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An Investigation of Techniques for Using Oxalic Acid to Reduce Varroa Mite Populations in Honey Bee Colonies and Package Bees

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AN INVESTIGATION OF TECHNIQUES FOR USING OXALIC ACID TO REDUCE

VARROA MITE POPULATIONS IN HONEY BEE COLONIES AND PACKAGE

BEES

by

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AN INVESTIGATION OF TECHNIQUES FOR USING OXALIC ACID TO REDUCE
VARROA MITE POPULATIONS IN HONEY BEE COLONIES AND PACKAGE
BEES

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I investigated strategies for reducing *Varroa* mite populations (*Varroa destructor*)
in honey bee colonies (*Apis mellifera*) using oxalic acid (OA). I examined the efficacy of
OA in both broodless colonies and colonies that contain brood. My data indicate that OA
is most effective at reducing *Varroa* populations when colonies are broodless because
repeated applications of OA did not significantly reduce mite populations in colonies
when brood was present. Next, I quantified the contact toxicity of OA to *Varroa* mites
and their honey bee hosts in laboratory bioassays. The results indicate that OA has a low
acute toxicity to honey bees and a high acute toxicity to mites. The toxicity data will help
guide scientists in delivering lethal dosages of OA to the parasite and in protecting its
host.

I also investigated how OA is distributed in honey bee colonies when applied
using the trickle method. For this study, I constructed nine divided Langstroth hives
using 3 different types of dividers that allowed trophallaxis, physical contact, or
fumigation. I treated bees on one side of the divider and then monitored mite mortality
on both the treated and untreated sides. Bee-to-bee contact was the primary route for OA
distribution. Finally, I developed a protocol for using OA to eliminate mites from
package bees. I made 97 packages of *Varroa*-infested adult bees. I sprayed an OA
solution directly on the bees through the mesh screen of the package cages using a
pressurized air brush. I quantified mite and bee mortality and estimated the optimum
dosage of OA to apply to package bees for mite control without injuring bees. The
application of 3 mL of a 2.8% OA sugar water solution per 1000 bees will allow
beekeepers to safely reduce populations of *Varroa* mites from packages prior to

installation. My research is significant because it offers beekeepers a safe, effective, and sustainable method for reducing *Varroa* populations.

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Introduction

Varroa jacobsoni was first described by Oudemans in 1904 as a natural ectoparasitic mite of the Eastern honey bee, *Apis cerana* F. Recently, Anderson and Trueman (2000) reported that *V. jacobsoni* is a complex of 2 different species that parasitize *A. cerana*. The original species, *V. jacobsoni*, encompasses 9 haplotypes that infest *A. cerana* in the Malaysia-Indonesia region. In contrast, the newly described species, *Varroa destructor*, includes 6 haplotypes that infest *A. cerana* on mainland Asia. Adult females of *V. destructor* are larger and less spherical than females of *V. jacobsoni*, and the 2 species are reproductively isolated (Anderson and Trueman 2002).

Movement of the European honey bee, *Apis mellifera* L. into areas where *A. cerana* is endemic let *V. destructor* transfer to *A. mellifera*, a far less resistant host (De Jong *et al*. 1982). *V. destructor* found on *A. cerana* in Asia exhibit commensalism, a host-parasite relationship that is advantageous to *V. destructor* but does not negatively affect *A. cerana* (Büchler 1994, Boecking *et al*. 1998). In contrast, *A. mellifera* colonies infested with *V. destructor* usually die in 3-5 years without beekeeper intervention (De Jong *et al*. 1982).

Since the discovery of *V. destructor* (*Varroa*) in the United States in 1987 (Anonymous 1987), the feral population of honey bees has dramatically declined (Kraus and Page 1995, Harbo and Hoopingarner 1997). Managed honey bee colonies have also been severely injured (Sammataro 1997, Martin 2001). Furthermore, *Varro*a has added considerable labor and expense to the cost of managing honey bees (Ellis 2001, Sanford

2001). *Varroa* is generally considered the most severe threat to beekeeping worldwide (De Jong 1990, Beetsma 1994, Martin 1998, Anderson & Trueman 2000).

Currently, 8 chemical treatments are registered in the United States to control *Varroa* mites: 1) Apistan® (fluvalinate), 2) CheckMite+® (coumaphos), 3) Mite-Away II® (formic acid), 4) Apicure® (formic acid), 5) Apiguard® (thymol), 6) ApiLife-Var® (blend of thymol, eucalyptol, menthol, and camphor), 7) Sucrocide® (sucrose octanoate), and 8) Hivastan® (fenpyroximate). Apistan® has been used extensively for *Varroa* control, achieving nearly 100% efficacy in susceptible mite populations (Faucon *et al.* 1995). Apistan® is sold in plastic strips impregnated with fluvalinate that are suspended between frames in the brood chamber for 6-8 weeks. They require contact with the cluster of bees to be effective. Apistan® was first registered in Nebraska in 1990 and reports of resistant mite populations emerged between 1996 and 1997 (Creger 2007). Fluvalinate resistance has been widely reported in the United States (Eischen 1995, 1998, Elzen *et al.* 1998, 1999, Macedo *et al.* 2002a,) and Europe (Milani 1994, Lodesani *et al.* 1995, Thompson *et al.* 2002). Today, many beekeepers have discontinued the use of Apistan® because its effectiveness has significantly decreased.

The prevalence of fluvalinate-resistant *Varroa* mite populations in the late 1990's led many U.S. beekeepers to begin using CheckMite+®, an organophosphate acaricide, as an alternative mite control agent. Like Apistan®, CheckMite+® is sold as plastic strips impregnated with a miticide (coumaphos) that are placed in the brood chamber for 6 weeks. Efficacy of 97-99% has been documented (10% coumaphos strips) (Milani and Iob 1998). CheckMite+® was first registered in Nebraska in 1999 and reports of resistant mite populations emerged between 2002 and 2003 (Creger 2007). By 2002,

several instances of coumaphos resistance were reported in the United States (Elzen and Westervelt 2002, Pettis 2004).

Mite-Away II® is a ready-to-use, single application formic acid pad that is effective at controlling both *Acarapis woodi* (tracheal mites) and *Varroa* mites. The pad is made from wooden fibers and contains 292 grams of 65% formic acid. The treatment period is 21 days and the outside daytime temperature should be between 50 and 79°F. Significant adult bee and brood mortality may occur if the daytime temperature exceeds 82°F during the treatment period. Several other formulations of formic acid have been shown to be effective but are not registered or available to beekeepers. Calderone (2000) was able to achieve 94% efficacy when treating colonies with 300 mL of 65% formic acid in a slow release evaporator, but effective and reliable control has proven elusive. Liquid formic acid can also be used to reduce *Varroa* populations.

Apicure® is formic acid in a gel-like base that slowly releases the active ingredient. Apicure® is sealed in plastic bags that are sliced open and placed in the hive for 3 weeks. Apicure® has limited use due to problems with packaging that prevent mail services from accepting it for shipment. For this reason, Mite-Away II® is the preferred *Varroa* product for those wishing to use formic acid.

Apiguard® is thymol in a slow-release gel matrix. Thymol is a naturally occurring substance derived from various species of thyme plants (genus *Thymus*). Thymol has proven efficacy against *Varroa* mites, tracheal mites, and chalkbrood. Apiguard® can be purchased in ready-to-use aluminum trays containing 50 g of thymol or purchased in 1 or 3 kg tubs. Fifty grams of Apiguard® is placed on top bars of the

brood chamber and a second 50 g treatment is applied after the first treatment has evaporated (2-4 weeks). The total treatment period is 4-6 weeks.

ApiLife-Var® is composed of a vermiculite tablet (5 x 9 x 1 cm) impregnated with a 20 gram mixture of thymol (76%), eucalyptol (16.4%), menthol (3.8%), and camphor (3.8%). Three tablets are placed on the top bars of brood combs and are left in place for 4-8 weeks. Imdorf *et al.* (1995) reported that 95% mite mortality was achieved if temperatures were optimal (between 15 and 21°C). ApiLife-Var® is most consistently effective when applied to hives that are contained in a single brood chamber. In contrast, its efficacy is less consistent when treating multiple story colonies. Imdorf *et al.* (1994, 1995) found that mite mortality varied considerably when treating multiple story colonies.

Sucrocide® is 40% sucrose octanoate. The label recommends spraying the bees clustered on each frame at 7-10 day intervals. Three treatments are recommended. Few data indicating the efficacy of Sucrocide® are available. However, Sheppard *et al.* (2003) reported that following a single treatment with sucrose octanoate esters (solution of 0.3% active ingredient in water; 1.5 ounces per frame), mite mortality ranged from 38% to 87%.

Hivastan® is a contact miticide that contains 0.3% fenpyroximate. The product is a thick, pliable formulation that can be formed into patties. The label recommends applying a 225 g patty of Hivastan® on the top of the brood frames on paper, wax paper, or cardboard. Two 225 g treatments per year per colony are permitted. Adult bees consume the patties, and in the process, get the material on their bodies and transfer it to other bees in the colony by physical contact. In typical circumstances, the initial 225 g

application will be consumed or removed in 6 weeks. The Hivastan® label precautions that a slightly higher incidence of adult bee mortality can occur during the initial 48-72 hours of the treatment period compared with untreated colonies.

Chemical resistance, the variable efficacies of current *Varroa* treatments, the adverse effects of treatments on bees, and the risk of hive and hive product contamination create a need for alternative treatment methods. Oxalic acid (OA) is extensively used for controlling *V. destructor* in Europe and Canada due to its high efficacy (>90%) and low risk of hive contamination (Charrière & Imdorf 2002, Special Supplement 2005). Its registration is pending in the United States. OA is applied to colonies by spraying or trickling a solution of OA in a 1:1 sugar water solution over the bees (Charrière & Imdorf 2002) or by sublimating crystals with heat. When spraying or trickling OA in Canada, the recommended application per hive is 50 mL of a solution containing 35 g OA dihydrate in one L of 1:1 sugar:water (w:v). When spraying or trickling OA, the maximum dose is 50 mL per hive, whether the bees are in nucs, single, or multiple brood chambers. When evaporating OA in Canada (vaporizer method), the recommended application is 1 g OA dihydrate per hive body (Langstroth hive body, 24.4 cm depth) (Special Supplement 2005). Although OA provides effective control of *V. destructor*, its mode of action is unknown. Further, only one study has quantified the contact toxicity of OA to *V. destructor* (Milani 2001), and the contact toxicity of OA to honey bees has not been determined. In short, OA is extensively used without knowing the basic toxicological properties of the compound to *V. destructor* or *A. mellifera*.

There are many important research opportunities surrounding OA. My dissertation research focuses on evaluating OA for reducing *Varroa* mite populations in

honey bee colonies and package bees, determining factors that influence efficacy, and investigating the mode of distribution of OA. The results from my research have important implications for the development and implementation of effective management strategies for controlling *Varroa* mites. Specifically, objectives for my work are:

1. To evaluate strategies for reducing *Varroa* mite populations using OA in both broodless and brood rearing colonies.

2. To quantify the contact toxicity of OA to *Varroa* mites and their honey bee host in laboratory bioassays.

3. To investigate factors that contribute to the distribution of OA in a hive.

4. To develop a protocol for using OA to reduce *Varroa* mite populations in package bees.

Literature Review

History of *Varroa* in the United States

Varroa destructor was first found in the United States in 1987 in Wisconsin (Anonymous 1987). Since their introduction, *Varroa* mites have spread rapidly across the United States. *Varroa* are spread locally by drifting of infested adult worker and drone adult bees, by the movement of swarms, and by bees robbing weakened colonies (De Jong 1990). Movement of infested colonies for wintering and crop pollination and the shipment of package bees and queens are probably responsible for the rapid dispersal of *Varroa* in the United States (de Guzman 1993). *Varroa* has greatly reduced populations of feral bee colonies in many parts of the United States (Kraus and Page 1995, Harbo and Hoopingarner 1997). Substantial losses of managed honey bee colonies have also been attributed to *Varroa* injury (Beetsma 1994, Sammataro 1997, Martin 2001, Sanford 2001).

The number of honey bee colonies in the United States has declined steadily since 1947. In 1947, the number of colonies peaked at 5.9 million (Hoff and Willett 1994). By 2006, the number of colonies had dwindled to 2.4 million (USDA 2007). Some of the decline may be attributed to urban growth and a reduction in forage plants due to monocultural farming practices and herbicides (NASS 1993). Recently, price competition from imports and the deleterious effects from the spread of both tracheal mites (*Acarapis woodi*) and *Varroa* mites have accelerated the drop in the number of managed colonies (NASS 1993). *Varroa* is considered the most severe threat to beekeeping worldwide (De Jong 1990, Beetsma 1994).

Lately, colony collapse disorder (CCD) has afflicted an estimated 23% of beekeeping operations across the United States (Cox-Foster *et al.* 2007). CCD has resulted in a loss of 50 to 90% of colonies in affected beekeeping operations over the winter of 2006-2007 (Cox-Foster *et al.* 2007). CCD is characterized by the inexplicable loss of a colony's adult bee population and few or no dead bees in or around the hive. The later stages of CCD are typified by colonies that have 5 or more frames of brood, honey reserves and a queen, but that lack sufficient adult bees to cover and care for the brood. The few bees that remain are frequently newly emerged bees. Although the cause of CCD has not been definitively proven, a team of scientists led by Diana Cox-Foster have found that Israeli acute paralysis virus of bees (IAPV) is strongly correlated with CCD (Cox-Foster *et al.* 2007). While the Cox-Foster *et al.* (2007) paper presents convincing evidence that CCD is related to the presence of IAPV, many questions about CCD remain unanswered. It is unknown whether IAPV acts alone or in concert with other factors such as *Varroa* mites, poor nutrition, exposure to pesticides, and climate. Other viruses associated with *Varroa* infestation include acute paralysis virus, Kashmir bee virus, and deformed wing virus (Sammataro 1997). Their contribution to the CCD problem and interaction with IAPV are under investigation (Cox-Foster *et al.* 2007).

Varroa biology

Adult female *V. destructor* are large (1.1 X 1.6 mm), reddish brown, dorsal-ventrally flattened external parasites of all castes of honey bees (De Jong 1990, Fries 1993). Adult female *Varroa* are phoretic on adult bees and are found principally on the underside of the abdomen between the overlapping abdominal sternites. *Varroa* feed on

haemolymph by piercing the intersegmental membranes with their chelicerae (De Jong

1990). *Varroa* only leave adult bees to enter brood cells where they reproduce and mate.

Mites invade worker and drone cells 15-20 hours and 40-50 hours preceding cell capping,

respectively (Boot *et al*. 1992). Drone larvae are preferred over workers, and drone

brood is often 8-10 times more infested than worker brood (Fuchs 1990).

To begin its reproductive phase, an adult female mite must be carried to a suitable

brood cell by a bee. The mite then transfers from the adult bee to the rim of the cell and

quickly moves inside quickly (Boot *et al*. 1994). After entering the cell, the mite

immediately buries itself underneath the bee larva and enters the larval food. At this

time, the mite assumes a characteristic position with its posterior ventral plates facing the

opening of the cell. The anterior dorsum, mouthparts, anterior ventral plates, and the

bases of its legs are submerged in the larval food. The peritremes protrude out of the

semi-liquid food, perpendicular to its surface (De Jong 1984). The mite remains in this

position up to 4 hours after cell capping or until the larval food is consumed. Once

liberated from the brood food by the feeding larva, the mite commences feeding on larval

haemolymph (De Jong 1984).

Approximately 60 hours after the cell is capped, the female mite lays a single,

male egg. Subsequent female eggs are laid at intervals of 30 hours. Female mites can lay

a maximum of 6 female eggs in a drone cell and 5 female eggs in a worker cell (Ifantidis

1983). The immature mites develop inside the egg. At this time, a 6-legged larva is

visible within. Approximately 1.5 days after the deposition of a female egg, an 8-legged

protonymph hatches. This stage lasts about 3 days, allowing the female mite to feed,

grow, and then molt to an 8-legged deutonymph. The deutonymphal stage (similar in

size and shape to the adult female) also lasts about 3 days. Both protonymphal and deutonymphal stages are subdivided into a mobile and immobile stage. About 7.5-8 days after the egg is laid, the female deutonymph molts into an adult (Ifantidis 1983, De Jong 1984, 1990).

The male mite develops from egg to adult in 5.5-6 days and is smaller, paler, and less sclereotized than the female. The adult male's chelicerae (mouthparts used for piercing the bee's integument by the female) are modified for sperm transfer, rendering it unable to feed. Mating takes place within the cell, and adult female mites leave with the emerging adult bees. The males and immature females that are present when the adult bee emerges perish (Ifantidis 1983, De Jong 1984, 1990). Studies of egg chromosome number have revealed that the first egg laid normally develops into a male and the subsequent eggs into females (Rehm and Ritter 1989). *Varroa* have a haplodiploid system of sex determination (arrhenotoky). Males arise from unfertilized eggs and have 7 chromosomes. In contrast, females arise from fertilized eggs and have 14 chromosomes (Ruijter and Pappas 1983).

There is a strong preference for mite reproduction in drone brood when both worker and drone brood are available (Boot *et al*. 1992, De Jong 1984, Fuchs 1990). The mechanisms governing this preference are not completely understood and are frequently debated. Le Conte *et al*. (1989) suggest that chemical stimuli may be important, as simple aliphatic esters isolated from drones attract mites. Boot *et al*. (1992) propose that the larger number of mites found in drone cells may be partly due to the longer period of mite invasion into drone brood. Physical differences between drone and worker cells may also influence mite distribution in brood cells. De Jong and Morse (1988) found that

raised cells (drone cells) are more attractive to mites than normal worker cells. Finally,

drone brood is capped about 2 days longer than worker brood. The adaptive advantage of

reproducing in drone brood is clear considering a greater number of mites are able reach

sexual maturity and mate due to the longer developmental period (21 days for workers

and 24 days for drones).

An important aspect of *Varroa* biology is disease transmission. *Varroa destructor*

feeds on the haemolymph of larvae, prepupae, pupae, and adult honey bees. The

female's piercing chelicerae effectively function as "dirty syringes," exposing its host to

many pathogens, including viral, bacterial, microsporidial, and fungal diseases (Ball

1994). Adult bee symptoms of *Varroa* infestation include a reduction in population,

deformed wings, bees crawling away from the hive that are unable to fly, and queen

supercedure. Brood symptoms include a spotty brood pattern and brood that appear

discolored and abnormally positioned. Symptoms may resemble European foulbrood,

American foulbrood, or sacbrood disease (Hung *et al.* 1995). Shimanuki *et al.* (1994)

proposed the name "parasitic mite syndrome" (PMS) for these symptoms. Several

viruses may be associated with PMS, but their role in PMS has not been established. The

most commonly observed viruses associated with *Varroa* infestation include sacbrood

virus (SBV), acute bee paralysis virus (ABPV), chronic bee paralysis virus (CBPV),

Kashmir bee virus (KBV), deformed wing virus (DWV), black queen cell virus (BQCV),

and Israeli acute paralysis virus (IAPV) (Sammataro 1997, Cox-Foster *et al.* 2007).

There is evidence that *Varroa* do not cause honey bee colonies to expire solely from

feeding. They also act indirectly by activating viruses and providing a port of entry for

bacterial and fungal diseases. Diseases may play a role that is equal or greater than mite feeding injury in colony death (Hung *et al.* 1995).

Varroa on *Apis cerana* vs. *Apis mellifera*

Varroa jacobsoni was first described by Oudemans in 1904 as a natural ectoparasitic mite of the Eastern honey bee *Apis cerana* F. Peng *et al*. (1987) discovered that *A. cerana* has evolved both physiological and behavioral adaptations to *V. jacobsoni* parasitism. *Apis cerana* worker bees are able to detect *Varroa* as they respond immediately to mite introduction. The presence of *Varroa* triggers a series of behavioral responses including auto-grooming, dancing to solicit nest mate grooming, nest mate cleaning, and group cleaning. Eventually, these behaviors lead to the removal of many mites from the adult bees and brood (Peng *et al*. 1987, Harris 2007).

Recently, Anderson and Trueman (2000) reported that *V. jacobsoni* is a complex of 2 different species that parasitize *A. cerana*. The original species, *V. jacobsoni*, encompasses 9 haplotypes that infest *A. cerana* in the Malaysia-Indonesia region. In contrast, the newly described species, *Varroa destructor*, includes 6 haplotypes that infest *A. cerana* on mainland Asia. Movement of *A. mellifera* by humans into areas where *A. cerana* is endemic in the 1950's enabled *V. destructor* to transfer to *A. mellifera*, a far less resistant host (De Jong *et al*. 1982). De Jong *et al*. (1982) reported that colonies parasitized by *Varroa* began dying in autumn of the first year of infestation. Frequently, all colonies in an apiary died in 3-5 years. Peng *et al*. (1987) discovered that grooming behavior occurs less frequently in *Varroa*-infested *A. mellifera* colonies, and that few mites are removed by grooming. Additionally, *A. mellifera* brood has a longer

developmental period (21 days for workers and 24 days for drones) than *A. cerana*, allowing *Varroa* to reproduce in both worker and drone brood. In contrast, *Varroa* mites parasitizing *A. cerana* reproduce almost exclusively in drone brood (developmental time of 22 days) because the developmental time of *A. cerana* worker brood (18 days) is insufficient for mite reproduction (Boot *et al.* 1997).

Adult female *Varroa* mites from different honey bee species show high phenotypic consistency, except for body size. Female mites infesting *A. cerana* are generally smaller than those infesting *A. mellifera*. Delfinado-Baker (1988) reported that the behavioral, physical, and physiological differences exhibited by *Varroa* on *A. cerana* and *A. mellifera* suggest that *V. jacobsoni* may be more than one species. Twelve years later, Anderson and Trueman (2000) were able to clearly demonstrate (through a comprehensive comparative study based on molecular techniques) that *V. jacobsoni* is actually 2 species: *V. destructor* and *V. jacobsoni*. It is now clear that both *V. jacobsoni* and *V. destructor* parasitize *A. cerana*, and that only *V. destructor* parasitizes *A. mellifera*.

