Mechanisms of *Varroa* Control by Thymol and Other Essential Oils

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Interpretive Summary:

Compared to other acaricides, essential oils are more environmental friendly and cost effective for varroa control. In this study three essential oils, thymol, origanum, and clove oils in different formulations were examined for their mechanisms for varroa control. 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 brood 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 brood cells treated with thymol crystals and β-cyclodextrin-encapsulated origanum oil were significantly lower than that of their controls. 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) reduced mite reproduction. Exploring the possibilities of using sub-lethal doses of essential oils for mite control can lead to reduced cost and chances of contamination, and reduced resistance development.

Objectives:

The objective of this study is to determine whether essential oils fed to bee larvae in a colony reduce varroa mite reproduction. Varroa mites are not exposed to oil or oil vapor directly but would come to contact and feed on larvae that have been artificially fed with known amounts of essential oil. We first determined the toxicity of each selected essential oil (type and formulation combination) to four day old bee larvae. Then we 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. We used doses that were relatively nontoxic to bee larvae and tested their effects on varroa mite by examining the number and statutes of varroa mite.

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 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 (Figure 1, 2, and 3). For toxicity studies (Figure 1 and 2), 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. 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 (Figure 3-4 and Table 1-5), 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 larval 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, was 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 Figure 1 and 2. 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 (Figure 3). 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 1995a,b). 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 fecundity = the number of viable adult female offspring divided by the number of mother mites (reproducing and non-reproducing)
Potential mite fecundity = 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 oisl 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 5) 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).

Results and Discussion:

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 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 dose (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 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 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 2).

Effects of clove oil on larvae and pupae

Only neat clove oil was tested and the larval survival rates are presented in Figure 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, $P > 0.05$).

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 dose was chosen from each oil/formulation to test their effects on mite infestation and reproduction. 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). 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 $P = 0.053$.

Effect on fertility

Actual fertility of mites are presented in Figure 4. Some mother mites died without any offspring (column E in Table 1). Little or no mite defecation was found in this type of brood cell. There were significant differences in actual fertility among the treatments (Figure 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 4, $F = 1.06$; df = 9,18; $P = 0.43$).

Effect on fecundity

Mite fecundity data are presented in Table 1. 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 2). Most cells were infested by one or two mother mites for the control and the oil treated larvae (Table 3), 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). 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).

Effect on reproductive rate

Actual reproductive rate of mites are presented in Table 4. 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 5. 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).

Discussion:

Different formulations of thymol and origanum oil have different toxicity to bee larvae (Figure 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 oils. 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). 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) 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 4, Table 1 and 4). 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 4, Table

1 and 4). Some non-reproducing mother mites died in cells without defecation (n3 in column e of Table 1) 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 1995a, b) 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 5). 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. 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.

