

INTERACTIONS OF VARROA, *Varroa destructor* Anderson and Trueman, WITH
CHALKBROOD, *Ascosphaera apis* (Maassen ex Claussen) Olive & Spiltoir, AND
NOSEMA, *Nosema apis* Zander, IN HONEY BEE, *Apis mellifera* L., COLONIES
TREATED WITH FORMIC ACID, AND THE INFLUENCE OF HIVE AND
AMBIENT CONDITIONS ON FORMIC ACID CONCENTRATION IN THE HIVE

A Thesis

Submitted to the Faculty

of

Graduate Studies

The University of Manitoba

by

David J. Ostermann

In Partial Fulfilment of the

Requirements of the Degree

of

Master of Science

Department of Entomology

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WITH CHALKBROOD, *ASCOSPHAERA APIS* (MAASSEN EX CLAUSSEN) OLIVE &
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DAVID J. OSTERMANN

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University

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Dedicated to my parents,
Fred & Marianne Ostermann

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ABSTRACT

Ostermann, David J. M.Sc., University of Manitoba, December 2002. Interactions of Varroa, *Varroa destructor* Anderson and Trueman, with Chalkbrood, *Ascosphaera apis* (Maassen ex Claussen) Olive & Spiltoir, and Nosema, *Nosema apis* Zander, in Honey Bee, *Apis mellifera* L., Colonies Treated with Formic Acid, and the Influence of Hive and Ambient Conditions on Formic Acid Concentration in the Hive. Major Professor: Robert W. Currie.

The interaction between the effects of varroa and formic acid treatments on colonies of honey bees was examined. Two field experiments were conducted over a two year period. In the first field experiment, colonies were made up with honey bees infested with varroa or treated with fluvalinate strips initially to eliminate varroa. Varroa and no-varroa treatment colonies were each subdivided into two groups then treated with formic acid or left untreated. The formic acid treatment consisted of 200 mL of 65% formic acid in a pin-perforated Ziploc® vegetable bag slow release application for 40 days. In the second field experiment, varroa treatments were created by infesting varroa-free packages of bees with 45 living varroa mites, and no-varroa treatment colonies were not infested initially with varroa. Varroa and no-varroa treatment colonies were each subdivided into three groups and exposed to a pour-on formic acid treatment, a slow release formic acid treatment, or left untreated. The pour-on formic acid treatment was 40 mL 65% formic acid applied to paper towels five times at seven day intervals. The slow release formic acid treatment consisted of 200 mL of 65% formic acid in a slit Ziploc® freezer bag. Varroa infestation, number of worker honey bees, and area of sealed worker brood per

colony were estimated over the course of the season each year. Additionally, in the second year field experiment, ambient temperature, relative humidity, and solar conditions, hive temperature and relative humidity, and formic acid concentration in hive air were measured at approximately 0900, 1400, and 1900 hours to assess the effects of ambient and hive conditions on hive formic acid concentration.

Formic acid application improved colony development by reducing the mean abundance of varroa when mean abundance was relatively high. However, when varroa were absent or mean abundance was relatively low, formic acid application diminished colony development. The formic acid applications in the second year field experiment reduced mean abundance of varroa by 9.4% in colonies treated with slow release formic acid and 29.3% in colonies treated with pour-on formic acid. In colonies treated with the pour-on formulation, there was a significantly higher average interday (1400 hours) formic acid concentration in hives not initially infested with varroa compared to hives which were initially infested with the mite on three of five application days. Similarly, there were significantly higher intraday (0900, 1400, and 1900 hours) concentrations of formic acid in hive air in no-varroa colonies than in varroa colonies treated with the pour-on application at 0900 and 1400 hours, on two of three days analyzed. Varroa's effect on colony size and activity may act with ambient conditions to influence concentration of formic acid in hive air with this formic acid formulation. In colonies treated with the slow release formulation, formic acid concentration in the hive at 1400 hours was positively correlated with hive and ambient temperature early in the application period. Ambient temperature was positively correlated with ambient solar condition during the

treatment period. In colonies treated with the pour-on formulation, interday hive concentration was positively, but weakly, correlated with fluctuations of hive temperature.

The effects of varroa and formic acid on chalkbrood and nosema infection were also examined in this two year experiment. Prevalence of chalkbrood and mean abundance of nosema were monitored over the course of the season each year. Laboratory bioassays were conducted to assess further the impact of formic acid on *Ascosphaera apis*. The fungus was exposed to formic acid in culture agar in two replicate bioassays. Mycelial and spore-producing mycelial growth areas were measured, spore ball and spore cyst diameters were measured, and number of spore cysts was estimated. The prevalence of chalkbrood may increase with increased mean abundance of varroa. Also, formic acid, applied as a spring treatment in the presence of varroa, may reduce chalkbrood prevalence through direct action and reduction in mite population. However, spring treatment with formic acid when varroa infestation was low tended to increase prevalence of chalkbrood. These trends were not statistically significant. Formic acid in agar significantly inhibited mycelial and spore-producing mycelial area growth as well as cyst production at high concentrations. Levels of nosema infection were not affected by varroa or formic acid treatments.

FOREWORD

This thesis is written in paper style. Chapter II is a review of literature necessary for understanding the topic of research. Chapters III and IV are individual manuscripts prepared as independent submissions to scientific journals. Both chapters will be submitted to the *Journal of Economic Entomology*. Chapter V is a general discussion of the research contained herein. The references cited in all chapters may be found at the end of this thesis.

CHAPTER 1

GENERAL INTRODUCTION

There are many parasites that attack honey bees. The fungus *Ascosphaera apis* (Maassen ex Claussen) Olive & Spiltoir, protozoan *Nosema apis* Zander, and ectoparasitic mite, *Varroa destructor* Anderson and Trueman, are parasites that infect the honey bee (*Apis mellifera* L.) around the world. All three parasites have detrimental effects on honey bee colonies; however, varroa is considered the greatest threat to world beekeeping (De Jong 1997).

It is not known exactly how *V. destructor* destroys colonies. It is believed that part of the damage is caused by microbial pathogens promoted by the mite, but interactions between *V. destructor* and *Ascosphaera apis* or *Nosema apis* are not well understood or documented. The complex activity of a honey bee colony combined with genetic and environmental factors, makes it very difficult to link colony damage to any specific parasite in a hive with multiple infections. Despite the complexity of the relationships, knowledge of parasite interactions with each other and with treatments is important to appreciate the exact nature of damage to colonies. Honey producers must know the source of damage before they can properly act against it.

Varroa is able to transport *N. apis* and *A. apis* spores (Glinski 1988; Liu 1996) and may promote chalkbrood (Glinski 1988) and nosema disease. Changes in honey bee physiology and general stress caused by varroa's feeding may also encourage disease development by *A. apis* and *N. apis*.

There is no clear effect of formic acid on chalkbrood or nosema infection.

Treatment with formic acid could have a direct effect on non-target parasites such as *A. apis* and *N. apis*, or it could reduce the diseases indirectly by reducing the level of varroa infestation. It is unclear what causes the great variation in concentration of formic acid in hives (Ostermann Chapter 3) and great variability in efficacy against varroa in colonies treated with the same formic acid application (Feldlaufer *et al.* 1997; Calderone 1999b), which likely affects colony well-being and perhaps disease development. With increasing occurrence of varroa resistance to fluvalinate (Elzen *et al.* 1999) and coumaphos (Elzen and Westervelt 2002), there is a need to understand how formic acid works and how it affects honey bee colonies.

This study is a contribution to the knowledge of how varroa interacts with chalkbrood and nosema in honey bee colonies treated with formic acid, how formic acid for varroa control impacts honey bee colonies, and how hive and ambient conditions influence formic acid concentration in the hive.

The following review of literature is an examination of the life cycles of *V. destructor*, *A. apis*, and *N. apis* to assess the overlap in time and place of the mite with the two microbial pathogens and to determine how they might interact. Formic acid literature is also discussed in this context.

CHAPTER 2

LITERATURE REVIEW

Varroa destructor

Introduction

The varroa mite, *Varroa destructor* Anderson and Trueman, is an ectoparasitic mite which feeds on the haemolymph of adult and immature honey bees (Anderson and Trueman 2000). Anderson and Trueman distinguished two haplotypes of *V. destructor* from 18 haplotypes of *V. jacobsoni* Oudemans. It is believed that *V. jacobsoni* passed from *Apis cerana* Fabricius onto *A. mellifera* in Asia in the first half of the 20th century (Crane 1988). While *V. destructor* infests only *A. mellifera*, *V. jacobsoni* infests both *A. mellifera* and *A. cerana* (Anderson and Trueman 2000). *Varroa destructor* was first discovered in the U.S. in 1987 (Southwick 1990) and soon after in Canada in 1990 (Anonymous 1991).

Varroa destructor can destroy a colony within a couple of years if left untreated. Feeding damage and loss of haemolymph from the host bee may cause weight loss, wing deformation, and death (De Jong *et al.* 1982; Schatton 1987; Ritter 1988; Marcangeli *et al.* 1992; Shimanuki *et al.* 1994). Workers infested with varroa have reduced haemolymph volume, total protein, and protein concentration during development (Weinberg and Madel 1985; Schatton and Engels 1988). The symptoms of varroa infestation may be caused in part by diseases which are transported by the mite, and/or which develop as a result of physiological changes caused by mite feeding.

Varroa has been associated with many microorganisms and viruses including

acute paralysis virus (Ball 1985; Ball 1988b), fungi (Liu and Ritter 1988) such as *Ascosphaera apis* (Glinski 1988; Liu 1996), protozoans (Liu and Ritter 1988) such as *Nosema apis* (Liu 1996), and bacteria (Liu 1996) (Table 1).

Life Cycle

Introduction

Varroa is highly adapted to survival and reproduction on the honey bee, *A. mellifera* (De Jong 1997). Its direct uptake and use of host bee haemolymph, and successful development of offspring in brood cells make it an efficient parasite. The life cycle of varroa consists of adult and immature bee-feeding stages (Fig. 1).

Cell entry

Fertilized female varroa enter cells containing five day old worker or seven day old drone larvae (Ifantidis 1988), about 20 or 40 h before capping, respectively (Boot *et al.* 1992). The mites prefer drone brood over worker brood and worker brood over queen cells; however, due to the relative scarcity of drone brood, the majority of varroa infest worker cells. The longer development time of a drone allows more mite offspring to reach maturity before the drone emerges from the cell (Boot *et al.* 1992). Greater attraction of varroa to drone cells than to worker cells may be attributed to drone kairomone (Goetz and Koeniger 1993), larger larva, shorter rim-to-larva distance (Goetz and Koeniger 1993; Calderone 1999a), raised rim cell (De Jong and Morse 1988; Ruijter and Calis 1988), or longer period of drone brood “attractivity” (Boot *et al.* 1991). Varroa is found in queen cells of heavily infested colonies (Harizanis 1991).

In the cell

Immediately after entering the cell, the foundress mite goes underneath the bee larva to the bottom of the cell (Boot *et al.* 1992) where she is “trapped” in the larval food, on her back, with her ventral side toward the opening of the cell (De Jong *et al.* 1982; Ifantidis 1988). Some larval jelly is consumed by the mite but its value to the mite is unknown (De Jong 1997). The two respiration tubes (peritremes), raised perpendicular to her body, allow her to breathe while submersed, and the mite’s hydrophobic epicuticle also keeps a thin layer of air on her dorsal scutum (Krantz and Baker 1982). As the bee larva eats the food at the bottom of the cell, varroa is freed from its sticky trap, and uses the opportunity to climb onto the larva (Donze *et al.* 1998a). Varroa may spend up to 15 h in the larval food of a worker cell and 45 h in the larval food of a drone cell (Ifantidis 1988). Approximately 5% of the mites that enter brood cells die in the jelly when the larval food is not consumed.

Feeding

The varroa mite begins feeding on larval haemolymph after being freed from the larval jelly (Donze *et al.* 1998a). The mite remains on the larva while the bee spins its cocoon, thus avoiding being trapped between the cocoon and the cell wall. The mite grows thicker and gains about one-third in weight, increasing from about 0.34 to 0.47 mg in 24 h (Steiner *et al.* 1994). In order to suck the haemolymph, varroa makes an incision in the integument of the individual bee with alternate movements of its saw-toothed chelicerae (Strick and Madel 1988). The volume of larval haemolymph ingested by a mite is about 0.86 μ L in 0.75 h to 1.45 μ L in 24 h (excrement included) (Tewarson 1983).

The mother mite actively pushes the third (metathoracic) set of legs of the pupa aside and creates a space for her male and female young to feed (Donze and Guerin 1994). Young mites rely on their mother to make an incision in the bee pupa's abdominal exoskeleton so they can feed (Steiner 1988). Sharing a "feeding hole" prevents unnecessary excessive damage to the host which the mites rely on to chew through the wax cap and emerge. *Varroa* never feeds on the head or thorax of the prepupa (Donze *et al.* 1998a). Protonymphs are at a competitive disadvantage at the communal feeding site (Donze and Guerin 1994). Competition among offspring at the feeding site may be the major factor in offspring mortality (Martin 1995).

Egg laying

The fertilized female mite lays her first egg after feeding at about 60 to 70 h after the brood cell is sealed (Ifantidis 1983; Steiner *et al.* 1994). Eggs are laid one at a time at intervals of about 30 h (Donze *et al.* 1998a). They are usually laid on the interior part of the cell wall where they are least likely to be disrupted or damaged by the developing bee. The total time available for mite development is 270 h in worker and 330 h in drone cells (Donze and Guerin 1994) so females lay up to seven eggs in a drone cell or 6 eggs in a worker cell (Ifantidis 1983). *Varroa* eggs hatch about 48 h after they are laid (De Jong *et al.* 1982) with males developing from unfertilized eggs and females developing from fertilized eggs (haplodiploidy). The first egg laid is usually male, and the remainder are female, but the last eggs laid often fail to reach maturity before the host bee matures and emerges from its brood cell. Due to high mortality of nymphs, one reproducing mite produces an average of about 1.3 to 1.4 new females in worker cells compared to about

2.2 to 2.6 in drone cells (Schulz 1984; Fuchs and Langenbach 1989). Males and immature females remain and die in the cell (Martin and Kemp 1997).

Varroa mites have two or three reproductive cycles in a lifetime (Martin and Kemp 1997). Fries and Rosenkranz (1993) observed that 13% of introduced female mites reproduced at least three times. The percentage of mother mites that produce viable offspring is 40 to 93% (Ritter and De Jong 1984; Fuchs and Langenbach 1989; Rosenkranz and Engels 1994; Kirsch and Rosenkranz 1998).

Mating

Unless more than one foundress enters the cell before it is capped, mating within the cell occurs between adult brother and sisters. Mating occurs just after ecdysis of the female (Donze *et al.* 1996) with young moulted females being preferred over older ones (Donze *et al.* 1998b). The number of spermatozoa stocked in the spermatheca of mated females increases with remating perhaps supporting the idea that sperm may be mixed if more than one male is present. The rendezvous location for mating is established by the mother mite at the site of faecal accumulation, usually near the bee's anal zone (Donze *et al.* 1998a).

Development

Male and female varroa mites develop from egg to larva to protonymph and then to deutonymph (Steiner 1988). Males mature from egg to adult in about 5.5 to 6.2 days and females mature in 6.5 to 6.9 days. The egg stage lasts one to two days for both sexes (Ifantidis 1983). Within the egg, the developing larva has six legs (De Jong 1979).

Varroa hatches as an eight-legged protonymph. The protonymph feeds on the immature

bee, grows, then molts to a deutonymph stage (Steiner 1988). The protonymph lasts three days in the male and four days in the female, while the duration of the deutonymph is one to two days (Ifantidis 1983). The final molt produces an adult varroa mite. Female mites that hatch in the summer live for approximately two to three months (Shabanov *et al.* 1978).

Phoresy

Following emergence from the cell, mature adult varroa mites live and feed on the adult honey bees and are said to be in the phoretic stage. The oval, dorso-ventrally flattened shape of varroa allows it to squeeze in between the abdominal tergites and sternites of the adult bee with its head toward the bee's body (Akimov *et al.* 1988). Phoretic mites are spread from colony to colony by various means such as drifting of infested adult workers and drone bees, transferring of bees and brood between colonies by beekeepers, and during robbing of honey in weak colonies by other bees (De Jong 1997).

The mites leave newly emerged bees rapidly in order to infest two day old bees (Le Conte and Arnold 1987). Furthermore, varroa prefer adult drones over adult workers and nurse bees over foragers (Hoppe and Ritter 1988). To distinguish between bees of different ages, varroa apparently uses geraniol, the main component of Nasonov pheromone. The forager's Nasonov gland pheromone has a strong repellent effect on varroa and this odour may influence the position of the mite on a bee. Alarm pheromone may also influence the behaviour and position of varroa (Liu 1991b). Varroa mites are usually found on the abdomen, especially between the overlapping abdominal sternites/sclerites (De Jong 1997). In heavily infested colonies, mites may also be seen on

top of the thorax of the host bee. The phoretic stage lasts about one to 10 days then females begin the cycle again and invade new brood cells (Boot *et al.* 1993).

A varroa population can grow dramatically in a short time. While Fries *et al.* (1991) claimed that a colony infestation of less than 10 female mites may increase 100 fold in a summer, Harbo and Harris (2001) observed varroa population growth from approximately 800 to about 1100 in 78 days. Korpela *et al.* (1992) documented varroa population increase from approximately 100 to about 12,000 mites in 26 months and from less than 10 to almost 6,000 mites in 38 months. Varroa's great reproductive success often translates into serious colony damage in a short period of time.

Damage

The amount of damage to bees and colonies depends on the degree of varroa infestation. Colonies infested with only a few hundred mites show little sign of damage and an infestation may go unnoticed. At this point, the mites are spreading among colonies (De Jong *et al.* 1982). Usually infestation increases continually until the host colony collapses. The increase in the varroa mite population in a colony can be so fast that colonies can be strong, produce a large crop of honey in late summer, then die by late fall (De Jong 1997). Varroa may also kill a colony over winter unless the colony is treated to control the mite (Korpela *et al.* 1992). Finley *et al.* (1998) reported that, in northeast regions of the U.S., during the winter and spring of 1995-1996, 25 to 80% colony loss was believed to be caused by tracheal (*Acarapis woodi* Rennie) and varroa mites.

When the mite population reaches 30 to 40% of the adult bee population, the number of adult bees declines rapidly (De Jong 1997). In heavily infested colonies, approximately 6% of bees have obvious deformities (De Jong *et al.* 1982). Marcangeli *et al.* (1992) found that about 25% of bees with one to three mites were severely damaged and many had deformed wings. Wing deformation appears to be the result of lost haemolymph or poor brood nourishment (Koch and Ritter 1991). The mean weight of infested workers is 30% less at emergence than uninfested workers (Schatton 1987).

Worker bees infested with varroa have reduced haemolymph volume, total protein, and protein concentration during development (Weinberg and Madel 1985; Schatton and Engels 1988). Varroa use unaltered bee haemolymph proteins in their oocytes and newly laid eggs (Tewarson 1983). Infestations of more than three mites per bee reduce the size of the fat body cells, while one to three mites per bee reduce the number of protein granules per cell (Drescher and Schneider 1988). Schneider and Drescher (1987) claimed that feeding by varroa inhibits the development of the hypopharyngeal glands of nurse bees and, as a result, brood is not nourished adequately and emerging bees may weigh less. Feeding by varroa appears to have less effect on drone bee protein levels than worker bee protein levels (Weinberg and Madel 1985). Varroa also reduce the emergence weight of drones and the size of their seminal vesicles and mucous glands (Ritter 1988).

Colonies infested with varroa mites (and sometimes tracheal mites) exhibit “parasitic mite syndrome” (PMS) (Shimanuki *et al.* 1994). The many symptoms of PMS include reduced adult bee population, queen supersedure, spotty brood, bees with

damaged wings, and sick brood with symptoms resembling sacbrood and European and American foulbrood diseases.

Vector potential

A number of microorganisms and honey bee diseases have been associated with varroa. Bacteria, fungi, protozoans, and a virus have been directly observed on or in varroa following natural interaction with contaminants (Table 1). These microorganisms were found in the groove between varroa's anal shield and the dorsal shield, on the surface of the leg and various parts of the ventral shields (Liu and Ritter 1988; Liu 1996), on the dorsal shield and idiosomal setae (Alippi *et al.* 1995), as well as on the mouthparts (Strick and Madel 1988). The sizes of these (external) microorganisms range from 0.90 to 7.9 μm . Microorganisms and the virus were also found in varroa's alimentary tract (Glinski 1988), in the rectum and surrounding tissue, gastric caecae, and malpighian tubules (Liu and Ritter 1988), and in the haemolymph, salivary glands, and faeces (Strick and Madel 1988). The sizes of these (internal) microorganisms/particles range from 0.35 to 2.5 μm . Clearly, the ability of varroa to harbour and transport potentially harmful microorganisms is considerable. Open wounds created by varroa feeding may allow for invasion by infectious material (Glinski and Jarosz 1992; Sammataro 1997).

Acute bee paralysis virus (APV) is one of the pathogens most commonly associated with varroa infestation. In Europe, APV is commonly present at low levels in apparently healthy adult bees. In colonies severely infested with varroa, the virus has been documented as being the primary cause of adult bee and brood mortality (Ball 1988a). It is believed that worker bees infect larvae by secreting APV from the salivary

glands into the larval food (Bailey 1976), and that varroa acquires the virus by feeding on severely infected adult bees then transfers it to other bees (Ball 1988a). Foreign proteins from the digestive enzymes of varroa that are introduced into the bee's haemolymph may activate or stimulate replication of APV (Ball 1985; Ball and Allen 1988). It is not clear whether varroa can activate or stimulate replication of other microorganisms with which it is known to interact.

Anatomy

Protonymphs are ovoid-shaped and measure about 500 μm maximal body width. They have three pairs of sternal setae. Sex determination of protonymphs requires dissection or examination of chromosomes (Steiner 1988).

The deutonymphal tritosternum is larger than that of the protonymph. Deutonymphs have five or six pairs of sternal setae. Male and female deutonymphs are easily distinguished by their different size and body shape (Steiner 1988). Male deutonymphs are small, round, and have relatively long legs, while female deutonymphs are large, have an oval shape, a broad idiosoma (body), and relatively short pedes.

Adults have the same number of sternal setae and teeth per chelicera as deutonymphs (Steiner 1988). An adult female varroa (Fig. 2) is larger (1167 μm long by 1709 μm wide) (Anderson and Trueman 2000) than a female deutonymph. The adult female is reddish-brown, has a dorso-ventrally flat, ovoid body shape. Lightly pigmented female mites have an exocuticle that is not completely hardened and they die shortly after emergence with the honey bee (Calatayud and Verdu 1995). Chelicerae of adult males are not capable of sucking haemolymph (Steiner 1988).

Factors Regulating *Varroa* Population Growth

Season

Marcangeli *et al.* (1995) noted that the greatest rate of varroa population growth occurred in the summer. Delaplane (1998c) observed that 39.9% of the mites reproduce in August while 56.2% of the mites reproduce in July. According to Kulincevic and Rinderer (1988), varroa mites reproduce at their highest rate in August and early September. The method of assessing varroa populations influences results; for example, as colony brood levels decrease and mites move from the reproductive stage in cells to the phoretic stage on adults, sampling adults detects mites with greater frequency. Varroa cannot reproduce when there is no brood, which is usually the situation in winter (as well as early spring and late fall), in temperate climates.

Reinfestation from mites entering from other colonies is low in the spring and increases in the fall with increased drifting and robbing (Sakofski *et al.* 1990). Drifting workers as well as drones can carry varroa between colonies. Mite reinfestation is greatest when nectar is scarce and bees steal honey from other colonies (Greatti *et al.* 1992). During the winter, mites are restricted to feeding on adult bees mostly and may move from one bee to another (Bowen-Walker and Gunn 1998). Approximately 40% of mites die either naturally or because the host bee dies and the mite is unable to move to another bee host in winter (Korpela *et al.* 1992).

Temperature

The core nest temperature of a normal colony of *A. mellifera* is 30 to 35°C (Eischen 1997). Varroa mites prefer temperatures of 26 to 33.5°C (Rosenkranz 1988; Le Conte *et al.* 1990; Kraus *et al.* 1998) which occur at the periphery of the brood, where drone brood is usually located. Slabezki *et al.* (1991) claimed that varroa populations reach the highest levels in hotter climates, but noted that the results may not only be due to temperature but to the availability of nectar and colony strength. Woyke (1987) found that mites remain outside sealed brood for a shorter time in colder areas, resulting in a more rapid varroa population increase.

Relative humidity

Due to their small size, varroa can spare very little water before becoming desiccated (Bruce *et al.* 1997). Varroa is likely to seek a high relative humidity environment or a new host to replenish lost water when the temperature is high or the relative humidity is low. Level of humidity influences mite reproduction. At 59 to 68% relative humidity, an average of 53% of mites produce offspring, while at 79 to 85% relative humidity, the percentage of mites that produce offspring ranges from zero to six per cent (Kraus and Velthuis 1997). Female varroa rarely reproduce at levels above 80% relative humidity. The relative humidity in a brood nest of *A. mellifera* is normally about 50 to 80% (unpublished data).

Ascosphaera apis

Introduction

Chalkbrood is a fungal disease that infects honey bee larvae (De Jong 1977) and sometimes pupae (Gilliam *et al.* 1983). It is caused by the heterothallic fungus, *Ascosphaera apis*. Despite its high potential for infection, chalkbrood is often considered a “minor” disease and not seriously detrimental to colonies (Thomas and Luce 1972; Gilliam and Taber 1973). *Ascosphaera apis* is believed to be an opportunistic pathogen that kills honey bee larvae only when they are stressed (Bailey 1967; Gilliam 1986b; Koenig *et al.* 1987; Flores *et al.* 1996; Medina and Vicario Mejia 1999).

The fungus was originally named *Pericystis apis* (Maassen 1916; Claussen 1921), then the name was changed to *Ascosphaera apis* variety *apis* (Spiltoir and Olive 1955), and finally to *Ascosphaera apis*, its present name (Skou 1972). Thomas and Luce (1972) first published a report of chalkbrood disease occurring in honey bees in the U.S., in California, in 1968. It was observed in British Columbia and Saskatchewan, Canada, for the first time in 1971 (Gochnauer *et al.* 1972).

Life Cycle

Infection

It is not known whether infection of larvae with chalkbrood occurs when spores are ingested with food (Bailey 1981), contact the body surface (cuticle or spiracles) from comb, or both (Gilliam *et al.* 1978; Glinski 1981; Delaplane 1998a). Most entomopathogenic fungi develop on the cuticle (Steinhaus 1963), but in *Apis mellifera* the

peritrophic membrane of the stomach is not an effective barrier to infection, so oral infection is possible (Bamford and Heath 1989b). Gilliam *et al.* (1978) observed the fungus growing from the mouth, the anus, and through the cuticle of mouth-inoculated larvae. On back-inoculated larvae, the fungus first appeared aurally at the point of inoculation then throughout the entire body, and the body became mummified. De Jong (1977) observed one to 84% infection in larvae "challenged" with approximately 2,000 spores of the fungus.

Larvae may be exposed to infectious spores a number of ways. Southwick (1994) claimed that chalkbrood disease is spread in stored pollen and is transferred from the guts of nurse bees to larvae. The transfer of *A. apis* spores from workers to larvae is probably the most common route of infection; however, spores may also be present in the cells prior to the queen laying the egg. Spores of *A. apis* may remain viable for at least 15 years (Toumanoff 1951).

Larvae appear to be most susceptible to infection when they are three to four days of age (De Jong 1977), but larvae may be killed by the fungus when they are only one to four days of age, and larvae 4.5 to 5.5 days old are also susceptible (Gilliam *et al.* 1978). Bailey (1967) claimed that the mycelium survives better in a three to four day old larva than in a younger one because the mycelium is aerobic and is deprived of oxygen in the digestive tract for a shorter time than in younger larvae. Symptoms of the disease (mummification) become evident when mycelia of *A. apis* break out of the hind end of the host's body (Bailey 1967), when larvae are five to 10 days of age, soon after they are sealed in the cell (De Jong 1977; Gilliam *et al.* 1978).