Detection methods and treatment thresholds

It is essential to assess the degree of *V. destructor* infestation in honey bee colonies to prevent colony injury or loss. Accurate estimation of mite population allows beekeepers to employ control measures when warranted and to save time and money when treatment can be delayed (Macedo and Ellis 2000). Commonly used detection methods for *Varroa* include ether roll, sugar roll, alcohol wash, brood examination, sticky boards placed on the bottom board, and acaricides with sticky boards. Detailed

descriptions of these methods can be found in USDA Agricultural Handbook Number

690, *Diagnosis of Honey Bee Diseases* (Shimanuki and Knox 2000).

Regardless of the detection method employed, assessing the degree of *Varroa*

infestation is crucial in determining if control measures are warranted. In the Midwest,

colonies with more than 0.12 mites-per-bee when brood is not present (in the fall) will

have increased winter mortality if mite populations are not reduced. Colonies with more

than 0.25 mites-per-bee will almost always perish in the winter (Ali and Ellis 2000).

When brood is present in mid-August, 0.03 or more mites-per-bee indicates that the

beekeeper should implement treatment measures as soon as possible. This may require

sacrificing honey production to conserve the colonies (Macedo and Ellis 2001).

Although the above treatment thresholds are a good indicator for *Varroa* treatment in the

Midwest, Delaplane (1998) noted that treatment thresholds can vary regionally.

Delaplane and Hood (1997) suggested that late-season acaricide treatments in first-year

colonies in the southeastern United States are justified when 300-bee ether roll levels are

15.1 ± 1.4 mites. Variation in the duration of the brood rearing period affects mite

population dynamics, and may explain why treatment thresholds have not been

established for most of the United States (Delaplane and Hood 1997). The lack of

treatment thresholds for all regions and the time required to determine *Varroa* infestation

result in many beekeepers applying annual or semi-annual prophylactic miticide

treatments (Strange and Sheppard 2001).

***Varroa* control**

Chemical control

Currently, 6 general use (Section 3) chemical treatments are used to control *V. destructor* in the United States: Apistan® (fluvalinate), Apicure® (formic acid), Mite-Away II® (formic acid), Sucrocide® (sucrose octanoate), Apiguard® (thymol), and ApiLife-Var® (76% thymol, 16.4% eucalyptol, 3.8% menthol, and 3.8% camphor). The first product registered for *Varroa* control in the United States was Apistan®. It achieved nearly 100% efficacy in susceptible mite populations (Faucon *et al.* 1995). Fluvalinate's target is the axonal transmission of nerve impulses. It alters the permeability of sodium channels and causes prolonged depolarization of nerve membranes. Apistan® was initially approved in 1990 as a Section 3 general use chemical, and its Section 3 registration remains active in the United States (U.S. EPA 2007a). However, the active ingredient, tau fluvalinate, was available to beekeepers under a Section 18 registration from 1987-1989. Liquid formulations of fluvalinate were applied to wooden strips that were placed in the brood chamber (Ellis *et al.* 1988). Currently, Apistan® is sold as impregnated plastic strips that are placed in the brood chamber for 6-8 weeks and act as a contact pesticide. Fluvalinate resistance has been documented in the United States (Eischen 1995, 1998, Elzen *et al.* 1998, 1999, Macedo *et al.* 2002a,) and Europe (Milani 1994, Lodesani *et al.* 1995, Thompson *et al.* 2002). Efficacy has been reported to drop to 10-70% in mite populations that have developed resistance (Pettis *et al.* 1998, Macedo *et al.* 2002a). Today, many U.S. beekeepers have discontinued the use of Apistan® due to loss of efficacy.

Apicure® is 65% formic acid in a gel-like base that slowly releases the active ingredient. Apicure® is sealed in plastic bags that are sliced open and placed in hives for 3 weeks. Formic acid is a fumigant that kills *Varroa* by respiratory inhibition (Imdorf *et al.* 1999. Since fumigant volatilization is temperature dependent, the results of treatment often exhibit variable results. Queen loss and brood damage are common side effects of overexposure to formic acid. Several other formulations of formic acid have been shown to be effective but are not registered or available to beekeepers. Calderone (2000) was able to achieve 94% efficacy when treating colonies with 300 mL of 65% formic acid in a slow release evaporator, but effective and reliable control has proven elusive. Although registered (Section 3), Apicure® is not available to most beekeepers due to packaging problems and the refusal of mail services to accept the current packaging for shipment. To date, Apicure® has not been widely distributed or applied. Mite-Away II® is the only formic acid based miticide currently available to U.S. beekeepers.

Mite-Away II® has a Section 3 registration that was approved on March 31, 2005 (U.S. EPA 2005). Mite-Away II™ is a ready-to-use, single application formic acid pad that is effective at controlling both tracheal and *Varroa* mites (Mite-Away II® U.S. label). The pad is made from wooden fibers and contains 292 grams of 65% formic acid. A 3.8 cm spacer rim must be placed over the brood chamber to accommodate the Mite-Away II® pad. The pad is placed in the hive directly over the brood chamber. The treatment period is 21 days and the outside daytime temperature should be between 50 and 79°F. The label recommends that applicators remove the pads from hives in the event of a heat wave (>82°F) within the first 7 days of treatment. Significant adult bee mortality, brood mortality, and absconding may occur if the daytime temperature exceeds

82°F during the treatment period. Like Apicure®, the formic acid present in Mite-Away II® acts as a fumigant and kills *Varroa* by respiratory inhibition (Imdorf *et al.* 1999), rendering its effectiveness highly variable. Formic acid is also hazardous to applicators and the EPA mandates stringent precautionary labeling for Mite-Away II®.

Sucrocide® (40% sucrose octanoate) is a general use *Varroa* treatment and is classified as a biopesticide by the EPA. Sucrose octanoate is a mixture of sugar esters that are manufactured from sucrose (table sugar) and an octanoic acid ester that is commonly found in plants and animals. The label recommends spraying both sides of each frame at 7-10 day intervals. Three treatments are recommended. The surfactant effect of sucrose octanoate esters de-waxes the cuticle of *Varroa*, causing desiccation (U.S. EPA. 2006a). No harmful effects to bees, humans, or the environment are expected from the use of Sucrocide® (U.S. EPA 2006a). Few data indicating the efficacy of Sucrocide® are available. However, Sheppard *et al.* (2003) reported that following a single treatment with sucrose octanoate esters (solution of 0.3% active ingredient in water; 1.5 ounces per frame), mite mortality ranged from 38% to 87%. Currently, Sucrocide® is available in all states and has a Section 3 registration (U.S. EPA 2006a).

Apiguard® received EPA approval for use in the United States in January 2006. Prior to United States approval, Apiguard® was only available in European countries. Currently, Apiguard® has a Section 3 registration in the United States (U.S. EPA 2006b). Apiguard® is thymol in a slow-release gel matrix. Thymol is a naturally occurring substance derived from various species of thyme plants (genus *Thymus*). Thymol has proven efficacy against *Varroa* mites, tracheal mites, and chalkbrood (Apiguard® U.S. label). Apiguard® can be purchased in ready-to-use aluminum trays containing 50 g of

thymol in a gel matrix or purchased in 1 kg or 3 kg tubs for beekeepers with many colonies to treat. The gel is placed in the hive using the dosing tools (scoop and spatula) that are supplied with the 1 kg and 3 kg tubs. The first dose requires the applicator to measure 50 g of the gel using the scoop and spatula. To do so, the applicator must completely fill the scoop with gel and then level the excess with the spatula. The gel is then spread to an even thickness on a waxy cardboard tray (supplied) and placed centrally on top of the brood frames. After 2 weeks, a second 50 g dose is applied using the methods described above. Apiguard® is left in the colony until it disappears completely from the tray or until supers are installed. Generally, the total treatment period lasts 4-6 weeks.

Registration was initially approved for ApiLife-Var® in 2003 under an EPA Section 18 Emergency Exemption for states requesting approval due to Apistan® and CheckMite+® resistance. Currently, ApiLife-Var® has a Section 3 registration in the United States. ApiLife-Var® is composed of a vermiculite tablet (5 x 9 x 1 cm) impregnated with a 20 gram mixture of thymol (76%), eucalyptol (16.4%), menthol (3.8%), and camphor (3.8%). The active ingredient, thymol, is a fumigant that effectively kills *Varroa* mites, but its mode of action is unknown. The tablets are placed on the top bars of brood combs and are left in place for 4-8 weeks. Imdorf *et al.* (1995) reported that 95% mite mortality was achieved if temperatures were optimal (between 15 and 21°C). ApiLife-Var® residues in hive products are not considered a health risk. The applicator should not get ApiLife-Var® on their hand and should not touch their eyes while treating. ApiLife-Var® is most consistently effective when applied to hives that are contained in a single brood chamber. In contrast, its efficacy is less consistent when

treating multiple story colonies. Imdorf *et al.* (1994, 1995) found that mite mortality varied considerably when treating multiple story colonies.

Two restricted use products are available for *Varroa* control in the United States, CheckMite+® (coumaphos) and Hivastan® (fenpyroximate). Registration was initially approved for CheckMite+® in 1998 under an EPA Section 18 Emergency Exemption for states requesting approval due to Apistan® resistance. The EPA has continued to renew the exemption and has made CheckMite+® available in a number of states on a year-to-year basis. The active ingredient, coumaphos, is an organophosphate and acts as an acetylcholinesterase inhibitor. This mode of action makes the use of coumaphos hazardous to applicators. Treatments must be applied at a time when bees are not producing a surplus honey crop. CheckMite+® is sold as plastic strips impregnated with miticide that are placed in the brood chamber for 6 weeks. Efficacy of 97-99% has been documented (10% coumaphos strips) (Milani and Iob 1998). There are also concerns about the risk of contaminating hive products, and a 4 week withdrawal period is required before adding surplus honey supers. Chemical resistant gloves must be worn when handling the strips. Coumaphos is highly lipophilic and residues are most likely to be found in beeswax. Coumaphos resistance has been reported in the United States (Elzen and Westervelt 2002, Pettis 2004) and Italy (Lodesani 1996).

Registration was approved for Hivastan® (fenpyroximate) in May 2007 under an EPA Section 18 Emergency Exemption (U.S. EPA 2007b). Fenpyroximate is a synthetic acaricide that inhibits the mitochondrial electron transport of complex I (NADH dehydrogenase) (Nauen and Bretschneider 2002). Hivastan® is a contact miticide that contains 0.3% fenpyroximate. The product is a thick, pliable formulation that can be

formed into patties. The label recommends applying a 225 g patty of Hivastan® on top of the brood frames on paper, wax paper, or cardboard. Two 225 g treatments per year per colony are permitted. Adult bees consume the patties, and in the process, get the material on their bodies and transfer it to other bees in the colony by physical contact (Hivastan® U.S. label). In typical circumstances, the initial 225 g application will be consumed or removed in 6 weeks. The Hivastan® label precautions that a slightly higher incidence of adult bee mortality can occur during the initial 48-72 hours of the treatment period compared with untreated colonies.

Biotechnical control

Chemical resistance, the variable efficacies of current *Varroa* treatments, the adverse effects of treatments on bees, and the risk of hive and hive product contamination create a need for alternative treatment methods. Lindberg *et al*. (2000) and Ali *et al*. (2002) recently evaluated several essential oils and related compounds including clove oil, benzyl acetate, thymol, carvacrol, methyl salicylate, and perillyl acetate as treatments for *Varroa*. The results indicated that the compounds they tested may not be highly effective under all conditions, but suggest that they could be a useful component of an integrated pest management approach. Charrière and Imdorf (2002) evaluated oxalic and lactic acids in Europe as an alternative *Varroa* treatment. Their results indicate that oxalic acid is effective in broodless colonies, but their protocol requires applying the material to the bees on each frame, and bees were harmed if overdosed. Oxalic and lactic acids can be applied by spraying or trickling on adult bees or by sublimation of the acid crystals in the hive.

Heat treatment (Tabor and Ambrose 2001), powdered sugar dusting (Aliano and Ellis 2005a, Fakhimzadeh 2000), pollen traps (Cakmak *et al*. 2002), and even electrical "zapping" (Huang 2001) have also been evaluated as *Varroa* treatment methods. Like other alternative *Varroa* treatments, the above methods exhibit potential as part of an integrated pest management strategy, but they are labor intensive and often less effective than other registered *Varroa* control products. If labor costs are considered, the treatments described above are more expensive than the alternatives.

One of the first products used to control *Varroa* was Sineacar, a mixture of powdered sugar (98.2%) and chloropropylate and bromopropylate (1.8%) (Ramirez 1994). The "clogging" of the ambulacrum with dust may explain why Sineacar knocked mites off adult honey bees without killing them first. The ambulacrum of adult female *Varroa* is a pretarsus with protractile, claw-like sclerites used for grasping the hairs of bees (Ramirez and Malavasi 1991). The claw-like sclerites of the ambulacrum enable mites to move rapidly on adult bees and other substrata. Macedo *et al*. (2002b) were able to achieve 92.9 ± 5.5% *Varroa* mite recovery by isolating bees from their nest and dusting them with powdered sugar. Similarly, Shah and Shah (1988) reported that a fine powder of wheat flour was effective at knocking *Varroa* off adult honey bees, but they did not indicate the percentage mite fall. In addition to dust adhering to the tarsal pads of *Varroa*, Macedo and Ellis (2001) suggest 2 more factors that may contribute to mite fall when bees are dusted with powdered sugar. They observed that dust stimulates the bees' grooming behavior and they proposed that it would result in greater mite removal. They also proposed that dust on the mite's body may stimulate it to release from its host to groom itself.

Fakhimzadeh (2000) evaluated powdered sugar as a tool for *Varroa* control. Fakhimzadeh applied sugar directly to adult bees in their nest and counted daily mite fall following treatment. He found post-treatment daily mite fall was significantly higher than pre-treatment daily mite fall. However, he did not determine the percentage of mites that were removed from the hives. Fakhimzadeh concluded that powdered sugar is useful in reducing mite infestation in a colony, but he indicates that his method still needs refining. In a related study, Fakhimzadeh (2001b) investigated the effects of powdered sugar on honey bee colony development. He applied sugar to the colony (10-20g) at 3-, 7-, and 14-day intervals. He observed that sugar particles did not enter the bee's spiracles or tracheal ducts. Also, the treatment had no obvious side effects on capped brood or the growth of the bee population. Additionally, Fakhimzadeh found that powdered sugar treatment did not cause queen loss or queen supercedure, even if the treatment was applied as frequently as every 3 days for one month.

Aliano and Ellis (2005a) developed a powdered sugar dusting technique that requires isolating a colony's adult bee population in a detachable box prior to powdered sugar application. By applying a bee repellent to a colony, Aliano and Ellis (2005a) forced adult bees into a detachable 'bee box' where the adult bee population was dusted with powdered sugar. Adult honey bee populations treated in this manner dropped 76.7 ± 3.6% of their mites. One drawback of the Aliano and Ellis (2005a) technique is that it is labor intensive and time consuming. Part-time beekeepers are more likely to adopt this technique than full-time beekeepers, especially those trying to reduce the use of chemical treatments. Aliano and Ellis (2005b) performed a follow up study to test the effects of powdered sugar entering brood cells when powdered sugar is applied to a hive. Their

results indicate that only large amounts (0.6 g per 152 cells) of powdered sugar applied directly to brood cells harms immature honey bees. Further, Aliano and Ellis (2005b) indicate that brood removal following management practices that introduce powdered sugar into colonies will be restricted to eggs.

Genetic control

Another approach to *Varroa* control is bee breeding. Spivak and Reuter (2001) reported that colonies bred for hygienic behavior maintained lower mite loads for up to one year without treatment. Ibrahim and Spivak (2005) showed that bees bred for suppression of mite reproduction (SMR) also express hygienic behavior. Harbo and Harris (2001) reported that single drone inseminated queens bred for SMR can significantly reduce mite populations. Recently, Harris (2007) discovered that honey bees bred for SMR resist the growth of *Varroa* mites by removing mite-infested pupae from capped brood. Harris (2007) named this behavior *Varroa*-sensitive hygiene (VSH) and describes VSH as "a multi-step process that involves detection, uncapping of the cell, and removal of the host."

Other heritable traits that have been associated with *Varroa* resistance include the duration of the capped period and the proportion of mites in brood cells (Harbo and Harris 1999). A disadvantage of intense selection for one trait is that selection for other valuable traits (honey production, gentleness, swarming, etc) may be reduced. Furthermore, intense selection for a single trait may narrow the genetic base over time. Finally, specific traits such as hygienic behavior and suppression of mite reproduction may be lost when a colony swarms or supercedes its queen. Resistant stock would lower

operating costs, reduce selection pressure for mite resistance to chemical treatments, and reduce the risks of chemical residues in honey and hive products. *Varroa* resistant lines probably will not eliminate the need for chemical treatments, but they may help beekeepers reduce the frequency of treatments and the selective pressures that promote miticide resistance (Ellis 2001).

Oxalic acid

Oxalic acid (OA) is a dicarboxylic acid and is approximately 10,000 times stronger than acetic acid. Oxalic acid is available in two formulations: anhydrous ($C_2H_2O_4$) (CAS # 144-62-7) and dihydrate ($C_2H_2O_4$ $2H_2O$) (CAS # 6153-56-6). Oxalic acid is commonly utilized in the industrial sector as a wood bleaching agent, as a mordant in dyeing processes, and as a surface pretreatment for stainless steels prior to applying a corrosion-inhibiting coating. Oxalic acid is also employed in the domestic sector as a household cleaner and rust remover. A structural sketch of anhydrous OA ($C_2H_2O_4$) is included below.

$$\text{HO} - \overset{\overset{\textstyle O}{\|}}{C} - \overset{\overset{\textstyle O}{\|}}{C} - \text{OH}$$
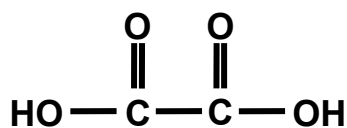
Oxalic acid is a common chemical in plants, but its physiological role is not completely understood. Scientists suggest that OA is involved in storage and regulation of calcium, seed germination, detoxification, ion balance, structural integrity, and insect repellence (EMEA 2004). Oxalic acid concentrations in plants range from 5 mg/kg up to 20,000 mg/kg dry weight (EMEA 2004). In humans, 30-70% (20-30 mg) of the OA excreted daily in urine originates from endogenous sources. The daily intake of OA in

the European diet averages 50 mg per day and is excreted in fecal material (Libert and Franceschi 1987, EMEA 2004). Dietary intake of OA varies widely based on the types of food eaten. Although most humans safely consume and excrete OA on a daily basis, excessive consumption of foods with high OA concentrations is a health concern because it has been implicated in several clinical disorders including cardiovascular disease and renal calculi (Singh *et al.* 1972).

The usefulness of OA as a *Varroa* mite control agent has been known since the end of the 20th century (Popov *et al.* 1989). The commercial availability and the low risk of hive product contamination render OA an attractive chemical for *Varroa* control. Numerous investigations of OA as a *Varroa* control agent have been conducted in European countries (Rademacher and Harz 2006). The European Agency for the Evaluation of Medicinal Products (EMEA) determined the maximum residue limit (MRL) for OA in honey to be 50 milliequivalents of free acid in December 2004 (Rademacher and Imdorf 2004). In all European countries, government approval is only given to a veterinary drug after the EMEA has determined the MRL of the active ingredient. Currently, every European country can apply for the legal approval of OA as a drug for honey bee colonies because the EMEA has determined the MRL for OA. If OA is applied properly, there is little risk of harmful residues in honey.

Oxalic acid is also a legal *Varroa* treatment in Canada. In 2005, Health Canada's Pest Management Regulatory Agency (PMRA) made an interim determination that OA can be safely applied to honey bee colonies to control *Varroa* provided that specific limitations and precautions are respected (Special supplement 2005). The Canadian Honey Council has provided the American Beekeeping Federation (ABF) their

registration data packet to expedite the registration of OA in the United States. The ABF is leading the registration process and is working with the United States Environmental Protection Agency to register OA as a biopesticide. Oxalic acid's registration is pending in the United States. My studies will contribute to the registration of OA and will guide beekeepers in how to apply the product safely and effectively once it is registered in the United States.

A review article by Rademacher and Harz (2006) summarizes over 50 references related to the use of OA in European countries. Their review article covers the efficacy of OA against *Varroa* and honey bee tolerance for the trickling, spraying, and evaporating application methods. The trickle and spray methods of OA application were employed in my dissertation research. I chose to exclude the evaporation method due to inherent hazards to the applicator. When OA sublimates, both OA and formic acid fumes are liberated and can permanently damage lung tissues if inhaled. Furthermore, the European literature regarding OA efficacy indicates that the trickle, spray, and evaporation methods of application are equally effective against *Varroa* mites.

Trickling method

The trickling method for applying OA is simple, quick, inexpensive, and effective. To trickle OA, a solution of OA in sugar water is applied with a syringe directly onto adult bees occupying the spaces between the combs. It takes about 1.5 minutes to treat a colony and costs 4-5 cents per hive to purchase the OA required. Rademacher and Harz's (2006) review found that most researchers recommend a dose of 5 mL per bee space (30-50 mL per hive). Oxalic acid is most effective during autumn or

when colonies are broodless and the temperature is above freezing. Oxalic acid is most effective when colonies have little or no brood because OA does not kill mites in sealed brood cells (Schuster and Schürzinger 2003).

Most research reviewed by Nanetti *et al.* (2003) and Rademacher and Harz (2006) found that a single autumn trickle treatment with a 3.0% OA sugar water solution (1:1 by weight) provided an efficacy of greater than 90% in Central Europe. Charrière and Imdorf (2002) found that a slightly more concentrated OA solution (3.5%) resulted in greater than 95% efficacy. They also tested the efficacy of a 3.0% OA sugar water solution with a lower sugar content (1:2; sugar:water by weight) and concluded that the solution with a 1:2 ratio of sugar:water seemed to have a negative influence of efficacy, although the difference was not significant.

Experiments in which OA solutions stronger than 3.5% were applied indicate that increasing the OA concentration above 3.5% does not increase efficacy (Charrière and Imdorf 2002, Nanetti *et al.* 2003). The most concentrated OA solutions examined ranged between 6 and 8% and reduced the efficacy when compared to a 3 or 3.5% solution (Liebig 1998, Nanetti *et al.* 2003). In Central Europe, adult bees tolerated a single autumn trickle treatment concentration up to 4.6% OA (Büchler 2002, Charrière and Imdorf 2002, Nanetti *et al.* 2003). Liebig (1998) quantified the impact on colonies when applying OA solutions with concentrations greater than 5% and described significant adult bee mortality, poor over-wintering ability, and impaired spring development.

