The Costs and Benefits of Resistance and Tolerance Behaviors against Varroa mite (*Varroa destructor* Anderson and Trueman) in Honey bee (*Apis mellifera* L.)

By

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GENERAL ABSTRACT:

Managed honey bee colonies face severe winter losses in northern climates. In my studies, interactions between genotypes of bees (genetically selected stock and unselected stock) with different levels of resistance and tolerance to varroa mites were assessed under a variety of treatment combinations to quantify effects of queen pheromone, acaricide treatment, wintering method, ventilation condition and pathogen infection on the costs and benefits associated with mite removal and mite-tolerance behaviors. In most of the experiments, mite-resistance caused greater varroa mite mortality within selected stock relative to unselected stock. Artificial and natural sources of queen pheromone caused greater varroa mite mortality within honey bee colonies relative to queenless colonies. While mite resistance had significant benefits, I showed that when producers selected colonies containing some mite resistance traits, it was traits associated with mite-tolerance and not mite-resistance were maintained and contributed to wintering success. Tolerance was effective at two levels of mites as obtained by late autumn treatment of colonies with oxalic but treatment did not improve wintering performance of either stock. Selected stock showed greater colony size, survival and resulted in more viable colonies in spring in comparison to unselected stock with similar initial mite levels (0.16 mites per bee). Selected stock showed greater relative wintering success than unselected stock when wintered indoors than when wintered outdoors but indoor wintering improved colony survival in both stocks relative to outdoor wintering. Carbon dioxide level increased within the winter bee cluster when colonies were maintained under restricted-ventilation (mean 3.82±0.031%, range 0.43-8.44%) and restricted ventilation increased mite mortality by 138% relative to standard-ventilation (mean 1.29±0.031%, range 0.09-5.26%), but restricted-ventilation did not affect bee

mortality in comparison to standard-ventilation. In a laboratory study, I showed that *Nosema* inoculation (with co-infections of *N. ceranae* and *N. apis)* suppressed the effectiveness of mite removal behavior within selected bees relative to unselected bees. *N. ceranae* was more abundant than *N. apis.* Bees with greater mite removal capacities had higher costs associated with varroa-resistance as indicated by greater bee mortality rates when inoculated with varroa but bee mortality was not affected in *Nosema* inoculated bees.

GENERAL INTRODUCTION:

The honey bee, *Apis mellifera* Linnaeus, is a vital component in ecosystem biodiversity and sustainability and is the most economically important pollinator of most crops can contribute to yield increases in 96% of entomophilous plants (Southwick and Southwick 1992; Klein et al. 2007). The contributions to pollination of the 600,000 managed-honey bee colonies in Canada have been estimated to be from CAN$1.3 billion to CAN $1.7 billion (Canadian Honey Council 2014). Therefore, global declines in honey bees, as well as other managed and wild bee pollinators have significant economic consequences (Currie et al. 2010; Potts et al. 2010).

In temperate climates, high winter colony mortality is an undeniable challenge for managed honey bee, colonies and is thought to be due to multiple infections with parasites - a parasite is "an organism that lives at its host's expense, obtaining nutriment from the living substance of the latter, depriving it of useful substance, or exerting other harmful influence upon it" (Bucher 1973) - and pathogens - a pathogen is "a microorganism capable of producing disease under normal conditions of host resistance and rarely living in close association with the host without producing disease" (Steinhaus and Martignoni 1967) - in association with a variety of other factors which induce stress (Ellis and Munn 2005; Higes et al. 2006; Oldroyd 2007). Varroa mite, *Varroa destructor* Anderson and Trueman, has been confirmed as a honey bee parasite with a main role in colony collapse, and thus it induces considerable cost to global apiculture (Currie et al. 2010; Guzman-Novoa et al. 2010; Neumann and Carreck 2010). To cope with this ectoparasite, apiarists typically have used chemical acaricides. However, the use of chemicals has several disadvantages: mites rapidly develop resistance to acaricides; their

disposal causes environmental pollution; they can cause residues in bee products; and they are costly to apply (Elzen et al. 1999; Wallner 1999). Genetically selected lines of bees have been advanced to moderate acaricide use in an attempt to improve colony survival through winter and to maintain levels of honey production and pollination services while relying on no or fewer chemical controls (Harbo and Hoopingarner 1997; Rinderer et al. 2001; Spivak and Reuter 2001; Ibrahim et al. 2007; Currie and Tahmasbi 2008; Danka et al. 2011). In this dissertation, groups of honey bee colonies that were selected through a co-operative breeding program for increased tolerance or resistance to varroa (referred to as "selected colonies") were compared to unselected colonies in lab and field experiments. The selected colonies were from stocks selected by the University of Manitoba and Manitoba Queen Breeders Association (MQBA), whereas "unselected colonies" were randomly chosen from the University campus bee yard pool from colonies headed with either local Manitoba queens, New Zealand queens or Hawaiian queens. The colonies used in my studies were selected for both ability to withstand high parasite loads without significant bee mortality (tolerance) during winter and the ability to reduce varroa mite burden through mite removal behavior (resistance).

This dissertation was written in manuscript style and consists of six chapters. In chapter 1, the literature review addressed details of the biology and management of the varroa mite and *Nosema*, costs and benefits of defensive mechanisms and reviewed what is known about interactions of colony environment, pathogens and their possible influence on resistance to varroa. In chapter 2, the effects of queen pheromone status on mite removal behavior in two different genotypes of bees under a simulated winter condition were quantified. In chapter 3, a field study assessed the effects of genotypes of

bee, acaricide treatment and wintering method (indoor vs. outdoor) on wintering success of two different genotypes of bees when exposed to high levels of varroa mite. In chapter 4, a set of field investigations was designed to characterize the effects of manipulated ventilation settings on atmospheric gases levels within the cluster of different genotypes of bees, and their interactive effects on varroa mite and worker bee mortality in indoor-wintering colonies. In chapter 5, I have assessed the interactive effects of co-infection with varroa and *Nosema* (*Nosema ceranae* Fries and *Nosema apis* Zander) on worker bees with different mite removal ability in a bioassay cage study. In chapter 6, the costs and benefits of mite-resistance and mite-tolerance behaviors under different management scenarios were discussed.

CHAPTER 1. LITERATURE REVIEW

Introduction:

High losses of *Apis mellifera* Linnaeus colonies have been associated with several biotic and abiotic factors. The main causes of winter mortality that are recognized throughout the world include high levels of ectoparasitic varroa mites (*Varroa destructor* Anderson and Trueman), tracheal mite [*Acarapis woodi* (Rennie)], small hive beetle (*Aethina tumida* Murray), fungi (*Nosema apis* Zander and *N. ceranae* Fries), viruses (e.g. ABPV, SBV and DWV), bacterial diseases (e.g. foulbrood), pesticides, miticides, queen age, poor genetic diversity, management stressors, genetically modified crops, bee nutritional fitness, and unusual weather (Table 1.1).

Of these factors, the varroa mite acting alone or in combination with other stressors is probably the most important cause of colony loss. *Varroa jacobsoni* Oudemans was initially identified in the Asian honey bees *Apis cerana* Fabricius in 1904 in Java, Indonesia and was subsequently described by Oudemans. This parasite was believed to have expanded its host range to include *A. mellifera* and spread throughout the world (Rath 1999; Sammataro et al. 2000). However, the parasite which was transferred to *A. mellifera* was actually a different species of varroa that has been described as *V. destructor* (Anderson and Trueman 2000). The original host, *A. cerana*, and the varroa mite have "adapted" to each other in a host-parasite relationship which rarely damages the colony (De Jong 1990; Anderson and Fuchs 1998; Anderson and Trueman 2000). However, in *A. mellifera* colony death in susceptible stock typically occurs within two-three years if colonies are not treated (Korpela et al. 1992; De Jong 1996).

To manage this ectoparasite, mite-infested colonies are typically treated with different kinds of acaricides. However, this approach has several disadvantages. The misuse and long-term application of acaricides has induced widespread acaricide resistance in mite populations (Lodesani et al. 1995; Hillesheim et al. 1996; Elzen et al. 1999), resulted in chemical residues in colonies and colony products (Faucon and Flamini 1990; Slabezki et al. 1991; Lodesani et al. 1992; Wallner 1999) and increased costs of honey production and pollination services. In order to avoid these disadvantages, it is necessary to develop alternative methods to manage varroa.

The use of resistant strains of bees has been suggested as an effective approach (Rinderer et al. 2001; Ibrahim et al. 2007; Currie and Tahmasbi 2008; Ward et al. 2008). The development of genotypes - "inheritable information that is internally coded, stored in DNA and carried by all living cells" (Gasche et al. 2003)- that are able to maintain mite populations below economic thresholds allows producers to keep healthier colonies, while minimizing difficulties and costs related to the use of miticides. Resistance mechanisms that have been identified as showing some success include grooming behavior, hygienic brood removal behavior, varroa sensitive hygiene (VSH), lower brood attractiveness and lower rates of mite reproduction within brood cells (Arechavaleta-Velasco and Guzman-Novoa 2001; Aumeier 2001; Ibrahim and Spivak 2006; Harbo and Harris 2009; Piccolo et al. 2010; Rinderer et al. 2010; Rosenkranz et al. 2010). Some lines of honey bees such as Russian bees and varroa sensitive hygiene bees (Harbo and Harris 2009; Rinderer et al. 2010; Danka et al. 2011) show better survival after being infested with varroa due to combinations of the behaviors listed above (Engels et al. 1986; Kulincevic and Rinderer 1988; Moosbeckhofer et al. 1988; Wallner 1990).

The literature review will address the biology and management of the varroa mite and *Nosema* spp., the costs and benefits of potential defence mechanisms against varroa, multiple mating and genetic diversity advantages in breeding plans, as well as how environmental effects interact with bee genetics to affect defensive behavior against varroa mites in honey bees.

Biological cycle and management:

Varroa mite:

Varroa is a species complex consisting of four or more species (*V. jacobsoni*, *V. destructor*, *V. underwoodi* Delfinado-Baker and Aggarwal, and *V. rindereri* de Guzman and Delfinado-Baker) and 18 haplotypes (Delfinado-Baker and Aggarwal 1987; Guzman-Novoa et al. 1999b; Anderson and Trueman 2000). *Varroa jacobsoni* consists of nine haplotypes that infest the Asian honey bee, *A. cerana*, in Malaysia–Indonesia. *Varroa destructor* consists of six haplotypes of mites that infest honey bee colonies on the mainland of Asia with different subsets of haplotypes being found in different geographical regions. The Korean haplotype of *V. destructor* is the most common and is found in North America, Europe, United Kingdom, Middle East, Africa and Asia (Anderson and Trueman 2000). The Japan haplotype (or Japan-Thailand strain) is also found in North America as well as South America, Japan and Thailand (Anderson and Trueman 2000; Navajas et al. 2010). *Varroa rindereri* has been reported from *A. koschevnikovi* Buttel-Reepen in Borneo, however, *V. underwoodi* has wide range of hosts (*A. cerana. A. mellifera, A. nigrocincta* Smith and *A. nuluensis* Tingek, Koeniger and Koeniger) and has been observed in southeast Asia (de Guzman and Rinderer 1999).

Matures and immatures of varroa feed on the hemolymph of adult and immature stages of honey bees. After a "phoretic" phase of 1-20 d on adult honey bees (Schulz 1984), mature female foundress mites enter brood cells containing 5th-instar bee larvae approximately 1 d before the cell is sealed (Fuchs and Muller 1988; Boot et al. 1992; Sammataro et al. 2000). The first egg is a male and is laid 60 h after cell sealing and completes development before females (protandry). Subsequent eggs are laid every 30 h and all develop into females. Varroa mites discriminate between cell types and prefer to enter drones cells over worker (Otten and Fuchs 1988; Fuchs 1992). However, reproductive success is higher in drone cells because the longer development period of drones allows more progeny to develop to maturity before the bees emerge (Ifantidis 1983; Rehm and Ritter 1989; Donze et al. 1996). Varroa mites nevertheless can successfully produce progeny in *A. mellifera* worker cells (Ifantidis 1983; Schulz 1984; Moosbeckhofer et al. 1988). In contrast, reproduction in worker cells is rare or absent in the original host, *A. cerana* (Koeniger et al. 1981; Koeniger et al. 1983).

Varroa parasitism decreases the weight of newly emerged bees, diminishes worker life span, reduces hypopharengeal glands, and reduces protein and carbohydrate levels in hemolymph (De Jong et al. 1982; Schneider and Drescher 1987). The varroa mite is also a vector of several pathogenic microorganisms of honey bees. It has been demonstrated that varroa can transmit chalkbrood spores [*Ascosphaera apis* (Maassen ex Claussen) Olive & Spiltoir] (Liu 1996) and viruses (Ball 1985; Chen et al. 2004; de Miranda and Fries 2008). The varroa mite also vectors *Hafnia alvei* Moller, which causes septicemia (Strick and Madel 1988) and transmits several other bacterial pathogens (Glinski and Jarosz 1992). Additionally, when honey bee colonies are infected

with varroa mite alone or in combination with viruses, reductions in morphological characters, brood rearing, foraging activity and honey production have been characterized (Schneider and Drescher 1987; Romero-Vera and Otero-Colina 2002; Gatien and Currie 2003; Garedew et al. 2004; Currie and Gatien 2006; Kralj and Fuchs 2006).

The impact of varroa on colonies varies with geography, climate and the strain of bees (De Jong et al. 1984; Anderson 1994). Therefore, data are required to establish economic thresholds to prevent colony collapse (Delaplane and Hood 1997) to protect spring colony population build up (Ostermann and Currie 2004) and to minimize losses in honey production (Gatien and Currie 2003). Thresholds are designed to prevent economic damage which "occurs when the value of damage to the host is greater than the cost of control" (Osteen 1993). A management program based on accurate sampling and treatments based on economic threshold should reduce the number of miticide applications, reduce the rate of development of mite resistance to acaricides, minimize the residues in bee products and reduce costs of treatment (Delaplane and Hood 1999). Varroa treatment thresholds associated with climate and area are based upon mean abundance of mite (mites per adult bee) or number of mites falling from the bee cluster on to a sticky board. In the Pacific northwest of the United States, 0.01 mites per bee (often reported as 1 mite per 100 bees or 3 mites per 300-bee ether roll) in April is recommended as the threshold for spring treatment. In this case the threshold is designed to prevent reductions in brood production and low adult bee populations in the following spring (Strange and Sheppard 2001). For the southeastern and northwestern United States, an August treatment threshold of 0.05 mites per bee (14-15 mites per 300-bee ether roll) is suggested to prevent colony loss in October (Delaplane and Hood 1999;

Strange and Sheppard 2001). Ellis and Baxendale (1996) recommended 0.02 mites per bee (6 or more mites per 300-bee ether roll) in August as economic threshold for Nebraska where the threshold is designed to allow colonies to enter winter with healthy bee population. Ostermann (2002) and Ostermann and Currie (2004) found relatively low levels of mite infestation reduce colony growth in the prairie region of Canada. This area has a short season for development and growth of honey bee colonies and honey production is significantly reduced when spring mite infestations are greater than 0.01 (1 mite per 100 bees) (Gatien and Currie 2003; Currie and Gatien 2006; Currie 2008). Producers in this region should treat infected colonies with mite levels greater than 0.03 mites per bee (3 mites per 100 bees) in late August to early September to prevent colony loss overwinter. Colonies with varroa mite levels less than 0.10 mites per bee (10 mites per 100 bees) in late October sampled at a time when no brood is present do not result in increased colony loss through winter if no other pathogens or parasites are present. However, a lower threshold is recommended for colonies which are infected with both tracheal mite and varroa mite (Currie 2008).

A number of treatment options are available to keep mites below the threshold. Management of varroa often involves using miticides such as synthetic pyrethroids (*t* - Fluvalinate and Flumethrin) (Ferrer-Dufol et al. 1991), amidine compounds (Amitraz) (Floris et al. 2001) and organophosphates (Coumaphos) (Milani and Iob 1998). Acaricide resistance to one or more of the these products has developed in most beekeeping regions (Milani 1994; Lodesani et al. 1995; Baxter et al. 1998; Elzen et al. 1998) and acaricide residues have been confirmed in bee colonies (Wallner 1999). A second approach uses “soft chemicals” such as formic acid, oxalic acid and botanical oils

to treat infested colonies (Imdorf et al. 1995; Charriere and Imdorf 2002; Bahreini 2003; Underwood and Currie 2004). Efficacy of these compounds often depends on their ability to spread within the hive and these dispersal mechanisms are often temperature dependent (Hoppe et al. 1989; Kraus and Berg 1994; Mutinelli et al. 1997a; Mutinelli et al. 1997b; Van Veen et al. 1998; Bahreini et al. 2004; Underwood and Currie 2007). Plant essential oils show some successes in managing honey bee parasitic mites (and several products are registered as acaricides), but they can have negative effects on colonies and variable efficacy that limits their use in apiculture (Imdorf et al. 1999; Melathopoulos et al. 2000a; Melathopoulos et al. 2000b). Biological controls have also been investigated. The potential of fungi *Hirsutella thompsonii* Fisher (Hypocreales: Ophiocordycipitaceae), *Beauvaria bassiana* (Ballsamo) Vuillemin (Hypocreales: Cordycipitaceae), *Verticillium lecanii* (Zimmerman) (Hypocreales: Clavicipitaceae), *Clonostachys rosea* (Link) Schroers, Samuels, Seifert and Gams (Hypocreales: Bionectriaceae) and *Metarhizium anisopliae* (Metschinkoff) Sorokin (Hypocreales: Clavicipitaceae) have been assessed in the laboratory and in the observation hives but are currently not reliable enough to be recommended as treatments (Chandler et al. 2001; Kanga et al. 2002; Peng et al. 2002; Shaw et al. 2002; James et al. 2006; Meikle et al. 2007; Hamiduzzaman et al. 2012). Cultural methods that trap varroa in brood (Calis et al. 1998; Wantuch and Tarpy 2009) or in screened bottom boards (Pettis and Shimanuki 1999; Hart and Nabors 2000; Ellis et al. 2001) do provide some level of control but usually have to be used in conjunction with acaricide treatments. Recently, molecular techniques such as RNA interference (RNAi) have been investigated to control varroa mite through silencing genes essential to the mites (Campbell et al. 2010). However,

none of the controls mentioned above are completely effective. The development of genetically resistant honey bee lines (Rinderer et al. 2001; Spivak and Reuter 2001) or lines with greater tolerance of varroa could allow producers to increase the treatment thresholds and/or to treat their colonies less often when less effective acaricides are applied.

Nosema:

Nosema apis and *N. ceranae* are endoparasitic microsporidian fungi that invade the digestive system of adult honey bees (Fries et al. 1996b). Zander (1909) isolated *N. apis* from European honey bee colonies in the early 1900's; however, the discovery of *N. ceranae* is more recent. Fries et al. (1996b) first reported *N. ceranae* spores isolated from *A. cerana* in China but it is now wide spread in the European honey bee (Higes et al. 2006; Huang et al. 2007) and has gradually replaced *N. apis* in *A. mellifera* colonies in many regions (Higes et al. 2006; Huang et al. 2007; Klee et al. 2007; Gisder et al. 2010). *Nosema ceranae* is also found in other species of *Apis* and *Bombus* (Fries 1993; Plischuk et al. 2009; Chaimanee et al. 2010; Suwannapong et al. 2010; Li et al. 2012). *Nosema* spores are typically identified and quantified by squashing bees and quantifying spore loads using a hemocytometer (Cantwell 1970; Shimanuki and Knox 1997). Since *N. ceranae* and *N. apis* spores are morphologically similar, it is difficult to quantify them through microscopic observation. Molecular techniques have been developed to identify and quantify *Nosema* infection (Klee et al. 2007; Martin-Hernandez et al. 2007; Higes et al. 2008; Chen et al. 2009a; Bourgeois et al. 2010; Hamiduzzaman et al. 2010). The use of molecular techniques to detect and quantify *Nosema* infection in honey bee colonies

provides better reliability and sensitivity relative to the standard microscopic method, also allows the detection of vegetative stages for both species at very low mean abundance (Higes et al. 2006; Klee et al. 2007; Martin-Hernandez et al. 2007). According to Hamiduzzaman et al. (2010) the minimum detection threshold for *Nosema* spores using the hemocytometer method is 50,000 spores per bee. Traver and Fell (2011a) showed 51% of samples with negative spore counts according to the standard microscopic method test positive in quantitative Real Time PCR.

Honey bee adults are infected when *Nosema* spores are ingested through infected food and water or through contact with infected hive equipment (Fries et al. 1996b; Higes et al. 2008). Growth patterns of *N. ceranae* and *N. apis* in the digestive tract are similar (Fries et al. 1996b). Mature spores germinate in the midgut of the host, inject their sporoplasm into the gut epithelial cell cytoplasm through the extrusion of a polar filament and then multiply producing millions of spores (Higes et al. 2007). Although both *Nosema* species infect gut epithelial cells, *N. ceranae* also occurs in Malpighian tubules, hypopharyngeal glands, salivary glands and fat bodies (Chen et al. 2009a). *Nosema* infection induces changes in the behavior and physiology of the honey bee (Goblirsch et al. 2013). Degenerated epithelial cells, reduced levels of fatty acids, proteins and digestive enzymes, diminished longevity, reduced bee populations, degenerated ovaries in queens, increased stress and altered polyethism have all been associated with *Nosema* parasitism in honey bee (Fries et al. 1992; Fries 1993; Dussaubat et al. 2010; Suwannapong et al. 2010; Goblirsch et al. 2013). *Nosema* also increases susceptibility to other pathogens as it affects the immune system of its host (Antunez et al. 2009).

Nosema ceranae may be more virulent than *N. apis* in some situations (Higes et al. 2007; Paxton et al. 2007; Forsgren and Fries 2010). In individual bees, *N. ceranae* showed a higher virulence then *N. apis* in bioassay cage study and infected bees died in as little as eight days after inoculation (Higes et al. 2007). However, in another lab study similar mortality rates were found for both *N. ceranae* and *N. apis* (Forsgren and Fries 2010). At the colony level, *N. ceranae* and *N. apis* induce the same mortality in infected bees suggesting there is no competitive advantage between the two species within hosts (Forsgren and Fries 2010). In many investigations, it has been concluded that *Nosema* parasitism is one of the major causes of colony loss (Nitschmann 1957; Tokarz et al. 2011). Highly virulent forms of *Nosema* and high colony mortality are consistently associated with *Nosema* infection in Spain (Martin-Hernandez et al. 2007; Higes et al. 2008; Higes et al. 2010b). However, some studies failed to confirm the role of *Nosema* infections in colony mortality or suggest *Nosema* may interact with other pathogens (Cox-Foster et al. 2007; Genersch et al. 2010; Gisder et al. 2010). Environmental differences may affect the impact and distribution of the two *Nosema* species in the honey bee colonies. According to Fries (2010) *N. ceranae* is better adapted to infect colonies in warmer climates and *N. apis* may be better adapted to infect colonies in colder regions.

Both *N. apis* and *N. ceranae* infections can be treated successfully with Fumagillin-B (Medivet Pharmaceuticals Ltd., Alberta, Canada) (Williams et al. 2008), Fumagillin-B is less effectives against *N. ceranae* than *N. apis* at lower doses of medicine (Huang et al. 2013). Lines of bees are being developed to minimize colony mortality in winter that results from exposure to varroa (Bahreini and Currie 2009), but little is known

about the influence of *Nosema* infection on defense against varroa and the effects of interaction between these parasites on winter colony loss. It is important to clarify how *Nosema* parasitism affects mite-defensive behavior in honey bees.

Defensive mechanisms, costs and benefits:

"Resistance is an adaptive response of the host to the presence of the parasite" (Langand et al. 1998). By definition resistance negatively affects the parasite and provides benefits for the host but may be costly for the host to maintain (Frank 1992; Langand et al. 1998). Reduced brood attractiveness (which prevents the parasite from invading brood cells at the optimal time), shorter post capping duration (which prevents varroa's offspring from completing development), hygienic brood removal (where workers remove or disrupt reproducing mites within brood cells) and grooming behavior (where adult bees remove phoretic mites from their bodies) all have negative effects on varroa (Arathi et al. 2000; Aumeier 2001; Ibrahim and Spivak 2006; Currie and Tahmasbi 2008; Piccolo et al. 2010). The benefits of behavioral resistance to the host that result from increased parasite death, lowered parasite reproduction or a reduction in prevalence of parasite would include increased worker and colony survival, and increased capacity for the colony to swarm and to produce reproductives (drones and queens) (Frank 1992; Langand et al. 1998; Rigby et al. 2002). The cost of such behavior involves loss of energy due to performing tasks related to resistance that may reduce measures of fitness in the host (Antonovics and Thrall 1994). There may also be fitness costs associated with maintaining traits for resistance when parasites are absent. In social insects, theory suggests the use of resistance as a defense should not occur when the abundance of

parasite is lower than a certain threshold, because the benefits could be lower than the expected costs of defense.

At low levels of parasitism, "tolerance" may be a more effective strategy (Jokela et al. 2000). Tolerance as a component of defensive mechanisms is defined as when the host organism sustains little damage in terms of crop yield or quality despite normal parasite or pathogen development (Schafer 1971). Tolerance has been observed in animals (Raberg et al. 2009), plants (Blouin et al. 2005) infected with viruses (Matthews 1970), insects (Painter 1951) and nematodes (Wallace 1963). Thus, tolerance to varroa would occur in honey bee colonies if bees endured severe parasites levels without activating defenses like grooming or causing severe losses in population. Africanized honey bees (*Apis mellifera scutellata* Lepeletier) also show greater tolerance to varroa when left untreated (Guzman-Novoa and Page 1999; Rosenkranz 1999).

Polyandry and benefits of genetic diversity:

Multiple mating by queens (polyandry) is common in social insects (Page 1986; Keller and Reeve 1994; Boomsma and Ratnieks 1996). A high level of polyandry is suggested for honey bees (*Apis* spp.) where mature queens mate with 8-27 males depending on the species (Woyke 1964; Palmer and Oldroyd 2000). During mating, *A. mellifera* queens receive an average of about six million sperm from each male (Kerr et al. 1962) which are mixed and stored in the queen's spermathecas for later utilization (Woyke 1964; Page and Metcalf 1982). Multiple mating increases the number of subfamilies within a colony (female offspring with the same father), so genetic diversity in the colony increases and genetic relatedness between sister nestmates decreases (Tarpy and Page 2002).

Sex determination in honey bees is based on a single locus with multiple alleles (Woyke 1963). Honey bees have a haplo-diploid reproduction system where fertilized eggs that possess two different alleles at the sex locus are heterozygous and develop into female workers and unfertilized eggs develop into haploid males (drones). If a queen mates with a closely related drone that shares the same sex allele the progeny will develop into a diploid male and is cannibalized within 72 h by workers (Woyke 1963). This reduces brood viability and negatively impacts colony fitness (Page 1980; Winston 1987). Multiple mating leads to increased colony fitness (Tarpy and Page 2002) because it reduces the risk of inbreeding depression.

High within colony genotypic diversity is also an important component of host defense against parasites in social insects. Polyandry increases genetic diversity within colonies of social bees (bumble bees and honey bees) and is thought to contribute to decreases in parasitic abundance, lower disease prevalence, lower colony mortality and increased fitness (Shykoff and Schmid-Hempel 1991; Schmid-Hempel 1998; Sherman et al. 1998; Schmid-Hempel and Crozier 1999; Tarpy and Page 2002; Tarpy and Seeley 2006). Genetically heterogeneous colonies are often parasitized less frequently and better able to prevent damage from parasites (Sherman et al. 1988; Schmid-Hempel 1998; Schmid-Hempel and Crozier 1999). For example transmission of the bumble bee trypanosome *Crithidia bombi* Lipa and Triggiani (Kinetoplastida: Trypanosomatidae) is lower in genetically heterogeneous groups of bees than in homogenous groups. The benefits of high genetic diversity within colonies to defend against parasites also benefit ants (Stuart and Page 1991; Snyder 1992; Carlin et al. 1993; Fraser et al. 2000), where lower mean abundance of parasites and pathogens occur within more diverse populations

(Sherman et al. 1988; Schmid-Hempel 1998; Baer and Schmid-Hempel 1999; Tarpy 2003). Increased parasite resistance associated with within colony high variability also has been confirmed in honey bee colonies for a small number of parasites and pathogens and viruses (Kulincevic 1986; Palmer and Oldroyd 2003; Tarpy 2003; Desai 2014).

Breeding for resistance to diseases and parasites:

Although, maintaining high within-colony and population level genetic diversity is important to prevent inbreeding and enhance defence against a wide array of stressors, the goal of most breeding programs is to create a genetically uniform stock with specific trait. Fortunately, it is possible to select for resistance or increased tolerance to parasites and pathogens in honey bee colonies, while maintaining enough genetic diversity to prevent problems with inbreeding depression. Honey bees have been selected for better resistance or tolerance to chalkbrood (Gilliam et al. 1988), tracheal mite (Gary et al. 1989; Danka and Villa 1996; de Guzman et al. 1998), *N. apis* (Rinderer et al. 1983; Woyciechowski and Krol 2001), American foulbrood (Rothenbuhler and Thompson 1956; Bamrick and Rothenbuhler 1961) and varroa mite (de Guzman et al. 1996; Harbo and Hoopingarner 1997; Bahreini and Currie 2007; Currie and Tahmasbi 2008). Most breeding programs for varroa resistance target traits that are present in varroa's original host (*A. cerana*) where varroa is usually present but remains at low non-damaging levels. Mechanisms of resistance in *A. cerana* that target varroa in brood include: infertility in female mites in worker brood, limited successful reproduction of mite in drone broods and decreased rates of parasitism in drone cells (Koeniger et al. 1983; Rath 1992; Tewarson et al. 1992; Rosenkranz et al. 1993). In *A. mellifera*, mite-resistant lines have

also been produced that target varroa in brood cells such as the suppressed mite reproduction line (SMR) later re-named the varroa sensitive hygienic line (VSH) (Harbo and Hoopingarner 1997; Harbo and Harris 1999; Harris 2007; Harris et al. 2010), the Minnesota hygienic line (Spivak and Reuter 1998; Spivak and Reuter 2001) and the Russian bee line (Rinderer et al. 2001; Rinderer et al. 2010). In colonies bred for VSH, the mites enter worker brood cells to feed and to reproduce but are either removed or have lower rates of reproductive success in worker cells. Mites may die in the cell without reproducing, produce no progeny, produce males only or produce progeny too late for them to mature (Harbo and Harris 1999). It is possible bees may also remove reproductive mites more often than non-reproductive mites (Ibrahim and Spivak 2004; Harbo and Harris 2005). Some stocks, like the USDA "Russian" stock have a variety of traits that work in concert to slow mite population growth and include resistance mechanisms in adult bees such as grooming (Rosenkranz and Liebig 2003; de Guzman et al. 2007; Buchler et al. 2010).

Apis cerana shows resistance to varroa in the adult stage by cleaning themselves more effectively through auto-grooming (self-grooming behavior) and allo-grooming (social-grooming behavior) where workers "dance" to encourage social grooming. *Apis mellifera* will also groom in response to external disturbance although it is not as effective as *A. cerana* in removing varroa (Fries et al. 1996a). In auto-grooming worker bees clean different parts of their own body with their legs, however, in allo-grooming the nest mates typically touch infected bees with their antenna and remove mites with their front legs and mandibles (Delfinado-Baker and Peng 1995). The "invitation dance" performed by worker bees (*A. mellifera*) when chalk dust is puffed onto the base of wings

involves vibrating body from side-to-side at a frequency of 4.2±0.2 Hz for 9.3±1.0 s while standing on the comb (Land and Seeley 2004). Grooming is not always successful because the flat oval shape of varroa's body is morphologically adapted to match its host and workers can not easily remove the parasite. Once a worker bee catches a mite, it sometimes bites it with its mandibles but this is not always the case and therefore the use of damaged mites to assess grooming efficacy has shown mixed results. Some malformations on mites are not a result of grooming and this also confuses assessments of previous research. Davis (2009) showed the irregular dimples on the idiosoma of varroa mites originate during mite development in brood cells and are not a result of crushing by worker mandible. However, other signs of damage appear to be created by bites from workers (Ruttner and Hanel 1992). Lodesani et al. (1996) found 10% of the mother mites (foundress) and 5% of daughter mites that enter the brood cells have damage on the dorsal shields. Damage to legs or mouthparts is more common than damage to the dorsal shield (Ruttner and Hanel 1992; Rosenkranz et al. 1997; Correa-Marques et al. 2000; Bahreini 2001). The rate of mite injury is recommended for use as a selection index for grooming response in breeding programs (Correa-Marques et al. 2000; Correa-Marques et al. 2002; Andino and Hunt 2011; Guzman-Novoa et al. 2012), but its validity is controversial because it is not always correlated with benefits to the host (Liebig 1997; Rosenkranz et al. 1997; Correa-Marques et al. 2000; Correa-Marques et al. 2002).

Since grooming behavior may be an effective resistance response in honey bees, one of the objectives in this thesis was to determine effects of environment, bee genetics

and pathogenic infections on the efficacy of grooming behavior in open-mated queens to inform development of breeding for this resistance mechanism in honey bee colonies.

Thermoregulation and ventilation:

Honey bees are able to balance the temperature within the nest through the use of various combinations of convection, evaporation and metabolic heat production (Kronenberg and Heller 1982; Fahrenholz et al. 1989; Van Nerum and Buelens 1997; Robert and Harrison 1998). Thermoregulatory behavior in winter bees involves individual physiological and behavioral feedback by worker bees which includes fanning behavior and clustering activity (Kronenberg and Heller 1982). Honey bees fan to evaporate water and circulate air in the colony when ambient temperature is high to cool the colony and also produce "metabolic" heat to maintain the brood nest temperature at about 35 °C (Esch 1960; Kronenberg and Heller 1982; Ritter 1982; Harrison 1987; Stabentheiner et al. 2002) and to avoid chill coma when ambient temperature is low (Esch 1960; Heinrich 1981; Stabentheiner et al. 2003). Mechanisms of dealing with cold temperature are of particular importance in northern regions.

At ambient temperatures below 10 °C individual honey bees immediately fall into chill coma (Hosler et al. 2000; Kovac et al. 2007). Honey bees display their lowest metabolic rate at chill coma and eventually it causes ionic imbalances, changes in cell membrane fluidity, reductions in efficiency of Na^+/K^--ATPase and disruptions in protein transport through cell membranes (Badre et al. 2005; Nilson et al. 2006). These induce ATP production and stop muscle and nerve function (Lighton and Lovegrove 1990). Bees that enter chill coma may be paralyzed and ultimately killed by long exposure to

low temperature. Sensitivity of individuals to chill coma depends on age, metabolic rate and protein content of bees. Pollen fed bees have lower chill coma temperature relative to controls not fed pollen (Free and Spencer-Booth 1960). Respiratory gases can also affect chilling responses in insects. In fruit flies carbon dioxide (CO_2) exposure increases chill coma recovery time and diminishes cold tolerance (Alonso et al. 2005; Nilson et al. 2006; Milton and Partridge 2008; MacAlpine et al. 2011) where lowered sensitivity to glutamate at the neuromuscular junction causes loss of motor ability (Badre et al. 2005). However, little is known about the effects of CO_2 on honey bee sensitivity to chill coma.

