The Potential Impact of Pathogens on Honey bee, *Apis mellifera* L., Colonies and Possibilities for their Control

By

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ABBREVIATIONS

Δ Ct	delta Cycle threshold
ABPV	Acute Bee Paralysis Virus
AFB	American Foulbrood
BLAST	Basic Local Alignment Search Tool
BPMS	Bee Parasitic Mite Syndrome
BQCV	Black Queen Cell Virus
CBPV	Chronic Bee Paralysis Virus
CCD	Colony Collapse Disorder
cDNA	Complementary DNA
CSBV	Chinese SacBrood Virus
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
dsRNA	double stranded RNA
DWV	Deformed Wing Virus
EFB	European Foulbrood
GFP-dsRNA	Green fluorescent protein-dsRNA
HBTM	Honey bee tracheal mite
IAPV	Israeli Acute Paralysis Virus
IBDS	Idiopathic brood disease syndrome
IRES	Internal Ribosome Entry Site
KBV	Kashmir Bee Virus
M-MLV RT	Moloney Murine Leukemia Virus Reverse Transcriptase
mRNA	messenger RNA
ORF	Open Reading Frame
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
qPCR	quantitative Polymerase Chain Reaction
RdRp	RNA Dependent RNA Polymerase
RISC	RNA Induced Silencing Complex
RNA	Ribonucleic Acid
RNAi	RNA Interference
RT-PCR	Real-Time Polymerase Chain Reaction
SBV	Sac Brood Virus
SID-1	Systemic Interference Defective
siRNA	small interfering RNA
TSBV	Thai Sac Brood Virus
UTR	Untranslated Region

ABSTRACT

Excessive honey bee colony losses all over the world are believed to be caused by multiple stressors. In this thesis, I characterized and quantified pathogen levels in honey bee colonies, studied their interactions with each other and with their associated parasite vectors, examined factors that influence their combined impacts on honey bees and developed methods to manage honey bee viruses so that colony losses can be minimized. My baseline study of virus prevalence and concentration in healthy and unhealthy (showing visible signs of disease) colonies in Canada showed that seven economically important viruses (DWV, BQCV, IAPV, KBV, SBV, ABPV, and CBPV) were all widely distributed in Canada. Differences in concentration and prevalence of some viruses were found between unhealthy and healthy colonies but these differences may have been due in part to seasonal or regional effects. Studies of the impact of viruses on worker bee populations over winter showed different factors were correlated with bee loss in different environments. Spring concentrations of DWV and mean abundance of *Varroa* (*Varroa destructor)* were positively correlated with bee loss and negatively correlated with spring population size in outdoor-wintered colonies. Fall concentration of IAPV was negatively correlated with spring population size of colonies in indoor-wintering environments but not in outdoor-environments. My study showed that it is important to consider location of sampling when associating pathogen loads with bee loss with *Nosema* and BQCV. Seasonal patterns of parasites and pathogens were characterized for each wintering methods (indoor and outdoor). My results revealed lower ABPV and *Nosema ceranae* prevalence and lower DWV concentration in genetically diverse than genetically similar colonies. I showed that within colony genetic diversity may be an important evolutionary adaptation to allow honey bees to defend against a wide range of

diseases. In laboratory studies, I showed that feeding DWV to larvae in the absence of *Varroa* causes wing deformity and decreased survival rates of adult bees relative to bees not fed DWV. Finally, I showed that RNA silencing can be used to reduce DWV concentrations in immature and adult bees, reduce wing deformity in emerging adults, and increase their longevity relative to controls.

GENERAL INTRODUCTION

Honey bee viruses are considered among the most serious pathogens affecting the beekeeping and pollination industry . Honey bee viruses interact with the *Varroa destructor* Anderson and Trueman, and other parasites and pathogens and thus, have potential to contribute to honey bee winter colony mortality in northern climates. The research reported in this thesis focused on using molecular techniques to identify and quantify bee viruses in order to characterize their impact on bee colonies and develop ways to minimize their impact on colony losses. The thesis is written in paper style with a general literature review (chapter 1), four manuscripts (chapter 2-5), and general discussion (chapter 6). The literature review provides the thesis framework and research objectives; the four manuscripts are written for publication in peer reviewed journals. In the general discussion, I deliberate on the salient findings and shortcomings and suggest future directions for research in the field.

Chapter 1 serves as introduction to my thesis. I reviewed the effect of viruses on honey bees and their interactions with the *Varroa* and fungal pathogens (*Nosema* spp.) in honey bees. I also reviewed the benefits of genetic diversity in honey bees and the role of RNA interference in insects.

Chapter 2 was a study to characterize the distribution and relative concentration of viruses within bee colonies in Canada. Colonies from across different geographical regions in Canada were tested for seven economically important honey bee viruses, deformed wing virus (DWV), black queen cell virus (BQCV), sac brood virus (SBV), Israeli acute paralysis virus (IAPV), Kashmir bee virus (KBV), acute bee paralysis virus (ABPV), and chronic bee paralysis virus (CBPV), by using conventional RT-PCR and

quantitative PCR (qPCR) (for DWV BQCV and IAPV). This was the first study to assess the distribution of bee viruses in different Canadian provinces. The findings showed that all seven viruses were common but DWV was the most prevalent virus. It was found at relatively high virus concentrations in many bee samples collected from beekeepers' colonies across Canada. Furthermore, viral loads of DWV were the only ones higher in unhealthy colonies than in healthy ones.

Chapter 3 was a field study designed to examine the relative impact of bee pathogens on bee mortality and characterize associations between pathogen and parasite interactions and winter colony losses, under two different overwintering management systems (indoor and outdoor). Long-term monitoring of beekeeper-managed colonies was conducted in commercial beekeeping operations to examine how winter environment and method of sampling bees affected the characterization of two parasitic mites, seven viruses, and *Nosema* and their impacts on bee population size over winter. Monitoring of colonies was performed over 9 months in Manitoba, Canada from fall 2009 and mid-winter 2009/2010 to spring 2010. Two viruses, DWV and BQCV, and one pathogen, *Nosema,* showed different seasonal pathogen profiles and patterns in indoor and outdoor-wintered colonies. Combinations of parasite and pathogen variables that were correlated with bee loss or spring colony size and also differed with the wintering method.

Chapter 4 was a field and laboratory study designed to address the effect of multiple mating on honey bee resistance to pathogens and parasites. I investigated the effect of manipulating genetic diversity on virus infections, nosema disease, grooming behaviour against *Varroa*, and bee survival. Instrumentally inseminated "genetically diverse" honey bee queens (multiple-mated) were compared with "genetically similar" (single-mated) queens and to a third set of queens that were open mated to unknown

numbers of unselected drones. My major finding was that queens inseminated with genetically diverse semen from *Varroa*-resistant colonies had reduced prevalence or concentrations of parasites and pathogens in about a quarter of the pathogen assays I studied relative to queens which were inseminated with semen from a single drone. The reverse pattern was never observed.

Chapter 5 reports on a laboratory study in which I developed a form of double-stranded RNA (dsRNA) against a honey bee virus, DWV, to assess the potential for using dsRNA as a control for honey bee viruses and to determine if potential reduction in viral loads could reduce deformities and improve bee survival. The findings of this experiment were published in *Insect Molecular Biology* and co-authored by Dr. Young-Jae Eu, Dr. Steve Whyard (Department of Biological Sciences), and my advisor, Dr. Rob Currie. This manuscript provided the first evidence that RNAi was effective in managing DWV in honey bees and that it reduced viral loads, reduced wing-deformities and improved honey bee survival relative to controls.

Chapter 6 is a general discussion of research contained herein.

CHAPTER 1. LITERATURE REVIEW

INTRODUCTION

The European honey bee, *Apis mellifera* L. (Hymenoptera: Apidae) is an ecologically and economically important species of honey bee found throughout the world (Fig. 1.1). Honey bees play a vital role in the global economy by producing commercial products such as honey, pollen, beeswax, and other hive products. They are also important crop pollinators and play a critical role contributing to the production of crops and human food security. One-third of the human diet is estimated to be dependent upon honey bee pollination (McGregor 1976; Delaplane and Mayer 2000). Their contribution to the economy exceeds CAD $2 billion annually in Canada (CHC 2012) and US$212 billion on a global basis (Klein et al. 2007; Aizen and Harder 2009; Gallai et al. 2009). The availability of healthy and strong populations of honey bees is critical for the managed pollination of food crops.

Honey bees are currently under threat because they are attacked by various parasites and pathogens including viruses, bacteria, fungi, microsporidia and mites that act in combination with other stresses to contribute to significant colony losses (Cox-Foster et al. 2007; Genersch 2010; Genersch et al. 2010; Ratnieks and Carreck 2010). Among the honey bee pathogens, viruses are one of the major threats to bee health and their interactions with *Varroa destructor* Anderson and Trueman have likely caused serious problems for the beekeeping industry (Genersch and Aubert 2010). Viruses are a group of obligate, intracellular parasites that are unable to grow or reproduce outside a host cell. They attack the host cell and use its biochemical machinery to replicate (producing virions), resulting in symptoms of infection or even death of the host (Ball and Bailey 1997). Viruses and microsporidia are the least understood of bee pathogens,

and there is not sufficient information about their role in the dynamics of underlying disease outbreaks (vanEngelsdorp et al. 2009).

In 2006, a syndrome identified as "colony collapse disorder" (CCD), resulted in colony losses of 50 to 90% in some beekeeping operations in the United States (Cox-Foster et al. 2007). CCD is characterized by the loss of most or all of a colony's adult bee population where few dead bees are found around the hive, but where frames of capped brood and surplus food reserves are present, sometimes with a laying queen and a few newly emerged attendant bees (Cox-Foster et al. 2007). An initial study conducted by Cox-Foster et al. (2007), correlated the presence of Israeli acute paralysis virus of bees (IAPV), KBV and *Nosema ceranae* Fries with CCD, but questions about the interactions between CCD, IAPV and other pathogens need to be explored. The possible causes of CCD-like symptoms, include *Varroa*, viruses, *Nosema* spp., stress on bees, and pesticide contamination, but no single factor seems to be consistently responsible for the large scale colony losses and disease outbreaks that have been observed all over the world at different time periods (vanEngelsdorp and Meixner 2010). High levels of colony loss occur in many parts of the world, often without "CCD-like symptoms", and have been attributed solely to *Varroa* infestations, but observations made in the 1980s suggest that certain honey bee viruses are responsible for much of the mortality observed in mite-infested colonies (Ball 1983).

Winter colony loss has also been high in Canada. Of the 600,000 honey bee colonies operated in Canada, Canadian beekeepers frequently have been losing close to 30% since 2006, which amounts to an unsustainable economic loss for the beekeeping and pollination industries at current market prices. In Canada, these bee colony losses

have been directly or indirectly attributed to *Varroa*, viruses, *Nosema* and interactions with other stressors that affect bees (Currie et al. 2010).

The honey bee Apis mellifera L.

There are nine species of honey bees within the Hymenopteran family *Apidae*. The genus *Apis* contains eight Asian species of honey bees and the European honey bee, *A. mellifera* (Fig. 1.1) (Hepburn and Radloff 2011), which is the focus of this study. There are several subspecies or races of the European honey bee, *A. mellifera* (Winston 1987). Some of the basic features of *A. mellifera* in temperate climates are described below.

A honey bee colony is composed of three castes, a single queen (fertile female), up to 60,000 workers (sterile), and a variable number of drones (males) depending on the time of the year. The honey bee queen mates with 10-25 males during flight, and then lays eggs in wax cells constructed by the workers (Palmer and Oldroyd 2000). Fertilized eggs produce workers that hatch after 3 days and develop into larvae. The larval period lasts for 6 days after which workers seal the fifth larval instar within the cell with a wax capping. The sealed-brood phase lasts for 11-12 days during which the larva spins a cocoon, pupates, and finally the adult emerges from its cell. Total development of a worker bee thus lasts for 20-21 days. Adult workers carry out most of the activities in the colony and feed brood, carry out hygienic and maintenance tasks (removing dead brood, building new combs, etc.), defend the colony, and finally forage for nectar and pollen (Winston 1987).

HONEY BEE PARASITES AND PATHOGENS

There are many parasites and pathogens that attack all stages of the honey bee life cycle including eggs, larvae, pupae, and adults of all three castes (workers, drones, and queens). These pests and pathogens acting alone, or in conjunction with each other, can adversely affect colony productivity and are capable of causing colony death (Morse and Flottum 1997). Although some honey bee parasites and pathogens do not cause obvious signs of disease, they can cause a variety of sub lethal effects and affect bee health, thereby shortening the lifespan of the infected bees under some conditions (Ball and Allen 1988; Martin 2001).

Figure 1.1 Honey bee *A. mellifera* with mite *V. destructor* on the abdomen (arrow) (Photo. S. Desai).

Varroa (Varroa destructor)

The parasitic mite, *Varroa destructor* Anderson and Trueman (Fig. 1), formerly known as *Varroa jacobsoni* Oud. expanded from its original host, *Apis cerana* F. to the European honey bee nearly 50 years ago (Anderson and Trueman 2000). The mite is now a major pest of *A. mellifera* throughout the world and is found in most regions in Canada. The adult female is 1.1-1.5 mm wide, weighs about 1 mg, is reddish brown in colour and dorsally flattened (De Jong 1997). Its life cycle has two phases. In the "phoretic" phase, it often attaches to the upper surface of the adult bees on the thorax or abdomen or between the overlapping abdominal sternites where it can feed. During the reproductive phase, the fertilized female mite enters brood cells and lays eggs, where its progeny mate inside sealed cells (mating occurs between adult brother and sisters) (Boot et al. 1992). Female *V. destructor* leave their adult bee hosts to enter brood cells about 20 h before they are sealed by worker bees (Boot et al. 1992). Within the cell, both the adult female mite and her newly emerged offspring feed on the developing honey bee pupae.

One of the key roles *Varroa* plays in the epidemiology of bee diseases is to enhance disease transmission. It has been demonstrated that *Varroa* can vector several bee viruses (Ball 1985; Chen et al. 2004c) and chalkbrood spores *Ascosphaera apis* (Massen ex Claussen) Olive and Spiltoir (Liu 1996). The relationships between mite infestation and virus infection, their combined impacts on different life stages of bees, and the overall effects on colonies are not clearly understood. If left untreated, heavy *Varroa* infestations inevitably kill the colony either through direct feeding action or through their associated pathogens (De Jong 1997). Viruses may play a role that is equivalent to or larger than *Varroa* feeding damage in affecting bee colony depopulation (Hung et al. 1995). Mite feeding helps spread viral infections among adult bees and

between adults and larvae. Feeding also weakens the bee immune system, making the bees more susceptible to virus and other diseases (Shen et al. 2005b; Yang and Cox-Foster 2005; Yang and Cox-Foster 2007).

Honey bee viruses

Most of the commonly observed single-stranded RNA viruses, including DWV, IAPV, SBV, BQCV, KBV, ABPV and CBPV, were present in European honey bees before they became associated with *Varroa* but typically remained as symptomless infections (Bailey and Ball 1991; Bowen-Walker et al. 1999). Even damaging bee viruses often do not cause noticeable morphological symptoms in bees, making it difficult to detect virus outbreaks and characterize their importance.

Most honey bee viruses are single-stranded RNA viruses and are very similar in size and shape, making them difficult to distinguish from each other using physical characteristics. However, recent advances in molecular techniques have facilitated the study of viruses. More than 20 small (20-30 nm) single-stranded RNA viruses have been described in honey bees so far. In addition, the complete genome sequences of at least 10 of these viruses have been determined (Evans and Schwarz 2011).

Viruses alone or in combination with *Varroa* can attack different stages of the honey bee life cycle. Seven viruses, namely, deformed wing virus (DWV), black queen cell virus (BQCV), sac brood virus (SBV), Israeli acute paralysis virus (IAPV), Kashmir bee virus (KBV), acute bee paralysis virus (ABPV), and chronic bee paralysis virus (CBPV), are the most common and economically important infections that have been documented to attack a variety of life stages of the honey bees and exhibit different symptoms (Table 1.1).

Table 1.1 Economically important honey bee viruses and their global distribution showing life stages affected, major symptoms and impacts on bees. §= see list of abbreviations for full names viruses (page ix).

Acronym of virus name §	Known distribution	Bee life stages affected	Symptoms (if any) associated with infection	References
DWV	All over the world	Egg, Larvae, Pupae and Adults	Bees emerge with wing deformities and early death of adults	(Ball and Allen 1988; Martin 2001; Highfield et al. 2009)
BQCV	USA, Canada, Europe, Asia	Larvae and Adults (association with *Nosema)*	Death of queen larvae and sometimes worker brood. Early death of adult workers	(Bailey et al. 1983; Bailey and Ball 1991)
IAPV	USA, Canada, Europe, Asia several other countries	Larvae, Pupae and Adults	Paralysis and death of colonies	(Cox-Foster et al. 2007) (Maori et al. 2007)
SBV	USA, Canada, Europe, Asia	Larvae and Adults	Death of larvae and earlier death of adults	(Bailey 1969; Bailey and Fernando 1972)
KBV	USA, Canada, Europe, Asia several other countries	Larvae, Pupae and Adults	Death of brood and adults	(Allen and Ball 1996; Ellis and Munn 2005)
ABPV	USA, Canada, Europe, Asia	Larvae, Pupae and Adults	Covert infection in adults and brood Death of brood and adults	(Allen and Ball 1996)
CBPV	USA, Canada, Europe, Asia	Larvae, Pupae and Adults	Death of adults	(Ball and Bailey 1997)

Viruses causing economic impact

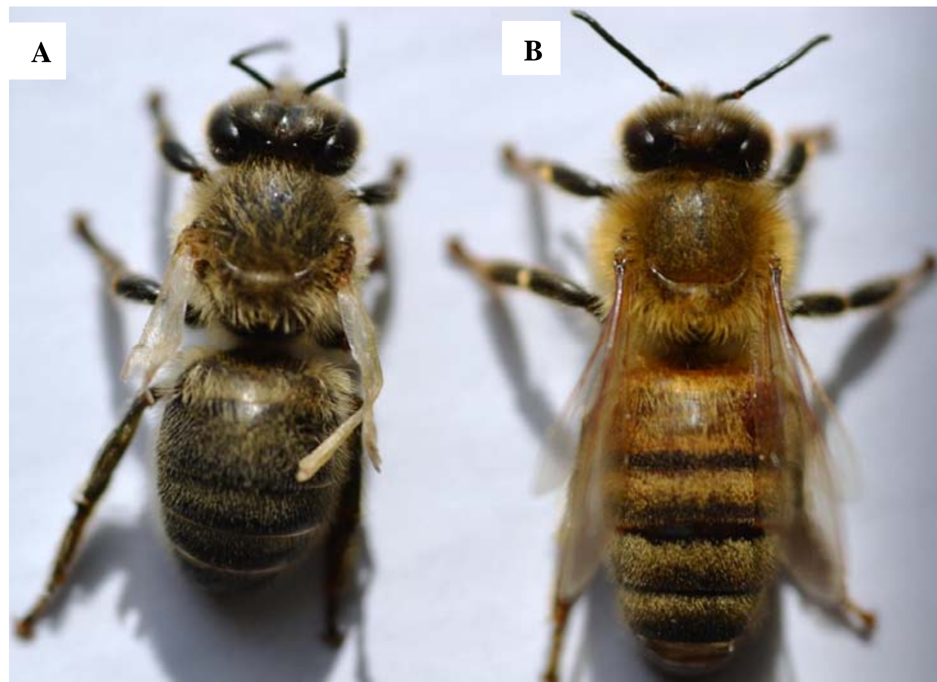
Deformed wing virus (DWV)

Recent advances in next generation sequencing techniques have broadened our knowledge on deformed wing virus, and led to our understanding of it as one of the most extensively dispersed and infectious bee viruses threating beekeepers (Highfield et al. 2009; de Miranda and Genersch 2010; Dainat et al. 2012a; Martin et al. 2012). DWV is one of the few bee viruses that cause visible disease symptoms in infected bees (Fig. 1.2 A). DWV was first isolated from diseased adult bees in Japan (Bailey and Ball 1991).

DWV typically causes disease symptoms such as shrunken, crumpled wings, reduced body size, and discoloration in adult bees and high concentrations of DWV are found in adult bees exhibiting deformed wing symptoms (Fig. 1.2 A). During the pupal stage, DWV multiplies slowly and is rarely fatal but affects wing development and results in early death of newly emerged adults (Chen et al. 2005; Yue and Genersch 2005; Chen et al. 2006b). DWV infections in bee colonies are often linked with infestations of *V. destructor* (Anderson and Trueman, 2000). Both laboratory and field studies show that the *Varroa* is an effective vector of DWV (Ball and Allen 1988; Martin et al. 1998; Bowen-Walker et al. 1999; Shen et al. 2005b). *Varroa* acquire the virus from infected bees and transmit it to uninfected larvae, which develop deformed wings during pupation and die earlier as adults because of the interaction between the virus and mite. Studies on virus status in Thailand and France found DWV is present in 100% of *Varroa* (Tentcheva et al. 2004b; Chantawannakul et al. 2006). Feeding by *Varroa* inoculates virus into the hemolymph of both immature and adult bees resulting in higher levels of exposure than would be found in mite-free colonies (Bowen-Walker et al. 1999; Di Prisco et al. 2011). Large proportions of workers in a colony can quickly become infected with DWV and

this virus has been implicated as a cause of mortality in overwintering bee colonies (Ball and Allen 1988; Martin 2001; Highfield et al. 2009).

Figure 1.2 (A) an adult bee exhibiting typical wing and body deformity symptoms, (B) an adult bee with normal body shape and normal wings (Photo. S. Desai).



There is debate on how and when DWV causes wing deformity in honey bees. Laboratory studies have demonstrated that high virus concentration at a critical stage of pupal development is very important in producing wing deformity (Mockel et al. 2011). Recently, Forsgren et al. (2012) discovered adult worker bees with symptoms of wing deformity in the absence of *Varroa* infestation, but this was isolated from a single beekeeper. This may have been due the strain of DWV in that apiary. Different strains of DWV exist and more virulent strains are favoured when *Varroa* is present (Martin et al. 2012).

Black queen cell virus (BQCV)

BQCV was isolated from dead larvae and prepupae of queen cells where the cell walls had turned black and partially decomposed (Bailey and Woods 1977). Recent studies show prevalence ranges from 30 – 86% (Tentcheva et al. 2004b; Berenyi et al. 2006; Kukielka et al. 2008; Kojima et al. 2011).

In the early stages of BQCV, the disease symptoms in larvae are similar to sac brood virus (SBV) infected larvae (Table 1.1). BQCV is believed to be transmitted to queen larvae via glandular secretions of nurse bees during feeding (Bailey and Ball 1991). The larvae change from white to pale yellow in appearance and have tough "sac like" skin. However, unlike in sac brood, the pupal stage of queens is most favourable for BQCV multiplication (Ball and Bailey 1997). BQCV infection is usually more prevalent during spring and summer, but goes unnoticed as adult bees do not display external symptoms (Tentcheva et al. 2004b). During spring and early summer, a high incidence of the virus infection causes problems in queen rearing colonies (Laidlaw 1979). In Australia, BQCV is the most common cause of death in queen larvae (Anderson 1993).

An association between BQCV and *Nosema apis* Zander has been demonstrated in some field and laboratory studies. Field surveys in Austria and France show that *N. apis* is present in 78% of BQCV-positive bee samples and that 75% of *N. apis* infected colonies are also infected with BQCV (Tentcheva et al. 2004b; Berenyi et al. 2006). Although BQCV does not multiply readily when ingested by either worker bees or larvae in the absence of *Nosema*, it replicates in abundance in adult bees when they are infected with *N. apis* (Bailey et al. 1983a). Since *N. apis* often cause gastrointestinal infections in bees, it is believed that infection of *N. apis* increases the susceptibility of the alimentary

tract to infection by BQCV, suggesting that the alimentary tract acts as an initial infection site for BQCV (Bailey et al. 1983a). When the incidence of *N. apis* infection is high during spring and summer, BQCV is more prevalent (Bailey et al. 1983a) and multiplies rapidly in adult bees (Genersch and Aubert 2010). Bee age structure may also be important as colonies with older adult worker bees often rear queen bees infested with BQCV and *N. apis* (Allen and Ball 1996). Although links between the BQCV and *N. apis* infections have been documented, the mechanism through which *N. apis* may facilitate transmission of BQCV infection is not known. *Varroa* are assumed to act as a vector for BQCV (Ball 1983) and detection of BQCV in *Varroa* collected from a Thai honey bee apiary supports this thought (Chantawannakul et al. 2006).

Sac brood virus (SBV)

SBV infection was first identified in the United States by G.F. White (1913) and is widely distributed (Table 1.1) (Bradbear 1988; Allen and Ball 1996; Ellis and Munn 2005). SBV infects both brood and adult bees, but 2-day old larvae are most susceptible to SBV infection (Bailey and Ball 1991). Larvae infected with SBV fail to pupate and form a fluid filled sac in which SBV multiplies, producing millions of SBV particles (Ball and Bailey 1997). Nurse bees acquire the virions while removing larvae killed by SBV and then infect young larvae by feeding them virus-contaminated food. SBV in adult bees is symptomless, but decreases life span (Bailey 1969; Bailey and Fernando 1972), and the virions build up in hypopharyngeal glands of nurse bees where they are transferred to other bees during food exchange. SBV also has strains that vary in virulence (Abrol and Bhat 1990). Thai sac brood virus (TSBV) is a different strain of SBV found in South Asia, first detected in Thailand in 1982. TSBV devastated the Asian

honey bee *Apis cerana* in India during 1991 (Abrol and Bhat 1990; Mishra and Dubey 1996).

Israeli acute paralysis virus (IAPV)

IAPV recently became one of the most "notorious" honey bee viruses, when a link was suggested between IAPV and Colony Collapse Disorder (CCD) colonies (Cox-Foster et al. 2007). IAPV was characterized for the first time in Israel from adult worker bees that showed trembling and shivering wings, where colonies had paralyzed and dead bees outside the hive (Maori et al. 2007). IAPV belongs to the family *Dicistroviridae* and its partially sequenced homology shows similarities to both Kashmir bee virus (KBV) and acute bee paralysis virus (ABPV) (de Miranda et al. 2010). IAPV may have gone unnoticed for a considerable time because sequence similarity with KBV and ABPV may have caused it to be mistaken for a strain of KBV (Hornitzky 1987; Anderson and Gibbs 1988; Anderson and East 2008; de Miranda et al. 2010). Analysis of stored honey bee specimens indicates that IAPV has been present in U.S. bee populations since at least 2002, although it was 2006 when it was first linked to colony losses (Chen and Evans 2007). IAPV not only infects *A. mellifera*; recently it has been identified in the Asian honey bee *A. cerana* in China (Ai et al. 2012; Yañez et al. 2012).

Varroa is an effective vector and activator of IAPV (Di Prisco et al. 2011) but foraging bees also spread virus-contaminated pollen to native bees (Singh et al. 2010). The incidence of IAPV is higher in individual bumble bees collected from locations closer to bee colonies affected by CCD than from locations farther from CCD affected-colonies (Singh et al. 2010). Greenhouse experiments show that IAPV transmission

between honey bees and bumble bees occurs in a short time and is bidirectional (Singh et al. 2010).

Kashmir bee virus (KBV)

KBV was isolated from Asian honey bees (*A. cerana*) collected in the northern state of India called Kashmir, hence the name (Bailey and Woods, 1977). Later in Australia, KBV was detected in *A. mellifera* (Bailey et al. 1979), even though *A. cerana* are absent from the region.. Laboratory experiments with *A. mellifera* worker bees and pupae showed that KBV replicates so fast that it can kill bees within 3 days from the day viral particles are injected into the hemolymph (Bailey et al. 1979). However, KBV does not cause severe infection in bees if virus particles are ingested with food. Larvae fed KBV develop into adults and the virus is "inapparent" in adult bees (Anderson and Gibbs 1988). KBV appears to be more virulent under laboratory conditions (Allen and Ball 1995). There are no clearly defined morphological disease symptoms that can be seen in adult honey bees under field conditions (Allen and Ball, 1995).

Chronic Bee Paralysis Virus (CBPV)

CBPV was one of the first viruses isolated from paralyzed honey bees. Before its identification, it was suspected that tracheal mite, *Acarapis woodi* (Rennie) was responsible for bee paralysis (Bailey et al. 1963). Two types of symptoms are associated with CBPV. First, and most common, is that the wings are dislocated, resulting in inability to fly often in combination with abnormal trembling, and swollen abdomens. Second, shiny, hairless and dark coloured bees struggle at the entrance to get inside the hive. The close contact of overcrowded bees breaks the hairs from the cuticle, allowing

CBPV to spread from diseased bees to healthy bees via their exposed epidermal cytoplasm (Ball and Bailey 1997). ABPV and CBPV infected bees share some symptoms, like trembling and inability to fly, but ABPV is more virulent and takes only 1 day to kill bees while CBPV takes several days (Bailey 1965b).

Acute Bee Paralysis Virus (ABPV)

Bailey et al. (1963) discovered ABPV when conducting CBPV infectivity tests in the laboratory. Both ABPV and CBPV viruses were isolated as a result of investigation of causative agents of paralysis in bees. When bees were experimentally inoculated with purified CBPV particles, the bees remained flightless and trembling for about 5-7 days before they died. In contrast, when healthy bees were injected with extract prepared from a group of apparently healthy bees and symptoms observed for 2-4 days, most of the bees became flightless and quickly died. Virus particles that were isolated from the extracts of those apparently healthy bees were the cause of this rapid paralysis, hence the designation of the name acute bee paralysis to distinguish it from CBPV (Bailey et al. 1963). ABPV affects both brood and adult stages of bees mainly during summer, but there are no morphological symptoms visible in bees that are infected, (Bailey 1965a; Bailey 1981). Although, occasionally infected larvae die before they are sealed in brood cells, those that survive emerge as "inapparently-infected" adult bees (Bailey and Ball 1991). ABPV and KBV are, serologically and pathologically closely related to each other. Symptoms and pathology of infections are similar. For example, both viruses persist as inapparent infections in bees and multiply quickly only when injected into the hemolymph of adult bees (Anderson 1991). The strains of KBV from Canada and Spain are serologically more closely related to ABPV than are other strains of KBV (Allen and Ball 1995).

However, molecular phylogenetic analysis found that KBV and ABPV share only 70% sequence homology and can be inferred to be different species, even though there is no clear geographical and ecological separation between the two viruses (Evans 2001; de Miranda et al. 2004).

Microsporidia: *Nosema*

Nosema disease, also called microsporidiosis, is an economically important and commonly encountered disease of *A. mellifera* caused by *Nosema* spp. belonging to the phylum Microsporidia (Bailey and Ball 1991; Fries et al. 1996). There are two species of *Nosema* commonly infecting honey bees: *N. apis* and *N. ceranae* obligate parasites that grow inside the digestive tract of bees and affect their health. Both have received widespread attention, but the latter species is relatively "new" and not as well understood. *Nosema ceranae* was first described in the Asian honey bee, *A. ceranae,* in China, by Fries et al. (1996). The role of *Nosema* in recent honey bee winter colony losses is unclear, but, recently it has been implicated as a potential cause of losses of *A. mellifera* in Europe (Higes et al. 2006), Asia (Huang et al. 2007), United States (Chen et al. 2008) and Canada (Williams et al. 2008).

Some laboratory studies show that *N. ceranae* is highly pathogenic when experimentally inoculated into *A. mellifera* bees (Higes et al. 2007). However, it does not appear to be highly virulent in all geographic regions (Gisder et al. 2010; Higes et al. 2013). Laboratory assays by Forsgren and Fries (2010) found no difference in virulence of *N. ceranae* and *N. apis* in individually infected honey bees. *N. ceranae* has been present in Manitoba, Canada from at least 1998 onwards and in Canada since 1994 (Currie et al. 2010). Although several studies demonstrate that *N. ceranae* is gradually

replacing *N. apis* in *A. mellifera* colonies (Klee et al. 2007; Paxton et al. 2007), the replacement is not universal and a study in Germany showed that *N. apis* is more prevalent than the *N. ceranae* (Gisder et al. 2010). In a recent two year study conducted in Spain, *N. ceranae* did not replace *N. apis* in honey bee colonies, and there was no evidence for competition between the two *Nosema* spp. (Martin-Hernandez et al. 2012).

Nosema infection spreads when bees swallow mature spores from pollen and honey contaminated with spores. Often in the case of *N. apis,* disease is associated with the presence of bee faeces inside the hive (Fries et al. 1996; Paxton et al. 2007; Higes et al. 2008a). Pollen collecting foragers that are infected with *N. ceranae* spores can also contaminate floral sources of pollen and thereby spread spores to different hives where they are later consumed by young bees (Higes et al. 2008a). After *Nosema* spores are ingested by bees, the polar filament of the *Nosema* spore germinates and penetrates the honey bee gut wall (Fries et al. 1996). The spore injects sporoplasm from the tubular polar filament and reproduces sexually inside the gut epithelial cells, producing more spores that eventually cause the cell to burst and release the mature spores back into the gut lumen (Fries et al. 1996). High levels of *N. apis* infection may also cause worker bee mortality during winter resulting in weaker spring colonies (Bailey and Ball 1991). *N. ceranae* is also associated with reduced honey production and increased winter mortality (Higes et al. 2006).

The most common method to assess *Nosema* abundance is by spore counts of pooled samples of bees (Cantwell 1970). The age cohort of bees that should be sampled in order to predict bee loss or future *Nosema* level is poorly understood. *Nosema* prevalence is higher in foragers or old worker bees than newly emerged bees and house bees (Smart and Sheppard 2012). Younger bees infected in fall subsequently undergo

overwintering and can spread spores to other bees. Infected bees with high *Nosema* levels can die during winter, but the winter environment may affect disease dynamics with *Nosema.* The *Nosema* spore count in outdoor-wintered colonies is slightly higher than in indoor winter colonies (Williams et al. 2010).

Increasing knowledge of host (*A. mellifera*), parasite (*V. destructor),* and pathogen (viruses, *Nosema* and bacteria) interactions has led to suggestions that viruses and nosema may contribute substantially to honey bee mortality (Ball and Allen 1988; Allen and Ball 1996; Brodsgaard et al. 2000). However, contradictory results from different studies have caused confusion about the relative importance of pathogens in different contexts and the extent to which each contributes to colony losses in winter. Recently, Dainat et al. (2012b) used long-term monitoring of colonies infected with parasitic mites and studied disease-causing agents and genes involved in bee immunity and, concluded that, *Varroa*, DWV, and *Nosema* pathogens all might be used as prognostic markers for winter colony losses.

Transmission

Spread of parasites and pathogens in a colony can occur though horizontal or vertical transmission depending on the disease or parasite involved. Horizontal transmission, involves the transmission of parasites between different individuals of the same generation of host or from individuals from different colonies. However, vertical transmission of parasites occurs from one generation to the next, from mother to offspring via egg (Chen et al. 2006a) or from father to offspring via sperm.

It has been experimentally proven that *Varroa* are effective vectors of several viruses and transmit KBV and DWV through both horizontal and vertical transmission

(Chen et al. 2004c). A subsequent study conducted by Shen et al. (2005b) further supports the role of *Varroa* as vector transmitting KBV among bees. SBV infection has also been associated with *Varroa* mite infestation. SBV occurs in a high proportion of adult bees from *Varroa* mite-infested colonies (Ball 1989; Berenyi et al. 2006). Detection of SBV in *Varroa* (Tentcheva et al. 2004b; Shen et al. 2005a; Chantawannakul et al. 2006) suggests *Varroa* also at least has the potential to transmit the virus in the bee colonies. *Varroa* are thought to sometimes act as a vector for BQCV (Bailey 1976). Tentcheva et al. (2004a) failed to detect BQCV in any of the *Varroa* they examined, but further studies to confirm the role of *Varroa* as a vector in BQCV transmission are necessary.

Through feeding behaviour, *Varroa* may play a role in activating viruses such as KBV to a pathogenic level. In the absence of *Varroa*, KBV persists as an inapparent infection (Bailey et al. 1979). But, high mite-infestation levels result in high viral titer in bee colonies (Hung et al. 1996). In some cases, the presence of *Varroa* alters the strain of virus present in bee populations. In Hawaii the recent introduction of *Varroa* has selected for a more virulent strain of DWV (Martin et al. 2012).

Varroa interacts with many viruses in ways that are not understood. The term `Bee Parasitic Mite Syndrome' (BPMS) has been used to describe the disease complex, which results when colonies are simultaneously infested with mites and multiple diseases (Shimanuki et al. 1994). Several viruses are associated with “bee parasitic mite syndrome” (BPMS) (Shimanuki et al. 1994), but their role in BPMS has not been established. Other syndromes that may be related to viruses include CCD (described earlier) (Cox-Foster et al. 2007) and Idiopathic Brood Disease Syndrome (IBDS) (vanEngelsdorp et al. 2013). The impact of *Varroa* and viruses varies with geography,

type of virus, strain of virus and strain of bees and their interactions with each other. Hence, detailed manipulative studies are required to quantify combinations of virus levels and *Varroa* that cause winter honey bee colony losses.

Control methods

Despite the threat presented by viruses, there are currently no adequate control measures available for beekeepers to reduce virus impact on colony health. Beekeepers can attempt to keep *Varroa* populations below thresholds to reduce virus transmission and minimize immune suppression, or they can apply cultural and chemical treatments to minimize the impact of other diseases and parasites so that lower stress on colonies may reduce the impact of viruses. Cultural treatments currently are limited to requeening, removal of virus infected equipment, and provision of good nutrition (Nasr 2012). Development of RNAi based treatments would provide an attractive alternative that might reduce the impact of viruses, and selective breeding for virus resistance may provide some benefit.

Control of Varroa

Control of the most serious parasite *Varroa*, would reduce a lot of the problems with associated diseases and environmental factors that contribute to colony loss. Control of *Varroa* has been shown to reduce DWV levels (Locke et al. 2012). Currently, several chemical treatments are registered to control *Varroa* in Canada, but the mites are resistant to some. Apistan® (fluvalinate) was the first chemical treatment to which mites developed resistance and resistance to Checkmite (coumaphos) followed shortly (Currie et al. 2010). Due to reductions in efficacy, their use has been reduced or discontinued by

beekeepers (Eischen 1995; Elzen et al. 1998; Currie et al. 2010) and they have been replaced by other acaricides, Apivar® (amitraz), Mite Away Quick Strips® (formic Acid), Thymovar® (thymol), and oxalic acid. Some acaricides can also increase susceptibility to viruses. Recently, a study by Locke et al. (2012) showed that treatment of *Varroa*-infected colonies with Apistan® initially increases DWV concentration relative to untreated colonies, although virus level decreases once mite levels are ultimately reduced by the acaricides. The use of acaricides may adversely affect bee immunity, thus leading bees to become more susceptible to DWV infection (Locke et al. 2012).

Since effective control of *Varroa* with acaricides cannot be guaranteed, alternative control measures are necessary to reduce stress caused by other viruses so that colonies can survive under higher mite levels. Control of viruses could also help mitigate the effects of other stresses such as pesticide residues, environmental change, poor nutrition and other factors that result in the suppression of immune defenses and poor colony health (Boncristiani et al. 2012).

Genetic diversity

Recent work has shown that high genetic diversity in honey bee colonies can help them to respond better to environmentally variable conditions (Mattila and Seeley 2007). Colonies with low genetic diversity (mated with only one or two drones) have a narrower range of behavioural response thresholds among their workers, which can lead to large variation in colony homeostasis if too many specialized workers are allocated to tasks (Myerscough and Oldroyd 2004). Such colonies can be expected to experience large fluctuations in expression of colony-level phenotypic responses. Genetically diverse

colonies have higher bee populations, construct comb faster, grow larger in size, and produce more honey than genetically uniform colonies (Mattila and Seeley 2007). Foragers of genetically diverse colonies are also better at communicating the presence of food resources through waggle dancing and take more foraging trips than those of genetically similar colonies.

There is a need to study how genetic diversity relates to resistance of parasites (*Varroa*) and diseases (virus, *Nosema*) in honey bees. The effect of genetic diversity on resistance to one bacterial brood disease has been examined. Seeley and Tarpy (2007) showed that genetically diverse colonies are more resistant to *Paenibacillus larvae* (White) Ash, Priest and Collins, which causes American foul brood disease, than genetically uniform colonies.

In addition to increased genetic diversity, selection and breeding of disease and parasite resistant bee strains could be an effective way to defend against viral pathogen attacks in honey bees. Despite the major impact of viruses on colony losses, currently there are no lines of bees have been developed specifically for resistance against bee viruses. There may be potential to select strains of bees resistant to one or more viruses while maintaining high genetic diversity. Various behaviors of honey bees, such as grooming behaviour, hygienic behaviour and suppressed mite reproduction (SMR), are important behavioral mechanisms of disease resistance against *Varroa* (Spivak and Gilliam 1998; Lapidge et al. 2002; Harbo and Harris 2005) and thus may indirectly help bees to tolerate or resist virus infection.