Spraying method

Like the trickle method, the spray method is most effective when colonies are broodless, and it should only be conducted when the temperature is above freezing. When implementing the spray method, a solution of OA and sugar water is sprayed onto the adult bees on both sides of each comb. The combs must be individually removed from their hive bodies for application and adult bees located on the hive walls and bottom board are sprayed as well. As a result, the spray method requires more time and labor than the trickle method.

Most studies of the spray method were conducted in Central Europe using broodless colonies with outside temperatures ranging from 5 to 12.3°C (Rademacher and Harz 2006). A single autumn spray treatment using a 3.0% OA sugar water solution (1:1 by weight) and doses of 3-4 mL per comb side provided efficacies of 97.3 to 98.8% (Charrière *et al.* 2004, Imdorf *et al.* 1995). These doses were tolerated well as none of the experimental colonies lost their queen and adult bee mortality was not significantly increased. However, Charrière *et al.* (2004) reported winter losses of 11 to 26% of adult bees. The adult bee losses were numerically higher than control colonies (12 to 16%), however the differences were not significant.

Other aspects of OA for Varroa control

Although OA provides effective control of *Varroa,* its mode of action is unknown. Furthermore, only one study has quantified the contact toxicity of OA to *Varroa* (Milani 2001), and the contact toxicity of OA to honey bees has not been determined. Milani (2001) quantified the toxicity of OA to *Varroa* mites collected from

bee brood. Milani reported that the 24 hour LD_{50} (95% CL) (median lethal density) for

Varroa mites collected from brood was 1.9 (1.49 to 2.36) $\mu g/cm^2$. One limitation of the

Milani (2001) study is that it did not quantify the toxicity of OA to phoretic *Varroa*

mites, the life cycle stage that is exposed to OA when colonies are treated (Schuster and

Schürzinger 2003). Interestingly, OA is extensively used without knowing its basic

toxicological properties to either *Varroa* mites or honey bees.

Recently, Fries (2006) answered an important question regarding OA application

techniques. The question was: which factor is critical for efficacy in *Varroa* control, the

total amount or the concentration of OA? Fries (2006) divided 89 honey bee colonies

into 3 experimental groups: trickle 30 mL sugar water solution, trickle 30 mL 3.2% OA

sugar water solution, and trickle 60 mL 1.6% OA sugar water solution. The results

showed that trickling 30 mL of a 3.2% OA solution is significantly more effective (92.2%

efficacious) than trickling 60 mL of a 1.6% OA solution (68.3% efficacious). Fries

(2006) clearly demonstrated that it is the concentration of OA that is critical for high

efficacy rather than the total amount of OA applied to a colony.

Research objectives

My overall dissertation objective was to develop techniques for using OA to suppress *Varroa* mites in the North Central Region (NCR) of the United States. When I started my Ph.D. research in 2004, OA treatment had not been tested in the United States as a *Varroa* suppression technique. Chapter 1 describes how I quantified the acute contact toxicity of OA to *Varroa* mites and their honey bee hosts in laboratory bioassays. Chapters 2 and 3 describe how I investigated the efficacy of OA in the NCR of the United States. Chapters 4 and 5 document my development of a protocol for using OA to eliminate mites from package bees. Finally, chapter 6 describes my methodology for investigating how OA is distributed in honey bee colonies when applied using the trickle method.

Chapter 1

Acute contact toxicity of oxalic acid to *Varroa destructor*

(Acari: Varroidae) and their *Apis mellifera* (Hymenoptera: Apidae) hosts in

laboratory bioassays

Abstract

I performed laboratory bioassays to characterize the acute contact toxicity of oxalic acid (OA) to *Varroa destructor* (Anderson & Trueman) and their honey bee hosts (*Apis mellifera*). Specifically, I conducted glass-vial residual bioassays to determine the lethal concentration of OA for *Varroa*, and I conducted topical applications of OA in acetone to determine the lethal dose for honey bees. The results indicate that OA has a low acute toxicity to honey bees and a high acute toxicity to mites. The toxicity data will help guide scientists in delivering optimum dosages of OA to the parasite and its host, and will be useful in making treatment recommendations. The data will also establish a baseline for OA susceptibility for both parasite and host to compare with future populations if *Varroa* mites exhibit resistance to OA.

Introduction

The objective of this study was to characterize the acute contact toxicity of OA to *Varroa* mites and their honey bee hosts in laboratory bioassays. Only one study has quantified the contact toxicity of OA to *Varroa* mites collected from brood cells (Milani 2001), and the contact toxicity of OA to honey bees has not been determined. One

limitation of the Milani (2001) study is that it did not quantify the toxicity of OA to phoretic *Varroa* mites. Oxalic acid does not kill mites in sealed brood cells (Schuster and Schürzinger 2003), thus the characterization of the contact toxicity of OA to phoretic *Varroa* mites has not been determined. Oxalic acid is extensively used without knowing the basic toxicological properties of the compound to *Varroa* mites or honey bees. The results of this research will be useful in formulating treatment recommendations for the North Central Region of the United States. The data will also establish a baseline for OA susceptibility for both parasite and host to compare with future populations if *Varroa* mites exhibit resistance to OA.

Materials and Methods

Collection of V. destructor

I collected adult worker bees from a single, mite-infested colony of Carniolan honey bees located on the University of Nebraska campus in February 2005. I shook the bees from each frame (12 frames total) through a funnel into a bulk bee box. I then subdivided the bees into six smaller wooden boxes that measured 17.75 cm long, 15.25 cm wide, and 10.15 cm deep (inside diameter). Each box had an 8-mesh screen on one of the 17.75 cm X 15.25 cm sides for ventilation and mite collection. I attached a pint jar of sugar water (1:1 by volume) to each box and stored the boxes at 15.6°C in complete darkness until needed for experimentation (no longer than 2 days).

I harvested mites from adult bees by applying 18 g of powdered sugar through the screen of each box (Aliano & Ellis 2005a). I inverted the boxes after bees were thoroughly coated with powdered sugar to collect mites (about 30 s). The boxes

remained inverted until mite fall ceased (about 20 min). Approximately 100 viable mites were recovered per cage. I gently brushed the powdered sugar off mites with a fine paint brush before transferring them to 20 mL glass scintillation vials (Wheaton Scientific, Millville, NJ) for OA exposure.

Glass-vial residual bioassays for V. destructor

I used techniques described by Plapp and Vinson (1977) and Macedo *et al*. (2002a) for conducting glass-vial residual bioassays. I prepared serial dilutions of oxalic acid dihydrate (>99% purity) (The Science Company, Denver, CO) (CAS # 6153-56-6) in acetone and conducted a preliminary range-finding bioassay to determine at least 3 concentrations that provided *Varroa* mortalities >0 and <100%. I prepared 7 concentrations of OA in acetone for the definitive bioassay (1.0, 0.3, 0.1, 0.03, 0.01, 0.003, and 0.001 mg/mL). I pipetted one-half mL of each solution into four, 20 mL glass scintillation vials for each treatment, including an acetone control. The 8 resulting OA concentrations that I tested in the definitive bioassay were 500, 150, 50, 15, 5, 1.5, 0.5, and 0.0 μg OA per vial. I rolled all vials (32 total) on their sides under a fume hood to evaporate the acetone while evenly coating the vials with OA. I promptly removed the vials after the acetone had evaporated (4 to 5 min). I gently brushed ten mites into each vial, screwed the cap on tightly, and placed the vials in a dark incubator for 24 h (26°C and 90% relative humidity). One vial of 10 mites constituted a replication and four vials (40 mites) were used for each of the 8 concentrations. I scored mite mortality 24 h later by examining mites under a light microscope. Mites were considered dead if they did not respond to probing with a small paint brush.

Topical application of OA to adult bees

I prepared serial dilutions of oxalic acid dihydrate (>99% purity) (The Science Company, Denver, CO) (CAS # 6153-56-6) in acetone and conducted a preliminary range-finding bioassay to determine at least 3 doses that provided honey bee mortalities >0 and <100%. Two to 7 day old bees from C.F. Koehnen & Sons Inc. (Glenn, CA) were shipped to the University of Nebraska in March 2005 and were used for the range-finding bioassay. I used the results from the range-finding bioassay to establish the definitive bioassay dosage levels.

I conducted the definitive bioassay in September 2005 using 2 to 7 day old bees that were obtained from brood frames kept in an incubator. I placed 10 bees in each of 32 Benton mailing cages with queen candy. One cage of 10 worker bees constituted a replication and 4 cages (40 bees) were tested for each treatment. I randomly assigned each cage to 1 of the 8 concentrations, and all 10 bees within a cage received the same treatment. I prepared a 200 mg/mL solution of OA in acetone. I dosed honey bees with 10.0, 8.0, 4.0, 2.0, 1.0, or 0.5 μL of the 200 mg/mL stock solution. These doses correspond to 2000, 1600, 800, 400, 200, and 100 μg OA per bee, respectively. I applied the doses to individual bee abdomens using a Hamilton microsyringe and repeating dispenser (Hamilton Company, Reno, NV) after anesthetizing bees with CO_2. I included a 10 μL control (acetone only) in the bioassay along with a dry control in which bees were anesthetized with CO_2 but were not treated with acetone. I held the bees at $21.7 \pm 0.4°C$ and $46.3 \pm 1.5\%$ relative humidity in darkness for 72 h except for brief periods when water was administered to the cages. I provided water twice daily to the bees

throughout the experiment by brushing the cage screens with several drops of water. I
evaluated mortality 24, 48, and 72 h after treatment.

Statistical analysis

I analyzed the results with Probit analysis (Finney 1971) using the POLO-PC
statistical software (LeOra Software 1991), and I took into account natural mortality.
The concentrations that I used for the Probit analysis of the mite bioassay data were
expressed as μg per vial. The doses that I used for the Probit analysis of the honey bee
bioassay data were expressed as μg per bee.

Results

V. destructor bioassays

The results for the definitive bioassay are summarized in Table 1.1. The natural
mortality for the definitive bioassay was 9.7 ± 3.4% after 24 h.

Honey bee bioassays

Doses of OA less than 100 μg per bee did not cause significant mortality after 48
h in the range-finding bioassay. Further, it was impossible to calculate the 24 and 48 h
LD_{50}'s for honey bees tested in the range-finding bioassay because significant mortality
did not occur until at least 72 h post-treatment. Aliquots of a 200 mg/mL solution of OA
in acetone were used to dose honey bees in both the range-finding and definitive
bioassays because solutions greater than 200 mg/mL clogged the microsyringe, making it
impossible to deliver accurate doses of OA. The results for the definitive bioassay are

summarized in Table 1.1. Only the 10 μL acetone control group was used for Probit

analysis of the definitive bioassay data because the 10 μL acetone control had slightly

more mortality than the dry control group. The natural mortality for the definitive

bioassay was $4.0 \pm 2.5\%$ after 48 h. I was unable to calculate a confidence interval for

the 72 h LD_{50} because all bees in the 2000, 1600, 800, and 400 μg OA per bee treatments

died after 72 h. The estimated 72 h LD_{50} for honey bees was 194.89 μg per bee, based on

the 100 and 200 μg OA per bee treatments.

Discussion

Milani (2001) is the only report that quantifies the toxicity of OA to *V. destructor*

collected from bee brood, where the 24 h median lethal density (OA density expected to

cause 50% mortality) for *V. destructor* collected from bee pupae with white eyes was 1.9

$\mu g/cm^2$ (95% fiducial limits = 1.49 to 2.36). The mites collected from brood were

exposed to OA for 4 h by placing them on glass disks that were sprayed with solutions of

OA. The mites were then transferred to clean glass Petri dishes at 32.5°C and 75%

relative humidity. My results indicate that the 24 h LC_{50} for phoretic mites is 5.12 μg per

vial. By assuming the area of a single, 20 mL scintillation vial treated with OA was

approximately 20 cm^2, the 24 h median lethal density in my study was calculated to be

0.26 $\mu g/cm^2$. One likely reason for the apparent higher toxicity in my study is that Milani

exposed mites to OA for 4 h versus 24 h in my bioassays. Further, the previous study

used mites collected from brood as experimental material, and I collected mites from

adult bees. Differences between mites collected from adults versus brood could have also

affected bioassay results. However, I believe it is preferable to use phoretic mites for

conducting OA bioassays because OA does not kill mites in brood when applied to a hive.

The mode of action for OA is not completely understood. My results suggest that OA may exhibit its lethal effects on mites via contact. However, I cannot rule out that some mite mortality was caused by exposure to OA vapors. I expect that mite mortality resulting from exposure to OA vapors was minimal because OA has a low volatility at room temperature (vapor pressure < 0.001 mm Hg at 25°C; melting point of 101-102°C) (Merck Index 1996). Charrière and Imdorf (2002) reported that OA mixed in 1:1 sugar water exhibits greater miticidal effectiveness than solutions with half as much sugar (1:2 sugar water). Further, Milani (2001) indicates that sucrose and glycerol are synergists of OA under laboratory conditions due to sucrose's ability to cause OA to become more hygroscopic. Perhaps the sugar water solution adheres better to bees, thus increasing mite exposure to OA.

The 400, 800, 1600, and 2000 μg per bee doses applied in the definitive bioassay completely covered the bees' abdomen with OA crystals. Further, the 2000 μg per bee dose that was delivered in 10 μL of acetone completely soaked the bees with some runoff. My results indicate that it is nearly impossible to kill 100% of a test population of adult bees in 24 h by topically applying OA in acetone. I conclude that OA has a relatively low acute toxicity to honey bees. There are several accounts in the literature of increased adult bee mortality as a result of OA application within hives (Charrière and Imdorf 2002, Imdorf *et al*. 1998), suggesting that OA may not exhibit its lethal effect on honey bees until more than 24 h after exposure.

A typical honey bee colony in the North Central Region of the United States has a November adult bee population of 35,000 bees (personal observation). According to the Canadian protocol (Special Supplement 2005), a beekeeper would apply a maximum of 2 g OA per hive for *Varroa* treatment (trickle or vaporizer methods). The resulting dose of OA per bee would have a maximum value of 57.1 µg per bee (2,000,000 µg OA / 35,000 bees). Results from acute exposure bioassays suggests that 57.1 µg per bee is considerably below the 48 h LD_{10} (176.68 µg per bee). My results showed that all bees dosed with 2000, 1600, 800, or 400 µg OA per bee died after 72 h. Bees subjected to the 100 µg OA per bee dose survived longer than 72 h and had mortality similar to the control treatment. My results roughly coincide with the recommended dose of OA per hive ($\leq$ 2 g) because doses $\leq$ 100 µg OA per bee in my laboratory bioassays did not cause significant mortality.

My data are significant because they quantify the dosage-mortality relationships for populations of both mites and bees that have not been exposed to OA. My data will facilitate future comparisons of toxicity if mite resistance to OA becomes evident. Further, these basic toxicological properties of the compound will help guide scientists in developing techniques for delivering optimum dosages to the parasite and its host.

Table 1.1 – Honey bee mortality responses to oxalic acid dihydrate when topically applied to bee abdomens in acetone and *Varroa destructor* mortality responses in glass-vial residual bioassays.

Honey bee mortality	n	Slope ± SE	LD_{10} (95% CL) (µg per bee)	LD_{50} (95% CL) (µg per bee)	LD_{90} (95% CL) (µg per bee)
24 h	280	2.87 ± 0.54	564.05 (95.22 to 877.92)	1,575.85 (1,087.44 to 2,962.72)	4,402.6 (2,541.78 to 48,848.75)
48 h	280	3.96 ± 0.54	176.68 (120.36 to 225.54)	372.01 (306.78 to 439.88)	783.27 (643.5 to 1,042.56)
Mite mortality	n	Slope ± SE	LC_{10} (95% CL) (µg per 20 mL vial)	LC_{50} (95% CL) (µg per 20 mL vial)	LC_{90} (95% CL) (µg per 20 mL vial)
24 h	320	2.28 ± 0.35	1.4 (0.63 to 2.26)	5.12 (3.47 to 7.0)	18.69 (13.14 to 31.7)

Chapter 2

Using fall treatments with oxalic acid to reduce *Varroa* mite populations in the

North Central Region of the United States

Abstract

Field trials were conducted on 52 honey bee colonies to quantify the efficacy of

oxalic acid (OA) against *Varroa* mites in the North Central Region (NCR) of the United

States. I performed the experiment in November 2004 on colonies that were located in 3

apiaries near Lincoln, NE. The experimental colonies had 1 or 2 hive bodies (24.4 cm

deeps) and contained less than 2 frames of brood. I randomly assigned the *Varroa*-

infested colonies to 3 treatment groups: 1) trickle OA, 2) spray OA, and 3) untreated. For

the trickle treatment, I applied 50 mL of a 3.5% OA sugar water solution to both single

and double story colonies using a 100 mL syringe (n = 17). For the spray treatment, I

applied 50 mL of a 3.5% OA sugar water solution to both single and double story

colonies by directly spraying the adult bee cluster from above and below using a 1 L,

non-pressurized sprayer (n = 18). The experiment also included a group of untreated

(control) colonies (n = 17). I determined the pre- and post-treatment *Varroa* infestation

levels for all colonies and calculated the percentage reduction in *Varroa* infestation 18

days post-treatment. The trickle treatment reduced *Varroa* infestation by 77.3 ± 14.1%

when compared to untreated colonies. The spray treatment reduced *Varroa* infestation by

80.2 ± 14.0% when compared to untreated colonies. The effectiveness of the trickle and

spray treatments was not significantly different. I conclude that OA is a viable treatment method for *Varroa* mites in the NCR of the United States.

Introduction

Honey bee colonies located in the North Temperate Zone (includes Great Britain, Europe, northern Asia, and North America) decelerate brood rearing in autumn to prepare for winter. In Nebraska, honey bee colonies have minimal brood by November, and by December, most colonies are broodless. The broodless condition of honey bee colonies located in temperate climate zones is ideal for OA application because OA does not kill mites in sealed brood cells (Schuster and Schürzinger 2003). Oxalic acid is most effective in broodless colonies because *Varroa* mites in brood cells are not exposed to treatments. During broodless periods, all mites are phoretic on adult bees and vulnerable to treatment.

The objective of this study was to investigate the usefulness of OA for reducing *Varroa* mite populations in broodless colonies in the NCR of the United States. At the time of this study, OA's efficacy had not been tested in the United States. In contrast, numerous field studies that quantified the efficacy of OA on broodless colonies had been published in the European literature and report ~90% efficacy against *Varroa* when using either the trickle or spray treatment methods (Imdorf *et al.* 1995, Charrière and Imdorf 2002, Nanetti *et al.* 2003, Charrière *et al.* 2004). My goal was to test the hypothesis that OA would have an efficacy of ~90% against *Varroa* mites as reported in the European literature.

Materials and Methods

Identification of experimental hives

I established 52 experimental colonies in 3 apiaries near Lincoln, NE in November 2004. The experimental hives were 1 or 2 stories (24.4 cm deeps) and contained less than 2 frames of brood.

Treatment and data collection

I randomly assigned the *Varroa*-infested colonies to 3 treatment groups: 1) trickle OA, 2) spray OA, and 3) untreated. I collected approximately 300 adult bees in alcohol from the 52 experimental hives on November 3rd, applied the OA treatments on November 4th, and collected the post-treatment alcohol samples on November 22nd, 2004. I estimated the pre- and post-treatment *Varroa* infestation levels using the alcohol wash method (Shimanuki and Knox 2000).

For the trickle treatment, I applied 50 mL of a 3.5% OA sugar water (sugar:water) (1:1) (w:w) solution to both single and double story colonies using a 100 mL syringe (n = 17). I trickled the OA solution from above the frames between each occupied bee-way and made an effort to maximize contact with the adult bee population. For the spray treatment, I applied 50 mL of a 3.5% OA sugar water solution (sugar:water) (1:1) (w:w) to both single and double story colonies by directly spraying the adult bee cluster from above and below using a 1 L, non-pressurized sprayer (n = 18). The untreated (control) colonies were left untouched (n = 17). I chose to treat the colonies with 50 mL of a 3.5% OA sugar water solution based on methods described by Rademacher and Harz (2006).

I inserted sticky boards and Checkmite+® strips in the hives from 2 of the apiaries (n = 25) on December 9th and removed them on December 13th, 2004. The experimental mite populations had not previously exhibited coumaphos resistance. I used the sticky board counts to determine the remaining mite populations in the experimental hives 35 days post-treatment.

Experimental design and statistical analysis

I designed my experiment as a randomized complete block design (RCBD) blocked by the apiaries in which the experimental hives were located (n = 3) to account for variance in the pre-treatment *Varroa* mite infestation levels. I used mites-per-100 adult bees as a response variable to test for differences in *Varroa* infestation between pre- and post-treatment experimental hives. I used the percentage reduction in *Varroa* infestation 18 days post-treatment as another response variable. For the final response variable, I examined the number of mites remaining 35 days post-treatment in hives treated with Checkmite+® strips and fitted with sticky boards.

I analyzed the data using PROC MIXED (SAS Institute 2006), and I separated means using a *t*-test (α = 0.05). I assumed random blocks, although the assumption of fixed blocks did not change the results. I applied the Kenwood-Rogers degrees of freedom adjustment, and used PROC UNIVARIATE and PROC GPLOT (SAS Institute 2006) to verify my assumptions of normality and constant variance.

Results

My assumptions of normality and constant variance were met. I used the Shapiro-Wilk test in the UNIVARIATE procedure of SAS to verify normality. The Shapiro-Wilk test indicated that my data were normal ($P = 0.3726$) when using either mites-per-100 adult bees or percentage reduction in *Varroa* infestation as response variables. In addition, a symmetric box-plot and a straight-lined normal probability plot confirmed normality. A plot of the residual versus the predicted values revealed no obvious patterns and was indicative of data that had constant variance.

