INFLUENCE OF THE WINTER ENVIRONMENT ON THE BIOLOGY AND CONTROL OF *VARROA DESTRUCTOR* ANDERSON AND TRUEMAN IN HONEY BEE COLONIES *APIS MELLIFERA* L.

BY

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A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

Department of Entomology

University of Manitoba

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FACULTY OF GRADUATE STUDIES

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Of

MASTER OF SCIENCE

Dedicated to Sarah Kozak,

for helping me install packages of angry bees at

four in the morning, and everything else.

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I hereby declare that I am the sole author of this thesis.

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Paul R. Kozak

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GENERAL ABSTRACT

This research examined the biology of *Varroa destructor* in honey bee hives and two potential treatments to control varroa during winter. One method examined the impact of brood removal on the efficacy of indoor formic acid fumigation of honey bee colonies. The other treatment used restricted ventilation in small honey bee clusters to assess the affect of increased CO_2 on varroa survival. The efficacies of low concentration indoor formic acid treatments were greater (85%) than previous studies. Brood removal had no impact on efficacy. Winter brood production in colonies was highly variable. The proportion of varroa within brood during winter was low (3%). Varroa reproduction in brood during winter (average 31%) was lower than previous studies. The overall proportion of the entire colonies varroa reproducing was quite low (average 1%). Restricted ventilation applied to honey bees, for short periods, at 25°C, increased varroa mortality without significant impact on bees.

GENERAL INTRODUCTION

The impact the parasitic mite, *Varroa destructor* Anderson and Trueman, can have on honey bee colonies in winter is a significant problem for beekeepers in Canada. Even low levels of varroa infestation in a colony can lead to economic losses and, if the infestation is high enough the entire colony population can perish (Gatien and Currie, 2003). Stress caused by varroa and other factors affecting the health of the colony may result in high colony mortality during winter. However, little is known about how varroa interacts with its host during extended winter periods, particularly with respect to how varroa may be able to use honey bee brood in winter.

Fully understanding the winter biology of varroa within the environment of *Apis mellifera* L. colonies is integral to controlling this serious pest. In particular, knowledge of how varroa utilizes honey bee brood (larvae) during winter, for reproduction and the proportion of the mite population infesting brood will contribute to our understanding of the spatial and population dynamics of varroa in the honey bee colony. There have been several studies published (Eguaras *et al.*, 1994; Martin, 2001) on varroa's biology in "winter" in other regions of the world, but no rigorous examination has been conducted in regions with extended periods of restricted brood rearing. This is also the first study to examine varroa's biology in relation to the winter storage conditions used by beekeepers on the Canadian prairies.

Traditionally, synthetic acaricides (including the compounds: fluavalinate and comaphos) have been used to control varroa infestations in honey bee colonies. However, new methods of control are needed, particularly methods that do not rely on synthetic acaricides as varroa have developed resistance to both fluvalinate and

comaphos (Elzen *et al.*, 1999; Floris *et al.*, 2001; Elzen and Westervelt, 2002). Two methods of varroa control will be explored in this thesis. The first method is indoor fumigation with formic acid during the period when colonies are stored indoors for winter. The second method is the use of modified atmospheres using elevated levels of CO_2 within the natural tolerances of honey bees to control varroa. Both of these methods rely on applying the knowledge of the winter environment to develop effective control measures of controlling varroa.

This thesis is written in scientific paper style and consists of three different research projects. The first chapter is a literature review that provides background information on the details of the biology of *V. destructor*, the environmental conditions of the honey bee hive during winter, and a brief background on the principles and practices employed in formic acid fumigation and modified atmospheres as methods to control arthropod pests.

The objective of the first paper was to examine the impact of brood rearing in winter on the efficacy of long-term, low-concentration formic acid fumigation during winter. By clarifying the relationship between the timing of brood rearing on treatment efficacy of formic acid fumigation and the potential for increases in the population of varroa mites overwinter, fumigation treatments can be refined to deliver minimal amounts of formic acid at the most appropriate times resulting in a more efficient fumigation treatment.

The objective of the second paper was to examine the amount of brood reared in varroa-infested colonies and how varroa is able to use this brood during winter. This

study addresses how varroa interacts with the adult and brood stages of its host during extended winter periods.

The third paper examines the potential application of modified atmosphere techniques to control varroa in clusters of honey bees. This study examined the role that the CO_2 levels found within the natural range in a honey bee cluster in winter may have on varroa mite mortality rates. The overall goal of this experiment was to find a level of atmospheric CO_2 and temperature that would cause significant varroa mortality without causing significant bee mortality.

LITERATURE REVIEW

Introduction

This study examines aspects of the biology and control of *Varroa destructor* Anderson and Trueman within the context of the winter cluster of the European honey bee, *Apis mellifera* L. The following literature review will discuss the conditions and characteristics found in a honey bee cluster in winter and discuss how this environment might affect varroa's utilization of honey bee brood during this time. This review will also discuss formic acid fumigation during indoor winter storage of honey bee colonies to control varroa infestations and the potential for new treatments for varroa control, such as the use of modified atmospheres, in particular, heightened levels of CO_2.

1 - Honey Bee Biology

1.1 - The Hive Environment of the Honey Bee

The hive environment is essential to survival of honey bee, *Apis mellifera* L. colonies in temperate regions of the world. The wax comb of the hive provides the substrate on which the bees walk and rest, and in which they lay eggs, rear brood and store nectar and pollen. This substrate is essential to support a large number of individuals in the colony and the copious amounts of food that bees must store for consumption over the winter. The European honey bee lives in colonies consisting of as many as 60,000 individuals at the height of summer, contained within a cavity that provides protection from the external environmental conditions and from predators (Seeley, 1977). This cavity also allows honey bees to maintain a relatively stable

microclimate in the colony where they can regulate the hive temperature and ventilation. This allows the colony to function under both hot summer and cold winter conditions.

1.2 - Winter Bees

Honey bees are active throughout winter, even in climates at Northern latitudes. They must collect and store enough food resources (nectar and pollen) in times of surplus to last the winter, when these resources are unavailable. During winter, worker bees within a colony differ physiologically from bees reared at other times of the year and are commonly referred to as "winter bees". Winter worker bees are characterized by having a longer lifespan than bees reared in summer (Mattila *et al.*, 2001). Spring bees live 30-40 days, summer bees live 25-30 days but winter bees live over 100 days, with a maximum range of between 212 days and 252 days (Milner, 1921; Fukuda and Sakagami, 1966; Sakagami and Fukuda, 1984). The bees that are reared in late fall may live longer because they have spent less time foraging then a typical summer bee. A worker bee's lifespan is related to how much of the glycogen reserves in the flight muscles are exhausted (Phillips and Demuth, 1914; Neukirch, 1992). Therefore, bees reared in late fall would likely have larger glycogen reserves. However, other physiological differences do occur; for example winter bees have lower levels of juvenile hormone and higher protein titre then summer bees (Fluri *et al.*, 1977). Enzyme activity also remains high in winter bees, suggesting that the aging process is suspended in winter (Johansson and Johansson, 1979).

Winter bees also exhibit differences in temperature tolerance and water balance compared to summer bees. They have a lower chill-coma temperature (Free and

Spencer-Booth, 1960), and lower body water content than summer bees (Johansson and Johansson, 1979). The latter may be a result of increased CO_2 in the hive, prompting the spiracles to open and release moisture from the bee (Johansson and Johansson, 1979). Within the winter cluster, the older workers are primarily responsible for maintaing the temperature of the colony. These workers can effectively increase their O_2 consumption, internal temperature and heat production while young worker bees can not (Allen, 1959). This is important because winter bees, generally being older and longer lived, must have the ability to adequately thermoregulate to ensure the survival of the colony.

1.3 - Optimal Body Temperatures for Bees

Although the body temperature of individual bees is largely dependant on the environmental temperature, bees can metabolically regulate their body temperature to a certain extent (Pirsch, 1923). The optimal temperature range for adult honey bees (workers and drones) is 30-35°C, with 35°C being ideal (Milner and Demuth, 1921; Cahill and Lustick, 1976; Stabentheiner *et al.*, 2003). As the ambient temperature decreases, metabolic activity in bees increases (Milner, 1921; Allen, 1959; Southwick and Mugaas, 1971; Cahill and Lustick, 1976; Southwick, 1991 a). When ambient temperatures fall below 10°C, an individual worker bee will go into a cold comatose condition (Free and Spencer-Booth, 1958; Southwick, 1991 b).

Drones do not play a major role in thermoregulation during winter. Unlike workers, drones are not able to regulate their body temperature as effectively and have body temperatures that are largely dependent on the temperature of the environment. Therefore, drones are not likely to contribute to the thermoregulation of the colony

(Cahill and Lustick, 1976). Accordingly, when a honey bee colony is preparing for winter in autumn, the workers force nearly all drones out of the colony.

1.4 - The Winter Cluster

Although individual honey bees thermoregulate at low temperatures, it is energetically "economical" for bees to group together and form a cluster to conserve heat (Cahill and Lustick, 1976; Kronenberg and Heller, 1982). This is initiated at 14-15°C (Milner and Demuth, 1921; Free and Spencer-Booth, 1958; Johansson and Johansson, 1969; Kronenberg and Heller, 1982; Southwick, 1991 b) whereby the entire colony makes up the cluster, forming one single large, spherical mass during cold temperatures (Phillips and Demuth, 1914; Delaplane, 1997). When bees form a cluster, collectively they can survive much lower environmental temperatures for longer periods of time than a single honey bee (Southwick, 1991 b).

The honey bee cluster can be described as a homeothermic "superorganism", where the cluster of individual ectothermic honey bees behaves in a way that is similar to an endothermic animal (Southwick, 1982). Essentially, the cluster protects the colony by reducing the surface area of the colony from exposure to the surrounding cold air. The bees at the outer edge of the cluster provide a compact barrier with their bodies, which traps the metabolic heat generated by bees in the center. The cluster loses heat by convection to circulating air, radiation to the surroundings and by direct conduction through the air in contact with the cluster (Wedmore, 1947). In addition to the insulation provided by the actual bodies of the bees themselves, the enclosed hive cavity with the

wax combs and honey stored in the combs also help to insulate the colony and provide the cluster with structural support (Anderson, 1948).

The physical components of the hive are essential to the survival of colonies in winter. Bees need the structure of the wax comb in the hive in order to form a cohesive cluster (Phillips and Demuth, 1914; Delaplane, 1997). The cluster occupies spaces in between the comb, above and below the frames (Phillips and Demuth, 1914). In addition, workers occupy the empty cells of the wax comb, further decreasing the empty space between individuals in the cluster (Southwick, 1991 b).

The cluster consists of different layers of bees that differ in their level of activity based on their position in the cluster. The outer layer or mantle of the cluster is tightly packed, while the core is loosely packed and the intermediate layer is moderately packed (Southwick, 1991 a). Under low temperatures, the thermal profile of the cluster consists of the outer edge (6.7°C), bees just inside the outer edge (12.8 to 13.3°C) and the core (33.3 to 34.4°C) (Owens, 1971). The exterior, or mantle of the cluster, traps the heat, insulating the cluster (Stabentheiner *et al.*, 2003). The outer shell of the cluster is kept at lower temperatures resulting in a low thermal conductance, whereby less heat is lost to the surrounding atmosphere (Southwick and Mugaas, 1971). The outer shell of the cluster is composed of worker bees close together with their heads facing inwards. This outer section can be several layers thick and act as an ideal insulator as the bees create dead air spaces with their interlacing thoracic hairs. Bees in the shell are less active than bees in the middle of the cluster (Phillips and Demuth, 1914). When bees on the surface of the cluster become too cold, they enter the center of the cluster to gain heat while individuals from the center replace them by moving out. This results in a continuous

exchange of bees from the surface to the core (Gates, 1914). The intermediate layer of the cluster has the greatest metabolic rates and is responsible for producing the majority of the heat (Simpson, 1961).

The ambient air outside the hive can range from –45°C in outdoor wintered colonies in Northern beekeeping regions of the world to 5°C in indoor winter storage facilities (Gruszka, 1998). To deal with these harsh conditions the winter cluster is dynamic, changing in size and density to suit the surrounding ambient temperature. The cluster contracts when it is colder and expands when the ambient temperature is warmer (Gates, 1914; Wedmore, 1947; Simpson, 1961; Owens, 1971; Southwick, 1982).

The temperature of the winter cluster is also regulated by the bees modifying their levels of food consumption and their metabolism during winter (Wedmore, 1947; Johansson and Johansson, 1969). In winter, honey bee colonies consume vast amounts of food to thermoregulate, often using up to 20kg of honey for a single colony (Seeley and Visscher, 1985). Winter bees metabolize honey, a carbohydrate rich fuel source, which provides energy for muscular activity (shivering), to generate heat (Phillips and Demuth, 1914; Milner, 1921; Wedmore, 1947; Southwick, 1991). As temperatures decrease, the bees in the colony consume more food (Free and Spencer-Booth, 1958). Larger clusters (9,480 to 23,394 individuals) have less metabolic variation than smaller clusters (2,000 to 3,000 individuals), most likely due to the buffering effect of the larger cluster (Southwick and Mugaas, 1971; Kronenberg and Heller, 1982; Southwick, 1982). During winter the cluster is vulnerable to starvation. If the cluster becomes separated from the food supply for prolonged periods the colony may starve to death (Wedmore, 1947). Varroa can also

interfere in this process as well, by weakening the bees that make up the cluster, thereby disrupting the formation of clusters in winter (Grobov, 1977).

The winter cluster also has the capacity to maintain brood at a constant temperature. If brood is present in a colony during winter, the cluster will center around it, forming an insulating layer around the brood on either side of the comb (Kronenberg and Heller, 1982). This provides the brood with high, stable temperatures and humidity (Southwick, 1991 a). Workers are able to warm brood cells by pressing their thorax against the cell capping to raise the temperature by up to 3.2°C (Bujok *et al.*, 2002). The ideal temperature of a brood nest is 34-35°C (Gates, 1914). The brood temperature rarely goes below 30°C, although temperatures as low as 25°C have been recorded in colonies (Simpson, 1961).

1.5 - Honey Bee Brood in Winter

Honey bees in northern regions of the world rear brood at a much lower quantities during winter, compared to the rest of the year (Seeley and Visscher, 1985). In honey bee colonies, brood rearing peaks throughout summer and then declines in early autumn (Seeley and Visscher, 1985). Between autumn and late-winter, there is often very little to no brood rearing within a colony, with November often having the lowest levels of brood production (Harris, 1980; Szabo, 1993; Nelson, 1995). In some Northern regions of the world (in particular, Great Britain), brood production occurs at a low level (slowly increasing) from November to February and increasing significantly towards late March or early April (Jeffree, 1956; Avitabile, 1978). In Northern latitudes, however brood rearing often increases slightly in December and then declines, though maintained at very

low levels until the end of March and early April (Harris, 1980). As the population of adults in a honey bee colony dwindles throughout winter, some brood production is initiated to replace these adults (Owens, 1971; Avitabile, 1978; Harris, 1980; Szabo, 1993; Nelson, 1995). The age profile of the colony changes over winter so that older bees predominate by March, but up to one-half of the colony's workers can be replaced during winter, at least when varroa is absent (Harris, 1980).

Although brood rearing requires vast quantities of food, which is limited in winter, winter brood rearing benefits the colony. Brood rearing in winter is an adaptation that allows colonies in many regions to have a larger population in early spring to take advantage of the floral resources early in the season and during the brief spring nectar flow. This allows a colony to produce enough food to survive to the next season (Wedmore, 1947; Seeley and Visscher, 1985) and to swarm in late spring, producing new honey bee colonies.

1.6 - Ventilation in the Honey Bee Hive

Honey bee colonies produce large amounts of CO_2 and moisture through respiration that results in in-hive levels of CO_2 that are higher than ambient levels (0.03% in air) due to the large numbers of bees are confined in an enclosed space (Nicolas and Sillans, 1989). Typically, the size of a cluster does not appear to have any effect on the CO_2 output of an individual bee (Free and Simpson, 1963). At ambient temperatures below 10°C, the CO_2 concentration in a cluster increases dramatically from the respiration given off as bees thermoregulate (Free and Simpson, 1963; Nagy and Stallone, 1976). Honey bees regulate the levels of CO_2 in the colony between 0.10% and

4.25% by collectively fanning their wings to exhaust air from the hive (Seeley, 1974). Larger colonies with more workers have better control of the CO_2 levels than smaller colonies (Seeley, 1974). The concentration of CO_2 in the winter cluster averages 4% (Simpson 1950; Simpson 1961) but honeybees will tolerate concentrations of up to 9% CO_2. At 10% CO_2, workers will begin to fan their wings to expel CO_2 from the hive and at concentrations of 40-45%, workers will be rendered motionless (Simpson, 1954; Simpson, 1961).

Honey bees detect increases in concentrations of CO_2 rather than decreases in O_2 (Seeley, 1974) through specialized receptors, called pit pegs, located on the antennae (Lacher, 1964). Individual bees can regulate CO_2 in their immediate environment by rhythmically opening and closing their spiracles in time with abdominal pumping producing a tidal flow of air that flushes stagnant air out of the trachea (Bailey, 1954).

While bees regulate the hive air to control the level of CO_2 they do not actively regulate their humidity (Simpson, 1961). Moisture escapes by diffusion from the cluster, facilitated by fanning (Simpson, 1961). At a colony level, bees can move large volumes of air by fanning their wings to create air currents (Wedmore, 1947). Generally, the air in a honey bee hive is changed approximately six times in an hour (Wedmore, 1947).

This presents a picture of the atmosphere of the hive as a dynamic system. However, this system can be affected in managed systems such as when large numbers of honey bee colonies are stored indoors during winter. In this case, forced ventilation is necessary for the removal of CO_2 and moisture from the building containing the hives (Wedmore, 1947). Large indoor winter storage facilities for honey bee colonies have ventilation systems, consisting of exhaust fans drawing in air from outside which is

further circulated to obtain an even distribution throughout the storage room (Gruszka, 1998). In the Canadian prairies, it is common for large-scale beekeeping operations to winter hundreds or even thousands of honey bee colonies stacked in rows in large indoor storage facilities. These facilities require a constant temperature of 4-6°C, continuous ventilation and constant darkness (Taber, 1997; Gruszka, 1998). In addition, colonies must be adequately fed in autumn so that there are sufficient food stores for the duration of winter storage. Inadequate quality or quantity of food stores, inadequate numbers of workers in a colony or the presence and severity of infestations of the microsporidian *Nosema apis* Zander, tracheal mites (*Acarapis woodi* Rennie), in addition to varroa mites can contribute to the death of a colony during winter. Colony death rates can be related to the compounded effects of these multiple stresses (Southwick, 1991 b).

2 - The Biology of Varroa

2.1 - Adaptations for Parasitism

The nest environment of the honey bee is also essential to the survival of *Varroa destructor* Anderson and Trueman, a parasitic mite that lives in association with cavity nesting honey bees. This mite was once restricted to Asia, where it parasitized and evolved on the Asian honey bee, *Apis cerana* Fabricius varroa has since switched hosts onto *A. mellifera* and has spread throughout most of the world, where it has become one of the most serious pests of the European honey bee (Sammataro *et al.*, 2000). Honey bee colonies infested with varroa experience economic damage. If colonies are not treated to reduce mite levels they will eventually perish (Gatien and Currie, 2003; Currie and Gatien, 2006).

Varroa destructor is in the order Acari, sub-order Mesostigmata and the family Varroidae (Delfinado and Baker, 1974). Adult female varroa mites are small oval-shaped (1065 μm long and 1575 μm wide) and are dark brown in colour (Oudemans, 1904). Varroa are covered by a tough sclerotized shield or idiosoma that protects the dorsal region of the body (Oudemans, 1904; Delfinado and Baker, 1974; Liu, 1982). The dorsal shield is coated in a hydrophobic epicuticle with multiple aristae (Akimov *et al.*, 1988) and surrounded by setae at the margins of the shield that are short and stout with pointed tips (Liu, 1982).

The body of varroa is well adapted to both the ectoparasitic phase of its lifecycle, where it must swiftly crawl around on the bodies of adult honey bees, and to its reproductive phase, in the restricted space of the brood cells (Liu, 1982; Akimov *et al.*, 1988). The shape of the idiosoma facilitates its use as a wedge to allow varroa to insert itself between the tergites and sternites of the host bee, and may also reduce the aerodynamic resistance of the mite on the bee during flight (De Jong *et al.*, 1982; Akimov *et al.*, 1988).

The legs of varroa are well adapted to phoresy, being short and stout (Oudemans, 1904; Liu, 1982). These legs are covered in setae that are longer than those on the dorsal shield (Oudemans, 1904; Liu, 1982) and have a pretarsus enlarged into a sucker-like pad or ambulacrum that divides into two lobes when fully expanded (Liu, 1982; Liu and Peng, 1990). The ambulacrum consists of tiny, protractile claw-like sclerites rather than a sucker-like structure or a sticky pad (Ramirez and Malavasi, 1991), which may be an adaptation for rapid movement, possibly by grasping hairs on the bee's body (Ramirez and Malavasi, 1991).

The mouthparts of varroa are made up primarily of a pair of chelicerae which function to grasp and pierce the integument of the honey bee (Griffiths, 1988). The three-segmented chelicerae are highly specialized with a movable segment modified into a saw-like blade used to make a neat incision into the body wall of the bee (Griffiths, 1988). Varroa also have paired palps covered in numerous distal, sensory hairs (Liu and Peng, 1990). These palps have a long, apotelic claw at the end, which may be used to cling to the host (Liu and Peng, 1990). The mandibles of varroa, on the other hand, are reduced and relatively short (Oudemans, 1904). The salivary stylets of the varroa are well developed on the lateral, internal walls of the hypostome. In this position they deliver and mix saliva with the bee's haemolymph (Griffiths, 1988).

The respiratory system of varroa consists of a tracheal network, totally independent of the host bee's respiration that is connected to a stigma on the ventral surface that functions like a spiracle (Akimov *et al.*, 1984; Richard *et al.*, 1990). Varroa mites also may use a specialized tube, called a peritreme, to obtain air from the environment (Pugh *et al.*, 1992; Liu, 1996). The peritreme is a mobile appendage-like structure positioned in between the third and fourth legs (Pugh *et al.*, 1992). The distal end of the peritreme has a slit-like opening and the interior is lined with a membrane that has tooth-like projections that may aid in respiration by increasing the surface area of the inner surface of the peritreme (Grobov, 1977; Liu, 1996). Gas exchange is thought to be adjusted, by changing the position of the peritreme, by raising or lowering the position of the peritrme. The peritreme may also function as a plastron to facilitate respiration when mites are within the brood cells (Pugh *et al.*, 1992).

2.2 - Taxonomy of Varroa

There are multiple species of varroa and many haplotypes that may differ in terms of their impact on their host (Anderson and Trueman, 2000). *Varroa jacobsoni* was first described by Oudemans in 1904, and thought to be the only species of varroa on *A. cerana*. Early on, researchers noticed that varroa mites had different impacts on their bee hosts in different regions of the world (Delfinado-Baker, 1974). Later, Anderson (1999) concluded that *V. jacobsoni* was a species-complex consisting of at least three distinct species. As a result, the species previously known as *V. jacobsoni* in North America was re-classified as *Varroa destructor* Anderson and Trueman. The less virulent form of *V. jacobsoni* remains restricted to its original host, *A. cerana*. *Varroa destructor* is the species which is found on *A. mellifera* throughout the world as well as on its original host, *A. cerana*. Delfinado-Baker (1988) had categorized varroa into three different biotypes that, Anderson and Fuchs (1998) demonstrated through DNA sequencing, were two genetically distinct populations with differing potential for reproduction on *A. mellifera*. In later tests by Anderson (1999), it was determined that there were four distinct genotypes based on geographic origins of the populations, an Indonesian-Malaysian genotype, a closely related genotype from the Northern Philippines and two other genotypes consisting of a mainland Asia genotype and a Sri-Lankan genotype. The two haplotypes of *V. destructor* present in North America are known as the Korean and Japan/Thailand haplotypes (Anderson and Trueman, 2000).

2.3 - Life Cycle of Varroa

Varroa mites are completely dependant on honey bees throughout their life cycle. Adult female mites live as ectoparasites on adult honey bees and then enter cells of honey bee larvae for reproduction. The female foundress mite enters the honey bee brood cell on the 5^{th} or 6^{th} day of brood development, right before the cells are to be capped with wax (Grobov, 1977). Once inside the cell, the female mite immerses itself in the brood food, dorsal side facing the bottom of the cell, with its peritreme raised, possibly to aid respiration (Martin, 1994). The bee larva consumes the brood food, freeing the mite, which then feeds on the developing bee larva (De Jong *et al.*, 1982).

As the honey bee larva is developing within the capped cell, the foundress mite makes an incision in the integument of the larva, known as the feeding site (Donzé *et al.*, 1998). This is important because the young mites cannot pierce the honey bee integument on their own (Donzé *et al.*, 1998). The foundress mite then deposits a mass of feces on the cell wall, called the fecal accumulation site, where the progeny of the foundress varroa will gather when not at the feeding site.

Sex determination in varroa is determined by haplo-diploidy. The foundress mite lays her first egg, which is a haploid male, in the brood cell 60 hours after the cell is capped. All subsequent eggs are diploid females laid at intervals of 26-32 hours (Ifantidis, 1983; Rehm and Ritter, 1989; Martin, 1994). Male mites require 6.9 days for complete development and females require 6.2 days (Rehm and Ritter, 1989). One female is able to lay five to six eggs (Martin, 1994), typically producing one viable male and one to two daughters per cycle in the time available within a worker brood cell (Rehm and Ritter, 1989). The eggs are laid at the bottom of the cell, hatching into a

larval protonymph stage, then molt into a deutonymph stage and finally an adult (Grobov, 1977). Protonymphs are small, white and round in overall shape while deutonymphs are white and resemble the adult mites in body shape (De Jong *et al.*, 1982).

Developing mites mate with their siblings inside the cell infested by a single foundress mite (Henderson *et al.*, 1986). If more than one foundress mite enters a cell, then the developing mites have the opportunity to mate with mites from the other parent. However, when higher numbers of mites infest a single cell reproductive rates decline due to competition between the varroa offspring for the pupa's haemolymph (Martin, 1995 a; Donzé *et al.*, 1996).

The behavior and life history of each of the two sexes of varroa is distinct. The small male varroa functions only to fertilize the female daughter mites and typically dies inside the brood cell (De Jong *et al.*, 1982). Female varroa offspring that successfully mate and mature within the brood cell will then attach and feed on adult bees before re-entering a new brood cell to then reproduce themselves. A single female varroa is capable of reproducing up to seven times, but typically will go through 2-3 reproductive cycles and can live up to 80-100 days (Ruijter, 1987; Calatayud and Verdu, 1994; Martin and Kemp, 1997).

Varroa mites prefer drone brood to worker brood because drone brood has longer development times (24 days for drones and 21 days for workers) and thus more developing female mites in each cell can reach maturity and thus survive, before the bee emerges from its cell (Boot *et al.*, 1991; Boot *et al.*, 1995). Typically, drone brood is attractive to varroa for two to three times longer than worker brood. However, drone brood is virtually absent during winter (Boot *et al.*, 1991; Boot *et al.*, 1992). Varroa cue

in to chemical attractants (or kairomones), in fatty acid esters to distinguish drone larvae from workers (Le Conte *et al.*, 1989). Semiochemicals found in the larval food of honey bees may also function as attractants for varroa to their larval host (Nazzi *et al.*, 2001).

Infestation of a brood cell by a female varroa does not always result in successful reproduction. Reproductive success is typically quantified by determining the number of eggs laid in the cell by the foundress mite which is called the "total reproductive rate" (Corrêa-Marques *et al.*, 2003) or the number of viable female offspring resulting from the foundress mite which is called the "effective reproductive rate" (Corrêa-Marques *et al.*, 2003). Harris *et al.* (2003) define "viable" females as those in which a foundress mite must have both male and female progeny within the first 12.5 days of the brood development cycle to permit the development, mating and full maturation of female varroa mites that emerge from cells. Not all female varroa mate successfully and thus would not be capable of producing viable female offspring themselves.