RNA interference

Viral disease outbreaks as well as inapparent viral infections can seriously affect the profitability of the beekeeping industry. Beekeepers are advised to take measures to limit viral infections, although chemotherapies for killing bee viruses are currently not available. RNA silencing, also known as post-transcriptional gene silencing (PTGS) or RNA Interference (RNAi) is a comparatively simple, rapid, and specific method for silencing gene function. RNAi reduces gene expression by causing degradation of the target mRNA. RNAi effects were first discovered in nematode *Caenorhabditis elegans* (Maupas) Doughert, by Fire, Mello and co-workers in 1998 who purified the RNA antisense, RNA sense and dsRNA. They showed the introduction of double-stranded RNA homologous to endogenous sequences rapidly leads to destruction of target mRNAs (Fire et al. 1998). The suppression of virus replication through RNA interference has recently been applied to a number of species including human beings, plants, and insects (Dzitoyeva et al. 2001; Huang et al. 2006; Van Rij and Andino 2006; Mao et al. 2007; Wolters and MacKeigan 2008). Typically in RNA interference, experimentally delivered or naturally present double-stranded RNA (dsRNA) is cleaved into small fragments of 21-23 base pair molecules (small interfering RNAs or siRNAs) by the intracellular endonuclease enzyme called Dicer (Ketting et al. 2001). The siRNAs antisense strands bind with RNA induced silencing complex (RISC) in the cell and cause specific degradation of the target mRNA (Martinez et al. 2002). Most honey bee viruses are single stranded RNA viruses and make excellent targets for this technique.

RNA silencing of Israeli Acute Paralysis Virus (IAPV) in honey bees was achieved by feeding virus-specific dsRNA to bees, which resulted in a reduction in viral load of IAPV and increased bee-to-brood ratio and nectar storage in laboratory conditions

(Maori et al. 2009). In a large-scale field study of control of IAPV in honey bee colonies using dsRNA specific to IAPV, there were reductions in viral level, higher bee survival rates, larger colony sizes and higher honey production relative to untreated colonies (Hunter et al. 2010). RNA interference has also been reported to reduce *N. ceranae* spore load in honey bees (Paldi et al. 2010) but has not been studied for use against all viruses and has not been developed for deformed wing virus in honey bees.

RATIONALE OF THIS STUDY

Honey bee viruses along with the *Varroa* are among the greatest threats to honey bees worldwide, as both severely affect colony health, cause declines in bee population and can ultimately result in colony death. Because *Varroa* populations have, developed resistance to most registered acaricides used to control the mite, there is a great need for alternative control methods for targeted management of the pathogens associated with *Varroa*. Management of these pathogens could help reduce the *Varroa*-related stresses on bee colonies, allowing colonies to survive when *Varroa* cannot be effectively controlled.

This thesis has the following objectives:

(1) To assess the distribution and relative concentration of seven viruses and to establish baseline data for the types and concentrations of viruses present in Canada in healthy and unhealthy colonies.
(2) To understand the seasonal patterns and interactions of mites, viruses and other pathogens and their effects on winter colony losses in two different

wintering environments, and to assess the relative impact of parasites and pathogens so that targeted control strategies can be developed for the most important groups of pathogens.

(3) To quantify the impact of high and low genetic diversity on the resistance of bees towards parasites, viruses and other pathogens.

(4) To study, whether RNAi could be used to reduce the effects of DWV infection in honey bees.

CHAPTER 2. OCCURRENCE AND QUANTIFICATION OF ECONOMICALLY IMPORTANT VIRUSES IN HEALTHY AND UNHEALTHY HONEY BEE, *APIS MELLIFERA* L., COLONIES IN CANADA

ABSTRACT

Honey bee winter colony loss is a serious problem that beekeepers face in Canada and in other temperate countries. RNA viruses infecting honey bees are one of the major threats to the beekeeping industry and pollinator conservation but their presence in different regions in Canada has not been characterized. The occurrence, quantification and distribution patterns of deformed wing virus (DWV), black queen cell virus (BQCV), Israeli acute paralysis virus (IAPV), sac brood virus (SBV), acute bee paralysis virus (ABPV), chronic bee paralysis virus (CBPV), and Kashmir bee virus (KBV) were characterized in 80 "healthy " honey bee colonies and 23 unhealthy (identifiable symptoms and signs of population decline) colonies by employing RT-PCR for virus identification and qPCR for quantification. Within representative samples from eight regions (Provinces) of Canada, 100% (n = 80) of colonies contained detectable levels of at least one virus as measured by RT-PCR. All seven viruses were common but the most prevalent viruses were DWV, which occurred in 90 -100% (proportion of colonies with detectable virus) of the samples, followed by BQCV 80-100%, IAPV 40–70%, KBV 10–40%, SBV 0–40%, ABPV 0–20% and CBPV 0–20%. SBV was the only virus that was more prevalent in unhealthy colonies (sampled in spring) from Manitoba (n = 23) than in healthy (sampled in fall) from colonies across Canada (n = 80) but seasonal or regional differences may have contributed to these differences. Prevalence of this virus did not vary between healthy (sampled in fall) and unhealthy colonies (sampled in spring) within Manitoba (n = 23). Multiple infections were ubiquitous with a few colonies having

simultaneous infection with as many as five viruses. Among the three viruses quantified by qPCR, DWV had the highest relative concentrations in pooled samples of worker bees and concentration ranged from 0.004 – 1.15 (log $2^{-\Delta Ct}$ concentrations). BQCV concentration ranged from 0.04 – 0.39 and IAPV ranged from 0.00016 – 0.27. DWV was the only virus that had lower concentrations in healthy colonies (sampled in fall) from across Canada and within Manitoba than in unhealthy colonies (sampled in spring) from Manitoba however, these differences could be due to different locations or timing of sampling. DWV was the only virus within healthy colonies that differed in concentration among provinces. Colonies in all provinces except British Columbia had similar concentrations of DWV. DWV concentration was higher in British Columbia than in Manitoba, Quebec, New Brunswick and Nova Scotia, but similar to that found in Alberta, Ontario and Prince Edward Island and unhealthy colonies from Manitoba. Differences in the concentration of the three viruses were found within the provinces of British Columbia and Alberta and within unhealthy colonies in Manitoba. BQCV was positively correlated with IAPV across all samples but no correlations were found between other virus pairs. My study provides the first major baseline study of viruses in Canadian honey bees.

INTRODUCTION

Recent losses in honey bee, *Apis mellifera* L., colonies in North America and around the world have greatly affected apiculture and the pollination industry (Underwood and vanEngelsdorp 2007; vanEngelsdorp et al. 2007; Aizen and Harder 2009; Currie et al. 2010; vanEngelsdorp and Meixner 2010). Large-scale loss of colonies in winter due to various diseases occurs in Canada, the USA, Europe, and other temperate regions (Currie et al. 2010; Neumann and Carreck 2010; Potts et al. 2010). In Canada, average colony losses increased from a historical average of 5-15% to over 36% in recent years with some commercial beekeepers losing up to 90% of their hives (Currie et al. 2010). These impacts are of considerable economic importance because the value of crops pollinated by honey bees is estimated to be up to CAD $2 billion annually in Canada (CHC 2012) and US $212 billion on a global basis (Gallai et al. 2009).

The mite, *Varroa destructor* Anderson and Trueman, is often associated directly or indirectly with these losses (Currie et al. 2010) and has become more problematic because *Varroa* resistance to acaricides such as fluvalinate and coumaphos is widespread (Eischen 1995; Elzen et al. 1998; Elzen et al. 1999; Currie et al. 2010). Among the other stresses affecting colony loss, bee viruses are thought to be one of the major threats and their transmission through *Varroa* has caused serious problems in the beekeeping industry (vanEngelsdorp et al. 2008; Highfield et al. 2009; vanEngelsdorp et al. 2009; Currie et al. 2010; Genersch et al. 2010; Guzman-Novoa et al. 2010). Virus transmission occurs through horizontal and vertical pathways (Chen and Siede 2007); however, transmission during feeding of *Varroa* on bee hemolymph typically has more impact on bee health than other transmission routes (e.g., food and sexual transmission). Both

mature and immature stages of *V. destructor* are effective vectors and activators of bee viruses (Shen et al. 2005b; Gisder et al. 2009; Mockel et al. 2011).

More than 20 different bee viruses have been detected in honey bees (Allen and Ball 1996; Ball and Bailey 1997; Evans and Schwarz 2011). Multiple virus infections have been reported in individual bees as well as in bee colonies (Chen et al. 2004b; Tentcheva et al. 2004b; Berenyi et al. 2006). Most of the bee viruses including the seven in this study are 30 nm isometric particles containing positive-sense, single-stranded RNA. The viruses most often thought responsible for severe disease outbreaks are distributed worldwide and include deformed wing virus (DWV), black queen cell virus (BQCV), sacbrood virus (SBV), Israeli acute paralysis virus (IAPV), Kashmir bee virus (KBV), chronic bee paralysis virus (CBPV), and acute bee paralysis virus (ABPV) (Chen and Siede 2007). Virus infections are found in all developmental stages of bees. Most of the bee viruses cause inapparent (lack of obvious morphological and behavioural symptoms) infections in bees carrying them. Of the common viruses, only DWV and CBPV produce obvious morphological symptoms in adult workers and SBV produces characteristic symptoms in larvae that are easily identifiable in field conditions.

All of the seven viruses listed above occur in adult workers. DWV is one of the most prevalent and is widely distributed all over the world. DWV multiplies slowly, and it is closely associated with the mite *V. destructor* (Ball and Allen 1988; Martin 2001; Highfield et al. 2009). It causes visible deformities in severely infected adult bees and is associated with high winter loss of bees (Highfield et al. 2009; Williams et al. 2009; de Miranda and Genersch 2010). Both BQCV and SBV cause visible symptoms in immature bees but also affect adults. BQCV is commonly found in adult bees throughout the world, and has been implicated as a cause of increased colony loss when found in

combination with *N. apis* (Chen and Siede 2007). SBV affects the honey bee brood resulting in swollen sac-like larvae and premature larval death, and also infects adult bees where it decreases life span without expressing obvious signs of disease (Bailey 1969; Bailey and Fernando 1972). IAPV, KBV, and ABPV are closely related to each other, forming a complex group within the family *Dicistroviridae* (de Miranda et al. 2010). Recently, IAPV received much attention throughout the world, as it has been linked to colony collapse disorder (CCD) in bees (Cox-Foster et al. 2007). IAPV causes bee paralysis and death of colonies, but its role as a specific cause of CCD is unclear (Maori et al. 2007). IAPV is not restricted to *A. mellifera* and occurs in the Asian honey bee *A. cerana* (Ai et al. 2012), dwarf bee *Apis florea* F. and the giant honey bee, *Apis dorsata* F. in India (Desai and Currie, unpublished data). KBV, first isolated from the Asian honey bee *Apis cerana* in Kashmir, India, is also found in *A. mellifera* from Canada, USA, Europe and several other countries (Allen and Ball 1996; Ellis and Munn 2005). There are no clearly defined disease symptoms associated with KBV in honey bee colonies under field conditions (Bailey and Woods 1977; Allen and Ball 1995). KBV has been strongly associated with *V. destructor,* is found in *Varroa*'s saliva (Shen et al. 2005a; Shen et al. 2005b) and has also been implicated in *Varroa*-induced colony losses (Todd et al. 2007; Ribiere et al. 2008). ABPV transmission also occurs through *V. destructor* feeding. ABPV is less prevalent than some of the other viruses and levels of virus vary with season. Although this virus produces no morphological symptoms in adults it results in characteristic paralysis (Allen and Ball 1996). CBPV-infected adults also show no morphological deformities, but exhibit paralysis similar to those infected with ABPV. CBPV-infected bees tremble and are unable to fly. They present as "dark-coloured" hairless-bees showing symptoms of paralysis and crawling at the entrance (Ball and

Bailey 1997). CBPV occurs sporadically and occasionally is associated with collapse of colonies. Unlike ABPV, CBPV takes several days to kill bees (Bailey and Ball 1991).

The distribution and relative concentration of viruses within bee colonies in Canada is poorly understood. In Canada, only three viruses KBV, DWV and IAPV have been previously reported in adult bees (Allen and Ball 1995; Berenyi et al. 2007; Palacios et al. 2008) and the distribution and impact of these viruses is not known. Recently, beekeepers have observed increased occurrence of symptoms such as twitching bees, dark and deformed bees, abnormally low worker populations, increased winter colony losses and collapse of entire colonies (Rhéal Lafrenière Personal communication), but the types of virus present and potential role that viruses might play in these events is unclear.

It is very difficult or impossible to differentiate and quantify virus infections through field symptoms but they can be diagnosed using RT-PCR and quantitative PCR (qPCR) (Chen et al. 2005; Chantawannakul et al. 2006; Tentcheva et al. 2006). The qPCR assay provides rapid, sensitive, specific and quantitative viral diagnosis, and is especially good for detecting virus at low concentrations and allows quantitation of mixed infections.

The purpose of this study is to characterize the occurrence and frequency of seven single stranded honey bee RNA viruses (DWV, BQCV, SBV, IAPV, KBV, CBPV and ABPV) by using conventional RT-PCR and to quantify the relative levels of DWV, BQCV, and IAPV from different geographical regions of Canada using qPCR. I investigated the distribution pattern of honey bee viruses in randomly collected samples of adult workers from healthy colonies and non-random samples from beekeeper-identified unhealthy colonies. My goal was to better understand the distribution and

relative concentration of these viruses in healthy and unhealthy colonies and to establish baseline data for the types and concentrations of viruses present in Canada.

MATERIALS AND METHODS

Honey bee sampling

Samples of bees were collected from honey bee colonies from five beekeepers in each of eight provinces within Canada by provincial apiarists and collaborators and immediately sent to the University of Manitoba, Winnipeg, Canada. Samples of approximately 100 live bees from each colony were shipped at ambient temperature by express mail. Bees from "healthy colonies" were obtained by collecting them from a total of ten hives (representing two colonies from each of five beekeepers) in each Province (n = 80). I also collected a separate set of bee samples (n = 23) from "symptomatic colonies" that had been identified by the provincial apiarist and local beekeepers within the Province of Manitoba, Canada. Colonies identified as "symptomatic" either had a heavy infestation of *Varroa*, had collapsed, had evidence of paralysed and dark coloured bees, were unexpectedly low in population, or had combinations of these symptoms. All adult honey bee samples from healthy colonies were collected and submitted for analysis between September 2009 and December 2009 and for unhealthy colonies between April to June each year 2009 and 2010. Upon arrival at the University of Manitoba, all the samples were stored immediately in a -80 °C freezer for further analysis.

RNA isolation, quantification and cDNA synthesis

A subset of adult worker honey bees (n = 10) from each hive sample was selected and the bees were pooled and crushed into a fine homogenous powder in a mortar cooled by liquid nitrogen. The RNA was extracted from 30 mg of ground honey bee material using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) following manufacturer's instructions. RNA samples were dissolved in DEPC-treated water in the presence of ribonuclease inhibitor and stored for further analysis at -80 °C. An average of 2 µg of total RNA in 20µL reaction volume was reverse transcribed using M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) used according to the supplier's recommendations.

PCR analysis

Polymerase chain reaction (PCR) reactions were performed in a PTC-100 Thermal Cycler (MJ Research Inc.). Amplification was performed in a 20 µL reaction volume consisting of: 2.5 µL of cDNA mixed with 5 µL 10X *Taq* buffer, 1 µL of 10 mM dNTP, 1 µL of forward primer and reverse primer each and 2.5 U of *Taq* polymerase, the volume adjusted with water. A control without template was included in all batches. Honey bee actin (DNA fragment), a housekeeping gene, was used as an endogenous control gene. The thermal cycling profiles were set as follows: the mixture was heated for 5 min at 95 °C, 31 PCR cycles at 95 °C for 30 s, between 55 and 58 °C (depending on the annealing temperature of the primer) for 30 s, 72 °C for 1 min. The reactions were completed by a final elongation step at 72 °C for 5 min and the reaction was then kept on hold at 4 °C. The primers for the PCR are listed in Table 2.1. The PCR products were electrophoresed in 1% agarose gel stained with ethidium bromide. The PCR products

were gel-extracted, ligated into pGEM-T easy plasmid vectors (Promega, Madison, WI, USA), and transformed into *E.coli* DH5α competent cells (Promega, Madison, WI, USA) according to the manufacturer's protocol. The transformed colonies positive for the presence of the insert were cultured and plasmid DNA was purified with the GenElute Plasmid Miniprep kit (Sigma-Aldrich, St. Louis, MO, USA). The plasmid clones were then sequenced using DNA sequencing services (Macrogen Inc., United States) and compared using NCBI's (National Center for Biotechnology Information) BLAST program. A BLAST search analysis confirmed that the seven cloned sequences resulted in a virus-specific product (data not shown).

qPCR analysis

Virus quantification by qPCR was performed in an ABI Prism 7300 real-time PCR machine system (Applied Biosystems, Foster City, CA, USA). Amplification was performed in 25 µL reaction volume consisting of 1 µL cDNA template (5 fold dilution), 1 µL of forward and reverse primer mix (100mM), 12.5 µL of 2X QuantiFast SYBR Green PCR kit (Qiagen, Valencia, CA, USA), the volume adjusted with water. Negative controls (reaction mix without cDNA template) were included in triplicate, in all batches, to check for contamination in any of the reagents. The qPCR was performed with a single cycle at 95 °C for 5 min, 40 PCR cycles at 95 °C for 15 s, 55 °C for 30 s, 72 °C for 30 s, followed by a dissociation step to determine the specificity of amplification. The qPCR reactions were performed in triplicate and the relative virus gene expressions were calculated using the $2^{-\Delta Ct}$ method, where Ct indicates the cycle threshold. Relative expression $2^{-\Delta Ct}$ was calculated; where $\Delta Ct = Ct_{(virus\ gene)} - Ct_{(\beta\text{-}actin)}$ (Persico et al. 2007). Honey bee actin (DNA fragment) was used as an internal control gene to normalize qPCR

experiments, since it is a stable gene and expressed in all honey bee cells. The primers for the qPCR are listed in Table 2.2.

Statistical analysis

The RT-PCR results were expressed as prevalence (the proportion of colonies with presence of virus) for DWV, BQCV, SBV, IAPV, KBV, CBPV, and ABPV. Proportions of colonies infected with different viruses in each province and comparisons between unhealthy colonies from Manitoba with healthy colonies from across Canada and healthy colonies from Manitoba were examined by Fisher's exact test (PROC FREQ, SAS 1999). The qPCR data were log $2^{-\Delta Ct}$ +1 transformed prior to analyses to improve the equality of variance, (Snedecor and Cochran 1980). In order to analyze the difference between virus levels of DWV, BQCV, and IAPV in different provinces, a multivariate analysis (MANOVA) was first performed using PROC GLM (SAS 1999). Univariate analysis (Welch's ANOVA) was than performed for each virus to assess differences in virus levels between provinces (Welch 1951). Means were separated by Tukey's test with the significance level of 0.05. Relative virus levels of DWV, BQCV, and IAPV for pooled values across all provinces for healthy colonies were compared with unhealthy colonies from Manitoba by orthogonal CONTRAST (SAS 1999).

Concentrations of DWV, BQCV and IAPV from the same samples were analysed by PROC MIXED using a repeated measures design with hive as the experimental subject and virus as a repeated measure (Carey 2012). An unstructured covariance matrix was used. Where significant interactions were observed between virus and provinces (including unhealthy and healthy colonies) interactions were partitioned by province using a slice statement. For provinces where differences in virus levels were observed,

differences among means within provinces were compared by Bonferroni corrected contrasts using Pdiff ($P<0.005$). Partial correlation coefficients for two viruses (controlling for the third virus) were generated in PROC GLM (MANOVA) (SAS 1991). Virus concentration data were presented as mean log $2^{-\Delta Ct}$ ± standard error.

RESULTS

Distribution pattern of seven bee viruses

A total of 103 honey bee samples from individual colonies were obtained from eight Canadian provinces. For each virus, the proportion of colonies infected with the virus was similar across all provinces within all healthy colonies ABPV $p = 0.274$; BQCV $p = 0.075$; CBPV $p = 0.062$; DWV $p= 1.0$; IAPV $p = 0.09$; KBV $p = 0.056$; SBV $p = 0.56$ (Fisher's exact test). Some of the viruses occurred more frequently than others (χ^2=153.95, df = 6, $P < 0.0001$). DWV was the most prevalent virus and was found in 90 to 100% (n = 10) of healthy colonies across all provinces and occurred at similar frequencies (100%, n = 23) in "symptomatic" colonies from MB (χ^2=0.17, df = 1, P = 0.68) (Fig. 2.1). BQCV was also very common and had similar prevalence in healthy colonies across all provinces 70-100% (n = 10) and unhealthy colonies from MB 91% (n = 23) (χ^2=0.04, df = 1, $P < 0.85$). IAPV was common but more variable in occurrence than some viruses, and ranged from 40-70% occurrence in healthy colonies across all provinces (n=10) and was at similar rates (40%) in unhealthy colonies (χ^2 = 1.77, df = 1, P = 0.18). It is of interest to note that SBV occurrence was higher in unhealthy colonies from Manitoba 44% (n = 23) than in the healthy colonies across all provinces 16% (n = 80) (χ^2 = 5.21, $P < 0.02$); however, SBV prevalence from healthy colonies within Manitoba was similar to healthy ones (χ^2 =1.89, df =1, P = 0.17) (Fig. 2.1). KBV was

also found at low levels of prevalence (10-40%) but was detected in all provinces except Alberta. ABPV and CBPV occurred at low frequencies (0-20% and 0-30% respectively), and were not consistently detected in all provinces (Fig. 2.1). KBV (χ^2 = 2.59, df =1, P = 0.11), ABPV (χ^2 = 0.29, df = 1, P = 0.59) and CBPV (χ^2 = 0.57, df = 1, P = 0.45) prevalence in healthy colonies across Canada did not differ from unhealthy colonies in MB. There were no differences in prevalence of any viruses among with unhealthy and healthy samples within Manitoba.

Relative virus levels of DWV, BQCV, and IAPV

A quantitative analysis of viruses (qPCR) was performed to examine the difference in relative concentration of the three most common viruses I found in Canada (DWV, BQCV and IAPV) (see above). Overall, virus levels differed among the different locations (healthy colonies in different provinces and the unhealthy colonies from Manitoba) (MANOVA, Wilks's λ = 0.47, F = 3.30, df = 24, 267.43, P < 0.0001) (Fig. 2.2). DWV levels accounted for 61.11% of the variability in virus concentration followed by BQCV (27.24%) and IAPV (11.65%). Univariate ANOVAs showed DWV varied between provinces (Welch ANOVA, $F_{8, 32.52}$ = 11.88, P = 0.0001) but BQCV and IAPV levels did not (BQCV, Welch ANOVA, $F_{8, 35.31}$ = 1.45, P = 0.21), (IAPV, Welch ANOVA, $F_{8, 32.43}$ = 2.14, P = 0.06) (Fig. 2.2). The DWV concentration in British Columbia (BC) was much higher than in Manitoba, Quebec, New Brunswick, and Nova Scotia, however, its level was similar to that found in Alberta, Ontario and Prince Edward Island (Tukey's test, P < 0.05) (Fig.2.2). DWV concentrations in all provinces other than BC were similar to each other.

Differences in relative concentration of the three viruses occurred within the provinces of AB (SLICE, df = 2, 94, F = 9.72, P < 0.0001), BC (SLICE, df = 2, 94, F = 12.10 P < 0.0001) and in unhealthy colonies within MB (SLICE, df = 2, 94 F = 27.97 P < 0.0001) (Fig.2.2). DWV concentration was higher than IAPV concentration within the provinces of AB, BC and in unhealthy colonies in MB (P < 0.05; Pdiff). BQCV concentration was lower than DWV concentration within the province of BC and in unhealthy colonies in MB (P < 0.05; Pdiff) but did not differ from DWV in AB (P > 0.05; Pdiff) (Fig.2.2). IAPV concentration was similar to BQCV in all provinces (P > 0.05; Pdiff). No difference in the concentrations of the three viruses occurred within healthy colonies in the rest of the provinces (P > 0.05; Slice).

Within Manitoba, relative virus loads of DWV were greater in unhealthy colonies than in healthy colonies (Tukey's test, P < 0.05) and DWV concentration was greater in unhealthy colonies from Manitoba than in healthy colonies for values pooled across all provinces (CONTRAST F = 18.42, df = 1, 94, P < 0.0001).

BQCV was positively correlated with IAPV virus levels across all samples (N = 103) (partial correlation coefficients (R = 0.31, P = 0.002). However, no other combinations of virus DWV - BQCV, DWV-IAPV showed significant correlations.

Simultaneous virus infections

Co-infections with multiple viruses in colonies were common and no samples were found with less than two viruses (Tables 3 and 4). Dual infection occurred in 33% of healthy colonies (Table 2.3) and was at a similar level in unhealthy colonies (30%) (Tables 2.3 and 2.4). The most prevalent dual infection was the pair of DWV and BQCV but the occurrence of this pair was at similar levels within the healthy colonies (24%) and

the unhealthy colonies (22%). Co-infections with three viruses were found in 48% of the healthy and 48% in unhealthy colonies. Four viruses were present at the same time in 18% of healthy colonies and 17% of unhealthy colonies. One colony in the unhealthy group and two colonies in the healthy colonies contained five viruses. Sac brood virus was the only virus that occurred at higher frequencies in unhealthy colonies across all provinces than in healthy colonies from Manitoba (see above). SBV occurred in combinations with DWV, BQCV and IAPV in healthy colonies and in combinations with DWV, BQCV, IAPV and CBPV in unhealthy colonies. Quantitative analysis of SBV was not performed in this study.

DISCUSSION

In this study, I characterized the prevalence of seven economically important single-stranded RNA viruses and quantified the variation in relative concentration of three (DWV, BQCV, and IAPV) across a broad geographic range of beekeeping regions in Canada. Since diagnostic procedures are commonly carried out on surviving colonies in spring and the value of this is unclear, I also compared virus composition and level between healthy and unhealthy (diseased) colonies within a region (Province of Manitoba) and within provinces in Canada. The viruses characterized in this study occur in *A. Mellifera* all over the world at varying frequencies, but comparatively little is known about which are the most prevalent and greatest potential threats, especially in northern beekeeping regions. My results show that most regions (as well as unhealthy and healthy colonies) had similar prevalence of viruses with only SBV differences in different samples, although it is not known if these differences were due to symptom class, sample timing or sample location. Multiple infections were ubiquitous and the most prevalent

viruses were DWV and BQCV. My results suggest identification of the mere presence or absence of bee viruses in colonies is not likely to be sufficient for assessing the impact of viruses on colonies or informing the development of successful management strategies for colonies since critical viruses are present in most colonies and their prevalence does not differ across the country or in healthy and unhealthy colonies. Although, it should be noted that the statistical power to detect differences in prevalence among provinces was low as only 10 colonies were sampled from each province.

One of the weaknesses of my study was that healthy colonies were randomly sampled in fall whereas unhealthy colonies were from different beekeepers and from a single province sampled in spring. These colonies were identified and sampled by beekeepers or extension personnel in spring when symptoms appeared. Colonies they selected were based on combinations of high *Varroa* population, small clusters of bees, sudden depopulation of bees, high numbers of twitching dead bees on the bottom board and other characteristics of dying colonies. Thus, observed differences in virus prevalence or concentration between unhealthy and healthy colonies could be due to seasonal differences, regional differences or differences in variety of other beekeeping management practices that affect colony health. However, data on unhealthy colonies provide important information on the pathogen profile and range of virus concentrations in collapsing colonies that will inform the design of future studies on the impacts of specific viruses. Beekeepers and extension personnel typically discover collapsing colonies in spring where attempts to a diagnosis can only be made from those colonies exhibiting symptoms that are still alive. The virus levels found may not represent an accurate picture of which pathogens are responsible for colony loss. Those viruses

remaining in collapsing colonies could be opportunistic pathogens that thrive when the colonies immune responses are weakened by other factors.

Of the potential viruses that infect bee colonies, DWV is emerging as one of the most serious and is frequently associated with severe winter loss of bees (Ball and Allen 1988; Martin 2001; Highfield et al. 2009; Dainat et al. 2012a). DWV is not restricted to *A. mellifera*; it also occurs in the Asian honey bee *Apis cerana* F., the dwarf bee *A. florea* (Allen and Ball 1996; Ellis and Munn 2005), bumble bees (Genersch et al. 2006) and the giant honey bee, *A. dorsata* (Zhang et al. 2012). DWV has recently been linked to honey bee colony declines in Africa, Asia, Europe, Middle East, North America and South America (Allen and Ball 1996; Chen et al. 2004a; Ellis and Munn 2005; Antunez et al. 2006; Berenyi et al. 2006; Berenyi et al. 2007; Ai et al. 2012). My results suggest the impact of this virus is worthy of further study in Canada as it was ubiquitous, often occurred at high levels in healthy colonies sampled in fall, and was at high levels in unhealthy colonies sampled in spring.

There were also significant differences in the relative concentrations of viruses among the different provinces of Canada as shown by the MANOVA analysis. Bee samples from British Columbia, for example had higher DWV concentration than healthy colonies in Manitoba, Quebec, New Brunswick, and Nova Scotia. There may be several reasons for these differences in virus concentrations between provinces. For example, treatment methods (acaricides) that reduce *Varroa* levels can influence DWV replication in diseased colonies and differences in the efficacy of mite control that occur in different regions could be responsible for some of the observed variation in DWV (Currie et al. 2010; Locke et al. 2012) but these require further study. Differences in climate or

beekeeping management practices may also be responsible for the variation and the low number of beekeepers sampled from each region may not be fully representative of provincial averages. I saw comparatively high concentrations of DWV in some provinces but high concentrations of DWV in adult workers also occur in the USA (Chen et al. 2004a), Thailand (Chantawannakul et al. 2006), and Europe (Dainat et al. 2012a).

In this study, quantitative analysis of relative virus showed DWV levels within BC, AB and unhealthy colonies in MB were higher than the other two most common viruses examined (BQCV and IAPV). This is in agreement with previous work in France that examined five of the same viruses as in my study and showed that DWV concentrations are generally higher than those of BQCV, SBV, ABPV, KBV, CBPV (Gauthier et al. 2007).

BQCV, was the second most prevalent virus and present in most of the colonies in every province sampled but the relative concentration was comparatively low relative to DWV. BQCV is also widespread in France (86%) (Tentcheva et al. 2004b), Austria (30%) (Berenyi et al. 2006), Spain (68%) (Kukielka et al. 2008) and Japan (77%) (Kojima et al. 2011), however, the contribution of this virus to colony loss is uncertain. It has been linked to losses when found in association with *Nosema* (Bailey et al. 1983a). In my study, *Nosema* was not quantified in either unhealthy or healthy colonies so potential relationships between nosema and BQCV could not be assessed.

The prevalence of IAPV is of special interest because it has been associated with colonies that die due to Colony Collapse Disorder (CCD) (Cox-Foster et al. 2007). CCD-like symptoms have since been diagnosed in many countries, however, subsequent research was shown that IAPV is not the sole reason for colony losses (vanEngelsdorp et al. 2008; vanEngelsdorp and Meixner 2010). Interactions of various stressors with

multiple pathogens are now generally thought to be involved in contributing to colony losses in different regions of the world (vanEngelsdorp et al. 2009). In my study, IAPV was common. It was detected in 43% of the colonies tested and at relatively high concentrations relative to other economically important viruses (Chapter 3). IAPV did not seem to be linked to colony loss, or symptoms associated with colony decline in this study, as prevalence and relative concentration of IAPV were similar in healthy and unhealthy colonies. IAPV occurs in Australia, USA, Israel, France, China and India (Chen and Evans 2007; Maori et al. 2007; Blanchard et al. 2008; Desai and Currie, Unpublished data). IAPV is prevalent in Australia, but as was the case in my study, is not linked to sudden colony mortality (Cox-Foster et al. 2007). In France, only 14% of the 35 apiaries tested are positive to IAPV, and the virus has not been definitively linked to severe winter losses (Blanchard et al. 2008).

Prevalence of CBPV, ABPV and KBV was comparatively lower in the colonies in my study when compared to DWV and BQCV; however, sporadic increases in prevalence of CBPV, ABPV, and KBV occur in European countries. At least one of these viruses (ABPV) is more often found at higher frequencies in mid-summer than in spring or fall (Aubert et al. 2008) and so if high levels of this virus were present, then it may have been missed by my sampling protocol as colonies were sampled only in fall and spring.

Our study showed SBV occurred more frequently in unhealthy colonies from Manitoba than healthy ones across all provinces. However, when comparing unhealthy colonies within Manitoba with healthy colonies, no differences were noted and differences between the unhealthy and healthy colonies may have been an artifact associated with differences in timing of sampling or regional variation. SBV has also

been reported from colonies that are weak in size France, Austria and Denmark (Tentcheva et al. 2004b; Berenyi et al. 2006; Aubert et al. 2008). Thai SBV a variant also causing disease, is also one of the major causes for severe colony losses in *A. cerana* (Abrol and Bhat 1990; Mishra and Dubey 1996; Ai et al. 2012). In this study, SBV only occurred in combination with DWV, BQCV, IAPV and CBPV. It is not known if SBV may have been a causal factor in colony losses in this study or if it is an "opportunistic pathogen" that increases in prevalence when colonies are weakened by other stressors. Further studies are required to assess the impacts of this virus alone, and in combination with those that co-occurred in unhealthy colonies.

All the colonies tested in my study had simultaneous infections with multiple viruses. There are similar reports of multiple infections from France (Tentcheva et al. 2004b), Austria (Berenyi et al. 2006), USA (Chen et al. 2004b), and China (Ai et al. 2012). Multiple virus infections have also been reported for queens, larvae, pupae and adults where up to four virus combinations can occur within a single individual (Chen et al. 2004b; Chen et al. 2006b). The dynamics of multiple virus infections and the interactions between viruses, *Varroa* populations and other stressors within bee colonies are poorly understood. Studies are needed to understand how interactions between these viruses affect colony survival.

The underlying causes of high rates of overwintering colony loss in the northern hemisphere remain unclear. In Canada, honey bee winter colony losses of 10% – 15% are considered "normal" from a historical perspective (CAPA 2013). However, *Varroa* infestation and interactions with other stressors such as viruses, *Nosema* and colony nutrition have significantly increased colony losses in recent years (Currie et al. 2010). Recent colony losses in Canada have ranged from 21% – 35% and are similar those found

in the US (35.8%) and in Europe (8 – 37%) (vanEngelsdorp and Meixner 2010). The ability to manage bee colonies to prevent colony loss will likely depend developing the ability to directly or indirectly control bee virus epidemics. Increased knowledge of the relative impact of viruses and how to manage viral disease infections is critical.

Our study provides important baseline data on virus prevalence and concentrations in healthy colonies. All seven viruses were common but DWV and BQCV were the most prevalent viruses in Canada. DWV was the only virus that varied in concentration with region and it was at high levels in unhealthy colonies. SBV was the only virus that differed in prevalence in the different sample locations. Management of honey bee virus infections, by better managing *Varroa* populations, providing better nutrition, reducing stresses on bees, reducing infection, breeding bees resistant to viruses or using dsRNA-based treatment methods, may offer substantial hope for successful management of various bee pathogens to prevent winter losses of colonies (Maori et al. 2009; Hunter et al. 2010; Desai et al. 2012). This study suggests that DWV and BQCV would be good candidates as models for examining the impact of virus control strategies under Canadian management conditions, because they are common and occur at moderate-to- high concentrations. The role that SBV plays as a possible contributor to colony loss also warrants further study.

Table 2.1 PCR primers used in the study

Virus specific primer set (5'-3')	Product size (bp)	Reference
ABPV-F: TTATGTGTCCAGAGACTGTATCCA ABPV-R: GCTCCTATTGCTCGGTTTTTCGGT	900	Benjeddou et al. (2001)
CBPV-F: AGTTGTCATGGTTAACAGGATACGAG CBPV-R: TCTAATCTTAGCACGAAAGCCGAG	455	Ribiere et al. (2002)
BQCV-F: TGGTCAGCTCCCACTACCTTAAAC BQCV-R: GCAACAAGAAGAAACGTAAACCAC	700	Benjeddou et al. (2001)
DWV-F: ATCAGCGCTTAGTGGAGGAA DWV-R: TCGACAATTTTCGGACATCA	702	Chen et al. (2004a)
KBV-F: GATGAACGTCGACCTATTGA KBV-R: TGTGGGTTGGCTATGAGTCA	415	Stoltz et al. (1995)
SBV-F: GCTGAGGTAGGATCTTTGCGT SBV-R: TCATCATCTTCACCATCCGA	824	Chen et al.(2004b)
IAPV-F: CCACCCCTCTCAAACAATCTCAAACA IAPV-R: AGATTTGTCTGTCTCCCAGTGCACAT	358	Maori et al.(2007)

bp = base pairs

Table 2.2 qPCR primers used in the study

qPCR Primers for bee viruses used in experiments	Product size (bp)	Reference
RT-BQCV-F: CCTGTATTCATGCATCTCAGA RT-BQCV-R:GCAACAAGAAGAAACGTAAACCAC	309	Chen personal communication
RT-DWV-F: CGAAACCAACTTCTGAGGAA RT-DWV-R: GTGTTGATCCCTGAGGCTTA	174	Chen personal communication
RT-IAPV-F: GCGGAGAATATAAGGCTCAG RT-IAPV-R: CTTGCAAGATAAGAAAGGGGG	586	Chen personal communication
β-actin-F: AGGAATGGAAGCTTGCGGTA β-actin-R: AATTTTCATGGTGGATGGTGC	181	Chen et al.(2004b)

Table 2.3 Frequencies of simultaneous virus infections in healthy honey bee samples n = 80.

No. of viruses	Type of infection	No. of samples	Percentage	Origin*
0-1	None	0	0	None
2	DWV, BQCV	19	24	PEI, MB, BC, QC, NB, NS
	DWV, IAPV	5	6	PEI, MB, QC, NB
	BQCV, IAPV	1	1	QC
	BQCV, KBV	1	1	NS
3	DWV, BQCV,IAPV	23	29	ON, AB, BC, QC, NB, NS,
	DWV, BQCV,KBV	5	6	ON, AB, NS
	DWV, BQCV,SBV	5	6	AB, MB
	DWV, BQCV,CBPV	2	3	MB, NB
	DWV, BQCV,ABPV	1	1	NB
	DWV, IAPV,KBV	1	1	QC
	BQCV,KBV,ABPV	1	1	NB
4	DWV, BQCV,IAPV, SBV	4	5	AB, PEI, MB
	DWV, BQCV,IAPV,KBV	3	4	PEI, MB
	DWV, BQCV,IAPV,ABPV	2	3	ON
	DWV, BQCV,KBV,CBPV	2	3	AB, BC
	DWV, BQCV,IAPV,CBPV	2	3	MB, BC
	DWV, IAPV,KBV,ABPV	1	1	NB
5	DWV,BQCV,IAPV,KBV,CBPV or DWV, BQCV, IAPV, SBV, CBPV	2	3	BC, PEI

*Abbreviations: AB, Alberta; BC, British Columbia; MB, Manitoba; NB, New Brunswick; NS, Nova Scotia; ON, Ontario; PEI, Prince Edward Island; QC, Quebec.

Table 2.4 Frequencies of simultaneous virus infections in unhealthy honey bee samples n = 23 from Manitoba.