RCBD with mites-per-100 adult bees as the response variable

I split the 3 treatment groups (trickle OA, spray OA, and untreated) into their pre- and post-treatment counterparts for this analysis. This resulted in 6 treatment estimates (pre-trickle, pre-spray, pre-untreated, post-trickle, post-spray, and post-untreated). The post-treatment estimates are derived from alcohol samples taken 18 days after the pre-treatment alcohol samples. The treatment effect was significant ($F = 3.71$, $df = 5, 96.1$, $P = 0.0041$). A summary of the treatment estimates reported as mites-per-100 adult bees is provided in Table 2.1. As illustrated in Table 2.1, the pre-trickle, pre-spray, and pre-untreated experimental colonies had *Varroa* infestations ranging from 7.8 to 11.4 mites-per-100 bees and were not significantly different from each other. In contrast, both the 18 d post-trickle and 18 d post-spray treatments had significantly fewer mites than the 18 d post-treatment group that received no treatment ($t = 2.52$, $df = 96$, $P = 0.0135$) ($t = 2.76$, $df = 96.1$, $P = 0.0070$). The 18 d post-trickle and 18 d post-spray *Varroa* infestations were not significantly different ($t = 0.21$, $df = 96.1$, $P = 0.8310$).

RCBD with percentage reduction in Varroa infestation 18 days post-treatment as the response variable

The treatment effect was significant ($F = 20.87$, $df = 2$, 47.1, $P = 0.0001$). See Table 2.2 for a summary of the treatment estimates reported as percentage reduction in *Varroa* infestation 18 days post-treatment. The trickle OA treatment significantly reduced *Varroa* infestation by 77.3 ± 14.1% when compared to untreated colonies ($t = 5.48$, $df = 46.8$, $P = 0.0001$). Similarly, the spray OA treatment significantly reduced *Varroa* infestation by 80.2 ± 14.0% when compared to untreated colonies ($t = 5.72$, $df = 47.2$, $P = 0.0001$). The effectiveness of the trickle OA and spray OA treatments were not significantly different ($t = 0.21$, $df = 47.2$, $P = 0.8377$).

RCBD with the number of mites remaining 35 days post-treatment as the response variable

I used the sticky board counts for mites falling during the first 4 days after CheckMite+® application to determine the remaining mite populations in a portion of the experimental hives 35 days post-treatment. The treatment effect was significant ($F = 13.59$, $df = 2$, 21.4, $P = 0.0002$). See Table 2.3 for a summary of the number of mites remaining 35 days post-treatment for each group. The trickle OA treatment significantly reduced *Varroa* infestation by 268.4 ± 60.4 mites when compared to untreated colonies ($t = 4.44$, $df = 21$, $P = 0.0002$). Similarly, the spray OA treatment significantly reduced *Varroa* infestation by 278.2 ± 60.8 mites when compared to untreated colonies ($t = 4.58$, $df = 21.5$, $P = 0.0002$). The number of mites remaining in the trickle OA and spray OA treatment colonies were not significantly different ($t = 0.16$, $df = 21.5$, $P = 0.8737$).

Discussion

I accept my hypothesis that OA would have an efficacy of ~90% against *Varroa* mites in the NCR of the United States. The trickle OA and spray OA treatments significantly reduced *Varroa* infestation by 77.3% (95% CI = 48.9 to 105.7%) and 80.2% (95% CI = 52.0 to 108.4%) when compared to untreated colonies, respectively. Although not statistically different, my results showed OA's average efficacy to be 10 to 13% lower than the European results which indicated ~90% efficacy against *Varroa* when using either the trickle or spray treatment methods on broodless colonies. One possible explanation as to why OA's efficacy was numerically lower in my study than European studies is that I waited 18 days to take the post-treatment alcohol samples. Several of the colonies had small patches of brood that may have increased *Varroa* infestation when they eclosed over the 18 day period between pre- and post-treatment samples. Perhaps I did achieve ~90% efficacy against *Varroa* mites on adult bees at the time of treatment, and the efficacy by day 18 was underestimated due to *Varroa* mites emerging from brood.

The effectiveness of the trickle OA and spray OA treatments was not significantly different. However, the trickle method may be preferable because it was less time and labor intensive yet had equivalent efficacy when compared to the spray method. It took about 1.5 minutes to treat a colony using the trickle method and about 5 minutes to treat a colony using the spray method. This includes removal of the lid and inner cover, application of OA, and reassembly of the hive.

My results reveal that the trickle OA and spray OA treatments significantly reduced *Varroa* infestation regardless of the response variable modeled (mites-per-100

bees, percentage reduction in *Varroa* infestation 18 days post-treatment, or the number of

mites remaining 35 days post-treatment). Furthermore, 77.3 to 80.2% reduction in

Varroa infestation is comparable to the efficacies of other *Varroa* control products on the

market. I conclude that OA is a viable treatment method for *Varroa* mites in the NCR of

the United States.

Table 2.1 – Treatment estimates with mites-per-100 adult bees as the response variable. Estimates with different letters indicate significant differences (*t*-test, $\alpha = 0.05$).

Treatment	Estimate ± Standard error	n
Pre-trickle	11.4 ± 5.6 a	17
Pre-spray	7.8 ± 5.6 a	18
Pre-untreated	10.8 ± 5.6 a	17
18 d Post-trickle*	1.8 ± 5.6 b	17
18 d Post-spray	1.1 ± 5.6 b	18
18 d Post-untreated	10.5 ± 5.6 a	17

* 18 d = 18 days post-treatment

Table 2.2 – Treatment estimates with percentage reduction in *Varroa* infestation 18 days post-treatment as the response variable. Estimates with different letters indicate significant differences (*t*-test, $\alpha = 0.05$).

Treatment	Estimate ± Standard error	n
Trickle OA	79.1 ± 14.4 a	17
Spray OA	82.0 ± 14.1 a	18
Untreated	1.8 ± 14.4 b	17

Table 2.3 – Treatment estimates with the number of mites
remaining 35 days post-treatment as the response variable.
Estimates with different letters indicate significant differences
(*t*-test, α = 0.05).

Treatment	Estimate ± Standard error	n
Trickle OA	31.9 ± 47.8 a*	8
Spray OA	22.1 ± 44.6 a	9
Untreated	300.3 ± 47.8 b	8

* The estimates represent the remaining mite populations 35
  days post-treatment that were recovered from sticky boards
  left in hives for 4 days with Checkmite+® strips present

Chapter 3

Using summer oxalic acid treatments to reduce *Varroa* mite populations in colonies

containing brood

Abstract

I conducted a field trial on 46 honey bee colonies to determine the efficacy of

oxalic acid (OA) against *Varroa* mites in hives containing brood. The experimental hives

were single story, queenright, and contained all stages of worker brood. I randomly

assigned the experimental hives to 4 treatment groups: 1.) untreated or control, 2.) 2.0%

OA, 3.) 3.5% OA, and 4.) 6.0% OA. For the OA treatments, I trickled 50 mL of a 2.0%,

3.5%, or 6.0% OA sugar water solution in the hives using a 100 mL syringe. Each

colony was treated 3 times at 1 week intervals. The experiment also included a group of

untreated colonies. I collected approximately 300 adult bees in alcohol to estimate the

pre- and post-treatment *Varroa* infestation level on adult bees. I measured the square

inches of capped worker brood, counted the number of frames that the adult bee cluster

occupied, and examined both worker and drone bee pupae from each hive. I counted the

number of adult female *Varroa* mites on excised bee pupae to estimate the pre- and post-

treatment *Varroa* infestation levels in brood. I also weighed the hives at the end of the

study to quantify the impact of OA treatment on weight gain. Repeated applications of

OA did not significantly reduce the *Varroa* infestation on adult bees or in brood

regardless of the concentration applied. The number of frames of adult bees, the square

inches of capped worker brood, and the average weight-per-hive were not negatively

affected by repeated applications of OA. My results do not support the use of OA as a summer treatment when colonies contain expanded brood nests. Although repeated treatments exhibited no harmful effects on bees, the paucity of significant mite population suppression indicates that repeated treatments are not useful in a *Varroa* management program.

Introduction

The broodless condition of honey bee colonies located in temperate climate zones is ideal for OA application because OA does not kill mites in sealed brood cells (Schuster and Schürzinger 2003). Oxalic acid is most effective in broodless colonies because *Varroa* mites in brood cells are not exposed to treatments. During broodless periods, all mites are phoretic on adult bees and vulnerable to treatment.

The leading objective of the current study was to investigate the usefulness of repeated mid-summer applications of OA for reducing *Varroa* mite populations in colonies containing expanded brood nests. While numerous studies of *Varroa* control with OA have been conducted in Europe, Asia, and Canada, few studies have been conducted in the United States. Most studies report ~90% efficacy against *Varroa* when using either the trickle or spray treatment methods (Imdorf *et al.* 1995, Charrière and Imdorf 2002, Nanetti *et al.* 2003, Charrière *et al.* 2004, Rademacher and Harz 2006). Schuster and Schürzinger (2003) reported that a single application of OA does not kill mites in sealed brood cells. My goal was to test the hypothesis that repeated applications of OA would significantly reduce *Varroa* mite populations in colonies containing brood. My theory was that by applying OA 3 times at one week intervals, mites in sealed brood

cells would eventually come in contact with OA as adult bees emerged over the 14 day treatment period and mites became phoretic on adult bees. I wanted to establish the efficacy of 2.0%, 3.5%, and 6.0% OA sugar water solutions. I hypothesized that the efficacy of OA against *Varroa* would increase as the concentration of the OA solution increased, and that 3 successive treatments at 7 day intervals would expose most of the *Varroa* population to OA. My final objective was to quantify the impact of repeated applications of OA on overall colony health by measuring the square inches of capped worker brood, by counting the number of frames containing adult bees, and by weighing the colonies at the end of the study.

Materials and Methods

Identification of experimental hives

I established 46 *Varroa*-infested honey bee colonies in 2 apiaries at the University of Nebraska-Lincoln's Agricultural Research and Development Center (ARDC) near Mead, NE as experimental units for this study. The experimental hives were single story Langstroth beehives (24.4 cm deep hive body) with a queen excluder and 1 or 2 supers (16.8 cm deep). The experimental colonies contained 3 to 6 frames of brood, a young Carniolan or Italian queen, and all frames were covered with bees.

Treatment and data collection

I randomly assigned the 46 experimental hives to 4 treatment groups: 1.) untreated or control, 2.) 2.0% OA, 3.) 3.5% OA, and 4.) 6.0% OA. The hives were queenright and contained all stages of worker brood (eggs, larvae, pupae). Not all hives

contained drone brood. I collected approximately 300 adult bees in alcohol to estimate the pre-treatment *Varroa* infestation level on adult bees (July 12). I applied the OA treatments on July 13, 19, and 27. I collected approximately 300 adult bees in alcohol to estimate the post-treatment *Varroa* infestation level on adult bees (August 9).

I measured the square inches of capped worker brood, counted the number of frames that the adult bee cluster occupied, and excised ~300 worker bee pupae and ~90 drone bee pupae from each hive (July 12 and August 9). I counted the number of adult female *Varroa* mites on excised bee pupae to estimate the pre- and post-treatment *Varroa* infestation levels in brood. I weighed the hives at the end of the study to quantify the impact of OA treatment on weight gain (August 9).

For the OA treatments, I trickled 50 mL of either a 2.0%, 3.5%, or 6.0% OA sugar water (sugar:water) (1:1) (w:w) solution over the cluster of bees using a 100 mL syringe. I trickled 5-6 mL of the OA solutions between each occupied bee-way and made an effort to maximize contact with the adult bee population. Each colony was treated 3 times at 1 week intervals (July 13, 19, and 27). The untreated (control) colonies were left untouched. I chose to treat the hives with 50 mL of the OA sugar water solutions based on European results reported by Rademacher and Harz (2006).

Experimental design and statistical analysis

I designed my experiment as an randomized complete block design (RCBD). I blocked by apiary (n = 2) in which the experimental hives were located to account for variance in the pre-treatment *Varroa* mite infestation levels. I used mites-per-100 adult bees pre- and post-treatment, percentage reduction in *Varroa* infestation 28 days post-

treatment, and mites-per-100 excised worker and drone pupae pre- and post-treatment as response variables. Other response variables included the number of frames of adult bees in each hive pre- and post-treatment, the square inches of capped worker brood pre- and post-treatment, and the post-treatment weight-per-hive.

I analyzed the mites-per-100 adult bee data, the mites-per-100 excised worker pupae data, the mites-per-100 excised drone pupae data, and the square inches of capped worker brood data using PROC GLIMMIX (SAS Institute 2006) and separated means using a t-test ($\alpha = 0.05$). I fit a generalized linear model (GLM) for each of the aforementioned response variables using a normal distribution. I also assumed random blocks, although the assumption of fixed blocks did not change the results. I framed my data as a factorial with dose and time as factors. Time had 2 levels: pre- and post-treatment. Dose had 4 levels: 0 (untreated), 2.0% OA, 3.5% OA, and 6% OA. For the above analyses, there were 8 total treatment combinations (pre-untreated, pre-2.0% OA, pre-3.5% OA, pre-6.0% OA, post-untreated, post-2.0% OA, post-3.5% OA, and post-6.0% OA). I applied the 'group=dose' option in PROC GLIMMIX to fit a separate variance for each dosage of OA. I used PROC UNIVARIATE (SAS Institute 2006) to verify my assumption of normality.

I also analyzed the number of frames of adult bees data using PROC GLIMMIX (SAS Institute 2006) and separated means using a t-test ($\alpha = 0.05$). This data set differed from the abovementioned response variables because the data were frame counts (1 to 9 frames). For this response variable, I fit a GLM and implemented the Poisson distribution in PROC GLIMMIX.

I analyzed the percentage reduction in *Varroa* infestation 28 days post-treatment data and the post-treatment weight-per-hive data using PROC GLIMMIX (SAS Institute 2006) and separated means using a *t*-test ($\alpha = 0.05$). I fit a GLM for the percentage reduction and weight-per-hive response variables using a normal distribution. I assumed random blocks, although the assumption of fixed blocks did not change the results. I considered 4 treatments for the percentage reduction in *Varroa* infestation 28 days post-treatment data (untreated, 2.0% OA, 3.5% OA, and 6.0% OA). The percentage reduction in *Varroa* infestation was calculated for each hive using the pre- and post-treatment mites-per-100 adult bee data. I considered 4 treatments for the post-treatment weight-per-hive data (post-untreated, post-2.0% OA, post-3.5% OA, and post-6.0% OA). I applied the 'group=treatment' option in PROC GLIMMIX to fit a separate variance for each treatment. I used PROC UNIVARIATE (SAS Institute 2006) to verify my assumption of normality.

Results

My assumption of normality was met for all response variables for which I fit a GLM using a normal distribution. These data sets produced a symmetric box-plot and a straight-lined normal probability plot that confirmed normality. A plot of the residual versus the predicted values revealed a problem with non-constant variance. The 'group=dose' option and the 'group=treatment' option that I employed in PROC GLIMMIX fixed the non-constant variance issue. After fitting a separate variance for each treatment, a plot of the residual versus the predicted values revealed no obvious patterns and was indicative of data that had constant variance.

Number of bees collected for adult and brood samples

The average number of adult bees collected in alcohol was 306.8 ± 41.2 (n = 92) for each pre- and post-treatment sample. The average was calculated using both pre- and post-treatment alcohol samples for each hive (46 experimental hives X 2 sample times = 92 adult bee alcohol samples). The average number of worker and drone brood cells excised for pre- and post-treatment sample is shown in Table 3.1.

RCBD with mites-per-100 adult bees as the response variable

There was no significant time*dose interaction ($F = 0.18$, $df = 3, 83$, $P = 0.9091$), and therefore, the main effects of time and dose were considered. The time effect was significant ($F = 20.69$, $df = 1, 83$, $P = 0.0001$). Prior to OA treatment, the hives had 2.5 ± 0.5 more mites-per-100 adult bees than after OA treatment regardless of the OA concentration applied ($t = 4.55$, $df = 83$, $P = 0.0001$). The dose effect was not significant ($F = 1.13$, $df = 3, 83$, $P = 0.3411$). See Table 3.2 for the treatment estimates with mites-per-100 adult bees as the response variable.

RCBD with percentage reduction in Varroa infestation 28 days post-treatment as the response variable

The treatment effect was not significant at $\alpha = 0.05$ ($F = 1.99$, $df = 3, 41$, $P = 0.1302$). See Table 3.3 for the treatment estimates with percentage reduction in *Varroa* infestation 28 days post-treatment as the response variable. Although the treatment effect was not significant at $\alpha = 0.05$, it is important to note that when using $\alpha = 0.1$, the 6.0% OA treatment significantly reduced *Varroa* infestation by $35.4 \pm 18.6\%$ when compared to untreated colonies ($t = 1.90$, $df = 41$, $P = 0.0640$). Similarly, when using the $\alpha = 0.1$

significance level, the 6.0% OA treatment significantly reduced *Varroa* infestation by

$29.0 \pm 16.1\%$ when compared to the 2.0% OA treatment ($t = 1.80$, $df = 41$, $P = 0.0792$).

RCBD with mites-per-100 excised worker pupae as the response variable

There was significant time*dose interaction ($F = 2.30$, $df = 3, 63$, $P = 0.0858$) at

the $\alpha = 0.1$ significance level, and therefore, the simple effects of time and dose were

considered. When only considering the post-treatment time effect, the untreated hives

had 0.7 ± 0.4 more mites-per-100 excised worker pupae than the hives that received the

3.5% OA treatment ($t = 2.01$, $df = 63$, $P = 0.0484$). When only considering the post-

treatment time effect, the untreated hives had 0.7 ± 0.3 more mites-per-100 excised

worker pupae than the hives that received the 6.0% OA treatment ($t = 2.18$, $df = 63$, $P =$

0.0329). When only considering the pre-treatment time effect, the hives slated to receive

the 2.0% OA treatment had 0.7 ± 0.3 more mites-per-100 excised worker pupae than the

hives slated to receive the 6.0% OA treatment ($t = 2.30$, $df = 63$, $P = 0.0245$). When only

considering the pre-treatment time effect, the hives slated to receive the 3.5% OA

treatment had 0.7 ± 0.3 more mites-per-100 excised worker pupae than the hives slated to

receive the 6.0% OA treatment ($t = 2.35$, $df = 63$, $P = 0.0220$). When only considering

the hives that received the 3.5% OA treatment, there were 1.0 ± 0.4 more mites-per-100

excised worker pupae prior to OA treatment than after OA treatment ($t = 2.92$, $df = 63$, P

$= 0.0049$). See Table 3.4 for the treatment estimates with mites-per-100 excised worker

pupae as the response variable.

RCBD with mites-per-100 excised drone pupae as the response variable

There was no significant time*dose interaction ($F = 0.32$, $df = 3, 41$, $P = 0.8114$), and therefore, the main effects of time and dose were considered. The time effect was not significant ($F = 1.23$, $df = 1, 41$, $P = 0.2734$). The dose effect was also not significant ($F = 0.36$, $df = 3, 41$, $P = 0.7826$). See Table 3.5 for the treatment estimates with mites-per-100 excised drone pupae as the response variable.

RCBD with the number of frames of adult bees in each hive as the response variable

There was no significant time*dose interaction ($F = 0.10$, $df = 3, 83$, $P = 0.9587$), and therefore, the main effects of time and dose were considered. The time effect was not significant ($F = 1.02$, $df = 1, 83$, $P = 0.3184$). The dose effect was not significant ($F = 0.13$, $df = 3, 83$, $P = 0.9443$). See Table 3.6 for the treatment estimates with the number of frames of adult bees in each hive as the response variable.

RCBD with the square inches of capped worker brood in each hive as the response variable

There was no significant time*dose interaction ($F = 0.42$, $df = 3, 83$, $P = 0.7390$), and therefore, the main effects of time and dose were considered. The time effect was not significant ($F = 1.37$, $df = 1, 83$, $P = 0.2444$). The dose effect was not significant ($F = 0.85$, $df = 3, 83$, $P = 0.4731$). See Table 3.7 for the treatment estimates with the square inches of capped worker brood in each hive as the response variable.

RCBD with the weight-per-hive (lbs) 28 days post-treatment as the response variable

The treatment effect was not significant ($F = 1.70$, $df = 3, 41$, $P = 0.1819$). See Table 3.8 for the treatment estimates with the weight-per-hive 28 days post-treatment as the response variable.

Discussion

I reject my hypothesis that repeated applications of OA would significantly reduce *Varroa* mite populations in colonies containing brood. There was an overall percentage reduction in the *Varroa* mite populations on adult bees regardless of treatment (Table 3.3). The mites-per-100 adult bee data coincide with the percentage reduction in *Varroa* infestation data as the hives had 2.5 ± 0.5 more mites-per-100 adult bees prior to OA treatment than after OA treatment. Again, treatment with OA did not significantly reduce the number of mites on adult bees regardless of the concentration applied (Table 3.2).

The 6.0% OA treatment significantly reduced *Varroa* infestation by $35.4 \pm 18.6\%$ when compared to untreated colonies ($\alpha = 0.1$ level of significance). Similarly, the 6.0% OA treatment significantly reduced *Varroa* infestation by $29.0 \pm 16.1\%$ when compared to the 2.0% OA treatment ($\alpha = 0.1$ level of significance). I do not consider the above treatment differences important because both the probability of Type I error and the variation in percentage reduction are large. Furthermore, the percentage reduction achieved with the 6.0% OA treatment is significantly less than other *Varroa* control products on the market.

I am puzzled by the result that the untreated colonies had a 37.4 ± 17.2% decrease in *Varroa* infestation. One possible reason that the *Varroa* population decreased on adult bees in control colonies is that one of the experimental apiaries was exposed to full sunlight for the majority of the day (n = 32). The average maximum daily temperature during the experiment was 35.1 ± 2.6°C (95.1 ± 4.6°F). Aliano and Ellis (2005a) report that heat significantly increases the number of mites that fall from adult bees at temperatures ≥ 35°C. I noticed large clusters of adult bees hanging on the outside of the colonies above the entrances that were fanning air currents to cool the colonies during hot periods of the day. Perhaps mites fell from the adult bee clusters outside of the hive and were not able to reenter the colony. Another possibility is that the internal colony temperature was not being adequately controlled by the bee population and mites fell or were injured due to extreme temperatures. One problem with the above explanations is that there was a significant decrease in *Varroa* infestation in the control colonies when only considering the apiary that had moderate shade (n = 14).