2.4 - The Spread and Distribution of Varroa

The "phoretic" lifestyle of varroa has contributed greatly to its ability to spread throughout apiaries all over the world and allows it to move from bee to bee and from colony to colony (Sakofski *et al.*, 1990; Liu, 1991 b; De Jong *et al.*, 1982). Varroa mites are able to easily transfer from one adult bee to another. This is of particular importance as the main method of infestation of new colonies is either by honey bees drifting (moving between hives) or when robbing food from other colonies occurs (Liu, 1991 a). Swarming further disperses potentially infested bees throughout an area and feral colonies that result can act as a natural reservoir of varroa to infest and/or reinfest

managed colonies (Rademacher, 1991). Practices in modern day apiculture have further facilitated the spread of mites from one region to another through sale of bees and the transport of large-scale colonies for pollination services (Grobov, 1977). Migratory beekeeping and sales of package colonies and queens are examples of these practices.

2.5 - Factors Affecting Varroa

Honey bees provide varroa mites with a consistent and regulated microenvironment within their hive cavities and inside the cluster of the colony. This regulated microenvironment allows varroa to survive the harsh conditions of winter. However, within this environment, there is a narrow range of conditions that varroa can tolerate. Varroa mites are hydrophilic by nature. They have a small body size with a large surface area to volume ratio, leading to extreme desiccation at high temperatures and low humidities (Bruce *et al.*, 1997). Varroa mites lose water rapidly; up to 2% per hour. However, mites can survive up to approximately 37% water loss during dehydration (Yoder *et al.*, 1999). Therefore, varroa prefer humid environments, such as inside a cluster of bees that has a relative humidity between 60% and 70% or a capped brood cell that has between 75% and 80% relative humidity (Wohlgemuth, 1957; Jay, 1965). When varroa mites are in brood cells, water loss is not a problem (Yoder *et al.*, 1999). Phoretic mites are more susceptible to desiccation but are provided the essential nutrients and moisture they require when they are attached to a host bee by feeding on the haemolymph. When varroa are in exposed sites and do not have access to haemolymph of a host bee, they are at risk of desiccation (Bruce *et al.*, 1997), but are able to survive

for a few days on the bottom boards in more humid locations under hive debris (De Guzman *et al.*, 1993).

Varroa also have limits on the range of temperatures they can tolerate. These tolerances vary seasonally and with mite age (Paetzold and Ritter, 1989). In summer, mites prefer a temperature range of 31.8 to 37.2°C but "winter mites" prefer 33 to 36.6°C. Temperature tolerance limits of summer mites range from 22 to 43°C, but winter mites tolerate temperatures from 16 to 43°C. The temperatures that varroa are exposed to depends on the temperatures within the bee hive and within the bee cluster. In the winter cluster, temperature ranges from 18 to 36°C (Paetzold and Ritter, 1989). Additionally, the optimal temperature for development of varroa mites corresponds to the brood temperature of *A. mellifera*, between 32.5°C and 33.4°C. Above 36.5°C, varroa reproduction is reduced and mites exposed for extended periods to 38°C will die (Le Conte *et al.*, 1990).

2.6 - Varroa In Winter

Varroa destructor evolved in the tropics as a parasite of the Asian honey bee, *A. cerana*. However, varroa has adapted remarkably well to the European honey bee, *A. mellifera*, throughout the temperate regions of the world, including areas that are characterized by prolonged and extremely cold winters. This is because both species of honey bees, including the new host, *A. mellifera*, nest in enclosed cavities. The honey bee hive and colony that inhabits it provides varroa with a constantly regulated 'micro tropical environment', even in winter (Ritter, 1988; Yoder *et al.*, 1999). The honey bee

cluster provides varroa with the appropriate temperature and humidity conditions within their survival range.

During winter varroa are present on adult bees at greater densities than summer, usually between the abdominal sternites due to lack of available brood in the colony (Smirnov, 1978; Bowen-Walker *et al.*, 1997). As the worker bees die throughout winter, some varroa are believed to transfer from their dying host to a new host bee, potentially leading to a concentration of varroa on a dwindling population of bees in a colony (Bowen-Walker and Gunn, 1998). However, other studies have shown that varroa lack the ability to transfer from a dying host and that levels of infestation remains stable over winter (Fries and Perez-Escala, 2001).

Without treatment, varroa infestations will increase throughout the season in a honey bee colony whenever brood is present (Ritter, 1988; Eguaras *et al.*, 1994). Increases in varroa numbers follow the yearly cycle of brood rearing in a honey bee colony. The reproductive success of varroa varies depending on the time of year (Sakofski *et al.*, 1990; Martin, 2001). The reproductive success of foundress mites ranges from 75-92% in summer (Martin *et al.*, 1998), 60-70% in autumn (Otten and Fuchs, 1990) and averages 51% in winter (Martin, 2001). During winter, brood provides varroa with a limited resource to infest, feed upon and use for reproduction (De Jong *et al.*, 1982; Martin, 2001). However, there are wide ranging reports on the ability of varroa to reproduce in winter, in different climates and in different beekeeping regions of the world. In Argentina, reproduction in varroa is lowest in spring and autumn, and higher in both winter and summer (Eguaras *et al.*, 1994). In Argentina, during "winter", the percentage of infested cells (25%) and the range of intensity of parasitism (1-6 mites/cell)

is much higher than summer (3.67% and 1-2 mites/cell respectively) (Eguaras *et al.*, 1994). The increased levels of brood parasitism in winter are thought to be a result of lower brood availability resulting in increased competition amongst varroa for the brood (Eguaras *et al.*, 1994).

In temperate climates, varroa produce fewer viable offspring per foundress in winter (avg. 0.5 mites/foundress) than in summer (avg. 1.0 mites/foundress) (Martin, 2001). Non-reproductive mites make up a higher proportion of the population (20% in winter, compared to 8% in summer) as a result of higher male mortality (42%, compared to 18% in summer) (Martin, 2001). The reason for increased male mortality in these studies is not known. Changes in host diet during winter, resulting from the different haemolymph composition of "winter" bees, may affect the nutritional requirements leading to the observed differences in reproductive capacity of varroa (Eguaras *et al.*, 1994). Further research is required to examine varroa's utilization of winter brood, particularly its reproductive success under Northern climatic conditions.

2.7 - The Effect of Varroa on Honey Bees

The adult female varroa live on the surface of adult honey bees as ectoparasites, usually underneath the sclerites on the abdomen and sometimes between the thorax and abdomen, where they pierce the integument of the honey bee and feed on the haemolymph (De Jong *et al.*, 1982; Akimov *et al.*, 1988; Ball, 1988). An individual honey bee may be infested with numerous varroa mites at any one time (Bowen-Walker *et al.*, 1997).

While feeding on honey bee pupae in the brood cell, varroa make perforations in the integument of the pupae consisting of two puncture wounds, most often occurring on the ventral side of the abdomen (Kanbar and Engels, 2004). Feeding by varroa results in tissue damage to pupae and causes a reduction in both the haemolymph protein concentration and volume. This results in a decrease in the size of fat body cells and ecdysteroid titers in pupae (Drescher and Schneider, 1988). The impact on pupae is often positively correlated with the level of varroa infestation in a colony (Glinski and Jarosz, 1984; Weinberg and Madel, 1985). Infested pupae with lowered levels of haemolymph components develop into adults that lack the physiological requirements to tolerate the stressful environmental conditions during winter (Amdam *et al.*, 2004).

As well as the direct physical effects of feeding, varroa are often vectors of a variety of diseases amongst honey bees. Varroa have been implicated in the transmission of bee viruses such as deformed wing virus, Kashmir bee virus, slow paralysis virus, acute paralysis virus and more recently Israeli acute paralysis virus (Ball, 1985; Ball and Allen, 1988; Liu, 1991; Sammataro, 1997; Cox-Foster *et al.*, 2007). These viruses may be present at low levels even in uninfested bees but are activated by the penetration of the bee integument as the mites feed (Ball and Allen, 1988). Varroa are also able to transmit bacterial infections when they feed (Strick and Madel, 1988; Glinski and Jarosz, 1992) and transmit microorganisms such as the fungal spores that cause chalkbrood infestations (Liu and Ritter, 1988; Liu, 1996).

Altogether, varroa mites have a considerable impact on their hosts. Honey bees infested with varroa have shorter life spans than individuals that are not infested (Southwick, 1990), and colonies that have high levels of infestation with varroa will

eventually perish (Korpela *et al.*, 1992; Gatien and Currie, 2003). A honey bee colony's infestation will increase throughout the season with the greatest population growth of varroa occurring in summer, when the highest levels of honey bee brood are available (Gatien and Currie, 2003; Currie and Gatien, 2006). The extent of the infestation and the season in which it occurs influence the impact of varroa on the longevity of bees. This is highlighted by the reduced survival of highly infested colonies throughout winter (Kovac and Crailsheim, 1988). Varroa mites may also disrupt the behavior of colonies, which can lead to a higher incidence of queen injury (Woyke *et al.*, 1994).

At a colony level, varroa have the potential to kill entire colonies of honey bees. Untreated colonies may be able to survive up to four to five years after initial infestation (Korpela *et al.*, 1992). However, this prediction does not take into account high initial infestations, additional and continued infestation from outside sources, the presence of viruses or additional diseases and parasites, and overall poor conditions for colonies. In these cases, colony collapse may occur much sooner. For example, colonies with both varroa and deformed wing virus have been predicted to perish within one year of initial infestation, dying the following winter or spring (Martin, 2001).

The impact of varroa on honey bees varies with climate and by geographic region (DeJong *et al.*, 1984). There are many reasons for this. The climate in northern regions of the world is harsh and may compound the physiological stresses that varroa place on a colony, resulting in higher colony mortality rates (De Jong *et al.*, 1984). Mite reproduction, and therefore population growth, may also differ across climatic regions (Corrêa-Marques, *et al.*, 2003) as a result of interactions between mite and honey bee genetics present within a particular region (Camazine, 1986). For example, Africanized

bees have several behavioral traits that may limit varroa reproduction, such as shorter periods of brood capping, a tendency to swarm more readily and frequently (resulting in breaks in brood rearing) and hygienic and grooming behaviors that are lacking in European bees (Moretto *et al.*, 1993; Arechavaleta-Velacsco and Guzman-Novoa, 2001). As well, mites in Africanized honey bee brood produce fewer viable offspring (40%) than in European bees (75%), resulting in lower reproductive success (Medina and Martin, 1999). Additionally, the variability in the biotypes of varroa in different regions and their differing reproductive capacity may also affect the impact of mites on their host (Medina and Martin, 1999).

3 - Varroa Control

3.1 - Formic Acid Treatments

The use of organic acids is one of the many methods of control for varroa. There are several organic acids that have been used in a number of different ways to control varroa infestations in honey bee colonies. The organic acids that have been used to treat varroa include formic acid, oxalic acid and lactic acid. Formic acid has been shown to be more effective at controlling varroa than either oxalic or lactic acid (Eguaras *et al.*, 1996). Formic acid is a clear, corrosive liquid that has a density of 1.22g/cm^3 at 20°C and a vapor pressure of 400 mmHg at 24°C, a melting point of 8.4°C and a boiling point of 100.7°C (Wagner, 1980). Formic acid is usually delivered as a liquid, often on an absorbent material, which is placed inside the honey bee hive where the liquid formic acid then evaporates into the hive environment (Gatien and Currie, 2003). The gaseous formic acid acts as a fumigant within the colony. Formic acid can also be delivered to

honey bee colonies through membrane delivery (Daniels *et al.*, 1999), gel matrices (Feldlaufer *et al.*, 1997; Kochansky and Shimanuki, 1999; Eguaras *et al.*, 2003; Elzen, 2003) and/or saturated plates or pads (Hoppe *et al.*, 1989 a; Mutinelli *et al.*, 1994; Currie and Gatien, 2006).

Presently, two methods are recommended for the delivery of formic acid to honey bee colonies in Canada; the slow release method and the pour-on method (Ostermann and Currie, 2004). The slow release method involves adding formic acid to an absorbent pad within a plastic sheath (Ostermann and Currie, 2004; Currie and Gatien, 2006). This allows the vapors to slowly release over a long period of time. This method has the advantage of requiring a single application, saving labour and time, and providing a continuous even application of formic acid. The other common method, the pour-on method, involves pouring liquid formic acid directly onto an absorbent material that is then left exposed within the hive. With the later method, the formic acid concentrations are often quite high during the initial application period and then dissipate rapidly afterwards (Ostermann and Currie, 2004). Therefore, additional applications at regular intervals are necessary to maintain formic acid vapors within the hive for an extended period of time. Within the pour-on treatment, differences in the initial volume of liquid applied, the number of applications and the interval between the applications and environmental conditions may affect treatment efficacy (Gatien and Currie, 2003; Ostermann and Currie, 2004; Currie and Gatien, 2006).

Generally, formic acid applications are recommended in spring or autumn when colonies are not producing honey. Formic acid applications are currently discouraged in summer as there will not be enough time for the formic acid in honey to dissipate to

acceptable levels before it is extracted (Bogdanov *et al.*, 1999). Removal of honey supers and any honey frames to be extracted for human consumption is also recommended before formic acid treatment is applied.

There are many advantages to using formic acid. First of all, it is inexpensive and readily available, making it particularly appealing for large-scale beekeeping operations that require many treatments (Liu, 1991; Mutinelli *et al.*, 1994). It can already be found in minute amounts in many food products and occurs naturally in plants, mammalian tissue and the venoms of some insects (Thompson, 1992). Also, formic acid is considered an innocuous substance in most environments as it can be biologically and chemically degraded, an important consideration since it is used in close association with food products (Kirk and Othmer, 1980). It also occurs naturally, in minute amounts, in honey, and up to 0.1% in honeydew honey. It will also dissipate from honey as time passes (Liu, 1991; Liu, 1992; Eischen, 1998 a; Bogdanov *et al.*, 1999). The threshold for detecting formic acid in honey by taste is 300-600 mg/kg (ppm) (Bogdanov *et al.*, 1999). Although formic acid penetrates beeswax easily, it quickly vaporizes and does not accumulate in wax (Liu, 1992).

A major advantage of formic acid over synthetic acaricide treatments is that formic acid has the ability to kill varroa inside brood cells. When highly concentrated formic acid (85%) is applied, it penetrates sealed brood cells and can kill the varroa with the majority of the brood surviving (Fries, 1989; Fries, 1991; Calderon *et al.*, 2000). The ability to kill mites in brood is important because when the appropriate concentrations of formic acid are used, all stages of mites in a colony (phoretic and reproductive) may be targeted with a single treatment. However, varroa have the capacity to survive

applications of formic acid with lower concentrations (65%) (Gatien and Currie, 2003). In many cases multiple applications with formic acid are necessary over more than one brood rearing cycle (21 days) to kill mites that may be "protected" in the brood cells and therefore which survived the treatment (Bracey and Fischer, 1989).

Formic acid also kills other mites, such as *Tropilaelaps clareae* and *Acarapis woodi* (Garg *et al.*, 1984; Hoppe *et al.*, 1989 b; Nelson *et al.*, 1994; Feldlaufer *et al.*, 1997; Hood and McCreadie, 2001). This is of particular importance as both varroa and tracheal mites can occur in a colony and these infestations with multiple pests can have devastating impacts on winter survival of colonies (Downey and Winston, 2000).

3.2 - Factors Affecting the Efficacy of Formic Acid

An even distribution of formic acid vapor is important for effective control of varroa. The most common placement of formic acid dispensers is either directly above and/or below the brood comb (Eguaras *et al.*, 2003). Formic acid treatments are more successful at higher temperatures (Bracey and Fischer, 1989; Calderone, 1999; Bahreini *et al.*, 2004). The concentration of formic acid in the hive is positively correlated with the ambient temperature (Skinner *et al.*, 2001; Ostermann and Currie, 2004). This can be a concern when treatments such as gel packs are used outdoors and the environmental temperature varies, thus affecting the efficacy of the treatment (Skinner *et al.*, 2001; Ostermann and Currie, 2004). Where treatments result in rapid volatilization, due to high temperatures, reapplication may be needed (Kochansky and Shimanuki, 1999).

There are various colony-related factors that can affect the efficacy of treatments with formic acid. The presence of brood in a honey bee colony can reduce the efficacy of

formic acid treatments (Eischen, 1998 b). Even the presence and the severity of varroa infestations can effect the concentrations of formic acid in colonies (Ostermann and Currie, 2004). Varroa-infested colonies have lower concentrations of formic acid in hive-air than colonies that are varroa-free suggesting that activity or disruption to the colony caused by varroa can affect the volatilization and/or the sorption of formic acid in the hive (Ostermann and Currie, 2004). The efficacy of formic acid treatments can also vary by season and time of year (Gatien and Currie, 2003; Currie and Gatien, 2006). In 1993, Gatien and Currie found that outdoor treatments at a concentration of 65% formic acid were more effective in fall and in 1994 and 1995 similar treatments were more effective in spring. More effective treatments in fall in some years may be due to less brood being available, resulting in less protection for varroa within capped brood and less potential for varroa to increase their population by reproducing (Gatien and Currie, 2003).

3.3 - Limitations of Formic Acid Treatments

Using formic acid to control varroa is not without its drawbacks. Formic acid can cause worker bee mortality (Nelson *et al.*, 1994; Elzen, 2003; Underwood and Currie, 2003), queen mortality (Fries, 1989; Underwood and Currie, 2004), capped and uncapped brood mortality (Fries, 1991; Bolli *et al.*, 1993) and reduce drone production and survival (DeGuzman *et al.*, 1999). Formic acid can also have non-lethal effects such as disturbance of worker bees in the colony, which display a fanning response at high concentrations (Nelson *et al.*, 1994; Gatien and Currie, 2003).

At a colony level, formic acid treatments can negatively impact colony growth and development but this may vary with the method of application. Ostermann and

Currie (2004) discovered that colony development was negatively impacted by slow-release delivery of formic acid when varroa abundance was low, while a pour-on treatment of formic acid had no impact on colony development or level of brood in the colony, irrespective of the level of varroa infestation.

In many studies, formic acid treatment has proven less effective than treatment with fluvalinate (although still effective at reducing the numbers of varroa) (Eischen, 1998; Calderone, 1999; Calderone and Nasr, 1999; Hood and McCreadie, 2001; Elzen, 2003; Gatien and Currie, 2003; Bahreini *et al.*, 2004) and efficacy is more variable than conventional treatments using synthetic acaracides (Elzen, 2003; Gatien and Currie, 2003; Ostermann and Currie, 2004; Currie and Gatien, 2006). In many of these studies, environmental conditions may have resulted in decreased volatilization at critical times and therefore decreased concentrations of formic acid within the hive environment (Ostermann and Currie, 2004). Subsequent treatments in the following season are recommended when varroa levels are not lowered below the economic threshold of 2 mites per 100 bees (Gatien and Currie, 2003; Currie and Gatien, 2006).

Formic acid can be a safety hazard for beekeepers, as it is caustic and corrosive (Kirk and Othmer, 1980). Formic acid is a powerful dehydrating agent and can cause blistering and serious burns to the skin. Vapors can cause irritation and damage to respiratory membranes (Kirk and Othmer, 1980). Some solutions to the hazard posed by formic acid are more convenient and user-friendly delivery methods for formic acid, such as pre-formulated applications like formic acid impregnated gel packets or absorbent packets (Feldlaufer *et al.*, 1997). Indoor fumigation of stored colonies is another application in which repeated exposure to formic acid is limited as beekeepers do not

have to be in the facility when bees are treated and beekeepers can treat large numbers of colonies simultaneously (Underwood and Currie, 2004).

3.4 - Performance of Fumigants

Fumigants have been used extensively to control arthropod pests. Monro (1969) defines a fumigant as "a chemical which, at a required temperature and pressure, can exist in the gaseous state in sufficient concentration to be lethal to a given pest organism". Fumigants typically enter the insect through the spiracles and act directly through the respiratory system.

In practical application of fumigants, dosage is defined as "the amount of fumigant applied, usually expressed as the weight of the chemical per volume of space treated" and concentration, as "the actual amount of fumigant present in the air space in any selected part of the fumigation system at any given time" (Monro, 1969). The concentration can be measured in terms of weight/volume (g/m^3), parts by volume or percentage by volume (Monro, 1969). Applications often need to consider exposure time as well as concentration in order to quantify mortality rates in the target organism. The concentraton-time product (CT), which is the concentration of the fumigant*time the fumigant is applied, usually expressed as mg hr/L, is often used as a standardized measure for recommendations related to arthropod control (Monro, 1969). The CT product provides a standard dosage for effective treatment, where the type of application can be modified. If a CT product is shown to be constant under different conditions, the time for fumigation can be extended while decreasing the concentration in a predicted

way, or alternatively, the concentration can be increased as the time for fumigation is decreased (Monro, 1969).

Environmental conditions have a large impact on fumigants. Temperature, and, to a lesser extent humidity, may influence fumigation of insects (Monro, 1969). Temperature is often positively correlated with insect mortality. Therefore, the time for many fumigation treatments can be decreased under the application of increased temperatures (Monro, 1969). At lower temperatures the effectiveness of the fumigant is often lowered, as the respiration rate of the insect decreases thereby reducing their exposure to the fumigants (Monro, 1969). As temperatures are lowered to 10°C, it becomes more difficult to kill insects. Below this temperature, many species can become weakened and will not actively take up the fumigant (Monro, 1969). At extremely high doses efficacy may be lowered due to an insect shutting down respiration in response to the shock of an initial high dose of a fumigant, thereby preventing uptake of the fumigant into the respiratory system (Estes, 1965; Bond *et al.*, 1969). Increasing the levels of CO_2 in the fumigation setting can synergize the action of fumigants as the CO_2 stimulates respiratory movements and the opening of spiracles in insects (Young and McDonald, 1970; Rajendran *et al.*, 1977).

The physical characteristics of the fumigant that affect its distribution within a fumigation chamber can be controlled through a number of methods. Generally, the heavier a fumigant is, the more time it will take to diffuse within a treated area. Heavier gases will sink to the bottom of a chamber and need to be agitated by means of a fan or multiple gas inlets to prevent settling or stratification of a fumigant (Monro, 1969). Fans can also function to increase the volatilization of the fumigant. Ventilation ducts can

improve the distribution of the fumigant in the chamber. Sealing of the fumigation chamber is important because leaks in the closed system will result in loss in the concentration of the fumigant and therefore lowered efficacy (Monro, 1969).

Sorption, (adsorption and absorption) also have a major effect on fumigant efficacy. Sorption is defined as "the total uptake of gas resulting from the attraction and the retention of molecules by any solid material present in the system" (Monro, 1969). Sorption is influenced by environmental variables. For example sorption is greater at lower temperatures. Adsorption is "when molecules of a gas remain attached to the surface of a material" (Monro, 1969). Absorption is "when the gas enters the solid or liquid phase and is held by capillary forces that govern the properties of solutions" (Monro, 1969). "Absorption is the most important physical factor modifying the penetration of fumigants" (Monro, 1969). As some of the fumigant is "lost" to the processes of sorption, (adsorption and absorption), it is necessary to add more fumigant to compensate for this. Thus materials; such as wax, honey and wood in a honey bee colony may affect the level of formic acid in hive air if they act as a "sink" for formic acid (Monro, 1969; Underwood and Currie, 2005; Bahreini and Currie, personal communication, 2006).

3.5 - Indoor Fumigation of Honey Bee Colonies with Formic Acid

Formic acid fumigation can be applied on a large scale in overwintering buildings, where the prolonged confinement of colonies indoors facilitates their treatment with sufficient doses of formic acid to limit varroa infestation (Underwood and Currie, 2003; 2004; 2005; 2007). Indoor fumigation in winter has the advantage of application

under controlled environmental conditions as temperature and ventilation can be regulated. Additionally, all colonies may be treated at once, minimizing the labour input required per colony (Underwood and Currie, 2004; 2005).

When hives are fumigated indoors with formic acid, the distribution patterns of the fumigant can affect treatment efficacy. During fumigation, the formic acid concentration of the treated room is higher than inside the treated hives and there is often considerable variation in the formic acid concentration within individual hives (Underwood and Currie 2004; 2005; 2007). It takes longer for the concentration of formic acid to build up in the hive air than in the room air, and formic acid concentration in hive air remains relatively high for up to 24 hours after indoor fumigation has ceased (Underwood and Currie, 2004). Within the hive itself, Underwood and Currie (2004) found no significant difference in the concentration of formic acid at the edge and the center of the honey bee cluster. Within rooms fumigated with formic acid, the concentration of formic in the room is relatively even at heights up to 1.5m (Underwood and Currie, 2004).

As colonies can be stored indoors during winter for several months there is a long potential window for the application of fumigation. Various applications with different durations and concentrations of formic acid have been tested for the effects on varroa mortality and colony health (Underwood and Currie, 2003; 2004; 2005; 2007). High concentrations of formic acid (41.2 ppm in room) delivered over 48 hours caused the greatest varroa mortality, but resulted in high levels of queen loss in colonies compared to medium (25.8 ppm in room) and low (11.9 ppm in room) formic acid concentration treatments (Underwood and Currie, 2004). In further studies, colonies were fumigated

for longer periods to determine the appropriate concentration and duration combination (CT product) that would result in adequate varroa control without high levels of worker mortality or queen loss using a standardized treatment of 471 ppm*days. It was shown that these of long-term, low-dose combination treatments of formic acid effectively reduce infestations of varroa while not affecting the mortality of workers or a queen in a colony (Underwood, 2005). While medium and high-short concentration treatments had high levels of efficacy (83% and 93%, respectively), the long-term, low-dose fumigation treatments resulted in lower efficacies of 60%. At the high and medium dosages, queen loss was observed and attributed to the acute affects of high in-hive concentrations of formic acid associated with increases in environmental temperatures during treatment (Underwood and Currie, 2005). Thus, low-concentration applications of formic acid have considerable merit.

Although indoor fumigation of colonies with formic acid has great potential to control levels of varroa, further studies are needed to examine the impact of brood rearing in colonies during winter on the efficacy of these treatments. Additionally, further modifications to the delivery of formic acid within the setup of overwintering buildings and standardization of colony sizes may result in greater treatment efficacies.

4 - Modified Atmospheres as a Control for Arthropod Pests

Pesticides have long been the dominant control for arthropod pests in agricultural settings. However, due to some of the undesirable effects and consequences of pesticides, such as pest resistance and potential health hazards, alternative methods of pest control are being sought. Alternative methods of control include physical control

where temperature, relative humidity or atmospheric composition of environment may be modified. One such technique relates to modification of the atmosphere of a space by altering the concentrations of CO_2, O_2 or N_2 to levels that are lethal to arthropods (Jay *et al.*, 1970; Aliniazee, 1971; White *et al.*, 1995). The use of modified atmospheres is effective, safe and economical (Aliniazee, 1971).

Using increased levels of CO_2 is particularly appealing because it can cause significant insect mortality (Jay *et al.*, 1970; Aliniazee, 1971; Spratt, 1975; Krishnamurthy *et al.*, 1986; White and Jayas, 1991; Mitcham *et al.*, 1994; Mann, 1998; Held *et al.*, 2000) resulting in up to 100% mortality in some trials to control stored products pests (Krishnamurthy *et al.*, 1986; Mitcham *et al.*, 1994). The ideal level of CO_2 for insect control is approximately 60% (Banks and Fields, 1995). However, CO_2 levels as low as 10% have been proven effective in killing stored grain beetles (Krishnamurthy *et al.*, 1986).

It is more effective to add additional CO_2 to a closed environment for arthropod control rather than limiting O_2 because this method maintains levels of CO_2 with more precision (Mellanby, 1934; Mann, 1998) and insects are much more vulnerable to high levels of CO_2 than low levels of O_2 (Jay *et al.*, 1970; Mitcham *et al.*, 1994).

A major cause of arthropod mortality from increased levels of CO_2 is related to inducing spiracles to open. In insects, spiracles remain open until accumulated CO_2 has diffused out of the tissues (Wigglesworth, 1935). Having the spiracles completely open for long periods of time causes significant water loss that often leads to death (Mellanby, 1934). Levels of 2% CO_2 in the air have proven sufficient to keep spiracles open permanently in some insects (Mellanby, 1934). Mortality of insects has been shown to

increase with a decrease in relative humidity when combined with high levels of ambient CO_2 (Jay *et al.*, 1970; Aliniazee, 1971; Spratt, 1975).

