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EXPLORING NEW METHODS FOR VARROA MITE CONTROL

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EXPLORING NEW METHODS FOR VARROA MITE CONTROL

By

Yu-Lun Lisa Fu

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ABSTRACT

EXPLORING NEW METHODS FOR VARROA MITE CONTROL

By

Yu-Lun Lisa Fu

The varroa mite, *Varroa destructor* Anderson & Trueman, is a serious ectoparasitic mite of *Apis mellifera* L. Once a honey bee colony is infested with varroa mites, the whole colony will die within two years and possibly within two months. To prevent the loss of honey bees from varroa damage, we explored new low cost and environmental friendly control methods to reduce varroa mites in the honey bee colonies. We tested 2.45 GHz microwave radiation, which is fast and does not contaminate the colonies, to control varroa mites in their phoretic and reproductive stages. However, adult bees and pupae were more susceptible to 2.45 GHz microwave radiation than the adult varroa mites. We conclude that 2.45 GHz microwave radiation is not an appropriate method to control varroa mite in the honey bee colonies. We also tested three essential oils, thymol, origanum, and clove oils in different formulations for examining their effects on mite reproduction. Neat clove oil, starch-encapsulated thymol, β-cyclodextrin encapsulated thymol, and β-cyclodextrin encapsulated origanum oil significantly reduced mite infestation. β-cyclodextrin-encapsulated origanum oil and thymol crystals significantly lowered mite reproduction. The results suggest that the tested essential oils can reduce mites by lowering their reproduction.

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Chapter 1: Literature Review

Varroa destructor (Acari: Varroidae) (Anderson and Trueman 2000), previously known as *V. jacobsoni* Oudemans, is currently the most serious threat to the European honey bee, *Apis mellifera* L. (Matheson 1993, 1995). Agricultural production in the U.S.A. and elsewhere depends heavily on honey bees for pollination (Robinson et al. 1989, Morse and Calderone 2000). Unfortunately, the varroa mite has seriously damaged the beekeeping industry in the United States since its invasion in 1987 (Anonymous 1987). It is possible to bring a large negative impact on the pollination of many fruits and crops and leads to a great agricultural economic loss (Roberson 2006).

I. Varroasis

The symptoms of varroa infestation are: (a) weakened colonies with a spotty brood pattern and other brood disease infections; (b) drone or worker brood cells with punctured cappings; and (c) adult bees with deformed legs and wings crawling on the combs or on the ground outside (Shimanuki et al. 1994, Sammataro et al. 2000). Brood mortality is increased because other pathogens (bacteria, fungi or virus) can invade pupae through the feeding site. The varroa mite transmits bee viruses such as acute paralysis virus, deformed wing virus, and Kashmir bee virus (Allen et al. 1986, Bowen-Walker et al. 1999, Bakonyi et al. 2002, Chen et al. 2004, Tentcheva et al. 2004). The pupae that are parasitized by varroa mites have lower level of immune-related gene transpripts (Gregory et al. 2005). Even if infested brood successfully emerge, behaviors of adult bees

can be disrupted by abnormal morphological features such as deformed wings, deformed legs, and shortened abdomens. Adult bees emerging from parasitized brood also have shorter life spans and lower body weight (De Jong et al. 1982). Because of these effects on workers and brood, untreated colonies usually die within 2 years of infestation, and in warmer climates colonies collapse within months.

II. Varroa Biology

Adult female varroa mites are 1.1 mm long and 1.7 mm wide (Figure 1.1A), flattened, hard, and reddish-brown. Adult male varroa mites are about 0.5 mm long and 0.6 mm wide (Figure 1.1B), not flattened, soft, and pale with a brownish tint. Varroa mites feed on hemolymph of both adult bees and pupae at different phases. In European honey bees, varroa mites preferentially infest drone brood; however, they also infest worker brood (Beetsma et al. 1999). The biology of varroa mites has been investigated thoroughly. The life cycle and reproduction of varroa mites and their genetics are summarized below.

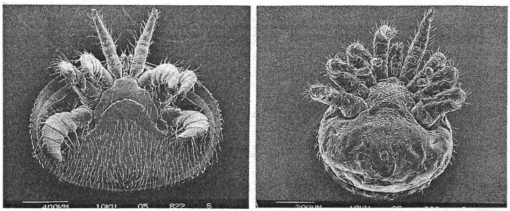

Figure 1.1. Electron scanning micrographs of *Varroa jacobsoni*, ventral views. A. adult female (bar = 400 µm). B. adult male (bar = 200 µm). (modified from De Jong, 1997)

II.A Life Cycle and Reproduction

The mite life cycle has two distinct phases. One is the phoretic phase (7-10 days during summer), during this phase the adult female mites feed on adult bees and they can move quickly over the bee's body or from one bee to another. The body shape of adult female mites is highly adapted to fit neatly under inter-segmental membrane of honey bee's abdomen and preferentially hide between the 3rd and 4th segments on the left side (Bowen-Walker et al. 1997). Their claws enable them to grasp on the hairs of the honey bees (Ramirez and Malavasi 1991) and their cuticles have been heavily sclerotized to reduce water loss (Martin 2001). During the phoretic stage, they prefer attaching to nurse bees over foragers and adult drones over adult worker bees. The other phase is the reproductive phase, about 13 days on worker brood and 16 days on drone brood. During this phase mites enter into the brood cells and complete their reproduction (Figure1.2). Fertilized female mites enter into brood cells

containing young larvae just before the cells are capped. First, they go to the bottom of the cells and immerse themselves in the remaining brood food. After the cells are capped, female mites start feeding on prepupae. About 60 h after a cell is capped, a female mite begins to lay the first egg (usually unfertilized, resulting in a male) and subsequent eggs (fertilized, resulting in females) are laid at intervals varying from 20-32 h in the drone brood (Martin 1995a) or 26-32 h in the worker brood (Martin 1994). They lay a total of 4 to 6 eggs in worker brood and usually they lay one additional egg in drone brood (Martin 1994, 1995a). The mite goes through four developmental stages: egg, protonymph, deutonymph, and the adult. Figure 1.3 shows the ontogenesis of male and female mites, both protonymph and deutonymph consist of two stages, the immobile stage and the mobile stage (Ifantidis 1983). The period from egg to adult takes 6 to 7 days for the female and 5 to 6 days for the male. Within 24 h after the last molting, the adult mites mate inside the capped honey bee brood cell. Because most cells are invaded by only one single mother mite, this means her offspring mate among themselves (brother-sister mating). Female mites emerge with newly eclosed adults and go through the phoretic phase again. Males are rarely seen alive outside the cells because they die from water loss shortly after the host bee ecloses. The development of mite progeny is synchronized with the development of pupae. When young honey bee emerges from the cell after pupation, the varroa mites also leave the cell to live on adult bees. During the broodless period, varroa mites can feed on adult bees and survive up to several months until the new brood cells are ready in the honey bee colonies. If mites

fall off adult bees, they usually only survive on their own for about 20 h (De Guzman et al. 1993).

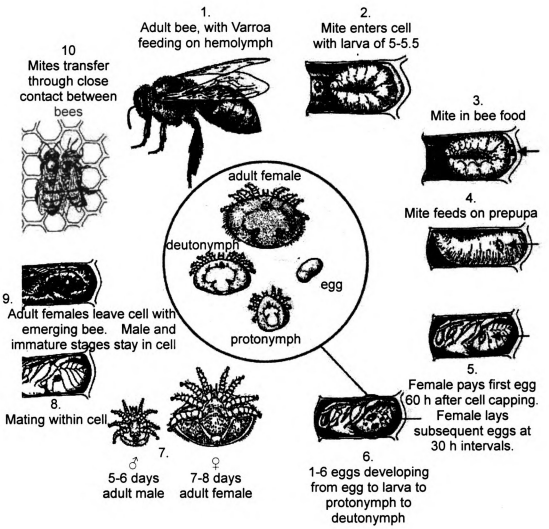

Figure 1.2. Life cycle of varroa mites, illustrated by Alexander. (USDA, ARS, BBII 1987).

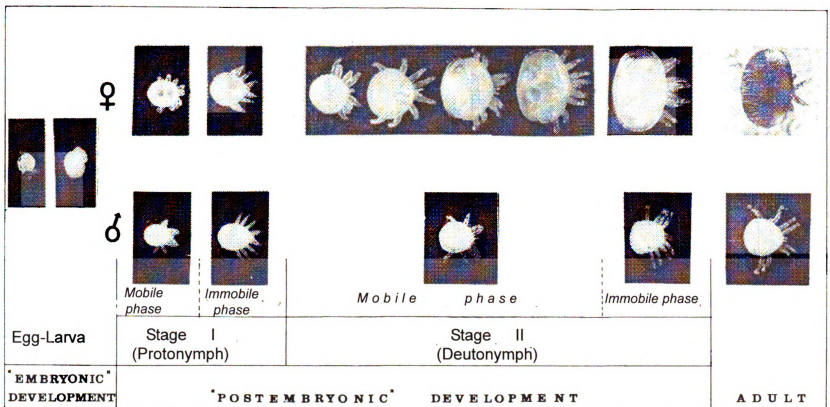

Figure 1.3. Ontogenetic development of the varroa mite (modified from Ifantidis 1983).

II.B Genetics of Varroa Mites in North America

For over 30 years, scientists and the general public thought the mite that switched from *Apis cerana* to *Apis mellifera* and the mite that still infests *Apis cerana* is the same species and is referred to as *Varroa jacobsoni* Oudemans (Oudemans 1904). Only recently Anderson and Trueman proposed that the mite that now infests *Apis mellifera* is a new species and named it *Varroa destructor* (Anderson and Trueman 2000). The naming of a new species is based on differences in morphology, geographical distributions, and genetics. Adult female *V. jacobsoni* appears less spherical in shape than adult female of *V. destructor.* Adult female *V. destructor* has larger mean body length (1.2 ± 0.03 mm) and width (1.7 ± 0.04 mm) compared to the mean body length (1.1 ± 0.03 mm) and width (1.5 ± 0.04 mm) of adult female *V. jacobsoni.* Only *V. destructor* has been found reproducing on *A. mellifera* in 32 different countries. *V. jacobsoni* mainly distributes in Asia and infests in Asian bee colonies. *V. destructor* has a 8 % difference in mtDNA from *V. jacobsoni.* Among the 5' 458 nucleotides of the mtDNA CO-I gene of *V. destructor* (Korea haplotypes) and *V. jacobsoni* (Java haplotypes), 30 nucleotides are different between them. The complete mitochondrial DNA (mtDNA) of *V. destructor* is available for addressing genetic questions and identifying the haplotypes from different localities (Evans and Lopez 2002, Navajas et al. 2002). So far, only two out of 18 different mitochondrial haplotypes of *V. destructor*, namely haplotypes Korea and Japan, have been found to be capable of reproducing on *A. mellifera.* mtDNA evidence suggests that the *V. destructor* Korea haplotype is the dominant type being found

on *A. mellifera* worldwide (Anderson and Trueman 2000, Garrido et al. 2003, Zhou et al. 2004). Using randomly amplified polymorphic DNA (RAPD), De Guzman et al (De Guzman et al. 1997, 1999) reported that both Russian (also called Korean type in recent publications) and Japanese genotypes of *V. destructor* were present in North America. Using a region of the mtDNA CO-I coding gene, Anderson and Trueman (2000) found that Japan-Thailand (=Japan) and Korea haplotypes of *V. destructor* were in the U.S. (95 samples from the U.S.). The results of RAPD and mtDNA indicates that North America had more than one introduction of *V. destructor.* Because Korea haplotypes are most common in the U.S., and motes cause great damage here, it suggests that the Korea haplotype is highly virulent on *A. mellifera* (De Guzman et al. 1999, Anderson and Trueman 2000).

Since 1987, after its first sighting in Wisconsin, varroa mite was quickly found in Florida in the same year and then found in 12 other states before 1991 (Sanford 2001). Until 2004, Hawaii was the only state considered to be free of varroa mites (Cullineу 2003, Reid 2004), however, varroa mites have been reported on the island of Oahu in Hawaii in April 2007 (Ramadan et al. 2007). The rapid spread of varroa mites to all states of the U.S. can be attributed to the movement of bees by humans. Once varroa mites are in a local area, robbing and drifting behaviors allow bees to transfer varroa mites from one colony to another (Rademacher 1991). Swarming is the only method for varroa to expand to new areas without human help, because a swarm may fly several km (around 300 m to 10 km) a way from the parental colony (Seeley and Morse 1977, Villa 2004).

III. Varroa Control Methods and Their Mechanisms

III.A Chemical Methods

The chemicals for varroa mites control have been classified according to their toxicity and efficacy. The "hard chemicals" can kill 99% of mites if mites are not yet resistant, these include fluvalinate, coumaphos, and amitraz. The soft chemicals usually kill 70-90% of mites; these include organic acids and essential oils. Generally, chemicals are applied to honey bee colonies during a time of no honey flow and broodless periods in order to prevent contamination of honey, and to increase the efficacy. Almost no chemicals can reach sealed brood cells to kill mites that are at the reproductive stage (with the exception of formic acid). So, treating during period of no brood or less brood would increase treatment efficacy.

III.A1 Hard Chemicals

Hard chemicals effectively control varroa mites in the honey bee colonies. However they are usually toxic to other animals, therefore not environmental friendly. They also tend to leave residues in bee products. Resistance is also easier to develop because of their higher mite-killing efficacy. Three major hard miticides are reviewed below.

III.A1-1 Apistan®

Apistan®, a tau-fluvalinate impregnated plastic strip, is a slow release polymer strip formulation designed for the control of varroa mites. First used in

Australia in 1988, Apistan® enjoyed widespread success in controlling non-resistant varroa mites. If used properly, its efficacy against non-resistant mite population is up to 98-100% (Hillesheim et al. 1996). This treatment is usually applied twice a year. It is used once in early spring before the main honey flow and once in late summer after honey harvest when the amount of brood diminishes. The treatment duration is 6 to 8 weeks. The strip should not be removed from the hive for at least 6 weeks and not be left in the hive for more than 8 weeks, in order to reduce residues in beeswax and to slow down the development of mite resistance. However, residues of fluvalinate have been detected in beeswax as well as in honey (Wallner 1995, 1999, Bogdanov 2006, Kamel and Al-Abbadi 2006). As is the case with pesticides directed against other pests, resistance development is inevitable. Fluvalinate resistance has been reported in Europe (Lodesani et al. 1995, Milani 1999, Floris et al. 2001b, Thompson et al. 2002,. 2003; Trouiller 1998), in Israel (Miozes-Koch et al. 2000) and in the U.S.A. (Baxter et al. 1998a, Elzen et al. 1998, Elzen et al. 1999a). The moderate level of mite resistance to fluvalinate in California mite populations has not resulted in a failure of Apistan® to control mites, but in Florida, Apistan® is less effective at controlling varroa mites (Elzen et al. 1999a). Fluvalinate is a synthetic pyrethroid that can alter gating properties of the sodium channel. Fluvalinate resistance in *V. destructor* is associated with the mutations of the sodium channel gene (Wang et al. 2002). The full-length sodium channel DNA of *V. destructor* is distinct from other insects (Wang et al. 2003) and the position corresponding to the leucine to proline mutation in mites perhaps explains the

higher sensitivity of varroa mites to fluvalinate, than honey bees whose sodium channel proteins already naturally contain a proline residue (Liu et al. 2006). No major differences of reproduction have been found between the resistant and susceptible mite populations (Martin et al. 2002). In addition to the problem of residues and mite resistance, less healthy drones, higher levels of drone mortality, and increased rate of queen supercedure have been observed in Apistan® treated colonies (Currie 1999, Rinderer et al. 1999).