No. of viruses	Type of infection	No. of samples	Percentage
0-1	None	0	0
2	DWV, BQCV	5	22
	DWV, IAPV	1	4
	DWV, CBPV	1	4
3	DWV, BQCV,SBV	5	22
	DWV, BQCV,IAPV	4	17
	DWV, BQCV,KBV	1	4
	DWV, BQCV,CBPV	1	4
4	DWV, BQCV,IAPV, SBV	3	13
	DWV, BQCV,IAPV, CBPV	1	4
5	DWV, BQCV,IAPV,SBV,CBPV	1	4

Figure 2.1. Prevalence of seven bee viruses in healthy colonies in eight provinces of Canada n = 10 (per province) and in unhealthy colonies from Manitoba n = 23

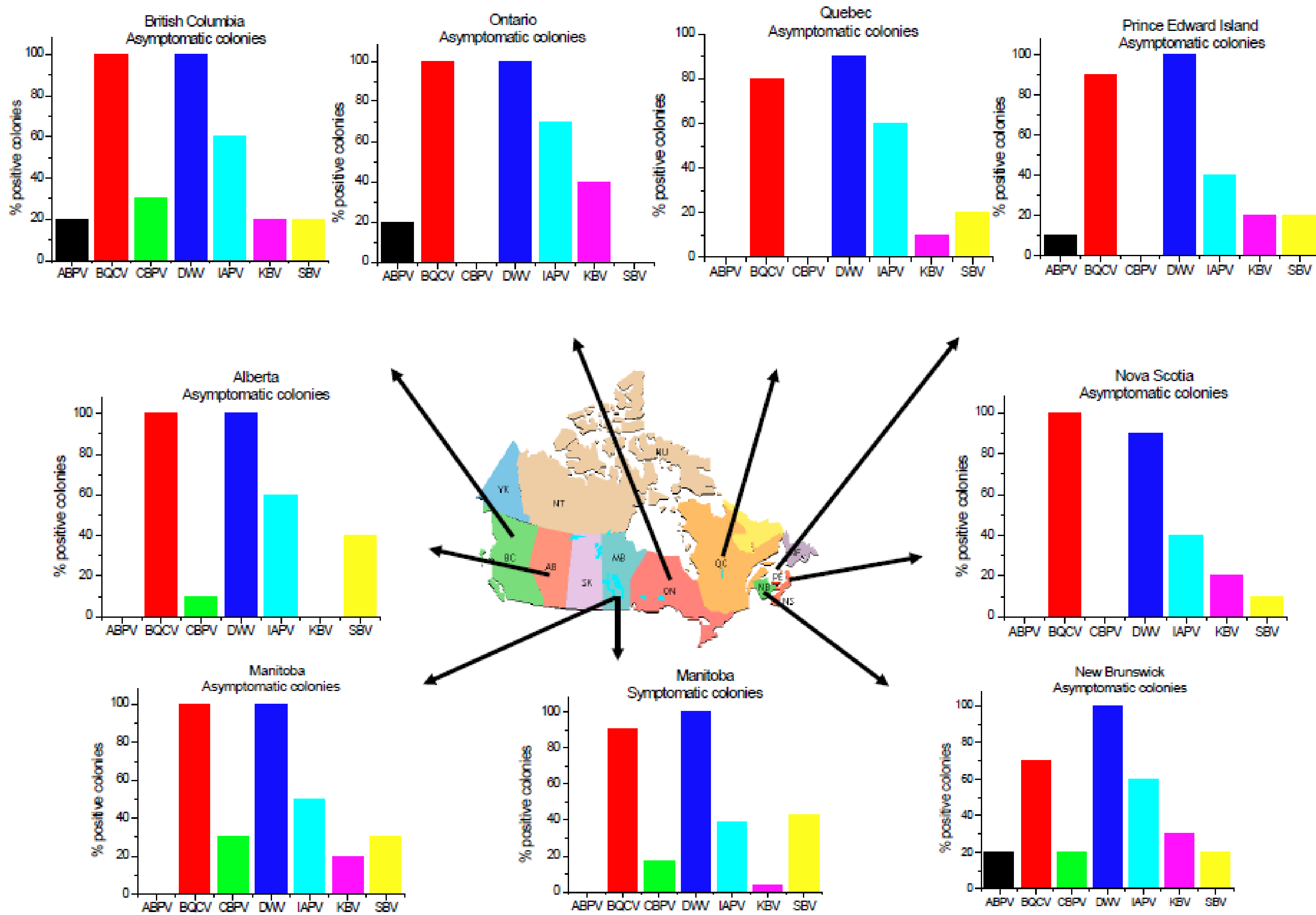
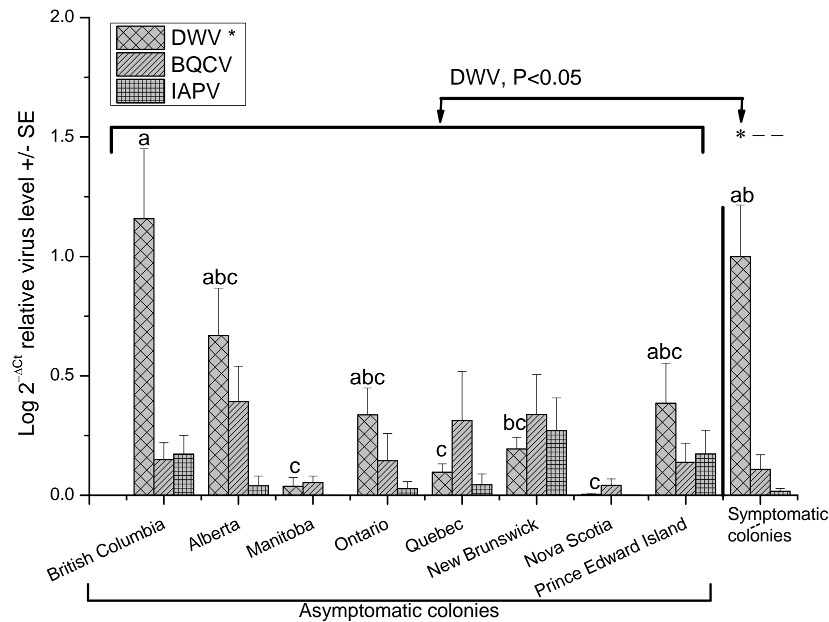


Figure 2.2. Relative viral concentration of DWV, BQCV, and IAPV among samples collected from healthy colonies in eight provinces in Canada and unhealthy colonies from Manitoba. Data are presented as mean $\log_{10} 2^{-\Delta Ct} \pm$ standard error. Letters above the bars with the same letter for each virus are not significantly different (Tukey's Studentized range test, $P > 0.05$). Horizontal line represents contrast between unhealthy colonies in MB and pooled values for all healthy colonies. For differences between relative virus concentrations within each province, see text.



CHAPTER 3. SEASONAL DYNAMICS OF HONEY BEE, *APIS MELLIFERA* L., VIRUS INFECTIONS, AND EFFECTS OF THEIR ASSOCIATIONS WITH OTHER DISEASES AND PARASITES ON COLONY LOSSES IN DIFFERENT WINTERING ENVIRONMENTS

ABSTRACT

Extreme winter losses of managed honey bee colonies are a major threat to the beekeeping and pollination industries across the Northern hemisphere, but the exact combinations of biotic and abiotic factors underlying winter colony loss remain debatable. Here I used long term monitoring of beekeeper-managed colonies in two environments (colonies wintered indoors or outdoors) and sampled adult bees to characterize the effects of two parasitic mites, seven viruses, and *Nosema* on honey bee colony mortality and population loss over winter. Colonies were sampled in fall 2009, mid-winter 2009/2010 and spring of 2010. Fall parasite and pathogen loads were similar in colonies that were wintered outdoors and in those wintered indoors, but outdoor-wintered colonies had greater changes in bee population score over winter.

Seasonal patterns in DWV, BQCV, and *Nosema* level also differed with the wintering environment. DWV levels decreased over winter for indoor-wintered colonies but remained at similar levels over winter for outdoor-wintered colonies. In spring, indoor-wintered colonies had lower DWV levels than outdoor-wintered samples despite having similar concentrations in fall. In contrast, BQCV concentration increased over winter in outdoor-wintered colonies and remained at similar levels for indoor-wintered colonies. The mean abundance of *Nosema* (millions of spores / bee) decreased over winter in indoor-wintered colonies but increased over winter for outdoor-wintered

colonies. The mean abundance of *Varroa* and concentration of SBV, KBV, and CBPV changed with season. *Varroa* mean abundance decreased over winter. SBV, KBV and CBPV concentrations increased. For most viruses, either entrance or brood area samples were reasonable predictors of colony virus load but there were significant season*sample location interactions for *Nosema* and BQCV, indicating that care must be taken when selecting samples from a single location. For *Nosema* spp., the fall entrance samples were better predictors of future infestation levels than were fall brood area samples.

For indoor-wintered colonies, IAPV concentration was negatively correlated with spring population size. For outdoor-wintered hives, spring *Varroa* abundance and DWV concentration were positively correlated with bee loss and negatively correlated with spring population size. Interactions between other pathogen pairs also differed with wintering method. Multivariate analyses for fall collected samples indicated DWV and spring-collected samples indicated SBV were associated with colony death.

INTRODUCTION

The beekeeping, and pollination industries worldwide are affected by the recent challenges to managed honey bee (*Apis mellifera* L.) colonies that have resulted in high winter losses of colonies. Honey bee colonies that diminish in population size to the point that they are not viable or die between late fall and early spring are referred to as "winter losses". Colony losses averaging 30 – 40% have been common in the U.S. and Europe (Cox-Foster et al. 2007; vanEngelsdorp and Meixner 2010). Unfortunately, Canadian beekeepers have also experienced similar levels of colony loss in managed honey bee colonies (Currie et al. 2010; Guzman-Novoa et al. 2010). These losses are now generally believed to result from interactions with multiple stressors that include parasitic mites, pathogens (viruses, bacteria, microsporidia parasitic fungi and parasitic mites), poor queen quality, low genetic diversity, pesticides and other environmental factors (Delaney et al. 2009; vanEngelsdorp et al. 2009; Currie et al. 2010; Genersch 2010; Meixner et al. 2010; Moritz et al. 2010; vanEngelsdorp and Meixner 2010; vanEngelsdorp et al. 2010; vanEngelsdorp et al. 2012).

Honey bee viruses are wide spread in Canada and some, such as deformed wing virus (DWV) and black queen cell virus (BQCV), are present in most colonies – often at high concentrations (Chapter 2). However, the roles that bee viruses and their interactions with other parasites, pathogens and environmental stressors play in contributing to winter colony losses are unclear. It is important to determine which of these pathogens, or groups of parasites and pathogens, has the greatest impact on winter mortality of honey bee colonies, under different wintering management (indoor and outdoor wintering) conditions. This information would assist in the development of effective management strategies.

The ectoparasitic mite, *Varroa destructor* Anderson and Trueman is known to cause winter loss of colonies when mite levels are greater than 10% in late fall (Gatien and Currie 1995; Gatien and Currie 2003; Currie and Gatien 2006; Currie 2008) and is a significant cause of winter losses of honey bee colonies in the northern hemisphere (Dahle 2010; Genersch et al. 2010; Guzman-Novoa et al. 2010; Schafer et al. 2010; van Dooremalen et al. 2012). *Varroa* can have synergistic interactions with other parasites such as the tracheal mite, *Acarapis woodi* (Rennie), thus resulting in winter losses occurring when even low levels of *Varroa* are present (Currie 2001; Downey and Winston 2001; Currie 2008). Multiple infestations of honey bee colonies with a variety of microbes might also play a role in winter colony mortality (Faucon et al. 2002; Ellis and Munn 2005; vanEngelsdorp et al. 2009; vanEngelsdorp and Meixner 2010; Dainat et al. 2012b, 2012a; Locke et al. 2012; vanEngelsdorp et al. 2012). Viruses may play a role in affecting bee colony population loss that is equivalent to, or larger than, direct *Varroa* feeding damage (Hung et al. 1995). However, very little is known about interactional effects between *Varroa* and pathogens of honey bees or how beekeepers can manage colonies to decrease the impact of such interactions in overwintering colonies. Three viruses in particular, (DWV, IAPV and ABPV) have often been linked with large scale overwinter losses (Cox-Foster et al. 2007; Highfield et al. 2009; Berthoud et al. 2010; Dainat et al. 2012a, 2012b). ABPV is suspected to be involved in colony losses in Europe (Berthoud et al. 2010; Genersch et al. 2010; Nguyen et al. 2011) but its role in colony loss in North America is less clear. Other viruses of importance include KBV, BQCV, SBV, and CBPV. Links between KBV and colony loss occur. For example, prevalence of KBV in CCD colonies is greater than in non-CCD colonies (Cox-Foster et al. 2007; Anderson and East 2008). BQCV is closely associated with the microsporidian

Nosema apis Z. and may work in concert with it to affect honey bee health but it is not thought to be a major factor in colony collapse in some areas of Europe (Dainat et al. 2012b). CBPV and SBV were the second most prevalent viruses identified in a study conducted in Belgium but neither were correlated with colony mortality.

One of the biggest challenges of studying virus pathogenesis in honey bees is linking infection by an individual virus to a particular set of economic impacts or disease symptoms. In field studies, honey bees are often infected by multiple viruses simultaneously, most of which usually persist as latent infections in the bee hosts (Chen et al. 2006b). In addition, virus infections in honey bees are often associated with non-viral pathogens and other parasites. Therefore, without the application of Koch's postulates, it is difficult to prove that specific symptoms are indeed caused by a particular virus and not the result of mixed virus infections particularly when viral loads in bees cannot be determined. Virus prevalence information is not adequate to predict colony loss (Chapter 2) so quantification of virus loads using sequence-based methods is essential to developing estimates of the potential impacts of individual viruses on infected bees (Chen et al. 2004a). Information as to how and when samples should be collected in order to best predict the disease impact of viruses is also required.

The other groups of parasites associated with colony losses in temperate countries are the microsporidian fungi, *N. apis* and *N. ceranae* (mentioned previously). Nosema has long been known as an economically important and commonly encountered disease (Bailey and Ball 1991; Fries et al. 1996). *Nosema ceranae* is described as a serious parasite of *A. mellifera* in Europe (Higes et al. 2006), Asia (Huang et al. 2007) and has been in Canada since at least 1994 (Currie et al. 2010). *Nosema ceranae* is associated with reduced honey production and increased winter mortality (Higes et al. 2006).

Nosema ceranae also inhibits immune system function in honey bees, which may lower resistance of the host against other pathogens (Antunez et al. 2009). A nominal treatment threshold of 1 million spores per bee is often recommended (Ostermann 2002), but this estimate was based upon *N. apis* and good thresholds do not exist for *N. ceranae*. BQCV is often positively correlated with the presence of *Nosema* spp. although the relationships of this pathogen-pair on colony winter loss and potential interactions with other parasites and pathogens in different wintering environments is unclear (Williams et al. 2010).

The level of *Nosema* is typically higher in foragers or old worker bees than newly emerged bees and house bees (Smart and Sheppard 2012) suggesting older workers may be better indicators of future disease impacts. Methods of sampling for *Nosema* may need to be refined to better predict its impacts and to develop thresholds for this organism. Similar issues related to sampling of bees need to be addressed with respect to both viruses and *Varroa*. Thus, studies on the best location in the hive for collecting samples for these groups of parasites and pathogens are required. Other studies assessing pathogen impact have collected bees only once in summer or fall, collected from experimentally managed colonies at a single research station, collected from weak, dead or healthy colonies and not prior to dwindling, and collected bees from colonies are wintered outdoors (Dainat et al. 2012a; Francis et al. 2013). Long term monitoring of indoor and outdoor-wintered colonies prior to symptoms of collapse is required to help identify the pathogens associated with colony losses.

Little is known about the effects of the winter environment on virus interactions. This can be examined in Canada where there are two primary types of wintering methods adopted by the beekeepers. Honey bee colonies can be wintered indoors in an environmentally controlled building, or outdoors, protected by insulation. For indoor-

wintering, honey bee colonies are stored in a building under complete darkness where temperatures are maintained at about 2 °C – 5 °C. Commercial overwintering buildings that may contain thousands of hives, are fitted with thermostatically controlled air circulation and ventilation systems, to remove excess heat and provide fresh air to the honey bees (Currie et al. 1998; Underwood and Currie 2004; Underwood and Currie 2007). In some cases, supplemental heat and air conditioning is used to help maintain constant temperatures (Currie 2001). For outdoor wintering, honey bee colonies are typically wrapped with insulation materials with a minimum of 15 cm of fiberglass insulation (R-value (thermal resistance) = 0.176 $°K·m^2/W$) on both the sides and on the top, and colonies are provided with a top entrance (Currie et al. 1998). Beekeepers may over winter bees in a single brood chamber or multiple brood chambers. For indoor-wintering, a majority of beekeepers in western Canada winter in single brood-chamber hives whereas for outdoor wintering, the majority winter in double brood chamber hives. Differences in susceptibility to *Varroa*, combinations of *Varroa* and tracheal mite and possibly *Nosema* may occur in colonies wintered indoors and those wintered outdoors and susceptibility to viruses may be similarly affected (Currie 2001, 2008; Bahreini and Currie 2009; Williams et al. 2010; Bahreini and Currie 2011).

The overall purpose of this study was to understand the seasonal dynamics and relative importance of parasites and pathogens on winter mortality under indoor and outdoor-wintering management systems and to determine if practical sampling methods can be developed to help predict their impact on colony survival over winter. The outcome of this study may help beekeepers optimize the management of parasites, diseases and their interactions to reduce winter mortality of honey bee colonies. I report

here the first assessment study of winter loss to examine viruses under different wintering management systems.

MATERIALS AND METHODS

Apiary and colony selection

Honey bee colonies were sampled from five different beekeeping regions in the Province of Manitoba, Canada (Eastern, Southwest, Northwest, Interlake, and Central). Five beekeepers that wintered bees using either an outdoor or indoor wintering management system were randomly selected from each region (except for regions in which beekeepers used only one wintering method) and asked to participate in this project (Fig. 3.1). For each beekeeper, three colonies were randomly selected from a single apiary site for inclusion in the study. Fifteen of these beekeepers practiced indoor wintering, and 10 of them practiced outdoor wintering. In my study, four beekeepers out of 15 that wintered indoors had double brood chamber colonies. While three beekeepers out of ten wintered that wintered outdoors had single brood chamber colonies. Hence, 45 colonies were wintered using indoor-wintering buildings (33 using single standard Langstroth brood chambers containing 9-10 frames and 12 in double brood chamber hives) (here after referred to as "indoor-wintered") (Currie et al. 1998) and 30 colonies were wintered outdoors (9 using single brood chambers and in 21 double brood chambers) (here after referred to as "outdoor-wintered"). Beekeepers were asked to follow their usual apicultural management techniques for controlling parasites, wintering and managing colonies. In fall, 14 of 15 producers that wintered indoors and 9 of 10 producers that wintered outdoors treated bees to control *Varroa*. Acaricides used were amitraz (Apivar ®) (11 producers), formic acid (various formulations) (10 producers),

oxalic acid (2 producers), and coumaphos (1 producer). In fall, 11 of 15 producers that wintered indoors and 4 of 10 producers that wintered outdoors treated bees with fumagillin to control *Nosema*.

Data and sample collection

For each of the 75 colonies, adult population (bee cluster size) was estimated in fall prior to wintering and in spring, when colonies were removed from the wintering building (indoor-wintered) or unwrapped (outdoor-wintered). In order to minimize disturbance to the colony, the honey bee population size was estimated from above and below, by counting the number of frame seams completely covered with bees and multiplying by 2,430 bees (Burgett and Burikam 1985).

Adult honey bee samples were collected from two locations within each test colony, to assess the levels of *Varroa*, tracheal mites, seven viruses, and *Nosema*: one at the "entrance" (front) of the hive and the other from a frame removed from the "brood area" (inside). In the fall, samples of approximately 250 bees were collected from the brood area inside the hive using 300 ml sample cups (at a time when most beekeepers would have already initiated or completed *Varroa* treatment). Additional samples of approximately 200 bees were collected from the entrance using a modified vacuum pump that sucked foragers and presumed "older" bees from the entrance into a cup. Bees were immediately transferred into 250 ml sample cups with a screened lid and the bees were provided with a sugar cube as a feed source to keep them alive until they could be returned to the lab. Information on management practices of each beekeeper was recorded (acaricides used, drug treatments and dates of application). All bee samples were stored immediately in a -80 °C freezer for further analysis. Colonies that were

sampled in fall were marked for resampling. All colonies were resampled in spring; only indoor-wintered colonies were resampled in mid-winter, as the beekeepers believed outdoor-wintered hives could not be unwrapped without causing damage to the colony. To collect mid-winter samples in the wintering buildings, the top lids of hives were opened and bee samples (approximately 250 bees) were carefully scooped into 300ml sample cups as quickly as possible to minimize colony disturbance. Spring sampling was done in the months of April and May when the hives came out of the over wintering building and outdoor-wintered colonies were unwrapped. In spring, I again collected adult worker bees, from two hive locations (brood area and entrance), measured the size of the honey bee cluster, counted number of live and dead colonies, and recorded any treatments that had been applied by beekeepers. Bee samples were collected from all surviving colonies and for weaker colonies with less than 300 bees, the surviving bees were sampled and the colony was marked as dead. Percent change in bee population was calculated by comparing fall and spring population scores.

Quantification of parasites and pathogens

The mean abundance of *Varroa* (mites per 100 bees) (Bush et al. 1997) on live bees and on dead bees from colonies that had died was assessed by the alcohol shake method (Gatien and Currie 2003). Tracheal mite prevalence was assessed by the thoracic slice method according to Delfinado-Baker (1984) using a subsample of 100 bees. *Nosema* spore mean abundance was assessed using a subsample of 100 bees, according to the methods of Cantwell (1970). Viruses were quantified as described below.

RNA extraction

Sub samples of 50 frozen adult bees were crushed in a mortar under liquid nitrogen. The total RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. RNA samples were dissolved in DEPC-treated water in the presence of ribonuclease inhibitor and stored at -80 °C for further analysis. The RNA quantity and purity was determined by spectrophotometer by measuring the absorbance at 260 nm and 280 nm. An average of 2 μg of total RNA was reverse-transcribed using M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) according to the supplier's recommendations.

qPCR analysis

A quantitative analysis (qPCR) was performed to examine the difference in relative virus concentration of seven single stranded RNA viruses (DWV, BQCV, SBV, IAPV, KBV, ABPV, and CBPV). The assays were performed in 20 μL volumes, containing 1μL cDNA (5 fold dilution), 10 μL SYBR green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), and 0.5 μL of each primer. Reaction volume was adjusted with water. Amplifications were performed in triplicate on an ABI Prism 7300 real time PCR machine (Applied Biosystems, Foster City, CA, USA) with the following PCR conditions a single cycle at 95 °C for 5 min, 40 cycles at 95 °C for 15 s, 58 °C for 30 s. An additional step of 72 °C for 30 s was added to measure the dissociation curve and data collection. Non-template controls (reaction mix without template) were included in triplicates in all batches. Honey bee actin (DNA fragment) was used as an endogenous control gene. Actin allows for the normalization of differences in cDNA reactions. The relative virus concentrations were calculated using the $2^{-\Delta Ct}$ method,

where Ct indicates the cycle threshold. Relative expression was calculated as $2^{-\Delta Ct}$; where $\Delta Ct = Ct_{(virus\ gene)} - Ct_{(\beta\text{-}actin)}$ (Persico et al. 2007). The primers for the qPCR are listed in Table 3.1. Virus prevalence was expressed as the proportion of colonies with detectable levels of each virus (ABPV, BQCV, CBPV, DWV, IAPV, KBV, and SBV).

Statistical analysis

Data for bee population size, virus concentration ($2^{-\Delta Ct}$) values, *Varroa* mean abundance, *Nosema* spore mean abundance, tracheal mite prevalence, and bee population loss over winter for each colony were analyzed as follows. Prior to doing the analysis the following transformations were applied: *Varroa* abundance (mites per 100 bees) and tracheal mite prevalence data were arcsine-transformed, bee population score data were square root + 0.5 transformed and *Nosema* levels were $\log_{10}$ transformed (Snedecor and Cochran 1980). The effect of wintering methods (indoor and outdoor) on the change in size of the colony population between fall and spring was analyzed by PROC MIXED (SAS 1999) using a repeated measures design with hives as subjects and season as a repeated measure using the REML statement (restricted maximum likelihood). Where significant interaction was observed ($P < 0.05$) between treatment factors, differences between means were compared by Bonferroni-corrected contrasts (SAS 1999).

Proportions of colonies infected with different parasites and pathogens in each season (fall and spring) and wintering method (indoor and outdoor-wintering) were compared using binary logistic regression (PROC CATMOD, SAS 1999). Significant pathogen *season interactions were found so separate analyses were performed on each pathogen. A separate analysis was performed for indoor wintered hives to compare mid-winter samples with fall and spring samples.

The response variables for the seven virus concentrations ($2^{-\Delta Ct}$), *Varroa* mean abundance, *Nosema* spore mean abundance and tracheal mite prevalence were analyzed using a mixed model ANOVA, PROC MIXED (SAS 1999) using the REML statement (restricted maximum likelihood). Wintering method (indoor and outdoor), sample location (entrance and brood area) and date (fall, winter and spring) were treated as fixed effects. Beekeepers (nested within region) and wintering method and hive (nested within beekeepers), region and wintering method and sample location (nested within beekeeper, region, wintering method and hive) were considered random effects. Data were analysed based on the following model:

$$y = \mu + w_i + s_j + + d_k + r_l + ws_{ij} + wd_{ik} + wr_{il} + sd_{jk} + wsd_{ijk} + b_{ilm} + h_{ilmn} + sh_{ijlmn} + e_{ijklmn}$$

where w is the effect of wintering method (*i* indexes indoor and outdoor), considered a fixed effect,

s_j = sample location (*j* indexes entrance and brood area), considered fixed,

d_k = date (*k* indexes fall, winter and spring), considered fixed,

r_l = region (*l* indexes Eastern, Southwest, Northwest, Interlake, and Central), considered fixed,

two and three-way interactions are denoted by letter combinations of the above main effects,

b_{ilm} = the effect of the mth beekeeper nested in the lth region and i^{th} wintering treatment, considered random,

h_{ilmn} = the effect of the nth hive nested in the mth beekeeper and the lth region and i^{th} wintering treatment, considered random

sh_{ijlmn} = the interaction of the jth sampling location with the nth hive nested in the mth beekeeper and the lth region and i^{th} wintering treatment, considered random, and

e_{ijklmn} = the residual error

Interactions with region were excluded from the model. Where significant interactions were observed, they were partitioned using the SLICE option in the LSMEANS statement with PROC MIXED by both wintering method and by season to compare differences between means within levels of treatments.

Correlations between bee loss (percent reduction in bee population as measured from fall to spring), colony population in spring and pathogen and parasite levels (as measured both in fall and spring), were analyzed using pooled values for brood area and entrance samples using simple correlations (PROC CORR, SAS Institute Inc., 1999). The partial correlations were calculated using multivariate ANOVA (PROC GLM with option MANOVA, SAS Institute Inc., 1999). The simple and partial correlations were adjusted for multiple testing using a false-discovery rate (FDR) procedure (Benjamini and Hochberg 1995; Storey and Tibshirani 2003). I calculated the *q*-values using a bootstrap technique with FDR and have included all values that met a cut off filter of 0.20 (Parker et al. 2012) using the QVALUE R-statistical software package http://genomics.princeton.edu/storeylab/ qvalue/windows.html (Storey 2002; Storey et al. 2004) or with simple or partial correlations with 0.05 or lower. Correlations are considered significant at q=0.05 but for exploratory purposes include those P values with 0.05 or lower. Correlations were carried out within each season and wintering method. For *Nosema,* the correlations between season (fall, winter and spring) and sampling location (brood area and entrance), were also analyzed using PROC CORR (SAS 1999).

Finally, a log-linear regression was carried out on fall and spring pathogens using pooled averages of brood and entrance samples. Data were analyzed by binary-logistic regression for each virus, using DataDesk 6·0 (Data Description, Inc., Ithaca, New York,

USA). Seventy-five hives were separated into two groups live=0 or dead=1, based on the observations taken in spring at the end of the experiment.

The effect of fall treatment with fumagillin on *Nosema* spore counts in spring (pooled brood area and entrance) was compared with hives not treated with fumagillin within indoor and outdoor-wintered colonies by ANOVA using PROC MIXED (SAS 1999). Since fumagillin was applied to individual hives, hives were treated as replicates for this analysis.

RESULTS

Change in colony size over winter

The relative change in bee population size from fall to spring was affected by wintering method as indicated by a significant wintering method *season interaction ($F = 138.12$, $df = 3, 72$ $P < 0.0001$; Fig. 3.2). Over the winter, the outdoor-wintered colonies suffered higher bee population loss (55%) than indoor-wintered ones (42%). In fall, outdoor-wintered colonies had higher populations than indoor-wintered colonies ($P < 0.05$, SLICE) but by spring colony populations for outdoor-wintered colonies and indoor were similar ($P<0.05$, SLICE) (Fig. 3.2). Overall, winter colony mortality (colonies that were dead after removal of winter wraps in spring) was 20% and did not differ with wintering method ($df = 1$, $\chi^2 = 0.70$, $P = 0.40$).

Prevalence of honey bee pathogens and parasites

There was a significant interaction between season*pathogen - parasite ($\chi^2 = 36.81$, $df = 9$, $P < 0.0001$) therefore separate analyses were performed on each pathogen and parasites. Prevalence of parasites and pathogens was similar in each wintering

method ($\chi^2 = 2.72$, df = 1, P = 0.10). *Varroa* were detected in 56-83% of colonies, but there was no difference in proportion of colonies with detectable mites among wintering methods ($\chi^2 = 1.26$, df = 1, P = 0.26) and among season ($\chi^2 = 0.25$, df = 1, P = 0.61) (Table 3.2). The honey bee tracheal mite was found in a low percentage of colonies (3% to 10%) and prevalence also did not vary with wintering method ($\chi^2 = 0.14$, df = 1, P = 0.70) or by season ($\chi^2 = 1.29$, df = 1, P = 0.25). *Nosema* spore prevalence increased from fall to spring seasons ($\chi^2 = 15.97$, df = 1, P < 0.0001) and within indoor-wintered colonies *Nosema* prevalence was higher in mid-winter than fall but remained at similar prevalence between mid-winter and spring (Table 3.2).

DWV and BQCV had the highest prevalence and were detected at similar frequencies in indoor and outdoor-wintered colonies (Table 3.2). Their prevalence did not change over winter (Table 3.2). Over both wintering methods, SBV prevalence increased from fall to spring ($\chi^2 = 11.08$, df = 1, P < 0.0009) and for indoor-wintered colonies (also sampled in mid-winter) prevalence increased from fall to mid-winter but remained at similar prevalence from mid-winter to spring. IAPV and KBV prevalence both increased over winter when averaged over both wintering methods (IAPV df = 1, $\chi^2 = 7.97$, P < 0.005, KBV df = 1, $\chi^2 = 8.03$, P < 0.005) but did not increase in prevalence from fall to mid-winter for indoor wintered colonies. CBPV and ABPV were detected in a comparatively low proportion of colonies and prevalence remained low the following spring (Table 3.2).

Mean abundance of pathogens and parasites

The analyses for effects of wintering method (indoor and outdoor), season and location of sampling within the colony (entrance vs. brood area) on the mean abundance of parasites and pathogens are summarized in table 3.3.

For *Nosema*, DWV and BQCV, there were significant interactions between season and wintering method (see Table 3.3) although for BQCV the three-way interaction between season, wintering method and sampling location was also significant. In fall, nosema, DWV and BQCV all had similar levels in indoor- and outdoor-wintered hives ($P > 0.05$ Slice) (Fig. 3.3 and 3.4). The mean abundance of *Nosema* (millions of spores / bee) decreased from fall to spring in indoor-wintered colonies ($F = 11.59$, $df = 1, 129$, $P < 0.0009$) but increased from fall to spring in outdoor-wintered colonies ($F = 29.48$, $df = 1, 129$, $P < 0.0001$(Fig. 3.3).

DWV concentration decreased from fall to spring in indoor-wintered colonies ($F = 9.06$, $df = 1, 121$, $P < 0.003$) but for outdoor wintered colonies DWV concentration was similar in spring and fall ($P > 0.05$ Slice). In spring DWV concentration was lower in indoor-wintered colonies ($F = 5.07$, $df = 1, 121$, $P < 0.02$), than outdoor-wintered colonies ($P > 0.05$ Slice). The wintering method*sampling method interaction was not significant (Table 3.3).

Significant season*wintering method*sampling location interactions were found only for BQCV (see Table 3.3). Overall, spring BQCV concentration was higher than fall (Fig. 3.3) ($F = 26.55$, $df = 1, 121$, $P < 0.0001$) but the seasonal differences that occurred in the outdoor wintered colonies were found only in entrance collected bees ($F = 29.61$, $df = 1, 121$, $P < 0.0001$). Fall BQCV levels were similar in indoor and outdoor-wintered colonies for both brood area and entrance samples (Fig.3.4), ($P > 0.05$ Slice).

Spring levels of BQCV were higher in outdoor-wintered hives than in indoor-wintered hives but only for entrance samples (F = 6.42, df = 3, 121, P < 0.0005). In outdoor-wintered colonies in spring, the virus levels in entrance samples were much higher than in brood area samples (F = 11.83, df = 3, 121, P < 0.0001). In outdoor-wintered colonies, the fall hive entrance samples of bees had much lower BQCV levels than the spring entrance samples (F = 14.15, df = 3, 121, P < 0.0001) (Fig. 3.4).

For *Nosema* there was also a significant season*sampling location interaction (Table 3.3). *Nosema* levels were higher in entrance than brood area samples in fall (F =11.91, df = 1, 129, P < 0.0008). However, no difference was seen between the two sampling locations in spring samples (P > 0.05, Slice). Brood area samples showed an increase in *Nosema* levels from fall to spring (F = 35.60, df = 1, 129, P < 0.0001), but entrance samples had higher *Nosema* levels in fall than in spring (F = 9.34 df = 1, 129, P < 0.003) (Fig. 3.5).

Varroa mean abundance (mites per 100 bees) was higher in fall than spring (Table 3.3, Fig. 3.6) but did not show any interactions with wintering method or sample location. Although, overall *Varroa* levels were low, mean abundance in individual hives ranged from 0 to 52.6% for indoor-wintered hives and 0 to 24.4% for outdoor wintered hives. In spring, *Varroa* ranged from 0 to 9.8% for indoor-wintered hives and 0 to 13.1% for outdoor wintered hives. The concentration of SBV, KBV, and CBPV also changed with season (Table 3.3), the seasonal patterns for each of these viruses were lower in fall and increased in spring (Fig. 3.6), but seasonal patterns for these viruses did not vary with wintering method or sample location (Table 3.3).

Correlation analyses

Correlations between parasites, pathogens, honey bee population loss and spring population size were examined using simple and partial correlations and showed different patterns for indoor and outdoor-wintered hives. Correlations with a *q*-value of less than 0.2 are included in table 3.4. Full tables can be found in appendix 1-4 (simple correlations) and appendix 5-8 (partial correlations). For indoor-wintered colonies, partial correlations showed that only IAPV was negatively correlated with spring population size (q = 0.01) (Table 3.4A). There were significant correlations between several combinations of virus pairs for indoor wintered hives. Positive simple and partial correlations were found between SBV and KBV (q = 0.003) and also between IAPV and CBPV (q = 0.003). Simple correlations showed IAPV was correlated with KBV (q = 0.004) but partial correlations between IAPV and KBV were not significant (q = 0.06).

Patterns were different for outdoor-wintered hives in fall. None of the variables were significantly correlated with either bee loss or spring population size after adjustments for false discovery rate were done (Table 3.4, B). However, in outdoor-wintered colonies, positive simple and partial correlations were found between *Varroa* and DWV (q = 0.006) and partial correlations showed *Nosema* was also positively correlated with DWV (q = 0.04).

Correlation analyses were also performed on parasite and pathogen levels in colonies that survived the winter and these data also showed different patterns for indoor- and outdoor-wintering. In spring indoor-wintered colonies, no significant correlations were found between any parasites or pathogens and bee loss (Table 3.4 C). However, simple correlations were found between *Nosema* and BQCV (q = 0.03). In spring, both simple and partial correlations showed IAPV levels were highly correlated with ABPV

both in indoor and outdoor wintered colonies. Similarly, KBV levels were positively correlated with CBPV both in indoor and outdoor wintered colonies (Table 3.4, C, and D).

For outdoor wintered colonies, partial correlations showed both *Varroa* (q = 0.006) DWV were positively correlated with bee loss (q = 0.008) and negatively correlated with spring population size (*Varroa* q = 0.02; DWV q = 0.001) (Table 3.4, D). For outdoor-wintered colonies in spring, both simple and partial correlations showed DWV levels were positively correlated with KBV (q = 0.001 simple; q = 0.005 partial) and CBPV (q = 0.0007 simple; q = 0.001 partial) in contrast to indoor-wintered hives where they were not. For outdoor-wintered colonies, both simple (q = 0.02) and partial (q = 0.006) showed *Nosema* levels were positively correlated with BQCV.

Seasonal Nosema correlations

Mean abundance of *Nosema* from each of the two sample locations within the hive (brood area or entrance) in fall was correlated with levels in colonies in mid-winter and spring and with each other to assess which sample location would result in a better prediction of future *Nosema* levels. There was a weak, but positive correlation between fall *Nosema* level of entrance-collected bees and fall *Nosema* level of brood area-collected bees (Table 3.5). However, fall entrance samples were better predictors of mid-winter *Nosema* levels than brood area samples. Fall entrance and brood area *Nosema* levels both showed weak negative correlations with spring *Nosema* spore levels, but fall brood area samples were marginally better at predicting spring brood area levels than were the fall entrance-collected samples. Mid-winter *Nosema* levels were highly correlated with the spring brood area samples but only weakly correlated with spring

entrance samples (Table 3.5). Interestingly, neither fall entrance-collected nor brood area-collected *Nosema* levels were correlated with spring entrance-collected levels.

Binary logistic regression

Multivariate analysis of colonies sampled in fall and spring for parasites and pathogens were related to the proportion of dead colonies using (binary logistic regression for colonies ranked = dead = 1 or live = 0). Analysis of fall sampling parameters showed that the proportion of dead colonies increases with increasing DWV ($F = 5.68$, $df = 1, 67$, $p < 0.02$). Analysis of spring sampling parameters showed that the proportion of dead colonies increases with increasing SBV ($F = 7.32$, $df = 1, 67$, $p < 0.009$) (for statistics related to other variables see Appendix 9).

Effect of fumagillin treatment

Fall fumagillin treatment suppressed *Nosema* for indoor-wintered colonies ($df = 1, 39$ $F = 15.17$, $P < 0.0003$) with colonies treated with fumagillin having lower *Nosema* (2.34 ± 0.91 SE million spores) than untreated ones (8.24 ± 1.50 million spores). Numerically, outdoor wintered, colonies showed a similar trend with lower spore counts in colonies treated with fumagillin (1.62 ± 2.31 million spores), than untreated colonies (8.86 ± 1.94 million spores), but the difference in suppression was not significant ($df = 1, 28$ $F = 3.64$, $P > 0.07$).

DISCUSSION

In this study I compared beekeeper-managed colonies across a broad geographic scale in two distinct environments: a "mild" stable environment (for colonies wintered

indoors) and a “harsh” fluctuating environment (for colonies wintered outdoors) (Currie et al. 1998). I sampled adult bees from in each hive before (two locations), during (one location for indoor-wintered colonies only), and after (two locations) winter to characterize the effects of two parasitic mites, seven RNA viruses, and *Nosema* on honey bee colony mortality and population loss over winter. The results showed that outdoor-wintered colonies had greater changes in bee population scores over winter than indoor-wintered colonies despite having a similar composition and level of parasites and pathogens prior to winter. Two viruses (DWV and BQCV) and one pathogen (*Nosema*) showed different seasonal patterns in indoor and outdoor-wintered colonies. Combinations of parasite and pathogen variables that were correlated with bee loss or spring size and each other also differed in the two wintering systems. Sample location affected assessment of *Nosema* and BQCV levels but did not affect assessment of other parasites or pathogens and this has implications for sampling to predict impacts of pathogens on colony loss.

Since 2006, honey bee winter colony losses in Canada have often exceeded 29% of the national total each winter (Currie et al. 2010; CAPA 2011) and these losses have been at levels equivalent to those found in the U.S. and Europe (vanEngelsdorp and Meixner 2010) where large scale collapses of colonies have also occurred. In the year of my broad scale study, overall death of colonies wintered in different environments was similar (20% loss) but the change in bee population score over winter was significantly greater in the outdoor-wintered colonies than in the indoor-wintered colonies. This was not likely a result of beekeeper management designed to achieve a specific spring population size. In northern regions, beekeepers often winter larger colonies (double brood chambers) outdoors and smaller colonies (single brood chamber) indoors as

occurred in this study. Thus, the larger fall populations found in outdoor-wintered colonies were expected. However, under this type of management, spring populations in healthy double brood chamber colonies would typically consist of 16,000-25,000 bees, whereas single brood chamber colonies would have lower populations (7,800 – 10,000 bees) (Beauchesne et al. 1978; Nelson 1999). This did not occur in this study where spring population size of the outdoor-wintered hives (mostly double brood chamber) and indoor-wintered hives (mostly single brood chamber) were similar.

The prevalence and concentrations of parasites and pathogens analyzed in this study were similar for the indoor and outdoor-wintered colonies prior to implementation of wintering management. No single parasite or pathogen was highly correlated with winter bee loss in either of the two wintering methods in my study. However, the interactions between parasites, pathogens, colony loss and spring population size were very different in each of the two wintering environments. Thus, the environment to which colonies were exposed, in combination with management practices of beekeepers, likely played a role in affecting the different interactions between parasites and pathogens that were observed. Indoor-wintered colonies were maintained in a comparatively mild, stable environment (5 °C) under total darkness for the entire winter period (November to March). In contrast, colonies wintered outdoors in my study were exposed to temperatures that ranged from -33 °C to 17 °C and would have been exposed to daily (or periodical) temperature fluctuations of up to 23 °C. Little is known about how environmental stressors interact with pathogen and parasite webs in honey bees at the colony level. Lab studies have shown that comparatively small variations in brood nest temperature (shifts from 30 to 33°) can influence the severity of viruses in developing bees (Di Prisco et al. 2011). Field studies have shown that wintering honey bees in the

more stable environments within wintering buildings allows colonies to survive winter under higher infestation levels of *Varroa*, either alone or in various combinations with tracheal mite and *Nosema* or other stressors (Currie 2001, 2008; Bahreini and Currie 2009; Williams et al. 2010; Bahreini and Currie 2011). However, before this work, interactions with viruses in different wintering environments have not been examined.