Carbon dioxide may also disrupt the physiological processes of insects. High levels of CO_2 in air result in high levels in the haemolymph of arthropods that are retained by a variety of potential mechanisms (Levenbook, 1950; Brooks, 1957). High levels of CO_2 can also affect the nervous system of insects by inducing depolization in neurons (Clark and Eaton, 1983), interfering with the neuromuscular transmission sequence (Hoyle, 1960) and extremely high levels of CO_2 (100%) can temporarily stop an insect's heartbeat (Edwards and Patton, 1965; Ward, 1971). In addition, an accumulation of CO_2 can upset the acid-base balance of the haemoplymph of insects that in turn can affect many physiological functions, such as ion transport and metabolism (Harrison, 2001).

Most modified atmosphere in insect control applications take place in closed systems such as storage situations. If there are leaks in these systems the levels of gases used cannot be controlled as effectively and the effects of the gases on the insects will be diminished. Therefore, it is important that systems where modified atmospheres are being applied be properly sealed and, if this cannot be achieved then more gas may be added to compensate for the leakage (White and Jayas, 1991).

Elevated levels of CO_2 have been used to control mites in agricultural settings, particularly in greenhouses (Held *et al.*, 2000). Small mites have a large surface area to volume ratio and thus are susceptible to desiccation. This water loss is limited in mites by a waxy layer on the surface of the body (Wharton and Richards, 1978). However, most terrestrial mites live in dry environments where they lose moisture to the air by

diffusion (Wharton and Richards, 1978; Arlian and Veselica, 1979). This presents a problem, as terrestrial mites need to conserve their body water. To compensate for this, the mites must actively take up water from their environment (Arlian, 1975). The varroa mite is provided with all the required moisture through feeding on honey bee haemolymph (Yoder and Sammataro, 1999), but when not attached to a host bee, they are at risk of desiccation (Bruce *et al.*, 1997). Unattached varroa lose more body moisture at high temperatures and low water vapor activities (Bruce *et al.*, 1997; Yoder and Sammataro, 1999). The ease at which varroa mites lose water to the environment provides an opportunity for their control, especially if modified atmospheres can alter varroa's respiration and cause desiccation.

Controlling varroa with modified atmospheres presents a challenge as varroa have adapted to the environment of the honey bee hive, with its increased levels of CO_2. The interior of the brood cell in particular has much higher levels of CO_2 and relative humidity than outside the cell (Jay, 1965). Varroa are thought to be able to survive the higher levels of CO_2 that they are exposed to inside the brood cell by adjusting the position of the peritreme (Pugh *et al.*, 1992). The peritreme may be used in combination with setae on the body surface to form a plastron to aid respiration while varroa are inside brood cells. The high levels of humidity (40-80%) within the sealed brood cell may also help to prevent desiccation that might otherwise occur (Jay, 1965). However, outside of the cell, varroa may be more susceptible to high CO_2 because the air outside the cell is less humid. Ultimately, the potential for control of varroa using modified atmospheres may hinge on varroa's existence in two very different environments during its lifecycle: the interior of the brood cell and the hive environment.

Varroa has only recently parasitized the European honey bee, *A. mellifera*, having evolved in association with the Asian honey bee, *Apis cerana* Fabricus. Although the two host species are very similar in their overall biology as cavity nesting honey bees, there are differences between the two species, such as *A. mellifera's* tolerance and adaptation to colder temperate climatic regions of the world and aspects of their environmental biology. These minor differences in the hive environment of its new host species may limit what varroa can tolerate within their naturally adapted thresholds.

The seasonal biology of *A. mellifera* and *V. destructor* and the conditions found within the honey bee hive and cluster provide an important opportunity for the control of varroa. The objectives of this study relate to the winter environment of honey bees and how varroa exists within this environment. The first objective of this study is to examine the role that brood rearing during winter in honey bee colonies has on the efficacy of low-concentration long exposure formic acid fumigation treatments for the control of varroa in honey bee colonies. The second objective of this study is to examine the level of brood (presence and abundance) in overwintered honey bee colonies and varroa's use of this brood. The third objective of this study is to determine whether modified atmospheric conditions can be applied to small clusters of honey bees to control the levels of varroa infestation within the normal range of conditions found within the honey bee cluster in winter.

CHAPTER 1. THE IMPACT OF BROOD REARING IN HONEY BEE COLONIES ON THE EFFICACY OF INDOOR FORMIC ACID FUMIGATIONS TO CONTROL INFESTATIONS OF *VARROA DESTRUCTOR* ANDERSON AND TRUEMAN DURING WINTER

ABSTRACT

Formic acid fumigation of honey bee colonies stored indoors during winter is a useful treatment for reducing infestations of varroa. However, the impact of honey bee brood in winter on the efficacy of these treatments is not known. This study examined the impact of brood removal on the mortality rates of varroa in low-concentration, long exposure time fumigation treatments with formic acid. In the fall of 2005 and 2006 twenty honey bee colonies with moderate to high-varroa infestations were randomly assigned to four treatment combinations consisting of two levels of fumigation (fumigated with formic acid or not fumigated) and two levels of brood removal (up to 1 frame removed or none). Daily measurements of formic acid concentration were taken from each colony and daily bottom board samples collected to quantify bee and varroa mortality. The results showed that brood removal treatments during early-winter in honey bee colonies had no impact on daily or cumulative varroa mortality. However, in both years varroa mortality was greater in fumigated colonies than unfumigated colonies. The level of efficacy differed between the two years of this study, most likely due to major differences with in-room (2005: 19.65 ± 0.67 ppm; 2006: 7.27 ± 0.51 ppm) and in-hive (2005: 8.0 ± 0.36 ppm; 2006: 2.81 ± 0.81 ppm) formic acid concentrations. The efficacies of the formic acid treatments were much higher in 2005 (brood removal 85.88

± 2.85%; no brood removal 88.15 ± 3.29%) than 2006 (brood removal 47.83 ± 8.47%, no brood removal 55.10 ± 1.85%).

INTRODUCTION

The parasitic mite *Varroa destructor* Anderson and Trueman is the most important pest in modern beekeeping. Even small levels of an infestation of *V. destructor* in a honey bee (*Apis mellifera* L.) colony can result in economic losses and will eventually lead to colony loss (Gatien and Currie, 2003; Currie and Gatien, 2006). The control of varroa is becoming increasingly difficult, as many populations of the mite are now resistant to some of the available treatments, such as synthetic acaricides (Elzen *et al.*, 1999; Mozes-Koch *et al.*, 2000). New methods of control for *V. destructor* are essential to the survival of apiculture as an industry, particularly methods that do not rely on synthetic acaricides and that minimize the numbers of applications necessary to reduce varroa infestations in honey bee colonies.

The use of organic acids (such as formic acid) has shown great promise for the control of *V. destructor*. Although formic acid is a caustic and corrosive liquid that requires caution to work with, it is a natural component of honey and considered an innocuous substance in most environments. This is because it can be biologically and chemically degraded and is volatile enough to decrease in concentration in honey as time passes (Liu, 1991). Formic acid is also inexpensive and readily available making it well suited to large-scale beekeeping operations that require large numbers of treatments.

In applications of formic acid, the formic acid vapors circulate within the hive and act as a contact fumigant. Formic acid can be delivered and distributed to honey bee colonies in a number of different ways including membranes (Daniels *et al.*, 1999), gel matrices (Feldlaufer *et al.*, 1997; Kochansky and Shimanuki, 1999; Eguaras *et al.*, 2003; Elzen, 2003), saturated plates or pads (Hoppe *et al.*, 1989; Mutinelli *et al.*, 1994;

Ostermann and Currie, 2004; Gatien and Currie, 2003; Currie and Gatien, 2006) and indoor fumigation of overwintered colonies (Underwood and Currie, 2004; 2005).

Formic acid fumigation can be applied on a large scale in overwintering buildings, where the prolonged confinement of colonies indoors facilitates their treatment with sufficient doses of formic acid to limit varroa infestation (Underwood and Currie, 2003). Indoor fumigation in winter has the advantage of allowing application under controlled environmental conditions as temperature and ventilation can be regulated, unlike in outdoor treatments where the ambient temperatures may greatly fluctuate and affect formic concentrations (Ostermann and Currie, 2004). Also, since all indoor-wintered colonies can be treated simultaneously with formic acid fumigation, there is much less labour input per colony.

As colonies can be stored indoors during winter for several months there is a long potential window for application. Various applications with different durations and concentrations of formic acid have been tested for their effects on varroa mortality and colony health (Underwood and Currie, 2003; 2004; 2005). Long-term, low-dose combination treatments (471 ppm*days) of 19 ppm formic acid for 27 days reduce infestations of *V. destructor* (by 60%) while not affecting the mortality of workers or causing queen mortality in colonies (Underwood, 2005). While fumigation treatments with higher concentrations and shorter durations (42 ppm for 10 days and 53 ppm for 9 days) result in greater efficacies (83% and 93% varroa mortality respectively) there is an increase in associated queen mortality under some ventilation conditions (Underwood and Currie, 2005). These queen losses in these colonies were attributed to the acute affects of high in-hive concentrations of formic acid that can be associated with increases

in environmental temperatures during treatment when ventilation is not controlled (Underwood and Currie, 2005).

Formic acid interacts with honey bees in a variety of ways, many of which are not fully understood. Under high concentrations and high temperatures formic acid has the potential to cause worker mortality (Nelson *et al.*, 1994; Elzen, 2003; Underwood and Currie, 2003), queen mortality (Fries, 1989; Underwood and Currie, 2004), capped and uncapped brood mortality (Fires, 1991; Bolli *et al.*, 1993) and can negatively influence drone production and survival (DeGuzman *et al.*, 1999). At a colony level, formic acid can disturb worker bees. Worker bees increase the air circulation in the hive by fanning their wings when in-hive concentrations of formic acid are high (Nelson, 1994). Formic acid treatments can also negatively impact colony development depending on the concentration and method of application (Ostermann and Currie, 2004). Even the presence of varroa can effect the concentrations of formic acid in colonies with more highly varroa infested colonies having lower concentrations of formic acid than colonies that are varroa-free (Ostermann and Currie, 2004). This suggests that activity or disruption of the hive activity caused by varroa may affect the volatilization and/or the sorption of formic acid in the hive.

Formic acid will also interact with the honey bee brood that may be present in the colony and can penetrate sealed brood cells in applications with very high concentrations of formic acid (85%) killing some of the varroa without causing high brood mortality (Fries, 1989; Fries, 1991; Calderon *et al.*, 2000). The ability to kill mites in brood is significant because when the appropriate concentration of formic acid is used, all stages of mites in a colony (phoretic and reproductive) can be targeted with a single treatment.

Lower concentrations of formic acid typical of registered treatments do not penetrate the brood capping consistently enough to cause high varroa mortality (Koeniger and Fuchs, 1988). Therefore, in most cases, multiple applications with formic acid are necessary over more than one brood rearing cycle to kill mites that are "protected" in the brood cells and survive treatments (Bracey and Fischer, 1989; Eischen, 1998 b). Although indoor formic acid fumigation of colonies is a useful method of reducing levels of varroa infestation in winter, the effect of winter brood rearing on the efficacy of these treatments in not known.

Honey bee colonies in northern regions of the world rear brood at a much lower levels in winter compared to the rest of the year (Seeley and Visscher, 1985). There is often very little to no fall brood rearing within a colony, with November often having the lowest levels of brood production (Harris, 1980; Szabo, 1993; Nelson, 1995). In latitudes with relatively mild and short winters, brood production slowly increases from November to February and increases significantly towards late March and early April (Jeffree, 1956; Avitable, 1978). Brood production in winter is initiated to replace worker bees as the population of adults in a honey bee colony dwindles throughout winter (Owens, 1971; Avitable, 1978; Harris, 1980; Szabo, 1993; Nelson, 1995). In more northerly latitudes slight increases in brood rearing in some colonies typically occurs in the period from December to mid January and are maintained at very low levels in some colonies until the end of March (Harris, 1980).

This limited brood during winter provides varroa with a valuable but extremely limited resource, which varroa can infest and use to reproduce (Martin, 2001). This brood may also provide a proportion of a colony's varroa population with physical

protection during winter from direct contact with fumigant treatment such as formic acid. Thus, winter brood rearing may affect treatment efficacy during low concentration formic acid treatments that are recommended for the control of varroa during winter.

The objectives of this study were to examine the impact of winter brood rearing in honey bee colonies on the efficacy of low-concentration, long-duration formic acid fumigation treatments to control *V. destructor* during indoor winter storage.

METHODS AND MATERIALS

Colonies of yellow strain *Apis mellifera* L. established from New Zealand packages and queens (Arataki Honey Ltd., P.O. Box 8016, Havelock North 4157, New Zealand) were selected based on the presence of moderate to high levels (1 to 46 mites per 100 bees) of varroa mite infestation. Colonies were sampled on 11 Nov., 2005 and on 20 Nov., 2006 by collecting approximately 150-200 bees, selected from the center frame of the brood nest of colonies. Varroa infestations were determined by alcohol wash technique as described in Gatien and Currie (2003). The bees from each individual sample were counted and put in 70% ethanol and vigorously shaken for 10 minutes while held in a wire screened basket within a 680ml plastic container on a mechanical shaker (Lab-line Instruments, Inc., Melrose Park, IL) for 10 min at 150 rpm to dislodge any mites from the bodies of the dead bees. The alcohol mixture was then filtered through fine mesh cloth (286*234 threads/cm^2) and the number of mites was counted.

In the fall of 2005 and 2006 twenty single super full-body Langstroth honey bee colonies were randomly assigned to one of four treatment combinations consisting of colonies fumigated with formic acid or left untreated. Within each fumigation treatment

half of the colonies received a brood removal treatment and the other half were left intact in winter storage.

On 16 Nov. in 2005, and 25 Nov. in 2006 colonies were moved into an indoor overwintering building, located at the University of Manitoba, Winnipeg, Manitoba, Canada (49° 54'N, 97° 14'W). The building was maintained at 2-7°C, in both years (Nov-March). Ten colonies each were randomly assigned (using manual techniques of drawing numbers from a hat) to two 3.0m x 2.7m x 1.7m treatment rooms, and placed in rows of five, along each wall. Colonies were placed on top of two empty super boxes, 54cm off the ground to facilitate sampling. The walls of each room were completely covered in vapor-barrier wrap (0.5 mil Polyester / 3 mil Polyolefin Platomer Coextrusion, Winpak Ltd., Winnipeg, Manitoba, Canada) and sealed at the seams with duct tape to prevent airflow between rooms. A hole (9.6mm) was drilled into the front of each hive and 6.4mm outer diameter (4.3mm inner diameter) polyethylene tubes were inserted directly into the center of the colony and connected to a tube leading outside the room. This allowed formic acid concentration from each colony to be sampled from outside the treatment room. Each colony had 52cm x 36cm x 4mm polyvinyl bottom boards covered with 30.2cm wide wax paper, that were inserted directly on top of the bottom board to collect any varroa mites or honey bees that fell from the winter cluster. Aluminum pan dead bee traps (19cm x 8cm) were fitted underneath the entrance of colonies to capture any worker bees or mites that fell out of the hive entrance. Colonies in both rooms received equal levels of sampling disturbance (daily bottom board removal) and were exposed to similar temperature and humidity levels. Both rooms were kept in complete

darkness for the duration of winter storage, except for an hour of sampling each day where a flashlight fitted with a red filter was used.

Brood removal and sampling was performed, before formic acid fumigation and after formic acid fumigation, in each year of the experiment (2005, 2006) in both the fumigated and control rooms. In 2005, pre-fumigation brood removal was done on 19 Jan. and the post-fumigation samples on 31 Mar. In 2006, pre-fumigation brood samples were taken on 21 Dec. and the post-fumigation samples on 30 Mar. The same rooms were used for the fumigation and control in both years of the study.

Five colonies were randomly selected (by manual method of drawing numbers from a hat) to have the brood removal treatment in each room. These colonies were opened and every frame in each colony was removed and examined for the presence of brood. Both sides of each frame were then photographed with a digital camera (Canon Powershot A520, 4.0 mega pixels) to quantify the brood area and the number of worker bees present. The brood removal treatment consisted of removing a single frame of brood (the frame with the largest amount of brood) from the selected colonies. The remaining frames were carefully returned to their original colony. Each frame selected for removal was brushed free of worker bees, taken back to the lab and stored in a freezer to quantify the level of brood and presence of varroa as part of another study. Techniques for dissection and sampling of brood cells are discussed in further detal in Chapter Two of this thesis.

Bottom board samples were collected daily from all colonies, in all treatments. In 2005, bottom board samples were collected from 17 Jan. to 20 Mar. In 2006, bottom board samples were collected from 17 Dec. to 27 Mar. Samples were collected prior to

fumigation, throughout the entire fumigation period and after fumigation by removing the bottom board samples from the colony and storing them in a freezer until samples could be processed. Any lives bees removed during sampling of the bottom boards were placed back in their original colony. All bottom board samples were processed in the lab where the number of dead mites and dead bees were quantified.

Each room had a (60cm x 51cm x 27cm) air-mixing chamber located directly outside of the rooms which provided the room with a constant supply of air (See Underwood and Currie, 2004 for a detailed schematic) through a 6.7cm radius polyethylene air circulation tube located in the center of the room, perforated with 10 evenly spaced 5cm diameter holes. A fan inside each air-mixing chamber (10cm diameter window fan, Holmes Products Corp., Milford, MA), provided an airflow of 106 L/sec. The air velocity from each of the holes in the tube was approximately 5 m/sec. Exhaust fans located on the opposite end of the room lead to the outside. These fans were controlled by a thermostat to expel air from the room at varying rates as the room temperature increased. Supplemental heat was also provided using 240 V electric wall mounted heater (Dimplex ®) with the thermostat set to 5°C.

Formic acid (35%) was delivered into the treatment room from plastic storage containers, (10 L, Aqua Pak ® #8905, Reliance Products Ltd., Winnipeg, Canada) through a 6.35mm x 4.3mm polyethylene tube that delivered the liquid formic acid directly to a 25.5cm x 25.5cm x 5.5cm humidifier pan (Genuine Wait ®) in the air-mixing chamber. Three paper humidifier plates (11.5cm x 16.7cm x 0.2cm, 64-3119-0, Dundas-Jafine Industries Ltd.) in the humidifier pans increased the surface area, facilitating formic acid evaporation. Each pan had a plastic float system (Genuine Wait

®) to control the rate of formic acid entering the pan, thereby preventing the pan from overflowing. Air blown over the pan by the fan evaporated the formic acid so it could be distributed throughout the room by the air tube ventilation system.

In both years, formic fumigation continued, uninterrupted throughout winter until immediately before colonies were moved out of storage. In 2005, fumigation began on 21 Jan. and ended on 29 Mar. for a total of 67 days. In 2006, fumigation began on 22 Dec. and ended on 25 Mar. for a total of 94 days. In both years, colonies were moved outdoors shortly after fumigation was terminated.

Formic acid was measured daily, using a Porta Sens II (Gas leak detector, Ati Technology) gas-sampling device using sensors (00-1038 acids sensor) sensitive to organic acids. The gas-sampling device was calibrated using drager tubes (formic acid 1-15 ppm 1a Part No. 6722701 ISO 9001; acetic acid 5-80 ppm 5a Part No.6722101 ISO 9001) with accompanying air pump (Accuro, hand held manual air pump, Drager, ARMM – F010). Gas samples were taken directly from tubes leading from the inside of each colony to the outside of the room where "outside" the end was completely sealed with a rubber septum to prevent air leakage. Gas sampling tubes were also located in the ventilation tube directly outside the air-mixing chamber, in the front of the room (along the floor) and in the back of room (along the floor).

In order to measure formic acid concentration, air was first drawn out of the tubes three times using a 60cc syringe to remove the stagnant air from the tubes. The input valve of the gas sensor (Porta Sens II) was then attached to each of the sampling tubes to obtain the concentration of formic acid in air. Air was drawn out using the methods listed above before taking gas samples using Drager tubes. Drager tubes were fitted into

a rubber tube with a needle at the end that was then pierced through the rubber septum of the sampling tubes and air was drawn through the tube using a manual air pump. The formic acid concentrations were measured prior to entering the treatment room, to minimize disturbance to fumigants in the room. Temperature and humidity were recorded daily from each room from a wall-mounted thermometer (Radio Shack Digital Thermo Hygro Cat No. 63-1013).

When entering the fumigated room an air hood respirator (Willson ® safety products P.O. Box 622, Reading PA 19603, R799 Air hood) with accompanying air supply hose connected to an air supply generator (ambient air breathing apparatus, AABA, model 49128, Willson ® AA01-11) was used to protect the eyes, skin, and the respiratory system from exposure to formic acid vapors.

In spring, the fumigated and non-fumigated colonies were moved outdoors, into separate apiary sites on the University of Manitoba campus that were separated by approximately 3km. This was done to prevent mixing of bees and varroa between treatments, through drift of bees or robbing behaviour. Samples of approximately 300 bees were taken from each colony, immediately after colonies were moved outside in spring to assess the levels of varroa mites as described above.

Each hive was then fitted with 37cm x 47cm plastic screened bottom board (Varroa-nator screen insert, Dimo's Tool and Die Ltd.) below which bottom board samplers (described above) were placed. Synthetic acaricide treatment was applied to kill most of the remaining mites in both the control and fumigated colonies to obtain an accurate estimate of the total varroa population remaining in each colony. In 2005, starting on April 4, fluvalinate (Apistan ® strips, Wellmark International) was used as the

final treatment and in the second year, starting on April 11, coumaphos (Checkmite+ ® Beehive pest control strips, Bayer Inc.) was used because some of the mites were suspected to have resistance to fluvalinate. In both cases, acaricide strips were used according to the label instructions. Bottom board samples were collected at 2 days, 9 days and 16 days after the acaricide treatments were applied to the colonies.

STATISTICAL ANALYSIS

The daily mortality rate of *V. destructor* was calculated by dividing the total number of varroa that fell on that day on bottom board samples, by the total varroa population present on the preceding sample day. This method used back calculating the end population of varroa to the initial population of varroa at the start of the experiment for each individual colony. The total varroa population at the end of the experiment was calculated by adding all the mites that fell during the spring acaricide treatment and all the mites that fell on all of the sampling days before, during and after fumigation.

The daily rate of bee mortality was calculated by dividing the total number of bees that fell on that day on bottom board samples by the total bee population on the preceding sample period day. This method used back calculating the end population of bees to the initial population of bees at the start of the experiment for each individual colony. The total bee population at the end of the experiment was calculated by adding all the bees that fell during the entire sampling period to the number remaining at the end of the experiment as assessed by photographic population assessments of every frame in each colony at the end of winter.

Cumulative varroa mortality was calculated by summing the number of varroa that died up to the final day of fumigation and dividing by the total mite population at the end of the experiment. Cumulative bee mortality was calculated by summing the number of bees that died up to the final day of fumigation and dividing by the total bee population. Mean abundance of varroa was calculated by dividing the total numbers of varroa in the colony by the total number of adult bees in the colony at the time of each sample period (before and after fumigation).

The effect of formic acid fumigation, brood removal, replication and date on daily worker mortality rate, cumulative worker mortality rate, daily varroa mortality rate and cumulative varroa mortality rate were analyzed (Proc GLM; SAS Institute Inc., 1999). All of the variables for this analysis were arcsine transformed prior to analysis, as they are proportions, but all data are presented as untransformed means. Separate ANOVA analyses were performed each year between fumigation treatment*brood treatment*replicate (Proc GLM; SAS Institute Inc., 1999).

Analysis of the experiment was set up using a before-after control-impact (BACI) design (Stewart-Oaten et al., 1986; Smith, 2002) in which hives were treated as replicates. A split-plot factorial analysis of variance was used, with fumigation level and brood treatment as main plot factors, hives as replicates and time as the subplot factor. Changes in mean mite abundance and daily mortality rate and cumulative mortality rate of varroa and bees were assessed using fumigation treatment*brood treatment*replicate interactions as the error term (Snedecor and Cochran, 1980). Where significant three-way interactions between fumigation treatment, brood removal treatment and time occurred, separate analyses were performed within each level of brood treatment (Proc

GLM; SAS Institute Inc., 1999). Mean varroa abundance and bee and varroa mortality rate values were arcsine transformed before analyses to improve equality of variance, as the variables are proportions.

RESULTS

Year 2005

Daily Bee Mortality

There was a significant three-way interaction between formic acid fumigation, brood removal treatment and date for daily bee mortality rate ($F = 2.82$; $df = 77, 587$; $P < 0.0001$). Therefore separate analyses were performed on each room. Within the formic acid fumigated room, brood treatment did not affect bee mortality over time ($F = 1.14$; $df = 77, 603$; $P = 0.2$) and bee mortality rate was similar in brood removal and unmanipulated colonies ($F = 1.09$; $df = 1, 603$; $P = 0.33$) but varied between sampling periods ($F = 4.86$; $df = 77, 603$; $P = 0.0001$) (Fig. 1.1 E, F). Within the control room bee mortality rates in brood removal and unmanipulated colonies differed over time ($F = 1.74$; $df = 77, 597$; $P = 0.0002$) (Fig. 1.1 G, H). Daily bee mortality was higher in brood removal colonies than in unmanipulated colonies ($F = 5.18$; $df = 1, 597$; $P = 0.05$) and also varied between sampling periods ($F = 3.69$; $df = 77, 597$; $P = 0.0001$) (Fig. 1.1 E, F, G, H).

Cumulative Bee Mortality

Fumigation treatment did not affect the cumulative honey bee mortality rate over winter as indicated by the fumigation treatment*time interval ($F = 1.93$; $df = 1, 16$;

P = 0.18) (Table 1.1 A). However, as expected cumulative bee mortality did increase over time (F = 77.75; df = 1, 16; P < 0.0001) Cumulative bee mortality rates in brood removal treatments differed in fumigated and non-fumigated rooms Room*Brood treatment (F =8.49; df = 1, 16; P < 0.01). In the fumigated room, bee mortality was significantly greater in the brood removal treatment than in unmanipulated colonies (F = 2350.43; df = 1, 16; P < 0.0001). Similarly, in the unfumigated room cumulative bee mortality increased relative to unmanipulated colonies as a result of the brood removal treatment (F = 3942.99; df = 1, 16; P < 0.0001) (Table 1.1 A, B).

Daily Varroa Mortality

There was a significant three-way interaction between formic acid fumigation, brood removal treatment and time for daily varroa mortality rate (F = 1.72; df = 77, 588; P = 0.0003). Fumigation treatment increased mite mortality rates relative to untreated colonies as indicated by a significant fumigation*date interaction (F = 3.90; df = 77, 588; P = 0.0001) (Fig. 1.2 E, F, G, H). Within the formic acid fumigated room, daily mortality of varroa was similar in brood removal and unmanipulated colonies over time (F = 1.14; df = 77, 607; P = 0.21) (Fig. 1.2 E, F). However, within the control room, brood removal treatments had greater mite mortality than in unmanipulated colonies (F = 1.38; df = 77, 597; P = 0.024) (Fig. 1.2 G, H).

Mean Abundance

Formic acid fumigation also reduced the mean abundance of mites relative to untreated colonies as indicated by a significant interaction between fumigation treatment

and time (F = 14.86; df = 1, 15; P = 0.0016) (Fig. 1.5 A). There was no difference between the fumigated and unfumigated treatments before fumigation was applied and a significant difference after fumigation (Fig. 1.5 A).

Year 2006

Daily Bee Mortality

There was no three-way interaction between formic acid fumigation, brood removal treatment and time for daily bee mortality rate (F = 0.57; df = 112, 854; P = 0.999). There was a significant interaction between formic acid treatment and time (F = 2.69; df = 1, 603; P = 0.0001) but no interaction between formic acid treatment and brood removal treatment (F = 0.1; df = 1, 603; P =0.759) and no interaction between brood treatment and time (F = 0.8; df = 1, 603; P = 0.928) for daily bee mortality. Daily bee mortality varied between sampling periods (F = 11.09; df = 77, 603; P = 0.0001) (Fig. 1.3 E, F, G, H).

Cumulative Bee Mortality

The cumulative bee mortality increased over time (F = 111.84; df = 1, 15; P < 0.0001) but was not affected by the fumigation treatment as indicated by the lack of interaction between formic acid fumigation and time (F = 0.63; df = 1, 15; P = 0.44) (Table 1.1 A, B).