III.A1-2 CheckMite+®

CheckMite+®, an organophosphate (coumaphos) impregnated plastic strip, is a slow release formulation for the control of varroa mites. Many states obtained EPA Section 18 emergency exemptions for the use of Checkmite+® as an alternative to control varroa mites, especially mites that are resistant to fluvalinate and amitraz (Elzen et al. 2000). The recommended method is to use one CheckMite+® strip for every 5 combs, applied once a year. The most effective time to apply Checkmite+® is whenever brood rearing is low. The best time could be in the spring before the first honey flow or in the fall after the last honey flow. The treatment duration is at least 42 days but not more than 45 days. Coumaphos is toxic to human and other vertebrates, so it is important to remove honey supers before application of CheckMite+® strips or to add honey supers two weeks after the strips are removed. Coumaphos residues can be detected in beeswax and honey after normal application (Wallner 1995, 1999), but the level found in honey is lower than the accepted maximum residue limit (MRL, 0.1 $mgkg^{-1}$) (Bogdanov

et al. 1998b, Bogdanov 2006, Karazafiris et al. 2008). There is still a risk of coumaphos using above the MRL because the transfer of coumaphos from beeswax into honey increases slowly over a few months (Kochansky et al. 2001). Coumaphos resistance has been detected in Europe (Spreaficom et al. 2001) and in the U.S.A. (Elzen and Westervelt 2002, Pettis 2004, Pettis and Jadczak 2005). Identifying coumaphos-resistant mites and using rotation schemes are important to prolong the use of coumaphos or to limit the spread of coumaphos resistant mites (Pettis and Jadczak 2005).

III.A1-3 Apivar®

Apivar® is a product with amitraz embedded in a slow-release plastic strip, with 97-99% efficacy against varroa mites (Baxter et al. 1998b). One strip of Apivar® is used for every 5 frames of bees. The strips are hung between the frames and left for at least 6 weeks. Amitraz is released when the bees rub against the strips. It is toxic to mites but safe for humans and bees. No or extremely low amount of amitraz residues is detected in the beeswax and the honey. Kamel and Al-Abbadi (2006) did not detect amitraz in the beeswax and honey using GC following Apivar® treatments and Martel et al. (2007) reported that no residues were found in honey or beeswax using HPLC following Apivar® treatments. Even after analyzing amitraz residues in beeswax after hydrolysis to 2,4-dimethylaniline by using a combination of SPME and GC/MS (with a detection limit of amitraz at 1 ng g^{-1}), wax samples from beekeepers and commercial foundations contained residues below 0.02 mg kg^{-1} (Leníček et al. 2006) which is

lower than its MRL (0.2 mg kg^{-1}) (Bogdanov 2006). Amitraz is volatile and unstable in honey, it degrades completely within 10 to 15 days (Jiménez et al. 1997, Korta et al. 2001). No amitraz residues higher than 0.01 mg kg^{-1} were detected in honey collected from Apivar® treated colonies (Floris et al. 2001a). In other words, amitraz is safe for bee products. However, resistance has been documented in the U.S.A. (Elzen et al. 1999b) and in Europe (Milani 1999). Most recently, slight amitraz resistance of varroa mites has been found in Mexico. Current LC_{50} of amitraz was approximately 2 to 3 times as high as the LC_{50} baseline established 9 years ago (Rodríguez-Dehaibes et al. 2005). People paid little attention to the issue of varroa mite resistance to amitraz since amitraz is a substitute for fluvalinate.

III.A2 Soft Chemicals

Soft chemicals usually are natural products extracted from plants. The advantage is that no persistent residues stay in bee products because of their volatility, although some soft chemicals could alter the taste of the honey if misused. Usually, its efficacy is lower and not consistent for controlling varroa mites in honey bee colonies. Commonly used organic acids and essential oils are reviewed below.

III.A2-1 Formic Acid

Formic acid is a natural substance found in small quantities in honey (Nelson and Mottern 1931, Mato et al. 2003). There are no residue accumulative problems in honey or other bee products (Bogdanov et al. 2002, Bogdanov 2006). Two formulations of formic acid are usually used to control varroa mites, either a liquid is poured onto an absorbent pad (Gatien and Currie 2003, Elzen et al. 2004) or a long-lasting, slow-release gel formulation (Feldlaufer et al. 1997, Lee 1998, Kochansky and Shimanuki 1999). Only the gel formulation is registered for mite control in the U.S.A. For absorbent pads, the concentration of formic acid should be diluted to 65% to reduce damage to the queens. The pad or gel is placed on the top of frames of the brood nest. The acid evaporates, and because the formic acid vapor is heavier than air, it sinks into the hive and kills varroa mites. The efficacy of formic acid for controlling mites is variable (50-100%) because it depends on methods of application (Feldlaufer et al. 1997, Satta et al. 2005, Underwood and Currie 2005), and environmental conditions (Calderone and Nasr 1999, Elzen et al. 2004, Ostermann and Currie 2004). It is generally applied during broodless period, either in spring or later summer. Formic acid has the advantage of killing mites in brood cells because it can penetrate beeswax capping (Fries 1991). However, dosage and dutration must be carefully regulated to avoid killing brood. Formic acid could inhibit oxygen consumption of not only varroa mites, but also bee brood and newly emerged bees (Bolli et al. 1993). Formic acid can kill bee brood directly or reduce brood care by interrupting the larval feeding behavior of nurses (Fries 1991, Bolli et al. 1993).

III.A2-2 Oxalic Acid

Oxalic acid is an organic acid that is commonly found in plants (Holmes and Kennedy 2000). Oxalic acid has been applied with trickling (= dribbling), evaporation, and spraying methods (Charrière and Imdorf. 2002, Rademacher and Harz 2006). Trickling is the most effective method to control varroa mites in honey bee colonies (Oliver 2006, Emsen et al. 2007). The general method is to mix oxalic acid in 50% sugar syrup (70 gram of acid in 1.67 L of syrup for spring/summer or 56 gram of acid in 1.67 L of syrup for fall/winter) and trickling the liquid between frames (Oliver 2006). Weather and brood conditions influence the efficacy of oxalic acid when applied by trickling; warmer temperature and smaller amount of brood result in increased mite drop (Bacandritsos et al. 2007). Mite-killing efficacy reaches 90 % when oxalic acid is applied during broodless periods in winter (Oliver 2006). On the other hand, relatively low efficacy ranging from 36-60% was found in broodright period when oxalic acid is applied in spring or summer (Oliver 2006, Rademacher and Harz 2006). This is largely because oxalic acid kills mites by acute contact toxicity and it cannot penetrate beeswax to kill mites inside brood cells. Laboratory bioassays indicate that oxalic acid has low acute contact toxicity to honey bees. The LC_{50} of mites measured 24 h after treatment is approximately 306 times lower than that of honey bees; so, oxalic acid is much more toxic to mites (Aliano et al. 2006). One paper reported that repeated spray applications of 3% oxalic acid in autumn and spring showed significantly negative effects on brood development and queen survival (Higes et al. 1999). Oxalic acid is safe for humans. Oxalic acid is a natural honey

constituent, and even in single or repeatedly treated colonies, the amount of residues of oxalic acid in honey is still lower than our regular intake from vegetables (Bogdanov 2006, Rademacher and Harz 2006). Although oxalic acid is safe and effective for varroa mite control and widely used by beekeepers, it has not been registered as a varroa control product in the U.S.A. yet (Oliver 2006).

III.A2-3 Thymol Based Products

Essential oils are inexpensive botanical products and pose the least threat to the environment or to human health (Isman 2006). Thymol and thymol blended with other essential oils offer better promise than other essential oil treatments (Imdorf et al. 1996, Calderone et al. 1997). A few thymol-based formulations have been registered for controlling varroa mites in different countries, for example Apilife VAR®, Apiguard®, and Thymovar®. Apilife VAR® consists of 76% thymol 16.4% eucalyptol, 3.8% menthol and 3.8% camphor in a vermiculite tablet. The tablets are applied at the end of the summer after honey is removed; they need to be reapplied several times, each time around 3 to 4 weeks (Imdorf et al. 1995, Imdorf et al. 1996). The efficacy for controlling varroa mites varies from 50% to more than 90% (Imdorf et al. 1995, Calderone et al. 1997, Imdorf et al. 1999, Ellis et al. 2001, Baggio et al. 2004). Apiguard® consists of only thymol and is formulated in a slow release gel matrix. An opened Apiguard® tray is placed on the top of frames in the hives and, 2 weeks later, it is replaced with a new tray for 2 to 4 weeks. The efficacy for varroa mite control varies from 49% to 99% (Baggio et al. 2004, Floris et al. 2004). Efficacy of Apiguard® also varies

with geographical locations. For example mite kill in Northern Italy is significantly lower than that in Central and Southern Italy (Baggio et al. 2004). Thymovar® also consists of only thymol and is formulated in a viscose sponge. Thymovar® is applied in two applications of 3 to 4 weeks each after honey is removed. The efficacy for varroa mite control varies from 82% to 100% (Baggio et al. 2004). Sublimation into vapor and direct contact are the two modes of reaching to varroa mites (Lindberg et al. 2000).

Many factors influence the action of thymol such as temperature (Imdorf et al. 1995), brood condition (Calderone et al. 1997, Gregorc 2005), application method (Baggio et al. 2004), and the combinations of environmental conditions such as different localities and climates (Imdorf et al. 1999, Melathopoulos and Gates 2003, Baggio et al. 2004). The highest efficacy is achieved when colonies are broodless and temperatures are between 15 °C and 20 °C (Imdorf et al. 1995). The presence of brood limits the efficacy of thymol-based control during summer (Calderone et al. 1997, Gregorc 2005). In addition, thymol-based products should not be applied when temperatures is high (> 30 °C) because higher temperature causes more rapid evaporation resulting in higher concentration of thymol vapor in hive which in turn causes adult and brood mortality (Imdorf et al. 1995, Melathopoulos and Gates 2003, Floris et al. 2004). These negative effects on colonies significantly reduce honey production (Mattila et al. 2000, Floris et al. 2004). Another potential problem is residues. Thymol is a fat-soluble molecule. As a result, thymol residues are found in higher amount in beeswax than in honey (Bogdanov et al. 1998a, Floris et al. 2004, Bogdanov 2006). The residues will

evaporate after combs and wax foundation are stored for a year. (Bogdanov et al. 1998a). The residues in wax can contaminate honey and influence the taste of honey. The taste threshold of thymol in acacia and rape honey is 1.1-1.3 mg kg^{-1} (Bogdanov et al. 1999). In Switzerland, the MRL of thymol residues in honey is set at 0.8 mg kg^{-1} because this level of thymol is not detectable in taste tests by consumers (Bogdanov 2006). The residues remain below the MRL when thymol-based products are used correctly (Bogdanov 2006). However, if treatments are carried out during the whole bee season or honey are produced during or right after treatments, thymol residues can be over the MRL and honey can taste astringent and medicine-like (Floris et al. 2004, Adamczyk et al. 2005, Bogdanov 2006). Thymovar® is currently registered in Europe but not in the U.S.A. The EPA has approved a Section 3 use permit for Apilife Var® since 2003 and Apiguard® since 2006 in the U.S.A.

III.A2-4 Other Essential Oils

More than 150 essential oils and the component of essential oils have been tested against mite. Imdorf et al. (1999) listed some essential oils that can reduce varroa mite populations. The mites are killed either by evaporation of essential oils or topical application of essential oils. Different oils have different toxicities to varroa mites and honey bees as well as different effects on behavior (repelling or attracting) and reproduction of mites (Imdorf et al. 1999). These results are summarized in Table 1.1, 1.2 and 1.3. Laboratory evaluations indicate that a large number of essential oils are effective in killing mites but they

are also detrimental to honey bees (Lindberg et al. 2000, Ruffinengo et al. 2007). Origanum oil, clove oil, wormwood flowers, peppermint oil were considered promising agents for varroa mite control through field and laboratory studies (Sammataro et al. 1998, Imdorf et al. 1999, Ariana et al. 2002, Al-Abbadi and Nazer 2003). Sour orange, lemon grass, and citronella oil could reduce mite infestation on adult bees and worker brood to 0 % after 3 or 4 week treatments (Abd El-Wahab and Ebada 2006). Neem oil and canola oil show some promise for varroa mite control, but they also significantly decrease honey bee brood which limits their usefulness to beekeepers (Melathopoulos et al. 2000a, Melathopoulos et al. 2000b). With the exception of thymol, information is lacking for other essential oils as to their effect on honey bee colony development, toxicity to adult bees or brood, and amount of residues. However, similar to thymol, the residues can contaminate bee products, especially changing the taste of the honey.

Table 1.1. Mortality of varroa mites and honey bees after being treated with various essential oils (from Imdorf et al. 1999)

Essential oil	MM. evap. (%)			MM top. (%)		BM evap. (%)		
	24 h	48 h	72 h	24 h	48 h	24 h	48 h	72 h
Control	0	1	11	0	4	0	1	1
Allylmustard	100			95	100			
Anise	5	80	100	55	60	0	2	5
Balm	0	85	100	15	15	2	5	8
Caraway	0	70	100	55	60	0	17	17
Cinnamon	100			60	100	0	2	7
Clove	100			100		0	0	2
Coriander	30	95	100	70	80	25	33	40
Dill	0	60	95	25	30	2	17	32
Eucalyptus	5	55	90	0	15	42	58	67
Fennel	0	40	100	50	60	2	2	2
Garlic	5	100		85	85	100	100	100
Geranium	5	55	95	70	70	0	2	2
Peppermint Jap.	20	95	100	30	35	2	12	25
Lavender	0	90	100	55	65	0	2	2
Marjoram	15	35	100	5	5	5	13	15
Onion	5	50	100	55	60	100	100	100
Orangeflowers	5	70	100	45	45	2	15	18
Oregano	0	90	100	80	90	3	43	87
Peppermint	40	95	100	15	15	10	48	48
Rosemary	10	45	100	15	15	2	3	3
Spearmint	0	100		55	55	2	15	17
Spik	0	10	95	10	10			
Tansy	15	75	100	15	25	7	17	17
Thyme	0	85	100	80	80	62	78	92
Wintergreen	50	100		5	10	0	5	7
Wormwood	0	25	100	15	15	7	63	95

MM evap.: mite mortality by evaporation of essential oil; MM top.: mite mortality by topical application of essential oil; BM evap.; bee mortality by evaporation of essential oil.

Table 1.2. Effects of essential oils on behavior and reproduction of varroa mites and on bee brood (from Imdorf et al. 1999)

Essential oil	Repellent-Attractant				Brood mortality	Mite reproduction
	Hoppe H.	Kraus B.	Bunsen J.D.	Colin M.E.	Bunsen J.D.	Bunsen J.D.
Ancna				R		
Balm		R			m	
Bergamot		R			h	
Caraway			R		s	
Cardamom		R			s	
Cedar		-	R		-	-
Celery		-	R		s	-
Chamomile		R				
Chenopodium				R		
Cinnamon	A	A			h	-
Citronella		R			s	
Clove	A	A			s	i
Coriander		R			S	
Dwarf pine		R				
Eucalyptus	R	R			s	
Fennel	-	R			h	
Fir needle		R	R		h	-
Galbanum			R		m	-
Geranium		R				
Grapefruit		R				
Juniper		-	R		s	-
Laurel		R				
Lavender	R	R	R		s	d
Lemon	R		-		m	i
Lily			-		s	i
Mandarin			R		s	-
Marjoram		R			s	
Melissa	R		R		s	
Mint		R				
Neem			R			-
Nerolidol			R		h	-
Nutmeg		R			s	
Peppermint	R	R	-		s	i
Pine			R		s	-
Rose		R			m	
Rosemary		R			l	
Sage			R	R	l	-
Sandal-wood		R				
Savory			R		h	
Thyme	R			R	h	-
Valerian		A				
Violet			R		s	-
Wine's yeast			R		m	-
Wintergreen		A	A		s	-
Wormwood	R					

R: repellent; A: attractant; s: small; m: medium; h: high; i: increase; d: decrease; -: neutral. Note that wintergreen oil is an essential oil with methyl salycilate as the main component, but it is also a common genetic name for pure methyl salycilate.

Table 1.3. Effects of different components of essential oils and organic substances on behavior and reproduction of varroa mites and on bee brood (from Imdorf et al. 1999)

Components of essentials oils and other organic substances*	Repellent-attractant			Brood mortality	Mite reproduction
	Hoppe H.	Kraus B.	Bunsen J.D.	Bunsen J.D.	Bunsen J.D.
Acethyl eugenol		A			
Anethole		R	R	h	-
Benzoic acid*		R			
Bornyl acetate*		R			
Camphor DL			-	s	d
Caryophyllene			R	m	-
Cinnamaldehyde		A			
Citral		R	R	m	-
Citronellal		R	R	s	-
Citronellol		R	-	m	d
Coumarin*		A			
Decenal*			R	m	-
Elemol		R			
Eucalyptol		R			
Eugenol	A	A	-	m	i
Geraniol		R			
Isoeugenol		R			
Linalool		-	-	h	d
Linalyl acetate			R	h	d
Menthol		R			
Menthone		A			
Nerolidol			R		
Octenal*			R	h	-
Phenyl acetylene*			R	s	-
Pinene		R	-	s	d
Terpineol			-	h	d

R: repellent; A: attractant; s: small; m: medium; h: high; i: increase; d: decrease; -: neutral (no effect).

