8-2004

Examining Varroa-resistant Honey Bee Queens from Commercial Breeders: Colony Productivity, Hygienic Behavior, Suppression of Mite Reproduction, and the Relationship of Juvenile Hormone III to Mite Abundance

Laura L. Bryant
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To the Graduate Council:

I am submitting herewith a thesis written by Laura L. Bryant entitled "Examining Varroa-resistant Honey Bee Queens from Commercial Breeders: Colony Productivity, Hygienic Behavior, Suppression of Mite Reproduction, and the Relationship of Juvenile Hormone III to Mite Abundance." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Entomology and Plant Pathology.

John A. Skinner, Major Professor

We have read this thesis and recommend its acceptance:

James P. Parkman, Carl Jones

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Examining *Varroa*-resistant Honey Bee Queens from Commercial Breeders: Colony Productivity, Hygienic Behavior, Suppression of Mite Reproduction, and the Relationship of Juvenile Hormone III to Mite Abundance

A Thesis Presented for the Master of Science Degree
The University of Tennessee, Knoxville

Laura L. Bryant
August 2004

Dedication

This thesis is dedicated to my mother, Lynne Webb, and to my fiancé and best friend, Edward Pausch. Their faith and support have strengthened me. I will be forever grateful for the love, wine, and laughter.

Acknowledgements

I would like to thank those whose help I have found "above-and-beyond" and without whom I would have certainly floundered. I extend most sincere appreciation to Michael Studer for his technical and field support (and for teaching me what at first seemed impossible) and to Dr. James Parkman who was there at every turn with informed answers, reliable advice, and a sarcasm that made me feel at home. I owe a debt of gratitude to Dr. John Skinner for showing patience and calm when I showed neither, and to Dr. Carl Jones for his wisdom and diplomacy at every critical juncture. Also, thanks to Aurora Canaday for her phenomenal work ethic and her willingness to endure bee stings and high temperatures long past time to go. Finally, a warm thank you to Pennie Long who sensed my apprehension on my very first day and kindly assured me that I was in a good place.

Abstract

This research was conducted to assess the performance of commercially bred honey bee queens sold as resistant to the parasitic mite, *Varroa destructor*. The study's objectives were to: 1) Compare honey and pollen stores and *V. destructor* infestation in colonies established with hybrid Russian, SMR, and control queens, 2) Determine levels of hygienic behavior and mite non-reproduction in the same colonies, and 3) Determine the relationship between juvenile hormone III in honey bee larvae and *V. destructor* reproduction.

In Part One, when honey, pollen, and *V. destructor* levels were measured, no significant differences were found among types of queens. The similarity of *V. destructor* levels among study colonies with hybrid queens suggests that hybridization has diminished the effectiveness of the mite-resistance found in artificially inseminated mite-resistant queens.

In Part Two, two traits associated with mite tolerance in honey bee colonies were measured, hygienic behavior and mite non-reproduction. Again, no significant differences were found in the levels of either trait among queen types. However, significant relationships were found between both traits and *V. destructor* concentrations in the colonies at the end of the season.

Data suggest that, while the levels of resistant traits in hybrid SMR and Russian queens available from commercial breeders do not differ significantly from controls, these traits are present in the honey bee population as a whole and contribute to lower parasite infestations.

In Part Three, the possible influence of honey bee juvenile hormone III levels on *V. destructor* reproduction was examined. A short test was conducted to determine juvenile hormone titers during the honey bee's fifth larval instar, a period coincident with initial mite feeding. Radioimmunoassay was used to detect juvenile hormone in the bees' hemolymph.

Positive relationships were found between juvenile hormone titers and *V. destructor* reproduction and between juvenile hormone titers and *V. destructor* concentration in the colonies at the end of the season. Results suggest that low host

juvenile hormone levels might diminish the reproductive capacity of the *Varroa* mite, both in terms of absolute non-reproduction and in reduced fecundity.

Recommendations are made to queen breeders for the increased use of *Varroa*-resistant drones in mating yards to ensure the preservation of resistant traits in hybrid queens. Broader studies of juvenile hormone and *V. destructor* reproduction are also recommended.

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Introduction

Varroa destructor (Anderson and Trueman 2000) is an ectoparasitic mite of the European honey bee (*Apis mellifera* L.). It is one of a small group of mites that reproduce exclusively in the brood of *Apis* spp. Specifically, it is part of a complex including at least one other species, *Varroa jacobsoni* Oud., which was described in 1904 in association with the Asian cavity-nesting honeybee, *Apis cerana* Fabr. *V. destructor* is native to the Asian mainland and is believed to have widened its host range to include *A. mellifera* in the 1950s. It is widely accepted that the introduction of *V. destructor* to *A. mellifera* resulted from the movement of colonies by *A. mellifera* beekeepers through mite-infested areas.

Because *V. destructor* and *A. mellifera* did not co-evolve, the parasite/host relationship is immature. *A. mellifera* has few defenses against *Varroa*, and as a result, the mite has thrived in the habitat of its new host's colonies. *V. destructor* reproduces within both worker and drone brood in *A. mellifera*, whereas in its natural host, it only reproduces in the drone brood (Anderson and Trueman, 2000; Koeniger 1981). This is significant because worker brood is far more abundant than is drone brood. The range of the mite has expanded because of some common practices of beekeeping, e.g. the transportation of colonies over long distances for crop pollination and the packaging and shipping of bees transcontinentally. The beekeeping industry has in fact, along with the natural swarming and robbing behaviors of honey bees, accelerated the dispersal and proliferation of *V. destructor* in *A. mellifera* colonies worldwide. The mite was eventually introduced to North America, through Florida, in 1987 (Sanford 1987), and today, only Australia is free of the honey bee parasite (Cunningham et al., 2002).

Economic losses caused by varoosis (the technical term for *Varroa* infestation) are difficult to quantify, because there are many factors that influence honey bee colony health. However, it is generally accepted that, once mites have been detected, colonies left untreated will collapse within two years. The susceptibility of *A. mellifera* to varoosis, together with the intrinsic value of *A. mellifera* as pollinators of commercial crops (estimated at $14.6 billion per year in the U.S.) (Morse and Calderone, 2000), suggest that the potential economic impact of *V. destructor* is very high. Contributing to

concern are studies, in both the U.S. and Europe, which have demonstrated *V. destructor* resistance to two of the most commonly used chemical miticides, fluvalinate (Milani, 1995; Elzen et al., 1998) and coumaphos (Massimo et al.2001; Elzen and Westervelt, 2002).

Because *V. destructor* is a considerable threat to American apiculture and agriculture, vast resources have been devoted to managing and researching it. Chemical treatments are currently the preferred method of control; however, concerns for cost, resistance, and hive product contamination preclude pesticides from long-term viability. Other management tactics, including various cultural controls have been explored, but are typically not cost-effective for the average large-scale beekeeping operation.

The most sustainable of the currently available *Varroa* control methods is the use of mite-resistant bees. Resistance to *V. destructor* has been found in *A. mellifera,* in a variety of forms, and controlled breeding programs have sought to select for resistant traits that are heritable (Harbo and Harris, 1999b; Rinderer et al, 1999). Two lines of bees have emerged from these programs, SMR (Suppression of Mite Reproduction) (Harris and Harbo, 2000) and Russian (Rinderer et al, 2000), and it has been the policy of the USDA and numerous university extension services to promote these lines as integrated pest management tools. However, there is limited data on the resistance levels of these bees when obtained through the usual channels, from commercial queen breeders. In addition, precise mechanisms of resistance within these bees have not yet been identified.

Mite Biology

V. destructor is a mesostigmatid, of the family Varroidae. The mature adult female is heavily sclerotized, ovoid, flattened (crab-like), and dark reddish-brown in color. At 1.1 mm long x 1.6 mm wide, the mature *V. destructor* female is, relative to the size of its host, one of the largest ectoparasites known. The adult male is smaller and more spherical (0.8 mm long x 0.7 mm wide), with a barely sclerotized, yellowish-green cuticle (Martin 2001a).

V. destructor has two distinct phases within its life cycle: the reproductive phase, which occurs only in capped honey bee brood cells, and the phoretic phase, which is spent out of the cell on the adult bee. Although it is not clear what induces it (see Juvenile Hormone), the reproductive phase begins with the female mite's entrance of a cell containing a developing 5th instar bee larva. Invasion occurs at a fairly constant rate during a 15-20 hour pre-capping period in worker brood and a 40-50 hour pre-capping period in drone brood (Boot et al., 1992). After the mite enters the cell, it climbs down the cell wall and immerses itself into the jelly-like brood food at the cell's bottom. The mite remains submerged, respiring through two peritremes (acting as snorkels) until the larva frees it by consuming the food. The mite is usually freed within the first six hours post-capping (Boot et al., 1994a).

Once released, the female mite begins to feed on the developing bee, using serrated chelicerae to tear the larva's integument and create a wound through which to access hemolymph. The mite lays her first egg 60-70 hours after the cell is capped (Infantidis, 1983). *V. destructor* is arrhenotokous (haplo-diploid); the male mite develops from unfertilized eggs and has only seven chromosomes, while the female mite develops from fertilized eggs and has 14 (Steiner et al., 1982). During her time in the cell, the mother mite lays up to six eggs. The first, unfertilized, egg is male. All subsequent eggs are female and are laid in approximate 30-hour intervals (Steiner et al., 1994; Donze and Guerin, 1994).

As an apparent adaptation to the time constraints of the capped brood period, *Varroa* mites have omitted the six-legged larval stage and, therefore, hatch directly into eight-legged protonymphs (Steiner et al, 1994). The protonymph stage lasts 52 hours for male mites, 30 hours for female. During this stage, male and female mites are similar in appearance, both are spheroid and translucent white; however, after molting to the deutonymph stage (which lasts approximately 75 hours for both sexes) female mites are their adult size and shape and are easily distinguished from the male (Harris and Harbo, 2001).

During the molt to the adult stage, the male mite's pointed chelicerae metamorphose into hollow tubes. This modification allows for sperm transfer to the

female, through a genital opening at the base of the third pair of legs (De Jong, 1997). Despite the lessened utility of the chelicerae, open wounds created by the female mites allow the male mite to continue feeding (Donzé and Guerin, 1994).