Daily Varroa Mortality

There was a significant three-way interaction between formic acid fumigation, brood removal treatment and time for daily varroa mortality rate ($F = 4.21$; $df = 112, 850$; $P = 0.0001$). Therefore, separate analyses were performed on each room.

Within the formic acid fumigated room brood treatment did not affect varroa mortality over time ($F = 1.05$; $df = 112, 874$; $P = 0.36$) and daily varroa mortality rate was similar in brood removal and unmanipulated colonies ($F = 0.07$; $df = 1, 874$; $P = 0.79$) but varied between sampling periods ($F = 6.42$; $df = 112, 874$; $P = 0.0041$) (Fig. 1.4 E, F). However, within the control room daily varroa mortality rates in brood removal and unmanipulated colonies differed over time ($F = 2.30$; $df = 112, 861$; $P = 0.0001$) (Fig. 1.4. G, H). Daily varroa mortality was higher in brood removal colonies than in unmanipulated colonies ($F = 9.06$; $df = 1, 861$; $P = 0.02$) but was consistent between sampling periods ($F = 2.28$; $df = 112, 861$; $P = 0.105$) (Fig. 1.4 E, F, G, H).

Mean Abundance

Formic acid fumigation reduced the mean abundance of varroa relative to that in unfumigated colonies as indicated by a significant interaction between fumigation treatment and date ($F = 15.1$; $df = 1, 12$; $P = 0.002$). There was no difference between the fumigated and unfumigated treatments before fumigation and a significant difference after fumigation (Fig. 1.5 B).

Formic Acid Concentrations

The in-room concentration of formic acid in the air of fumigated rooms was much higher in 2005 than in 2006. The average concentration of the formic acid adjacent to the ventilation tube was 25.7 ± 0.6 ppm in 2005 and only 10.9 ± 0.6 ppm in 2006. The average formic acid concentration of the front of the fumigated room was 12.6 ± 0.6 ppm in 2005 and 9.2 ± 0.6 ppm in 2006. The formic acid concentration at the back of the room was 19.7 ± 0.7 ppm in 2005 and 7.3 ± 0.5 ppm in 2006. The average in hive concentrations in the two years reflected the trends seen in the concentrations of the hive air with the hive average in 2005 (8.0 ± 0.4 ppm) greater than 2006 (2.9 ± 0.2 ppm).

There was a significant difference between the in-room formic concentration by year ($F = 29.57$; $df = 1, 228$; $P < 0.0001$) but no significant difference by day ($F = 0.17$; $df = 98, 228$; $P = 1.0$). There was a significant interaction between replicate and year in in-hive formic acid concentration ($F = 38.66$; $df = 18, 1887$; $P < 0.0001$). Overall in-hive formic acid concentration was significantly greater in 2005 as shown by a significant difference between in-hive formic acid concentration by year ($F = 43.72$; $df = 1, 1887$, $P < 0.0001$) (Fig. 1.1 A, B, C, D; Fig. 1.3 A, B, C, D).

Temperature and Humidity

The temperature and humidity was similar in both rooms in both years. The average relative humidities were 47.5 ± 1.0% in the formic acid fumigated room in 2005, 40.9 ± 0.8% relative humidity in the control room in 2005, 47.1 ± 1.1% relative humidity in the formic acid fumigated room in 2006 and 40.8 ± 1.1% relative humidity in the control room in 2006. The average temperatures were 5.5 ± 0.2°C in the formic acid

fumigated room in 2005, 5.6 ± 0.2°C in the control room in 2005, 5.6 ± 0.2°C in the formic acid fumigated room in 2006 and 6.1 ± 0.1°C in the control room in 2006.

Efficacies of Formic Acid Fumigation

The efficacies were very different between the two years of the study. The average treatment efficacy in 2005 was 87.01 ± 0.03% in all fumigated colonies (Min 69.1%; Max 94.9%; N =10) and 51.15 ± 0.06% in all fumigated colonies in 2006 (Min 7.0%; Max 78.1%; N = 10). There was a significant treatment effect relative to untreated colonies as determined by the treatment*time interaction (Fig. 1.5 A, B).

DISCUSSION

This study examined the relationship between the efficacy of formic acid fumigation treatments for the control of varroa and brood rearing in honey bee colonies stored indoors during winter. Efficacy of formic acid fumigation was not affected in colonies with and without brood removed. However, in both years brood removal treatments affected varroa mortality in unfumigated colonies. The level of efficacy associated with fumigation treatments differed between the two years of this study, most likely due to major differences with in-room formic acid concentrations. The results suggest low levels of in-hive formic acid concentrations (avg 2.81; max 8.3 ppm) in 2006 may be close to the threshold below which varroa can survive formic acid fumigation. Over the two years of this study in unfumigated colonies with no brood removal, the natural varroa mortalities (0.34% daily and 13% cumulative) were lower than the levels of bee mortality (0.5% daily and 17% cumulative). These differences in mortalities

between parasite and host show how varroa infestations may increase over winter as a result of differences in the proportional death rates of the two species.

Very low amounts of brood were removed from the colonies selected to have the brood removal treatment in 2005. The proportions of the colonies that had brood were very low (only 2 out of 10 colonies had brood), while the brood present was in very low levels (mean of 19 sealed brood cells) and contained very few mites (a total of 25 varroa mites were removed, mean of 12.5 varroa/colony with brood) (see Chapter 2). However in 2006, substantially more brood was removed from colonies than in 2005. The proportion of colonies that had brood present was greater (seven out of ten colonies), while the amounts of brood removed (mean of 184 sealed brood cells), and the numbers of varroa mites removed (total mites 719, mean of 102 varroa/colony with brood) were all much higher. The difference in timing (17 Dec., 2006 compared to 20 Jan., 2005) of the pre-fumigation brood removal between years may have influenced the amount of brood removed. The stressful nature of the brood removal treatment and the resulting disturbance to the colony likely contributed to the increased bee mortalities in these colonies during both years of the study. Similar levels of mid-winter disturbance of honey bee colonies are known to affect bee survival (Underwood and Currie, 2004).

In both years, the formic acid concentration of the treated room was higher than inside the treated hives and there was often considerable variation in the formic acid concentration between individual hives. The concentration of formic acid also took longer to build up in the hive air than the room air. These same trends were seen in previous indoor fumigation studies conducted in overwintering buildings (Underwood and Currie, 2004; 2005; 2007). This trend for formic acid concentration to increase in

hives over time is likely due to the reduction in sorption of formic acid by components of the hive; such as wood, honey and the bodies of the bees themselves as the hive environment becomes progressively saturated with formic acid (Underwood and Currie, 2007; Bahreni and Currie, personal communication, 2006). The fluctuations in formic acid levels inside and outside the hives in both years of the study were positively correlated and likely most affected by changes in patterns of room ventilation associated with the use of an exhaust fan to maintain a constant room temperature of 4-7°C.

The in-room formic acid concentration was consistently higher in 2005 (mean of 20 ppm; max 30 ppm) than 2006 (mean of 7 ppm; max 24 ppm). This may have been due in part, to increased rates of room ventilation in 2006, as the ventilation was set to maintain the room temperature at 4 to 6°C. The increased ventilation in 2006 may have been caused by thermostat settings that resulted in the fans in each room constantly running to maintain the rooms at the appropriate temperature. Similar trends of decreasing formic acid concentration resulting from increased treatment room ventilation induced by high outside temperatures, or artificial heating of treatment rooms, were demonstrated by Underwood and Currie (2005; 2007). In those experiments, formic acid concentrations were nearly halved when there was a higher level of ventilation of the treatment room. The results suggest producers may need to monitor and adjust formic acid concentrations in years where excessive fan ventilation occurs.

The resulting average and maximum in-hive concentrations of formic acid were also greater in 2005 (mean 7.97 ppm, max 14.05 ppm) than in 2006 (mean 2.81 ppm, Max 8.3 ppm). The in-hive concentrations of formic acid in 2006 were much also lower than in similar indoor low-concentration, long-duration fumigations that achieved

moderate efficacy (avg. 6.0 ppm, max 17.6 ppm) (Underwood and Currie, 2005) and are much lower than many of the in-hive levels (averages of 10 to 70 ppm) achieved in previous outdoor trials with formic acid, where it was quantified (Feldlaufer *et al.*, 1997; Ostermann and Currie, 2004).

This study tracked the daily mortality of varroa and bees in response to a constant indoor fumigation treatment of formic acid over extended periods resulting in a detailed representation of the relationship between fluctuating levels of formic acid and varroa mortality over winter. In both years of the study in fumigated colonies, brood removal treatments showed a slight increase (but not significant) in the daily varroa mortality rates relative to unmanipulated colonies. This was likely due to increased numbers of bees dying due to the stress of the brood removal and not a direct effect on the mites themselves. In both years the large increases in varroa mortality at the end of winter when bees were removed from winter quarters were due to the impact of the synthetic acaricide treatment used to kill all of the remaining mites in the colonies and unrelated to experimental treatments.

The fumigated colonies exhibit very different trends in daily varroa mortality rates between the two years of the study. In the fumigated colonies in 2005, there is an immediate increase in the varroa mortality while in 2006, varroa mortality rates were much lower and fluctuated throughout the winter. The occasional increases in daily varroa mortality rates observed, particularly in late-winter in 2006, likely represent a response in varroa mortality to temporary increases of the in-hive formic acid concentration associated with changing ventilation patterns in the room and not an effect of brood. Fluctuations in daily varroa mortality in both years appear to match

fluctuations in both the room and average hive formic acid concentrations, with somewhat of a lag. This lag may represent a slight delay in mortality in response of varroa to the transient increase in levels of formic acid in the hive environment. Concentrations of formic acid were likely fluctuating around the threshold to which varroa are susceptible. Experiments on small clusters of varroa infested bees under carefully controlled environmental conditions demonstrated that varroa are susceptible to concentrations of 5 ppm and above but that concentrations of 40 and 80 ppm were the most effective at reducing varroa at the full ranges of temperatures that exist within the honey bee environment in winter (Underwood and Currie, 2003).

Varroa may also receive different levels of exposure to formic acid at different locations within the winter cluster. During winter, honey bees cluster together to form a compact cluster of individuals in the colony with distinct layers. Temperature differs within these distinct layers with the interior being the warmest (25-35°C) and the exterior being much cooler (6.7-13.3°C) (Cahil and Lustick, 1976; Southwick, 1991 a). The daily fluctuations in varroa mortality may reflect exposure of the mites on bees at the periphery of the cluster that are more directly exposed to formic acid. The relationship between the location of bees in the cluster and their relative exposure to formic acid is likely complex. Mites on bees located in the center of the cluster may be somewhat protected from exposure to formic acid if concentrations in the center are lower. However, bees in the cluster will cycle from the center to the outside throughout the winter (Gates, 1914) so mites that may have been protected in the center of the cluster will eventually be exposed. Underwood and Currie (2005) demonstrated that formic acid concentrations are not significantly different between the centers of winter clusters and on the edges of clusters,

but there was a clear trend toward lower levels of formic acid in the center of clusters. This trend may represent a crucial difference when in-hive formic acid levels are extremely low, as was the case in this study throughout most of 2006.

Differences in susceptibility of varroa to formic acid may also be a consequence of the position of varroa on the adult bees. The preferred location for varroa on the body of an adult bee during winter is between the 3rd and 4th abdominal tergites (Bowen-Walker *et al.*, 1997). At this position varroa are partially covered as they are wedged between the overlapping abdominal tergites, possibly affording varroa some protection from direct contact with formic acid vapors. Varroa located at different positions on adult honey bees or varroa that move momentarily from this position may be at increased risk of exposure to increased levels of fumigant. As little is known about the dynamics of varroa's movement within the winter cluster, further studies may reveal relationships between varroa's location on their host and their susceptibility to fumigants.

The overall efficacies in both years reflected a clear relationship between in-hive concentrations of formic acid and exposure time. In 2005, the infestations were significantly reduced in the fumigated colonies (87% efficacy) (19.65 ppm*65 days = CT 1277). In 2006, the infestations in the fumigated colonies were reduced relative to untreated colonies but efficacy was low (51% efficacy) (7.27 ppm*99 days = CT 719). In 2006, additional acaricide treatments would have been required after fumigation in early spring to further reduce the varroa infestations below the economic threshold (> 2 mites/100 bees) (Currie and Gatien, 2006) to prevent further colony damage. The efficacies achieved in 2005 were much higher than those attained by Underwood and Currie (2005) (60% efficacy) (19 ppm*27 days = CT 513) under similar conditions. Both

years of our study had longer durations of fumigation and higher CTs than their study but this did not always result in a higher efficacy. The lower efficacy in the 2006 fumigation suggests that exposure to critical concentrations of formic acid are required to effectively increase varroa mortality rates. The fumigation trial in 2006 showed that if the in-hive formic acid levels do not reach the appropriate levels, then fumigation treatments had low efficacy even with treatments that last over three months.

Previous studies have shown that varroa can become concentrated on bees within a colony over winter as varroa may transfer from dying bees to live bees in a dwindling population of bees in a colony (Smirnov, 1978; Bowen-Walker *et al.*, 1997) but other studies have shown stable infestations (Fries and Perez-Escala, 2001) or decreasing infestations over winter (Underwood and Currie, 2007). Accurate data on mortality rates of bees and mites during winter are lacking. Daily death rates of varroa over winter used for population modeling have been estimated at 0.2% (Martin *et al.*, 1998) to 0.4% (Calis *et al.*, 1999; Fries *et al.*, 1994). In unfumigated colonies without brood removal, daily varroa mortality was 0.34% or 3 varroa/day based on samples collected over four months when little to no brood was present. The average bee mortality rate was 0.5% or 19 bees/day, which was greater than the varroa mortality rate in these colonies. Over the winter period the cumulative varroa mortality rate in untreated colonies was less (13%) than the cumulative bee mortality rate (17%) and much lower than previous estimates of cumulative varroa mortality over winter (40%) (Korpela *et al.*, 1992).

Some mortality models of varroa during winter (e.g. Fries and Perez-Escala, 2001) predict stable infestations over winter due to similar bee and varroa mortalities. However, our results demonstrate that the mean abundance changed over the winter and

that this change was likely due to differences in bee and mite mortality rates. Trends in our study are supported by larger sample sizes than in previous studies and using daily mortality measurements taken over most of the winter. In addition, our study incorporated detailed population assessments of both the entire bee and varroa population of each colony over time in addition to alcohol wash samples, both of which demonstrated similar trends. The data demonstrated that infestations over winter can either increase (2006) or slightly decrease (2005) and that this change in infestation depends on the variation in the bee and varroa mortalities in relation to each other. Differential mortality of bees and mites could occur because of the differences in positions of the winter cluster in relation to the bottom board, which may allow varroa to leave dead or dying bees on the bottom board and access live bees in the cluster (Fries and Perez-Escala, 2001). Variation in varroa mortality rates could result from varroa's preference for young bees (Le Conte and Arnold, 1989; Bowen-Walker *et al.*, 1997; Kuenen and Calderone, 1997; Pernal *et al.*, 2005). The varroa population of a colony during winter could become concentrated on the bees as a result of varroa switching from older host bees (which are more likely to die) to younger host bees (Harris, 1980).

Concentration of varroa on bees in winter could also result from reproduction of varroa in winter brood (Fries and Perez-Escala, 2001). However, brood production in this study would have added only slightly to the varroa population (see Chapter 2). Due to the overall limited reproductive capacity of varroa in honey bee colonies throughout winter, the increase in mean abundance was likely the result of higher levels of bee mortality in relation to the varroa mortality. The average daily varroa mortality in untreated colonies (0.34%) was greater than the bee mortality rate (0.2%) (see Chapter 2).

The potential for the mean abundance of varroa to greatly increase over winter reinforces the need for colonies to have their varroa infestations reduced in fall to a level well below the thresholds where colonies will collapse (10 mites per 100 bees) (Currie and Gatien, 2006). Increasing concentrations of mites on bees could in turn cause greater bee mortality rates. The capacity of these infestations to increase during winter reinforces the need for adequate control of varroa infestations in fall. If the short treatment window to apply acaricides for varroa control doesn't allow adequate treatment, then indoor fumigation during winter storage of honey bee colonies would allow beekeepers the opportunity to reduce mite levels below the economic injury level during winter.

The results of this research showed that the limited production of brood during winter in honey bee colonies did not affect the efficacy of low-concentration, long-duration formic acid fumigation treatments to control varroa infestations. This study demonstrated how closely cumulative varroa mortality was associated with slight changes in concentrations of formic acid, most likely due to changes in ventilation patterns in the treatment rooms. This underscores the importance of consistently maintained room conditions during fumigation. The high efficacy achieved in low-concentration, long duration formic acid fumigations in 2005 was higher (84%) than in previous studies and than in 2006 of this study. These results suggest that accumulation of large concentration*time products will not cause high mortality of varroa if a critical concentration threshold is not achieved. Additionally, the results indicated that slight shifts in the balance of bee and varroa mortalities during winter may be responsible for

observed decreases or increases in the mean abundance of varroa in untreated honey bee colonies over winter.

Fig. 1.1 Daily bee mortality in honey bee colonies in 2005 A) and B) Room air concentrations of formic acid (ppm) C) Mean colony concentration of formic acid (ppm) in colonies with brood removal D) Mean colony concentration of formic acid (ppm) in colonies without brood removal E) Daily rate of bee mortality in fumigated colonies with brood removal F) Daily rate of bee mortality in fumigated colonies without brood removal G) Daily rate of bee mortality in unfumigated colonies with brood removal H) Daily rate of bee mortality in unfumigated colonies without brood removal. For (C) to (H) values represent means ± standard error, N = 5 hives per treatment combination.

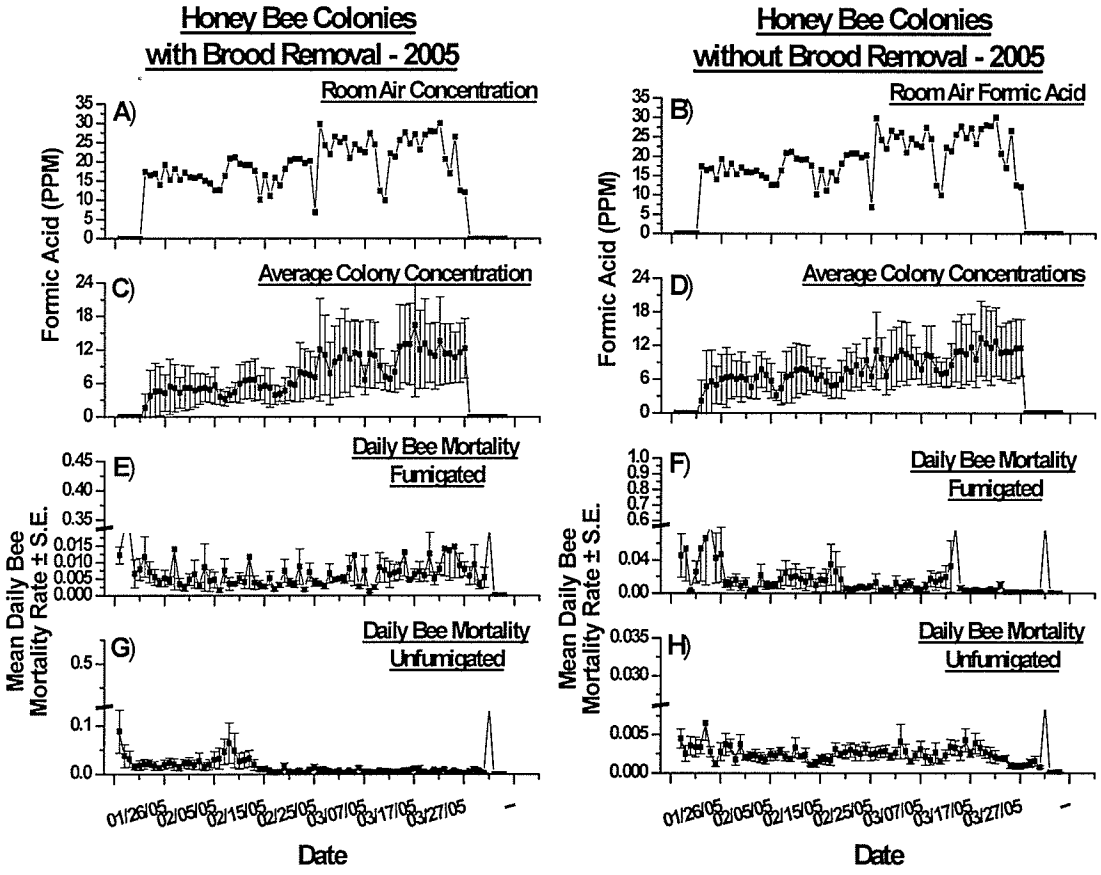


Fig. 1.2 Daily varroa mortality in honey bee colonies in 2005 A) and B) Room air concentrations of formic acid (ppm) C) Mean colony concentration of formic acid (ppm) in colonies with brood removal D) Mean colony concentration of formic acid (ppm) in colonies without brood removal E) Daily rate of varroa mortality in fumigated colonies with brood removal F) Daily rate of varroa mortality in fumigated colonies without brood removal G) Daily rate of varroa mortality in unfumigated colonies with brood removal H) Daily rate of varroa mortality in unfumigated colonies without brood removal. For (C) to (H) values represent means ± standard error, N = 5 hives per treatment combination.

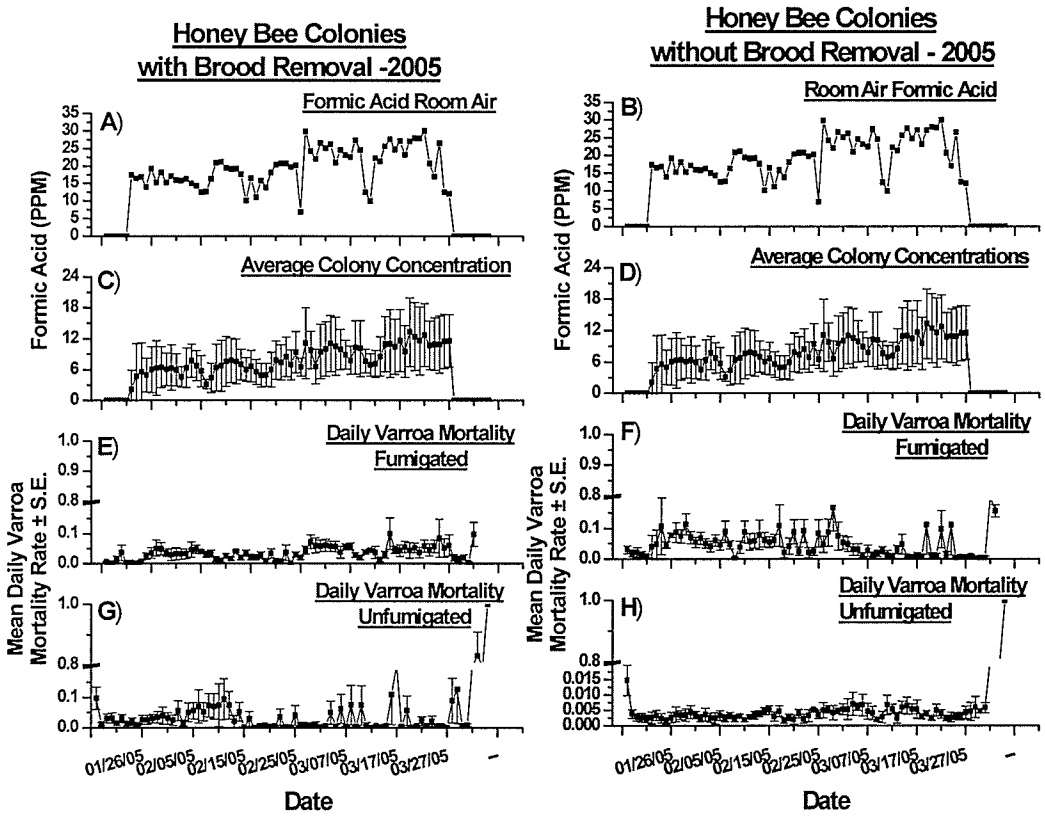


Fig. 1.3 Daily bee mortality in honey bee colonies in 2006 A) and B) Room air concentrations of formic acid (ppm) C) Mean colony concentration of formic acid (ppm) in colonies with brood removal D) Mean colony concentration of formic acid (ppm) in colonies without brood removal E) Daily rate of bee mortality in fumigated colonies with brood removal F) Daily rate of bee mortality in fumigated colonies without brood removal G) Daily rate of bee mortality in unfumigated colonies with brood removal H) Daily rate of bee mortality in unfumigated colonies without brood removal. For (C) to (H) values represent means ± standard error, N = 5 hives per treatment combination.

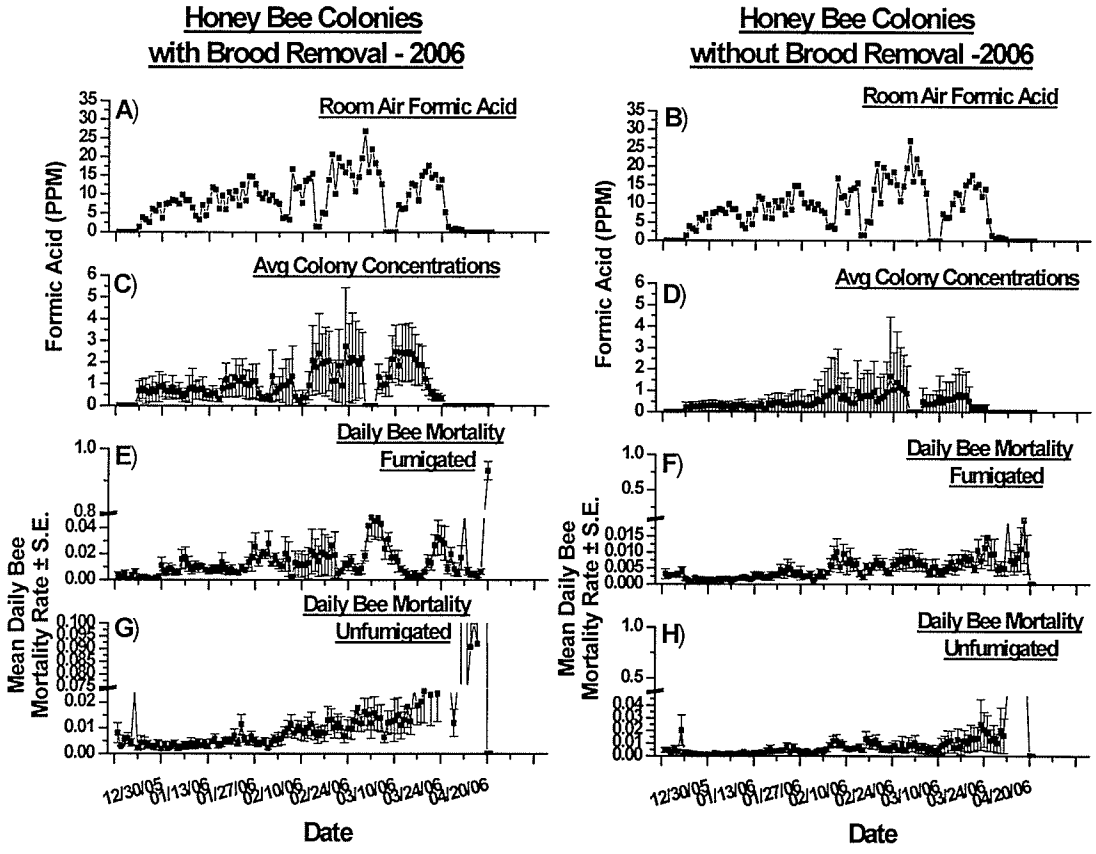


Fig. 1.4 Daily varroa mortality in honey bee colonies in 2006 A) and B) Room air concentrations of formic acid (ppm) C) Mean colony concentration of formic acid (ppm) in colonies with brood removal D) Mean colony concentration of formic acid (ppm) in colonies without brood removal E) Daily rate of varroa mortality in fumigated colonies with brood removal F) Daily rate of varroa mortality in fumigated colonies without brood removal G) Daily rate of varroa mortality in unfumigated colonies with brood removal H) Daily rate of varroa mortality in unfumigated colonies without brood removal. For (C) to (H) values represent means ± standard error, N = 5 hives per treatment combination.