III.B Cultural Methods

Cultural controls are management related methods to reduce varroa mite populations without using chemicals. Usually, they are less effective when used alone but can be applied as part of an integrated pest management (IPM) for effective varroa mite control. Three cultural methods are described below.

III. B1 Drone Trapping

Varroa mites have a strong preference for invading drone brood, mainly because drone brood is capped about 3 days longer than worker brood, so mother mites have a higher fitness on drone brood compared to worker brood (De Jone 1997). The infestation rate to drone cells is 7-11 times higher compared to worker brood (Fuchs 1990, Boot et al. 1995). When drone brood is sealed, it can be taken out of the colony and put in a deep freezer overnight to kill the mites and drone brood. Drone brood trapping is much more efficient (95%) than worker brood trapping to reduce varroa mites (Calis et al. 1999a). Using drone-trapping during broodless period could increase its efficacy to reduce mite populations (Wilkinson and Smith 2002). Drone trapping has been used successfully in field colonies (Fries and Hansen 1993, Calis et al. 1999b, Calderone 2005). The main disadvantage of using drone-trapping is that it is time and labor intensive, in addition to requiring a large freezer.

III. B2 Screen Bottom Board

Screen bottom boards are used to detect and trap varroa mites. A screen bottom board is constructed as a piece of wire-mesh screen separated from the bottom where sits a piece of sticky paper or a board coated it with crisco or vaseline. Mites can fall through the mesh but workers cannot pass through to reach the sticky paper or board. It is reported that up to 50% of varroa mites falling off the adult bees to the hive bottom are still alive and they could return to infest brood by reattaching to passing bees in a hive with a regular hive bottom (Lobb and Martin 1997, Webster et al. 2000). However, with a screen bottom board installed, mites are caught by the sticky paper and removed from the reproducing population. This method by itself has relatively low effectiveness to control varroa population (14-28% efficacy, Pettis and Shimanuki 1999, Ellis et al. 2001), however, its efficacy of controlling varroa mites increases when other treatments are used to dislodge varroa mites, such as acaricide application, essential oil treatments, or using resistant bees (Ostiguy et al. 2000, Ellis et al. 2001).

III. B3 MiteZapper®

The newly developed MiteZapper® is basically an improved method for drone trapping. Varroa mites are more attracted to drone brood (see previous section) and they are also sensitive to temperature. Mites cannot survive at a temperature of 44 °C (BrØdsgaard and Hansen 1994). MiteZapper® is a frame with a heating element embedded inside drone foundations. Once the drone

cells are sealed, a beekeeper can directly connect two terminals outside the hive to a car battery for 5 minutes until the temperature reaches around 45 °C. Thus mites will be killed in the drone cells and then worker bees will naturally remove dead or dying drone brood, similar to bees removing freezed-killed brood (Spivak 1996). The advantages of using the MiteZapper® are (1) beekeepers do not need to open the colony to remove the drone brood, (2) no chemical residues contaminate the bee products, and (3) the developing of mite resistance to miticides may be decelerated. MiteZapper® can be used during summer when drone brood exist and it is possible to engineer the MiteZapper® to improve its effectiveness in the future (Huang 2001).

III.C Use of Resistant Bees

Some lines of *A. mellifera* are resistant to varroa mites due to behavioral and/or physiological traits. Physiological characteristics include shorter capping period, and the ability of nurse bees to detect varroa mites inside brood cells. Behavioral characteristics include the ability of nurse bees to remove varroa mites from sealed brood and adult bees (Harbo and Harris 1999, Spivak and Boecking 2001). Beekeepers are encouraged to use resistant bee strains when possible because they can better tolerate varroa infestation and survive longer without acaricide treatments.

III.C1 Varroa-sensitive hygiene (VSH) bees

These bees have been called "suppression mite reproduction" (SMR) bees because it was thought mite reproduction was suppressed by worker brood (Harbo and Hoopingarner 1997). Researchers selected for the SMR trait by breeding bees which had mother mites with less offspring. This trait was recently renamed as VSH for varroa-sensitive hygiene (Danka et al. 2008). VSH bees are capable of detecting and removing most reproductive mites from the brood cells by their hygienic behaviors (Harbo and Harris 2005, Ibrahim and Spivak 2006), thus leaving only the mother mites that did not reproduce, giving the false impression that mite reproduction was suppressed. VHS bees are at least 4 times more likely to remove mite-infested brood than commercial controls. Removal of mite-infested pupae by VHS bees is associated with the age of postcapping infested pupae. Mite-infested pupae within 5 days postcapping release stimuli to trigger cell opening by VHS bees, but the ability of VHS bees to remove infested pupae is similar to control bees when the age of infested pupae are over 5.6 days postcapping (Harris 2007). VSH bees have significantly lower varroa infestations than Italian bees but have similar honey production. Beekeepers also have similar satisfaction levels with VSH bees compared to Russian bees and Italian bees (Danka et al. 2008).

III.C2 Hygienic (HYG) bees

Hygienic (HYG) bees are a line of bees bred by Spivak (Spivak 1996, Spivak and Reuter 1998), selected by breeding colonies which show good cleaning behavior toward freeze-killed brood. These bees can quickly detect, uncap, and remove diseased brood from colonies. HYG bees are different from VSH bees in that these bees remove any diseased brood (e.g. chalkbrood, American fouldbrood), not just varroa-infested brood. Hygienic bees remove significantly more pupae infested with mites than non-hygienic bees (Spivak 1996, Spivak and Reuter 2001). Overall, hygienic bees can successfully defend against low levels of varroa mite infestation, but they still need treatments to prevent colony collapse from high mite infestations (Spivak and Reuter 2001). The size of adult population, amount of worker brood, brood pattern, and the honey production are similar to commercial bees (Spivak and Reuter 2001, Ibrahim et al. 2007). Crosses are made between HYG and VSH bees (HYG/VSH), these bees have increased resistance against varroa, but their amount of brood is lower compared to that of VSH or commercial bees (Ibrahim et al. 2007).

III.C3 Russian bees

"Russian bees" are a line of bees imported to the U.S.A. because of their high resistance to varroa mites, originally derived from Primorsky Territory of Far-Eastern Russia (Danka et al. 1995, Rinderer et al. 2001, Rinderer et al. 2003, Harris and Rinderer 2004). Varroa mite infestations are significant lower in brood cells and on adult bees in Russian bee colonies possibly because Russian bees have been exposed to varroa mites for 45 to 100 years, (Danka et al. 1995). Hybrid between Russian and European bees has been commonly used to control varroa mites, but the pure Russian bees have the maximum varroa resistance (Harris and Rinderer 2004). When combined with other mire-reducing methods such as screen bottom board and organic acids, Russian bees are highly efficient in their resistance against varroa mites (Rinderer et al. 2003).

Chapter 2: Exploring Microwave as A Control Method

Abstract

Varroa destructor Anderson & Trueman (Acari: Varroidae) is a serious ectoparasitic mite of the European honey bee, *Apis mellifera* L. Because it is known that varroa mites are more sensitive to high temperature compared to honey bees, we explored the possibility of using microwave radiation as a new control method. We used a 2.45 GHz microwave oven to test the sensitivity of adult bees, pupae, and varroa mites to microwave radiation. Adult bees, including foragers and nurses, were highly sensitive to microwave; over 50 % of worker bees died after 4 s of microwaving treatment. Pupae were more tolerant of microwave radiation; nevertheless, their mortality was twice that of varroa mites. Microwaving pupae for 20 s killed most mites and pupae. We determined water content of bees and mites to see if different water content was causing their differential sensitivities to microwave radiation. The water content of mites was 70 ± 1.2 % which was similar to foragers (70 ± 1 %), but significantly lower than nurses (77 ± 0.7 %) and worker pupae (78 ± 0.2 %). Water content therefore did not explain the high sensitivity of foragers to microwave. The varroa mite has about 11 times higher surface to volume ratio than the bee. This should enable the mite to dissipate heat faster, and give it a higher tolerance to microwave radiation. The higher mite mortality observed when the mites were on pupae might be caused by heat generated by bees. We conclude that 2.45 GHz microwave radiation is not an appropriate method to control varroa mite in the

honey bee colonies.

Key words: varroa mite, *Varroa destructor*, *Apis mellifera*, microwave radiation, honey bee pest control

Introduction

The varroa mite, *Varroa destructor* Anderson & Trueman (Acari: Varroidae), previously known as *V. jacobsoni* Oudemans, is an ectoparasite of the honey bee (*Apis mellifera* L.) and distributed worldwide. Since its introduction to the U.S.A. in 1987, the varroa mite has seriously damaged the beekeeping industry. (Anonymous 1987). Based on USDA-NASS reports, honey bees contribute to the production of approximately $200 million in honey annually and pollinate $34 billion worth of fruit and vegetable crops (Roberson 2006). In some areas of the U.S.A., infestation by this introduced mite have reached epidemic proportions; with annual mortalities of colonies soaring to as high as 50 %-80 % (Kraus and Page 1995, Finley et al. 1996).

Chemical control agents include organic acids, essential oils, and Apistan® (fluvalinate) treatment (Sammataro et al. 2000, Ellis 2001). CheckMite+® (coumaphos) received a Section 18 registration in 47 States in U.S. before 2007, allowing a limited, short-term, and single-use application (EPA 2007). Coumaphos is an organophosphate pesticide that is toxic to human and other animals; hence it may be eliminated by the U.S. Environmental Protection Agency. Also, resistance to coumaphos has been recorded in both Italy and the United States (Elzen and Westervelt 2002, Vedova 1997). Fluvalinate strips (Apistan®) were the most effective chemical in controlling varroa mite in apiaries but mite resistance to fluvalinate has been reported in Europe, California and Florida (Milani 1995, Baxter et al. 1998a, Elzen et al. 1998). Thus, apart from the

potential to contaminate bee products, another serious shortcoming of miticide application is the eventual emergence of resistance. Costs of miticides such as CheckMite+® and Apistan® are $2 per strip and up to four strips are required per colony, and two treatments are required each year.

Alternative control methods have been used in apiaries. For example, smoke, bottom screen boards, resistant bees, and so on (Sammataro et al. 2000, Hoopingarner 2001, De Jong 1997), but most are labor intensive and impractical. In lightly infested colonies, varroa mites can be treated with smoke produced by tobacco leaves/stems or other plants, the smoke causes mites to dislodge from bees and the falling mites can be captured by a screen bottom board (Eischen 1997, Eischen and Wilson 1998). Drone brood can be used to trap and remove varroa mites because mites prefer infesting drone brood (Fries and Hansen 1989). Hygienic and grooming behaviors of honey bees can eliminate mites by removing infested brood or by autogrooming and allogrooming (Büchler 1992, Boecking and Spivak1999). In some honey bee subspecies or strains, varroa mites show lower fecundity and infection rates. This might be due to mutual adaptation between the host and parasite (De Jong 1997). Using those varroa- resistant bees such as varroa-sensitive hygiene trait (VSH) can reduce the varroa mite problem by combining with other integrated pest management (IPM) methods (Sammataro and Needham 1996).

Heat treatment at 42-48°C is another option to reduce varroa mite populations, but adult bees must be removed (Khrust 1978, Komissar 1978. De

Jong 1997). Otherwise, the temperature and treatment duration have to be controlled carefully to avoid injuring the brood. Usually, heating is used in combination with chemical methods to achieve effective treatment. The MiteZapper® is a new invention for varroa mite control (Huang 2001), which is based on the principles of drone preference of varroa mites and heat treatment to kill the mites. Our goal is to develop non-chemical and economical tactics to control varroa mites.

The US Federal Communications Commission allocates five frequencies for industrial, scientific, and medical applications: 13.56, 27.12, and 40.68 MHz in the radio frequency range, and 914 MHz and 2.45 GHz in the microwave range. Radio frequency and microwave treatments have been used to control woodworms, weevils, and other postharvest pests such as codling moth larvae, Indian meal moth larvae, navel orangeworm larvae, and Mexican fruit flies (Nelson and Payne 1982, Andreuccetti et al. 1994, Hallman and Sharp 1994, Nelson 1996, Ikediala et al. 1999, Wang and Tang 2001, Wang et al. 2003, 2006,). High-power microwave radiation at 10.6 GHz was tested to control maize weevil in white wheat (Halverson et al. 1996). Low cost is the main advantage of this type of control (Wang et al. 2006).

The *goal* of this study was to explore the feasibility of using microwave as a method for varroa mite control. Our objectives were to determine the microwave effect on 1) foraging bees, 2) randomly sampled adult bees with mites, 3) capped brood with mites, and 4) both adult bees and capped brood with mites. We also

measured the water content of adult bees, brood, and varroa mites to determine whether there is a relationship between water content and sensitivity to microwave.

Materials and Methods

Collection of honey bees and varroa mites

Adults and pupae of honey bees , and varroa mites were collected from the apiary at Michigan State University, East Lansing, Michigan (42.75° N, 84.46° W), in the summer of 2006 and 2007. Bees and mites were from colonies maintained according to standard beekeeping procedures. Adult foragers and nurses were taken from over-wintered colonies that had large populations of varroa mites. Adult bees were collected by an insect vacuum (BioQuip, CA), and put on ice for 5 min for anesthetization. The bees were weighed or counted and put into 10×13×15 cm plexiglass cages. Varroa mites were collected by opening drone pupal cells and brushing of mites from the pupae, or from nurses by "sugar dusting" (Fakhimzadeh 2001). This involved shaking bees inside a glass container with a screen lid after adding 2 teaspoons of confectioner's sugar and allowing the dislodged mites falling through the screen.

Microwave treatment

All *microwave* experiments were done using a commercial microwave oven (Proctor Silex 87008, Washington, NC) with a maximum output of 600W, frequency 2.45 GHz (wavelength 122 mm). Before conducting experiments, we

used water to verify the working status of the oven by constructing a standard curve of microwave duration and temperature increase. At the maximum power level, there was a linear relationship between microwave duration and temperature increase. Other power levels gave inconsistent results because the power output was regulated indirectly by the duration during which microwave was produced. For example, at 10 % power, the microwave was on for 1 second for every 10 seconds, thus 2 seconds and 19 seconds would yield the same energy. Therefore, all experiments were performed at the maximum power output. To distribute microwave radiation evenly, all the experimental materials, including adult bees, pupae, and mites, were put in the center of the rotating tray in the microwave oven. After microwaving, both control and treated adult bees were provided with 50 % sugar water and kept at 34 °C and 75 % relative humidity, so were pupae and varroa mites.

Mortality evaluation

We classified bees unable to move in a coordinated manner as "dead," for example those too weak to crawl but still able to move their legs when touched. Dead pupae were defined as those not emerging from the capped cells more than 3 days after their expected time of emergence. Held for one week after microwave treatment, the newly emerged bees were continuously recorded daily and then pupae mortality calculated. We classified mites as dead when they did not move their legs when touched by an insect pin under a dissect microscope. We used soapy water to wash off living mites from dead or live adult bees. We

opened capped brood cells to count mites under a dissect microscope. Both bee and mite mortality were determined 24 h after treatments.

Experiment 1: Forager sensitivity to microwave

Foragers were collected on 26 July, 31 July, and 2 August 2006 and put into Plexiglas cages (100 bees per cage). We assumed that the cage did not absorb the microwave energy differentially among different treatments. There were five treatments: 0 (control), 1, 4, 7, and 10 s microwave duration, with three cages per treatment (colony 2 only had two cages for the control). The experiment was replicated in three colonies. A total of 4,400 foragers was used.

Experiment 2: Relative sensitivity of nurses and mites to microwave

Adult bees were collected from inside the brood nest (hereafter referred to as "nurses") from heavily mite-infested colonies on 20, 23, and 25 October 2006. Each cage contained bees totaling 20 g, about 170 ~ 200 bees. Varroa mites were "phoretic" mites which were on the body of adult bees. Based on the results of the previous experiment, 4 s microwave treatment was used. The bee and mite mixture received microwave radiation for 4 s (treated) or 0 s (control). Each experiment was replicated 2 to 5 times for each colony and repeated in 4 colonies. After treatment, we put each cage on a piece of white paper (15×12 cm) centered on a piece of sticky contact paper (30 × 24 cm) to catch the escaping mites.