There are three morphologically distinct stages of spore germination in *A. apis* (Heath and Gaze 1987; Bamford and Heath 1989b): activation, enlargement (or swelling), and germ tube production. Heath and Gaze (1987) showed the importance of carbon dioxide for spore activation, the first stage of germination, which is distinguished by changes in the spore coat which permits the entry of substances which were excluded during dormancy. At less than 5% CO₂ in air, about half the spores were activated, while at 12.5% CO₂, virtually all spores were activated. Thomas and Luce (1972) found that spores germinate but mycelia are not produced in anaerobic cultures. Bignell and Heath (1985) showed that the gut of a fifth-instar honey bee larva is aerobic, concluding that anaerobiosis is not the stimulus which causes ascospores to germinate in the gut. The observations of Gilliam *et al.* (1978), where the fungus grew on back-inoculated larvae, also seem to support Bignell and Heath. It is not clear whether the change in CO₂ level or perhaps a related change in O₂ level is responsible for these observations. In laboratory-raised larvae, Bamford and Heath (1989b) observed activated and enlarged spores 18 h post-inoculation and germ tubes 24 h post-inoculation.

The presence of chalkbrood spores alone may be insufficient to cause the disease (Bailey 1967; Heath 1982). Gilliam (1986b) claimed that *A. apis* may reside in honey bee colonies without causing overt signs of chalkbrood disease until the proper conditions exist.

Sexual interaction and development

Ascosphaera apis is a heterothallic organism. Sporulation occurs on the mycelium when two hyphae of opposite sex/mating type come together and a spore cyst

begins to develop (Thomas and Luce 1972). Spiltoir and Olive (1955) described the mating types and the interaction process. When male and female cultures are paired, the female mycelium produces an ascogonium, which is made up of a trichogyne, a nutriocyte, which later nourishes the developing ascogenous system, and a stalk-like base (Fig. 3). The male hyphae produces receptive papillae, often at the point of contact, but no sex organs. During sexual interaction, the trichogyne fuses with the male hypha but does not involve fusion of the nuclei (plasmogamy). Hyphal cells are partitioned by septae. After plasmogamy, a cylindrical mass of protoplasm, the primary ascogenous hypha, containing male and female nuclei, passes from the trichogyne into the nutriocyte, where it develops a thin wall around itself and grows rapidly to produce an ascogenous system of croziers and asci. Asci are produced by crozier proliferation within the nutriocyte. The asci become aggregated into balls and each ascus produces numerous (asco)spores. At this time, the much enlarged nutriocyte is nearly devoid of protoplasm and is now referred to as the spore cyst.

Dead larvae infected with *A. apis* become mummified as the fungal mycelia completely replace their bodies (Thomas and Luce 1972). At first, the fungus is restricted to the lumen of the hind end of the midgut (Heath 1982). By 48 h post-inoculation, hyphae grow from the lumen and penetrate the peritrophic membrane and epithelial cells of the gut wall (Bamford and Heath 1989b). Germ tubes within the ventricular lumen grow directly towards the epithelium rather than first filling the lumen with hyphae. Carrera *et al.* (1987) observed that the hyphae did not seem to have a preferential pathway and penetrated the next tissue in its path. Hyphae grow throughout the haemocoel after

the epithelial cells have been penetrated, growing around tracheal tubes rather than penetrating them. The hyphae attack the fat body and multiply into cysts late in the infection process (Gilliam 1986a). The fungus does not appear to penetrate the anterior portion of the body; the larval head is not mummified (Heath 1982). According to Glinski (1981), the fungus replicates in mummified and non-mummified larvae.

If opposite sex types interact (sexual reproduction), many spore balls within dark olive or brown spore cysts are formed (Southwick 1994) on the outside of the dead larvae (Bailey 1981). Spored (black) mummies may be covered with spore cysts containing 100 million (Bailey 1967) to 300 million (Nelson and Gochnauer 1982) spores. When mature, the spore cyst splits open and releases the spore balls (Thomas and Luce 1972). The spore balls in turn break down releasing the individual spores.

Mating type

Both mating types can infect and kill honey bee larvae (Gruszka 1998). The two mating types (thalli) of *A. apis* are often referred to as male and female or denoted as + and -. Claussen (1921) observed that spore balls of *A. apis* contain both + and - spores in equal quantities (1:1). Christensen and Gilliam (1983) found that the percentage of mummies with + only mating type predominated (46%) over - only mating type (14%) as well as mummies containing both + and - mating type (40%). Similarly, Rose *et al.* (1984) found that 44% of white mummies contain + mating type only while 27% contain - mating type only and 29% contain both + and - mating types.

There are a number of explanations for mummies containing a single mating type. One is that the larva ingested one spore or was invaded by the hyphae from one spore

(Rose *et al.* 1984). A second explanation is that the larva ingested many spores but one thallus was more vigorous and dominated or out-competed the other (Christensen and Gilliam 1983). A third explanation is that one mating type had an advantage over the other as a result of particular gut or external conditions. The first explanation is unlikely as many spores are usually required for mycosis by the fungus (De Jong 1977).

Damage

Chalkbrood can kill all larvae in a colony (Anderson 1938); however, this is extremely rare (Heath 1982). Most infected colonies have diseased larvae in less than 10 cells (Nelson *et al.* 1977). When 5% of the larvae are killed it may lead to a reduced honey crop (Mehr *et al.* 1976). The disease may also reduce winter survival of colonies due to a reduced proportion of young worker bees going into winter.

It is unclear exactly how the disease causes damage to honey bee hosts. Tissue-destroying enzymes (Alonso *et al.* 1993), not direct competition for primary nutrients (Gochnauer and Margetts 1979), result in the death of the host. Alonso *et al.* (1993) found evidence of enzymes such as N-acetyl-B-glucosamidase, which can break down molecules of N-acetyl-B-glucosamine, in 74.4% of *A. apis* isolates. Chitin, the main component of the cuticle, is a polymer composed of N-acetyl-B-glucosamine. The enzyme, N-acetyl-B-glucosamidase therefore, may act to pierce the cuticle with the help of pressure from the hyphae. Gilliam and Lorenz (1993) analyzed 19 enzymes produced by *A. apis* strains. All strains produced alkaline phosphatase, butyrate esterase, leucine aminopeptidase, acid phosphatase, and B-glucosidase. Enzyme activity appears to be

very important to the progression of the fungus and development of the disease.

Anatomy

Taxonomy of fungi is based mostly on vegetative and sexual features. The basic taxonomic characteristics used for classification among Ascosphaerales include the spore cyst (ascoma or ascocarp), spore ball (group of spores), spore (ascospore) dimensions, and spore cyst ornamentation (Maghrabi and Kish 1987). Each mature spore cyst may contain up to 100 spore balls (Mossadegh and Alizadeh 1995) with five to nine spore balls across the diameter of a cyst (Bissett *et al.* 1996). A spore cyst measures 47 to 140 μm and a spore ball measures 9 to 19 μm (Rose *et al.* 1984; Shimanuki and Knox 1991; Graham 1992). The cyst wall is thin and easily ruptured (Mossadegh and Alizadeh 1995). The brownish colour of the cyst gives sporulated mummies their dark appearance. Each spore ball is made up of hundreds of spores. Spores are hyaline (transparent), smooth, ellipsoidal and straight to slightly curved (Bissett *et al.* 1996). The size of a spore is about 1.4 x 2.5 μm (Rose *et al.* 1984; Maghrabi and Kish 1987; Bissett *et al.* 1996). Spores can be as long as 2.6 to 4.0 μm (Spiltoir and Olive 1955; Christensen and Gilliam 1983; Shimanuki and Knox 1991; Graham 1992).

Spiltoir and Olive (1955) reported that there were no important structural differences between male and female mycelia when they were maintained separately, and no sex organs were produced when they were apart. Neither the pigmentation nor growth rates are consistently different between all + and - strains (Heath 1982).

Factors Affecting Chalkbrood Development

Season

Chalkbrood is usually most evident in the spring (Bailey 1967). In the spring, colonies are growing rapidly and the ratio of brood to adult bees is the largest, and the disease may be promoted when larvae are chilled immediately after they are sealed in their cells. Similarly, in U.S. apiaries, chalkbrood was most prevalent in April, May, and June (Menapace and Wilson 1976), but De Jong (1977) indicated that chalkbrood peaks in July. Gilliam (1986b) reported that high levels of chalkbrood infection in November seem to be related to nutritional stress.

Temperature

Ascosphaera apis appears to be well adapted to life within the bee colony, where the temperature of the brood area is maintained at approximately 35°C (Bamford and Heath 1989a). The optimum temperature is 25°C for mummification (Flores *et al.* 1996), 30°C for cyst formation (Maurizio 1935), and 31-35°C for spore germination (Bamford and Heath 1989a). The minimum and maximum temperatures for growth of *A. apis* are 5°C and 42°C, respectively (Mossadegh and Alizadeh 1995) but the fungus can withstand freezing. Hale and Menapace (1980) observed normal growth of *A. apis* after exposure to -12°C for 12 months.

Temperature may affect larval susceptibility to the fungus. According to Bailey (1967) and Flores *et al.* (1996), larvae are most susceptible to chalkbrood if they ingest spores of *A. apis* when they are three to four days old and then are chilled briefly when five or six days old, immediately after being sealed in their cells. Bailey (1967) suggested

that chilling allows oxygen to enter the gut and reactivate the mycelia, which then continue to grow when the brood temperature is restored to 35°C. Flores *et al.* (1996) found that chalkbrood infection increased from 2.2 to 29%, when larvae were chilled. It has been suggested that chilling is the reason that affected larvae are usually found on the periphery of the brood area (Gilliam and Vandenberg 1997), but chilling alone is not the sole cause as mummies are often found throughout the brood.

Relative humidity

High humidity or moisture may favour chalkbrood infestation. Flores *et al.* (1996) found that maintaining sealed brood in an incubator at 30°C and 87% humidity induced a higher percentage of mummification (7.75%) than at 68% humidity (0.95%). De Jong (1977) and Herbert *et al.* (1987) stated that excessive hive moisture may be a factor which triggers *A. apis* development in a hive, yet a dry hive is not necessarily without chalkbrood.

Sources of infection

In colonies heavily infected with chalkbrood disease, many spore-covered mummies may be found in brood cells and on the bottom board. Spivak and Reuter (1998) observed an average (believed to be natural) infection of approximately one to 10 mummies per frame with capped brood in colonies in June and September. Worker bees remove mummies from brood cells and drop them onto the bottom board of the hive, sometimes concentrating them to one side or corner. There may be 500 to 1000 mummies on the bottom board of a hive. Eventually the mummies are usually moved out of the hive. Workers move the mummies with their mouthparts and likely spread the spores

further through food or physical contact unless the spores are filtered out via the proventricular valve of the honey stomach (Dustmann 1993).

Spivak and Gilliam (1998) stated that pollen is the major source of *A. apis*. Spores of *A. apis* have been found in pollen loads (Koenig *et al.* 1987), and healthy colonies that are fed pollen collected from colonies with chalkbrood may become infected with the disease (Menapace 1978). The fungal spores may also be found at water sources or on flowers (Koenig *et al.* 1987). The minute size of the spores makes them very easy to transport.

Spores of *A. apis* may be transferred by many means including by beekeepers and bees. Herbert *et al.* (1977) demonstrated transmission of chalkbrood by contaminated queens, adult workers, and unsealed or sealed brood. The spores may be carried on the outside as well as the inside of the honey bees. De Jong and Morse (1976) found *A. apis* in the honey sac of adult worker bees, and showed that spores are passed between adults and from brood-feeding adults to brood in food exchange. Nelson and Gochner (1982) isolated *A. apis* from the midgut of adult bees, but not from the honey sac. The fungus was not detected in ripened honey, freshly stored nectar, royal jelly, sterile swabs from empty brood cells after mummy removal, or in the gut of healthy pupae.

Other diseases

Herbert *et al.* (1987) and Shimanuki *et al.* (1992) stated that other diseases, such as European foulbrood (EFB), may weaken or stress a colony and make it more susceptible to chalkbrood disease. There also has been speculation that antibiotics such as oxytetracycline, which beekeepers use to control EFB or American foulbrood (AFB) in

their hives, may contribute to increased chalkbrood infestation. It has been suggested that antibiotics may disrupt the equilibrium of the intestinal flora and allow the fungus to develop; however, there is no research to support this hypothesis (Scott-Dupree 2000). Menapace and Wilson (1979) found that oxytetracycline did not inhibit or enhance chalkbrood infections in hives. Gochnauer *et al.* (1979) found that tetracycline had no effect on *A. apis* when placed on culture medium with the fungus.

Nutrition

Inadequate and old pollen may influence larval susceptibility to chalkbrood. De Jong (1976) claimed that colonies with inadequate pollen supplies were more susceptible to chalkbrood, although the basis for this claim was not clearly presented. Chu *et al.* (1992) thought that nutritional stress may be a predisposing factor for chalkbrood infection because starved larvae exhibited the highest rate of infection (Chu 1988).

Interaction Between Chalkbrood and *Varroa*

There is evidence that varroa promotes chalkbrood disease. Glinski (1988) found that the percentage of colonies with chalkbrood, increased from 13.5 to 52.3% in varroa-infested colonies, and from 10.0 to 18.8% in varroa-free colonies in approximately two years. Furthermore, the number of mummies per colony was 1.2 to 4.2 times greater in varroa-infested colonies than in colonies that were free of the mite. Glinski (1988) claimed that the ectoparasite may physically transfer spores throughout the hive, increase larval exposure to the chalkbrood spores by puncturing their protective cuticle, and impair the immune system of bees via stress caused by the mite. Glinski (1988) observed *A. apis*

on the body and inside varroa (Table 1). Similarly, Liu (1996) found up to 3,598 chalkbrood spores on the surface of the mite. In contrast, Medina and Vicario Mejia (1999) observed no correlation ($r=0.46$; $p=0.158$) between the number of varroa mites and chalkbrood mummies in normal and collapsing colonies. Medina and Vicario Mejia (1999) concluded that varroa could play an indirect role in increasing chalkbrood in collapsing colonies by increasing colony stress.

Chalkbrood transfer could also be enhanced by varroa mites that fall from honey bees to the bottom board. While varroa may acquire spores from any contaminated source, in colonies heavily infected with chalkbrood disease, the bottom board is often the greatest source of contamination. Fallen varroa may contact bottom board mummies and subsequently pick up infectious material on their dorsal and ventral surfaces as well as their legs (Table 1). Pettis and Shimanuki (1999) observed that approximately five living mites fell to the bottom board per day in June, while approximately 225 living mites fell to the bottom board per day in September. Consequently, these mites could vector the disease when they return to bees or brood to continue their life cycle

Ascosphaera apis spores may be transported from the surface of varroa to any place visited by the mite. A spore-contaminated foundress entering the cell of a five day old worker larva may expose the larva to disease for the first time. Immediately after entering the cell, the foundress goes underneath the bee larva to the bottom of the cell into the larval food (Boot *et al.* 1992) where she may spend up to 15 h in a worker cell (Ifantidis 1988), and during which time spores from the mite's surface could contaminate the food. At this point any food ingested by the immature bee could result in chalkbrood

infection, although it is not known how many spores are required to induce the disease symptoms. De Jong (1977) documented a wide range of mummification success with approximately 2000 spores. It is known that 4.5 to 5.5 day old larvae are susceptible to the fungus (Gilliam *et al.* 1978). Interestingly, the observation of Martin (1995), that mites entered and attempted to breed in cells containing larvae which subsequently died due to chalkbrood, may be explained by varroa-vectored infection.

Furthermore, it is probable that both mating types would be present among the thousands of spores that varroa can transport. Therefore, the resulting mummy would likely be sporulated and harbour hundreds of millions of infectious spores. A spored black mummy poses a greater future threat to the colony than a non-spored white mummy.

After being freed from the larval food, the foundress mite moves onto the larva to feed (Donze *et al.* 1998a) where it could ingest *A. apis*-infected haemolymph. There is also evidence that some "larval jelly" is directly consumed by varroa (De Jong 1997). Once inside the mite and following emergence, it seems possible that *A. apis* spores could be transmitted to, or between, adult workers in the same way that varroa transmit viral and bacterial agents (Ball 1988b; Glinski and Jarosz 1990). Microbial spores have been observed to cause infection when introduced with haemolymph into healthy bees (Sokolov and Grobov 1963). The emerged foundress mite has a great potential to spread chalkbrood as it may enter up to two more host cells to complete two more reproductive cycles before it dies (Martin and Kemp 1997).

It is unclear how daughter mites might interact with the fungus given the progression of the fungus at the time the first daughter begins to feed on the host bee. The first female varroa egg hatches to a feeding protonymph approximately seven days after the mother enters the cell of the five day old larva; [20 h to cell capping (Boot *et al.* 1992) + 60 h for first egg (Ifantidis 1983) + 30 h for first female egg (Donze *et al.* 1998a) + 48 h for female egg to hatch (De Jong *et al.* 1982)]. De Jong (1977) and Gilliam *et al.* (1978) claimed that development of the fungus in the host becomes evident when the larvae are five to 10 days of age, soon after they are sealed in the cell, while (Bamford and Heath 1989b) found that the hyphae of *A. apis* began growing from the lumen and penetrate the epithelial cells of the gut wall at 48 h post-inoculation. Since the bee will have developed into a pupa by the time the first daughter begins feeding, there does not appear to be a window of opportunity for daughter varroa, if carrying the fungus, to infect the developing bee with chalkbrood when she feeds. However, there may be an opportunity for progeny mites to acquire *A. apis* (mycelia or spores), depending on the progression of the fungus in the host at the time of feeding.

Varroa may also promote chalkbrood disease by impacting the nutritional state of the host bee. The reduced total protein, protein concentration (Weinberg and Madel 1985; Schatton and Engels 1988), and number of protein granules per cell (Drescher and Schneider 1988) resulting from varroa feeding and direct use of unaltered proteins (Tewarson 1983) may cause nutritional stress which is believed to make larvae more susceptible to chalkbrood infection (De Jong 1976; Chu 1988; Chu *et al.* 1992).

Diminished nutritional health may also hinder larval defense against the fungus (Chu

1988).

The reduced worker populations in varroa infested colonies (Shimanuki *et al.* 1994; De Jong 1997) likely increases the level of chalkbrood disease. Higher worker mortality caused by feeding varroa would reduce the cluster size, increase the ratio of brood to workers, and expose outer brood to cooler conditions. Chilled brood may then become infected with the fungus which is usually present and believed to be opportunistic. Furthermore, the indirect elimination of bees caused by other varroa-promoted diseases could amplify the effect and lead to even more colony stress.

There is no evidence to suggest that *A. apis* coevolved with *V. destructor*. The fungus and the mite do not have a long documented history of association. One white mummy containing the fungus which was obtained from a drone cell of a colony of varroa's original host, *Apis cerana*, is believed to have been infected after the introduction of infected *A. mellifera* (Gilliam *et al.* 1993).

Interaction Between Chalkbrood and Formic Acid

There is no registered control agent for use against chalkbrood disease in the U.S. (Vaughan and Shimanuki 1997) or Canada (Scott-Dupree 2000). Ethylene oxide is registered by the U.S. E.P.A. for general treatment of diseased beehives. Fumigation of infected combs with the compound kills the fungus (Thomas and Luce 1972); however, the mode of action of ethylene oxide against chalkbrood disease is not known.

Acidic conditions are known to affect *A. apis* spore germination. Bamford and

Heath (1989a) found that lower pH's, equivalent to those found in honey (3.2 to 4.5), pollen (3.6 to 4.6), and brood food (4.0 to 4.3), drastically reduced spore enlargement and germ-tube production. Activation, enlargement and germ-tube production were independent of pH between 5.0 and 7.8, equivalent to the acidity of larval haemolymph and gut contents.

It appears that *A. apis* spores might be vulnerable to acid treatment, but it is not known how vulnerable, or whether reduced pH also affects mycelial development. Flores *et al.* (1995) treated spores of *A. apis* with trichloroisocyanuric acid inside honey bee hives then cultured the fungus *in vitro* and claimed that efficacy of the treatment was dependent on the number of spores and the degree of agglutination in spore balls. The fungus is probably similarly susceptible to formic acid vapours on the bottom board and in sealed brood cells as formic acid is known to have an effect on the contents of sealed brood cells. Calderon *et al.* (2000) observed up to 77% effectiveness against varroa mites in capped brood cells of Africanized honey bees. It is important to note that *A. apis* spores are well protected by their thick outer cover and highly resistant to surrounding conditions (Southwick 1994), and it is not known what effect formic acid vapours may have on *A. apis* within a larva. Finally, the possibility that formic acid could stress young larvae (Bolli *et al.* 1993) or older larvae (Fries 1991), such that chalkbrood infection may be more likely, must also be considered.

Nosema apis

Introduction

Nosema disease is caused by the microsporidian endoparasite, *Nosema apis*. It infects the epithelial cells of the ventriculus (midgut) of adult honey bees and is generally considered a serious disease (Fries 1988b; Delaplane 1998b). Colonies infected with *N. apis* are less productive (Cantwell and Shimanuki 1969; Fries *et al.* 1984), show an increased rate of winter loss (Fries 1988a), and have increased queen supersedure (Farrar 1947).

Nosema apis has a very wide distribution. The disease has been recorded in North, Central, and South America, the Caribbean, Europe, Africa, Asia, and Australia (Matheson 1997).

Life Cycle

There appear to be two types of *N. apis* spores (Fries *et al.* 1992; Graaf *et al.* 1994). One type of spore, adapted to survive outside the host bee, is durable and has a thick endospore. These durable spores are primarily responsible for transmitting the disease between host bees and exhibit extracellular germination believed to be triggered by digestive fluids of the intestine (Bailey 1955). Spores of the second type have a relatively thin endospore and are believed to be responsible for multiplication of the endoparasite in the epithelium of bees (Fries *et al.* 1992). Germination of these spores is intracellular.

A honey bee becomes infected with *N. apis* when it ingests spores (Fries 1988b).

The spread of vegetative stages (merozoite) of *N. apis* between cells is not likely (Fries 1989). Fries (1988b) found that an average of 94.3 spores are required to infect a honey bee (29 to 344 spores; 95% c.i.). El-Shemy and Pickard (1989) found no spores in workers less than eight days of age while 70% of bees of 22 days of age and older contained spores of the protozoan.

Development of the protozoan is intracellular and probably tissue specific (Fries 1989). It is generally accepted that midgut epithelial cells of adults are commonly attacked by *N. apis*; however, Gilliam and Shimanuki (1967) observed attack of larval haemolymph cells by the protozoan on a slide in a laboratory bioassay. *Nosema apis* spores have been found in the haemolymph, pharyngeal gland, and thoracic gland of infected bees. And healthy bees can become infected with nosema disease when spores of the protozoan are introduced into the bee with haemolymph (Sokolov and Grobov 1963).

The developmental stages of *N. apis* are as follows: first merogony, second merogony, sporogony, sporoblasts, then spores (Fries 1989). Merozoites are produced via cytokinesis division in the host cytoplasm within 24 h of infection (Fries *et al.* 1992). The merozoites mature into sporonts, each sporont divides into two sporoblasts, each sporoblast matures into the final stage of development, the spore (Fig. 4) (Fries 1989). All developmental stages of *N. apis* contain two nuclei and are called diplokaryotic (Gray *et al.* 1969).

The life cycle of *N. apis* is not fully understood and could possibly be asexual as there is no sign of meiotic activity (Fries 1989). The endoparasite completes

development from ingestion of spores to production of new spores within 48 to 60 h (Kellner 1980). Spores of the parasite germinate when stimulated by digestive fluid, shortly after entering the ventriculus (Bailey 1955). Olsen *et al.* (1986) found that germination of epithelial spores including sporoplasm formation is completed within three seconds. During germination, a hollow polar filament, which is coiled within the spore, is discharged. It may penetrate an epithelial cell if it is thrown out in the right direction and close enough to the ventricular epithelium (Fries 1988b). Epithelial cells of the ventriculus are infected when the vegetative stage is introduced via the polar filament (Bailey 1955). The sporoplasm enters the host cell through the elastic hollow polar filament and receives a plasma membrane during this process (Weidner *et al.* 1984). The sporoplasm's plasma membrane may come from the spore polaroplast organelle membrane (Fig. 5).

Infection is usually initiated in the posterior part of the ventriculus then spreads to the anterior part (Fries 1988b). This may be due to increased probability of infection at the narrow junction of the ventriculus and proctodeum (posterior end) where the polar filament is more likely to penetrate an epithelial cell. In heavily infected midguts, all epithelial cells may contain spores of *N. apis* (Liu 1984). The parasite is probably able to spread from one epithelial cell to another (Fries 1989). Infected epithelial cells become distended and are voided into the gut lumen (Bailey 1981). Uninfected cells are also shed into the gut (Liu 1990). The breakdown of the voided cell appears to be due to an enzymatic process. The newly formed spores then germinate and spread the infection throughout the gut. Spores which have extruded their filament and sporoplasm are

termed "empty". Hartwig (1976) suggested that bacterial flora are involved in the development of *N. apis*.

The number of spores per bee peaks within two to four weeks post infection (Cantwell and Shimanuki 1969; Fries 1988c). The guts of infected honey bees may each contain 30 to 50 million spores (Bailey 1981). Individual feral honey bees have been found to contain one to five spores of *N. apis* (Gilliam and Taber 1991), which would probably be undetected in most studies.

The natural turnover of adult bees lowers *N. apis* infection in the colony due to reduced exposure to the disease (Moeller 1972; Malone *et al.* 1992). Newly emerged bees are subjected to lower levels of spores in the hive and thus sustain lower infections.

Damage

Nosema disease damages colony performance and individual bees. Fries *et al.* (1984) observed that honey yield was reduced by 50% at severe infections of 15 million spores per bee where 48% bees were infected. Similar devastating effects were noted by Pickard and El-Shemy (1989) who observed that infection levels of greater than 50% were associated with colony death and debilitation. Colonies fed spores of *N. apis* in sucrose syrup collected almost half as many pollen pellets as control colonies (Anderson and Giacon 1992). The reduction in pollen collection is likely due to diminished colony size as a result of nosema infection.

Reduced longevity is a serious effect of nosema on host workers. The lifespan of individually infected bees is 32 to 60% as long as noninfected bees (Hassanein 1953).

Overwinter loss of colonies is related to nosema disease (Fries 1988a; Graham 1992). In general, a greater proportion of worker bees become infected with *N. apis* than drones or queens (Bailey 1972). This is likely the result of increased exposure due to the cleaning activities of young house worker bees.

Nosema is also linked to queen supersedure and 50 to 100% of queens with nosema may be superseded (Farrar 1947; Furgala 1962). The replacement is probably due to the queen being weakened by the disease and not laying eggs sufficiently or maintaining adequate control over her colony. Heavily infected queens produce eggs that shrivel and fail to hatch. Nosema-inoculated queens lay eggs normally for one to seven weeks, then cease suddenly (Farrar 1947). Liu (1992) reported that oocytes in the ovary of queen honey bees degenerate after seven days of infection by *N. apis*, with evidence of resorption, and soon after she is superseded. The resorption process may be due to starvation or competition between oocytes for available protein. Nosema-infected queens become sluggish and may remain in the hive for several days before dying, or they may crawl, or be carried by workers out of the hive to die (Farrar 1947).

Pathological changes to the hypopharyngeal glands in worker bees infected with *N. apis* (Wang and Moeller 1971) appear to be responsible for reduced brood rearing (Farrar 1947; Moeller 1962). Moeller (1962) observed that infected colonies produce 12% less sealed brood than uninfected colonies. *Nosema apis* may disintegrate the cytoplasm of the glands which secrete royal jelly, which is a component of brood (Wang and Moeller 1971).

Workers infected with nosema disease start their orientation flights, guard duties

(Wang 1969), and foraging at an earlier age than healthy workers (Hassanein 1953). Adult bees infected with *N. apis* start foraging approximately 10 days earlier (day 7) than normal bees (day 17). These findings support the idea that colony labour is divided according to worker life expectancy, where workers with shorter life expectancy do riskier tasks such as foraging. It is also possible that younger workers take up the task because of older workers dying from infection with the protozoan. Structural changes to the corpora allata may cause infected bees to forage at an earlier age than healthy bees. Corpora allata cells of nosema-infected bees have electron-dense chromatin material in the nucleus, mitochondria are smaller, and neurosecretory granules are often absent in the axon (Liu 1986). The corpora allata produce juvenile hormone which regulates bee foraging behaviour.