To avoid chill coma at the individual level worker bees increase their metabolic activity collectively. The main colony level response to low temperature is to form a cluster where bees are tightly grouped together. The bee cluster is formed when environmental temperature drops below 14 °C. Temperature is maintained between 8-12 °C in the periphery of the colony and averages 21.3 °C (12-35 °C) in the core during wintering (Wilson and Milum 1927; Simpson 1961; Johansson and Johansson 1979; Szabo 1985; Fahrenholz et al. 1989). Worker movement within winter clusters of bees is affected by ambient temperature. In order to minimize heat loss from the cluster bees decrease the cluster size (by up to 55% in terms of volume) and cluster surface area (by up to 40%) by tightly packing bees in the outer surface of the cluster (Severson and Erickson 1990; Stabentheiner et al. 2003). Individual bees move from colder temperature to warmer ones and vice versa so that there is a continued exchange of bees between the cold outer surface and warm center of the winter cluster. These thermoregulatory methods help honey bees remain active at very low temperatures while reducing energy consumption and preventing bees from succumbing to the cold.

While the colony is regulating temperature it must continually bring oxygen (O_2) into the hive for respiration and CO_2 must be expelled or it can reach levels that would be toxic to the bees. In wingless social insects, such as termites, ventilation is conducted via special nest structures which allow cool fresh air to circulate through the nest to avoid overheating and prevent high CO_2 concentration in the nest (Jackson 1957; Luscher 1961). In honey bees ventilation is a cooperative social activity where the internal nest environment is regulated through fanning behavior (Southwick and Moritz 1987). High CO_2 triggers increased fanning response and acts as the regulatory "switch" as O_2 depletion alone does not encourage fanning (Seeley 1974). Worker bees have a CO_2 sensor receptor on their antenna and use it to respond to changes in CO_2 level (Hazelhoff 1941; Lacher 1967b; Seeley 1974). Oxygen is controlled indirectly as its level is inversely correlated with changes in CO_2 in the cluster. A typical colony circulates an average of 0.42±0.01 L of air per minute during summer (Southwick and Moritz 1987). Honey bee colony-level ventilation involves two phases, an active expiration phase which is facilitated by fanning behavior and a passive inspiration phase without fanning.

Honey bees produce different amounts of CO_2 at different temperatures. Free and Simpson (1963) found CO_2 production in the bee cluster was lowest at ambient temperatures of 10 °C, and increases above or below this temperature. Worker bees react to lower temperatures by increasing their metabolic rate as discussed earlier (Free and Spencer-Booth 1958; Allen 1959) which in turn, increases CO_2 output. Rates of 111-117 µg of CO_2 per hour were reported for 6-10 °C (Milner and Demuth 1921; Free and Simpson 1963). Carbon dioxide concentration in the hive fluctuates greatly and is correlated with changes in temperature (Free and Simpson 1963; Nagy and Stallone

1976). The CO_2 concentration in the honey bee cluster ranges from: 0.2- 9.9% (Simpson 1950; Hallund 1956; Taranov and Mikailov 1960; Simpson 1961; Free and Simpson 1963; Seeley 1974; Van Nerum and Buelens 1997; Sugahara and Sakamoto 2009; Kozak and Currie 2011) under different conditions with higher levels occurring within winter clusters under colder temperatures.

According to Van Nerum and Buelens (1997) higher CO_2 levels (hypercapnia) in winter induce hypoxia (low O_2) in bees. At summer temperatures honey bees keep O_2 levels at close to 15% by controlling CO_2 concentration in the cluster and through frequent ventilation for cooling purposes. In winter with higher levels of CO_2 and lower levels of O_2 (sometimes less than 7.5%), bees switch to an ultra low metabolic rate that is similar to deep diapause in insects but it is temporary in nature (Van Nerum and Buelens 1997). They intersperse this with periods of fanning to circulate air through the cluster when required. The mechanism of inducing hypoxia in the winter cluster allows for efficient thermoregulation, lower water loss and lower food consumption while allowing bees to move as required for food acquisition or thermoregulation.

Changes in the dynamics of colony ventilation may affect varroa mite mortality. Previous studies show that honey bees are capable of reducing mite loads through winter and that differences in temperature, humidity and CO_2 levels in caged-bee studies may be associated with changes in varroa mite mortality (Currie and Tahmasbi 2008; Kozak and Currie 2011). Restricted-ventilation also appears to increase mite fall from bee clusters (Underwood and Currie 2005;2007) but the effects of restricted-ventilation, CO_2 and other environmental factors on varroa mite mortality in full size colonies and colonies with different levels of grooming ability needs to be quantified.

Objectives:

Because of the serious problems currently associated with the use chemical treatments for varroa, breeding lines of honey bees resistant to mites is recommended to help producers maintain the levels of mite infestation below economic thresholds (Rinderer et al. 2010). Mite resistant lines of bees could help minimize the risks and costs associated with acaricide use, improve bee health and decrease winter colony loss. Reduction of mite populations through selection for grooming behavior shows promise. Mite load reductions of up to 60% through wintering may be feasible under environmental conditions and wintering management systems in Canada (Underwood and Currie 2002). Currie and Tahmasbi (2008) identified genetic strains of bees that reduce mite load over winter through a resistant response, but the effectiveness of this behavior may be affected by interactions with other pathogens and ambient conditions. The long-term objective of this study was to develop effective and economical methods to reduce the impact of varroa mite on honey bees under Canadian apicultural management systems. The specific objectives of this thesis were to examine how factors related to environment and pathogenic infection interact with bee genetics to affect the ability of colonies to better tolerate or reduce their varroa mite population, to assess the potential biological costs (as measured by assessing worker longevity) and to determine if mite-resistant stock can be used in combination with acaricide treatments with lower efficacy to increase treatment thresholds to prevent winter loss.

Table 1.1: The main causes of winter honey bee colony loss which can act alone or in combination with each other.

Cause (s)	Locality	Literature source (s)
Varroa mite (*Varroa destructor*)	Canada, USA, Europe, Middle East, Japan	(Korpela et al. 1992; Currie et al. 2010; Dahle 2010; Gajger et al. 2010; Genersch et al. 2010; Guzman-Novoa et al. 2010; Topolska et al. 2010; Dainat et al. 2012a; vanEngelsdorp et al. 2012; van der Zee et al. 2014)
Tracheal mite (*Acarapis woodi*)	Canada, USA	(Furgala et al. 1989; Otis and Scott-dupree 1992; Downey et al. 2000; Currie 2001; Currie 2008)
Nosema (*Nosema apis, N. ceranae*)	Canada, USA, Europe	(Downey et al. 2000; Higes et al. 2006; Cox-Foster et al. 2007; Higes et al. 2009b; Bacandritsos et al. 2010; Currie et al. 2010; Gajger et al. 2010; Paxton 2010; Santrac et al. 2010; Topolska et al. 2010)
Small hive beetle (*Aethina tumida*)	USA	(Schafer et al. 2010)
Viruses (e.g. ABPV, SBV and DWV)	Canada, USA, Europe	(Berthoud et al. 2010; Carreck et al. 2010; Currie et al. 2010; Martin et al. 2010; Topolska et al. 2010; Dainat et al. 2012b; Desai 2014)
Queen age/ Genetic diversity	USA, Europe	(Brodschneider et al. 2010; Ellis et al. 2010; Gajger et al. 2010; Genersch et al. 2010; Tarpy and Pettis 2013; van der Zee et al. 2014)

Pesticides / Varroacides / Genetically modified crops	Canada, USA, Europe	(Desneux et al. 2007; Frazier et al. 2008; Chauzat et al. 2009; Nguyen et al. 2009; Brodschneider et al. 2010; Chauzat et al. 2010; Ellis et al. 2010; Medrzycki et al. 2010; vanEngelsdorp and Meixner 2010)
Bee nutritional fitness	Canada, USA, Europe	(Stankus 2008; Brodschneider et al. 2010; Currie et al. 2010; Ellis et al. 2010; Gajger et al. 2010; vanEngelsdorp and Meixner 2010)
Bacterial diseases	USA, Europe	(Chauzat et al. 2010; vanEngelsdorp and Meixner 2010)
Mangement stress	Canada, USA, Europe, Middle East	(Johansson and Johansson 1971; Berthoud et al. 2010; Currie et al. 2010; Ellis et al. 2010; Gajger et al. 2010; Giray et al. 2010)
Unusual weather	Canada, Middle East	(Currie et al. 2010; Giray et al. 2010)

CHAPTER 2. THE EFFECT OF QUEEN PHEROMONE STATUS ON VARROA MITE REMOVAL FROM HONEY BEE COLONIES WITH DIFFERENT GROOMING ABILITY

ABSTRACT:

The objective of this study was to assess the effects of honey bee (*Apis mellifera* Linnaeus) genotype and queen pheromone status on mortality rates of varroa mites (*Varroa destructor* Anderson and Trueman), mite damage, and mortality rates of honey bees. Twenty-four small queenless colonies were established in five-frame colonies containing either stock selected for grooming behavior against varroa (high rates of mite removal) (n=12) or unselected stock (n=12). Within each genotype four colonies were randomly assigned to be treated with one of three queen pheromone status treatments: (1) caged-mated queen, (2) a synthetic queen mandibular pheromone lure (QMP) (Pseudo Queen), or (3) queenless with no queen substitute. Colonies were randomly assigned to positions in an environmental chamber and held under constant darkness at 5 °C. The results showed overall mite mortality rate was greater in stock selected for grooming than in unselected stock. There was a short term transitory increase in bee mortality rates in selected stock when compared to unselected stock. The presence of both queen pheromone treatments (caged-mated queen and QMP) increased the mite mortality rate relative to queenless colonies but the effect was significant only on the first day of the experiment. Daily mite mortality rate was constant across dates for queenless colonies but varied within both mated-queen and QMP treatments over time. Queen pheromone treatment did not affect the proportion of damaged mites under these conditions. The

effects of genotype of bees on mite damage varied with time. On day one of the experiment the proportion of mites damaged was greater in unselected stock than in selected stock but the opposite trend was observed on day 5. Over all of the treatments, 39 percent of mites that fell from the bee cluster showed visible signs of damage. Damage to the idiosoma was found in 78% of injured mites, and damage to the legs was found in 51% of injured mites. Mites from selected and unselected stocks showed similar types of damage. In conclusion, this study showed differential mite removal of different stocks under low temperature but damage to mites was unrelated to mite mortality. The presence of queen pheromone from either caged-mated queens or QMP enhanced mite removal from clusters of bees relative to queenless colonies over short periods of time and increased the variation in mite mortality over time relative to colonies without queen pheromone. Queen status should be considered when designing experiments using bioassays for grooming response.

INTRODUCTION:

European honey bee (*Apis mellifera* Linnaeus) colonies act as a social superorganism in which workers, queens and drones perform different tasks related to colony growth, maintenance and reproduction (Winston 1987; Pankiw and Page 2001). Genetic components strongly affect a variety of honey bee behaviors such as defensive behavior (Guzman-Novoa and Page 1993; Pankiw and Page 2001), foraging and pollen hoarding behavior (Hellmich et al. 1985; Pesante et al. 1987), cleaning behavior (Robinson and Page 1988), swarming (Winston 1980) and mite-resistance behavior (Spivak 1996; Currie and Tahmasbi 2008; Harris et al. 2010; Rinderer et al. 2010; Guzman-Novoa et al. 2012). Although workers within colonies are typically progeny of a single queen, many genetically distinct worker subfamilies exist in each colony as a result of polyandrous mating because queens concurrently utilize sperm from several drones to fertilize their offspring (Page and Metcalf 1982; Laidlaw and Page 1984; Page 1986). Subfamilies often differ in their ability to perform behaviors like guarding at the nest entrance, removing dead bees from the nest, pollen foraging (Robinson and Page 1988) and grooming nest mates (Frumhoff and Baker 1988). External stimuli (environment) and stimuli within the hive (nutrition and pheromones) can interact with bee genetics to affect behavior and task performance (Pankiw et al. 1994; Mattila and Otis 2006; Currie and Tahmasbi 2008).

Pheromones play a major role in communication and social maintenance within colonies of social insects (Pankiw et al. 1994; Pankiw et al. 1995; Vander Meer et al. 1998; Winston and Slessor 1998; Pankiw and Page 2000). Honey bee queen pheromones influence various colony functions including queen production (Butler and Fairey 1964;

Butler and Simpson 1967; Winston et al. 1991), swarm suppression (Winston et al. 1991), attraction of workers during swarming (Velthuis and Es 1964; Butler and Simpson 1967), drone attraction (Gary 1961a; Butler and Fairey 1964), worker attraction to the queen (Gary 1961b; Zmarlicki and Morse 1964), pollen and nectar foraging (Currie et al. 1992; Higo et al. 1992; Naumann et al. 1994), comb building and brood rearing (Free 1987), orientation at the colony entrance (Ferguson and Free 1981), guarding behavior (Moore et al. 1987), hygienic behavior (Rothenbuhler 1964) and grooming (Post et al. 1987). Interactions between bee genetics and responses to pheromones are known to occur. For example, colony genotype affects pheromone-based retinue responses (Pankiw et al. 1994), mating behaviors (Collins 1979; Pankiw et al. 1995) and ovary activation in worker bees (Barron et al. 2001).

Synthetic queen mandibular pheromone (QMP) in the form of pseudo queen (PQ) (Pseudo Queen, Contech Enterprises Inc., Victoria, British Colombia, Canada) is able to mimic many of the effects of natural queen pheromone in the absence of a queen (Pankiw and Page 2003). For example, synthetic queen pheromone influences defensive behavior (Gervan et al. 2005), queen mating (Pettis et al. 1993), sucrose responsiveness (Pankiw and Page 2003) and comb building (Ledoux et al. 2001). But, it is not known if QMP affects grooming behavior against varroa mites (*Varroa destructor* Anderson and Trueman) in honey bees. Since assays of grooming behavior are typically carried out on individuals or groups of bees in queenless conditions, it is important to know what effects queen pheromone may have on grooming success and if this varies in different stocks of bees.

Grooming behavior is one of several defense mechanisms that honey bees use against the varroa mite (Bozic and Valentincic 1995; Arechavaleta-Velasco and Guzman-Novoa 2001; Currie and Tahmasbi 2008) and grooming response of bee genotypes is affected by external conditions (Currie and Tahmasbi 2008). Grooming can be assessed by indirect measure of mite fall from clusters of bees (Currie and Tahmasbi 2008), direct measure of bee behaviors (Andino and Hunt 2011) or by quantifying damage to mites (Wallner 1994; Andino and Hunt 2011; Guzman-Novoa et al. 2012). During grooming worker bees may injure mites with their mandibles (Moosbeckhofer and Derakhshifar 1986; Morse et al. 1991; Ruttner and Hanel 1992; Boecking et al. 1993; Moretto et al. 1993; Wallner 1994; Rosenkranz et al. 1997), but the type of body part injured and amount of damage is highly variable (Ruttner and Hanel 1992; Rosenkranz et al. 1997). Correa-Marques et al. (2000) found no correlation between the percentage of injured mites, resistance behavior and mite infestation level, suggesting that evaluation of the proportion of injured mites on bottom boards is not a consistently reliable measure of resistance. The use of damage criterion to assess grooming of varroa may be useful in some cases but may not categorize all behavioral components that can result in successful grooming events.

The objective of this study was to assess the effect of queen pheromone status on mite mortality rates, mite injury and bee survival within two groups of honey bee colonies with different grooming ability.

MATERIALS AND METHODS:

The experiment was carried out at the University of Manitoba, Winnipeg, Manitoba, Canada (49°54' N, 97°14' W). Bees from European honey bee colonies used in this study are referred to as "selected" or "unselected" stocks. "Selected" stock was obtained from the Manitoba Queen Breeders Association from a pool of colonies that had been selected for a combination of criterion related to resistance (ability to reduce mite load overwinter) and tolerance (ability to tolerate high mite infestations with below average bee loss). Four "unselected" control stock colonies were chosen that were originally headed by New Zealand queens from a single supplier (Arataki Honey, Havelock North, New Zealand). To minimize genetic variation among colonies, one hive from "selected" stock with a high level of grooming response was chosen through a pre-bioassay test at 25 °C and 55-65 RH% (for more details see Currie and Tahmasbi 2008) and bees from the four New Zealand colonies were pooled to form source bees for making the unselected stock colonies. In summer 2007, small colonies were established from the selected stock (n = 12) and unselected stock (n = 12) in nucleus hives (5 frame standard Langstroth hive bodies) with an average of 5,538±153 mixed-age worker bees and average mite loads that were below 1.65 mites per 100 bees. Within each stock the twelve independent queenless colonies were then randomly assigned to one of three "queen pheromone status" treatments: (1) caged-mated queen, (2) synthetic QMP lure, or (3) queenless with no queen or pheromone substitute. Each mated-queen was caged in a JZ/BZ plastic queen cage (QC-800, Mann Lake, Hackensack, MN, USA) and placed between two frames in the center of the colony. The QMP treatment was queenless but contained a Pseudo Queen Lure as a queen substitute. The lure was placed in the center

of the colony. All colonies (n = 24) were then randomly assigned to locations in a temperature-controlled environmental chamber (208 x 208 x 273 cm = 11.81 m^3 in the Animal Science/Entomology building, University of Manitoba) and held in constant darkness at 5 °C. The temperature (°C) and relative humidity (%) inside the room were monitored using a HOBO C-8® (Onset Computer Corporation, Bourne, MA, USA) data logger.

All colonies were inoculated with 70 live varroa mites. Mites for this experiment were collected from a separate set of highly infested colonies using a modification of the carbon dioxide (CO_2) method (Ariana et al. 2002). In this method, bees infested with varroa were placed in a box with a screen bottom and put in a Rubbermaid (3.5 L TakeAlongs™, Rubbermaid, Mississauga, ON, Canada) container. The container was "then agitated at 400 rpm for 10 min on a Labline® (Fisher, Ottawa, ON, Canada) orbital shaker table" while being exposed to CO_2 (5 $L.min^{-1}$) (for more details see Currie and Tahmasbi 2008). Mites falling onto the bottom of the container were collected and placed in Petri dishes lined with a moist paper towel. The mites were then introduced into the small hives by a fine-tipped paint brush through a wire mesh screen (8 squares per inch) that covered the top of the hive and prevented bees from flying out. The mites were placed directly on the bees. To monitor mite and bee mortality, a piece of white poster board (19 x 61 cm) completely covered with wax paper was placed on the bottom board of each hive so varroa mites and worker bees that fell from bee cluster could be collected on daily basis. Each hive had a completely open bottom entrance (19 x 2.5 cm). Additional dead bees and mites were collected outside of hive entrance in a dead bee trap (consisting of an Aluminum, three-sided tray, 29 x 19 x 8 cm).

To assess bee population size in colonies at the end of the experiment, colonies were viewed visually from both top and bottom and the numbers of frame seams completely covered with bees was scored (each frame seam = approximately 2430 worker bees) (Burgett and Burikam 1985; Underwood and Currie 2005). The mean abundance of varroa mites [arithmetic mean of the number of varroa mites per bee (Bush et al. 1997; Rozsa et al. 2000)] in each hive was estimated on the final sample date by collecting a sample of adult bees (200-300 worker bees) and using an alcohol wash technique to remove the mites from the bees (for more details see Gatien and Currie 2003). Mites were removed from dead bees according to Gatien and Currie (2003) by agitating bees in 70% ethanol for 10 min using a Labline® orbital shaker (Fisher, Ottawa, ON, Canada) rotating at 200 rpm. After each sample was shaken, the basket with bees was removed and varroa mites in the alcohol and on the dead bees were counted. In order to confirm that equal numbers of mites were present in each treatment group at the beginning of the experiment, I calculated the initial mean abundance. This was done by adding all mites that dropped during the experiment to those remaining on live bees at the end of the experiment (as measured by alcohol wash). Initial mean abundance was determined by dividing the total number of mites by the total number of bees present in the colony on day zero. The rates of daily worker bee and varroa mite mortality were calculated using the following equation (Martin 1998):

$$\text{Daily mortality rate} = 1 - [(1 - a/100) \wedge 1/b]$$

where a denotes percentage of bees or mites lost and b represents length (day) of each sampling period. The "dead mite" values include the number of mites that fell from the bee cluster onto the bottom board, mites found on dead bees on the bottom board and

mites found in the dead bee trap. The "dead bee" values consist of the number of bees that fell onto bottom board and into the dead bee trap. The varroa mites removed from bottom boards and dead bee traps of each hive were classified as to the types of injury found on the idiosoma (dorsal shield), ventral shield, mouthparts, legs or a complex of these parts (Fig. 2.1). Normal "dimples" that can occur on mites were not categorized as damage (Davis 2009). The proportion of injured mites was assessed by dividing the total number of injured mites by the total number of varroa mites that dropped from the bee cluster onto the bottom board of each hive.

STATISTICAL ANALYSIS:

The daily mortality rates of worker bees and varroa mites and the proportion of injured mites were analyzed by a repeated measures analysis of variance (ANOVA) using a compound symmetry covariance structure with stocks of bees and queen pheromone status as main effects and day as repeated measure (PROC MIXED, SAS Institute Inc. 2011). Categorical comparisons among different injury categories in different genotypes of bees and treatments were analyzed by the maximum likelihood method (PROC CATMOD, SAS Institute Inc. 2011). Proportions were arcsine transformed prior to analyses (Snedecor and Cochran 1980). All data are presented as untransformed means. Where significant interactions were observed, they were partitioned using the SLICE option in LSMEANS statement and differences among treatment means were compared using Tukey's test (PDiff, PROC MIXED, SAS Institute Inc. 2011).

RESULTS:

Daily mite mortality rate was higher in the selected stock than the unselected stock ($F = 4.54$; df = 1, 18; $P = 0.05$) (Fig. 2.2), but there was no interaction between genotype of bees and queen pheromone treatment ($F = 0.37$; df = 2, 18; $P = 0.69$) and the three way interaction between genotype of bees*queen pheromone*time was not significant ($F = 1.18$; df = 8, 72; $P = 0.32$). For daily mite mortality rate there was a significant queen pheromone treatment*time interaction ($F = 2.27$; df = 8, 72; $P = 0.01$). Colonies with QMP or caged-mated queens had similar mite mortality rates but both were higher than queenless colonies (Fig. 2.3). LSMEANS slices of the pheromone treatment*time interaction by day showed that significant differences between treatments occurred only on the first day of experiment. Slices by treatment showed mite mortality rate was stable in queenless colonies but fluctuated over time for both caged-mated queen and QMP treatments (Fig. 2.3) (Table 2.1).

The overall daily rate of bee mortality did not differ between different pheromone source treatments (mated-queen: 0.0041±0.0014, QMP: 0.0032±0.0014 and queenless: 0.0038±0.0014) ($F = 0.07$; df = 2, 18; $P = 0.93$) or with genotype of bees (selected bees: 0.0053±0.0012; unselected bees: 0.0021±0.0012) ($F = 4.12$; df = 1, 18; $P = 0.06$). However, the genotype of bees*time interaction was significant ($F = 2.98$; df = 4, 72; $P = 0.03$). Bee mortality in selected bees was higher than in unselected bees only during the first 2 d of the experiment (Fig. 2.4). The interactions between queen pheromone treatment*genotype of bees ($F = 0.61$; df = 2, 18; $P = 0.55$), queen pheromone treatment*time ($F = 0.26$; df = 8, 72; $P = 0.98$), and queen pheromone treatment*genotype of bees*time ($F = 0.74$; df = 8, 72; $P = 0.65$) were not significant.

Measurement of the proportion of damaged mites averaged over all treatment combinations showed a significant genotype of bees*time interaction ($F = 4.46$; df = 4, 72; $P = 0.003$). The proportion of injured mites was higher in unselected bees than in selected bees on the first day of the experiment but the reverse trend was seen on day five (Fig. 2.5). The proportion of injured mites in different pheromone source treatments (mated-queen: 0.20±0.08, QMP: 0.25±0.08 and queenless: 0.33±0.08) was similar ($F = 0.42$; df = 2, 18; $P = 0.67$). The most frequent category of damage was injury to the idiosoma (dorsal shield) followed by damage to the legs, with damage to the mouthparts being rare. Complexes of either idiosoma, ventral shield and legs or idiosoma, ventral shield, legs and mouthparts occurred in 11 to 20% of cases (other possible combinations showed no damage) (Table 2.2). The proportion of damaged mites in different injury categories was similar between different genotypes of bees ($\chi^2 = 1.64$; df = 4; $P = 0.80$), and among queen pheromone treatments ($\chi^2 = 1.03$; df = 8; $P = 0.99$) and there was no significant genotypes of bees*queen pheromone treatment interaction ($\chi^2 = 3.62$; df = 8; $P = 0.89$).

DISCUSSION:

In this study, the effects of queen pheromone status and genotype of bees on mite mortality rates of *V. destructor* were assessed in honey bee colonies. As expected, colonies established from stock selected for increased rates of grooming displayed higher mite mortality rates when compared to colonies established from unselected stock. The difference in mite mortality between stocks could be a result of higher grooming, higher loss of bees infested with mites or other factors that influence mite mortality. When

worker bees were treated with different queen pheromone treatments under simulated winter conditions, queen pheromone addition (through the use of PQ lures or caged-mated queens) affected mite mortality rates relative to those in queenless colonies in two different ways. First, pheromone addition caused a short (1 d) transitory increase in mite mortality relative to queenless colonies. Second, both the queen pheromone and caged-mated queen treatments increased the daily variability in mite mortality rates over time relative to the queenless colonies where mortality rates were similar over time.

The effects of queen mandibular pheromone on grooming against varroa have not been studied. However, Naumann (1991) has shown bees initiate a range of grooming behaviors after coming in contact with queen pheromone. These same grooming behaviors may be related to the enhanced rates of varroa removal from the colony that I observed when bees were exposed to the two queen pheromone treatments, but I was not able to observe bees directly in my experiment.

The short-duration effects of queen pheromone on mite mortality rates relative to queenless colonies may be related to changes in release rates from both PQ lures and caged-mated queens. Pseudo queen is a synthetic pheromone used commercially to improve queen survival in mating nuclei, inhibit swarming or as a temporary queen replacement (Winston and Slessor 1992; Pettis et al. 1993). Mean daily release rates of pheromone averaging 0.3 to 1.4 queen equivalents of QMP are released from PQ over a five-day period, but the amount released decreases over the first two days (Gervan et al. 2005). The amount of QMP released from PQ was not measured in my study, but the diminished response after one day suggests a dose-related reduction may have been at least partly responsible for lower mite mortality rates found after day one. Similar effects

may have occurred with the caged-mated queens. Queens were caged in this experiment to prevent confounding effects that would result from brood production by the queen and to prevent queen movement so that it would make pheromone dispersion similar to the stationary lure. Worker bees directly or indirectly (through queen-to-bee and bee-to-bee contacts) receive and disperse pheromone from the queen. Thus, a queen "running free" through the colony is better able to disperse pheromone (Naumann et al. 1991; Gervan et al. 2005). Higher variability in mite mortality rates that I observed in both the queen and pheromone treatments relative to queenless colonies may have been related to uneven fluctuation dispersion of pheromone from the lure or caged-mated queens. However, I did not measure daily release rates or have a free-running queen treatment in my experiment so this could not be assessed.

In addition to releasing short-term behaviors like retinue behavior in worker bees (Winston and Slessor 1992), queen mandibular pheromone acts as a primer pheromone having long-term effects on bee physiology and subsequent age-based behaviors of honey bees. Juvenile hormone titer, for example, is lower in QMP-treated worker bees (Kaatz et al. 1992; Pankiw et al. 1998) and juvenile hormone affects age polyethism in honey bees (Robinson 1987). If longer term effects associated with grooming resulting from pheromone application occur, they would not likely be evident in my study due to the short duration of the experiment. I can not exclude the possibility that age-based changes resulting from queen pheromone influence colonies ability to remove mites from their cluster if exposed to QMP for longer periods, but this requires further study.

Genotype of bees influences worker response to queen pheromone in some contexts. For example, Pankiw et al. (1994) showed New Zealand queens show higher

retinue responses than Australian queens in QMP retinue bioassays. My experiment showed the synthetic QMP (in the form of the PQ Lure) had similar effects on mite mortality rates in colonies established from two different sources of bees although further testing on colonies with a broad range of genetic diversity is required. If QMP influences grooming across a wide array of genetic sources of bees it may have a role as an alternative or a supportive tool to improve management of varroa mites in commercial operations, but this needs testing in commercial operations on longer time scales.

Currie and Tahmasbi (2008) showed mite removal from caged bees is affected by interactions between environmental conditions and the genotype of bees. Under conditions that simulated winter temperatures of 5 °C, grooming was less effective and there appeared to be a transient cost associated with grooming at low temperatures (Currie and Tahmasbi 2008). In the larger colonies utilized in this study at low temperature (5 °C), I found greater mite removal in colonies established from the selected stock than in the colonies established from unselected stock. There was also a short transitory cost in terms of increased bee mortality rates in the selected stock when compared to the unselected stock in the first two days of the experiment. However, the overall bee mortality rates did not differ between these two treatments. The increased rates of bee mortality in selected stock may have contributed to increased mite mortality if those bees that died were also mite infested. However, this was not likely to have been responsible for increased mite mortality in the queen pheromone treatments as queen treatment did not affect bee mortality rates. Longer term studies are required to get a true measure of the potential costs of resistance.

The use of mite damage as an indicator to select and to breed resistant stocks with enhanced grooming is still debatable (Liebig 1997; Boecking and Spivak 1999; Correa-Marques et al. 2000). In numerous studies, higher percentages of injured mites found on the bottom board of the hives are suggested as criterion for selection for grooming responses that provide resistance to varroa (Ruttner et al. 1984; Peng et al. 1987; Moosbeckhofer 1992; Correa-Marques et al. 2000; Arechavaleta-Velasco and Guzman-Novoa 2001). Guzman-Novoa et al. (2012) showed significant correlations between the proportion of injured mites and mite removal rate at the colony level. They also found mite-resistant strains of bees show higher mite damage than unselected genotypes. Correa-Marques et al. (2002) suggest mite damage is not sufficient to explain resistance of Africanized bees (*Apis mellifera scutellata* Lepeletier) against varroa mite. My results were mixed as the proportion of injured mites was greater in unselected colonies than in selected colonies early in the experiment but the reverse was true later in the experiment. From the results of their study and mine it appears that successful grooming can occur without visible damage to mites. Other mechanisms that increase mite fall from clusters without resulting in visible mite damage may be present. In my study the reason why the proportion of mites damaged fluctuated with time in selected colonies relative to unselected colonies could be related to temperature. My study was conducted at 5 °C and mites that fell to the bottom board as a result of "grooming" or some other mechanisms would have difficulty relocating a host. The cold temperature would interfere with host seeking and questing behaviors by the mite and reduce the number of potential host bees on the bottom board (as they would remain in the winter cluster at this temperature). Thus, mites that are removed by bees in these conditions may be less likely to be

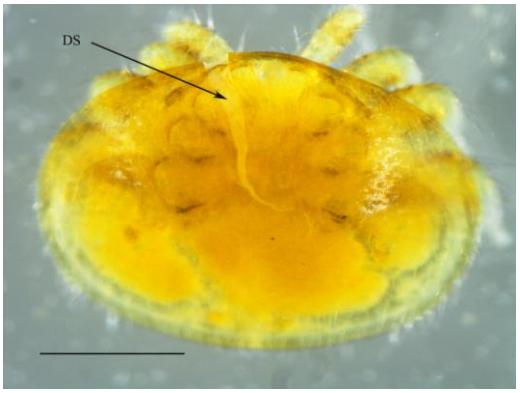
damaged after removal from individual bees (when they are on comb) or removal from the colony (on the bottom board of the hive). Other studies that showed positive relationship between mite damage and resistance to mites were conducted at warmer temperatures where undamaged mites may have been more likely to relocate potential host bees and be removed multiple times or be damaged by bees after being removed from the bee's body.

Correa-Marques et al. (2000) classified mite damage into six categories and show injured legs are more frequent than other types of damages. Phoretic varroa mites use the front legs to attach to the body of host, and these legs are therefore most likely to be subjected to breakage during attempts to remove the mites by the worker bees (Zaitoun et al. 2001). Several studies confirmed that damage to legs is the most frequent category of injury [19.3% (Boecking and Ritter 1993), 22.8% (Zaitoun et al. 2001), 23% (Bahreini 2001), 25% (Rosenkranz et al. 1997), 46-47.4% (Correa-Marques et al. 2002), 30-50% (Ruttner and Hanel 1992) and 54-72% (Correa-Marques et al. 2000)] and damage to legs, alone or in conjunction with other damage categories, in my study was also frequent (11-51%). It has been suggested that injured mite legs are indicative of an active defense against varroa (Ruttner and Hanel 1992) but my results do not agree as injury categories did not differ with genotype of bees. The type and degree of mite damage also did not vary with queen pheromone treatment. The most frequent category of "unique" injury occurred on the dorsal shield (47%). In a study on Africanized and European honey bee colonies, approximately 16% and 37-47% of the mites had injured dorsal shields, respectively (Correa-Marques et al. 2000). In addition to worker-worker grooming, damage to the body of varroa mites can also result from hygienic brood

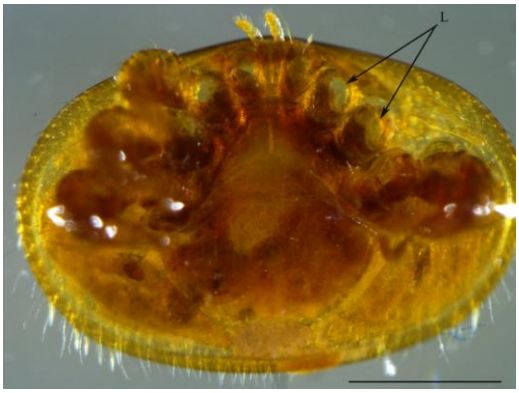
removal behaviors (usually distinguished from auto- and allo-grooming of adults) or other commensal animals or scavengers in the hive (Rosenkranz et al. 1997; Guzman-Novoa and Page 1999; Harbo and Harris 1999; Davis 2012). In my experiment, hives were broodless and maintained under controlled conditions, that excluded external sources of damage from scavengers. Therefore, damage that was observed would be due to grooming responses of bees during the process of removing mites or damaging them after they were removed.

In summary, my study showed the presence of queen pheromone or caged-mated queen caused transient increases in mite mortality relative to queenless colonies that lasted for about a day, and queen pheromone treatments increased the variability in mite mortality rates over a period of five days relative to queenless treatments. My findings revealed that the colonies from selected stock removed more varroa mites than the colonies from unselected stock under low temperature. Injury signs on the mites' bodies commonly associated with grooming behavior were not reliably linked to grooming success as measured by mite mortality rates. My results suggest assessment of mite damage may not be reliable as a selective criterion for breeding programs under low temperatures. Further studies are needed to define the range of environmental conditions where it might be useful.