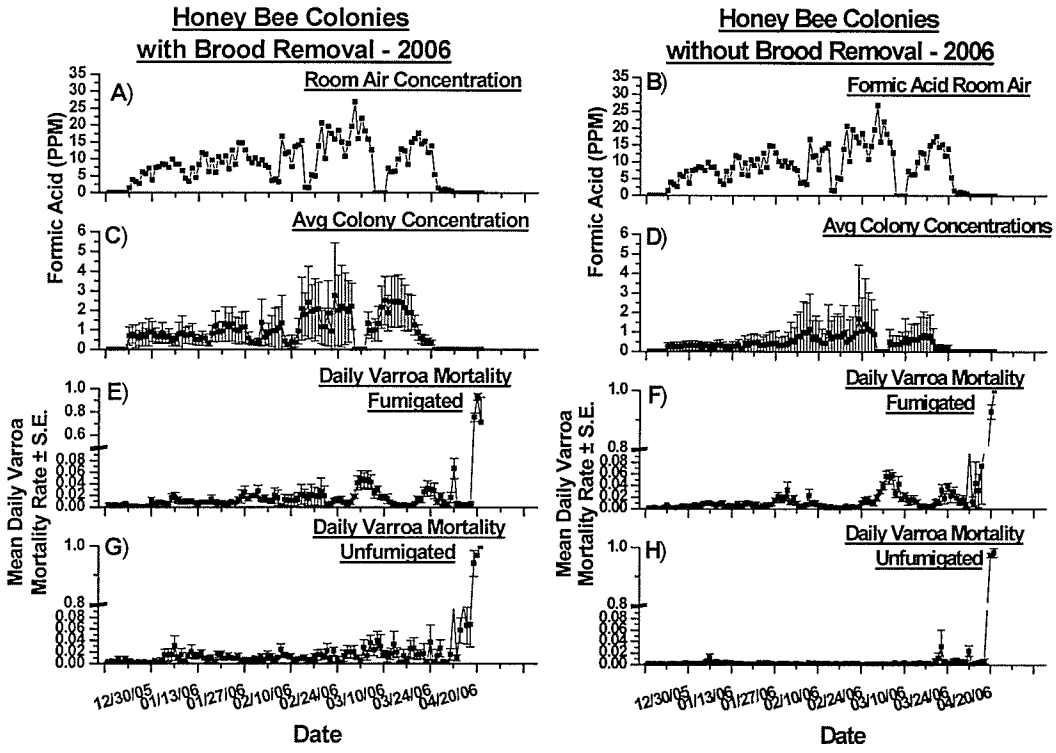


Fig. 1.5 Mean abundance of varroa in honey bee colonies fumigated with low concentration formic acid with brood removal, fumigated colonies without brood removal, unfumigated colonies with brood removal and unfumigated colonies without brood removal before and after winter. A) Colonies in 2005 B) Colonies in 2006. Asterisks indicate a significant treatment effect relative to untreated colonies as determined by the treatment*time interaction ($P < 0.05$).

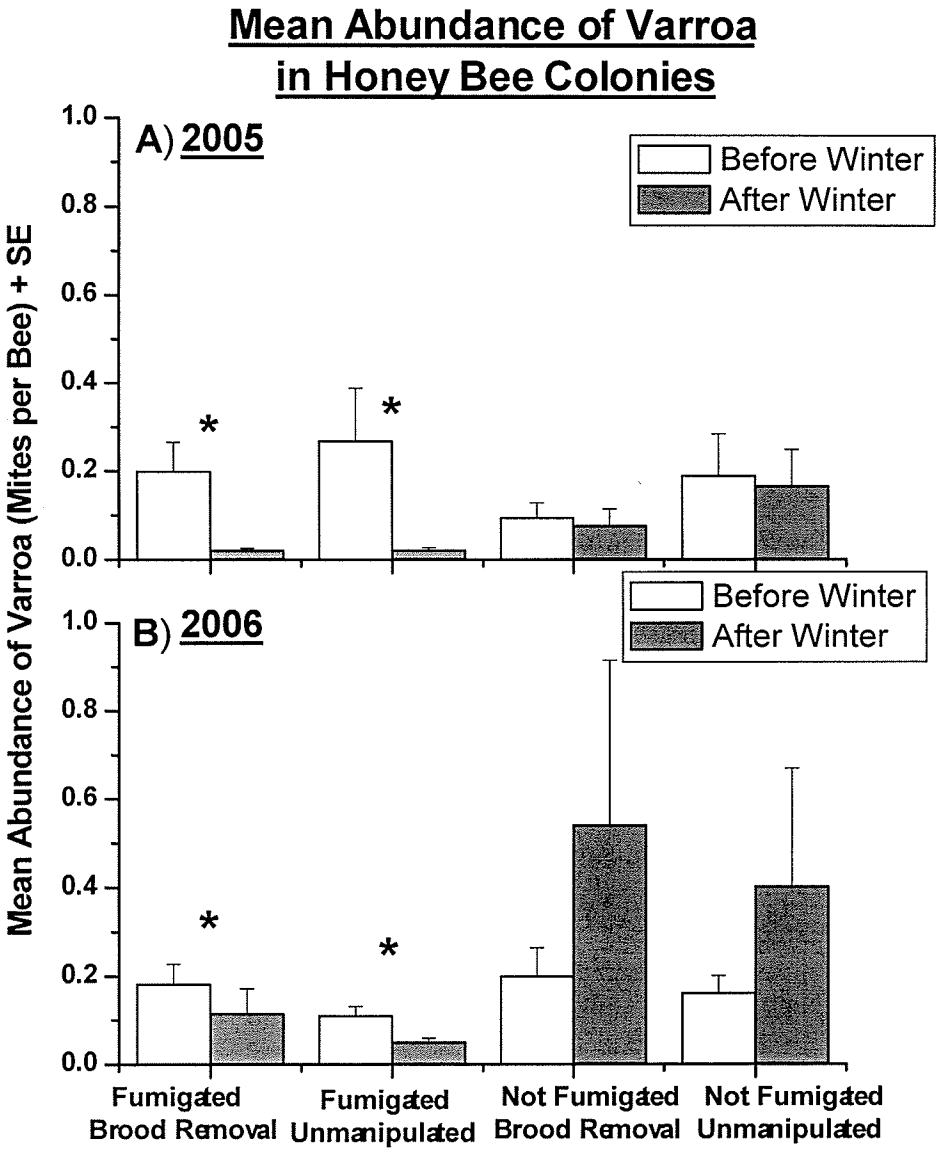


Table 1.1 Overall mean (± standard error) honey bee and varroa mortality rates throughout both years of winter sampling in fumigated and unfumigated colonies: daily number of varroa dropping on bottom board, daily varroa mortality rate, cumulative varroa mortality rate, daily numbers of bees dropping on bottom board, daily bee mortality rates, cumulative bee mortality rate. A) All colonies fumigated with low concentration, long duration formic acid in 2005 and 2006 B) Unfumigated colonies with brood removal and without brood removal in both years

A) **Fumigated honey bee colonies**

	2005; N = 10	**2006; N = 10**
	Mean	**Mean**
Daily Varroa Drop	13.52 ± 1.03 (Min 0, Max 256)	7.19 ± 0.30 (Min 0, Max 71)
Daily Varroa Mortality	0.043 ± 0.002 (Min 0, Max 0.5)	0.014 ± 0.001 (Min 0, Max 0.120)
Cumulative Varroa Mortality	0.70 ± 0.011 (Min 0.023, Max 1.0)	0.37 ± 0.01 (Min 0.01, Max 0.86)
Daily Bee Drop	14.34 ± 0.7 (Min 0, Max 311)	23.36 ± 0.63 (Min 0, Max 176)
Daily Bee Mortality	0.01 ± 0.0012 (Min 0, Max 0.43)	0.006 ± 0.0004 (Min 0, Max 0.17)
Cumulative Bee Mortality	0.34 ± 0.011 (Min 0.035, Max 0.94)	0.20 ± 0.006 (Min 0.02, Max 0.85)

B) **Unfumigated honey bee colonies**

	With brood removal 2005; N = 10	Without brood removal 2006; N = 10
	Mean	**Mean**
Daily Varroa Drop	3.22 ± 0.14 (Min 0, Max 71)	3.00 ± 0.19 (Min 0, Max 102)
Daily Varroa Mortality	0.021 ± 0.002 (Min 0, Max 1.0)	0.0034 ± 0.0002 (Min 0, Max 0.15)
Cumulative Varroa Mortality	0.43 ± 0.011 (Min 0.013, Max 1.0)	0.13 ± 0.003 (Min 0.0086, Max 0.39)
Daily Bee Drop	19.50 ± 0.57 (Min 0, Max 167)	18.56 ± 0.63 (Min 0, Max 176)
Daily Bee Mortality	0.012 ± 0.0007 (Min 0, Max 0.22)	0.005 ± 0.00032 (Min 0, Max 0.1)
Cumulative Bee Mortality	0.35 ± 0.01 (Min 0.02, Max 1.0)	0.17 ± 0.005 (Min 0.015, Max 0.87)

CHAPTER 2. *VARROA DESTRUCTOR'S* UTILIZATION OF HONEY BEE BROOD DURING WINTER.

ABSTRACT

This study was conducted to determine the precise dynamics of brood utilization by varroa under extended periods of limited brood rearing during winter. Frames of brood were removed from honey bee colonies stored in a honey bee wintering facility over a three year period in early, mid and late-winter. The levels and stages of brood present and levels of varroa in the brood were quantified for each colony. Detailed population assessments of the varroa and bee populations were also taken for each hive using bottom board and alcohol wash samples. The overall amount of brood found in this study throughout all sampling periods (between December and late March) in winter was very limited with a slight overall trend toward more brood in early-winter (December) than mid or late-winter (February to March). The majority of varroa survived as ectoparasites on adult bees in the colony, even though brood was available to infest. During winter, only 23% of brood cells were infested with varroa. The proportion of mites in brood that reproduced (31%), was lower than in different temperate regions of the world with shorter winters. However, overall reproductive rates of varroa in colonies were very low. Only 2% of the total population of varroa in the colony was reproducing at any one time and the number of colonies with brood averaged 44% (range 0% to 80%). This research demonstrates that varroa are under-utilizing the honey bee brood in winter, in both infestation and reproduction.

INTRODUCTION

During winter, honey bee brood provides *Varroa destructor* Anderson-Trueman with a resource to infest, feed upon and use for reproduction. Previous studies have shown that the proportion of brood infested and the reproduction of varroa in brood is much lower than the levels in summer, while the crowding of varroa in brood is much greater in winter (Eguaras *et al.*, 1994; Martin, 1995; Martin, 2001). However, regional differences in varroa, honey bee genetics and climate can influence the reproduction of varroa (Correa-Marques et al., 2003) as well as the levels and duration of brood rearing during winter. Therefore these previous studies carried out in different regions of the world may not necessarily reflect the precise dynamics of how varroa uses brood in northern regions with extremely cold and prolonged winters.

Varroa mites are completely dependant on honey bees throughout their lifecycle. Varroa feed on both the adult and developing honey bee. Adult female varroa mites live as ectoparasites on adult honey bees and then enter the cells of honey bee larvae in order to reproduce.

Varroa mites evolved in association with the Asian honey bee, *Apis cerana* Fabricius, and have recently transferred to the European honey bee, *Apis mellifera* L., where varroa has exploited the overall similarities between the two host species, particularly in terms of nesting biology and brood rearing. However, there are some major differences in varroa's interaction between the two species. First of all, in *A. cerana*, varroa primarily use the drone brood to reproduce. This is because in the worker brood of *A. cerana*, the development time is too short for varroa to rear offspring to maturity in sufficient numbers that would result in a population increase

(Boot *et al.*, 1995). *Apis cerana* also exhibit heightened hygienic behavior and are able to detect and remove worker cells infested with varroa (Boot *et al.*, 1995). Varroa will still infest worker cells in *A. cerana*, even though there is minimal reproduction occurring (Rath, 1999). While in *A. mellifera*, varroa will use both drone and worker brood to successfully reproduce, although varroa still prefer drone brood as the longer development of the drone brood allows more varroa offspring to develop to maturity (Boot *et al.*, 1995; Boot *et al.*, 1992; Rath, 1999). The level of observed reproduction in worker brood is 80% in *A. mellifera* and only 10% in *A. cerana* (Boot *et al.*, 1992). For these reasons, varroa levels are suppressed in *A. cerana* and the parasite is able to coexist with its original host without causing major harm. In the new host, *A. mellifera*, varroa is a very serious pest. However, the way in which varroa uses the brood of *A. mellifera* for reproduction is not fully understood, especially with respect to geographic and seasonal variations.

The behavior and life history of the two sexes of varroa on *A. mellifera* is distinct. The small male varroa functions only to fertilize the female sister mites and typically dies within the brood cell (De Jong, 1982). The female varroa offspring that successfully mature within the brood cell will leave the brood cell with the emerging bee. The recently emerged female varroa will then attach and feed on adult bees before re-entering another brood cell to reproduce. A single female varroa is capable of reproducing up to seven times in its lifetime, but typically will go through 2-3 reproductive cycles and can live up to 80-100 days (Ruijter, 1987; Calatayud and Verdu, 1994; Martin and Kemp, 1997). One female varroa is able to lay five to six eggs for each period she enters a brood cell (Ruijter, 1987; Martin, 1994), typically producing one male and three female

daughters per cycle in a worker cell, of which one to two viable daughter mites typically survive (Rehm and Ritter, 1989; Martin, 1994).

Varroa mites prefer drone brood to worker brood because drone brood has longer development times (24 days for drones and 21 days for workers) and thus more female mites in each cell can reach maturity before the bee emerges from its cell (Boot *et al.*, 1991; Boot *et al.*, 1995). Drone brood infestations result in two to three viable female offspring being produced per infested cell and worker brood infestations typically produce one to two per cell, averaging 1.45 (Schulz, 1984; Fuchs and Langenbach, 1989; Martin, 1994). Drone brood are attractive to varroa for two to three times longer than worker brood (Boot *et al.*, 1991; Boot *et al.*, 1992). Varroa cue in to chemical attractants or kairomones in fatty acid esters to distinguish drone larvae from workers (LeConte *et al.*, 1989). Semiochemicals found in the larval food of honey bees may also be a significant attractant of varroa to their larval host (Nazzi *et al.*, 2001).

Foundress mites enter the brood cell of honey bees on the 5^{th} or 6^{th} day of brood development, right before the cells are to be capped with wax (Grobov, 1977). The foundress mite lays her first egg, which is a haploid male, in the brood cell 60 hours after the cell is capped. All subsequent eggs are diploid females laid at intervals of 26-32 hours (Ifantidis, 1983; Rehm and Ritter, 1989; Martin, 1994). Male mites require 6.9 days to complete development and females require 6.2 days (Rehm and Ritter, 1989). Diploid eggs hatch into a larval protonymph stage, then a deutonymph stage and finally an adult (Grobov, 1977). Protonymphs are small, white and round in overall shape while deutonymphs are white and resemble the adult mites in shape (DeJong, 1982).

In cells infested by a single foundress mite, developing mites mate with their siblings inside the brood cell (Henderson *et al.*, 1986). If more than one foundress mite enters a brood cell, then the developing mites have the opportunity to mate with mites from the other parent(s). When high numbers of mites infest a single cell, reproductive rates decline due to competition between the offspring for the resources of the pupa's haemolymph, at the feeding site (Martin, 1995; Donze *et al.*, 1996). As more foundress mites enter a brood cell, there is a decline in the number of eggs produced per mite. The presence of four or more foundress mites results in mortality of the developing varroa offspring (Martin, 1995).

Reproductive success can be quantified by determining the number of eggs laid in the cell by the foundress mite called the "total reproductive rate" (Corrêa-Marques *et al.*, 2003) or the number of viable female offspring resulting from the foundress mite which is called the "effective reproductive rate" (Corrêa-Marques *et al.*, 2003). Harris *et al.* (2003) defines "viable" females as those in which a foundress mite has both male and female progeny within the first 12.5 days of the brood development cycle. This permits development, mating and full maturation of female varroa mites that emerge from cells. When a female varroa has successfully mated and is viable the idiosoma will swell, visibly stretching the intersegmental membranes (Martin, 1994).

Not all varroa successfully mate. Unmated females would be incapable of producing viable female offspring. This is often due to male mortality in cells (Martin, 1994). There may be a variety of factors contributing to male mortality, including environmental conditions. Temperatures in the brood nest are crucial for the survival of developing varroa offspring. LeConte *et al.* (1990) demonstrated that survival of

offspring is reduced at temperatures above 36.5°C. Unsuccessful reproduction is more common in winter due to increased male mortality (Martin, 2001). Perhaps this is due to differences in the seasonal temperature ranges of the honey bee colony.

In winter, the relationship between varroa and the available honey bee brood affects the spatial and population dynamics of varroa infestations in honey bee colonies. Varroa's use of honey bee brood can affect potential control measures for varroa during or leading up to winter. For example, varroa may be protected if they are present in the brood from any form of fumigation treatment that may be applied to colonies in winter (Underwood and Currie, 2005). As well, varroa may be protected within the brood at different times during winter. Therefore, specific information on the location and activities of varroa within the honey bee colony at any one time during winter may be used by beekeepers to determine the appropriate timing and duration of control treatments for varroa during winter. Information on the reproduction and seasonal biology of varroa in a specific region and season may also be used to provide a more detailed assessment of the population dynamics of varroa infestations throughout the season. This information may also be used with the existing information on regional economic thresholds to revise existing treatment guidelines for beekeepers.

This study examined the level and composition of brood production in honey bee colonies stored indoors during winter and how varroa uses this brood. This study was conducted over a three-year period where colonies were sampled in early, mid and late-winter. Brood was examined for the presence of varroa and the levels of varroa reproduction taking place. This study is the first to comprehensively examine the

relationship between varroa and honey bee brood during winter under periods of prolonged winter.

MATERIALS AND METHODS

Colonies of the yellow strain of *Apis mellifera* established from New Zealand packages and queens (Arataki Honey Ltd., P.O. Box 8016 Havelock North 4157, New Zealand) were selected that had moderate to high levels (1 to 46 mites per 100 bees) of varroa mite infestation. Colonies were sampled on 11 Nov., 2005, on 20 Nov., 2006 and 9 Nov., 2007 by collecting approximately 150-300 bees, selected from the center of the brood nest into a sample cup. Varroa mite levels were determined by the alcohol wash technique (Gatien and Currie, 2003). The bees from each individual sample were put in 70% ethanol and vigorously shaken for 10 minutes while held in a screened basket within a 680ml plastic container on a mechanical shaker (Lab-line Instruments, Inc., Melrose Park, IL) for 10 min at 150 rpm in order to dislodge any mites from the bodies of the dead bees. The alcohol mixture containing the mites was then filtered through fine mesh cloth (286*234 threads/cm^2) and the number of mites was quantified.

On 16 Nov., 2005, 25 Nov., 2006 and 14 Nov., 2007 colonies were moved indoors into a storage facility building, located at the University of Manitoba, Winnipeg, Manitoba, Canada (49° 54'N, 97° 14'W) for the duration of winter. Colonies were weighed with an electric scale immediately before colonies were placed indoors for storage in fall and immediately after colonies were moved outdoors in spring.

During winter storage, the colonies were maintained at (5°C) under complete darkness. In 2005 and 2006, colonies had 52cm x 36cm x 4mm polyvinyl boards covered

with 30.2cm wide wax paper, inserted through the entrance that rested directly on top of the bottom board to collect any varroa mites or honey bees that fell from the winter cluster. Aluminum pans (19cm x 8cm) were inserted underneath the entrance of colonies to capture any worker bees or mites that fell out of the hive entrance.

Bottom board samples were taken on a daily basis in the first two years of the study (2005, 2006) from 17 Jan., 2005 to 20 Mar., 2005 and from 17 Dec., 2005 to 27 Mar., 2006. The mites in the bottom board samples were identified and classified as immatures if their exoskeleton was light tan, white or clear in colour, indicating that the exoskeleton has not yet completed the hardening and darkening phase (Infatidis, 1983). The presence of immature mites was used as an indication of the occurrence of brood rearing during winter (Martin and Kemp, 1997).

Acaricide treatments were applied to all the colonies immediately after winter storage, on 4 Apr. in 2005 (Apistan ®) and on 11 Apr. in 2006 (coumaphos ®) to obtain an accurate estimate of the remaining varroa population in each colony. This was added to the cumulative varroa drop over winter to obtain the total population of varroa in each colony so that daily mortality rates of immature mites could be calculated.

Brood sampling was performed on two dates in years 2005 and 2006 and three dates in 2007. In 2005, "mid-winter" brood samples were taken on 19 Jan. (N = 9) and "late-winter" samples on 31 Mar. (N = 10). In 2006, "early-winter" brood samples were taken on 21 Dec. (N = 10) and the "late-winter" brood samples on 30 Mar. (N = 9). In 2007, the "early-winter" samples were taken on 24 Dec. (N = 6), the "mid-winter" samples were taken on 21 Feb. (N = 10) and the "late-winter" samples were taken on

13 Apr. (N = 24). During the years 2005 and 2006, the same colonies were sampled in both early and late (or mid and late) winter. During 2007, three groups of colonies were sampled in either the "early" and "late-winter", "mid" and "late-winter" or only "late-winter" periods.

Colonies were sampled by removing each frame and determining the amount of brood and number of workers present. Both sides of each frame were photographed with a digital camera (Canon Powershot A520, 4.0 mega pixels) to allow later quantification of the area and stages of brood on each frame and to quantify the colony population. In cases where more than one frame with brood was present the frame with the greatest proportion of capped brood was removed. All brood frames were brushed free of worker bees, taken back to the lab and stored in a freezer for later analysis.

For each brood comb sample, every cell in the frame was inspected and the number of eggs, unsealed larvae, and sealed brood (larvae, pupae and teneral adults) were recorded. All of the capped cells were opened with dissecting tweezers and examined. The stage of honey bee development and age of pupae were determined, based on eye color (Jay, 1962) and recorded. Following brood removal, each cell was thoroughly examined for the presence of varroa mites with an otoscope (3.5 V Hal, Welch Allyn ®, Skaneateles Falls, N.Y., USA, 13153) and an external light source (Chiu technical corporation model F0 – 50, 252 Indian head Rd., Kings Park, N.Y., USA, 11754). Then a damp cotton swab was inserted into the brood cell several times to collect all the mites from each cell. The number of varroa mites, life stages (egg, protonymph, deutonymph, adult) and gender of each individual was recorded for each cell. Varroa in all life stages were identified according to Infantidis (1983).

Varroa was considered to have reproduced successfully in a brood cell if there were both male and female varroa offspring present and the female offspring would have time to sufficiently develop to maturity before the brood cell was opened (Harris *et al.*, 2003). This was determined by the stage of the honey bee brood and how many days were left until the fully developed bee left the cell. In the case of honey bee pupae eye colour was used to determine age and the time before the brood cell would be opened based on established development times (Jay, 1962).

Calculations

Numbers of 'eggs', 'larvae' and 'capped brood' were determined by direct counts on each brood frame that was sampled. The total population of bees in each colony was estimated by visual inspection of each frame using photographs taken during winter sampling. In 2005 and 2006, the total population of varroa in a colony was estimated by summing the number collected during bottom board sampling and the number killed following acaricide treatments. In 2007, the total varroa population was estimated by multiplying fall infestation levels as estimated by alcohol wash by population estimation of colonies obtained through cluster estimates as used in (Underwood and Currie, 2005). The number of varroa in brood was calculated from the total number of varroa found in all brood cells on a given date. Brood utilization (Bush *et al.*, 1997) was calculated by taking the total number of varroa present in the brood and dividing by the total number present in the colony. Prevalence (Bush *et al.*, 1997) was calculated by dividing the number of brood cells containing at least one mite by the total number of brood cells. 'Reproduction in brood' was calculated as the proportion of varroa in the brood that were

successfully reproducing (as defined above). 'Reproduction in hive' was calculated as the proportion of the total varroa population in the colony that were reproducing. 'Intensity' (Bush et al., 1997) was calculated from the total number of varroa foundresses in the brood divided by the total number of infested brood. Mean intensity was calculated by averaging the number of varroa foundresses per infested host brood cell. 'Chalkbrood' prevalence was calculated by dividing the number of cells infested with chalkbrood by the total number of sealed brood cells. The 'Chalkbrood Cells Infested with Varroa ' indicates the number of brood cells infested with chalkbrood that were also infested with varroa.

STATISTICAL ANALYSIS

Data were analyzed separately for each variable (Proc Mixed; SAS Institute Inc., 1999) with the individual, timing of sampling in early-winter (Dec.), mid-winter (Jan.-Feb.) and late-winter (Mar.-Apr.) and year (2005, 2006 and 2007) as treatments in an unbalanced ANOVA design. Hive (year) was treated as a random effect. The variables based on proportions, 'proportion of colony mites in brood', 'prevalence', 'reproduction (brood)' and 'reproduction (hive)' and 'intensity' were all arcsine transformed prior to analysis. The means and standard errors are reported as untransformed data.

Simple correlations were performed between the variables 'bee population' and 'eggs', 'larvae', 'capped brood', 'brood utilization', 'prevalence', 'intensity', 'reproduction in brood' and 'reproduction in hive'; the variables 'varroa population' and

'eggs', 'larvae', 'capped brood', 'mean abundance (brood)', 'prevalence', 'intensity' and 'reproduction in brood' (Proc Corr; SAS Institute Inc., 1999).

RESULTS

Eggs

In colonies with winter brood, the mean number of honey bee eggs per colony differed with time of sampling during winter, but the effect was not consistent in all years ($F = 18.12$; $df = 2, 23$; $P < 0.0001$). In 2006 numbers of eggs were higher in 'early-winter' than in 'late-winter' ($F = 66.78$; $df = 1, 23$; $P < 0.0001$) but similar in 'mid' and 'late-winter' in 2005 and 'early', 'mid' and 'late-winter' in 2007 ($F = 0.48$; $df = 2, 23$; $P = 0.62$). The number of eggs was much higher in 2006 (685.2 ± 119.15) than 2007 (92 ± 54.81) in early-winter ($F = 24.75$; $df = 1, 23$; $P < 0.0001$) but numbers of eggs were similar in all years in 'mid' ($F = 1.71$; $df = 1, 23$; $P = 0.204$) and 'late-winter' ($F = 1.15$; $df = 2, 23$; $P = 0.335$) (Table 2.1 B).

Larvae

In colonies, with winter brood, the mean number of honey bee larvae also differed with time of sampling during winter, but the effect was not consistent in all years ($F = 6.89$; $df = 2, 20$; $P = 0.0053$). In 2006, mean numbers of larvae were greater in 'early-winter' than 'late-winter' ($F = 27.17$; $df = 1, 20$; $P < 0.0001$) but numbers of larvae were similar in 'mid' and 'late-winter' in 2005 ($F = 1.22$; $df = 1, 20$; $P = 0.28$) and 'early', 'mid' and 'late-winter' in 2007 ($F = 2.7$; $df = 2, 20$; $P = 0.092$). Numbers of larvae were higher in 2006 than 2007 in 'early-winter' ($F = 7.25$; $df = 1, 20$; $P = 0.014$)

but were similar among years for 'mid' ($F = 0.27$; $df = 1, 20$; $P = 0.61$) and 'late-winter' ($F = 1.14$; $df = 2, 20$; $P = 0.34$) (Table 2.1 C).

Capped Brood

In colonies with winter brood the sealed brood stage is utilized by varroa (Fig. 2.1 B). The mean number of sealed brood cells differed with time of sampling during winter, but the effect was not consistent in all years ($F = 4.57$; $df = 2, 23$; $P = 0.021$) (Fig. 2.1). In 2006, brood was more abundant in 'early-winter' than in 'late-winter' ($F = 15.59$; $df = 1, 23$; $P = 0.0006$) but brood abundance was similar in 'early', 'mid' and 'late-winter' in 2005 ($F = 0.00$; $df = 1, 23$; $P = 0.96$) and 2007 ($F = 0.45$; $df = 2, 23$; $P = 0.64$). 'Early-winter' brood rearing ($F = 2.56$; $df = 1, 23$; $P = 0.12$) and 'mid-winter' brood rearing ($F = 1.31$; $df = 1, 23$; $P = 0.26$) were similar in all years. However, in 'late-winter' ($F = 4.31$; $df = 2, 23$; $P = 0.026$) the amount of capped brood was greater in 2007 than in either 2005 or 2006 (Fig. 2.1 B).