Heavy infections of nosema disease are often correlated with dysentery (Fries 1997) and faeces spotted on the front of the hive (Delaplane 1998b). Identifying dysentery is not a good way to diagnose nosema disease (Bailey 1981), as faeces may or may not contain *N. apis* spores (Delaplane 1998b). If faeces are contaminated with spores, then dysentery can quickly spread *N. apis* throughout the hive. Therefore, conditions which promote dysentery, such as feeding low quality sugars, should be minimized.

Many other physiological changes to the host bee have been linked to nosema disease infection. The disease has been associated with a reduction in total protein, lipids, glucose and fructose content in the haemolymph of infected bees (Tomaszewska 1979). Infection with the disease reduces the ability of the bee to store protein in the fat

body (Lotmar 1939). Liu (1984) found a lack of zymogen granules and aggregation of ribosomes in the infected host cell indicating cessation of digestive enzyme secretion in infected bees. Craig *et al.* (1989) observed unidentified proteins in the haemolymph of the honey bee in response to *N. apis*, perhaps created by physiological reactions to parasitism, damage of the midgut epithelium, or products from *N. apis*. Zhrebkin (1976) reported a significant relationship between the activity of the enzyme chymosin and the percentage of bees with nosema disease.

Anatomy

The taxonomic status of microsporidian spores, including spores of *N. apis*, is most commonly determined by the size and shape of the spore, host and tissue specificity, and the number of sporoblasts produced from a sporont (Weidner 1970). Scanning electron microscopy and freeze-etching techniques are also used (Liu and Liu 1974). The structure of spores of *N. apis* observed in queens is similar to that observed in worker bees (Liu 1984).

Mature spores of *N. apis* are egg-shaped (ovoid) with pointed anterior poles (Liu and Liu 1974). The size of a spore of *N. apis* is 4 to 6 μm long by 2 to 4 μm wide (Liu 1984; Shimanuki and Knox 1991; Graham 1992; Fries 1993) and, according to Liu (1984), immature spores are larger than mature spores. Freeze-etching revealed that the most distinct internal feature is the shape and arrangement of the polaroplast and polar filament (Liu and Liu 1974). The polar filament is connected to the inner side of the apical portion of the spore wall by the anchoring disc (Kellner *et al.* 1983). Liu (1984)

observed more than 32 coils of the polar filament. It may be as long as 400 μm (Morgenthaler 1963). The sporoplasm, the germ of the spore, includes the cytoplasm and the nucleus material (Vavra and Sprague 1976).

The spore wall is triple-layered and consists of the exospore, an outer electron dense layer, the endospore, an electron transport middle layer, and the plasma membrane (Kellner *et al.* 1983). The endospore is thicker than the exospore and is comprised of two layers, the inner layer being much thinner and electron dense (Shabanov 1973). The endospore may contain chitin (Liu 1973). The plasma (cytoplasmic) membrane is the lining of the sporoplasm and consists of two membranes.

Factors Affecting Nosema Development

Season

A typical nosema-infected colony has high nosema levels in the spring, low levels during the summer, a small peak in the fall and a slow rise of infection during the winter (Fries 1988c). The level of infection increases rapidly in the spring as brood-rearing starts while flight and foraging are still limited. Dyess and Wilson (1978), Hassanein (1953), and Doull and Cellier (1961) observed a similar seasonal trend; however, there is some discrepancy regarding overwinter infection. Pickard and El-Shemy (1989) claimed that there was no absolute annual cycle for nosema infection levels; instead there was a cycle of relative infection levels. Often the greatest rate increase of infection occurs at the beginning of spring while the greatest rate decrease of infection occurs at the end of the spring.

Temperature

Production of *N. apis* spores in the honey bee ventriculus is strongly dependent on environmental factors. Anderson (1990) observed that nosema disease was more common in the wetter, windward than hot, dry, leeward regions, in Fiji. According to Lotmar (1944), development of the disease is inhibited in workers at temperatures below 30°C and above 35°C. In contrast, Woyciechowski and Czekonska (1999) found that infection of *N. apis* was higher at 25°C than 35°C. Cantwell and Lehnert (1968) observed a sharp reduction in the infectivity of nosema spores heated above 55°C in a moist environment.

Incidence of nosema may be related to colony activity, which is affected by environmental conditions. Levels of the disease may increase when infected foraging bees, that carry more spores than other bees in the colony, are confined to the colony (Anderson 1990). Infected bees may spread the disease via trophalaxis or by defecating inside rather than outside the hive. Conversely, in drier, warmer regions, infected foraging bees may fly longer and die quicker, possibly reducing exposure of the colony to the disease. Levels of the disease are also relatively high in places such as Canada, where winter is relatively long, and colonies are confined for several months without the benefit of cleansing flights (Szabo and Heikel 1987).

Sources of infection

The primary source of *N. apis* spores is faecal matter on contaminated comb which younger bees ingest when they clean comb (Bailey 1981). Fries (1988a) observed that old comb increased nosema disease, and wax renewal helped control the disease.

Since the faecal matter of diseased bees contains infectious spores, hive confinement, without defecation flights, greatly aggravates nosema disease (Moeller 1972; Pickard and El-Shemy 1989). Like chalkbrood, nosema disease can infect honey bees at water collecting sites (Fries 1997). The disease can be spread from any contaminated source to bees, then passed between bees.

There is a higher incidence of nosema disease in the forager and guard bees at the entrance and top of the hive than other colony workers (Wang and Moeller 1969). El-Shemy and Pickard (1989) found the following relationship between bee position and number of spores per bee: flying bees (8.93×10^6), entrance bees (6.77×10^6), and central comb bees (0.31×10^6). Less infection near the brood and queen, during warm weather when bees are flying freely, may serve to protect the queen from nosema infection. In the winter, Moeller (1956) observed that nosema-infected bees congregate in the warm parts of the cluster in and immediately above the brood area, perhaps increasing the queen's exposure to the disease.

Other diseases

Nosema has been associated with a number of diseases and microorganisms. *Nosema apis* has been associated with the amoeba, *Malpighamoeba mellificae* (Liu *et al.* 1987), the bacteria, *Hafnia alvei* (Grobov 1974), and many viruses including Kashmir bee virus (Anderson 1990), bee virus Y (Bailey *et al.* 1983), black queen cell virus, filamentous virus, and chronic paralysis virus (Grobov 1974). In some situations, it appears that infection with *N. apis* increases susceptibility to other diseases (Grobov 1974; Bailey *et al.* 1983; Liu *et al.* 1987).

Nutrition

The protein supply of the host also appears to affect nosema development. Providing adequate pollen to colonies is part of good management to combat the effects of nosema disease (Graham 1992). Supplemental feeding of high quality pollens reduces the level of infection probably by increasing the number of honey bees and promoting stronger colonies (Kleinschmidt and Furguson 1989).

Interaction Between *Nosema* and *Varroa*

The evidence that varroa promotes nosema is not as strong as that which suggests promotion of chalkbrood by the mite. *Nosema apis* has never been found within varroa. Spores of the protozoan, however, have been found on the cuticle of varroa (Liu 1996), demonstrating clear interaction of the two organisms. Orantes Bermejo and Garcia Fernandez (1997) observed that colonies with the highest infestation of varroa had more nosema spores per individual bee, though the results were not statistically significant.

There is little overlap in the life cycles of *V. destructor* and *N. apis*. Varroa prefer adult bees around two days of age (Le Conte and Arnold 1987) while *N. apis* does not infect adults under eight days old (El-Shemy and Pickard 1989). Most nosema-infected adults are 22 days of age and older. Gontarski (1951) tried unsuccessfully to infect young adults up to four days of age. In fact, nosema disease is mostly concentrated in foragers, the oldest bees. Perhaps *N. apis* has never been found in varroa because the mite generally feeds on adults with little or no nosema infection. There is also no exposure of varroa to the protozoan from immature bees. Although Gilliam and Shimanuki (1967)

observed that *N. apis* attacked larval haemocytes when spores are added to larval haemolymph on a microscope slide, the disease has only been documented in adult bees.

The size of a *N. apis* spore (4.0 to 6.0 μm by 2.0 to 4.0 μm) (Liu 1984; Graham 1992; Fries 1993) may prevent its uptake by *V. destructor* during feeding.

Microorganism spores which have been found within the mite range in size from 0.35 to 2.5 μm (Glinski 1988; Liu and Ritter 1988), suggesting an inability of the mite to ingest substances the size of a *N. apis* spore (Table 1). The literature suggests that varroa could transfer the disease with haemolymph if the mite was able to ingest the spore (Sokolov and Grobov 1963).

Like chalkbrood, nosema disease is associated with stress conditions. Reduced bee haemolymph protein due to varroa feeding (Weinberg and Madel 1985; Schatton and Engels 1988) may create conditions of nutritional stress believed to favour progression of nosema disease. Furthermore, because *N. apis* also negatively impacts the nutritional status of the bee (Lotmar 1939; Tomaszewska 1979), the amount of stress on the nutritional state is compounded when both parasites are present in the colony. It is also believed that feeding causes general stress directly to the bee and that the mite promotes other diseases which stress the colony.

Nosema apis was observed in *Apis cerana* from Thailand in 1989-1990 (Yakobson *et al.* 1992). The history of the relationship between *N. apis* and *A. cerana* is not well documented and it is not known if there are any evolutionary ties between *N. apis* and *V. destructor*.

Interaction Between *Nosema* and Formic Acid

There is only one product registered in Canada for controlling nosema disease. Fumagillin is the active ingredient and Fumidil B® is the trade name of the product. Fumagillin is an antibiotic that provides excellent control of nosema disease with low worker bee toxicity (Moffett *et al.* 1969) and no significant affect on queen survival (Webster 1994). The precise action of the drug on the host and parasite is not known; however, fumagillin inhibits the replication of DNA (Hartwig and Przelecka 1971) and RNA (Jaronski 1972), and may alter fatty acid metabolism (Liu 1973). The antibiotic is effective against the nosema parasite but not effective against spores deposited in faecal matter throughout the hive (Wyborn and McCutcheon 1987).

It appears that *N. apis* is sheltered from acidic ambient conditions by the midgut of adult honey bees. As an adult bee grows older, its midgut pH decreases. The pH of the midgut of an emerging bee is about 7.5, about 6.5 in a 10 day old bee, and about 4.9 in a 36 day old bee (Gontarski 1951). The alimentary canal of a honey bee has a strong buffer capacity, and it is very difficult to influence the pH environment of its gut. Due to this "extraordinarily stable" gut pH, development of the parasite is not effected by feeding an acidic or alkaline sugar solution. Once inside the bee, a spore of *N. apis* is essentially protected from any adverse conditions unless the bee dies.

The effects of acids on *N. apis* infectivity have been examined in two previous studies. Formic acid fumigation was treated against exposed *N. apis* and there was no effect of the acid on spore infectivity. Fries (1990) treated faeces-stained combs with four applications of 15 mL 85% formic acid from below and 24 h between treatments.

Bees were then fed treated spore suspensions and became infected with *Nosema* disease. In another study, acetic acid was tested against the protozoan and found that the acid can prevent infection. Ruijter and Steen (1989) fumigated exposed *N. apis* spores with 100 mL 96% acetic acid on cotton wool from above and observed that the spores did not induce infection following this treatment. In the same study, fumigation of the acetic acid from a dish did not affect spore infectivity. These mixed results of acids on the infectivity of exposed spores suggest that the protozoan is vulnerable to acid treatment but that the type of acid, dose, and/or method of application may influence results.

Formic Acid

Introduction

Formic acid is a relatively inexpensive treatment that is gaining interest as varroa resistance to fluvalinate (Elzen *et al.* 1999) and coumaphos (Elzen and Westervelt 2002) increases. Formic acid has been used in Europe to control varroa since the late 1970's (Liu 1991a). Formic acid is the only product which controls varroa in capped brood as well as on bees (Fries 1991; Calderon *et al.* 2000), although releasing constant amounts of other acaricides over an extended period also solves the problem of exposing mites in sealed brood to treatment (Koeniger and Fuchs 1988).

Formic acid is a natural component of honey. Liu *et al.* (1993) found that the naturally occurring formic acid content in honey varies with the floral source and may range from 9 to 152 parts per million (ppm) (0.2 to 3.3 $\mu\text{mol/g}$ honey). Honey in hives treated for tracheal mite control with 22 g of 65% formic acid per super, repeated three

times at one week intervals, may contain up to 492 ppm (10.7 $\mu\text{mol/g}$ honey).

The mode of action of varroa control via formic acid is not known for certain; however, Bolli *et al.* (1993) found that it inhibited oxygen consumption of varroa mites as well as young larvae *in vitro*.

Depending on the formulation, formic acid concentrations in the hive usually range from zero to 100 ppm (Feldlaufer *et al.* 1997; Skinner *et al.* 2001; Ostermann Chapter 3). Short exposure to a concentration of about 80 ppm (0.16 mg/L air) formic acid maximizes varroa control and minimizes honey bee mortality. Underwood and Currie (2002b) observed approximately 2% mite survival and about 67% worker bee survival after two days of exposure to 80 ppm formic acid at 35°C under laboratory conditions.

Efficacy and Application Methods

Results of formic acid treatments are often highly variable. Efficacy of formic acid against the mite vary from 51 to 100% (Hoppe *et al.* 1989; Fries 1991; Feldlaufer *et al.* 1997; Calis *et al.* 1998; Eischen 1998; Calderone 1999b; Calderone and Nasr 1999). Factors that may affect the efficacy of a formic acid treatment include method of application (Feldlaufer *et al.* 1997), positioning of the acid (Hoppe *et al.* 1989), and ambient temperature (Von Posern 1988; Bracey and Fischer 1989; Feldlaufer *et al.* 1997; Calderone 1999b). Although Liu (1991a) stated that the amount of formic acid vaporization is relatively unaffected by temperature, levels of formic acid in colonies do increase during the warmer parts of the day and drop during the cooler parts of the day

(Feldlaufer *et al.* 1997). According to Von Posern (1988), low temperatures reduce the efficacy of the acid, while high temperatures with formic acid kill bees and the queen.

Methods of applying formic acid include short-term repeated liquid application (Manitoba Agriculture and Food 1999), long-term slow release liquid application (Gatien and Currie, unpublished data), and long-term slow release gel application (Feldlaufer *et al.* 1997).

Disadvantages

Formic acid may have a negative impact on the well-being of the honey bee colony. Bee mortality of up to 30% has been reported with formic acid treatment (Von Posern 1988). Larvae appear to be most vulnerable to formic acid. Bolli *et al.* (1993) found that young larvae were most susceptible to formic acid due to inhibited oxygen consumption which may then cause larval feeding and brood care to decrease. Fries (1991) found that old larvae, during developmental transition from stretch larva to pupa, were most susceptible to formic acid exposure. Von Posern (1988) reported mortality of five and 10% of queens with formic acid treatment. DeGuzman *et al.* (1999) found that formic acid reduced drone brood survival and production. In contrast, Hoppe *et al.* (1989) observed no lasting damage to bees or brood, and no loss of queens.

Formic acid is also very dangerous to handle. It can burn the skin and damage the respiratory system of the handler. Safety precautions must be taken and the treatment must be applied using the proper equipment. Metal parts exposed to formic acid can become corroded (Von Posern 1988).

Summary

There is clear evidence that varroa interacts with microorganisms including *N. apis* and *A. apis* (Glinski 1988; Liu 1996). The life cycle of varroa, which includes feeding periods on both immature and mature honey bees, overlaps in time and place with the life cycles of both the adult disease protozoan, *N. apis*, and larval disease fungus, *A. apis*. The evidence for promotion of chalkbrood by varroa is strong, while the relatively large size of a spore of *N. apis* may limit its spread by the mite.

There is no clear effect of formic acid on nosema or chalkbrood infection, nor is it clear what causes the great variation in concentration of formic acid in hives and wide range of efficacy against varroa in colonies which are treated with the same formic acid application (Feldlaufer *et al.* 1997; Calderone 1999b). It is believed that spores of *N. apis* and *A. apis* are protected from formic acid treatment once inside the bee but may be vulnerable when exposed on the hive substrates depending on application. Ambient conditions may influence formic acid hive concentration which in turn affects varroa infestation level and may indirectly affect colony disease development and promotion by the mite.

The objectives of this thesis were to assess the interaction between formic acid treatment and varroa infestation on colony size, to assess the effects of treatments, hive and ambient factors on formic acid concentration in hive air, to assess the potential for varroa to increase chalkbrood and/or nosema disease in a honey bee colony, and to assess whether formic acid might directly or indirectly affect chalkbrood and/or nosema infections.

Table 1. Microorganisms and a virus on/in varroa following natural interaction*.

Microorganism/ Particle	Tissue	Microorganism/ Particle Size	Amount of Microorganism/ Particle	Location	Reference
<i>Ascospaera apis</i>	varroa cuticle	1.4x2.5 μ m (Maghrabi and Kish 1987) (spore)	up to 3,598 spores per mite	Canada	Liu 1996
<i>Ascospaera apis</i>	varroa body surface and alimentary tract	1.4x2.5 μ m (Maghrabi and Kish 1987) (spore)		Poland	Glinski 1988
<i>Ascospaera apis</i>	varroa cuticle	1.4x2.5 μ m (Maghrabi and Kish 1987) (spore)			Puerta <i>et al.</i> 1990
<i>Ascospaera</i> - like	varroa cuticle; in groove between the anal shield and the dorsal shield	approx. 0.9x2.5 μ m (spore)	1 ball	Brazil and Germany	Liu and Ritter 1988
<i>Aspergillus</i> - like (fungus)	varroa cuticle; on surface of the legs and the ventral surfaces of the mite	2.5-7.9 μ m dia. (spore)		Brazil	Liu and Ritter 1988
fungus	penetrating the varroa cuticle	(mycelium)	network		Liu and Ritter 1988
<i>Nosema apis</i>	varroa cuticle	(spore)		Canada	Liu 1996
<i>Rickettsia</i> -like (protozoan)	varroa rectum and surrounding tissue	0.35-0.46 x 0.75-1.63 μ m		Germany	Liu and Ritter 1988

Microorganism	Tissue	Microorganism Size	Amount of Microorganism	Location	Reference
<i>Paenibacillus larvae</i> White (AFB/bacteria)	varroa dorsal shield and idiosomal setae	approx. 1.2x2.7 μm (spore)		Argentina	Alippi <i>et al.</i> 1995
<i>Serratia marcescens</i> Bizio (bacteria)	varroa integument and internally		integument: up to 150 cells internally: up to 61,200 cells ²		Glinski and Jarosz 1990
bacteria	varroa gastric caecae	0.41-0.62 x 0.80-2.3 μm (spore)		Germany	Liu and Ritter 1988
bacteria	varroa malpighian tubules	0.41-0.65 x 1.1-2.4 μm (spore)		Germany	Liu and Ritter 1988
Acute paralysis virus	varroa		55-1259 ng APV/mite (where 1×10^{-5} ng sufficient for bee infection)	Germany	Ball 1988b

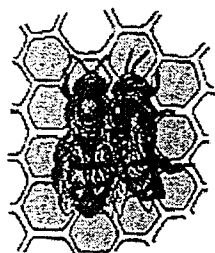
*where natural interaction refers to (1) varroa from colony and examined, (2) varroa from artificially injected or fed (a microorganism) mature or immature bees, and examined.

¹ brown-eyed worker pupae were injected with 3 μL of a *Hafnia* suspension ($15\text{-}20 \times 10^4$ cells/ μL) through the dorsal thorax then fed upon by adult varroa (Strick and Madel 1988).

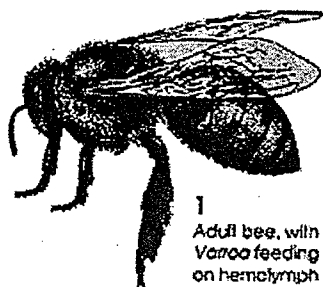
² adult worker honey bees were starved for 8 h then fed sugar containing about 20,000 *S. marcescens* cells/mL. The number of *S. marcescens* on the body surface or inside varroa was evaluated by agar plate colony count after 36 h of mite feeding (Glinski and Jarosz 1990).

Fig. 1. Life cycle of *Varroa destructor* (Fries 1997).

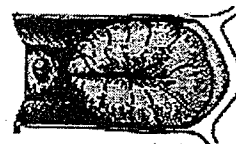
Illustrated by B. Alexander



10
Mites transfer through
close contact between bees.



1
Adult bee, with
Varroa feeding
on hemolymph



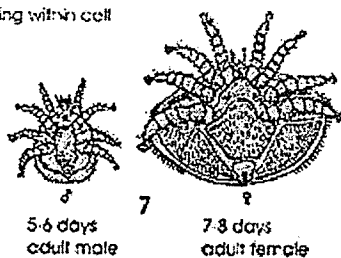
2
Mite enters cell with
larva of 5 to 5-1/2 days.



9
Adult females leave cell
with emerging bee.
Male and immature stages
stay in cell.

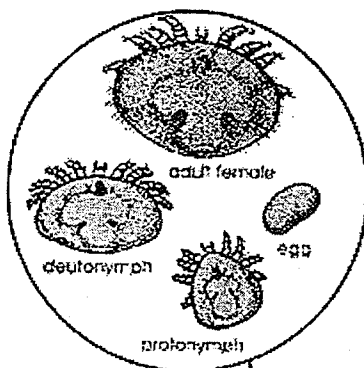


8
Mating within cell



5-6 days
adult male

7-8 days
adult female



6
1-6 eggs developing
from egg to larva
to protonymph to
deutonymph



3
Mite in bee food

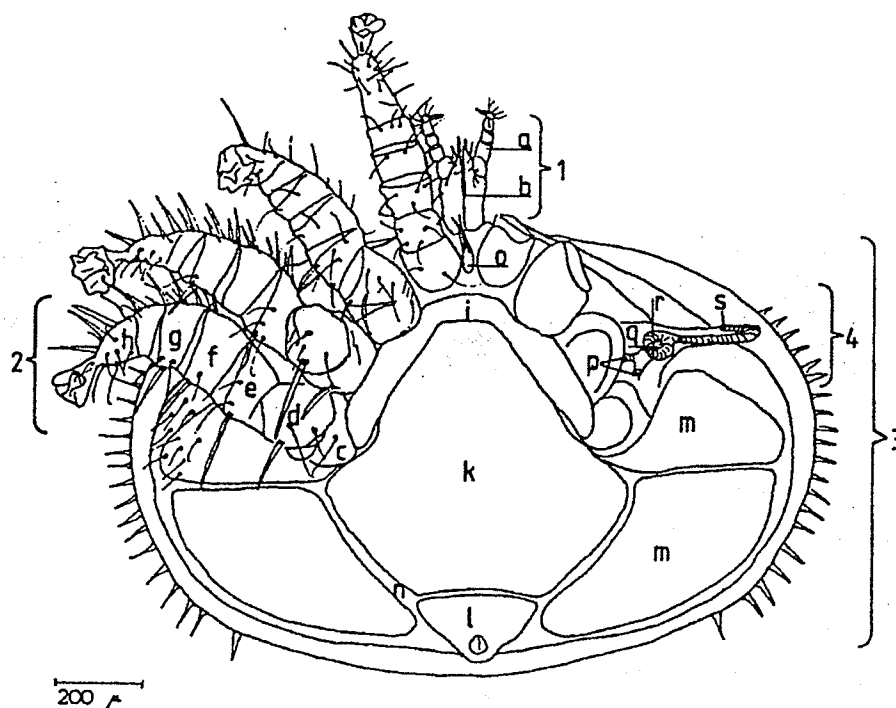


4
Mite feeds on prepupa.



5
Female lays first egg
60 hours after cell
capping. Female lays
subsequent eggs at
30-hour intervals.

Fig. 2. Anatomy of *Varroa destructor* (Ritter 1981).



- | | |
|---------------------|---|
| 1. Gnathosoma: | a = pedipalp |
| | b = gnathosmal tube |
| 2. Leg: | c = coxa |
| | d = trochanter |
| | e = femur |
| | f = genu |
| | g = tibia |
| | h = tarsus with pretarsus
(setae and ambulacrum) |
| 3. Idosoma: | i = sternal shield |
| | k = genitoventral shield |
| | l = anal shield with anal valves |
| | m = metapodal shields |
| | n = interscutal membrane |
| | o = tritosternum |
| 4. Breathing organ: | p = tracheal trunks |
| | q = stigma |
| | r+s = peritreme |

Fig. 3. Basic structure of *Ascospaera apis*. Ascogonium following plasmogamy, showing nutriocyte (n), primary ascogenous hypha (p), and trichogyne (t) (Spiltoir and Olive 1955).

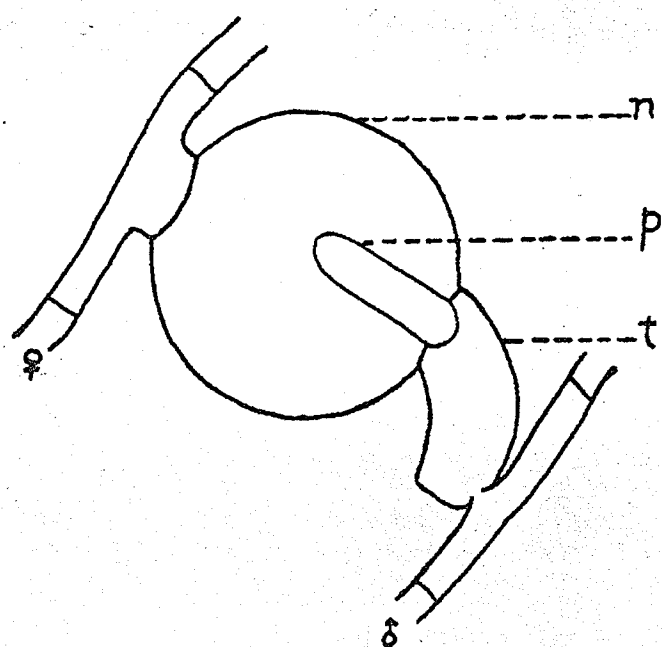


Fig. 4. Suggested life cycle of *Nosema apis* (Fries 1993).

