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Genetic Stock Identification Of Production Colonies Of Russian Honey Bees

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Keywords: Russian honey bee, production, queen, genetic stock identification

In 2008, the USDA-ARS Honey Bee Breeding, Genetics, and Physiology Laboratory (USDA-ARS HBB Lab) fully released the Russian honey bee stock to the Russian Honey Bee Breeders Association (RBBA). Each year, members of the RBBA are required to have their breeding stock genetically certified, to conduct tests of honey production, and assess pest and pathogen (*Varroa* mites, tracheal mites and *Nosema ceranae*) status.

When the stock was released the stock diversity was characterized and a suite of genetic markers was identified that would be used for later stock certification (Bourgeois and Rinderer 2009, Bourgeois *et al.* 2010). A summary of the procedures used to certify breeding stock is given below. The members of the RBBA often use different times and mating apiaries to produce the

production queens that they use in honey production apiaries and market to other beekeepers. We were curious about the genetic nature of their production queens. Hence, in Autumn 2009 and Spring 2010, we asked beekeepers of the RBBA to submit samples of worker bees from their production queens to determine the level of Russian alleles in the production colonies.

A total of 5 of the 8 certified members of the RBBA submitted samples for this study. All bees were submitted as live bees and were frozen upon receipt. DNA was then extracted according to published protocols (Bourgeois *et al.* 2010). Russian and non-Russian alleles were identified as previously described in Bourgeois *et al.* (2010). Briefly, 8 individual bees per colony were genotyped with 12 microsatellite and 5 single nucleotide polymorphism (SNP) markers. These markers created a genetic fingerprint of each bee. After genotyping was completed, the data were visually inspected to identify bees that had “drifted” into the colony. This was done

using the identification of the queen's alleles. All bees that do not have one of the queen's two alleles for each locus were considered to have "drifted" and were eliminated from the analysis pool. This procedure is a component of the standard procedure used for testing certification samples for Russian honey bees.

After drifting bees were identified and removed from the data set, ONCOR software (<http://www.montana.edu/kalinowski>) was used to determine genetic stock identification. The software algorithm compared the genetic fingerprint of the test bees to the genotypes of bees that comprise a baseline sample of Russian and non-Russian honey bees from commercial operations throughout the U.S. The software provided the probability of assignment of each bee to either the Russian or Non-Russian group. The minimum acceptable threshold for assignment to the Russian group is held at 70% for each bee for stock certification. Because the production colonies are openly mated and are not held to the strict standards required for the propagation of breeder stock (i.e., isolated mating yards and strict control of drone source colonies), we would expect to see lower levels of Russian alleles in production queens. Dilution of the stocks' alleles is not uncommon in production yards where open mating and drift are likely to occur.

Overall, colonies had a mean probability of assignment to the Russian group of 0.66 ± 0.04 . This compares favorably with a recent report of stock assessment in commercial operations (Spivak *et al.* 2009) using Minnesota hygienic bees. An assessment of commercial apiaries using the Minnesota Hygienic stock of honey bees 10 years post-release showed that 24-29% of sampled colonies exhibited the hygienic trait at a high enough rate to be considered as good potential breeder colonies (Spivak *et al.* 2009). In the Russian production colonies, 48% had an average assignment value of > 0.7 meaning that 48% of these colonies could serve as potential breeder colonies.

The variability in probability of assignment was high among colonies and among beekeepers (Figure 1). Stock assignments of individual colonies ranged from 0.95 to the Non-Russian group to 1.0 to the Russian group. SAS 9.2 was used for all data analyses. Group assignment values were consistent among beekeepers ($P > 0.05$) with all operations having a large degree of variation among colonies (Fig. 1). A comparison of the production colonies with certified breeder colonies for each beekeeper showed that breeder colonies have higher percentages of Russian alleles (0.94 ± 0.01 ; $P < 0.001$). This was expected, because the RBBA members must follow strict guidelines while setting up their mating yards. The production yards are not held to the same restrictions. However, RBBA members work only with Russian bees in their apiaries. The



Figure 1. Genetic stock identification of production colonies of Russian honey bees. Bars represent mean \pm S.E. for colonies from five Russian honey bee breeders. The horizontal line signifies the minimum assignment threshold for the certification of breeder stock that is used by the Russian Honey Bee Breeders Association.

primary source of non-Russian alleles would be from nearby apiaries or feral colonies. In other words, some level of introgression of non-Russian alleles is expected in openly mated production colonies.

The high level of Russian alleles in the production colonies is an indication that the bees should exhibit the characteristics of the USDA-ARS stock of Russian honey bees. Even in the presence of non-Russian alleles, the continuous selection that the Russian stock undergoes through activities of the RBBA should maintain the positive stock characteristics.

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A Spring Evaluation Of Thymol Formulated In A Sucrose Dust For The Control Of *Varroa destructor*, a Parasite Of The Honey Bee (*Apis mellifera*) In Alberta, Canada

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SUMMARY

We evaluated thymol formulated in a sucrose dust, as a control for the *Varroa* mite (*Varroa destructor*) under the cool spring

conditions of northern Alberta, Canada. Our study compared two different application schedules of this formulation (four applications of 30 g at 7 d intervals or two applications of 60 g at a 14 d interval,

per colony) to a commercial thymol product, Apilife VAR[®], or an untreated control group receiving sucrose without thymol. The number of *Varroa* mites falling on adhesive bottom boards was counted for 30 d during the treatment period. Treatments were then removed, highly effective synthetic acaricide strips (amitraz) were applied and the number of residual mites was counted for an additional 12 d. Overall, the thymol treated groups had higher mite counts during the treatment period compared to the untreated group, while the reverse was observed during the post-treatment interval. During the treatment period counts differed significantly on only two dates. On these dates we observed that all the thymol treatments had significantly higher counts compared to the untreated group, but only the application of 60 g of dust at the 14 d interval had similar high counts compared to Apilife VAR[®]. Nonetheless, the percentage of *Varroa* counted during the treatment period, compared to the total counted during both the treatment and post-treatment period, was similarly high for all the thymol treatments (>94%). Our results suggest that spring treatments with thymol dust provide a good alternative to Apilife VAR[®] and that reducing the application frequency of dust from four to two applications does not reduce treatment efficacy.

Keywords: Thymol, Apilife VAR[®], honey bee, *Varroa* mite, acaricide

Introduction

The *Varroa* mite, *Varroa destructor* Anderson and Trueman, is considered the most damaging parasite of honey bees (*Apis mellifera* L.) (van Engelsdorp and Meixner 2009). To date, infestations have largely been managed using four synthetic acaricides (fluvalinate, flumethrin, coumaphous and amitraz), however, mite resistance to all four has evolved across the major beekeeping regions of the world (reviewed in Rosenkranz et al. 2009). Furthermore, acaricide residues have accumulated in honey and beeswax, generating concerns for not only human health but also for the health of the colony (reviewed in Rosenkranz et al. 2009).

Naturally-derived acaricides have been developed as alternatives to synthetic products, not only because their novel modes of action would be useful in managing *Varroa* mite populations that are resistant to synthetic ingredients, but also because they frequently are less persistent in the hive (reviewed in Imdorf et al. 1999). Thymol, a naturally-derived monoterpenoid, has pronounced selective toxicity against *Varroa* mites as compared to its honey bee host (Lindberg et al. 2000, Gashout and Guzmán-Novoa 2009). Consequently, two thymol-based commercial fumigants have been registered in the U.S., Apilife VAR[®] and Apiguard[™]. No thymol treatments are currently registered in Canada.

The current formulations of thymol, including products like Apilife VAR[®], do not provide consistent control of *Varroa* mites (reviewed in Imdorf et al. 1999). In an effort to improve thymol efficacy, Emsen et al. (2007) compared a number of novel formulations, most notably a sucrose-based dust, to a previously developed formulation in which the thymol was impregnated in vermiculite blocks, similar to that of Apilife VAR[®]. The authors observed that while the efficacy of the thymol formulated in dust against *Varroa* mites was not different from that of the vermiculite formulation, only the dust formulation demonstrated a significantly higher efficacy compared to the other formulations in the study.