A final explanation for why the *Varroa* population decreased on adult bees in the control colonies is that mites may have migrated to drone brood as it became available during the study. Approximately half of the colonies did not have appropriately aged drone brood to sample for mites at the end of the study (n = 21) (pupae with purple eyes) but the majority of the colonies had recently capped drone brood present.

I reject my hypothesis that the efficacy of OA would increase as the concentration of the OA solution increased in colonies containing brood. As Table 3.3 illustrates, the efficacy of OA numerically increased as the OA concentration increased, but the differences were not significant. It is interesting that the treatment standard errors

decreased as the OA concentration increased. The fact that the variance in *Varroa* infestation went down as OA concentration increased makes biological sense because more of the mites theoretically died as more OA was applied.

The analysis of the mites-per-100 excised worker pupae data is difficult to interpret because there were significantly more mites in the hives slated to receive the 2.0% and 3.5% OA treatment than the hives slated to receive the 6.0% OA treatment. Although the untreated hives had 0.7 more mites-per-100 excised worker pupae than the hives that received the 3.5% OA treatment or the 6.0% OA treatment, the differences may be fictitious because the pre-treatment *Varroa* infestation in worker brood was not constant among the treatments (Table 3.4). In contrast, the mites-per-100 excised drone pupae data indicates that the *Varroa* infestation in drone brood was not significantly different between the treatment groups before or after OA application (Table 3.5). I place more importance on the mites-per-100 excised drone pupae data because *Varroa* mites preferentially parasitize drone brood over worker brood, and drone brood is often 8-10 times more infested than worker brood (Fuchs 1990). Similar to the mites-per-100 adult bee data and the percentage reduction in *Varroa* infestation data, the mites-per-100 excised drone pupae data indicates that OA did not significantly reduce *Varroa* mite populations in colonies containing brood.

The final objective of this experiment was to quantify the impact of repeated applications of OA on overall colony health by measuring the square inches of capped worker brood, by counting the number of frames containing adult bees, and by weighing the colonies at the end of the study. As illustrated in Tables 3.6, 3.7, and 3.8, the number of frames of adult bees, the square inches of capped worker brood, and the average

weight-per-hive were not significantly influenced by repeated applications of OA, regardless of the OA concentration. My results indicate that summer bees can tolerate larger amounts of OA than reported in the literature for autumn or winter bees. Liebig (1998) and Charrière (2001) quantified the impact on autumn or winter colonies when applying OA solutions with concentrations greater than 5% and described significant adult bee mortality, poor over-wintering ability, and impaired spring development. My data suggests that 3 summer trickle treatments with 6.0% OA at one week intervals did not have a negative impact on colony health.

I conclude that repeated applications of OA did not significantly reduce *Varroa* mite populations in honey bee colonies when brood was present. My results confirm the observations that OA is less effective when significant brood is present (Fuchs 1990, Gregorc 2001). One possible reason for the poor efficacy of OA in the presence of brood may be that brood provides a robust buffer that counteracts phoretic mite mortality. Another reason for the poor efficacy of mid-summer use of OA may be inadequate distribution of OA among the adult bee population. Unlike OA treatments in the fall or early winter, mid-summer bees do not cluster, which may result in OA missing the majority of adult bees. Furthermore, mid-summer use of OA may result in rapid evaporation of the OA crystals due to heat and excessive fanning of the hive by adult bees for cooling purposes. My results do not support the mid-summer use of OA to reduce *Varroa* populations which agrees with observation in Europe (Rademacher and Harz 2006).

Table 3.1 – Average number of brood cells excised for pre- and post-treatment sample. The estimates were calculated using both the pre- and post-treatment brood samples for each hive.

Brood type	Estimate ± Standard error	n*
Worker	304.7 ± 75.6	86
Drone	88.9 ± 38.1	60

* 43 of the 46 experimental hives had available worker brood to sample
* 30 of the 46 experimental hives had available drone brood to sample

Table 3.2 – Treatment estimates with mites-per-100 adult bees as the response variable. The estimates represent the *Varroa* infestation of adult bees prior to and after treatment with OA. Estimates with different letters indicate significant differences (*t*-test, $\alpha = 0.05$).

Treatment	Estimate ± Standard error	n
*Pre-untreated	3.8 ± 1.4 a	12
Pre-OA 2.0%	4.3 ± 1.5 a	12
Pre-OA 3.5%	3.5 ± 1.5 a	11
Pre-OA 6.0%	3.2 ± 1.2 a	11
*Post-untreated	1.9 ± 1.4 b	12
Post-OA 2.0%	1.6 ± 1.5 b	12
Post-OA 3.5%	0.5 ± 1.5 b	11
Post-OA 6.0%	0.9 ± 1.2 b	11

* Average pre-treatment estimate summed over OA
  concentration = 3.7 ± 1.2 mites-per-100 adult bees
* Average post-treatment estimate summed over OA
  concentration = 1.2 ± 1.2 mites-per-100 adult bees

Table 3.3 – Treatment estimates with percentage reduction in *Varroa* infestation 28 days post-treatment as the response variable. Estimates with different letters indicate significant differences (*t*-test, $\alpha = 0.05$).

Treatment	Estimate ± Standard error	n
Untreated	37.4 ± 17.2 a	12
OA 2.0%	43.7 ± 14.5 a	12
OA 3.5%	55.7 ± 13.8 a	11
OA 6.0%	72.8 ± 8.0 a	11

Table 3.4 – Treatment estimates with mites-per-100 excised worker pupae as the response variable. The estimates represent the *Varroa* infestation of worker brood prior to and after treatment with OA. Estimates with different letters indicate significant differences (*t*-test, α = 0.05).

Treatment	Estimate ± Standard error	n[3]
[1]Pre-untreated	0.8 ± 0.3 ab[4]	10
Pre-OA 2.0%	1.3 ± 0.3 b	11
Pre-OA 3.5%	1.3 ± 0.3 b	5
Pre-OA 6.0%	0.6 ± 0.3 a	10
[2]Post-untreated	1.0 ± 0.3 a	10
Post-OA 2.0%	0.7 ± 0.3 ab	11
Post-OA 3.5%	0.3 ± 0.3 b	5
Post-OA 6.0%	0.4 ± 0.3 b	10

[1] Average pre-treatment estimate summed over OA concentration = 1.0 ± 0.2 mites-per-100 excised worker pupae

[2] Average post-treatment estimate summed over OA concentration = 0.6 ± 0.2 mites-per-100 excised worker pupae

[3] Hives with non-detectable mites pre- and post-treatment were eliminated from the analysis

[4] This table does not consider comparisons between treatments above and below the double line

Table 3.5 – Treatment estimates with mites-per-100 excised drone pupae as the response variable. The estimates represent the *Varroa* infestation of drone brood prior to and after treatment with OA. Estimates with different letters indicate significant differences (*t*-test, $\alpha = 0.05$).

Treatment	Estimate ± Standard error	n[3]
[1]Pre-untreated	10.6 ± 5.7 a	6
Pre-OA 2.0%	8.6 ± 6.0 a	8
Pre-OA 3.5%	9.6 ± 8.4 a	3
Pre-OA 6.0%	12.5 ± 6.6 a	8
[2]Post-untreated	10.6 ± 5.7 a	6
Post-OA 2.0%	7.3 ± 6.0 a	8
Post-OA 3.5%	2.0 ± 8.4 a	3
Post-OA 6.0%	6.2 ± 6.6 a	8

[1] Average pre-treatment estimate summed over OA concentration = 10.3 ± 5.3 excised drone pupae

[2] Average post-treatment estimate summed over OA concentration = 6.5 ± 5.3 excised drone pupae

[3] Hives with zero mites pre- and post-treatment were eliminated from the analysis

Table 3.6 – Average number of frames of adult bees in each hive. Estimates with different letters indicate significant differences (*t*-test, $\alpha = 0.05$).

Treatment	Estimate ± Standard error	n
*Pre-untreated	6.8 ± 0.8 a	12
Pre-OA 2.0%	7.2 ± 0.8 a	12
Pre-OA 3.5%	7.2 ± 0.8 a	11
Pre-OA 6.0%	7.6 ± 0.8 a	11
*Post-untreated	7.8 ± 0.8 a	12
Post-OA 2.0%	8.0 ± 0.8 a	12
Post-OA 3.5%	7.5 ± 0.8 a	11
Post-OA 6.0%	7.8 ± 0.8 a	11

* Average pre-treatment estimate summed over OA concentration = 7.2 ± 0.4 frames of adult bees

* Average post-treatment estimate summed over OA concentration = 7.8 ± 0.4 frames of adult bees

Table 3.7 – Average square inches of capped worker brood in each hive. Estimates with different letters indicate significant differences (*t*-test, α = 0.05).

Treatment	Estimate ± Standard error	n
*Pre-untreated	308.0 ± 39.7 a	12
Pre-OA 2.0%	294.4 ± 47.8 a	12
Pre-OA 3.5%	263.0 ± 50.6 a	11
Pre-OA 6.0%	322.5 ± 40.7 a	11
*Post-untreated	364.3 ± 39.7 a	12
Post-OA 2.0%	332.0 ± 47.8 a	12
Post-OA 3.5%	294.8 ± 50.6 a	11
Post-OA 6.0%	314.2 ± 40.7 a	11

* Average pre-treatment estimate summed over OA concentration = 297.0 ± 32.9 square inches of worker brood

* Average post-treatment estimate summed over OA concentration = 326.3 ± 32.9 square inches of worker brood

Table 3.8 – Average weight-per-hive 28 days post-treatment. Estimates with different letters indicate significant differences (*t*-test, $\alpha = 0.05$).

Treatment	Estimate (lbs) ± Standard error	n
Untreated	117.3 ± 23.5 a	12
OA 2.0%	111.9 ± 23.2 a	12
OA 3.5%	97.3 ± 22.1 a	11
OA 6.0%	112.7 ± 22.1 a	11

Chapter 4

Oxalic acid – a prospective tool for reducing *Varroa* mite populations in package bees: Part I – Laboratory evaluation

Abstract

Numerous studies have investigated using oxalic acid (OA) to control *Varroa* mites in honey bee colonies. In contrast, techniques for treating package bees with OA have not been investigated. The goal of this study was to develop a protocol for using OA to reduce mite infestation in package bees. I made 97 mini packages of *Varroa*-infested adult bees. Each package contained 1613 ± 18 bees, 92 ± 3 mites, and represented an experimental unit. I prepared a 2.8% solution of OA by mixing 35 g OA with 1 L of sugar water (sugar:water) (1:1) (w:w). Eight treatments were assigned to the packages based on previous laboratory bioassays which characterized the acute contact toxicity of OA to mites and bees. I administered the treatments by spraying the OA solution directly on the bees through the mesh screen cage using a pressurized air brush and quantified mite and bee mortality over a 10 day period. My results support applying an optimum volume of 3.0 mL of a 2.8% OA solution per 1000 bees to packages for effective mite control with minimal adult bee mortality. The outcome of my research provides beekeepers and package bee shippers guidance for using OA to reduce mite populations in package bees.

Introduction

Numerous studies have investigated using oxalic acid (OA) to control *Varroa* mites in honey bee colonies. In contrast, techniques for treating package bees with OA have not been investigated. A review of literature regarding the efficacy of OA (Rademacher and Harz 2006) indicates that broodless colonies are ideal for OA application. Oxalic acid does not kill mites in sealed brood cells (Schuster and Schürzinger 2003), and it is less effective when brood is present (Fuchs 1990, Gregorc 2001). Oxalic acid is most effective in broodless colonies because *Varroa* mites are phoretic on adult bees, and mites inevitably come in physical contact with OA when it is applied.

Treating package bees with OA is a logical extension of the usefulness of OA as a *Varroa* mite control agent because all mites present in packages are phoretic on adult bees. The goal of this study was to develop a protocol for using OA to reduce mite infestation in package bees. This study establishes an optimum volume of a 2.8% OA solution for spraying on package bees to provide effective mite control while minimizing adult bee mortality.

Materials and Methods

Stocking of packages

I shook 25 kg of *Varroa*-infested adult honey bees from 20 source colonies into a bulk bee box on the evening of May 25th, 2006. The source colonies were located at the University of Nebraska-Lincoln's Agricultural Research and Development Center (ARDC) near Mead, NE. I had previously caged the queens from the source colonies to

ensure that all mites were phoretic on adult bees (May 5). The source colonies were 2 or 3 stories high and were composed of a mixture of Italian and Carniolan bees. I added 1 frame of honey, 2 frames of pollen and nectar, 1 frame of unsealed brood, and 1 frame with 15 caged queens to stabilize the bulk bee box. In addition, I fit the bulk bee box with top feeders that contained 6 L of light syrup and 3 L of water. I maintained the bulk box overnight to allow the bees to cluster and the mite population to become equally distributed.

The next day, I made 97 packages of adult bees by subdividing the adult bee population in the bulk bee box (May 6). The dimensions of the packages were 10.2 cm long, 8.3 cm wide, and 17.8 cm high (Figure 4.1). I provided each package with a 0.5 L scoop of adult bees. Each package contained 1613 ± 18 bees and 92 ± 3 mites. I moved the packages to a dark, air-conditioned laboratory where I fit each package with a 0.5 L top feeder containing light syrup (sugar:water) (1:1) (w:w). I placed 3 large fans in the laboratory to remove CO_2 and to prevent the packages from overheating. The daily temperature and relative humidity inside the laboratory averaged $23 \pm 2°C$ (n = 6) and $63.6 \pm 9\%$ (n = 6), respectively.

Determination of the OA concentration and volumes to apply to the packages

The Canadian Honey Council has provided the American Beekeeping Federation their registration data packet to expedite the registration of OA in the United States. As a result, the recommended concentration of the OA solution that will appear on the United States label will most likely be identical to the Canadian label. Therefore, I chose to test one concentration of OA based on the Canadian treatment recommendations for honey

bee colonies. The Canadian label recommends dissolving 35 g of OA dihydrate in 1 L of warm syrup made from a mixture of sugar and water (sugar:water) (1:1) (weight:volume) (Special Supplement 2005). This mixture results in a 2.8% OA sugar water solution by weight (sugar:water) (1:1) (w:w).

I used the bioassay data from chapter 1 to determine the maximum volume of OA to apply to the packages. The bioassay data estimated the 48 hour LD_{50} for adult honey bees to be 372 μg per bee (95% CL = 307 to 440 μg per bee) (Table 1.1). I picked a high volume of 15 mL of a 2.8% OA solution per package to approximate a dose that was slightly less than the 48 hour LD_{50} for adult honey bees (equivalent to 9.3 mL per 1000 bees). A volume greater than 15 mL may have resulted in the premature death of packages so I decided to err on the side of caution. My calculations were as follows: 35 g of OA in 1 L of sugar water results in approximately 0.035 g OA/mL solution. 15 mL of OA solution X 0.035 g OA/mL solution = 0.525 g OA per package (525,000 μg per package). 525,000 μg per package / 1618 bees per package = 325 μg per bee.

I chose 8 volumes of OA to apply to the packages: 0, 1, 3, 5, 7, 9, 11, and 15 mL of a 2.8% OA solution per package. The above volumes are equivalent to 0.0, 0.6, 1.8, 3.1, 4.6, 5.4, 6.7, and 9.3 mL of a 2.8% OA solution per 1000 bees, respectively.

Treatment and data collection

I randomly assigned the 97 packages to the 8 treatment groups defined above. I prepared a 2.8% solution of OA by mixing 35 g OA with 1 L of sugar water (sugar:water) (1:1) (w:w). I administered the treatments by spraying the OA solution directly on the bees through the mesh screen cage using a pressurized air brush (King Spark Hardware &

Tool Corporation, model AB-105, Taichung County, Taiwan) (Figure 4.2). I pipetted the appropriate volume of OA solution into the air brush gravity cup (paint cup) for each treatment and sprayed the adult bee cluster until the solution was gone. I made an effort to maximize OA contact with the adult bees by spraying both sides of the screen cages.

I placed sticky boards in the packages prior to OA application to monitor mite fall. I replaced the sticky boards 3, 6, and 10 days post-treatment to estimate 3, 6, and 10 day *Varroa* mite mortality. In addition, I recovered dead bees 3, 6, and 10 days post-treatment by removing them from the packages (Figure 4.3). I chilled the packages at 2°C for 20 minutes to force the bees to cluster and facilitate dead bee recovery (3, 6, and 10 days post-treatment). The packages had a sliding screened side that allowed me to remove dead bees from the bottom of the packages while leaving the cluster of live bees undisturbed. After counting the number of dead bees at each time interval, I washed the bees in alcohol to recover the mites that had fallen into the pile of dead bees instead of landing on the sticky boards below. I added the number of mites collected on the sticky boards to the number of mites collected from the dead bees to estimate the *Varroa* mite mortality for each time interval.

At the end of the experiment, I killed and counted the remaining bees in the packages by spraying them with 70% alcohol. In addition, I recovered the remaining mites using the alcohol wash method (Shimanuki and Knox 2000). I was able to calculate the total number of bees and mites present in each package prior to OA application by destructively sampling the packages at the end of the experiment. Specifically, I added the number of mites collected from sticky boards and dead bees 3, 6, and 10 days post-treatment to the number of mites collected from live bees at the end of

the experiment to calculate the total number of mites present in each package prior to experimentation. Similarly, I added the number of dead bees collected 3, 6, and 10 days post-treatment to the number of live bees remaining at the end of the experiment to calculate the total number of bees present in each package prior to experimentation. This enabled me to calculate the proportion of mites and bees killed in each package 3, 6, and 10 days post-treatment. The 97 packages contained a total of 9,309 mites and 162,934 bees.

Experimental design and statistical analysis

I designed my experiment as a completely randomized design (CRD). I used the proportion of mites and bees killed 3, 6, and 10 days post-treatment as response variables. I used PROC GLIMMIX to analyze both the mite and bee proportion data and used a binomial response distribution (SAS Institute 2006). I fit a generalized linear model (GLM) with a binomial response distribution implemented in PROC GLIMMIX. I separated means using a *t*-test ($\alpha = 0.05$). In addition to the PROC GLIMMIX analysis, I fit the same model using the NLMIXED procedure (SAS Institute 2006) to calculate the lethal doses (LD's) for both mites and bees 3, 6, and 10 days post-treatment.

Results

Lethal doses of OA for mites and bees

Tables 4.1 and 4.2 lists honey bee and *Varroa* mite mortality responses to a 2.8% OA solution when sprayed on package bees, respectively. Note that Tables 4.1 and 4.2 list the LD_{50} 3, 6, and 10 days post-treatment. In addition, Table 4.2 lists the LD_{90} and

LD_{95} for mites while Table 4.1 lists the LD_{10} and LD_{15} for honey bees because my

objective was to choose a dose that provided effective mite control while minimizing

adult bee mortality.

CRD with the proportion of mites killed 3, 6, and 10 days post-treatment as the response variable

I modeled the factors time, volume, and the time*volume interaction. The

time*volume interaction was not significant ($F = 0.06$, $df = 14, 267$, $P = 1.0000$). The

time effect was also not significant ($F = 0.27$, $df = 2, 267$, $P = 0.7655$), indicating that

mite mortality 3 days post-treatment was not significantly different than mite mortality 6

or 10 days post-treatment. The volume effect was significant ($F = 10.09$, $df = 7, 267$, $P =$

0.0001). Table 4.3 lists the main effect estimates of the proportion of mites killed for

each of the 8 volumes. Volumes ≥ 3.1 mL of a 2.8% OA solution per 1000 bees resulted

in $\geq 94\%$ mite mortality.   In addition, increasing the volume above 3.1 mL per 1000 bees

did not significantly increase mite mortality. Figure 4.4 illustrates the *Varroa* mite

mortality response to a 2.8% OA solution when sprayed on package bees. The standard

error bars were intentionally not included in Figure 4.4 because they overlap for each

time interval and clutter the graph. The standard error for each volume is listed in Table

4.3.

CRD with the proportion of bees killed 3, 6, and 10 days post-treatment as the response variable

I modeled the factors time, volume, and the time*volume interaction. The time*volume interaction was not significant ($F = 0.05$, $df = 14, 267$, $P = 1.0000$). The time effect was not significant ($F = 2.61$, $df = 2, 267$, $P = 0.0756$), indicating that bee mortality 3 days post-treatment was not significantly different than bee mortality 6 or 10 days post-treatment. The volume effect was significant ($F = 7.67$, $df = 7, 267$, $P = 0.0001$). Table 4.4 lists the main effect estimates of the proportion of bees killed for each of the 8 volumes. In general, volumes ≥ 4.6 mL of a 2.8% OA solution per 1000 bees caused significant adult bee mortality when compared to untreated packages. Figure 4.5 illustrates the adult bee mortality response to a 2.8% OA solution when sprayed on package bees. The standard error bars were intentionally not included in Figure 4.5 because they overlap for each time interval and clutter the graph. The standard error for each volume is listed in Table 4.4.

Discussion

The data collected from this experiment indicate an optimum volume of a 2.8% OA solution that provides effective mite control while minimizing adult bee mortality when sprayed on package bees. I recommend applying 3.0 mL of a 2.8% OA sugar water solution (sugar:water) (1:1) (w:w) per 1000 bees to packages for > 90% mite control and no significant bee mortality. The OA solution should be applied to packages once the adult bees have clustered to maximize contact.

I chose to make my recommendation for treating package bees with OA based on the 6 day *Varroa* mite LD_{90} because the 3 day and 10 day time intervals are not realistic.

For example, bees must remain in their package for at least 3 days before they

biologically become a swarm (personal observation). In general, beekeepers do not

install packages 3 days after they were created because of additional shipping transit

time. The 10 day time interval is also unrealistic because significant bee mortality occurs

if packages are left caged for 10 days. The 6 day time interval is the most realistic

because it factors in the 3 days that are necessary for a package to become a swarm and it

accounts for shipping transit time.

My recommendation for treating packages with OA is based on achieving > 90%

mite mortality and < 10% adult bee mortality as described below. Table 4.2 indicates

that the 6 day *Varroa* mite LD_{90} was 2.98 mL of a 2.8% OA solution per 1000 bees. In

addition, Table 4.3 indicates that there was no significant difference between the

proportions of mites killed on package bees when volumes ≥ 3.1 mL per 1000 bees were

applied (0.94 to 0.99). From the mite mortality perspective, 3.0 mL per 1000 bees is the

ideal volume to apply to packages.