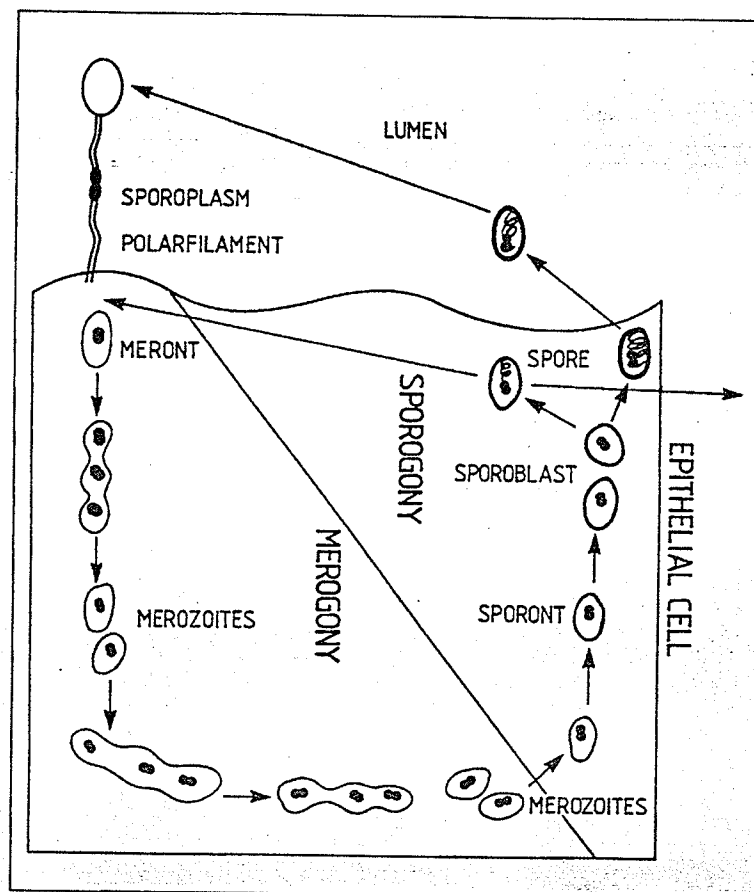
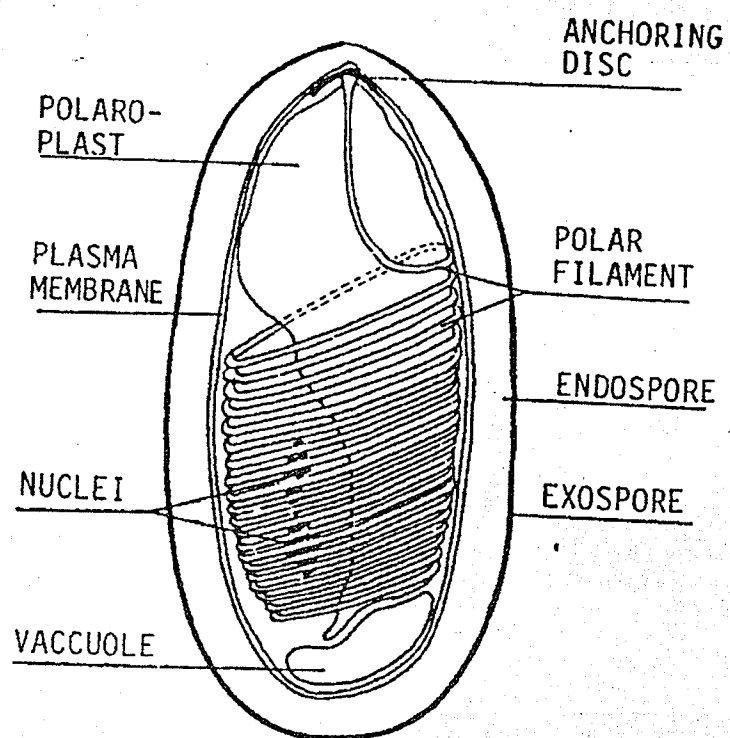


Fig. 5. General structure of Microsporidia, showing polar filament used to penetrate the host membrane (Fries 1993).



CHAPTER 3

The Impact of Formic Acid for Control of the Honey Bee Ectoparasite, Varroa, *Varroa destructor* Anderson and Trueman, on Honey Bee, *Apis mellifera* L., Colonies, and the Influence of Hive and Ambient Conditions on Formic Acid Concentration in the Hive

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The Impact of Formic Acid for Control of the Honey Bee Ectoparasite, *Varroa*, *Varroa destructor* Anderson and Trueman, on Honey Bee, *Apis mellifera* L., Colonies, and the Influence of Hive and Ambient Conditions on Formic Acid Concentration in the Hive

Abstract

The interaction between the effects of varroa and formic acid treatments on colonies of honey bees was examined. Two field experiments were conducted over a two year period. In the first field experiment, colonies were made up with honey bees infested with varroa or treated with fluvalinate strips initially to eliminate varroa. Varroa and no-varroa treatment colonies were each subdivided into two groups then treated with formic acid or left untreated. The formic acid treatment consisted of 200 mL of 65% formic acid in a pin-perforated Ziploc® vegetable bag slow release application for 40 days. In the second field experiment, varroa treatments were created by infesting varroa-free packages of bees with 45 living varroa mites, and no-varroa treatment colonies were not infested initially with varroa. Varroa and no-varroa treatment colonies were each subdivided into three groups and exposed to a pour-on formic acid treatment, a slow release formic acid treatment, or left untreated. The pour-on formic acid treatment was 40 mL 65% formic acid applied to paper towels five times at seven day intervals. The slow release formic acid treatment consisted of 200 mL of 65% formic acid in a slit Ziploc® freezer bag. Varroa infestation, number of honey bees, and area of sealed worker brood per colony were estimated over the course of the season in each field experiment. In the second year, the effects of treatments, hive and ambient conditions on formic acid concentration in hive air were also examined. Formic acid concentration in the hive was measured with

Dräger® detector tubes at 0900, 1400, and 1900 hours; hive temperature and relative humidity were measured with HOBO® data loggers, and ambient temperature, relative humidity, and solar conditions were measured at a nearby weather station.

Formic acid application improved colony development by reducing mean varroa abundance when infestation level was relatively high. However, when varroa were absent or mean abundance was relatively low, formic acid application suppressed colony development. Over the entire season, formic acid applications reduced varroa mean abundance by 9.4% in colonies treated with slow release formic acid and 29.3% in colonies treated with pour-on formic acid.

Colonies treated with the pour-on formulation had significantly higher formic acid concentration in hive air of colonies not initially infested with varroa than in colonies that were initially infested with the mite on three of five application days. Similarly, there were significantly higher intraday concentrations of formic acid in hive air in no-varroa treatment colonies than in varroa treatment colonies treated with the pour-on application at 0900 and 1400 hours, on two of three days analyzed. Varroa may affect colony size and/or worker activity level and may act in combination with ambient conditions to influence the concentration of formic acid in the hive. In colonies treated with the slow release formulation, formic acid concentration in the hive at 1400 hours was positively correlated with hive and ambient temperature early in the application period. Ambient temperature was positively correlated with ambient solar condition during the treatment period. In colonies treated with the pour-on formulation, interday hive concentration was positively, but weakly, correlated with fluctuations of hive temperature. In hives treated

with the slow release application, the mean concentration ($\pm$ SE) at 1400 hours declined from 44 ± 8 ppm on the day of application to 18 ± 4 ppm on the 11th day post application. The mean formic acid concentration during this period was 23 ± 2 ppm, with a range of 0 to 82 ppm (0.00 to 0.16 mg/L). In hives treated with the pour-on application, mean formic acid concentration of all treatment dates was 69 ± 8 ppm at 1400 hours, but individual hives ranged from 5 to 222 ppm (0.01 to 0.44 mg/L). Mean daily formic acid concentration in pour-on hives ranged from 113 ± 21 ppm to 19 ± 4 ppm depending on the day when application occurred.

Introduction

Varroa destructor Anderson and Trueman is an ectoparasitic mite which feeds on the haemolymph of adult and immature honey bees (*Apis mellifera* L.). This very serious pest can destroy colonies that are left untreated. Part of this damage may be caused by diseases which the mite promotes (Ostermann Chapter 4; Ball 1988a; Shimanuki *et al.* 1994).

Formic acid is a relatively inexpensive treatment for varroa which is gaining popularity as resistance of the mite to other treatments increases (Elzen *et al.* 1999; Elzen and Westervelt 2002). Efficacy of formic acid against the mite varies from 51 to 100% (Hoppe *et al.* 1989; Fries 1991; Feldlaufer *et al.* 1997; Calis *et al.* 1998; Eischen 1998; Calderone 1999b; Calderone and Nasr 1999). Factors that may affect the efficacy of a formic acid treatment include method of application (Feldlaufer *et al.* 1997), positioning of the acid (Hoppe *et al.* 1989) and ambient temperature (Bracey and Fischer 1989;

Feldlaufer *et al.* 1997; Calderone 1999b). Although Liu (1991a) stated that the amount of formic acid vaporization is relatively unaffected by temperature, levels of formic acid in colonies increase during the warmer parts of the day and drop during the cooler parts of the day (Feldlaufer *et al.* 1997). According to Von Posern (1988), low temperatures are thought to reduce the efficiency of the acid, while application of formic acid when temperatures are high can kill bees and the queen.

Formic acid is usually applied as a liquid poured onto an absorbent pad (Gatien and Currie, unpublished data) or as some form of long-term slow release application (Gatien and Currie, unpublished data; Feldlaufer *et al.* 1997).

In some situations formic acid may have a negative impact on the well-being of the honey bee colony. Larvae appear to be most vulnerable to formic acid. Bolli *et al.* (1993) found that young larvae were most susceptible to formic acid, while Fries (1991) found that old larvae, during developmental transition from stretch larva to pupa, were most susceptible. Young larvae are killed because their oxygen consumption is inhibited, but prolonged treatment with formic acid may also hurt brood care by decreasing larval feeding by nurse bees (Bolli *et al.* 1993). Formic acid can cause worker mortality of up to 30% and queen mortality of five to 10% (Von Posern 1988). Formic acid also reduces drone brood survival and production (DeGuzman *et al.* 1999). In contrast, Skinner *et al.* (2001) observed no reduction of adult bee or brood populations, and Hoppe *et al.* (1989) found no lasting damage to bees or brood, and no loss of queens.

The main objectives of this study were to assess the interaction between formic acid treatment and varroa infestation on colony size, and to assess the effects of

treatments, hive and ambient factors on formic acid concentration in hive air.

Methods

This study consisted of two field experiments conducted in 1999 and 2000. Both field trials were designed to assess the effects of formic acid and varroa mite levels on honey bee colonies. The 1999 and 2000 field experiments are designated as experiment 1 and experiment 2, respectively.

Experiment 1

Honey bees for the first year trial came from colonies wintered at the University of Manitoba campus yard. On 8 June, 1999, frames of honey bees were manually shaken from their frames, through a funnel, into standard wooden shipping packages placed on a scale. Approximately 0.91 kg of a yellow strain of bees was shaken into each of 32 packages. Packages were randomly assigned to four treatment groups as follows: (1) varroa and formic acid, (2) varroa and no formic acid, (3) no-varroa and formic acid, and (4) no-varroa and no formic acid. Varroa infestation levels of all 32 packages were determined, and the packages were sorted by infestation level and randomly assigned to four treatment groups such that the average infestation level in each group was approximately equal. To create varroa and no-varroa treatments, half of the packages were treated with Apistan® strips¹ (7.6 cm x 2.54 cm) for 24 h before the packages were hived. Colonies treated with fluvalinate started the experiment apparently free of the

¹a.i. 10% fluvalinate; Wellmark International, Bensenville, Illinois, U.S.

mite, but the average infestation level of no-varroa colonies increased over the course of the experiment. Mated queens of a yellow strain were purchased from New Zealand for each package.

On 10 June, 1999, the packages were shaken into standard Langstroth hives. All colonies were inoculated with chalkbrood and nosema on 23 June, 1999. Colonies were artificially infected with chalkbrood by using a spouted plastic bottle to spray broodnest frames with 75 mL of sugar syrup (1 part sucrose sugar: 1 part deionized water by weight) containing three black chalkbrood macerated mummies. Mummies were collected from hive entrances and bottom boards of infected hives in the spring of 1999 and frozen for about two weeks before use. Colonies were infected with nosema disease by spraying 100 mL of sugar syrup (1 sucrose sugar: 1 deionized water by weight) containing 1×10^8 *N. apis* spores on the brood frames of each hive after Malone *et al.* (1992). *Nosema apis* spores were obtained from bees scooped from infected hives.

Hives were located at four separate sites at the Glenlea Research Station, 20 km south of Winnipeg, Manitoba, Canada. There were eight hives at each site and each site was separated by 75 m to 1500 m. Within an apiary, hives were approximately 1.5 m apart and were arranged in a row or semicircle, with offset entrances, to reduce drift. Each nine-frame brood chamber contained two frames of honey and two frames of pollen. The varroa and formic acid treatment colonies were located by trees which partly shaded the colonies during the formic acid application.

The formic acid treatment consisted of exposing colonies to a slow-release formulation of 200 mL of 65% formic acid, on a felt pad, sealed within a pin-perforated

Ziploc[®] vegetable bag² (27 cm x 28 cm). The formic acid bags were placed on the top bars of the top hive box under the inner cover lid. The inner cover gap was 19 mm deep. The formic acid treatment was applied 8 July, 1999 and removed 16 August, 1999, after 40 days.

Varroa infestation, number of bees and area of brood per colony were estimated every week from 23 June to 3 November, 1999. Varroa infestation for each colony was determined from a sample of approximately 100 bees, collected in a cup from the brood cluster of colonies at least 3,500 bees strong. The bees were counted and washed in 70% ethanol for 10 minutes to dislodge varroa from the bees. Number of bees per colony was estimated by comparing frames of bees with pictures of known numbers of bees on frames. Brood area per colony was estimated using a transparent plexiglass grid. The grid was held in front of each frame containing brood and sealed worker brood area was measured in cm² then later translated into number of cells, where 1 cm² = 4.35 worker brood cells. Observations prior to 8 July represent pretreatment observations.

Statistical Analysis

The effect of varroa and formic acid treatments on amount of worker brood and number of honey bees per colony was analyzed by ANOVA (Snedecor and Cochran 1980). Varroa infestation was analyzed as the mean number of mites in the sample divided by the number of bees in the sample, or mean abundance (Bush *et al.* 1997). Data were analyzed as a split-plot analysis of variance, with varroa treatment and formic

²S.C. Johnson and Son, Limited, Brantford, Ontario, Canada.

acid treatment as main-plot factors, date as a sub-plot factor, and hive as a replicate within each treatment group. Main-plot factors and interactions between them were examined using varroa*formic acid*replicate as an error term. Where significant interactions were found, single degree of freedom contrasts were used to assess the effect of formic acid treatments on the number of workers per colony and amount of worker brood per colony within varroa treatments for each date. Results were adjusted with the Bonferroni correction ($\alpha=0.05$).

Experiment 2

Twenty-four 1.82 kg packages from Australia were each split and hived into 48 standard Langstroth brood chambers on 2 May, 2000. Each hive received approximately 0.91 kg of bees and the original queen or a separately-caged queen from the same supplier. All bees were of a yellow strain. Packages were hived into standard Langstroth brood chambers. Each nine-frame brood chamber contained two frames of honey and two frames of pollen.

Every second hive in the formic acid treatment groups was modified to allow sampling of formic acid from the brood chamber without opening the hive. A hole was drilled into the back center of each bottom hive box and a piece of polyethylene tubing³ (approximately 30 cm long x 0.43 cm inside diameter) sealed with a rubber sleeve stopper⁴ was inserted into each hole. The open end of the tube reached into the middle of

³Anderson Barrows Metals Corporation, Palmdale, CA, U.S.

⁴Fisher Scientific Limited, Ottawa, 03-215-5.

the hive box while the end with the rubber sleeve stopper stuck out of the back of the hive. A HOBO[®] H8 logger⁵ was placed on the hive top bars, under the cover of each hive, and programmed to record hive temperature and relative humidity every 30 mins. from 18 May to 10 August, 2000. A cable ran from the logger to outside the hive so data could be extracted without opening the hive. On 19 June, 2000 a second brood box was added beneath the first brood box of all hives. Supers were added to all hives each time that one hive required a super, up to five hive boxes high.

Six treatments were randomly assigned to the 48 colonies as follows: (1) varroa and slow-release formic acid, (2) varroa and pour-on formic acid, (3) varroa and no formic acid, (4) no-varroa and slow-release formic acid, (5) no-varroa and pour-on formic acid, and (6) no-varroa and no formic acid. All colonies were inoculated with chalkbrood on 6 June, 2000 as described under Experiment 1.

Varroa treatment colonies were inoculated with 45 mites each on 16 May, 2000, and no-varroa treatment colonies were not inoculated with mites. Mites were collected from live honey bees at the University of Manitoba campus apiary using a water-wash technique (Currie, personal communication). Specifically, frames of bees from colonies known to be infested with varroa were shaken into a plastic bag and the bees were then lightly anesthetized with gaseous CO₂. The immobilized bees were scooped into a small plastic container and covered with a screen lid. The container with bees was immersed into a large pail filled with water and lined with mesh straining cloth and shaken vigorously to remove mites from the bees. Mites that were counted were kept on

⁵Onset Computer Corporation, Cape Cod, MA, U.S.

dampened paper towel in petri dishes in a cooler until they could be introduced into colonies. Half of the colonies received no mites, but average infestation level of all treatment groups increased over the course of the experiment.

The slow-release formic acid treatment was similar to the formic acid treatment in Experiment 1 except Ziploc[®] freezer bags⁶ (27 cm x 28 cm) were used in place of vegetable bags, and three vertical slits were cut (1 cm x 22 cm per slit) from one side of each bag. The pour-on formic acid treatment consisted of 40 mL of 65% formic acid poured onto five or six stacked paper towels (13.4 cm x 23.2 cm surface area) on the hive top-bars. The pour-on acid treatment was applied at approximately 0830 hours on five separate applications at a seven day interval. Both formic acid treatments were first applied 8 June, 2000 and slow release bags were removed 17 July, 2000 after 40 days. Slow-release freezer bags were weighed post-treatment to determine the total amount of vaporization.

Colonies were examined and sampled every second week from 6 June to 10 October, 2000 to assess hive strength. Observations before 8 June represent pretreatment observations.

Dräger[®] detector tubes⁷ and hand pump were used to determine the concentration of formic acid in the air of every second hive ($n = 4$ hives per treatment) of the colonies exposed to each formic acid treatment. The entire volume of the tube was drawn out before each reading. Formic acid concentration in hive air was determined from three full

⁶S.C. Johnson and Son, Limited, Brantford, Ontario, Canada.

⁷Acetic acid (1-80 ppm); Lübeck, Germany, E.U.

pumps of the hand pump, except for concentrations beyond 80 ppm which were estimated based on the reading after the first pump. Measurements were taken from the hives on 8, 9, 10, 11, 12, 13, 14, 15, 19, 22, 26, 29 June, 3, and 6 July at approximately 1400 hours, and additionally at approximately 0900 and 1900 hours on 8, 15, and 29 June.

Hive temperature and relative humidity data were downloaded from the HOBO® data loggers when they were almost full, using a notebook computer and a serial port cable. Ambient temperature, relative humidity, and solar conditions were measured every 15 mins. from a nearby weather station. Colonies of all treatment groups were positioned for equal access to the sun.

Statistical Analysis

The effect of varroa and formic acid treatments on amount of worker brood and number of honey bees per colony was analyzed by ANOVA (Snedecor and Cochran 1980). Varroa infestation was analyzed as the mean number of mites in the sample divided by the number of bees in the sample, or mean abundance. Data were analyzed as a split-plot analysis of variance, with varroa treatment and formic acid treatment as main-plot factors, date as a sub-plot factor, and hive as a replicate within each treatment group. Main-plot factors and interactions between them were examined using varroa*formic acid*replicate as an error term. Where significant interactions were found, single degree of freedom contrasts were used to assess the effect of formic acid treatments on the number of workers per colony and amount of worker brood per colony within varroa treatments for each date. Results were adjusted with the Bonferroni correction ($\alpha=0.05$).

The effect of varroa and formic acid treatments on inter- and intraday formic acid concentration in hive air was analyzed by ANOVA (Snedecor and Cochran 1980). For interday analysis, data were analyzed as a split-plot analysis of variance. Varroa and formic acid treatments were main-plot factors, date was a sub-plot factor, and hive was a replicate within each treatment group. Main-plot factors and interactions between them were examined using varroa*formic acid*replicate as an error term. Single degree of freedom contrasts were used to assess the effect of varroa on formic acid concentration in hive air within formic acid treatments for each date. Results were adjusted with the Bonferroni correction ($\alpha=0.05$). For intraday analysis, data were analyzed as a split-plot analysis of variance. Varroa and formic acid treatments were main-plot factors, date and time were sub-plot factors, and hive was a replicate within each treatment group. Main-plot factors and interactions between them were examined using varroa*formic acid*time*replicate as an error term. Single degree of freedom contrasts were used to assess the effect of varroa on formic acid concentration in hive air within formic acid treatments at each measurement time for each date. Results were adjusted with the Bonferroni correction ($\alpha=0.05$).

Inter- and intraday formic acid concentrations in hive air were correlated with hive and ambient conditions and number of honey bees per colony. Simple correlations were calculated to quantify strength of relationships between variables (Snedecor and Cochran 1980).

The formic acid concentration for the period of treatment (concentration X time product) was estimated by regression. Models were chosen based on fit (primarily) as

well as simplicity. For slow release hives, formic acid concentration X time product for 28 days post application was estimated using a logarithmic model ($y = a - b \ln(x+c)$). For pour-on hives, formic acid concentration X time product for seven days post application was estimated using a sigmoidal model ($y = A2 + (A1-A2)/(1 + (x/x0)^p)$) then multiplied by five to determine the amount for the five weeks of pour-on treatment application.

Results

Experiment 1

In the first field experiment, mean abundance of varroa ($\pm$ SE) over the course of the experiment was significantly greater in colonies initially infested with the mite, or varroa treatment colonies (0.053 ± 0.007), than in colonies not initially with the mite, or no-varroa treatment colonies (0.012 ± 0.002) ($F=118.16$; $df=1,7$; $P>F<0.0001$) (Fig. 6a, b). On the final day of the experiment average varroa abundance was 0.112 ± 0.030 in colonies initially infested with varroa and 0.035 ± 0.010 in colonies not initially infested with varroa. Since weaker colonies were not sampled for varroa on all sampling dates, statistical analyses of seasonal effects were not conducted; however, the mite abundance data that were collected from colonies with more than 3,500 bees are presented in Fig. 6. The three-way interaction between the effects of varroa, formic acid, and sampling period on mean abundance of varroa was not significant ($F=0.12$; $df=1,7$; $P>F=0.7408$).

The three-way interaction between the effects of varroa, formic acid, and sampling period on the number of workers per colony was significant ($F=2.39$; $df=16,112$; $P>F=0.0042$) (Fig. 7a, b). In colonies initially infested with varroa, the average number

of bees per colony tended to be greater in colonies treated with formic acid than in colonies not treated with formic acid; however, the difference was not significant on any measurement date. In colonies not initially infested with varroa, the average number of bees per colony was significantly lower in colonies treated with formic acid than in colonies not treated with formic acid on each of the following measurement dates: 4 August, 1999 ($F=13.74$; $df=1,112$; $P>F=0.0003$), 11 August ($F=19.34$; $df=1,112$; $P>F<0.0001$), 18 August ($F=16.44$; $df=1,112$; $P>F<0.0001$), 25 August ($F=47.07$; $df=1,112$; $P>F<0.0001$), 1 September ($F=46.42$; $df=1,112$; $P>F<0.0001$), 8 September ($F=44.62$; $df=1,112$; $P>F<0.0001$), 22 September ($F=32.74$; $df=1,112$; $P>F<0.0001$), 29 September ($F=30.97$; $df=1,112$; $P>F<0.0001$), 6 October ($F=23.71$; $df=1,112$; $P>F<0.0001$), 13 October ($F=24.27$; $df=1,112$; $P>F<0.0001$), 20 October ($F=16.40$; $df=1,112$; $P>F<0.0001$), 27 October ($F=15.86$; $df=1,112$; $P>F=0.0001$), and 3 November ($F=14.89$; $df=1,112$; $P>F=0.0002$).

The three-way interaction between the effects of varroa, formic acid, and sampling period on the amount of worker brood was significant ($F=2.42$; $df=14,97$; $P>F=0.0060$) (Fig. 8a, b). In colonies initially infested with varroa, the average amount of worker brood per colony tended to be greater in colonies treated with formic acid than in colonies not treated with formic acid; however, the difference was not significant on any measurement date. In colonies not initially infested with varroa, the average amount of worker brood per colony was significantly lower in colonies treated with formic acid than in colonies not treated with formic acid on each of the following measurement dates: 28 July, 1999 ($F=26.53$; $df=1, 97$; $P>F<0.0001$), 4 August ($F=20.76$; $df=1,97$; $P>F<0.0001$),

11 August ($F=17.88$; $df=1,97$; $P>F<0.0001$), 18 August ($F=29.12$; $df=1,97$; $P>F<0.0001$), 25 August ($F=10.67$; $df=1,97$; $P>F=0.0015$), 1 September ($F=21.33$; $df=1,97$; $P>F<0.0001$), and 8 September ($F=17.11$; $df=1,97$; $P>F<0.0001$).

The first and second observations of the trial were not included in the statistical analysis since they were recorded prior to formic acid treatment application. The data from both pretreatment periods are included in the figures.

Experiment 2

In the second field experiment, mean abundance of varroa ($\pm$ SE) over the course of the experiment was significantly greater (0.009 ± 0.001) in colonies initially infested with the mite, or varroa treatment colonies, than in colonies not initially with the mite, or no-varroa treatment colonies (0.003 ± 0.001) ($F=16.05$; $df=1,14$; $P>F=0.0013$) (Fig. 6c, d). On the final day of the experiment mean abundance of varroa was 0.035 ± 0.006 in varroa treatment colonies and 0.018 ± 0.008 in no-varroa treatment colonies. Formic acid application did not have a significant effect on the level of varroa infestation per colony ($F=0.98$; $df=2,14$; $P>F=0.3999$); however, there was a trend towards a reduction of varroa levels in colonies treated with either formulation of formic acid. In the second year field experiment, the slow release formic acid application decreased overall average varroa mean abundance by 9.4% to 0.007 ± 0.001 , while the pour-on application decreased varroa mean abundance by 29.3% to 0.005 ± 0.001 , compared to the control group level of varroa infestation of 0.007 ± 0.002 . The three-way interaction between the effects of varroa, formic acid, and sampling period on varroa infestation level was not significant

($F=0.76$; $df=14,31$; $P>F=0.6977$).

The three-way interaction between the effects of varroa, formic acid, and sampling period on the number of workers per colony was significant ($F=3.24$; $df=16,108$, $P>F=0.0001$) (Fig. 7c, d). In colonies initially infested with varroa, the average number of bees per colony was significantly greater in colonies treated with formic acid than in colonies not treated with formic acid on the following measurement dates: 1 August, 2000 ($F=19.33$; $df=2,108$; $P>F<0.0001$), 15 August ($F=26.86$; $df=2,108$; $P>F<0.0001$), 29 August ($F=18.86$; $df=2,108$; $P>F<0.0001$), 12 September ($F=21.49$; $df=2,108$; $P>F<0.0001$), 26 September ($F=34.38$; $df=2,108$; $P>F<0.0001$), and 10 October ($F=19.29$; $df=2,108$; $P>F<0.0001$). In colonies not initially infested with varroa, the average number of bees per colony was significantly lower in colonies treated with formic acid than in colonies not treated with formic acid on the following measurement dates: 1 August, 2000 ($F=10.10$; $df=2,108$; $P>F<0.0001$), 15 August ($F=10.83$; $df=2,108$; $P>F<0.0001$), 29 August ($F=9.41$; $df=2,108$; $P>F=0.0002$), 12 September ($F=15.34$; $df=2,108$; $P>F<0.0001$), and 26 September ($F=9.15$; $df=2,108$; $P>F=0.0002$).

The three-way interaction between the effects of varroa, formic acid, and sampling period on the amount of worker brood was significant ($F=2.38$; $df=16,108$, $P>F=0.0044$) (Fig. 8c, d). In colonies initially infested with varroa, the average amount of worker brood per colony was significantly greater in colonies treated with formic acid than in colonies not treated with formic acid on the following measurement dates: 29 August ($F=15.50$; $df=2,108$; $P>F<0.0001$), 12 September ($F=16.43$; $df=2,108$; $P>F<0.0001$), and 26 September ($F=18.40$; $df=2,108$; $P>F<0.0001$). In colonies not initially infested with

varroa, the average amount of worker brood per colony was significantly lower in colonies treated with formic acid than in colonies not treated with formic acid on the following measurement dates: 29 August ($F=6.40$; $df=2,108$; $P>F=0.0024$), and 26 September ($F=8.80$; $df=2,108$; $P>F=0.0003$).

The first observations of the trial were not included in the statistical analysis since they were recorded prior to formic acid treatment application. The pretreatment observations are included in the figures.

Interday Formic Acid Hive Concentration

Overall differences in the formic acid concentration in hive air between slow release and pour-on applications were not significant ($F=3.17$; $df=1,3$; $P>F=0.1733$). However, in hives treated with the slow release formulation of formic acid, the concentration of formic acid in the hive air tended to decrease gradually over the period of the application. In hives treated with the pour-on formulation, formic acid concentration increased sharply on days of application and quickly dissipated by the next day. In hives treated with the slow release application, the mean concentration ($\pm$ SE) at 1400 hours declined from 44 ± 8 ppm on the day of application to 18 ± 4 ppm on the 11th day post application. The mean formic acid concentration during this period was 23 ± 2 ppm, with a range of 0 to 82 ppm (0.00 to 0.16 mg/L). In hives treated with the pour-on application, mean formic acid concentration of all treatment dates was 69 ± 8 ppm at 1400 hours, but individual hives ranged from 5 to 222 ppm (0.01 to 0.44 mg/L). Mean daily formic acid concentration in pour-on hives ranged from 113 ± 21 ppm to 19 ± 4

ppm depending on the day when application occurred.