In this study, most beekeepers (23 of 25) treated colonies for *Varroa*. Overall, the average *Varroa* levels in late fall were well below the fall economic threshold for *Varroa* of > 3% in early fall and >10% in late fall that can result in significant winter colony mortality in this region of Canada (Gatien and Currie 2003; Currie and Gatien 2006; Currie 2008). It is likely some samples were taken before residual effects of the acaricide treatments brought mite levels fully under control as *Varroa* levels decreased further before spring. However, despite the acaricide treatments, there were still a few colonies that were detected at the time of fall sampling that were well above treatment thresholds in both indoor and outdoor treatment groups. The mites in these colonies may have escaped treatment due to acaricide resistance, which is common in Canada (Currie et al. 2010) or may have immigrated to colonies through drifting or robbing bees. It should also be noted that some colonies could have been above the threshold prior to my sampling but had low mite levels when samples were collected and processed for parasites and pathogens as a result of acaricide treatment. The extent to which this might have occurred could not be quantified, as beekeepers did not know their mite levels prior to treatment.

Varroa on its own has major impacts on colony survival. *Varroa* feeding activity directly affects adult worker bees by removing their hemolymph and depleting protein and lipid reserves, which shortens their life span (Weinberg and Madel 1985). Despite

the relatively low mite levels in my study, there was a positive correlation between *Varroa* mean abundance and bee loss and negative correlation between *Varroa* mean abundance and spring population size for outdoor-wintered hives (when sampled in spring). Others have found high rates of winter bee loss caused by *Varroa*, but usually in association with much higher mite levels (>10 mites per 100 bees) (Currie and Gatien 2006; Currie et al. 2010; Dainat et al. 2012b; Guzman et al. 2010). *Varroa* and honey bee tracheal mites are also known to interact synergistically in enhancing bee losses (Currie 2001; Downey and Winston 2001), but tracheal mites did not have a significant effect on bee loss in this study.

It is not known if the dynamics of *Varroa* feeding and its role as a vector and activator of viruses would be substantially different in clusters of bees wintered indoors and those wintered outdoors. It is likely that lower levels of environmental "stress" associated with indoor wintering may have contributed to the different patterns that were observed. *Varroa* mites are effective vectors of many viruses and play a major role in activating DWV, KBV, ABPV, IAPV, SBV, ABPV, and CBPV to pathogenic levels in honey bees (Bailey et al. 1979; Ball 1989; Bailey and Ball 1991; Shen et al. 2005b; Yang and Cox-Foster 2005; Berenyi et al. 2006; Chantawannakul et al. 2006; Tentcheva et al. 2006; Yang and Cox-Foster 2007). *Varroa* weaken the bee's immune systems, making them more susceptible to viruses, and act as effective vectors to spread viruses within colonies (Kovac and Crailsheim 1988; De Jong 1997; Amdam et al. 2004; Yang and Cox-Foster 2007). *Varroa* and DWV together affect storage lipoproteins (vitellogenin) necessary for winter survival (Dainat et al. 2012a) and affect immune system function (Ghosh and Karin 2002; Li and Verma 2002; Nazzi et al. 2012). DWV alone could also be damaging. DWV replicates in various tissues such as the fat body (Fievet et al. 2006)

and can replicate in immature and adult bees and increase bee mortality even in the absence of *Varroa* (Chapter 5). Of the viruses tested, only DWV was associated with bee loss and low spring population size. I did not find any other direct correlations between *Varroa* and other viruses, which are often linked to poor bee health in different regions.

Varroa was correlated with DWV for fall samples in outdoor-wintered colonies. The high *Varroa* populations that were in some of my colonies would likely increase the chance of transmission of DWV and increase the susceptibility of bees to the virus (Yang and Cox-Foster 2005; Yang and Cox-Foster 2007; Schroeder and Martin 2012). Others have shown that viruses such as DWV can remain at high levels even after *Varroa* has been removed by acaricide treatment and these interactions with viruses are now thought to be a major factor associated with colony loss (Highfield et al. 2009). This is also likely to have occurred in my study to some extent as the most of mites were controlled by acaricide treatments but could have been at high levels prior to treatment.

DWV was the most common virus found in colonies in my study and was at much higher concentrations than other viruses in fall sampling periods. DWV levels declined from fall to spring in indoor-wintered colonies and remained stable from fall to spring in outdoor-wintered colonies. Spring DWV levels were significantly higher in outdoor-wintered than in those wintered indoors. Reductions in DWV from fall to spring in indoor-wintered hives may have been the result of either lower *Varroa* levels found in spring than in fall, highly infected bees dying and being removed from the colony over winter (Dainat et al. 2012a) or possibly the result of population turnover in the colonies through brood rearing during in indoor-wintering during winter and early spring. The outdoor wintering environment seemed to favour "maintenance" of DWV. It is not known why or even if this happened, but interactions with *Nosema* or other viruses may

have played role in facilitating DWV maintenance in outdoor-wintered colonies. Reductions in *Varroa* from fall to spring were not likely involved since *Varroa* was also lower in spring than in fall, during indoor-wintering where virus concentration declined over winter. Perhaps greater stress associated with the outdoor wintering environment suppressed immune responses in the bees. It is also possible that the acaricides used by beekeepers to control *Varroa* levels influenced DWV replication (Locke et al. 2012); however, the same types of acaricides were applied in both environments.

Spring DWV concentration was associated with winter bee loss and low spring population size for outdoor wintering thereby building upon the growing evidence implicating DWV as a cause of colony losses in many environmental and bee-management contexts (Chen et al. 2004a; Highfield et al. 2009; Cornman et al. 2012; Dainat et al. 2012b, 2012a; Martin et al. 2012; Francis et al. 2013). Although DWV appeared to affect bee loss in combination with *Varroa*, it also was correlated with other pathogens. For outdoor-wintered colonies in spring, DWV was positively correlated with KBV and CBPV. It is possible DWV may make bees more susceptible to other virus infections; however, this requires further study.

Previously, IAPV has been associated with CCD (Cox-Foster et al. 2007) and has been demonstrated to cause mortality in honey bees (Maori et al. 2007). IAPV causes increased risk for colony collapse in association with *N. apis* (Cox-Foster et al. 2007), but associations with CCD are not always correlated with *Nosema* (Cornman et al. 2012). IAPV is not consistently linked with CCD-like symptoms (Cornman et al. 2012). In my study, the relationship between IAPV and spring population size also differed with the wintering environment. IAPV levels did not change with season but I did find marginal positive correlations between fall IAPV and bee loss (q =0.06) and negative correlations

between IAPV and spring population sizes of colonies (q =0.01) for colonies wintered indoors. Interestingly, no correlations between IAPV and bee loss parameters were evident in spring. Cornman et al. (2012) did not find a link between bee loss and IAPV, but sampled colonies only after declines associated with colony collapse had occurred. This may explain in part why IAPV was not linked with bee loss in their study. If I had sampled only in spring, I also would not have found any correlation between IAPV and bee loss. However, environmental influences may have also affected links between IAPV and bee loss as I saw no link with IAPV and bee loss-related parameters in outdoor-wintered hives. Colonies in the outdoor-wintering environment may have succumbed to the presence of other stressors before IAPV could exert any effects on mortality.

Interactions between IAPV and other pathogens also varied with the wintering environment. For indoor-wintered colonies, I found positive correlations between IAPV and the viruses KBV and CBPV in fall and between IAPV and ABPV in spring. Whereas, for outdoor-wintered colonies, IAPV was positively correlated with ABPV only in spring. CBPV concentration increased from fall to spring in both wintering methods. Partial correlations of CBPV were marginally correlated with spring bee loss (q =0.06) but only in outdoor wintered hives. I also found KBV concentrations increased from fall to spring; however, KBV was not correlated with bee loss in my study. ABPV, KBV and IAPV are closely related viruses belong to the family *Dicistroviridae* that have been linked to poor bee health (de Miranda et al. 2010; Francis et al. 2013) and are usually damaging with very low levels of virus particles (Schroeder and Martin 2012). Recently, Francis et al. (2013) showed that combinations of ABPV, KBV and IAPV and DWV levels were very high in untreated colonies that died during winter, compared to survivors which treated with acaricides in Denmark. Their virus levels of ABPV, KBV and IAPV

and DWV were more closely correlated with *Varroa* population in untreated colonies than in colonies treated with acaricides with low *Varroa*. ABPV is wide spread in Europe, where colony prevalence ranges from 8% to 69% and it has been linked to colony death (Allen and Ball 1996; Berthoud et al. 2010; de Miranda et al. 2010; Nguyen et al. 2011). However, I found low prevalence (up to 16%) of ABPV in colonies, very low concentrations relative to other viruses and no association with *Varroa* in my study. ABPV concentrations did not change from fall to spring. ABPV is often found at low levels, as it kills the pupae along with the *Varroa* entrapped inside the cell and this prevents further spread and multiplication of the virus (Schroeder and Martin 2012).

Sac brood virus is generally thought of as a disease of immature bees (brood), and typically occurs at low levels in spring, peaks in mid-summer and declines in fall following natural brood cycles (Bailey 1981). In adult bees, the virus is also at lower levels in fall (Tentcheva et al. 2004b) but little is known about the seasonal dynamics of this virus over winter. In my study, SBV prevalence increased dramatically from fall to spring and was also very high in colonies sampled in mid-winter even though little brood would be present in colonies at that time. SBV concentrations also were higher in spring than in fall. Sac brood was the only virus strongly associated with colony death and was also the only virus that had higher prevalence in unhealthy colonies from Manitoba than in healthy colonies across Canada (Chapter 2). It is not known if SBV plays a direct role in colony death or if it is an opportunistic pathogen that is favoured when colonies are succumbing to other stresses. However, SBV was not correlated with any other parasites or pathogens linked to bee losses. Cornman et al. (2012) found SBV is correlated with IAPV but only in colonies not expressing symptoms of CCD. Although IAPV was linked to low spring populations for indoor-wintered colonies in my studies, I did not see any

correlations between SBV and IAPV. In indoor-wintering environments, fall SBV concentrations were correlated with KBV levels, but Cornman et al. (2012) did not find an association between SBV and KBV. Cornman et al. (2012) also found that in non-CCD colonies with *Nosema*, SBV is correlated with ABPV and CBPV, but I did not find any relationship between SBV and any of the pathogens.

Although *Nosema* plays a role in honey bee colony losses in some European countries (Higes et al. 2009; Higes et al. 2010) its role in contributing to colony losses is controversial (Le Conte et al. 2010). Recent studies from Germany (Genersch et al. 2010) and Switzerland (Dainat et al. 2012a), found that neither *N. ceranae* or *N. apis* affect colony loss but this conflicts with outcomes from studies in Spain, where *N. ceranae* is one of the key parasites in colony losses (Higes et al. 2008b; Higes et al. 2009; Higes et al. 2010). *Nosema* was not correlated with bee loss in this study in either environment, even though spore counts were well above the nominal threshold in all three seasons. Although unadjusted partial correlations in fall outdoor-wintered colonies suggested *Nosema* was negatively correlated with spring population size, the *q*-values were not significant (q =0.2). Manipulative experiments where colonies are exposed to different levels of infection in the absence of fumagillin treatments are needed to clarify the true impact of this disease on winter colony survival.

One possible drawback in my study is that use of *Nosema* spore counts would not fully estimate the level of *Nosema* spp. infection in bees as the method ignores the vegetative stage of the disease which occurs inside gut and other tissues (Meana et al. 2010). Quantitative analysis (qPCR) would detect the vegetative state as well as spores, but the association between spore counts and qPCR varies between studies (Bourgeois et al. 2010; Traver and Fell 2011). Colony level mean abundance estimates based upon

both spore counts or qPCR can both be biased by the presence of a few highly infected individuals. Neither spore counts or qPCR provide direct measures of *Nosema* prevalence on a per bee basis but prevalence has been shown to be highly correlated with mean abundance at least when large enough samples of bees are taken (50 bees) (Smart and Sheppard 2012). My *Nosema* spore counts were based upon samples of 100 bees and this should help to further reduce bias in my study (Marques and Cabral 2007).

Our study showed that *Nosema* mean abundance increased over winter for outdoor-wintered hives and decreased over winter for indoor-wintered hives. Williams et al. (2010) also compared *Nosema* in indoor- and outdoor-wintering environments in a smaller study (3 beekeeping operations) Although they did not show an overall effect of wintering method on *Nosema* intensity, they did find higher levels of *Nosema* in outdoor-wintered colonies than in indoor-wintered colonies for one operation. Fall treatments of *Nosema* with fumagillin were carried out in all of their colonies. In my study, 11 of 15 producers that wintered indoor, and 4 of 10 producers that wintered outdoors treated with fumagillin in fall for control of *Nosema*. For producers using indoor-wintering environments, those fall treatments resulted in lower spring *Nosema* spore counts than in producers that did not treat hives. However, in producers with outdoor-wintered hives spring *Nosema* spore counts did not significantly differ between those who treated with fumagillin and those that did not. This suggest that observed differences in the season pattern of *Nosema* spore abundance for the two wintering environments may have been partly the results of differences in the residual efficacy of fumagillin in the two environments or a greater capacity for *Nosema* spores to replicate in colonies that are wintered outdoors compared to those wintered indoor.

Significant partial correlations between *Nosema* and DWV were observed for outdoor-wintered colonies in fall. Since spring DWV was associated with bee loss over winter and low spring population size in outdoor-wintered hives, this suggests a synergistic interaction between these pathogens may be occurring. Since *Nosema* suppresses the immune system in workers, the higher levels of DWV that were observed in spring outdoor-wintered hives could be related to inability to effectively control *Nosema* in outdoor wintered hives (Antunez et al. 2009). Although it should be noted that *Varroa* is also correlated with DWV, *Varroa* may also be a factor affecting DWV concentration, (see earlier discussion), this needs further study. *Nosema ceranae* also causes severe damage to the mid-gut epithelial cells (Fries 2010). Mid-gut damage might facilitate exchange of viral pathogens across the gut wall and into the haemolymph (Bromenshenk et al. 2010) but antagonistic interactions may also occur (Costa et al. 2011). Similar to my study, Cornman et al. (2012) found a correlation between DWV and *N. ceranae* in colonies showing CCD-like symptoms but not in non-CCD colonies. However, Costa et al. (2011) found a negative correlation between DWV and *N. ceranae* in mid-gut cells of honey bees in laboratory cage studies and Martin et al. (2013) found no evidence for synergistic correlations between DWV and *N. ceranae* in Hawaiian colonies. Environmental influences could explain some of the differences as other studies did not have their colonies exposed to long periods of confinement or "harsh" wintering conditions.

Significant positive correlations were found between *Nosema* and BQCV for both indoor and outdoor wintered colonies in spring. Partial correlations between *Nosema* and BQCV in spring were only significant for outdoor-wintered colonies. BQCV is commonly associated with *Nosema* and this combination of these pathogens is thought to

cause greater mortality in bees than either on its own (Bailey et al. 1983a, 1983b). However, I did not see significant correlations between *Nosema* and bee loss or spring population size in either wintering environment. Marginal negative correlations between spring population and BQCV (q =0.08) suggest BQCV may play a role in contributing to lower bee population but partial correlations between BQCV and spring population were not significant.

Of the seven viruses, parasites and pathogens tested, sampling location affected mean abundance estimates of only *Nosema* and BQCV. My data suggest that for fall and spring sampling for *Varroa*, DWV, SBV IAPV, KBV, ABPV, and CBPV sampling from either entrances or brood area would produce similar estimates of colony level mean abundances. For *Nosema,* fall spore counts were dramatically different in entrance and brood area samples and although entrance samples may not best represent the total pathogen load in colonies at the time of sampling or impacts on colonies, they were better predictors of future pathogen levels in mid-winter and fall than were brood area samples. *Nosema* spore counts were likely higher in entrance-bee samples because foraging bees caught at entrance tend to be older bees and older bees are more likely to be infested with higher *Nosema* spores (Higes et al. 2008b; Smart and Sheppard 2012).

BQCV levels differed with season, sample location and wintering method, indicating care must be taken when sampling for this pathogen and extrapolating interpretation of results of other studies where single samples are taken from only one location in the hive. My data suggest that in fall, BQCV could be sampled from either entrance or brood area samples for both in indoor and outdoor-wintered colonies and provide similar estimate of colony-level mean abundance. However, in spring, mean abundance of BQCV differed with sample location for outdoor wintered hives; thus,

colonies should be sampled from a combination of entrance and brood area samples to give a more realistic estimate of colony level mean abundance. BQCV shows a seasonal pattern that was similar to *Nosema,* in that the seasonal response differed with wintering method. For BQCV there was no change in mean abundance from fall to spring for indoor-wintered colonies and increase in mean abundance from fall to spring outdoor-wintered colonies. This is not unexpected since *Nosema* and BQCV are often highly correlated, as they were in spring samples outdoor-wintered colonies (Tentcheva et al. 2004b; Berenyi et al. 2006).

In conclusion, my study showed that colonies under similar initial parasite and pathogen loads experience lower rates of bee loss in indoor-wintering management than in outdoor-wintering management. This suggests producers should consider the use of indoor wintering as a management tool to reduce winter loss when . I showed that parasite and pathogen interactions and seasonal changes in mean abundance differed in the two different wintering environments. Fall IAPV level was negatively correlated with spring population but only for indoor wintered colonies. Spring *Varroa* and DWV levels were positively correlated with bee loss and negatively correlated with spring population but only for outdoor-wintered hives. SBV was the only virus significantly associated with colony death over winter for both wintering methods. Sampling location in the hive needs to be considered when interpreting the pathogen load of colonies for *Nosema* and BQCV and for estimating their impact on colony populations. For these pathogens, the best location for sampling differs between pathogens and seasons. Further experiments are urgently required to better predict bee population losses that result from the interaction of honey bee viruses and to develop management practices that will reduce their impact on colonies.

Figure 3.1. Map showing regions from which bee samples were collected. Dots represent the locations of samples collected from beekeepers (5 beekeepers per region). Below the sampling scheme is a time line indicating when fall, mid-winter and spring samples were taken. Samples taken at each sample date are listed below the bar.

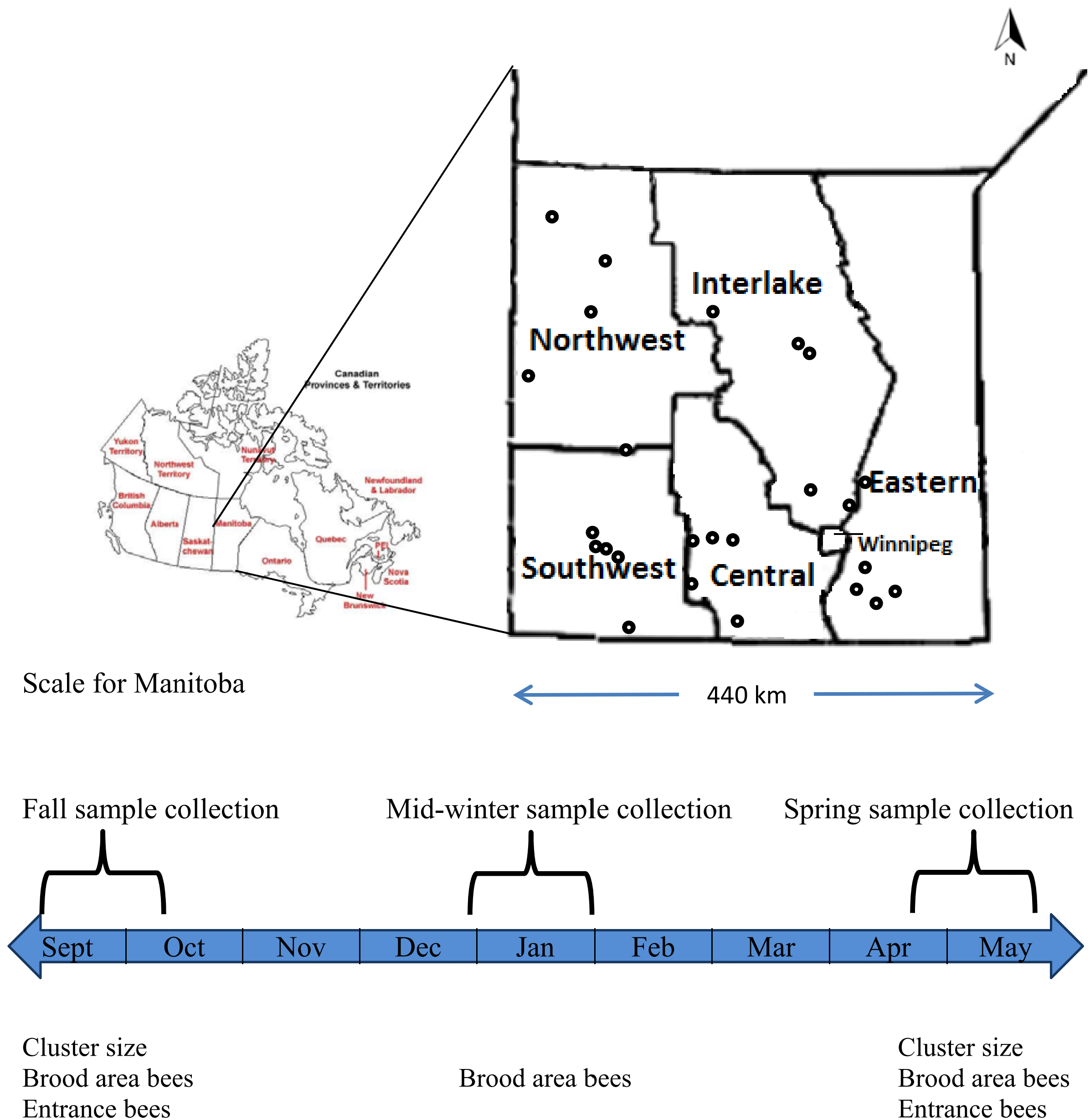


Table 3.1. Primers used for the used for PCR analysis and qPCR analysis for quantification.

Sl.No	Primers for bee viruses used in experiments	Product size (bp)	Reference
1	RT-DWV-F: CGAAACCAACTTCTGAGGAA RT-DWV-R: GTGTTGATCCCTGAGGCTTA	174	(Li et al. 2011a)
2	RT-BQCV-F: CCTGTATTCATGCATCTCAGA RT-BQCV-R: GCAACAAGAAGAAACGTAAACCAC	310	Chen personal communication
3	RT-SBV-F: GTAGTCCAGTGCCCGATGTGT RT-SBV-R: GCACCAAAAGTACCTCCCAA	165	Chen personal communication
4	RT-IAPV-F: GCGGAGAATATAAGGCTCAG RT-IAPV-R: CTTGCAAGATAAGAAAGGGGG	586	Chen personal communication
5	RT-KBV1 F: GGTGTAGCAGCCATATTCG RT-KBV1 R: CTCGCAAGTCTCCTAATTCG	157	This work
6	RT-CBPV F: TAAGTCGTTGTCGTATCAC RT-CBPV R: AAGCAGTCATTCGTTATCC	168	This work
7	RT-ABPV-F: ATAACACGATGTTACCCGA RT-ABPV-R: TCTTCTAAAGTACTGCGCTTG	146	Chen personal communication
8	β-actin-F: AGGAATGGAAGCTTGCGGTA β-actin-R: AATTTTCATGGTGGATGGTGC	181	Chen et al. (2005)

Figure 3.2. Change in colony population score (frames of bees, 1 frame= ~ 2,430 bees) from fall to spring, for indoor (- ■-) and outdoor (—●—) wintered colonies. Means followed by the same letter are not significantly different (P > 0.05; Bonferroni).

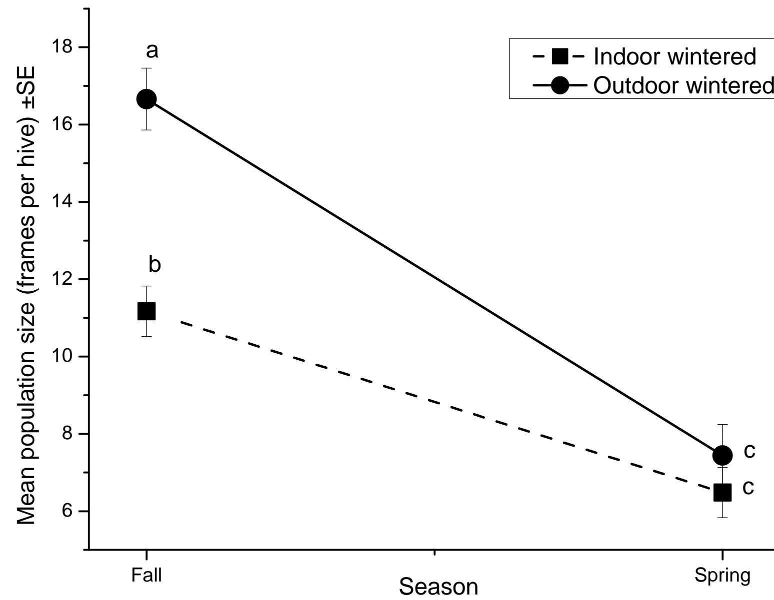


Table 3.2 Proportion of colonies (in two wintering methods) with detectable levels of parasites or pathogens as measured by the alcohol wash method for *Varroa* on adult bees collected in the brood area (250 bees) and from colony entrances (200 bees approximately), spore count for *Nosema* spp (from 100 bees), thoracic slice method for tracheal mite (100 bees), and RT-PCR for viruses using 50 bees for brood area and 10 bees for entrance samples.

Wintering method	Season	Number of colonies	*Varroa* (%)	Tracheal mite (%)	*Nosema** (%) #	DWV (%)	BQCV (%)	SBV* (%) #	IAPV* (%)	KBV* (%)	CBPV (%)	ABPV (%)
Indoor	**Fall**	45	69	4	53a	100	98	40a	13	11	13	2
	Mid-winter	45	56	9	87b	87	82	84b	33	18	13	16
	Spring	40	58	10	90b	98	88	73b	20	20	13	10
Outdoor	**Fall**	30	83	3	47	100	100	50	13	13	10	3
	Spring	26	73	8	96	96	92	85	50	46	35	8
Pooled	**Fall**	75	76	3.5	50	100	99	45	13	12	11.5	2.5
Pooled	**Spring**	66	65.5	9	93	97	90	79	35	33	24	9

*= Overall seasonal change in prevalence (see results for statistics) from fall to spring averaged over both wintering methods.
#= Comparisons within indoor-wintered colonies for changes in seasonal prevalence (see results for statistics) proportions followed by the same letter within indoor-wintering hives are not significantly different (P > 0.05).

Table 3.3. Effect of wintering method, regions, sampling location in the hive and season on the relative levels of parasites and pathogens in honey bee colonies. Table 3.3. contd… (next page)

	Wintering method			Region			Sample location			Wintering method* sampling location		
Parameter§	*F*	df	*P*	*F*	df	*P*	*F*	df	*P*	*F*	df	*P*
Varroa	0.67	1, 19	0.42	0.08	4, 19	0.99	2.24	1,64	0.14	0.06	1, 64	0.81
HBTM	0.00	1, 19	0.97	1.00	4, 19	0.43	0.00	1,65	0.99	0.07	1, 65	0.79
Nosema	0.38	1, 19	0.54	0.65	4, 19	0.64	**7.31**	**1, 73**	**0.008**	1.49	1, 19	0.23
DWV	2.24	1, 19	0.15	0.67	4, 19	0.62	0.63	1, 73	0.43	1.14	1, 73	0.29
BQCV	0.04	1, 19	0.84	1.98	4, 19	0.14	**6.87**	**1, 73**	**0.01**	**4.27**	**1, 73**	**0.04**
SBV	0.11	1, 19	0.74	1.02	4, 19	0.42	0.12	1, 73	0.73	0.05	1, 73	0.83
IAPV	1.71	1, 19	0.21	1.13	4, 19	0.37	0.03	1, 73	0.87	2.58	1, 73	0.11
KBV	0.62	1, 19	0.44	0.37	4, 19	0.83	1.81	1, 73	0.18	2.48	1, 73	0.12
CBPV	4.29	1, 19	0.05*	0.87	4, 19	0.50	0.06	1, 73	0.80	0.74	1, 73	0.39
ABPV	3.32	1, 19	0.08	1.54	4, 19	0.23	0.95	1, 73	0.33	0.84	1, 73	0.36

§= see abbreviations for full names (page ix).
F = *F* value, df = degrees of freedom, *P* = P value
* P=0.052

Table 3.3. contd…

	Season			Season* sampling location			Season * Wintering method			Season *Wintering method* sampling location		
Parameter§	*F*	Df	*P*	*F*	df	*P*	*F*	df	*P*	*F*	df	*P*
***Varroa*†**	**16.86**	**1, 65**	**0.0001**	-	-	-	0.01	1, 65	0.91	-	-	-
HBTM†	0.35	1, 68	0.55	-	-	-	0.51	1, 68	0.48	-	-	-
Nosema	**40.43**	**1,129**	**0.0001**	**4.08**	**1,129**	**0.045**	**4.2**	**1,129**	**0.04**	0.00	1,129	0.95
DWV	1.61	1,121	0.21	2.17	1,121	0.14	**6.57**	**1,121**	**0.01**	0.03	1,121	0.86
BQCV	**26.29**	**1,121**	**0.0001**	**5.90**	**1,121**	**0.02**	**10.71**	**1,121**	**0.001**	**5.8**	**1,121**	**0.02**
SBV	**8.65**	**1,120**	**0.003**	1.19	1,120	0.27	0.72	1,120	0.39	0.84	1,120	0.36
IAPV	2.91	1,121	0.09	0.03	1,121	0.87	0.04	1,121	0.83	2.58	1,121	0.11
KBV	**5.84**	**1,121**	**0.02**	1.60	1,121	0.20	1.59	1,121	0.20	2.18	1,121	0.14
CBPV	**16.38**	**1,121**	**0.0001**	0.09	1,121	0.76	3.59	1,121	0.06	0.76	1,121	0.39
ABPV	1.31	1,121	0.26	0.99	1,121	0.32	0.83	1,121	0.36	0.87	1,121	0.35

§= see abbreviations for full names (page ix).
† *Varroa* and honey bee tracheal mite were not sampled from entrances in fall.

Figure 3.3. Interactions between season (spring and fall) and wintering method (indoor and outdoor) (see Table 3.3) for DWV and BQCV concentrations (left axis) and mean abundance of *Nosema* (right axis) (± standard error). Results of significant slices for each virus are indicated above the bars for slices by wintering method and below the graph for slices by season, (horizontal lines indicate significant slices). Data represent pooled values for brood area and entrance samples.

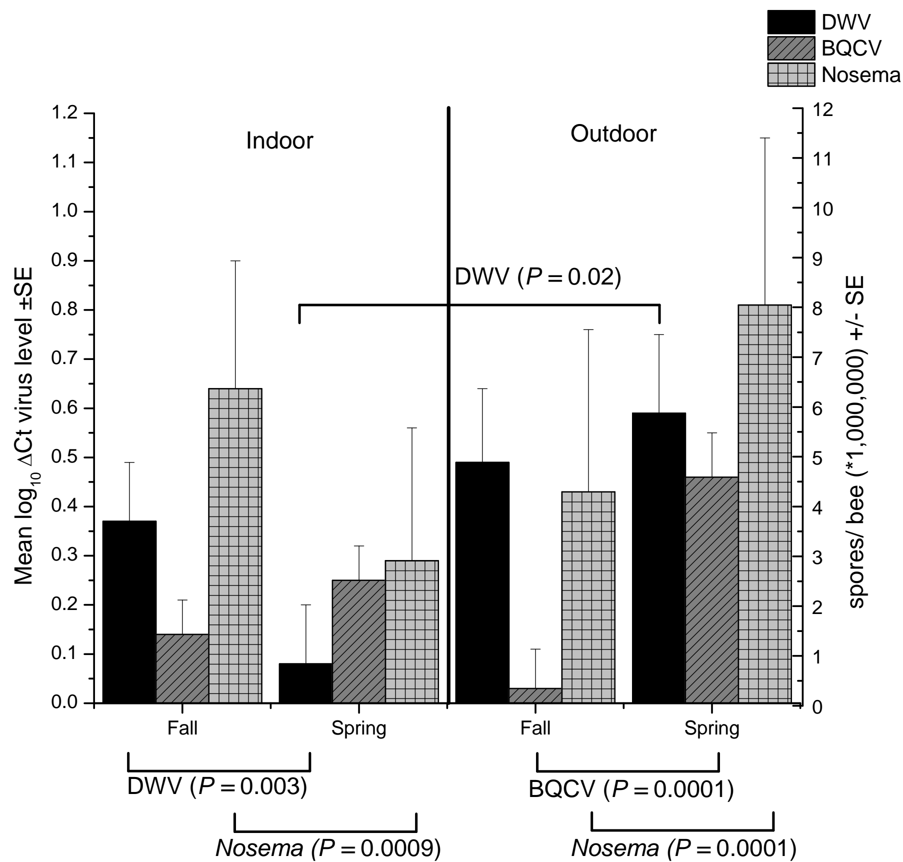


Figure 3.4. Interaction between season (spring and fall), wintering method (indoor and outdoor), and sampling location (brood area and entrance) (see Table 3.3) for BQCV concentrations (± standard error). Results of significant slices are indicated above the bars for wintering method and below the graph for season and wintering method, (horizontal lines indicate significant slices).

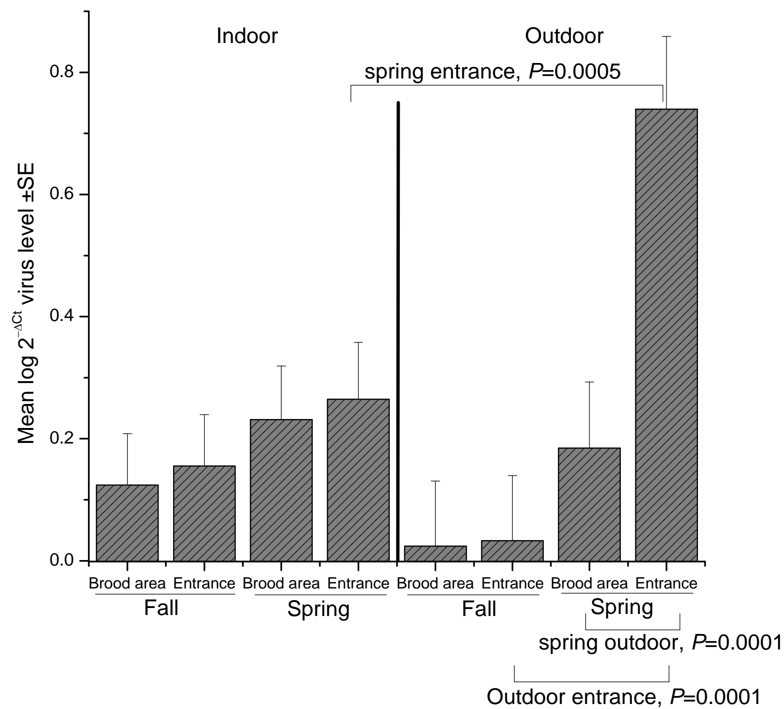


Figure 3.5. Interaction between season (fall and spring) and sampling location (brood area and entrance) (see Table 3.3) on mean abundance of *Nosema* (± standard error). Results of slices are indicated above the bars for sampling location and below the bars for season, (horizontal lines indicate significant slices). Data represent pooled values for indoor and outdoor samples.

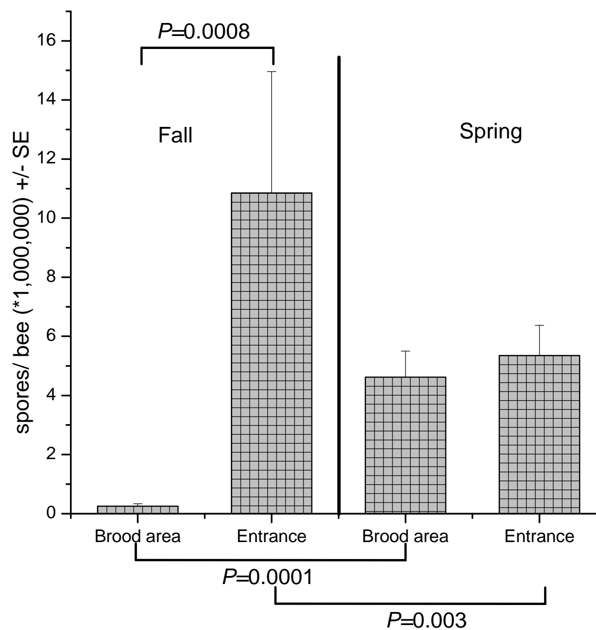


Figure 3.6. Effect of season (fall and spring) on mean abundance of *Varroa* (left axis) and concentrations of viruses (SBV, KBV and CBPV) (right axis) (± standard error) (Table 3.3). Data represent pooled levels for both wintering methods (indoor and outdoor) and both sampling locations (brood area and entrance). Means followed by the same letter within a parasite or pathogen group are not significantly different (P > 0.05, Slice).

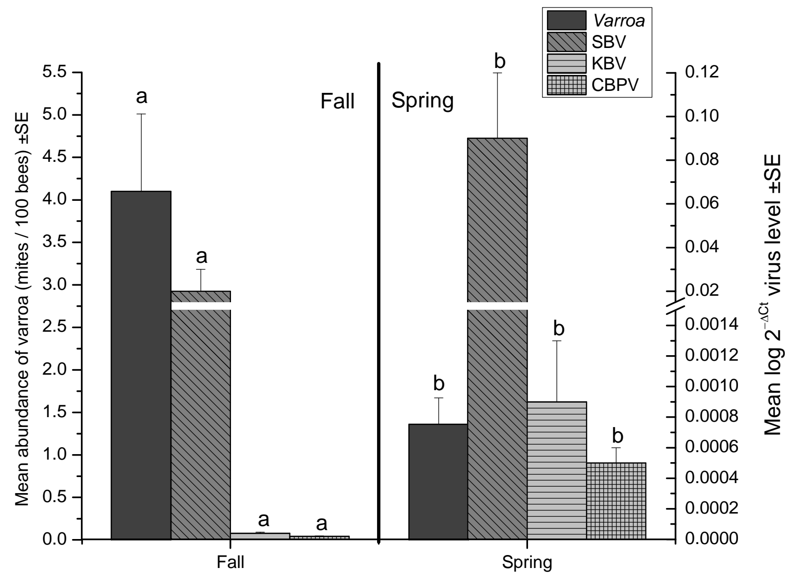


Table 3.4. Simple and partial correlations (Pearson's) between colony parasite (mean abundance of *Varroa* and prevalence of tracheal mite) and pathogen (mean abundance of *Nosema,* log $2^{-\Delta Ct}$ of DWV, BQCV, IAPV, SBV, KBV, CBPV and ABPV) levels, percent bee loss over winter and spring colony population size across all samples in fall and spring for indoor and outdoor wintering colonies. Brood area and entrance samples were pooled prior to analysis. Full tables can be found in appendix 1-8. Significant correlations are in boldface (q < 0.05).

(A) Indoor-wintering: Fall parasite and pathogen levels with each other, and bee loss and spring population size.