Bee Population

The bee populations of colonies were similar among years ($F = 0.13$; $df = 1, 26$; $P = 0.72$) and did not vary significantly between 'early' to 'late-winter' ($F = 3.72$; $df = 2, 8$; $P = 0.072$) (Table 2.1 A).

Varroa Population

The total varroa population was similar in all years ($F = 0.76$; $df = 2, 45$; $P = 0.47$) and did not change in the 'early' to 'late-winter' periods ($F = 2.21$; $df = 2, 21$; $P = 0.13$). Over all sampling periods and years, the average varroa population in colonies was 989 ± 69 varroa/colony, and ranged from 84 to 2, 630 varroa/colony. There was no interaction between year and sampling period ($F = 2.67$; $df = 2, 21$; $P = 0.093$) (Fig. 2.1 A).

Brood Utilization

The proportion of the entire varroa population reproducing in brood was similar among years ($F = 0.15$; $df = 2, 24$; $P = 0.86$) and sampling periods ($F = 4.61$; $df = 2, 5$; $P = 0.07$) (Fig. 2.3). There was no interaction between year and sampling period ($F = 2.88$; $df = 2, 5$; $P = 0.15$) (Fig. 2.2 A).

Prevalence

The prevalence of varroa in brood was similar among years ($F = 0.76$; $df = 2, 24$; $P = 0.48$) and between sampling periods ($F = 0.53$; $df = 2, 5$; $P = 0.62$). There was no interaction between year and sampling period for prevalence of varroa in brood ($F = 0.55$; $df = 2, 5$; $P = 0.61$) (Fig. 2.2 B).

Intensity

The intensity of varroa parasitism in brood was greater in 2007 (0.8 ± 0.11) than either 2005 (0.23 ± 0.1) or 2006 (0.64 ± 0.18) ($F = 6.57$; $df = 2, 24$; $P = 0.0053$) but was

similar among sampling periods (F = 0.98; df = 2, 5; P = 0.44). There was no interaction between year and sampling period for the intensity of varroa parasitism in brood (F = 2.34; df = 2, 5; P =0.19) (Fig. 2.2 C).

Reproduction in Brood

The proportion of *V. destructor* within brood cells that reproduced was greater in 2007 (0.24 ± 0.44) than 2005 (0.002 ± 0.002) and 2006 (0.075 ± 0.44) (F = 8.58; df = 2, 24; P = 0.0015) but was similar between sampling periods (F = 1.28; df = 2, 5; P = 0.36). There was no significant interaction between years and sampling period for the proportion of reproduction in brood (F = 1.57; df = 2, 5; P = 0.3) (Fig. 2.3 A).

Reproduction in Hive

The proportion of the total varroa population that successfully reproduced was similar between years (F = 1.98; df = 2, 24; P = 0.16) and between sampling periods (F = 1.97; df = 2, 5; P = 0.23). There was no interaction between years and sampling period for the proportion of the entire varroa population that reproduced (F = 0.67; df = 2, 5; P = 0.55) (Fig. 2.3 B).

Proportion of Brood Cells with Chalkbrood

The proportion of brood cells infested with chalkbrood infested with varroa was greater in 2006 than 2005 and 2007 (F = 5.92; df = 2, 24; P = 0.01) but was similar among sampling periods (F = 0.88; df = 2, 5; P = 0.48). There was no significant

interaction between year and sampling period for the proportion of brood cells with chalkbrood infested with varroa ($F = 2.98$; $df = 2, 5$; $P = 0.14$) (Table 2.2 A).

Chalkbrood Cells Infested with Varroa

The proportion of cells with chalkbrood was greater in 2006 than 2005 and 2007 ($F = 10.98$; $df = 2, 24$; $P = 0.0004$) but was among timing in winter ($F = 1.99$; $df = 2, 5$; $P = 0.23$). There was no significant interaction between years and sampling period for the proportion of the proportion of brood cells with chalkbrood ($F = 1.94$; $df = 2, 5$; $P = 0.24$) (Table 2.2 B).

Immature Varroa Collected from Bottom Board Samples

Both the proportion of immature brood and average number of immature varroa collected from bottom board samples in 2006 was greatest in early January with a second smaller peak in mid February (Fig. 2.4).

DISCUSSION

The amount of brood found in this study throughout all sampling periods in winter was very limited between November and early April. Brood rearing was variable in colonies, typically occurring in different colonies at different times in winter with a slight trend toward more brood rearing in early-winter than mid or late-winter. The proportion of the varroa population found within the brood at any one time was typically limited to about 3% of the population (Maximum 12%) with the majority of the varroa present as ectoparasites on adult bees within the cluster at any one time. The prevalence

of varroa in brood was also low, averaging 23% and was relatively consistent throughout all years and sample periods. Despite low availability of brood, the intensity of brood infestation was very low, averaging 1.38 mites/brood cell having relatively few cells with multiple infestations. The proportion of varroa that reproduced in the brood was lower than previous studies (31%). However, the overall proportion of the varroa population in colonies that were reproducing at any one time was very low averaging only 1%. The results of this study are an important contribution to the understanding of how varroa uses the brood of its newly acquired host, *Apis mellifera*, during prolonged periods of limited brood rearing where varroa encounter a shortage of suitable hosts for reproduction.

In the absence of varroa, a typical honey bee colony produces very little brood throughout the winter, with many temperate regions reporting that the lowest levels of brood occur in November, and typically increasing from December to March (Jeffree, 1956; Avitable, 1978). In northern latitudes, in the absence of varroa, brood rearing is severely curtailed in honey bee colonies and remains at a very low level throughout mid winter and throughout most of late-winter (February to March) (Harris, 1980; Szabo, 1993; Nelson, 1995).

This study examined brood rearing in colonies infested with varroa, at levels near or above the fall economic threshold specific to northern North America (Gatien and Currie, 2003). In this study, the overall level of sealed brood found in colonies during winter was low (overall average 78 cells per colony) and the proportion of colonies sampled that had at least some sealed brood present was quite variable ranging from 0% to 80% and averaged 44% over all sample periods and all three years. There were also large variations in the presence of sealed brood between different colonies at any one

time during winter. Brood levels in this study did not reflect the trends previously reported for uninfested honey bee colonies in Southern temperate regions of North America where brood steadily increases throughout the winter until early spring in honey bee colonies (Jeffree, 1958; Avitable, 1978) but were similar to those uninfested colonies in North temperate regions that have slightly more (but very low numbers of) sealed brood in early-winter than mid or late-winter (Harris, 1980; Szabo, 1993; Nelson, 1995).

The immature varroa drop in the bottom board samples in 2006, which is indicative of brood rearing (Martin, 1994), showed the greatest presence of the immature mites between the end of December to late January with a drop in levels in late January to February and very low levels of immature varroa drop later in winter. In late-winter up to 20% of colonies exhibited the presence of immature mites. Late-winter mite drop occurred sporadically and no immature varroa were found after 17 March. Brood rearing occurs intermittently in colonies rather than continuously throughout the winter, as demonstrated by bottom board samples.

Although larger colonies might be expected to produce more brood than smaller colonies, we found no correlation between colony size and the number of sealed brood cells in a colony. We also expected that colonies with higher levels of varroa infestations in the fall would have less brood, possibly as a result of the stress from a high parasite load. However, the correlations failed to show any significant relationship between colony infestation and the level of brood.

During summer, the proportion of the colony's varroa population present in the brood typically ranges from 60% to 74% at any one time (Harris *et al.*, 2003). In this study, the proportion of the colony's total mite population found inside the brood during

winter was very low (3%). This is not surprising, as the small amount of brood found in winter colonies in this study would not have the capacity to support a large proportion of the varroa population, especially since the average population of varroa present in these colonies was quite high (overall average 990 mites/colony). Although we would have expected colonies with higher levels of brood to contain a higher proportion of the colony's varroa population, correlations did not reveal a relationship between the available brood and prevalence nor did the population of varroa in a colony result in a higher level of the total population of varroa present in the brood.

This study showed that at any time in winter, when there was brood present in moderate to highly infested colonies, the majority of mites were still living as ectoparasites on workers in the cluster even though brood was available. The low levels of brood found during winter place severe constraints on the numbers of varroa that are able to occupy the brood. This is important because when short-term control treatments for varroa are applied in the winter (such as formic acid fumigation), the majority of the varroa population will be directly exposed to the fumigant. The overall trend in all three years was for a higher proportion of varroa in the colony to infest brood in early-winter than in late-winter. Therefore, short-term fumigation treatments would be more effective in mid or late-winter when there are fewer varroa protected inside the available brood.

Brood is a limited resource in winter. When inside brood cells, varroa are given the protection of a constantly maintained microenvironment and have the opportunity to reproduce. Therefore, it was surprising that we did not find more brood infested. During winter, the prevalence of varroa in brood (brood cells infested / total brood) ranges from 6 to 42% in England (Martin, 2001). In our study, there was a greater range of

prevalence (3% to 71%). However, overall the average prevalence was consistent, (23%) over all sample periods and all years despite high variability in brood area. The lower levels of prevalence may indicate that there may be costs to varroa associated with infesting brood during this time. During winter, varroa are typically found between the ventro-lateral tergites of the abdomen in worker bees (Bowen-Walker *et al.*, 1997). Perhaps the risk of being groomed off their host by leaving this position (Delfinado-Baker *et al.*, 1992) to locate and infest a brood cell in winter is too high. If a varroa mite falls off its bee host in winter onto the bottom board, the low temperatures outside the cluster may prevent that varroa from crawling back into the cluster and may result in death, as varroa are susceptible to low temperatures (Paetzold and Ritter, 1989).

Additionally, the seasonal dynamics of the honey bee colony in winter may interfere with varroa's behavior to locate brood. During winter, honey bees form a tightly packed cluster. The tightly packed cluster may physically obstruct varroa from effectively reaching the brood. The dynamics of such a closely packed cluster during winter may also alter the stimuli required by varroa to locate the brood, possibly by reducing the levels of circulating brood pheromones. Varroa require these chemical stimuli to infest the brood (Le Conte *et al.*, 1989). Additionally, higher levels of CO_2 within the winter cluster (Van Neurem and Buelens, 1997) may also interrupt varroa's detection of brood.

Previous studies have shown the intensity of infestation (number of varroa mites per infested brood cell) increases when brood rearing declines. Eguaras *et al.* (1994) found that the range in intensity of varroa infesting brood cells in Argentina increases from 1-2 mites/cell in summer to 1-6 mites/cell in winter, in South America. In our

study, the intensity of varroa parasitism on brood was relatively consistent throughout the winter in all three years, averaging 1.4 mites/cell. We would have expected the intensity of brood infestation to increase as the brood availability decreased. There was no relationship between the mean abundance of varroa on adult bees and intensity in brood cells. However, there was a slight positive correlation between the total size of the mite population in a colony and the intensity, and a moderately strong positive correlation between prevalence and intensity. Therefore, as the population of varroa in a colony increases, so does the infestation of the brood. Additionally, as the proportion of brood infested increases, so does the density of the varroa infesting the brood.

There was also a moderate positive correlation between intensity and the proportion of varroa that reproduced. This was not expected, as high levels of intensity typically result in lower levels of reproduction (Eguaras *et al.*, 1994; Martin, 1995). However, the average intensity levels in mid-winter in our colonies were lower than intensities in other studies where reproduction was noticeably affected (Eguaras *et al.*, 1994; Martin, 1995). In this study, despite a high mean abundance of mites on adult bees, levels of intensity were moderate and appeared to have no influence on the reproductive output of the mites.

Regional differences in strains of varroa, honey bee genetics and climate, can all influence the reproductive success of varroa (Correa-Marques *et al.*, 2003). Therefore, information on the reproductive biology of varroa in different regions and seasons is of great importance. The proportion of varroa in brood cells that reproduce ranges from 75% to 92% in late spring to summer and 60% to 70% in autumn in temperate climates in Europe (Otten and Fuchs, 1990; Martin, 2001). Harris *et al.* (2003) found that (53% to

87%) of varroa in brood cells reproduce in summer in the Southern United States while the proportion of varroa that reproduce in winter brood in England is typically 55% (Martin, 2001). In this study 31% of the mites that entered cells reproduced on average but the proportion that reproduced was highly variable ranging from 0 to 82%. These levels are lower levels than found in previous studies in winter (Martin, 2001). We expected the proportion of varroa that reproduced to increase with increased brood area and this did indeed occur. Also, the proportion of cells with reprodcuctive varroa, tended to be higher in early-winter than mid or late-winter.

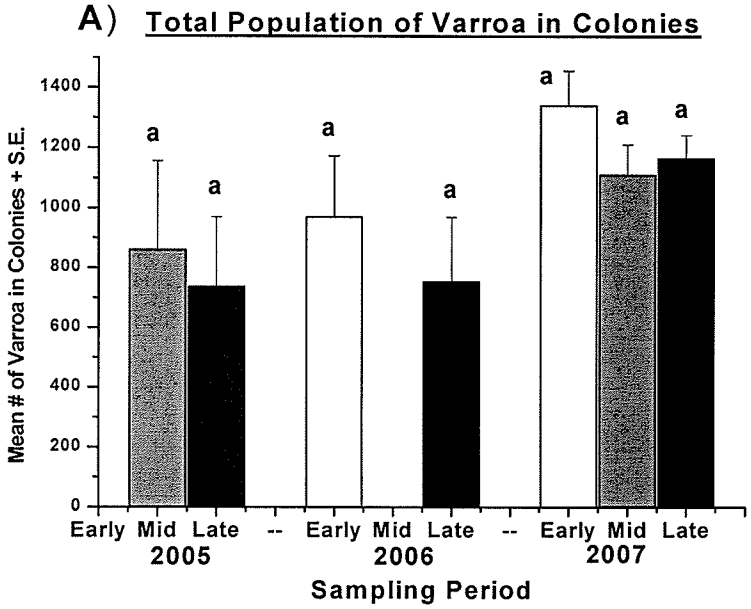
When the entire varroa population of the colony is considered, it is evident that reproductive success in winter is very low. The highest proportion of the varroa population in the colony that would be reproducing during winter at any one time was only 2% and the average proportion of the entire varroa population reproducing at any time, over all three years was only 1%. The data suggest that the low level of reproduction in combination with low levels of available honey bee brood would greatly restrict any potential for substantial increases in the varroa population to replace the varroa population that would be dying during this time.

Martin (2001) attributed non-reproduction in varroa during winter to male mortality. However, there are examples where fully fertile varroa do not reproduce in infested brood cells (Ruijter, 1987). Perhaps varroa's response to the infestation of brood in winter is similar to the adaptations associated with infestation of worker brood in its original host species, *A. cerana*, where varroa are primarily provided protection from grooming and nutritional requirements and where the need for reproduction may be secondary (Rath, 1999). Cell invasion without reproduction in these circumstances may

be a strategy to delay reproduction until brood conditions are more favorable, especially since varroa have a fixed capacity for reproduction during their lifespan, usually five to six reproductive cycles and the capacity to produce a maximum of approximately 30 eggs (Ruijter, 1987). Lower reproduction by varroa may also be due to seasonal differences in nutrition of the bee brood resulting in differences the capacity for varroa development (Boot *et al.*, 1999).

The results of this study contribute to the knowledge of the seasonal biology, population dynamics and host-parasite interactions between *Varroa destructor* and *Apis mellifera*. This study provides new information on the winter biology of varroa during winter, under conditions of prolonged brood shortage. This research demonstrates there is large variation in the seasonal pattern of brood rearing in colonies highly infested with varroa. Varroa reproduction occurred at much lower levels than previously reported in other studies despite the presence of brood during most time periods in winter. Overall, the proportion of the entire varroa population in a colony reproducing during winter was quite low (1%) and would not be sufficient to allow increases in varroa population over winter. This research provides important information on how varroa uses brood in winter and contributes to a more thorough understanding of the reproductive biology and spatial distribution of mites within the honey bee colony in winter.

Fig. 2.1 A) Mean total population of varroa and B) Mean numbers of sealed brood cells in honey bee colonies during winter (+ standard error) by sampling period (early = 21 Dec., 2006, 24 Dec., 2007; mid = 19 Jan., 2005, 21 Feb., 2007; late = 31 Mar., 2005, 30 Mar., 2006, 13 Apr., 2007) and year. N represents the number of colonies with brood present / total number of colonies sampled. Means followed by the same letters within years are not significantly different by sampling period ($P < 0.05$).



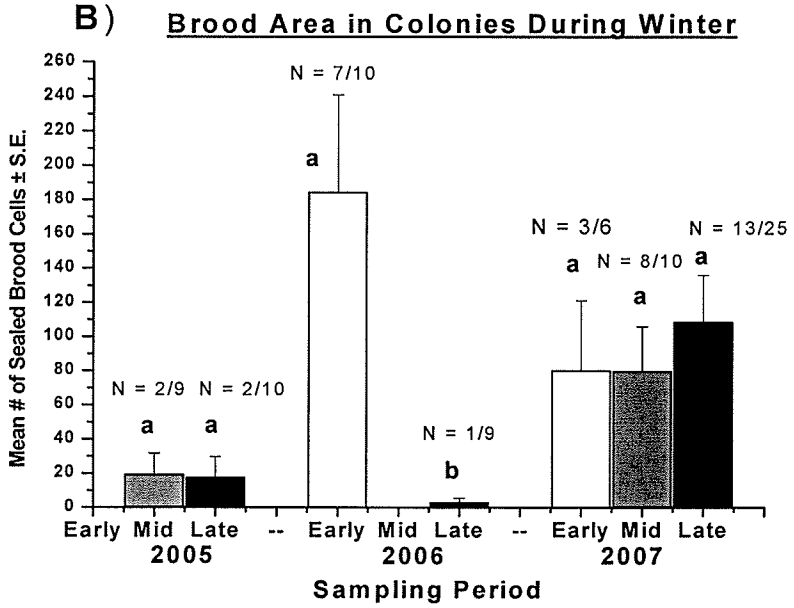


Fig. 2.2 A) The mean proportion of the total varroa population found in brood cells. B) Prevalence of varroa in brood and C) Intensity of varroa in brood in honey bee colonies during winter (+ standard error) by sampling period (early = 21 Dec., 2006, 24 Dec., 2007; mid = 19 Jan., 2005, 21 Feb., 2007; late = Mar. 31, 2005, Mar. 30, 2006, 13 Apr., 2007) and year. Means followed by the same letters within years are not significantly different by sampling period ($P < 0.05$).

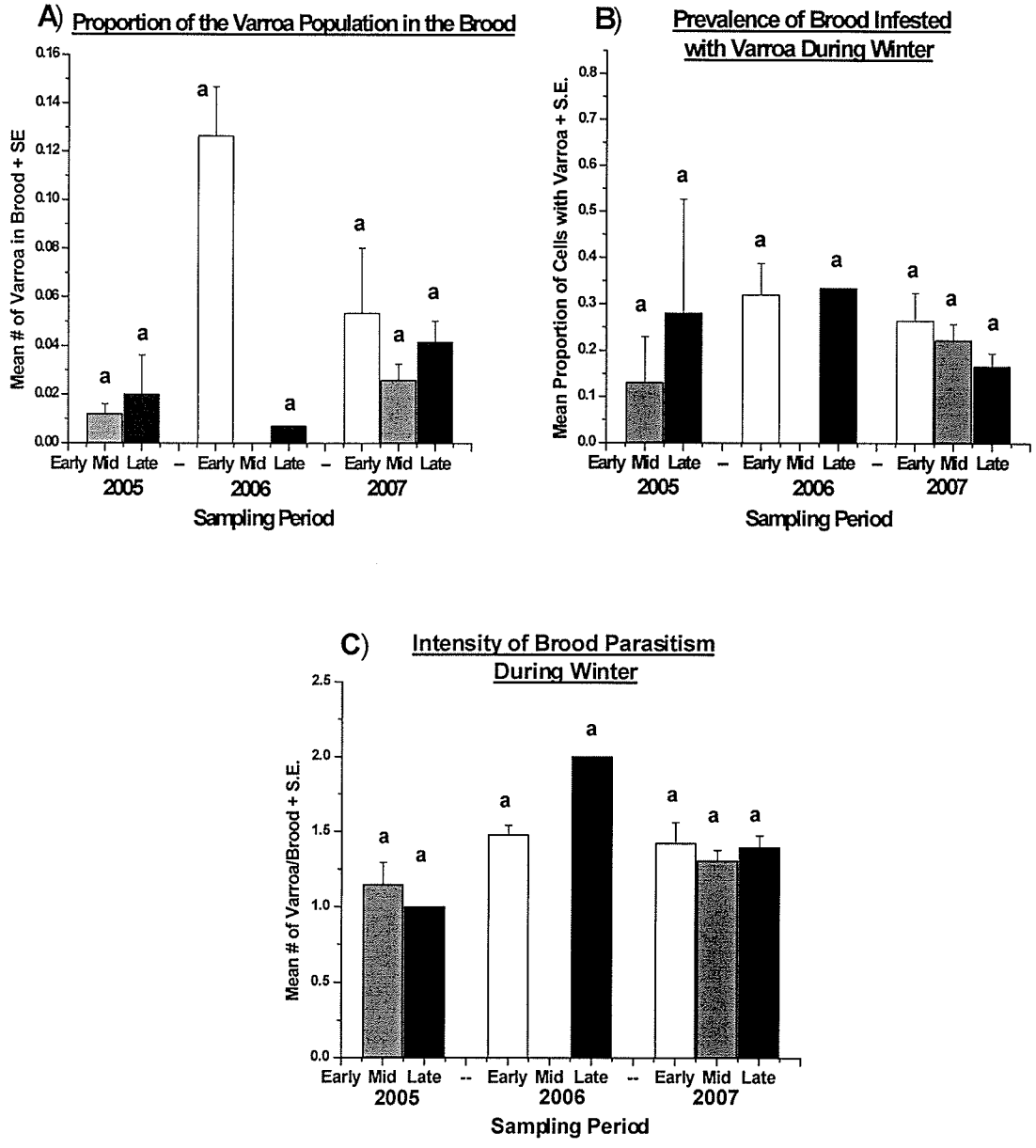
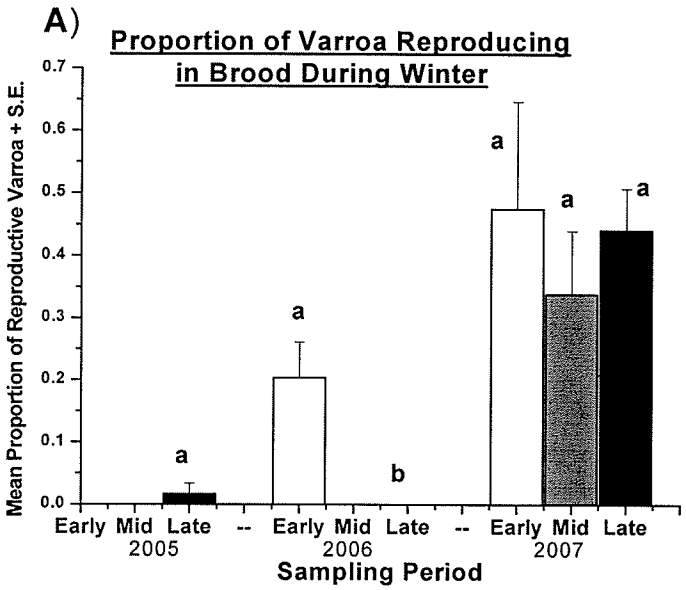


Fig. 2.3 A) Mean proportion of varroa that entered brood cells that reproduced and B) Proportion of the total population of varroa that reproduced on each sample date in honey bee colonies during winter (+ standard error) by sampling period (early = 21 Dec., 2006, 24 Dec., 2007; mid = 19 Jan., 2005, 21 Feb., 2007; late = 31 Mar., 2005, 30 Mar., 2006, 13 Apr., 2007) and year. Means followed by the same letters within years are not significantly different by sampling period ($P < 0.05$).



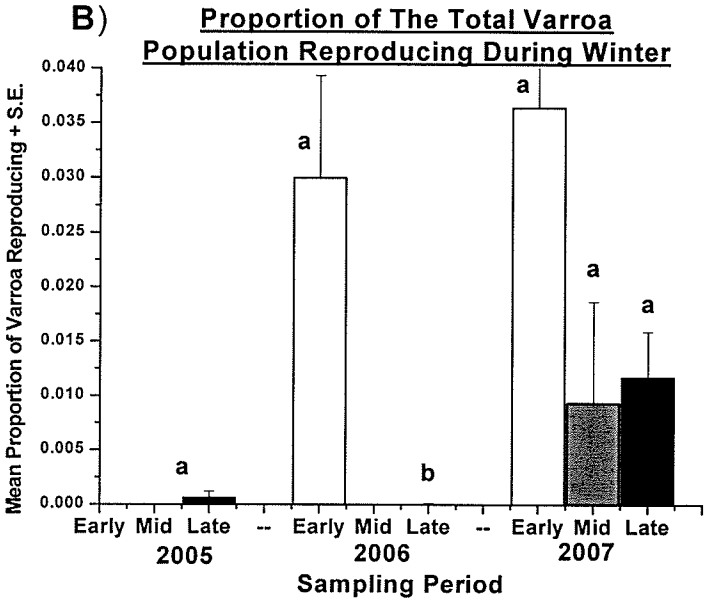


Fig. 2.4 A) Proportion of colonies (N = 10) with immature varroa collected on bottom board samples by date in winter of 2006 and B) mean number of (± standard error) immature (light tan, white or clear colored) varroa collected on bottom board samples by date in winter of 2006.

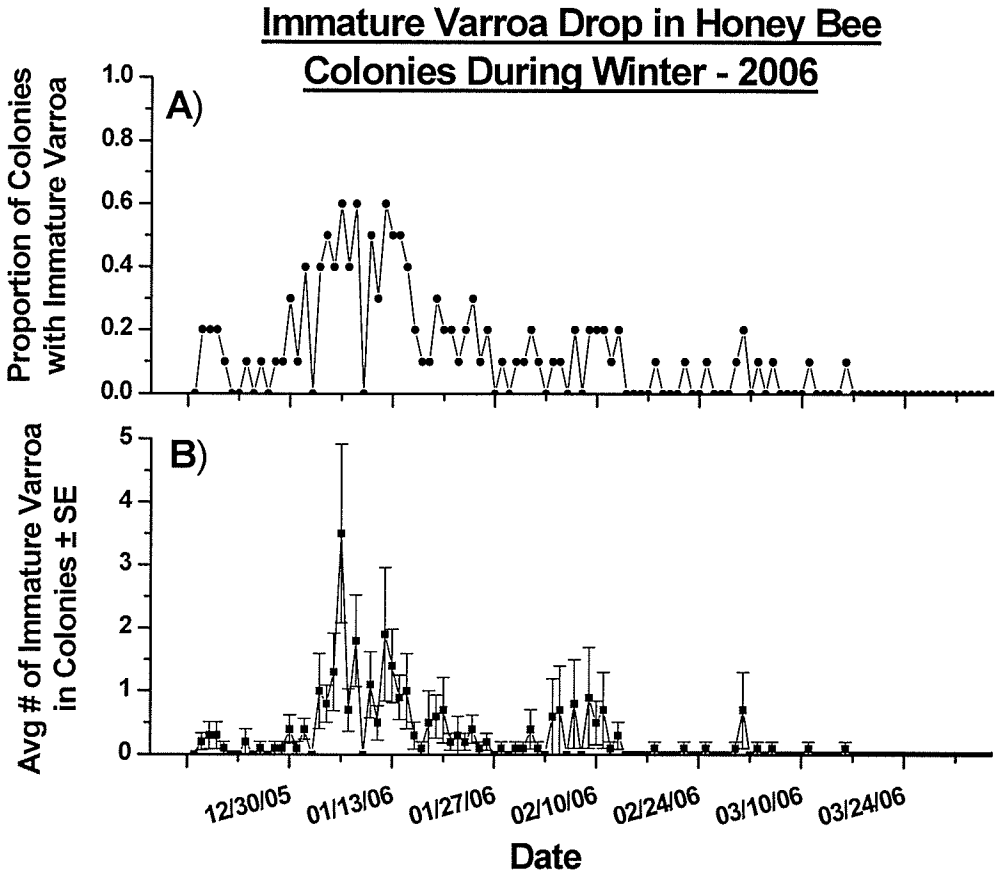


Table 2.1 A) Mean worker bee population during winter B) Mean number of eggs during winter C) Mean number of larvae in honey bee colonies during winter (± standard error) by sampling period (early = 21 Dec., 2006, N = 10, 24 Dec., 2007, N = 6; mid = 19 Jan., 2005, N = 9, 21 Feb., 2007, N = 10; late = 31 Mar., 2005, N = 10, 30 Mar., 2006, N = 6, 13 Apr., 2007, N = 24) and year.