For slow release hives, the formic acid concentration X time product for the 27 day post application period was estimated using a logarithmic model ($y = a - b \ln(x+c)$; $r^2 = 0.723$) to be 277 ppm days (Fig. 9). For pour-on hives, the formic acid concentration X time product was estimated for the five week application period using a sigmoidal model ($y = A2 + (A1-A2)/(1 + (x/x0)^p)$; $r^2 = 0.975$) to be 258 ppm days (Fig. 10). Data points are presented in Figs. 9 and 10 as means at each time of formic acid hive concentration measurement. Average per cent weight loss from the slow release felt bags at the end of the 40 day treatment period was 91.6% and the odour clearly indicated that some formic acid remained in the felts.

There was a significant three-way interaction between the effects of varroa, formic acid, and measurement date on interday formic acid hive concentration ($F=7.04$; $df=11,32$; $F>P<0.0001$) (Fig. 11). In the pour-on treatment hives, formic acid concentration tended to be higher in no-varroa treatment colonies than in varroa treatment colonies. Single degree of freedom contrasts between formic acid and measurement date revealed significant differences in formic acid hive concentration between varroa and no-varroa colonies that were treated with the pour-on formulation of formic acid on the following dates: 8 June ($F=77.31$; $df=1,32$; $P>F<0.0001$), 22 June ($F=88.65$; $df=1,32$; $P>F<0.0001$), and 29 June ($F=44.30$; $df=1,32$; $P>F<0.0001$). Formic acid was applied on each of these days. No significant differences ($P>0.05$) were observed in the formic acid hive concentration between varroa and no-varroa treatments on the other two dates of pour-on application, 15 June ($F=1.62$; $df=1,32$; $P>F=0.2127$) and 6 July ($F=0.21$;

df=1,32; $P>F=0.6519$). In hives treated with slow release formic acid, concentrations were not significantly different ($P>0.05$) between varroa and no-varroa treatments on any of the measurement dates.

In colonies treated with slow release formic acid, the concentration of formic acid in the hive air, measured at approximately 1400 hours, was positively correlated with ambient temperature during the first seven days post application ($r=0.52711$; $P<0.0001$), but not during the period 11 to 28 days post application ($r=0.16579$; $P=0.2821$) (no observations were made between eight and 11 days post application) (Fig. 11). Ambient temperature was negatively correlated with ambient relative humidity ($r=-0.85678$; $P<0.0001$) and positively correlated with ambient solar conditions ($r=0.60852$; $P<0.0001$) during the 29 day measurement period. Interday slow release formic acid concentration was also positively correlated with hive temperature ($r=0.42065$; $P=0.0017$) during the first seven days post application, but not during the period 11 to 28 days post application ($r=-0.13338$; $P=0.4183$). Slow release hive temperature was negatively correlated with hive relative humidity ($r=-0.59454$; $P<0.0001$) during the 29 day measurement period. Ambient temperature and ambient relative humidity were significantly correlated with average slow release hive temperature ($r=0.74418$; $P<0.0001$, and $r=-0.63558$; $P<0.0001$, respectively) and slow release hive relative humidity ($r=-0.55021$; $P<0.0001$ and $r=0.73244$; $P<0.0001$, respectively) during the 29 day measurement period.

In colonies treated with pour-on formic acid, concentration of formic acid in the hive air, measured at approximately 1400 hours, was not significantly correlated with ambient temperature during the first seven days post application ($r=0.17001$; $P=0.1941$),

nor during the period 11 to 28 days after the first pour-on application ($r=0.16097$; $P=0.3025$) (Fig. 11). However, interday pour-on formic acid concentration was positively correlated with hive temperature seven days after the first pour-on application ($r=0.23479$; $P=0.0170$) and during the period 11 to 28 days after the first pour-on application ($r=0.30458$; $P=0.0470$). Pour-on hive temperature was negatively correlated with hive relative humidity ($r=-0.22515$, $P=0.0175$) during the 29 day measurement period.

Formic acid hive concentration at 1400 hours was not significantly correlated with the number of honey bees in colonies treated with the pour-on formulation ($r=-0.208$; $P=0.3411$) nor slow release formulation ($r=-0.071$; $P=0.7418$).

Intraday Formic Acid Hive Concentration

There was no significant four-way interaction between the effects of varroa, formic acid, date, and measurement time on intraday formic acid hive concentration ($F=1.67$; $df=4,12$; $P>F=0.2216$); however because separate pour-on formulations were conducted under different environmental condition on each date, the results of the effects of varroa and formic acid treatments on interday formic acid hive concentration were examined (Fig. 12). Single degree of freedom contrasts revealed significantly higher concentrations of formic acid at 0900 and 1400 hours in no-varroa treatment hives than in varroa treatment hives, that were exposed to the pour-on formulation of formic acid, on two of the three days; 0900-8 June ($F=18.13$; $df=1,12$; $P>F=0.0011$), 1400-8 June ($F=51.96$; $df=1,12$; $P>F<0.0001$), 0900-29 June ($F=10.92$; $df=1,12$; $P>F=0.0063$), and

1400-29 June ($F=59.57$; $df=1,12$; $P < 0.0001$). Formic acid was applied on each of these days. Formic acid hive concentrations were not significantly different between varroa and no-varroa treatments on 1900-8 June ($F=4.07$; $df=1,12$; $P > F=0.0665$), 1900-29 June ($F=2.41$; $df=1,12$; $P > F=0.1465$), 0900-15 June ($F=1.60$; $df=1,12$; $P > F=0.2302$), 1400-15 June ($F=1.09$; $df=1,12$, $P > F=0.3178$), 1900-15 June ($F=1.54$; $df=1,12$; $P > F=0.2379$). In hives treated with slow release formic acid, concentrations were not significantly different between varroa treatments at any of the measurement times on any date.

In colonies treated with slow release formic acid, intraday fluctuation in concentration of formic acid in the hive air, measured at approximately 0900, 1400, and 1900 hours, was not significantly correlated with ambient temperature ($r=-0.12397$; $P=0.2995$) nor ambient solar conditions ($r=-0.04766$; $P=0.6910$) (Fig. 12). Ambient temperature was negatively correlated with ambient relative humidity ($r=-0.97657$; $P<0.0001$) and positively correlated with ambient solar conditions ($r=0.58357$; $P<0.0001$). Intraday slow release formic acid concentration was not significantly correlated to hive temperature ($r=0.00251$; $P=0.9845$) nor hive relative humidity ($r=-0.06144$; $P=0.6324$). Hive temperature was negatively correlated with hive relative humidity ($r=-0.63199$; $P<0.0001$).

In colonies treated with pour-on formic acid, intraday fluctuations in concentration of formic acid in the hive air, measured at approximately 0900, 1400, and 1900 hours, was not significantly correlated with ambient temperature ($r=0.07051$; $P=0.5562$) but was positively correlated with ambient solar conditions ($r=0.40792$;

$P=0.0004$) (Fig. 12). Intraday pour-on formic acid concentration in hive air was not significantly correlated with hive temperature ($r=0.14897$; $P=0.2117$) nor hive relative humidity ($r=0.06662$; $P=0.5782$). Hive temperature was negatively correlated with hive relative humidity ($r=-0.31895$; $P=0.0067$).

Discussion

There were clear benefits of appropriate use of formic acid treatment in both field experiments when varroa were at relatively high levels in colonies. Formic acid application improved colony development by reducing mean abundance of varroa when infestation was relatively high. However, when mean abundance of varroa was relatively low or varroa were absent, formic acid application suppressed colony development. These findings support the results of other studies where treatment with formic acid improved colonies which were heavily infested with the mite (Hoppe *et al.* 1989), but that formic acid can also have a negative effect on colony well-being (Gatien and Currie, unpublished data; Von Posern 1988; Fries 1991; Bolli *et al.* 1993; DeGuzman *et al.* 1999). Although the negative impact of spring application of formic acid on colony growth was clearly evident in each year of our study, spring treatments with slow release formic acid using the same application methods employed in our study did not always impact colony growth (Currie 2001).

Formic acid reduced the level of varroa infestation; however, the results were highly variable, which is consistent with the results of other studies (Feldlaufer *et al.*

1997; Calderone 1999b). Seasonal reductions of overall mean varroa abundance of 9.4% with the slow release application and 29.3% with the pour-on application of formic acid were below the percentage infestation reductions of 51 to 100% reported by others (Hoppe *et al.* 1989; Fries 1991; Feldlaufer *et al.* 1997; Calis *et al.* 1998; Eischen 1998; Calderone 1999b; Calderone and Nasr 1999). This is also much lower than similar treatments that were carried out earlier in the spring and later in the fall (Gatien and Currie, unpublished data) using the same methods and similar environmental conditions. The low efficacy we observed may have been due to the timing of our treatments, which occurred much later in the season than would occur in colonies being managed for honey production in our region. Late season honey robbing probably increased mean abundance of varroa in colonies initially infested with varroa and exposed to slow release formic acid, the treatment group which had the greatest average number of bees per colony, and this reduced the overall efficacy of the formic acid. Mean abundance of varroa in all treatment groups, including the no-varroa treatment, increased over the course of the experiment probably due to the spread of varroa between treatment groups as a result of robbing and/or drifting. Although the data on the effect of formic acid on varroa levels in the first year field experiment were not analysed because sampling of small colonies might impede their development, the trend in mean varroa abundance in the different treatment groups was similar to that of the 2000 field experiment.

In the 1999 field experiment, formic acid application, in colonies initially infested with varroa and treated with formic acid, may not have been as effective as in the 2000 field experiment, because these colonies were shaded until mid afternoon. Our second

year field experiment results and others have shown that ambient temperature has an effect on formic acid hive concentration (Von Posem 1988; Bracey and Fischer 1989; Feldlaufer *et al.* 1997; and Calderone 1999b).

The mean formic acid concentrations in hives ($\pm$ SE) treated with the slow release application in our experiment ranged from 44 ± 8 ppm on the day of application to 18 ± 4 ppm on the 11th day post treatment. The decline in formic acid concentration in our slow release formulation was similar to that found by Skinner *et al.* (2001) when using 200 g of 65% formic acid gel in a plastic bag (56.3 ± 7.7 ppm at 1 day post treatment and 2.8 ± 0.9 ppm at 13 days post treatment), and with 250 mL of 65% formic acid on a Homosote board in a vegetable bag (35.9 ± 6.0 ppm at 1 day post treatment and 14.7 ± 2.3 ppm at 13 days post treatment). Although the three treatment methods are quite similar, differences in concentrations among the treatments may be attributable to method of application, environmental conditions or colony size.

In our study, the weight of the slow release felt bags was reduced by 91.6% after 40 days. This was greater than the weight loss of 63.1% from natural fiber sheathing board in a vented vegetable bag observed by Calderone and Nasr (1999), but they measured weight loss after only 30 days. It is not clear how much formic acid remained in our application felts after 40 days since the concentration of formic acid likely decreased over the treatment period due to unequal vaporization rates of formic acid and water.

Mean formic acid concentrations in hive air of colonies treated with the pour-on formulation were much higher and dropped more quickly than that which were observed

by Feldlaufer *et al.* (1997) in hives treated with the same application. On 8 June, the first day of pour-on application, our mean formic acid hive concentration ($\pm$ SE) was 58 ± 12 ppm at 0900 hours, 113 ± 21 ppm at 1400 hours, and 47 ± 9 ppm at 1900 hours, and dropped to 13 ± 5 ppm by 1400 hours on the following day. Overall, the highest pour-on formic acid hive concentration was estimated at 222 ppm. Comparatively, Feldlaufer *et al.* (1997), who made eight measurements per day, observed a peak in formic acid hive concentration around 1700 hours, and lower levels in the morning and at night, on the day of and the day after application, all within a range of 0 to 20 ppm. Part of the great difference of findings may be attributable to our use of single brood chambers up to 19 June versus the use of double brood chambers with the monitoring tube in the lower chamber. However, we continued to observe relatively high formic acid hive concentrations after the addition of second brood chambers. Ambient temperatures, position of the treatment, absorbent application medium, and method of hive air monitoring appear to be similar in both studies.

The large surface area of evaporation (13.4 cm x 23.2 cm x 2 sides) from the fully saturated paper towels was likely the major reason for the much higher concentrations of formic acid in hive air we observed with our pour-on application compared to our slow release application (1 cm x 22 cm x 3 slits). Both treatment methods used the same amount (200 mL) of 65% formic acid; however, despite the substantial difference in method of application, the concentration X time product for formic acid in the air of slow release treated hives (276.8 ppm days) and pour-on treated hives (258.2 ppm days) was similar. On average, the amount of formic acid lost from hives was similar in each

treatment.

The great variation in concentration of formic acid in hive air that was observed between colonies treated with the same formic acid application on different dates, even between different groups of colonies treated with the same formic acid application under the same environmental conditions, is likely due to a number of factors. Ambient temperature is a factor which may act in combination with other environmental, hive, and colony factors to influence the concentration of formic acid in the hive. The significant differences in the formic acid concentration of varroa and no-varroa treatment hives when the pour-on application was used suggest that colony size may influence the rate of evaporation with this formulation. Similar effects were not seen with the slow release formulation. It is unlikely that varroa would have a direct effect on the evaporation rate of formic acid. Varroa may act by diminishing colony size and/or activity, resulting in lower average hive temperature. This could reduce formic acid hive concentration in those colonies. These effects appeared to be present only early in the application period at 0900 and 1400 hours on 8 and 29 June. Non-significant differences at 1900 hours on 8 and 29 June were probably due to a rapid rate of evaporation from the pour-on absorbent paper towel, leaving little formic acid in the hive late in the day on those two days. While formic acid hive concentration at 1400 hours was not correlated with the number of honey bees per colony in either the pour-on or slow release formulation, comparison of formic acid hive concentrations on 15 June when hive temperatures were relatively low, to formic acid hive concentrations on 8 and 29 June when hive temperatures were higher, showed that the possible effect of colony size on the volatilization of formic acid may be

evident only on days with higher ambient temperatures. This may in turn, be related to greater colony activity on those days.

Our analysis of inter- and intraday variance of formic acid hive concentration suggests that hive and ambient conditions combine to influence concentration particularly during the first day of the pour-on application. With the slow release formulation, interday formic acid concentration measured at approximately 1400 hours was correlated with ambient and hive temperature during the first seven days post application, but not during the period 11 to 28 days post application. When the formic acid concentration of the solution in the slow release formulation dispenser was high, it was more readily affected by fluctuations in hive and ambient conditions. With the pour-on formulation, interday formic acid concentration measured at 1400 hours was not correlated with ambient temperature during either the seven day period post application or during the period 11 to 28 days post application, but was correlated with hive temperature during these two time periods, although the correlations were relatively weak.

Within a single day, on average, formic acid hive concentrations for both slow release and pour-on formulations appeared to be correlated with ambient and hive temperatures. On days of pour-on formic acid application, formic acid concentration in hive air tended to be low at 0900, high at 1400, and low at 1900 hours. While the mid-day increase in formic acid concentration may be due to greater volatilization of formic acid at higher temperatures or greater colony activity, the late-day drop in formic acid concentration in hives treated with the pour-on formulation that was observed on all treatment dates was likely due to a combination of depletion of formic acid from the

absorbent paper towel and hive temperature or hive activity effects. The intraday slow release graph of 8 June suggests that, when the evaporation rate of formic acid early in the day was slower, the late-day decline in formic acid concentration in hive air of pour-on treated hives was not as severe.

Similar but less dramatic daily trends were observed in the slow release treatments. This also suggests that the combination of hive temperature and/or hive activity plays a role in affecting the amount of formic acid in hive air. As the treatment progressed and the concentration of formic acid within the bags declined, the overall concentration of formic acid in hive air and temperature effects were much lower.

Our results show the benefits of using formic acid when mean varroa abundance in honey bee colonies is relatively high. Treatment with formic acid when varroa infestation was relatively high improved colony development. However, application of formic acid when varroa were absent, or infestation was low, suppressed brood production and colony growth. The concentration of formic acid in hive air varied substantially with the treatment method but the overall dose to which the colony was exposed, as measured by the concentration X time product, was quite similar. The amount of formic acid released by treatment devices was influenced by ambient conditions, as well as colony size, which in turn was affected by the presence of varroa.

Fig. 6. Mean varroa abundance with varroa and formic acid treatments: varroa in experiment 1 (a), no-varroa in experiment 1 (b), varroa in experiment 2 (c), and no-varroa in experiment 2 (d). The arrow indicates the time of formic acid application. In both field experiments, varroa colonies were infested with known levels of the mite while no-varroa colonies were free of the mite. In the first field experiment, half of the colonies were exposed to 200 mL of 65% formic acid (slowrels FA) on a felt pad in a perforated vegetable bag on the hive top bars for 40 days in the spring while the other colonies acted as controls (no FA). In the second field experiment, colonies were exposed in the spring to either 200 mL of 65% formic acid on a felt pad in a slit freezer bag for 40 days (slowrels FA) or 40 mL of 65% liquid formic acid applied five times at a seven day interval to paper towels on the hive top bars (pouren FA), while the remaining third of colonies acted as controls (no FA).

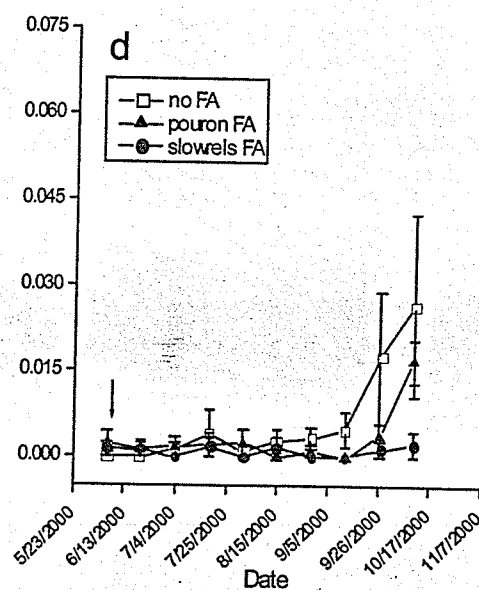
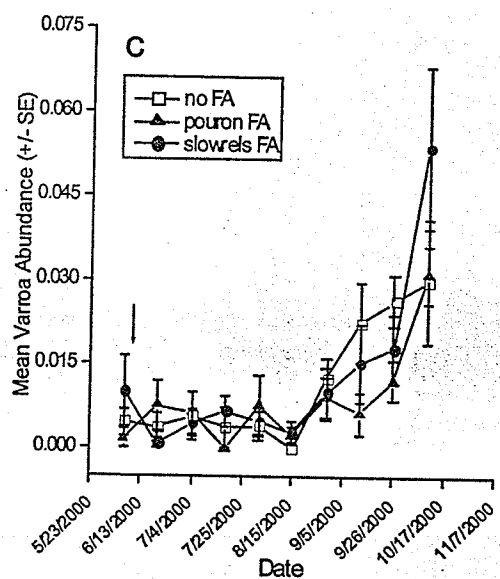
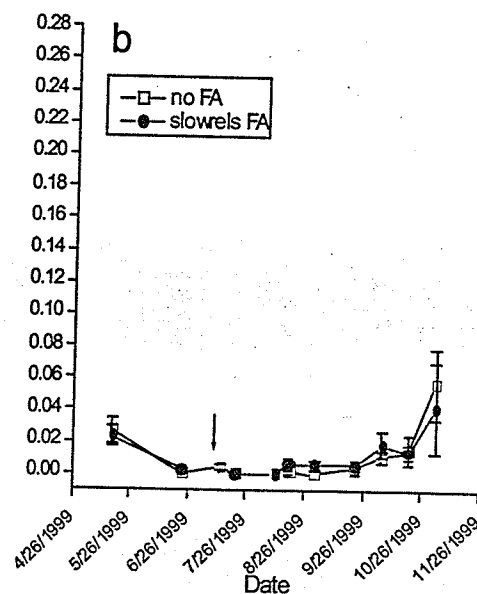
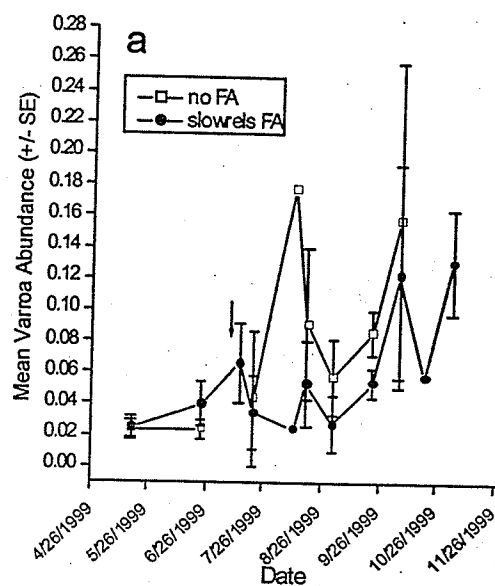


Fig. 7. Number of adult worker bees per colony for varroa and formic acid treatments: varroa in experiment 1 (a), no-varroa in experiment 1 (b), varroa in experiment 2 (c), and no-varroa in experiment 2 (d). The arrow shows the time of formic acid application. See Fig. 6 for description of treatments.

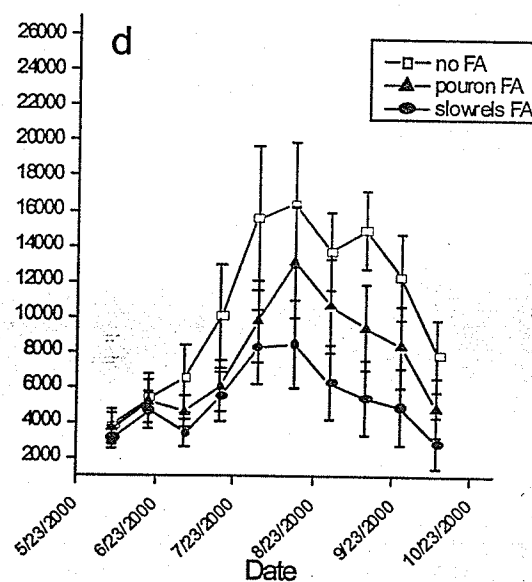
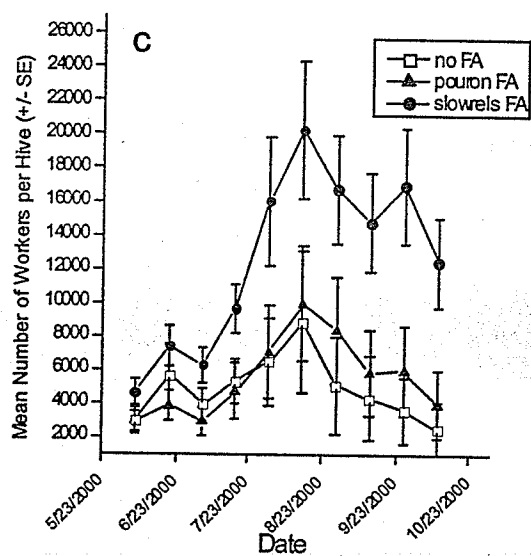
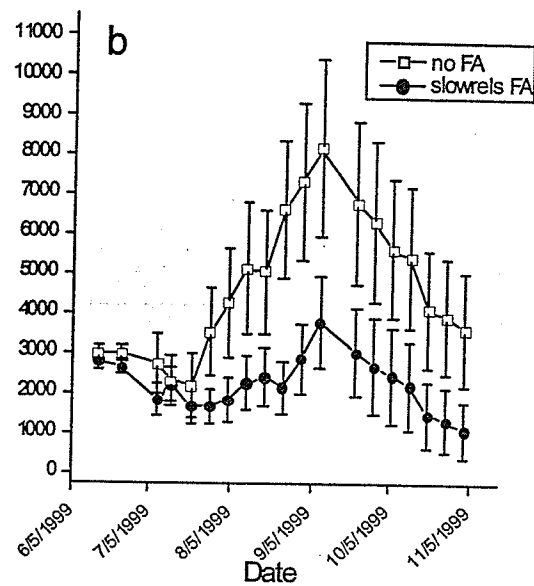
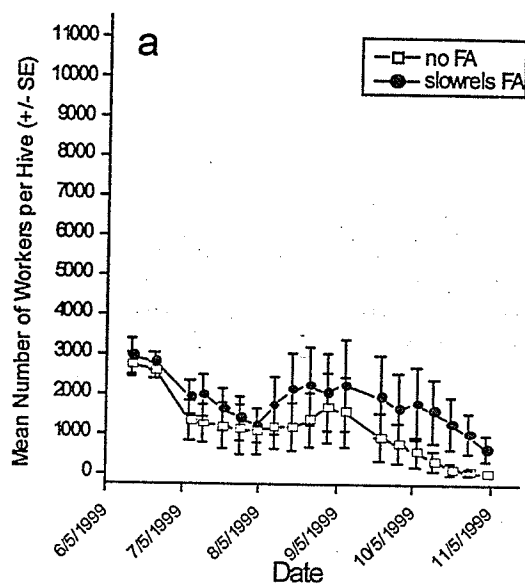


Fig. 8. Amount of worker brood per colony for varroa and formic acid treatments: varroa in experiment 1 (a), no-varroa in experiment 1 (b), varroa in experiment 2 (c), and no-varroa in experiment 2 (d). The arrow indicates the time of treatment application. See Fig. 6 for description of treatments.

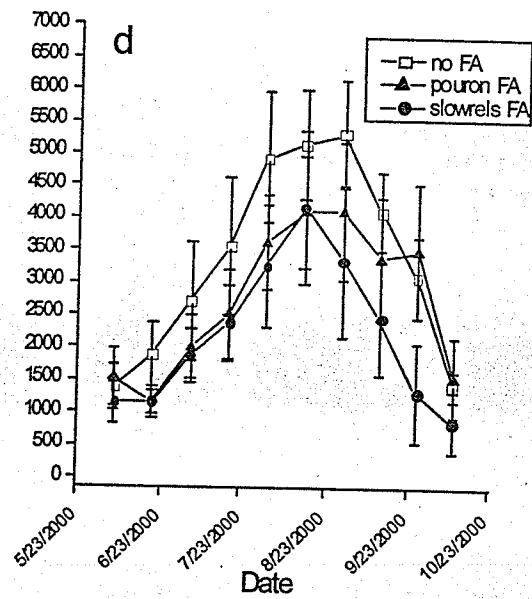
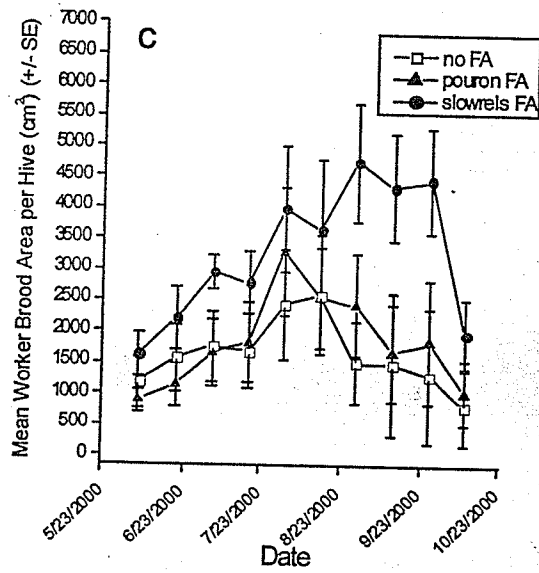
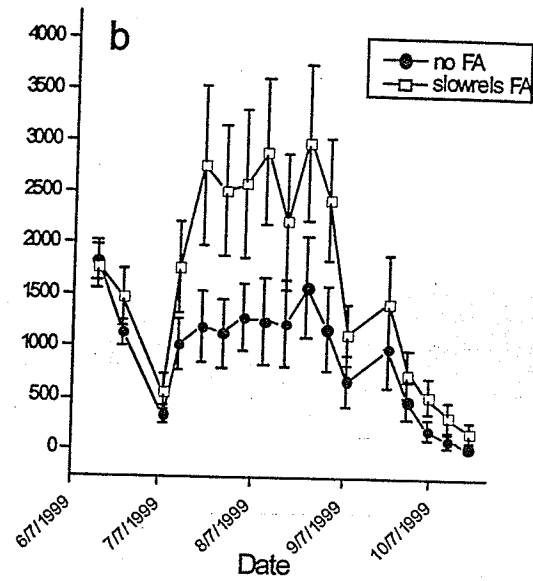
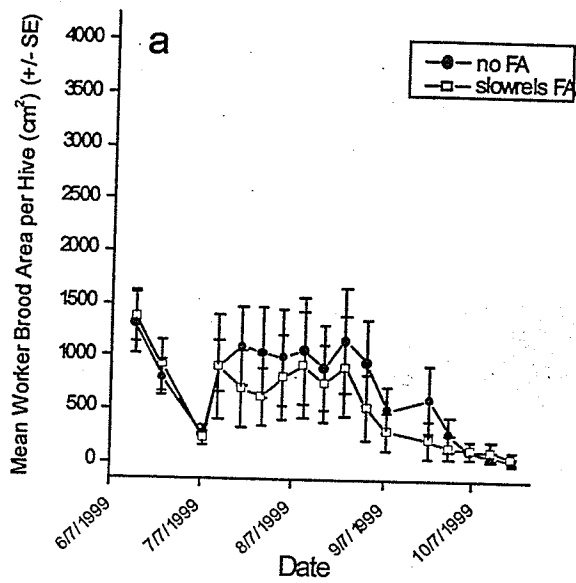


Fig. 9. Concentration of formic acid in hives treated with the slow release formulation in experiment 2. The equation predicts the decrease in the concentration of formic acid in hive air over time. The points represent mean concentrations of formic acid in hive air at 1400 hours on measurement dates (n=8 per sample).