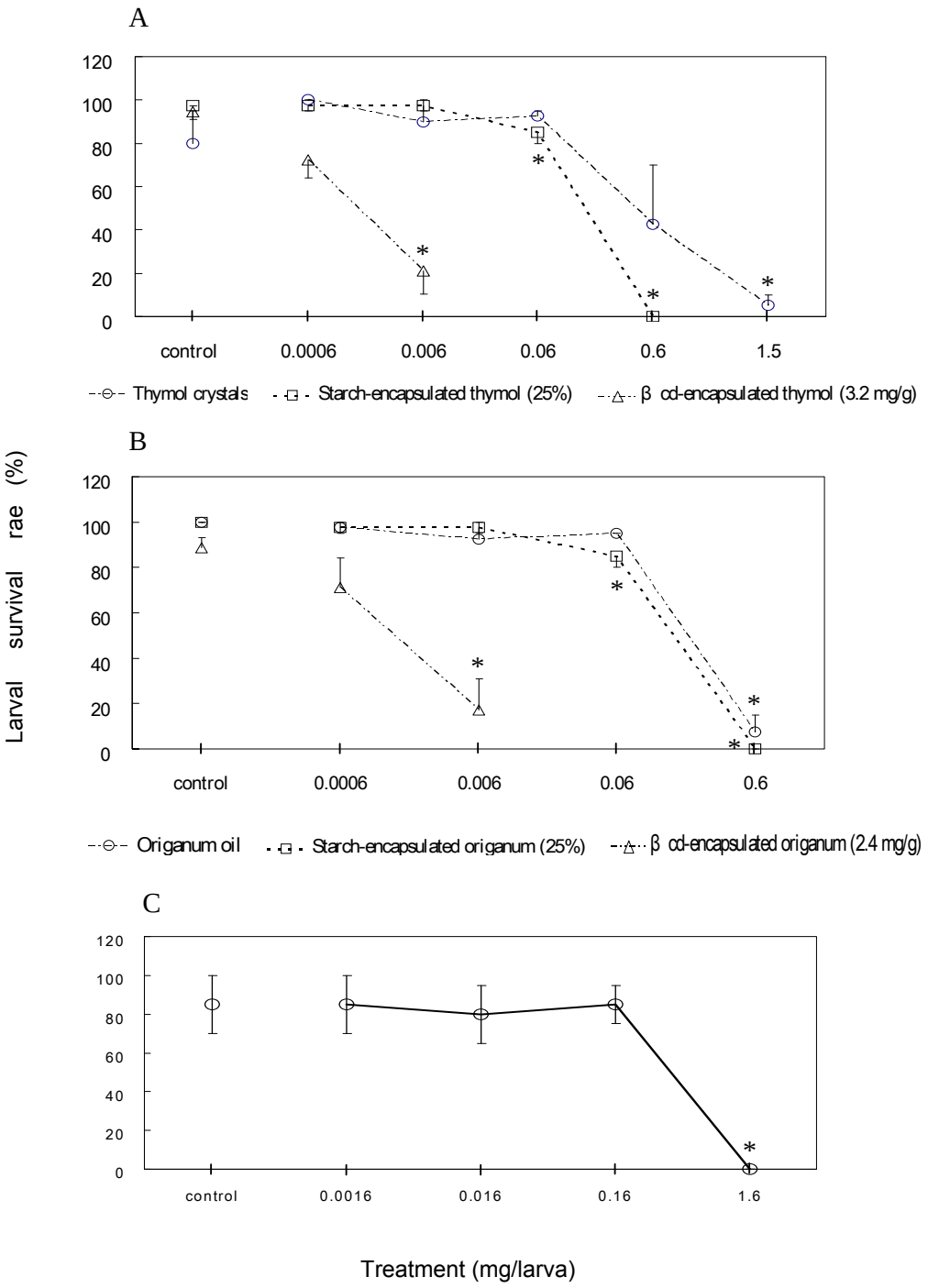

Figure 1. Survival rates of larvae and pupae (mean ± SE) after 4 day old larvae were fed with thymol (A), origanum oil (B) in various formulations and neat clove oil (C). For the neat oils, 10% ethanol in 30% sugar syrup was fed as a control. For starch-encapsulated and β-cyclodextrin-encapsulated oils, 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, $P < 0.05$).

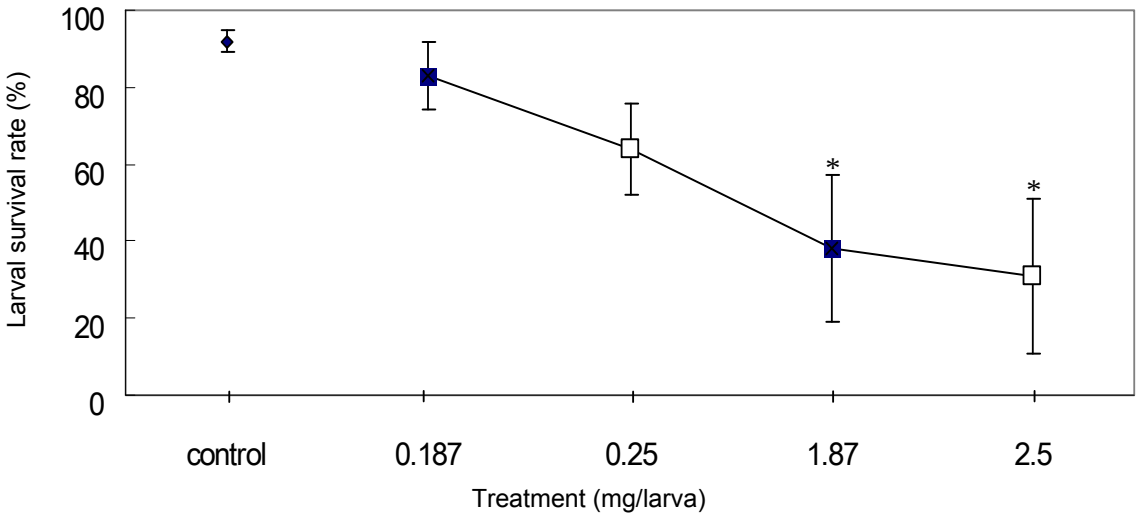

Figure 2. Effect of β-cyclodextrin on larval survival. Thirty percent sugar syrup was used as a control for βcd. Experiments replicated in 3 colonies. Means marked with a * are significantly different from the control (LSMEANS, $P < 0.05$).