After the female *Varroa* molts to the adult stage, it mates with the brother mite in the cell. In optimum environments, the male and two to three of the female mites reach reproductive maturity before the honey bee uncaps the cell. Tests have demonstrated, though, that given mortality and infertility rates, the mean number of viable female offspring per foundress mite is less than two (Infantidis, 1983; De Ruijter, 1987). Multiple matings are required to fill the spermatheca, after which the female mite can no longer accept sperm, thereby preventing any subsequent mating (Donzé et al, 1996). A newly mated female *Varroa* mite does not, however, lay eggs in the cell in which it has mated. Sperm maturation occurs in the female reproductive tract, and 4-13 days are required for the complete development of prosperm to spermatozoa. (Alberti and Hänel, 1986; Harris and Harbo, 1999). Under natural conditions, the female *V. destructor* averages three reproductive cycles per mite (Martin and Kemp, 1997). The reproductive behavior of *V. destructor* dictates that the only opportunity for genetic recombination occurs in instances of mutation or when more than one foundress mite invades a brood cell.

When the fully developed honey bee emerges (after 20-21days), the phoretic phase begins for those female mites that have reached maturity. The male mite, which is small and soft, and which has no functioning chelicerae, does not survive long outside the cell. Nor do immature female mites. The hard and flattened adult female, however, is equipped to survive in the open colony. With sharp claws and numerous ventral setae, which act as Velcro® with the honey bee's branched hairs, the mite maintains a firm hold, attaching itself to the bee's abdomen and feeding at the intersegmental membranes (Martin, 2001a).

The duration of the phoretic phase is dependent on the availability of brood cells as well as on the number of adult bees coming in contact with those brood cells. (It is, perhaps, for this reason that *Varroa* appear to prefer nurse bees) (De Jong, 1997). At times when brood production is ample, as in early summer, the phoretic period averages

4-6 days. During the winter, when production slows or stops**,** the phoretic period may last many months (De Ruijter, 1987). There is a disagreement in the literature as to whether a phoretic period is necessary for successful mite reproduction. De Ruijter (1987) published a study demonstrating that mites kept from feeding on adult bees are still capable of reproduction. However, another study since then has indicated that phoresy is a requirement. (Beetsma et al., 1999).

Finally, though much of the research on *Varroa* and *A. mellifera* has been done with worker brood, *V. destructor* exhibits a strong preference for drone brood (Boot et al, 1994b). A number of explanations are plausible: the mite is simply retaining an inherent behavior (it prefers drone brood in its original host, *A. cerana,* as well); the mite is showing preference for the larger larva; the drone cell size is larger, therefore the mite is less likely to be injured; or the duration of the capped cell period is longer (averaging 24 days), giving mites longer time to develop (De Jong, 1997).

Damage from *Varroa destuctor*

Because *V. destructor* is so large relative to the size of *A. mellifera*, it has been widely accepted that the parasite's feeding weakens individual bees and thus cripples a colony over time. With the acceleration of a mite population, too few healthy bees remain to sustain the colony. Symptoms of heavy mite infestation include: spotty brood patterns, uncapped brood, visible mites on backs of bees, queen supercedure, and deformed wings.

In 1982, De Jong et al. reported that individual bees from mite-infested cells weigh 6 to 25 percent less than bees from un-infested cells. Infested bees lose three percent of their body water per parasite and also have lower abdominal concentrations of carbohydrates and lower head and abdominal concentrations of protein than do unparasitized bees (Bowen-Walker and Gunn, 2001). The mean lifespan of *Varroa*-parasitized bees is 34 to 68 percent shorter than those from cells without mites (Schneider, 1986).

In addition, *V. destructor* has been proven to serve as a vector several naturally occurring honey bee viruses (Ball, 1985; Kulincevic et al.,1990; Martin, 2001b). Two

viruses in particular, acute paralysis virus (APV) and deformed wing virus (DWV) have been linked to the collapse of millions of colonies, although before *Varroa* became epidemic, they were never associated with colony deaths. It is unlikely that the mites trigger virus multiplication, but rather that they serve as a route of transmission (Sumpter and Martin, 2004). Using radioactive labeling, it was shown that hemolymph from one bee can be transferred to another when a mite changes hosts, and that the quantities transferred are greater than what would be expected from simple mouthpart contamination (Bowen-Walker and Gunn, 2001). Sumpter and Martin (2004) have developed a model to determine the mite load at which a virus becomes epidemic, which might be useful as a guide for acaricide treatment.

Apis mellifera Tolerance

Although *Apis mellifera* did not co-evolve with *V. destructor*, some *A. mellifera* are less susceptible than others to injurious infestation. Numerous examples of *Varroa* tolerance have been cited over the years; however, consistency has been elusive. Discussion of mite tolerance in *A. mellifera* is best undertaken by organizing potential factors, keeping in mind that clear demarcation is not always possible. For these purposes, the areas of research are: *Mite Genotype, Climate*, *Bee Genotype, Bee Behavior,* and *Juvenile Hormone.*

Mite Genotype

It has only recently been determined that *V. jacobsoni* comprises multiple genotypes (Anderson and Fuchs, 1998; De Guzman et al., 1999; Anderson and Trueman, 2000). Anderson and Trueman (2000) reported genotypic and phenotypic variation as well as reproductive isolation in *Varroa* mites infesting *A. cerana* throughout Asia. They determined that *V. jacobsoni* is a complex of at least two different species encompassing 18 haplotypes (mites with distinct mitochondrial DNA CO-I gene sequences)—nine *V. jacobsoni*, six *V. destructor,* and three undetermined. *Varroa jacobsoni* haplotypes were found in colonies in the Malaysia-Indonesia region of Asia, while the six *V. destructor* haplotypes were traced to the Asian mainland. The three others, that were undetermined,

were found in the Phillipines. Of the 18 haplotypes found, only two reproduce successfully within *A. mellifera* colonies; both are *V. destructor*. The two were given names based on their probable origins: Korea and Japan/Thailand.

The results pointed to the Korea haplotype as the most widespread, found in Europe, Africa, the Middle East, and North and South America. The Japan haplotype was collected from Japan, Thailand, and North and South America. Anderson and Trueman suggested that these two mites differ in virulence, noting that in Brazil (where bees are *Varro*a-tolerant), the Japan type was most commonly found. Since then, however, the majority of *V. destructor* collected from Brazil have been the Korea haplotype (Garrido et al., 2003).

Anderson and Trueman (2000) concluded that the bulk of the research findings attributed to *Varroa jacobsoni*, are primarily applicable to *Varroa destructor*.

Climate

Varroa destructor reproduction is dependent on honey bee brood production. When brood production is slow or has stopped (in the winter), the mite cannot reproduce and must resort to an extended phoresy. However, *V. destructor* is most vulnerable during the phoretic phase. Activity within the hive, host foraging, falling to the bottom of the hive, and bee grooming behaviors are all hazards that mites face outside of the brood cell. It follows then that, in regions where summers are shorter and mites are forced to spend more time on adult bees, *Varroa* populations reach injurious levels at a slower rate (Ritter, 1988; Kulinçeviç et al, 1988).

However, in tropical climates, where there is no downtime in brood production and mite levels would be expected to be at their worst, colony collapse from *Varroa* infestation is rare and chemical control is unnecessary. Studies in Brazil have cited lowered mite reproduction in tropical and subtropical climates as a possible explanation (Engels et al., 1986; Moretto et al., 1991).

In support of that theory, studies in which conditions have been controlled *within the brood cell* demonstrate a direct effect of temperature and humidity on mite reproduction. The temperature range for optimum mite reproduction is 32.5-33.4ºC

(90.5-92.12°F). At temperatures above 36°C, mite reproduction slows, and at 38°C, mites begin to die (Le Conte et al., 1990). Relative humidity also directly correlates with mite reproduction at rates of up to 70 percent relative humidity. At 80 percent, however, mites stop reproducing altogether (Le Conte et al., 1990; Kraus and Velthuis, 1997).

Because honey bees maintain fairly constant temperature and humidity levels within the hive, it is hard to know the relationship between conditions within the brood cell and those that are ambient. However, in a recent study in the southern U.S. that measured mite population growth over ten years, growth was inversely correlated to the percentage of days per year in which ambient temperatures reached ≥ 35°C. Also, the growth rate was directly correlated to the average daily relative humidity (Harris et al., 2003).

More evidence to support the relationship between ambient conditions and mite impact lies in the fact that tolerance found in South American colonies could not be duplicated in other climates. When colonies of *Varroa*-resistant Italians were found on an island in Brazil (De Jong and Soares, 1997) scientists were unable to reproduce that resistance using same bee lines in Germany (Corrêa-Marques et al., 2002). Also, European honey bee colonies that were resistant in Uruguay performed no differently than domestic colonies when imported to Poland and France (Hoopingarner, 2001). It is likely then, that high average temperatures and relative humidity exceeding 80 percent are factors contributing to *A. mellifera* tolerance of *Varroa* in the tropics.

In addition, mite populations in tropical climates have been shown to be more stable overall than in temperate zones. In one study of apiaries in Brazil, colonies in the warmest regions had mean mite infestations that varied only 2.5 to 5 percent over the year, while those in cooler regions (at higher elevations) varied up to 27 percent (Moretto et al, 1991). A test conducted in the UK showed that male mite mortality increases 24 percent in the winter (Martin, 2001c). The reason for these fluctuations is unknown, but may be related to hormonal changes within colonies as weather shifts.

Finally, temperate honey bee colonies may be more susceptible to the harmful effects of *V. destructor* than tropical colonies because of their need for an adequate population of "winter bees" (those that must survive from fall until spring to insure

colony survival). Because mite parasitization shortens the honey bee lifespan (Schneider, 1986), it is possible that too few bees survive through spring, thereby undermining the effort needed to sustain the colony.

Bee Genotype

The most consistently reported *Varroa*-tolerance in *A. mellifera* colonies is in tropical regions of Brazil, where the African sub-species *A.m. scutella* was introduced in 1956 (De Jong, 1997). The African bees have, through time, mixed with the existing *A.m. ligustica* colonies (EHB) and produced a hybrid known as the Africanized honey bee (AHB). Though Brazil has a climate conducive to limiting mite populations, there is evidence that bee genetics are a factor in the tolerance as well (Camazine, 1986).

Mites in AHB colonies in Brazil are reported to have much lower fertility levels (50%), where fertility is defined as *whether a mite lays eggs* (Rosenkrantz, 1999), than do the average EHB colony in Europe (80-90%) (Rosenkrantz and Engles, 1994). In a study that compared EHB and AHB colonies at the same site in Brazil, the *percentage infestation* in AHB colonies was also significantly lower than in the EHB colonies (Moretto and Mello, 1999).