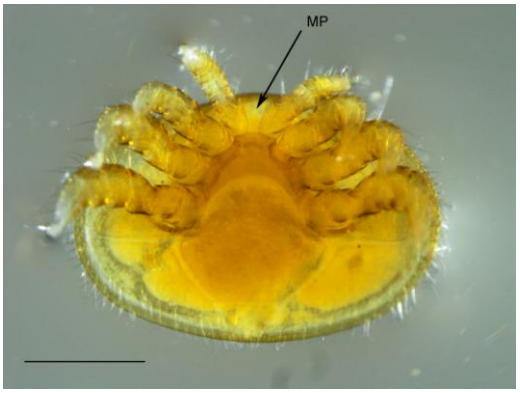
Fig. 2.1: Injuries in adult *V. destructor* collected from the bottom of hives. Mites that fell from bee cluster showed signs of damage to dorsal shield (DS) (*i*), legs (L) (*ii*), mouth parts (MP) (*iii*) and complex damage to legs, dorsal and ventral (VS) shields (*vi*). The scale bars represent 0.5 mm.



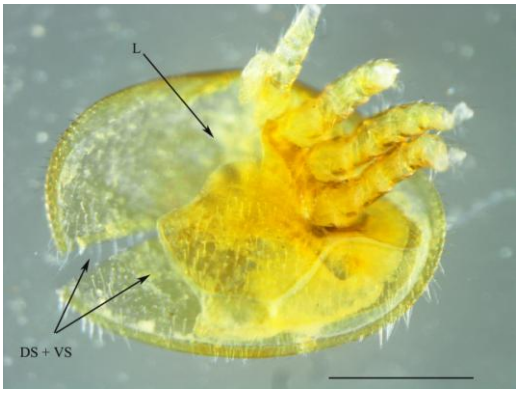
i



ii



iii



vi

Fig. 2.2: Mean daily mortality rate of varroa mites in selected bees and unselected bees. Vertical lines on each bar indicate ± standard error (SE). Means followed by the same letter between different genotypes of bees are not significantly different.

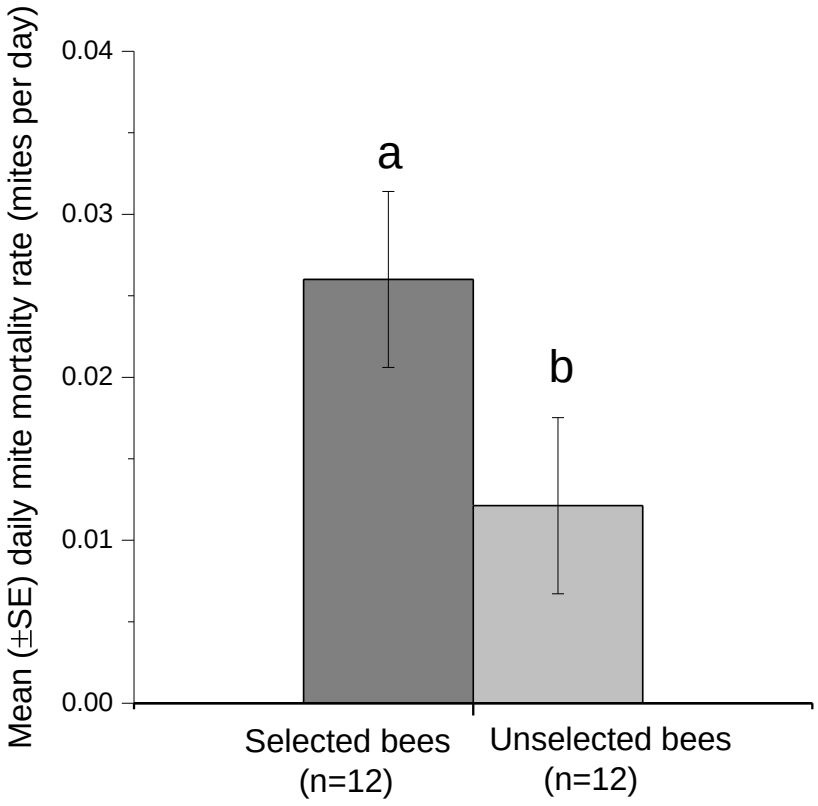


Fig. 2.3: Effects of queen pheromone treatment and time on mean daily mortality rate of varroa mites. Vertical lines on each bar indicate ± standard error (SE). Asterisk (on legend) indicates a significant difference within queen treatments ($P < 0.05$, Slice) and *ns* represents a non-significant difference among periods within treatments (see text for results of LSMEANS slice option). Means followed by the same letter among days within queen treatments and within days among queen treatments (horizontal line) are not significantly different.

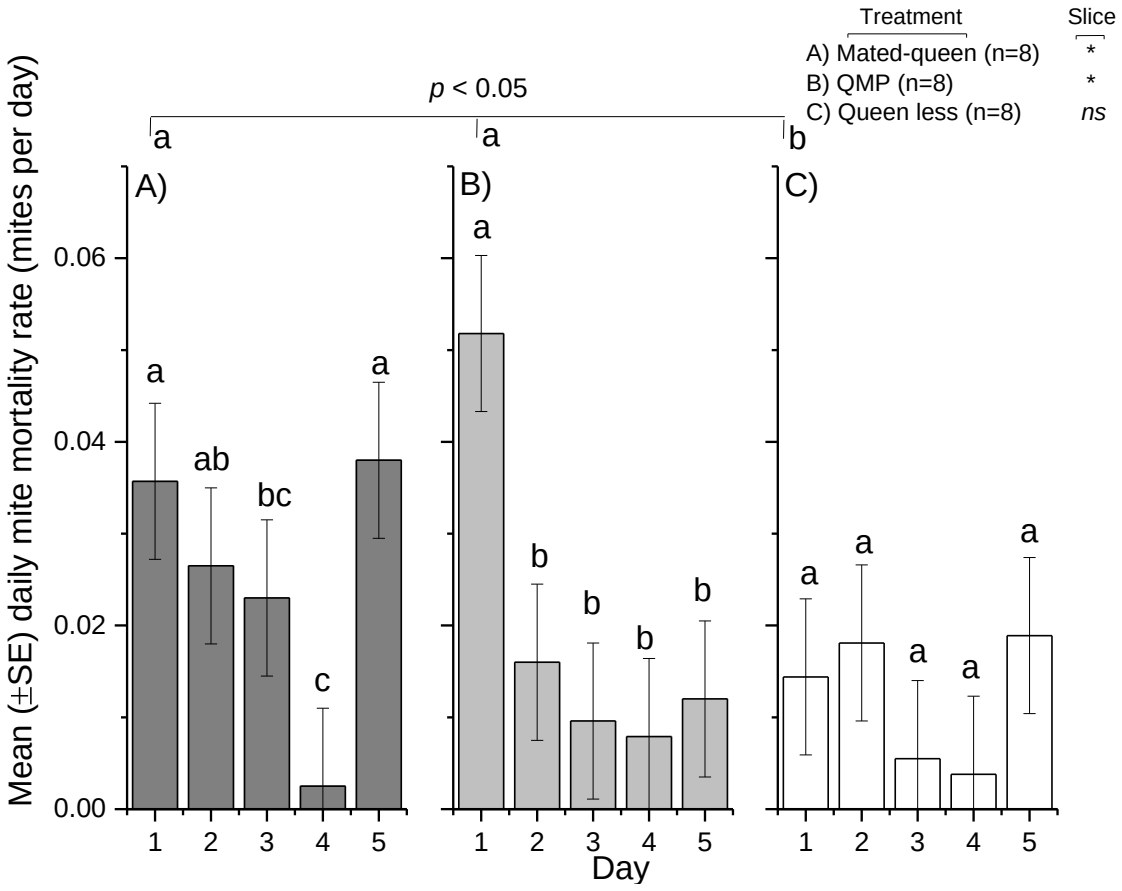


Table 2.1: Summary of LSMEANS slice option results for the significant treatment*time interaction by day and by queen pheromone treatment for daily varroa mite mortality rate.

Slice by day	F	df	*P*
1	3.64	2, 72	0.037
2	0.11	2, 72	0.895
3	0.07	2, 72	0.932
4	0.48	2, 72	0.623
5	2.59	2, 72	0.082
Slice by treatment	**F**	**df**	***P***
Queenless	1.28	4, 72	0.288
Caged-queen	8.09	4, 72	<0.0001
QMP	11.00	4, 72	<0.0001

Fig. 2.4: Mean daily worker bee mortality in different genotypes of bees during experiment. Vertical bars on each point indicate ± standard error (SE). Means followed by the same letter among genotype of bees are not significantly different.

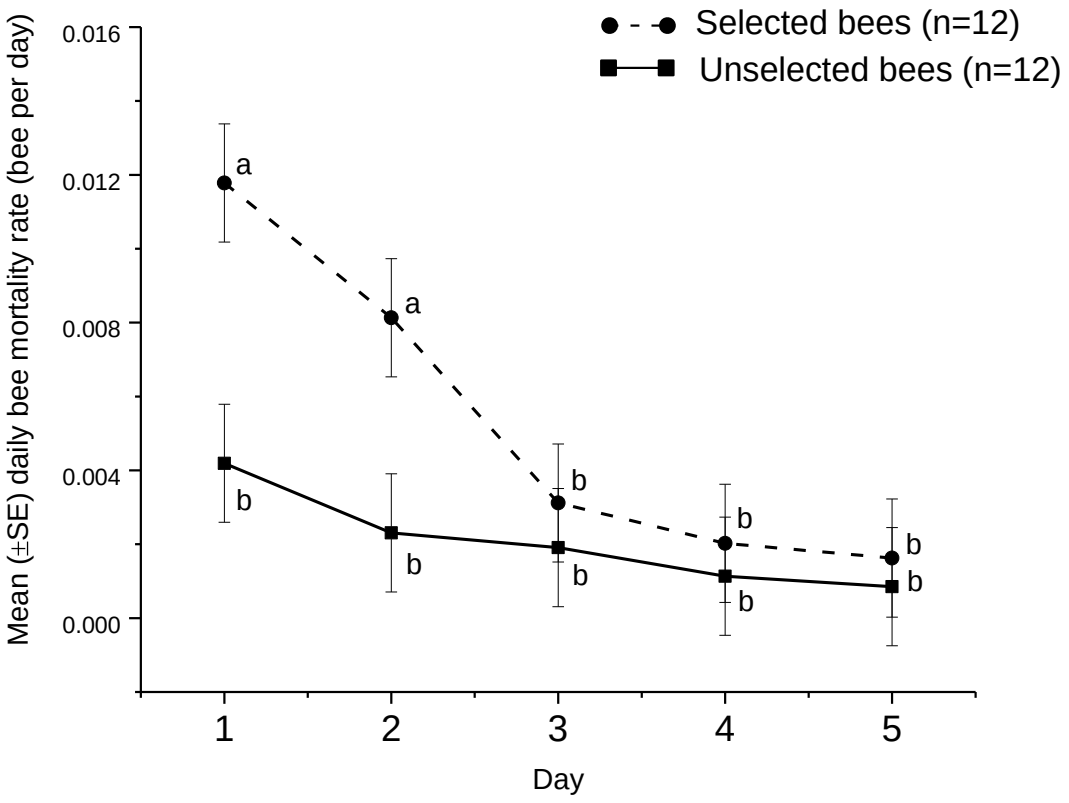


Fig. 2.5: Mean proportion of injured mites collected from bottom boards of selected and unselected stocks during simulated winter storage. Vertical lines on each bar indicate ± standard error (SE). Asterisks (on bars) indicate significant difference within genotype of bees ($P < 0.05$, Slice).

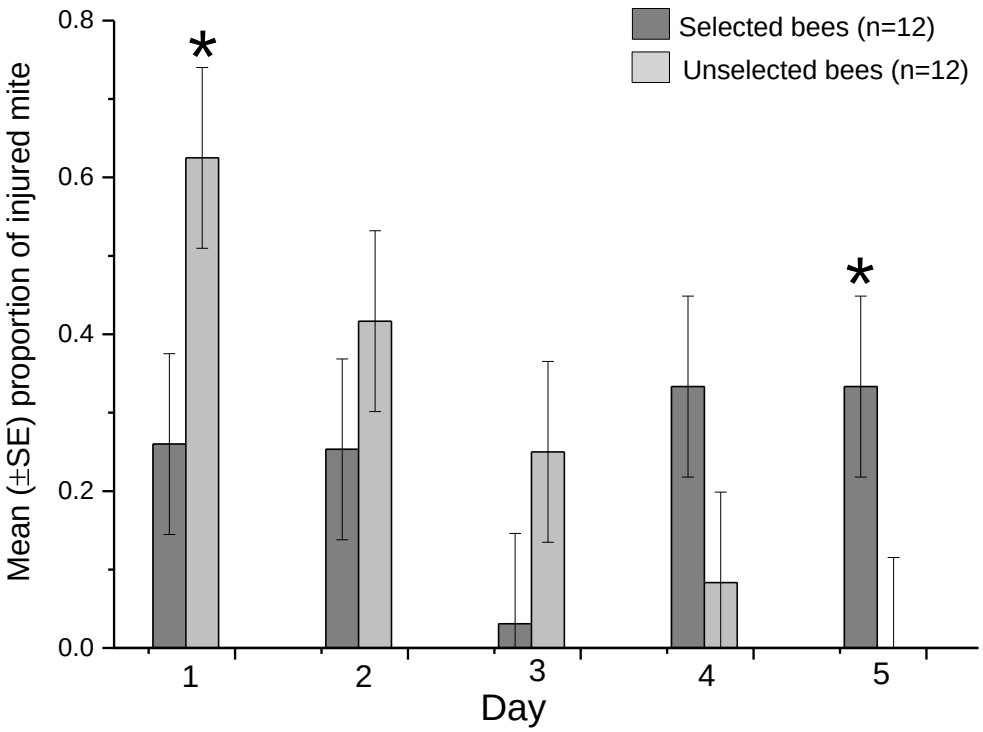


Table 2.2: Proportion of mites with various combinations of different injuries. Injuries were found on the dorsal shield (DS), ventral shield (VS), legs (L) and mouthparts (MP) or combination of the above (+) on *V. destructor* collected from bottom boards of selected and unselected stocks during simulated winter storage. (*n*) represents the number of mites examined in each category.

Bee genotype	Injured mites % (*n*)					
	DS	DS +VS +L	DS +VS +MP +L	L	MP	Total
Selected	64 (18)	14 (4)	11 (3)	11 (3)	0 (0)	100 (28)
Unselected	32 (10)	16 (5)	20 (6)	29 (9)	3 (1)	100 (31)

CHAPTER 3. INFLUENCE OF HONEY BEE GENOTYPE AND WINTERING METHOD ON WINTERING PERFORMANCE OF *VARROA DESTRUCTOR* (MESOSTIGMATA: VARROIDAE) INFECTED HONEY BEE (HYMENOPTERA: APIDAE) COLONIES IN NORTHERN CLIMATES

ABSTRACT:

The objective of this study was to assess the effectiveness of a cooperative breeding program designed to enhance winter survival of honey bees (*Apis mellifera* L.) when exposed to high levels of varroa *(Varroa destructor* Anderson and Trueman*)*. The impact of late autumn varroa mite control on winter colony loss in two genotypes of bees was quantified in outdoor-wintered and indoor-wintered colonies. Colonies of stock selected against varroa and unselected stocks were established with similar total numbers of mites and mean abundance of mites averaging 1,548±208 mites per colony and 0.164±0.019 mites per bee, respectively, which is well above the recommended late autumn economic threshold for the Canadian prairie region. Half of the colonies from each stock were randomly assigned to be treated with late autumn oxalic acid treatment or to be left untreated. Colonies within each acaricide treatment combination (genotype and acaricide) were then randomly assigned to be wintered either indoors (n = 37) or outdoors (n = 40). Late autumn treatment with oxalic acid reduced the spring mean abundance of varroa mites (0.057±0.011 mites per bee) relative to that found in untreated colonies (0.091±0.011 mites per bee) but did not increase colony survival or the proportion of viable colonies found in spring. Autumn worker bee populations of colonies were similar in selected colonies (11,042±1,065 bees) and in unselected colonies (9,681±851 bees).

Genotype of bees affected colony survival and the proportion of commercially viable colonies in spring, as indicated by greater rates of colony survival and commercially viable colonies for selected stock (43% survived and 33% were viable) in comparison to unselected stock (19% survived and 9% were viable) across all treatment groups. Indoor wintering improved spring worker bee population size, proportion of colonies surviving and proportion of commercially viable colony relative to outdoor wintering (73% of selected stock and 41% of unselected stock survived, during indoor wintering). For indoor wintering, autumn mean abundance of mites in selected colonies that survived winter was not significantly different (0.14±0.02 mites per bee) than in unselected colonies that survived winter (0.10±0.02 mites per bee), but colonies from selected stock that survived winter had higher numbers of mites (1,301.15±242.62 mites per colony) than unselected stock (958.50±268.23 mites per colony). Selected stock showed better "tolerance" to varroa as the selected stock also maintained higher bee populations relative to unselected stock. However, there was no evidence of "resistance" in selected colonies (greater mite removal) as the mean abundance of mites was not reduced in selected stock relative to unselected stock in untreated group and mite mortality rates in mid-winter were similar in both stocks. Collectively, this experiment showed that cooperative breeding programs by beekeepers can improve tolerance to varroa through continual selection and this can help minimize colony loss through winter. Overall, colony wintering success of both genotypes of bees was better when colonies were wintered indoors than when colonies were wintered outdoors.

INTRODUCTION:

Varroa destructor (Anderson and Trueman) causes serious impacts to managed honey bee, *Apis mellifera* Linnaeus, colonies that vary with geographical regions, climate, bee genotype and the haplotypes of mite (De Jong et al. 1984; Anderson 1994; Sammataro et al. 2000; Medina-Flores et al. 2014). Varroa affects immature and mature stages of worker honey bees lowering adult body mass, protein content, hypopharengeal gland size, and worker longevity (De Jong et al. 1982; Schneider and Drescher 1987; Bowen-Walker and Gunn 2001). In drones it affects seminal vesicle weight and sperm counts (Rinderer et al. 1999; Duay et al. 2002). Varroa also influences antiviral defense mechanisms and immune system function causing symptoms such as deformed wings and a variety of other effects at the individual level, that culminate in a colony-level effects (Aronstein et al. 2012; Nazzi et al. 2012). At the colony-level, *V. destructor* affects brood rearing, foraging ability (Janmaat et al. 2000; Romero-Vera and Otero-Colina 2002; Garedew et al. 2004; Kralj and Fuchs 2006), adult population growth (Ostermann and Currie 2004), honey production and wintering ability (Currie 2001; Gatien and Currie 2003; Currie and Gatien 2006).

Winter loss of colonies is one of the most serious challenges affecting managed honey bee colonies throughout the world and is a major problem in northern climates (Korpela et al. 1992; Brodschneider et al. 2010; Currie et al. 2010; Dahle 2010; Genersch et al. 2010; Guzman-Novoa et al. 2010; Le Conte et al. 2010; Topolska et al. 2010; Vejsnaes et al. 2010). At northern latitudes (> 49°N) such as in the Canadian prairies, honey bee colonies can be wintered under outdoor- or indoor-management systems (Currie et al. 1998; Underwood and Currie 2004;2007), and colony mortality can vary

with wintering method (Currie 2001; Currie 2008; Williams et al. 2010b). Infection with parasites and pathogens can have a significant role in winter mortality of colonies (Currie 2001; Downey and Winston 2001; Ellis and Munn 2005; Higes et al. 2006; Oldroyd 2007; Currie 2008; vanEngelsdorp and Meixner 2010). However, very little is known about how genotype of bees affects wintering success when interacting with varroa mites and other honey bee parasites or pathogens in different wintering environments.

Treatment thresholds can assist producers in determining optimal timing of treatments, reducing the number of applications, decreasing the risk of developing resistance to acaricides, reducing honey and wax contamination, reducing the cost of mite control, increasing colony productivity and preventing colony loss (Delaplane and Hood 1999; Strange and Sheppard 2001; Currie and Gatien 2006). Thresholds for varroa vary with regions and climates (Delaplane and Hood 1997) and the type of economic impact they are designed to prevent. For varroa they typically are based upon sampling measures of the number of mites per adult bee (mean abundance) or number of mites falling from the colony on to a sticky board. August thresholds of 5 to 13 mites per 100 bees are recommended in the southern United States to prevent reductions in autumn population size (Delaplane and Hood 1997; 1999). Late autumn thresholds of 3 mites per 100 bees (in Nebraska) (Ellis and Baxendale 1996) and 1 mite per 100 bees (for the pacific northwest United Sate) (Strange and Sheppard 2001) are recommended but in those cases are designed to prevent losses associated with slow population build up in the following spring. In the prairie regions of Canada, early autumn (late August to early September) thresholds of ≥ 3 mites per 100 bees and late autumn (October-November) thresholds of > 10 mites per 100 bees are designed to prevent winter colony loss (Currie

2001; Gatien and Currie 2003; Currie and Gatien 2006; Currie 2008). However, these thresholds differ when tracheal mites are present (Currie 2008). Likewise, in Ontario, Canada, an early autumn treatment threshold of 3 mites per 100 bees is suggested to prevent winter loss but values of 5 mites per 100 bees or lower are suggested for late autumn (Guzman-Novoa et al. 2010).

Several non-acaricidal management approaches focus on slowing mite population build up and thus delay the time until treatment thresholds are reached (Delaplane et al. 2005) including biotechnical methods (drone brood trapping, screened bottom boards and sticky bottom boards) (Calis et al. 1998; Wilkinson and Smith 2002; Charriere et al. 2003; Calderone 2005; Wantuch and Tarpy 2009) and the development of lines with forms of resistance to varroa (e.g. Varroa Sensitive Hygienic line, Minnesota hygienic line and Russian Bee line) (Spivak and Reuter 2001; Ibrahim and Spivak 2006; Harris 2007). Another approach is to select for traits that would allow colonies to survive the winter when mite levels are above normal treatment thresholds. This could occur if colonies were able to reduce their mite load over winter (resistance) or were better able to tolerate high populations of mites without significant host mortality (tolerance). In Manitoba, Canada, a cooperative breeding program designed to enhance colony survival when exposed to varroa in autumn was implemented in 2002 (as described below), but the effectiveness of this type of selection has not been evaluated under controlled conditions. The breeding program was designed to select for resistance or tolerance within colonies from local populations that were adapted to regional management and climatic conditions.

The objective of this study was to assess the effectiveness of a cooperative breeding program designed to enhance winter survival of bees when exposed to varroa. I exposed two genotypes of bees (selected and unselected) to mite levels above the late autumn economic threshold, manipulated late autumn varroa mite levels within each stock through the application of an oxalic acid fumigation treatment, and assessed colony wintering success under two wintering environments (indoor and outdoor wintering). Colonies of each genotype were evaluated in spring for metrics that would indicate improvement in resistance or tolerance against varroa relative to unselected stock.

MATERIALS AND METHODS:

The experiment was carried out at the University of Manitoba, Winnipeg, Manitoba, Canada (49°54' N, 97°14' W). Bees used in the study that are referred to as "selected" stock were from European honey bee (*A. mellifera*) colonies headed by queens from the Manitoba Queen Breeders Association program. The breeding program was designed to select for increased colony winter survival and colony bee population size when exposed to varroa mites.

The cooperative breeding program was implemented on an annual basis since 2002. Each year, "selected queens" were shipped to the University of Manitoba by cooperating producers from the Manitoba Queen Breeders Association, where they were established in varroa mite-infested hives and evaluated under equivalent management and climatic conditions for the following criteria. Colonies were sampled each autumn just prior to being placed in a wintering building and each spring upon removal from winter storage. On each sampling date, colonies were scored to assess population size and

samples of 250-300 bees were removed from the center of each brood nest to determine mite level (as described in chapter 2). Colonies were then ranked, based upon autumn and spring cluster size (when exposed to pressure from varroa), change in cluster size over winter, change in varroa abundance over winter and change in total numbers of varroa in the colony over winter. Beekeepers were allowed to choose colonies from ranked lists within each category and select queens with characteristics that most suited their breeding goals. They then grafted queens from this stock to incorporate into their own operations, where it would be allowed to open mate with their own stock. In subsequent years, stock that had desirable economic traits as (determined by each producer) would then be sent back to the University for further screening and the process was repeated on an annual basis for five years.

In my study, bees categorized as "unselected" control stock were either from colonies donated from Manitoba honey producers (n=28) or from bees headed by New Zealand queens (n=19) from a single supplier (Arataki Honey, Havelock North, New Zealand). Colonies from selected stock (n = 30) and unselected stock (n = 47) were chosen and housed in single Langstroth hive bodies. Within each stock colonies were randomly assigned into two groups that would either receive a late autumn treatment (November 13, 2007) with oxalic acid (n = 39) or be left untreated (n = 38). Half of the colonies within each treatment combination were then randomly assigned to be wintered indoors (n = 37) or outdoors (n = 40). Oxalic acid (1 g) was applied according to label directions using a Varrox®-vaporizer (Andermatt BioVet AG, Grossdietwil, Switzerland) which was introduced into the hive via the entrance. Heat was used to sublimate the acid so that fine crystals were distributed through the hive (Radetzki et al. 2000). During the

five minute treatment period, the entrance to the hive was plugged with burlap pieces. Following each treatment the apparatus was cooled down to ambient temperature and refilled for the next application. Colonies were fed syrup treated with Fumagillin-B in the autumn and treated with Oxytet-25 on September 28, October 4 and October 17, 2007 according to label recommendations (both drugs were obtained from Medivet Pharmaceutical Ltd., High River, AB, Canada).

For indoor wintering, colonies with a completely open bottom entrance (36 x 2.5 cm) were randomly assigned to one of two treatment rooms in the overwintering building at the University of Manitoba and maintained in constant darkness. The wintering chambers were 170 x 270 x 300 cm in size as described in Underwood and Currie (2004). Each room simulated the air flow conditions of a commercial wintering building with a fan jet air distribution system (Currie et al. 1998; Underwood and Currie 2004;2007). Temperature inside the treatment rooms was controlled by a base board heater and a thermostat maintained temperature at 5-7 °C. Temperature in the room was monitored with temperature probes (HOBO C-8® data logger, Onset Computer Corporation, Bourne, MA, USA). Outdoor-wintered colonies were wrapped in groups of four with entrances facing outwards using standard methods for the region with a minimum of 15 cm of fiberglass insulation materials [R-value (thermal resistance) = 0.176 $°K.m^2.W^{-1}$)] on the sides and top and covered with black polyethylene sheet (Currie et al. 1998). All hives were also provided with a top entrance (5.5 x 2 cm) associated with the inner cover of the colony as was the case for indoor-wintered colonies.

Colonies were sampled on 7 November 2007 prior to wrapping (for outdoor wintering) or moving bees (for indoor wintering) and again on 9 April 2008 (after

unwrapping or removing them from the building) to determine worker bee population, colony weight, mean abundance of varroa mites and prevalence of tracheal mites [arithmetic mean of the number of worker bees infected with tracheal mite (Bush et al. 1997)]. To assess bee population size, all colonies were scored visually as described in chapter 2. Colonies were categorized as commercially viable if the cluster score in April 2008 was 3 or higher (colonies with less than 3 frames are classed as weakened and need supplemental management) (Chaudhary and Nasr 2007). The mean abundance of varroa in each hive was estimated by collecting a sample of adult worker bees (200-300) from the brood chamber and using an alcohol wash technique as described in Gatien and Currie (2003). Prevalence of tracheal mite in colonies was estimated by the thoracic slice method (Delfinado-Baker 1984).

Mites and bees that fell from indoor-wintering colonies during winter were monitored using poster board as described in chapter 2 to assess daily mortality rates. Sampling took place during five consecutive periods between January 18 and April 8 in winter 2008 [Period 1: January 18-31 (14 d), Period 2: February 1-18 (18 d), Period 3: February 19 - March 3 (14 d), Period 4: March 4-25 (22 d), and Period 5: March 26 - April 8 (12 d)]. Additional dead bees and dead mites that exited the hive were collected in a dead bee trap affixed to the hive entrance as described in chapter 2. Bees and mites that fell onto the bottom board and dead bee trap of hives were also subjected to alcohol wash and those bees and mites were included when calculating mortality rates. Bees and mites were not collected from outdoor-wintered colonies during mid-winter as the colonies were inaccessible and sampling them would have been too disruptive to colony

survival. The rates of daily worker bee and varroa mite mortality during each period were calculated (Martin 1998) as described in chapter 2.

STATISTICAL ANALYSIS:

Pretreatment (before assignment of oxalic acid and wintering treatments) mean abundance of varroa, total numbers of varroa (a derived variable obtained by multiplying mean abundance by the total number of bees in the hive), bee population score and initial hive weights in each genotype were compared by ANOVA (PROC MIXED, SAS Institute Inc. 2011). The effects of genotype of bees, oxalic acid treatment and wintering method on worker bee population score and percentage weight loss of colonies over winter was also analyzed by ANOVA using a repeated measures analysis of variance using an autoregressive heterogeneous covariance structure, with genotype of bees, wintering method and oxalic acid as main effects, colonies as subjects and season as a repeated measure (PROC MIXED, SAS Institute Inc. 2011). Due to high mortality in outdoor-wintered stock comparisons of pre- and post-treatment varroa mean abundance level and total numbers of mites in surviving hives were carried out only for indoor-wintered colonies. These data were also analyzed by a repeated measures analysis of variance using an autoregressive heterogonous covariance structure but with wintering method removed from the model (PROC MIXED, SAS Institute Inc. 2011). Proportions were arcsine transformed prior to analysis (Snedecor and Cochran 1980). All data are presented as untransformed means. Where significant interactions were observed between treatment factors, differences among treatment means were partitioned using an analysis of simple effects (Slice procedure, PROC MIXED, SAS Institute Inc. 2011).

The proportions of colonies surviving and proportion of viable colonies among different genotypes of bees, oxalic acid treatment and wintering method were analyzed by the maximum likelihood method (PROC CATMOD, SAS Institute Inc. 2011).

RESULTS:

Pre-oxalic treatment samples of workers collected from hives in autumn 2007 showed that the mean abundance ($F = 0.01$; df = 1, 75; $P = 0.907$) and total number of varroa per colony ($F = 0.02$; df = 1, 75; $P = 0.877$) was similar in both genotypes of bees. Mean abundance of varroa averaged 0.159±0.031 mites per bee in selected stock and was 0.167±0.025 mites per bee in unselected stock (Fig. 3.1 A). The total number of varroa mites in the colony averaged 1,587±335 mites per colony in selected stock and 1,520±268 mites per colony in unselected stock (Fig. 3.1 B). Selected stock and unselected stock also had similar cluster scores (selected stock: 4.5±0.4 frames of bees per colony; unselected stock: 4.0±0.4 frames of bees per colony) ($F = 1.0$; df = 1, 75; $P = 0.321$) and similar autumn colony weights (selected stock: 30.13±0.78 kg; unselected stock: 29.91±0.64 kg) ($F = 0.02$; df = 1, 69; $P = 0.879$).

Analysis of mean abundance of varroa in surviving indoor-wintered colonies showed a significant treatment*season interaction ($F = 11.78$; df = 1, 16; $P = 0.003$) (Fig. 3.2 A-D). Partitioning the treatment*season interaction by season, showed surviving colonies in all treatment combinations had similar mite levels in autumn (Slice, $F = 0.00$; df = 1, 16; $P = 0.97$), but significant differences were found among treatments in spring (Slice, $F = 8.14$; df = 1, 16; $P = 0.01$) (Fig. 3.2 A and C). Mite levels were reduced from autumn to spring in colonies treated with oxalic acid ($F = 13.6$; df = 1, 16; $P = 0.002$), but

not in untreated colonies (F = 0.77; df = 1, 16; *P* = 0.39). Genotype of bees did not affect mean abundance of varroa (F = 0.11; df = 1, 16; *P* = 0.75) and there were no interactions between genotype of bees and other factors (*P* > 0.05). The over all, mean abundance of varroa in the colonies that eventually died (n = 55) over winter was 0.183±0.026mites per bee in autumn 2007. Late autumn prevalence of tracheal mites in samples collected before oxalic acid treatment was zero percent in all treatment combinations.

Analysis of total numbers of varroa in surviving colonies showed a significant decrease from autumn to spring (F = 4.52; df = 1, 16; *P* = 0.05), but there was also a significant genotype of bees*oxalic acid treatment interaction (F = 4.42; df = 1, 16; *P* = 0.05) (Fig. 3.2 E and G). Partitioning the interactions by oxalic acid treatment showed differences in total numbers of mites between genotypes within untreated colonies (Slice, F = 6.87; df = 1, 16; *P* = 0.02), but not within treated colonies (Slice, F = 0.37; df = 1, 16; *P* = 0.55) in spring (Fig. 3.2 G). Oxalic acid-treated colonies had fewer mites than in untreated colonies within selected stock, but both acaricide treatments had similar numbers of mites within the unselected stock in spring (Slice, F = 0.81; df = 1, 16; *P* = 0.38) (Fig. 3.2 G).

For indoor-wintered colonies, daily mite and bee mortality rates were estimated during five periods in colonies (from January to March) and both showed significant differences among periods (mites: F = 7.30; df = 4, 132; *P* < 0.0001; bees: F = 3.75; df = 4, 132; *P* = 0.0063) (Fig 3.3), however daily bee and mite mortality rates were similar in both genotypes of bees (mites: F = 0.94; df = 1, 33; *P* = 0.340; bees: F = 0.00; df = 1, 33; *P* = 0.951), and in both acaricide treatments (mites: F = 0.51; df = 1, 33; P = 0.481; bees: F = 0.06; df = 1, 33; *P* = 0.807), and there was no significant genotype of bees*acaricide

treatment interaction (mites: F = 0.10; df = 1, 33; *P* = 0.759; bees: F = 0.17; df = 1, 33; *P* = 0.681).

Over all colonies, spring cluster scores of worker bees were greater in selected stock (11,402±1,180 bees per colony) than in unselected stock (6,480±1,419 bees per colony) (Slice, F = 13.85; df = 1, 69; *P* = 0.0004) and were greater in indoor-wintered colonies than in outdoor-wintered colonies. However, there was a significant genotype of bees*wintering method interaction (F = 71.00; df = 1, 69; *P* = 0.03). Selected stock wintered indoors had significantly higher cluster scores than unselected stock (Slice, F = 16.94; df = 1, 69; *P* = 0.0001) but both stocks had similar spring cluster scores when wintered outdoors (Slice, F = 1.23; df = 1, 69; *P* = 0.27) (Fig. 3.4 A). Oxalic acid treatment did not affect spring cluster scores (Slice, F = 0.23; df = 1, 69; *P* = 0.63) and there were no interactions between oxalic acid treatment and any other factors (*P* > 0.05, Slice).

Both genotype of bees and wintering method had effects on colony survival and the proportion of colonies classed as commercially viable in spring. Colony survival over winter was greater for selected stock than for unselected stock (CATMOD, χ^2 = 5.88; df = 1; *P* < 0.0001), and indoor-wintered colonies had greater survival than outdoor-wintered colonies (CATMOD, χ^2 = 15.20; df = 1; *P* < 0.0001), but the relative differences between genotypes of bees varied with wintering method as indicated by significant genotype of bees*wintering method interaction (CATMOD, χ^2 = 28.68; df = 1; *P* < 0.0001) (Fig. 3.4 B). The proportion of commercially viable colonies found in spring showed similar trends. More viable colonies were found for selected stock than for unselected stock (CATMOD, χ^2 = 7.08; df = 1; *P* < 0.008) and indoor-wintered resulted

in more viable colonies than outdoor-wintered (CATMOD d, $\chi^2 = 7.99$; df = 1; $P < 0.005$). Relative differences between genotypes of bees for colony viability in spring also varied with wintering method as indicated by significant genotype of bees*wintering method interaction (CATMOD, $\chi^2 = 40.17$; df = 1; $P < 0.0001$) (Fig. 3.4 C).