A) Bee population

	Early	Mid	Late
2005	X	4656.67 ± 822.35	5233.8 ± 1023.21
	X	(Min 531, Max 8338)	(Min 531, Max 11,141)
2006	6160.2 ± 819.07	X	5826.22 ± 814.18
	(Min 2000, Max 9891)	X	(Min 2000, Max 9028)
2007	X	X	X
	X	X	X

B) Eggs

	Early	Mid	Late
2005	X	62.22 ± 42.39	106.2 ± 84.3
	X	(Min 0, Max 341)	(Min 0, Max 838)
2006	685.2 ± 119.15	X	0
	(Min 0, Max 194)	X	(Min 0, Max 0)
2007	92 ± 54.81	107.7 ± 39.06	104.13 ± 25.86
	(Min 0, Max 342)	(Min 0, Max 349)	(Min 0, Max 393)

C) Larvae

	Early	Mid	Late
2005	X	78.11 ± 55.49	4.5 ± 4.5
	X	(Min 0, Max 473)	(Min 0, Max 45)
2006	174.1 ± 44.19	X	0
	(Min 0, Max 371)	X	(Min 0, Max 0)
2007	44.83 ± 22.97	89.9 ± 22.58	40.75 ± 11
	(Min 0, Max 138)	(Min 0, Max 195)	(Min 0, Max 163)

Table 2.2 A) Mean proportion of brood cells infested with chalkbrood B) Mean proportion of varroa in brood in chalkbrood infested brood cells in honey bee colonies during winter (± standard error) by sampling period (early = 21 Dec., 2006, N = 10, 24 Dec., 2007, N = 6; mid = 19 Jan., 2005, N = 9, 21 Feb., 2007, N = 10; late = 31 Mar., 2005, N = 10, 30 Mar., 2006, N = 6, 13 Apr., 2007, N = 24) and year.

A) Brood Cells with Chalkbrood

	Early	Mid	Late
2005	X X	0.03 ± 0.03 (Min 0, Max 5)	0 (Min 0, Max 0)
2006	0.06 ± 0.03 (Min 0, Max 0.24)	X X	0.12 ± 0.12 (Min 0.13, Max 0.13)
2007	0.02 ± 0.02 (Min 0, Max 0.07)	0.003 ± 0.002 (Min 0, Max 0.01)	0.02 ± 0.01 (Min 0, Max 0.08)

B) Chalkbrood Cells Infested with Varroa

	Early	Mid	Late
2005	X X	0 (Min 0, 0)	0 (Min 0, 0)
2006	0.05 ± 0.02 (Min 0, Max 0.13)	X X	0.13 ± 0.12 (Min 0.13, Max 0.13)
2007	0 (Min 0, Max 0)	0 (Min 0, Max 0)	0.001 ± 0.001 (Min 0, Max 0.01)

Table 2.3 Correlations between variables 1) Eggs 2) Larvae 3) Capped Brood 4) Mean abundance of brood 5) Prevalence 6) Intensity 7) Varroa reproduction in brood 8) Varroa reproduction in colonies and A) Bee populations during winter B) Varroa population during winter C) Sealed brood cells during winter D) Intensity of varroa infesting brood cells in honey bee colonies during winter (± standard error).

	Correlations		A		B		C		D	
	Mean	**N**	**R**	**Sig**	**R**	**Sig**	**R**	**Sig**	**R**	**Sig**
1	**169.6 ± 31.19**	**78**	+0.29	0.08	+0.17	0.15	X	X	X	X
2	**61.8 ± 11.49**	**75**	+0.21	0.24	+0.03	0.83	X	X	X	X
3	**78.0 ± 13.55**	**78**	+0.01	0.96	+0.15	0.21	X	X	X	X
4	**0.33 ± 0.04**	**36**	-0.14	0.39	+0.3	0.07	-0.11	0.54	X	X
5	**0.23 ± 0.02**	**36**	+0.14	0.68	+0.32	0.06	-0.08	0.66	X	X
6	**1.38 ± 0.04**	**36**	-0.02	0.91	+0.33	0.05	-0.02	0.93	X	X
7	**0.31 ± 0.04**	**36**	-0.46	0.14	-0.15	0.37	+0.25	0.14	+0.19	0.26
8	**0.01 ± 0.004**	**36**	-0.43	0.16	-0.16	0.33	+0.49	0.003	+0.33	0.05

CHAPTER 3. EXAMINING THE IMPACT OF MODIFIED ATMOSPHERES ON INFESTATIONS OF *VARROA DESTRUCTOR* IN CLUSTERS OF HONEY BEES

ABSTRACT

In this study, reduced ventilation was applied to small clusters of bees under controlled conditions to determine if lowered ventilation and increased levels of CO_2 could increase the mortality rates of varroa. Two experiments were performed at two different temperatures (10°C and 25°C). Both experiments compared varroa mortality between high, medium and low rates of ventilation. The clusters of bees (~300 worker bees) in bioassay cages with 40 introduced varroa mites were placed into glass chambers and were randomly assigned to one of the three ventilation treatments within incubators set at either of the two temperatures. Bee and varroa mortality and the levels of CO_2 concentration were measured in each of the experimental chambers. In both experiments CO_2 levels increased with a decrease in ventilation with CO_2 reaching a maximum of (1.2 ± 0.45%) at 10°C and (2.13 ± 0.2%) at 25°C. Both humidity and CO_2 were higher at 25°C. Bee mortality was similar in all ventilation rate treatments at 10°C and 25°C. Varroa mortality increased slightly (14%) at high ventilation in the 10°C experiment. Varroa mortality increased greatly (55%) under the low ventilation treatment in the 25°C experiment. This study demonstrates that restricted ventilations, resulting in high levels of CO2 in the surrounding environment of small clusters of honey bees, has the potential to increase the mortality of varroa.

INTRODUCTION

The parasitic mite *Varroa destructor* Anderson and Trueman is the most important pest in modern beekeeping. Even low infestations of varroa mites in a honey bee (*Apis mellifera* L.) colony can result in economic losses and will eventually lead to colony death (Currie and Gatien, 2006). The control of varroa is becoming increasingly difficult, as many populations of varroa are now resistant to one or more classes of synthetic acaricides (Elzen *et al.*, 1998; Mozes-Koch *et al.*, 2000). New methods of control for varroa using integrated pest management approaches that do not rely exclusively on toxic chemicals are essential to the survival of apiculture as an industry.

The environment of the honey bee hive is one that is constantly changing and regulated by the activities of the workers of the colony. Within the hive air is exchanged approximately six times an hour (Wedmore, 1947). Varroa has adapted to living within this dynamic environment of the enclosed honey bee hive. Varroa evolved in close association with the Asian honey bee, *Apis cerana* Fabricius, before recently transferring to *A. mellifera*. Like *A. mellifera*, *A. cerana*, is also a cavity nesting bee. Both species regulate an enclosed space to maintain the temperature within appropriate ranges (Dyer and Seeley, 1991). Both species characteristically respond to changes in environmental temperatures by increasing their rate of respiration (Verma and Edwards, 1971). Thus humidity and CO_2 levels within the hive have the potential to fluctuate substantially.

While honey bees regulate air in the hive to control CO_2, they do not actively regulate humidity (Simpson, 1961). The relative humidity within a cluster of honey bees is typically between 60%-80% (Wohlgemuth, 1957) but can be as low as 45% within the center of a winter cluster (Van Nerum and Buelens, 1997). The level of in-hive humidity

varies as the worker bees fan their wings to circulate the air throughout the hive to control CO_2 (Simpson, 1961).

Within the hive environment of *A. cerana* and *A. mellifera*, there is a narrow range of humidities which varroa can tolerate. Varroa are hydrophilic by nature. They have a small body size with a large surface area to volume ratio, leading to extreme water loss at high temperatures and low humidities (Bruce *et al.*, 1997). Varroa lose water rapidly, up to 2% per hour when not attached to a host bee. Beyond 37% water loss, varroa will die (Yoder *et al.*, 1990). When varroa are outside the brood cell, their moisture requirements are met by feeding on the haemolymph of their host bee. However, as ectoparasites, when separated from their adult host bee, varroa are subject to desiccation (Bruce *et al.*, 1997).

Within the enclosed hive environment, a typical *A. mellifera* colony produces levels of CO_2 and moisture substantially higher than the air outside the hive (0.035% CO_2) (Milner, 1921; Nicolas and Sillans, 1989). Honey bees maintain the concentrations of CO_2 in the colony at 0.10% to 4.25% in summer and 4% to 6% in winter (Simpson 1950; Simpson 1961; Seeley, 1974; Van Nerum and Buelens, 1997). However, levels of CO_2 within the cluster may often exceed these seasonal ranges. During winter, honey bee colonies will tolerate concentrations of up to 9% CO_2 (Simpson 1950; Simpson 1961; Seeley, 1974; Van Nerum and Buelens, 1997).

Honey bees are sensitive to high levels of CO_2 and can detect increases in CO_2 (rather than decreases of O_2) (Seeley, 1974; Van Nerum and Buelens, 1997) through specialized receptors located on the antennae called pit pegs (Lacher, 1964). Like many other insects, individual bees can regulate the CO_2 levels in their immediate environment

by rhythmically opening and closing their spiracles in time with abdominal pumping. This produces a tidal flow of air from the spiracles to flush out stagnant air laden with CO_2 (Bailey, 1954). At the colony level, honey bees will fan their wings in large numbers to flush air out of the hive when the in-hive CO_2 levels reach 10%. Larger colonies with more workers available to fan have better control of the CO_2 levels than do smaller colonies (Seeley, 1974). Even with the air being constantly cycled through the hive, the levels of CO_2 within the hive are higher than ambient levels.

Honey bees are well adapted to living in conditions with relatively high levels of CO_2. During winter, the entire honey bee colony is confined within the hive for several months and under low temperatures forms a compact cluster to thermoregulate. When the ambient temperatures drop below 10°C, respiration within the enclosed spaces leads to an accumulation of CO_2 (Free and Simpson, 1963; Nagy and Stallone, 1976). Levels of CO_2 within the center of the cluster are much higher (6%) than at the outside of the cluster (3%) (Van Nerum and Buelens, 1997). This relationship between environmental temperature, respiration and CO_2 levels within the cluster is also seen in the dynamics of a swarm outside the hive with the core of the cluster reaching a CO_2 level of up to 2% (Nagy and Stallone, 1976).

Although the biology and nest architecture of *A. mellifera* and *A. cerana* are similar, little is known about the dynamics of CO_2 management within the hive environment of *A. cerana*. However, as *A. cerana* is adapted to much warmer climates than *A. mellifera* (Dyer and Seeley, 1991) the in-hive CO_2 levels may not reach the same extremes as in *A. mellifera,* which is confined for long periods of time within the hive environment throughout winter leading to a buildup of respiratory gasses. Varroa

evolved to tolerate a range of CO_2 levels in the environment of *A. cerana.* However, the potential differences in CO_2 ranges of the hive environment between the two host species of bee, and the potential limitations of CO_2 tolerance in varroa in the environment of *A. mellifera* may leave varroa vulnerable to changes in ambient CO_2 concentrations that exceed their level of tolerance.

If there is a difference in the susceptibility of bees and varroa to CO_2 levels, this may have the potential to be used as a modified atmosphere control technique. The use of modified atmospheres has been used successfully in the control of stored product insects. This method of control uses elevated concentrations of atmospheric gases such as N_2 or CO_2 that are high enough to cause significant mortality in a target pest species. High levels of CO_2 are commonly applied in stored products systems where they can cause significant insect mortality (Aliniazee, 1971; Spratt, 1975; Krishnamurthy *et al.*, 1986; White and Jayas, 1991; Mitcham *et al.*, 1994; Mann, 1998; Held *et al.*, 2000).

Arthropod mortality in modified atmospheres is caused in part by desiccation. High levels of ambient CO_2 will induce some arthropods to open their spiracles (Wigglesworth, 1935). If spiracles are open for extended periods of time, significant water loss can occur, leading to the death of the target species (Mellanby, 1934). Typically the levels of CO_2 in modified atmosphere treatments are quite high, ideally around 60% or higher (Banks and Fields, 1995). However, CO_2 levels as low as 10% have been effective in killing insects in storage settings (Krishnamurthy *et al.*, 1986) and levels as low as 2% CO_2 in the air are sufficient to keep insect spiracles open (Mellanby, 1934). Reducing the relative humidity in the presence of high CO_2 levels enhances

desiccation, thereby increasing the efficacy against stored products pests (Aliniazee, 1971; Spratt, 1974).

In addition to water loss, high levels of CO_2 may cause toxicity in arthropods through a number of mechanisms. High levels of CO_2 can affect the nervous system in insects by inducing depolarization in neurons (Clark and Eaton, 1983) and interfering with the neuromuscular transmission sequence (Hoyle, 1960). High levels of CO_2 may also be retained in the haemolymph in arthropods (Levenbook, 1950; Brooks, 1957) that can upset the acid-base balance, which in turn can affect numerous physiological and cellular functions (Harrison, 1994; 2001; Slama, 1994). Extremely high levels of CO_2 (100%) can even temporarily stop an insect's heartbeat (Edwards and Patton, 1965; Ward, 1971). However, the exact mechanisms through which CO_2 kills specific insect species are, for the most part poorly understood.

Although varroa have adapted quite well to living within the hive environment of *A. mellifera* during winter the effects of high levels of CO_2 on varroa mortality rates are not known. The goal of this research was to examine the effect of hive ventilation rates on humidity, CO_2 concentration, and varroa mortality rates in caged worker bees. If varroa has greater susceptibility to the acute physical effects of CO_2 than *A. mellifera* during winter this may be exploited as a physical method of control by managing the storage and ventilation conditions of honey bee colonies during winter storage.

MATERIALS AND METHODS

Worker bees were collected from colonies of similar genetic background established using a yellow strain of New Zealand queens (Arataki Honey Ltd., P.O. Box

8016, Havelock North 4157, New Zealand). Within each replicate all bees used were collected from a single colony. However, different colonies were used to supply the bees for different replicates. All colonies were located on a beeyard at the University of Manitoba campus, Winnipeg, Manitoba, Canada (49° 54'N, 97° 14'W). Two sets of experiments were conducted, one at 10°C and another at 25°C. The 10°C experiment was replicated ten times on 19 Jul., 2005 (N = 2), 1 Aug., 2005 (N = 2), 8 Aug., 2005 (N = 2), 15 Aug., 2005 (N = 2) and 29 Aug., 2005 (N = 2). The 25°C experiment was replicated 14 times, on 19 Jul., 2005 (N = 2), 1 Aug., 2005 (N = 2), 8 Aug., 2005 (N = 2), 15 Aug., 2005 (N = 2), 29 Aug., 2005 (N = 2), and 5 Sep., 2005 (N =4).

Bees were collected from frames within the brood chamber of selected colonies anesthetized using CO_2 and divided into groups of approximately 300 bees by weight. The groups of bees where placed into wooden bioassay cages measuring 20cm x 11.5cm x 9cm outside, bees were contained within a 14.5cm x 8.7cm x 5cm space within the cage. The front of each cage was covered with hardware cloth (3 squares/cm) and the back was covered using a piece of plastic comb (Perma-comb system ®) filled with 60ml 2:1 sucrose:water solution as a food supply for the bees. The floor of the cage was constructed of hardware cloth under which was an 8cm x 4.8cm x 1.5cm drawer that was used to collect any varroa falling from the cluster of bees.

Bioassay cages were then stored in an incubator for approximately 36 h and kept at a constant temperature of 24°C, in complete darkness to allow the bees to acclimate to the cages and the environment of the incubators. During this period bees were fed using two 50ml feeding tubes (Fisherbrand ®) covered with fine mesh cloth , where one of the tubes contained sucrose:water solution (2:1) and the second contained water. Both tubes

were placed into holes at the top of the bioassay cages where bees could access the contents directly through a hardware cloth screen.

Varroa were collected from highly infested colonies that were maintained at the University of Manitoba campus beeyard using methods developed by Currie and Tahmasbi (personal communication). Frames of bees were removed from the brood chamber of colonies, anesthetized using CO_2 and then transferred into a 28cm x 18cm x 6cm wire mesh box which was enclosed in a 32cm x 20cm x 8cm plastic container with a sealed lid. Moist paper towels were placed beneath the wire box to catch any live varroa mites that fell through the mesh grating. The container was then placed on top of an orbital shaker table (model No. 3520, Lab-line Instruments Inc., Lab-line Plaza, Melrose Park, IL 60160) for a period of ten minutes at 150 rpm to dislodge the mites from the bees. During the shaking period, carbon dioxide was delivered into the container through a tube and regulated by a flowmeter to provide a flow of 10 L/min of CO_2 into the container. An open valve on the opposite side of the container allowed excess gas to be voided from the container. The varroa collected were immediately transferred to the honey bees in each bioassay cage using a moist, fine-tipped paint brush. Forty mites were introduced into each of the bioassay cages. After a period of approximately one hour, the sampling tray at the bottom of the bioassay cage was opened to check for any mites that may have initially fallen off the surface of bees. Any live mites found in the bottom tray were reintroduced and the process was repeated again.

Within each replicate, bioassay cages were randomly assigned to one of three ventilation treatments (high, medium and low ventilation rates), within each of four different incubators (Percival Manufacturing Company, Boone, Iowa 50036). Ventilation

rate treatments were established by randomly assigning cages of bees (using a manual method of drawing numbers from a hat) to one of three glass 8.5 L dessicator chambers (Fisher Scientific Canada, 111 Scotia Court, Willby Ont. Canada, L1M SJ6) located in each incubator. Dessicator chambers containing the bees in the bioassay cages were sealed with a 30cm x 30cm x 5mm thick Plexiglass® sheet placed on the rim of the chamber, which was lined with petroleum jelly (Vaseline ®) to provide an airtight seal. Each lid had three tubes running through the surface. A large tube (outer diameter 6.35mm, inner diameter 4.32mm) regulated by a flowmeter (Dwyer ® Series RMA Rate-master ® flowmeter, Dwyer Instruments Inc., 102 Highway 212, Michigan City, IN, 46360), provided a common source of air to all chambers from a filtered air line located in the lab. All air was filtered through a Husky ® Mini, "general purpose" filter (21 – SCFM flow capacity @ 90 – PSI). Flowmeters with the range of 0–10 L/min (Dwyer ®) were used to supply the high rate of ventilation. Flowmeters with a range of 0 – 2 SCFH (Dwyer ®) were used to supply the medium and low rates of ventilation. Flowmeters for each ventilation treatment were set at the following rates of ventilation: high (360 L/h), medium (42.5 L/h) and low (14 L/h). A second tube (outer diameter 6.35mm, inner diameter 4.32mm) leading into the chamber was sealed with a septum to allow air sampling within the chamber. The third tube (outer diameter 21.16mm, inner diameter 11.11mm) led outside the incubator to exhaust air from the system, allowing for a constant flow of air in and out of the system.

Bees were maintained under total darkness throughout the experiment except when incubators were opened briefly when bioassay cages were sampled. Hobo ® data loggers were placed within each dessicator chamber and within each incubator to monitor

the temperature (°C) and humidity (g/m^3) at 30 minute intervals. The values for temperature and absolute humidity were averaged by day using measurements taken by the Hobo sensors at half hour intervals. Temperature data were not collected at 25°C on 19 Aug. for the low ventilation treatment (rep five) and absolute humidity was not measured at 10°C on 8 Aug. for the medium ventilation treatment (rep one), 15 Aug. (rep three), 29 Aug. (rep five) and at 25°C on 8 Aug. (rep two), 15 Aug. (rep two), 29 Aug. (rep four) for the low ventilation treatment (rep five), and the medium ventilation treatment (rep six), 5 Sep. and for the low ventilation treatment 29 Aug. (rep five). The missing data points are due to Hobo sensors running out of battery power.

On 15 Aug., 29 Aug. and 5 Sep. air samples were collected from each dessicator chamber using a 60cc syringe piercing the rubber supper septum of the sampling tube. Air was drawn into each syringe three times, with the first two volumes voided and the last sample saved for analysis. Each syringe was sealed, labeled and stored in a 20 L Coleman ® "cooler" for transport to the Federal Cereal Research Center Lab, Agriculture and Agri-Food Canada, (located on the University of Manitoba Campus) for analysis. Carbon dioxide was quantified by gas chromatography using an Elmer Sigma 3B gas chromatograph with a thermal conductivity detector and Porapak N packed column connected to a Hewlett-Packard 3380S integrator. The injector and oven were maintained at 75°C, the detector at 150°C and inlet pressure was 270 kPa. Gas was injected through a 1ml fixed-volume sample valve to standardize analysis. All samples were analyzed 30 minutes to one hour after the samples were collected.

Bioassay cages were sampled to assess mite and bee mortality two days after cages were sealed in the chambers. Bioassay cages were removed from the dessicator

chambers, sampling drawers were opened and any dead mites that had dropped through the screen into the drawer were quantified. Dead mites were also collected from the surface of the bioassay cages and the interior of the dessicator chambers. At the end of the experiment, bioassay cages were opened inside 61cm x 30cm x 30cm Plexiglass® shelters and all live bees were collected and sampled by alcohol wash (Gatien and Currie, 2003) to collect any remaining mites. The bioassay cages were then completely dismantled and every part was examined for mites.

Daily varroa mortality was calculated as the number of dead varroa collected in the dessicator, divided by the entire population of varroa in the bioassay cage, determined at the end of the experiment (by inspection of the bottom drawer of the bioassay cage) and divided by the number of sampling days. Daily worker mortality was analyzed as the number of dead worker bees on a particular sample day divided by the entire population of bees in the bioassay cage, determined at the end of the experiment.

STATISTICAL ANALYSIS

The effect of ventilation rate on average varroa mortality rate, average worker mortality rate, CO_2 concentration, temperature (°C), absolute humidity (g/m^3), with each treatment combination was analyzed (Proc GLM; SAS Institute Inc., 1999) separately for the 10°C and 25°C temperature experiments. Varroa and worker mortality rate data were analyzed in a randomized complete block design, with ventilation rate as the treatment and replicate as the block.

The effect of ventilation treatment on CO_2 concentration, temperature (°C) and absolute humidity (g/m^3) within each chamber over time was analyzed as a split-plot

design. Ventilation rate and replicate were treated as the main plot factors using ventilation rate*replication as the error term. Day and interactions of treatments with day were treated as sub-plot factors. Carbon dioxide concentration, worker mortality and varroa mortality were arcsine transformed prior to statistical analyses to improve the equality of variance.

RESULTS

Temp 10°C

At 10°C, the average temperature within chambers was not affected by ventilation treatments ($F = 0.04$; $df = 2, 10$; $P > 0.9619$) but did vary slightly (avg. 9.12 ± 0.01°C, ranging from 8.23 to 14.09°C) among replications on different dates ($F = 8.89$; $df = 5, 10$; $P = 0.002$).

Changes in ventilation rate affected both the humidity level (g/m^3) ($F = 214.17$; $df = 2, 7$; $P < 0.0001$) and the CO_2 level ($F = 9.58$; $df = 2, 6$; $P < 0.0136$). Humidity and CO_2 levels were similar in all replicates ($F = 1.07$; $df = 5, 7$; $P > 0.4498$; and $F = 3.59$; $df = 3, 6$; $P = 0.055$), respectively. Levels of both humidity and CO_2 were higher in treatments with reduced ventilation than in those with maximum ventilation (Fig. 3.1 A, B). Ventilation treatment also affected varroa mite mortality rate ($F = 5.07$; $df = 2, 9$; $P < 0.0335$) but did not affect honey bee worker mortality rate ($F = 3.22$; $df = 2, 9$; $P < 0.088$) (Fig. 3.1 C). Varroa mortality rates were similar across all replicates ($F = 1.8$; $df = 9,9$; $P = 0.19$), while there was significant variance in worker mortality rate in different replicates ($F = 5.8$; $df = 9, 9$; $P = 0.01$) (Fig. 3.1).

Temp 25°C

At 25°C, the average temperature within chambers was not affected by ventilation treatments ($F = 2.62$; $df = 2, 17$; $P > 0.10$) but did vary slightly (avg 23.31 ± 0.02°C, ranging from 20.19 to 27.12°C) among replications on different dates ($F = 27.23$; $df = 9, 17$; $P < 0.0001$).

Changes in ventilation affected both the humidity level (g/m^3) ($F = 15.31$; $df = 2, 12$; $P < 0.0005$) and the CO_2 level within chambers ($F = 9.58$; $df = 2, 6$; $P < 0.014$). Humidity and CO_2 levels were similar in all replicates ($F = 1.18$; $df = 9, 12$; $P > 0.29$; and $F = 3.59$; $df = 3, 6$; $P > 0.055$, respectively.) The levels of both humidity and CO_2 were higher in treatments with reduced ventilation than with maximum ventilation (Fig. 3.2 A, B). Ventilation treatment also affected varroa mite mortality ($F = 16.44$; $df = 2, 25$; $P < 0.0001$) but did not affect worker mortality ($F = 2.41$; $df = 2, 25$; $P > 0.11$) (Fig. 3.1 C). Varroa mortality was greater at low ventilation rates than at higher ventilation rates (Fig. 3.1 C). Both varroa mortality ranges and worker bee mortality ranges varied slightly among replicates ($F = 3.87$; $df = 13, 25$; $P < 0.002$) and ($F = 2.52$, $df = 13, 25$; $P < 0.023$) respectively (Fig. 3.2).

DISCUSSION

This study showed that modification of the atmosphere in the environment of the honey bee cluster can increase the daily mortality rate of varroa. In both temperature experiments, there was a significant increase in the CO_2 concentrations as the ventilation rate decreased. In the 25°C experiments, the varroa mortality increased substantially as the ventilation rate decreased. This was not the case in the 10°C experiment where a

slight increase in mortality was observed under high ventilation conditions. However, the varroa mortality rates at 10°C under high ventilation were still relatively low and were comparable to the lowest levels of varroa mortality in the 25°C experiment. At both temperatures, the bee mortality was similar in the different ventilation treatments.

Carbon dioxide levels in the atmosphere outside a honey bee hive average 0.035% (Nicolas and Sillans, 1989) but within a typical honey bee colony CO_2 levels ranges from 0.1% to 4.25% in summer and 4% to 6% in winter (Simpson, 1950; Simpson, 1961; Seeley, 1974; Van Nerum and Buelens, 1997). In our study, CO_2 levels were also much higher in the chamber air than the levels found in ambient air. The overall trend for both temperature experiments was for CO_2 concentration to increase with a decrease in ventilation. This demonstrates that by limiting the ventilation to honey bees in chambers, CO_2 levels can be effectively controlled within the natural thresholds of a typical honey bee cluster.

The highest level of CO_2 in any of the ventilation treatments in this study was $2.5 \pm 0.07\%$ CO_2, which is within the range of a typical honey bee cluster in summer and in winter (Simpson, 1950; Simpson, 1961; Seeley, 1974; Van Nerum and Buelens, 1997). However, the levels of CO_2 were sampled from a position relatively far away from the bees within the chamber. Van Nerum and Buelens (1997) demonstrated that the CO_2 levels are much higher in the center of a cluster (6%) of bees than outside the cluster (3%). Therefore the levels of CO_2 in the center of the clusters of bees used in this study may underestimate the maximum value but are likely equivalent to that found in air inside the hive. The small size of the clusters in this study may also have prevented them from accumulating the high levels of CO_2 (6%) found within a full-sized colony.