Experiment 3: Relative sensitivity of bee pupae and mites to microwave

A modified brood frame was used in this experiment. Only one side had brood cells; the other side had wax cells was removed and covered with a wooden board to prevent cell building. The queen in each colony was forced to lay eggs on the modified frame for 24 h. After 24 h, the queen was transferred to another cage so she could no longer lay eggs in the experimental frame. On the ninth day we marked mature larvae that were being capped by workers. Six h later we checked the brood again and marked any cell that was partially capped 6 h ago but now totally capped. Only these cells,capped within 6 h were used as recipient cells for mite transfer. We opened a small opening by an insect pin and transferred one mite into a cell by a small brush (size 00, sable, Loew-Cornell) and then the opening was sealed with melted beeswax. The frames with transferred mites were incubated at 34°C, 50 % RH for 8 days. We cut each comb which had no cells on the other side (due to modification) into small pieces, each containing 100 brown-color pupae with an electric saw one day before conducting microwave experiment. The cells on the edge were removed because of possible damage during cutting. Each piece was placed in a wire-screened cage and incubated at 34°C, 75 % RH. Microwave treatments were conducted two days before adult eclosion (dark brown pupae). The cages received microwave radiation either for 20 s, or 0 s (control). Mite mortality was evaluated 24 h after microwave exposure. Half of the cells (50) on each 100-sealed-brood piece were opened under a dissection microscope. The numbers of living and dead mites at all stages were recorded. These stages

include the mother mite, daughter mites at protonymph, deutonymph, and adult stages and males. Some mite-transferred cells contained more than one mother mite due to natural infestation prior to mite transfer. The remaining 50 pupae on each piece were held for 1 wk to check for pupal mortality. This experiment was repeated in 3 colonies.

Experiment 4: Relative sensitivities of adult bees, pupae, and mites to microwave

We collected foragers, 3-day-old bees, and white-eyed pupae from colonies on 13, 23 and 29 September 2006. To evaluate the microwave sensitivities of bees and reproductive mites in brood cells, we transferred varroa mites into 20 of the 40 capped brood cells (one mite per cell). Reddish-brown female mites were obtained from drone pupae. Mite transfer was done as described in Experiment 3. There were three treatments: 0 (control), 3 and 5 s microwave exposure. During the experiment, adult bees and pupae were placed in separate containers but exposed to microwave radiation simultaneously. Foragers (30) and 3-day-old bees (30) were housed in a cage; white-eyed pupae (40) with 20 transferred mites were house in a glass jar. Mite mortality was checked 24 h after microwave exposure by opening the 20 mite-transferred pupal cells under a dissection microscope. The remaining 20 pupae were held for another week to check for pupae mortality.

Water content of bees and mites

Adult bees and pupae were collected for water content from four colonies. Two mite-free colonies from the Michigan State University Student Organic Farm were used on 9 August 2006 and two colonies with high mite infestation were used from the Michigan State University apiary on 15 September 2006. We collected seven castes/stages of bees from each colony (worker larvae, worker pupae, nurses, foragers, drone larvae, and drone pupae and adult drones; 5 or 10 each). Each sample was put in a pre-weighed 1.5 ml Eppendorf tube. Each tube with sample was then weighed, dried in an oven (80 °C, 24 h), then weighed again. The difference in weight before and after drying was considered to be water content.

Adult female varroa mites were collected from drone brood or directly from newly emerged bees from heavily infested colonies by sugar dusting. Mites (50-65) were placed in pre-weighed 1.5 ml Eppendorf tubes; and four tubes were used for determining mite water content. The total number of mites was determined after water content determination.

Statistical analysis

All data from experiments with more than two treatments were analyzed by analysis of variance (ANOVA) using General Linear Models (Proc GLM) in SAS 9.1.3 (SAS Institute 2006). Mortality and water content data were transformed by taking the square root first, then taking its arcsin to obtain normal distribution

before ANOVA. Duncan's multiple range test (Duncan's MRT) was used to detect differences among treatments after ANOVA found a significant effect. For experiments involving only two treatments (sensitivity to microwave of nurses vs. varroa, and pupae vs. varroa), Student t-tests were used.

Results

Experiment 1: Forager sensitivity to microwave

The purpose of this experiment was to find the longest microwave duration that bees can tolerate and use that duration for subsequent experiments to test for its effect on mites. There was significant difference in sensitivity to microwave among the three different colonies ($F = 8.76$; $df = 2, 29$; $P = 0.001$) as well as among the treatments ($F = 156.37$; $df = 4, 29$; $P < 0.0001$). The treatment difference was consistent across all colonies because the interaction between colony and treatment was not significant ($F = 1.18$; $df = 8, 29$; $P = 0.36$). We therefore present the data pooled across the colonies and analyzed the effect of microwave duration. Foragers experienced high mortality (55 ± 7 %), after being microwaved for only 4 s. Mortality reached 93 % after 10 s (Figure 2.1). Only 1 s microwave treatment did not significantly increase forager mortality compared to the control. These data suggest that honey bees are highly sensitive to microwave radiation.

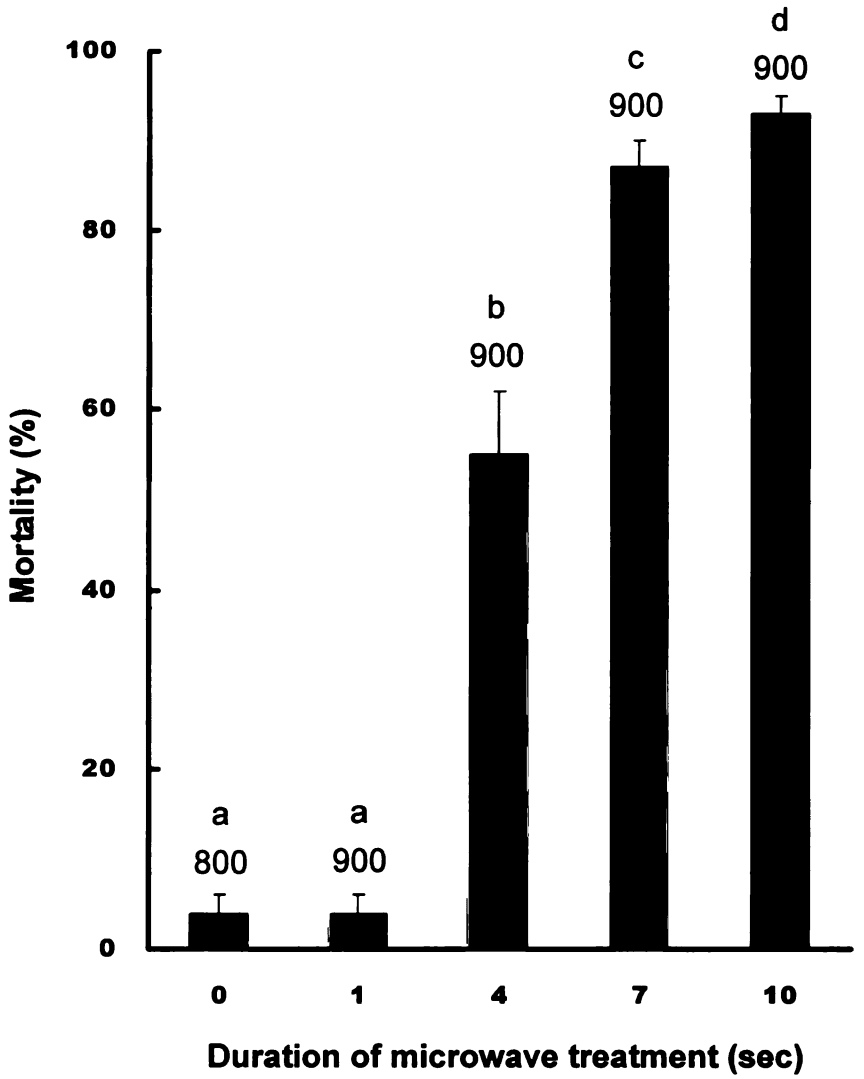

Figure 2.1. Mean (+SE) mortality of foragers after treating with microwave of various durations. Means with different letters are significantly different from each other (Duncan's multiple range test, $P < 0.05$). The number on top of each bar indicates the number of workers tested. Data are based on 3 different colonies.

Experiment 2: Relative sensitivity of nurses and mites to microwave

The hosts of phoretic mites are nurses so that it is important to understand the relative sensitivity of nurses and mites to microwave. Twenty grams of nurses contained 183 ± 2 bees and carried from 15 to 88 varroa mites among four colonies (42 ± 4). Microwave duration and organism type showed significant effects (microwave duration: $F = 302.38$; $df = 1, 9$; $P < 0.0001$; and organism type: $F = 263.48$; $df = 1, 9$; $P < 0.0001$) (Figure 2.2). The interaction between microwave duration and organism type were significant ($F = 200.07$; $df = 1, 9$; $P < 0.0001$). Mortality of honey bee workers was 56 ± 3 % after 4 s microwave treatment, similar to the first experiment without mites (55 ± 7 %). This mortality was highly significantly different (t-test, $P < 0.0001$) from that of the control (1 ± 1 %). Varroa mortalities also were significantly different (t-test, $P < 0.01$) between the treatment (2 ± 1 %) and control (0 + 0 %), however this is mainly due to the 0 % mortality and its lack of variation. The 2 % increase can be argued as biologically insignificant. In the 4 s microwave treatment, the mortality was significantly different between varroa mites and worker bees (t-test, $P < 0.05$).

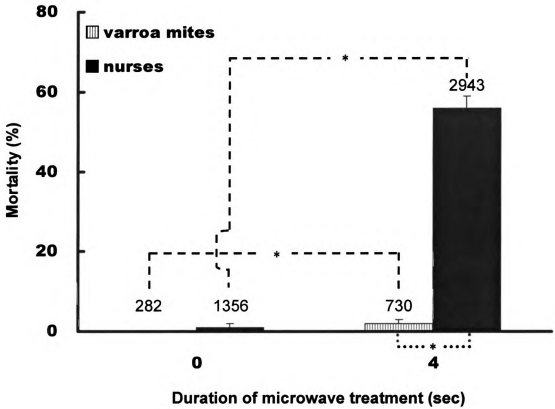

Figure 2.2. Mean (+SE) mortality of nurses and varroa mites when untreated (0 s) and after 4 s microwave treatment. Mortality of 4 s treated nurses are significantly higher than that of untreated nurses (t-test, $P < 0.05$). Mortality of 4 s treated mites are significant higher than that of untreated mites (t-test, $P < 0.05$). After 4 s microwave treatment, the mortality of nurses is significant higher than that of mites (t-test, $P < 0.05$). The number on top of each bar is the number of workers tested. Data are based on 3 different colonies.

Experiment 3: Relative sensitivity of pupal bees and mites to microwave

The difference in mortality between two organisms, worker pupae and mites (both immature and adults), and microwave duration (0 s and 20 s) were not significant (organism type: F = 3.04; df = 1, 2; $P = 0.223$; microwave duration: F = 6.09; df = 1, 2; $P = 0.132$). There was no interaction between organism and microwave duration (F = 1.95; df = 1, 2; $P = 0.297$) (Figure 2.3A). Likewise, there was no significant difference between the mortality of mites and that of pupae in the 20 s microwave treatment after the mortalities were adjusted by subtracting the natural mortality in the control for both organism types (t-test, $P > 0.05$) (not shown in the Figure).

When only adult mites are considered, excluding the immature mites, microwave duration showed significant effect (F = 18.35; df = 1, 2; $P = 0.05$) but organism types was not significant effect (F = 0.09; df = 1, 2; $P = 0.792$). There was no interaction between microwave duration and organism (F = 0.47; df = 1, 2; $P = 0.565$) (Figure 2.3B). In 20 s microwave treatment, the mortality of mites was lower than that of pupae but not significantly different (t-test, $P > 0.05$). Likewise, there was no significant difference between the mortality of adult mites and that of pupae in the 20 s microwave treatment after the mortalities were adjusted by subtracting the natural mortality in the control for both organisms (t-test, $P > 0.05$) (not shown in the Figure).

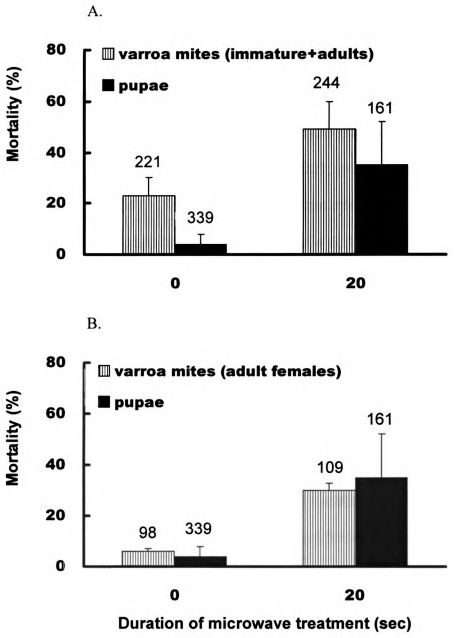

Figure 2.3. Mean (+SE) mortality of worker pupae and varroa mites of various stages (A) or adult female mites only (B) when untreated (0 s) and after 20 s microwave treatment. The number on top of each bar is the number of pupae or mites tested. The mortality of adult female mite in 20 s microwave treatment is significantly higher than that of the control (t-tests, $P < 0.05$). Data are based on 3 different colonies.

Experiment 4: Relative sensitivities of adult bees, pupae, and mites to microwave

ANOVA revealed no significant variation among colonies ($F = 2.87$; $df = 2, 12$; $P = 0.09$) but microwave duration and organism type effects were significant (microwave duration : $F = 75.81$; $df = 2, 12$; $P < 0.0001$; and organism type: $F = 38.88$; $df = 3, 12$; $P < 0.0001$). There was a significant interaction between microwave duration and organism type ($F = 20.85$; $df = 6, 12$; $P < 0.0001$). Therefore, we compared mortality among different organism under each microwave duration using Duncan's MRT (Figure 2.4). For the control group, the mortality of worker pupae (14 ± 6 %) was significant higher than that of the other three organisms, foragers, nurses, and mites, all which showed 0 mortality. In 3 s and 5 s treatments, the mortality of foragers and nurses were not significantly different from each other, but both were significant higher than that of mites. We also compared the mortality among three microwave durations (0, 3, 5 s) within each organism using Duncan's MRT. The mortality of foragers and nurses were significantly higher after 3 s and 5 s microwave treatment compared to the control. The mortality of pupae and varroa mites were not significant different among the three microwave durations.

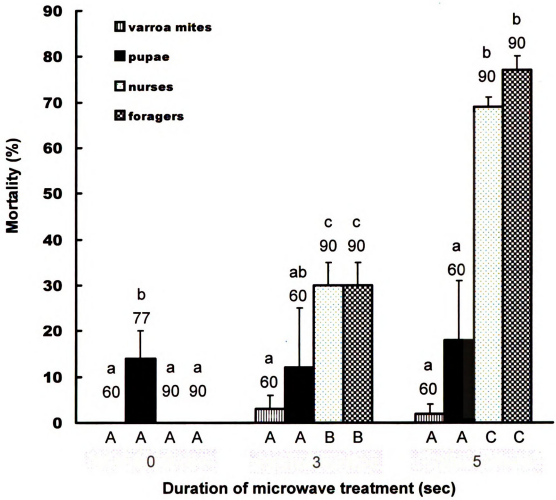

Figure 2.4. Mean (+SE) mortality of varroa mites and various stages of honey bees (pupae, nurses and foragers) after 0, 3 or 5 s of microwave treatment. Means with different letters are significantly different from each other (Duncan's multiple range test, $P < 0.05$). The lower case letter on the top of each bar indicates comparisons for various organisms under each microwave duration. The upper case letter on the bottom of each bar indicates comparisons for various microwave durations under each organism. The number on top of each bar is the number of workers and mites tested. Data are based on 3 different colonies.