Percentage colony weight loss over winter averaged over all colonies, was higher in selected colonies than unselected colonies (F = 4.09; df = 1, 69; $P = 0.047$) (Fig. 3.5). However, percentage weight loss over winter was not affected by oxalic acid treatment (F = 0.00; df = 1, 69; $P = 0.960$), or wintering method (F = 2.25; df = 1, 69; $P = 0.139$) and there were no interactions between genotype of bees, acaricide treatment or wintering method.

DISCUSSION:

This study characterized the presence of resistance and tolerance to varroa mites in genetically selected stocks that originated from a cooperative breeding program and measured wintering success of those stocks relative to unselected stock under varroa mite levels that were above the late autumn economic threshold for the region. Colonies of each genotype were treated with acaricide in late autumn or left untreated and then wintered under two different environments: indoor- or outdoor-wintering under Canadian prairie climatic conditions. Acaricide reduced mean abundance of mites in treated groups relative to untreated groups and brought mite levels below the threshold in half the colonies but the reduction in mites did not improve winter performance of treated colonies in either genotype. However, selected stock showed a greater bee population size, wintered while maintaining higher mite population levels (if left untreated) and had

greater colony survival when compared to unselected stock. Selected stock showed no evidence of increased "resistance" towards varroa, as there was no reduction in mite population over winter relative to unselected stock, but did show increased "tolerance" against varroa mite. Overall, indoor-wintering improved wintering success of colonies in comparison to outdoor-wintering but there were significant interactions between wintering method and genotype. Selected stock performed better than unselected stock under indoor-wintering but was not significantly better than unselected stock under outdoor wintering at very high mite infestation levels.

Although adult grooming behavior has been suggested as a mechanism associated with host defense against mites (Moretto et al. 1991; Moretto et al. 1993; Boecking and Spivak 1999), it is not consistently associated with effective resistance (Fries et al. 2006). Currie and Tahmasbi (2008) showed high-grooming stock reduces a colony's mite load (mean abundance) over winter during indoor-wintering, thus acting as a "resistance" mechanism that could be of value in reducing colony loss. In my study, the potential for the presence of "resistance" against varroa was evaluated in selected and unselected genotypes by assessing two metrics that could be related to resistance: the potential for a reduction in the mean abundance of mites over winter and daily mortality rates of mites during mid-winter. Although, "grooming" potential was one of the criteria evaluated in the colony selections provided to the cooperative queen breeders participating in the study, my results showed it was not a trait that was retained in the producer's stock after they "reselected" based upon economic performance (primarily honey production) or other criteria that were important within their own operations. Based on this study, grooming behavior was not likely a significant factor contributing to the increased colony

survival that was observed, because neither genotype decreased mite populations through winter as would be expected if enhanced grooming behavior was present.

Another form of host defense against parasites is "tolerance" (Raberg et al. 2009). Mite-tolerance of honey bee colonies in the context of this study is defined as the ability of the host to coexist with higher varroa mite burdens over winter while minimizing the impact on winter survival and population size of the host colony. Some forms of tolerance may be present in honey bees. The coexistence of honey bee with *V. destructor* has been demonstrated in North America (de Guzman et al. 1996), South America (Ruttner et al. 1984; Guzman-Novoa et al. 1999a; Rosenkranz 1999), North Africa (Boecking and Ritter 1993), and Europe (Fries et al. 2006; Le Conte et al. 2007). However, the relative contributions of resistance and tolerance to colony survival are not fully understood. De Guzman et al. (1996) showed several lines of bees do have greater tolerance to varroa than Louisiana stock under summer condition in Florida, United States. In this study, I found that untreated groups of colonies from the selected stock wintered with significantly higher mite burden (numbers of mites per colony) than unselected colonies and selected stock also had higher overall spring population sizes and colony survival. This showed that under the extended winter periods on the Canadian prairie, selected colonies tolerated higher levels of varroa mites than unselected colonies. The factors associated with this increased mite tolerance are unknown, but likely involve a combination factors that enhance winter survival of bees or minimize the impact of mites on the host.

In this investigation, "unselected colonies" were chosen that would have had little selection pressure from mites, and that came from two sources that were broadly

representative of stock used by commercial beekeepers: local stock from Canadian beekeepers who had not been breeding stock for varroa resistance, and imported stock from a traditional supplier in New Zealand that also would be expected to have little resistance or tolerance to varroa. Variations in the haplotype of varroa from different origins could vary in "virulence" and thus, potentially affect the overall capacity for the degree of tolerance against parasites in bees within different genotypes of bees but this is unlikely. The varroa in Canada are of the Japan-Thailand and Korea haplotypes (Anderson and Trueman 2000). Bees from New Zealand would probably have had little selection pressure from *V. destructor*, although the Korea haplotype occurs there (Zhang 2000). Even though I did not attempt to determine haplotype of mites in my study, varroa was still being effectively controlled up to the time of the experiment and there would have been little selection pressure for reduced virulence on the part of the mites. Multiple infections with parasites-pathogens of different levels of virulence and their interactions with other factors could have impacted my results but attempts were made to control as many of these factors as possible. For example, interactions between varroa and tracheal mite result in much greater colony mortality than varroa alone (Currie 2001; Downey and Winston 2001). Additionally, *Nosema* and viruses are sometimes correlated with colony winter losses or reduced spring population sizes and interactions with these pathogens can be directly or indirectly correlated with varroa (Martin 2001; Nordstrom 2003; Martin-Hernandez et al. 2007; Dainat et al. 2012a; Desai 2014). Effects observed in my study were likely a result of varroa (or possibly interactions between varroa and viruses) as tracheal mite was sampled and not present at detectable levels, *Nosema* was controlled using Fumagillin, and other aspects of colony nutrition and colony

management were standardized across all treatments. I did not measure viruses in this study, so it is possible that some mechanisms associated with increased tolerance that were observed could be related to better defence against viruses rather than mites or could be less effective if more virulent strains of virus were present (Locke et al. 2014).

In *A. mellifera* colonies where beekeeper management of varroa may constrain natural selection, most infested colonies typically die after three years in Nordic climates (Korpela et al. 1992). If colonies are left unmanaged in an isolated region, an "adaptive" host-parasite relationship can establish. For example, winter colony mortality in isolated Nordic populations of "unmanaged" bees infested with varroa decreases from 76% after three years of infestation to 19% by the end of six years of coexistence under cold climates (Fries et al. 2006). The mechanisms behind this adaptation are not fully understood but some factors (e.g. increased propensity to swarm, lower densities of colonies in the landscape) may not be completely compatible with commercial beekeeping. It has been suggested that beekeeper management of parasites may remove the selection pressure that can lead to adaptation on the part of bees, mites or both (Fries et al. 2006). My findings suggest that several years of selection of colonies under pressure by varroa followed by selection of colonies under commercial beekeeping practice, allowed some degree of host tolerance in my experimental colonies. This was evidenced by a higher winter survival of colonies, and larger spring populations in the selected stock relative to the unselected stock and strongly supports the hypothesis that a component of adaptation such as increased parasite tolerance can be improved in stock selected through this type of breeding program. However, further studies are required to

understand factors behind this adaptation and more research is required to attempt to further improve colony tolerance and resistance against varroa mites.

In my experiment, I exposed colonies to average pre-treatment mite infestations (16% to 17%) that were well above the late autumn apiary-based damage thresholds of 5% to 10% typically recommended in Canada for varroa (Currie 2001; Gatien and Currie 2003; Currie and Gatien 2006; Currie 2008; Guzman-Novoa et al. 2010). In the year of my study, commercially managed colonies in Manitoba experienced average losses of 28% through winter 2007-2008, which was less than national average of 35% (Currie et al. 2010). Selected stock in this study had greater winter loss (57%) relative to that of the Provincial average but was subjected to a very high stress associated with the exposure to high autumn varroa. However, the high colony winter loss I observed (76%) in unselected stock was in line with what would be expected for the region under similar mite infestation levels (Currie 2001; Currie and Gatien 2006) and consistent with that found in many other regions when colonies are exposed to high levels of varroa in autumn (Strange and Sheppard 2001; Akyol and Yeninar 2011; Dainat et al. 2012b; van Dooremalen et al. 2012). In addition to causing colony death, autumn varroa mite levels above treatment thresholds can influence bee populations and brood rearing in spring (Delaplane and Hood 1997; Strange and Sheppard 2001; van Dooremalen et al. 2012). Surviving colonies may be too small to be commercially viable without considerable input of labor by beekeepers or supplementation with addition of substantial numbers of bees. In some surveys, "weak" colonies are reported as a component of colony loss. Chaudhary and Nasr (2007) reported an average of 30% winter loss among colonies in Alberta, Canada, where 15% of the "lost" colonies were associated with small non-viable

colonies in spring. In this study, colonies with three or more frames completely occupied by worker bees in April were counted as commercially viable (Chaudhary and Nasr 2007) and genetically selected stock also showed significantly higher proportions of commercially viable colonies relative to unselected colonies.

Indoor wintering is a widely adopted management strategy in some regions of Canada and is utilized in other countries with cold climates (Fingler 1980; Murrell and McDonald 1986; Stalidzans et al. 2007). Several studies now show significantly higher colony mortality or bee loss occurs in outdoor-wintering relative to indoor-wintering when multiple pests and pathogens are present (Currie 2001; Williams et al. 2010a; Desai 2014). Surveys of commercial beekeepers in Manitoba in years with high bee losses also show higher losses for outdoor colonies (42%) than for indoor colonies (29%) but it is often difficult to separate beekeeper management effects from wintering method effects in this type of data (Lafreniere 2011). Also, differences in bee survival with different wintering methods are not always evident in survey data. Outdoor wintering losses of 32% and indoor losses of only 26% have been reported for colonies in Alberta, Canada, in years where high colony loss is linked to high levels of varroa, *Nosema* and adverse weather conditions in winter and spring (Hartman and Nasr 2008). In order to more clearly address the question about the value of indoor-wintering in mitigating bee loss, I attempted to control most variables and limit the focus of my study to one parasite (varroa). Colonies were randomly assigned to each wintering treatment after being exposed to high but similar mite infestation levels. I showed a clear benefit to indoor-wintering over outdoor-wintering at high (untreated) and moderate (oxalic acid treated) varroa levels for both selected and unselected stocks as indicated by higher spring bee

populations, higher colony survival, and higher numbers of commercially viable colonies when colonies were placed under high parasite pressure from varroa.

There are a number of factors that could affect interpretations of the mite population assessments in this study. In the overwintering period, mites may die, be groomed from a host and drop from the winter cluster of bees, or leave the colony attached to a dead host (De Jong 1990; Fries et al. 1991). Under warm conditions, varroa mites that fall onto bottoms of hives are able to relocate to new host bees and make their way back to the bee cluster. Thus, daily removal of boards could influence mite mortality. However, the latter was unlikely to have occurred as this study was carried out under winter conditions at 5-7 °C and most mites that fell from the cluster would likely become immobile at that temperature before having a chance to find a live host and re-enter the bee cluster (Fries and Perez-Escala 2001). Anywhere from 3% to 50% of varroa mites die over winter (Fries et al. 1991; Moosbeckhofer 1991; Korpela et al. 1992). The numbers of mites that die on bees that either leave on their own, or are removed from the colony, is difficult to assess in outdoor environments (Moosbeckhofer 1991; Korpela et al. 1992). However, in my experiment, I was able to quantify mortality rates of both bees and mites within the hive and those removed from the hive through the use of bottom board sampling and dead bee traps in the wintering building. The daily rates of mite mortality are typically higher in summer (0.005-0.007 mites per day per colony) (Boot et al. 1992; Martin and Kemp 1997) relative to those in winter (0.001-0.004 mites per day per colony) (Moosbeckhofer 1991; Korpela et al. 1992). In this study, daily mite mortality rates of 0.0018±0.0002 mites per day per colony were in the expected range and bee mortality was also similar to other studies (Martin 1998; Underwood and Currie

2005). Although neither the genotype of bees nor the oxalic acid treatment affected the daily mite or daily bee mortality rates, mortality rates did vary with different time periods during winter but declined at a similar rate. Differences in bee and mite mortality rates can contribute to changes in the mean abundance over winter. For example, Bowen-Walker et al. (1997) showed that differential death rates for bees and mites in the United Kingdom can result in increases in mite infestation level (mean abundance) from 13% to 37% during October and February. The small differences between bee and mite mortality rates in this study did not result in changes in the mean abundance of mites throughout winter in the untreated colonies. In my colonies, mite levels were very consistent between January (12.4%) and April (12.3%) which is similar to the finding of Fries and Perez-Escala (2001), who also evaluated death rate of bees and mites based on sampling from inside and outside the colony in a Swedish climate. Winter brood rearing can occur in colonies in the period from November to early April under Canadian prairie conditions. Differences in this parameter could also influence assessment of mite populations as the total mite population could be underestimated. I did not measure winter brood rearing, but it is known that under indoor wintering, only a small proportion of mites are found in winter brood and mite reproduction within winter brood is very low (Kozak 2008) and would not likely have a major impact on my results. Given the large differences in spring bee population between selected and unselected stock, it is surprising that I did not detect genotype-related differences in bee mortality rates using the white board technique. It is possible that differences in mortality outside of our measurement period contributed to this discrepancy or that some of the bees were not

captured in the dead bee traps but this latter source of error would likely have been proportional across treatment groups.

In this study, oxalic treatments were used in broodless periods in late autumn to bring mite levels below the typical economic threshold of 10 mites per 100 bees that is recommended for the region (Currie 2008). Although high efficacy usually occurs when oxalic is applied in broodless periods (Radetzki et al. 2000; Charriere and Imdorf 2002; Rademacher and Harz 2006), I found oxalic fumigation reduced mite infestations by only about half in surviving colonies. Since winter assessments of mite mortality rate (which began in January) did not detect differences between oxalic-treated and untreated hives, it is likely that most of the treatment related mite mortality occurred between the late autumn treatment and January and that there was little to no residual effect of the oxalic treatment beyond that period. Dainat et al. (2012a) show similar natural mite drop within surviving colonies through January and April, following treatment with oxalic acid in November. Autumn acaricide treatments with other products such as thymol, that reduce varroa infestation when applied earlier in the fall, result in higher winter colony survival (van Dooremalen et al. 2012) but I did not see a benefit in terms of increased colony survival or increased spring population size in either genotype of bees with my late autumn treatment. Pupae that are infested with varroa in the autumn do not complete all of the physiological processes that are necessary to allow them to survive periods as "winter bees" (Amdam et al. 2004). The late-autumn acaricide treatment with organic acid in my study would not have prevented any varroa-related damage to pupae because brood was absent or minimal at the time of treatment. The results suggest that reductions in mite infestations on adult bees resulting from oxalic treatment were either insufficient

to relieve varroa-related stress and to improve winter success (Gregorc and Planinc 2002) or that the damage done by varroa feeding on developing or adult bees was too great to allow any type of compensatory recovery in the adult stage once the intensity of varroa feeding was reduced. Most studies using oxalic vaporizers in late autumn report no adverse effects on colony performance when mite levels are comparatively low (Rademacher and Harz 2006) and I did not see significant negative effects associated with the use of oxalic under the high mite infestation levels in my study.

Selective breeding programs usually focus on producing a line of bees designed to maximize a small number of traits associated with factors such as greater bee populations, honey production, increased parasite and pathogen resistance or tolerance, lower swarming and winter colony strength (Rinderer et al. 2001; Buchler et al. 2010; Gisder et al. 2010; Rinderer et al. 2010). Honey bees with some forms of resistance or tolerance to varroa have been developed that show greater survival through summer and autumn in comparison with local stock in United States (Primorski, Russian) and Europe (Rinderer et al. 2001; Buchler et al. 2010). Several lines have been developed that slow mite population growth through removal of varroa in brood (Varroa Sensitive Hygiene, Minnesota hygienic line) or possible disruption of mite reproduction in brood (Spivak and Reuter 2001; Ibrahim and Spivak 2006; Harris 2007). However, these lines may not be best adapted for management in different beekeeping regions with different climatic conditions. The approach used in this study was to attempt to enhance traits associated with resistance and tolerance in local queen breeding operations by having queen breeders rear queens from local stock that had been repeatedly exposed to autumn selection pressure from varroa over a period of five years and evaluated for traits

associated with resistance and tolerance that could enhance wintering success. Based on outcomes of this study the impact of this breeding program has resulted in improved wintering success of selected stock relative to unselected stock when used in conjunction with indoor-wintering, and has been successful in incorporating traits associated with increased tolerance but not with increased resistance. Although the only colonies that survived during outdoor wintering were from the selected stock, the survival rate did not differ significantly from that of the unselected stock due in part to low sample size. It is possible that the stock might provide some enhanced level of tolerance from varroa during outdoor wintering if mite infestation levels closer to the damage threshold were used but this requires further testing. Throughout the 5-year breeding program in Manitoba, queen breeders bred queens in their operations and then reselected colonies based on economic traits such as increased honey production (a primary selection criteria for all producers) and other management traits that were considered desirable in their operations. Resistance traits associated with enhanced mite removal from colonies were not retained. This could be due to resistance traits associated with grooming having lower levels of heritability (Moretto et al. 1993; Stanimirovic et al. 2010) than those associated with tolerance (although the heritability of tolerance traits has not been measured) or that the re-selection of colonies by beekeepers for economic characteristics did not result in the retention of these resistance traits. Since resistance to varroa through enhanced mite grooming can be effective in reducing mite infestation levels over winter (Currie and Tahmasbi 2008) it is a further trait that could be pursued for incorporation into local stock if that trait is also found to be compatible with commercial beekeeping

requirements. However, incorporation of this trait using the "cooperative breeding" approach may require more intensive selection or selection over a longer period.

A variety of treatment thresholds in late autumn have been recommended for successful overwintering and to minimize winter loss when honey bee colonies are infested with either multiple or single parasite. In the Canadian Prairie, an apiary-based treatment threshold for varroa of 10 mites per 100 bees in late-October is the limit colonies can tolerate without experiencing significant colony loss (Currie 2008). My experimental hives were established with a relatively high level of varroa mites, and in the absence of tracheal mite, varroa mite-tolerant colonies experienced 57% winter loss when infected with a mean abundance of varroa well above recommended treatment thresholds, compared to 81% loss in unselected stock. However, when indoor wintering was used that loss was reduced in selected stock to only 27% and to only 59% in unselected stock. Although producers would suffer less economic damage from colony collapse under high levels of varroa if they used selected stock my data indicate they still need to treat varroa-tolerant stock to prevent damage when exposed to mites above thresholds. Furthermore, late treatments, at least using oxalic acid, would not be successful in mitigating damage that would occur. The use of indoor wintering could be used to help mitigate losses from high levels of varroa in both stocks selected for increased tolerance to varroa and in unselected stocks.

Fig. 3.1: Box plots of pretreatment mean abundance of varroa mites (A) and total mites (B) in all colonies from selected and unselected stocks prior to initiation of acaricide and wintering treatments. Black diamond indicates individual observations, empty circle represents mean. Median, 10th, 25 th, 75 th and 90 th percentiles are indicated on figures. Current late autumn apiary-based threshold for mean abundance is indicated by arrow and dotted line. Means followed by the same letters between genotypes of bees are not significantly different.

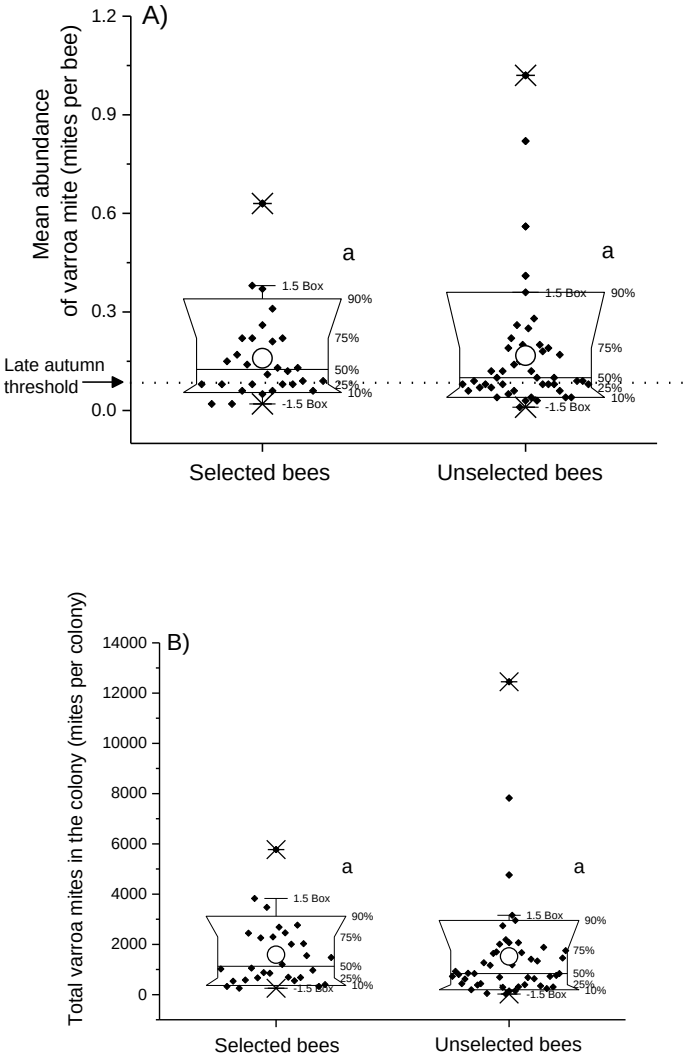


Fig. 3.2: Autumn and spring mean abundance (A-D) and total numbers (E-H) of varroa for colonies that survived to spring for selected and unselected stocks that received a late autumn treatment with oxalic acid (treated) or were left untreated. Half of the colonies were wintered indoors and half outdoors. *n* represents the number of surviving colonies within each treatment combination. NA indicates no colonies survived to be sampled. Vertical bars on each column indicate ± standard error (SE). Dashed line represents current recommended apiary-based treatment thresholds for late autumn. Horizontal lines represent significant simple effects comparisons between autumn and spring for indoors ($P < 0.05$) (due to high colony mortality outdoor-wintered colonies were not included in comparisons among means but data for surviving colonies are presented). Means followed by the same letter for differences in mean abundance for acaricide treatment within season are not significantly different ($P < 0.05$, Slice). Asterisks indicate significant genotype of bees*oxalic acid treatment interaction (see text for explanation).

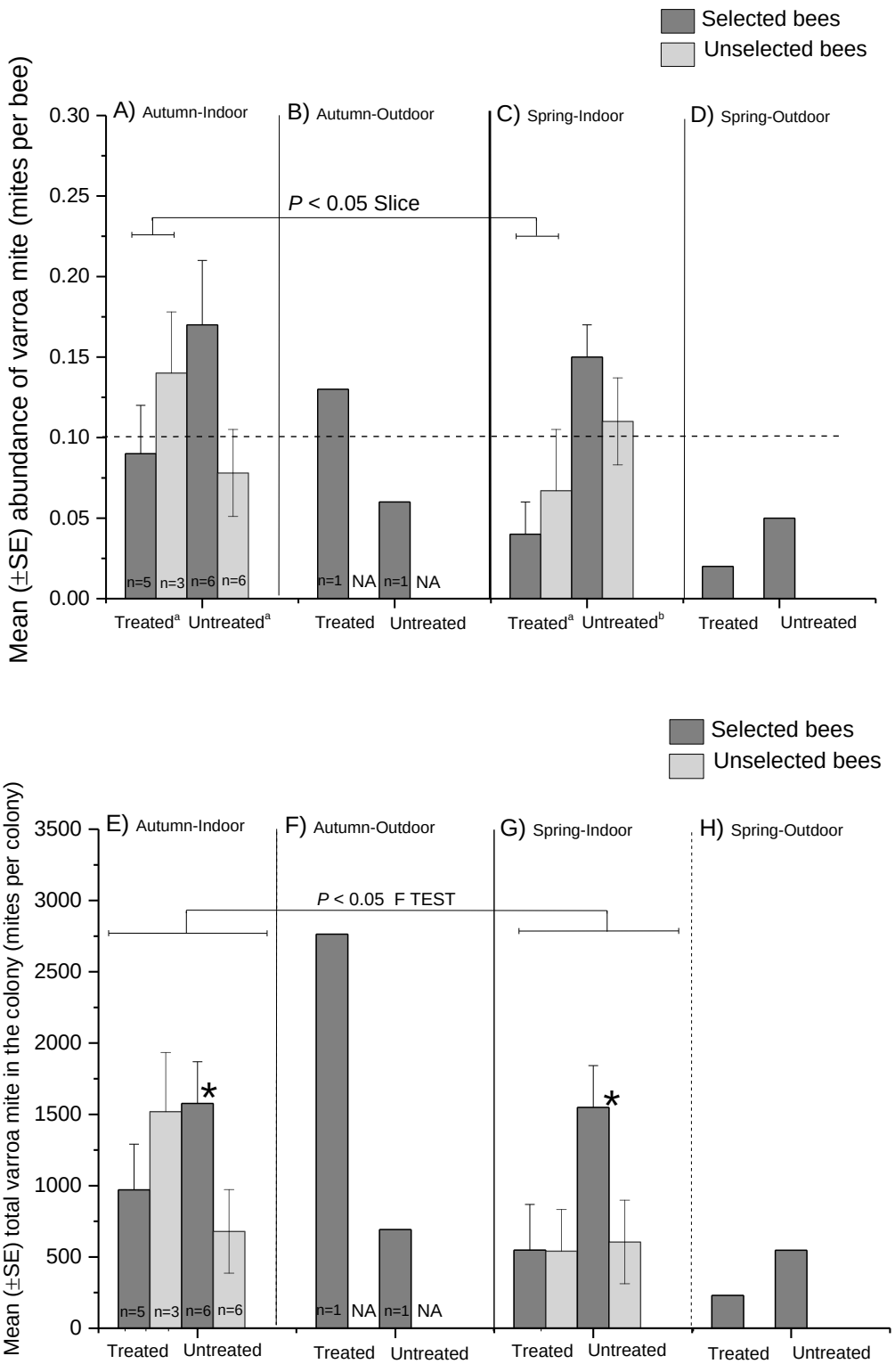

Selected bees
Unselected bees
A) Autumn-Indoor
B) Autumn-Outdoor
C) Spring-Indoor
D) Spring-Outdoor
P < 0.05 Slice
Mean (±SE) abundance of varroa mite (mites per bee)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
n=5
n=3
n=6
n=6
n=1
NA
n=1
NA
Treated[a]
Untreated[a]
Treated
Untreated
Treated[a]
Untreated[b]
Treated
Untreated
Selected bees
Unselected bees
E) Autumn-Indoor
F) Autumn-Outdoor
G) Spring-Indoor
H) Spring-Outdoor
P < 0.05 F TEST
Mean (±SE) total varroa mite in the colony (mites per colony)
3500
3000
2500
2000
1500
1000
500
0
*
*
n=5
n=3
n=6
n=6
n=1
NA
n=1
NA
Treated
Untreated
Treated
Untreated
Treated
Untreated
Treated
Untreated

Fig. 3.3: Mean daily varroa mite and worker bee mortality rates for five periods during mid-winter of 2008 in colonies that received a late autumn treatment with oxalic acid or were left untreated (for indoor-wintering only). Data represent pooled values for all treatments [Period 1: January 18-31 (14 d); Period 2: February 1-18 (18 d); Period 3: February 19 - March 3 (14 d); Period 4: March 4-25 (22 d); Period 5: March 26 - April 8 (12 d)]. Vertical bars on each point indicate ± standard error (SE). Means followed by the same letter among periods are not significantly different.

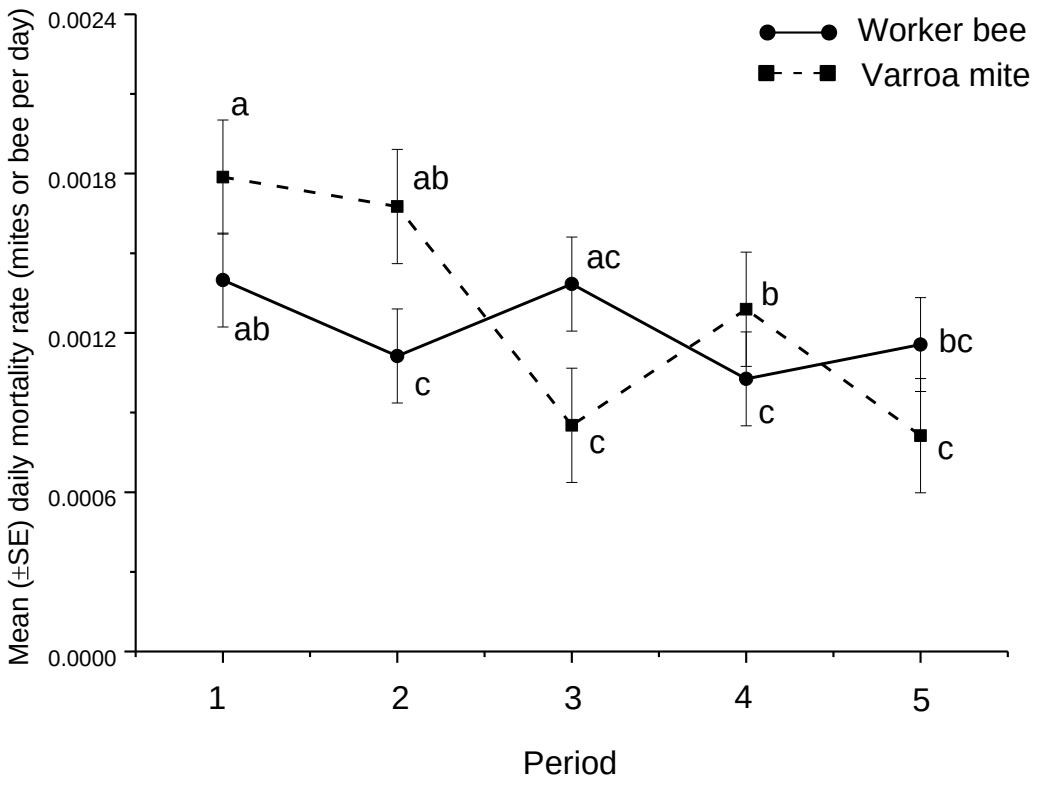


Fig. 3.4: Average bee population score in selected and unselected stocks in spring 2008 (A) for colonies wintered either indoors or outdoors. Dashed line represents spring colony size that would be considered as viable for commercial production. Proportion (%) of colonies that survived to spring (B) and proportion (%) of commercially viable colonies in spring (C) in selected and unselected stocks (pooled for both wintering methods). *n* represents the number of colonies within each genotype of bees. Genotype*wintering = interaction between honey bee genotype and wintering method. Vertical bars on each column in graph A indicate ± standard error (SE). Means followed by the same letter between genotypes of bees or between wintering methods are not significantly different.

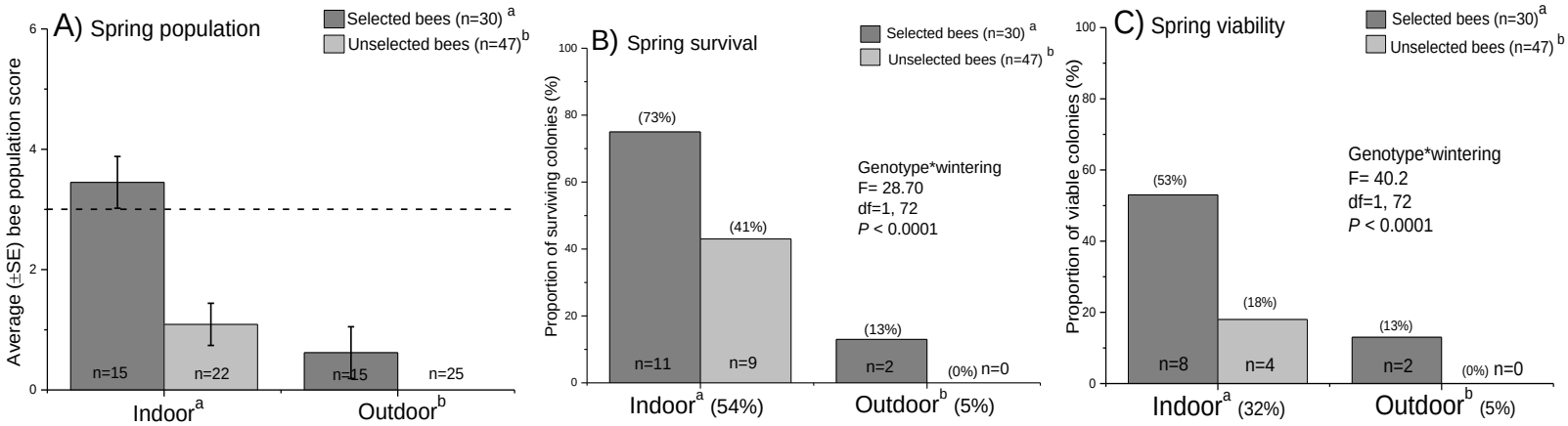
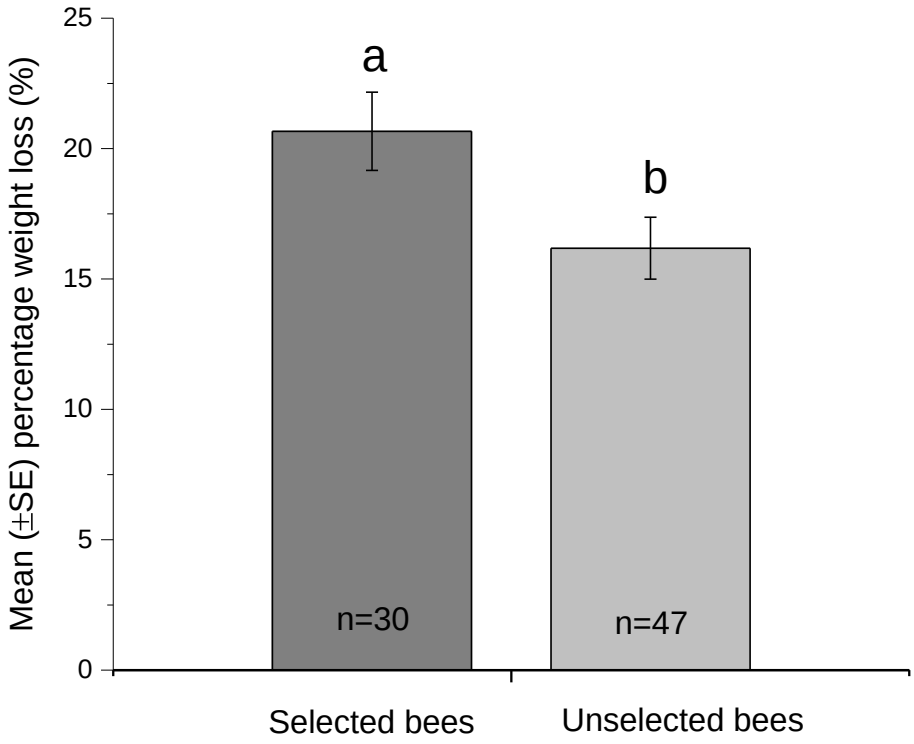


Fig. 3.5: Mean percentage weight loss from autumn to spring for all colonies from selected and unselected stocks. Vertical bars on each column indicate ± standard error (SE). *n* represents the number of colonies within each genotype of bees. Means followed by the same letter between genotypes of bees are not significantly different.