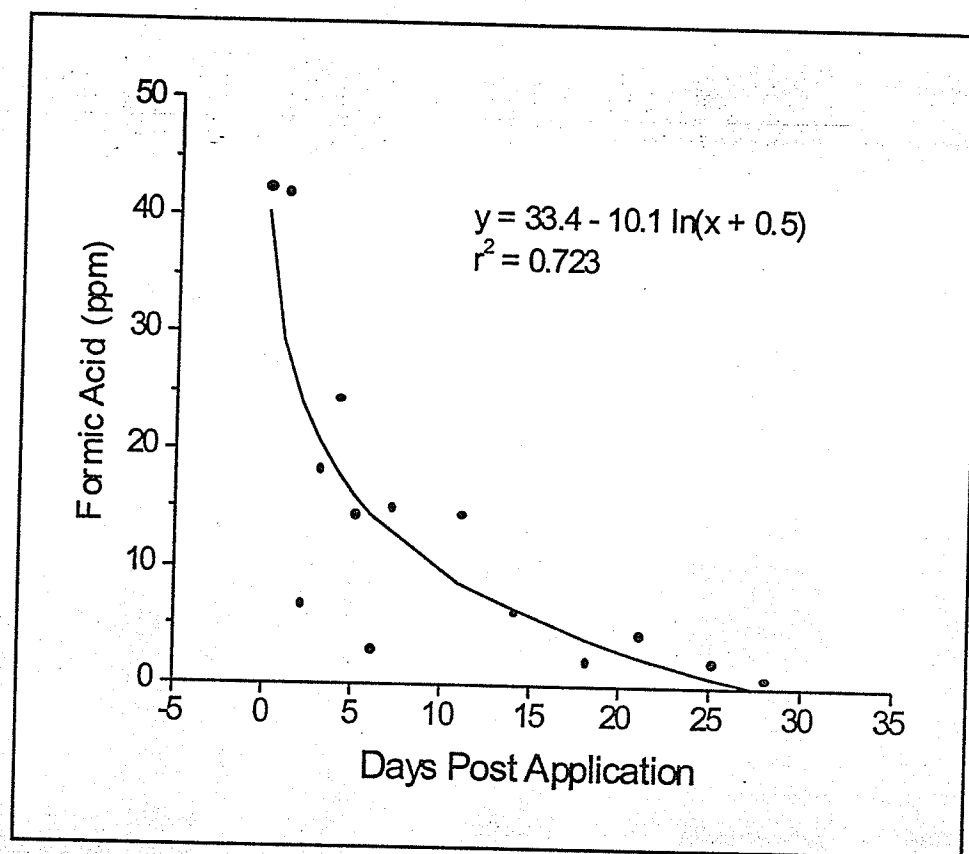


Fig. 10. Concentration of formic acid in hives treated with the pour-on formulation for a one week treatment period in experiment 2. The equation predicts the concentration of formic acid in hive air over time. The points represent mean concentrations of formic acid in hive air at 0900, 1400, and 1900 hours on the days of application and at 1400 hours on the other measurement dates (n=8 per sample).

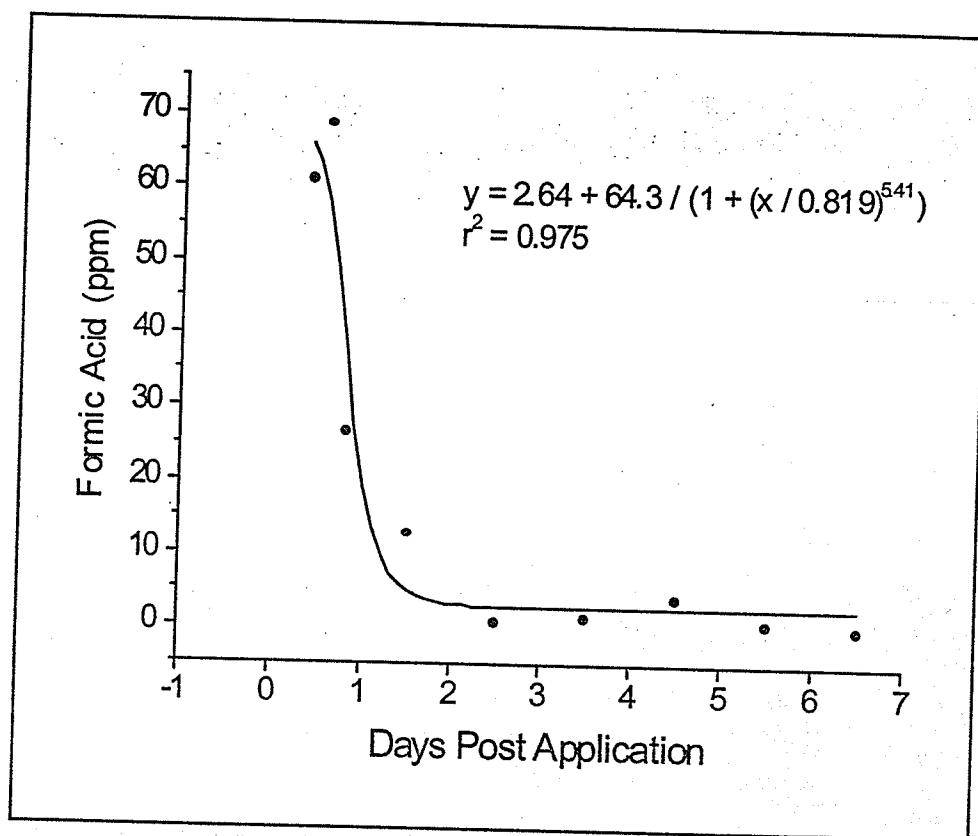


Fig. 11. Interday concentrations of formic acid in hive air of colonies with varroa and formic acid treatments in experiment 2: pour-on and varroa (a), pour-on and no-varroa (b), slow release and varroa (c), and slow release and no-varroa (d). The arrows indicate the times of formic acid application. See Fig. 6 for description of treatments (n=4 for each treatment at each time and on each day).

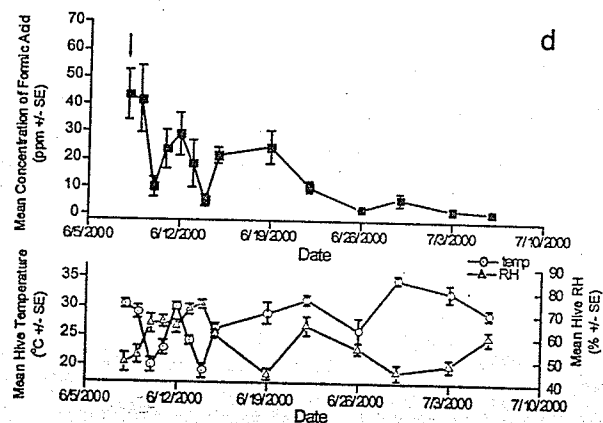
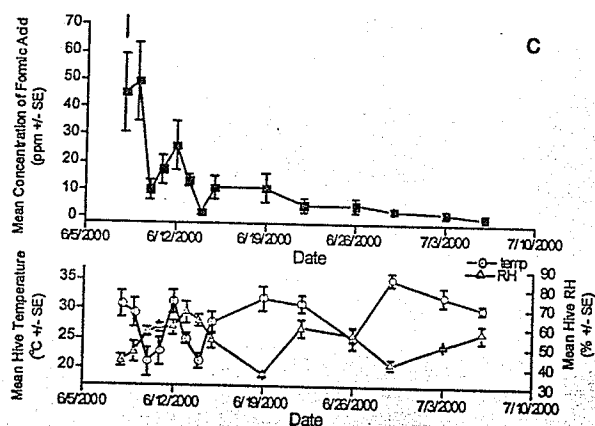
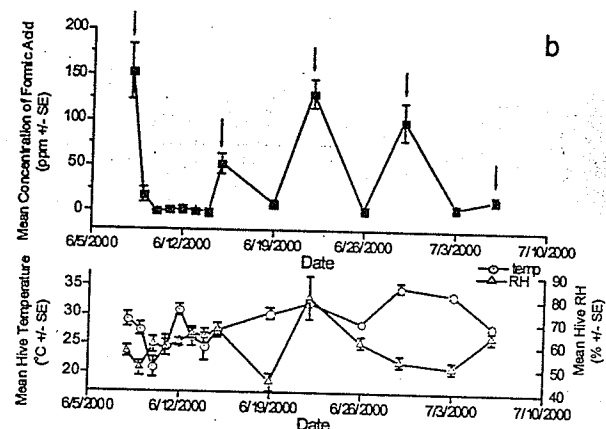
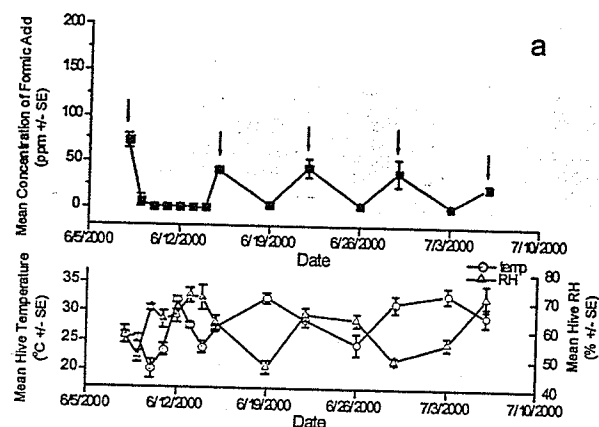
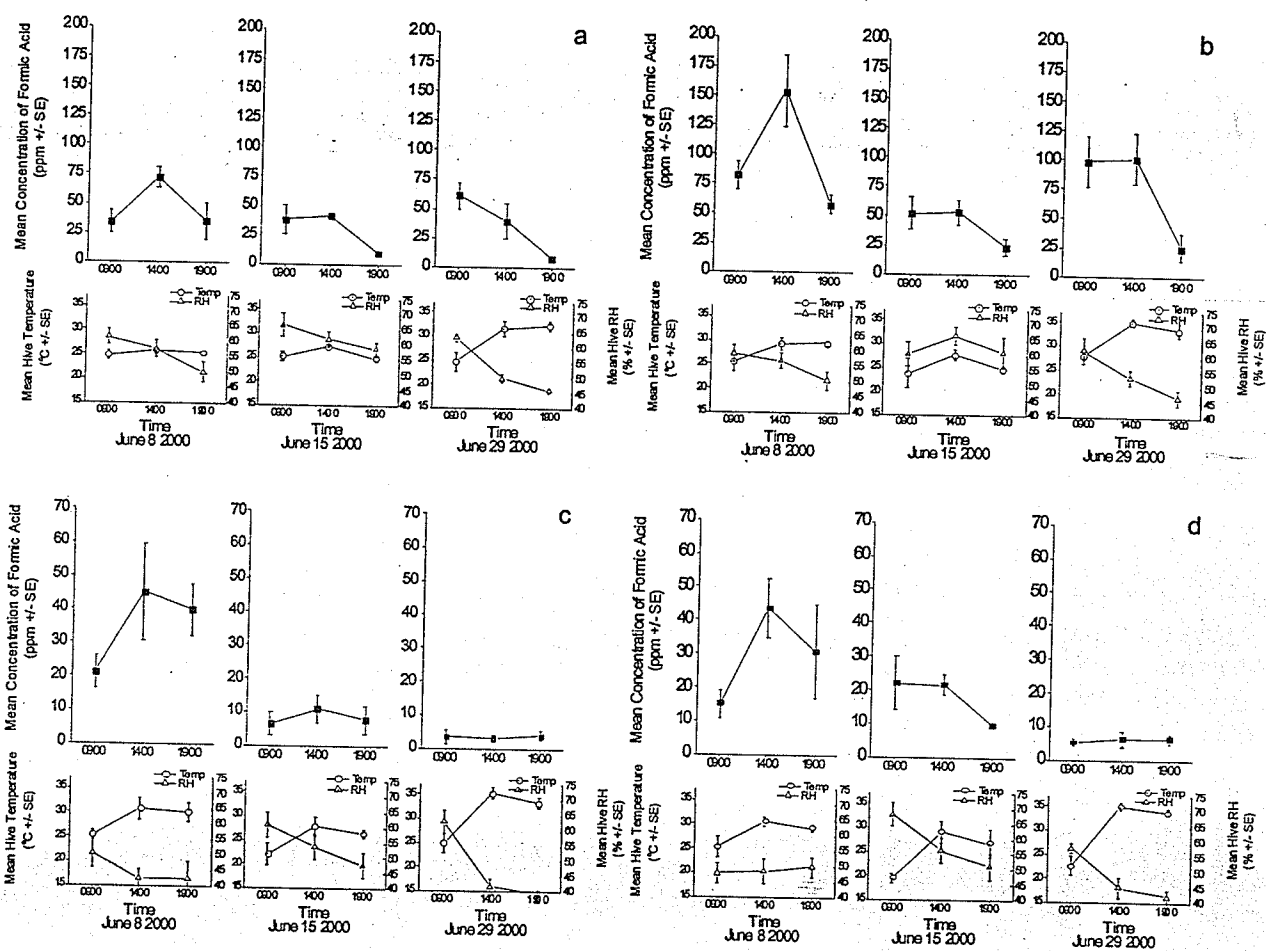


Fig. 12. Intraday concentration of formic acid in hive air on three days with varroa and formic acid treatments: pour-on and varroa (a), pour-on and no-varroa (b), slow release and varroa (c), and slow release and no-varroa (d). See Fig. 6 for description of treatments. Measurements were recorded at approximately 0900, 1400, and 1900 hours on 8, 15, and 29 June (n=4 for each treatment at each time and on each day).



CHAPTER 4

The Interactions of Varroa, *Varroa destructor* Anderson and Trueman, with Chalkbrood, *Ascosphaera apis* (Maassen ex Claussen) Olive & Spiltoir, and Nosema, *Nosema apis* Zander, in Honey Bee, *Apis mellifera* L., Colonies Treated with Formic Acid

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The Interactions of Varroa, *Varroa destructor* Anderson and Trueman, with Chalkbrood, *Ascosphaera apis* (Maassen ex Claussen) Olive & Spiltoir, and Nosema, *Nosema apis* Zander, in Honey Bee, *Apis mellifera* L., Colonies Treated with Formic Acid

Abstract

The effects of varroa infestation and formic acid treatment on chalkbrood and nosema infection were examined. Two field experiments were conducted over a two year period. In the first field experiment, colonies were made up with honey bees infested with varroa or treated with fluvalinate strips to initially eliminate varroa. Varroa and no-varroa treatment colonies were each subdivided into two groups then treated with formic acid or left untreated. The formic acid treatment consisted of 200 mL of 65% formic acid in a pin-perforated Ziploc® vegetable bag slow release application for 40 days. In the second field experiment, varroa treatments were created by infesting varroa-free packages of bees with 45 living varroa mites, and no-varroa treatment colonies were not infested initially with varroa. Varroa and no-varroa treatment colonies were each subdivided into three groups and exposed to a pour-on formic acid treatment, a slow release formic acid treatment, or left untreated. The pour-on formic acid treatment was 40 mL 65% formic acid applied to paper towels five times at seven day intervals. The slow release formic acid treatment consisted of 200 mL of 65% formic acid in a slit Ziploc® freezer bag containing boot felt. Mean varroa and nosema abundance, chalkbrood prevalence, number of honey bees, and area of sealed worker brood per colony were estimated over the course of the season in each field experiment.

Laboratory bioassays were conducted to assess directly the impact of formic acid

on *Ascosphaera apis*. Fungus cultures were treated with different concentrations of formic acid. Total mycelial growth area and spore-producing mycelial growth area were measured, spore cyst and spore ball diameters were measured, and number of spore cysts was estimated for each treatment. In field experiments, chalkbrood prevalence tended to increase with increased mean abundance of varroa. Formic acid, applied as a spring treatment in the presence of varroa, tended to reduce chalkbrood prevalence through direct action and reduction in mite population. However, spring treatment with formic acid when varroa was at low levels, tended to increase prevalence of chalkbrood. These trends were not statistically significant. In laboratory experiments, formic acid in agar inhibited *A. apis* mycelial growth area, spore-producing mycelial growth area, and cyst production significantly at 750 ppm and non-significantly at 250 ppm. Neither varroa infestation nor formic acid treatment had an effect on the levels of nosema infection in either year of the experiment.

Introduction

Varroa destructor Anderson and Trueman is an ectoparasitic mite which feeds on the haemolymph of adult and immature honey bees (*Apis mellifera* L.). This very serious pest can destroy colonies within a couple of years if left untreated. Part of this damage may be caused by diseases which the mite is believed to promote.

A wide variety of disease organisms including bacteria, fungi, protozoans, and a virus have been observed on or within varroa following natural interaction with contaminants (Ball 1988b; Glinski 1988; Liu and Ritter 1988; Strick and Madel 1988;

Alippi *et al.* 1995; Liu 1996; Sammataro 1997). Liu (1996) found as many as 3,598 spores of *Ascosphaera apis* (Maassen ex Claussen) Olive & Spiltoir on the surface of a mite. Apparently the ability of varroa to harbour and transport potentially harmful microorganisms is considerable. Changes in honey bee physiology and general stress caused by varroa's feeding may also encourage development of disease. The parasitic mite syndrome (PMS) exhibited by colonies infested with *V. destructor* and sometimes tracheal mite, *Acarapis woodi* Rennie, is defined by symptoms such as queen supersedure and spotty brood (Shimanuki *et al.* 1994); however, the number of pathogens contributing to this syndrome is not fully understood.

Formic acid is a relatively inexpensive treatment for varroa which is gaining popularity as resistance of the mite to other treatments increases. Using formic acid against mites in the hive may also reduce levels of chalkbrood and nosema by acting on the pathogens directly or by reducing the number of mites which could promote the diseases. Efficacy of formic acid against varroa mites ranges from 51 to 100% mortality (Fries 1991; Feldlaufer *et al.* 1997; Calis *et al.* 1998; Eischen 1998; Calderone 1999b; Calderone and Nasr 1999). Formic acid is usually applied in some form of slow release formulation (Feldlaufer *et al.* 1997; Gatien and Currie, unpublished data), or liquid pour-on formulation (Manitoba Agriculture and Food 1999).

The two main objectives of this study were to assess the potential for varroa to increase the level of chalkbrood and/or nosema disease in a honey bee colony, and to assess whether formic acid might directly or indirectly affect chalkbrood and/or nosema infections.

Methods

This study consisted of two field experiments conducted in 1999 and 2000. Both field trials were designed to assess the effects of formic acid and varroa mite level on honey bee colonies. The 1999 and 2000 field experiments are designated as experiment 1 and experiment 2, respectively.

Experiment 1

Honey bees for the first year trial came from wintered colonies located at the University of Manitoba campus yard. On 8 June, 1999, frames of honey bees were manually shaken from their frames, through a funnel, into standard wooden shipping packages placed on a scale. Approximately 0.91 kg of a yellow strain of bees were shaken into each of 32 packages. Packages were randomly assigned to four treatment groups as follows: (1) varroa and formic acid, (2) varroa and no formic acid, (3) no-varroa and formic acid, and (4) no-varroa and no formic acid. Varroa infestation levels of all 32 packages were determined, and the packages were sorted by infestation level and randomly assigned to four treatment groups such that the average infestation level in each group was approximately equal. To create varroa and no-varroa treatments, half of the packages were treated with Apistan® strips⁸ (7.6 cm x 2.54 cm) for 24 h before the packages were hived. Colonies treated with fluvalinate started the experiment apparently free of the mite, but the average infestation level of no-varroa treatment colonies

⁸a.i. 10% fluvalinate; Wellmark International, Bensenville, Illinois, U.S.

increased over the course of the experiment. Mated queens of a yellow strain were purchased from New Zealand for each package.

On 10 June, 1999, the packages were shaken into standard Langstroth hives. All colonies were inoculated with chalkbrood and nosema on 23 June, 1999. Colonies were artificially infected with chalkbrood by using a spouted plastic bottle to spray 75 mL of sugar syrup (1 part sucrose sugar: 1 part deionized water by weight) containing three macerated black chalkbrood mummies onto broodnest frames. Mummies were collected from hive entrances and bottom boards of infected hives in the spring of 1999 and frozen for about two weeks before use. Colonies were infected with nosema disease by spraying bees on the brood frames of each hive with 100 mL of sugar syrup (1 sucrose sugar: 1 deionized water by weight) containing 1×10^8 *N. apis* spores after Malone *et al.* (1992). *Nosema apis* spores were obtained from bees scooped from infected hives.

Hives were located at four separate sites at the Glenlea Research Station, 20 km south of Winnipeg, Manitoba, Canada. There were eight hives at each site and each site was separated by 75 m to 1500 m. Within an apiary, hives were approximately 1.5 m apart and were arranged in a row or semicircle, with offset entrances, to reduce drift. Each nine-frame brood chamber contained two frames of honey and two frames of pollen. The varroa and formic acid treatment groups of colonies were located by trees which partly shaded the colonies for the duration of the formic acid application.

The formic acid treatment consisted of exposing colonies to a slow-release formulation of 200 mL of 65% formic acid, on a felt pad, sealed within a pin-perforated

Ziploc® vegetable bag⁹ (27 cm x 28 cm). The formic acid bags were placed on the top bars of the top hive box under the inner cover lid. The inner cover gap was 19 mm deep. The formic acid treatment was applied 8 July, 1999 and removed 16 August, 1999, after 40 days.

Varroa infestation, chalkbrood and nosema infection, number of bees and area of brood per colony were estimated every week from 23 June to 3 November, 1999. Varroa infestation for each colony was determined from a sample of approximately 100 bees, collected in a cup from the brood cluster of colonies at least 3,500 bees strong. The bees were counted and washed in 70% ethanol for 10 minutes to dislodge varroa from the bees. Chalkbrood infection was measured by counting the number of chalkbrood mummies in open cells per hive. Nosema infection of each colony was measured by estimating the mean number of *N. apis* spores per bee using a haemocytometer. The sample of approximately 100 bees used to determine varroa infestation was also used to determine nosema infection level. Number of bees per colony was estimated by comparing frames of bees with pictures of known numbers of bees on frames. Brood area per colony was estimated using a transparent plexiglass grid. The grid was held in front of each frame containing brood and sealed worker brood area was measured in cm² then later translated into number of cells, where 1 cm²=4.35 worker brood cells. Observations prior to 8 July represent pretreatment observations.

⁹S.C. Johnson and Son, Limited, Brantford, Ontario, Canada.

Statistical Analysis

The effect of varroa and formic acid treatment groups on colony chalkbrood and nosema infection levels were analyzed by ANOVA (Snedecor and Cochran 1980). Varroa infestation was analyzed as the mean number of mites in the sample divided by the number of bees in the sample, or mean abundance (Bush *et al.* 1997). Chalkbrood infection was analyzed as the mean per cent of brood cells containing chalkbrood mummies, or prevalence, and was transformed using an arcsine transformation. Nosema infection was analyzed as the mean number of spores per sample bee in millions, or mean abundance. Data were analyzed as a split-plot analysis of variance, with varroa treatment and formic acid treatment as main-plot factors, time as a sub-plot factor, and hive as a replicate within each treatment group. Main-plot factors and interactions between them were examined using varroa*formic acid*replicate as an error term. Duncan's multiple range test was used for means separation. The three-way interaction of varroa, formic acid, and time was examined using single degree of freedom contrasts. Results were adjusted with the Bonferroni correction ($\alpha=0.05$).

Experiment 2

Twenty-four 1.82 kg packages from Australia were each split and hived into 48 standard Langstroth brood chambers on 2 May, 2000. Each hive received approximately 0.91 kg of bees and the original queen or a separately-caged queen from the same supplier. All bees were of a yellow strain. Packages were hived into standard Langstroth brood chambers. Each nine-frame brood chamber contained two frames of honey and

two frames of pollen.

Six treatments were randomly assigned to the 48 colonies as follows: (1) varroa and slow-release formic acid, (2) varroa and pour-on formic acid, (3) varroa and no formic acid, (4) no-varroa and slow-release formic acid, (5) no-varroa and pour-on formic acid, and (6) no-varroa and no formic acid. All colonies were inoculated with chalkbrood on 6 June, 2000 as described under Experiment 1.

Varroa treatment colonies were inoculated with 45 mites each on 16 May, 2000, and no-varroa treatment colonies were not inoculated. Mites were collected from live honey bees at the University of Manitoba campus apiary using CO₂ gas and a water-wash setup. Specifically, frames of bees from colonies known to be infested with varroa were shaken into a plastic bag and the bees were then lightly anesthetized with gaseous CO₂. The immobilized bees were scooped into a small plastic container and covered with a screen lid. The container with bees was immersed into a large pail filled with water and lined with mesh straining cloth and shaken vigorously to remove mites from the bees. Mites that were counted were kept on dampened paper towel in petri dishes in a cooler until they could be introduced into colonies. Half of the colonies received no mites, but average infestation level of all treatment groups increased over the course of the experiment.

The slow-release formic acid treatment was similar to the formic acid treatment in Experiment 1 except Ziploc[®] freezer bags¹⁰ (27 cm x 28 cm) were used in place of

¹⁰S.C. Johnson and Son, Limited, Brantford, Ontario, Canada.

vegetable bags, and three vertical slits were cut (1 cm x 22 cm per slit) from one side of each bag. The pour-on formic acid treatment consisted of 40 mL of 65% formic acid poured onto five or six stacked paper towels (13.4 cm x 23.2 cm surface area) on the hive top-bars. The pour-on acid treatment was applied at approximately 0830 hours on five separate applications at a seven day interval. Both formic acid treatments were applied 8 June, 2000 and removed 17 July, 2000, after 40 days. Slow-release freezer bags were weighed post-treatment to determine the total amount of vaporization.

Colonies were examined and sampled every second week from 6 June to 10 October, 2000 to assess nosema and chalkbrood infection, varroa infestation, and hive strength. Observations before 8 June represent pretreatment observations.