Table 4.4 indicates that volumes ≥ 4.6 mL per 1000 bees caused significant adult

bee mortality when compared to untreated packages. From the bee data perspective, 3

mL per 1000 bees is the ideal volume because it does not cause significant bee mortality.

The 3.1 mL per 1000 bee volume resulted in 10% greater bee mortality than untreated

packages, however, the mortality was not significantly different from untreated packages

(Table 4.4).

The easiest method for preparing a 2.8% solution of OA is to mix 35 g OA

dihydrate with 1 L of warm sugar water (Special Supplement 2005). Although a

pressurized air brush was used to apply small volumes of the OA solution in this study,

beekeepers may use a non-pressurized spray bottle to apply the OA. Simply add the appropriate volume to the bottle and spray the adult bee cluster within the package until the solution is gone. Another option for OA application is a MeterJet™ Spray Gun Kit. The MeterJet™ Spray Gun can be attached to a Solo®, D.B. Smith®, or SP Systems™ sprayer and delivers a precise metered volume of solution with each pull of the trigger. The MeterJet™ Spray Gun Kit can be purchased from various forestry supply companies such as Forestry Suppliers Inc. (http://www.forestry-suppliers.com/product_pages/View_Catalog_Page.asp?mi=1853) (accessed January 1, 2008).

My recommendation for treating package bees is useful because it provides beekeepers and package bee shippers guidance for using OA to reduce mite populations from package bees or bulk bee boxes. My data indicate that small deviations in the amount of OA applied to packages result in drastically different outcomes. For example, although the 3.1 mL per 1000 bees volume did not cause significant bee mortality, the 4.6 mL per 1000 bees volume caused significant bee mortality. Therefore, the applicator should emphasize accurate measurements when mixing and applying OA to package bees.

Table 4.1 – Honey bee mortality responses to a 2.8% OA solution when sprayed on package bees.

Honey bee mortality	n	LD_{50} (95% CL) (mL per 1000 bees)	LD_{15} (95% CL) (mL per 1000 bees)	LD_{10} (95% CL) (mL per 1000 bees)
3 d	162,934	7.97 (7.93 to 8.01)	3.80 (3.77 to 3.84)	2.60 (2.56 to 2.64)
6 d	162,934	7.45 (7.41 to 7.48)	3.28 (3.24 to 3.31)	2.08 (2.04 to 2.12)
10 d	162,934	6.16 (6.13 to 6.19)	2.00 (1.96 to 2.03)	0.79 (0.75 to 0.84)

Table 4.2 – *Varroa* mite mortality responses to a 2.8% OA solution when sprayed on package bees.

Mite mortality	n	LD_{95} (95% CL) (mL per 1000 bees)	LD_{90} (95% CL) (mL per 1000 bees)	LD_{50} (95% CL) (mL per 1000 bees)
3 d	9,309	3.83 (3.74 to 3.91)	3.25 (3.18 to 3.33)	1.57 (1.51 to 1.62)
6 d	9,309	3.55 (3.47 to 3.64)	2.98 (2.91 to 3.05)	1.29 (1.23 to 1.35)
10 d	9,309	3.41 (3.32 to 3.49)	2.83 (2.76 to 2.90)	1.14 (1.08 to 1.20)

Table 4.3 – Estimates of the proportion of *Varroa* mites killed
when package bees were sprayed with a 2.8% OA solution.
Estimates with different letters indicate significant differences (*t*-
test, $\alpha = 0.05$).

Volume (mL per 1000 bees)	Estimate ± Standard error	n
0.0	0.07 ± 0.04 a	36
0.6	0.22 ± 0.07 b	36
1.8	0.76 ± 0.07 c	36
3.1	0.94 ± 0.04 d	42
4.6	0.97 ± 0.03 d	36
5.4	0.99 ± 0.02 d	36
6.7	0.98 ± 0.03 d	30
9.3	0.99 ± 0.02 d	39

Table 4.4 – Estimates of the proportion of honey bees killed when packages were sprayed with a 2.8% OA solution. Estimates with different letters indicate significant differences (*t*-test, $\alpha = 0.05$).

Volume (mL per 1000 bees)	Estimate ± Standard error	n
0.0	0.05 ± 0.04 a	36
0.6	0.07 ± 0.04 a	36
1.8	0.10 ± 0.05 ab	36
3.1	0.15 ± 0.05 ab	42
4.6	0.22 ± 0.07 bc	36
5.4	0.21 ± 0.07 bc	36
6.7	0.35 ± 0.09 c	30
9.3	0.79 ± 0.06 d	39

Figure 4.1 – Rear view of package highlighting the sticky board compartment used to quantify mite mortality over time.

Figure 4.2 – Application of the OA solution using a pressurized airbrush.

Figure 4.3 – Left: The packages were chilled at 2°C for 20 minutes to force the bees to cluster. Right: front view of the package highlighting the sliding screened side used to collect dead adult bees.

Figure 4.4 – *Varroa* mite mortality response to a 2.8% OA solution when sprayed on package bees.

Proportion of mites killed

Volume of 2.8% OA solution (in mL per 1000
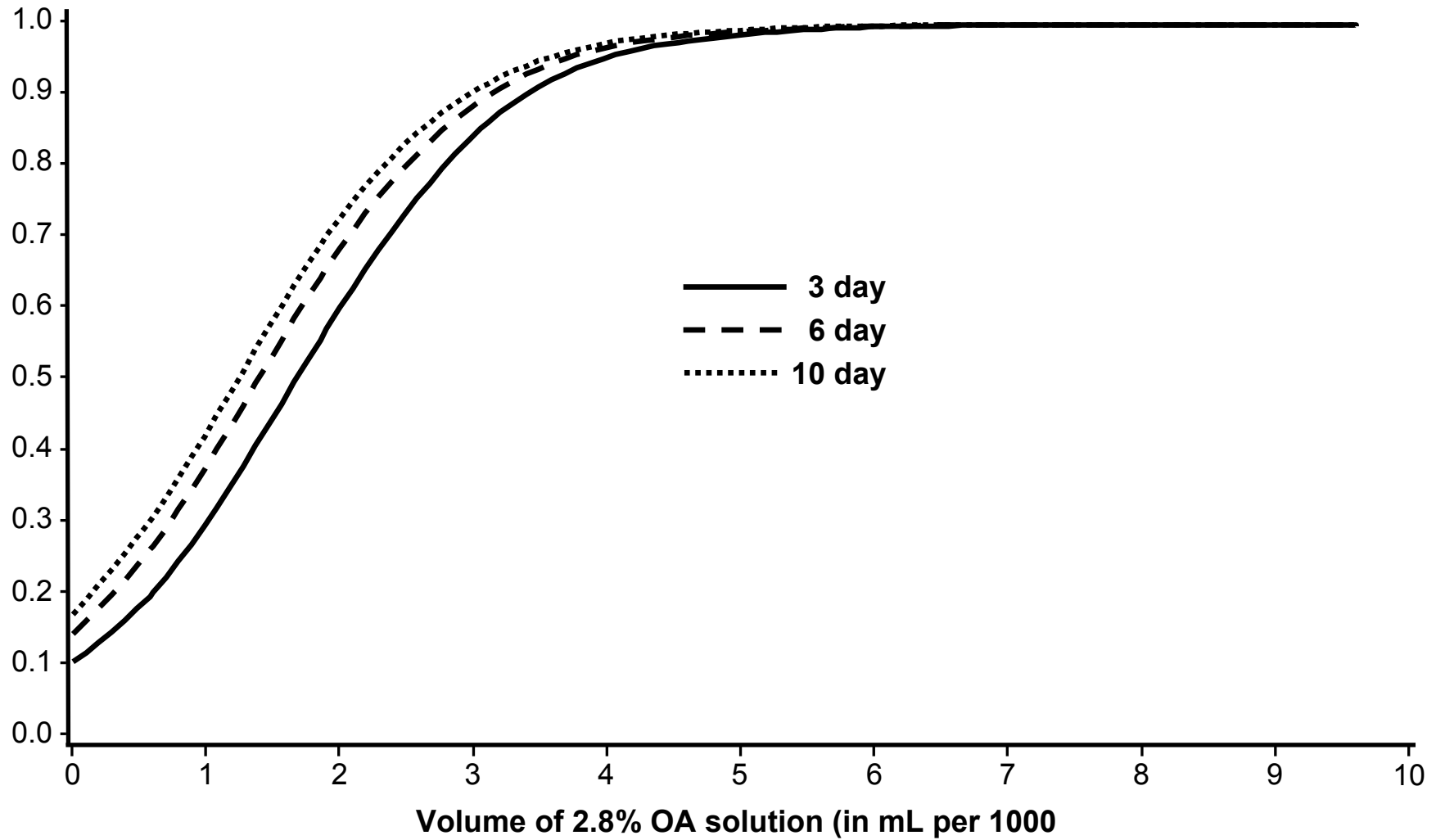
Figure 4.5 – Honey bee mortality response to a 2.8% OA solution when sprayed on package bees.

Proportion of bees killed

Legend:
— 3 day
– – 6 day
···· 10 day

Volume of 2.8% OA solution (in mL per 1000
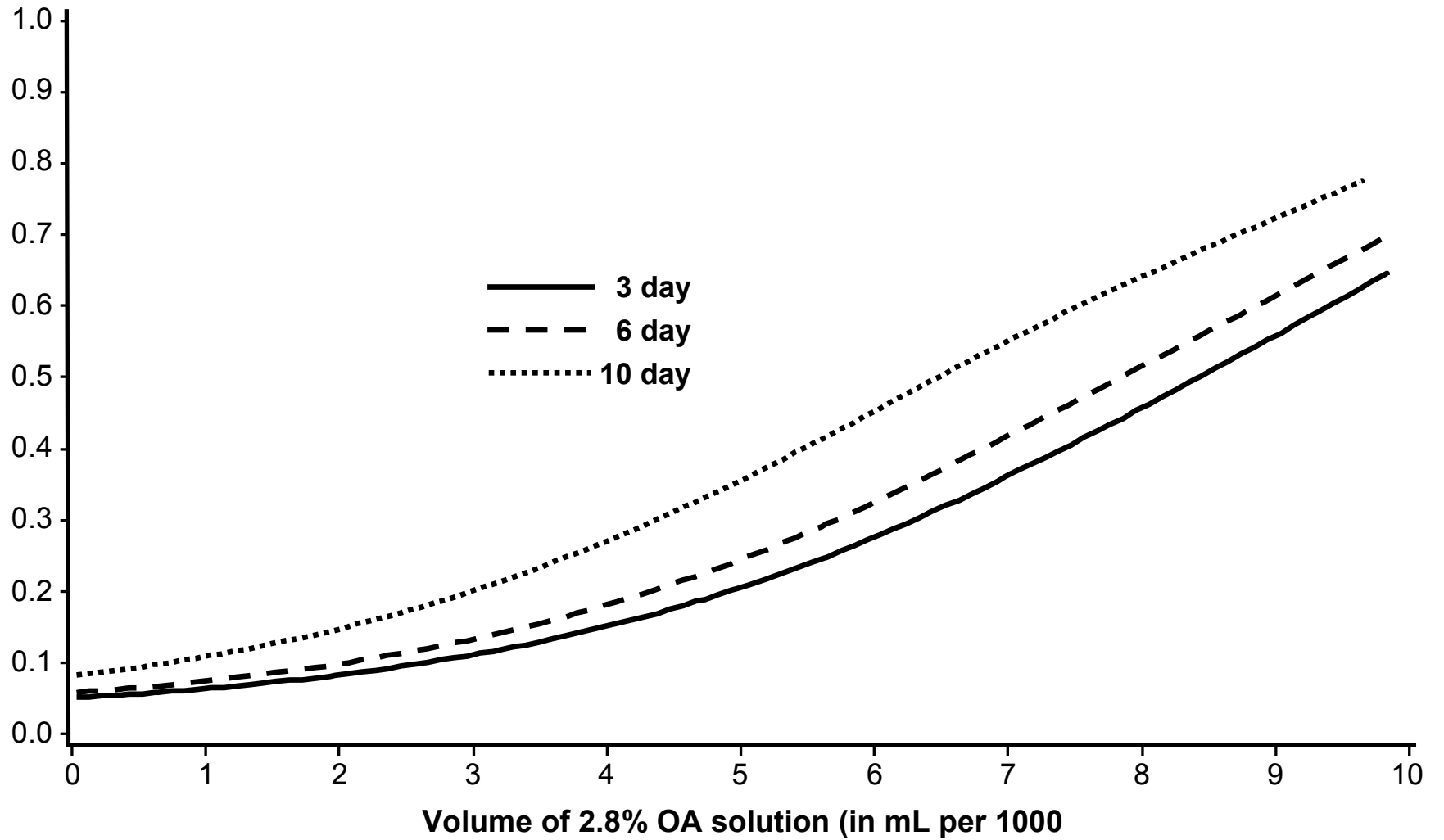
Chapter 5

Oxalic acid – a prospective tool for reducing *Varroa* mite populations in package bees: Part II – Field evaluation

Abstract

Results of research reported in chapter 4 were used to establish an optimum volume of a 2.8% oxalic acid (OA) solution to spray on package bees to reduce *Varroa* mite populations while minimizing adult bee mortality. The two studies described in this chapter are field trials of package bee treatment protocols. The first (preliminary study) was a feasibility study conducted prior to the laboratory studies reported in chapter 4. The second (secondary study) was conducted after the laboratory studies reported in chapter 4. In both studies I made 2 lb *Varroa*-infested packages and treated them with OA prior to installation. OA treatment significantly reduced *Varroa* infestation when compared to untreated packages, and OA's efficacy was dependant on the concentration and volume applied. The outcome of my research provides beekeepers and package bee shippers guidance for using OA to reduce mite populations in package bees.

Introduction

Numerous studies have investigated using oxalic acid (OA) to control *Varroa* mites in honey bee colonies. In contrast, techniques for treating package bees with OA have not been previously investigated. In chapter 4, I established an optimum volume of OA for spraying on package bees to provide effective mite control while minimizing

adult bee mortality (3.0 mL of a 2.8% OA sugar water solution (sugar:water) (1:1) (w:w) per 1000 bees). I derived the optimum volume recommendation from a laboratory experiment where 97 cages containing approximately 1600 adult bees were treated with OA. The two studies reported in this chapter (chapter 5) comprise the field component of my research on using OA to reduce *Varroa* mite populations in package bees. My laboratory and field studies combine to provide package bee treatment recommendations that beekeepers or package bee shippers can safely use to reduce *Varroa* mites in package bees.

The two experiments reported in this chapter are subsequently referred to as the *preliminary study* and the *secondary study*. The preliminary study was conducted before the laboratory evaluation described in chapter 4. At the time I conducted the preliminary study, European and Canadian recommendations for dissolving OA in sugar water to treat colonies were not established. Most research reviewed by Nanetti *et al.* (2003) and Rademacher and Harz (2006) found that a single autumn trickle treatment with 50 mL of a 3.0% OA sugar water solution (1:1) (w:w) provided an efficacy of greater than 90% in Central Europe. For my preliminary study, I chose a conservative concentration and volume of OA for package bee treatment to minimize the risk of adult bee mortality. I chose to apply 25 mL of a 2.0% OA sugar water solution (sugar:water) (1:1) (w:w) per 2 lb package to approximate a dose that was less than the 48 hour LD_{10} for adult honey bees (equivalent to 3.9 mL per 1000 bees) reported in chapter 1. The results of the preliminary study, $62.5 \pm 15.8\%$ *Varroa* infestation reduction, led me to conduct the laboratory studies (chapter 4) and subsequent secondary study to find the optimum volume and concentration of OA to apply to package bees.

At the time the secondary study was conducted, the Canadian Honey Council had provided the American Beekeeping Federation their OA registration data packet to expedite its registration in the United States. As a result, the recommended concentration of the OA solution that will appear on the United States label will most likely be identical to the Canadian label. Therefore, I used a concentration of OA for the secondary study that was based on the Canadian treatment recommendations for honey bee colonies. The Canadian label recommends dissolving 35 g of OA dihydrate in 1 L of warm syrup made from a mixture of sugar and water (sugar:water) (1:1) (weight:volume) (Special Supplement 2005). This mixture results in a 2.8% OA sugar water solution by weight (sugar:water) (1:1) (w:w). I used my results from chapter 4 regarding the volume of a 2.8% OA solution to apply to packages for > 90% mite control and no significant bee mortality. The goal of the secondary study was to verify the laboratory-based protocol for treating package bees with OA to reduce *Varroa* populations. The end product would be a field-tested recommendation for treating package bees or bulk bee boxes that would be useful to beekeepers and package bee shippers.

Materials and Methods

Preliminary study

Stocking of packages

I shook 18 kg of *Varroa*-infested adult honey bees from 12 source colonies into a bulk bee box on the evening of May 15th, 2005. The source colonies were located at the University of Nebraska-Lincoln's Agricultural Research and Development Center (ARDC) near Mead, NE. I had previously caged the queens from the source colonies to

ensure that all mites were phoretic on adult bees (April 25). The source colonies were 2

stories high and were composed of a mixture of Italian and Carniolan bees. I added 1

frame of honey, 2 frames of pollen and nectar, 1 frame of unsealed brood, and 1 frame

with 20 caged queens to stabilize the bulk bee box. In addition, I fit the bulk bee box

with top feeders that contained 6 L of light syrup (sugar:water) (1:1) (w:w) and 3 L of

water. I maintained the bees in the bulk box overnight to allow the bees to cluster and the

mite population to become equally distributed.

The next day, I made 19 packages of adult bees by subdividing the adult bee

population in the bulk bee box (May 16). The package cages were standard, 2 lb cages

made of pine wood and 8-mesh hardware cloth. They contained a caged queen and a

feeder can containing light syrup (sugar:water) (1:1) (w:w). The average weight of the

adult bees in 8 randomly chosen packages was 1.9 ± 0.2 lbs (0.8 ± 0.1 kg). After

transferring bees from the bulk cage to individual packages, I held them in a dark, cool

warehouse for 72 hours.

Establishing the volume of OA to apply to the packages

I used the bioassay data from chapter 1 to determine a conservative dose of OA to

apply to the packages without injuring adult bees. The bioassay data estimated the 48

hour LD_{10} for adult honey bees to be 177 µg per bee (95% CL = 120 to 226 µg per bee)

(Table 1.1). I picked a volume of 25 mL of a 2.0% OA sugar water solution

(sugar:water) (1:1) (w:w) per package to approximate a dose that was less than the 48

hour LD_{10} for adult honey bees (equivalent to 3.9 mL per 1000 bees). A volume greater

than 25 mL may have resulted in the premature death of packages. My calculations were

as follows: 25 g of OA in 1 L of sugar water results in approximately 0.025 g OA/mL

solution (2.0% OA sugar water solution). 25 mL of OA solution X 0.025 g OA/mL

solution = 0.625 g OA per package (625,000 µg per package). 625,000 µg per package /

6500 bees per package = 96 µg per bee.

Treatment and data collection

I randomly assigned the 19 packages to 2 treatment groups: 1.) untreated and 2.)

OA treatment. I prepared a 2.0% solution of OA by mixing 25 g OA with 1 L of sugar

water (sugar:water) (1:1) (w:w). I administered the treatments by spraying 25 mL of the

OA solution directly on the bees through the mesh screen cage using a non-pressurized

sprayer (spray bottle) 72 hours after the packages were made. I measured 25 mL of the

OA solution in a graduated cylinder, poured the liquid into the spray bottle, and sprayed

the adult bee cluster until the solution was gone. I made an effort to maximize OA

contact with the adult bees by spraying both sides of the screen cages. I sprayed the

untreated group with 25 mL of sugar water (sugar:water) (1:1) (w:w).

I installed the packages in single-story Langstroth beehives (24.4 cm deep hive

body) approximately 8 hours after treatment in an apiary near Lincoln, NE. I furnished

each hive with 8 empty frames with foundation and a division board feeder containing 1

gallon of syrup (sugar:water) (2:1) (w:w). The entrances to the hives were reduced to

approximately 2.5 cm to reduce drifting and discourage robbing behavior. As the

experiment progressed over a 7 week period, the hives were provided with a queen

excluder and 1 or 2 supers (16.8 cm deep) as needed.

I collected 8 samples of approximately 300 adult bees in alcohol from the bulk

bee box while stocking the packages to estimate the pre-treatment *Varroa* infestation

level on adult bees. I also collected approximately 300 adult bees in alcohol 1 and 3

weeks after package installation to estimate the post-treatment *Varroa* infestation level on

adult bees. I estimated the pre- and post-treatment *Varroa* infestation levels using the

alcohol wash method (Shimanuki and Knox 2000). All mites were phoretic on adult bees

1 week after package installation because appropriately aged brood for mite invasion was

not yet present in the hives. In contrast, the hives had brood of all ages (eggs, larvae, and

pupae) 3 weeks after package installation. I measured the square inches of capped

worker brood and counted the number of frames that the adult bee cluster occupied 3 and

6 weeks after package installation. I weighed the hives at the end of the study to quantify

the impact of OA treatment on weight gain (7 weeks after package installation).

Experimental design and statistical analysis

I designed my experiment as a completely randomized design (CRD). I used

mites-per-100 adult bees 1 and 3 weeks after package installation, the percentage

reduction in *Varroa* infestation 1 and 3 weeks after package installation, the number of

frames of adult bees in each hive 3 and 6 weeks after package installation, the square

inches of capped worker brood 3 and 6 weeks after package installation, and the average

weight-per-hive 7 weeks after package installation as response variables.

I analyzed the mites-per-100 adult bees data, the percentage reduction in *Varroa*

infestation data, the number of frames of adult bees data, and the square inches of capped

worker brood data using PROC GLIMMIX (SAS Institute 2006) and separated means

using a *t*-test (α = 0.05). I fit a generalized linear model (GLM) for each of the

aforementioned response variables using a normal distribution except for the number of

frames of adult bees data. This data set differed from the other response variables

because the data were frame counts (1 to 9 frames). For this response variable, I fit a

GLM and implemented the Poisson distribution in PROC GLIMMIX.