CHAPTER 4. THE POTENTIAL OF BEE-GENERATED CARBON DIOXIDE FOR CONTROL OF VARROA MITE (MESOSTIGMATA: VARROIDAE) IN INDOOR OVERWINTERING HONEY BEE (HYMENOPTERA: APIDAE) COLONIES

ABSTRACT:

The objective of this study was to manipulate ventilation rate to characterize interactions between genotypes of honey bees (*Apis mellifera* L.) and ventilation setting on varroa mite (*Varroa destructor* Anderson and Trueman) mortality in overwintering honey bee colonies. The first experiment used colonies established from selected bees (n = 6) and unselected bees (n = 6) to assess mite and bee mortality and levels of carbon dioxide (CO_2) and oxygen (O_2) in the bee cluster when kept under a simulated winter condition at 5 °C. The second experiment, used colonies from selected bees (n = 10) and unselected bees (n = 12) that were exposed to either standard-ventilation or restricted-ventilation (in a Plexiglas chamber) to assess the influence of restricted air flow on winter mortality rates of varroa mites and honey bees. Restricted-ventilation colonies were sampled during the following periods: 6 d of stabilization (14.4 $L \cdot min^{-1} \cdot hive^{-1}$), 2 d as pre-treatment (14.4 $L \cdot min^{-1} \cdot hive^{-1}$), 16 d as treatment (0.24 $L \cdot min^{-1} \cdot hive^{-1}$) and 2 d as post-treatment (14.4 $L \cdot min^{-1} \cdot hive^{-1}$). Colonies exposed to standard-ventilation were sampled concurrently, in the same periods, but the rate of air flow was consistently 14.4 $L \cdot min^{-1} \cdot hive^{-1}$ over time. The experiment was repeated in early, mid and late winter. In experiment 2, concentrations of CO_2 and O_2 in the core of the bee cluster and peripheral environment of hive, daily rates of varroa mite and worker bee mortality and changes in mean abundance of varroa mite and *Nosema* spores over time were quantified. The first

experiment showed that the average CO_2 and O_2 concentrations in the bee cluster were similar in both genotypes of bees. Mean daily mite mortality was significantly greater in selected bees than unselected bees, and selected bees had lower bee mortality relative to unselected bees. Under CO_2 concentrations averaging less than 2% there was no correlation between CO_2 and varroa mite mortality when colonies were placed under low temperature. Carbon dioxide was negatively correlated with O_2 in the bee cluster. The maximum CO_2 concentration and minimum O_2 concentration in the bee cluster were 9.3% and 12.01%, respectively, in pre-experiment. When ventilation was restricted mean CO_2 level (3.82±0.31%, range 0.43-8.44%) increased by 200% relative to standard-ventilation (1.29±0.31%, range 0.09-5.26%) within the 16 d treatment period. The overall mite mortality rates and the reduction in mean abundance of varroa mite over time was greater under restricted-ventilation (37±4.2%) than under standard-ventilation (23±4.2%) but not affected by genotype of bee. Selected bees showed overall greater mite mortality relative unselected bees. Restricting ventilation increased mite mortality, but did not affect worker bee mortality relative to that for colonies under standard-ventilation. In conclusion, these findings suggested that restricted-ventilation has potential to suppress varroa mite in overwintering honey bee colonies via a low cost and environmentally friendly measure.

INTRODUCTION:

Carbon dioxide (CO_2) has a vital role in arthropod physiology. In addition to its role in respiration it influences longevity and behaviors such as orientation, reproduction and cold resistance (Engels and Ramamurty 1976; Barrozo and Lazzari 2004). The concentration of CO_2 in social insect colonies varies considerably (Dietein 1985; reviewed in Nicolas and Sillans 1989). Carbon dioxide concentration ranges from 1-2% in ant colonies (Portier and Duval 1929; Nielsen et al. 2003), 0.8-5.2% in termite colonies (Luscher 1961; Matsumoto 1977) and 0.2-9.9% in honey bee colonies (Hallund 1956; Taranov and Mikailov 1960; Free and Simpson 1963; Seeley 1974; Sugahara and Sakamoto 2009).

It is known that the honey bee (*Apis mellifera* L.), as a superorganism, is well adapted to thermoregulate and manage the hive microclimate in order to survive under severe winter conditions in temperate climates (Fahrenholz et al. 1989; Stabentheiner et al. 2003; Stabentheiner et al. 2010). These changes in colony-level ventilation can affect levels of respiratory gases in the cluster. The level of CO_2 in the bee cluster correlates with changes in temperature (Free and Simpson 1963; Nagy and Stallone 1976). However, bees are able to adjust atmospheric CO_2 concentration to some degree and maintain concentrations of 0.1-4.25% in small colonies and 5-6% in full size wintered colonies (Seeley 1974; Van Nerum and Buelens 1997). The level of CO_2 concentration in the cluster in winter (5-6%) is higher than in summer (0.5-2%) (Van Nerum and Buelens 1997). There also may be daily fluctuation in CO_2 within the hive. Ohashi et al. (2009) found two peaks in CO_2 concentration in honey bee colonies in early morning (approximately 5%) and another in afternoon (4%) in late summer due to daily changes in

bee ventilation behavior. Oxygen level is typically inversely correlated with CO_2 (Nagy and Stallone 1976; Southwick and Moritz 1987). However, bees can cause O_2 concentrations of around 15% in the winter cluster even when ambient O_2 is greater than 15% (Van Nerum and Buelens 1997).

Little is known about the effects of colony-level respiratory changes on varroa. Varroa mites evolved in conjunction with a tropically-adapted species of honey bee, *Apis cerana* Fabricius, prior to transferring to the European honey bee (Anderson and Trueman 2000). Little is known what ranges or durations of CO_2 concentrations might be typically found in *A. cerana*, but due to the warmer tropical climates these bees inhabit, the concentrations are likely to be lower than that found in bee colonies in the more temperate climates where *A. mellifera* typically occurs. If the "phoretic" stage of varroa is susceptible to the higher levels of CO_2 found in wintering clusters of *A. mellifera*, it may provide opportunities for controlling the mite through manipulation of CO_2 levels or enhancing the effectiveness of other defensive responses such as grooming.

Carbon dioxide fumigation has been suggested as a non-polluting and an environmentally-friendly tactic for control of grain pests because it produces no residue in food products (Aliniazee 1971; White and Jayas 1991). The potential to control varroa mite with CO_2 through manipulation of ventilation has been investigated in caged-bee studies. Kozak and Currie (2011) showed increased CO_2 levels (1.1-2.5% in the air surrounding the cluster) enhance varroa mite mortality in small clusters of caged-bees at 10 °C and 25 °C. However, nothing is known about the influence natural bee-generated CO_2 would have on mite mortality at the levels of gas that would be found in wintering bee clusters under the colder temperatures experienced during indoor wintering.

Although CO_2 fumigation has been suggested for control of some honey bee pests, for example the greater wax moth, *Galleria mellonella* Linnaeus, in beekeeping equipment (Shimanuki and Knox 1997) and varroa mite in colonies (Kozak and Currie 2011), it can have negative effects on bees (Ebadi et al. 1980; Mardan and Rinderer 1980) and could "flare" populations of other pathogens. Czekonska (2007) found that *Nosema apis* Zander spores rapidly proliferate when caged-bees are inoculated with CO_2-treated *Nosema* spores which, in turn, dramatically decreases survival rates of *Nosema*-inoculated bees. She speculated that higher colony-level CO_2 concentrations in spring might contribute to increases in *Nosema* infection levels that are often observed (Czekonska 2007). This could pose potential problems for winter fumigation with CO_2 for varroa if it also facilitates *Nosema* development. There is currently no information about how high CO_2 might affect microsporidian diseases in overwintering honey bee colonies. Other aspects of honey bee behavior or physiology could also be influenced by CO_2 manipulation. Some physiological and behavioral aspects of honey bees are affected by changes in CO_2 concentration such as the development of hypopharyngeal glands, fat body, wax glands, ovaries, corpora allata, brain chemistry, juvenile hormone titer, pollen collection and consumption, hoarding behavior, polyethism, lifespan and egg production in queens (Ribbands 1950; Mardan and Rinderer 1980; Buhler et al. 1983; Koywiwattrakul et al. 2005; Czekonska 2009). Ribbands (1950) proposed that CO_2 may affect colonies by regulating change from nursing to foraging tasks, thus influencing colony level effects such as less swarming and honey production. In queens, exposure to CO_2 causes earlier oviposition and stimulates synthesis of vitellogenin in virgin queens (Engels and Ramamurty 1976). CO_2 is used to narcotize queens during instrumental

insemination (Mackensen 1947; Ebadi et al. 1980; Chuda-Mickiewicz et al. 2012). Thus it could be disadvantageous to implement CO_2 based fumigation on a commercial scale if the CO_2 negatively influences colony performance.

Although much is known about the critical roles of ventilation and thermoregulation in wintering honey bees, little information is available on how variation in respiratory gases could affect ectoparasitic mites in honey bee genotypes that vary with regard to resistance against mites. The goals of this study were to manipulate ventilation rate in order to increase bee-generated CO_2 levels in the bee cluster; and to assess interactions between restricted-ventilation (elevated CO_2) and genotype of bees on varroa mite mortality rates, worker mortality rates and reduction in mean abundance of varroa and *Nosema* during indoor wintering of honey bee colonies.

MATERIALS AND METHODS:

The experiments were carried out at the University of Manitoba, Winnipeg, Manitoba, Canada (49°54' N, 97°14' W). Two experiments were conducted to examine the effects of restricted-ventilation on varroa mite and worker bee mortality. Experiment 1 was performed in summer 2008 and experiment 2 in winter 2011. In each of the two experiments, all bees were from European honey bee (*A. mellifera*) colonies headed by either queens from the Manitoba Queen Breeders Association that had been characterized as "selected " stock (selected for mite-resistance or mite-tolerance through a cooperative breeding program as described in chapter 3 but after four additional years of selection) or from colonies headed by either New Zealand queens (Arataki Honey, Havelock North, New Zealand) or Hawaiian queens (Kona Queens, Captain Cook, HI, USA) classified as

"unselected" stock. The objective of experiment 1 was to characterize mite and bee mortality, and to measure CO_2 and O_2 levels in the bee cluster in selected and unselected stocks maintained under a simulated winter condition. The objectives of the second experiment were to assess the influence of restricting ventilation (from the standard rate of 14.4 $L \cdot min^{-1} \cdot hive^{-1}$ to 0.24 $L \cdot min^{-1} \cdot hive^{-1}$) on the levels of CO_2 and O_2, varroa load reduction and mortality of mite and bee in selected and unselected colonies during indoor wintering.

i) Experiment 1:

In the summer of 2008, small hives (5-frame standard Langstroth) were established from colonies at the University of Manitoba that contained mixed-age worker bees from either selected stock (n = 6) or unselected stock (n = 6). Each small hive was queenless but contained a plastic release device containing synthetic queen mandibular pheromone (Pseudo Queen, Contech Enterprises Inc., Victoria, BC, Canada) as a queen substitute. All colonies were inoculated with 200 live varroa mites as described in chapter 2. The small hives (n = 12) from each of the two stocks (selected and unselected) were randomly placed in a temperature-controlled chamber (208 x 208 x 273 cm = 11.81 m^3 in the Animal Science/Entomology building, University of Manitoba) and held in constant darkness at 5 °C for 6 d. Carbon dioxide and O_2 concentrations (%) were measured twice per day, morning and afternoon, in room air and inside the cluster of bees using a headspace gas analyzer (Model 900141, Bridge Analyzer Inc., Alameda, CA, USA). The temperature (°C) and absolute humidity ($g.m^{-3}$) inside the room were monitored every 2 h using HOBO C-8® (Onset Computer Corporation, Bourne, MA, USA) data loggers to

monitor average daily fluctuations of temperature and humidity. Each hive had a completely open bottom entrance (19 x 2.5 cm). To measure CO_2 and O_2 concentrations, polyethylene air sample tubes (outer diameter: 6.35 mm; inner diameter: 4.32 mm, Watts company, North Andover, MA, USA) were fitted with one end positioned at either the center of the bee cluster or in the room space and with the other end of the tubes on the outside the room so gases could be sampled without entering the room as described by Underwood and Currie (2004) for sampling of formic acid. Tubes were sealed with a rubber sleeve stopper (Size No. 11-1/2, 10.70 mm length, plug diameter 5.16-6.73 mm, Red, Fisher Limited, Ottawa, ON, Canada). Air samples were collected after removing "dead air" from the tubes using a disposable 50 ml syringe before using the headspace gas analyzer to quantify CO_2 and O_2.

Daily varroa mite and worker bee mortality rates were assessed based on dead bees and dead mites collected from bottom board of hives and dead bee traps as described in chapter 2. At the end of the experiment the bee population was assessed visually as described in chapter 2. Mites remaining in the colony were determined by multiplying the total number of bees in the colony by the percentage mite infestation as determined by alcohol wash as described in chapter 2.

ii) Experiment 2:

In order to tightly control ventilation for this experiment and simultaneously monitor respiratory gases and bee activity a special Plexiglas chamber was constructed. The Plexiglas chamber (65 x 130 x 130 cm = 1.0985 m^3, Acrylic sheet with 6.0 mm thickness, Evonik Industrial, Parsippany, NJ, USA) was designed and constructed with a volume of

air approximately equal to the space required for overwintering hives (Currie et al. 1998). The air flow to the chamber was provided by a heavy duty air compressor (Speedaire , Model 4B233E, 135 PSI, 30 gallons, Grainer Inc., IL, USA) supplied with an air compressor filter (HDA70400AV, Husky, Mexico). Inlet air flow to chamber was regulated by a flow meter (CF-4000, 50 PSI, National Torch Tip Co., Pittsburgh, PA, USA). The flow meter was connected to the chamber via a clear vinyl tube (outer diameter: 9.52 mm, inner diameter: 6.35 mm, Watts company, North Andover, MA, USA). The Plexiglas chamber was located in a small treatment room of overwintering building as described in chapter 3 (Fig. 4.1). Air flowed throughout the small room via a ventilation tube at a rate of 14.4 $L \cdot min^{-1} \cdot hive^{-1}$ (Underwood and Currie 2004). Temperature inside the treatment room was controlled as described in chapter 3.

Three to four small hives were placed in the ventilation chamber during experiments. The effect of hive position in the chamber (2 hives in top row vs. 2 hives in bottom row) on the amount of CO_2 in the bee cluster was tested in a "pre-experiment" assay. In the "pre-experiment", small hives (n = 4) from unselected stock were randomly placed at each of four positions in the chamber (hives were placed two rows and two columns) and maintained for 7 d under a low ventilation rate (0.24 $L \cdot min^{-1} \cdot hive^{-1}$) in a temperature-controlled chamber at 5 °C as described in experiment 1. Then, the positions of hives were switched for the next 7 d. These queenless hives (supplied with Pseudo Queen as a queen substitute) were fitted with polyethylene air sample tubes in the center of the cluster as described in experiment 1. In the pre-experiment, only CO_2 and O_2 concentrations (%) in the bee cluster were measured and mite and bee mortality were not assessed.

Experiment 2 compared selected and unselected stocks under either standard- or restricted-ventilation using standard Langstroth nucleus hives with a five-frame capacity. The experiment was repeated using four colonies from selected-stock and four colonies from unselected-stock on each of the first two trials (trial 1: February 6 - March 3, 2011; trial 2: March 10 - April 4, 2011). On the third trial (April 9 - May 4, 2011) there were only two selected and four unselected colonies (n = 10 selected colonies and 12 unselected colonies). Different colonies were used on each trial. Selected and unselected colonies were randomly positioned in either a small room within the overwintering building (2 selected colonies + 2 unselected colonies) or were arranged in the Plexiglas chamber (2 selected colonies + 2 unselected colonies) at 5 °C. Once the hives were placed in the chamber or in the room, polyethylene air-sampling tubes (6.35-4.32 mm) were fitted to the center of the bee cluster in each hive and inside the spaces of the Plexiglas ventilation chamber and wintering building room to allow for gas sampling as described earlier. The colonies were maintained in the room (standard-ventilation) or in the chamber (restricted-ventilation) for 26 d (6 d as stabilizing period, 2 d as pre-treatment period, 16 d as treatment period and 2 d as post-treatment period). Inlet air flow to the chamber was controlled with a flow meter set on 0.24 $L \cdot min^{-1} \cdot hive^{-1}$ (as determined by preliminary experiments) during 16 d of treatment. However, hives in the chamber were exposed to standard-ventilation (14.4 $L \cdot min^{-1} \cdot hive^{-1}$) during stabilizing, pre-treatment and post-treatment periods. Colonies under standard-ventilation were sampled in the same way as for restricted-ventilation, but the rate of air flow was consistently 14.4 $L \cdot min^{-1} \cdot hive^{-1}$ over all periods. Carbon dioxide and O_2 concentrations were measured two times per day, morning and afternoon, as described in experiment 1.

Daily mite and bee mortality rates were quantified as described in chapter 2. The main door of the chamber during the experiment was sealed to maintain gas balance inside the chamber except for a short period when dead bees and dead mites were collected from either bottom board of hives or dead bee traps. In the Plexiglas chamber this was done through a small gate (48 x 18 cm) on the main door of the chamber. In this experiment, the mean abundance of varroa mites and *Nosema* spores [arithmetic mean of the number of spores per bee (Rozsa et al. 2000)] were also assessed. Mean abundance of varroa at the end of the stabilization period (day 7 and 8) and at the end of the ventilation manipulation (day 25 and 26) was determined by dividing the total number of mites present at the time by the total number of bees. Mean abundance of *Nosema* spores was assessed in samples of 100 bees collected from each colony at the beginning and end of each trial. Samples were processed according to the method of Cantwell (1970) using maceration and homogenization of the bees in water (1 ml distilled water per bee) then the number of *Nosema* spores in each bee (million spores per bee) was counted on a hemocytometer (Bright-Line®, American Optical, Buffalo, NY, USA). To estimate the total number of mites remaining in the colony, hives were treated with an acaricide (Apistan®, Wellmark International, Guelph, ON, Canada) for 4 weeks to collect all remaining mites on the bees after each trial was over. Then, the initial number of mites in each colony at the beginning of the experiment was calculated by the summing all mites that fell during the trial and after acaricide treatment. The initial number of worker bees in the hives was also estimated by summing the number of bees that fell during each trial and adding this number to the number of live bees remaining after the trial. Bee population size at the end of each trial was visually scored as described in chapter 2.

STATISTICAL ANALYSES:

In the first experiment, the daily mortality rates for worker bee and varroa mites and concentrations of CO_2 and O_2 were analyzed by a repeated measure analysis of variance (ANOVA) using an autoregressive covariance structure with genotype of bees as main effect (PROC MIXED, SAS Institute Inc. 2011). In the pre-experiment, the effects of hive position in the chamber on CO_2 and O_2 levels were analyzed by a repeated measure analysis of variance (ANOVA) using an autoregressive covariance structure with position of hive as main effect (PROC MIXED, SAS Institute Inc. 2011). For experiment 2, the percent change in mean abundance of varroa from the beginning of treatment (on day 7 and 8 of the pre-treatment period) to the end of treatment (on day 25 and 26 of the post-treatment period) was calculated and analyzed by a two-way ANOVA with ventilation and genotype of bees as main factors (PROC MIXED, SAS Institute Inc. 2011). The percentage change in log transformed mean abundance of *Nosema* spores over the entire experiment was analyzed by the same method. For changes in mean abundance of varroa and *Nosema*, "trial" (the time of winter when experiment 2 was replicated) and interactions between "trial" and main effect factors were removed from the model because they were not significant. A before and after control impact (BACI) design was used to assess the effects of the ventilation treatment and genotype of bees on bee and mite mortality rates and CO_2 and O_2 concentrations, where colonies within each chamber were treated as replicates and the interaction between main effects and period were used as criteria to declare significant treatment effects (Stewart-Oaten et al. 1986; Smith 2002; Underwood and Currie 2005). Ventilation setting, genotype of bees, period and "trial" were treated as main effects and period and day within periods were treated as repeated

measures with colony as the subject. Non-significant higher order interactions were deleted from the model. An autoregressive covariance structure was used for the analysis. Where significant interactions were found an analysis of simple effects was used to test for differences within trials and periods (Slice procedure, PROC MIXED, SAS Institute Inc. 2011). Prior to analyses, concentrations of CO_2 and O_2 were square root transformed, but mean abundance of varroa and the rates of mite and bee mortality for each day were arcsine transformed. All data was presented as untransformed means. The correlations among CO_2 and O_2 concentrations, daily mortality of bees and mites were analyzed using partial correlation controlling for all other factors (PROC CORR,SAS Institute Inc. 2011).

RESULTS:

i) Experiment 1:

In the experiment simulating winter conditions, the results showed that the concentration of gases measured in the core of the bee cluster was consistent for both genotypes of bees throughout the experiment. The average CO_2 concentration in the bee cluster was similar in selected bees (average: 1.42±0.14%; max: 3.90%) and unselected bees (average: 1.39±0.14%; max: 2.62%) ($F = 0.04$; df = 1, 10; $P = 0.840$) and did not vary over time (genotype of bees*time: $F = 1.24$; df = 5, 50; $P = 0.303$) (Fig. 4.2 A). The average O_2 concentration was also similar in the cluster of selected bees (19.50±0.12%) and unselected bees (19.54±0.12%) ($F = 0.07$; df = 1, 10; $P = 0.303$) and did not vary with time (genotype of bees*time: $F = 1.35$; df = 5, 50; $P = 0.258$) (Fig. 4.2 B). Carbon dioxide was negatively correlated with O_2 ($r = -0.96$, $P < 0.0001$) in the bee cluster.

Under these ventilation conditions neither daily mite mortality nor daily bee mortality were correlated with CO_2 concentration ($P > 0.05$).

Daily mite morality rate was greater in selected bees (0.029±0.004) than in unselected bees (0.015±0.004) (F = 20.06; df = 1, 10; $P = 0.0012$), but did not vary with time (genotype of bees*time: F = 2.28; df = 5, 50; $P = 0.060$). Bee mortality rates were significantly lower in selected stock (0.004±0.001) than unselected stock (0.009±0.001) (F = 9.85; df = 1, 10; $P = 0.011$). The two-way interaction between bee genotype and time was not significant indicating that treatment differences in bee mortality were consistent during the experimental period (F = 0.07; df = 5, 50; $P = 0.620$).

ii) Pre-experiment:

The "pre-experiment" showed that the position of hives in the chamber (top row vs. bottom row) did not affect CO_2 or O_2 concentrations in the honey bee cluster. Mean CO_2 concentration in colonies in the top row (3.54±1.17%) and in the bottom row (4.84±1.17%) was similar (F = 1.83; df = 1, 3; $P = 0.269$) and did not vary through the experiment (position*time: F = 1.01; df = 13, 25; $P = 0.468$). Mean O_2 concentration in colonies in the top row (17.55±1.03%) and in the bottom row (16.39±1.03%) was also similar (F = 2.88; df = 1, 3; $P = 0.189$) and did not vary over time (position*time: F = 1.01; df = 13, 25; $P = 0.472$). The greatest concentration of CO_2 and the lowest concentration of O_2 in the core of the cluster were 9.3% and 12.01%, respectively.

iii) Experiment 2:

Overall, concentration of CO_2 in the bee cluster differed within ventilation settings (restricted: 2.85±0.23% vs. standard: 1.43±0.23%) (F = 24.01; df = 1, 10; *P* = 0.0006). However, genotype of bees (selected: 1.99±0.25% vs. unselected: 2.29±0.22%) did not affect the concentration of CO_2 in the bee cluster (F = 0.76; df = 1, 10; *P* = 0.403) during the 26 d of experiment. There was a significant interaction between ventilation and period in CO_2 concentration (F = 4.79; df = 3, 42; *P* = 0.0058). The three-way interaction ventilation*trial*period was not significant (F = 1.77; df = 12, 42; *P* = 0.086). The mean CO_2 concentration in the bee cluster during the 16 d treatment period was significantly higher under restricted-ventilation (3.82±0.31%) than under standard-ventilation (1.29±0.31%) (F = 41.44; df = 1, 10; *P* < 0.0001), but this variable did not differ over days within ventilation period (ventilation setting*time: F = 0.45; df = 15, 270; *P* = 0.963). Carbon dioxide concentration in both genotypes of bees (selected: 2.43±0.33%, unselected: 2.67±0.28%) was similar through all treatment periods (F = 0.27; df = 1, 10; *P* = 0.616) (Fig. 4.3). The maximum concentration of CO_2 in the core of the bee cluster was 8.44% and 7.76% in restricted- and standard-ventilation, respectively.

During the 26 d of experiment, the concentration of O_2 in the bee cluster also differed within ventilation settings (restricted: 17.76±0.18% vs. standard: 19.03±0.18%) (F = 29.35; df = 1, 10; *P* = 0.0003), and genotype of bees did not affect the concentration of O_2 in the bee cluster (selected: 18.45±0.19% vs. unselected: 18.34±0.16%) (F = 0.21; df = 1, 10; *P* = 0.66). However, the three-way interaction ventilation*trial*period was significant (F = 6.28; df = 12, 42; *P* < 0.0001). Partitioning by trial and period showed significant differences in O_2 concentration between ventilation treatments (Table 4.1)

(Fig. 4.5 A). Average O_2 concentration measured in the bee cluster during the 16 d treatment period was significantly lower under restricted-ventilation (17.00±0.21%) than under standard-ventilation (19.16±0.21%) ($F = 51.08$; df = 1, 10; $P < 0.0001$), but this variable was similar between selected bees (18.10±0.22%) and unselected bees (18.07±0.19%) ($F = 0.01$; df = 1, 10; $P = 0.916$). The minimum concentrations of O_2 in the core of bee cluster were 13.40% and 15.48% in restricted- and standard-ventilation, respectively.

Ventilation setting and genotype of bees both affected overall mite mortality rate during the experiment. Over all periods and trials, daily varroa mortality was greater in the restricted-ventilation colonies (0.031±0.003) than in the standard-ventilation colonies (0.013±0.003) ($F = 14.63$; df = 1, 10; $P = 0.0033$), and was higher in selected bees (0.027±0.003) relative to unselected bees (0.016±0.003) ($F = 5.03$; df = 1, 10; $P = 0.049$) (Fig. 4.4). However, the three-way interaction ventilation*trial*period was significant ($F = 2.35$; df = 12, 42; $P = 0.021$). Partitioning by trial showed significant treatment effects as indicated by changes in ventilation with period within the latter two trials (late winter) but not the first trial (Table 4.1) (Fig. 4.5 B). Overall, ventilation treatment had a significant effect on the change in mean abundance over the 16 d of restricted-ventilation ($F = 5.27$; df = 1, 18; $P = 0.03$) relative to standard-ventilation indicating an overall significant treatment effect. Mean abundance of mites decreased 37±4.2% under restricted-ventilation relative to only 23±4.2% under standard-ventilation. However, honey bee genotype did not affect the change in mean abundance over the same time span ($F = 2.78$; df = 1, 18; $P = 0.113$).

Overall, ventilation setting and genotype of bees did not affect bee mortality rate. Daily bee mortality did not differ between ventilation treatments (restricted: 0.0020±0.0002 vs. standard: 0.0016±0.0002) ($F = 3.86$; df = 1, 10; $P = 0.078$), or between genotypes of bees (selected: 0.0019±0.0002 vs. unselected: 0.0017±0.0002) ($F = 0.98$; df = 1, 10; $P = 0.35$). However, the interaction among ventilation, trial and period was significant ($F = 3.47$; df = 12, 42; $P = 0.0013$). Partitioning by trial showed a significant interaction between ventilation and period within the first and second trials, but bee mortality differed between the two ventilations only for the stabilizing period in the first trial and differences between treatments within all other trials were not significant (Table 4.1) (Fig. 4.5 C).

Percentage change in mean abundance of *Nosema* spores over the duration of the experiment did not differ with ventilation treatment ($F = 0.02$; df = 1, 18; $P = 0.89$) or genotype of bees ($F = 2.12$; df = 1, 18; $P = 0.16$) and there was no interaction between period and ventilation through the experiment ($F = 0.01$; df = 1, 18; $P = 0.92$).

Measures of CO_2 concentration, O_2 concentration, temperature and absolute humidity in the bee cluster or in the spaces of Plexiglas chamber and treatment room are summarized in table 4.2.

DISCUSSION:

In this study, I showed that restricting ventilation to honey bee colonies during winter resulted in greater CO_2 levels and lower O_2 levels within the cluster and was associated with higher CO_2 levels, lower O_2 levels and higher humidity in the air space surrounding the colony relative to colonies held under standard-ventilation. When honey

bee colonies were exposed to restricted-ventilation during winter, the overall rate of varroa mite mortality increased relative to colonies held under standard-ventilation but overall bee mortality rates were not affected. Although greater mite mortality rates were found in bees selected for resistance to varroa relative to unselected bees**,** there was no interaction between the ventilation treatment and bee genotype.

A number of factors correlated with the restriction of ventilation could be responsible for the increased mite mortality rates that were observed. Kozak and Currie (2011) showed increases in humidity and CO_2 occur when caged-bees are held under restricted-ventilation and result in higher varroa mortality when ventilation is manipulated at high temperatures (25 °C). However, in contrast to my results, they found that when caged bees are subjected to restricted-ventilation at low temperature (10 °C) greater mite mortality occurs at the highest ventilation rate with the lowest CO_2 and humidity. The greater mite mortality at high ventilation rates in that experiment was attributed to a higher probability of mite desiccation associated with low humidity in the bioassay chambers, which in turn, could affect mite survival and the ability of mites to relocate their host in the bioassay chamber (Kozak and Currie 2011). My experiment was conducted at slightly lower temperatures (~6-7 °C), with much larger colonies and conducted within a wintering buildings rather than in a small scale incubator trial. I found the highest mite mortality in the restricted-ventilation treatment which had the highest humidity in room air (absolute humidity was 12 $g \cdot m^{-3}$ under restricted-ventilation and only 7 $g \cdot m^{-3}$ under standard-ventilation). The high humidity in air surrounding the colonies likely resulted from moisture that accumulated in the chamber air space due to respiration of bees (Currie et al. 1998). Although, I did not measure humidity in the core

of the bee cluster it is likely that clusters also had high humidity as CO_2 is positively correlated with humidity in wintering bees (Van Nerum and Buelens 1997). Since high levels of humidity under constant ventilation do not increase mite mortality rates and since CO_2 at very high concentrations can be used to remove varroa from anesthetized bees (Currie and Tahmasbi 2008) it is likely that high levels of CO_2 (or low levels O_2) rather than high humidity were primarily responsible for the increased rates of mite mortality. However, humidity cannot be excluded as a possible contributor to the increased mite mortality that was observed.

Hypercapnic (high CO_2 in hemolymph) and hypoxic (low O_2) conditions affect insect longevity (Calderon and Navarro 1979; reviewed in Nicolas and Sillans 1989) and influence gas exchange and ventilation mechanisms in insects (Anderson and Ultsch 1987), and thus could be expected to have effects on either varroa or wintering honey bees. The mode of action for effects of CO_2 on varroa is not clearly understood. However, CO_2 is known to influence feeding and locomotion activities in other arthropods by blocking signals at the neuromuscular junctions (Badre et al. 2005). This level of interference with the nervous system of varroa could be enough to result in greater numbers of mites dying, falling from the cluster or being more susceptible to grooming behavior of bees. In some contexts, varroa is well-adapted to tolerate high levels of CO_2, such as when female foundresses bury themselves in the food found in sealed brood cells where they are exposed to much higher levels of CO_2 (Bishop 1923) but where they likely can respire successfully with the aid of the peritreme (Strube and Flechtmann 1985). However, phoretic mites, which appeared to be affected by much

lower levels of CO_2 (less than 5%), likely cannot use the same mechanism in a different behavioral context.

There are several factors that could affect colony CO_2 levels. Low ambient temperature increases endothermic activity in honey bee clusters, increasing metabolic rates in honey bees and O_2 consumption by colonies (Free and Spencer-Booth 1959; Nagy and Stallone 1976; Stabentheiner et al. 2003). This is followed by higher CO_2 concentrations in bee clusters especially when temperatures are below 10 °C (Free and Spencer-Booth 1959; Nagy and Stallone 1976). However, CO_2 is regulated. Honey bees possess antennal sensory organs to distinguish different concentrations of CO_2 (Lacher 1967a) and have the capacity to respond to differences in CO_2 concentration of less than 0.5% (Walla 1948). Fanning behavior is used to circulate fresh air into the colony in response to high CO_2 concentrations in the colony (Seeley 1974) and is typically initiated when CO_2 concentrations exceed 3.5% (Hazelhoff 1954). Van Nerum and Buelens (1997) show the CO_2 level in small hives varied seasonally with summer bees having 0.5-2% CO_2 and winter colonies having 5-6%. Colony size may influence CO_2 regulation with larger colonies being better able to regulate CO_2 than very small colonies (Seeley 1974; Van Nerum and Buelens 1997). Video tape analysis of colonies in preliminary experiments I conducted confirmed fanning behavior was induced as expected when high levels of CO_2 occurred in the experimental hives in the ventilation chamber used in this study (personal observation, data not shown). The concentration of CO_2 in the bee clusters in this study were within the range of published values but somewhat lower than those often reported for wintered colonies. Carbon dioxide concentration averaged only 1.4% when honey bee colonies were maintained under standard-ventilation, but was

much higher (~4%) under restricted-ventilation. This difference is in agreement with the study by Kozak and Currie (2011), which showed that restricting ventilation rate in caged bees in a confined system increases CO_2 concentration surrounding the bee cluster. My results extend this finding and show that similar effects can be achieved in colonies during indoor-wintering at low temperatures. The highest level of CO_2 in my experiments (9.3%) was similar to the highest levels reported in the literature (9-9.9%) (Hallund 1956; Free and Simpson 1963). In experiment 2, the standard-ventilation treatment utilized a ventilation system typical for an overwintering building (Currie et al. 1998; Underwood and Currie 2004). This system likely prevented the CO_2 from accumulating in the room space where CO_2 levels (~0.01%) were similar to that in commercial buildings (Currie, personal observation) but slightly lower than CO_2 levels in peripheral environment of hives (0.03%) (Southwick 1987; Nicolas and Sillans 1989). However, CO_2 concentration in the chamber space under restricted-ventilation (averaged 1.4%) was numerically higher than under standard-ventilation during the treatment period. Absolute humidity in the room space was numerically higher under restricted-ventilation than under standard-ventilation but well within the range expected in commercial facilities (30-70%) (Fingler 1980; Currie et al. 1998). Oxygen concentration was slightly lower in the air space surrounding hives when restricted-ventilation was applied than under standard-ventilation.

Fahrenholz et al. (1989) showed different age groups of bees can differ in their ability to thermoregulate. This did not appear to be a factor in my study although age distribution likely varied between experiments, within each experiment all treatment group colonies were composed of bees with a similar age distribution. In experiment 1,

my colonies were created in mid-summer and would have consisted of a high proportion of young bees which should be less capable of producing heat and would have a lower resting metabolism than winter bees (Stabentheiner et al. 2003; Stabentheiner et al. 2010). Experiment 2, in contrast, was set up using colonies in mid-winter with an older population of mixed bees and would be expected to have had a high proportion of endothermic winter bees with higher resting metabolism (Southwick 1982). Despite this difference in age distribution between experiments, mean CO_2 concentration using "winter bees" under standard-ventilation in experiment 2 had CO_2 concentrations (1.43%) that were similar to colonies composed of "summer bees" (1.41%) in experiment 1. Both experiments were carried out under low temperature. This suggests that honey bees with different physiology may actively control atmospheric gases at similar levels when clustering in broodless conditions under low temperature.