Water content

Because microwave produces heat by vibrating molecules that have electric dipoles (water and other irons), and water is the predominant molecule in organisms, we determined the water content of mites and bees to see if differences in water content can explain their differential sensitivities to microwave. Varroa mites contained significantly less water than honey bee pupae and larvae (both drone and workers), and also nurses (Duncan's MRT, $P < 0.01$), but contained similar water levels (Duncan's MRT, $P > 0.05$) as foragers, and drones (Table 2.1). The average biomass of individual varroa mite was 395 ± 11.9 µg, which is consistent with Garedew et al. (2004) who estimated the biomass of varroa mites from worker brood as 395 $\pm$ 43 µg.

Table 2.1. The biomass and water content of mites and bees of various stages. Means of water content followed by different letters are significantly different from each other (Duncan's multiple range test, $P < 0.05$). Data are based on 4 different colonies.

Organisms	Sample size (No.)	Biomass (Means ± SE) (mg)	Water content (Means ± SE) (%)
Worker Larvae	30	118.1 + 3.9	77.96 ± 0.38 a
Worker Pupae	30	139.9 + 2.1	77.81 ± 0.19 ab
Nurses	29	100.4 ± 2.9	77.17 ± 0.68 ab
Foragers	30	98.2 ± 3.0	69.59 ± 0.97 c
Drone Larvae	27	208.2 ± 20.2	76.67 ± 1,16 ab
Drone Pupae	30	308.1 ± 2.1	75.22 ± 0.46 b
Adult Drones	30	236.4 ± 7.2	71.98 ± 0.88 c
Adult female Varroa	223	0.395 ± 0.012	70.10 ± 1.28 c

Discussion

We conducted three experiments to test relative sensitivity of mites and bees of various castes/stages to microwave radiation in order to explore the possibility of using microwave for controlling varroa mites. The pupae and mites were highly tolerant to microwave radiation. Foragers contained less water than pupae, yet foragers were much less tolerant of microwave, as indicated by their higher mortality at the 5 s microwave treatment in experiment 4 (Figure 2.4). Foragers contained the same amount of water as mites (Table 2.1), yet their mortality was much higher than that of mites (Figure 2.2). Similarly, foragers contained less water than nurses, but there was no significant difference between the two types of bees in mortality after microwave treatment. Therefore, differential sensitivity to microwave does not seem to be related to different water levels in different organisms.

Pupae are highly tolerant to microwave radiation compared with adult bees. Over 50 % nurses died after 4 s microwave treatment (per 200 bees, Figure 2.2), yet only about 35 % pupae died after 20 s microwave treatment (per 100 pupae, Figure 2.3). In experiment 4, pupal mortality after 5 s microwave treatment were below 20 %, while both foragers and nurses had over 70 % mortality (Figure 2.4). These results suggest that honey bee pupae have a higher tolerance to microwave than adult honey bees. This could be due to honey bee pupae having higher heat tolerance than adults, similar to other insects such as silkworms, house flies, and blow flies (Joy and Gopina 1995, Tiwari et al. 1995,

Tiwari et al. 1997). Honey bee pupae might also be able to lose heater faster due to a higher evaporation rate because pupal cuticle is yet not fully chitinized.

Varroa mites also have much lower mortality after microwave treatment compared with adult bees (Figure 2.4). Previous studies have shown that mites are less tolerant to heat compared to honey bees (Hoppe and Ritter 1986, Rosenkranz 1987, Appel and Büchler 1991), so the difference we observed here must be specifically due to the way microwave heats organisms. One possible reason is that the smaller body size (0.25 mm height x 1.7 mm long diameter, 1.1 mm short diameter) gives varroa mites a much higher surface to volume ratio. Assuming that a mite is an elliptic cylinder, the surface to volume ratio is 8.29 (surface to volume ratio is calculated as [(1.7 mm/2)(1.1 mm/2) $\pi \times 2$ + 0.3168×0.25 mm]/ (1.7 mm/2)(1.1 mm/2) $\pi \times 0.25$ mm]). For adult bee (13 mm height, 6.6 mm diameter), the surface to volume ratio is 0.75 if we assume a cylinder approximate to its volume (surface to volume ratio is calculated as [(6.6 mm/2)$^2\pi \times 2$ + 6.6$\pi \times 13$ mm]/(6.6/2)$^2\pi \times 13$ mm]). Thus there is about a 11 fold difference in their surface to volume ratios, enabling mites to cool off more efficiently by radiation and convection. It is also possible that varroa mites and bees of various stages have different dielectric constants which affect heating by microwave. Besides water content, other molecules such as other dipoles and ions (e.g. salts) also affect the dielectric properties of biological materials (Ryynänen 1995), hence their ability to respond to microwave radiation. Dielectric properties of other insects and their suitabilities for being controlled by microwave have been studied (Nelson and Payne 1982, Colpitts et al. 1992,

Andreuccetti et al. 1994, Ikediala et al. 2000, Wang et al. 2003), but so far there are no data on either the honey bee or the varroa mite.

In Experiment 3, the high mortality of total varroa mites in the control group might be due to the death of males and female protonymph during experimental manipulations e.g. cutting brood into pieces by table saw. Martin (1994) also observed that the later nymph of mites often failed to develop and subsequently die in worker brood as the bee developed. Adult varroa mites showed very low mortality (< 5%) after microwave, when they were attached to adult bees (Figure 2.2), but moderate mortality (~ 30 %) when they were sealed with pupae, and at a longer microwave duration (Figure 2.3). It is possible that mites attached on pupae have more limited ways to lose heat. For example they have almost no convection (no air flow inside sealed cells), and radiation is also limited because they are spatially close to the pupae which are receiving microwave radiation and maintaining a high temperature. Previous studies mentioned that varroa mites begin to be affected above 38°C (De Jong 1997) and die at 44°C after 4 hours (Appel and Büchler 1991, BrØdsgaard and Hansen 1994). We measured temperature inside brood cell by a thermocouple thermometer after 20 s microwave treatment. The average temperatures inside inner capped cell reached 43.7 ± 0.4 °C. In contrast, the air temperature inside a microwave remains low (a mere increase of 1.4 ± 0.4 °C after 20 s microwave treatment), so mites attached to adult bees can lose heat both through convection and radiation, resulting a lower mortality.

In summery, we discovered that even though varroa mites are known to be more sensitive to heat when heat was provided conventionally, they are not more sensitive to microwave radiation. In fact, they are less sensitive to microwave radiation compared to honey bees. We therefore conclude that 2.45 GHz microwave is not a feasible method for varroa mite control. Any heat treatment to mites must be provided by conventional methods such as through an oven (BrØdsgaard and Hansen 1994, Tabor and Ambrose 2001) or through an embedded heater in the drone foundation (Huang, 2001).

Chapter 3: Exploring the Use of Essential Oil to Lower Mite Reproduction

Abstract

Compared to other acaricides essential oils are more environmental friendly and cost effective agents for varroa control. In this study three essential oils, thymol, origanum, and clove oils in different formulations, were examined for their effects on mite reproductions. We tested various concentrations of the three essential oils in different formulations to obtain a dose that did not harm honey bee larvae, then used this dose to study the effect of oils on rates of mite infestation and reproduction. Infestation rates of varroa mites in larval cells treated with neat clove oil, starch-encapsulated thymol, β-cyclodextrin encapsulated thymol, and β-cyclodextrin encapsulated origanum oil were significantly lower than that of their controls. Actual fertility, actual fecundity, and actual reproductive rate of varroa mites in larval cells treated with thymol crystals and β-cyclodextrin-encapsulated origanum oil were significantly lower than values for control groups. However, potential fertility, potential fecundity, and potential reproductive rate of varroa mites were not significantly different among brood cells treated with the two oils and the control. These results suggest that thymol and origanum oil prevented some mother mites from initiating reproduction, but did not decrease the reproduction of other mites that were reproducing. Essential oils did not delay the development of mite offspring. We concluded that some essential oils (neat clove oil, starch-encapsulated thymol, β-cyclodextrin-encapsulated thymol, and β-cyclodextrin-encapsulated origanum oil) reduced mite infestation and others (thymol crystals and β-cyclodextrin-encapsulated origanum oil) reduce mite

reproduction. Exploring the possibilities of using sub-lethal doses of essential oils for mite control can lead to reduced cost, chances of contamination, and reduced resistance development.

Key words: varroa mite, *Varroa destructor,* honey bee, *Apis mellifera*, essential oil, thymol, origanum oil, clove oil, mite reproduction

Introduction

Varroa mite, *Varroa destructor* (Acari: Varroidae) (Anderson and Trueman 2000), previously known as *Varroa jacobsoni* Oudemans, is currently the most serious threat to the European honey bee, *Apis mellifera* L. It has quickly spread worldwide (Matheson 1993, 1995) and negatively impacts pollination of many fruits and crops, leading to a great economic loss (Roberson 2006). The varroa mite has seriously damaged the beekeeping industry in the U.S.A. since their introduction in 1987 (Anonymous 1987).

Although varroa mites preferentially infest drone brood, they also infest worker brood of the European honey bee. Both immature and adult varroa mites feed on pupal hemolymph in capped brood (De Jong, 1997). Mite infestation increases mortality of pupae because pathogens (bacteria, fungi and viruses) can invade at the feeding site of pupae. The varroa mite also is a vector of bee viruses (Allen et al. 1986, Bowen-Walker et al. 1999, Bakonyi et al. 2002, Chen et al. 2004, Tentcheva et al. 2004). Even if infested pupae successfully emerge as adults; they can have shrunken wings due to deformed wing virus, shortened life spans (De Jong et al. 1982), and other changes in their behaviors, such as reduced learning capacity (Kralj and Fuchs 2006, Kralj et al. 2007). Infested colonies eventually develop parasitic mite syndrome (Sammataro et al. 2000) and die. Therefore, beekeepers must use acaricides to reduce mite population and prevent the death of colonies.

Currently, varroa mite control is largely based on the use of synthetic

acaricides. The most commonly used acaricide was Apistan®, with fluvalinate, a pyrethroid, as its active ingredient. However, resistance of varroa mite to pyrethroid has been documented in Europe (Lodesani et al. 1995, Thompson et al. 2002, Thompson et al. 2003), and the U. S. (Baxter et al. 1998a, Elzen et al. 1998). An organophosphate acaricide, coumaphos (Checkmite+®), has been used to control varroa mites after resistance to fluvalinate was discovered in varroa mites (Elzen et al. 2000). Varroa resistance to coumaphos has been reported in the U.S. and other countries (Spreaficom et al. 2001, Elzen and Westervelt 2002, Pettis 2004, Pettis and Jadczak 2005). Residues of both fluvalinate and coumaphos have been found in honey, beeswax (Wallner 1995, Bogdanov 2006) and pollen (Chauzat et al. 2006). These problems led to the development of alternative control methods for varroa mites such as essential oils (Imdorf et al. 1996, Sammataro et al. 1998).

Essential oils are extracted from natural aromatic plants and are less toxic to bees and other animals so they are more environmental friendly; they also less than synthetic acaricides (Isman 2000, 2006). About 150 essential oils have been tested for varroa mite control. The degree of toxicity and repellency (Colin 1990, Imdorf et al. 1999) of essential oils to varroa mites vary with oil's chemical composition and environmental conditions (Imdorf et al. 1999, Melathopoulos et al. 2000a, El-Zemity et al. 2006, Ruffinengo et al. 2007). Currently, two commercial products are based on the essential oil thymol for varroa control, Apilife VAR® and Apiguard. Although thymol residue can be found in honey, it does not affect honey taste when thymol residues are below 0.8- 1.1 mg /kg honey (Bogdanov et

al. 1999, Bogdanov 2006) and this chemical decreases rapidly through evaporation (Bogdanov et al. 1998b, Bogdanov et al. 1999, Floris et al. 2004). Beeswax can contain more thymol residue than honey, but the residue decreases rapidly when wax foundations and combs are exposed to air during storage (Bogdanov et al. 1998, Bogdanov et al. 1999, Floris et al. 2004).

Many field studies have shown that some essential oils can control varroa mites effectively (Chiesa 1991, Calderone et al. 1997, Al-Abbadi and Nazer 2003), but the mode of actions of essential oils remains unknown. Essential oils can have acute toxicity to phoretic mites by either direct contact or through vapor (Lindberg et al. 2000). The current study aims to determine whether smaller doses of essential oils can inhibit mite reproduction or be passed to the next generation to cause mortality or delay in development of mite offspring.

There are three possible mechanisms for an essential oil to inhibit mite reproduction. First, essential oil might be passed to larval food from nurses and resuce infestation of phoretic mites on worker brood. Second, essential oil could be absorbed into larval hemolymph, picked up by feeding mites, after which the oil could affect the mother mite directly. Non-lethal doses of essential oil might disrupt the reproduction of mother mites or interrupt their physiological functions such as muscle contraction. Third, the effect of oil could be on the offspring generation. For example, offspring could have trouble mating successfully so they are not fertile, or the development of offspring might be delayed, such that fewer daughter mites would mature when the host bee ecloses, or the offspring

may be killed by the oil during early development.

The objective of this study is to evaluate the effects of essential oils on varroa mite infestation and reproduction after the mother mites fed on the larvae, possibly ingesting essential oils through larval/pupal hemolymph. Varroa mites are not exposed to oil or oil vapor directly but would contact and feed on larvae artificially fed with known amounts of essential oil. We determined the toxicity of each selected essential oil (type and formulation combination) to four day old bee larvae. Then we used doses that were relatively nontoxic to bee larvae and tested their effects on varroa mite infestation and reproduction. Finally, we examined the number and statutes of varroa mite offspring and analyzed their population composition after treating with essential oils.

Materials and Methods

Collection of honey bee larvae and varroa mites

Honey bee larvae were collected from the apiary at Michigan State University, East Lansing, Michigan (42.75° N, 84.46° W), in summer 2007. Bees and mites were from colonies maintained according to standard beekeeping procedures. Over-wintered colonies or colonies started as packages (purchased spring of 2007) were used in this study.

Varroa mite populations were monitored every month since May 2007. Phoretic mites were removed by shaking nurses with confectioner's sugar inside a mason jar (Fakhimzadeh 2001). Nurses were collected from over-wintered

colonies with high varroa mite populations. Bee larvae of similar ages were obtained by confining a queen in a cage (24.5×5×35 cm, China) for 24-48 h. The cage had queen-excluding grids on both sides which allowed workers but not the queen to cross. After 24-48 h, the frame with eggs was taken out, and the queen was confined in the cage with another frame. This prevented the queen from laying more eggs on the experimental frame. The experimental frame remained in the colony until the larvae were 4 days old.

Chemicals

Essential oils in various formulations were provided by S.A.F.E. Research & Development, LLC (Tucson, AZ) and from the USDA Honey Bee Research Laboratory at Tucson, AZ. Thymol had three formulations, thymol crystals; 25 % starch-encapsulated thymol (hereafter referred to as starch-thymol), and β-cyclodextrin-encapsulated thymol (3.2 mg thymol/ gram β-cyclodextrin, βcd-thymol). Origanum oil had three formulations, neat origanum oil, 25 % starch encapsulated origanum oil (starch-origanum oil), and β-cyclodextrin-encapsulated origanum oil (2.4 mg origanum oil / gram β-cyclodextrin, βcd-origanum oil). Clove oil was provided as neat oil only. β-cyclodextrin (βcd) was used as the control for βcd-thymol and βcd-origanum oil. We did not use starch as a negative control because previous studies have shown that starch is not toxic to honey bees (Herbert et al. 1980). For the highest (stock) concentrations of solutions, thymol crystals, origanum oil and clove oil were dissolved in 99.5 % ethanol first, then mixed in 30 % sugar syrup, the final

aqueous solution contained 10 % ethanol and 30 % sugar. Lower concentrations of oils were made by serially diluting the stock solutions using 10 % ethanol and 30 % sugar solution. All encapsulated oils, i.e. starch-thymol (25 %), βcd-thymol, starch-origanum oil (25 %), βcd-origanum oil, and βcd were dissolved in 30 % sugar syrup.

Feeding bees with essential oils

Four-day-old larvae were fed the chosen essential oils at different doses (Table 3.1 and 3.2). For toxicity studies (Table 3.1), each larva was fed 10 µl food containing oils using an Eppendorf micropipette (Eppendorf Research®, Westbury, NY) for neat and encapsulated oils at different doses, except starch-encapsulated oils at the highest dose, which was fed 30 µl food (Table 3.1). Starch-thymol and starch-origanum oil were prepared as 20 µg oils/µl feeding solutions and 30µl (containing 0.6 mg thymol or origanum oil) was fed to each larva because their low solubility did not allow a concentration as high as 60 µg/µl. Four doses of βcd controls were used as controls for βcd-thymol or βcd -origanum oil.