We investigated the efficacy of the dust formulation of thymol

developed by Emsen et al. The objective of this work was not only to confirm their findings under different environmental and apicultural conditions, specifically the cool spring conditions in the Aspen Parkland zone of Alberta, Canada, but also to compare the effect of reducing the frequency of treatment application.

Materials and Methods

Acaricides. Thymol experimental dust treatments were prepared by Medivet Pharmaceutical Ltd. (High River, AB, Canada) and were previously described by Emsen et al. (2007). The thymol was finely powdered and mixed into confectionary sugar at a rate of 0.2 g of thymol per gram of total powder. The commercial thymol standard was Apilife VAR[®] (Chemicals Laif, Vigonza, Italy) which consisted of 11 g tablets containing 74.08% (wt:wt) thymol, 16.00% eucalyptus oil and 3.7% L-menthol. Apilife VAR[®] was applied according to the manufacturer's treatment recommendation of three applications of one tablet at 7d intervals.

Colony set-up. The experimental colonies were obtained from a pool of overwintered commercial colonies located near Grimshaw, Alberta, Canada (56° 11' N – 117° 36' W). In mid-April 2009, 40 single-chamber, Langstroth-hives were identified with similar high levels of *Varroa* mite infestation on the 29 April (9.1% ± 1.1 (SE), alcohol wash of phoretic *Varroa* from ~ 150 brood nest bees, Currie and Gaten 2006) and with comparable worker bee populations (adult workers covering at least six frames, with at least two frames of brood) and were moved to an apiary, where they were randomly assigned to ten replicates of four treatment groups: 1) thymol dust (4 × 30 g), 2) thymol dust (2 × 60 g) 3) commercial thymol standard (Apilife VAR) and 4) untreated control. A total of 120 g of the dust formulation was applied to each colony on a 25 x 10 cm piece of newspaper placed in the frame top bars right above the brood. The thymol dust was applied to colonies at two different frequencies: 1) four applications of 30 g at 7 d intervals (4 × 30 g) (Emsen et al. 2007) or 2) a previously untested schedule of two applications of 60 g at 14 d intervals (2 × 60 g). The untreated control colonies were provided with sucrose dust that was formulated without thymol.

The first application of treatments was on 8 May 2009 and coincided with the blossoming of willow (*Salix spp.*), the first major source of natural forage in the region. Immediately before the treatments were applied, 30 x 40 cm paper sheets coated with hydrogenated vegetable oil (Crisco[®]) were placed on the bottom boards of each colony to collect dislodged *Varroa* mites. Bees were restricted from contacting the adhesive surface with a 2 mm-mesh hardware cloth. The adhesive sheets were replaced at three to five-day intervals and the adult mites adhering to the surface were counted and recorded. The four week- treatment period was followed by a 12 d post-treatment period (9 – 21 June) in which all residual thymol or sucrose was removed from the colonies and replaced with controlled-release plastic strips containing 3.33% (wt:wt) amitraz (Apivar[®], Véto-pharma S.A., France), an acaricide that kills >80% of the *Varroa* in a colony (Floris et al. 2001). The number of *Varroa* mites counted on the adhesive sheets during the post-treatment period was an estimate of the size of the population of mites not killed by the experimental treatments.

We estimated the overall treatment effectiveness for each colony by calculating the number of mites counted during the treatment period as a percentage of the total counted (treatment period + post-treatment period, Figure 1).

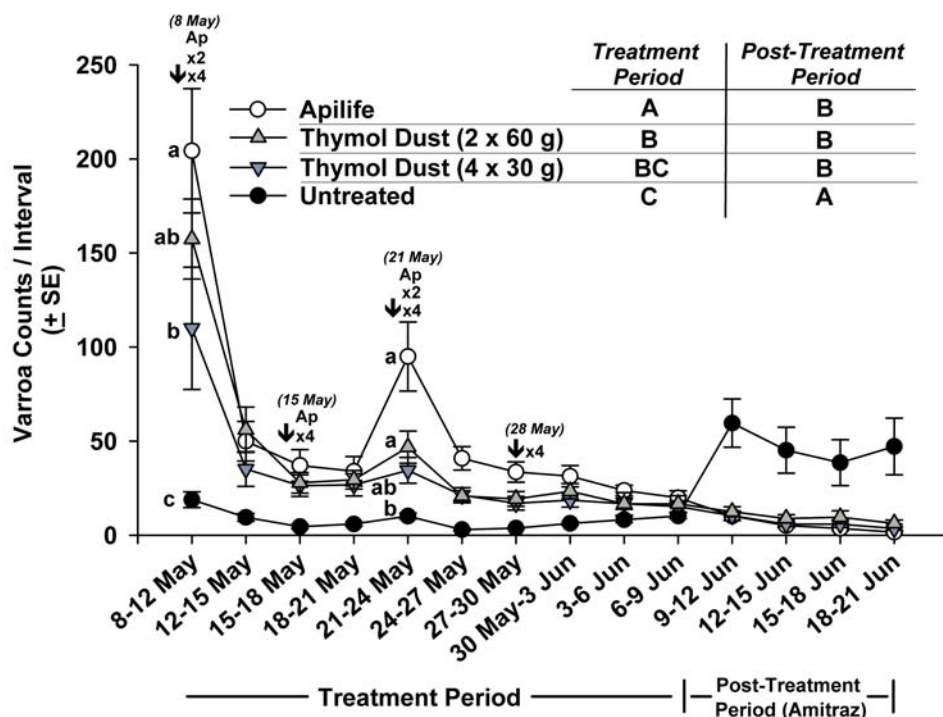


Figure 1. Average interval varroa mite counts on grease-coated boards for colonies not treated, treated with Apilife VAR® (Ap), a commercial thymol treatment, or treated with 120 g of thymol dust applied across four dates 7 d apart (x4) or two dates 14 d apart (x2). Means followed by the same lower case letter indicate no significant difference in counts for specific dates during the treatment period (Tukey-Kramer, $\alpha = 0.05$). Although there were significant time \times treatment interactions during the treatment period (see text), overall means across this period followed by the same uppercase letter indicate no significant difference (Tukey-Kramer, $\alpha = 0.05$). Means across the four dates of the post-treatment period were combined for the comparison as the treatment \times time interaction was not a significant source of variation (see text) and the same upper case letter indicate no significant differences (Tukey-Kramer, $\alpha = 0.05$).

Temperature - We measured the ambient temperature in the apiary every two hours during the treatment period by placing a StowAway® TidbiT™ temperature logger (Onset Computer Corporation Bourne, MA, USA) in a shaded spot within the apiary location.

Statistical Analysis. We tested the hypotheses that *Varroa* mite counts among the treatment groups did not differ either in the treatment period or post treatment period, within each three- to five-day interval, or by the interaction of treatment and interval using a repeated measures mixed model analysis of variance (PROC MIXED, SAS version 9.2, Littell *et al.* 1996). We used the PDMIX800 macro (Saxton 1998) to get Tukey-Kramer adjusted mean letter groupings for each period, and when significant treatment by date interactions were detected, within each period. We tested the hypothesis that percent efficacy did not differ among the treatments using a one way analysis of variance, separating treatment means using Tukey-Kramer (PROC GLM, SAS version 9.2, Scholotzhauer and Littell 1987).

Results

Varroa mite counts underwent significant change through both the treatment period (Figure 1, May 9 – June 9) ($F = 56.88$; $df = 9,324$; $P < 0.001$) and the post-treatment period ($F = 9.98$; $df = 3,108$; $P < 0.001$), in spite of there being no evidence of difference in pre-treatment phoretic *Varroa* population ($F = 2.19$; $df = 3,36$; $P = 0.1057$). There was a large increase in counts during the first week of treatment, then again following the warming of ambient temperatures beginning on 21 May, but only among the thymol treated colonies (time \times treatment, $F = 7.02$; $df = 27,324$; $P < 0.001$; treatment, $F = 12.92$; $df = 3,36$; $P < 0.001$). We observed ten-times the *Varroa* mite counts among Apilife VAR® colonies compared to untreated colonies on 12 May and twenty-times the counts on 24 May. Only the thymol dust treatment applied twice at a rate of 60 g had counts that did not statistically differ from those of Apilife VAR®. We also, however, observed higher counts among

colonies receiving four 30 g applications of thymol dust compared to untreated colonies.