Some studies suggest genotype might influence fanning behavior. Jones et al. (2004) showed different thermoregulation and fanning thresholds exist within subfamilies of bees involved in temperature regulation in genetically diverse colonies and genetically uniform colonies. More genetically diverse colonies are better able to respond to changes in environmental disturbance and have better thermal homeostasis than genetically uniform colonies. In my study, the two genotypes of colonies tested were established with "winter bees" from open mated queens which should have had similar age structure and levels of genetic diversity. I saw no evidence of differences in fanning or thermoregulation between genotypes that were selected for resistance to varroa relative to unselected colonies as neither CO_2 or O_2 varied with genotype during either of the two experiments. This suggests that although the stocks differed in mite removal capacity,

they showed a similar response in ability to fan and to regulate metabolic gases in the cluster.

Other factors which could influence the ventilation of colonies relate to changes that influence colony state. For example, the presence or absence of a queen and brood could also alter the dynamics of temperature regulation in the winter bee cluster and cause changes in bee activity and fanning behavior (Chapter 2; Andino and Hunt 2011). My experiments were conducted using queenless and broodless colonies fitted with pseudo-queen pheromone lures, which should be similar to typical wintering colonies which have little brood at that time of year (Chapter 3; Kozak 2008). Interactions with parasites and pathogens are also possible but unlikely to have been a factor. Schafer et al. (2011) show multiple infestations with varroa mites and small hive beetle (*Athena tumida* Murray) affect the thermoregulation activities in winter bee clusters. However, in my study small hive beetle was absent. I did not measure viruses in this study, and significant differences in the numbers of other parasites or pathogens in the colonies were not found.

While hypercapnic conditions stimulate antennal receptors in worker bees, hypoxia does not (Southwick and Moritz 1987). Thus, O_2 is indirectly controlled in the bee cluster (Van Nerum and Buelens 1997). Van Nerum and Buelens (1997) found honey bees tend to maintain the core O_2 concentration at around 15% in winter bee clusters, even when ambient O_2 concentrations are higher. This may be of benefit in helping bees maintain lower metabolic rates in the winter cluster. My measurements showed higher average O_2 concentration in bee clusters than those found by Van Nerum and Buelens (1997), under both standard-ventilation (19%) and restricted-ventilation

(17%). As others have noted, I found O_2 concentration was inversely correlated with CO_2 concentration (Seeley 1974; Van Nerum and Buelens 1997). Even though O_2 concentration decreased to a minimum of 13% in my study, it did not have a significant effect on worker bee mortality rates. Part of the greater varroa mortality I observed might be associated with the low O_2 availability, or synergistic effects of hypoxia and hypercapnia that are known to contribute to increased mortality in grain pests (Calderon and Navarro 1979). However, reactions of varroa to hypoxia and hypercapnia have not been studied.

Varroa is a main cause of economic damage to honey bee colonies during winter in Canada (Currie et al. 2010). Mid-winter treatments could be used to prevent winter colony loss when bees are stored during indoor wintering (Underwood and Currie 2003), but also are of potential value in ensuring that varroa is below the spring mite treatment thresholds of 2 mites per 100 bees recommended for in the prairie region of Canada to prevent losses in honey yield (Currie and Gatien 2006). Treatment of indoor-wintered colonies with natural bee-generated CO_2 has the potential to treat many colonies simultaneously with minimal labor and equipment costs and with no acaricide costs. One risk associated with indoor-wintering treatments using formic acid fumigation is increased queen loss (Underwood and Currie 2008). Since queenless colonies supplemented with queen lures were used in my study, any queen loss associated with CO_2 treatment could not be assessed. This would have to be examined in a full scale trial in wintering buildings before the treatment should be considered for use by commercial beekeepers. The level of efficacy using restricted-ventilation was lower than that found with indoor fumigation of wintering buildings with formic acid (Underwood and Currie

2004;2007) but the low cost and low hazard to beekeepers compared with the use of formic acid may make this an attractive management option. In order to improve the reliability and efficacy of varroa mite control with restricted-ventilation further work on the combinations of CO_2 or O_2 concentration (dose) and exposure time necessary to bring mean abundance of mites below spring economic threshold is required.

Carbon dioxide narcosis has many effects on the behavior and physiology of bees (Ribbands 1950; Ebadi et al. 1980; Mardan and Rinderer 1980; Buhler et al. 1983; Koywiwattrakul et al. 2005). It is possible that the use of restricted-ventilation with high levels of CO_2 for treating varroa mite could influence factors associated with the longevity of bees during winter such as worker vitellogenin gene expression (Koywiwattrakul et al. 2005), or juvenile hormone titer in the hemolymph of worker bees (Buhler et al. 1983). High levels of CO_2 affect cold tolerance of *Drosophila melanogaster* Meigen (Nilson et al. 2006; Milton and Partridge 2008) but are not likely to affect honey bees within the range found in winter clusters (Van Nerum and Buelens 1997). Free and Spencer-Booth (1960) showed that CO_2 treatment does not influence worker bee chill-coma temperature. Similarly, Kozak and Currie (2011) did not find higher bee mortality when elevated CO_2 concentrations occurred in the short duration of their experiment. In this study, CO_2 levels were within the range of those found in "normal" wintering colonies of honey bee. Although the duration of exposure to those levels may have been higher, I found only a small difference in bee mortality rates between ventilation treatments and only during the stabilizing period in the first trial. This was not attributed to a treatment effect as it occurred prior to manipulation of ventilation and there were no differences in CO_2 or O_2 concentrations between ventilation

treatments through this period. It is likely that handling manipulations associated with placing the colonies in the ventilation chamber contributed to the pre-treatment difference in bee mortality rate. The bee mortality rate during the restricted-ventilation period was similar within both treatments (0.00174 bees per day per colony) and close to those found in previous indoor-wintering studies when overwintering full size colonies placed under a standard-ventilation (Chapter 2).

In conclusion, this study showed that the use of restricted-ventilation during indoor-wintering of honey bees has potential to decrease varroa mite levels in colonies without significant effects on bee mortality. In this study, mite-infested colonies were treated with CO_2 during a 16 d period. Although reductions in mean abundance averaged only 37%, this method may be sufficient to reduce mean abundance of mite below 2% recommended for spring mite treatment in the prairie region of Canada to prevent losses in honey yield (Currie and Gatien 2006) if used in conjunction with other integrated pest management approaches. The technique is attractive because it would be possible for beekeepers to manipulate the ventilation systems without costly modifications to their ventilation facilities in overwintering buildings. This approach is a low cost, environmentally-friendly approach to control of varroa with no danger of residue accumulation in bee products. In general, the results showed that treatment of varroa - infested honey bee colonies with natural bee-generated CO_2 has potential for controlling varroa mites in overwintering colonies, but more research is needed to standardize this method on a commercial scale before it should be recommended for application in wintering buildings.

Fig. 4.1: Setup of experiment 2. Small hives from selected stock and unselected stock were arranged either in a Plexiglas ventilation chamber (PC) for application of restricted-ventilation (RV) or in a wintering room for standard ventilation (SV). Carbon dioxide and O_2 concentrations (%) in the bee cluster and spaces of chamber and room were measured through vinyl tubes via a gas analyzer (GA). During the restricted-ventilation (treatment period) inlet air flow into the chamber was regulated using a flow meter (FM) set on 0.24 $L \cdot min^{-1} \cdot hive^{-1}$. The standard-ventilation rate was set to 14.4 $L \cdot min^{-1} \cdot hive^{-1}$ in the wintering room and in the Plexiglas chamber (under standard-ventilation).

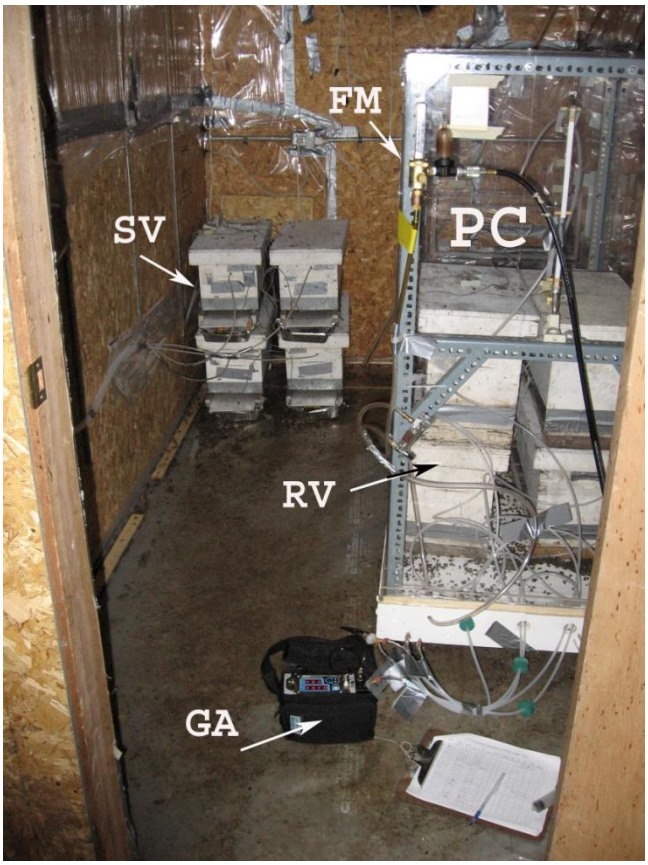


Fig. 4.2: Mean CO_2 (A) and O_2 (B) concentrations (%) in the bee cluster within selected stock and unselected stock in experiment 1. Hives were located in a temperature-controlled environmental chamber and held in constant darkness at 5 °C. Each point represents an average of two measurements (morning and afternoon). Vertical bars on each point indicate ± standard error (SE). Means followed by the same letter between genotypes of bees are not significantly different.

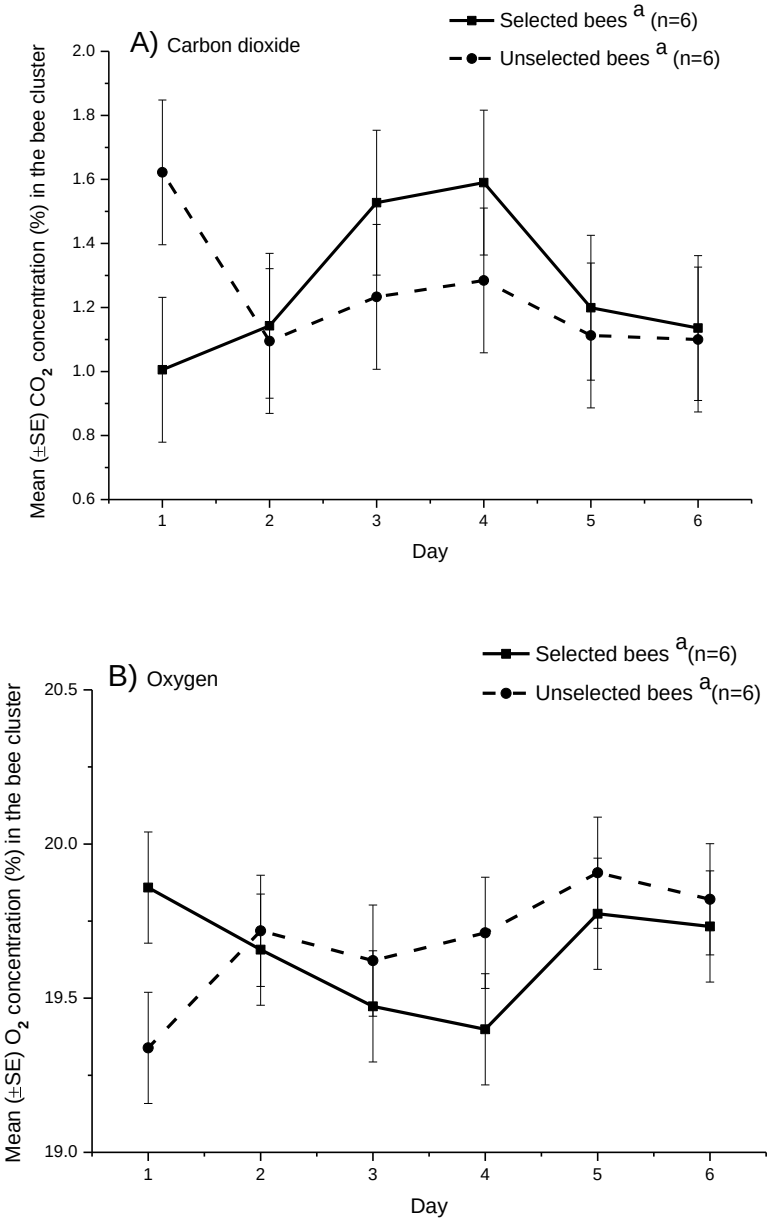


Fig. 4.3: Mean (± SE) CO_2 concentration (%) in the core of the cluster of either selected bees or unselected bees in restricted-ventilation (n = 11) (A) and in standard-ventilation (n = 11) (B) treatment groups over the 26 d of experiment-2. Restricted-ventilation involved 6 d as a stabilizing period (14.4 $L \cdot min^{-1} \cdot hive^{-1}$), 2 d as a pre-treatment period (14.4 $L \cdot min^{-1} \cdot hive^{-1}$), 16 d as a treatment period (0.24 $L \cdot min^{-1} \cdot hive^{-1}$) and 2 d as a post-treatment period (14.4 $L \cdot min^{-1} \cdot hive^{-1}$), however, the rate of air flow in standard-ventilation was consistently 14.4 $L \cdot min^{-1} \cdot hive^{-1}$ over all periods. Each point represents pooled values for two measurements (morning and afternoon) and are averages for all three trials. Vertical bars on each point indicate ± standard error (SE). Asterisks (on legend) indicate significant differences between ventilations within each period ($P < 0.05$, Slice) and *ns* represents a non-significant difference between ventilations within each period. Means followed by the same letter between ventilation settings are not significantly different.

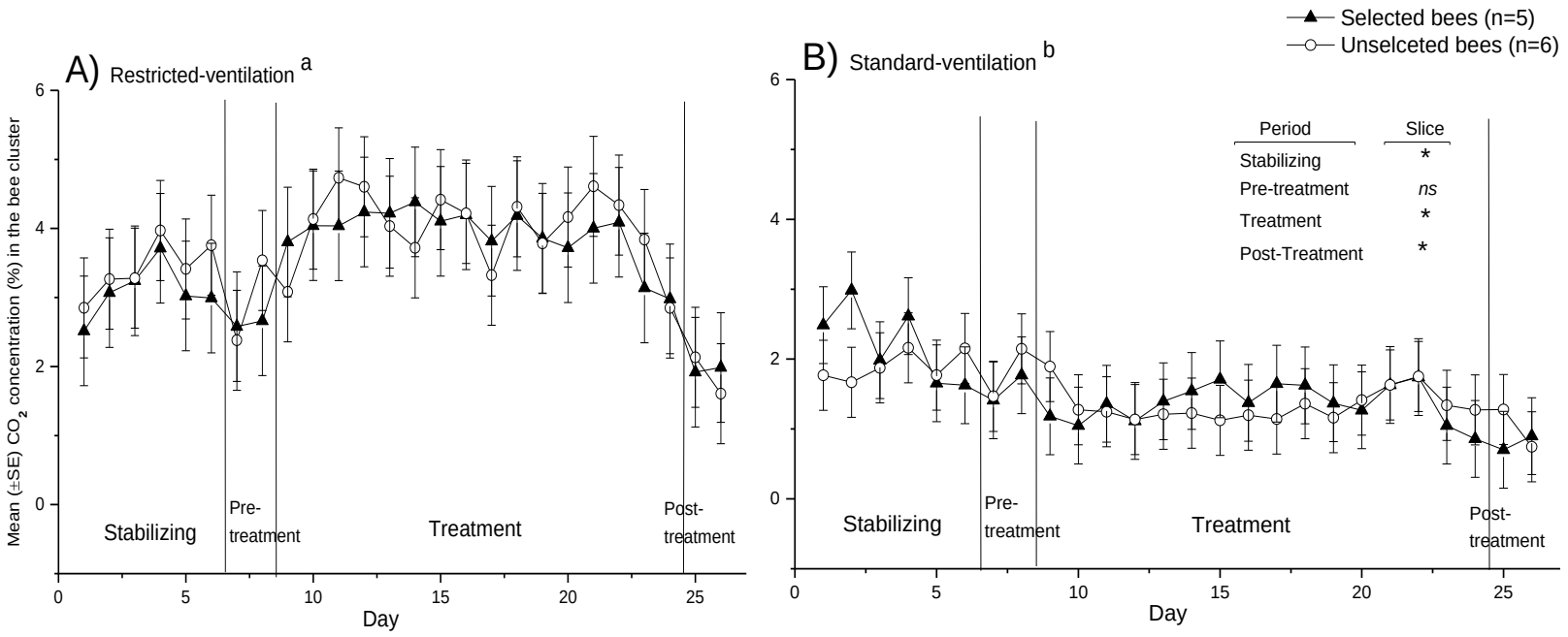

A) Restricted-ventilation a
B) Standard-ventilation b
Selected bees (n=5)
Unselceted bees (n=6)
Mean (±SE) CO2 concentration (%) in the bee cluster
Day
Stabilizing
Pre-treatment
Treatment
Post-treatment
Period
Slice
Stabilizing *
Pre-treatment ns
Treatment *
Post-Treatment *

Fig. 4.4: Mean (± SE) daily mortality rate of varroa mite in selected bees and unselected bees in the restricted-ventilation (A) (n = 11) and standard-ventilation (B) (n = 11) within the 26 d of experiment-2. Restricted-ventilation involved 6 d as a stabilizing period (14.4 $L \cdot min^{-1} \cdot hive^{-1}$), 2 d as a pre-treatment period (14.4 $L \cdot min^{-1} \cdot hive^{-1}$), 16 d as a treatment period (0.24 $L \cdot min^{-1} \cdot hive^{-1}$) and 2 d as a post-treatment period (14.4 $L \cdot min^{-1} \cdot hive^{-1}$), however, the rate of air flow in standard-ventilation was consistently 14.4 $L \cdot min^{-1} \cdot hive^{-1}$ over all periods. Vertical bars on each point indicate ± standard error (SE). Means followed by the same letter between ventilation settings or between genotypes of bees are not significantly different.

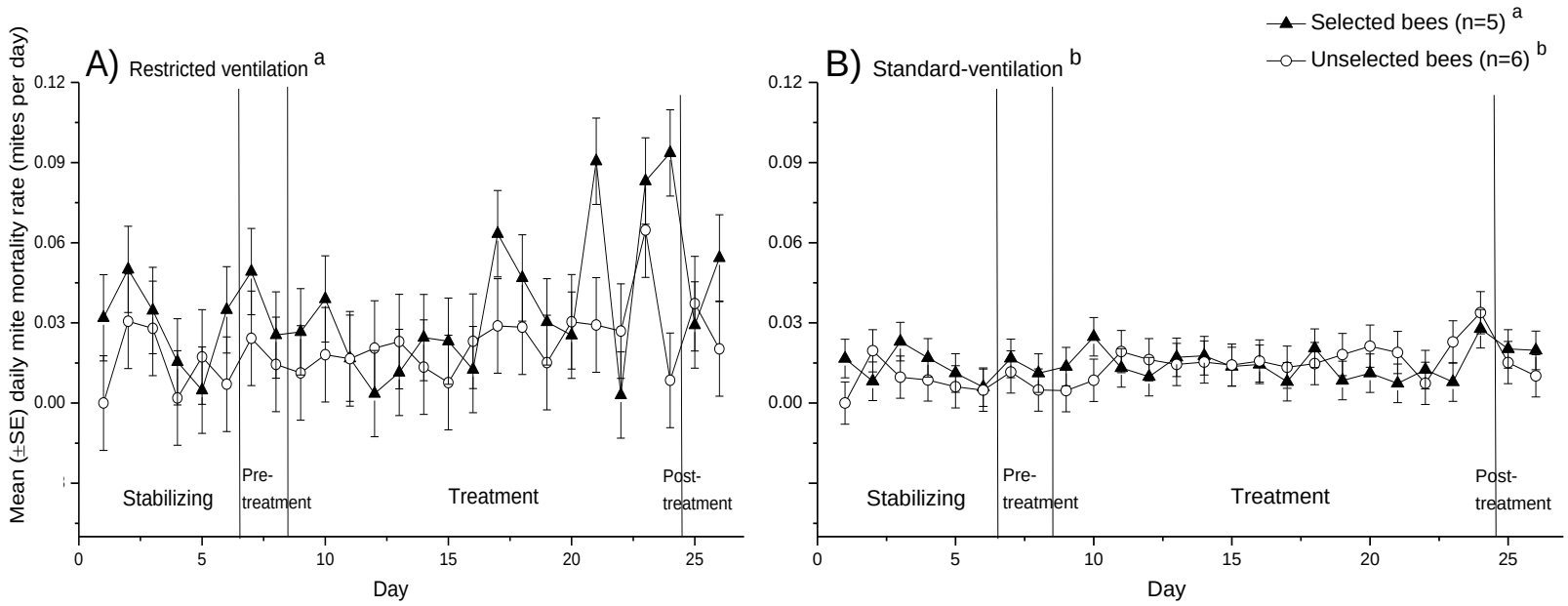


Table 4.1: Partitioning results for the significant ventilation*trial*period interaction by trial and period for O_2 concentration in the bee cluster and daily varroa mite and bee mortality rates in experiment 2 ("-" under period indicates slice only by trial).

	Trial	Period	F	Df	*P*
Oxygen concentration	1	Treatment	47.46	1, 42	<0.0001
	1	Post-treatment	5.93	1, 42	0.019
	2	Stabilizing	19.72	1, 42	<0.0001
	2	Treatment	47.28	1, 42	<0.0001
	3	Treatment	8.02	1, 42	0.007
Daily mite mortality	2	Treatment	5.66	1, 42	0.022
	3	Stabilizing	4.53	1, 42	0.039
	3	Treatment	6.32	1, 42	0.016
	3	Post-treatment	17.64	1, 42	0.0001
Daily bee mortality	1	Stabilizing	8.10	1, 42	0.007
	1	-	3.99	7, 42	0.002
	2	-	2.25	7, 42	0.049

Fig. 4.5: Mean (± SE) concentrations (%) of O_2 in the bee cluster (A), and daily varroa mite (B) and bee (C) mortality rates in wintering colonies in experiment 2 which were located in either a Plexiglas ventilation-controlled chamber (restricted-ventilation, n = 11) or in a standard wintering room (standard-ventilation, n = 11). Restricted-ventilation involved 6 d as a stabilizing period (14.4 $L \cdot min^{-1} \cdot hive^{-1}$), 2 d as a pre-treatment period (14.4 $L \cdot min^{-1} \cdot hive^{-1}$), 16 d as a treatment period (0.24 $L \cdot min^{-1} \cdot hive^{-1}$) and 2 d as a post-treatment period (14.4 $L \cdot min^{-1} \cdot hive^{-1}$), however, the rate of air flow in standard-ventilation was consistently 14.4 $L \cdot min^{-1} \cdot hive^{-1}$ over all periods. Vertical bars on each column indicate ± standard error (SE). Asterisks indicate significant difference between restricted-ventilation and standard-ventilation in each trial ($P < 0.05$, Slice).

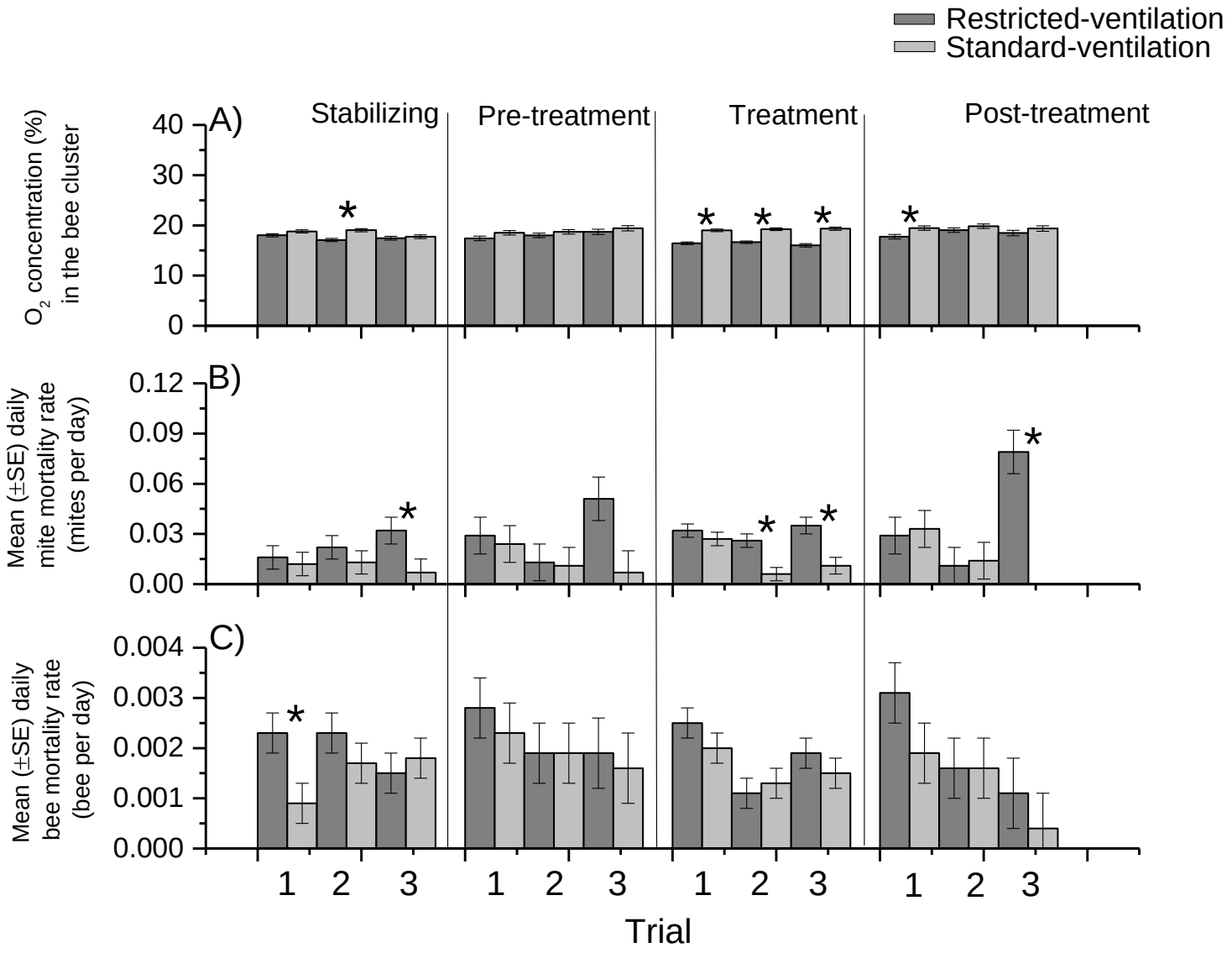

Restricted-ventilation
Standard-ventilation
A)
B)
C)
Stabilizing
Pre-treatment
Treatment
Post-treatment
O2 concentration (%) in the bee cluster
Mean (±SE) daily mite mortality rate (mites per day)
Mean (±SE) daily bee mortality rate (bee per day)
0
10
20
30
40
0.00
0.03
0.06
0.09
0.12
0.000
0.001
0.002
0.003
0.004
1 2 3 1 2 3 1 2 3 1 2 3
Trial

Table 4.2: Mean (±SE) concentrations (%) of CO_2 and O_2 (in chamber space/room space), temperature (°C) (in the bee cluster and in chamber space/room space), and absolute humidity ($g.m^{-3}$) (in chamber space/room space) in wintering colonies in experiment 2 which were located in either a Plexiglas ventilation-controlled chamber (restricted-ventilation, n = 11) or in a standard wintering room (standard-ventilation, n = 11). Restricted-ventilation involved 6 d as a stabilizing period (14.4 $L \cdot min^{-1} \cdot hive^{-1}$), 2 d as a pre-treatment period (14.4 $L \cdot min^{-1} \cdot hive^{-1}$), 16 d as a treatment period (0.24 $L \cdot min^{-1} \cdot hive^{-1}$) and 2 d as a post-treatment period (14.4 $L \cdot min^{-1} \cdot hive^{-1}$), however, the rate of air flow in standard-ventilation was consistently 14.4 $L \cdot min^{-1} \cdot hive^{-1}$ over all periods. Data are averaged across all three trials but were not analyzed.

Ventilation setting	Period	Ventilation rate ($L \cdot min^{-1} \cdot hive^{-1}$)	CO_2 (%) in space (chamber/ room)	O_2 (%) in space (chamber/ room)	Temperature (°C) in the bee cluster	Temperature (°C) in space (chamber/ room)	Absolute humidity ($g \cdot m^{-3}$) in space (chamber/ room)
Restricted ventilation	Stabilizing	14.4	0.015±0.025	20.01±0.038	17.57±0.96	6.86±0.14	7.69±0.28
	Pre-treatment	14.4	0.015±0.041	19.98±0.065	21.04±1.36	6.29±0.20	8.06±0.25
	Treatment	0.24	1.399±0.018	18.64±0.025	20.52±0.96	6.77±0.09	12.14±0.18
	Post-treatment	14.4	0.093±0.041	19.02±0.065	22.00±1.36	6.27±0.20	8.79±0.25
Standard ventilation	Stabilizing	14.4	0.008±0.025	20.05±0.038	21.13±0.89	7.06±0.13	5.86±0.28
	Pre-treatment	14.4	0.016±0.041	20.07±0.065	17.04±1.27	6.73±0.18	6.91±0.25
	Treatment	14.4	0.007±0.018	20.05±0.025	19.46±0.61	6.58±0.08	6.66±0.18
	Post-treatment	14.4	0.012±0.041	20.25±0.065	17.38±1.27	6.18±0.18	5.72±0.25

CHAPTER 5. THE INFLUENCE OF *NOSEMA* (MICROSPORA: NOSEMATIDAE) ON HONEY BEE (HYMENOPTERA: APIDAE) RESISTANCE AGAINST VARROA MITE (MESOSTIGMATA: VARROIDAE)

ABSTRACT:

The objectives of this study were to quantify the costs (as measured by bee mortality) and benefits (as measured by mite mortality) of co-parasitism with varroa (*Varroa destructor* Anderson and Trueman) and *Nosema* (*Nosema ceranae* Fries and *Nosema apis* Zander) on honey bees (*Apis mellifera* L.) with different mite removal capacity. Newly-emerged worker bees from either high-mite-removal-rate (high-MR) bees or low-mite-removal-rate (low-MR) bees were confined in forty bioassay cages and incubated at 25 °C and 55-65% RH during 19 d. Caged-bees with different mite removal ability and varroa infestation level were either inoculated with *Nosema* [*Nosema* (+) group] on the afternoon of day 4 or were left un-inoculated [*Nosema* (-) group]. Caged-bees were inoculated with 40 live varroa mites [varroa (+) group] on the afternoon of day 11or were left untreated [varroa (-) group]. This established four treatment combinations within each *Nosema* treatment group: low-MR varroa (-), high-MR varroa (-), low-MR varroa (+) and high-MR varroa (+), each with five replicates. Daily mite and bee mortality rates during 8 d post-mite inoculation, mean abundance of varroa mites and mean abundance of *Nosema* spores (in live bees and dead bees) at the end of experiment were assessed. Results showed that overall mite mortality in high-MR bees (0.12±0.02 mites per day) was significantly greater than in the low-MR bees (0.06±0.02 mites per day). Worker bee mortality was affected by interactions between mite removal rate and *Nosema* treatment. In the *Nosema* (-) group bee mortality was greater in high-MR bees

than low-MR bees but only when bees had a higher mite burden. *Nosema* parasitism and mite removal rate both affected the final mean abundance of mites. Overall, high-MR bees in the *Nosema* (-) group showed greater reductions in mean abundance of mites over time compared with low-MR bees, when inoculated with additional mites. However, high-MR bees could not reduce mite load as well as in the *Nosema* (-) group when fed with *Nosema* spores. Mean abundance of *Nosema* spores in live bees and dead bees of both strains was significantly greater in the *Nosema* (+) group. Molecular analyses confirmed the presence of both *Nosema* species in inoculated bees but *N. ceranae* was more abundant than *N. apis* and unlike *N. apis* increased over the course of the experiment. Collectively, this study showed differential mite removal ability among different genotype of bees, however, *Nosema* infection suppressed varroa removal success in high-MR bees.

INTRODUCTION:

Global honey bee (*Apis mellifera* Linnaeus) colony loss has been found to be due to interactions between different stressors (Cox-Foster et al. 2007; Currie et al. 2010; Neumann and Carreck 2010). Although *Nosema ceranae* Fries and *Varroa destructor* Anderson and Trueman have been recognized as two potential contributors to this global crisis, the main cause of the high colony collapse is still controversial (Cox-Foster et al. 2007; vanEngelsdorp et al. 2009; Currie et al. 2010).

Nosema species (*N. ceranae* and *N. apis* Zander) are intracellular endoparasites of adult honey bees infecting the cell lining the midgut (Forsgren and Fries 2010). *N. ceranae* was originally reported from the Asian honey bee *Apis cerana* Fabricus (Fries et al. 1996b) but in recent decades this endoparasite has been found in European honey bees *A. mellifera* across the world (Higes et al. 2006; Klee et al. 2007). *N. ceranae* is dominant over *N. apis* in many regions (Klee et al. 2007; Paxton et al. 2007; Chen et al. 2009b; Giersch et al. 2009) but some regions remain where *N. apis* has not been yet replaced by *N. ceranae* (Gisder et al. 2010; Forsgren and Fries 2012). This may be associated with faster reproduction of *N. ceranae* or the inability of host immune system to cope with *N. ceranae* infection (Chen et al. 2009b; Martin-Hernandez et al. 2011). Single- and co-infection of *N. ceranae* and *N. apis* occur in both *A. mellifera* and *A. cerana* colonies with higher prevalence of *N. ceranae* (Klee et al. 2007; Chen et al. 2009b). Although *N. apis* is only found in *A. cerana* and *A. mellifera*, *N. ceranae* has a broader host range and also infects *Apis koschevnikovi* Enderlein, *Apis florea* Fabricus, *Apis dorsata* Fabricus and some species of bumble bees, *Bombus* spp. (Fries 1993;

Plischuk et al. 2009; Chaimanee et al. 2010; Suwannapong et al. 2010; Li et al. 2012; Furst et al. 2014).