In studies outside of Brazil, AHB colonies have proven tolerant, but not to the same degree. In Costa Rica, no significant differences were found between AHB colonies and AHBxEHB hybrids when mite fecundity, fertility, and viable offspring were measured (Calderone et al., 2003). However, AHB had an overall greater percentage of mites that produced no progeny at all. Incidentally, the study's authors found higher levels of mite non-reproduction in EHB than had been previously reported and also lower levels than what was expected in AHB. These results suggest the influence of climate.

AHB moved northward into Mexico in 1992, where, again, they survived without treatment for *Varroa* (Vandame, 1996). Vandame compared AHB and EHB colonies in coastal areas and found that EHB colonies collapsed within two years of infestation, while the AHB were tolerant—although not as tolerant as those in Brazil. The AHB colonies in Mexico had more than twice as many mites per hive as their Brazilian counterparts, and the mite numbers fluctuated far more than in the tropics. In another

study, in the Yucatan, mite fertility levels in the Mexican AHB colonies were more comparable to EHB in Europe than to AHB in Brazil (Medina et al., 2002). Still, the mean infestation rate never reached injurious levels.

This review would be remiss without mentioning a recently published paper that expresses concern for the comparability of much of the data from AHB studies (Corrêa-Marques, 2003). The paper cites a lack of standardization in the measurement of mite fertility, noting that some researchers report whether eggs are laid, some report number of progeny, and others reports number of viable offspring. In their efforts to determine the parameter that most accurately reflects mite impact, the authors decided upon Effective Reproduction Rate (ERR), which is defined as the "the number of viable females per female that had invaded the worker brood in singly infested cells." By this measure, they determined that the ERR in Africanized bees was 0.64 in Brazil and 0.73 in Mexico. In EHB in Europe, the ERR is 1.01.

Bee Behavior

Adult bees have two behaviors that potentially contribute to suppressing mite levels: grooming and hygienic. During grooming behavior, bees remove phoretic mites from themselves and from each other. This behavior is thought to be a factor in the ability of *V. destructor's* natural host, *A. cerana*, to tolerate infestation, though reports vary widely as to the percentage of mites dislodged (Peng et al., 1987; Fries et al., 1996). It is likely that grooming behavior is most valuable in those instances when mites are actually damaged by the removal. Otherwise, it is probable that mites removed from a bee fall unharmed to the bottom of the hive from which they can climb onto another bee.

Because grooming is not considered a major factor in *A. mellifera* tolerance to *Varroa*, most behavioral research has focused on hygienic behavior. Hygienic bees are those that detect, uncap, and remove diseased brood from cells (Rothenbuhler, 1964), including those infested with *Varroa* (Peng, 1987; Boecking and Drescher, 1991; Spivak, 1996). Hygiene is recognized as being a valuable tool against two honey bee diseases, American Foulbrood (Rothenbuhler, 1964) and chalkbrood (Spivak and Gilliam, 1998; Spivak and Reuter, 1998). Unlike grooming, hygiene does little direct physical damage to

mites; however, the premature uncapping of the brood cell interrupts *V. destructor's* reproductive cycle.

Hygiene is not a factor in *A. cerana's* control of mite infestation, as *V. destructor* invades only drone brood (which has very thick capping) in its natural host. Therefore, hygiene is an apparent adaptation by *A. mellifera* to *V. destructor* infestation and is heritable (Boecking et al., 2000). When the effects of hygiene were tested on *Varroa*-infested domestic honey bees in the U.S., results suggested that hygienic behavior is a tolerance mechanism when mite levels are low and that it can possibly play a role in delaying injurious levels. However, at infestation rates >15 percent (in brood and on adults), it has little impact on mite populations (Spivak and Reuter, 2000). Hygienic behavior, in concert with other tolerance factors, might explain the prolonged survival of *A. mellifera* colonies in Brazil, Tunisia, and the Primorsky region of Russia (De Jong, 1997; De Guzman et al., 2001).

Juvenile Hormone

Because mites are in a previtellogenic phase when they enter the brood cell, it is reasonable to speculate that factors within the cell induce oogenesis. *Varroa* lays its first egg ~60 hours post-capping only if it has been in contact with a 5th instar bee larva within the first 24 hours (Steiner et al., 1994).

Titer determinations of juvenile hormone III (JH) indicate 5ng/ml peaks in the drone brood of both *A. cerana* and *A. mellifera* during the 60-hour post-capping period and a 3-7ng/ml peak in *A. mellifera* worker brood. Only in *A. cerana* workers, where *V. destructor* cannot reproduce, do JH levels not reach 1ng/ml (Hanel and Koeniger, 1986). Also, Hanel (1983) showed that when 5th instar larvae are treated topically with JH, the number of mite offspring increase significantly.

Other studies have discounted the possible role of JH in mite reproduction. When JH levels of 5th instar larvae were examined in colonies known to have differing mite reproduction (EHB and AHB), no significant differences were reported (Rosenkrantz et al., 1990). Also, Rosenkrantz et al. (1993) found no differences in JH levels between *A. mellifera* and *A. cerana* worker brood during the critical post-capping period,

contradicting the Hanel and Koeniger (1986) findings and suggesting that *V. destructor's* inability to reproduce in *A. cerana* workers has nothing to do with hormone differences. In both of these studies, however, JH samples were gathered from multiple larvae, pooled, and presented as an average. It is possible then, that these results might not reflect the impact of varying hormone levels in individual bees.

In a more recent study, scientists examined the behavior of *Varroa* in mid-cycle (Garrido and Rosenkrantz, 2003). When mites were taken from brood cells in which they had already begun reproduction and placed into newly capped cells (5^{th} instar larvae), 77 percent started the reproductive cycle from the beginning. This was demonstrated by the presence of the male as first offspring. When reproducing mites were placed into cells that contained older pupae, only six percent started the reproductive cycle again. These results would seem to point to a stimulus present primarily in the newly capped host.

Also, though it has not been investigated, it is possible that JH levels within the adult bee affect *Varroa* oogenesis. Hanel and Koeniger (1986) proposed a two-fold influence of JH, which complements Beetsma et al.'s (1999) finding that a phoretic period is necessary for successful mite reproduction. Furthermore, Rutz et al. (1976) showed that in temperate climates, JH levels in young workers (those preferred by mites) rise steadily as the summer progresses. The rising hormone levels, then, are coincident with the typical rise in mite populations.

Breeding for *Varroa* Tolerance

SMR Bees

In 1995, the USDA began gathering colonies of honey bees that demonstrated resistance to *Varroa*. Their intention was to assemble genetic sources for a program in which tolerant bees would be selectively bred, then made available to beekeepers throughout the country. In their first test, 43 colonies were established in Michigan and Louisiana, each with an artificially inseminated queen produced from colonies surviving *Varroa.* During the ten-week study, colonies were tested for four tolerance-related variables: capping period, hygiene, grooming, and non-reproduction. Of the four, only non-reproduction was highly correlated to changes in mite population. Non-reproduction,

as mentioned above, was defined as mite-infested brood cells containing purple-eyed pupae (~15 days) or older in which mites were dead, had not laid eggs, produced only male mites, or had offspring too young to reach maturity before the cell was uncapped (Harbo and Hoopingarner, 1997).

Non-reproduction became the basis for the selective breeding program after Harbo and Harris (1999a) confirmed that it was a heritable trait. The trait itself became known as the Suppression of Mite Reproduction (SMR), and selective breeding eventually produced colonies that had up to 90 percent non-reproducing mites (Harris and Harbo, 2000).

To examine the performance of SMR bees performed when queens were not artificially inseminated but allowed to mate naturally with unselected drones, Harbo and Harris (2001) conducted another test in Louisiana. Three types of colonies were established using queens that were either: 1) purely resistant (RxR), 2) partially resistant (RxC, resistant mother mated to unselected drone) or 3) control (CxC, not genetically predisposed to resistance). They found that the colonies with naturally mated resistant queens (RxC) had a higher percentage of non-reproducing mites, fewer mites per hundred cells, and lower final mite populations than the control colonies. They also found that they had significantly better growth in bee population. The purely resistant queens did not factor in the results as they were either not accepted by their colonies or did not produce enough brood. This poor performance was attributed to inbreeding.

Because they determined that free-mated queens could confer resistance and therefore be helpful to beekeepers, the USDA-ARS at Baton Rouge developed a project designed to disseminate the SMR trait. They agreed to select for breeding stock, which was then sent to a commercial breeder, specifically Glenn Apiaries in California. Glenn Apiaries agreed to instrumentally inseminate, sell, and ship pure SMR queens to beekeepers and other breeders for the production of partially resistant queens (Harbo and Harris, 2002). Due to inbreeding, the pure queens were determined not to be suitable for establishing productive field colonies, (Harbo and Harris, 2001).

The mechanism of the SMR trait is still not understood. However, it is believed to be additive (Harbo and Harris, 2002) and it takes six weeks following installation of

the queen before resistance is expressed (Harris and Harbo, 1999b). Results of one study support Hanel and Koeniger's (1986) hypothesis of a two-fold host influence on *Varroa*. When eggs and young larvae were exchanged between colonies with highly non-reproductive mites and highly reproductive mites, results indicated that both adult and larval feeding are factors. The most highly non-reproductive mites (83%) were those that fed on adults and larvae from non-reproductive colonies; the second most non-reproductive (64%) were those that fed on adults from non-reproductive colonies, but fed on larvae from reproductive colonies. Those that fed on adults from reproductive colonies and larvae from non-reproductive were 18% non-reproductive, and finally, those that fed on larvae and adults from reproductive colonies were only 8% non-reproductive (Harbo and Hoopingarner, 1997).

When queens are exchanged between susceptible and SMR colonies, the introduction of the SMR queen precipitates a decrease in mite population (Harris and Harbo, 2000). Conversely, when an SMR queen is replaced with a susceptible queen, mite populations increase. Both changes require five to six weeks before they are measurable. The SMR trait is manifested primarily in an increase in dead mites (which are entrapped by the cocoon) and more dramatically, in the percentage of live mites with no progeny. In SMR colonies, up to 65 percent of live mites do not lay eggs, compared to the 10-15 percent that do not in normal colonies.