Fall Indoor (N = 45)							
Simple correlations				**Partial correlations**			
Variable pair§	**r**	**P- value**	***q*-value**	**Variable pair§**	**R**	**P-value**	***q*-value**
Bee loss-*Varroa*	+0.33	0.03	0.17				
Bee loss- DWV	+0.31	0.04	0.19	Bee loss- DWV	+0.32	0.04	ns
Bee loss-IAPV	+0.32	0.03	0.17	Bee loss-IAPV	+0.43	0.004	0.06
Pop-*Varroa*	-0.41	0.006	0.06	Pop- *Varroa*	-0.37	0.02	0.11
Pop-DWV	-0.40	0.006	0.06	Pop-DWV	-0.36	0.02	0.11
Pop-IAPV	-0.30	0.04	0.19	**Pop-IAPV**	**-0.50**	**0.008**	**0.01***
Varroa -DWV	+0.45	0.02	0.14	*Varroa* -DWV	+0.37	0.02	0.11
BQCV-KBV	+0.41	0.005	0.06	BQCV-KBV	+0.36	0.02	0.11
SBV-KBV	**+0.56**	**0.0001**	**0.003***	**SBV-KBV**	**+0.56**	**0.0001**	**0.003***
IAPV-KBV	**+0.53**	**0.0002**	**0.004***	IAPV-KBV	+0.42	0.005	0.06
IAPV-CBPV	**+0.63**	**0.0001**	**0.003***	**IAPV-CBPV**	**+0.61**	**0.0001**	**0.003***
KBV-CBPV	+0.35	0.02	0.14				

(B) Outdoor-wintering: Fall parasite and pathogen levels with each other, bee loss and spring population size

Fall Outdoor (N = 30)							
Simple correlations				**Partial correlations**			
Variable pair§	**r**	**P-value**	***q*-value**	**Variable pair§**	**R**	**P-value**	***q*-value**
				Bee loss-*Varroa*	+0.44	0.02	ns
Bee loss- DWV	+0.43	0.02	ns	Bee loss- DWV	+0.45	0.02	ns
Pop-*Varroa*	-0.39	0.03	ns	Pop-*Varroa*	-0.41	0.04	ns
Pop-DWV	-0.41	0.02	ns	Pop-DWV	-0.46	0.02	ns
				Pop-*Nosema*	-0.41	0.03	ns
***Varroa* -DWV**	**+0.70**	**0.0001**	**0.006***	***Varroa* -DWV**	**+0.71**	**0.0001**	**0.006***
IAPV-ABPV	+0.50	0.005	0.10	IAPV-ABPV	+0.45	0.01	ns
Nosema-DWV	+0.55	0.002	0.06	***Nosema*-DWV**	**+0.58**	**0.002**	**0.04***

Pop=population, ns= *q*-value > 0.2, r = correlation coefficient

*= *q*-values of 0.05 or lower are considered significant

§= see list of abbreviations for full names of parasites and pathogens (page ix)

P-value=Pearson's simple and partial correlations

q-value= FDR adjusted *q*-values with cut off of 0.2

table 3.4 contd….

(C) Indoor wintering: Spring parasite and pathogen levels with each other, bee loss and spring population size.

Spring Indoor (N = 41)							
Simple correlations				**Partial correlations**			
Variable pair§	**r**	**P- value**	***q*-value**	**Variable pair§**	**r**	**P- value**	***q*-value**
Pop-BQCV	-0.43	0.006	0.08	Pop-BQCV	-0.37	0.02	ns
IAPV-ABPV	**+1.00**	**0.0001**	**0.003***	**IAPV-ABPV**	**+1.00**	**0.0001**	**0.003***
KBV-CBPV	**+0.81**	**0.0001**	**0.003***	**KBV-CBPV**	**+0.80**	**0.0001**	**0.003***
***Nosema*-BQCV**	**+0.47**	**0.002**	**0.03***	*Nosema*-BQCV	+0.45	0.005	0.11

(D) Outdoor-wintering: Spring parasite and pathogen levels with each other, bee loss and spring population size

Spring outdoor (N = 29)							
Simple correlations				**Partial correlations**			
Variable pair§	**r**	**P- value**	***q*-value**	**Variable pair§**	**r**	**P- value**	***q*-value**
Bee loss-*Varroa*	+0.33	0.06	0.12	**Bee loss-*Varroa***	**+0.61**	**0.0008**	**0.006***
				Bee loss- DWV	**+0.59**	**0.001**	**0.008***
				Bee loss-CBPV	+0.41	0.04	0.12
				Pop-*Varroa*	**-0.55**	**0.003**	**0.02***
				Pop-DWV	**-0.71**	**0.0001**	**0.001***
				Pop-CBPV	-0.48	0.01	0.06
***Varroa* –DWV**	**+0.53**	**0.003**	**0.01***	*Varroa* –DWV	+0.40	0.04	0.12
Varroa –HBTM	+0.39	0.04	0.09				
				HBTM-CBPV	-0.42	0.03	0.11
DWV-KBV	**+0.65**	**0.0002**	**0.001***	**DWV-KBV**	**+0.63**	**0.0005**	**0.005***
DWV-CBPV	**+0.78**	**0.0001**	**0.0007***	**DWV-CBPV**	**+0.71**	**0.0001**	**0.001***
BQCV-KBV	**+0.45**	**0.01**	**0.03***	**BQCV-KBV**	**+0.55**	**0.004**	**0.02***
IAPV-ABPV	**+0.72**	**0.0001**	**0.0007***	**IAPV-ABPV**	**+0.77**	**0.0001**	**0.001***
KBV-CBPV	**+0.81**	**0.0001**	**0.0007***	**KBV-CBPV**	**+0.79**	**0.0001**	**0.001***
CBPV -ABPV	+0.44	0.02	ns	CBPV -ABPV	+0.43	0.03	0.10
***Nosema*-BQCV**	**+0.50**	**0.006**	**0.02***	***Nosema*-BQCV**	**+0.61**	**0.001**	**0.006***

Pop=population, ns= *q*-value > 0.2, r = correlation coefficient
*= *q*-values of 0.05 or lower are considered significant
§= see list of abbreviations for full names of parasites and pathogens (page ix)
P-value=Pearson's simple and partial correlations
q-value= FDR adjusted *q*-values with cut off of 0.2

Table 3.5. Pearson's correlation analyses of *Nosema* levels in entrance and brood area samples collected in fall, mid-winter and spring for colonies wintered indoors. Significant correlations are in boldface.

Parameter		**Fall brood area**	**Winter**	**Spring entrance**	**Spring brood area**
Fall entrance	R	**+0.37**	**+0.76**	+0.02	**+0.30**
	P- value	**0.001**	**0.0001**	0.88	**0.01**
Fall brood area	R		**+0.53**	+0.17	**+0.42**
	P- value		**0.0002**	0.18	**0.0004**
Winter	R			**+0.39**	**+0.71**
	P- value			**0.02**	**0.0001**
Spring entrance	R				**+0.76**
	P- value				**0.0001**

R = correlation coefficient

CHAPTER 4. GENETIC DIVERSITY WITHIN HONEY BEE COLONIES AFFECTS PATHOGEN LOAD AND RELATIVE VIRUS LEVELS IN HONEY BEES, *APIS MELLIFERA* L.

ABSTRACT

The evolution of polyandry (multiple mating) in Hymenoptera is one of the most highly debated topics in evolutionary sociobiology. Various hypotheses have been proposed to explain the benefits of polyandry. One hypothesis suggests that because multiple mating increases genetic diversity among colony members, colony resistance to a wide range pathogens and parasites is increased and thus colony fitness is also increased. I investigated the effect of manipulating genetic diversity on colony population size, fall *Varroa* population, *Varroa* and bee mortality rates, virus prevalence and concentration, and prevalence of *Nosema apis* Z. and *Nosema ceranae* F. The experiment was carried out from spring 2007 to summer 2008. Sister queens selected for resistance to *Varroa* were each inseminated with either mixed semen from 12 drone sources (genetically diverse colonies GDC) or semen from 12 individual drones (Genetically similar colonies produced through single drone insemination GSC), and colonies from these queens were compared to colonies from a third set of unrelated queens that were open-mated. When exposed to parasites and pathogens, open mated colonies (OMC) had larger worker populations before winter than GDCs and GSCs but GDCs did not differ from GSCs. GSCs produced fewer *Varroa* per colony than those with greater genetic diversity, but the mean abundance on adult bees was similar. The bees of GDCs had higher *Varroa* mortality rates than those of GSCs in the field study. Within the instrumentally inseminated treatments, three pathogens occurred at lower

prevalence (acute bee paralysis virus (ABPV) and *N. ceranae*) or concentrations (deformed wing virus (DWV) in GDCs than in GSCs. None of the other pathogens studied occurred at higher frequencies or concentrations in GDCs than in GSCs. Both GDCs and GSCs that had been selected for resistance to *Varroa* had lower DWV concentration in spring when compared to unselected open mated colonies (OMCs). This study showed that colonies inseminated by multiple drones and having greater within-colony genetic diversity showed reduced prevalence or concentrations in about a quarter of the pathogen assays relative to genetically uniform colonies and the reverse pattern was never observed. The diminished levels of pathogen challenge therefore may be a selection pressure favouring the retention of polyandry in honey bees. This study supports the hypothesis that polyandry may partly be an important adaptation to defend against parasites and diseases in honey bee colonies.

INTRODUCTION

Honey bee queens and many other social species mate with multiple males (polyandry). The evolution of polyandry in highly social insects, such as *Apis* (Hymenoptera), is a widely debated topic in evolutionary sociobiology (Page 1980; Laidlaw and Page 1984; Fuchs and Moritz 1998). The number of mates can vary substantially between species. Honey bee (*Apis mellifera* L.) queens, for example, mate during a restricted period when six-to-10-days old, typically mate with 10 – 25 drones, and store the sperm in their spermatheca (reviewed by Currie 1987; Palmer and Oldroyd 2000). Other *Apis* species also have very high mating frequencies. For example, *Apis dorsata* F. queens mate with 27 to 107 drones (Wattanachaiyingcharoen et al. 2003) and *Apis florea* F. queens mate with 13 to 19 drones (Palmer and Oldroyd 2001).

High mating frequency (Page 1986) and synchronized use of sperm packets from numerous mates results in heterogeneous worker populations within colonies, where several subfamilies of different genetic makeup are present in the colony at any given time (Laidlaw 1974; Page and Metcalf 1982; Laidlaw and Page 1984). Each worker subfamily shares genes from the queen and whatever drone contributed sperm to that egg during fertilization. The different subfamilies form a homogenous bee cluster, but individual subfamilies often differ in their propensity to perform certain behaviors like defending the nest entrance, collecting pollen, cleaning dead bees from the nest (Robinson and Page 1988), and grooming of nest mates (Frumhoff and Baker 1988).

Several hypotheses have been presented to explain the probable benefits of multiple mating in honey bee queens. First, genetic diversity reduces the risk of producing diploid males (Page 1980). Diploid males are produced when closely related

drones and queens mate as a result of a sex determination system where fertilized eggs (normally female) that share the same allele for sex determination produce diploid males (Page 1980; Ratnieks 1990; Tarpy and Page 2002). Diploid drone eggs laid by the queen are eaten by workers and, thus, colonies with high levels of inbreeding have greatly reduced brood viability and lower fitness (Currie 1987; Beye et al. 2003). In some eusocial insects such as *A. mellifera*, multiple mating reduces the probability of producing diploid males, and the fitness benefits are often thought to be one of the factors favouring the evolution of polyandry.

Second, genetically diverse colonies have more subfamilies of worker bees and are, thus, more behaviorally diverse, which could increase colony-level efficiency and fitness with respect to acquiring food resources (Oldroyd et al. 1991; Oldroyd et al. 1992; Mattila and Seeley 2007). Considerable evidence supports this hypothesis. Genetically diverse colonies have higher bee populations, construct comb faster, and produce more honey than genetically uniform colonies (Mattila and Seeley 2007). Foragers from genetically diverse colonies also recruit nestmates to food more frequently through waggle dancing and, thus, go on more foraging trips than genetically similar colonies (Mattila et al. 2008). Genetically diverse colonies of the harvester ant *Pogonomyrmex occidentalis* Cresson (Hymenoptera: Formicidae) also forage for a longer time than colonies with lower diversity, showing that diverse ant workers are ecologically predominant (Cole et al. 2010). A genetically diverse colony has more flexibility to respond positively to highly variable environmental conditions. In contrast, colonies with low genetic diversity have a narrow range of behavioral thresholds among their workers. For example, genetically similar colonies may allocate inappropriate numbers of workers

to tasks required by the colony because the particular genotypes present may not have an optimal task threshold (Robinson 1992; Myerscough and Oldroyd 2004). Such colonies can experience large fluctuations in the expression of a colony-level phenotypic response.

Third, it has been hypothesized that high genetic diversity that results from multiple mating may lower the occurrence of disease infections (parasites and pathogens) (Sherman et al. 1988; Schmid-Hempel and Loosli 1998; Baer and Schmid-Hempel 1999, 2001; Tarpy 2003; Tarpy and Seeley 2006; Seeley and Tarpy 2007). According to Shykoff and Schmid-Hempel (1991b), who worked on the *Bombus*–parasite system, close kinship in bees can be "a boon and a bane", because the likelihood of getting a parasitic infection (mites or diseases) from close kin is much higher than from a single individual. Colonies with little or no genetic diversity, living together in close kinship, are advantageous from the parasite's perspective, and will likely make for an environment easily exploited by pathogens and parasites (Schmid-Hempel and Loosli 1998). Genetically heterogeneous colonies can better resist parasites under certain conditions (Brown and Schmid-Hempel 2003; van Baalen and Beekman 2006). In bumble bees, *Bombus terrestris* L. (Hymenoptera: Apidae) populations having high genetic diversity are closely linked to lower numbers of parasites (Shykoff and Schmid-Hempel 1991a; Liersch and Schmid-Hempel 1998; Schmid-Hempel and Loosli 1998; Baer and Schmid-Hempel 1999). Schmid-Hempel and Crozier's (1999) exhaustive comparative study on 119 species of ants showed that high genotypic diversity inside the colonies correlates with lower parasite loads.

Most of the studies on honey bees that tested the plausibility of the "parasite hypothesis" have been done on immature stages of bees. These studies examined

bacterial and fungal diseases such as American foulbrood (*Paenibacillus larvae*) (White), Ash, Priest and Collins), European foulbrood (*Melissococcus plutonius* White), and chalkbrood (*Ascosphaera apis* (Massen ex Claussen) Olive and Spiltoir) (Palmer and Oldroyd 2003; Tarpy 2003; Tarpy and Seeley 2006; Seeley and Tarpy 2007; Invernizzi et al. 2009). Seeley and Tarpy (2007) showed that genetically diverse colonies had greater resistance to *P. larvae* than did genetically uniform colonies. None of the above studies have focused on the effect of within-colony genetic diversity on viruses of adult bees, or their interactions with parasitic mites such as *Varroa*, or with other adult diseases such as *Nosema*.

Studies on the role of genetic diversity in host defence are also important in a practical sense. Current challenges to honey bee health are affecting the beekeeping, agriculture, and pollination industries worldwide. In recent years, much research on causes of colony losses has focused on the role of multiple pests and pathogens. Some speculate low genetic diversity in honey bee populations in North America may contribute to disease susceptibility (Delaney et al. 2009; Meixner et al. 2010; vanEngelsdorp and Meixner 2010; vanEngelsdorp et al. 2010; vanEngelsdorp et al. 2012).

Honey bees have evolved a variety of mechanisms to use against pests and pathogens. They possess innate immune responses that benefit colonies by reducing pathogen replication within hosts (Wilson-Rich et al. 2009), but have lower numbers of immune-response genes compared to solitary species of “model insects” such as fruit flies (*Drosophila* spp.) and mosquitoes (*Anopheles* spp.) (Evans et al. 2006). Thus, bees may use more of a collective response to fight against parasites and pathogens, which includes

forms of behavioral or social immunity (Wilson-Rich et al. 2009; Evans and Spivak 2010; Wilson-Rich et al. 2012). Efficient defensive mechanisms are important because immune responses and behavioural defenses both have associated fitness costs, and can reduce colony productivity (Evans and Pettis 2005).

For honey bees, grooming is an important defensive mechanism. Grooming behavior is a form of social immunity involving removal of mites from workers' bodies that may result in death of the mites (Peng et al. 1987). Various studies have assessed the outcome of successful grooming through direct (Peng et al. 1987; Hoffmann 1993; Arechavaleta-Velasco and Guzman-Novoa 2001; Aumeier 2001) and indirect assays (Büchler et al. 1992; Hoffmann 1993, 1995; Lodesani et al. 2002; Moretto 2002; Currie and Tahmasbi 2008) and have shown that grooming is a successful defense behaviour against the *Varroa*. I evaluated grooming against *Varroa* (method to measure grooming intensity as described by Currie and Tahmasbi (2008)), and assess reductions in the mean abundance of *Varroa* over time and changes in the proportion of mites falling to the bottom of colonies of bees during cage and colony-level assessments.

The pathogens examined in this study vary in virulence and methods of transmission. Viruses can be transmitted horizontally (between different workers of the same generation) or vertically (from mother to offspring during egg development through the follicle cells or after completion of egg development) (Chen and Siede 2007). Virus transmission also occurs during mating (Chen et al. 2006a; de Miranda and Fries 2008) and the fact that each queen mates with 10 or more drones increases the potential for horizontal transmission of viruses from the semen of infected drones to queens, and contributes to potential for transovarial transmission of viruses from queens to their eggs.

Defensive mechanisms could disrupt either pathway or act in combination with innate mechanisms of immunity to reduce the impact of viruses. In the absence of some form of individual or social immunity, increased virus prevalence or concentration would be predicted in colonies headed by multiple-mated queens relative to those mated by a single drone. This study allowed for the occurrence of both vertical (queens to eggs through ovaries; drones to sperm) and horizontal (e.g. semen from drones into the queen's spermatheca, food sharing, *Varroa* transmission, fecal exchange between workers) routes of transmission to occur.

There are seven economically important honey bee viruses DWV, BQCV, IAPV, SBV, KBV, CBPV and ABPV. At least three of these (DWV, BQCV, and IAPV) can be transmitted horizontally and vertically (Chen et al. 2006a; Yue et al. 2007; de Miranda and Fries 2008; Mockel et al. 2011). DWV typically causes symptoms such as malformed wings, reduced body size, and discoloration in adult bees and developmental abnormalities. DWV reduces worker foraging efficiency and reduces worker longevity (Bailey and Ball 1991; Ball and Bailey 1997). BQCV infection of adult bees is common during the spring and summer, but goes unnoticed because the adult bees have no external symptoms (Tentcheva et al. 2004b). It has been associated with increases in winter bee losses often when interacting with *Nosema* (Bailey et al. 1983a). IAPV has been linked to Colony Collapse Disorder (CCD) (Cox-Foster et al. 2007) and causes symptoms such as trembling, shivering wings, paralyzed bees, and dead bees outside the hive.

Nosema is an important fungal pathogen that affects adult bees. It may act alone or interact with *Varroa* and viruses to cause impacts on colonies. It has been implicated

as a possible cause of colony losses of *A. mellifera* in some areas of Europe (Higes et al. 2006), Asia (Huang et al. 2007), and the United States (Chen et al. 2008). Two different species of microsporidian parasites are involved, *N. apis* and *N. ceranae*. Both species cause nosemosis in adult honey bees (Fries et al. 1996; Chen et al. 2009). Virulence of *Nosema* may vary with different haplotypes (Williams et al. 2008) and different responses to *N. ceranae* infestations occur in different strains of bees (Rinderer et al. 2012). *Nosema* affects worker bee longevity, queen ovaries, winter colony survival, spring brood rearing and honey production (Rinderer and Sylvester 1978; Anderson and Giacon 1992; Faucon et al. 2002; Higes et al. 2006).

We tested the hypothesis that the level of within-colony diversity influences resistance to a suite of parasites and pathogens. I examined the relationship between genetic diversity and variation in resistance behaviour (grooming) against the *Varroa*, viruses, and nosema disease. By creating genetically diverse and similar colonies through insemination and comparing these two groups of bees with open mated queens, I assessed the following: (1) three defense variables against *Varroa* in colonies with different levels of diversity: (i) mite population build up prior to wintering, (ii) mean abundance of mites on adult bees, and (iii) effectiveness of bee grooming against mites.; (2) the effect of genetic diversity on (i) relative viral prevalence a of seven viruses (DWV, BQCV, IAPV, SBV, KBV, CBPV and ABPV) and (ii) viral concentration of three (DWV, BQCV, and IAPV) ; and (3) the effect of genetic diversity on prevalence of *N. apis* and *N. ceranae*.

MATERIALS AND METHODS

Queen rearing and drone selection

The experiment was conducted at the University of Manitoba (UM) campus apiary, Winnipeg (49°54'N, 97°14'W), Manitoba, Canada, in 2007 – 2008. Queens that were used to produce genetically diverse and genetically similar queens were daughters of one queen that had been inseminated with semen from a single drone, so 75% of their genes were related. The queens were reared following the standard procedures described in Laidlaw and Page (1997). Queens were produced by grafting larvae (< 36 h old) into artificial queen cups, which were then placed in a queenless starter colony. On day 12 (after grafting), capped queen cells were placed in an incubator and queens that emerged were placed in individual cages and stored in a queen bank (Cobey 2005). Seven-days after queen emergence, virgin queens were collected from the queen bank for artificial insemination. Drone source colonies were selected from mite resistance stocks that were part of the UM selective breeding programme for resistance to the mite *Varroa destructor*. These stocks were not related to the maternal source or to each other. Drones were captured at hive entrances when they returned to the hive. Only mature drones were selected for use in insemination.

Artificial Insemination

Artificial insemination was done with a Schley II insemination device using a Harbo syringe (Harbo 1985). Queens were inseminated with ~1.0 µL of semen either from single drones using 12 different drone source colonies or from a mixture of semen from 12 different drone source colonies. Thirty queens were inseminated with mixed semen from 12 drones to produce genetically diverse colonies (GDCs), and 17 queens

were single-drone inseminated to create genetically similar colonies (GSCs). Also created was a group of eight queens, which were not reared from inseminated queens and not selected for disease resistance. This group was open-mated with an unknown number of drones, it is referred to as open-mated colonies (OMCs, n = 8) and were produced in the same season as the instrumentally inseminated queens but their number of mating's and time of mating could not be determined. The inseminated queens were introduced into colonies on 19 July 2007. The colonies had similar worker populations, quantities of brood, and food stores. One month following release, 24 queens were successfully established in the GDCs treatment group, and 11 were established in the GSCs group and eight in the OMCs.

Colony management and sample collection

Colonies were managed according to standard practices. Additional supers were provided as required to permit colony growth, and colonies were treated with Oxytet-25 (Medivet Pharmaceutical Ltd., High River, AB, Canada) according to label direction to suppress American foul brood disease. Sucrose syrup was fed as required. Colonies were treated in early fall with Fumagilin-B antimicrobial agents to manage *Nosema* according to label recommendations.

Once inseminated queens were established in colonies, they were allowed to lay eggs for > 12 weeks (so that most of the workers in the colony would be progeny of the experimental queens at the time of assessment). Hives were opened and scored visually from both top and bottom to count the numbers of frames completely covered with bees to assess colony population size (Burgett and Burikam 1985). On 1 November 2007, samples of ~300 worker bees were collected from the brood area of each colony to assess

the mean abundance of *V. destructor*, presence of tracheal mite *Acarapis woodi* (Rennie), and mean abundance and species of *Nosema* spp. spores. A separate sample of ~50 bees was collected and stored at -80 °C and used to assess the viral load (described below).

The mean abundance of mites on live bees and dead bees (mites/100 bees) (Bush et al. 1997) was assessed by the alcohol-shake method (Gatien and Currie 2003). Mean abundance of *Nosema* spores, was assessed according to Cantwell (1970). Tracheal mite prevalence was assessed by the thoracic slice method (Delfinado-Baker 1984). Tracheal mites were not found and thus were not included in further analyses. On 7 November 2007 the colonies were transferred to an over-wintering building (OWB) for storage during winter, according to standard practice for the region, and were held under constant darkness at 5 °C and ventilation rate of 106 L/s (Underwood and Currie 2004).

Daily Varroa and bee mortality rates

Colonies from the different treatments were randomly assigned to one of two small 3.0 m × 2.7 m × 1.7 m rooms in the OWB, and placed in rows of four, along each wall on November 7 (Currie et al. 1998; Underwood and Currie 2004). The rooms were thermostatically controlled and fitted with data loggers (HOBO data logger, Onset Computer Corporation, Bourne, MA, USA) and maintained at 5 °C in complete darkness.

Mite and bee mortality were assessed in mid-winter so results could be obtained before any colony death occurred. A polyvinyl board (52 cm × 36 cm × 4 mm) covered with wax paper (30.2 cm wide) was inserted above the bottom board of each hive to collect any *Varroa* or honey bees that fell from the winter cluster and subsequently died. “Bottom board” (polyvinyl board) samples were collected eight times at 8–13 day intervals (total of 85 days) from 7 January to 31 March 2008. The mites and bees falling

onto the wax paper and in the dead bee traps (29 × 19 × 8 cm aluminum trays fitted on the hive entrance to collect additional bees that left the hive) were then counted, (screens were not used in conjunction with sampling boards, and boards were not removed daily to allow mites and bees to return to the cluster if they were still alive). Colonies were moved outdoors on 8 April 2008. Daily mite and bee mortality rates were calculated using the formula.

$$\text{Daily mortality rate} = \left[1 - \sqrt[t]{\left(1 - \frac{Dead}{Initial\ N}\right)}\right]$$

Where *initial N* is number at the beginning of the sample period, *dead* is the number that die during the sample period, and *t* is the number of days in the sample period. Total numbers of bees and mites in the hive on 7 January 2008 were estimated by summing mites and bees that fell during winter with those remaining in the hive in spring, so that mite and bee mortality could be determined. Colonies with live bees typically remove all dead bees thus, no attempt was made to sample live mites on dead bees because they were rare or absent. Colonies were also assessed for each of the variables (mean abundance of *V. destructor*, presence of *A. woodi* and virus concentration for DWV, BQCV, and IAPV) after removal from winter storage in spring of 2008.

Grooming behaviour—Bioassay cage study

Grooming behaviour (Currie and Tahmasbi 2008) of bees from surviving colonies of GDCs and GSCs was assessed in the summer of 2008. OMCs were not included in this experiment. Mixed-age worker honey bees were collected from the brood area of surviving colonies and placed in 20.5 cm × 8.0 cm × 11.2 cm wooden bioassay cages.

Groups of 150 mixed-age workers were established from the surviving colonies (GDC n = 16 colonies; GSC n = 6) which at the time of collection had low parasite abundance (mite infestation was estimated by alcohol wash technique); the bees were anesthetized with CO_2, counted, and placed in a cage as described in Underwood and Currie (2003). Two cages were established from each parent colony (44 cages in total). In the cage, the bees were confined in a 15.8 cm × 5.0 cm × 8.0 cm space containing a piece of plastic honey comb. The front of the cage was covered with a fine with 8-mesh screen (3 squares / cm) and the removable floor was made of 8 mesh metal screen with 8 squares / cm. Below the floor was a drawer that could be removed to count fallen mites and bees without allowing live bees to escape (Figure 4.1). The cage top had two 3.5 cm diameter holes into which 50 mL centrifuge feeder tubes were placed. The lid of each centrifuge tube had a 2 cm diameter hole cut into it that was covered with a fine mesh (24.6 squares / cm). One feeder tube contained 67% sugar solution in distilled water the other contained distilled water alone. Each tube was refilled when necessary. Sugar syrup and water were fed *ad libitum*.

The bees in each cage were inoculated with 30 live *Varroa* that were obtained from highly infested colonies by a modification of the CO_2 method (Ariana et al. 2002) as described in Currie and Tahmasbi (2008). In this method, infested bees with *Varroa* were anesthetised with CO_2 until all of the bees were knocked down; they were then placed in a 25 cm × 17 cm × 5 cm tray made of 8 mesh (3 mm square/cm) hardware cloth that was enclosed in a sealed 3.8 L TakeAlongs™ (Rubbermaid®, Mississauga, Ontario, Canada) container lined with damp paper towel. Bees were then agitated at 200 rpm for 10 min on a Labline® (Fisher, Ottawa, Ontario, Canada) orbital shaker table while CO_2 was released into the container at 5 L / min. Mites falling through the screen onto the paper towel

were collected with the tip of a soft paint brush, placed in Petri dishes lined with moist towel, and covered with lids until they could be introduced into the bioassay cages (Currie and Tahmasbi 2008). The cages were then randomly placed in two temperature-controlled incubators at 25 °C and 55–65% relative humidity for 5 d with no interior lighting. On each day, fallen mites were removed from the bottom of cages and counted and the mites were classified as live or dead. Daily mite and bee mortality rates were calculated using the formula as described above. The range of temperature and humidity within each incubator was monitored using HOBO C-8® (Onset Computer Crop., Bourne, Massachusetts, USA) data loggers.

RNA extraction

A subset of adult worker honey bees (n = 10) from each hive sample, were cooled in liquid nitrogen, and were crushed into a fine, homogenous powder by using a mortar and pestle. The total RNA was extracted from 30 mg of ground honey bee material using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) following manufacturer's instructions. An average of 2 μg of total RNA in 20 μL reaction volume was reverse-transcribed to produce cDNA using M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) according to the supplier's recommendations.

Virus analysis — PCR analysis for presence of viruses

Reaction conditions for the PCR assays were as follows: 2.5 μL of cDNA was mixed with 5 μL 10X *Taq* buffer, 1 μL of 10 mM dNTP, 1 μM of forward primer and 1 μM reverse primer (0.5 μM), and 0.25 μL of *Taq* polymerase. The thermal cycling profiles were set as follows: the mixture was heated for 5 min at 95 °C, 31 PCR cycles at 95 °C for 30 s,

55-60 °C for 30 s, 72 °C for 1min. The reactions were completed by a final elongation step 72 °C for 5 min. The PCR products were electrophoresed in a 1.2% agarose gel stained with GelRed™ Nucleic Acid Stain (Biotium Inc. Hayward, CA, USA).

qPCR analysis for viruses (DWV, BQCV, and IAPV)

The assays were performed with 1 µL cDNA template (5 fold dilution), 0.5 µL of forward and reverse primer mix (100 mM), 12.5 µL of 2X Quantifast SYBR green mix (Qiagen) and the volume adjusted to 25 µL with water. Non-template controls (water) were included in triplicate in all batches. Amplifications were performed in triplicate on an ABI Prism 7300 real time PCR machine (Applied Biosystems, Foster City, CA, USA) with a single cycle at 95 °C for 5 min, 40 PCR cycles at 95 °C for 15 s, 55 °C for 30 s, 72 °C for 30 s, followed by dissociation step to determine the specificity of amplification. The qPCR relative virus gene expressions were calculated using the $2^{-\Delta Ct}$ method, where Ct indicates the cycle threshold. As an internal control cDNA corresponding to honey bee β-actin was used. Relative expression was $2^{-\Delta Ct}$; where ΔCt = average $Ct_{(virus\ gene)}$ - average $Ct_{(\beta\text{-}actin)}$ (Persico et al. 2007). The primers for the qPCR are listed in Table 4.1.

DNA extraction for Nosema

Samples collected from each group in fall of 2007 were stored at -80 °C and samples of 10 bees from each hive were immersed in liquid nitrogen and crushed using mortar and pestle to get a homogenous sample. DNA was extracted from pooled samples of bees from each hive using a total DNeasy® Blood and Tissue Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol.

PCR analysis for Nosema

DNA extracted from each sample was amplified separately with three sets of *Nosema* primers. Amplification was done using HotStar *Taq* polymerase (Qiagen Inc., Valencia, CA, USA). All PCRs were done using a DNAEngine® Peltier thermal cycler (Bio-Rad, Hercules, CA, USA) in 25 µL total volume, containing a reaction mixture of 5 µL total DNA, 5 µL PCR buffer, 0.5 µL dNTP, 2.5 mM $MgSO_4$, 2.5 µL of each sense and antisense primer, and 0.25 µL HotStar *Taq* polymerase. The thermal cycling profile was as follows: initial denaturation of 5 min for 95 °C, 35 cycles of 1 min at 95 °C, annealing for 1 min at 55 °C, and extension for 1 min at 68 °C. The reactions were terminated with a final extension step at 72 °C for 5 min. The PCR amplification products were electrophoresed on 1.2% agarose (Invitrogen, Carlsbad, CA, USA) gel and stained with GelRed™ Nucleic Acid Stain (Biotium Inc. Hayward, CA, USA). PCR fragments were purified using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. The gel-extracted and purified DNA fragments were ligated into pGEM-T Easy plasmid vectors (Promega, Madison, WI, USA). The ligated vector was transferred into *E.coli* DH5α competent cells (Promega, Madison, WI, USA), according to the manufacturers protocol. The *Nosema* primers for the PCR are listed in Table 4.1.

Statistical analyses

Repeated measures analysis of variance (PROC MIXED) was used to examine, bee cluster scores, total number of mites in the colony, and DWV, BQCV and IAPV concentration with season as repeated measure (SAS 1999). Data for fall mean abundance of *Varroa* was analyzed by ANOVA. Mean mite abundance data were

arcsine-transformed and bee population score data were square-root transformed prior to analyses (Snedecor and Cochran 1980). Where significant interactions were observed between treatment and season, differences between means were compared by Bonferroni-corrected contrasts. Average daily *Varroa* and bee mortality rates in wintered colonies were square-root transformed and analyzed by ANOVA using colonies as subjects with PROC MIXED (SAS 1999). Daily *Varroa* and mite mortality in the cage study were analyzed as a repeated measures design with colony as the experimental subject and replicate cages on each hive as a repeated measure. The DDFM Satterthwaite approximation was used, as data were not homogeneous. For mixed model analyses, a compound symmetry covariance structure was used. Virus prevalence (the proportion of colonies with detectable levels of each virus) data were compared for DWV, BQCV, IAPV, KBV, SBV, ABPV, and CBPV with Fisher`s exact test using PROC FREQ (SAS 1999). Virus data are presented as mean log $2^{-\Delta Ct}$ +1± standard error. Data for prevalence of *N. ceranae* and *N. apis* (proportion of colonies with detectable levels as determined by PCR) were analyzed by Fisher`s exact test, using PROC FREQ (SAS 1999).

RESULTS

Effect of genetic diversity on bee cluster size and Varroa levels of colonies established in summer

For bee population score, the interaction between queen treatment and season was not significant (df = 2, 40; F = 1.88, P = 0.16) but bee population averaged over both seasons was affected by queen treatment (df = 2, 40; F = 6.65, p < 0.003) and season (df = 1, 40; F = 76.9, P < 0.0001). Bee populations in colonies with open mated queens were higher than in GSCs and GDCs (Bonferroni P < 0.05) and GSCs and GDCs did not differ

from each other (Bonferroni $P > 0.05$) (Fig. 4.2 A). Over all treatments, bee populations were smaller in spring than in fall (Bonferroni $P < 0.05$).

For total mites in the hive, there was a significant interaction between queen type and season (df = 2, 40; $F = 4.66$, $P < 0.02$) (Fig.4.2, B). In late fall, GDCs had similar numbers of mites to colonies headed by open-mated unselected-queens and had more mites than GSCs (Bonferroni $P > 0.05$) (Fig. 4.2, B). The total number of mites in the hive in GDCs decreased over the winter (Bonferroni $P < 0.05$) (Fig. 4.2, B). In contrast, in GSCs reductions in total numbers of mites were not significant and in OMCs total numbers of mites tended to increase (Bonferroni $P > 0.05$) (Fig. 4.2, B). Mean abundance of *Varroa* in fall was similar in all three treatments (Bonferroni $P > 0.05$) (Fig. 4.3).

Winter daily Varroa mite and bee mortality rates in full size colonies

The daily mite mortality rate in GDCs was higher than in GSCs ($F = 12.24$; df = 1, 33; $p < 0.001$) (Fig. 4.4). OMCs were not assessed for this variable. The daily bee mortality rate was similar in bees from GDCs and GSCs ($F = 2.03$; df = 1, 33; $p < 0.16$) (Fig. 4.5).

Varroa mite and bee mortality rates -Bioassay cage study

Mean daily mite mortality rate was significantly different in bees from GDCs and GSCs ($F = 6.82$, df = 1, 20; $p = 0.02$) (Fig. 4.6A). However, mean daily bee mortality in GDCs did not differ from GSCs ($F = 0.74$, df = 1, 20; $p = 0.40$) (Fig. 4.6 B).

Effect of genetic diversity on viruses: prevalence of viruses in colonies

The prevalence of DWV, BQCV, IAPV, KBV, and CBPV was similar in GDCs, GSCs, and OMCs (Fig.4.7). DWV (98%) and BQCV (93%) were the most common viruses and had similar prevalence in colonies sampled from GDCs, GSCs, and OMCs groups ($n = 43$) (Fig. 4.7). IAPV prevalence (mean = 49%), KBV prevalence (mean = 19%) and CBPV prevalence (mean = 4%) were also similar in groups of bees from different queen types. Virus prevalence differed with queen type for SBV and ABPV. For SBV, prevalence was similar in GDCs and GSCs but both of these were lower than in OMCs. ABPV was not detected in GDCs or OMCs but was found more often in GSCs although only at low frequency (Fig. 4.7).

Effect of genetic diversity on relative virus levels

There were significant genotype*season interactions for concentrations of DWV ($F = 3.38$, $df = 2, 40$; $p = 0.04$) and BQCV ($F = 4.22$, $df = 2, 40$; $p = 0.02$) but not for IAPV ($F = 0.07$, $df = 2, 40$; $p = 0.94$). Differences in the concentration of virus in colonies with different levels of genetic diversity occurred within season only with DWV. In fall, bees that originated from GDCs had lower DWV levels relative to bees that originated from GSCs and OMCs (Fig. 4.8). In spring, DWV concentration in GDCs was also much lower than in OMCs in spring (Fig. 4.8). GSCs had lower DWV virus levels than OMCs in spring but not in fall (Fig. 4.8). BQCV and IAPV were at similar levels in all three treatment groups in both fall and spring.

Effect of genetic diversity on Nosema prevalence

Nosema apis and *N. ceranae* were present singly or as co-infections in both GDCs and GSCs but *N. ceranae* was slightly more common (Fig. 4.9). *Nosema apis* prevalence was similar in GDCs and GSCs (χ^2 = 0 .58, p > 0.445); however, *N. ceranae* prevalence differed significantly between GSCs and GDCs (χ^2 = 4.71, p < 0.03) (Fig. 4.9). Only two colonies had undetectable levels of *Nosema* spp., and both of these were GDCs.

DISCUSSION

The results of this study support the hypothesis that increased genetic diversity among colony members that results from multiple mating by the colony's queen, increases the colonies resistance to pathogens and parasites. Sister queens inseminated with multiple drones (GDCs) were compared to those inseminated with single drones (GSCs) and with unrelated queens that were open-mated (OMCs). Colony population sizes were not affected by queen treatment between GDCs and GSCs. Although GSCs produced fewer *Varroa* per colony than GDCs, the mean abundance on adult bees was similar and GDCs had higher mite mortality rates than GSCs over winter. Some pathogens occurred at lower prevalence (ABPV, *N. ceranae*) or concentrations (DWV) in GDCs than in GSCs in fall, none of the other pathogens occurred at higher frequencies or concentrations in GDCs than in GSCs within the instrumentally inseminated treatments. By spring, both GSCs and GDCs had lower relative DWV concentrations than OMCs but did not differ from each other.

Several studies on a variety of social insects have shown genetically diverse worker populations may have an advantage over genetically similar with regard to reducing the prevalence or impact of parasites and pathogens on colony members and

colony fitness (Hamilton 1987; Sherman et al. 1988; Schmid-Hempel and Loosli 1998; Baer and Schmid-Hempel 1999, 2001; Tarpy 2003; Tarpy and Seeley 2006; Seeley and Tarpy 2007). Mating with multiple males produces increased diversity in the colony population, which benefits colony phenotype associated with defensive factors such as grooming behaviour, hygienic behaviour and other mechanisms that reduce transmission or reduce parasites and pathogens (deGuzman et al. 1996; Spivak and Downey 1998; Seeley and Tarpy 2007). These studies used field and cross-infection experiments, and in some cases, the pathogen of interest was manipulated by artificially infecting the colonies. I did not see significant differences in bee population scores between GDCs AND GSCs in the different queen treatments. Mattila and Seeley (2007) showed genetically diverse colonies built comb faster, grew larger, and produced more honey than genetically similar colonies, although their bees were not significantly exposed to pathogens. My colonies may not have had enough time to maximize population build up before brood rearing shut down in the winter. However, the power in my experiment was large enough to detect differences between OMCs and the other two queen types. Differences in colony growth rates can have consequences for the colony's ability to withstand parasitism and disease. Large colonies have greater brood production than small colonies, which favours *Varroa* reproduction (Wilkinson and Smith 2002). My study showed total numbers of mites in the hive were greater in GDC and OMC treatments than in GSC treatments in late fall. However, this increased level of mites in the colony may not have significantly affected parasite "pressure" at the individual bee level. Large colonies may grow more mites than small colonies, but if colony growth rate is greater than or equal to mite growth rate the consequences for individual bees (feeding pressure by parasites) may be similar or lower. My study showed the mean abundance of

Varroa, a measure of the parasite load experienced by individual bees, was similar among colonies with different levels of genetic diversity.