For varroa mite infestation and reproduction experiment (Table 3.2), we prepared seven feeding solutions, one dose for each type/formulation of oil, and fed 10 µl to each larva. The food was carefully delivered into a cell near the larva mouthpart. The feeding solutions were vortexed before feeding each larva to completely mix the oil in the solutions.

We left at least one row of cells untreated between treatments to reduce the possibility of cross-contamination of different oils. Larvae were returned to the colony immediately after feeding. Each feeding regime, which includes four to five treatments for one frame, were finished within 2 hours. A solution of 10 % ethanol in 30 % sugar syrup was used as control for the three neat oils, and 30 % of sugar syrup as control for the encapsulated oils. The concentration of essential oil in the hemolymph from which varroa mites were feeding would be different from the dose we applied to larvae due to dilution in hemolymph and possible degradation. We therefore sampled larval hemolymph to measure its concentration of oil. These samples are currently being measured and we are not able to include them in this version of the report, but they will be included in the final manuscript.

The positions of each cell containing a 4 day old larva were marked on a letter-sized transparent plastic sheet, fixed to the frame by three pins. Different treatments were marked by different colors or symbols. The sheet was then removed after feeding, while the positions of pins were marked on the frame. After 5 days, the plastic sheet was placed on the brood comb again to map the positions of each treatment when the cells were checked for larval survival.

Toxicity tests of essential oil to honey bee larvae and pupae

The purpose of this experiment was to find a dose for each oil that is relatively nontoxic to bees to conduct the next study. Four-day-old larvae were obtained from three colonies on 28 July, 1, 3, 4, 11, 10, 24, 29 August, and 11

September 2007 and fed with essential oils. Doses of thymol with three formulations and clove oil were selected based on results of a previous study (Lindberg et al. 2000). All the tested doses were presented in Table 3.1. Two doses of βcd, 0.187 and 1.87 mg/larva, were used as controls for two doses of βcd-thymol because 0.0006 and 0.006 mg thymol were encapsulated in 0.187 and 1.87 mg βcd respectively. Two doses of βcd, 0.25 and 2.5 mg/larva were used as controls for two doses of βcd-origanum oil because 0.0006 and 0.006 mg origanum oil were encapsulated in 0.25 and 2.5 mg βcd respectively. Each treatment had 20 larvae and the experiment was repeated in 2 or 3 colonies. We checked larval survival as described 5 days later, then incubated the puape at 34 °C, 65 % RH until adult eclosion when numbers of emerged adult bees were counted.

Effect of essential oil on varroa mite infestation and reproduction

Four-day-old-larvae were collected from three colonies on 4, 5, 7, 9 October 2007 and treated with essential oils. Based on the toxicity data to honey bees, we selected one appropriate dose for each formulation of oils (Table 3.2). βcd was applied to larvae either as control of βcd-thymol (0.1875 mg/larva), or as control of βcd-origanum oil (0.25 mg/larva), in both cases the oil concentration was 0.0006 mg/larva even though βcd concentration was different. We used three different controls for larvae receiving essential oils: natural (no artificial feeding), 10 % ethanol in 30 % sugar syrup, and 30 % sugar syrup. Each treatment had 50 larvae and the experiment was repeated in 4 colonies.

Feeding procedure was the same as the toxicity experiment. We checked for varroa infestation and offspring status when pupae turned dark brown (1 day before emergence). The rate of infestation was calculated as the numbers of cells with mites divided by the numbers of examined cells (number of cells with mites + number of cells without mites).

We also recorded the numbers and status (whether live or dead, except for eggs whose status we could not determine) of: eggs, male immature and adults, female protonymph, deutonymph and adults and the location of mite defecation from 3 colonies on 5, 7, 9 October 2007 to evaluate mite reproduction. We distinguished immature males from newly hatched female protonymphs by the description of Ifantidis (1983). Daughter mites in general are lighter in color compared to mother mites. When many adult reddish-brown females were present in a cell, and their colors are similar, the number of males and adult female offspring were used as a guide to determine the number of mothers. The remainder reddish-brown adult females were count as daughter females. For example, if there were 5 mature adult mites in one cell, and there were 3 males, most likely three mother mites invaded the cell and 2 were daughter mites. This is because in general one mother mite produces one male, the first offspring, the remainder all being females. The number of exuvia of deutonymph is another indicator to separate the mother and her daughter mites: for example, if there were 3 mature adult mites but only one deutonymph skin, then most likely 2 mother mites invaded the cell and one produced a mature daughter mite (Ifantidis 1983, Martin 1994, Martin 1995b). The numbers of examined cells were less

than the treated cells because some larvae or pupae were removed by nurse bees in colonies. Mite reproduction was evaluated by mite fertility (percentage of sampled cells with egg laying female mites), mite fecundity (number of eggs per mother mite), and mite reproductive rates (number of viable female offspring per mother mite). The following parameters were calculated below according to previous studies (Ifantidis 1984, Alattal et al. 2006):

Mite fertility:

Actual mite fertility= the number of brood cells with reproducing mites (i.e. mother with offspring) divided by the number of cells with mother mites including dead and living mother mites.

Potential mite fertility= the number of brood cells with reproducing mites divided by the number of cells with living mother mites.

Mite fecundity:

Actual mite fecundity = the number of offspring (egg, male and female) divided by the number of mother mites (reproducing and non-reproducing)

Potential mite fecundity = the number of offspring (egg, male and female) divided by the number of reproducing mother mites

Mite reproductive rates:

Actual mite reproductive rate = the number of viable adult female offspring divided by the number of mother mites (reproducing and non-reproducing)

Potential mite reproductive rate = the number of viable adult female offspring divided by the number of reproducing mother mites

Mite density was denoted as the number of total mites (including offspring plus mother) in one worker brood cell. It was an indicator combing both the infestation (more than one mother per cell) and fecundity. The proportions of various stages and status of mites in different treatments were used to evaluate the effect of selected oils on offspring. The proportion in each treatment was denoted as the number in each category which was classed by mite stage, status, and gender (Table 3.7) divided by the total number of mites examined in each treatment.

Statistical analysis

Prior to statistical analysis, percentage data were transformed by taking its square root then its arcsin. Untransformed values are shown in the tables and figures. Analysis of variance (ANOVA) was conducted by using General Linear Model (GLM) for almost all experiments and all pairwise comparisons to the control were performed by determining whether the 95 % confidence intervals of least square means overlap with one another (LSMEANS option in SAS). Analysis of covariance (ANCOVA) was performed for mite fecundity, with number of mother mites per cell as a covariable. Multivariate analysis of variance (MANOVA) was performed for data of mite population compositions to determine whether oil treatment had effects on the proportion of different mite stages, status (live or dead), and genders. All statistical analyses were performed by SAS (SAS institute 2006).

Table 3.1. Tested doses of various essential oils for feeding 4 day old larvae

Treated Chemicals	Concentrations of Food solution (mg/µl)	Feeding volume (µl)	Doses (mg/larva)
Thymol crystals**	0, 0.00006, 0.0006, 0.006, 0.06, 0.15	10, 10, 10, 10, 10, 10	0, 0.0006, 0.006, 0.06*, 0.6, 1.5
Starch-encapsulated Thymol (25%)	0, 0.00006, 0.0006, 0.006, 0.02	10, 10, 10, 10, 30	0, 0.0006, 0.006, 0.06, 0.6
β-cyclodextrin encapsulated thymol (3.2 mg/gram)	0, 0.00006, 0.0006	10, 10, 10	0, 0.0006, 0.006
β-cyclodextrin as control for β-cyclodextrin encapsulated thymol	0, 0.0187, 0.187	10, 10, 10	0, 0.187, 1.87
Origanum oil**	0, 0. C00006, 0.0006, 0.006, 0.06	10, 10, 10, 10, 10	0, 0.0006, 0.006, 0.06, 0.6
Starch-encapsulated origanum oil (25%)	0, 0.00006, 0.0006, 0.006, 0.02	10, 10, 10, 10, 30	0, 0.0006, 0.006, 0.06, 0.6
β-cyclodextrin encapsulated origanum oil (2.4 mg/gram)	0, 0.00006, 0.0006	10, 10, 10	0, 0.0006, 0.006
β-cyclodextrin as control for β-cyclodextrin encapsulated origanum oil	0, 0.025, 0.25	10, 10, 10	0, 0.25, 2.5
Clove oil**	0, 0.00016, 0.0016, 0.016, 0.16	10, 10, 10, 10, 10	0, 0.0016, 0.016, 0.16*, 1.6

* The best dose for killing varroa mites in Lindberg et al. 2000.

** Dissolved into 10% ethanol in 30% sugar syrup. Other encapsulated formulations of oils were dissolved in 30% sugar syrup (without **)

Table 3.2. The doses for feeding 4 day bee larvae in experiments measuring infestation rate and reproductive rate

Oil Type	Formulation	Feeding dose (mg/larva)
Thymol	Thymol crystals*	0.15 mg
	Starch-encapsulated thymol	0.06 mg
	β-cyclodextrin- encapsulated thymol (3.2 mg/gram)	0.0006 mg
	β-cyclodextrin (control of βcd-thymol complex)	0.187 mg
Origanum	Origanum oil*	0.06 mg
	Starch-encapsulated origanum oil	0.06 mg
	β cyclodextrin encapsulated origanum oil (2.4 mg/gram)	0.0006 mg
	β-cyclodextrin (control of βcd-origanum oil)	0.25 mg
Clove	Clove oil*	0.16 mg

* Dissolved into 10% ethanol in 30% sugar syrup. Other encapsulated formulations of oils were dissolved in 30% sugar syrup (without **)

Results

Toxicity tests of essential oil to honey bee larvae and pupae

Effects of three thymol formulations on larvae and pupae

Survival rates of larvae treated with the three formulations of thymol are presented in Figure 3.1A. Both oil formulation and dose effects on larval survival rates were significant (oil formulation: $F = 8.01$; $df = 2,15$; $P = 0.004$, and dose: $F = 24.3$; $df = 5,15$; $P < 0.0001$). For thymol crystals, the highest dosage (1.5 mg/larva) yielded a significantly lower larval survival rate compared to the control (LSMEANS, $P < 0.05$), but the second highest dose (0.6 mg/larva) was not significant (LSMEANS, $P > 0.05$). For starch-thymol, both the highest (0.6 mg/larva) and the second highest dose (0.06 mg/larva) significantly reduced larval survival rates compared to the control (LSMEANS, $P < 0.05$). For βcd-thymol, the highest dose (0.006 mg/larva) significantly lowered larval survival rate compared to the sugar syrup control (LSMEANS, $P < 0.05$). Survival rates of βcd-thymol and βcd treated larvae were not significantly different across the doses (ANOVA, $F = 1.38$; $df = 1,10$; $P = 0.27$). The βcd at the dose of 1.87 mg/larva, which was the control for βcd-thymol at the dose of 0.006 mg/larva, significantly resuced larval survival rate compared to the sugar syrup control (LSMEANS, $P < 0.05$; Figure 3.2). Nearly all mortality occurred at the larval stage, therefore the cumulative mortality of pupae (including larval mortality) and larval mortality were nearly identical for thymol, origanum oil and clove oil treated larvae. We therefore only presented larval mortality here.

Effects of three origanum oil formulations on larvae and pupae

Survival rates of larvae treated with the three formulations of origanum oil are presented in Figure 3.1B. Both oil formulation and dose significantly affected larval survival rates (oil formulation: $F = 7.35$; $df = 2,14$; $P = 0.006$, and dose: $F = 21.31$; $df = 4,14$; $P < 0.0001$). For neat origanum oil, the highest dose (0.6 mg/larva) significantly reduced larval survival rate compared to the control (LSMEANS, $P < 0.05$). For starch-origanum oil, the highest dose (0.6 mg/larva) and the second highest dose (0.06 mg/larva) significantly reduced larval survival rates compared to the control (LSMEANS, $P < 0.05$). For βcd-origanum oil, the highest dose (0.06 mg/larva) significantly reduced larval survival rate compared to the sugar syrup control (LSMEANS, $P < 0.05$). Survival rates of larvae treated with βcd-origanum oil and those treated with βcd were not significantly different from each other and across the doses (ANOVA, $F = 0.006$; $df = 1,10$; $P = 0.94$). The βcd at the dose of 2.5 mg/larva, which was the control of βcd-origanum oil at the dose of 0.006 mg/larva, significantly reduced larval survival rate compared to the sugar syrup control (LSMEANS, $P < 0.05$, Figure 3.2).

Effects of clove oil on larvae and pupae

Only neat clove oil was tested and the larval survival rates are presented in Figure 3.1C. All larvae died after feeding with 1.6 mg/larva clove oil, this survival rate was significantly lower than that for the control (LSMEANS, $P < 0.05$). There was no significant difference in larval survival rates between each of the other three doses and the control (LSMEANS, $P > 0.05$).

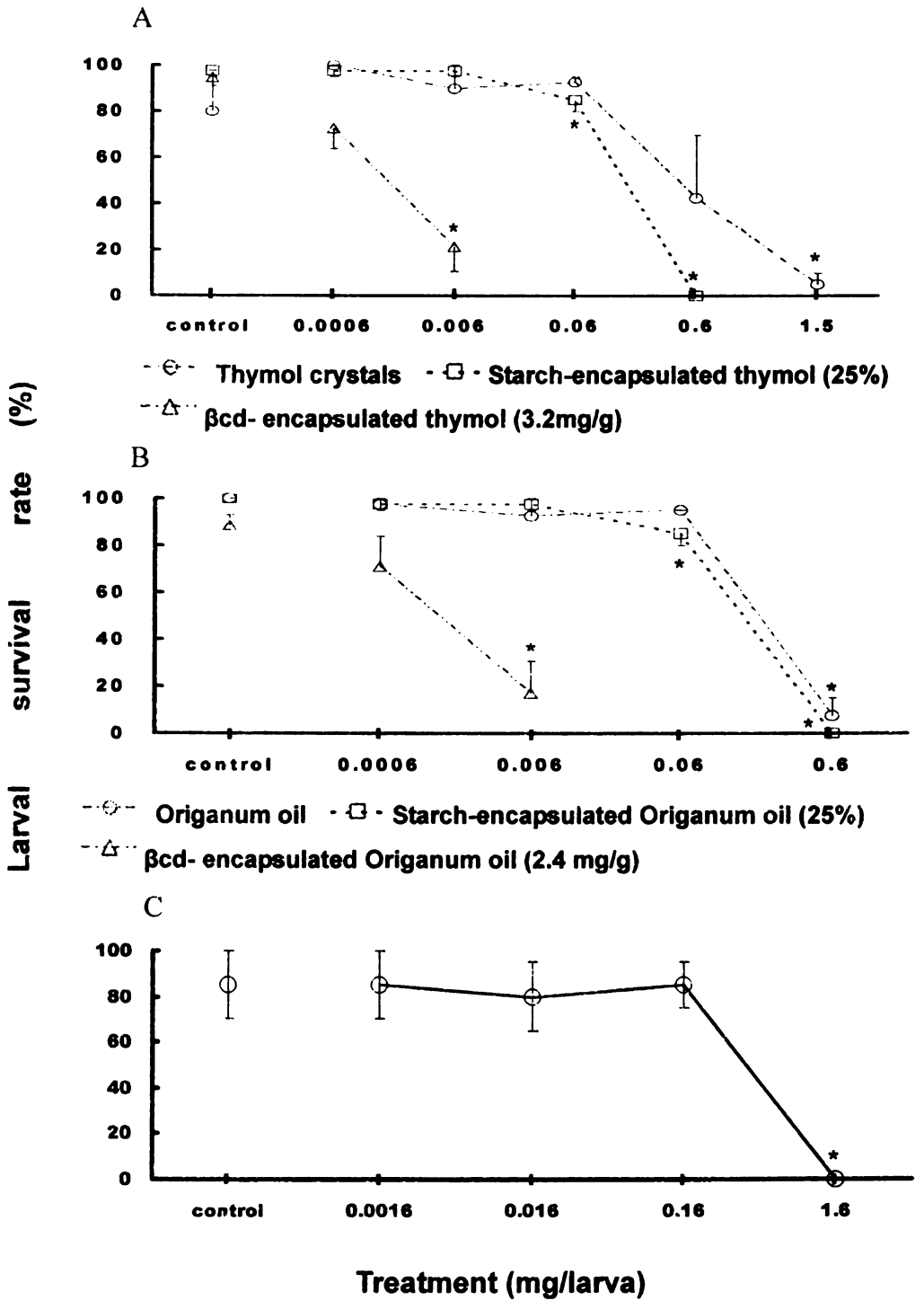

Figure 3.1. Survival rates of larvae and pupae (mean ± SE) after 4-day-old larvae were fed with thymol (A), origanum oil (B) in various formulations and neat clove oil (C). For the neat oils, 10% ethanol in 30% sugar syrup was fed as a control. For starch-encapsulated and β-cyclodextrin-encapsulated oils, 30% sugar syrup was used as a control. The experiment with βcd-encapsulated oils was replicated in 3 colonies; others oils in 2 colonies. Means marked with * are significantly different from the control (LSMEANS, $P < 0.05$).