Also, Harbo and Harris (1999) found that the mites not laying eggs after entering the brood cell have only ten percent of the normal volume of sperm in the seminal receptacle (Harris and Harbo, 1999). This is due either to lack of maturation of the prosperm or to poor mating. In 55 percent of the non-laying mites examined, no form of sperm was found, suggesting that non-mating was responsible and that the brother mite in the cell of origin either died or was not stimulated to mate. The same study also looked more closely at mites with offspring too young to reach viability. They determined that colonies with higher percentages of non-reproduction had lower overall fecundity in those mites that *were* reproductive. Their conclusion was that even mites that do mate successfully might be affected by unknown factors influencing *Varroa*-tolerance.

Russian Bees

In 1995, Danka et al. reported that *A. mellifera* colonies in the Primorsky region of far-eastern Russia might be *Varroa* tolerant. European settlers moved their colonies to the area in the mid 1800s, where *A.cerana* was already living with the parasite. The mite likely transferred to the new host at that time, resulting in, according to Rinderer et al., (2001a), the longest known association of *V. destructor* and *A. mellifera*. This extra time spent habituating to the mite is likely a factor in the Russian bees' tolerance.

One hundred Primorsky region queens were imported to the U.S. in 1997 and were quarantined at Grand Terre Island, Louisiana (Rinderer et al., 1997) until 1998. Colonies were established by the USDA near their bee labs in Baton Rouge and were monitored for *Varroa* tolerance. Based on initial evaluations, 40 queens were selected as breeder queens, from which a Russian bee stock was created and studied—with the long-term goal being a new *Varroa*-tolerant line that would be made available to American beekeepers (Rinderer et al., 1999). In field assays in Louisiana, Iowa, and Mississippi, the Russian bees averaged ~50 percent fewer mites than the control (Rinderer et al., 2001c). When tested for honey production, the majority of the Russian colonies met or exceeded industry standards (Rinderer et al., 2001b).

In another study, Rinderer et al. (2001a) tested the daughter queens of imported Russians for two years to determine whether their tolerance was heritable and if so, which factors contributed to it. In both years, the Russian colonies had significantly fewer mites and fewer colony collapses than the domestic colonies (18 deaths in domestic vs. 3 in Russian). Also, the Russian colonies had fewer mites invading cells, meaning more time spent on adult bees. Congruently, the dead mites in the Russian colonies showed 14 percent more grooming damage than did those in domestic colonies. Since then, two studies have also demonstrated that Russian colonies are also more hygienic than domestic colonies (DeGuzman et al.2001, Wilson et al., 2002).

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Part 2
Honey and Pollen Storage and *Varroa* Population Growth in Colonies with Open-mated Mite-resistant Queens from Commercial Breeders

This article will be submitted for publication in *Bee Culture* and was authored by Laura Bryant, John A. Skinner, James Parkman, Michael Studer, and Carl Jones. My use of "we" hereafter refers to the co-authors and myself. My contributions to this work include, but are not limited to, literature review, mite sampling, assistance with strength assessments, statistical analyses, and composition.

Abstract

In eastern Tennessee, during summer 2003, field trials were conducted to determine *Varroa* resistance and productivity of colonies from open-mated, mite-resistant queens obtained from multiple commercial breeders. Russian, SMR, and Italian (control) queens were compared for honey and pollen stores, as well as *Varroa* resistance and queen acceptance. Mite-resistant queens available from commercial breeders varied little from controls in all areas tested. There were no statistically significant differences among them. To preserve *Varroa*-resistance in their stock, breeders marketing mite-resistant queens should maintain sufficient numbers of mite-resistant drones in mating yards**.**

Introduction

The parasitic bee mite, *Varroa destructor* (Anderson and Trueman) remains the greatest threat to beekeeping worldwide and to the billions of dollars in pollination services that the honey bee, *Apis mellifera* L., contributes to the U.S. economy each year (Morse and Calderone, 2000). Because *Varroa* mites have demonstrated resistance to many of the chemicals used to manage them (Elzen et al., 1998; Elzen and Westervelt, 2002), finding sustainable control methods has become imperative.

After studies demonstrated that some *A. mellifera* colonies tolerate mite infestation better than others, several researchers began to focus on the honey bee itself as a tool for managing *Varroa*. The presence of heritable traits in tolerant bees provided a basis for the selective breeding of *Varroa*-tolerant queens (Harbo and Harris, 1999a, b; Rinderer et al., 1999; 2001a). Two USDA programs have bred queens that are now commercially available as a means of managing mite populations—Russian and Suppression of Mite Reproduction (SMR). Both bee types have demonstrated significant resistance to *Varroa* in field trials (Harbo and Harris, 1999b, 2001; Rinderer et al., 2001a) and both have been made available to the public through cooperative breeding

arrangements. These arrangements provide that the USDA supply pure SMR or Russian queens to participating breeders, who in turn, maintain and expand the "stock" for distribution to other commercial breeders and to beekeepers. Pure, instrumentally inseminated queens range in price from $75-500, while hybrid queens, which are available from numerous commercial sources, cost $10-20.

In the case of SMR bees, buying pure queens for populating colonies is not recommended, because inbreeding has impacted brood production and, thus, overall colony productivity (Harbo and Harris, 2002). Studies have demonstrated, however, that SMR hybrids, which tend to be healthier, are still resistant to *Varroa* (though to a lesser degree) and are normally productive (Harbo and Harris, 2001). Pure Russian queens can be put directly into colonies and have demonstrated high productivity (Rinderer et al., 2001b); however, the cost of pure queens is prohibitive to most beekeepers. And, because studies with Russians have been conducted with only pure stock, it is difficult to predict the performance of the more accessible hybrid queens. Furthermore, when buying a hybrid queen of either type from the numerous breeders not affiliated with the USDA, it is difficult to know how many generations removed that queen is from an instrumentally inseminated resistant queen.

Because the installation of new queens into an apiary is often a significant investment of time and money, this study was conducted to provide beekeepers with information on the performance of the hybrid Russian and SMR queens they might be considering. Queens tested were from commercial breeders not affiliated with the USDA, and the characteristics measured included productivity (honey and pollen stores), queen acceptance, and *Varroa* resistance.

Materials and Methods

Colony Set Up

In spring 2003, 45 study colonies were established from existing colonies in three apiaries in eastern Tennessee. In one apiary, colonies were maintained in Illinois hive bodies, while at the other two, a combination of Illinois, deep, and shallow hive bodies

were used. Each apiary contained 15 study colonies: five re-queened with SMR queens, five with Russian queens, and five with Italian queens (used as control). All queens used were obtained from commercial breeders; however, to minimize the potential impact of an atypical contribution from any one breeder, multiple sources were used for each queen type. Colonies at each site were set up and maintained in the same manner, resulting in three replications. In instances of supercedure, a second queen from the same source was installed.

Measuring Honey and Pollen Stores

In June, when queens had been established for at least one month, baseline colony strength assessments were conducted via frame-by-frame visual inspections. For each frame, the proportions of honey and pollen, as well as capped and uncapped brood, were recorded (Skinner et al., 2001). Proportion values were converted to square inches to account for the differences in hive box sizes. Strength assessments were conducted every six weeks until late October. No honey or pollen was harvested during the study. Results were reported as percent changes from the baseline value.

Queen Acceptance

Queens were clearly marked before installation into the study colonies. Colonies were checked two weeks after re-queening for presence of marked queens. Thereafter, presence of marked queens was verified during every strength assessment. Because colonies were prevented from swarming, the absence of a marked queen was considered a result of supercedure. Although in most cases, queen supercedure was followed by the successful installation of a second queen of the same type and from the same breeder, the original marked queen was determined "not accepted". Queen acceptance was reported as the percentage of the original 15 queens (per type) that were accepted.

Varroa Resistance

Varroa resistance was reported as the rate of mite population growth (RMPG). Mite populations were sampled using the sticky bottom board method of collecting

natural mite drop (Fries et al., 1991; Parkman et al., 2002). A bottom board was placed in each colony for three days every three weeks from June through September. Collected mites were counted using a gridded light table. Out of concern that the mite collection data alone did not reflect mite infestation in colonies, a concentration value was created: the ratio of mites collected to colony strength. Size of the brood area—the amount of capped and uncapped brood—was chosen as the best indicator of colony strength, because numbers of adult bees and quantity of food stores are highly variable. The amount of capped and uncapped brood was determined in the manner described above for honey and pollen, through visual inspection of every frame. Mite concentration for each colony was recorded as the number of mites collected in three days per square inch of brood.

Because most colonies had been managed for *Varroa* prior to this study, initial mite concentrations were very low. Final mite concentrations were determined at the end of September when the study was concluded, or in the cases of colonies that succumbed to *Varroa*, the last date that data were collected.

Finally, to determine the growth rate of *Varroa* populations, initial mite concentration was subtracted from the final concentration. That figure was divided by the total number of days between final and initial sampling dates to provide the RMPG.

Statistical Analyses

Colonies were set up as a randomized complete block. Measurements of honey, pollen, and RMPG were analyzed using single factor analyses of variance (ANOVA), using bee types as treatments and apiaries as replications (PROC ANOVA, SAS Institute, 2002). Queen breeder was not used as a factor. Because colony numbers were not equal for every sampling period, each date was analyzed separately.

Results

Honey Stores

Honey stores were low throughout the season for all study colonies. Although no honey was harvested, only 6.7% of colonies had more honey in October than they did in

June. No significant differences in honey stores were found among apiaries (P = 0.3516; 0.1563; and 0.2084) or bee types (P = 0.4897; 0.5158; 0.0816) during any of the three post-baseline sampling periods (Table 2.1). Graphical comparison of mean percent change of honey stores illustrates that bee types performed similarly throughout the summer (Figure 2.1).

Pollen Stores

During the first sampling period, the mean percent increase in pollen stores varied only 3.26% (± 1.07 S.E.) among bee types. The only statistically significant difference found was among apiaries during the last sampling period. Among bee types, there were no differences (Table 2.1); however, the Italian control colonies were the only bees to have more pollen stores in October than in June (Figure 2.2).

Queen Acceptance

Of the 15 control queens installed, 14 of 15 (93.33%) were accepted. In the SMR colonies, 13 of 15 (86.67%) queens were accepted. Of the Russians, 11 of 15 (73.33%) were accepted.