Varroa mite counts increased again at the beginning of the post-treatment period (June 9) when amitraz strips were applied, but, this was due to mite counts in the untreated colonies only (time \times treatment, $F = 1.84$; $df = 9,108$; $P = 0.068$; treatment, $F = 10.18$; $df = 3,36$; $P < 0.001$).

The percentage of mites counted during the treatment period for the three thymol treatments were not different from one another, but were different from the untreated colonies (Figure 2, $F = 37.48$; $df = 3,36$; $P < 0.001$).

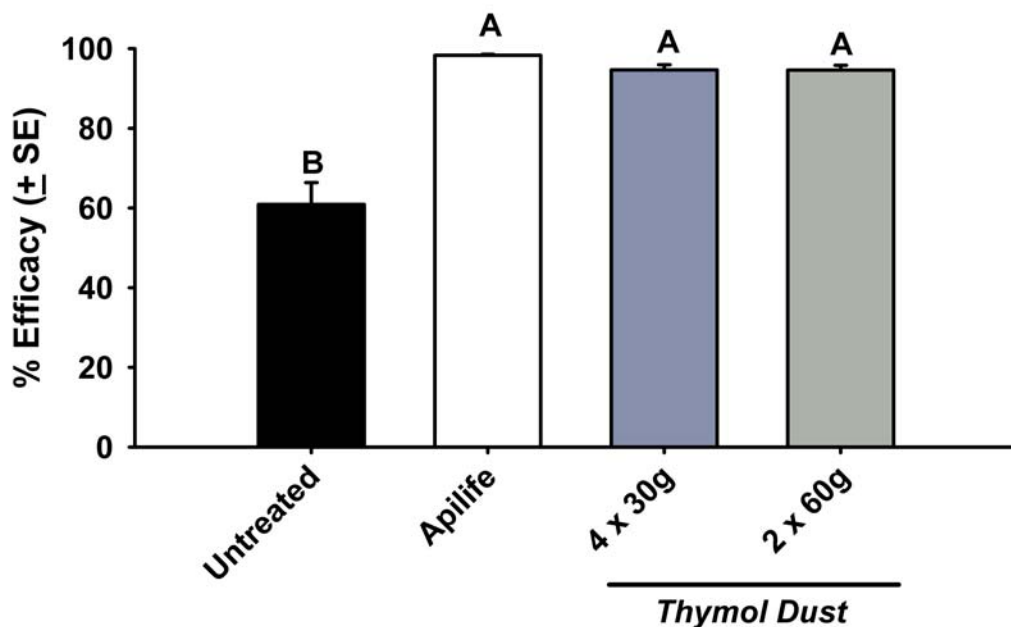
The average temperature through the treatment period was 8.6 ± 9.0 (SD)°C. There was considerable variation throughout this period, with the highest temperature recorded at 32.2°C on 3 June and the lowest at -9.4°C on 18 May (Figure 3).

Discussion

The application of thymol treatments resulted in a significant increase in *Varroa* counts compared to untreated colonies. This trend was reversed after the 30 d treatment period, when acaricide strips were placed among all the colonies, and untreated colonies alone exhibited a significant increase in *Varroa* counts. Furthermore, the proportion of the total number of *Varroa* counted among the thymol treatments during the treatment period exceeded 94%, compared to the 60% among the untreated colonies. Combined these results suggest that thymol treatments significantly increased *Varroa* mortality.

In general we cannot reject the hypothesis that the three thymol treatments were similar to one another and this suggests that thymol dust is a suitable alternative to Apilife VAR®. It should be noted, however, that there were two treatment period count intervals when we could not specifically reject this hypothesis (9-12 May and 21-24 May). For both intervals we found that only the thymol dust applied across two rather than four dates did not differ from the Apilife VAR® treatment. This suggests that reducing the number

Figure 2. The average percentage of the overall number of *Varroa* mites that were counted during the treatment period for colonies not treated, treated with Apilife VAR®, a commercial thymol treatment, or treated with 120 g of thymol dust applied across four dates 7 d apart or two dates 14 d apart. Means followed by the same letter indicate no significant difference in percentages (Tukey-Kramer, $\alpha = 0.05$).



of thymol dust applications in half, which reduces treatment labour costs, does not reduce efficacy. Moreover each dust treatment application took less labour to apply to colonies than the Apilife VAR® treatment.

The labour cost associated with thymol dust treatment application could be further decreased by eliminating the use of the newspaper platform. Consequently the necessity of this step with respect to treatment efficacy and safety to bees should be investigated. Since powdered sucrose is also a matrix for applying antibiotics to colonies, labour costs could be further rationalized by combining thymol and antibiotics in a single application treatment. The possibility of cross-compatibility with commonly used antibiotics should thus be investigated, although tempered with strategies to eliminate unnecessary prophylactic antibiotic applications.

These results should be considered noteworthy given the unseasonably cool spring conditions under which the treatments were evaluated. The temperature observed in our study was below that recommended for thymol treatments generally (reviewed in Imdorf et al. 1999) and, specifically, below the 12°C threshold indicated on the U.S. label for Apilife VAR® below which, the label suggests, the control of *Varroa* mites may be reduced. Furthermore the temperatures which Emsen et al. (2007), for example, found dust treatments to be effective in their study in Ontario in September (14.5°C) was almost twice the average temperature registered in this study. Specifically the temperatures in our study were markedly cool four to nine days into the treatment (12-21 May), when we observed an average temperature of 4.02°C, which is well below the 30 year average of 9.75°C. The return of seasonal temperatures over the next interval, 21-24 May, coincided with a period when *Varroa*

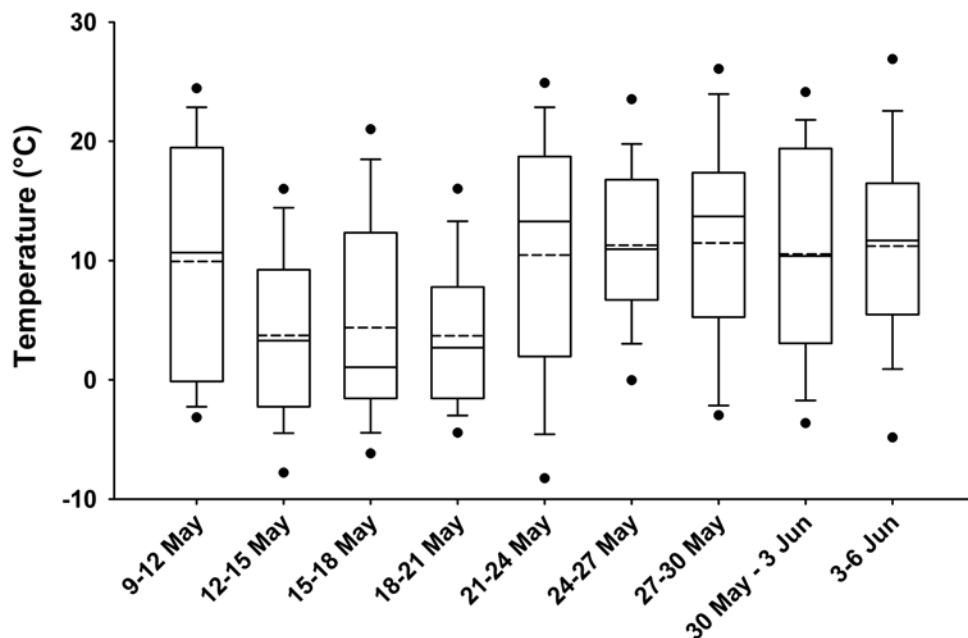


Figure 3. Box plots of ambient temperature for each varroa mite counting interval in the experimental apiary during the treatment period (9 May – 9 June 2009). The mean for each interval is represented by the hashed line and the median by the solid line inside each box. The 25th and 50th percentile is represented by the bottom and top of the box, 10th and 90th percentile by the whiskers outside the box and the 5th and 95th percentile represented by dots outside each box.

mite counts dramatically increased, suggesting low temperatures constrained efficacy. Consequently, it is reasonable to hypothesize that under more seasonal spring conditions treatment efficacy could have been higher. The possibility of control at low temperatures is of significance to beekeepers in the region as many prefer treating for *Varroa* in the spring due to time and labor constraints following honey harvest. It remains to be seen, however, whether reducing the frequency of thymol dust from four applications 7 d apart to two applications 14 d apart will continue to provide similar treatment efficacy at higher ambient temperatures.