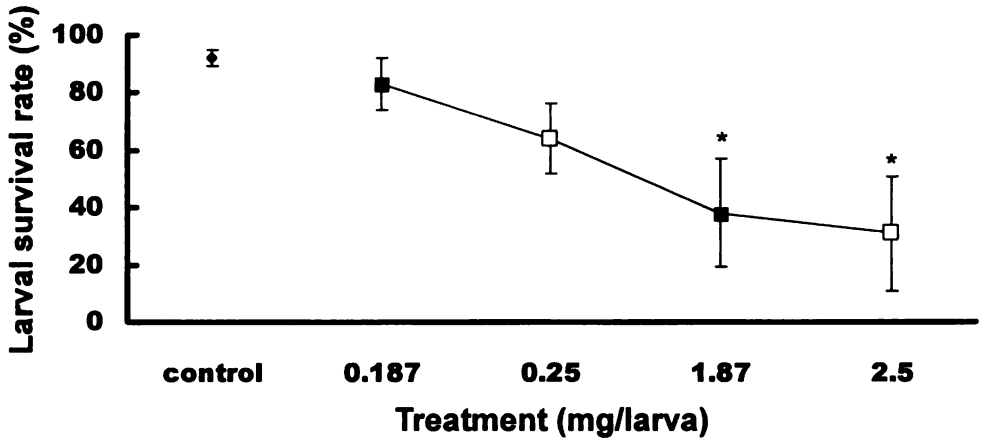

Figure 3.2. Effect of β-cyclodextrin on larval survival. Thirty percent sugar syrup was used as a control for βcd. Experiments replicated in 3 colonies. Means marked with a * are significantly different from the control (LSMEANS, $P < 0.05$).

- ■ βcd 0.187 mg/larva was the control for βcd-encapsulated thymol 0.0006 mg/larva
- ■ βcd 1.87 mg/larva was the control for βcd-encapsulated thymol 0.006 mg/larva
- □ βcd 0.25 mg/larva was the control for βcd-encapsulated origanum 0.0006 mg/larva
- □ βcd 2.5 mg/larva was the control for βcd-encapsulated origanum 0.006 mg/larva

Effect of essential oils on varroa mite infestation and reproduction

Effect on infestation rates

Based on the results of toxicity tests on bee larvae, one dosage was chosen from each oil/formulation to test their effects on mite infestation and reproduction (Table 3.2). The number of examined brood was less than 50 for each treatment per colony because some larvae were removed by bees (as reflected by the low mortality during larval stage). Varroa infestation rates of 0.25 and 0.1875 mg/larva βcd treated larvae (N = 44 and 42, respectively) were 27 % and 45 %

respectively. Rates of mite infestation were significantly different among the ten treatments including three controls (ANOVA, F = 3.06; df = 9,27, $P = 0.012$) (Figure 3.3). Varroa infestation rate for βcd-origanum oil treated larvae was the lowest and significantly differed from that of its control, sugar syrup (LSMEANS, $P < 0.05$). Varroa infestation rates for starch-thymol and βcd-thymol treated larvae were significantly lower than its control, sugar syrup (LSMEANS, $P < 0.05$). Clove-oil-treated larvae were significantly less infestated than control, 10 % ethanol in 30 % sugar syrup (LSMEANS, $P < 0.05$). There was no significant difference in mite infestation rates between larvae fed sugar syrup and 10 % ethanol in sugar syrup (LSMEANS, $P = 0.97$). The infestation rate of the un-fed control ("natural") was significantly lower than larvae fed with sugar syrup at $P = 0.053$.

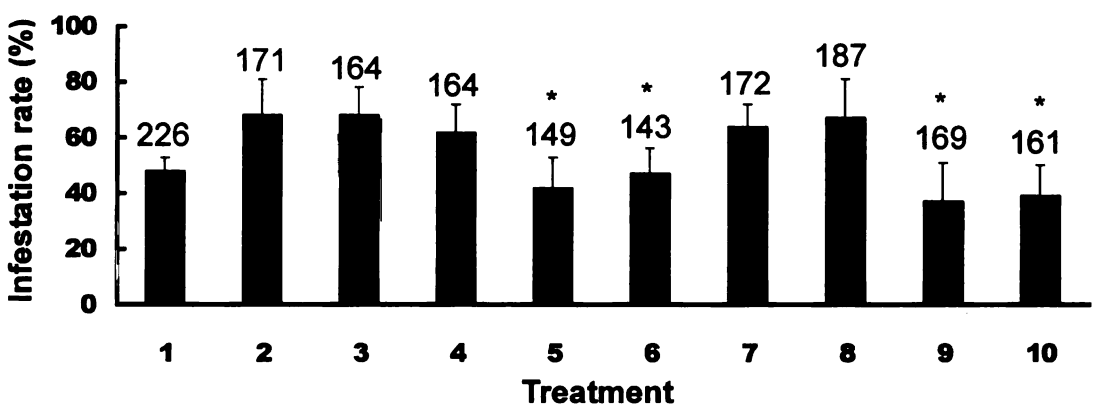

Figure 3.3. Infestation rates (mean ± SE) of varroa mites in different treatments. Treatments: **1.** Natural (no artificial feeding), **2.** 30% sugar syrup, **3.** 10% ethanol in 30% sugar syrup, **4.** Thymol 0.15 mg/larva, **5.** Starch-encapsulated thymol (25%) 0.06 mg/larva, **6.** βcd-encapsulated thymol (3.2 mg/g) 0.0006 mg/larva, **7.** Origanum oil 0.06mg/larva, **8.** Starch-encapsulated origanum (25%) 0.06 mg/larva, **9.** βcd-encapsulated origanum oil (2.4 mg/g) 0.0006mg/larva, **10.** Clove oil 0.016 mg/larva. The number on the top of each bar is the sample size. Treatment 2 was the control for treatment 5, 6, 8, and 9. Treatment 3 was the control for treatment 4, 7, and 10. Bar with * on top indicate it is significantly different from the control (LSMEANS, $P < 0.05$). N = 4 colonies.

Effect on fertility

Actual fertility of mites are presented in Figure 3.4. Some mother mites died without any offspring (column E in Table 3.3). Little or no mite defecation was found in this type of brood cell. There were significant differences in actual fertility among the treatments (Figure 3.4, F = 3.3; df = 9,18; $P = 0.0149$). Actual fertility was significantly lower in thymol-crystal-treated brood and in βcd-origanum-oil-treated brood, compared to the control (LSMEANS, $P<0.05$). Potential fertility of mites did not differ among the treatments and the controls (Figure 3.4, F = 1.06; df = 9,18; $P = 0.43$).

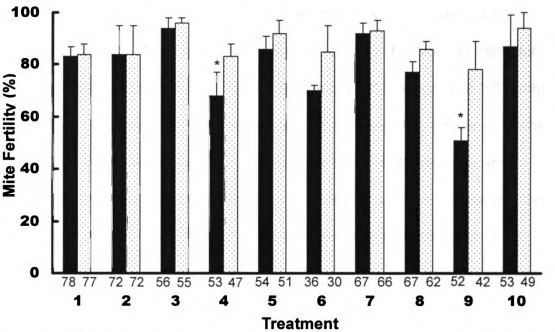

Figure 3.4. Fertility (mean ± SE %) of varroa mites in different treatments. Treatment legend the same as Figure 3. The number on the bottom of each bar is the sample size of sampled cells. ■ Actual fertility: % was calculated from total sampled cells which included the cells with living mother mites and the cells with dead non-reproducing mother mites. ⬚ Potential fertility: % was calculated from cells with living mother mites only. Pairewise comparisons between a treatment and its control were conducted for actual and potential reproduction rate separately. Means marked a * are significantly different from their control (LSMEANS, $P < 0.05$). Experiment was replicated in 3 colonies.

Effect on fecundity

Mite fecundity data are presented in Table 3.3. ANCOVA indicated significant difference among all the treatments in actual fecundity ($F = 2.59$; df = 9,18; $P = 0.04$) but there was no significant difference in potential fecundity ($F = 1.06$; df = 9,18; $P = 0.43$). The actual fecundity of mites in brood treated with thymol crystals, and βcd-origanum oil was significantly lower than that of their control (LSMEANS, $P < 0.05$). The number of mother mites per cell significant reduced mite fecundity (ANCOVA, $F = 15.89$, df = 1,555, $P = 0.0005$ for actual fecundity and $F = 39.65$, df = 1,446, $P < 0.0001$ for potential fecundity). Mean number of mother mites per cell in various treatments was not significantly different (Table 3.4). Most cells were infested by one or two mother mites for the control and the oil treated larvae (Table 3.5), the overall average was 1.6 mother mite per cell. The fecundity per mother decreased when the number of mother mites per cell increased (Table 3.5). Only around 4 % sampled cells were invaded by more than four mother mites in all treatments. The highest number of mother mites was found in clove oil treatment, two cells sampled from clove treatment were invaded by six mother mites, their fecundity was extremely low (0.42 ± 0.08 offspring per mother).

Table 3.3. Mite fecundity in different treatments

Treatment*	Sampled cells (n_1)†	Total progeny of mite	Female mites of parental generation (mother mites)				Fecundity	
			Total	Reproducing	Non-reproducing		Actual fecundity (progeny/total mites)	Potential fecundity (progeny /reproducing mites)
					Living	Dead (n_2)††	Mean ± SE (a/b)	Mean ± SE (a/c)
		a	b	c	d	e		
1	78 (65)	300	122	104	15	3 (0)	2.54±0.17 (2.46)	3.05±0.14 (2.88)
2	72 (63)	317	124	114	9	1 (0)	2.59±0.18 (2.56)	2.96±0.16 (2.78)
3	56 (53)	264	106	101	2	3 (0)	2.64±0.19 (2.49)	2.80±0.18 (2.61)
4	53 (38)	162	87	65	9	13 (5)	1.96±0.22 ** (1.86)	2.73±0.20 (2.49)
5	54 (47)	204	78	69	5	4 (3)	2.71±0.21 (2.62)	3.12±0.18 (2.96)
6	36 (25)	96	53	37	7	9 (6)	1.92±0.28 (1.81)	2.77±0.25 (2.59)
7	67 (60)	333	127	120	6	1 (1)	2.67±0.17 (2.62)	2.99±0.14 (2.78)
8	67 (53)	236	107	87	14	6 (3)	2.26±0.18 (2.21)	2.86±0.15 (2.71)
9	52 (28)	125	81	45	19	17 (7)	1.65±0.24 ** (1.54)	3.07±0.22 (2.78)
10	53 (47)	225	93	83	5	5 (1)	2.90±0.24 (2.42)	3.28±0.21 (2.71)

* Treatment legends the same as Figure 3.3.

** Pairwise comparisons conducted within the same column and ** indicates that it is significantly different from the control (LSMEANS, $P < 0.05$).

† Sample size: the number of sampled cells for mite reproduction. Larger number before (n_1): the number of cells with mother mites; (n_1) : the number of cells with mother mites and offspring

†† n_2 : the number of mites without defecation on cell wall among those died without ever reproduce.

Table 3.4. Mean number of mother mites per cell in various treatments

	Treatments*									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
*Total female mites of parental generation** (mother mites/cell)*										
	1.56	1.72	1.89	1.64	1.44	1.47	1.89	1.59	1.55	1.75
*Reproducing female mites of parental generation*** (mother mites/cell)*										
	1.56	1.80	1.90	1.71	1.46	1.48	2.00	1.63	1.60	1.76

* Treatment legends the same as Figure 3.3.
** Total female mites of parental generation: data were from the cells contained reproducing and non-reproducing mother mites in each treatment.
*** Reproducing female mites of parental generation: data were from the cells contained reproducing mother mites only in each treatment.
No significance between treatments and controls.

Table 3.5. The relationship between fecundity and the number of mother mites per cell under different treatments

Offspring (n)‡	Treatments*									
	1	2	3	4	5	6	7	8	9	10
Actual fecundity										
Mother**/cell										
1	2.58 (50)	2.63 (41)	3.27 (22)	2.10 (29)	2.92 (36)	2.14 (21)	2.70 (27)	2.32 (41)	1.78 (32)	3.46 (28)
2	2.80 (15)	2.59 (16)	2.07 (21)	1.82 (17)	2.13 (12)	1.65 (13)	2.85 (24)	2.20 (15)	1.75 (12)	2.64 (18)
3	2.30 (10)	2.56 (9)	2.60 (10)	2.17 (4)	2.67 (6)	1.33 (2)	2.36 (12)	2.41 (9)	0.90 (7)	2.00 (4)
4	1.50 (3)	2.38 (6)	2.25 (3)	1.08 (3)			2.38 (4)	0.00 (1)	1.75 (1)	.
5								2.00 (1)		0.80 (1)
6										0.42 (2)
Potential fecundity										
Reproducing mother***/cell										
1	3.23 (40)	3.27 (33)	3.43 (21)	3.21 (19)	3.39 (31)	3.21 (14)	3.65 (20)	3.17 (30)	3.56 (16)	3.88 (25)
2	3.23 (13)	2.77 (15)	2.29 (19)	2.38 (13)	2.55 (10)	2.15 (10)	2.85 (24)	2.36 (14)	2.63 (8)	2.97 (16)
3	2.30 (10)	2.56 (9)	2.60 (10)	2.17 (4)	2.67 (6)	2.67 (1)	2.36 (12)	2.71 (8)	2.11 (3)	2.67 (3)
4	2.25 (2)	2.38 (6)	2.25 (3)	1.63 (2)			2.38 (4)	.	1.75 (1)	.
5								2.00 (1)		0.80 (1)
6										0.42 (2)

*Treatment legend the same as Figure 3.3.

** Total female mites of parental generation: data were from the cells contained reproducing and non-reproducing mother mites in each treatment.

*** Reproducing female mites of parental generation: data were from the cells contained reproducing mother mites only in each treatment.

‡ (n) : n = sample size, the number of sampled cells.

Effect on reproductive rate

Actual reproductive rate of mites are presented in Table 3.6. Actual reproductive rates among different treatments were nearly statistically significant ($F = 1.39$; $df = 9,18$; $P = 0.055$). By pairwise comparisons, the actual reproductive rate was significantly lower in βcd-origanum oil treated brood, compared to the control (LSMEANS, $P < 0.05$). Actual reproductive rate for thymol–crystal-treated-brood did not differ from that of the control but nearly statistically significant (LSMEANS, $P = 0.07$). Potential reproductive rate of mites did not differ between any of the treatments and the controls ($F = 0.93$; $df = 9,18$; $P = 0.53$).

Effect on offspring

The oil treatments did not significantly reduce mite populations, because overall the densities of mites per cell from different treatments were not significantly different ($F = 1.34$; $df = 9,18$; $P = 0.2842$). Starch-thymol treated larvae had the lowest mite density (5.15 ± 0.92 mites/cell); neat-origanum-oil-treated larvae had the highest mite density (7.07 ± 0.39 mites/cell).

Mite population compositions classified by stage, gender, and status of different treatments are presented in Table 3.7. MANOVA indicated that there was no significant difference among treatments in the proportions of various stages: egg, immobile/dead protonymph, dead deutonymph, dead light brown

adult daughter, mobile light brown adult daughter, dead reddish brown adult female, immobile/dead immature males, dead mature male, and mobile mature male in seven different treatment groups and the controls (MANOVA: Wilks' lambda F = 1.30, df = 108, 63.5; $P = 0.126$). We conducted pairwise comparisons between the treatment and its control within each category because we expected that the proportions might have slight differences within each category. There were significantly lower proportions of mobile protonymph (ANOVA: F = 2.39; df= 9,18; $P= 0.055$) in starch-thymol, βcd-thymol, and βcd-origanum-oil treated larvae compared to sugar syrup control (LSMEANS, $P < 0.05$). There were significantly lower proportions of immobile/alive deutonymph (ANOVA: F = 2.24; df= 9,18; $P= 0.051$) in starch-origanum oil and βcd-origanum oil treated larvae compared to sugar syrup control (LSMEANS, $P < 0.05$). There were significantly lower proportions of reddish brown mobile adult females in clove-oil-treated larvae compared to its control, 10 % ethanol in sugar syrup (LSMEANS, $P < 0.05$).