Varroa Resistance

Four of the study colonies collapsed due to Varoosis: two control colonies, one SMR colony, and one Russian. Conversely, three colonies had negative RMPGs: one control colony and two SMR. No apiary effect on RMPG was observed (df = 2,35; F = 1.08; P = 0.3534), and there were no significant differences in mean RMPG among bee types (Italian = 0.0058; SMR = 0.0037; Russian = 0.0083) (df=2,35; F = 0.74; P = 0.4852). *Varroa* concentrations for each date are provided in Figure 2.3.

Discussion

Honey production was low for all bee types, though the cause for this is unknown. In the Knoxville area, during the first two weeks of both April and May, rainfall was two to three times greater than average (Logan, 2004). Perhaps foragers were kept inside for

Table 2.1 Honey and Pollen ANOVA

		Bee Type			Apiary		
		df	F	P	df	F	P
Honey	**6/10-7/24**	2,35	0.73	0.4897	2,35	1.08	0.3516
	7/24-9/4	2,29	0.68	0.5158	2,29	2.00	0.1563
	9/4-10/30	2,26	2.81	0.0816	2,26	1.69	0.2084
Pollen	**6/10-7/24**	2,35	0.27	0.7665	2,35	1.68	0.2028
	7/24-9/4	2,29	1.27	0.2988	2,29	6.64	0.0047
	9/4-10/30	2,26	2.01	0.1584	2,26	1.39	0.2706

Single-factor ANOVA among SMR, Russian, and control colonies for honey and pollen, summer 2003.

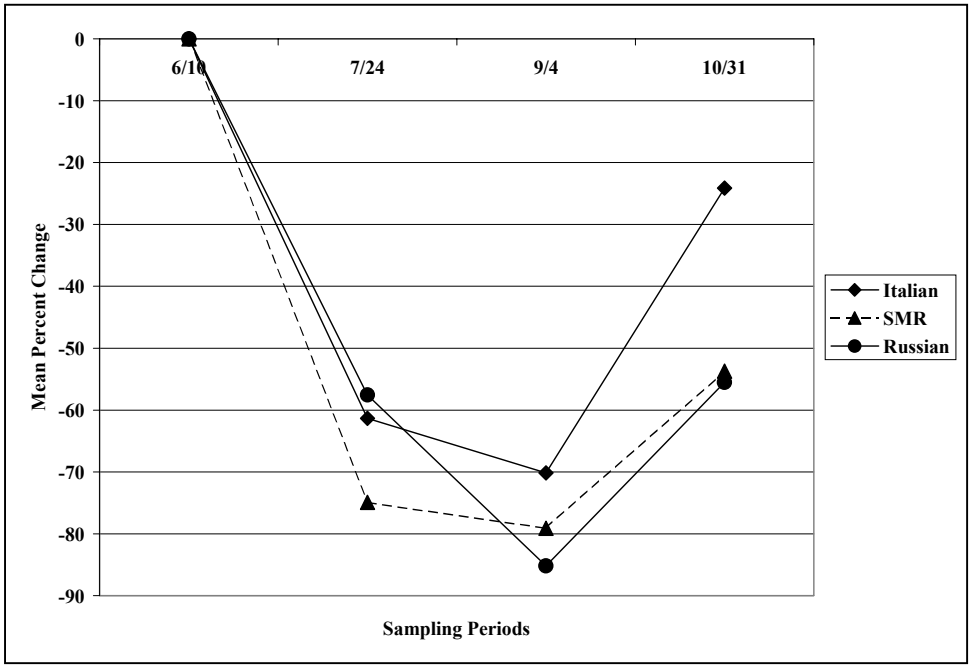

Figure 2.1 Change in honey stores across summer 2003 for Italian, SMR, and Russian honey bees in three apiaries in Tennessee.
Mean percent change in honey stores per bee type. No significant differences were found during any period.

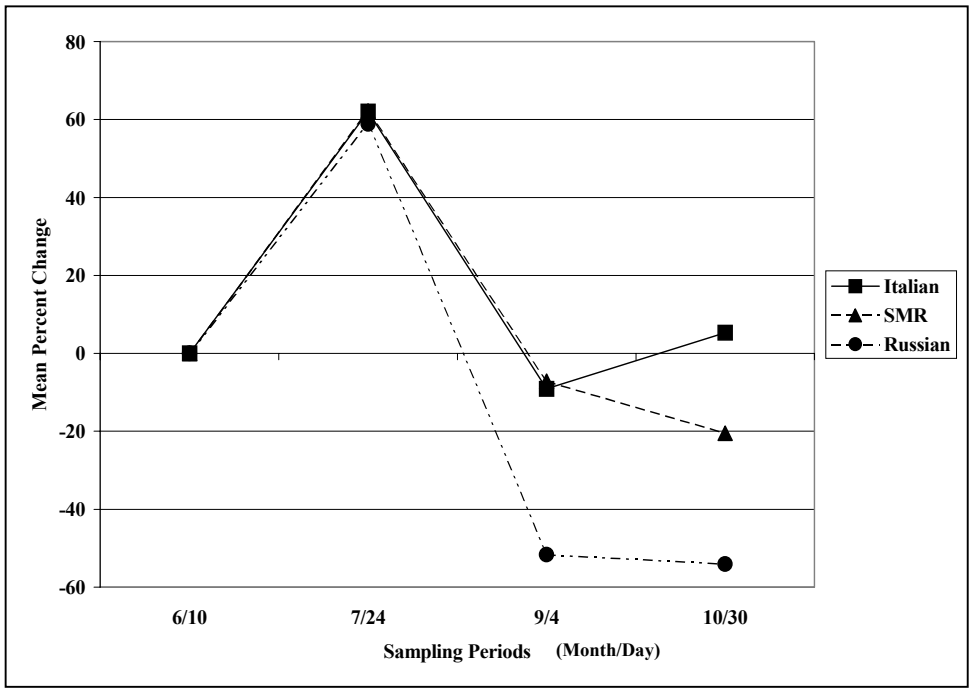

Figure 2.2 Change in pollen stores across summer 2003 for Italian, SMR, and Russian honey bees in three apiaries in Tennessee.
Mean percent change in pollen stores per bee type. No significant differences were found during any period.

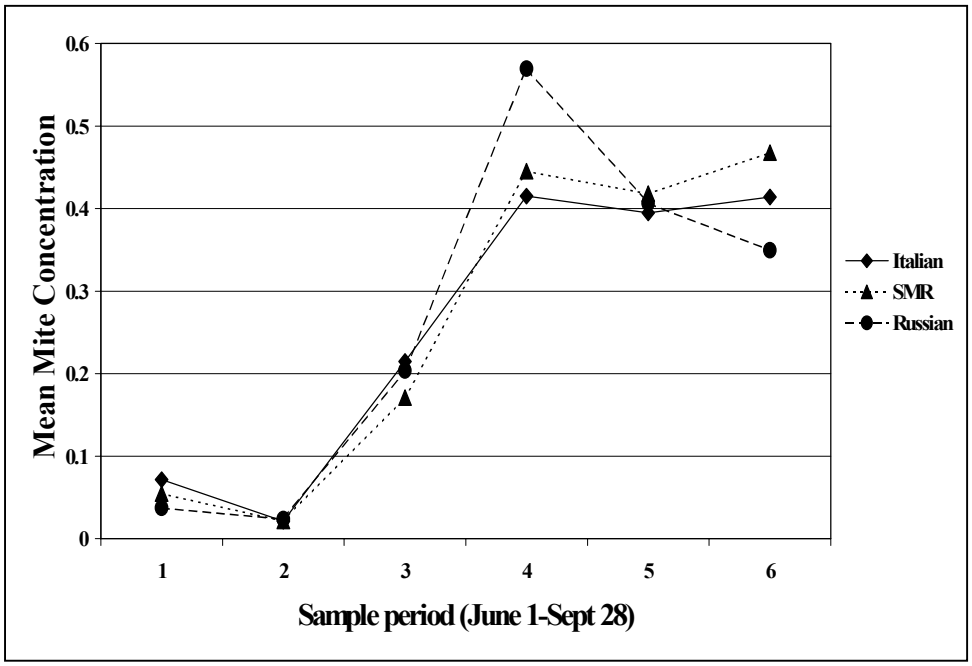

Figure 2.3 *Varroa* concentrations in summer 2003 for Italian, SMR, and Russian honey bees in three apiaries in Tennessee.
Mean mite concentrations (mites collected/colony strength) per bee type. No significant differences were found among bee types during any sampling period.

too long during the nectar flow. We can only speculate the long-term impact this might have had in terms of brood build-up and consequently, the number of foragers to gather food. We do know that, before the fall flowering season, many colonies were in danger of starvation regardless of bee type. Despite the overall low volume of honey stores, it seems apparent from the mean values that all bee types responded similarly to available nectar sources.

This was also true for pollen. During the first six weeks of colony monitoring, pollen stores were nearly identical. Later in the summer, when pollen sources became scarce, stores decreased for all bee types. There were no statistically significant differences among colonies; however, Russian colonies had the greatest percent loss of pollen stores and seemed to have the most trouble recovering. By the end of the season, the control colonies were the only colonies to build pollen stores back to spring levels.

Conclusions about queen acceptance could not be made, because a replicated trial was not conducted to specifically study this factor. Furthermore, though queen breeder was not a variable in the design, data suggest that queen acceptance in this experiment was more a function of the bee source, rather than the type.

Finally, as with honey and pollen, there were no differences among bee type in *Varroa* population growth. Differences, when they were found, occurred on a colony level and appeared to be independent of bee type or apiary site. Other results of this study have indicated that the variation in mite resistance of colonies within the same bee type might be correlated to variations of juvenile hormone levels (see Part 4).

Our results suggest that there are few differences among open-mated queens obtained from commercial breeders (though our data do not reflect differences in individual queens). This may reflect the lack, or paucity, of resistant drones in and near mating yards. Commercial breeders hoping to preserve *Varroa* resistance in their stock should saturate congregation areas with sons of resistant queens and/or isolate mating yards until *Varroa*-resistance becomes more thoroughly integrated into the gene pool. Beekeepers specifically seeking resistant queens for *Varroa* management should purchase daughters of pure queens from trusted sources in hopes of ensuring that the "resistant" queens they are purchasing have mated with resistant drones.