- 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

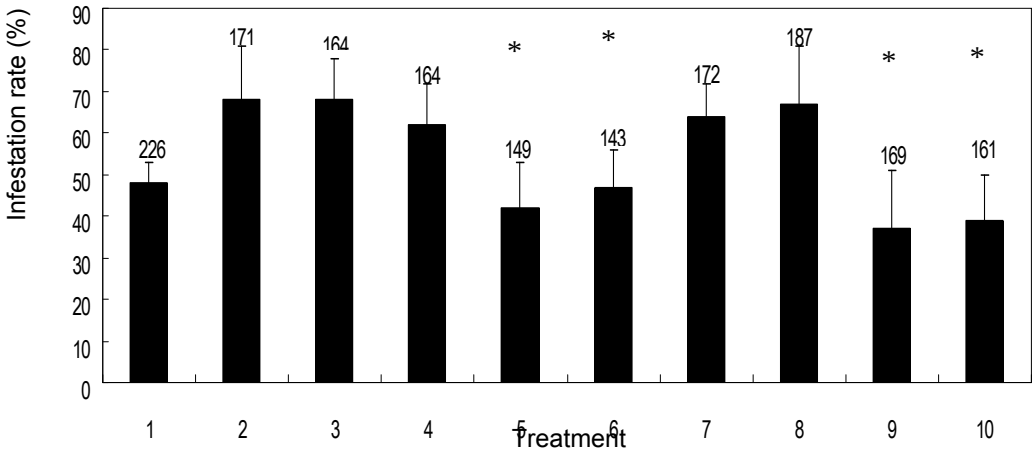

Figure 3. Infestation rates (mean ± SE) of varroa mites in different treatments. Treatments: **1.** Natural (no artificial feeding), **2.** 30% sugar syrup, **3.** 10% ethanol in 30% sugar syrup, **4.** Thymol 0.15 mg/larva, **5.** Starch-encapsulated thymol (25%) 0.06 mg/larva, **6.** βcd-encapsulated 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. A bar with * on top indicates that it is significantly different from the control (LSMEANS, $P < 0.05$). N = 4 colonies.

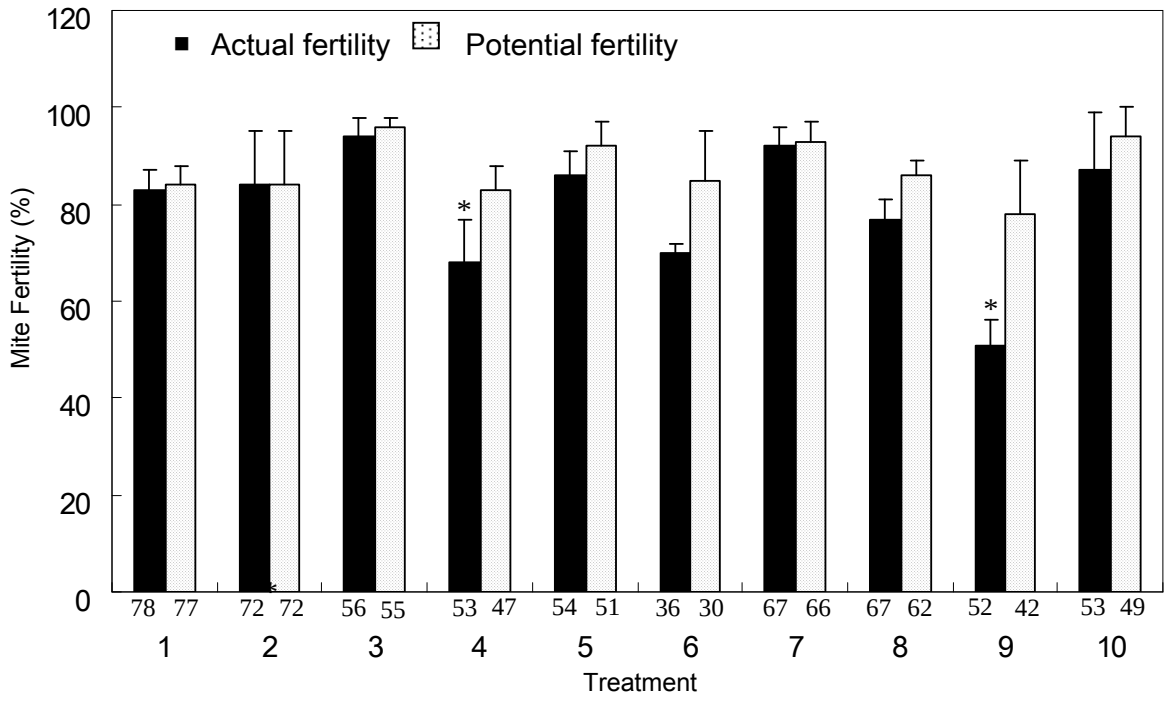

Figure 4. Fertility (mean ± SE %) of varroa mites in different treatments. Treatment legend the same as Figure 3. The number on the bottom of each bar is the sample size of sampled cells. ■ Actual fertility: % was calculated from total sampled cells which included the cells with living mother mites and 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.

Table 1. Mite fecundity in different treatments

Treatment*	Sampled cells (n_1)†	Female mites of parental generation (mother mites)					Fecundity	
		Total progeny of mite	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.

** 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 2. 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.
** 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. 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.
** 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.

Table 4. 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.

** 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 5. 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.

** 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 5. (Continued, horizontally)

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

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Recent Publications:

Fu, Yu-lun, Z. Y. Huang, B. LeBlanc, G. Wardell, Effect of Thymol and Other Essential Oils on Reproduction and Invasion of Varroa Mites. In preparation.