We counted a high percentage of *Varroa* mites, exceeding 94%, falling onto adhesive boards during the treatment period for all the thymol treatments. This percentage, in a sense, is comparable to estimates of percentage efficacy calculated in other *Varroa* mite acaricide trials. Making comparisons to these studies is problematic as our approach likely overestimates efficacy because it is based on a post-treatment interval of only twelve days, whereas the complete treatment interval for the Apivar acaricide strips is 56d. This is strongly suggested when comparing the efficacy estimates for the untreated colonies in this study (61%) to those of Emsen *et al.* (2007) (~20%). Nonetheless, we are confident that while this may lead to an overestimate of efficacy, the post-treatment counts nonetheless reflect the numbers of mites remaining in the colony, as short-term acaricide counts are correlated to the counts across the entire span of the treatment (Calderone 1999). Furthermore, we suggest that since the rate of post-treatment *Varroa* drop remained relatively unchanged among the untreated colonies compared to the thymol treated groups, which declined, that we are likely overestimating the efficacy of the untreated group to a greater extent than we are for the thymol treatments.

Conclusion and Recommendations

Thymol dusting appears to be a promising alternative to vermiculite-based formulations. We recommend the continued testing of thymol dust formulations across different regions of North America in order to better predict the consistency of *Varroa* mite control beekeepers could expect. Nonetheless, our results suggest that better efficacy may be attained when average temperatures are above 10°C. Although reducing the treatment application frequency from four applications at 7 d intervals to two applications at 14 d intervals shows promise for reducing the labour costs of dust formulations, we recommend continued research to confirm this finding in other apicultural settings.

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Combining An Artificial Break In Brood Rearing With Oxalic Acid Treatment To Reduce Varroa Mite Levels

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Summary

Sixty-two colonies were set up to test the hypothesis that *Varroa* control with oxalic acid (OA) can be enhanced by combining treatment with late summer requeening. Each colony consisted of two full depth boxes with nine frames per box. All colonies were queen right at the beginning of experiment and had brood present. Approximately 300 adult bees were collected pre- and post-treatment to calculate mites-per-bee. This experiment consisted of four treatment groups, requeen plus OA, requeen only, OA only, and controls (untreated). Queens were caged in the requeen treatment groups five days prior to placing a sealed queen cell in the colonies. This provided a period of 18-21 days without egg lying, which allowed most of the brood present to emerge.

A 1.7 oz (50 ml) application of a 3.0% OA sugar water solution (sugar:water) (1:1) (w:w:w) was applied to the requeen plus OA, and OA only treatment groups. The OA solution was trickled from

above the frames into the bee-ways with a 3.4 oz (100 ml) syringe. Post-treatment alcohol samples were taken four days after treatment. The results indicate that combining late summer requeening with OA treatment significantly reduced mites compared to untreated colonies, colonies that are requeened but not treated, and colonies only treated with OA.

Although we had a significant decrease in the treatment group that was requeened and treated with OA, we found evidence that the presence of brood is not the only factor affecting the efficacy of OA treatments.

Keywords: *Apis mellifera*, *Varroa destructor*, honey bees

Introduction

The *Varroa* mite (*Varroa destructor*) was first detected in North America in 1987 (Anonymous 1987). It is currently the most

destructive bee pest in North America (Sanford 2001). *Varroa* is an obligate ectoparasitic mite of the honey bee (*Apis mellifera* L.) that feeds on both adult bees and brood. It injures them both by feeding and by facilitating infection with viruses and microorganisms (Martin 2001). We investigated the hypothesis that combining an artificial break in the brood cycle in late summer with an oxalic acid (OA) treatment would provide a greater reduction in *Varroa* mite infestation than treatment alone or a break in the brood cycle without treatment. Synthetic miticides have been used frequently by beekeepers to control *Varroa* mites with differing degrees of success. However, *Varroa* mites have shown the ability to develop resistance to synthetic miticides. All of the synthetic miticides used in beehives are lipophilic in nature, and can accumulate in beeswax (Wallner 1999). Oxalic acid (OA) has been used in Europe and Canada extensively for the control of *V. destructor* with a high degree of success. OA is applied to colonies by spraying the adult bees on each frame, by trickling a solution of OA in 1:1 sugar water between the frames (Charrière and Imdorf 2002, Special Supplement 2005) or by sublimating oxalic acid crystals with heat (Special Supplement 2005). OA has been used to reduce *Varroa* mite infestations for approximately 20 years (Popov *et al.* 1989), but it is largely ineffective when brood is present (Charrière and Imorf 2002).

Queen replacement (requeening) is a best management practice that creates an artificial break in a colony's brood cycle (Laidlaw and Page 1997). A break in the brood cycle can then be used to control swarming by allowing thorough nest cleaning (Miller 1917) or to reduce diseases in the colony like sac brood (Scott Dupree 1996). In addition, a break in the brood cycle and introduction of a new queen can be used at the start of a main honey flow to release adult bees from brood rearing and therefore increase honey production (Killion 1981, Taylor 1977).

Materials and Methods

The 62 experimental colonies were located at the University of Nebraska-Lincoln's Agricultural Research and Development Center (ARDC) near Mead, Nebraska. The experiment was conducted from August 12th to September 4th, 2007. The colonies were a mixture of *A. mellifera carnica* and *A. mellifera ligustica* (obtained from C.F. Koehnen and Sons, Glenn, California). Each colony consisted of two full depth Langstroth hive bodies with nine frames each. All colonies were queen right at the beginning of experiment and had brood present. Mite infestation levels were equalized by moving frames of sealed brood among the colonies approximately three weeks prior to the beginning of the experiment. We randomly assigned the 62 experimental colonies to four treatment groups, requeen plus OA, requeen only, OA only, and controls (untreated). The treatments were applied to colonies distributed in four different apiary locations at the Agricultural Research and Development Center.

Oxalic acid treatments

A 1.7 oz (50 ml) application of a 3.0% OA solution in sugar water (1:1) (w:w) was applied to the requeen plus OA, and OA only treatment groups. The two-story colonies were separated and each half received approximately 0.85 oz (25 ml) of the OA solution. The OA solution was trickled from above the frames into the bee-ways with a 3.4 oz (100 ml) syringe and an effort was made to maximize adult bee contact with the solution. OA treatment occurred later in the afternoon when most of the colony's adult bee population was present.

Requeening

The queens in the requeen treatment groups were caged 5 days prior to inserting queen cells, and were killed when the queen cells were inserted. Queen cells normally take 10-14 days from queen emergence to the start of egg laying (Spivak and Rueter 1997), so this technique provided 18-21 days without egg lying in the treatment groups that were requeened. This protocol allowed most of the brood present in the colonies to emerge prior to OA treatment resulting in exposure of all or most of the *Varroa* mites to the OA treatment.

Estimating mite numbers

We collected approximately 300 adult bees from each colony to estimate mite populations. We used the alcohol wash technique to estimate the number of mites per adult bee (Shimanuki and Knox 2001). Adult bee samples were collected three times: 1) initial samples were taken before there was a break in the brood cycle, 2) pre-treatment samples were taken after the break in brood to estimate the *Varroa* population before OA treatments were applied, 3) post-treatment samples were taken four days after OA application.