Statistical Analysis

The effect of varroa and formic acid treatment groups on colony chalkbrood and nosema infection levels were analyzed by ANOVA (Snedecor and Cochran 1980). Varroa infestation was analyzed as the mean number of mites in the sample divided by the number of bees in the sample, or mean abundance. Chalkbrood infection was analyzed as the mean per cent of brood cells containing chalkbrood mummies, or prevalence, and was transformed using an arcsine transformation. Nosema infection was analyzed as the mean number of spores per sample bee in millions, or mean abundance. Data were analyzed as a split-plot analysis of variance, with varroa treatment and formic acid treatment as main-plot factors, time as a sub-plot factor, and hive as a replicate within each treatment group. Main-plot factors and interactions between them were

examined using varroa*formic acid*replicate as an error term. Duncan's multiple range test was used for means separation. The three-way interaction of varroa, formic acid, and time was examined using single degree of freedom contrasts. Results were adjusted with the Bonferroni correction ($\alpha=0.05$).

Laboratory Experiment

Ascosphaera apis was treated with formic acid in two experiments to assess the impact of formic acid on different stages of *A. apis*. The fungus was treated with formic acid in culture dishes containing agar. Total mycelial growth area, spore-producing mycelial growth area, and spore cyst and spore ball diameter were measured. The number of spore cysts per dish was estimated. The effect of formic acid vapours on germination of *A. apis* was also assessed.

Culture Growth Trials

Potato dextrose agar with yeast extract (PDAY) was prepared by dissolving 50 g of potato dextrose agar and 5 g of yeast extract in 1.25 L of deionized water. The solution was autoclaved for 15 min, cooled in the autoclave for 1 h, then cooled at room temperature for about 1 h. Culture plates (100 mm x 15 mm) were poured under a fume hood on a weigh scale (30 g PDAY per plate). Formic acid was added to the liquid agar using a micropipette and mixed using a flame-sterilized metal stirrer. Formic acid concentrations in the agar were approximately 0 ppm (controls), 250 ppm, 500 ppm, and 750 ppm. One mycelial plug or spore plug (6 mm diameter) from a parent *A. apis* culture

was placed at the center of each agar dish. Plugs were taken from clean and pure parent cultures. Parent cultures that exhibited average growth and that were not previously treated with formic acid were selected and refrigerated for 24 h to halt growth of the fungus and allow time for preparation of the next experiment. Culture plates were covered partially with a lid and exposed to CO₂ for approximately 10 to 15 sec, then covered completely and sealed with Parafilm M®. Four replicates of each treatment were prepared for both mycelial plugs and spore plugs (32 dishes total) and incubated at 30°C for 168 h (7 days). The experiment was repeated twice.

After seven days, *A. apis* growth of mycelia and spore-producing mycelia were traced using a transparency sheet, then measured using a small transparent plexiglass grid with 0.25 cm² divisions. Mycelial area was measured as the area of mycelial growth. Spore area was measured from the spore plug cultures as the area of the mycelia which actively produced spore cysts. This area was black and originated from the spore plug at the center of the culture. The area of the plug (approximately 0.25 cm²) was subtracted from the measured growth areas of each culture.

Spore Cyst and Spore Ball Counts and Measurements

The culture with agar was divided into pieces of approximately 5 cm² and scooped into a 250 mL beaker using a metal scoop. Ten mL deionized water and a 2 cm stirring bar were added to the mixture. A glass petri dish (100 mm x 20 mm) was used to cover the beaker. The mixture was boiled for 10 minutes and stirred until the mixture began to boil. An aliquot of the solution was extracted with a micropipette and placed on both

sides of a haemocytometer. The solution was then examined under a microscope at 400x magnification. The number of spore cysts (intact) in the entire 3 mm x 3 mm (9 mm²) counting chamber of the haemocytometer was determined. Total spore cyst culture count in approximately 30 mL of homogenized culture mixture was estimated by multiplying the sample count by 33,000. The sizes of spore cysts and spore balls were determined using a ruled eye piece calibrated to the known measurements of the haemocytometer grid (where 1 unit=2.94 µm). Measurements were estimated to approximately ± 1.5 µm.

Spore Germination Test

Ten black chalkbrood mummies were collected from an infected hive at the apiary at the University of Manitoba. Each of the 10 mummies were divided into four pieces. Two of the pieces from each mummy were each added to 0.5 mL distilled water in separate mortar bowls and macerated with a pestle. The spore mixture from each sample was poured onto a glass slide, labeled, and randomly assigned to either a formic acid or control treatment. The remaining two intact mummy pieces were placed in small plastic weighing dishes, labeled, and randomly assigned to the formic acid or control treatment. All ten replicates of each treatment were placed on a clipboard and put into either a formic acid-treated or control room at a height of 1.0 m for six days. Formic acid was introduced into the room using an apparatus which consisted of a fan over a pan with a float, fed from a plastic bag containing 85% formic acid (Underwood and Currie 2002a). The dimensions of each treatment room were 1.75 m wide x 3.05 m deep x 2.7 m high. Formic acid concentration ranged from 40 to 64 ppm (0.08 to 0.13 mg/L) during the

course of treatment. Temperature and relative humidity in the formic acid room ranged from 10.2 to 14.5 °C and 50.4 to 80.9%, respectively, while temperature and relative humidity in the control room ranged from 5.4 to 7.8 °C and 29.1 to 40.7%, respectively.

After six days, samples were removed and prepared for incubation. A solution of Sabaraud Dextrose Broth with yeast extract (SDBY) was made with 7.5 g SDB, 2.5 g yeast extract, and 250 mL deionized water. The spores were washed off the slides with 5 mL SBDY solution into a mortar then poured into a small beaker. Each mummy piece was individually macerated with a pestle in a mortar with 5 mL SBDY solution then poured into a small beaker. Each beaker mixture was exposed to CO₂ through a tube from a tank of CO₂ for 60 sec. sealed with Parafilm M[®], and incubated at 30°C for three days.

Following incubation, each mixture was examined for spore germination by removing a drop and examining it on a microscope slide under 1000x magnification. The presence of germinated spores was recorded.

Statistical Analysis

For the laboratory bioassays, total mycelial growth area, spore-producing mycelial growth area, spore cyst and spore ball diameter, and number of spore cysts were determined for each plate. Spore cyst and spore ball diameters were analyzed based on the average value per dish. Data were analyzed as a randomized complete block ANOVA (Snedecor and Cochran 1980), with formic acid concentration as the treatment and the date when experiments were repeated as a blocking factor. Formic acid had four levels

and block had two levels. Formic acid treatment differences were examined using Duncan's multiple range test ($\alpha=0.05$).

Results

Experiment 1

In the first field experiment the three-way interaction between the effects of varroa, formic acid, and sampling period on mean abundance of varroa was not significant ($F=0.12$; $df=1,7$; $P>F=0.7048$). Mean abundance of varroa ($\pm$ SE) over the course of the experiment was significantly greater in colonies initially infested with the mite, or varroa treatment colonies (0.053 ± 0.007), than in colonies not initially infested with the mite, or no-varroa treatment colonies (0.012 ± 0.002) ($F=118.16$; $df=1,7$; $P>F<0.0001$) (Fig. 6a, b). On the final day of the experiment, average varroa abundance was 0.112 ± 0.030 in varroa treatment colonies and 0.035 ± 0.010 in no-varroa treatment colonies. Since weaker colonies were not sampled on all sampling dates statistical analysis of seasonal effects were not conducted; however, the mite mean abundance data that were collected, from colonies with more than 3,500 bees, are presented in Fig. 6.

Chalkbrood Disease

In the first field experiment, there was no significant effect of either the formic acid treatment ($F=1.01$; $df=1,6$; $P>F=0.3546$) or the varroa mite treatment ($F=0.08$; $df=1,6$; $P>F=0.9650$) on the percentage of brood cells containing chalkbrood mummies, or prevalence of chalkbrood (Fig. 13a, b). The overall average percent of brood affected

by chalkbrood in a colony ($\pm$ SE) was $1.2 \pm 0.2\%$ in colonies not treated with formic acid and $1.6 \pm 0.3\%$ in colonies treated with formic acid. The average prevalence of chalkbrood infection was $1.8 \pm 0.3\%$ in varroa treatment colonies and $1.0 \pm 0.2\%$ in no-varroa treatment colonies.

The three-way interaction between the effects of varroa, formic acid, and sampling period on chalkbrood infection level was not significant ($F=0.18$; $df=6,18$; $P>F=0.996$). However, there was a trend towards higher levels of chalkbrood in colonies that had higher infestations of varroa. In colonies initially infested with varroa, the overall average prevalence of chalkbrood ($\pm$ SE) was $1.8 \pm 0.4\%$ in the colonies not treated with formic acid, and $1.8 \pm 0.3\%$ in the colonies treated with formic acid. However, in colonies not initially infested with varroa, the average prevalence of chalkbrood was only $0.7 \pm 0.3\%$ in the colonies not treated with formic acid, and $1.3 \pm 0.3\%$ in the colonies treated with the formic acid treatment.

Nosema Disease

Mean abundance of nosema declined steeply two weeks into the trial and was low throughout the season. There was no effect of formic acid ($F=0.40$; $df=1,5$; $P>F=0.5560$) or varroa ($F=0.19$; $df=1,5$; $P>F=0.6819$) on the average number of nosema spores per adult worker (Fig. 14a, b). Over the course of the season, the overall average nosema infection level ($\pm$ SE) was $0.70 \pm 0.16 \times 10^6$ spores per bee in colonies not treated with formic acid and $0.61 \pm 0.15 \times 10^6$ spores per bee in colonies treated with formic acid. The overall average nosema infection level was $0.70 \pm 0.19 \times 10^6$ spores per bee in colonies

initially infested with varroa and $0.62 \pm 0.14 \times 10^6$ spores per bee in colonies not initially infested with varroa.

The three-way interaction between the effects of varroa, formic acid, and sampling period on nosema infection level was not significant ($F=0.157$; $df=5,8$; $P>F=2.06$). In colonies initially infested with varroa, the overall average level of nosema infection ($\pm$ SE) was $0.86 \pm 0.37 \times 10^6$ spores per bee in the colonies not treated with formic acid, and $0.59 \pm 0.18 \times 10^6$ spores per bee in the colonies treated with formic acid. In colonies not initially infested with varroa, the average level of nosema infection was $0.67 \pm 0.16 \times 10^6$ spores per bee in the colonies not treated with formic acid, and $0.62 \pm 0.23 \times 10^6$ spores per bee in the colonies treated with the formic acid treatment.

The first and second observations of the trial were not included in the statistical analysis since they were recorded prior to formic acid treatment application. The data of both pretreatment periods are included in the figures.

Experiment 2

In the second field experiment, mean abundance of varroa ($\pm$ SE) over the course of the experiment was significantly greater (0.009 ± 0.001) in colonies initially infested with the mite, or varroa treatment colonies, than in colonies not initially with the mite, or no-varroa treatment colonies (0.003 ± 0.001) ($F=16.05$; $df=1,14$; $P>F=0.0013$) (Fig. 6). On the final day of the experiment, mean abundance of varroa was 0.035 ± 0.006 in varroa treatment colonies and 0.018 ± 0.008 in no-varroa treatment colonies. Formic acid application did not have a significant effect on the level of varroa infestation per colony

($F=0.98$; $df=2,14$; $P>F=0.3999$); however, there was a trend towards a reduction of varroa levels in colonies treated with either formulation of formic acid. In 2000, the slow release formic acid application decreased overall average varroa infestation over the course of the season by 9.4% to 0.007 ± 0.001 , while the pour-on application decreased varroa infestation over the course of the season by 29.3% to 0.005 ± 0.001 , compared to the control group level of varroa infestation of 0.007 ± 0.002 . The three-way interaction between the effects of varroa, formic acid, and sampling period on varroa infestation level was not significant ($F=0.95$; $df=14,44$; $P>F=0.5259$).

Chalkbrood Disease

In the second field experiment, there was no significant effect of either the formic acid treatment ($F=1.57$; $df=2,14$; $P>F=0.2422$) or the varroa mite treatment ($F=0.470$; $df=1,14$; $P>F=0.5060$) on the prevalence of chalkbrood (Fig. 13c, d). The overall average percentage of brood infected with chalkbrood in a colony ($\pm$ SE) was $0.35 \pm 0.06\%$ in colonies not treated with formic acid, $0.19 \pm 0.05\%$ in colonies treated with slow release formic acid, and $0.16 \pm 0.04\%$ in colonies treated with pour-on formic acid. The average prevalence of chalkbrood infection was $0.20 \pm 0.04\%$ in varroa treatment colonies and $0.27 \pm 0.04\%$ in no-varroa treatment colonies.

The three-way interaction between the effects of varroa, formic acid, and sampling period on chalkbrood infection level was not significant ($F=1.64$; $df=14,44$; $P>F=0.0973$). However, there was a trend towards lower levels of chalkbrood in colonies treated with formic acid. In colonies initially infested with varroa, the average prevalence

of chalkbrood ($\pm$ SE) was $0.35 \pm 0.10\%$ in colonies not treated with formic acid, $0.13 \pm 0.07\%$ in colonies treated with slow release formic acid, and $0.16 \pm 0.04\%$ in colonies treated with pour-on formic acid. In colonies not initially infested with varroa, the average prevalence of chalkbrood was $0.36 \pm 0.07\%$ in colonies not treated with formic acid, $0.25 \pm 0.06\%$ in colonies treated with slow release formic acid, and $0.17 \pm 0.06\%$ in colonies treated with pour-on formic acid.

Nosema Disease

There was no effect of formic acid ($F=1.49$; $df=2,12$; $P>F=0.2648$) or varroa ($F=0.45$; $df=1,12$; $P>F=0.5138$) on the mean abundance of nosema (Fig. 14c, d). The mean abundance of nosema ($\pm$ SE) was $0.58 \pm 0.09 \times 10^6$ spores per bee in colonies not treated with formic acid, $0.79 \pm 0.12 \times 10^6$ spores per bee in colonies treated with slow release formic acid, and $0.62 \pm 0.10 \times 10^6$ spores per bee in colonies treated with pour-on formic acid. The average nosema infection level was $0.70 \pm 0.08 \times 10^6$ spores per bee in colonies initially infested with varroa and $0.64 \pm 0.09 \times 10^6$ spores per bee in colonies not initially infested with varroa.

The three-way interaction between the effects of varroa, formic acid, and sampling period on nosema infection level was not significant ($F=0.52$; $df=12,30$; $P>F=0.9112$). In colonies initially infested with varroa, the overall average level of nosema infection ($\pm$ SE) was $0.73 \pm 0.14 \times 10^6$ spores per bee in the no formic acid colonies, $0.72 \pm 0.13 \times 10^6$ spores per bee in the slow release formic acid colonies, and $0.64 \pm 0.15 \times 10^6$ spores per bee in the pour-on formic acid colonies. In colonies not initially infested with varroa, the

overall average of nosema infection was $0.49 \pm 0.11 \times 10^6$ spores per bee in the no formic acid colonies, $0.90 \pm 0.22 \times 10^6$ spores per bee in the slow release formic acid colonies, and $0.60 \pm 0.14 \times 10^6$ spores per bee in the pour-on formic acid colonies.

The first observations of the trial were not included in the statistical analysis since they were recorded prior to formic acid treatment application. The pretreatment observations are included in the figures.

Laboratory Experiments

Ascosphaera apis mycelial growth area ($F=46.23$; $df=3,54$; $P>F<.0001$) and spore-producing mycelial growth area ($F=3.93$; $df=3,25$; $P>F=0.0199$) were inhibited by formic acid in laboratory bioassays. Formic acid suppressed mycelial growth area relative to controls in agar at 500 ppm and 750 ppm (Fig. 15). Formic acid suppressed spore-producing mycelial growth area at 750 ppm (Fig. 16). Similarly, *A. apis* cyst production on agar was significantly lower than the controls at 750 ppm ($F=3.54$; $df=3,24$; $P>F=0.0296$) (Fig. 17).

Ascosphaera apis spore ball size ($F=2.60$; $3,23$; $P>F=0.2983$) and spore cyst size ($F=0.24$; $df=3,23$; $P>F=0.8708$) were not significantly affected by formic acid in agar at concentrations up to 750 ppm (Figs. 18 and 19, respectively).

Chalkbrood germination was not affected by room fumigation of formic acid at 40 to 64 ppm for six days. Germination of the spores on the slides and the spores from intact mummy pieces, from nine of the 10 chalkbrood mummies, in both the formic acid and control treatment groups, was observed. Spores from the tenth chalkbrood mummy did

not germinate after either the formic acid or control treatments.

Discussion

Prevalence of chalkbrood tended to increase with increased mean varroa abundance, though not significantly. Formic acid applied as a spring treatment in the presence of varroa tended to reduce prevalence of chalkbrood, though not significantly. However, formic acid applied as a spring treatment when varroa was at low levels tended to increase the prevalence of chalkbrood. Formic acid in agar inhibited *A. apis* mycelial growth area, spore-producing mycelial growth area, and cyst production significantly at 750 ppm and non-significantly at 250 ppm, and had no effect on the size of cysts or spore balls at any tested concentration. Neither varroa infestation nor formic acid treatment had an effect on the levels of nosema infection in either year of the experiment.

Promotion of chalkbrood by varroa appeared to depend on the mite infestation level. Mean abundance of varroa was an average of two to four times higher in experiment one than in experiment two. In the first field experiment, when the overall average varroa abundance ($\pm$ SE) was relatively high (0.053 ± 0.007) compared to experiment two, the prevalence of chalkbrood was 75.5% higher on average in colonies initially infested with varroa than in colonies with no initial varroa infestation. In the second field experiment, when average varroa abundance was relatively low (0.009 ± 0.001), the prevalence of chalkbrood was 25.2% lower on average in varroa treatment colonies than in no-varroa treatment colonies. When infestation is relatively high, varroa may facilitate the ability of chalkbrood to infect larvae or increase the susceptibility of the

brood. It is known that honey bee larvae are susceptible to chalkbrood infection at the time when varroa enter their cells (Gilliam *et al.* 1978). The mites frequently fall to the bottom board (Pettis and Shimanuki 1999) where they can potentially acquire many *A. apis* spores (Liu 1996). Varroa could also increase the level of chalkbrood by increasing the susceptibility of honey bees to infection with the disease. The reduced total protein, protein concentration (Weinberg and Madel 1985; Schatton and Engels 1988), and number of protein granules per cell (Drescher and Schneider 1988) resulting from varroa feeding and use of unaltered proteins (Tewarson 1983) may cause nutritional stress which is believed to make larvae more susceptible to chalkbrood infection (De Jong 1976; Chu 1988; Chu *et al.* 1992). The reduction in number of bees per colony infested with varroa could also reduce the cluster size thereby exposing outer brood to cooler conditions that are associated with increased potential for infection (Bailey 1967).

Mixed results were also documented in previous studies in which the interaction between chalkbrood and varroa was examined. Glinski (1988) found that the percentage of colonies with chalkbrood, increased from 13.5 to 52.3% in varroa-infested apiaries, and from 10.0 to 18.8% in varroa-free apiaries in approximately two years. Furthermore, the number of mummies per colony was 1.2 to 4.2 times greater in varroa-infested colonies than in colonies that were free of the mite. However, the percentage of brood cells containing chalkbrood mummies in colonies infested with the mite and the varroa infestation levels were not presented in their report. Medina and Vicario Mejia (1999) observed no significant correlation between the number of varroa and chalkbrood mummies per colony, in bottom board traps of normal and collapsing colonies, but

concluded that varroa could play an indirect role in increasing chalkbrood by increasing colony stress.

In our second field experiment, formic acid reduced prevalence of chalkbrood directly as well as indirectly through a reduction in mite population. In 2000, in colonies initially infested with varroa and treated with slow release or pour-on formic acid, chalkbrood prevalence decreased by 61% and 55%, respectively, relative to colonies not treated with formic acid. In colonies not initially infested with varroa and treated with slow release or pour-on formic acid, chalkbrood prevalence decreased by 29% and 53%, respectively, relative to colonies not treated with formic acid. A similar trend was not observed in the first field experiment but high levels of colony stress may have contributed to the differences in chalkbrood prevalence between experiments. In 1999, colonies initially infested with varroa and treated with formic acid had 3% less chalkbrood than colonies not treated with formic acid. In colonies not initially infested with varroa, formic acid treatment increased chalkbrood prevalence by 81% over colonies not treated with formic acid. It appears the additional stress of formic acid exposure on colonies (Von Posern 1988; Fries 1991; Bolli *et al.* 1993) may promote prevalence of chalkbrood when colonies are already stressed by relatively high levels of chalkbrood and nosema. Large variation in the levels of chalkbrood disease between similarly treated colonies is not uncommon (Gilliam *et al.* 1983). The greater variability of chalkbrood prevalence that we observed in varroa treatment colonies compared to no-varroa treatment colonies was probably due to the progressive death of colonies that resulted from the combination of mite and disease pressure over the season.

In laboratory bioassays, *A. apis* growth was suppressed by formic acid in agar, particularly at high concentrations. Formic acid significantly inhibited mycelial and spore-producing mycelial area growth as well as cyst production at 750 ppm in agar. Overall, mycelial and spore-producing mycelial growth areas decreased as formic acid concentration in agar increased. Spore cyst and spore ball sizes did not change as the formic acid concentration in agar increased. Fumigating sporulated chalkbrood mummies and *A. apis* spores with 40 to 64 ppm formic acid for six days at a low temperature had no apparent effect on spore germination of the fungus. The differences of temperature and relative humidity in the formic acid treatment and control rooms likely had no effect on *A. apis* because of the fungus' tolerance to a wide range of temperature (Hale and Menapace 1980; Mossadegh and Alizadeh 1995) and humidity (De Jong 1977; Herbert *et al.* 1987). Formic acid in agar had no effect on the number of spores within a spore ball or the number of spore balls within a spore cyst. Therefore, if formic acid does control *A. apis* in the field, it would likely be via the inhibition of mycelial growth and subsequent cyst production, rather than by diminishing the spore arsenal within individual cysts. The great variability of spore cyst and ball counts and measurements of samples taken from the same culture mixture demands modification to the methods used in our laboratory bioassays. Taking sample aliquots while the culture mixture is being stirred rather than shortly thereafter would probably reduce the variability of same culture mixture observations.

The laboratory results showed that formic acid concentration greater than 250 ppm is required to control chalkbrood. Control of chalkbrood by formic acid in the hive

may be poor and/or sporadic because the concentration of acid rarely reaches the dose required to suppress chalkbrood. Formic acid concentrations in hive air may reach up to 250 ppm, but the range is usually 0 to 100 ppm, depending on the formulation and the number of days post application (Ostermann Chapter 3; Feldlaufer *et al.* 1997; Skinner *et al.* 2001). Formic acid is a natural component of honey. Some evidence suggests that concentrations of formic acid in honey may reach levels high enough to suppress chalkbrood growth. The naturally occurring formic acid content in honey varies with the floral source and may range from 9 to 152 ppm, but honey in hives treated with 65% formic acid for tracheal mite control may contain up to almost 500 ppm (Liu *et al.* 1993). The reason why the influence of formic acid on chalkbrood development in colonies is so variable may be because the required dose-exposure time combination is not usually achieved.

In our study, mean abundance of nosema was not affected by spring treatments with formic acid. However, Underwood and Currie (2002a) have shown that the level of nosema found in honey bee workers, can be suppressed by winter fumigation with high doses of formic acid. Fries (1990) tested formic acid fumigation against exposed *N. apis* and found no effect of the acid on spore infectivity when faeces-stained comb was treated with 4 applications, in 24 h intervals, of 15 mL 85% formic acid from below. Honey bees that were then fed spores from the treated comb became infected with nosema disease. However, Ruijter and Steen (1989) fumigated exposed *N. apis* spores with 100 mL 96% acetic acid on cotton wool from above and observed that the spores did not induce infection in workers. In the same study, treatment of *N. apis* spores with acetic acid from

a dish did not affect spore infectivity. Gontarski (1951) found that feeding solutions of acidic or alkaline sugar solution did not prevent nosema infection nor reduce the number of *N. apis* spores produced by infected honey bees. These mixed results of acids on the infectivity of exposed spores are evidence that the protozoan is vulnerable to acid treatment but that the type of acid and method of application may influence results.

Concentrations of formic acid used in our colonies were not effective in suppressing nosema when applied as a spring application. Varroa mites did not appear to enhance the development of nosema in colonies. *Nosema apis* spores have been found on the surface of varroa (Liu 1996) but it is not known whether the mite can carry enough spores to increase the probability of infection. Fries (1988b) found that the average number of spores required for nosema infection was 94.3. The protozoan has been found in the haemolymph of honey bees (Sokolov and Grobov 1963), and may even attack larval haemocytes (Gilliam and Shimanuki 1967), but spores of *N. apis* have never been documented within *V. destructor*. The size of a *N. apis* spore (4.0 to 6.0 μm by 2.0 to 4.0 μm (Liu 1984; Graham 1992; Fries 1993)) likely prevents its uptake by *V. destructor* during feeding. Spores which have been found within the mite range in size from 0.35 to 2.5 μm , so the mite may be unable to ingest substances the size of a *N. apis* spore (Glinski 1988; Liu and Ritter 1988). Although the literature suggests that varroa could transfer the disease in the haemolymph if the mite was able to ingest the spore (Sokolov and Grobov 1963), we saw no evidence of an increase of nosema abundance associated with varroa populations.

Varroa's interaction with *A. apis* appears to promote the fungal disease and should

be considered in the evaluation of damage caused by the mite. In addition to slightly reducing the mean abundance of varroa, treatment with formic acid reduced chalkbrood prevalence. Although formic acid in the hive air did not reach concentrations that our laboratory trials indicated were required for significant inhibition of fungal growth, it is possible that chalkbrood disease may be directly inhibited at lower concentrations in full size colonies.

Fig. 13. Chalkbrood prevalence with varroa and formic acid treatments: varroa in experiment 1 (a), no-varroa in experiment 1 (b), varroa in experiment 2 (c), and no-varroa in experiment 2 (d). The arrow indicates the time of treatment application. See Fig. 6 for description of treatments.

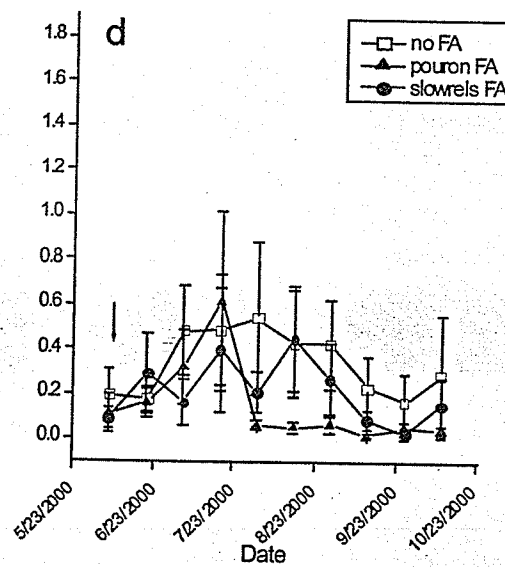
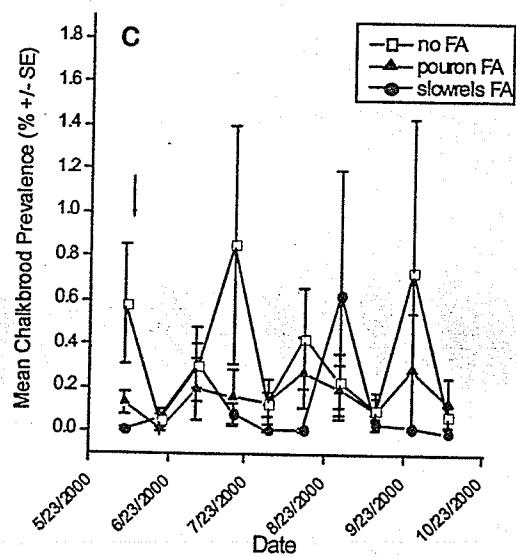
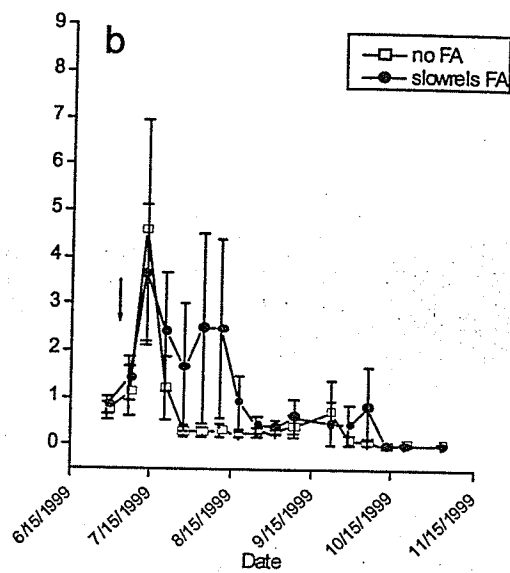
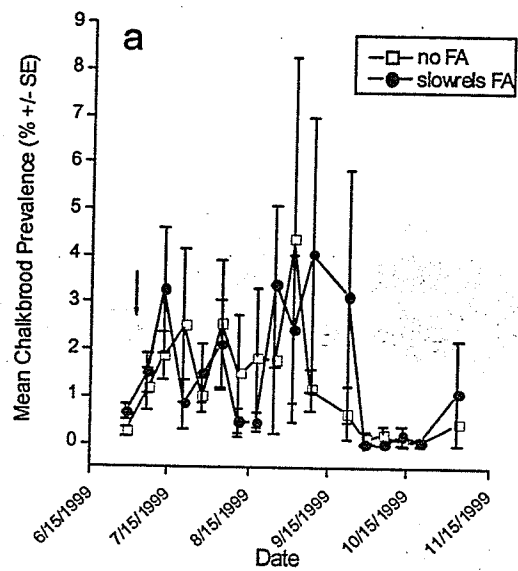


Fig. 14. Mean nosema abundance with varroa and formic acid treatments: varroa in experiment 1 (a), no-varroa in experiment 1 (b), varroa in experiment 2 (c), and no-varroa in experiment 2 (d). The arrow shows the time of formic acid application. See Fig. 6 for description of treatments.