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Part 3
Field Trials of Hygienic Behavior and Suppressed Mite Reproduction in Open-mated Honey Bee Queens from Commercial Breeders

This chapter will be submitted for publication in *American Bee Journal* and was authored by Laura Bryant, John A.Skinner, James Parkman, Michael Studer, and Carl Jones. My use of "we" hereafter refers to the co-authors and myself. My contributions to this chapter include, but are not limited to, project proposal, literature review, hygiene assays, suppressed mite reproduction assays, colony sampling, statistical analyses, and composition.

Abstract

In eastern Tennessee, during summer 2003, field trials were conducted with colonies of open-mated, mite-resistant queens obtained from multiple commercial breeders. We compared Russian, SMR, and Italian (control) queens to quantify two resistance factors, hygienic behavior and suppressed mite reproduction. No significant differences were found among the selected stock for either hygiene or mite reproduction. A significant correlation was discovered between the levels of both resistance factors and the final mite concentrations in the colonies. Results of this study suggest that hygiene and suppression of mite reproduction are present at low levels in the honey bee population as a whole, but that it is difficult to actively choose one over the without investing in queens from controlled breeding programs.

Introduction

Since the introduction of *Varroa destructor* (Anderson and Trueman) to the U.S. in 1987, beekeepers and bee researchers have sought effective, long-term methods of reducing mite damage to honey bee (*Apis mellifera* L.) colonies. Because studies have shown that some bee colonies tolerate *Varroa* infestation better than others, it follows that the most desirable, and sustainable, mite control tactic is to use the most resistant bees. To that end, researchers have set out to find resistance that is heritable and that can therefore be bred into honey bee populations through time.

USDA breeding programs have produced two types of bees that are now commercially available as a means of combating *Varroa*: Suppression of Mite Reproduction (SMR) (Harbo and Harris, 1999b) and Russian (Rinderer et al., 2000). The USDA has provided selectively bred stock to cooperating breeders, who since then have produced breeder queens for dissemination to other breeders and to beekeepers.

The Russians are a *line* of honey bee, brought to the U.S. from the Primorsky region of Russia after they were observed to be more tolerant to mite infestation than other *A. mellifera* colonies (Danka et al., 1995). A SMR bee on the other hand, is not from a *line* of bees but is, theoretically, any bee that possesses the SMR trait. Both types show significant resistance to *Varroa* in field assays (Rinderer et al., 2001a, 2001b; Harris and Harbo, 2000; Harbo and Harris, 2001), but the effectiveness of commercially bred and sold forms is unknown.

In addition, their mechanisms of resistance are not completely understood. Research with SMR bees has concentrated on mite reproduction within the brood cell (Harris and Harbo, 1999, 2000) while studies on Russians have focused on behavioral resistance, specifically hygienic behavior (DeGuzman et al., 2002).

In the SMR studies, mite reproduction was measured in terms of non-reproduction—mites that enter a cell to reproduce yet yield no viable offspring (Harris and Harbo, 1999). This likely involves the *mites*' physiological response to an, as yet, unknown host factor(s). Alternatively, hygienic behavior is a *host* behavioral response, and is already considered a valuable defense against Amercian foulbrood and chalkbrood (Spivak and Reuter, 1998b). Hygienic bees detect and remove diseased brood from cells (Rothenbuhler, 1964) and, consequently, interrupt mite reproduction. Both forms of resistance are heritable (Harbo and Harris, 1999a; Boecking et al., 2000) and are desirable traits to incorporate into an apiary.

Because re-queening colonies can be a significant investment of time and money, it is important to know whether commercially available queens possess the traits for which they are sought. We conducted field trials of open-mated SMR and Russian queens that are readily accessible from commercial breeders. We tested these colonies for levels of suppressed mite reproduction and hygienic behavior. We then related these resistance factors to two measures of mite success: **mite concentrations** at the end of the season and **mite population growth rate**. In addition, we conducted a simple test to determine whether colony size plays a role in hygienic behavior.

Materials and Methods

Colony Set Up

In spring 2003, 45 study colonies were established from existing colonies in three apiaries in eastern Tennessee. At one apiary, colonies were maintained in medium (Illinois) hive bodies; in the other two, colonies were maintained in Illinois, deep, and shallow hive bodies. Each apiary had 15 study colonies: five were re-queened with SMR queens, five with Russian queens, and five with Italian queens (used as the control). All queens used were obtained from commercial breeders; however, to minimize the potential impact of an atypical contribution from any one breeder, multiple sources were used for each queen type. Colonies at each site were set up in the same manner, resulting in three replications. In instances of supercedure, a second queen from the same source was installed.

Measuring Mite Concentration and Mite Population Growth Rate

Mite populations were sampled using the sticky bottom board method of collecting natural mite drop (Fries et al., 1991; Parkman et al., 2001). Mites were collected for three days, every three weeks. Afterward, the bottom boards were placed over a light table and the mites were counted. Out of concern that the sample data alone did not reflect mite infestation in colonies, we created a concentration value, the ratio of mites sampled to colony strength. We chose the size of the brood area as the best indicator of colony strength, because the number of adult bees present on a given day and the volume of food stores are highly variable. Size of brood area was assessed routinely by frame-by-frame visual inspection and was recorded as proportions of a frame (Skinner et al., 2001). Proportions were converted to square inches to equalize different sized hive boxes; therefore for each colony, mite concentration = mites sampled/per sq. inch of brood. Mite concentrations were monitored every three weeks for six months, until late September. Because most colonies had been managed for *Varroa* prior to this study, initial mite concentrations were very low.

To determine the growth rate of *Varroa* populations, we subtracted the initial mite concentration from the final and divided that figure by the number of days in the sampling period.

To relate colony size to hygienic behavior, we visually inspected each colony early in the morning on the day of or near each hygiene assay to get the best estimate of adult population. Estimating the number of adults and brood per frame in the same manner as above provided a size index used to correlate with hygienic levels.

Determining Non-reproduction

In late summer/early fall, suppressed mite reproduction (non-reproduction) was quantified by examining capped brood cells that had been invaded by only one mite. Only cells with purple-eyed bee pupae (~15 days post capping) were considered because, at that stage of pupal development, only mite progeny beyond the protonymph stage have had time to mature before the bee emerges from the cell (Harris and Harbo, 1999). At least 20 singly infested cells from each colony were examined, and mites were considered non-reproductive if: they were dead, had laid no eggs, had only male progeny, or had no progeny beyond the protonymph stage. This test was conducted two times, one month apart. Non-reproduction was measured as a percentage of mites that had entered cells, but had not produced viable offspring.

Measuring Hygienic Behavior

In July, hygienic behavior was measured using the freeze-killed brood assay, which has proven a reliable screen for the hygiene response (Spivak and Downey, 1998). In each colony, one frame of capped brood was removed and laid horizontally on a supportive base. A 3" diameter section of double-lipped PVC pipe was then pressed into a solid patch of brood, creating a seal. Any empty cells within the pipe's circumference were counted and recorded. Then, using a Styrofoam cup, ~ 10 ounces of liquid nitrogen were poured into the pipe, freezing and killing the enclosed brood. Before replacing the frame into the colony, it was marked and left to thaw for 5-10 minutes. Colonies were checked 48 h later for amount of brood removed from the test patch (Spivak and Reuter,

1998a). This test was performed twice within two weeks. Colonies were considered hygienic only if they removed ≥ 95 % of the dead brood both times. We recorded and averaged results from each colony, including those that were not hygienic, for analysis of variance among bee types and for hygiene/mite success correlations.

Statistical Analyses

Single-factor analysis of variance (ANOVA) was performed for the ten colonies found to be hygienic, using bee type as treatment and apiaries as replications (SAS institute, 2002). The same ANOVA procedure was used for *all* colony hygiene results (n=37), including those that removed < 95 % dead brood.

The mite non-reproduction assay was treated as separate experiment, because it was performed later in the summer and involved fewer colonies. A single-factor ANOVA was used for this experiment in the same manner described for the hygiene assay; bee type was used as the treatment and apiary as the replication.

Simple linear regression was used to determine the relationship between the two resistance factors (hygienic behavior and suppressed mite reproduction) and the two measures of mite success (mite concentration and mite growth rate), resulting in four separate analyses.

To determine the relationship between hygiene and colony strength, results of the two hygiene assays were not averaged, as above. Results of each test were correlated to the colony strength on or near the day the test was performed. A correlation analysis was used to determine the Pearson's correlation coefficient for hygienic behavior and colony size.

Results

Hygienic Behavior

Among Colonies Of the 45 colonies studied, the freeze-kill assay was performed the requisite two times on 37 (12 Control, 13 SMR, and 12 Russian). This was due either to colony collapse or to insufficient brood. Ten colonies were hygienic, distributed among bee types and apiary sites. No significant differences in bee type were found in the

hygienic colonies (df = 2,9; F = 0.97; P = 0.4254). When hygiene levels (%) for *all* 37 colonies were considered, no significant differences were found among bee types (df = 2,36; F = 1.35; P = 0.274) or apiaries (df = 2,36; F = 1.08; P = 0.358) (Table 3.1). Mean hygiene levels for the 37 study colonies = 78.28 %.

Relationship to Mite Success There was a significant relationship between hygienic behavior and final mite concentration (df = 1, 36; P = 0.018) (Figure 3.1), but not between hygienic behavior and mite population growth rate (df = 1,36; P = 0.612) (Figure 3.2).

Relationship to Colony Size There was no correlation between size of the colonies and whether they were hygienic (df = 1,77; r = -0.052; P = 0.647).

Mite Non-reproduction

Among Colonies The two assays for non-reproduction were averaged, despite unexpected sizable differences in non-reproduction in some colonies between the first and second test. Also, in some cases, we felt that taking brood would be detrimental to colony health, so no test was performed. No significant differences were found among apiaries (df = 2,28; F = 2.09; P = 0.145) or bee types (df = 2,28; F = 0.03; P = 0.969) (Table 3.2). Mean Non-reproduction = 27.97 %.

Relationship to Mite Success As with hygienic behavior, there was a significant correlation between mite non-reproduction and final mite concentration (df = 1,28; P = 0.009) (Figure 3.3), but not between mite non-reproduction and mite growth rate (df = 1,28; P = 0.094) (Figure 3.4).