High within-colony genetic diversity could provide better defence against parasite or pathogens through either social or innate immunity if it allowed colonies to maintain multiple cohorts of workers specializing in different defence tactics or with different immune responses to microbes. Differences in parasite, fungus, or virus prevalence and concentration in colonies with different levels of genetic diversity could occur through differences in the ability of different queen types to reduce transmission of these noxious species, reduce their reproduction within the colony, or by increased rates of removal from the colony. For example, bees with heavy virus infections could simply leave the colony or die and be removed by house-cleaning activities of undertaker bees. Transmission between colonies can be affected by a bee's associated parasites. *Varroa* can vector many of the viruses and assist in horizontal transmission but viruses can be spread to different colonies (Shen et al. 2005a), in the presence or absence of *Varroa*, by drifting bees, robbing, removal of dead and dying bees, and through cultural management by beekeepers moving diseased frames between colonies (the latter did not occur in this study). Possible defences by GDCs to reduce between-colony transmission of *Varroa* in natural environments would likely be related to colony defensive mechanisms that reduce robbing or result in better orientation ability (nest recognition) that would reduce drift. However, little is known about mechanisms genetically resistant bees can use to reduce virus spread between colonies, other than by reducing mite load or viral load on returning scout and foraging bees. The GDCs in my study had greater mite mortality over winter than GSCs and thus overall reductions in population levels of the parasite could

potentially spread fewer *Varroa* and associated pathogens between colonies. However, the spread of *Varroa* and viruses between colonies was not specifically studied.

Social immunity mechanisms include responses, such as hygienic brood removal behaviours (detection and removal of infected brood) (Wilson-Rich et al. 2009) and grooming of nest mates (Currie and Tahmasbi 2008). Differences in the relative viral prevalence and disease loads that occurred between the GDCs and GSCs may also have been caused indirectly as a result of social immunity related to seasonal differences in *Varroa* mortality in the GDCs and GSCs caused by differential grooming behaviours or some other combination of behaviours (e.g. hygienic behaviour, *Varroa* sensitive hygiene, etc.). Hygienic behaviour of worker bees is an important aspect of the honey bee's social immunity and it is effective in reducing the impact of American foulbrood, chalk brood, *Nosema*, and *Varroa* in colonies (Rothenbuhler 1964; Peng et al. 1987; Spivak and Reuter 1998). Although mean abundance of mites was similar in GDCs and GSCs, the impact of those mites in transmitting or activating viruses could be disrupted if they were affected by grooming behavior of bees that contributed to differences in mite mortality rates. Hygienic brood removal in my study was not examined because little or no brood was present in my colonies in late fall.

Varroa mortality rates were higher in GDCs than GSCs in the field study, supporting the theory that increased colony genetic diversity benefits defence against parasites. Mite mortality rates were measured rather than mite drop because differences in colony size would affect number of mites and bees falling from colonies. The specific mechanisms resulting in increased mite mortality were not observed, but enhanced grooming was likely involved since it was one of the criteria used in selection of this

stock (Currie and Tahmasbi 2008). Differences in mite mortality rates may also have been influenced by slight differences in bee mortality rates, if mites on dead bees were unable to return to the cluster. However, differences in bee mortality rates occurred only in wintered colonies and not in the caged bee study. The presence of *Varroa* triggers a series of behavioral responses in adult workers, including self-grooming, grooming dance calls, and nest-mate group cleanings that occur at higher frequencies in resistant strains (Peng et al. 1987; Currie and Tahmasbi 2008; Guzman-Novoa et al. 2012; Rinderer et al. 2012). The proportions of workers in a colony that are required to perform effective grooming are not known, but a small cohort in a GDC would likely be effective. In high-grooming colonies, small groups of specialist workers are better able to groom than other worker bees (Winston and Punnett 1982; Bahraini and Currie, personal communication; Frumhoff and Baker 1988). The proportion of grooming specialists in GDCs and GSCs was not assessed in this study. Differences in mite mortality rates between the instrumentally inseminated and OMCs were not assessed in my field experiment. However, the two inseminated treatment groups that were selected for resistance to *Varroa* both had fewer mites per colony than in OMCs (that were not selected for resistance to *Varroa*) by spring. This result suggests that, although high genetic diversity is important in helping colonies resist parasites, it is still possible to select for resistance against targeted groups of pathogens as long as relatively high diversity within and between colonies is maintained. However, there may be costs associated with grooming that are only partially mitigated by higher genetic diversity. Results of my field study showed that bee mortality was also slightly higher in GDCs than GSCs in field experiments, which may relate to the increased costs associated with grooming at low temperatures (Currie and Tahmasbi 2008).

The inseminated lines in this study were selected for resistance to *Varroa*, so it is possible that aspects of social immunity that are affecting *Varroa* also help in the suppression of certain viruses through direct or indirect means. *Varroa* is a vector and activator of DWV (Bowen-Walker et al. 1999), and differences in the levels of *Varroa* are known to influence concentrations of DWV in colonies (Dainat et al. 2012a). However, differences in *Varroa* were not likely responsible for all of the differences in virus concentration that were observed in colonies with different queen types. In this study, fall *Varroa* levels were lower in GSCs than in GDCs, but the mean abundance of mites was similar in GDCs and GSCs suggesting differences in *Varroa* abundance likely were not responsible for differences in virus levels. Total numbers of mites in OMCs were also greater than in GSCs in fall, and for viruses, both SBV prevalence and DWV concentration were greater in the OMCs than in the GDCs. BQCV and IAPV concentration and the prevalence of five other viruses, other than ABPV, did not vary between the inseminated queen treatments in my experiment, which suggests that immune responses are not equally effective against all viruses. It may also suggest that more opportunistic viruses only develop to high concentrations and cause impacts when the host's social or individual immune responses are compromised by other factors.

Viruses can transfer horizontally from drone to queen through the sperm during mating, and then vertically through the queen to her offspring. Thus, one might expect multiple mating could increase risks associated with virus transmission because queens could store virus-laden sperm in their spermatheca when multiple mating. However, this possibility is unlikely. Vertical transmission of DWV from infected queens or drones to their progeny results in less-virulent infections, often producing covert infections with no

obvious symptoms (Chen and Siede 2007; de Miranda and Fries 2008; de Miranda and Genersch 2010). The reasons are unclear; DWV can replicate within all developmental stages of bees (Yue and Genersch 2005; Boncristiani et al. 2009). There was no evidence of increased prevalence of any of the seven viruses, or increased concentrations of DWV, BQCV, and IAPV, in worker progeny from GDCs compared to worker progeny from GSCs. OMCs did have greater prevalence of SBV than GSCs in fall and greater concentrations of DWV in spring. However, OMCs also had greater prevalence of SBV and greater concentrations of DWV (in spring and fall) than the selected genetically diverse lines. This difference in virus was more likely related to levels of *Varroa*, or other differences in the capacity to defend against viruses that may have existed in the GDCs than in the OMCs, than a result of high levels of virus being introduced to queens through sperm. It is also possible, that immunity related factors providing resistance to the viruses are passed from drones to queens during mating to counteract negative effects of the multiple mating and reduce the viral levels.

Differences in the immune responses of individual bees within GDCs and GSCs could also contribute to the observed differences in virus prevalence and concentration. The immune system of insects is simpler than that of humans, although both have cellular and humoral defenses (Gillespie et al. 1997). Very little is known about the immune response of honey bees to viruses (Azzami et al. 2012). However, bees can maintain viral infections without showing obvious disease symptoms (Bailey et al. 1963), suggesting some kind of antiviral mechanisms prevent virus replication. Some viruses such as ABPV do not induce humoral or cellular responses and RNAi based mechanism may be more important for viral immunity (Azzami et al. 2012). Some studies show no relationship between hyperpolyandry and immune system function in colonies where

queens mate with very large numbers of drones (Wilson-Rich et al. 2012), but other studies have suggested increased within-colony genetic diversity could be beneficial in enhancing defences against pathogens (Lee et al. 2013).

Our data are consistent with the idea that differences in viral replication between colonies with different levels of genetic diversity are mediated by the insect's immune system. If a pathogen gains entry into the insect hemocoel, then it may be attacked by cellular and humoral immune mechanisms that suppress viral reproduction (Casteels 1997). Activating the host's immune system is costly, so it may be beneficial if only small numbers of certain subfamilies respond. Systems activated to attack a parasite or pathogen by specific subfamilies may improve pathogen loads at the colony level, leading to continued selection for multiple mating as a way to defend against multiple disease threats.

Studies on the effect of genetic diversity on gut pathogens have shown conflicting results. Recently, Mattila et al. (2012) characterized bacterial communities in bee guts and bee bread samples and found bees from GDCs had more diverse bacterial communities (1105 species), with a higher percentage of potentially beneficial species and a lower percentage of potentially pathogenic species, than bees from GSCs (781 species). Woyciechowski and Krol, (2001), studied the effect of colony genetic diversity on *N. apis* and did not find any difference between the GDCs and GSCs. Similarly, there were no significant differences in the prevalence of *N. apis* in this study. However, GSCs had higher prevalence (proportion of colonies infected) of *N. ceranae* GDCs. *N. apis* and *N. ceranae* were present alone or as a co-infection in both genetically diverse and genetically similar bees, and *N. ceranae* was the most common infection of the two species of *Nosema*. The primary focus of this study was to look at viruses and *Varroa*

grooming, so colonies were fed Fumagillin-B in early fall to suppress replication of *Nosema* during fall management. Therefore, spore abundance levels were low and not examined as the power of detection for *Nosema* prevalence was lower than by the more sensitive technique RT-PCR that I used to quantify prevalence. In addition, species of *Nosema* could not be easily identified from spores. Transmission of nosema disease occurs from contaminated food (nectar, pollen, and water), cleaning fecal matter from combs in infected colonies, robbing, or swarming (Fries 1988; Fries et al. 1996; Fries and Camazine 2001). Increased hygienic behaviour helps suppress spore transmission within colonies (Evans and Spivak 2010), and could explain why my GDCs had lower *N. cerana* prevalence than GSCs; however, these behaviours were not assessed. Further studies are required to examine effects genetic diversity involving *Nosema* spp.

In conclusion, this study has demonstrated that genetic similarity within honey bee colonies is associated with lower colony populations in fall, and higher prevalence or concentrations of some pathogens and parasites when compared to colonies with high levels of colony genetic diversity. This result supports theories that suggest polyandry may have evolved or have been favoured because of more effective suppression of pathogens and parasites. My results suggest that selection for resistance against *Varroa* and viruses is possible, but high levels of within-colony genetic diversity need to be maintained to help colonies deal with multiple threats. Future research is needed to study how genetic diversity directly affects immunocompetence, transmission, and suppression of parasites and pathogen levels.

Table 4.1. Primers used in the study for viruses (PCR and qPCR) and *Nosema* (PCR)

PCR primers for virus

Virus specific primer set (5'-3')	Product size (bp)	Reference
ABPV-F: TTATGTGTCCAGAGACTGTATCCA ABPV-R: GCTCCTATTGCTCGGTTTTTCGGT	900	(Benjeddou et al. 2001)
CBPV-F: AGTTGTCATGGTTAACAGGATACGAG CBPV-R: TCTAATCTTAGCACGAAAGCCGAG	455	(Ribiere et al. 2002)
BQCV-F: TGGTCAGCTCCCACTACCTTAAAC BQCV-R: GCAACAAGAAGAAACGTAAACCAC	700	(Benjeddou et al. 2001)
DWV-F: ATCAGCGCTTAGTGGAGGAA DWV-R: TCGACAATTTTCGGACATCA	702	(Chen et al. 2004b)
KBV-F: GATGAACGTCGACCTATTGA KBV-R: TGTGGGTTGGCTATGAGTCA	415	(Stoltz et al. 1995)
SBV-F: GCTGAGGTAGGATCTTTGCGT SBV-R: TCATCATCTTCACCATCCGA	824	(Chen et al. 2004b)
IAPV-F: CCACCCCTCTCAAACAATCTCAAACA IAPV-R: AGATTTGTCTGTCTCCCAGTGCACAT	358	(Maori et al. 2007)
β-actin-F: AGGAATGGAAGCTTGCGGTA β-actin-R: AATTTTCATGGTGGATGGTGC	181	(Chen et al. 2004b)

qPCR primers for virus

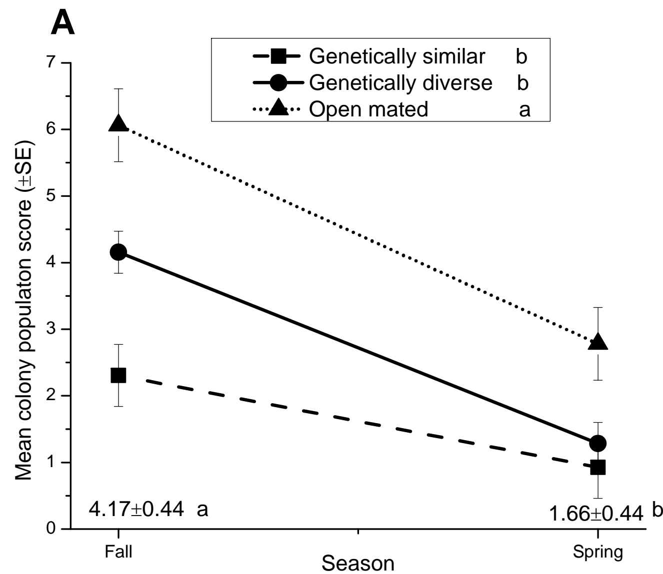
Virus specific primer set (5'-3')	Product size (bp)	Reference
RT-BQCV-F: CCTGTATTCATGCATCTCAGA RT-BQCV-R: GCAACAAGAAGAAACGTAAACCAC	309	Judy Chen (personal communication)
RT-DWV-F: CGAAACCAACTTCTGAGGAA RT-DWV-R: GTGTTGATCCCTGAGGCTTA	174	Judy Chen (personal communication)
RT-IAPV-F: GCGGAGAATATAAGGCTCAG RT-IAPV-R: CTTGCAAGATAAGAAAGGGGG	586	Judy Chen (personal communication)
β-actin-F: AGGAATGGAAGCTTGCGGTA β-actin-R: AATTTTCATGGTGGATGGTGC	181	(Chen et al. 2004b)

PCR primers for *Nosema*

Nosema **specific primer set (5'-3')**	Product size (bp)	Reference
Nosema F: GGCAGTTATGGGAAGTAACA *Nosema* R: GGTCGTCACATTTCATCTCT	208	(Chen et al. 2008)
N. Ceranae F: CGGATAAAAGAGTCCGTTACC *N. Ceranae* R: TGAGCAGGGTTCTAGGGAT	401	(Chen et al. 2008)
N. Apis F: CCATTGCCGGATAAGAGAGT *N. Apis* R: CACGCATTGCTGCATCATTGAC	250	(Chen et al. 2008)

Figure 4.1. Wooden bioassay cages used for the grooming experiments. Below each cage is a screened floor with a drawer that allows fallen mites to be removed from the cage.

Figure 4.2. Changes in the bee population score over winter (A) and changes in the total *Varroa* population in the hive over winter (B). Honey bee colonies were headed by queens that were genetically diverse (n = 24 colonies), genetically similar (n = 11 colonies) or had open mated queens (n = 8 colonies). (A) For bee population score, the decline in population was similar in all three queen treatments, but population was affected by queen treatment and season. (B) There was a significant queen treatment*season interaction for total numbers of mites in the colony (see text). Means among queen types and seasons (bottom of graph A) or as indicated on legend of (A) by the same letter are not significantly different; asterisk indicates difference between seasons within queen type (SAS PROC MIXED; Bonferroni P > 0.05).



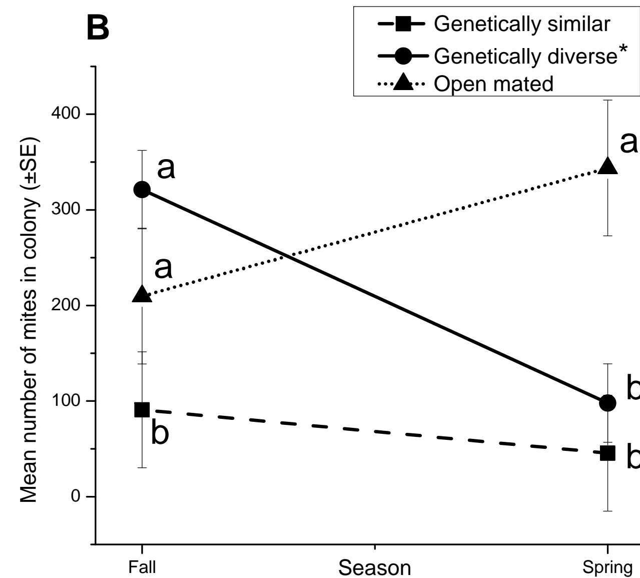


Figure 4.3. Mean abundance of *Varroa* mites in honey bee colonies collected from genetically diverse (n = 24), genetically similar (n = 11), and open-mated (n = 8) hives in fall 2007. Mean abundance of *Varroa* was similar in all three queen treatments (F = 2.19, df = 2, 40 p > 0.125). Treatments with the same letter above bars are not significantly different (SAS PROC MIXED; Bonferroni corrected, P > 0.05).

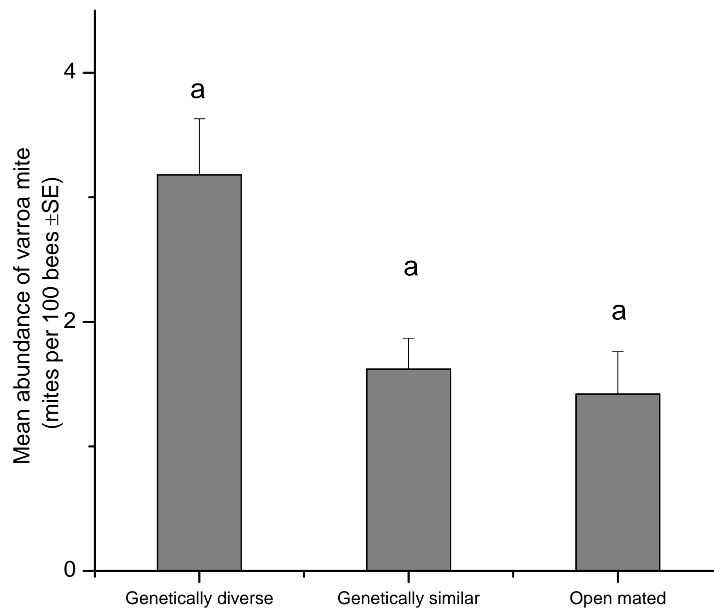


Figure 4.4. Mean daily mite mortality rate (mites/mite/day) (±standard error) over winter (January – March 2008) in GDCs (n = 24) and GSCs (n = 11) differed significantly ($F = 12.24$; $df = 1, 33$; $p < 0.0001$). Treatments with the same letter above bars are not significantly different (SAS PROC MIXED;).

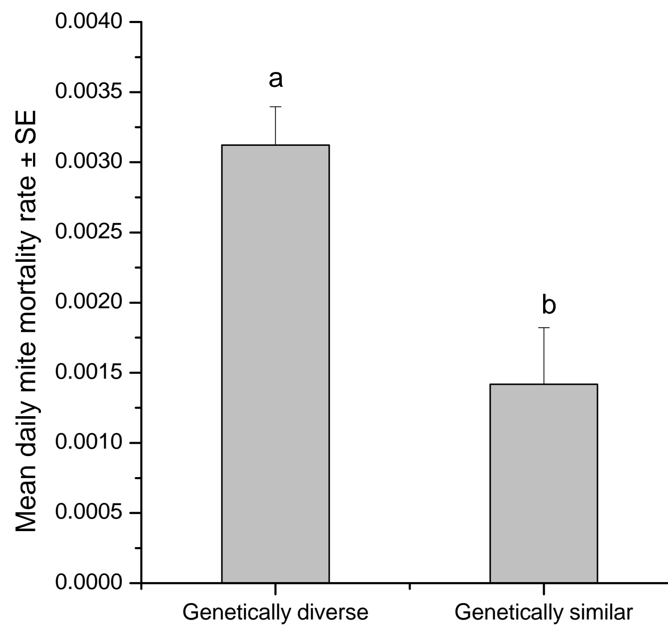


Figure 4.5. Mean daily bee mortality rate (bees/bee/day) (±standard error) over winter (January – March 2008) in GDCs (n = 24 colonies) and GSCs (n = 11 colonies) were similar ($F = 2.03$; $df = 1, 33$; $p < 0.16$). Treatments with the same letter above bars are not significantly different (SAS PROC MIXED;).

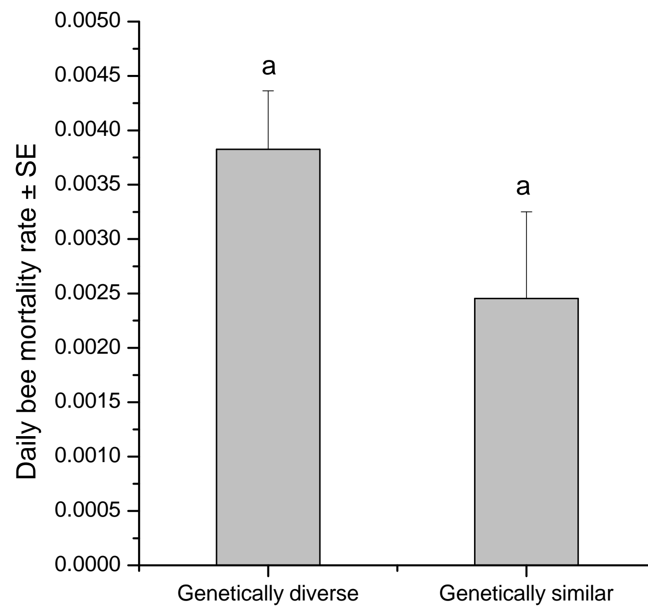


Figure 4.6. Bee and mite mortality rate as assessed in caged bees in the laboratory. The mite mortality rate (mites/mite/day) between GDCs (n = 24 colonies) and GSCs (n = 12 colonies) were significantly different (A), however, mean daily bee mortality rate (bees/bee/day) between queen types was similar (B). Treatments with the same letter above bars (within A and B) are not significantly different (SAS PROC MIXED;).

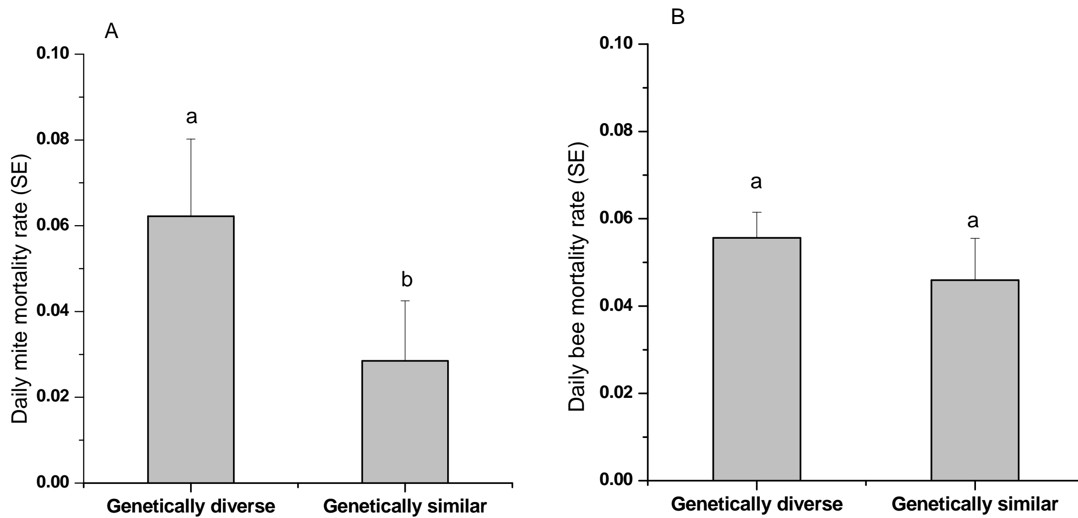


Figure 4.7. Proportion of honey bee colonies (GDC, GSC, OMC) with detectable level of viruses in fall 2007. Differences in frequency of occurrence between queen types within each virus are shown in legend (χ^2 values and associated p-value for each virus; where significant differences occurred they are marked with an asterisk). Viruses with the same letter above bars within a virus are not significantly different. §= see list of abbreviations for full names of parasites and pathogens (page ix).

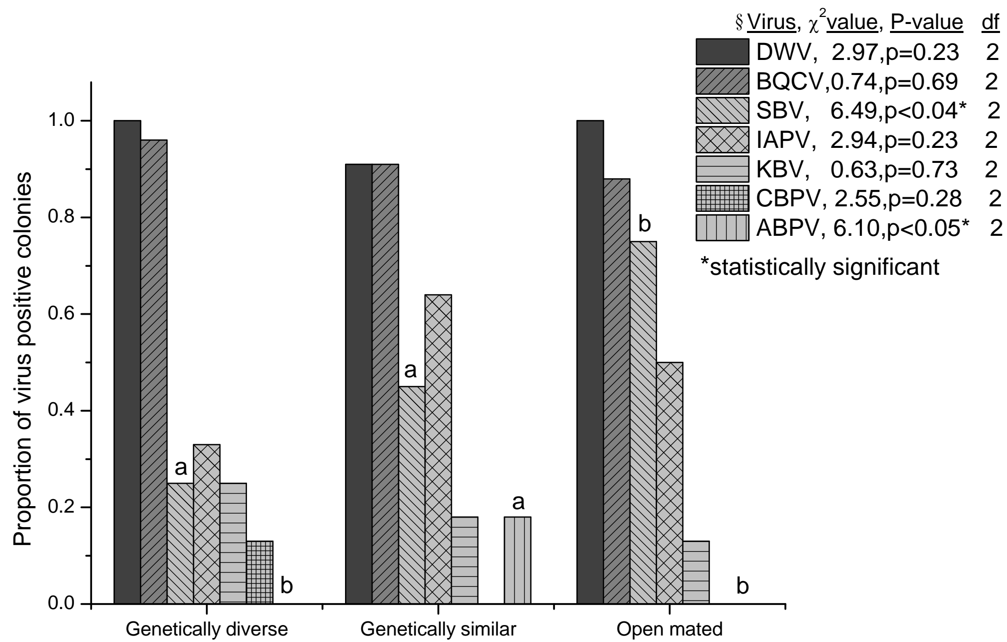


Figure 4.8. Relative change in mean (±SE) concentration of DWV, BQCV, and IAPV among samples collected from GDCs (n = 24), GSCs (n = 11) and OMCs (n = 8) in (A) fall 2007 and (B) spring 2008. Treatments with the same letter above bars within virus and season are not significantly different (PROC MIXED; Bonferroni adjusted, P > 0.05).

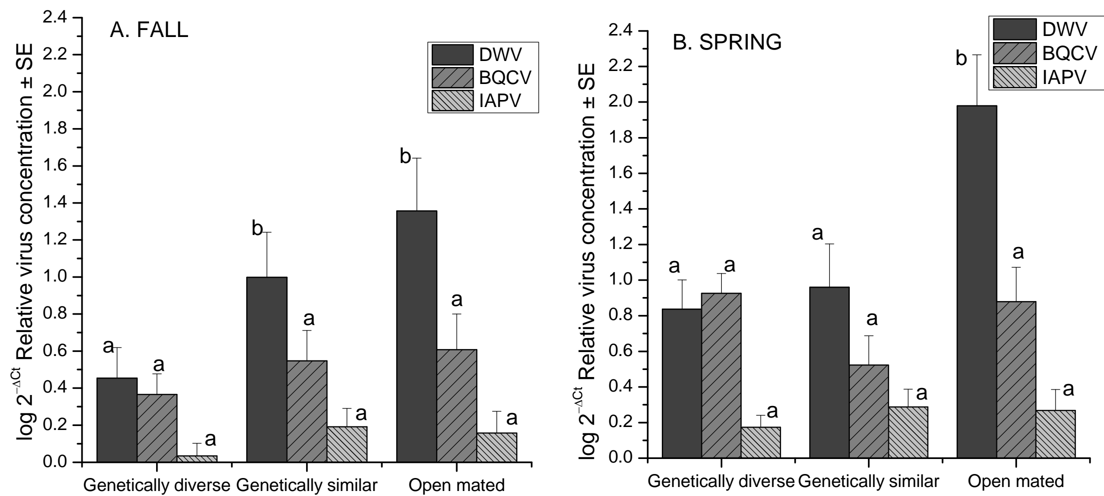
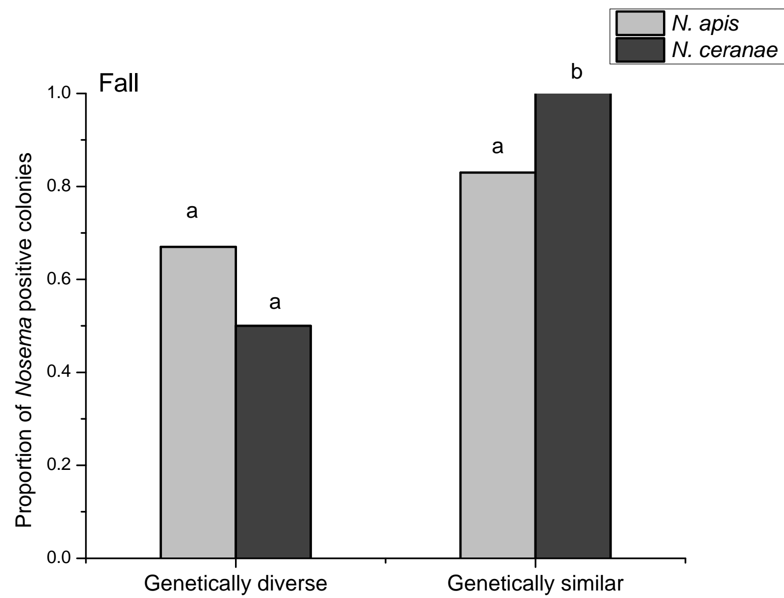


Figure 4.9. Prevalence of *Nosema* infection in GDCs (n = 15) and GSCs (n = 8) in fall 2007. The prevalence of *N. apis* was similar in GDCs and GSCs. The prevalence of *N. ceranae* was significantly different between GSC (100%) and GDC (50%). Treatments with the same letter above bars within *Nosema* species are not significantly different (P > 0.05).



CHAPTER 5. REDUCTION IN DEFORMED WING VIRUS INFECTION IN LARVAL AND ADULT HONEY BEES (*APIS MELLIFERA* L.) BY DOUBLE-STRANDED RNA INGESTION*

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Desai SD, Eu YJ, Whyard S, Currie RW. 2012. Reduction in deformed wing virus infection in larval and adult honey bees (*Apis mellifera* L.) by double-stranded RNA ingestion. Insect Molecular Biology 21(4):446-455.

ABSTRACT

Deformed wing virus (DWV) is a serious pathogen of honey bees, *Apis mellifera* L., that is vectored by a parasitic mite *Varroa destructor* Anderson and Truman. The virus is associated with wing deformity in unhealthy bees and premature death and reduced colony performance in asymptomatic bees. Here, I report on suppression of DWV infection by feeding both first instar larvae and adult *A. mellifera* with a double-stranded RNA construct (DWV-dsRNA) specific to DWV in DWV-inoculated bees. I fed DWV-dsRNA by mixing it into larval food or to sugar syrup fed to adult bees. Larvae were observed larvae for 21 days and adults for eight days to assess the effects of DWV-dsRNA treatment on mortality, wing deformity and virus concentration in larvae and adult bees. Bees fed DWV and DWV–dsRNA were compared with bees fed DWV only, bees fed DWV with a “negative control” green fluorescent protein (GFP)-dsRNA, and bees not fed DWV or any dsRNA. For the larval feeding experiment, an additional control with bees fed DWV-dsRNA with no virus was included. I showed that feeding DWV to larvae causes’ wing deformity in adult bees in the absence of mites and decreases survival rates of adult bees relative to bees not fed DWV. Feeding larvae with

DWV-dsRNA in advance of and following inoculation with virus reduced DWV viral level and reduced the frequency of wing deformity of emerging adults relative to larvae fed DWV or DWV with GFP-dsRNA, but did not affect survival to the adult stage. This was likely a result of RNA silencing. Feeding DWV-dsRNA did not affect survival to the adult stage, which suggests that dsRNA is non-toxic to larvae. Feeding adult workers with DWV-dsRNA in advance of inoculation with virus increased their longevity and reduced DWV concentration relative to controls treated with DWV+ GFP dsRNA or DWV only.

INTRODUCTION

The honey bee, *Apis mellifera* L. (Hymenoptera: Apidae), is the world's most economically important insect pollinator, with over one-third of the human diet estimated to depend on honey bee pollination (McGregor 1976; Delaplane and Mayer 2000) with a market value that exceeds US$212 billion on a global basis (Gallai et al. 2009). Honey bees also play a vital role in the global economy by producing honey, pollen, beeswax and other hive products. Honey bees are under attack by a variety of parasites, pathogens and pests, including mites, bacteria, microsporidia and viruses, which alone or in combination with other stresses contribute to significant colony losses and consequent reductions in crop pollination (vanEngelsdorp et al. 2009). Large-scale colony losses and disease outbreaks of honey bee colonies have occurred sporadically in the past (Underwood and vanEngelsdorp 2007; vanEngelsdorp et al. 2007) but in 2006, the term Colony Collapse Disorder (CCD) was used to describe the strange phenomenon of huge honey bee colony losses reported throughout most of the United States. Catastrophic winter losses of colonies were subsequently observed in Canada, the United Kingdom and other parts of the world (Currie et al. 2010; Genersch et al. 2010; Neumann and Carreck 2010). A variety of causes have been suggested, including pathogens, parasites, stress and pesticide contamination (Cox-Foster et al. 2007; Genersch 2010; vanEngelsdorp et al. 2009), but to date, no single factor can be linked to all the colony losses across the world.

Viruses are one of the major threats to honey bee health, and the interactions of viruses with *Varroa* mites (*Varroa destructor* Anderson and Truman) (Acari: Varroidae) have caused serious problems in the beekeeping industry (Ball and Bailey 1997). Of the many viruses that affect bees, deformed wing virus (DWV) is one of the most common

and is associated with severe winter loss of bees and visible deformities in infected bees (Ball and Allen 1988; Highfield et al. 2009; Martin 2001). DWV was first isolated from deformed adult bees in Japan (Bailey and Ball 1991) but now it has a worldwide distribution (Allen and Ball 1996; Ellis and Munn 2005). DWV is not restricted to *A. mellifera*; it also occurs in the Asian honey bee *Apis cerana* F. the dwarf bee *Apis florea* F. (Allen and Ball 1996; Ellis and Munn 2005), bumble bees (Genersch et al. 2006) and the giant honey bee, *Apis dorsata* F. in southern India (Desai and Currie, unpublished data). DWV can be transmitted vertically and horizontally and persists in all stages of bee development, including the eggs, larvae, pupae and adults (Bailey and Ball 1991; Chen et al. 2006a). DWV multiplies slowly during the immature stages of honey bees, and although it rarely kills the pupa, it reduces the life span of the subsequent adult stage.

Severe symptoms, resulting in developmental abnormalities including deformed wings in adults, are associated with simultaneous DWV and *V. destructor* infestations (Ball and Bailey 1997). When DWV-infected *V. destructor* mites feed on bees, the virus typically causes symptoms such as shrunken or crumpled wings, reduced body size, and discoloration in adult bees (Bailey and Ball 1991; Ball and Bailey 1997). Typically, virus levels are much higher in unhealthy adults, with titers 3.7 times higher than those observed in larvae (Chen et al. 2005). To date, DWV-induced deformities have not been observed without either an associated with *V. destructor* feeding, or inoculation through other means (de Miranda and Genersch 2010). Mockel et al. (2011) produced wing deformities in adult bees by artificially injecting DWV into the pupae under lab conditions in the absence of mites, which suggests that very high titers ($> 1 \times 10^7$ copies) of DWV or the replication of DWV inside the *Varroa* are necessary for the development of deformed wings.

DWV is a small (30 nm) virus, with a single stranded RNA genome that encodes three major structural proteins, and belongs to the recently established genus *Inflavirus* of the picorna-like family Iflaviridae (Lanzi et al. 2006). The genome organization of DWV consists of a single open reading frame (ORF) flanked by a long untranslated region (5' UTR) and a highly conserved 3' UTR. Both the 5' and 3' UTR have important functions in regulating the replication and translation of the genome (Belsham 2009; Nakashima and Uchiumi 2009; Roberts and Groppelli 2009). The functional domains of DWV include a helicase, a highly conserved RNA-dependent RNA polymerase (RdRp), two capsid protein domains and a 3C-protease (Fujiyuki et al. 2004; Ongus et al. 2004; Lanzi et al. 2006).

Like picornaviruses, DWV replicates its single-stranded RNA within the cytoplasm of its host's cells by producing a double-stranded RNA intermediate (Lescar and Canard 2009). This characteristic of DWV makes it amenable to control through RNA interference (RNAi), also known as RNA silencing or post-transcriptional gene silencing. RNAi is a comparatively simple, rapid and specific method of silencing gene function by causing degradation of the target mRNA. RNAi was first discovered in the nematode *Caenorhabditis elegans* (Maupas) by Fire et al. (1998), who showed that introduction of double-stranded RNA (dsRNA) homologous to endogenous sequences rapidly leads to destruction of target mRNAs (Fire et al. 1998). RNAi has revolutionized genomics and drug discovery because of its efficiency and high specificity. Experimentally delivered or naturally present dsRNA is typically cleaved into small fragments of 21-23 base pair molecules called "small interfering RNAs" (siRNAs) by the intracellular endonuclease enzyme, Dicer (Ketting et al. 2001). The siRNAs are incorporated into the RNA-induced silencing complex (RISC), which uses the siRNA

antisense strand to find and promote the specific degradation of complementary target mRNAs (Martinez et al. 2002). RNA interference has been used to suppress viruses in a number of species, including humans, plants, animals and insects (Dzitoyeva et al. 2001; Huang et al. 2006; Van Rij and Andino 2006; Mao et al. 2007; Wolters and MacKeigan 2008) and recently in bees to suppress viruses and microsporidia (Maori et al. 2009; Liu et al. 2010; Paldi et al. 2010). RNA silencing of Israeli acute paralysis virus (IAPV) in honey bees was achieved in the lab. Feeding virus-specific dsRNA to bees, results in dramatically improved performance-related variables such as bee-to-brood ratio and honey yield (Maori et al. 2009). Hunter et al. (2010) conducted a large-scale field study to control IAPV in honey bee colonies using dsRNA specific to IAPV, and observed higher bee survival rates, larger colony sizes and higher honey production than in untreated colonies. RNAi has also been used in bees to disable the honey bee microsporidium *Nosema ceranae* F. (Paldi et al. 2010).

In this study, I assessed whether RNAi could be used to suppress or reduce DWV levels in honey bees. An ability to reduce virus-related stress could increase colony survival and possibly allow bees to tolerate higher levels of *Varroa* by reducing overall stress. I assessed whether feeding virus-challenged honey bee larvae and adults a form of dsRNA against DWV can reduce the lethal or sublethal effects of DWV infections and evaluated the practicality of using dsRNA to protect honey bees from DWV.

MATERIALS AND METHODS

Honey bee larvae and In vitro larval rearing

Larval rearing techniques described below were used to produce larvae with high levels of DWV to use in inoculation, and for experiments to expose bees and larvae to RNAi treatments. Worker honey bee larvae were collected from a single colony. The honey bee queen was confined with workers from her colony for 24 h on a single honey bee comb using a modified queen excluder cage (46 × 24 × 6 cm) and allowed to lay eggs. After three days, freshly hatched larvae, less than 24 h old, were grafted onto larval food and individually reared (1 larva per cell) in 24-well cell culture polystyrene plates (Costar®3524, USA) as described by Patel et al. (2007). Deformed wing virus and RNAi treatments were applied by pipetting them into the artificial diet (Peng et al. 1992) prior to feeding it to the larvae (as described below). The larvae in each treatment were monitored to assess morphological development and survival over a period of 21 days or until the adult's eclosed from pupa. Larvae were transferred to fresh food in a fresh plate on a daily basis, examined under the microscope, and scored as dead or alive based upon movement and colour change in the larvae. Larvae with pale yellow or dark color and no movement of spiracular tracheal openings were scored as dead, then collected and stored in -80 °C. Adults that emerged from pupae were scored, classified into deformed and normal winged groups, and stored at -80 °C for further analysis. All efforts were made to minimise the disturbance and damage to the larvae during *in vitro* larval rearing.