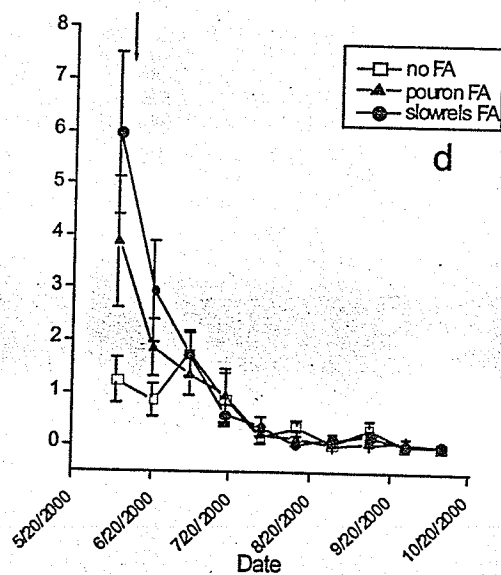
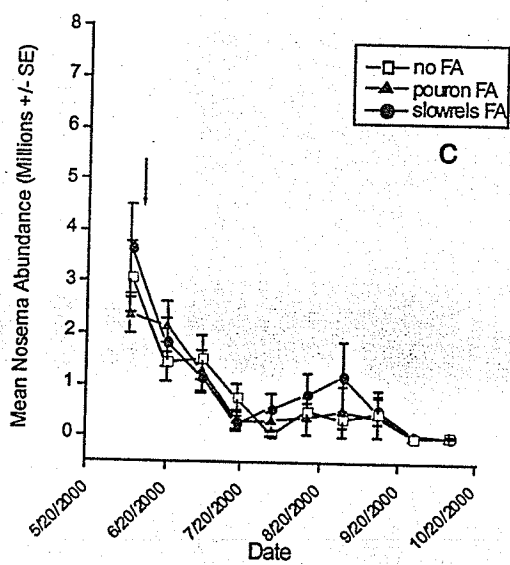
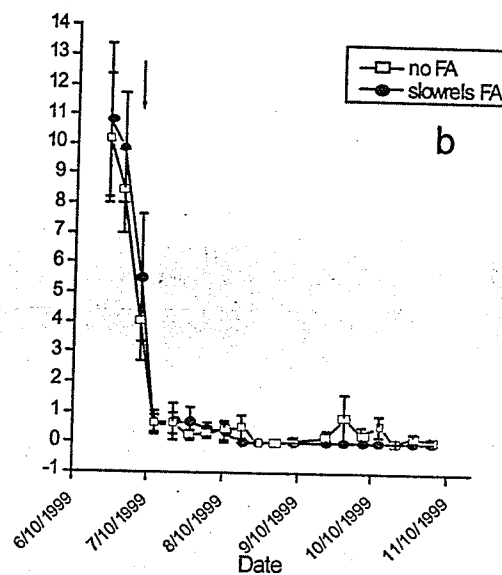
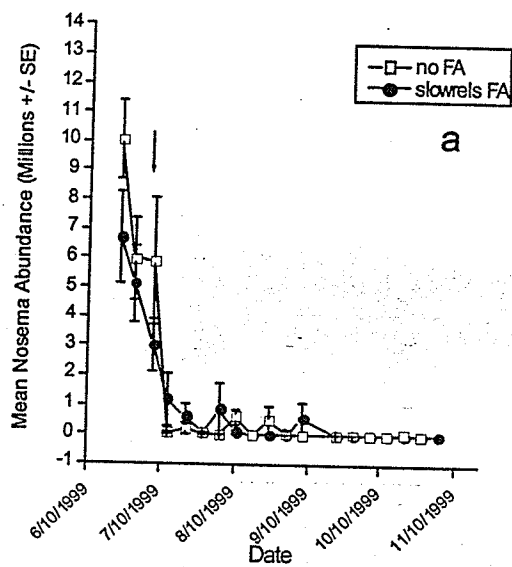


Fig. 15. *Ascosphaera apis* mycelial growth on potato dextrose with yeast extract agar inoculated with formic acid. Individual cultures were measured following incubation at 30°C for 168 h. Means with the same letter are not significantly different (Duncan's multiple range test; $\alpha=0.05$).

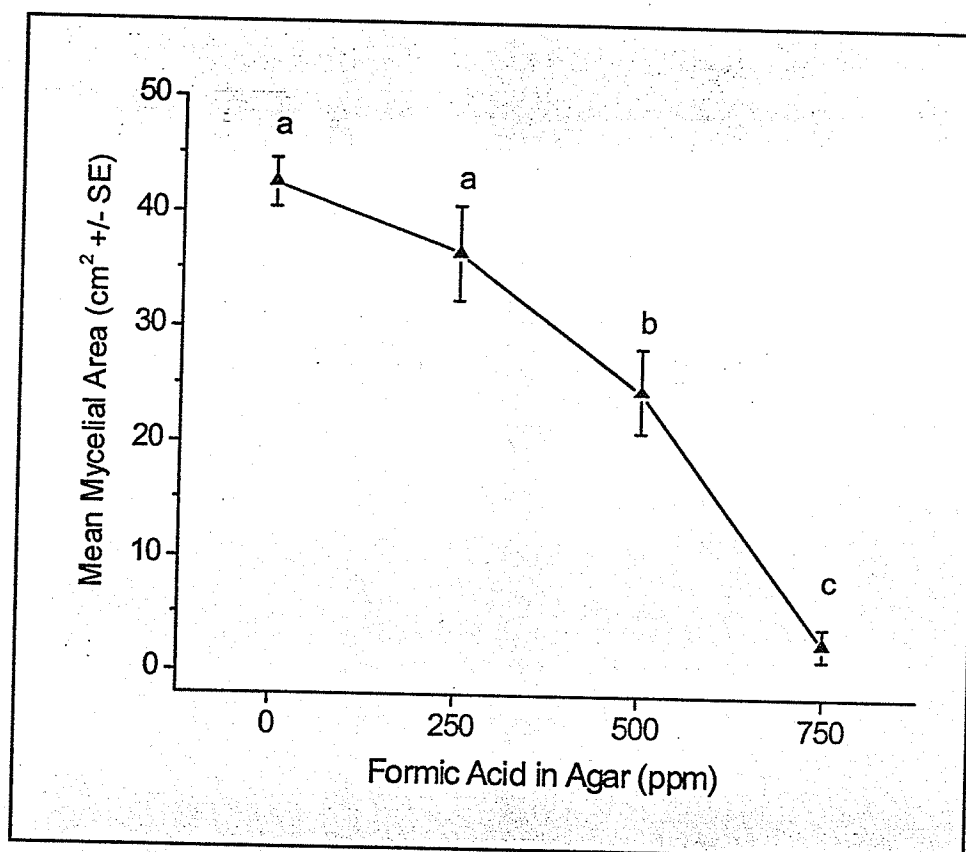


Fig. 16. *Ascosphaera apis* growth of spore-producing mycelia on potato dextrose with yeast extract agar inoculated with formic acid. Area of spore-producing mycelia of individual cultures was measured following incubation at 30°C for 168 h. Means with the same letter are not significantly different (Duncan's multiple range test; $\alpha=0.05$).

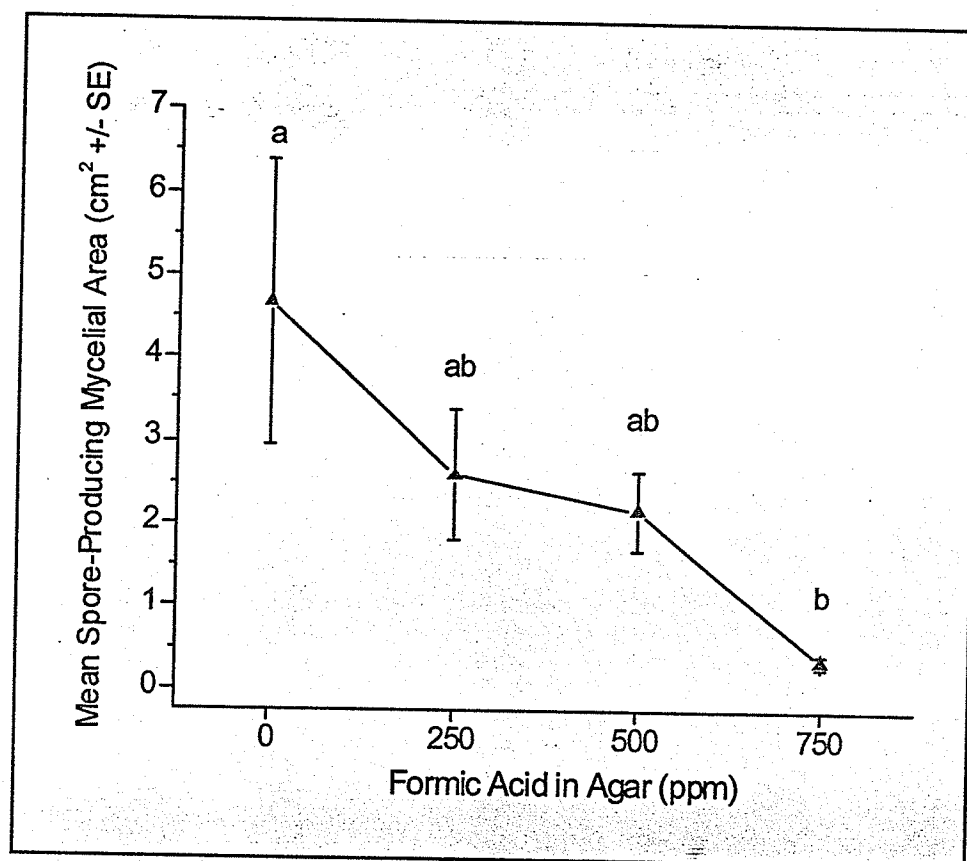


Fig. 17. *Ascosphaera apis* cyst production on potato dextrose with yeast extract agar inoculated with formic acid. Following incubation, cultures were homogenized, samples were examined with a microscope at 400x magnification and the number of cysts per culture was estimated. Means with the same letter are not significantly different (Duncan's multiple range test; $\alpha=0.05$).

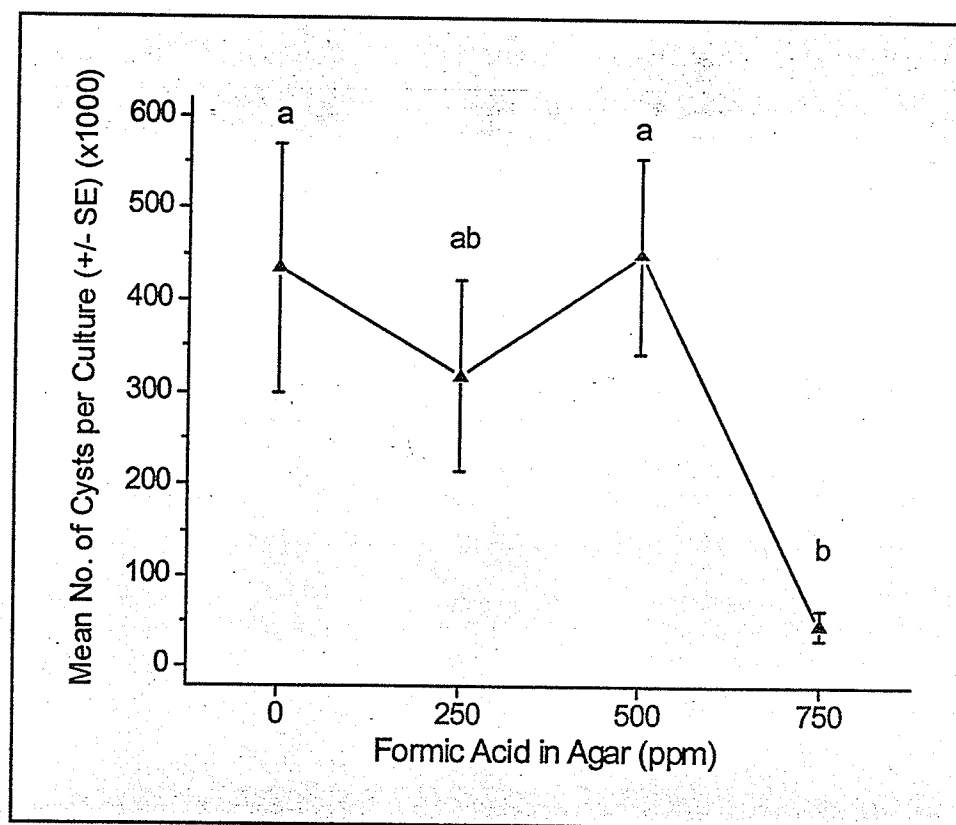


Fig. 18. Size of *Ascosphaera apis* spore ball on potato dextrose with yeast extract agar inoculated with formic acid. Following incubation, cultures were homogenized, samples were examined with a microscope at 400x magnification and the size of spore balls was estimated. Means with the same letter are not significantly different (Duncan's multiple range test; $\alpha=0.05$).

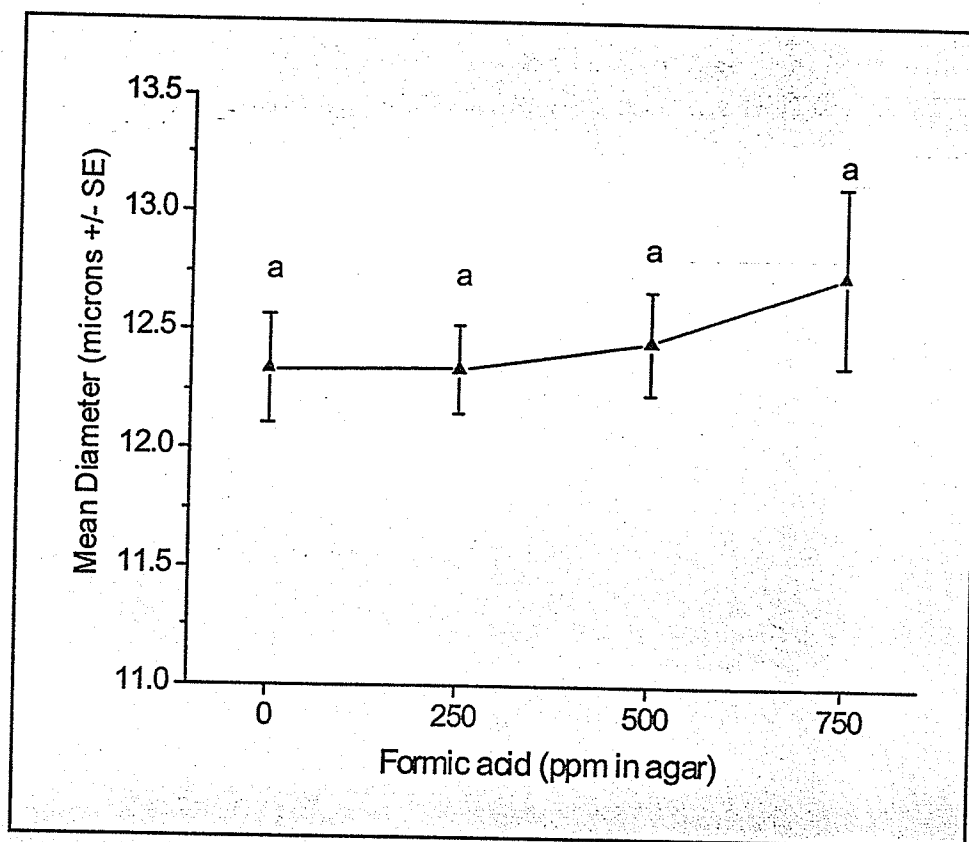
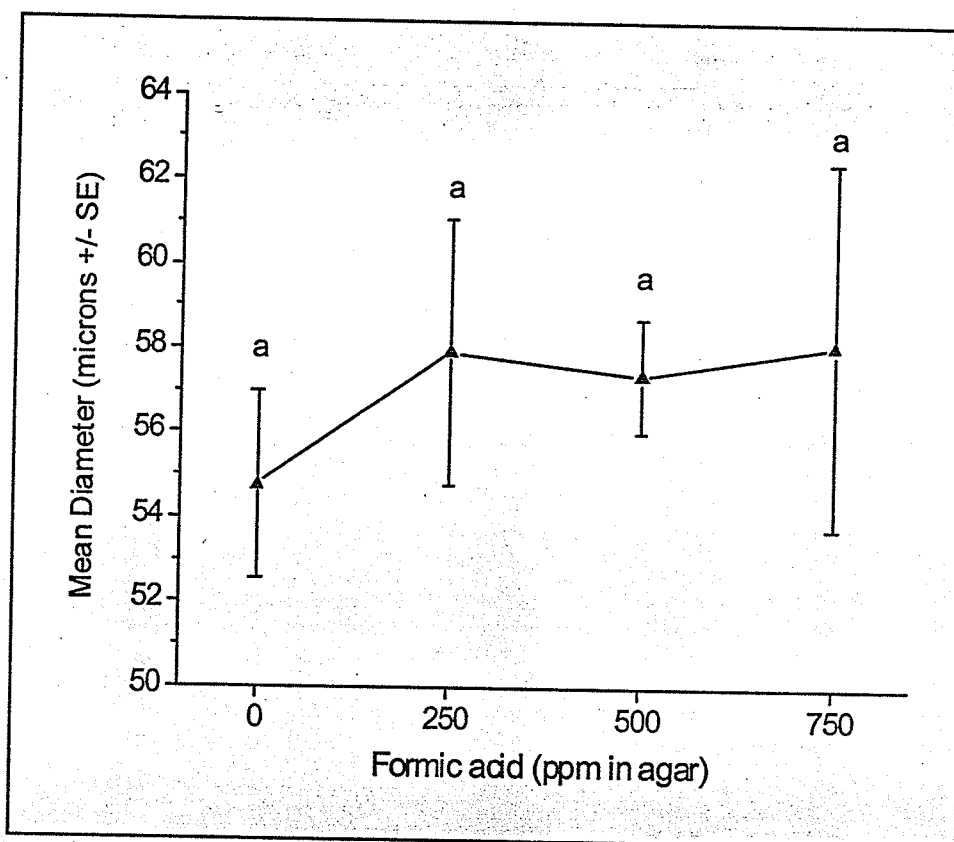


Fig. 19. Size of *Ascosphaera apis* spore cysts on potato dextrose with yeast extract agar inoculated with formic acid. Following incubation, cultures were homogenized, samples were examined with a microscope at 400x magnification and the size of spore cysts was estimated. Means with the same letter are not significantly different (Duncan's multiple range test; $\alpha=0.05$).



CHAPTER 5

GENERAL DISCUSSION

The serious damage to honey bee colonies that occurs when varroa are present has prompted a need to understand precisely how the ectoparasite impacts colonies and how the damage can be managed or controlled. The complex relationships between varroa and microbial parasites, and the great variability of the effect of formic acid in honey bee colonies, has made this a challenging problem.

Levels of chalkbrood infection may increase with increased varroa infestation, while levels of nosema infection were not affected by varroa. There is clear evidence that varroa naturally interacts with *N. apis* and *A. apis*. (Glinski 1988; Puerta *et al.* 1990; Liu 1996). The life cycle of varroa overlaps in both time and place with the life cycles of adult disease protozoan *N. apis* and larval disease fungus *A. apis*. The different responses of chalkbrood and nosema to varroa may be due to the sizes of spores, differences in their mode of infection, and the extent of their interaction with the mite. While both microorganisms are associated with stress conditions, a spore of *A. apis* is relatively small and its potential to interact with varroa is considerable. Varroa may facilitate the ability of chalkbrood to infect larvae or increase the susceptibility of the brood. It is known that honey bee larvae are susceptible to chalkbrood infection at the time when varroa enter their cells (Gilliam *et al.* 1978). The mites fall to the bottom board everyday (Pettis and Shimanuki 1999) where they can potentially acquire many *A. apis* spores (Liu 1996). Varroa could also increase the incidence of chalkbrood by increasing the susceptibility of honey bees to infection with the disease (Medina and Vicario Mejia 1999). The reduced

total protein, protein concentration (Weinberg and Madel 1985; Schatton and Engels 1988), and number of protein granules per cell (Drescher and Schneider 1988) resulting from varroa feeding and use of unaltered proteins (Tewarson 1983) may cause nutritional stress which is believed to make larvae more susceptible to chalkbrood infection (De Jong 1976; Chu 1988; Chu *et al.* 1992). The reduction in bee population caused by varroa feeding could also reduce the cluster size and expose outer brood to cooler conditions and potential infection.

We also showed the effects of formic acid treatments on chalkbrood and nosema. The field experiments revealed that formic acid, applied as a spring treatment in the presence of varroa, may reduce the level of chalkbrood infection through direct action and reduction in mite population. However, spring treatment with formic acid when varroa infestation is low may increase prevalence of chalkbrood. In laboratory experiments, formic acid in agar inhibited *A. apis* mycelial and spore-producing mycelial growth areas as well as cyst production significantly at 750 ppm and non-significantly at 250 ppm. Although formic acid in hive air does not reach the concentrations required for significant inhibition of growth of the fungus, chalkbrood disease may be inhibited directly at lower formic acid concentrations. Formic acid probably impacts *A. apis* by inhibiting mycelial growth and subsequent cyst production. Formic acid treatment probably also reduces the level of chalkbrood infection by decreasing the level of disease-promoting varroa when infestation is relatively high. Levels of nosema infection were not affected by formic acid treatment. The effect of formic acid or other acids on nosema or chalkbrood infection is not clear from the literature; however, it is believed that spores of *N. apis* and *A. apis* may

be vulnerable when exposed on the hive substrates depending on the method of application (Ruijter and Steen 1989), but are protected from formic acid treatment once inside the bee (Gontarski 1951):

The benefit of appropriate use of formic acid treatment on colony development was clearly evident in both field experiments. Formic acid application improved colony development by reducing the level of varroa infestation when infestation was relatively high. When varroa were absent or varroa infestation level was relatively low, formic acid application suppressed colony development probably by negatively impacting honey bee larvae (Fries 1991; Bolli *et al.* 1993), and perhaps making them more susceptible to chalkbrood infection.

The great variation in concentration of formic acid in hive air that was observed between colonies treated with the same formic acid application on different dates, even between different groups of colonies treated with the same formic acid application under the same environmental conditions, is likely due to a number of factors. Ambient temperature is a factor which may act in combination with other environmental, hive, and colony factors to influence the concentration of formic acid in the hive. The significant differences in the formic acid concentration of varroa and no-varroa treatment hives when the pour-on application was used suggest that colony size may influence the rate of evaporation with this formulation. Similar effects were not seen with the slow release formulation. It is unlikely that varroa would have a direct effect on the evaporation rate of formic acid. Varroa may act by diminishing colony size and/or activity, resulting in lower average hive temperature. This could reduce formic acid hive concentration in

those colonies.

Our analysis of inter- and intraday variance of formic acid hive concentration suggests that hive and ambient conditions combine to influence concentration particularly during the first day of the pour-on application. With the slow release formulation, interday formic acid concentration measured at approximately 1400 hours was correlated with ambient and hive temperature during the first seven days post application, but not during the period 11 to 28 days post application. When the formic acid concentration of the solution in the slow release formulation dispenser was high, it was more readily affected by fluctuations in hive and ambient conditions.

Within a single day, formic acid hive concentrations for both slow release and pour-on formulations appeared to be correlated with ambient and hive temperatures. On days of pour-on formic acid application, formic acid concentration in hive air tended to be low at 0900, high at 1400, and low at 1900 hours. While the mid-day increase in formic acid concentration may be due to greater volatilization of formic acid at higher temperatures or greater colony activity, the late-day drop in formic acid concentration in hives treated with the pour-on formulation that was observed on all treatment dates was likely due to a combination of depletion of formic acid from the absorbent paper towel and hive temperature or hive activity effects.

Similar but less dramatic daily trends were observed in the slow release treatments. This also suggests that the combination of hive temperature and/or hive activity plays a role in affecting the amount of formic acid in hive air. As the treatment progressed and the concentration of formic acid within the bags declined, the overall

concentration of formic acid in hive air and temperature effects were much lower.

Knowledge of how varroa causes damage and how formic acid affects mite and honey bee hive populations helps honey producers make appropriate management decisions. The understanding of how to use alternative treatments for varroa such as formic acid will continue to be very important in the future as mites develop resistance to other registered controls.

Conclusions

- Prevalence of chalkbrood may increase with increased varroa infestation.
- Mean abundance of nosema was not affected by varroa infestation.
- Formic acid applied as a spring treatment may reduce the prevalence of chalkbrood through direct action and reduction in mite population in the presence of varroa; however, when varroa are absent or varroa infestation is low, spring treatment with formic acid may cause an increase in the prevalence of chalkbrood.
- Formic acid spring application did not affect the mean abundance of nosema.
- Formic acid applications reduced overall average varroa infestation by 9.4% in colonies treated with slow release formic acid and 29.3% in colonies treated with pour-on formic acid.

- Formic acid significantly inhibited *A. apis* mycelial and spore-producing mycelial area growth as well as cyst production at high concentrations, and appeared to impact chalkbrood negatively at concentrations to which it may be exposed in the hive. The mode through which formic acid controls *A. apis* appears to be via the inhibition of mycelial growth and subsequent cyst production, rather than by diminishing the spore arsenal within individual cysts.
- Formic acid application improved colony development by reducing the level of varroa infestation when infestation level was relatively high. However, when varroa were absent or infestation level was relatively low, formic acid application reduced colony population growth.
- In colonies exposed to the slow release formulation, interday concentration of formic acid in the hive was significantly correlated with hive temperature, relative humidity, and ambient temperature, relative humidity, solar conditions, up to eight to 11 days post application.
- In colonies treated with the pour-on formulation, there was a significantly higher average formic acid concentration in hives not initially infested with varroa compared to hives which were initially infested with the mite, suggesting that varroa's effect on colony size and/or activity may act with ambient conditions to influence formic acid hive concentration with this formic acid formulation.

Future Research

Recommendations:

- That chalkbrood infection is included in the evaluation of damage caused by varroa.
- That attention is paid to research apiary surroundings to ensure that colonies are exposed to similar environmental conditions if consistency of formic acid concentration in the hive between all research apiaries is desired.
- That an applicator/dispenser of formic acid is developed which releases consistent amounts of formic acid into a hive, and can be adjusted for appropriate and predictable treatment exposure, to maximize pest control while minimizing harm to honey bees.

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