Van Nerum and Buelens (1997) demonstrated that the concentrations of CO_2 change quite dramatically in small clusters of bees during an 18 hour period. Honey bees respond to increasing levels of CO_2, by using large numbers of workers to periodically fan their wings to expel air from the colony, thus reducing the levels of CO_2 in the hive environment (Simpson, 1954; 1961). Seeley (1974) demonstrated that numbers of fanning bees increase as CO_2 increases in a colony and that worker fanning begins at CO_2 concentrations as low as 1%. We measured CO_2 at only one time of day; however the CO_2 levels in the chambers may have fluctuated over time due to changes in the activity of fanning in the clusters of bees over time.

The trend of absolute humidities (g/m^3) increasing with a decrease in ventilation was also likely the result of moisture given off as the bees respond, building up in the air of the chamber. There was also a trend for humidity to increase as temperatures increased. Experiments performed by Currie and Tahmasbi (2008) under similar conditions that prevented CO_2 buildup found little or no varroa mortality associated with different humidity levels. However, in this study during the 10°C experiment, varroa mortality increased slightly at the lowest levels of both CO_2 and humidity, possibly because varroa that fell from bees were susceptible to dehydration (Bruce *et al.*, 1997). Varroa have a large surface area to volume ratio, making them vulnerable to desiccation (Schmidt-Nielsen, 1984; Yoder *et al.*, 1999). Yoder *et al.* (1999) found that adult varroa are able to withstand up to 37% weight loss by desiccation, losing approximately 2% body water/hour. Bruce *et al.* (1997) found that varroa lost the most moisture more readily at high temperatures and low humidities. However, in the 25°C experiment the highest levels of varroa mortality were associated with the highest levels of absolute

humidity (g/m^3). Therefore, desiccation was unlikely to be the main cause of mortality in varroa. This suggests CO_2 increase in the chamber may be responsible for the greater varroa mortality that was observed. In modified atmosphere treatments with CO_2, a major cause of mortality in the target species is through desiccation due to the prolonged opening of spiracles leading to water loss on insects exposed to CO_2 (Aliniazee, 1971; Spratt, 1975; Krishnamurthy *et al.*, 1986). This was clearly not the case in our study because mortality was greater at higher humidities. Similarly, Currie and Tahmasbi (2008) found that absolute humidity had no affect on varroa mortality under similar experimental conditions, although the ranges of absolute humidity in our study (2.25 to 8.04 g/m^3 at 10°C and 5.0 to 15.6 g/m^3 at 25°C) were much greater than theirs (5.3 to 10.9 g/m^3). The results of this study, using modified atmospheres, suggest it is unlikely that desiccation is the primary cause of mortality of varroa in this study.

The main cause of varroa mortality in these experiments is likely the acute effects of increased levels of CO_2 in the haemolymph due to the inability of varroa to effectively control the levels of CO_2 in their surroundings. The mode of action may include depolarization in neurons (Clark and Eaton, 1983), interfering with the neuromuscular transmission sequence (Hoyle, 1960) and/or disruption to the acid-base balance resulting in a lowered pH of the haemolymph (Harrison, 1994, 2001; Slama, 1994). However, most of the research on these physical effects of elevated CO_2 in arthropods is not fully understood and has not been carried out on *V. destructor*. The difference between the mortalities between the two experiments may be due to a difference in varroa's susceptibility to CO_2 at different thresholds for toxicity of CO_2 may vary. Additionally,

in the low ventilation treatments at 25°C, CO_2 levels were much higher than at 10°C (2.13% CO_2 vs 1.2% CO_2, respectively).

Terrestrial arthropods respire through extensive traheal networks (Wigglesworth, 1935; Slama, 1988). Typically, arthropods rely on diffusive ventilation to rid themselves of an accumulation of CO_2 in the traheal system (Slama, 1988). This is accomplished by CO_2 diffusing along gradients of high to low concentrations, from the trahcea to the surrounding air (Slama, 1988).

Both European honey bees and varroa live in environments with elevated levels of CO_2. However, there may be a crucial difference in how each species regulates the CO_2 levels in their immediate environment. At a colony level, honey bees are able to control the CO_2 concentrations of the hive by fanning their wings to create air currents to void accumulations of CO_2 from the interior of the hive (Seeley, 1974). On an individual level, honey bees can adjust their immediate CO_2 concentrations by pumping the abdomen to flush out air from their trachea in response to high concentrations of CO_2 (Bailey, 1954). This is known as discontinuous ventilation and is used by a variety of different insects to control respiration and water loss (Slama, 1988; Lighton and Lovegrove, 1990; Slama 1994). In honey bees, the spiracles are normally closed, but they will occasionally open and close, creating a tidal flow of air in and out of the thoracic spiracles by respiratory pulses (which are regulated by the nervous system) (Lighton and Lovegrove, 1990). At temperatures above 12°C, honey bees will switch to this type of respiratory ventilation from diffusive ventilation (Lighton and Lovegrove, 1990). Diffusive ventilation is insufficient for maintaining acid-base balances at high CO_2 levels in some insects and acari, therefore, respiratory pulses are thought to be

responsible for preventing the buildup of acids in the haemolymph of insects, in environments of high CO_2 (Slama, 1994). Because not all arthropods are able to adequately utilize discontinuous ventilation, particularly under differing environmental conditions (Lighton, 1994; Hadley, 1994), it is possible that varroa do not have the ability to greatly alter their surrounding levels of CO_2. This may leave varroa vulnerable to increases in CO_2 levels in the environment of the honey bee hive.

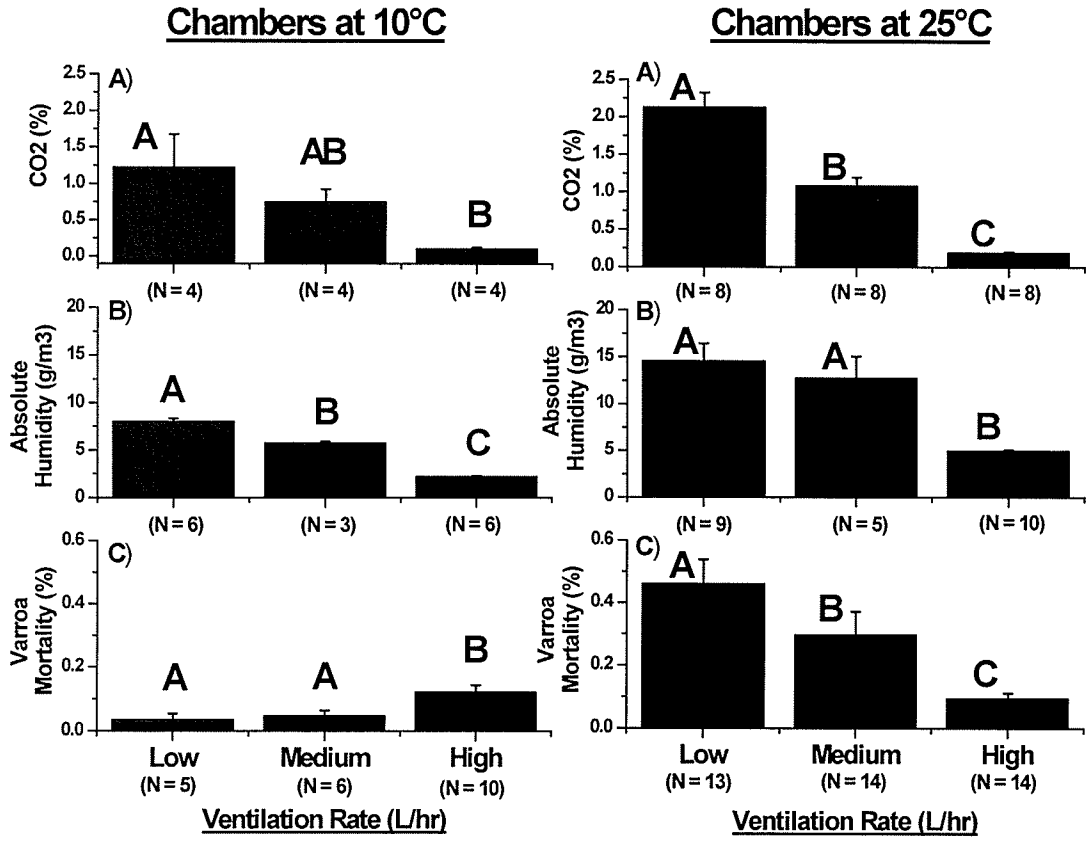
The respiratory system of varroa consists of a tracheal network, totally independent of the host bee's respiration (Richard *et al.*, 1990). Varroa mites use a mobile appendage-like structure, called a peritreme, to obtain air from the environment (Liu, 1996). Although gas exchange can be adjusted by changing the position of the peritreme, (Pugh *et al.*, 1992) it is not known whether varroa have the capability to actively flush air out of their tracheal system to expel high levels of CO_2 like honey bees. Changing the position of the peritreme may have limited success in allowing varroa to tolerate CO_2 levels. Varroa's ability to control its ambient CO_2 may be described as passive diffusion compared to the active control that honey bees are able to use via discontinuous ventilation. The potential inability of varroa to actively alter their CO_2 levels within their own microenvironment may put them at risk to possible acute effects of CO_2. This vulnerability to CO_2 may not apply in all contexts in the hive. Varroa spend prolonged periods of time (12 days in workers and 14 days in drones) within the brood cells of honey bees to reproduce (Infitidis, 1983). This is an environment consisting of high humidity and high CO_2 levels (Bishop, 1923). However, varroa are quite well adapted to this environment. Additionally, the honey bee larva has increased levels of dissolved CO_2 in their haemolymph. These heightened levels result in a lower

pH of the larval haemolymph (pH 6.8) (Bishop, 1923). To equalize the levels of pH in the haemolymph and protect against the harmful effects of a lowered pH, larvae may produce buffers that are exploited by varroa when feeding on brood. If there are differences in the haemolymph composition of adult and larval honey bees, then perhaps varroa are not protected by the buffering effects of the haemolymph while outside the brood cell, making them susceptible to higher levels of ambient CO_2 than within the brood cells.

Further research is needed, particularly with larger groups of bees to determine if achieving increased varroa mortality by exposing them to increased levels of CO_2 is possible at a colony level. This research would also need to address the potential effects of CO_2 on the survival of worker bees and queens. The presence of a queen may change the dynamics of the colony activity and fanning behavior of the workers in the hive environment.

This research shows the potential to control varroa in honey bees using the natural buildup of CO_2 generated by a cluster of worker bees in reduced ventilation settings. The results from this study show promise for the application of modified atmospheres to control varroa in large-scale indoor overwintering buildings used to store honey bee colonies as ventilation can be easily controlled. If successful, this could provide a low-cost, non-chemical control for varroa.

Fig. 3.1 Honey bee clusters in chambers held at 10°C (Chambers at 10°C) or at 25°C (Chambers at 25°C) three different rates of ventilation (low 14 L/h, medium 42.5 L/h and high 360 L/h) A) Mean CO_2 concentration of chambers (+ standard error) B) Mean absolute humidity (g/m^3) (+ standard error) of chambers C) Mean proportion of varroa mortality per day (+ standard error) of total varroa in cluster. Vertical lines on bars indicate + standard error. N = number of cages per treatment combination. Means followed by the same letter within ventilation levels are not significantly different by ventilation treatment.



GENERAL DISCUSSION

The environment of the honey bee hive is essential to the survival of honey bee colonies during winter. This environment is made up of the hive, the bodies of the bees (in the form of the cluster) and the environmental conditions, including the temperature and CO_2 regulated by bees. The environment of the hive allows honey bees to rear limited amounts of brood throughout winter and also provides the conditions that allow parasites such as *Varroa destructor* to survive in honey bee colonies year round. *V. destructor* causes serious harm to honey bee colonies throughout the year and high levels of this pest are a major cause of colony mortality over winter. However, the dynamics of how *V. destructor* interacts with its newly acquired host are not fully understood, particularly during winter in regions of the world with extended periods of limited brood rearing. Therefore a more complete understanding of colony dynamics of bees during winter and the biology of varroa within this environment is essential to develop methods to control varroa infestations during this critical period.

The objectives of this thesis further the understanding of the winter environment of honey bees and how aspects of the environment may be used to control infestations of *V. destructor* during winter. The first objective of this study was to examine the role that brood rearing during winter in honey bee colonies has on the efficacy of low level formic acid fumigation treatments for the control of varroa in honey bee colonies. The second objective of this study was to examine how varroa utilizes the limited amounts of brood produced during winter in moderately to highly infested honey bee colonies. The third objective of this study was to determine whether modified atmospheric conditions created through the use of reduced ventilation rates would increase levels of CO_2 enough to

potentially control the levels of varroa infestation in small clusters of bees. Altogether, the seasonal biology of *A. mellifera* and *V. destructor*, the conditions found within the hive environment, the dynamics of the honey bee cluster and the management of storage conditions during winter provide an unique opportunity for the control of varroa during a time when colonies are inactive and can be treated economically using limited labor.

Brood in Winter

Brood rearing occurs during winter in honey bee colonies in temperate regions of the world, although at much lower levels than other seasons (Jeffree, 1956; Avitable, 1978; Harris, 1980; Seeley and Visscher, 1985; Szabo, 1993; Nelson, 1995). The brood found in honey bee colonies during winter in this study were limited and their presence in colonies varied considerably in different years of the study. Although previous research has shown that varroa will infest and reproduce in brood during winter at much lower levels compared to summer (De Jong *et al.*, 1982; Otten and Fuchs, 1990; Eguaras *et al.*, 1994; Martin, 2001; Harris *et al.*, 2003) this study is the first to examine how varroa utilizes brood under conditions of extended periods of limited brood rearing and the dynamics of varroa reproduction at a population level. The proportion of varroa found in winter brood in this study (31%) was much lower than previously reported (55%), in milder temperate climates of the world (Martin, 2001). The detailed population assessments of honey bee and varroa populations in the colonies in this study revealed that the overall proportion of varroa reproducing in the colony at any one time is actually quite low, typically averaging only 1% in colonies throughout winter. This is, at least, partially the combined result of the lower levels of host brood being available during this

time (avg. 78.04 ± 13.55 capped brood), the lower levels of infestation of this brood (23% prevalence) and the lower levels of reproduction in this brood (31% reproduction). Therefore, the overall capacity for the population of varroa to increase, in absolute numbers, during winter is limited.

Varroa Mortality and Reproduction During Winter

Although levels of brood and varroa reproduction were low in colonies that were sampled, there were increases in mean abundance of varroa of untreated colonies over the winter of 2006. Our data suggest that this was likely due to differing rates of varroa mite and bee mortality during winter. Previous studies have suggested that increases in the mean abundance of varroa in honey bee colonys result from varroa transfer from dying bees to live bees in a dwindling population of bees in a colony (Smirnov, 1978; Bowen-Walker *et al.*, 1997). We observed similar trends in our data that indicated that these increases in mean abundance of varroa may be due to the differential mortality of bees and mites. The average daily varroa mortality rate in our study as measured by bottom board samples in unfumigated colonies without brood removal over the two years of the fumigation study was 0.0034 ± 0.0002 or 3.0 ± 0.19 varroa/day based on averages of varroa numbers dropping. Other studies have estimated similar death rates of varroa during winter at 0.004 (Fries *et al.*, 1994; Calis *et al.*, 1999) and 0.002 (Martin *et al.*, 1998). However, the average daily bee mortality rates of 0.005 ± 0.0003 (or 18.56 ± 0.63 bees/day) were greater than those found for varroa. Overall, in both years of the study, the cumulative bee mortality in a colony during winter was greater (17 ± 1%) compared to (13 ± 0.3%) in varroa.

The levels of brood produced during winter in colonies in this study may have slightly increased the bee population, replacing dying bees throughout winter. The varroa reproduction occurring within this brood would have slightly increased the varroa population. However, the limited reproductive capacity of both bees and varroa throughout winter strongly suggests any increase in the mean abundance of varroa or bees overwinter was likely the result of higher bee mortality rates relative to varroa mortality. The average daily varroa mortality in untreated colonies in this study was 0.34% compared to the overall estimated rate of reproduction 0.2%. Accordingly, the mortality rate of varroa would exceed the birth rate in these colonies. Therefore, the increases in colony infestation could not be solely attributed to varroa reproduction during winter.

Mortality models of varroa during winter by Fries and Perez-Escala (2001) predicted similar bee and varroa mortality rates in winter resulting in a stable infestation of varroa over winter. However, our results demonstrate that the infestation level has the capacity to change considerably over the winter period due to differences in mortality rates between bee and varroa populations. These varroa infestations can either increase (2006) or slightly decrease (2005) in honey bee colonies as shown by the changes in the average infestation of the control colonies (unfumigated, no brood removal) in both years of the fumigation experiment. Whether the infestation increases, decreases or remains stable over the winter may depend on the potential for variation in the bee and varroa mortalities relative to each other.

The controversy of whether varroa infestations increase, decrease or remain stable during winter is an important aspect of the seasonal population dynamics of varroa.

Compared to previous studies, this study incorporated detailed assessments of the entire varroa and honey bee populations of individual colonies as well as varroa mortality sampled continuously throughout winter providing a more comprehensive picture of the population dynamics of varroa during winter. These overwinter studies also incorporated detailed assessments of brood in winter and mite reproductive rates, which previous studies ignored.

Formic Acid Treatments in Relation to Brood in Winter

During periods of high levels of brood rearing within the colony (60 to 74%) of the colony's varroa are present within the brood at any one time (Harris *et al.*, 2003). The presence of brood in a colony affects the spatial-distribution of varroa in a colony as varroa infest brood to reproduce they leave their adult bee hosts. The presence of honey bee brood in colonies has been known to affect the efficacy of formic acid treatmens to control varroa in outdoor summer conditions (Eischen, 1998 a). During our long-term fumigation treatments removal of the limited brood found during winter did not affect the efficacy of low-level formic acid fumigation treatments in either year. The levels of brood found over three years of sampling revealed that brood rearing is limited (avg 77 brood cells) and highly variable throughout winter (ranging from 0 to 470 brood cells). Overall, during brood rearing in winter, the majority of the varroa population in a colony (97%) was found outside of the available brood living as ectoparasites on adult bees in the winter cluster. However, the levels of varroa infesting brood were as high as 20%. Due to the sporadic nature of brood rearing within individual colonies in winter, the majority of the varroa in a colony would eventually be exposed to formic acid fumigation

treatments because the low-concentration, long-duration fumigation treatments lasted much longer than the capped brood stage (12 days) during which varroa may have been "protected" from formic acid vapor.

Although high-concentration, short duration fumigation treatments were not tested in relation to brood removal in this study, the small proportion of a colony's varroa population (3%) usually found within the brood and the low levels of brood found in this study suggest that only small proportions of a colony's varroa population would be protected by the limited levels of brood in winter.

Though brood production was variable over the three years of sampling in early, mid and late-winter, there was a slight overall trend toward more brood in early-winter. These results suggest that if treatments are to be scheduled to avoid the presence of brood in colonies, fumigation should take place from January to February to potentially avoid the slight increase in varroa populations during early-winter. However, it should be noted that delaying treatments may prolong the damage that varroa may inflict on a colony over winter, which may potentially risk colony mortality.

Formic Acid Efficacy

This study demonstrated that long-duration, low-concentration formic acid fumigation can achieve much greater levels of efficacy (84% in 2005) than reported in previous studies (60%) (Underwood and Currie, 2005). Colonies treated in 2005 resulted in varroa infestations well below the regional economic thresholds of varroa infestation (2%), where acaricide treatments in spring and would not likely be needed for futher varroa control (Gatien and Currie, 2003; Currie and Gatien, 2006). Therefore, treatment

for varroa could be delayed until the following fall if no reinfestation occurred over summer in these colonies. This makes long-duration, low-concentrations all the more appealing, as relative to high concentration treatments, there is no impact on worker mortality and queen mortality (Underwood and Currie, 2005). However, minimum concentrations would have to be maintained in long-duration, low concentration treatments. In the second year of the fumigation study the efficacy was lower (51%) than reported in previous studies (60%) (Underwood and Currie, 2005), even though the colonies in 2006 were fumigated for longer, and had higher CT values than reported in previous studies under similar conditions (Underwood and Currie, 2005). This highlights the importance of maintaining sufficient levels of in-room and in-hive concentrations at any one time. The lower in-hive concentrations of formic acid in 2006 were likely below the threshold required to achieve adequate varroa mortality. Lower concentrations were likely due in part to high rates of ventilation in the treatment rooms as a result of fan setting used in 2006 to prevent the overheating of rooms.

In outdoor applications of formic acid treatments, variabilities in the environmental conditions can affect efficacy (Skinner *et al.*, 2001; Ostermann and Currie, 2004). The stability of the controlled storage conditions of indoor overwintering buildings provides a substantial advantage in maintaining constant environmental conditions relative to outdoor-treated colonies (Underwood and Currie, 2004; 2005). However, the results of 2006 suggest that better temperature control through the use of air conditioning systems and/or better adjustment of ventilation levels is still required to provide consistent results.

Any manipulations of the ventilation system in indoor storage conditions of honey bee colonies may results in changes in CO_2 which may interact with the mode of action of formic acid. Increasing the levels of CO_2 increases the efficacy of many fumigants used to control arthropod pests by inducing spiracles to open in arthorpods, thereby increasing the uptake of fumigants through the respiratory system (Monro, 1969). The full range of interaction between low-concentration, long-duration formic acid fumigation and increased levels of CO_2 within a typical honey bee cluster is presently unknown. Examining the potential interaction between formic acid fumigation and the heightened levels of CO_2 in colonies during winter may reveal any important relationships between the uptake of fumigants in both honey bees and varroa within locations of the winter cluster experiencing increased levels of CO_2. Additionally, formic acid may influence the levels of CO_2 in the cluster as the worker bees will respond to high concentrations of formic acid by fanning their wings, thereby decreasing the levels of CO_2 in the cluster (Nelson *et al.*, 1994).

Controlling Varroa with Modified Atmospheres

The results of the bioassay studies with reduced ventilation demonstrated that varroa is susceptible to increased levels of CO_2 present in the environment of the honey bee. These increased levels of CO_2 produced in our bioassay system were within the range of a typical winter cluster (2–6%) (Simpson, 1954; Simpson, 1961; Seeley, 1974; Van Nerum and Buelens, 1997). While the colonies used in these studies were small (~300 bees), the results show great promise for the application of modified atmospheres to full-sized honey bee colonies within the management scheme of indoor overwintering

storage conditions. However, detailed studies are needed to determine the thresholds of ventilation that affect colony survival within reduced ventilation storage settings, as the application of inadequate ventilation could potentially result in the build up of CO_2 that is high enough to cause colony mortality. Determining the underlying causes of mortality in *V. destructor* from increased levels of CO_2, whether from desiccation or disruption of the acid-base or other physiological processes is also needed. It seems more likely that the causes are related to physiological changes or disruption of pH as we saw increases in varroa mortality in treatments with increased levels of CO_2 even at relatively high humidities suggesting that dessication is likely not the cause of death in varroa. When the overall tolerance and survival thresholds for ventilation and CO_2 levels are determined for individual colonies these modified atmospheres may be applied to large-scale overwintering storage conditions, as a potential non-chemical treatment for varroa.

Modified atmosphere treatments typically require storage conditions containing the target pests that are completely sealed to achieve and maintain high levels of CO_2 (White and Jayas, 1991). However, completely sealing an overwintering building would not be necessary. Even with ventilation removing a buildup of CO_2 from the interior of overwintering buildings, additional CO_2 is constantly being generated by honey bee colonies themselves. Therefore, if this type of treatment were applied to the set up of a typical overwintering building, adequate ventilation levels that achieved levels of CO_2 within the tolerance of honey bees would have to be incorporated into a management strategy to prevent suffocation of honey bee colonies (Gruzska, 1998).

This research examined the winter biology of *V. destructor* in conditions of extended periods of restricted brood rearing and the winter biology of *A. mellifera* in

relation to its parasite *V. destructor*. This research also explored alternative techniques for controlling varroa during this critical period for colony survival by incorporating treatments that use either organic acids or physical controls instead of synthetic acaricides to which *V. destructor* have become largely resistant. This research applies the principles of integrated pest management, where aspects of the parasite and host biology, environmental conditions of the host, management of the host and the host tolerances are all incorporated into novel pest management strategies to reduce the levels of varroa infestations in honey bee colonies.

CONCLUSIONS

1. Brood removal treatments had no impact on the efficacy of low-concentration, long duration indoor formic acid fumigation treatments for the control of varroa in honey bee colonies.

2. Formic acid efficacy was much greater in our low-concentration, long duration indoor formic acid fumigation treatments than in previous studies.

3. Effective varroa control requires minimum in-hive levels of formic acid. Ventilation and continued purging of the room air may greatly influence the formic acid concentration in room and hive air.

4. The presence and timing of winter brood production in varroa infested honey bee colonies is highly variable among years, seasons and colonies. There seems to be a slight overall trend for increased levels of brood in early-winter (December).

5. The proportion of the varroa population of a colony found within the brood at any one time during winter is typically low (3 ± 0.51%), though can average up to 20%, with the majority of the varroa present as ectoparasites on the adult bees within the cluster.

6. The prevalence of varroa in the brood during winter was 23% ± 2.35% and consistent throughout all sampling periods over a three-year period.

7. The intensity of brood infestation was quite low (Avg 1.38 ± 0.04 mites/brood cell) and consistent throughout all sampling periods with only small numbers of multiple cell infestations even though the available brood was extremely limited and the varroa populations were high.

8. The proportion of varroa reproducing in the brood during winter was lower than reported in previous studies (Avg 31% ± 4.4%).

9. The overall proportion of varroa in colonies that were reproducing at any one time was quite low during winter (Avg 1% ± 0.4%).

10. At 25°C, in applications of restricted ventilation to honey bee clusters, for short periods (two days), decreased ventilation increased the mortality of varroa without having a significant impact on bee mortality.

11. At 10°C, in applications of restricted ventilation to honey bee clusters, for short periods (two days), increased ventilation slightly decreased the mortality of varroa without having a significant impact on bee mortality.

12. Reduced ventilation rates at both high (25°C) and low (10°C) temperatures significantly increased the CO_2 concentrations in small clusters of honey bees.

RECOMMENDATIONS FOR FUTURE RESEARCH

1. Further studies on the factors (e.g. viruses) that influence variability in brood rearing during winter in honey bee colonies infested with varroa. Levels of honey bee brood in winter are variable and colonies rear brood sporadically (see Chapter Two). Further sampling of bees and brood in large numbers of honey bee colonies in different times during winter may reveal potential relationships between brood rearing and viral loads of colonies.

2. Studies on the role of brood and queen pheromones within the winter cluster on orientation of varroa and on the stimuli that varroa use to locate brood during winter. The spatial dynamics of the cluster are such that the bees are clustered very tightly, all throughout winter (Southwick 1991 b). In this environment, the bodies of the bees themselves may interfere with the distribution of the circulating brood pheromones. This may infere with varroa's infestation of the brood and may account for the lower levels of infestation of honey bee brood by varroa in winter (see Chapter Two). Additionally, the lower quantity of brood reared during winter may negatively influence the brood locating behavior of varroa.

3. Studies examining the potential interactions of CO_2 and formic acid within the winter cluster of a honey bee colony. As CO2 has been shown to enhance the efficacy of fumigants, treatments applying formic acid fumigation indoors under

environments of high CO2 may increase varroa control (Young and McDonald, 1970; Rajendran *et al.*, 1977). These studies could use restricted ventilation, through modificateion of the ventilation system of the storage facility or the addition of CO2 gases from an external source in addition to an indoor formic acid fumigation treatment.

4. Studies on the whether formic acid fumigation during winter may be able to reduce the levels of different bee viruses present in a colony. Varroa are continually feeding on bees throughout winter and therefore likely have the capacity to transmit virsuses within the colony during the time colonies are stored indoors. Applying treatments of formic acid during this time to control the levels of varroa may also result in a lower viral load of worker bees in spring, compared to untreated colonies. Initial infestation levels and viral loads of colonies would have to be quantified in this study prior to, and following, formic acid treatments.

5. Studies examining the effects of long-term exposure to increased levels of CO_2 levels on the long-term survival of varroa in winter in honey bee colonies. These high levels of CO_2 may be achieved by adding CO_2 directly into colonies from an external source or by restricted ventilation applied to the ventilation system of the storage facility. These studies would assess whether modified atmospheres could be used as a treatment for varroa in the context of full sized colonies.

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