Preparation of DWV

Virus extraction was done according to Bailey and Ball (1991). Briefly, the infected larvae were ground in 5 ml phosphate buffered saline (PBS, pH 7) with a mortar and pestle on ice. The solution was centrifuged at 10,000 rpm for 10 min, the supernatant was collected and was then ultra-centrifuged at 40,000 rpm for 30 min at 4 °C. The concentration of the virus in the supernatant was determined using a qPCR comparative Ct (cycle threshold) (Δ Ct) method (Chen et al. 2005) to ensure that the insects were treated with the same amount of virus in each replicate experiment. Serial dilutions of the DWV lysate were prepared using PBS, and were quantitatively compared with a known quantity of plasmid DNA containing the DWV RdRp gene (see below), to estimate the genome equivalents in the DWV lysate. Quantitative PCR reactions were performed in triplicate on a ABI Prism 7300 machine (Applied Biosystems) using a QuantiFast SYBR Green PCR kit (Qiagen). A control without template was included in all batches. The PCR programme profile began with a single cycle heated for 5 min at 95 °C, 40 PCR cycles at 95 °C for 15 s, 55 °C for 30 s, 72 °C for 30 s. To determine and measure the dissociation curve the PCR products were heated to 95 °C. The RdRp DNA fragment was amplified using the primers dwv-qF 5' CCTGGACAAGGTCTCGGTAGAA, and dwv-qR 5' ATTCAGGACCCCACCCAAAT and the PCR amplification results were normalized to a housekeeping gene product, *A. mellifera* actin (DNA fragment), using primers listed in Table 5.1. Serial dilutions of DWV lysate in PBS were prepared and orally fed to the bee larvae (described above) and to the adult bees in a 67% sucrose artificial liquid diet to determine the optimum dose of virus that would result in wing deformity and mortality relative to control bees that were not fed DWV lysate. DWV lysate diluted 1:10 in PBS was used for both larval and adult bee virus inoculation

experiments. The presence of DWV in the lysate and absence of black queen cell virus (BQCV), Israeli acute paralysis virus (IAPV), sacbrood virus (SBV), acute bee paralysis virus (ABPV), chronic bee paralysis virus (CBPV) and Kashmir bee virus (KBV) were confirmed by RT-PCR using primers listed in Table 2.1 and Table 5.1.

dsRNA preparation

Viral RNA was purified from a preparation of DWV using a RNeasy Mini Kit (Qiagen) RNA extraction kit and complementary DNA (cDNA) was prepared using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Invitrogen). A 700 bp region of the RdRp gene (corresponding to bases 8565-9355 of GenBank: AY292384.1) was PCR-amplified from the cDNA using primers listed in Table 5.1. The PCR product was initially ligated into the PCR cloning vector pDrive (Qiagen) and its identity was confirmed by DNA sequencing. A search of the honey bee genome using BLAST found no similarities to any honey bee sequences. The RdRp gene fragment was then excised from pDrive using *Kpn*I and *Xba*I and ligated into a similarly digested dsRNA transcription vector pL4440 (provided by Andrew Fire, Stanford University, Palo Alto, CA), which contains two convergent T7 promoters that flank the plasmid's multiple cloning site. A 431 bp segment of the green fluorescence protein (GFP) gene (provided by Beeologics LLC, Miami, FL, USA) was similarly cloned into pL4440, to be used to produce GFP-dsRNA that would serve as a negative control to account for any general, non-specific effects of activating the RNAi pathway. The dsRNA was synthesized using the MEGAscript® RNAi Kit from Ambion (Austin, TX. USA). The final preparation of dsRNA was dissolved in nuclease free water and then quantified at 260 nm using a spectrophotometer. The primer pairs used for the experiment are listed in Table 5.1.

Virus inoculation and dsRNA treatment methods

The virus and dsRNA were provided in larval food to test whether the DWV-dsRNA targeted DWV transcripts in larvae. Thirty freshly hatched larvae (<12 hours old) were fed for each of the five treatments in the larval experiment: Treatment 1 (T1) DWV-dsRNA + DWV; Treatment 2 (T2) GFP-dsRNA + DWV; Treatment 3 (T3) DWV only; Treatment 4 (T4) Control/no treatment; and Treatment 5 (T5) DWV-dsRNA only, with no DWV. All dsRNA treatments were mixed with larval food at 1 µg dsRNA per larvae per day and fed for the six-day feeding period. All treatments that included virus were inoculated with 400 µl of DWV lysate containing 10^5 genome equivalents of DWV in larval food on the evening of day three. Virus-free controls were fed larval food lacking virus throughout the six-day feeding period. The entire experiment was done twice (n= 60 larvae per treatment).

On day 21, all adults were scored for mortality and wing deformity in all bees in the experiment was assessed after death or after emergence as an adult. All bees that were collected were immediately frozen in liquid nitrogen and stored in a -80 °C freezer. I randomly selected a subset of 10 bees for RNA extraction from the DWV-dsRNA + DWV, GFP dsRNA + DWV, and DWV only and those selected were all processed to measure the subsequent viral titers resulting in 5 to 7 bees from each treatment that were successfully analyzed by using qPCR.

A similar dsRNA feeding experiment was conducted on adult bees. Fifty adult bees were placed in each bioassay cage (outer dimensions 20.5 × 8.0 × 11.2 cm) and held at 25 °C in a temperature controlled flight room (Pernal and Currie 2001). The treatments were the same as those described above for the larval feeding experiment, but given the lack of negative effects of the dsRNA on the bees, the DWV-dsRNA with no virus

treatment was not included. Double-stranded RNA (50 µg of dsRNA mixture) treatments (T1 and T2) were fed to bees daily in 3 ml of 50% sucrose solution for six days, at a dose of approximately 1 µg per bee per day. Virus inoculation occurred on day three (for T1, T2, and T3) by adding 750 µl of DWV lysate (see above) to the sucrose diet. Control bees were fed at the same rate with sucrose diet containing no virus. Daily bee mortality rates were assessed for eight days, and samples of dead bees were collected daily. Dead and live bees were immediately frozen in liquid nitrogen and stored at -80 °C. I selected some bees for RNA extraction and these were processed to measure the subsequent viral titers using qPCR. Due to the high cost associated with feeding large numbers of workers with dsRNA constructs, the entire experiment was done twice (2 cages per treatment). At the end of the experiment 5 bees were randomly selected from each cage for qPCR.

RNA extraction and cDNA synthesis

To quantify virus load, the frozen bee samples from the larval and adult bee experiment were crushed in a mortar under liquid nitrogen. The total RNA was extracted from 30 mg of ground honey bee material using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions with following modification; i) After homogenization crushed samples were centrifuged for 8 minutes, with RNeasy Lysis Buffer (buffer RLT) and β-mercaptoethanol (β-ME) to remove the excess debris and ii) the elution volume was 20 µL. RNA samples were dissolved in diethylpyrocarbonate (DEPC)-treated water in the presence of ribonuclease inhibitor and stored at -80 °C for further analysis. The RNA quantity and purity were determined by spectrophotometer by measuring the absorbance at 260 nm and 280 nm. An average of 2.5 µg of total RNA was reverse transcribed to produce cDNA using Moloney Murine

Leukemia Virus Reverse Transcriptase (M-MLV RT, Invitrogen, Carlsbad, CA, USA) according to the supplier's recommendations.

PCR Analysis

PCR assays were done as follows: 2.5 microliters (µl) of cDNA was mixed with 5 µl 10X *Taq* buffer, 1 µl of dNTP, 1 µM of sense primer and 1 µM anti sense primer. The thermal cycling profiles were as follows, the mixture was heated for 10 min at 95 °C, 31 PCR cycles at (95 °C for 15 s, 56 °C for 30 s, 72 °C for 1 min), and the reactions were completed by a final elongation step for 5 min at 72 °C. A control without template was included in all batches. The PCR products were electrophoresed in a 1% agarose gel and stained with ethidium bromide. The PCR products were ligated into pGEM-T easy plasmid vectors (Promega, Madison, WI, USA) and the ligated vector transformed into *E. coli* DH5α competent cells (Promega, Madison, WI, USA) according to the manufacturer's protocol. Those colonies containing plasmids with inserts (white bacterial colonies) were selected and plasmid DNA was purified with the GenElute Plasmid Miniprep kit (Sigma-Aldrich, St. Louis, MO, USA). All PCR fragments and purified DNA were sequenced by the DNA sequencing services Macrogen Inc., Rockville, MD, USA.

qPCR analysis

Total RNA was extracted from the adult bees as described above. After RNA purification, cDNA was produced using M-MLV RT (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The efficiency of each primer set was first validated by constructing a standard curve through serial dilutions. The DWV

quantification by real time PCR was carried out in triplicate on an ABI Prism 7300 machine (Applied Biosystems, Foster City, CA, USA) using QuantiFast SYBR Green PCR kit (Qiagen, Valencia, CA, USA). A control without template was included in all batches. The PCR programme profile began with a single cycle heated for 5 min at 95 °C, 40 PCR cycles at 95 °C for 15 s, 55 °C for 30 s, 72 °C for 30 s. To determine and measure the dissociation curve the PCR products were heated to 95 °C. Real Time PCR reactions were performed in triplicate, and data were processed according to the cycle threshold value (Ct) with honey bee β-actin (DNA fragment) used as an internal control to corresponding samples obtained using the relative quantification $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The ΔCt values indicate the difference in the Ct values between DWV gene and the β-actin gene. The change in the gene expression is calculated as $2^{-\Delta\Delta Ct}$. The data are expressed as the relative amount of viral RNA levels compared with internal control as a standard. I used a single bee extract to assess the quantity. The primers used in the experiments are presented in Table 1.

Statistical analysis

The effect of treatments on survivorship of larvae and adult bees was analyzed by survival analysis using the non-parametric Kaplin-Meier (product limit) SAS LIFETEST procedure (SAS 1999; Gardiner 2010). Wing deformity data were analyzed using the using maximum likelihood estimation of parameters for log-linear models initially with replicates and experiment in the model PROC CATMOD procedure (SAS 1999). No significant treatment * experiment interaction was observed, so data for both experiments were pooled. Standard errors generated by CATMOD are based on binomial proportions. Pre-planned differences between treatment groups were assessed using Bonferroni

adjusted contrasts. The real -time PCR data are presented as log-transformed values (Snedecor and Cochran 1980). For adult bees, relative virus titers ($2^{-\Delta Ct}$) among treatments were analyzed as a split plot ANOVA with treatment*cage as the error term. Differences among means were compared by Scheffe's (SAS 1999).

RESULTS

Effects of DWV and dsRNA treatments on survival

Laboratory-reared bees

Feeding DWV-dsRNA to larvae of laboratory-reared bees did not adversely affect their survival, as both untreated and dsRNA-treated bees showed similar survivorship (68 ± 5% and 71 ± 6%, mean ± SE respectively) over the 21 d of observation (Fig. 5.1). Relative to bees without virus, survival of bees that were fed DWV in their diets as larvae dropped dramatically to 31 ± 6% ($P < 0.05$). The survival of bees fed DWV and DWV-dsRNA was 45 ± 6% at the end of the 21-day feeding trail, which was not significantly different than either the control treatment or the other treatments where bees were fed DWV alone or in combination with the negative control GFP-dsRNA (Fig. 5.1, $P < 0.05$).

Adult bee survival

Bees that were fed DWV-dsRNA had higher survival than bees that did not receive preventative dsRNA treatments against DWV (DWV-dsRNA vs. GFP-dsRNA and DWV only) (Wilcoxon $P < 0.05$). When adult bees were fed DWV without preventative dsRNA treatments, the survival rates at the end of the experiment ranged from 3 ± 2% to 22 ± 5% (in DWV only and unrelated dsRNA GFP respectively) (Fig.

5.2). Bees fed with virus-free sugar syrup had the highest survival (86 ± 3%) and had higher survival than bees fed DWV-dsRNA (Fig. 5.2, Wilcoxon $P < 0.05$).

Effects of DWV and dsRNA treatments on deformities

One of the frequently observed symptoms of DWV infection in bees is wing deformity. Significant differences in wing deformity were observed in the different treatments ($df = 4$, $\chi^2 = 16.98$, $P < 0.002$). A small percentage of bees in the negative controls and bees treated with dsRNA alone showed some wing deformities, whereas a considerably higher percentage of bees showed deformities when fed DWV as larvae (both treatments with high deformities vs. the rest) ($df=1$, $\chi^2 = 9.62$, $P < 0.002$, Fig. 5.3). In contrast, larvae fed DWV-dsRNA showed significantly reduced frequencies of wing deformities relative to other virus-fed (GFP dsRNA + DWV and DWV only) treatments ($df = 1$, $\chi^2 = 3.96$, $P < 0.05$), and wing-deformity was comparable to levels observed in the negative controls ($df = 1$, $\chi^2 = 0.03$, $P > 0.87$) (Fig. 5.3). In contrast, feeding bees the negative control GFP-dsRNA did not have any positive effect in reducing wing-deformity in the DWV-infected bees ($df = 1$, $\chi^2 = 0.12$, $P > 0.73$). In addition to wing deformities, many virus-infected bees also had bloated abdomens (Fig.5.4).

qPCR analysis of DWV concentration

The concentration of DWV was measured in a semi quantitative manner using qPCR to examine whether the DWV-dsRNA ingested by bee larvae inhibited replication of DWV. Differences between treatments were significant ($F_{4, 12} = 30.90$, $P < 0.05$) (Fig. 5.5). The viral concentration when DWV-dsRNA was absent was much higher than when it was present ($p < 0.05$). Wing deformities were highest in the treatment groups with the

highest levels of virus ($p < 0.05$); however, within treatments; there was no difference in DWV levels between bees with the deformed-winged and normal-winged phenotype within any treatment group ($p > 0.05$) (Fig. 5.5).

Virus concentration in adult bees was also assessed. In adult bees, treatments had an effect on relative virus concentration ($F_{2, 3} = 28.96$, $P < 0.0001$). Feeding adults DWV-dsRNA also reduced the level of virus, relative to bees fed either GFP-dsRNA and bees not fed dsRNA at all ($p < 0.05$; Fig. 5.6).

DISCUSSION

In this study, I have shown that feeding DWV-specific dsRNA to bees reduced the adverse effects of subsequent DWV infections. Bee larvae fed DWV-dsRNA prior to feeding on solutions containing DWV had significantly lower levels of virus than larvae fed either virus alone or larvae fed a negative control GFP-specific dsRNA. Similarly, adults that were fed DWV-dsRNA before DWV inoculations showed lower levels of DWV virus infection. The mechanism for this suppression is likely to be RNAi but this needs further validation. In related studies, dsRNA feeding has been demonstrated to confer resistance to IAPV (Maori et al. 2009) and SBV (Liu et al. 2010) viruses in honey bees, and my study now adds to the growing evidence that dsRNAs can offer potential prophylactic treatments against honey bee pathogens.

DWV infections in bees can be spread either vertically, from parent to offspring, or horizontally from one infected individual to another. Vertical transmission of DWV from infected queens or drones to their progeny is generally considered less virulent, often producing covert infections with no obvious symptoms (Chen and Siede 2007; de Miranda and Fries 2008; de Miranda and Genersch 2010). These inconspicuous

infections may however, enable long-term persistence of the virus in the population (Burden et al. 2003; Bonsall et al. 2005; Hails et al. 2008) and could lead to much more severe outbreaks if the virus is then transmitted horizontally. Horizontal transmission of DWV has previously only been correlated with the presence of the bee ectoparasite, the *Varroa* mite, *Varroa destructor*. While DWV can replicate within all developmental stages (Boncristiani et al. 2009; Yue and Genersch 2005), it is speculated that DWV must replicate inside *Varroa* to high titers and be inoculated into larvae or pupae to induce the characteristic deformed wings symptom observed in overt infections (Koch and Ritter 1991; Gisder et al. 2009). Recently, Mockel and colleagues (2011) demonstrated a direct association of DWV with the deformed wing phenotype. By artificially injecting DWV into honey bee pupae between the third and fourth abdominal segments, typical of *Varroa* feeding sites, they induced the development of deformed wings in adult bees. They noted that there was a correlation with high numbers of DWV particles found in the heads of the bees and the deformed wing phenotype, as infections with less than 1×10^7 virus particles within the head induced no deformities, while infections greater than 1×10^7 was prerequisite for wing deformation. In my study, I demonstrated for the first time that either adult or larval bees could acquire the virus through feeding. Although virus-contaminated food has been suspected as a mode of infection (Yue and Genersch, 2005), particularly as bees feed their larvae and each other, it had previously not been demonstrated that ingested virus could lead to systemic infections capable of inducing the stereotypical wing deformities or bloated abdomens that I observed here.

How and why DWV induces the visible deformity in the developing wings is not fully understood. Given that most wing deformities arise from *Varroa* infections during the larval or pupal phase, it is likely that the virus infection is disrupting normal wing

development during these critical developmental periods. In honey bee larvae, wing buds begin to emerge on the ventral side of the spiracles in the thoracic region (Snodgrass 1984). DWV has been detected in all body parts of deformed bees (Boncristiani et al. 2009; Yue and Genersch, 2005), but characteristic wing deformity symptoms are suspected to occur only if DWV has multiplied in localized tissues during wing development. During the early wing development, clusters of cells grow underneath the larval cuticle, usually in pocket-like structures of the epidermis. In the last larval stage, wings push out of the wing-pocket beneath the hypodermis and expand under the ventral side of the old cuticle of the larvae (Comstock and Needham 1899). Later in the pre-pupal stage, the wing disk elongates and forms folded flaps along the spiracles (Comstock and Needham 1899; Snodgrass 1984). If DWV levels are high in these regions during these developmental stages, conspicuous wing deformation may arise. Bees can be infected with DWV but exhibit no wing deformity, and presumably they acquired the virus after the critical phase of wing development, either through food exchange or transmission by *Varroa*.

The fact that DWV replication and onset of visible symptoms was likely inhibited by oral delivery of DWV-specific dsRNA suggests that the dsRNA was readily absorbed by gut epithelium and transferred to other tissues. A growing number of species of insects have been observed to take up dsRNA through the diet (Turner et al. 2006; Bautista et al. 2009; Maori et al. 2009; Shakesby et al. 2009; Whyard et al. 2009; Zhang et al. 2010; Li et al. 2011b), and several studies have likewise tried feeding dsRNA to honey bees (Amdam et al. 2003; Nelson et al. 2007; Marco Antonio et al. 2008; Liu et al. 2010). Given that hemolymph titers of virus and the wing deformity frequency were reduced in dsRNA-fed bees, the dsRNA must have spread systemically through the

insect. Many insects have similarly shown systemic RNAi following ingestion of the dsRNA, including flour beetles (Tomoyasu and Denell 2004), termites (Zhou et al. 2008), honey bees (Aronstein et al. 2006), and most recently the brown plant hopper (Zha et al. 2011). Systemic RNAi appears to be mediated by an RNA transporter, SID-1 (systemic interference defective), first observed in the nematode *Caenorhabditis elegans* (Tabara et al. 1998), but subsequently, closely related SID-1-like genes have been identified in a wide variety of organisms reviewed in (Huvenne and Smagghe 2010), including honey bees (Aronstein et al. 2006; Weinstock et al. 2006). The uptake of dsRNA, targeting viruses, has been observed previously in bees. Maori et al. (2009) reported an approximate 100-fold reduction in the level of IAPV RNA in dsRNA-ingesting *A. mellifera* adult bees compared to controls. Liu et al. (2010) demonstrated that Chinese sac brood virus (CSBV) genes *VP1* and *SBV1* mRNA levels were reduced up to 80 to 95% in dsRNA-ingesting larvae compared to untreated *Apis cerana* bees. My qPCR results similarly prove that dsRNA can be introduced through feeding and that it can spread systemically to reduce viral replication in the hemolymph.

DWV is one of the main viruses infecting bees. Adult bees with deformed wing symptoms typically die soon after emergence, while those with normal wings but still with high titers of DWV have a very short life span (Yang and Cox-Foster 2007). DWV multiplies slowly, without killing the larvae or pupae of honey bees, thereby allowing the *Varroa* to complete its development on bee pupae and spread the virus further thus ultimately cause significant mortalities in honey bee colonies (Martin 2001; Sumpter and Martin 2004). Several studies have concluded that DWV in association with high *Varroa* infestation is a major cause of colony losses (Bowen-Walker et al. 1999; Ribière et al. 2008; Sumpter and Martin 2004). Highfield et al. (2009) showed that even if the *Varroa*

population is controlled, high levels of DWV might play a major role in causing winter loss of colonies in the United Kingdom. More research is needed to optimize the efficacy of RNAi treatments against DWV to further improve honey bee survival rates. The prospect of using RNAi-based treatments to protect honey bee hives from DWV and other virus infections through feeding it to bees in sugar syrup feeders, offers considerable hope for the successful mitigation of various bee pathogens.

Table 5.1. Primers used in the experiment

Primer (5'-3')	Product size (bp)	Reference
Honey bee β-actin real time PCR Actin F: 5- AGGAATGGAAGCTTGCGGTA-3 Actin R: 5- AATTTTCATGGTGGATGGTGC-3	181	(Chen et al. 2004b)
DWV: dsRNA synthesis T7RdRp-F: 5- **TAATACGACTCACTATAGGGCGA**CACCTGGAACATCGGGTAAG-3 T7RdRP-R: 5- **TAATACGACTCACTATAGGGCGA**AGAGAACTCGGACAAAGGC-3	700	This work
GFP: dsGFP synthesis F: 5- **TAATACGACTCACTATAGGGCGA**GCCAACACTTGTCACTACTTTCTCTT-3 R: 5- **TAATACGACTCACTATAGGGCGA**AGGTAATGGTTGTCTGGTAAAAGGAC-3	431	(Maori et al. 2009)
DWV RT-PCR DWV-F: 5-ATCAGCGCTTAGTGGAGGAA-3 DWV-R: 5-TCGACAATTTTCGGACATCA-3	702	(Chen et al. 2004b)
DWV qPCR DWV RT-F: 5- CGAAACCAACTTCTGAGGAA -3 DWV RT-R: 5- GTGTTGATCCCTGAGGCTTA -3	174	This work

Figure 5.1. The effect of treatments on survivorship of laboratory-reared bee larvae during the 21-day rearing period. Larvae were treated with no virus [deformed wing virus (DWV)-double-stranded (ds) RNA], were untreated (Controls), or were treated with DWV-dsRNA + DWV; green fluorescent protein (GFP)-dsRNA + DWV; or virus only (DWV). Treatments followed by same letter are not significantly different (Log-Rank test P > 0.05 and Wilcoxon rank test P > 0.05 of the LIFETEST procedure) (SAS, 1999).

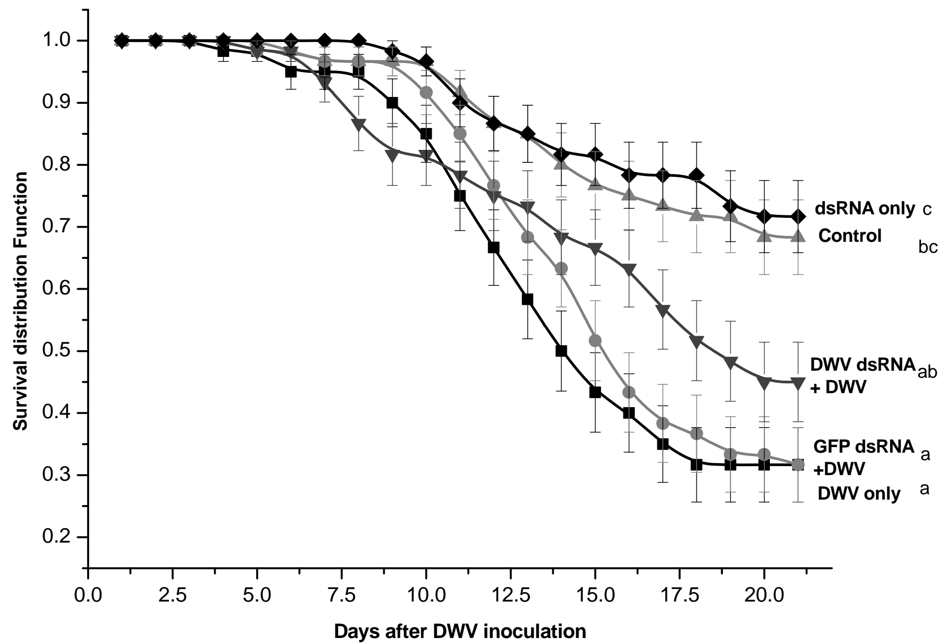


Figure 5.2. The effect of treatments on survivorship of adult bees during the 8-day adult feeding experiment. Bees were untreated (Control) or treated with: deformed wing virus (DWV)-double-stranded (ds) RNA+ DWV; unrelated green fluorescent protein (GFP)-dsRNA + DWV; or virus only (DWV). Treatments followed by same letter are not significantly different (log-rank test P > 0.05 and Wilcoxon test P > 0.05 of the LIFETEST procedure) (SAS, 1999).

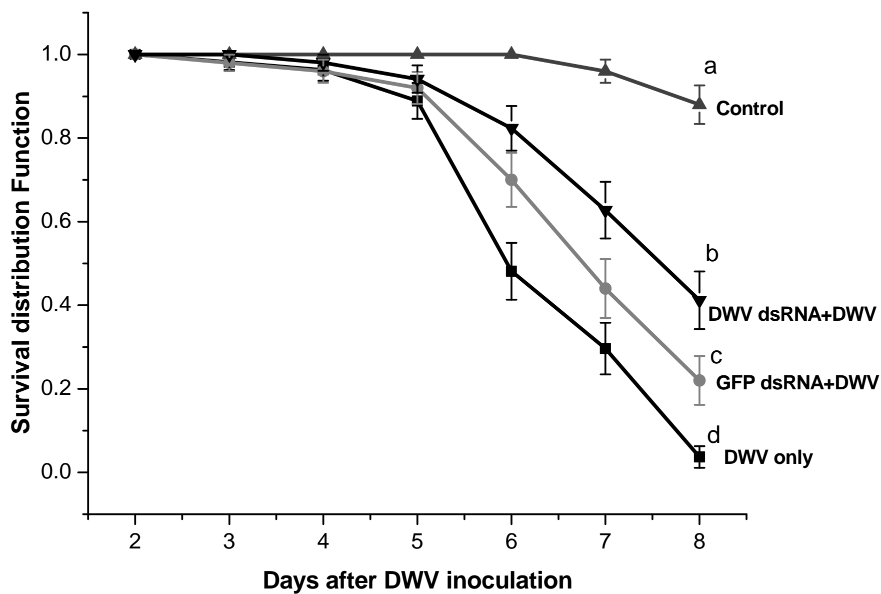


Figure 5.3. The effect of treatments on proportion of adult bees with deformed wings (SE) that emerged from laboratory reared bees on day 21. Larvae were treated with deformed wing virus-double-stranded RNA with no virus (DWV-dsRNA); or untreated (Control), or were treated with: DWV-dsRNA + DWV; unrelated GFP-dsRNA + DWV; and virus only (DWV). Frequency of wing deformity differed between treatments (CATMOD maximum likelihood) ANOVA, Likelihood Ratio- chi-squared, SAS, 1999).

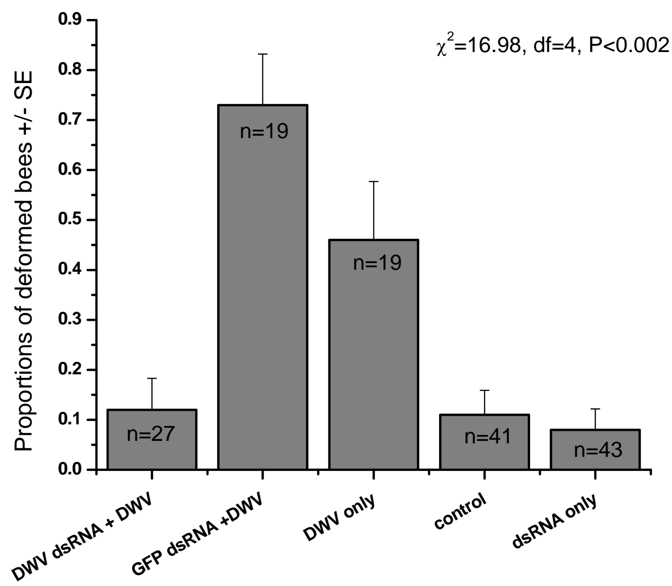


Figure 5.4. Effect of the DWV-dsRNA on symptoms of wing deformity: (A) Deformed wing virus (DWV)-double-stranded (ds) RNA was fed to the larvae: no symptoms of wing deformity; (B) Unrelated green fluorescent protein (GFP)-dsRNA and DWV were fed to the larvae: arrows show symptoms of wing deformity on adult bees; and (C) when only DWV was fed to the larvae: the arrows show symptoms of wing deformity on adult bees. Bees with bloated abdomen are also evident in (B) and (C).

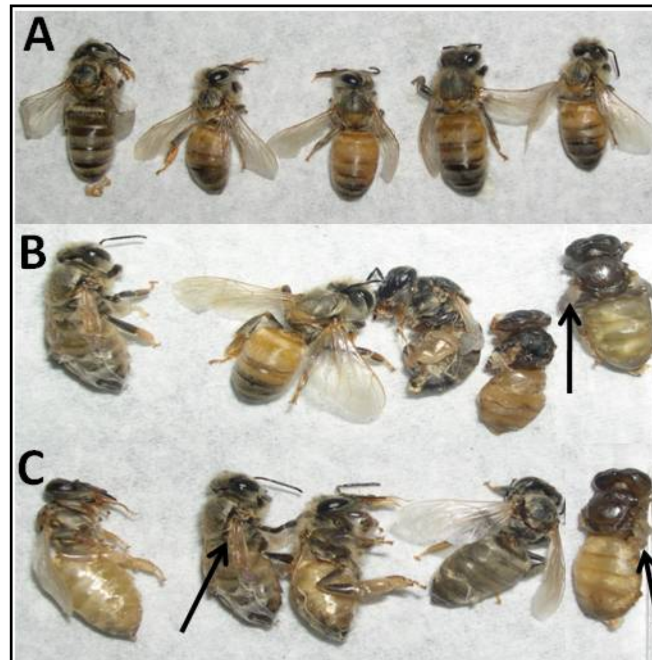


Figure 5.5. The effect of deformed wing virus (DWV)-double-stranded (ds)RNA and GFP-dsRNA on relative DWV concentration in treatments where larvae were fed with DWV. A subset of the adult bees that emerged on day 21 showing deformed wings or normal wings were analyzed. There is a significantly lower ($P < 0.05$) DWV concentration between the DWV-dsRNA treated bees and bees treated with green fluorescent protein (GFP)-dsRNA or DWV alone. Bars with same letters are not significantly different (Scheffe's multiple comparisons, $P > 0.05$).

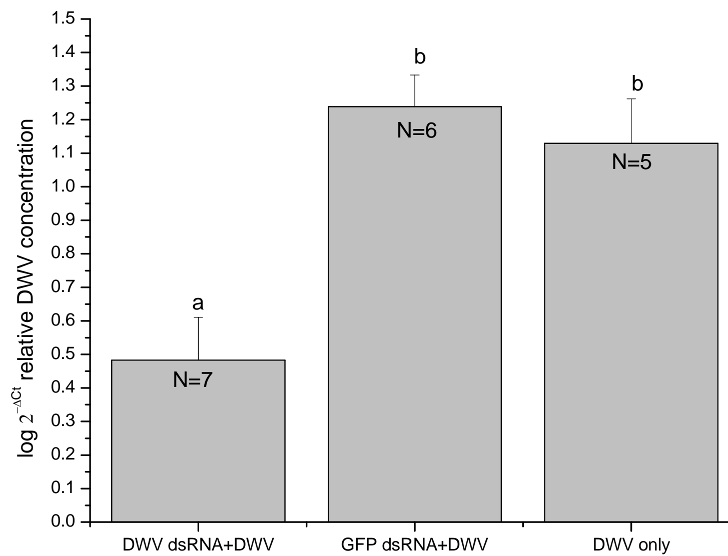
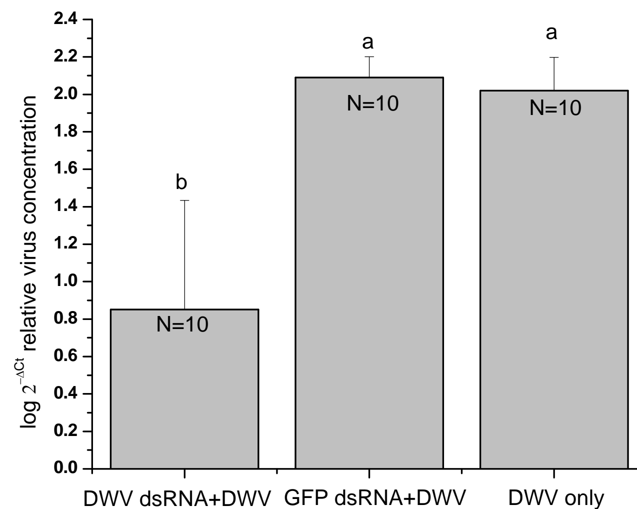


Figure 5.6. Relative deformed wing virus (DWV) concentration (log) in adult honey bees exposed to different treatments. Measurements are quantitative reverse transcription-PCR by using the comparative cycle threshold method. DWV quantification was done on single bees collected on day 8. The data show DWV and green fluorescent protein (GFP)-dsRNA fed bees had high DWV loads compared to bees fed DWV-dsRNA. There is a significantly lower ($P < 0.05$) DWV concentration between the DWV-dsRNA treated bees and bees treated with GFP-dsRNA or DWV alone. Bars with same letters are not significantly different (Scheffe's multiple comparisons, $P > 0.05$).



CHAPTER 6. GENERAL DISCUSSION

The beekeeping industry in Canada and around the world has faced a number of obstacles in the past few years. Beekeepers have frequently reported higher than usual winter loss of colonies since the fall of 2006. This study examined the likely impact of pathogens on honey bee *Apis mellifera* L. colonies and feasibility for their control. A series of laboratory and field experiments was conducted to address the main objectives. My main interest was to 1) study the occurrence, distribution and concentration of viruses in Canada. 2) understand the role of bee parasites and pathogens on bee losses in different winter environments in northern climates; 3) assess the effect of within colony genetic diversity on viruses and their interactions with other parasites and pathogens; and 4) determine if RNA silencing can be used to prevent deformed wing virus (DWV) infection in adult bees and larvae using double-stranded RNA feeding through food source. This study has led to a number of contributions to the existing knowledge on impact of bee pathogens and use of RNAi technology to control viruses in honey bees.

Impact of parasites and pathogens on bee loss

Viruses

Like other animals, honey bees face a huge number of threats from pathogens and parasites, which act in combination with other stressors to contribute to significant colony losses. Among honey bee pathogens, single-stranded RNA viruses are one of the major threats to colony health and their interactions with *Varroa* have likely caused serious problems in the beekeeping industry (vanEngelsdorp et al. 2009; Genersch et al. 2010). Viruses can attack bees in different stages of their lives, and in turn, can influence outcomes of other parasites and pathogens. Many RNA viruses cause asymptomatic

infections, and bees carrying virus often exhibit no morphological symptoms. Prior to this work, little was known about the distribution of economically important viruses in Canada and no baseline studies had been conducted. In this study, I showed that all seven viruses I sampled for were found in most beekeeping regions in Canada with multiple infections within colonies being ubiquitous. The most prevalent virus was DWV and occurred in 98 to 100% of the samples, followed by BQCV 70-100%, IAPV 40-70%, KBV 10-40%, SBV 0-40%, ABPV 0-20% and CBPV 0-30% (Chapter 2). In this study, quantitative analysis of relative virus levels showed DWV concentration varied throughout the country with BQCV and IAPV being at similar concentrations in different provinces. DWV was the only virus that was found at higher concentrations in unhealthy colonies (sampled in spring) than in healthy colonies (sampled in fall) within Manitoba and across Canada, but seasonal or regional differences may have contributed to these differences. SBV prevalence was higher in unhealthy colonies (sampled in spring) in Manitoba than in healthy colonies (sampled in fall) across Canada but did not differ from healthy colonies (sampled in fall) within Manitoba but these differences may have been due in part to seasonal or regional effects. DWV has recently been linked to honey bee colony declines throughout the world (Allen and Ball 1996; Chen et al. 2004a; Ellis and Munn 2005; Antunez et al. 2006; Berenyi et al. 2006; Berenyi et al. 2007; Ai et al. 2012; Martin et al. 2012; Francis et al. 2013) and my study suggests it may also be a virus of concern in Canada. The fact that my survey showed that it was common and potentially damaging made it an ideal target for later studies with RNAi (Chapter 5).

To understand the impact of a suite of parasites and pathogens on honey bee colonies in two different wintering environments, a broad scale study was designed, in which samples were collected from 75 colonies from randomly selected commercial

beekeepers in Manitoba over three seasons (fall, mid-winter and spring). In the broad scale Manitoba study, the prevalence of viruses in indoor-wintered colonies and in outdoor-wintered colonies were similar (Chapter 3). I found similar patterns for virus prevalence to those I found in Canada as a whole, with the exception of SBV, which showed higher prevalence in the Manitoba study.

In this study, I showed that DWV, BQCV and *Nosema* concentration exhibited different seasonal patterns in indoor and outdoor-wintered colonies. However, unlike many of the other viruses DWV concentrations declined over winter in indoor-wintered colonies, while they were unchanged in outdoor-wintered colonies. Previous studies have shown that DWV is a potential cause of colony losses in many environmental and bee management contexts (Chen et al. 2004b; Cornman et al. 2012; Dainat et al. 2012a, 2012b; Francis et al. 2013; Highfield et al. 2009; Martin et al. 2012). My results support this view from the survey study and within the context of outdoor wintering in the Manitoba study. DWV concentrations were higher in symptomatic colonies than in healthy colonies) both within the Province of Manitoba and in comparison with all colonies across Canada (Chapter 2) but these differences may have been due in part to seasonal or regional effects. Similarly, Highfield et al. (2009) showed that DWV load is lower in asymptomatic honey bees than in *Varroa*-infected symptomatic bees. However, their samples were collected from a single colony, while I did my virus analysis from pooled samples of mixed 10 adult worker bees from each hive. Further studies are required to quantify all viruses (instead of the three DWV, BQCV and IAPV) from different provinces from Canada, to look at the impact of other viruses such as SBV, which showed higher prevalence in mid-winter and spring than fall samples and spring

SBV concentration correlated with dead colonies (Chapter 3). Such studies should examine bees collected in both spring and fall to control for seasonal effects (Chapter 3).

I found significant correlations between DWV, *V. destructor* and "bee loss" (Chapter 3) in outdoor wintered colonies. *Varroa* likely contributes to a variety of different mechanisms that can affect winter bee loss, and its role as a vector of viral infections, in particular deformed wing virus, is well documented (Cox-Foster et al. 2007; Yang and Cox-Foster 2007; Martin et al. 2012). *Varroa* not only vectors viruses, it reduces genetic diversity of DWV strains and increases the prevalence of more virulent strains of virus (Martin et al. 2012). This could have implications for development of resistance to forms of RNAi targeted against DWV. This question was not a focus of my study but should be addressed in further research.

Overt DWV infections are consistently associated with infestations by *V. destructor* (Martin 2001), whereas in the absence of the mite DWV seems to exist as a covert infection (de Miranda and Genersch 2010). For fall outdoor-wintered colonies, in my study DWV and *Varroa* were positively correlated (both simple and partial correlations). The mite acts as a mechanical vector to transmit the viruses however DWV may also be able to replicate within mites (Yue and Genersch 2005; Ongus et al. 2004). DWV is one of the few bee viruses that cause distinct morphological symptoms in infected adult bees. DWV infection causes typical disease symptoms such as shrunken, crumpled wings, reduced body size, and discoloration in adult bees but also infects eggs, larvae, and pupae. DWV infections in bees can be spread either vertically, from parent to offspring, or horizontally from one infected individual to another. Wing deformity was thought to be related to the DWV titers within the parasitizing mites and thought to require inoculation by mites during feeding (Gisder et al. 2009; Bowen-Walker et al.

1999). However, in my study, I demonstrated that adult bees and larvae can also acquire the virus through feeding and show increased viral titers, increased wing deformities, and shortened life spans in infected bees (Chapter 5). Here, I fed only DWV to bee larvae in lab conditions and even though no *Varroa* were present on the bees the infection led to high wing deformity in emerging adults showing that a linkage between the virus and wing deformity in the absence of mites existed (Chapter 5). The fact that concentrations of virus increased in the absence of mites could have epidemiological implications if heavily contaminated bees can also transfer virus during feeding of susceptible larvae. This needs to be tested experimentally.

Viruses can be highly correlated with each other and other pathogens. Correlations between DWV and ABPV have been shown in several studies and linked to colony death in association with *Varroa* when colonies are sampled in spring (Berthoud et al. 2010). However, in this study, I did not find any correlation between the two, and ABPV was not correlated with bee loss. For both indoor and outdoor wintered colonies in spring, positive correlations were found between *Nosema* and BQCV. However, partial correlations showed this relationship held only for outdoor-wintered colonies. BQCV showed a seasonal pattern that was similar to *Nosema*. BQCV levels did not change in mean abundance from fall to spring for indoor-wintered colonies, however there was an increase in mean abundance from fall to spring outdoor-wintered colonies. Similarly, previous studies have showed that BQCV and Nosema are frequently associated and highly correlated with each other (Yue and Genersch 2005; Berenyi et al. 2006). Factors that may have affected *Nosema* (see later discussion) may also influence patterns with BQCV if a causal relationship exists between the two.