Experimental design and statistical analysis

Our experimental design was a randomized complete block design (RCBD). Apiary was the blocking factor, and mites-per-bee ratio was the response variable. We analyzed the data using PROC GLIMMIX (SAS Institute 2006) and separated means using a *t*-test ($\alpha = 0.05$).

Results

There was no significant difference in the number of mites-per-bee among the treatment groups prior to beginning the experiment. This is shown in the first column of Table 1. The treatment groups ranged from a high of 0.04 ± 0.05 mites-per-bee to a low of 0.02 ± 0.02 mites-per-bee. There was also no significant effect for apiary location. Since up to 80% of the mites are in sealed brood when available (Martin 2001), it is understandable that there would be an increase in the number of mites on adult bees with a break in the brood cycle. This was evident when we observed an increase in mite numbers in all four treatment groups prior to OA treatment (Table 1). Post-treatment samples showed that *Varroa* mite numbers on adult bees decreased significantly in both the requeen only and the requeen plus OA treatment groups. The requeen only treatment was significant at $\alpha = 0.05$ ($t = 2.04$, $df = 168$, $P = 0.0429$). The requeen plus OA treatment provided the greatest reduction in mite infestation with a drop in infestation from 0.2 ± 0.05 to 0.04 ± 0.05 mites-per-bee ($t = 2.65$, $df = 168$, $P = 0.008$)(Figure 1).

Treatment	Mites-per-bee before break	Mites-per-bee after break	Reps (n)
Requeen + OA	0.04 ± 0.05 a ¹	0.20 ± 0.05 a	16
Requeen only	0.02 ± 0.02 a	0.10 ± 0.02 b	16
OA only	0.03 ± 0.02 a	0.08 ± 0.02 bc	16
Untreated	0.03 ± 0.01 a	0.05 ± 0.01 c	14

Table 1- ¹Means in a column followed by different letters represent significant differences (*t*-test, $\alpha = 0.05$, $df = 114$).

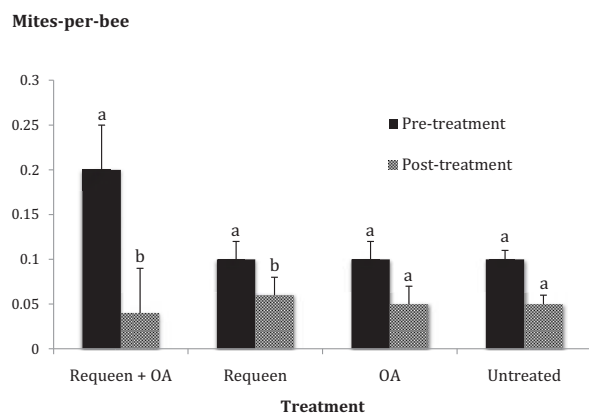


Figure 1. Estimated number of mites-per-bee before and after treatment for the 4 treatment groups. Sample times bearing different letters for each treatment group were significantly different (*t*-test, $\alpha = 0.05$).

Discussion

Combining late summer requeening with an oxalic acid treatment significantly reduces *Varroa* mite levels in honey bee colonies. Colonies that were only requeened had a significant drop in mite infestation, but the difference was not as large as occurred when OA treatment and requeening were combined. Colonies that were treated with only OA and untreated controls did not exhibit a significant drop in mite infestation. Although mite levels were significantly reduced, we did not achieve the degree of efficacy that Aliano (2008) achieved with late fall OA treatments. The difference we observed may be due to the presence of some brood, to OA not being distributed efficiently (bees do not form a tight cluster in August) or to evaporation due to warm ambient temperatures.

Our research shows that an artificial break in brood rearing accomplished by requeening can be exploited to enhance OA efficacy in late summer treatments. This experiment along with previous work, suggest that the presence of brood is not the only factor that limits OA treatment efficacy at warmer temperatures.

It would be helpful to investigate why late summer treatment of broodless colonies is less effective than early winter treatment of clustered bees. We speculate that temperature, humidity or reduced distribution are possible factors affecting OA's efficacy. Repeating this experiment using sublimation as an application method would result in more uniform distribution and would help clarify the distribution question. Placing colonies in climate controlled chambers for treatment would help clarify the role of temperature, humidity and evaporation.

Conclusions and Recommendations

The protocol used in this experiment has multiple benefits as a beekeeping management strategy. The results demonstrate that an artificial break in the brood cycle can be used to increase the effectiveness of different miticides in late summer treatments. In the Midwest, late summer is the optimal time for rearing and mating queens. This strategy provides colonies with young mated queen for the following spring. The colonies will fill the brood nest with food stores (nectar and pollen), when available, thus increasing the odds of the colony being able to overwinter. The benefits described above also provide beekeepers with optimal colonies for early spring pollination of crops such as almonds. Finally, if OA becomes a legal treatment in the United States it will allow the beekeeper to reduce *Varroa* mites with a low cost miticide.

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The Effect Of Oxalic Acid On Honey Bee Queens

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Summary

An experiment was conducted to determine if oxalic acid (OA) has a lethal or sub-lethal effect on honey bee (*Apis mellifera* L.) queens. Single-story Langstroth hives were fitted with dividers to house three three-frame colonies providing a total of 45 three-frame colonies. Each of the colonies had a separate entrance. Each colony was stocked with one frame of sealed brood, one empty comb, and 1 frame of honey and pollen. Sufficient adult bees were added to cover the frames. The colonies were randomly assigned to one of

the three treatment groups: 1) control (1 μ L of acetone), 2) 18 μ g OA per μ L (low dose), or 3) 180 μ g OA per μ L (high dose). Treatments were applied by first anesthetizing the queens with CO₂ and then applying the OA solution to the abdomen with a Hamilton micro syringe. The high dose was equal to the 48-hour LD₁₀ for worker bees (Aliano *et al.* 2006). Four response variables were measured for all colonies: queen survival, eggs laid in 24-hours, percent brood viability, cm² of sealed brood. The high dose (180 μ g OA) queens exhibited a percent brood viability of 46.7 \pm 7.35%, which

was significantly lower than in both the controls (untreated) and the low dose treatment (18 µg OA) groups. However, we found no significant difference in the amount of sealed brood following OA treatment. The reduction in brood viability appears to be temporary as colonies continued to develop normally. The dosage required to cause a measurable affect was higher than a queen would receive in a hive treated with 50 ml of a 3.0 % OA solution.

Keywords: *Apis mellifera*, *Varroa destructor*, oxalic acid, honey bee queen

Introduction

The *Varroa* mite (*Varroa destructor*) was first detected in North America in 1987 (Anonymous 1987). It is currently the most destructive bee pest in North America (Sanford 2001). *Varroa* is an obligate ectoparasitic mite of the honey bee (*Apis mellifera* L.) that feeds on both adult bees and brood. It causes injury by feeding and facilitating infection with viruses and microorganisms (Martin 2001). Synthetic miticides have been used frequently by beekeepers to control *Varroa* mites with differing degrees of success. However, *Varroa* mites have shown the ability to develop resistance to synthetic miticides. All of the synthetic miticides approved for beekeeping are lipophilic in nature, and they accumulate in beeswax (Wallner 1999). Oxalic acid (OA) has been used in Europe and Canada extensively to control *V. destructor* with a high degree of success. OA is applied to colonies by spraying the adult bees on each frame, by trickling a solution of OA in a 1:1 sugar water syrup between the frames (Charrière and Imdorf 2002, Special Supplement 2005) or by sublimating oxalic acid crystals with heat (Special Supplement 2005). Oxalic acid (OA) has been used to reduce *Varroa* mite populations for approximately 20 years (Popov *et al.* 1989), but it is largely ineffective when brood is present (Charrière and Imorf 2002). Even though extensively used in Europe and Canada oxalic acid's effect on the reproductive members of a colony is relatively unknown. This study was conducted to see if there are any sub-lethal effects on honey bee queens when used to control *Varroa* mites.