Worker bees are infected and the infection can spread within and between colonies through the transfer of spores. *Nosema* spores can be consumed while eating contaminated food (nectar, pollen and water) or when cleaning fecal residues from combs in infected colonies, and the infection spread when bees move between colonies as a result of robbing, drifting, swarming (Fries 1988; Fries and Camazine 2001). Infected bees can transfer *Nosema* spores to other workers, the queen (Higes et al. 2009a) and drones (Traver and Fell 2011b). *Nosema* alters aspects of both honey bee behavior and physiology (Genersch et al. 2010; Higes et al. 2010a; Le Conte et al. 2010; Goblirsch et al. 2013). *Nosema* infection causes energetic stress in infected bees which increases hunger followed by increases in food intake and less food sharing within nestmates (Mayack and Naug 2009; Naug and Gibbs 2009). *Nosema* also affects the metabolism of infected bees by degenerating epithelial ventricular cells, thus influencing hemolymph levels of fatty acids and vitellogenin, affecting enzymes secretion and protein content of hypopharyngeal glands (Wang and Moeller 1970; Suwannapong et al. 2010; Alaux et al. 2011; Chaimanee et al. 2012; Matasin et al. 2012; Goblirsch et al. 2013). Additionally, *Nosema* infection inhibits immune system function in honey bees (Antunez et al. 2009) which may decrease resistance of the host against other pathogens. *Nosema* infection also results in the degeneration of queen ovaries and reduced queen pheromone production (Hassanein 1951; Liu 1992; Alaux et al. 2011). Collectively, infection with *Nosema* often prevents bee population build up in spring, decreases bee longevity and consequently reduces colony survival (Higes et al. 2009b; Botias et al. 2013; Goblirsch et

al. 2013). Although, the distribution, mean abundance, seasonal cycle and epidemiology of *N. ceranae* and *N. apis* are becoming better understood (Klee et al. 2007; Martin-Hernandez et al. 2007; Copley et al. 2012; Traver et al. 2012), there is little information on how *Nosema* infection interacts with mite-resistance responses under different levels of varroa infestation .

Several mechanisms of resistance against varroa have been developed in commercial lines that include increased removal of mites from brood, suppression of mite reproduction and increased rates of mite removal through grooming (Spivak and Reuter 2001; Ibrahim and Spivak 2006; Harris 2007). Currie and Tahmasbi (2008) showed that mite removal is affected by genotype of bees and environmental conditions. Interactions between pathogens and parasites could influence potential benefits of host-resistance mechanisms in honey bees or increase the costs associated with defence against *V. destructor*. Therefore, the goals of this study were to determine the effects of combinations of varroa and *Nosema* parasitism on groups of bees with different mite removal rates, and to quantify the benefits (in terms of the differences in the levels of mite mortality rates) and potential costs (in terms of differences in bee mortality rates).

MATERIALS AND METHODS:

The experiments were carried out at the University of Manitoba, Winnipeg, Manitoba, Canada (49°54' N, 97°14' W) in summer 2011. All bees used in this bioassay study were provided from European honey bee (*A. mellifera*) colonies headed by either queens from the Manitoba Queen Breeder Association (MQBA) that had been characterized as "selected" bees with high mite removal (high-MR) rates (selected for mite-resistance or

mite-tolerance through a cooperative breeding program as described in chapter 3 - but after four additional years of selection) or for "unselected" bees with low mite removal (low-MR) rates from colonies headed by New Zealand queens (Arataki Honey, Havelock North, New Zealand).

Experiment 1: Screening mite removal behavior

"High-MR" and "low-MR" bees for experiment 2 were chosen by pre-screening 6 colonies from the MQBA stock and 8 from the New Zealand stock, respectively, using a cage bioassay (Currie and Tahmasbi 2008). Cages that had approximately 150 bees (three cages per colony), low levels of varroa and undetectable levels of *Nosema* spores were inoculated with 30 mites each to quantify mite removal ability. From this prescreening, I selected two colonies with "highest" mite removal rates (obtained from the MQBA stock) and three colonies with "lowest" mite removal rates (from the New Zealand stock) that were used to populate cages for experiment 2.

Experiment 2: Effects of Nosema *and varroa inoculation*

In order to collect relatively "*Nosema*-free" worker bees, frames of sealed brood with no detectable levels of *Nosema* were removed from colonies selected in experiment 1, and individually confined in brood emergence cages (47 x 25 x 6 cm) with screen on both sides and kept in an incubator at 32±2 °C with 65-75% RH. Groups of approximately ~160 newly emerged bees were weighed (21±1 g) and confined in each bioassay cage (20.5 x 8.0 x 11.2 cm). Cages were designed with a screened bottom through which mites that fell from the bees were collected on a daily basis. A piece of

aluminum foil (15 x 20 cm) coated in Vaseline® was placed under each cage to collect any mites that escaped from the cage. Two 50 ml centrifuge feeder tubes were placed on top of each cage, containing either distilled water or sucrose solution (2:1 w/w) (Underwood and Currie 2003; Kozak and Currie 2011). All bioassay cages were randomly positioned in temperature-controlled incubators at 25 °C and 55-65% RH in the dark for 19 d.

In experiment 2, cages with different mite removal rates and varroa infestation levels were either challenged with *Nosema* [*Nosema* (+)] or not [*Nosema* (-)]. In the *Nosema* (+) group, there were two treatment factors: factor 1= mite removal rate (high or low); and factor 2= varroa infestation level (high or low) (n=5 cages per treatment group). The *Nosema* (+) and *Nosema* (-) treatment groups were performed over two time periods, due to limitations of space in incubators and ability to provide enough newly-emerged bees with different mite removal rates. Within each mite removal rate factor, cages were randomly assigned to be inoculated with additional varroa as the "varroa (+) group" (n=10) or were left un-inoculated as the "varroa (-) group" (n=10). The *Nosema* (+) treatment group [high mite removal- varroa (-) and high mite removal- varroa (+); low mite removal- varroa (-) and low mite removal varroa (+)] was incubated August 3-17. The *Nosema* (-) group [same treatment combinations as for *Nosema* (+)] was incubated August 25-September 8. For both *Nosema* treatment groups, all cages were starved for 2 h on the afternoon of day 4 and bees only in the *Nosema* (+) group were inoculated with *Nosema* spores. Bees were inoculated by feeding them 20 ml of sucrose solution (2:1 w/w) containing 2 x $10^{\mathbf{6}}$ fresh-prepared *Nosema* spores [a mix of *N. apis* (9 x 10^6 gene copy per ml) and *N. ceranae* (39 x 10^6 gene copy per ml) confirmed by

quantitative real time PCR (qRT-PCR)] *ad libitum*. Bees in the *Nosema* (-) group were fed *Nosema*-free sucrose solution *ad libitum*. Half of the cages within each mite removal group in each period were then randomly assigned to be infested with 40 live varroa mites [varroa (+) group] on the afternoon of day 11 or were left untreated [varroa (-) group]. All caged bees were fed *Nosema*-free sucrose solution (2:1 w/w) *ad libitum* throughout the experiment (Fig. 5.1).

Isolation of Nosema *spores and quantification by hemocytometer:*

Nosema spores used in inoculation were obtained from a group of 400-500 worker bees from a separate set of highly infected colonies at the University bee yard. These bees were caged and incubated at 25 °C and 70-75% RH for 10 d. *Nosema* spores were obtained according to Webster et al. (2004). Diseased bees were immobilized by exposure to pure CO_2, macerated in distilled water, and the supernatant filtered through Whatman 4 filter paper. The filtered supernatant was centrifuged at 1500 rpm for 5 min to produce a pellet (Hartwig and Przelecka 1971), which was re-suspended in distilled water to produce a spore solution. *Nosema* spores were counted using a hemocytometer chamber as described in chapter 4.

Inoculation and quantification of mites:

Mites for the bioassay test were collected from a separate set of highly-infested colonies as described in chapter 2, and then introduced into cages according to the method of Currie and Tahmasbi (2008). Any mites that initially fell through to the bottom of cages during inoculation were re-introduced into the cages again after 2 h post mite inoculation

(p.m.i). The range of temperature (°C) and relative humidity (%) within each incubator were monitored using HOBO C-8® (Onset Computer Corporation, Bourne, MA, USA) data loggers. Any live mites and bees within each cage were counted at the end of experiment. Each cage was unsealed under a sleeved Plexiglas box (30 x 30 x 60 cm), where live and dead bees were separated and placed in labeled sample containers containing ethanol 75%. Mites that fell from cages were assessed as a metric associated with resistance whereby bees reduced the overall mite infestation level in the bee cluster. During 15 d post *Nosema* inoculation (p.n.i), mites and bees that fell from cages were monitored daily. However, the date of varroa inoculation was considered "day 0" of the experiment for the calculation of daily mite and bee mortality rates and mean abundance of mites as described in chapter 2.

Quantification of Nosema *in live bees using hemocytometer and qRT-PCR:*

Nosema spore mean abundance (million spores per bee) in live bees and dead bees at the end of experiment was assessed by hemocytometer as described above. The mean abundance of *N. apis* and *N. ceranae* DNA in live bees at the end of experiment was also quantified through qRT-PCR as described below. The frozen samples of worker bees were crushed in liquid nitrogen using a mortar and pestle. DNA was extracted from 100 µl of homogenized bee samples using the DNeasy blood and tissue kit (Cat. no. 69504; Qiagen, Valencia, CA, USA) following the manufacturer's protocols. The products were quantified using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). DNA was then run through 1% agarose gel electrophoresis (Embi Tec, Run one™, Electrophoresis cell, San Diego, CA, USA) to confirm quality. Primers

218MITOC-F and 218MITOC-R (Invitrogen by Life Technologies ™**,** Life Technologies Inc., Burlington, ON, Canada) were used to amplify 218 bp products of *N. ceranae*. Primers 321APIS-F and 321APIS-R (Invitrogen by Life Technologies ™) were used to amplify 321 bp products of *N. apis* (Martin-Hernandez et al. 2007). To confirm that the selected primers were able to amplify the target genes, extracted DNA from positive samples was examined with specific primers for *N. ceranae* and *N. apis* in conventional PCR. The PCR reaction mixture included 10 µl 5 x PCR buffer, 1 µl 10mm dNTP (deoxyribonucleotide triphosphate), 2 µl mixed primer (F+R), 0.5 µl Taq, 34.5 µl DNA-free ddH_2O and 2 µl extracted DNA in a total volume of 30 µl. The modified thermal cycling (Martin-Hernandez et al. 2007) was one cycle at 95 °C for 5 min (initial denaturation), followed by 35 cycles at 94 °C for 30 s (denaturation), 62 °C for 30 s (annealing) and 72 °C for 45 s (extension), followed by one cycle at 72 °C for 7 min (final extension), using the DNA Engine® Peltier Thermal PCR System (PTC-0200, Bio-Rad, Mississauga, ON, Canada). All mixed primers were tested with a negative control containing no template DNA. All samples were run through 1% agarose gel electrophoresis (Embi Tec). The PCR products were purified using QIAquick Gel Extraction kit (Cat. no. 28704; Qiagen). Purified DNA of *N. apis* and *N. ceranae* were each cloned into PDrive vector using the QIAGEN® PCR cloning kit (Cat. no. 231122; Qiagen) following the manufacturer's protocols. I purified plasmid DNA using the Gen Elute™ Plasmid Miniprep kit (Cat. no. PLN10; Sigma-Aldrich, St. Louis, MO, USA) and sent them for sequencing at Macrogen (Rockville, MD, USA). The results of sequences were tested using the GenBank database. The gene copy numbers for plasmid and plasmid plus vector were calculated based on a standard curve. The standard curves for

N. apis and *N. ceranae* were generated separately based on serial dilution. The efficiency of amplifications were calculated for each reaction based on the slope of the standard curve ($E = 10^{-1/slope}-1$). The nucleic acid quantification for *N. apis* and *N. ceranae* were performed for all samples by using qRT-PCR (Applied Biosystems 7300, CA, USA). Each 20 µl reaction included 10 µl 2 x power SYBR Green PCR Master buffer (Applied Biosystems), 1 µl primer (F+R), 1 µl DNA template and 8 µl ddH_2O. To quantify *N. apis* and *N. ceranae* amplifications, Na-321 primer (Martin-Hernandez et al. 2007) and Nc-104 primer (Bourgeois et al. 2010), respectively, were used (both primers were obtained from Invitrogen by Life Technologies ^{TM}). All qRT-PCR were run based on the following thermocycler program: one cycle of initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 30 s and annealing at 63 °C for 30 s. Negative and positive templates were run for each plate. Gene copy number of *N. apis* and *N. ceranae* for each sample were calculated using the C_t values (mean three replicates) against the standard curve (Appendix 1).

STATISTICAL ANALYSIS:

In experiment 1, the screening experiment to compare mite and bee mortality rates in each of the two stocks, the data were analyzed using a repeated measures ANOVA with rep (cages within colonies) as a repeated measure and colony as the experimental unit. In experiment 2, the variables for mite and bee mortality rates, mean abundance of varroa mites, and mean abundance of *Nosema* spores were analyzed using a mixed model ANOVA in which mite removal rate (high-MR and low-MR), varroa treatment [varroa (+) group and varroa (-) group] and *Nosema* challenge level [*Nosema* (+) group and *Nosema* (-) group] were treated as main factors, cages were experimental units and

colony (within mite removal) was treated as a random effect. A repeated "group= varroa" statement was used to relax the assumptions to allow unequal residual variance across varroa groups (PROC MIXED, SAS Institute Inc. 2011). Non-significant interactions were removed from the model. Proportions for mite mortality rate, bee mortality rate and mean abundance of varroa were arcsine transformed, and *Nosema* spore and gene copy mean abundance data were log transformed prior to analyses (Snedecor and Cochran 1980). All data are presented as untransformed means. Where significant interactions were found an analysis of simple effects was used to test for differences within bees with different mite removal rates, varroa treatments and *Nosema* treatments (Slice procedure, PROC MIXED, SAS Institute Inc. 2011).

RESULTS:

Experiment 1:

Prescreening showed the MQBA stock had higher mite mortality rates (0.081±0.004) than the New Zealand stock (0.057±0.003) ($F = 24.7$; df = 1, 12; $P = 0.0003$). "High-MR" colonies that were selected from the pool of MQBA stock had mite mortality rates of 0.090±0.004 and "low-MR" colonies from the New Zealand stock had mite mortality rates of 0.052±0.003 ($F = 49.12$; df = 1, 3; $P = 0.006$). Overall bee mortality rate averaged (0.020±0.002) and did not differ between high-MR and low-MR colonies ($F = 0.33$; df = 1, 3; $P = 0.61$).

Experiment 2:

Similarly in experiment 2, over all cages, greater mite mortality was found in high-MR cage treatments than in low-MR cage treatments ($F = 6.77$; df = 1, 24.5; $P = 0.016$) and

this response did not vary significantly with either varroa (F = 0.01; df = 1, 24.5; *P* = 0.936) or *Nosema* (F = 0.01; df = 1, 24.5; *P* = 0.904) treatment. There were no significant interactions with mite removal rate and varroa treatment (F = 0.01; df = 1, 24.5; *P* = 0.929) or with mite removal rate and *Nosema* treatment (F = 0.03; df = 1, 23.7; *P* = 0.859). The interaction between, *Nosema* treatment*varroa treatment also was not significant (F = 3.95; df = 1, 23.1; *P* = 0.059) (Fig. 5.2 A).

Overall, across all treatments, mite removal rate (F = 6.95; df = 1, 33; *P* = 0.0127) and *Nosema* treatment (F = 5.04; df = 1, 33; *P* = 0.0316) affected bee mortality rate following inoculation with mites (p.m.i). Across all treatment groups, varroa treatment (F = 3.95; df = 1, 33; *P* = 0.0552), and the interaction of *Nosema* treatment* varroa treatment (F = 3.20; df = 1, 33; *P* = 0.0826) did not affect daily bee mortality rate. In the *Nosema* (+) group, there were no differences in bee mortality with mite removal rate or varroa level treatments (P > 0.05). However, in the *Nosema* (-) group, there was a significant interaction between mite removal rate and varroa treatment for daily bee mortality rate (F = 15.2; df = 1, 15.8; *P* = 0.013) (Fig. 5.2 B). Partitioning by mite removal treatment and varroa inoculation level showed that the varroa inoculation treatments both had higher bee mortality than the un-inoculated treatments and that the high-MR colonies had greater bee mortality than the low-MR colonies but only when inoculated with high levels of varroa (Table 5.1) (Fig. 5.2 B).

Inoculation with varroa mite (F = 327.39; df = 1, 31.9; *P* < 0.0001) and *Nosema* spores (F = 5.34; df = 1, 31.8; *P* = 0.0275) both had overall effects on the final mean abundance of varroa on bees. The varroa -inoculated colonies still had higher mite infestations by the end of the experiment and *Nosema*-inoculated bees had higher mite

infestations than un-inoculated bees (Fig. 5.3). However, for final mean abundance of varroa , there were significant interactions among mite removal rate and *Nosema* treatments (F = 8.20; df = 1, 31.8; *P* = 0.0074) and among varroa and *Nosema* inoculation treatments (F = 8.89; df = 1, 32.3; *P* = 0.0054) (Fig. 5.3). Partitioning the varroa**Nosema* interaction by varroa inoculation treatment showed similar final mite infestations in the *Nosema* (-) and *Nosema* (+) groups for un-inoculated bees [varroa (-) group], but in varroa inoculated bees [varroa (+) group] final mite infestations were higher in the *Nosema* (+) group than the *Nosema* (-) group (Table 5.1). Partitioning the mite removal rate treatment**Nosema* interaction showed differences within the un-inoculated group (F = 9.34; df = 1, 17.7; *P* = 0.0069) but not in the inoculated group (F = 0.88; df = 1, 17.7; *P* = 0.362). Within the *Nosema* (-) group, there was a significant mite removal rate*varroa inoculation interaction (F = 9.81; df = 1, 9.39; *P* = 0.01) for final mean abundance of varroa, where high-MR colonies had lower final mean abundance than low-MR colonies when inoculated with varroa but not when cages were left with "low" infestation levels (Table 5.1).

Mean abundance of *Nosema* spores at the end of experiment was greater in the *Nosema* (+) group than in the *Nosema* (-) group for both live bees (F = 72.24; df = 1, 31; *P* < 0.0001) (Fig. 5.4 A) and dead bees (F = 19.95; df = 1, 29.6; *P* < 0.0001) (Fig. 5.4 B). Both species of *Nosema* were present. Mean abundance of DNA for *N. ceranae* was greater in the *Nosema* (+) group than in the *Nosema* (-) group (F = 31.96; f = 1, 24.8; *P* = 0.0001) (Fig. 5.5 A). In *N. apis* mean abundance of DNA was overall similar in bees inoculated with *Nosema* spores and in those that were not (F = 0.94; df = 1, 30.2; *P* = 0.3411) (Fig. 5.5 B), however, this trait was higher in the *Nosema* (+) group than in the

Nosema (-) group only in high-MR bees (mite removal rate**Nosema* treatment: $F = 5.53$; df = 1, 18.9; $P = 0.03$).

DISCUSSION:

This study characterized the influence of a mixed-species *Nosema* infection on groups of honey bee workers with different mite removal abilities. The results showed that *A. mellifera* worker bees selected for their ability to reduce their mite burden (high-MR bees) had higher mite mortality rates than low-MR bees whether inoculated with high levels of *Nosema* or varroa. Overall, both high-MR and low-MR bees had higher bee mortality rates when inoculated with *Nosema*. High-MR bees had similar bee mortality to low-MR bees when not inoculated with varroa, or when inoculated with varroa and *Nosema*. However, when inoculated with varroa but not *Nosema,* high-MR bees had greater bee mortality rates than low-MR bees. High-MR bees decreased their mite burden more than low-MR bees when *Nosema* was at low levels but not when they were also inoculated with *Nosema*. To my knowledge, this is the first study to examine the influence of *Nosema* infection on mite removal behavior in honey bees. The results suggest that the effectiveness of mite removal activity was impaired when the bees were infected with high levels of *Nosema*.

Both environmental and genetic factors affect varroa removal rates in honey bees (de Guzman et al. 1996; Currie and Tahmasbi 2008), but it is not known how endoparasitism affects this behavior. My experiment was conducted in a cage study at temperatures of 25 °C that provide optimal conditions to detect differences in mite removal between high- and low-grooming bees (Currie and Tahmasbi 2008). The initial

mean abundance of varroa averaged 33% in the varroa (+) group, which is a level that is effective in inducing mite removal behavior within caged bee populations (Bahreini, unpublished data). Under these conditions, high-MR bees caused the mean abundance of mites to decrease more than in low-MR bees, when bees were inoculated only with varroa mites but not inoculated with *Nosema*. This confirms the results of Currie and Tahmasbi (2008) which were carried out in the absence of *Nosema*. In contrast, the final mean abundance of varroa was similar in both high- and low-MR bees when varroa-infested bees were inoculated with *Nosema* spores. This suggests that the pathogenic infection suppressed grooming behaviors. There are some possible reasons for this phenomenon. *N. ceranae* suppresses immune genes expression and enhances host susceptibility to other pathogenic infections (Antunez et al. 2009). Similarly, bees weakened by *Nosema* may also have lower capabilities to defend against ectoparasitic mites. *Nosema* infection also causes changes in the physiology of bees which accelerate age polyethism (Naug and Gibbs 2009; Alaux et al. 2011; Goblirsch et al. 2013). If age of bees is related to grooming frequency, it is possible that disruption of worker polyethism could influence grooming success.

Higher levels of bee mortality provide metrics that indicate "biological costs" that have been associated with mite removal behavior in honey bees under low temperature (Currie and Tahmasbi 2008). In this study, high-MR bees had much higher mortality than low-MR in the *Nosema* (-) bees indicating a potential cost associated with resistance behavior. However, this was not observed in the *Nosema* (+) group where mite removal was less effective (no reductions in mean abundance were observed) and *Nosema* had a significant impact on host death.

Differential mortality of bees and mites could also result in changes in the mean abundance that are unrelated to direct grooming behaviors. Bees within the cluster that are infected with both varroa and *Nosema* could die at greater rate than uninfected bees in the same cage, resulting in reductions in the mean abundance of varroa. In this study, mites that fell directly from the bee cluster and those that were attached to a dead host were all used to calculate daily mite mortality rate. Although bee mortality rates within high-MR bees inoculated with varroa were greater than in low-MR bees, the mean abundance of mites in high-MR bees was still lower than in low-MR bees. This indicates that although some mite mortality could be associated with mites leaving the cluster of bees on dying hosts, other factors such as grooming likely contributed to the greater reductions in mean abundance that were observed in high-MR bees. Increases in the mean abundance of mites are also possible. Varroa is known to be able to switch host bees within the cluster. Two scenarios can be expected when host bees infected with varroa die that would allow varroa to survive. First, varroa might leave dying bees and use questing behavior to locate new hosts, thus accumulating on remaining bees. Bowen-Walker et al. (1997) showed that varroa can leave host bees before workers die and drop from the winter cluster. Second, varroa may prefer to leave diseased hosts if they can identify them, and accumulate on healthy bees. *Nosema* alters the metabolic activities in workers (Wang and Moeller 1970; Dussaubat et al. 2012), inducing energetic stress (Mayack and Naug 2009) and causes malnutrition in the host that could potentially produce cues that could be recognized by ectoparasites. Varroa mites are able to switch hosts and move between different strains of bees under laboratory conditions (Bahreini, unpublished data) so this could have occurred within caged-bees in this study.

In my study, caged-bees were fed with a mix of *N. apis* and *N. ceranae* spores. Mean abundance of *Nosema* spores was quantified using traditional spore counting as well as molecular methods. I found some cages in the *Nosema* (-) group that showed no spores in live bees using the hemocytometer method. This confirms the results of previous studies suggesting a lower sensitivity of standard spore counting relative to molecular methods (Hamiduzzaman et al. 2010; Traver and Fell 2011a). Final gene copy abundance levels of *N. apis* in live bees were lower than the initial gene copy levels in food but final levels of *N. ceranae* in food increased over time in live bees. Differences in the final quantity of *Nosema* of each species in inoculated and un-inoculated groups may have been affected by differences in the initial numbers of spores I used for inoculation. Neither *N. apis* nor *N. ceranae* were found alone in any cages, but molecular analysis showed higher abundance for *N. ceranae* than *N. apis* in the inoculum for the *Nosema* (+) group treatment. Some *Nosema* was also present in un-inoculated bees in my study. In un-inoculated bees, *N. apis* levels were considerably lower than that of *N. ceranae* and similar levels of both of these pathogens would be in the bees that were ultimately inoculated with *Nosema* at the beginning of the experiment.

Caged bees were inoculated with approximately 1.4×10^{4} spores per bee which is within the range of suggested levels for *Nosema* inoculation (Forsgren and Fries 2010; Martin-Hernandez et al. 2011; Porrini et al. 2011). As expected, the mean abundance of *Nosema* spores in live bees increased in the *Nosema* (+) groups over the15 d of the experiment, to between 1.2 to 1.7 million spores per bee with controls [*Nosema* (-)] averaging only 0.03 to 0.09 million spores per bee. *N. ceranae* and *N. apis* normally show similar growth patterns when bees are infected with 10^{4} spores per bee and can

reach 30 million spores during 10-12 d p.n.i. in the ventriculus (Fries 2010). However, in the full body extracts of bees I found greater growth of *N. ceranae* than for *N. apis* in inoculated bees as indicated by final gene copy numbers in inoculated cages. Martin-Hernandez et al. (2009) investigated whole abdomen extracts of infected-bees and also found faster growth for *N. ceranae* spores than *N. apis* when inoculated with 10^5 spores per bee and incubated at 33 °C. The differences in relative increases in abundance of *N. ceranae* and *N. apis* may be related to differences in the virulence of *Nosema* species in different regions (Paxton et al. 2007) or differences in species-specific susceptibility of host bees (Fontbonne et al. 2013). *Nosema* (Antunez et al. 2009) and varroa (Gregory et al. 2005; Yang and Cox-Foster 2005) both suppress immune system function in infected bees. In my study, the number of gene copies or spores of *Nosema* were at similar levels between varroa (-) and varroa (+) within *Nosema* (+) group suggesting that immune suppression resulting from varroa parasitism did not significantly contribute to *Nosema* build up in co-infected bees.

As expected, inoculation with *Nosema* increased the bee mortality rate. Since bees in my study had mixed infections of both species, the contributions of each to effects on mortality cannot be assessed. It is likely that much of the bee mortality resulting from inoculation is linked to *N. ceranae* since it was the predominate species and the only one that increased over the experiment. Several studies show higher virulence for *N. ceranae* than *N. apis* (Higes et al. 2007; Paxton et al. 2007; Mayack and Naug 2009; Martin-Hernandez et al. 2011), but in some studies similar bee mortality occurs with each species of *Nosema* (Forsgren and Fries 2010). *Nosema* spore levels at the end of my experiment had reached the recommended nominal threshold of 1 million spores per bee for *N. apis*

that is recommended at the colony level. However, thresholds have not been established for *N. ceranae* and little is known about the interactions between varroa and *Nosema*. Orantes Bermejo and Garcia Fernandez (1997) found higher *Nosema* intensity in colonies highly infested with varroa, but mite infestation did not affect *N. apis* prevalence. Hedtke et al. (2011) found a positive relationship between varroa mite levels in colonies in autumn and *N. apis* infection level in the following spring. In the controlled cage study, *Nosema* levels were not affected by varroa inoculation in my experiment. Bee mortality within bee sources with different mite removal abilities was similar when exposed to *Nosema*. Although, interactions between varroa and pathogens may contribute to colony losses, such as has been found for varroa and Deformed wing virus (DWV) (Nazzi et al. 2012), my findings indicated no interactive effect between *Nosema* and varroa on worker bee longevity, at least during this short term study. Energetic stress due to *Nosema* infections increases hunger rate and leads to enhance the risk of death (Mayack and Naug 2009). Therefore, it is possible the unlimited sucrose available in my experiment may have mitigated the negative effects of some interactions. Although infected-bees were fed with fresh *Nosema*-free sucrose *ad libitum* over time, higher bee mortality still occurred in the *Nosema* (+) group in comparison to control bees.

Genotype of bees influences the degree of host tolerance or susceptibility to a variety of bee pathogens. In my experiment all bees in the *Nosema* (+) group were inoculated with equivalent numbers of spores. Final abundance of all *Nosema* spores in live bees was similar within both groups of bees (high-MR and low-MR) but spore counts do not capture all stages of *Nosema*. Molecular analysis showed a significant interaction between the mite removal rate treatment and *Nosema* inoculation treatments for mean

gene copy number within *N. apis* but not within *N. ceranae*. High-MR bees had higher relative levels of *N. apis* compared to low-MR bees when inoculated with *Nosema* but had lower relative levels of *N. apis* compared to low-MR bees when not inoculated with *Nosema*. These differences may be due to slight differences in the susceptibility of high-MR bees to microsporidian endoparasitism in comparison to low-MR bees or possibly competitive effects between the two species within individual bees or cages. The actual mechanism behind this phenomenon is not clear, but more investigations are required to clarify this finding. Resistance to *Nosema* has been linked to changes in immune gene expression that decrease the virulence of *Nosema* (Huang et al. 2012). Different susceptibility to *Nosema* infection has also been found within native bees (*Apis cerana* Fabricius, *Apis dorsata* Fabricius and *Apis florea* Fabricius) and European bees (*A. mellifera*) (Huang et al. 2012; Chaimanee et al. 2013; Fontbonne et al. 2013).

The age of host affects the development and impact of *Nosema* on its host. In this study, bees were established from newly emerged bees and followed for a period of 15 days by which time measurable effects related to spore production and bee longevity would be expected. About half of the young bees died when inoculated with *Nosema* spores, which is in line with other laboratory experiments where bee mortality typically ranges from 50-100% (Higes et al. 2007; Martin-Hernandez et al. 2011; Aufauvre et al. 2012; Dussaubat et al. 2012; Smart and Sheppard 2012; Goblirsch et al. 2013). This bioassay cage study focused on the effects of pathogenic infections on resistance mechanisms in young honey bees, but little is known about how this might affect mite removal in colonies of older overwintering bees with different physiology. Mortality

rates of 2-week old bees collected from hives is typically lower (0-22%) when fed with a mix of *N. ceranae* and *N. apis* spores through 14 d p.n.i (Forsgren and Fries 2010).

In conclusion, I showed that *Nosema* inoculation reduced the effectiveness of mite-removal capabilities in co-infected bees. Future investigations are needed to clarify microsprodian diseases impacts on resistance mechanisms at colony scale.

Fig. 5.1: Experimental procedure. Worker bees in the *Nosema* (+) group were fed with 20 ml of sucrose solution (2:1 w/w) containing 2 x 10^6 fresh-prepared *Nosema* spores (a mix of *N. apis* and *N. ceranae) ad libitum* on the afternoon of day 4. The varroa (+) group were infested with 40 live varroa on the afternoon of day 11 to increase the mean abundance of mites. Afterward, all cages were fed with *Nosema*-free sucrose solution (2:1 w/w) *ad libitum* until the end of experiment.


fed with *Nosema*-free sucrose


fed with *Nosema* spores


inoculated with varroa mite

Group		Day																	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Nosema (+)	Varroa (+)																			
	Varroa (-)																			
Nosema (-)	Varroa (+)																			
	Varroa (-)																			

Fig. 5.2: Mean (±SE) daily mortality rates of varroa (A) and worker bees (B) in different treatments within high-MR bees and low-MR bees during 8 d p.m.i. The boxplots indicate the standard error (length of box), mean (solid dot), median (horizontal line inside box), 5^{th} and 95^{th} percentiles (lower and upper vertical lines). Means followed by the same letter between mite removal rate (legend), between *Nosema* treatments and between mite removal rate within treatments are not significantly different.

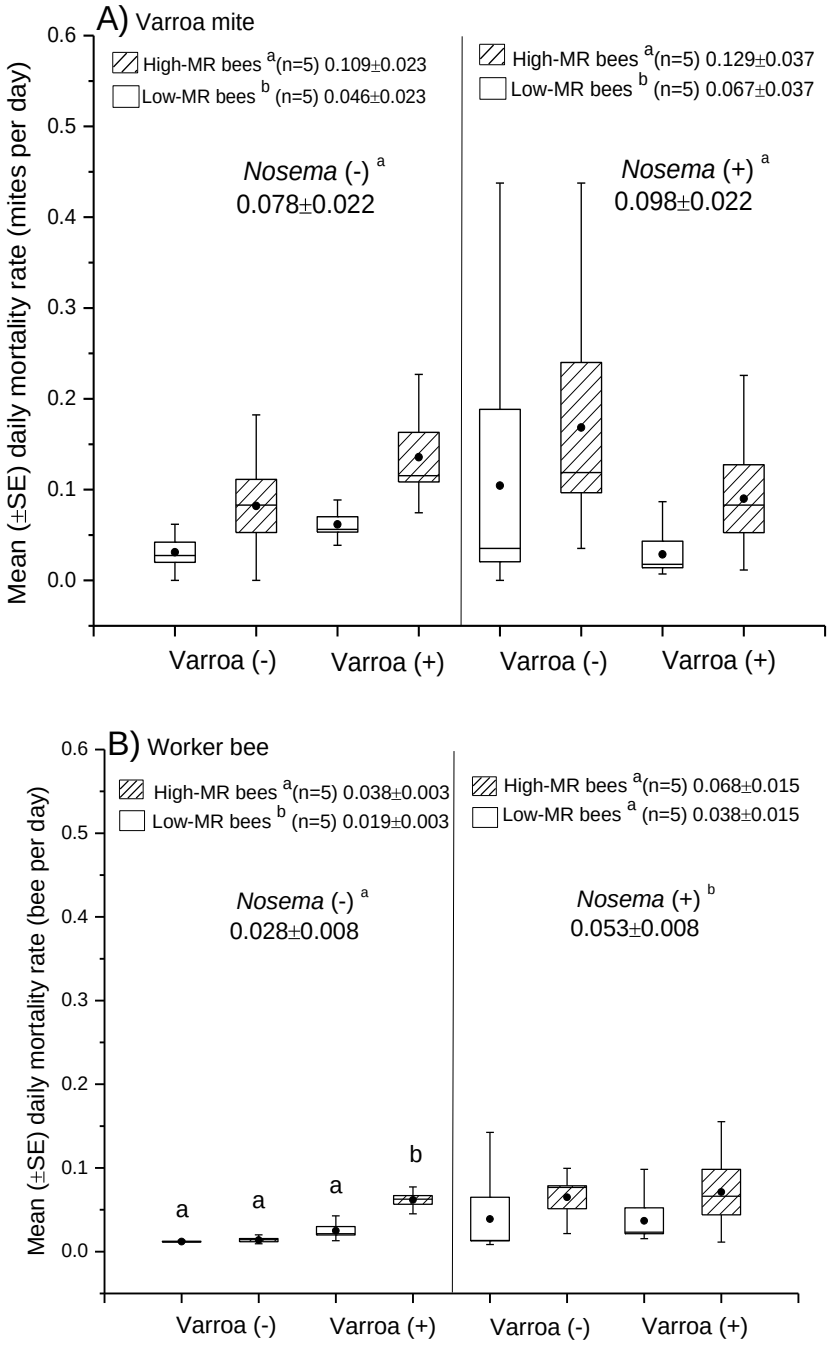

A) Varroa mite
High-MR bees a(n=5) 0.109±0.023
Low-MR bees b (n=5) 0.046±0.023
High-MR bees a(n=5) 0.129±0.037
Low-MR bees b (n=5) 0.067±0.037
Nosema (-) a
0.078±0.022
Nosema (+) a
0.098±0.022
Mean (±SE) daily mortality rate (mites per day)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Varroa (-)
Varroa (+)
Varroa (-)
Varroa (+)
B) Worker bee
High-MR bees a(n=5) 0.038±0.003
Low-MR bees b (n=5) 0.019±0.003
High-MR bees a(n=5) 0.068±0.015
Low-MR bees a (n=5) 0.038±0.015
Nosema (-) a
0.028±0.008
Nosema (+) b
0.053±0.008
Mean (±SE) daily mortality rate (bee per day)
a
a
a
b
Varroa (-)
Varroa (+)
Varroa (-)
Varroa (+)

Table 5.1: Partitioning of significant interactions between mite removal*varroa and *Nosema**varroa sliced by mite removal rate (high- or low- MR), varroa treatment [varroa (+) or varroa (-)] or *Nosema* treatment [*Nosema* (+) or *Nosema* (-)] for the variables daily bee mortality rate and final mean abundance of mite.