CBPV has been associated with colony losses in Europe, but in this study, I did not find any correlation between CBPV and bee loss. Ribiere et al. (2002) showed that fewer particles of CBPV than of ABPV are required in *Varroa* to induce clinical symptoms in adult bees. However, I was able to detect low levels of virus with qPCR and I did not observe the paralysis, trembling and crawling symptoms associated with CBPV in my study. Prevalence of CBPV, ABPV and KBV were relatively low in my samples; however, sporadic increases in CBPV, ABPV and KBV have been observed in European countries, one of these (ABPV) is more often found at higher frequencies in midsummer and it is possible that if high levels were present then this have been missed by my sampling protocol (Chapter 2). The three viruses ABPV, KBV and IAPV have been linked to poor bee health (Francis et al. 2013), and all three are genetically, serologically and biologically related to each other (de Miranda et al. 2010). In this study, IAPV was positively correlated with ABPV both in indoor- and outdoor-wintered colonies. However, I found low prevalence (up to 16%) of ABPV, (up to 46%) of KBV and (up to 50%) of IAPV. These viruses were at low concentrations in colonies relative to other viruses in my study. Francis et al (2013) showed that ABPV, KBV and IAPV occurring in combination along with DWV were linked with colony losses. However, neither ABPV nor KBV were positively correlated with bee loss in this study. Different interactions among each of the seven viruses (DWV, BQCV, SBV, IAPV, KBV, CBPV and ABPV) occurred in the two different wintering environments (indoor and outdoor) over time (fall, winter and spring) suggesting that as yet unknown abiotic and biotic factors may affect their transmission and replication in different wintering environments.

SBV was the only virus in this study that showed differences in prevalence in different samples from the Canada-wide survey (Chapter 2). SBV was also associated

with colony mortality (Chapter 3). In my study, SBV prevalence increased dramatically from fall to spring and was high in colonies sampled in mid-winter and remained high in spring (Chapter 3). Seasonal changes of SBV in the United Kingdom show low levels in spring, peaks in mid-summer and declines in fall that follow the natural brood cycle (Bailey 1981). However, the results in my study were mixed with respect to SBV. Unhealthy colonies within Manitoba did not differ from healthy colonies in the same province (Chapter 2) and the fact that prevalence increased from fall to spring (Chapter 3) suggests the differences in spring collected unhealthy colonies in MB and fall collected samples across Canada (Chapter 2) were likely due to seasonal effect. Simple correlations also did not show a relationship between SBV. It is not known if SBV may have been a causal factor in colony loss or if it is an "opportunistic pathogen" that increases in prevalence when colonies are weakened by other stressors. This requires further investigation.

Previous field studies have shown that wintering is more stable in indoor wintering environments and allows colonies to survive winter under higher infestation levels of *Varroa*, either alone or in various combinations with tracheal mite and *Nosema* or other stressors (Bahreini and Currie 2009; Currie 2001; Currie 2008; Williams et al. 2010). This is beneficial because it reduces colony replacement costs. However, mine is the first broad scale study which looked at interactions with viruses in two different wintering environments. In this study, no single parasite or pathogen was highly correlated with colony loss in either of the two winter environments although significant correlations between parasites, pathogens, and bee loss did occur. I found that although the prevalence and concentrations of parasites and pathogens analysed in this study were similar prior to implementation of wintering management and during winter, their impact

on colonies and interactions with each other were different in the two environments. The environment to which colonies were exposed likely played a role in affecting the different impacts of parasites and pathogens that were observed, although beekeeper management practices related to drug feeding may have also played a role (with respect to *Nosema*).

Winter makes survival for many insects a challenge. In northern latitudes, honey bee colonies can be wintered in indoor or outdoor management systems. A minimum of 15000 bees are typically recommended as a suitable population size to survive the severe wintering in western Canada, to maintain a minimum of 32 °C inner cluster temperature (Harris 2009). In my study (2009-2010), I found similar percentage of dead colonies in indoor and outdoor-wintered colonies, however the rate of bee loss was much higher for outdoor-wintered colonies than for indoor-wintered colonies. A survey of producers in Manitoba in the same year, reported lower losses for indoor-wintered colonies (29%) than for outdoor wintered colonies (37%) (Lafrenière 2011), also a similar trend was observed in 2010-2011 where indoor wintered bee loss was 28%, and outdoor wintered bee loss was 42% (Lafrenière 2011). Differences in the proportion colonies that died between my randomly sampled producers (20% died when wintered indoor and 20% died for outdoor wintering) and the survey in the same year could be due to survey bias. It is possible that a higher proportion of producers that had high losses responded to the voluntary survey than did beekeepers with low losses. Beekeepers may also have reported losses that may have occurred later in spring after my study was complete. However, it is interesting to note that the survey results support my study with respect to the differential impact of indoor and outdoor-wintering. The results of all these studies suggest beekeepers could reduce the impact of parasites and pathogens in bees by wintering their colonies indoors rather than outdoors. Indoor wintering honey bee colonies probably lowers some of the

associated stresses on colonies allowing them to better tolerate the impact of pathogens and parasites and still survive the winter with lower percentage bee loss than outdoor-wintered colonies. However, controlled studies that manipulate pathogen level in each environment are required to clarify the impacts of different stressors.

The causes of CCD and more generally, colony losses, on a global scale are a debated topic; several parasites and pathogens have been "shortlisted" as reasons for sudden loss, including, *Varroa destructor,* DWV, IAPV and *Nosema ceranae* (Cox-Foster et al. 2007; Higes et al. 2009; Highfield et al. 2009; Guzman-Novoa et al. 2010; Dainat et al. 2012b, 2012a; Martin et al. 2012). However, different factors appear to be important in different geographic areas. Australia for example, where *Varroa* is absent, has not recorded CCD-like events in spite of the common presence of IAPV in honey bees. IAPV has been present in the USA since 1994 and *Nosema ceranae* in Canada at least since 1994 (Chen and Evans 2007; Currie et al. 2010), however the increased frequency of major colony deaths began in 2006 and has continued since that time (Currie et al. 2010, vanEngelsdorp and Meixner 2010). No single factor has been identified as the cause of CCD, but increased levels of acaricide resistance in *Varroa*, synergistic interactions between multiple groups of pathogens, parasites, pesticides and the environment may have all affected the bee loss (Currie et al. 2010). A study by Bromenshenk et al. (2010) claimed the interaction between Iridovirus and *N. ceranae* may be important in causing bee mortality but subsequent studies shed doubt on this claim (Tokarz et al. 2011). Several viruses have been directly linked to colony death in different parts of the world, under different conditions. My large-scale field study (Chapter 3) has yielded a considerable amount of information, which helps us better understand the synergistic interaction of virus-*Varroa* and virus-virus and *Nosema*-virus

dynamics in bee colonies in northern climates. Understanding the transmission of viruses, as well as the distribution of viruses in the colonies and among the bees, opens possibilities for developing management techniques or breeding resistant bees to suppress targeted group of pathogens. The findings suggest that *V. destructor,* DWV, IAPV and possibly SBV, affected colony population losses during winter or spring population size. Correlations between *Nosema* and low spring population size (outdoor-wintering), CBPV and bee loss, CBPV and low spring population size (outdoor-wintering in spring) and BQCV and low spring population (indoor-wintering in spring), *Varroa* and DWV with bee loss and low spring population size (indoor-wintering fall) were also observed and may be worth further investigation but my false discovery rate (FDR) analysis indicated these may have been due to chance. Interactions between these viruses and *Varroa* may lead to colony death through both injection and activation of virus particles into hemolymph (Ribiere et al. 2008, Yue and Genersch 2005) and by secondary suppression of immune system by *Varroa* (Cox-foster et al. 2007). However, studies that manipulate the levels of each of these parasites and pathogens are required to establish causal relationships with colony loss.

Nosema

A third group of organisms I investigated are the fungi, *Nosema apis* and *Nosema ceranae*. *Nosema* is an obligate parasite that grows inside the digestive tract of bees and reduces its life span. *Nosema* is an economically important and commonly encountered disease of *Apis mellifera* caused by *Nosema* spp. (Bailey and Ball 1991). *Nosema* infection induces change in both the behaviour and physiology of the honey bee. It was important to include in my study because it increases honey bee susceptibility to other

pathogens due to effects on the immune system of the host (Antunez et al. 2009). However, the impact of colony level infections of *N. ceranae* on colony loss is controversial. Studies in Spain show *N. ceranae* is a virulent parasite and that the infection eventually causes colony collapse unless controlled with antibiotics (Martin-Hernandez et al. 2007; Higes et al. 2008b; Higes et al. 2009). However, in this study, the results were less clear. *Nosema* was positively correlated with BQCV in both indoor and outdoor-wintered hives in spring as has been shown by others (Bailey et al. 1983a). Partial correlations between *Nosema* and BQCV in spring were significant only for outdoor-wintered colonies. Sample location affected assessment of *Nosema* and BQCV levels and this has implications for sampling to predict impacts of pathogens on colony loss. *Nosema* was also correlated with DWV in outdoor-wintered colonies in fall. Unlike the viruses I studied, *Nosema* is not known to be vectored by *Varroa*. Worker bees are infected with nosema disease when taking contaminated food, rubbing each other, drifting between hives, swarming or hive cleaning (Fries 1988; Fries et al. 1996; Fries and Camazine 2001; Higes et al. 2008a), and can horizontally transfer *Nosema* to the queen (Higes et al. 2009).

Nosema spore counts in worker bees peak when the bees are from 9-24 days old (El-Shemy and Pickard 1989), but *Nosema* spore intensity is higher in foragers than newly emerged bees and house bees (Smart and Sheppard 2012). My study showed that foragers collected at the entrance of the hive might be better predictors of future infestation levels during winter. Although brood area samples in fall may better represent the overall load of spores in the colony at that point in time (Chapter 3). This study has helped develop better sampling recommendations for *Nosema* and suggested beekeepers should be sampling bees at the entrance of the hive in fall in order to better predict winter

Nosema levels and make decisions about control. The use of brood area samples, which are currently used in Manitoba for making management decisions, may not appropriate as it could cause beekeepers to avoid treating when their *Nosema* levels may warrant treatment. For either method, good treatment thresholds still need to be developed.

Impact of polyandrous mating

The effect of genetic diversity on colony fitness measures has been studied and it is known that genetically diverse colonies can perform better than genetically similar colonies in several contexts (Tarpy 2003, Tarpy and Seeley 2006, Mattila and Seeley 2007). However, prior to my work the impacts of multiple mating on resistance to viruses and their interactions with other parasites had not been studied. Honey bees are excellent model insects to study the impact of multiple mating on parasites and pathogens as they can be artificially inseminated to create queens mated with known levels of males (drones). In this study, I produced 24 genetically diverse queens and 11 genetically similar queens, using instrumental insemination techniques and controlled for the volume of sperm that was inseminated. Several hypotheses have been put forth to explain the probable benefits of multiple mating in honey bee queens. I attempted to address the question as to whether higher within colony genetic diversity may lower the prevalence or intensity of disease infections (parasites and pathogens) when colonies are exposed to multiple pathogens. A honey bee's ability to resist disease depends on many different factors.

Since GDCs consist of multiple subfamilies that could differ in their propensity to perform different tasks in the colony (Page and Metcalf, 1982, Laidlaw and Page, 1984),

they have the potential to defend against a broader array of pathogens. This is because their subfamily structure can allow the colony to possess a greater array of potential defenses than in GSCs. Studies demonstrating that increased diversity benefits defense usually examine single pathogens such as American foulbrood or Chalk brood in honey bees and *Crithidia bombi* Lipa and Triggiani in bumble bees (Sherman et al. 1988; Schmid-Hempel 1998; Schmid-Hempel and Crozier 1999; Seeley and Tarpy 2007). A robust test of this hypothesis with multiple parasites and pathogens has not been done, so I examined the effects of increased genetic diversity on two parasitic mites, seven RNA viruses and two fungi (*Nosema* spp.).

Although *A. mellifera* did not co-evolve with *V. destructor*, some genetic strains are more resistant than others, so the possibility of having different subfamilies with different levels of resistance is likely. I demonstrated the benefit of greater within colony diversity on defense against *Varroa* by assessing *Varroa* mortality rates using both cage studies and full size colonies in winter. The outcomes of this series of experiments are presented in chapter 4. Although GDCs had higher *Varroa* mortality than in GSCs, the daily worker bee mortality was also higher in the full size colony experiment that was carried out at 5 °C in winter. However, there was no difference in daily bee mortality after they were placed in bioassay cages in laboratory conditions at 25 °C. This difference in bee mortality rates between the two experiments may represent a transient cost to resistance that has been shown to occur at low temperatures (Currie and Tahmasbi 2008). This study also showed that GDCs showed lower prevalence of (acute bee paralysis virus (ABPV and *N. ceranae*) and lower concentrations of DWV (in fall sampling) than GSCs. Both GDCs and GSCs that had been selected for resistance to

Varroa had low relative deformed wing virus (DWV) concentration in spring when compared to unselected OMCs.

This suggests that although increased genetic diversity may be beneficial in an evolutionary context, it may still be possible to select strains of bees with resistance to critical groups of parasites and pathogens as long as some genetic diversity is maintained. In my study, GDCs had similar bee populations to that of GSCs. In contrast to the results of some studies (Mattila and Seeley 2007) that show colonies with increased genetic diversity have higher population sizes. One possible reason for similar worker population between both groups in my study might be related to the timing of queen introduction in my experiment. Queens may not have been established in colonies early enough to reach peak summer populations that would make differences between GDCs and GSCs more apparent. Differences in population may also have been evident if I had larger sample sizes, although the power in my experiment was sufficient to detect differences between OMCs and the other two queen types. Geographic variation in forage availability and other location-dependent factors could also affect colony diversity responses.

Overall, my results were consistent with the hypothesis that GDCs lead to better colony defense against parasites and pathogens relative to GSCs and supported the hypothesis that retention of polyandry in honey bees may be of benefit to allow honey bees to defend against pathogen challenge.

RNAi as control method

Another major achievement of my thesis was quantifying the impact of deformed wing virus on bees in the absence of mites and developing a method for using RNAi to control deformed wing virus. In this study, I used dsRNA that I developed to control

DWV. I selected the RNA dependent RNA polymerase (RdRp) region of the DWV gene to construct several forms of dsRNA against DWV and tested one of these on bees infected with DWV. This study aimed to interfere with DWV gene expression and replication in honey bees and suggests that this mechanism may be used to block pathogen replication within an insect host and, thus, block disease transmission**.**

Several studies including this one, have now shown that *Varroa*, in combination with DWV is often associated with colony losses (Bowen-Walker et al. 1999; Sumpter and Martin 2004; Ribiere et al. 2008). A more recent study from Highfield et al. (2009) found that, DWV plays a major role in affecting the health of the honey bees, independent of *Varroa* and DWV alone can potentially kill hives. They also found a significant correlation between DWV viral load and overwintering colony losses in the United Kingdom. Similarly, I have found significant correlations between fall DWV concentration and spring colony mortality in Manitoba (Chapter 3). In this study, I showed that feeding DWV to larvae causes wing deformity in adult bees in the absence of *Varroa* and decreases survival rates of adult bees. Feeding larvae with dsRNA specific to DWV in advance of inoculation with virus suppressed DWV viral replication, reduced wing deformity in adult bees relative to bees fed DWV or DWV with an unrelated form of dsRNA, and increased survival rates of adult bees. The double stranded RNA did not negatively affect bee survival (Chapter 5). RNA interference has now been used in bees to suppress viruses and microsporidia (Liu et al. 2010; Maori et al. 2009; Paldi et al. 2010). I showed that RNAi can be fed and used as a preventative antiviral strategy in honey bees. With reduced ability to suppress the *Varroa* due to its resistance to a wide variety of acaricides, the ability to control viruses directly could be of considerable

benefit as it could allow beekeepers to tolerate higher mite levels without experiencing economic loss. This still need to optimized and tested in full size colonies in field tests.

The experiments outlined above will help to increase our understanding of how viruses affect life span of honey bees and how we can use recent advances in molecular biology to develop methods to better manage bees to control viruses. The technology should help beekeepers to manage honey bees while reducing the probability of colony loss.

SUMMARY

In conclusion, my study is the first to record the presence of ABPV, CBPV, BQCV, and IAPV in Canada and I also found previously described DWV, SBV, and KBV in my samples and quantified the relative concentrations of all of these viruses (3 in Canada and 7 in Manitoba). I found that honey bee viruses are widespread and colonies are typically co-infected with two or more viruses. The results of my thesis indicate that interactions between the *Varroa*, bee viruses, other pathogens and wintering management methods may be linked to winter colony losses in northern climates and they may have greater importance in bee loss than previously thought. The outcome of my study showed that two viruses (DWV and BQCV) and one pathogen (*Nosema*) showed different seasonal patterns in indoor and outdoor-wintered colonies. The results showed that indoor-wintered colonies had lower rates of bee loss over winter than outdoor-wintered colonies despite having comparable parasites and pathogens levels in fall. Sample location affected assessment of *Nosema* and BQCV levels but did not affect assessment of other parasites or pathogens and this has implications for sampling to predict impacts of pathogens on colony loss. My study showed that multiple

insemination of queens resulted in lower prevalence of ABPV, and *N. ceranae* and lower concentration of DWV than in queens mated just once. *Varroa* mortality rates were also higher in GDCs than GSCs in the field study, supporting the theory that increased colony genetic diversity benefits defence against parasites and pathogens. My study demonstrated that virus concentration was lower in laboratory-reared larvae and adults fed dsRNA specific to DWV than in untreated bees fed only DWV and that feeding RNAi as a preventative treatment to larvae reduced levels of wing deformity in emerging workers and feeding it to adults increased bee survival rates relative to bees infected with DWV and not fed RNAi. The efforts made in my thesis increase our understanding of how pathogens affect honeybee colony survival, how viruses interact with different pathogens and parasites in different wintering environments, and how recent advances in molecular biology may help us to predict the impact of viruses on bees and develop methods to control them.

In summary, my results have several implications for commercial management of diseases and parasites that will help prevent winter loss of honey bee colonies. Our knowledge of honey bee viruses in Canada has been greatly limited due to lack of a baseline study of virus prevalence and concentrations. Currently beekeepers often submit samples to have assessments done on prevalence of viruses in their apiaries. My studies show that virus occurrence in colonies or prevalence at the apiary level, will not likely be of use for beekeepers as a predictive management tool. Quantitative analysis of viruses will be required and thresholds based upon this will have to be established. However, relationships between parasites and pathogens are complex and thresholds that consider concurrent effects of multiple parasites and pathogens will be required. Of the parasites

and pathogens studied, my results suggest *Varroa,* DWV, IAPV and SBV are of greatest concern in relation to bee loss over winter and colony death. Beekeepers should develop management strategies to minimize the individual or collective impact of these parasites and pathogens on colony loss. My study showed that colonies wintered indoor had lower rates of bee loss over winter than colonies wintered outdoors despite having equivalent parasites and pathogens levels in fall. This suggests that beekeepers should consider indoor-wintering as a management option to minimize colony loss. I also showed that in sampling their colonies to assess pathogen load, beekeepers need to be aware of the effect of sample location on the estimates of pathogen load in colonies. For most parasites and pathogens sample location did not significantly affect interpretation of results suggesting beekeepers could sample from the brood nest or entrance. However, results from entrance samples and brood nest samples did differ for BQCV and *Nosema*. For *Nosema* I showed that beekeepers should sample bees at the entrance of the hive in fall in order to better predict winter *Nosema* levels and make decisions about control. There is some evidence that beekeepers could benefit by maintaining genetically diverse colonies but that selection for better colony defense against parasites and pathogens will still be beneficial as long as some genetic diversity is maintained. Breeding programs for parasite disease resistance should ensure adequate genetic diversity is maintained. I also show DWV-RNAi has great promise for control of DWV and RNAi technology could become an effective tool for managing viruses in beekeepers colonies in the future.

THE NEED FOR FUTURE RESEARCH

Arising from my thesis are several questions for future research.

1. Further experiments are urgently required to address how viruses affect honey bees in different environments and refine sampling protocols to better predict bee population losses that result from the interaction of honey bee parasites and pathogens. With this information we could establish economic thresholds to predict and manage colony loss associated with viruses.
2. The identification of the specific genes that influence social immunity would not only improve our understanding of its mechanisms, but also shed new light on the evolution of collective defense in insect societies.
3. Future studies to look at the genes, which play a role in immunocompetence and their potential to provide resistance against viruses, would be worth investigating.
4. We need to identify and characterize DWV coat proteins, which are important for the recognition of the host cell and infection. Research to identify the receptor of the virus will help us to suggest some strategies to neutralize or inactivate viral infection of honey bee cells.
5. More research is needed in full size colonies to optimize the efficacy of RNAi treatments against honey bee viruses.

APPENDICES

Appendix 1. Simple correlations (Pearson's) for fall indoor-wintered colonies between colony parasites (mean abundance of *Varroa* and prevalence of tracheal mite), pathogens (mean abundance of *Nosema*, log $2^{-\Delta Ct}$ of DWV, BQCV, IAPV, SBV, KBV, CBPV, and ABPV) levels, bee population score and bee loss. r = correlation coefficient, *P*= P-value, N=45

Parameter§		***Varroa***	***Nosema***	**HBTM**	**DWV**	**BQCV**	**SBV**	**IAPV**	**KBV**	**CBPV**	**ABPV**
Population	*r*	**-0.41**	+0.05	-0.08	**-0.40**	-0.02	+0.10	**-0.30**	+0.14	+0.04	+0.10
	P	**0.006**	0.74	0.58	**0.006**	0.89	0.52	**0.04**	0.35	0.80	0.53
Bee loss	*r*	**+0.33**	-0.08	+0.05	**+0.31**	+0.05	+0.01	**+0.32**	+0.05	+0.06	-0.15
	P	**0.03**	0.59	0.73	**0.04**	0.74	0.94	**0.03**	0.75	0.71	0.33
Varroa											
	r	1	+0.02	-0.12	**+0.34**	-0.16	-0.07	+0.15	-0.12	-0.04	-0.16
	P		0.88	0.43	**0.02**	0.31	0.64	0.32	0.45	0.77	0.30
Nosema											
	r		1	+0.02	-0.16	+0.01	-0.08	-0.08	-0.10	-0.05	+0.02
	P			0.92	0.29	0.96	0.58	0.61	0.51	0.76	0.90
HBTM	*r*			1	+0.03	-0.09	-0.04	+0.15	-0.04	-0.06	-0.10
	P				0.87	0.56	0.78	0.34	0.81	0.70	0.50
DWV	*r*				1	+0.17	+0.04	+0.22	-0.18	-0.08	+0.06
	P					0.26	0.78	0.14	0.22	0.58	0.72
BQCV	*r*					1	-0.02	+0.13	**+0.41**	+0.09	-0.09
	P						0.90	0.41	**0.005**	0.55	0.57
SBV	*r*						1	+0.08	**+0.56**	-0.09	-0.09
	P							0.60	**<.0001**	0.56	0.56
IAPV	*r*							1	**+0.53**	**+0.63**	+0.00
	P								**0.0002**	**<.0001**	0.99
KBV	*r*								1	**+0.35**	-0.04
	P									**0.02**	0.78
CBPV	*r*									1	+0.09
	P										0.58

§= see list of abbreviations for full names of parasites and pathogens (page ix).

Appendix 2. Simple correlations (Pearson's) for fall outdoor-wintered colonies between colony parasites (mean abundance of *Varroa* and prevalence of tracheal mite), pathogens (mean abundance of *Nosema*, log $2^{-\Delta Ct}$ of DWV, BQCV, IAPV, SBV, KBV, CBPV, and ABPV) levels, bee population score and bee loss. r = correlation coefficient, *P*= P-value, N=30

Parameter§		***Varroa***	***Nosema***	**HBTM**	**DWV**	**BQCV**	**SBV**	**IAPV**	**KBV**	**CBPV**	**ABPV**
Population	*r*	**-0.39**	-0.30	-0.16	**-0.41**	-0.12	+0.12	+0.15	-0.20	0.29	+0.06
	P	**0.03**	0.10	0.40	**0.02**	0.55	0.51	0.43	0.29	0.12	0.73
Bee loss	*r*	+0.34	+0.33	+0.19	**+0.43**	+0.00	-0.11	-0.09	+0.23	-0.27	-0.01
	P	0.06	0.08	0.32	**0.02**	0.99	0.55	0.63	0.22	0.15	0.96
Varroa											
	r	1	+0.32	-0.02	**+0.70**	-0.01	-0.06	+0.19	+0.12	+0.01	+0.10
	P		0.08	0.93	**<.0001**	0.95	0.73	0.30	0.52	0.96	0.59
Nosema											
	r		1	-0.09	**+0.55**	-0.04	+0.03	-0.06	-0.08	+0.09	+0.34
	P			0.62	**0.002**	0.85	0.86	0.74	0.67	0.64	0.07
HBTM	*r*			1	+0.07	-0.06	-0.05	-0.10	-0.05	-0.07	-0.13
	P				0.71	0.77	0.80	0.58	0.81	0.70	0.49
DWV	*r*				1	-0.24	+0.14	+0.15	+0.09	+0.00	+0.03
	P					0.21	0.47	0.41	0.63	1.00	0.87
BQCV	*r*					1	-0.04	-0.15	-0.07	-0.12	+0.05
	P						0.84	0.44	0.70	0.51	0.80
SBV	*r*						1	+0.31	+0.09	-0.12	+0.22
	P							0.10	0.65	0.54	0.24
IAPV	*r*							1	+0.35	+0.19	**+0.50**
	P								0.06	0.30	**0.005**
KBV	*r*								1	+0.03	+0.09
	P									0.89	0.64
CBPV	*r*									1	+0.13
	P										0.51

§= see list of abbreviations for full names of parasites and pathogens (page ix).

Appendix 3 Simple correlations (Pearson's) for spring indoor-wintered colonies between colony parasite (mean abundance of *Varroa* and prevalence of tracheal mite), pathogen (mean abundance of *Nosema*, log $2^{-\Delta Ct}$ of DWV, BQCV, IAPV, SBV, KBV, CBPV, and ABPV) levels, bee population score and bee loss. r = correlation coefficient, *P*= P-value. N=40

Parameter§		***Varroa***	***Nosema***	**HBTM**	**DWV**	**BQCV**	**SBV**	**IAPV**	**KBV**	**CBPV**	**ABPV**
Population	*r*	+0.03	+0.06	+0.03	-0.05	**-0.43**	-0.12	+0.01	-0.05	-0.11	+0.02
	P	0.88	0.70	0.86	0.75	**0.006**	0.48	0.97	0.75	0.49	0.90
Bee loss	*r*	+0.01	+0.02	-0.06	-0.02	+0.30	+0.06	+0.12	+0.21	+0.25	+0.11
	P	0.95	0.91	0.73	0.89	0.06	0.71	0.47	0.19	0.13	0.52
Varroa	*r*	1	-0.07	-0.12	-0.04	-0.22	+0.01	+0.16	+0.07	-0.05	+0.17
	P		0.67	0.45	0.82	0.17	0.96	0.31	0.66	0.75	0.31
Nosema	*r*		1	-0.10	-0.05	**+0.47**	-0.18	+0.07	-0.10	-0.24	+0.08
	P			0.52	0.75	**0.002**	0.27	0.65	0.55	0.13	0.64
HBTM	*r*			1	-0.01	-0.05	-0.10	-0.05	-0.08	-0.13	-0.06
	P				0.94	0.78	0.52	0.74	0.64	0.44	0.72
DWV	*r*				1	-0.12	-0.09	+0.18	-0.10	-0.06	+0.18
	P					0.45	0.60	0.27	0.56	0.69	0.27
BQCV	*r*					1	+0.07	-0.07	-0.09	-0.12	-0.08
	P						0.67	0.66	0.59	0.45	0.64
SBV	*r*						1	-0.06	+0.14	+0.25	-0.05
	P							0.73	0.37	0.13	0.77
IAPV	*r*							1	-0.06	-0.08	**+1.00**
	P								0.70	0.63	**<.0001**
KBV	*r*								1	**+0.81**	-0.05
	P									**<.0001**	0.77
CBPV	*r*									1	-0.06
	P										0.70

§= see list of abbreviations for full names of parasites and pathogens (page ix).

Appendix 4 Simple correlations (Pearson's) for spring outdoor-wintered colonies between colony parasite (mean abundance of *Varroa* and prevalence of tracheal mite), pathogen (mean abundance of *Nosema*, log $2^{-\Delta Ct}$ of DWV, BQCV, IAPV, SBV, KBV, CBPV, and ABPV) levels, bee population score and bee loss. r = correlation coefficient, *P*= P-value. N=29

Parameter§		***Varroa***	***Nosema***	**HBTM**	**DWV**	**BQCV**	**SBV**	**IAPV**	**KBV**	**CBPV**	**ABPV**
Population	*r*	-0.23	+0.24	+0.13	-0.20	+0.22	-0.20	-0.10	-0.21	-0.26	-0.28
	P	0.22	0.21	0.49	0.31	0.25	0.31	0.60	0.28	0.17	0.14
Bee loss	*r*	+0.35	-0.24	-0.08	+0.25	-0.22	+0.19	+0.13	+0.24	+0.28	+0.27
	P	0.06	0.22	0.69	0.19	0.25	0.32	0.52	0.22	0.14	0.15
Varroa	*r*	1	-0.09	**+0.39**	**+0.53**	+0.04	-0.19	+0.11	+0.19	+0.21	+0.16
	P		0.64	**0.04**	**0.003**	0.83	0.32	0.55	0.31	0.28	0.41
Nosema	*r*		1	+0.12	-0.15	**+0.50**	-0.25	-0.04	+0.03	-0.12	-0.01
	P			0.55	0.44	**0.006**	0.20	0.86	0.89	0.55	0.94
HBTM	*r*			1	+0.21	-0.16	-0.08	-0.06	-0.07	-0.08	-0.12
	P				0.28	0.42	0.69	0.75	0.70	0.68	0.54
DWV	*r*				1	-0.04	+0.10	+0.02	**+0.65**	**+0.78**	+0.31
	P					0.85	0.62	0.91	**0.0002**	**<.0001**	0.11
BQCV	*r*					1	-0.14	+0.13	**+0.45**	+0.08	+0.15
	P						0.46	0.50	**0.01**	0.67	0.43
SBV	*r*						1	-0.06	+0.08	+0.15	+0.17
	P							0.76	0.67	0.43	0.39
IAPV	*r*							1	-0.04	-0.04	**+0.72**
	P								0.82	0.85	**<.0001**
KBV	*r*								1	**+0.81**	+0.28
	P									**<.0001**	0.15
CBPV	*r*									1	**+0.44**
	P										**0.02**

§= see list of abbreviations for full names of parasites and pathogens (page ix).

Appendix 5. Partial correlations (Pearson's) for fall indoor-wintered colonies between colony parasite (mean abundance of *Varroa* and prevalence of tracheal mite), pathogen (mean abundance of *Nosema*, log $2^{-\Delta Ct}$ of DWV, BQCV, IAPV, SBV, KBV, CBPV, and ABPV) levels, bee population score and bee loss. r = correlation coefficient, *P*= P-value, N=45

Parameter§		*Varroa*	*Nosema*	HBTM	DWV	BQCV	SBV	IAPV	KBV	CBPV	ABPV
Population	*r*	-0.37	0.11	-0.17	-0.36	-0.03	0.07	-0.50	-0.03	-0.09	0.10
	P	0.015	0.49	0.28	0.02	0.87	0.66	0.001	0.86	0.56	0.55
Bee loss	*r*	0.27	-0.17	0.09	0.32	0.15	0.14	0.43	0.30	0.11	-0.25
	P	0.08	0.27	0.57	0.04	0.34	0.38	0.00	0.06	0.49	0.11
Varroa											
	r	1	-0.04	-0.10	0.37	-0.12	-0.03	0.23	-0.02	0.01	-0.14
	P		0.82	0.52	0.02	0.45	0.86	0.15	0.92	0.93	0.39
Nosema											
	r		1	0.13	-0.30	0.03	-0.06	-0.03	0.07	0.00	-0.01
	P			0.40	0.06	0.87	0.72	0.85	0.67	1.00	0.94
HBTM	*r*			1	0.14	-0.08	-0.05	0.16	-0.17	-0.08	-0.08
	P				0.36	0.61	0.74	0.31	0.28	0.61	0.61
DWV	*r*				1	0.18	0.06	0.30	-0.10	-0.07	0.01
	P					0.26	0.72	0.06	0.54	0.64	0.97
BQCV	*r*					1	-0.09	0.02	0.36	0.03	-0.09
	P						0.58	0.91	0.02	0.85	0.58
SBV	*r*						1	-0.01	0.56	-0.16	-0.09
	P							0.95	0.0001	0.32	0.58
IAPV	*r*							1	0.42	0.62	0.04
	P								0.005	0.0001	0.78
KBV	*r*								1	0.27	0.01
	P									0.08	0.93
CBPV	*r*									1	0.10
	P										0.54

§= see list of abbreviations for full names of parasites and pathogens (page ix).

Appendix 6. Partial correlations (Pearson's) for fall outdoor-wintered colonies between colony parasite (mean abundance of *Varroa* and prevalence of tracheal mite), pathogen (mean abundance of *Nosema*, log $2^{-\Delta Ct}$ of DWV, BQCV, IAPV, SBV, KBV, CBPV, and ABPV) levels, bee population score and bee loss. r = correlation coefficient, *P*= P-value, N=30

Parameter§		***Varroa***	***Nosema***	**HBTM**	**DWV**	**BQCV**	**SBV**	**IAPV**	**KBV**	**CBPV**	**ABPV**
Population	*r*	-0.41	-0.41	-0.09	-0.46	-0.04	0.11	0.05	-0.37	0.18	0.05
	P	0.04	0.03	0.66	0.02	0.83	0.60	0.82	0.06	0.38	0.82
Bee loss	*r*	0.44	0.39	0.13	0.45	0.04	-0.01	0.02	0.35	-0.26	0.14
	P	0.02	0.05	0.52	0.02	0.84	0.95	0.94	0.07	0.19	0.49
Varroa											
	r	1	0.29	-0.08	0.71	-0.02	-0.02	0.13	0.18	-0.05	0.04
	P		0.14	0.68	0.0001	0.94	0.91	0.51	0.37	0.79	0.84
Nosema											
	r		1	-0.09	0.58	-0.01	0.06	-0.16	-0.10	-0.01	0.30
	P			0.65	0.002	0.96	0.77	0.44	0.63	0.97	0.13
HBTM	*r*			1	0.03	-0.09	-0.02	-0.12	0.01	-0.05	-0.15
	P				0.87	0.65	0.93	0.56	0.95	0.82	0.44
DWV	*r*				1	-0.26	0.15	0.15	0.11	0.00	0.01
	P					0.18	0.45	0.46	0.58	1.00	0.97
BQCV	*r*					1	-0.05	-0.14	-0.08	-0.10	0.05
	P						0.80	0.49	0.70	0.61	0.80
SBV	*r*						1	0.35	0.03	-0.12	0.24
	P							0.08	0.87	0.55	0.23
IAPV	*r*							1	0.37	0.11	0.45
	P								0.06	0.60	0.02
KBV	*r*								1	-0.02	0.07
	P									0.92	0.73
CBPV	*r*									1	0.04
	P										0.82

§= see list of abbreviations for full names of parasites and pathogens (page ix).

Appendix 7. Partial correlations (Pearson's) for spring indoor-wintered colonies between colony parasite (mean abundance of *Varroa* and prevalence of tracheal mite), pathogen (mean abundance of *Nosema*, log $2^{-\Delta Ct}$ of DWV, BQCV, IAPV, SBV, KBV, CBPV, and ABPV) levels, bee population score and bee loss. r = correlation coefficient, *P*= P-value, N=40

Parameter§		***Varroa***	***Nosema***	**HBTM**	**DWV**	**BQCV**	**SBV**	**IAPV**	**KBV**	**CBPV**	**ABPV**
Population	*r*	-0.17	0.21	-0.05	0.01	-0.37	-0.26	-0.06	-0.01	-0.03	-0.05
	P	0.32	0.21	0.75	0.93	0.02	0.12	0.71	0.97	0.85	0.78
Bee loss	*r*	0.14	-0.05	-0.10	-0.01	0.24	0.10	0.13	0.19	0.18	0.12
	P	0.42	0.75	0.56	0.94	0.16	0.58	0.45	0.26	0.29	0.49
Varroa											
	r	1	0.04	-0.20	0.00	-0.16	-0.03	0.16	0.13	0.04	0.17
	P		0.80	0.23	1.00	0.34	0.87	0.33	0.43	0.83	0.33
Nosema											
	r		1	-0.14	-0.11	0.45	-0.13	0.05	-0.14	-0.34	0.05
	P			0.42	0.53	0.01	0.45	0.77	0.41	0.04	0.75
HBTM	*r*			1	-0.05	0.01	0.00	-0.29	0.01	-0.03	-0.29
	P				0.78	0.96	0.98	0.08	0.96	0.86	0.08
DWV	*r*				1	-0.15	-0.05	0.16	-0.11	-0.09	0.16
	P					0.37	0.75	0.34	0.53	0.58	0.35
BQCV	*r*					1	0.13	-0.01	-0.13	-0.21	-0.01
	P						0.44	0.97	0.43	0.22	0.95
SBV	*r*						1	0.00	0.13	0.26	0.01
	P							0.99	0.45	0.12	0.94
IAPV	*r*							1	-0.01	-0.03	1.00
	P								0.94	0.87	0.0001
KBV	*r*								1	0.80	0.00
	P									0.0001	0.99
CBPV	*r*									1	-0.01
	P										0.96

§= see list of abbreviations for full names of parasites and pathogens (page ix).

Appendix 8. Partial correlations (Pearson's) for spring outdoor-wintered colonies between colony parasite (mean abundance of *Varroa* and prevalence of tracheal mite) and pathogen (mean abundance of *Nosema*, log $2^{-\Delta Ct}$ of DWV, BQCV, IAPV, SBV, KBV, CBPV, and ABPV) levels, bee population score and bee loss. r = correlation coefficient, *P*= P-value, N=29

Parameter§		*Varroa*	*Nosema*	HBTM	DWV	BQCV	SBV	IAPV	KBV	CBPV	ABPV
Population	*r*	-0.55	0.27	0.08	-0.71	0.16	-0.21	-0.14	-0.34	-0.48	-0.32
	P	0.004	0.19	0.69	0.0001	0.43	0.29	0.50	0.09	0.01	0.12
Bee loss	*r*	0.61	-0.37	0.08	0.59	-0.24	0.19	0.19	0.34	0.41	0.33
	P	0.001	0.06	0.70	0.001	0.25	0.36	0.35	0.09	0.04	0.10
Varroa											
	r	1	-0.09	0.27	0.40	-0.07	-0.19	0.06	0.04	0.04	0.14
	P		0.68	0.18	0.04	0.74	0.36	0.76	0.84	0.86	0.50
Nosema											
	r		1	0.10	-0.29	0.61	-0.22	-0.03	0.01	-0.17	0.00
	P			0.61	0.14	0.001	0.28	0.89	0.94	0.40	0.98
HBTM	*r*			1	-0.28	-0.12	-0.09	-0.06	-0.29	-0.42	-0.22
	P				0.16	0.56	0.65	0.79	0.15	0.03	0.29
DWV	*r*				1	0.09	0.15	0.06	0.63	0.71	0.31
	P					0.66	0.48	0.77	0.0005	0.0001	0.12
BQCV	*r*					1	-0.09	0.05	0.55	0.21	0.21
	P						0.65	0.82	0.004	0.30	0.29
SBV	*r*						1	-0.02	0.10	0.17	0.15
	P							0.91	0.63	0.42	0.48
IAPV	*r*							1	-0.05	-0.02	0.77
	P								0.80	0.94	0.0001
KBV	*r*								1	0.79	0.25
	P									0.0001	0.215
CBPV	*r*									1	0.43
	P										0.03

§= see list of abbreviations for full names of parasites and pathogens (page ix).

Appendix 9. Results for the binary logistic regression analysis on the fall and spring parasites and pathogens compared with live and dead colonies.

Fall

Factors §	df	F	P
Varroa	1, 67	0.09	P = 0.76
HBTM	1, 67	0.19	P = 0.68
Nosema	1, 67	0.51	P = 0.38
DWV	**1, 67**	**5.68**	**p = 0.02***
BQCV	1, 67	0.07	p = 0.77
SBV	1, 67	2.91	P = 0.09
IAPV	1, 67	0.16	p = 0.70
KBV	1, 67	0.92	p = 0.33
CBPV	1, 67	0.89	p = 0.34
ABPV	1, 67	2.10	p = 0.14

Spring

Factors §	df	F	P
Varroa	1, 67	1.58	P = 0.12
HBTM	1, 67	0.27	P = 0.62
Nosema	1, 67	0.53	P = 0.38
DWV	1, 67	1.09	p = 0.29
BQCV	1, 67	2.96	p = 0.09
SBV	**1, 67**	**7.32**	**P = 0.009***
IAPV	1, 67	0.02	p = 0.90
KBV	1, 67	0.43	p = 0.51
CBPV	1, 67	0.35	p = 0.55
ABPV	1, 67	0.13	p = 0.71

§= see list of abbreviations for full names of parasites and pathogens (page ix).

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