Materials and methods

Construction of divided hives

We designed and built 15 single-story Langstroth hives that were fitted with dividers to house three three-frame colonies providing a total of 45 three-frame colonies. The dividers were made of 1/8" plywood and provided a bee-tight seal between the sides of the hive body, the inner covers, and the bottom board. Each divide had a separate inner cover, which allowed examination of one colony without disturbing the others. Each of the colonies had a separate entrance. The two colonies to the outside of the hive body had entrances on one side and the middle colony had an entrance on the opposite side of the hive body. This was done to reduce drifting between the individual colonies.

Stocking the divides

We stocked the 15 divided hives by splitting colonies from an apiary located at the University of Nebraska-Lincoln East Campus. The parent colonies were composed of a mixture of *A. mellifera carnica* and *A. mellifera ligustica* honey bees (obtained from C.F. Koehnen and Sons, Glenn, California). Each colony was stocked with 1 frame of sealed brood, one empty comb, and one frame of honey and pollen with sufficient adult bees to cover the frames. This resulted in three frames for each colony and nine frames total

for the entire Langstroth hive. The entrances to each colony were sealed and the hives were moved to a cool (21-24°C) dark storage building for 12 hours. The following morning the hives were moved to the University of Nebraska-Lincoln Agricultural Research and Development Center, approximately 35 miles north of Lincoln, Nebraska. A ripe queen cell (ready to emerge) was then placed in each colony. The hives were left untouched for 2 weeks, which gave the virgin queen time to emerge, mate and begin laying eggs.

Treatment and data collection

The queens in the 45 three-frame colonies were randomly assigned to one of the three treatment groups: 1) control (1 µL of acetone), 2) 18 µg OA per µL (low dose), or 3) 180 µg OA per µL (high dose). The high dose was equal to the 48-hour LD₁₀ for worker bees reported by Aliano *et al.* (2006). The low dose was calculated to be 10 fold lower than the LD₁₀. Serial dilutions of OA dihydrate (>99% purity) (The Science Company) (CAS no. 6153-56-6) in acetone were prepared. The 45-mated queens were located and placed in individual cages. The queens were anesthetized with CO₂ one at a time. The corresponding treatments were applied to the abdomen using a Hamilton micro syringe and repeating dispenser (Hamilton Company, Reno, NV). The queens were then returned to their colonies and monitored for seven weeks.

Queen survival was measure by locating the queen in each colony seven, 14, and 21 days after OA treatment. The queens were each marked on the thorax with a unique color of enamel paint (Dadant & Sons Inc.) when they received the OA treatment. The markings made it possible to determine if the original queen was still present.

To measure queen productivity all of the frames in the colony were removed individually and a piece of clear plexiglass with a 1" X 1" grid was placed over the frame. The cm² of sealed brood were then estimated. The measurements were taken approximately three weeks after treatment.

To examine the egg laying rate, the queens were caged to one side of an empty frame for 24-hours. The cages measured 17.8 cm X 17.8 cm and covered approximately 1165 worker cells. The cages were built with eight-mesh wire and had plastic queen excluder covering the center of the cages so that worker bees would be able to tend to the queen. After 24-hours, the queens were released and the eggs on the frame were counted. To measure percent brood viability, the frames that the queens were caged on were removed from the colonies after 10 days and the sealed brood cells were counted. The number of sealed brood cells was then divided by the number of eggs originally laid in the 24-hour period.

Experimental design and statistical analysis

The experimental design was a completely randomized design (CRD). Four response variables were measured for all colonies: 1) queen survival, 2) eggs laid in 24-hours, 3) brood viability, 4) square centimeters of sealed brood. We analyzed the data using PROC MIX (SAS Institute 1999) and separated means using a *t*-test ($\alpha = 0.05$).

Results

Queen survival

Before OA treatment occurred 14 of the 45 queens were lost. This was due to some of the queens not emerging or not returning from mating flights. Even though the LD₁₀ for workers reported by Aliano *et al.* (2006) was used as the high dose, there was no loss of due to the treatment of OA.

Treatment	Estimates ± std. error	N
High Dose	639.3 ± 335.6 a ¹	11
Low Dose	768.8 ± 443.1 a	12
Control	846.8 ± 489.7 a	8

Queen productivity

The three treatment means for queen productivity are shown in Table 1. There was no significant difference in the cm² among the three treatments ($F = 0.60, df = 28, P = 0.5561$). The amount of sealed brood present for the 3 treatments ranged from 226 to 1852 cm².

Egg production

The three treatment means for egg production are shown in Table 2. There was no significant difference in the number of eggs laid in 24 hours among the three treatments ($F = 0.49, df = 28, P = 0.6194$). The highest number of eggs laid in 24 hours was in the high dose at 679. Both the control and low dose treatments had queens that laid no eggs.

Treatment	Estimates ± std. error	N
High Dose	400.27 ± 225.01 a ¹	11
Low Dose	387.58 ± 186.56 a	12
Control	315.75 ± 156.86 a	8

Table 2. Mean egg laying estimates during the 24-hours following treatment with one of two concentrations of oxalic acid.

¹Means followed by different letters represent significant differences (*t*-test, $\alpha = 0.05$).

Brood viability

The treatment effect was significant for brood viability ($F = 7.93, df = 26, P = 0.002$). There was a significant difference in brood viability between the control and the high dose treatments ($t = 3.12, df = 26, P = 0.0043$)(Figure 1). Similarly, there was a significant difference in brood viability between the high dose and low dose ($t = -3.60, df = 26, P = 0.0013$). There was no significant difference between the controls and the low dose.

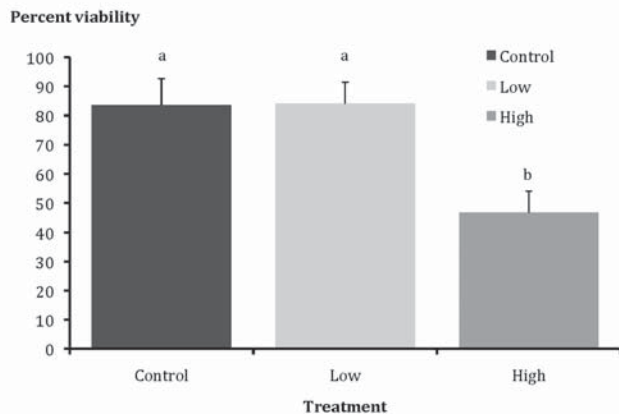


Figure 1. Mean percent viability for brood laid by queens treated with one of two concentrations of oxalic acid. Treatments bearing different letters were significantly different (*t*-test, $\alpha = 0.05$).

Table 1. Mean queen productivity estimates (cm² of brood by treatment) for queens that received one of two concentrations of oxalic acid.

¹Means followed by different letters represent significant differences (*t*-test, $\alpha = 0.05$).

Discussion

The queens in the high dose (180 µg OA) treatment demonstrated a mean brood viability of 46.7 ± 7.35% which is significantly lower than both the control (untreated) and the low dose treatment (18 µg OA) queens' mean brood viability. However, since we observed no significant difference in brood production following OA treatment, the reduction in brood viability seems to have little effect on the colonies' development and may be temporary. The low dose treatment (18 µg OA) more closely resembles what a queen would be exposed to when a colony is treated with OA (trickling method), and it did not result in any reduction in egg laying or brood production. There was also no significant reduction in brood viability between the low dose and controls. Interestingly, there was no difference in queen survival for any of the three treatments, and no queens died during the experiment.

The results also indicate some interesting trends. Queens in both of the OA treatment groups (high and low dose) laid a higher number of eggs in a 24-hour period than untreated queens. Although the difference was not significant, this trend is possibly a response by the queen to the stress of exposure to OA. However, the cm² of sealed brood present 3 weeks later was numerically lower in the OA treatment groups than in the control group. Although our results indicated a trend, the differences between treatments were not significant.

Some remaining questions to answer include: 1) is there a difference in how workers, drones and queens respond to OA exposure? 2) at exactly what developmental stage is brood viability affected? 3) does OA affect sperm stored in the spermatheca?

Conclusions and Recommendations

The results of this study support the recommendation that beekeepers should be careful not to exceed the recommended dose of OA. The results also suggest that there may be a difference in the ability of workers and queens to tolerate OA exposure with queens possibly being less vulnerable to injury. A larger test would be required to verify this possible difference.