Discussion

The results of this study lead us to three conclusions:

The first is that there is no relationship between the size of the colony and level of hygienic behavior. Several studies have shown that hygiene is a heritable trait (Boecking et al., 2000; Spivak and Reuter, 2000); nevertheless, it seemed worthwhile to investigate whether the size of the workforce contributed to the behavior. Our results indicate that colony size is not a factor. In fact, some of the most hygienic colonies in our study were

Table 3.1 Hygienic levels (%) in colonies with open-mated SMR, Russian, and Italian (control) queens in three apiaries in Tennesee

	Control	SMR	Russian
Site 1	76.20	86.75	94.30
	77.45	**99.05**	**98.30**
	97.35	70.70	57.70
	63.35	84.45	
	96.05	79.20	
Site 2	71.90	58.90	56.40
	97.65	**95.10**	70.25
	73.65	74.38	87.50
	77.25	75.75	43.95
	70.65		
Site 3	67.05	69.35	50.05
	77.45	90.90	79.40
		100.00	**98.75**
		99.30	**98.75**
			31.20

Mean hygienic levels in study colonies after both assays (hygienic colonies are in bold). No significant differences were found among bee types (P=0.274) or apiaries (P=0.358)

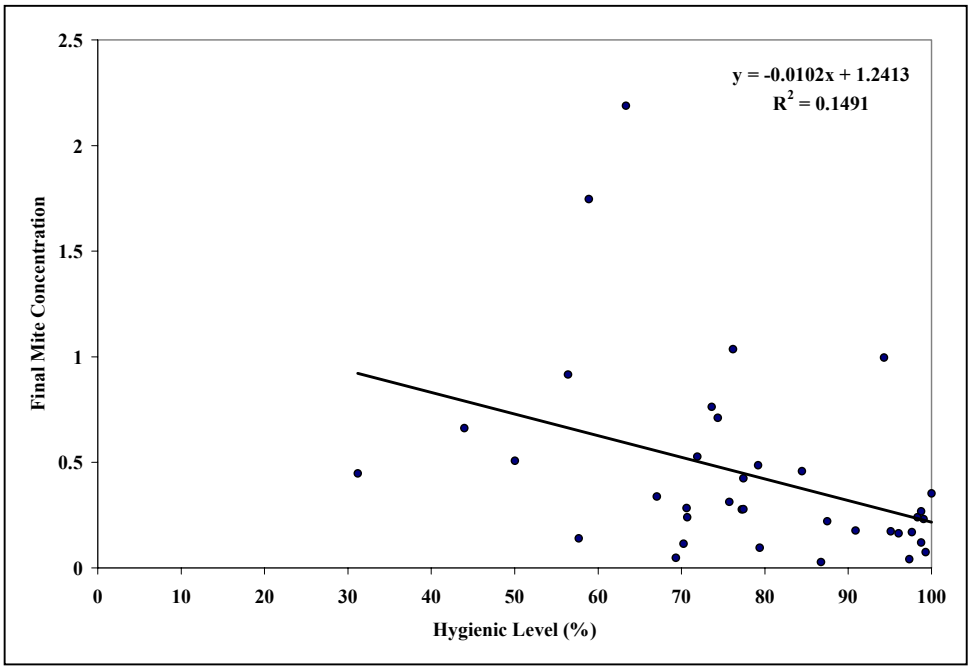

Figure 3.1 Linear regression analyses between mean hygiene levels and final mite concentrations in colonies

The mean hygienic level (from two assays per colony (n = 37)) was significantly related to the final *Varroa* concentrations (mites sampled/ per square inch of brood) in colonies (P = 0.018).

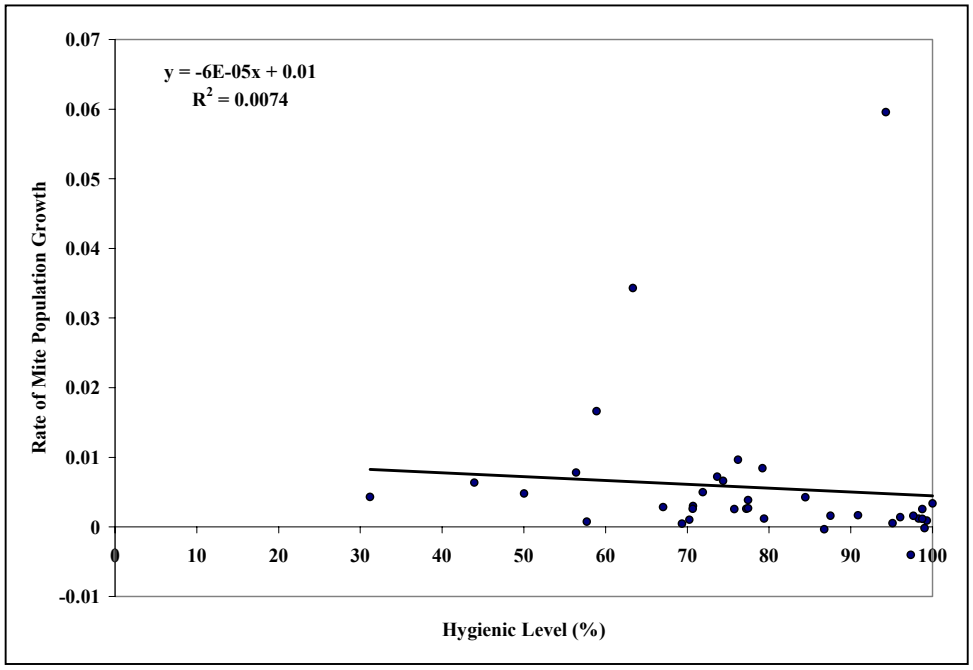

Figure 3.2 Linear regression analyses between mean hygiene levels and rate of mite population growth in colonies

The mean hygienic level (from two assays per colony (n = 37)) was not related to the rate of *Varroa* population growth ((final mite concentration – initial)/ days in sampling period) (P=0.612).

Table 3.2 Mean number of non-reproducing mites per bee type

	Mean Mite Non-reproduction (%)	Stan. Error	Percent Range
Control	26.49	4.14	37.93 (17.50-55.43)
SMR	27.38	3.56	33.78 (11.76-45.54)
Russian	28.50	2.65	31.30 (11.20-42.50)

Cells of purple-eyed pupae that were infested with one foundress mite were classified as non-productive if: 1) mites were dead, 2) there were only male offspring, or 3) there were no progeny beyond the protonymph stage. No significant differences were found among bee types (P = 0.969).

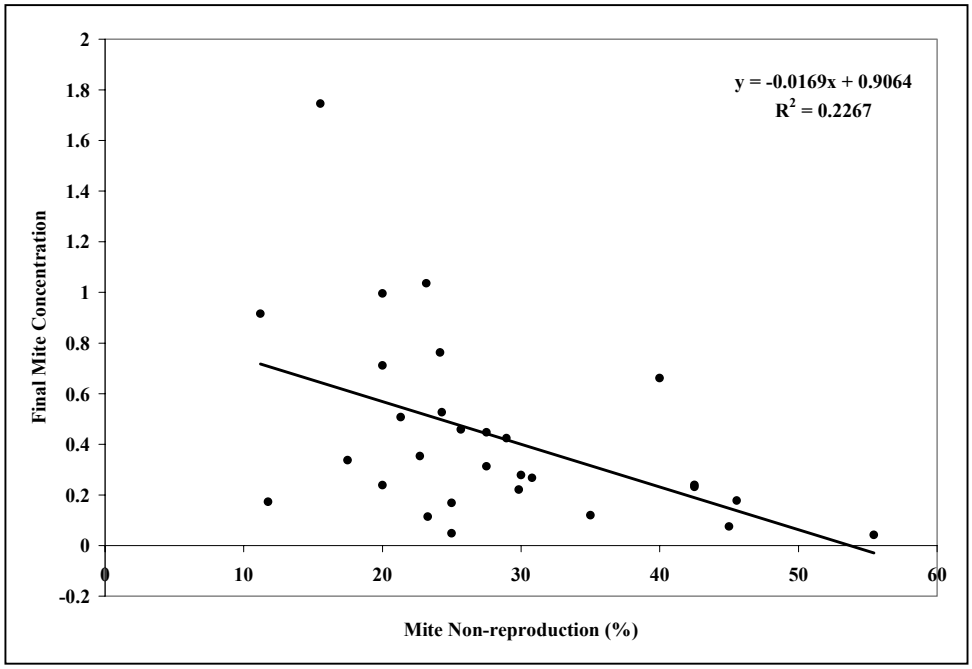

Figure 3.3 Linear regression analyses between non-reproductive mites and final mite concentration in colonies

The percent of non-reproductive mites (mites that produced no viable offspring) in a colony was significantly related to the final mite concentrations (mites sampled/square inches of brood) ($P = 0.009$).

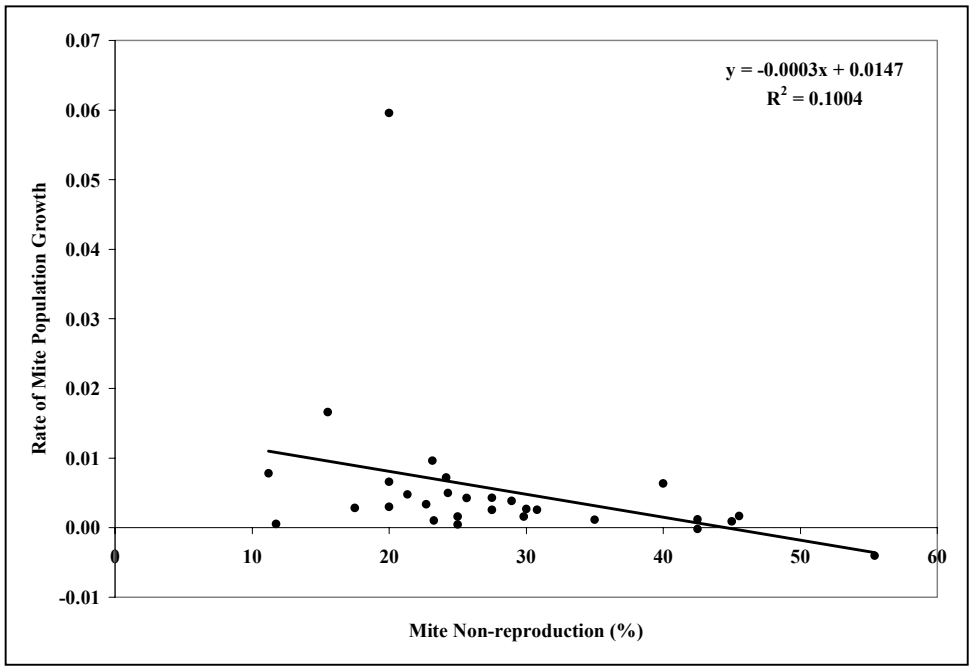

Figure 3.4 Linear regression analyses between non-reproductive mites and the rate of mite population growth in colonies

There was not a significant relationship between the percent of non-reproductive mites in a colony (mites that produce no viable progeny) and the rate of Varroa population growth ((final mite concentration-initial)/ number of days in a sampling period) (P=0.094).

the smallest. This lends further evidence of genetic predisposition to hygienic behavior.