I framed my data as a factorial with time and treatment as factors. Time had 2

levels: 1 and 3 weeks (or 3 and 6 weeks depending on the response variable). Treatment

had 2 levels: untreated and OA treatment. I applied the 'group=trt' option in PROC

GLIMMIX to fit a separate variance for the 2 treatment levels. I used PROC

UNIVARIATE (SAS Institute 2006) to verify my assumptions of normality and equal

variance (except for the number of frames data as this distribution was Poisson).

I also analyzed the average weight-per-hive data using PROC GLIMMIX (SAS

Institute 2006) and separated means using a *t*-test (α = 0.05). This data set differed from

the abovementioned because it was not a factorial. It was a CRD with two treatments:

untreated and OA treatment.

Secondary study

The materials and methods that I used for the secondary study were identical to

those described for the preliminary study except for the differences listed below:

1. *Stocking of packages*

    a. I shook 29 kg of *Varroa*-infested adult honey bees from 20 source colonies

        into a bulk bee box in September 2006.

b. I made 31 packages of adult bees by subdividing the adult bee population in the bulk bee box. Each package was placed on a scale and the scale was tared. I then stocked the package with adult bees until it weighed 2 lbs. The average weight of the adult bees present in the packages was 2.0 ± 0.1 lbs (0.9 ± 0.03 kg) (n = 31).

2. *Establishing the volume of OA to apply to the packages*

   a. I used my recommendation from chapter 4 regarding the optimum dosage of OA to apply to packages for > 90% mite control and no significant bee mortality.

      i. The recommended volume that I applied was 3.0 mL of a 2.8% OA sugar water solution (sugar:water) (1:1) (w:w) per 1000 bees.

      ii. I also included a slightly higher dose to monitor the effects on adult honey bees (4.5 mL of a 2.8% OA sugar water solution (sugar:water) (1:1) (w:w) per 1000 bees). I chose this dose to assess the risk associated with over-application.

3. *Treatment and data collection*

   a. I treated the packages the same day that they were stocked with bees.

   b. The experiment was terminated 8 days after package installation.

   c. I prepared a 2.8% solution of OA by mixing 35 g OA with 1 L of sugar water (sugar:water) (1:1) (w:w).

   d. I administered the treatments using a Solo® backpack sprayer with a 3 gallon chemical tank and piston pump system. I furnished the sprayer with a MeterJet™ spray gun. The spray gun was calibrated to deliver either 7.0 or

10.5 mL of OA solution with each pull of the trigger depending on the treatment.

  i. Three pulls of the trigger with the MeterJet™ spray gun calibrated to deliver 7.0 mL per pull resulted in 21.0 mL per package (3.0 mL per 1000 bees treatment).

  ii. Three pulls of the trigger with the MeterJet™ spray gun calibrated to deliver 10.5 mL per pull resulted in 31.5 mL per package (4.5 mL per 1000 bees treatment).

  e. I collected 10 samples of approximately 300 adult bees in alcohol from the bulk bee box while stocking the packages to estimate the pre-treatment *Varroa* infestation level on adult bees.

4. *Experimental design and statistical analysis*

  a. I did not measure the square inches of capped worker brood or weigh the hives.

  b. I used mites-per-100 adult bees 8 days after package installation, the percentage reduction in *Varroa* infestation 8 days after package installation, and the number of frames of adult bees 8 days after package installation as response variables.

  c. I analyzed the mites-per-100 adult bees data, the percentage reduction in *Varroa* infestation data, and the number of frames of adult bees data using PROC GLIMMIX (SAS Institute 2006), and I separated means using a *t*-test ($\alpha = 0.05$). I fit a GLM for each of the aforementioned response variables using a normal distribution except for the number of frames of adult bees

data. This data set differed from the other response variables because the data were frame counts (1 to 9 frames). For this response variable, I fit a GLM and implemented the Poisson distribution in PROC GLIMMIX.

i. The mites-per-100 adult bees data were designed as a CRD with 4 treatments: 1.) bulk bee box pre-treatment, 2.) untreated, 3.) 3.0 mL per 1000 bees OA solution, and 4.) 4.5 mL per 1000 bees OA solution.

ii. The percentage reduction in *Varroa* infestation data and the number of frames of adult bees data were designed as CRD's with 3 treatments: 1.) untreated, 2.) 3.0 mL per 1000 bees OA solution, and 3.) 4.5 mL per 1000 bees OA solution.

Results

Preliminary study

My assumption of normality was met for all response variables for which I fit a GLM using a normal distribution. These data sets produced a symmetric box-plot and a straight-lined normal probability plot that confirmed normality. A plot of the residual versus the predicted values revealed a problem with non-constant variance. The 'group=trt' option that I employed in PROC GLIMMIX fixed the non-constant variance issue. After fitting a separate variance for each treatment, a plot of the residual versus the predicted values revealed no obvious patterns and was indicative of data that had constant variance.

Treatment*time factorial with mites-per-100 adult bees as the response variable

I modeled the factors treatment, time, and the treatment*time interaction. The treatment*time interaction was significant ($F = 7.65$, df $= 1, 34$, $P = 0.0091$), and therefore, the simple effects of treatment and time were considered.   See Table 5.1 for the treatment estimates of mites-per-100 adult bees 1 and 3 weeks after package installation. One week after package installation, hives established from the OA-treated packages had 5.1 ± 1.7 fewer mites-per-100 bees than hives established from the untreated packages ($t = 3.08$, df $= 34$, $P = 0.0041$). In contrast, there was no significant difference in *Varroa* infestation between hives established from OA-treated packages and hives established from untreated packages 3 weeks after package installation ($t = 0.84$, df $= 34$, $P = 0.4059$).   When only considering the untreated packages, there were 6.9 ± 1.4 more mites-per-100 bees 1 week after package installation than 3 weeks after package installation ($t = 5.58$, df $= 34$, $P = 0.0001$). Similarly, when only considering the OA-treated packages, there were 2.2 ± 1.2 more mites-per-100 bees 1 week after package installation than 3 weeks after package installation ($t = 1.87$, df $= 34$, $P = 0.0501$).

The *Varroa* infestation in the bulk bee box was not significantly different than the *Varroa* infestation in the hives established from untreated packages 1 week post-treatment ($t = 0.30$, df $= 15$, $P = 0.7693$).

Treatment*time factorial with percentage reduction in Varroa infestation as the response variable

I modeled the factors treatment, time, and the treatment*time interaction. The treatment*time interaction was significant ($F = 7.59$, df $= 1, 34$, $P = 0.0094$), and therefore, the simple effects of treatment and time were considered.   See Table 5.2 for

the treatment estimates of the percentage reduction in *Varroa* infestation 1 and 3 weeks after package installation. One week after package installation, OA treatment significantly reduced *Varroa* infestation by $70.6 \pm 23.0\%$ when compared to hives established from the untreated packages ($t = 3.07$, df $= 34$, $P = 0.0042$). In contrast, there was no significant difference in *Varroa* infestation between hives established from OA-treated packages and hives established from untreated packages 3 weeks after package installation ($t = 0.85$, df $= 34$, $P = 0.4008$). When only considering the untreated packages, the 3 week *Varroa* infestation diminished $96.1 \pm 17.2\%$ compared to the *Varroa* infestation 1 week after package installation ($t = 5.58$, df $= 34$, $P = 0.0001$). Similarly, when only considering the OA-treated packages, the 3 week *Varroa* infestation diminished $30.7 \pm 16.3\%$ compared to the *Varroa* infestation 1 week after package installation ($t = 1.88$, df $= 34$, $P = 0.0503$).

Treatment*time factorial with the number of frames of adult bees as the response variable

I modeled the factors treatment, time, and the treatment*time interaction. The treatment*time interaction was not significant ($F = 0.12$, df $= 1, 34$, $P = 0.7356$), and therefore, the main effects of treatment and time were considered. The treatment effect was not significant ($F = 0.06$, df $= 1, 34$, $P = 0.8096$). The time effect was significant ($F = 11.00$, df $= 1, 34$, $P = 0.0022$). See Table 5.3 for the treatment estimates of the number of frames of adult bees 3 and 6 weeks after package installation.

Treatment*time factorial with the square inches of capped worker brood as the response variable

I modeled the factors treatment, time, and the treatment*time interaction. The treatment*time interaction was not significant ($F = 0.01$, df = 1, 34, $P = 0.9071$), and therefore, the main effects of treatment and time were considered. The treatment effect was not significant ($F = 0.01$, df = 1, 34, $P = 0.9321$). The time effect was also not significant ($F = 1.26$, df = 1, 34, $P = 0.2702$). See Table 5.4 for the treatment estimates of the square inches of capped worker brood 3 and 6 weeks after package installation.

CRD with the weight-per-hive (lbs) 7 weeks after package installation as the response variable

The treatment effect was not significant ($F = 1.53$, df = 1, 17, $P = 0.2334$). See Table 5.5 for the treatment estimates of the weight-per-hive 7 weeks after package installation.

Secondary study

My assumption of normality was met for the 2 response variables for which I fit a GLM using a normal distribution. These data sets produced a symmetric box-plot and a straight-lined normal probability plot that confirmed normality. A plot of the residual versus the predicted values revealed a problem with non-constant variance. The 'group=trt' option that I employed in PROC GLIMMIX fixed the non-constant variance issue. After fitting a separate variance for each treatment, a plot of the residual versus the predicted values revealed no obvious patterns and was indicative of data that had constant variance.

CRD with mites-per-100 adult bees 8 days after package installation as the response variable

The treatment effect was significant (F = 121.86, df = 3, 37, P = 0.0001). See Table 5.6 for the treatment estimates of mites-per-100 adult bees 8 days after package installation. Colonies established from the untreated packages had 2.2 ± 0.9 more mites-per-100 bees than the bulk bee box (t = 2.36, df = 37, P = 0.0237). Colonies established from the packages treated with the low OA volume (3.0 mL per 1000 bees) had 7.7 ± 0.7 fewer mites-per-100 bees than the bulk bee box (t = 10.44, df = 37, P = 0.0001). Similarly, colonies established from the packages treated with the high OA volume (4.5 mL per 1000 bees) had 7.6 ± 0.8 fewer mites-per-100 bees than the bulk bee box (t = 10.06, df = 37, P = 0.0001). Colonies established from the packages treated with the low OA volume had 9.9 ± 0.6 fewer mites-per-100 bees than the untreated packages (t = 16.25, df = 37, P = 0.0001). Likewise, colonies established from the packages treated with the high OA volume had 9.7 ± 0.6 fewer mites-per-100 bees than the untreated packages (t = 15.61, df = 37, P = 0.0001). There was no significant difference in *Varroa* infestation between colonies established from the packages that were treated with the low OA volume and the packages that were treated with the high OA volume (t = 0.50, df = 37, P = 0.6178).

CRD with the percentage reduction in Varroa infestation 8 days after package installation as the response variable

The treatment effect was significant ($F = 136.95$, df = 2, 28, $P = 0.0001$). See Table 5.7 for the treatment estimates of the percentage reduction in *Varroa* infestation 8 days after package installation. Colonies established from the packages treated with the low OA volume (3.0 mL per 1000 bees) had $104.5 \pm 6.4\%$ fewer mites than the colonies established from the untreated packages ($t = 16.25$, df = 28, $P = 0.0001$). Colonies established from the packages treated with the high OA volume (4.5 mL per 1000 bees) had $102.8 \pm 6.6\%$ fewer mites than the colonies established from the untreated packages ($t = 15.59$, df = 28, $P = 0.0001$). There was no significant difference in *Varroa* infestation between colonies established from the packages that were treated with the low OA volume and the packages that were treated with the high OA volume ($t = 0.48$, df = 28, $P = 0.6349$).

CRD with the number of frames of adult bees 8 days after package installation as the response variable

The treatment effect was not significant ($F = 0.08$, df = 2, 28, $P = 0.9278$). See Table 5.8 for the treatment estimates of the number of frames of adult bees in hives 8 days after package installation.

Discussion

Preliminary study

Oxalic acid treatment significantly reduced *Varroa* infestation by 71% when compared to hives stocked with untreated packages one week after installation. Although

this statistic is encouraging, the standard error of the estimate was ± 23%. As a result, the efficacy of OA was highly variable and unreliable. An explanation for the high variance in efficacy is that the packages may have received different volumes of the OA solution. It was impossible to deliver exactly 25 mL of the OA solution per package with a non-pressurized spray bottle. Furthermore, I only weighed a subsample of the packages to estimate the number of adult bees present, resulting in variable dosages. I addressed both of these issues in the secondary study by using a MeterJet™ spray gun to accurately apply the OA solution and by weighing each package prior to treatment.

Another drawback of the preliminary study is that I treated the packages the same day that I installed them. This may have reduced the efficacy of OA against mites because the length of OA exposure before installation was brief. In contrast, I maximized exposure time by treating the packages the same day they were made for the secondary study. This ensured that mites would be exposed to OA for at least 72 h before installation in hives.

Despite OA's variable efficacy when applied to package bees in the preliminary study, treatment did not negatively affect the adult bee population, the amount of capped worker brood, or the hive weight at the end of the study. Furthermore, all hives remained queenright throughout experimentation.

I designed the preliminary experiment so that all mites would be phoretic on adult bees 1 week after package installation. This ensured that the 1 week post-treatment sample would provide an accurate comparison between the *Varroa* infestation in the bulk bee box and the *Varroa* population in each hive. Furthermore, the 1 week post-treatment sample verified that the *Varroa* infestation in the bulk bee box (7.3 ± 1.0 mites-per-100

bees) was not significantly different than the *Varroa* infestation in the hives established from untreated packages (7.8 ± 1.2 mites-per-100 bees). This confirmed that mites had not invaded brood and that my experimental protocol regarding the use of the bulk bee box as a pre-treatment estimate of *Varroa* infestation was justified.

I predicted that the hives would have brood of all ages (eggs, larvae, and pupae) 3 weeks after package installation because the packages were installed with healthy, mated queens. An interesting result from the preliminary study was that the *Varroa* infestation on adult bees significantly declined in all experimental hives 3 weeks after package installation, regardless of treatment (Tables 5.1 and 5.2). This statistic indicates that mites readily invade appropriately aged brood to begin reproduction in hives started from package bees, and highlights the importance of reducing *Varroa* populations in packages prior to installation.

Secondary study

An unexpected result from the secondary study was that although no *Varroa* reproduction could have occurred, the *Varroa* infestation in hives established from the untreated packages significantly increased 8 days after package installation (Table 5.6). This observation can be explained by the presence of robbing bees during the installation process. Upon reaching the apiary, the packages were immediately inundated with bees from an unknown source. The packages had a single layer of bees clinging to the outside of the cages. I quickly installed the packages because daylight was running out and the bees had consumed most of the syrup in their feed cans. I released the queens and sealed the hive entrances to ensure no additional bees could invade the hives. Despite my

efforts, some foreign bees entered each hive during installation. The next morning, robbing bees were no longer a problem and I opened each hive entrance approximately 1.3 cm to allow the bees to fly.

I terminated the secondary study after collecting the samples of adult bees 8 days after package installation. Although each hive had approximately 2 frames of adult bees, all hives were queenless. I found 3 dead queens inside of their hives that had battered wings and were stripped of body hair. The robbing bees must have balled the queens while the package bees were still wet from the installation process. In hindsight, I should have left the queens in their cages overnight instead of releasing them into the hives when foreign bees were present. Regardless, the secondary study provides evidence that invading bees that drift into or rob a hive have the potential to significantly increase the *Varroa* infestation.

It is interesting that the colonies established from the packages treated with the low or high volume of OA had approximately 100% fewer mites than the colonies established from the untreated packages (remember that percentage reduction in *Varroa* infestation was calculated using the bulk bee box as the pre-treatment sample). This makes sense considering that the untreated packages increased in *Varroa* infestation by about 20% and the treated packages had about an 80% reduction in *Varroa* infestation (80% reduction in treated hives compared to a 20% increase in untreated hives = 100% reduction in *Varroa* infestation). My results suggest that the treated packages were nearly devoid of *Varroa* mites prior to installation, and like the untreated packages, acquired mites due to the invasion of foreign, mite-infested bees.

The secondary study was conducted in the fall (September) when strong colonies are prone to attempt to rob small or weak colonies. I did not anticipate robber bees because there were no other apiaries in the vicinity, suggesting that the robbing bees originated from feral colonies or a distant apiary. In reality, beekeepers in temperate climates normally install package bees in the spring when robbing is less likely to occur.

The problem that occurred with robbing bees in the secondary study caused me to prematurely terminate the experiment. As a result, it was impossible to collect the same type of data reported in the preliminary study (3 week adult bee samples, square inches of capped worker brood, hive weight, and queen survival). Consequently, the data gathered from the secondary study will not be submitted for publication in a scientific journal, but was included in the dissertation for completeness.

Overall conclusions

The use of the MeterJet™ for OA application was quick, easy, and accurate. If beekeepers use my recommendation for treating package bees described in chapter 4, I strongly suggest the employment of a MeterJet™ spray gun used in conjunction with a backpack sprayer. Chapters 4 and 5 provide strong evidence that deviations in both the concentration and volume of the OA solution applied will significantly affect efficacy and adult bee tolerance. Also, it is important to weigh each package, cage, or bulk bee box before OA treatment to accurately calculate the volume of OA to apply. The applicator should emphasize accurate measurements when mixing and applying OA to package bees to maximize efficacy and minimize adult bee injury.

My chapter 4 results support applying an optimum volume of 3.0 mL of a 2.8% OA solution per 1000 bees for effective mite control with minimal adult bee mortality. The outcome of my field trials suggests that OA can be used safely to reduce mites in package bees, however, the study should be repeated due to the problem that occurred with robbing bees during installation.

Table 5.1 – *Preliminary study*. Treatment estimates with mites-per-100 adult bees 1 and 3 weeks after package installation as the response variable. Estimates with different letters indicate significant differences (*t*-test, α = 0.05).

Treatment*	Estimate ± Standard error	n
1 week–untreated	7.8 ± 1.2 a	9
1 week-OA 2.0%**	2.7 ± 1.1 b	10
3 week-untreated	0.9 ± 0.3 c	9
3 week-OA 2.0%**	0.5 ± 0.3 c	10

* The *Varroa* infestation in the bulk bee box was 7.3 ± 1.0 (n=8) mites-per-100 bees.

** The packages were sprayed with 3.9 mL per 1000 bees of a 2.0% OA sugar water solution by weight (sugar:water) (1:1) (w:w).

Table 5.2 – *Preliminary study*. Treatment estimates with percentage reduction in *Varroa* infestation 1 and 3 weeks after package installation as the response variable. Estimates with different letters indicate significant differences (*t*-test, $\alpha = 0.05$).

Treatment	Estimate ± Standard error*	n
1 week-untreated	-8.0 ± 16.7 a	9
1 week-OA 2.0%**	62.5 ± 15.8 b	10
3 week-untreated	88.1 ± 4.4 c	9
3 week-OA 2.0%**	93.2 ± 4.1 c	10

* Percentage reduction in *Varroa* infestation was calculated using the mite infestation in the bulk bee box as the pre-treatment baseline (7.3 ± 1.0 mites-per-100 bees).

** The packages were sprayed with 3.9 mL per 1000 bees of a 2.0% OA sugar water solution by weight (sugar:water) (1:1) (w:w).

Table 5.3 – *Preliminary study*. Average number of frames of adult
bees in hives 3 and 6 weeks after package installation. Estimates
with different letters indicate significant differences (*t*-test, α = 0.05).

Treatment	Estimate ± Standard error	n
3 week-untreated	3.3 ± 0.6 a	9
3 week-OA 2.0%*	3.7 ± 0.6 a	10
6 week-untreated	5.9 ± 0.8 b	9
6 week-OA 2.0%*	5.8 ± 0.8 b	10

* The packages were sprayed with 3.9 mL per 1000 bees of a 2.0%
  OA sugar water solution by weight (sugar:water) (1:1) (w:w).

Table 5.4 – *Preliminary study*. Average square inches of capped worker brood in hives 3 and 6 weeks after package installation. Estimates with different letters indicate significant differences (*t*-test, α = 0.05).

Treatment	Estimate ± Standard error	n
3 week-untreated	282.0 ± 48.8 a	9
3 week-OA 2.0%*	272.9 ± 40.8 a	10
6 week-untreated	226.4 ± 48.8 a	9
6 week-OA 2.0%*	227.8 ± 40.8 a	10

* The packages were sprayed with 3.9 mL per 1000 bees of a 2.0% OA sugar water solution by weight (sugar:water) (1:1) (w:w).

Table 5.5 – *Preliminary study*. Average weight-per-hive 7 weeks after package installation. Estimates with different letters indicate significant differences (*t*-test, α = 0.05).

Treatment	Estimate (lbs) ± Standard error	n
Untreated	91.3 ± 5.0 a	9
OA 2.0%*	82.9 ± 4.7 a	10

* The packages were sprayed with 3.9 mL per 1000 bees of a 2.0% OA sugar water solution by weight (sugar:water) (1:1) (w:w).

Table 5.6 – *Secondary study*. Treatment estimates with mites-per-100 adult bees 8 days after package installation as the response variable. Estimates with different letters indicate significant differences (*t*-test, α = 0.05).

Treatment	Estimate ± Standard error	n
Bulk bee box*	9.4 ± 0.7 a	10
Untreated	11.6 ± 0.6 b	10
3.0 mL per 1000 bees**	1.7 ± 0.2 c	11
4.5 mL per 1000 bees**	1.9 ± 0.2 c	10

* The bulk bee box estimate represents the pre-treatment baseline for *Varroa* infestation as the samples were obtained as the packages were made.

** The packages were sprayed with a 2.8% OA sugar water solution by weight (sugar:water) (1:1) (w:w).

Table 5.7 – *Secondary study*. Treatment estimates with percentage reduction in *Varroa* infestation 8 days after package installation as the response variable. Estimates with different letters indicate significant differences (*t*-test, α = 0.05).

Treatment	Estimate ± Standard error*	n
Untreated	-22.5 ± 6.0 a	10
3.0 mL per 1000 bees**	81.9 ± 2.2 b	11
4.5 mL per 1000 bees**	80.3 ± 2.6 b	10

* Percentage reduction in *Varroa* infestation was calculated using the mite infestation in the bulk bee box as the pre-treatment baseline (9.4 ± 0.7 mites-per-100 bees).