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Analysis of Bacterial Pathogens in Virginia Honey

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Abstract

The presence of microorganisms in honey samples from beekeepers throughout the State of Virginia (USA) was investigated to determine the prevalence of *Clostridium botulinum* (van Ermengem) and *Paenibacillus larvae* (White). Several studies have shown honey to contain microorganisms such as spore forming bacteria and yeasts. *C. botulinum* has been linked to cases of infant botulism, and *P. larvae* is the etiological agent of American Foulbrood (AFB) in honeybees, *Apis mellifera* L. Out of 92 honey samples submitted by beekeepers from around the state, only three (3.2%) were found to contain *P. larvae*, and no *C. botulinum* was found.

Keywords: *Paenibacillus larvae*, *Clostridium botulinum*, American Foulbrood Disease

Introduction

The low pH, high sugar content, and low water content contribute to the antimicrobial activity of honey (Iurlina and Fritz, 2005). According to White (1963) the pH of honey ranges from 3.4 to 6.1 with an average of 3.9 and an optimum moisture content of 17.2%. Glucose oxidase also contributes to the bacterostatic and bactericidal properties of honey by producing hydrogen peroxide and gluconic acid when honey is diluted. These compounds influence the volatile conditions that prevent growth and germination of microorganisms in honey (White, 1963). However, several studies have shown honey to contain microorganisms such as spore forming bacteria and yeasts (Finola et al., 2005; Iurlina and Fritz, 2005). Microorganisms are not typically able to grow in honey, but may be able to survive in a static or spore stage. Spore forming bacteria are potentially dangerous because spores are not destroyed during the extraction and processing of honey and can persist in honey indefinitely. Two spore forming bacteria of particular concern are *Clostridium botulinum* and *Paenibacillus larvae* because of the potentially harmful diseases they produce (Arnon et al., 1978; Shimanuki, 1997).

C. botulinum, a Gram positive anaerobe, is the causative agent of botulism, a neurotoxic disorder. *C. botulinum* spores have been found in honey and have been linked to several infant botulism occurrences (Arnon et al. 1978). Children less than one year of age have an immature intestinal flora that cannot prevent spore germination or production of the botulin neurotoxins after the spores have been ingested (Nevas et al. 2002). Previous studies have indicated that the incidence of *C. botulinum* in honey samples is usually low (Monetto et al. 1999), but can range from 2% to 24%, with an average prevalence of about 10% (Nevas et al., 2006). However, Iurlina and Fritz (2005) did not find *C. botulinum* in any of the honey samples they tested from Argentina.

P. larvae is a gram positive aerobe and the etiological agent of American Foulbrood Disease (AFB) in honeybees, *Apis mellifera*

(Shimanuki 1997). AFB is a highly contagious disease that affects only the larvae of honeybees, but can be detrimental to a colony and may result in hive death, if it is not caught early in the disease outbreak cycle (Lauro et al. 2003). The spore is the only infectious stage, yet can cause disease after the ingestion of as few as 10 spores by a larva (Brodsgaard et al. 1998). AFB is easily spread to healthy hives though the transfer of spores by robbing or drifting bees. Furthermore, Antúnez et al. (2004) indicated that there is a correlation between the first occurrence of AFB in a hive and the spread of the disease to other colonies in close proximity. Since *P. larvae* is easily transmissible and highly destructive to colonies, it is of considerable socioeconomic importance to beekeepers (Lauro et al. 2003).

Studies on the prevalence of *C. botulinum* and *P. larvae* have not been conducted on Virginia honey, although several studies have reported on the occurrence of both pathogens in honey in the United States. The most recent study (Solomon and Lilly 2001) reported a 13% prevalence of *C. botulinum* in U.S. honey, while Alippi (1995) reported an 8% incidence of *P. larvae* in U.S. honey. Steinkraus and Morse (1992) reported an 8.5% incidence level for *P. larvae* in some U.S. and Canadian honeys. The focus of this study was to identify the incidence of *C. botulinum* and *P. larvae* in Virginia honeys and identify any associations with respect to pathogen distribution within the state.

Materials and Methods

Sample sources. A total of 92 honey samples were examined for the presence of *C. botulinum* and *P. larvae* spores. The honey samples were submitted by local beekeepers in different areas of Virginia (Figure 1), following requests for honey samples at local and state association meetings, and in the state association newsletter.

Moisture content and pH. An Atago honey refractometer (Atago USA, Bellevue, WA) was used to determine the moisture



Figure 1. The locations within Virginia USA from which honey samples were submitted for the analysis of the pathogenic bacteria, *Paenibacillus larvae* and *Clostridium botulinum*. Squares indicate the location of positive *P. larvae* samples.

content of each honey sample. The pH of 58 of the honey samples was taken following the procedures outlined in the AOAC Official Method (Method 962.19) for honey acidity (AOAC 2002). An Accumet pH meter (model 805MP; Allied Fisher Scientific, Pittsburg, PA) was used for the pH determinations after calibration using Orion buffers (pH 7.00 and 4.01) (Thermo Electron Corporation Waltham, MA). All measurements were performed at 25°C. Since low pH and low moisture content both contribute to the antibacterial properties of honey, they were examined to determine if there was any correlation between high pH or moisture content and bacterial growth.

Bacterial Detection. Honey samples were warmed to 35°C in a water bath prior to mixing and analysis. A 1 ml sub-sample was removed from each beekeeper sample and diluted 1:2 (w/v) in sterile distilled water. The samples were then centrifuged at 12,000 x g for 30 min. The supernatant was discarded and the pellet was streaked onto one Brain Heart Infusion plate supplemented with 1% thiamine (BHI+T) and one Columbia Blood agar plate. *P. larvae* requires thymine in the media in order to survive; it is not capable of growth on minimal media, but can sometimes grow on Colombia Blood agar plates (Heyndrickx et al. 1996). Plates were incubated at 37°C for up to 72 hours to allow sufficient time for growth of any spore forming bacteria present in the honey. Plates that had observable growth were further analyzed to determine the species of bacteria. Classification of bacterial growth was based on characteristic colony morphology on BHI+T and Colombia Blood agar plates (Heyndrickx et al., 1996). Cell morphology was also used as a diagnostic tool in identification through use of a standard Gram stain method and a spore stain using malachite green with basic fuchsin as a counter stain. Samples suspected to be *P. larvae* were subjected to further investigation.

Catalase and Plagemann tests. All cultures suspected to be *P. larvae* based on colony and cell morphology were analyzed by catalase and Plagemann tests (Kilwinski et al. 2004). For the catalase test, a small portion of the colony from the BHI+T plate was transferred onto a clean petri plate and mixed with a drop of 3% H₂O₂. Production of air bubbles indicated a positive reaction; no production of air bubbles or weak/delayed production indicated a negative reaction. *P. larvae* is negative or weakly positive for catalase (Kilwinski et al., 2004). For the Plagemann test, blood agar slants were inoculated and sealed with Parafilm. After 10 days of incubation at 37°C on a blood slant, growth was examined for the presence of giant whips (Hansen and Brodsgaard, 1999) using phase contrast microscopy.

C. botulinum detection. Ten samples suspected to contain *C. botulinum* were selected based on the observation of spores when the raw honey sample was examined either by Gram stain or a spore stain and no aerobic growth. These samples were diluted 2:1 (w:v) in sterile DI water and centrifuged at 12,000 x g for 3 min. The supernatant was discarded. The pellet was transferred to 19 ml of thioglycolate medium and initially incubated under anaerobic conditions at 35°C for 48 hours. Samples that appeared turbid were then streaked onto BHI agar roll tubes and incubated under anaerobic conditions at 35°C for 48 hours. Samples which were not turbid were incubated further at 35°C for a total of 5 days and new colonies were isolated. From the BHI+T agar roll tubes, each sample was inoculated into TPYG medium (Appendix A) (Nevas et al. 2002). Positive samples were then subjected to a cellular fatty acid analysis by gas chromatography and fatty acid profiles compared to a library of known organisms (Moore et al. 1994). Prior to GC cellular fatty acid analysis the samples were concentrated by

centrifugation, washed, and then frozen.

