsdorp, D., J. D. Evans, L. Donovall, C. Mullin, M. Frazier, J. Frazier, D. R. Tarpy, J. Hayes Jr, and J. S. Pettis. 2009a. "Entombed Pollen": A new condition in honey bee colonies associated with increased risk of colony mortality. Journal of Invertebrate Pathology 101: 147-149.

vanEngelsdorp, D., J. D. Evans, C. Saegerman, C. Mullin, E. Haubruge, B. K. Nguyen, M. Frazier, J. Frazier, D. Cox-Foster, Y. Chen, R. Underwood, D. R. Tarpy, and J. S. Pettis. 2009b. Colony Collapse Disorder: A descriptive study. PloS ONE 4: e6481.

vanEngelsdorp, D., N. Speybroeck, J. Evans, B. K. Nguyen, C. Mullin, M. Frazier, J. Frazier, D. Cox-Foster, Y. Chen, D. R. Tarpy, E. Haubruge, J. S. Pettis, and C. Saegerman. 2010b. Weighing risk factors associated with bee Colony Collapse Disorder by classification and regression tree analysis. Journal of Economic Entomology 103: 1517-1523.

Woodward, M. 2005. Epidemiology. Study Design and Data Analysis. Champman & Hall/CRC, New York.

Zar, J. H. 1996. Biostatistical Analysis. Prentice Hall, Upper Saddle River.

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Select Publications

vanEngelsdorp, D., J. Hayes Jr, R. M. Underwood, D. Caron and J. S. Pettis. 2011 A survey of honey bee colony losses in the United States, fall 2009 to spring 2010. *Journal of Apicultural Research*.

vanEngelsdorp, D., N. Speybroeck, J. Evans, B. K. Nguyen, C. Mullin, M. Frazier, J. Frazier, D. Cox-Foster, Y. Chen, D. R. Tarpy, E. Haubruge, J. S. Pettis, and C. Saegerman. 2010. Weighing risk factors associated with bee Colony Collapse Disorder by classification and regression tree analysis. *Journal of Economic Entomology*. 103:1517-1523

Donovall, L. R., and D. **vanEngelsdorp**. 2010**.** A Checklist of the Bees (Hymenoptera: Apoidea) of Pennsylvania. Journal of the Kansas Entomological Society 83: 7-24.C.

vanEngelsdorp, D., J. Hayes Jr, R. M. Underwood, and J. S. Pettis. 2010. A survey of honey bee colony losses in the United States, fall 2008 to spring 2009. *Journal of Apicultural Research*. 49: 7 -14.

vanEngelsdorp, D., and M. D. Meixner. 2010. A Historical Review of Managed Honey Bee Populations in Europe and the United States and the Factors That May Affect Them. *Journal of Invertebrate Pathology.* 103: S80-S95.

vanEngelsdorp, D**.,** and L. R. Donovall. 2009. Note: New Plant Host Record and First Record of the Burrower Bug Sehirus cinetus (Palisot de Beauvois) Hemiptera:Cydnidae)Associating with Honey Bees, *Apis mellifera* L. (Hymenoptera: Apidae). *Proceedings of the Entomology Society of Washington.* 111: 903-906.

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

vanEngelsdorp, D., J. D. Evans, L. Donovall, C. Mullin, M. Frazier, J. Frazier, D. R. Tarpy, J. Hayes Jr, and J. S. Pettis. 2009. "Entombed Pollen": A new condition in honey bee colonies associated with increased risk of colony mortality. *Journal of Invertebrate Pathology*. 101: 147-1