** The packages were sprayed with a 2.8% OA sugar water solution by weight (sugar:water) (1:1) (w:w).

Table 5.8 – *Secondary study*. Average number of frames of adult bees in hives 8 days after package installation. Estimates with different letters indicate significant differences (*t*-test, $\alpha = 0.05$).

Treatment	Estimate ± Standard error	n
Untreated	1.9 ± 0.2 a	10
3.0 mL per 1000 bees*	1.8 ± 0.3 a	11
4.5 mL per 1000 bees*	2.0 ± 0.2 a	10

* The packages were sprayed with a 2.8% OA sugar water solution
  by weight (sugar:water) (1:1) (w:w).

Chapter 6

Bee-to-bee contact drives oxalic acid distribution in honey bee colonies

Abstract

I constructed 10 divided hives to study the distribution of oxalic acid (OA). I split experimental colonies into 2 equal sections with 1 of 3 divider types. The first divider allowed trophallaxis to occur between adult bees on each side, but did not allow physical contact. The second divider did not allow trophallaxis or physical contact. The third divider allowed both physical contact and trophallaxis between the 2 sides. All 3 dividers allowed gas exchange of volatile materials. The objective was to investigate factors that contribute to the distribution of OA in a hive by monitoring *Varroa* mortality. I trickled 40 mL of a 3.5% OA sugar water solution on one side of the divider. I used sticky boards to quantify mite fall before, during, and after OA treatment on both treated and untreated sides. Trophallactic interactions and fumigation did not significantly influence the distribution of OA. Bee-to-bee contact was the primary route for OA distribution.

Introduction

The bioassay data from chapter 1 indicate that OA has a high acute toxicity to *Varroa* mites. I hypothesize that OA may kill *Varroa* mites via contact. The 24 hour LC_{50} (95% CL) for phoretic *Varroa* mites was 5.12 (3.5 to 7.0) μg of OA per 20-mL vial. The toxicity of OA to *Varroa* mites collected from bee brood was quantified by Milani (2001) who reported that the 24 hour LD_{50} (95% CL) (median lethal density) for *Varroa*

mites collected from brood was 1.9 (1.49 to 2.36) $\mu g/cm^2$. My bioassay results from chapter 1 and results from Milani (2001) suggest that OA has a high acute toxicity to mites. The high acute toxicity of OA to *Varroa* mites in glass-vial residual bioassays suggests that OA readily kills mites that come in physical contact with the crystals although some mite mortality could have been caused by exposure to OA vapors within the scintillation vials.

The objective of the current study was to identify factors that contribute to the distribution of OA in a hive and to test my hypothesis that OA kills mites via contact. I evaluated the importance of fumigation, trophallaxis, and direct contact when trickling OA. The results will give beekeepers and researchers insight as to how OA is distributed in hives. My results will provide guidance for selecting application techniques that maximize the efficacy of OA.

Materials and Methods

Construction of divided (split-unit) hives

I designed and built 10 divided single-story Langstroth hives in June, 2005. My hives resembled standard, single story Langstroth beehives. I modified the boxes by splitting them into 2 equal sections that held 4 frames each (Figure 6.1). I separated the sections using 1 of 3 different dividers. All dividers had a 2 X 46.5 cm wooden frame that formed bee-tight seals between the sides of the hive body, the inner cover, and the bottom board. The first divider (single-screen divider) had a 585 cm^2 area in its center made from 8-mesh screen and it allowed trophallaxis and gas exchange between bees on the 2 sides. The second divider (double-screen divider) had a 585 cm^2 area in its center

made from 2 pieces of 8-mesh screen that were separated by a 2 cm gap. It allowed gas exchange, but did not allow trophallaxis between the 2 sides. The third divider (queen excluder divider) had a 585 cm^2 area in its center made from plastic queen excluder that allowed worker bees to move freely between the 2 sides. It allowed trophallaxis, gas exchange, and physical contact between the 2 sides. The 3 dividers described above are shown in Figure 6.2. The fourth divider was a solid-wood divider that did not allow adult bee interaction or gas exchange between the 2 sides.

All divided colonies had a separate entrance for each side.   The entrances faced opposing directions to minimize the drift of adult bees from side-to-side. In addition, the bottom board was fitted with 8-mesh screen that allowed mites to fall onto a sticky board placed below the screen. This allowed me to independently monitor mite fall on each side of the divider (Figure 6.1).

I designed the divided hives to allow me to examine the distribution of OA by treating one side and monitoring the resulting mite fall in both the treated and untreated sections. I expected similar mite fall on the sides that were treated with OA regardless of divider. My intention was to correlate mite fall on the untreated section with divider type. The design of my dividers allowed me to restrict the amount of adult bee interaction between each half-unit and ranged from complete isolation (solid divider) to minimal isolation (queen excluder divider) as described above.

Stocking of hives

I stocked the 10 divided hives by splitting *Varroa*-infested colonies from an apiary located at the University of Nebraska Agricultural Research and Development

Center on June 29, 2005. The apiary was composed of a mixture of Carniolan and Italian honey bees (*Apis mellifera*). At this time, all hives were given a solid-wood divider. I furnished each side of the divided hive with a frame of capped brood, a frame of honey, a frame of pollen, and an empty frame with foundation. This resulted in 4 frames for each side and 8 frames for the entire divided Langstroth hive. I transferred adult bees to the units directly on the combs from which the splits were made. I immediately sealed the hives and moved them approximately 35 miles to the University of Nebraska-Lincoln East Campus. I placed a 15 day old queen cell in each side of the divided colonies the following day (June 30). I left the hives untouched for approximately 2 weeks. This period allowed mites to emerge from brood cells and gave the virgin queens time to mate and begin laying eggs.

I randomly assigned 9 of the 10 hives to 3 treatment groups. I assigned 3 hives to each of 3 treatment groups (single-screen divider, double-screen divider, and queen excluder divider). I removed the solid-wood dividers that I used when the units were stocked and replaced them with the appropriate dividers listed above. I also verified that each side of the divided colony was queenright and that sealed brood was not present. I only included divided hives that had successfully reared a queen on each side in this experiment. I used queen cells to make each side queenright resulting in hives void of capped brood during treatment. This ensured that all mites present in the hives were phoretic on adult bees and vulnerable to OA treatment.

I left the remaining 10th hive untouched with its solid-wood divider in place. I did not include the solid-wood divider hive in the statistical analysis of the experiment. I

used this hive as a control colony to monitor natural mite fall. Prior to experimentation, I placed sticky boards in all hives for a 48 h period to monitor natural mite fall (July 13).

Treatment and data collection

I replaced the sticky boards prior to OA application (July 15). I treated one side of the 10 divided hives with 40 mL of a 3.5% OA sugar water solution (sugar:water) (1:1) (w:w). I trickled the OA solution from above the frames between each occupied bee-way using a 100 mL syringe and made an effort to maximize contact with the adult bee population. I chose this dose based on a review article for treating colonies with minimal capped brood (Rademacher and Harz 2006).

I replaced the sticky boards and counted mite fall at 2, 4, and 6 days post-treatment (July 17, 19, and 21). I placed a Checkmite® strip in each half-hive to quantify remaining mites (July 21) (the experimental mite population had not previously exhibited coumaphos resistance). I replaced sticky boards every 48 hours until no mites were detectable (July 23 and 25). The use of the Checkmite® strips allowed me to quantify the total number of mites in each hive prior to OA application. I added the total number of mites recovered 2, 4, and 6 days after OA treatment to the number of mites recovered after placing Checkmite® strips in the hives. This enabled me to calculate the post-treatment percentage mite fall at 2, 4, and 6 days.

Replication

I replicated the entire experiment to increase the power of my tests (September 2005). The materials and methods were similar to those listed above. The only

difference was that I did not add queen cells to the hives. Instead, I allowed the bees to rear a queen from a small patch of eggs that I deliberately left when stocking the units. Like adding queen cells, allowing the units to rear their own queen ensured that the hives would be void of capped brood during experimentation.

Experimental design and statistical analysis

I designed my experiment as a split-plot design. The whole plot factor was divider type (single-screen, double-screen, and queen excluder) and the whole plot unit was the entire hive. The split-plot factor was treatment with OA (treated and untreated) and the split-plot unit was a half hive. I used the percentage reduction in *Varroa* infestation 2, 4, and 6 days post-treatment as my response variable. I blocked by the month in which the experiment was conducted (July and September) to account for variance in the total mite infestation between the two replicates.

I analyzed the data using PROC MIXED (SAS Institute 2006) and I separated means using a *t*-test ($\alpha = 0.05$). I assumed random blocks, although the assumption of fixed blocks did not change the results. I used the Kenwood-Rogers degrees of freedom adjustment. I used PROC UNIVARIATE and PROC GPLOT (SAS Institute 2006) to verify my assumptions of normality and constant variance.

Results

Forty eight hour pre-treatment mite fall

The 48 h pre-treatment mite fall was 30.4 ± 4.0 mites per split-unit hive in the July replicate (n = 20) and 45.8 ± 6.9 mites per split-unit hive in the September replicate

(n = 20). The pre-treatment mite fall was not significantly different for the sides scheduled to receive OA versus the sides scheduled to be left untreated for either replicate ($t = 0.25$, $df = 36$, $P = 0.8048$).

Total mite infestation

The total number of mites recovered per split-unit hive was 389 ± 52 mites (n = 18) for the July replicate. The total number of mites recovered per split-unit hive was 665 ± 52 mites (n = 18) for the September replicate. The total *Varroa* infestation in the September replicate was 276 ± 73 mites greater per split-unit hive than the July replicate ($t = 3.76$, $df = 34$, $P = 0.0006$).

Randomized Complete Block Design (RCBD) split-plot in time

My assumptions of normality and constant variance were met. I used the Shapiro-Wilk test in the UNIVARIATE procedure of SAS to verify normality. The Shapiro-Wilk test indicated that my data were normal ($P = 0.5722$). In addition, a symmetric box-plot and a straight-lined normal probability plot confirmed normality. A plot of the residual versus the predicted values revealed no obvious patterns and was indicative of data that had constant variance.

In total, there were 18 split-unit hives that were sampled at 2, 4, and 6 days post-treatment (54 observations per replicate). Fifty-four observations in the July replicate plus 54 observations in the September replicate sum to 108 total observations. The response variable was percentage reduction in *Varroa* infestation. See Table 6.1 for a summary of the *F* tests for the RCBD split-plot in time effects.

There was significant divider*treatment interaction ($P = 0.0001$). The factor 'time' was not part of this interaction so I analyzed the main effect for time. The time effect was significant ($P = 0.0001$). There was a $43.1 \pm 3.6\%$ (n = 36), $51.4 \pm 3.6\%$ (n = 36) and $58.5 \pm 3.6\%$ (n = 36) reduction in *Varroa* infestation at 2, 4, and 6 days after OA application. The above means represent the average mite fall per split-unit hive regardless of divider type or treatment.

Significantly more mites fell by day 6 than by days 2 or 4. Explicitly, $8.4 \pm 1.8\%$ more mites fell by day 4 versus day 2 ($t = 4.73$, $df = 75$, $P = 0.0001$), $7.1 \pm 1.8\%$ more mites fell by day 6 versus day 4 ($t = 3.98$, $df = 75$, $P = 0.0001$), and $15.4 \pm 1.8\%$ more mites fell by day six versus day 2 ($t = 8.71$, $df = 75$, $P = 0.0001$).

RCBD split-plot on 6 day percentage mite fall

The analysis of the RCBD split-plot in time confirmed that it was appropriate to only model the 6 day percentage mite fall because more mites fell by day 6 than days 2 and 4. To simplify my model, I removed the time factor and used 6 day percentage mite fall as the sole response variable in my subsequent data analysis. This reduced the total number of observations from 108 to 36 (108 total observations / 3 time intervals = 36 observations for 6 day percentage mite fall). See Table 6.2 for a summary of the *F* tests for the RCBD split-plot on 6 day percentage mite fall.

There was significant divider*treatment interaction ($P = 0.0001$). I did not consider the main effects of divider and treatment because of the significant interaction term. Rather, I analyzed the simple effects to draw conclusions about these two factors. Table 6.3 is a summary of the 6 treatment means reported as percentage reduction in

Varroa infestation. Treatment combinations in the divider/treatment column with 'OA treated side' indicate that OA was applied. Treatment combinations in the divider/treatment column with 'untreated side' indicate that OA was not applied. For example, the 6 divided hives with a single-screen divider averaged 73.3 ± 7.5% mite reduction on the side that was treated with OA and 22.6 ± 7.5% mite reduction on the side that was left untreated.

The sides that were treated with OA had significantly more mite fall than the untreated sides for all 3 dividers. When only considering the units with single-screen divider, sides that were treated with OA had 50.7 ± 5.4% greater mite fall than the sides left untreated ($t = 9.33$, $df = 15$, $P = 0.0001$). When only considering the units with double-screen divider, sides that were treated with OA had 59.6 ± 5.4% greater mite fall than the sides left untreated ($t = 10.96$, $df = 15$, $P = 0.0001$). When only considering the units with queen excluder divider, sides that were treated with OA had 15.9 ± 5.4% greater mite fall than the sides left untreated ($t = 2.92$, $df = 15$, $P = 0.0105$).

There was no difference in the percentage mite fall on the sides that were treated with OA for all three dividers. When only considering the sides that were treated with OA; units with double-screen dividers had 4.4 ± 7.8% greater mite fall than units with queen excluder dividers ($t = 0.55$, $df = 22.2$, $P = 0.5849$), units with double-screen dividers had 11.5 ± 7.8% greater mite fall than units with single-screen dividers ($t = 1.46$, $df = 22.2$, $P = 0.1579$), and units with queen excluder dividers had 7.1 ± 7.8% greater mite fall than units with single-screen dividers ($t = 0.91$, $df = 22.2$, $P = 0.3742$).

When only considering the untreated sides, units with the queen excluder divider had significantly more mite fall than units containing either single- or double-screen

dividers. When only considering the untreated sides, units with the queen excluder divider had 39.3 ± 7.8% greater mite fall than units containing double-screen dividers ($t = $ 5.01, $df = 22.2$, $P = 0.0001$) and units with the queen excluder divider had 42.0 ± 7.8% greater mite fall than units containing single-screen dividers ($t = 5.35$, $df = 22.2$, $P = $ 0.0001). The percentage mite fall on the untreated sides was not significantly different for units containing the single-screen versus the double-screen dividers ($t = 0.34$, $df = $ 22.2, $P = 0.7403$).

Discussion

I accept my hypothesis that OA kills mites via contact. Only the queen excluder divider permitted worker bees to move freely and allowed physical contact between the 2 sides. As Table 6.3 illustrates, bee-to-bee contact was the primary route for OA distribution because divided hives with queen excluders had significantly more mite fall (65%) on their untreated sides than divided hives with single-screen or double-screen dividers.

As expected, the percentage mite reduction was not significantly different on the sides of the divided hives that were treated with OA regardless of divider type. My intention was to correlate mite fall on the untreated side with divider type. Divided hives with single-screen and double-screen dividers averaged 23 and 25% mite fall on their untreated sides after 6 days, respectively. The 2 control colonies with solid-wood dividers (one control per replicate) averaged 29% (n = 2) mite fall on their untreated sides after 6 days. The 29% mite fall on the untreated sides of the control colonies containing solid-wood dividers corresponds with the mite fall on the untreated sides of the single-

screen and double-screen divided hives (23 and 25%). Trophallactic interactions and fumigation did not significantly influence the distribution of OA as single-screen, double-screen, and solid wood divided hives had similar mite fall on their untreated sides.

Significantly more mites fell 6 days after OA application than 2 or 4 days after OA application. This statistic may be interpreted several ways. One interpretation is that OA has residual activity against *Varroa* for at least 6 days post-treatment. Another interpretation is that a portion of the *Varroa* mites exposed to OA experience a drawn-out death. My data from chapter 1 and Milani (2001) demonstrate that OA has a high acute toxicity to mites in laboratory bioassays 24 hours post-treatment. Chapter 1 and Milani (2001) do not quantify the chronic toxicity of OA to *Varroa* because of the impossibility of sustaining mite populations for long periods of time away from their honey bee hosts. Perhaps the chronic toxicity of OA for phoretic mites in the hive environment is significantly less than the acute toxicity reported in chapter 1 and Milani (2001).

One important assumption of my experiment was that the single-screen divider allowed trophallaxis to occur between adult bees on each side. This assumption held true throughout experimentation as I observed adult bees performing proboscis extensions and trophallactically interacting between the single-screen dividers. The role of trophallaxis in the distribution of Perizin (coumaphos) in honey bee colonies was investigated by van Buren et al. (1992) who divided hives into 3 compartments with screens and traced the amount of coumaphos transferred between the sections via trophallaxis. Although trophallactic interactions were of minor importance in the distribution of coumaphos, the authors indicate that trophallaxis was occurring between the screened sections of the hive.

Anecdotal observations from beekeepers suggest that adult honey bees will ingest sugar water feed containing OA. I noticed small, pea-size pools of the OA sugar water solution on the top bars of several hives up to 6 days after OA application. I did not observe ingestion of the OA solution by adult bees and the pools eventually evaporated. If the anecdotal observation that bees will ingest sugar water containing OA is true, my results suggest that the concentration must be lower than 3.5% OA by weight. My results only apply to the trickle method with a 3.5% OA sugar water solution (1:1) (w:w). The distribution of OA in honey bee colonies when the vaporizer method is used was not tested in my study. My results give beekeepers and researchers insight as to how OA is distributed in hives and provide guidance for selecting application techniques that maximize the efficacy of OA.

Table 6.1 – *F* tests for RCBD split-plot in time.

Effect	Numerator df	Denominator df	F	P
divider	2	14	5.9	0.0142
treatment	1	75	853.8	0.0001
divider*treatment	2	75	75.8	0.0001
time	2	75	38.1	0.0001
divider*time	4	75	0.9	0.4567
treatment*time	2	75	0.1	0.8994
divider*treatment*time	4	75	0.2	0.9341

Table 6.2 – *F* tests for RCBD split-plot on 6 day percentage mite fall.

Effect	Numerator df	Denominator df	F	P
divider	2	14	6.8	0.0086
treatment	1	15	179.6	0.0001
divider*treatment	2	15	18.0	0.0001

Table 6.3 – Percentage reduction in *Varroa* infestation 6 days post-treatment. Estimates with different letters indicate significant differences (*t*-test, $\alpha = 0.05$).

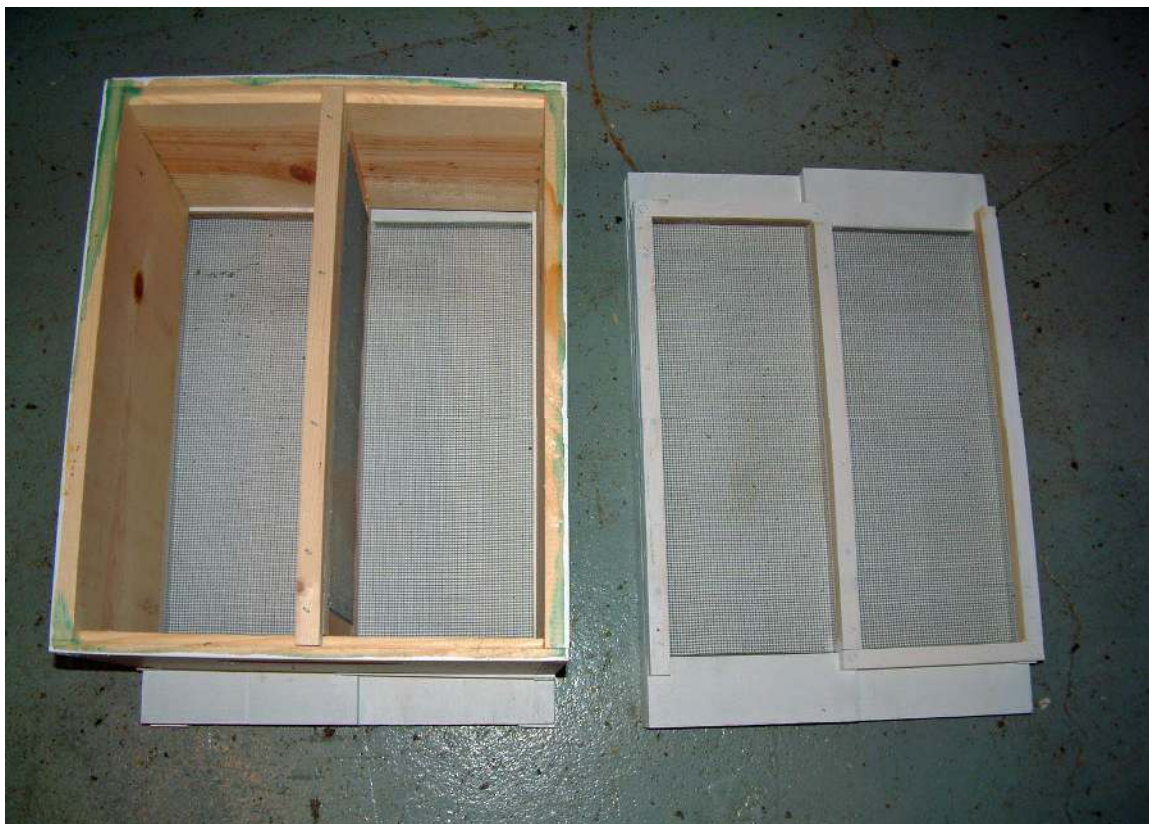| Divider type / Treatment	Estimate ± Standard Error	n
single screen / oa* treated side	73.3 ± 7.5 a	6
double screen / oa treated side	84.8 ± 7.5 a	6
queen excluder / oa treated side	80.5 ± 7.5 a	6
single screen / untreated side	22.6 ± 7.5 b	6
double screen / untreated side	25.2 ± 7.5 b	6
queen excluder / untreated side	64.6 ± 7.5 c	6

* oxalic acid

Figure 6.1 – Left: split-unit Langstroth hive with single-screen divider. Right: screened bottom board with opposing entrances.

Figure 6.2 – Left to right: double screen divider and queen excluder divider.

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