Cellular Fatty Acid Identification (CFA). The frozen pellets from the PYGT cultures were thawed and the cells were lysed and saponified with 1.0 ml of basic methanol (45 g of NaOH, 150 ml of methanol, 150 ml of deionized water). The samples were then heated in a boiling water bath for five min, mixed, heated in the boiling water bath for an additional 25 min and then cooled. The anaerobe cell constituents were methylated and the methylated components were then extracted following the procedures of Moore et al. (1994). Each extract was washed once with a three ml solution containing 5.4 g of NaOH in 450 ml of deionized distilled water saturated with NaCl.

A 2ul portion of the washed extract was analyzed on an HP-5890A gas chromatograph (Hewlett-Packard Co., Palo Alto, CA.) equipped with a fused-silica capillary column, a flame ionization detector, a model HP 6763 autosampler, and a model HP-3392A integrator (Hewlett-Packard). Gas flow rates were 400 ml/min for air, 30 ml/min for hydrogen, and 30 ml/min for nitrogen. Temperatures were 250°C for the injection port and 300°C for the detector. After injection, the oven temperature was increased from 170 to 270°C at a rate of 5°C/min and then from 270 to 310°C at a rate of 30°C/min, held at 310°C for 2 min, and then returned to 170°C before the next sample was injected. A standard mixture containing known fatty acids (FAs) (C9 through C20 straight-chain FAs and 2-OH, C10, 3-OH C14, 3-OH C14, and 2-OH C16 FAs) was chromatographed at the beginning of each day on which samples were analyzed and after each set of 10 samples.

The Sherlock Microbial Identification System version 4.0B software package (MIS), (MIDI, Inc., Newark, DE) (13) was used to identify the peaks (by retention time) and to determine the area, the ratio of area to height, the equivalent chain length (ECL), the total area, and the total area for named or listed compounds. The (MIS) software package was used to calculate the percentage of area for each named or listed compound compared with the total area of the compounds detected. Compounds were identified using the Moore Broth library, version 3.90 (released in 1995) for anaerobes.

Results and Discussion

The pH of honey samples fell between 3.70-5.26, with an average pH of 4.19 (± 0.31 SD). All pH values fell within the mid-range of values reported by White (1992) for US honeys. The moisture content of the honey samples fell between 13.6 –19.4% with an average of 17.0%; only four samples (4.3%) had a moisture content greater than 18.6%, the U.S. standard for Grade A honey. Moisture levels above 19.0% increase the likelihood of fermentation (White 1992, Finola et al. 2007). No significant correlations were found between pH, moisture content, and bacterial growth on either media.

Out of 92 honey samples tested, 46 (50.0%) samples had growth on either BHI+T plates, Colombia Blood plates, or both. From these, 11 (23.9%) plates had colony characteristics that matched *P. larvae*. Five (10.8%) of these colonies had either a negative or weakly positive result to the catalase test and were examined microscopically for the presence of giant whips after the Plagemann test was conducted (Figure 2). Giant whips were present in three (3.2%) of the honey samples, confirming the presence of *P. larvae*.

Ten samples were suspected to contain *C. botulinum* spores based on the microscopic analysis. Several colonies with different morphology were observed after growth on a BHI agar roll tube.



Figure 2. Gram stain giant whips. Sediment from the Plagemann test was Gram stained and found to be Gram-positive. Giant whips, indicative of *P. larvae*, were observed in three samples.

Each colony with a different morphology was subjected to fatty acid analysis by GC, but none of the fatty acid profiles were indicative of *C. botulinum*. The identity of the organisms was not confirmed, but could indicate the presence of other obligate anaerobes in the honey samples. These results are consistent with those of Iurlina and Fritz (2005) who did not find *C. botulinum* in any of the samples they tested. The results are also in agreement with Piana et al. (1991) who found unidentified anaerobic spores in their samples. Additional studies are needed to identify the unknown anaerobic bacteria in honey.

Brevibacillus laterosporus was identified in two cultures through microscopic analysis and cell morphology. *B. laterosporus* has a characteristic appearance identified as a Gram-positive canoe-shaped body that stains darkly with a large spore that is not penetrated by stain during a Gram stain (Figure 3). During the spore stain, the spores appear green, while the cells appear pink. *B. laterosporus* was not of primary concern because it is a microbe that is found throughout soil, but does not have any pathogenic implications.

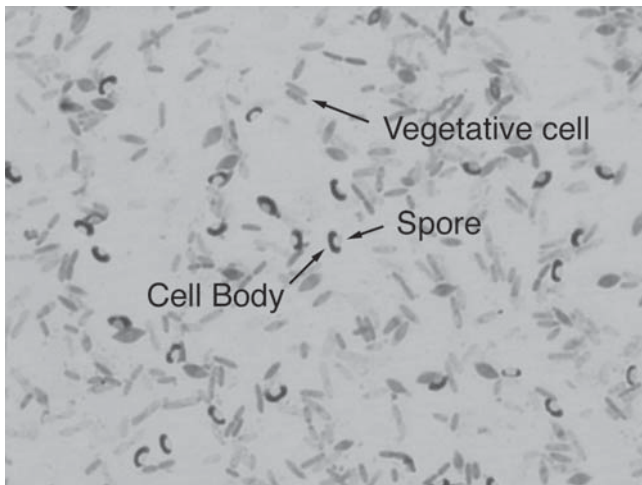


Figure 3. *Brevibacillus laterosporus* has a characteristic canoe-shape when observed under a Gram stain. *B. laterosporus* are Gram-positive rods that appear dark, but the spores can not hold a stain and are clear, resulting in the characteristic shape.

The low level of *P. larvae* (3.2% of all samples) found in Virginia honey reflects the low incidence of AFB in hives (3%) in the state (Tignor 2009). Both Steinkraus and Morse (1992) and Pernal and Melathopolos (2006) reported significantly higher incidence levels of *P. larvae* in U.S. and Canadian honeys. The initial higher number of samples (11 samples, 12.0% of all samples) that had bacterial colony characteristics similar to those described for *P. larvae* indicates the need for confirmatory tests, such as the Plagemann test, before drawing conclusions as to the identity of the organism. The development of PCR assays for the detection of *P. larvae* in honey samples provides an alternative approach for the detection and confirmation of contaminated honey (Govan et al. 1999, De Graaf et al. 2001, Bakonyi et al. 2003).

The absence of *C. botulinum* in any honey samples is consistent with reports from several other studies on honey (White 1992, Iurlina and Fritz 2005). However, this result could also be due to several factors such as a low incidence of *C. botulinum* in the soil in Virginia or to a lack of detection from an uneven distribution or low numbers of spores in honey (Midura et al., 1979). Efforts were made to obtain a homogenous mixture of each honey sample; however, multiple sub-samples might better achieve this goal and yield different results. A knowledge of the levels of *C. botulinum* in Virginia soils may also give a better indication of the accuracy of these results, since Nevas et al. (2006) found a correlation between *C. botulinum* spores found in honey and the surrounding environment.

Conclusions and Recommendations

Honey can serve as a vector medium for both honey bee and human disease organisms (Sturtevant 1932, Arnon 1980). However, the low levels of pathogenic bacteria in the honey samples analyzed in this survey indicate that the honey produced and extracted by beekeepers in Virginia has a very low probability of causing health problems to humans or bees. The low incidence of *P. larvae* in honey also suggests that the movement of honey frames between hives by beekeepers (within their own yards) is relatively safe as long as the hives do not exhibit signs of AFB.

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Appendix A: TPYG formula

Peptone	0.5 g
Trypticase	0.5 g
Yeast extract	1.0 g
Resazurin	0.4 ml
Cysteine	0.05 g
Glucose	0.5 g
Salt solution	4.0 ml
Hemin solution	1.0 ml
Vitamin K1	0.2 ml
Distilled water	100 ml



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