Variables	Interaction	Sliced by	F	Df	*P*
Daily bee mortality rate	MR*varroa	High-MR	85.12	1, 14.6	0.001
		Low-MR	4.53	1, 13.4	0.0524
		Varroa (+)	29.23	1, 3.16	0.0108
		Varroa (-)	67.04	1, 3.16	0.852
Mean abundance of mite	*Nosema**varroa	*Nosema* (+)	245.32	1, 30.7	< 0.0001
		Nosema (-)	103.70	1. 32.8	< 0.0001
		Varroa (+)	11.74	1, 31.5	0.0017
		Varroa (-)	0.06	1, 31.9	0.814
	MR*varroa [in *Nosema* (-)]	Varroa (+)	14.74	1, 9.25	0.0038
		Varroa (-)	2.19	1, 9.48	0.316

Fig. 5.3: Mean (±SE) abundance of varroa mite at the end of experiment in different treatments within high-MR bees and low-MR bees. The boxplots indicate the standard error (length of box), mean (solid dot), median (horizontal line inside box), 5th and 95th percentiles (lower and upper vertical lines). Means followed by the same letter between mite removal rate (legend) and between *Nosema* treatments are not significantly different.

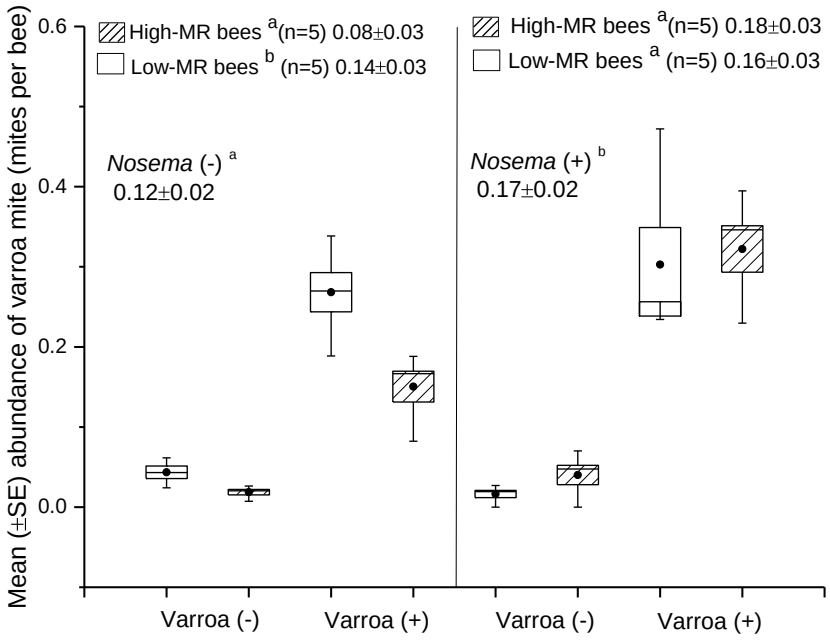
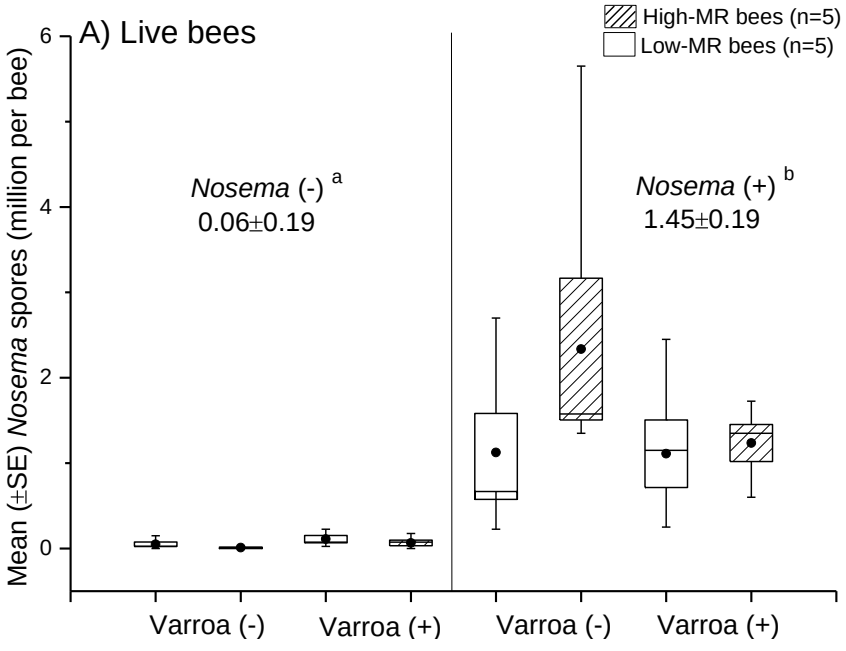


Fig. 5.4: Mean (±SE) *Nosema* spores (million per bee) in live bees (A) and dead bees (B) at the end of experiment in different treatments within high- MR bees and low-MR bees. The boxplots indicate the standard error (length of box), mean (solid dot), median (horizontal line inside box), 5^{th} and 95^{th} percentiles (lower and upper vertical lines). Means followed by the same letter between *Nosema* treatments are not significantly different.


A) Live bees
High-MR bees (n=5)
Low-MR bees (n=5)
Mean (±SE) Nosema spores (million per bee)
6
4
2
0
Nosema (-) a
0.06±0.19
Nosema (+) b
1.45±0.19
Varroa (-)
Varroa (+)
Varroa (-)
Varroa (+)

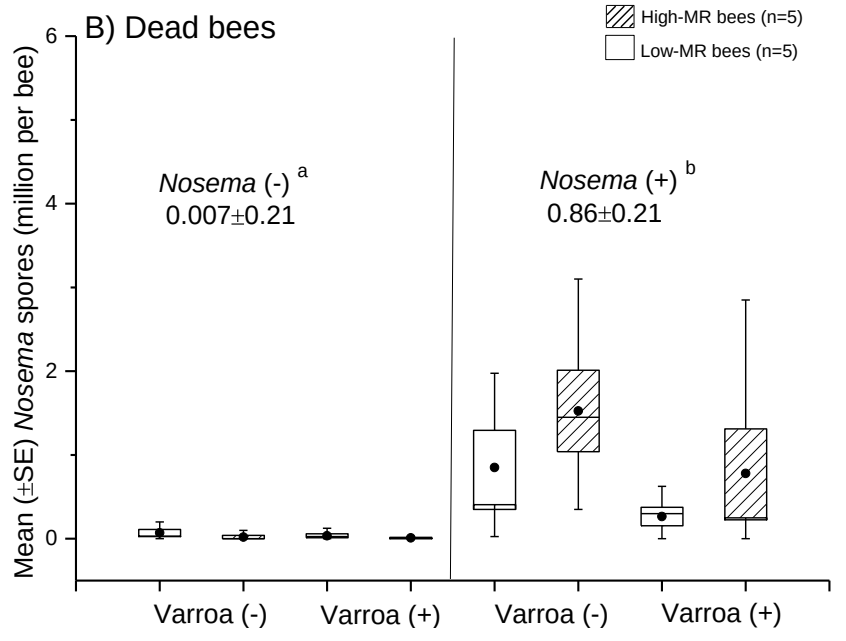
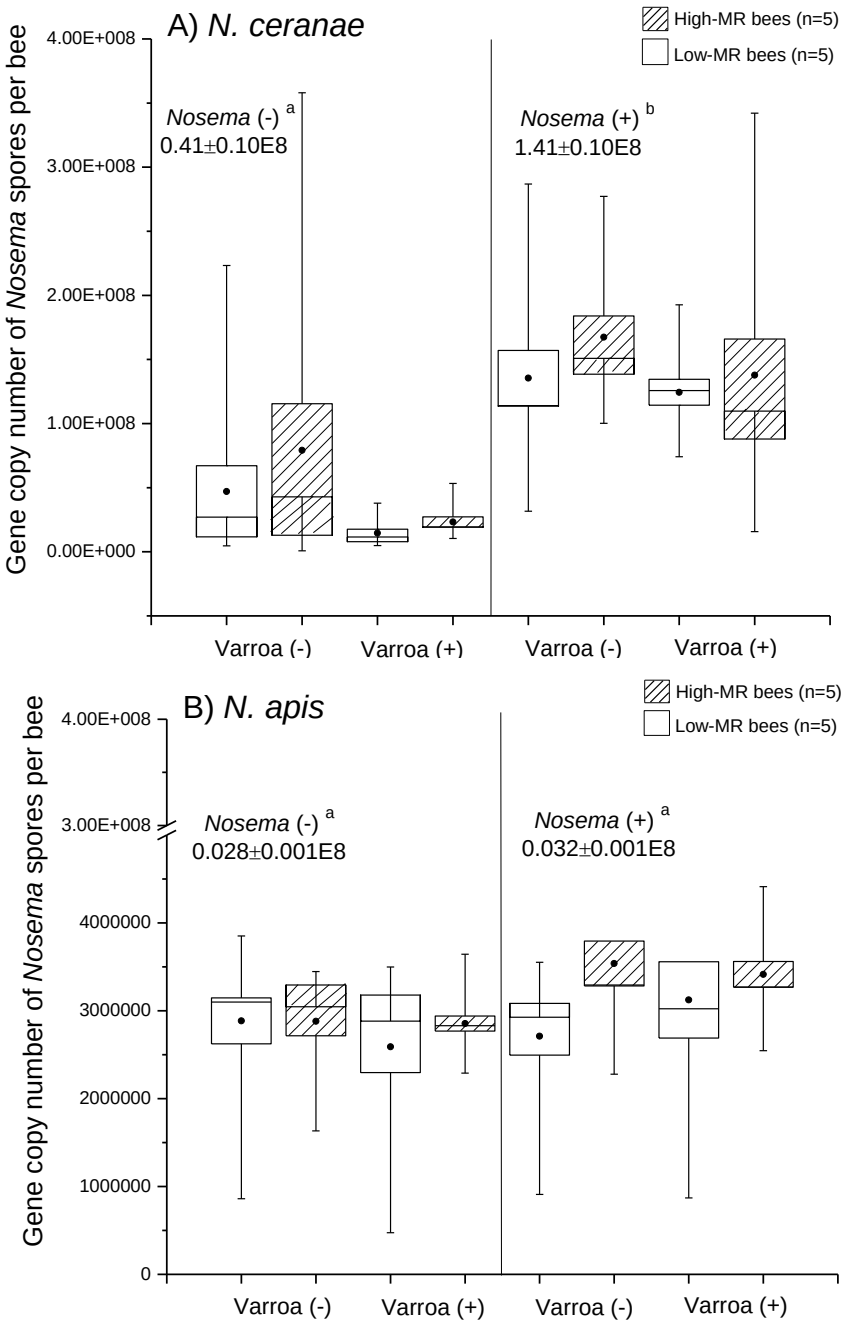

B) Dead bees
High-MR bees (n=5)
Low-MR bees (n=5)
Mean (±SE) Nosema spores (million per bee)
6
4
2
0
Nosema (-) a
0.007±0.21
Nosema (+) b
0.86±0.21
Varroa (-)
Varroa (+)
Varroa (-)
Varroa (+)

Fig. 5.5: Mean (±SE) gene copy number of *N. ceranae* (A) and *N. apis* (B) per live bee which were collected at the end of the experiment in different treatments. The boxplots indicate the standard error (length of box), mean (solid dot), median (horizontal line inside box), 5^{th} and 95^{th} percentiles (lower and upper vertical lines). Means followed by the same letter between *Nosema* treatments are not significantly different.


A) N. ceranae
High-MR bees (n=5)
Low-MR bees (n=5)
Gene copy number of Nosema spores per bee
4.00E+008
3.00E+008
2.00E+008
1.00E+008
0.00E+000
Nosema (-) a
0.41±0.10E8
Nosema (+) b
1.41±0.10E8
Varroa (-)
Varroa (+)
Varroa (-)
Varroa (+)
B) N. apis
High-MR bees (n=5)
Low-MR bees (n=5)
Gene copy number of Nosema spores per bee
4.00E+008
3.00E+008
4000000
3000000
2000000
1000000
0
Nosema (-) a
0.028±0.001E8
Nosema (+) a
0.032±0.001E8
Varroa (-)
Varroa (+)
Varroa (-)
Varroa (+)

CHAPTER 6: GENERAL DISCUSSION:

Varroa destructor Anderson and Trueman is a critical factor influencing overwintering honey bee (*Apis mellifera* L.) colony losses in northern climates (Currie et al. 2010; Guzman-Novoa et al. 2010; Akyol and Yeninar 2011). Through the past few decades, lines of honey bees that either "resist" or "tolerate" ectoparasitic mites at some level have been developed (de Guzman et al. 1996; Harbo and Harris 2009; reviewed in Buchler et al. 2010). Similarly, the University of Manitoba and Manitoba Queen Breeders Association (MQBA) used a cooperative breeding program to attempt to increase the frequency of mite-resistance or mite-tolerance traits in local stocks. However, little is known about how internal and external factors affect resistance or tolerance behaviors or what traits would be most beneficial in different management contexts. Collectively, the goals of several lab and field studies conducted in this dissertation were to develop more effective and economical approaches to minimize the impact of varroa parasitism on overwintering honey bee colonies using stock selected for resistance and tolerance to varroa. In this thesis, I evaluated the costs and benefits of mite removal and mite-tolerance behaviors and factors that may affect the efficiency of these behaviors in honey bee workers through several lab and field experiments as described below. I evaluated the effects of queen pheromone, different wintering environments, restricted-ventilation during wintering and level of varroa and *Nosema* infection on their interactions with selected and unselected stocks.

Benefits of mite resistance and mite-tolerance behaviors:

Prior to my study, the level of success in incorporating mite resistance or tolerance traits using MQBA's cooperative breeding approach had not been comprehensively evaluated.

In this thesis, I assessed potential "benefits" of selection by using mite mortality rates and reduction in mite abundance as indicators of mite resistance and the ability of bees to survive with greater mite burdens as indicators of the benefits association with mite-tolerance. The results of my experiments, where honey bees were exposed to high mite levels under different experimental conditions, suggest both mite removal and mite-tolerance traits can be improved within Manitoba stock through cooperative breeding by queen producers. The results of chapters 2 and 3 showed some benefits of mite removal and mite-tolerance behaviors, respectively. When colonies were artificially inoculated with additional mites under a simulated winter environment at 5 °C (Chapter 2) or infested stock was tested in a wintering building (Chapter 4), I found evidence of resistance through enhanced mite removal (probably through grooming). Benefits of mite removal were also shown in the cage study (Chapter 5) where bees selected for high mite removal rate (high-MR) decreased the initial mean abundance of mites by 55%. Potential benefits of grooming for increasing mite removal rates have been shown in different contexts (Rinderer et al. 2001; Currie and Tahmasbi 2008; Andino and Hunt 2011; Danka et al. 2011; Guzman-Novoa et al. 2012). My findings showed that honey bees responded with greater mite removal when exposed to additional parasite load (Chapters 2 and 6), which is in agreement with Guzman-Novoa et al.'s (2012) results who evaluated self-grooming (auto-grooming) when bees were inoculated with a single mite but also found a negative correlation between mite mortality and mean abundance of mite at the colony scale in some genotypes of bees in Ontario, Canada. Levels of mite inoculation that I used in my cage studies were chosen because they were effective in stimulating enhanced mite removal behavior. There may be a "threshold" number of

mites which induces mite removal response in the bee populations (Personal observations) but this was not quantified within the context of this thesis. However, it is not known how individual bees would be able to sense the overall mite density in the cluster or how mite introduction to a group of bees induces the onset of mite removal behavior within clusters of honey bees. It is possible that chemical cues, vibration or thermal signals release grooming response in the bee cluster. This requires further investigation. Successful reductions in mite load through enhanced mite removal have been previously observed in overwintering colonies (Currie and Tahmasbi 2008), and in chapter 2 under simulated winter conditions, but my study using colonies selected by MQBA producers did not show reduction in mite levels over winter (Chapter 3). These traits may not have been retained in producer stock if the behaviors associated with enhanced mite removal had lower rates of heredity than other traits or were less compatible with other traits that are considered economically important by producers– such as honey production. Such traits may be less likely to be retained unless specifically included as a priority in selection criteria at the producer level.

I found that worker bees with different genotypes both showed enhanced mite removal rates when stimulated with queen pheromone. In most laboratory studies, mite removal behavior has been quantified within bee populations without queen pheromone (Aumeier 2001; Currie and Tahmasbi 2008; Andino and Hunt 2011). Andino and Hunt (2011) suggested queen pheromone was unnecessary to stimulate mite removal behavior in small groups of bees. My results support this conclusion (Chapter 2) but importantly, showed that presence of queen pheromone (caged queens or pheromone lures) enhanced parasite removal within colonies.

I also assessed the rate of injured mites in small colonies from two different genotypes of bees which were inoculated with varroa mites under low temperature (5 °C). Several studies suggest the proportion of injured mites on the bottom of a cage or hive be used as an indicator for grooming success (Moosbeckhofer 1992; Bienefeld et al. 1999; Andino and Hunt 2011; Guzman-Novoa et al. 2012). However, under low temperature, I found similar overall rates of mite damage in selected stock vs. unselected stock and interactions over time showed use of these criteria could result in selection for lower rates of mite removal if colonies were assessed for short periods at the wrong time. Therefore, my results suggest the proportion of injured mites should not be considered as a reliable index to assess mite removal success in wintering studies.

Mite-tolerance is a form of host-parasite co-evolution that enables the host to coexist with parasites without significant negative effects on the host or parasite. Tolerance can be a favorable strategy at low levels of parasite infection, as the costs of defense might outweigh the benefits (Vandame et al. 2002). Different forms of tolerance against varroa mites may operate within honey bees (Ruttner et al. 1984; de Guzman et al. 1999; Le Conte et al. 2007) but little is known about the tolerance of bees against varroa during winter. My study is the first to examine tolerance in wintering bees in Canada and showed selected stock derived from a breeding program in Manitoba was able to sustain heavier varroa loads than unselected stocks under indoor-wintering conditions (Chapter 3). The selected stock had larger colony sizes, greater colony survival and provided more commercially viable colonies in spring compared to the unselected stock. I do not know the physiological or behavioral reasons for the

occurrence of greater mite-tolerance in the selected stock used in my wintering study (Chapter 3) this needs to be investigated in future research.

Costs of defensive behaviors against varroa:

I used the rate of bee mortality as a metric to quantify costs of mite removal or mite-tolerance mechanisms. Costs of resistance against natural enemies have been quantified in some organisms such as plants (Bergelson and Purrington 1996), snails (Webster and Woolhouse 1999), birds (Sheldon and Verhulst 1996) and arthropods (Groeters et al. 1994; Vandame et al. 2002; Currie and Tahmasbi 2008). Costs of resistance activities vary with environmental conditions in plants (Cipollini et al. 2003) as well as in honey bees (Currie and Tahmasbi 2008). For example, Currie and Tahmasbi (2008) showed a short transient biological cost for grooming behavior under low temperature (10°C), however, they found no significant difference in the cost (bee mortality) at 25 °C and 34 °C. Similarly, my findings (Chapter 2) showed that bees selected for higher mite removal rate also have higher bee mortality rates for short periods (the first 2 d of the experiment) at low temperature (5 °C). However, my bioassay cage studies (Chapter 5) showed greater biological costs associated with mite removal under high parasite loads can also occur at higher ambient temperatures (25 °C). Thus, in the presence of high parasitic mite levels, honey bee's defensive reactions to a varroa can sometimes result in costs (increased bee mortality) for performing resistance behavior. Cage studies (Chapter 5) indicated these costs can pay off in terms of reduced mite burden. However, my field studies (Chapter 3) showed that tolerance can also be a successful method to allow bees to increase winter survival under high mite loads and was the factor that contributed most

to wintering success after the first five years of re-selection of stock for commercial traits by queen producers.

Interaction with modified ventilation rates:

The aim of experiments that I have addressed in chapter 4 was to assess the effects of manipulating ventilation in wintering buildings on the levels of respiratory gases in the bee cluster and their impact on varroa mites in stock that was selected for mite resistance and in unselected stock. Previous studies suggested that manipulated ventilation could be used to increase mite mortality rates during indoor wintering management but this had not been experimentally tested on full scale colonies and the effectiveness of manipulating ventilation rates on respiratory gasses in clusters of full scale colonies was unknown (Underwood and Currie 2005; Kozak and Currie 2011). My study showed that restricting ventilation during wintering in indoor chambers can increase varroa mortality rates but this method has similar advantages for both selected and unselected stocks. I showed that restricted-ventilation increased carbon dioxide (CO_2) concentration and decreased oxygen (O_2) concentration within the winter bee cluster, and increased humidity in the chamber air surrounding the colony. These changes in ventilation caused higher varroa mite mortality relative to colonies under standard-ventilation rates. Under restricted-ventilation, the rate of mite mortality was increased when the CO_2 level increased to ~ 4%, O_2 level decreased to 17% and absolute humidity in the area surrounding the colony increased to an average of 12 $g \cdot m^{-3}$. The real mechanism behind this increased mite mortality is not completely understood, but direct and indirect effects of CO_2 or O_2 on varroa mites and possibly fanning behavior of bees are likely

responsible. Increased humidity is unlikely to be related to increased mite mortality as lower levels of humidity typically increase mortality in insects exposed to CO_2 (Aliniazee 1971) and possibly with varroa under conditions of low temperature (Kozak and Currie 2011). Also, increasing humidity in cage studies does not affect varroa mite mortality (Currie and Tahmasbi 2008). Since CO_2 influences the nervous system of arthropods (Van Dijken et al. 1977), the enhanced CO_2 levels in my experiment may have resulted in sublethal effects on mites due to impaired locomotory behavior or have had direct effects causing mortality since mites are much smaller than bees. The possibility that lower availability of O_2 for short times might have contributed to mite death cannot be excluded. Varroa mites are able to tolerate lower levels of O_2 because they respire through the peritreme (Strube and Flechtmann 1985) when they are trapped in larval food in sealed brood, but little is known about critical O_2 levels in varroa mites and their ability to tolerate relatively lower O_2 concentrations within the bee cluster. High CO_2 or low O_2 could cause mites to fall from the cluster and die on the bottom of hives where it would be difficult for them to relocate a new host under low temperature. If this is the cause of increased mite mortality, it seems that resistant stock with enhanced mite rates did not synergize the rate of mite removal in conjunction with restricted-ventilation. Meanwhile, elevated CO_2 in the bee cluster did not result in costs to the colony in terms of an impact on worker bee survival. The benefits of restricted-ventilation were reflected in lower mean abundance of mites at the end of the treatment period without measurable cost for treated colonies. This method would benefit beekeepers by providing a low cost measure to control varroa mites in overwintering buildings via simple modification of ventilation facilities. Further experiments are needed to determine the influence of

enhanced CO_2 on queen, brood and other parasites [e.g. tracheal mite (*Acarapis woodi* (Rennie))] and pathogens [e.g. *Nosema* (*N. cerana* Fries and *N. apis* Zander) and viruses] on a commercial scale in full size overwintering colonies.

Interaction with pathogenic infection:

The influence of *Nosema* parasitism on the effectiveness of mite-resistance behavior in worker bees against varroa mite was quantified in chapter 5. As in studies of full size colonies in chapter 2 that were not inoculated with *Nosema*, the experiment in chapter 5 showed greater efficiency of mite removal behavior in high-MR bees when artificially infested with varroa. However, mite removal in high-MR bees was no better that in low-MR bees when the bees were inoculated with *Nosema* spores. Lower longevity is known to result from infections with *Nosema* (Goblirsch et al. 2013) and varroa (Kralj and Fuchs 2006). Similarly, results obtained in the bioassay cage study (Chapter 5) showed higher bee mortality when bees were inoculated with *Nosema*, varroa or both. *Nosema* increases the susceptibility of honey bees to other pathogenic infections (Antunez et al. 2009), but I did not observe additive interactions between *Nosema* and varroa. Varroa (Contzen et al. 2003) and *Nosema* (Mayack and Naug 2009) both induce energetic stress in infected bees. Since mite removal behavior requires bees to invest energy it may be less effective in *Nosema*-infected bees because of the lower levels of available energy resulting from immune system defences. The biological or pathological reasons for this phenomenon are not clear, but there could be a trade-off between mite-resistance and *Nosema*-resistance in high-MR bees. Trade-offs between resistance and susceptibility to parasites or pathogens have been demonstrated in other species (reviewed in Carton et al. 2005).

This possibility of a trade-off between the benefits of mite resistance and possible costs associated with susceptibility to *Nosema* should be clarified. The results of my laboratory study could have implications for the management of mite-resistant honey bee stock and suggest that resistance mechanisms may operate more effectively if *Nosema* is kept under control through the use of cultural methods or application of drug treatments.

Interaction with wintering method and acaricide treatment:

Heavy varroa mite pressure in late autumn alone or in combination with other stressors usually significantly decreases the ability of bees to over winter (Strange and Sheppard 2001; Currie 2008; Dainat et al. 2012a). However, my findings have shown the benefits of selected stock through their ability to tolerate higher varroa mite pressure when the mean abundance of mites in late autumn was above recommended threshold (Chapter 3). Overall, colonies from selected stock had larger populations and suffered less colony loss (a significant benefit) in comparison to unselected stock. This has important potential for improving the wintering success of commercial beekeeping operations. In my study, I chose "unselected" control colonies from local colonies provided by Manitoba beekeepers and colonies headed by queens imported from New Zealand (Chapters 2-5) or Hawaii (Chapter 4) that had not been selected for mite-tolerance or mite-resistance. Since neither the Hawaiian, New Zealand or local stock had been exposed to high pressure from varroa at the time of my study, it was hypothesized that they would have a low degree of resistance or tolerance against varroa than stock subjected to selection if in fact selection was successful. This was confirmed in all my experiments where the unselected stock showed either lower resistance or tolerance than the selected stock.

Since most commercial Manitoba beekeepers in Manitoba use colonies headed by these same queen "unselected" sources, the controls would have been broadly representative of commercial bee populations in Manitoba. Therefore, my results suggest adoption of local stock that has been selected for resistance and tolerance to mites and wintering performance could have significant benefits in improving colony winter survival in years when autumn control of varroa is poor and mite levels are high going into winter.

In my experiment to assess stock under different wintering methods (Chapter 3), I manipulated the levels of varroa mites in experimental colonies using oxalic acid in late autumn which allowed me to compare winter performance of different genotypes of bees with heavy and moderate mite infestation levels under two different wintering methods. The treatments were applied during periods when colonies would have very little to no brood. Although previous studies recommend oxalic acid to control varroa mites during broodless period (Mutinelli et al. 1997b; Gregorc and Planinc 2002), the results showed that late autumn fumigation with oxalic acid reduced the mean abundance of varroa by only about half. That level of mite reduction did not benefit colony size or survival in either the selected stock or the unselected stock and the costs of treatment would not be justified in this case. Oxalic acid treated colonies that did survive would still have mite levels that would require treatment the following spring (Currie 2008).

Standard management practices for beekeeping (e.g. colony nutrition and treatment) were carried out across all colonies in the wintering experiment to minimize stressors other than varroa (Chapter 3). Other parasites or pathogens such as tracheal mite (Furgala et al. 1989) or *Nosema* (Higes et al. 2009b) that have been linked to winter colony mortality were absent or well below economic thresholds (Ostermann 2002; Villa

et al. 2009) in late autumn. Therefore, potential interactions with those factors on costs and benefits of mite-tolerance behavior were not likely factors in my experiment. I did not assess the role of viral infections in colony mortality, but viruses alone or in combination with varroa have been suggested to cause winter loss in overwintering colonies and are often at high levels when colonies also have high levels of varroa mite in late autumn (Highfield et al. 2009; Berthoud et al. 2010; Desai 2014). For example, Desai (2014) suggested the combinations of varroa and deformed wing virus (DWV) contribute to increased winter mortality of bees in Manitoba. Therefore, the greater survival of selected stock, I observed, could be associated with a combination of tolerance to varroa and viruses. Further studies are required to understand the role of both varroa and viral infections on mite-tolerance and mite-resistance mechanisms in overwintering colonies.

Honey bee colonies face biotic and abiotic stressors during winter that affect their wintering ability thus, different wintering management approaches could affect a colony's ability to over winter under pressure of parasites and pathogens. In this thesis, interactions between varroa and two different genotypes of bees on wintering performance (survival rate and proportion of commercially viable colonies) were assessed under two different wintering systems (Chapter 3). I found a significant interaction between genotypes of bees and wintering environment on the degree of wintering success. During indoor wintering colonies showed greater relative size in spring, greater rates of winter survival and had more colonies of a commercially viable size in selected stock than in unselected stock that was wintered outdoors. Previous studies often show advantages of indoor wintering over outdoor wintering in cold

climates but that work did not test colonies under high varroa pressure and did not use stock selected for resistance to varroa (Stalidzans et al. 2007; Williams et al. 2010a; Desai 2014). Desai (2014) showed similar colony loss (20%) in indoor- and outdoor-wintered colonies in Manitoba under normal commercial management of unselected stocks of bees but, with lower winter bee mortality for indoor-wintered hives, Lafreniere (2011) reported higher mortality within outdoor-wintered colonies (42%) relative to indoor-wintered ones (29%) in a survey of winter success in the same geographic area. But results of surveys in prairie regions often indicate outdoor wintering can be very successful in many seasons (Currie, personal communication). In my carefully controlled study, colonies wintered indoors had 54% survival rate compared with only 5% for colonies wintered outdoors when maintained under high pressure of varroa. MQBA stock wintered indoors had 73% survival.

In general, use of indoor-wintering systems to mitigate losses from high parasite and pathogen loads appears to be a good strategy to improve winter colony survival. Use of colonies selected for varroa tolerance and resistance through a cooperative breeding program, in combination with wintering indoors would be beneficial because it would provide more colonies for producers and pollination services in spring. Use of restricted-ventilation management in a wintering building may be useful as part of an integrity pest management approach for varroa. Use of selected stock would reduce costs associated with labor needed to repopulate hives and producers would have to purchase fewer packages of replacement bees.

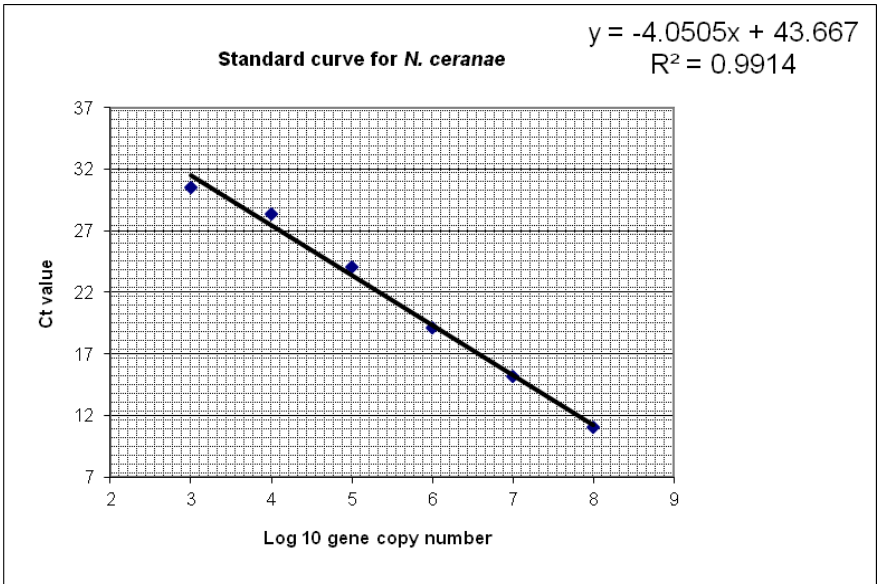
PROPOSAL FOR FUTURE RESEARCH:

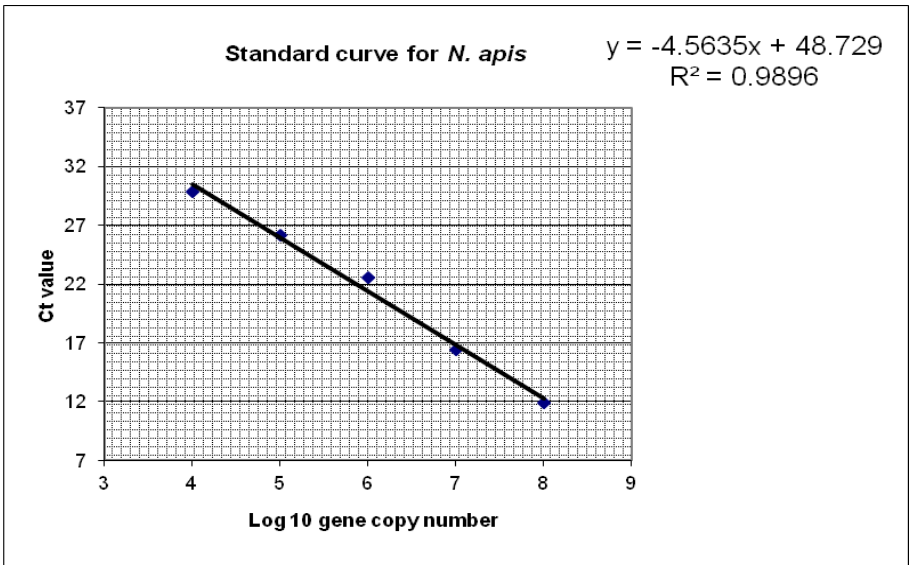
My findings suggest potential guide for future research:

- Future experiments should be carried out on the interactions between genotype of bees and environmental factors to gain a better understanding of tolerance and resistance in a complex of traits against varroa mites and other pathogens (e.g. viruses) within honey bee stocks.
- Further investigations are required to more clearly address the role of mite-resistance mechanisms in reducing varroa infestation levels over winter, and to improve mite removal traits within local stock that also meet the needs of commercial beekeeping requirements.
- In order to improve the efficiency of varroa mite control with bee-generated CO_2, more work on the combinations of CO_2 and O_2 concentrations (dose) and exposure time (CT product) are necessary to standardize this method for application on a commercial scale and possible adverse interactions with other integrated pest management approaches need to be assessed before it should be recommended for application in wintering buildings.
- More studies are needed to clarify impacts of different *Nosema* species on mite-resistance and mite-tolerance mechanisms within different genotypes of bees at the colony scale during winter.

APPENDIX:

Appendix 1: Standard curves for *N. ceranae* and *N. apis* were generated by using serial dilution of the plasmid DNA in quantitative real time PCR (qRT-PCR) assay. Standard curves were made using the calculated C_t (mean three replications) against the log of serial dilutions.





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