Table 3.6. Reproductive rate varroa mites in different treatments

	Actual reproductive rate [viable female offspring/reproducing mother mites]				Potential reproductive rate [viable female offspring/(reproducing+ non-reproducing mother mites)]			
Treatment*	n‡	Min.	Max.	Mean ± SE	n‡	Min.	Max.	Mean ± SE
1	78	0	5	1.4 ± 0.2	65	0	5	1.7 ± 0.2
2	72	0	5	1.5 ± 0.2	63	0	5	1.7 ± 0.2
3	56	0	4	1.5 ± 0.2	53	0	4	1.6 ± 0.2
4	53	0	3	1.0 ± 0.1	38	0	3	1.4 ± 0.2
5	54	0	4	1.6 ± 0.2	47	0	4	1.8 ± 0.2
6	36	0	4	1.0 ± 0.2	25	0	4	1.4 ± 0.2
7	67	0	4	1.6 ± 0.2	60	0	4	1.7 ± 0.2
8	67	0	4	1.2 ± 0.1	53	0	4	1.5 ± 0.2
9	52	0	4	0.9 ± 0.2**	28	0	4	1.6 ± 0.2
10	53	0	5	1.7 ± 0.2	47	0	5	1.9 ± 0.2

* Treatment legends the same as Figure 3.3.

** Pairwise comparisons conducted within the same column and ** indicates that it is significantly different from the (LSMEANS, $P < 0.05$).

‡ n = sample size, the number of sampled cells.

Table 3.7. The proportions of various stages and status of mites in different treatments. The data included all genders, stages, and status. The proportion was calculated by the numbers in each category (specific stage and status) divided by the total population numbers. Data from three different colonies.

Treatment*		*(Gender)*	Female mites							
	Sample size†	*(Stage)*								
		Egg	Protonymph		Deutonymph		Light brown adults		Reddish brown adults	
		(Status)	Immobile/dead	Mobile	Dead***	Immobile/alive	Dead	Mobile	Dead	Mobile
Controls										
1	*65(388)*	0.04±0.02	0.05±0.02	0.01±0	0±0	0.20±0.01	0.01±0.01	0.17±0.04	0.02±0.01	0.28±0.02
2	*63(416)*	0.04±0.01	0.06±0.02	0.03±0	0±0	0.18±0	0.02±0.02	0.19±0.02	0.04±0.02	0.26±0.02
3	*53(355)*	0.03±0.01	0.06±0.01	0.02±0.01	0.01±0.01	0.16±0.03	0.01±0.01	0.18±0.02	0.03±0.02	0.28±0.01
Thymol										
4	*38(222)*	0.03±0.01	0.08±0	0.03±0.01	0±0	0.17±0.01	0.04±0.04	0.12±0.05	0.07±0.02	0.26±0.01
5	*47(269)*	0.02±0.01	0.06±0.01	0.01±0.01 **	0±0	0.19±0.03	0.01±0.01	0.19±0.03	0.04±0.01	0.26±0
6	*25(130)*	0.02±0	0.10±0.03	0.01±0.01 **	0±0	0.16±0.01	0.02±0.02	0.16±0.02	0.06±0.04	0.27±0.03
Origanum oil										
7	*60(436)*	0.03±0.01	0.06±0.01	0.01±0	0±0	0.17±0.01	0.01±0.01	0.18±0.02	0.05±0.02	0.29±0.02
8	*53(317)*	0.02±0.01	0.11±0.05	0.01±0	0±0	0.11±0.01 **	0.02±0.01	0.21±0.03	0.02±0.01	0.29±0.02
9	*28(165)*	0.04±0.03	0.09±0.04	0±0 **	0±0	0.12±0.03 **	0.04±0.02	0.15±0.01	0.02±0.01	0.29±0.02
Clove oil										
10	*47(298)*	0.04±0.02	0.09±0.05	0.01±0.01	0±0	0.16±0.01	0±0	0.23±0.05	0.05±0.03	0.24±0.02 **

*Treatment legend the same as Figure 3.3.

** Pairwise comparisons conducted within the same column and ** indicates that it is significantly different from the (LSMEANS, $P < 0.05$). Comparisons were conducted within one category (specific stage under the same status).

***Its color turned dark so we counted as dead deutonymph.

†Sample size was the total number of examined alive pupae with reproduced mites; the number in (*n*) was the total mites examined on each treatment from three colonies.

Table 3.7. (Continued)

Treatment*	*(Gender)*	Male mites		
Sample size†	*(Stage)*	Immature	Mature	
	(Status)	Immobile/dead	Dead	Mobile
Controls				
1 *65 (388)*		0.05±0.01	0.02±0	0.14±0.02
2 *63 (416)*		0.05±0	0.01±0.01	0.14±0.02
3 *53 (355)*		0.07±0.02	0.02±0.01	0.11±0.01
Thymol				
4 *38 (222)*		0.07±0.03	0.03±0.02	0.09±0.03
5 *47 (269)*		0.09±0.01	0.03±0.01	0.11±0.01
6 *25 (130)*		0.09±0.02	0.02±0.01	0.09±0.02
Origanum oil				
7 *60 (436)*		0.06±0.02	0.03±0.01	0.12±0.02
8 *53 (317)*		0.09±0.02	0.01±0.01	0.09±0.02
9 *28 (165)*		0.04±0.02	0.02±0.01	0.19±0.06
Clove oil				
10 *47 (298)*		0.05±0.03	0.04±0.02	0.09±0.01

Discussion

Different formulations of thymol and origanum oil have different toxicity to bee larvae (Figure 3.1 A and B). The degree of toxicity of various formulations of thymol and origanum oil had a similar rank order: βcd encapsulated > starch encapsulated ≧ neat oil. The high toxicity of βcd-thymol orβcd-origanum oil is mainly due to the toxicity of βcd itself, because the βcd control also killed larvae at a similar rate as βcd-thymol or βcd-origanum oil. The toxicity of the starch-origanum oil and neat origanum oil is almost the same, suggesting that starch alone is not toxic to honey bees. Starch is a natural product that has been used as a carrier for medicine or pest control agents (McGuire et al. 1994, Chandler et al. 1995, USDA 2005, Daramola and Falade 2006), and, bioassays also indicate that starch is not toxic to tested insects (Weissling et al. 1991).

Worker bees apparently removed brood that ingested too much essential oil. In a laboratory feeding experiment (data not shown), larvae could survive at the highest dose (0.6mg/larva) of thymol crystals/ starch-thymol and origanum oil/ starch-origanum oil, although with smaller larval size on the fifth day. This contrasts with field experiment showing that no larvae survived at the highest dose of these oils, except for larvae fed with thymol crystals which had a 40 % survival rate. Based on this observation, we assume that adult bees removed the larvae fed with 0.6 mg thymol or 0.6 mg origanum oil, perhaps because normal larva odors or pheromones were affected. Sub-lethal effects of essential

oils such as feeding deterrence and delayed growth found in other insects (Isman 1999, Kostyukovsky et al. 2002) could contribute to abnormal development of bee larvae. These abnormal larvae could survive under laboratory rearing conditions but may be removed by workers in the colonies. Larva can survive when fed 0.16 mg clove oil in the field, but 1.6 mg clove oil per larva caused 100 % mortality in both laboratory and field experiments. Larvae seem to be the most sensitive stage to essential oils, because after larvae were capped, nearly all eclosed successfully.

Thymol, origanum oil, and clove oil have the potential to reduce mite infestation. In the infestation and reproductive rate experiments, βcd-origanum oil, starch-thymol, βcd-thymol, and clove oil deterred varroa mites from infesting because their rates of infestation were significantly lower than the two controls (30 % syrup or 10 % ethanol in 30 % syrup) (Figure 3.3). Several essential oils have been reported to disturb mite's orientation at non-lethal doses (Kraus et al. 1994, Imdorf et al. 1999, Ruffinengo et al. 2005). Kraus et al (1994) found that 23 out of 32 tested essential oils exhibited a clear repellent effect on varroa mites, and 7 oils had a clear attractant effect on them. They discovered that origanum oil had a repellent effect and clove oil had a highly attractant effect. Infestation rate of varroa mites was significantly decreased when wax foundation contained 0.1 % marjoram oil, but the rate was significantly higher when clove oil was used (Kraus et al. 1994, Imdorf et al. 1999). Thyme, which contains thymol, also showed a repellent effect on varroa (Colin 1990, Imdorf et al. 1999). These studies are consistent with our finding that thymol and origanum oil reduced varroa mite

infestation. This deterrent effect could either be due to residual larval food containing oils, or due to changed odor of larvae after feeding with oils. Our finding of lowered varroa infestation rate in clove oil fed brood (Figure 3.3) is inconsistent with previous studies reporting increased infestation (Kraus et al. 1994, Imdorf et al. 1999). It is possible that effect of oil could vary depending whether the oil is in beeswax (Kraus et al. 1994), or in larval food or on surface of larvae (this study).

Thymol crystals and βcd-origanum oil can suppress varroa population by decreasing actual fertility, actual fecundity, and actual reproductive rates of mites (Figure 3.4, Table 3.3 and 3.6). However, once the mites reproduced, the given oils will not affect mite reproduction at all because potential fertility, potential fecundity, and potential reproductive rates were not significantly different among treatments (Figure 3.4, Table 3.3 and 3.6). Some non-reproducing mother mites died in cells without defecation (n3 in column e of Table 3.3) in oil fed larvae but not in the control. This suggests that the reduction of mite reproduction for the infested mother mites is mainly derived from mite-kill by poisoning. We do not have evidence to conclude that clove oils have any effect on mite reproduction. Our results do not support the notion that clove oil could increase mite reproduction. Bunsen (1991, cited by Imdorf, 1999) documented that 5 out of 71 essential oils (including clove oil) increased mite reproduction. The number of mother mites per cell is a key factor to influence mite fecundity across the treatments and controls. Many of our larvae had multiple mother mites per cell, and in these cells, there was a negative relationship between the number of

mother mites and the average fecundity per mother. This is consistent with previous findings (Martin 1994, Martin 1995b) that found decreasing fecundity with increasing number of mothers sharing a cell.

Overall, the essential oil treatments do not delay the development of the young mites because most mite offspring can become adults despite treatment. In addition to evaluating mite reproduction, we also determined the population composition of mite populations in different treatments (Table 3.7). We found little difference in the composition of mite population in protonymph mobile stage and deutonymph immobile/alive stage between larvae treated with oils and the controls, about 15-20 % daughter mites became adults which is similar to controls. Most older offspring developed to the immobile deutonymphs and adults in all treated larvae. Only a small portion of young mites died in the early stage in our experiment, similar to what Martin (1994) found under natural conditions. It seems essential oils did not cause significant mortality to nymph stages of mites in our study because the proportions of dead nymphs in the treatments did not significantly differ from controls.

We observed several types of abnormal behaviors of mites when they were fed on brood with oils. These included (1) mature daughter mites lying with their ventral side up on the cell bottom with legs trembling, (2) mature daughter and male mites repeatedly falling from the cell wall because they were unable to grab the substrate, and (3) mature males unable to attach to mature daughter mites so no successful mating took place. Those observations suggest that female or

male offspring show neurotoxic responses after intaking non-lethal doses of essential oils which might discourage mite pre-mating behavior. Then neurotoxic responses suggest that the target site of these essential oils is the the nervous system. The molecular mode of actions of essential oils in mites and most insects remains unclear. Some essential oil monoterpenes competitively inhibit acetylcholinesterase in the stored product insects and some essential oils bound to octopaminergic target site to mimic the action of octopamine in some pests (Enan 2001, Kostyukovsky et al. 2002, Shaaya and Rafaeli 2007). Thymol has been reported to be a positive GABA-modulating and GABA-mimetic substance capable of interacting with human GABA(A) receptor and *Drosophila melanogaster* homomeric RDLac GABA receptors which were expressed in *Xenopus laevis* oocytes (Priestley et al. 2003). Unraveling the mode of actions of essential oils in varroa mites could be a future research topic. This knowledge can enable us to understand the reasons of low toxicity of these oils to vertebrate and invertebrate animals, to develop new formulations for improving their acaricidal potency, and to develop new application procedures for reducing the cost.

Thymol, origanum, and clove oils were used in this because they have been proven as effective agents for controlling varroa mites both in laboratory and field evaluation (Chiesa 1991, Calderone et al. 1997, Sammataro et al. 1998, Lindberg et al. 2000, Al-Abbadi and Nazer 2003, El-Zemity et al. 2006). The thymol-based commercial products Apilife VAR, Apiguard, and Thymovar provide high efficacy for varroa control (Melathopoulos and Gates 2003, Baggio et al. 2004). Our

results here suggest that low doses of essential oils can affect mite infestation and reproduction, even though oil is presented to mites through host hemolymph.

This is the first study to use an indirect method of applying essential oils to varroa mites to investigate their effects on varroa mite reproduction. In summary, essential oils might regulate mite populations at two levels. Essential oils can reduce mite infestations. Because this is a "choice" experiment, i.e. the invading mites had many types of brood cells from which to choose. Further studies are needed to determine if this deterrent effect would persist when mites do not have a choice (e.g. when the entire colony was treated with one type of oil). When the mother mites have invaded brood cells, thymol and origanum oil could reduce actual reproduction of mites by causing early death of some mother mites. Once the mother mites survived to reproduce offspring, we did not find any difference in their fertility, fecundity or reproductive rate. Therefore, the reduction of parental mite reproduction seems due to mite-kill of oil itself. There is some evidence that thymol or origanum oil can reduce mite reproduction in the offspring generation. In order to answer the question of whether essential oils can lower mite reproduction on the second generation, one could collect the viable adult daughter females from the treated larval brood cells to examine their ovarian development using histochemistry or examine spermatozoa in their seminal receptacles. Exploring the possibilities of using sub-lethal doses of essential oils for lower mite reproduction can reduce cost of mite control, chances of contamination, and slow down resistance development.

Appendix 1

Record of Deposition of Voucher Specimens*

The specimens listed on the following sheet(s) have been deposited in the named museum(s) as samples of those species or other taxa, which were used in this research. Voucher recognition labels bearing the Voucher No. have been attached or included in fluid-preserved specimens.

Voucher No.: 2008-04

Title of thesis or dissertation (or other research projects):

Exploring new methods for varroa mite control

Museum(s) where deposited and abbreviations for table on following sheets:

Entomology Museum, Michigan State University (MSU)

Other Museums:

Investigator's Name(s) (typed)
Yu-Lun Lisa Fu

Date 2008-04-20

*Reference: Yoshimoto, C. M. 1978. Voucher Specimens for Entomology in North America. Bull. Entomol. Soc. Amer. 24: 141-42.

Deposit as follows:

Original: Include as Appendix 1 in ribbon copy of thesis or dissertation.

Copies: Include as Appendix 1 in copies of thesis or dissertation.
Museum(s) files.
Research project files.

This form is available from and the Voucher No. is assigned by the Curator, Michigan State University Entomology Museum.

Appendix 1.1

Voucher Specimen Data

Page 1 of 1 Pages

Species or other taxon	Label data for specimens collected or used and deposited	Number of: Eggs	Larvae	Nymphs	Pupae	Adults ♀	Adults ♂	Other	Museum where deposited
Apis mellifera (L.)	Michigan, Ingham Co., East Lansing MSU Campus, Apiary ex. Honey bee hive				5	5			MSU
Varroa destructor	Michigan, Ingham Co., East Lansing MSU Campus, Apiary ex. Honey bee hive			10		10	10		MSU

(Use additional sheets if necessary)

Investigator's Name(s) (typed)

Yu-Lun Lisa Fu

Date 4/20/2008

Voucher No. 2008-04

Received the above listed specimens for deposit in the Michigan State University Entomology Museum.

Curator Date 8/15/2008

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