Secondly, there are no significant differences in hygiene or in the levels of mite reproduction among the bee types in this study. We were just as likely to find highly hygienic behavior in a control colony as we were in a "resistant" colony. Likewise with suppressed mite reproduction. We attribute this to two possibilities: 1) In the "resistant" queens, desired traits were diluted in generations subsequent to P_1 through open matings with non-resistant drones and 2) in the control queens, desired traits had been added via their incorporation into the honey bee gene pool. This has serious implications for beekeepers, because queens advertised as SMR or Russian usually cost more than Italian (non-resistant) queens.

Finally, the correlations of hygienic behavior and suppressed mite reproduction with the final mite concentrations in colonies attest to the value of these two resistance factors. Despite having used open-mated queens, where progeny gentoype may vary greatly from that of the queen, we found cause-and-effect relationships. That we found no significant differences in these traits between queen types is not necessarily an indictment on the breeding operations or on the persistence of the traits. Optimistically, it could be that the breeding programs are working and that the resistant traits are slowly being incorporated into the honey bee gene pool. Assuming this is the case, we feel that continued use of resistant queens is of long-term benefit to the beekeeping industry. For those beekeepers actively seeking one trait over the other, however, investment in queens from controlled breeding programs is recommended. Resistant traits should be maintained in open-mated queens by isolating breeding yards or saturating congregation areas with resistant drones.

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Part 4
L5 Juvenile Hormone Titers in Honey Bee Colonies with Varying Mite Infestations

This chapter will be submitted for publication in the *Journal of Apiculture Research* and was authored by Laura Bryant, John A. Skinner, Zachary Huang, James Parkman, Michael Studer, and Carl Jones. My use of "we" hereafter refers to the co-authors and myself. My contributions to this chapter include, but are not limited to: project proposal, literature review, colony monitoring and sampling, non-reproduction tests, hemolymph extraction, statistical analysis, and composition.

Abstract

Varroa destructor is currently the most serious threat to American beekeeping and to the economically important pollination services of the European honey bee, *Apis mellifera.* Though bees resistant to *Varroa* have been reported in the literature, mechanisms of resistance are not completely understood. The purpose of this study was to re-examine the role of host juvenile hormone III (JH) on mite reproduction, specifically the role of JH during the 5th larval instar. In September 2003, nine honey bee colonies with varying mite concentrations were chosen for JH titer determinations, and larvae ages were estimated to ~24 h post-capping, when JH levels peak. Hemolymph was extracted from ten larvae per colony and analyzed per individual, using radioimmunoassay. Juvenile hormone titers were compared to the final mite concentrations of the colonies and with the non-reproduction levels of mites in each colony. Regression analyses of JH titers with final mite concentrations and with mite non-reproduction indicated significant relationships. Significant relationships also exist between intra-colony JH variance, mite concentrations, and mite non-reproduction. These data support the hypothesis of an influence on mite reproduction by host JH levels.

Introduction

Varroa destructor (Anderson and Trueman) is an ectoparasitic mite that reproduces in the brood cells of two honey bees, *Apis cerana* and *A. mellifera.* In the U.S., where *A. mellifera* pollination services are valued at $14.6 billion a year (Morse and Calderone, 2000), research has focused on finding sustainable methods for controlling mite populations. Selective breeding programs have successfully produced bees with resistance to *Varroa* (Harbo and Harris, 2000; Rinderer et al., 2000); however, the mechanisms of resistance are still unknown.

Varroa enters the cell of a fifth instar bee larva (L5) 0-18 hours before it is capped, while its oocytes are still in a previtellogenic phase. It begins to feed on the

larva's hemolymph ~24 h later (6-24 h after the cell is capped). Mite vitellogenesis starts 10-25 h post capping, and embryogenesis begins ~30 h post capping (Steiner et al., 1994). In experimental conditions, a mite does not lay eggs if inserted into a cell after the larva has begun spinning its cocoon (~24-30 h post-capping)(Beetsma and Zonnefeld, 1992; Steiner et al., 1994). Also, mites already into their reproductive cycles start them over when transferred from cells of older pupae into those with newly capped larvae (Garrido and Rosenkrantz, 2003). These facts are suggestive of the influence of the larval host on mite reproduction during a critical period of the L5 phase.

Several researchers have theorized an association between mite oocyte development and juvenile hormone III (JH) levels of the larval host. In honey bees, JH is associated with the accumulation of vitellogenin in the hemolymph (Pinto et al., 2000) and with the regulation of the division of labor (Robinson et al., 1989; Huang et al., 1994). JH levels in capped worker brood are highest during the L5 stage, increasing sharply at ~18 h post-capping and peaking at ~30 h (Rembold and Hagenguth, 1980; Rosenkrantz et al., 1993), the approximate time frame of mite vitellogenesis and embryogenesis.

JH is a common hormone in arthropods, and there is evidence that in at least some species of Acari, it affects reproduction (Connat et al., 1983; Oliver et al., 1985). Hanel (1983) found that exogeneous application of JH to L5 honey bee larvae significantly increases the number of *Varroa* offspring. Also, Hanel and Koeniger (1986) reported that *A. cerana*, *Varroa*'s original, tolerant host, has significantly lower JH levels during the first day post-capping than *A. mellifera*.

However, when Rosenkrantz et al. (1990) examined JH titers in *A. mellifera ligustica* (susceptible to mite damage) with those of Africanized bees (tolerant) in a study in Brazil, no significant differences were found. In another study, Rosentkrantz et al. (1993) contradicted the previous findings that JH in *A. cerana* L5 brood is lower than in *A. mellifera*. They concluded that a species-specific JH adaptation to mite parasitization was unlikely. There is evidence, however, that larval genotype does impact JH titer (Robinson et al., 1989; Elekonich et al., 2003)

We wanted to re-examine the role of L5 JH in mite reproduction because: 1) the current data are conflicting; 2) previous studies have used pooled samples, which do not reflect variance within sub-populations of colonies; and 3) JH levels are affected by environmental changes (Huang and Robinson, 1995) and could therefore be anomalous in tropical climates like Brazil. Our objective was to determine the relationship, if any, between mean colony L5 JH titers, mite reproduction, and mite infestation levels in late summer in eastern Tennessee and to examine the impact of intra-colony JH variation.

Materials and Methods

Mite Concentrations

In 2003, the *Varroa* populations in 45 *A. mellifera* colonies in eastern Tennessee were evaluated. The colonies were established as a part of a larger study comparing commercially bred *Varroa*-resistant bees and included open-mated Russian (Rinderer et al., 2000), SMR (Harris and Harbo, 2000), and Italian queens. Sampling was conducted every three weeks, using the sticky bottom board method of collecting natural mite-drop (Fries, 1991; Parkman et al., 2002). Sticky boards were placed in colonies for three days, after which collected mites were counted using a gridded light table.

Because colony growth was inconsistent among colonies, raw sample data did not accurately reflect mite infestation. And, because most, but not all colonies began the season with mite populations of zero (based on sticky board samples), calculations for *change* in mites sampled resulted in deceivingly inflated numbers. A meaningful value (mite concentration) for comparing mite infestation at summer's end was derived using the number of mites sampled relative to colony strength. The amount of capped and uncapped brood was chosen as the indicator of colony strength and was assessed during frame-by-frame visual inspections conducted every six weeks (Skinner et al., 2001). Numerical values (proportions of a frame converted to square inches) were assigned to quantities of brood present. Mite concentrations were reported in terms of number of mites sampled/per square inch of brood. All initial concentrations were very low, ≤ 0.05. In September, otherwise healthy colonies with varying mite concentrations were chosen for JH analysis.

Mite Reproduction

Mite reproduction was measured in terms of *non*-reproduction, which occurs when female mites enter cells (foundress mites), but do not produce viable offspring. Only cells with purple-eyed bee pupae were examined, because by this stage of development, the reproductive success of foundress mites is accurately predicted by the presence of female deutonymphs (Harris and Harbo, 1999). At least 20 singly infested cells from each colony were examined, and mites were considered non-reproductive if: mites were dead, were alive but laid no eggs, had only male progeny, or had no progeny beyond the protonymph stage. Reproduction assessments were made in late August and in late September, and were reported as percentages of singly infested cells that contained non-reproductive foundress mites.

JH Titers

In September, nine colonies (three of each queen-type) with varying mite concentrations and reproduction were chosen for JH titer determination. Larval age was determined using marked transparencies on the brood frame. Brood cells containing 4th instar larvae were monitored every six hours for capping. At 24 h post-capping (+/- 3 h), brood frames were removed from colonies and brought to the lab, where hemolymph from ten larvae was extracted under a dissecting scope. Hemolymph was collected with micro-capillary tubes and transferred to Teflon®-capped culture tubes containing 500μl acetonitrile. Samples were kept at -20ºC until they were sent, on dry ice, to Michigan State University for analysis. JH titers were determined using radioimmunoassay (Huang et al., 1994; Huang and Robinson, 1995). Extractions were completed within a three-week period before the end of September**.**

Statistical Analyses

Results of the two mite non-reproduction assays were compared using a simple t test. The non-reproduction data sets were averaged and related to final mite concentration in colonies using the SAS correlation procedure (SAS Institute, 2002).

Results of colony JH titers were compared using the SAS ANOVA single-factor procedure in a linear additive mathematical model. Analyses were conducted using colonies as treatment and bee type as replication.

Regression analyses were performed using four values for each colony: final mite concentrations, mean mite-non reproduction, mean JH titer, and mean variance in JH titer. Both SAS and Microsoft 2000 Excel software were used.

Results

Mite Concentrations and Mite Non-reproduction

Colony mite concentrations by late September ranged from 0.042-0.92 mites sampled/per sq. inch of brood (Table 4.1). Because both sample sets of non-reproduction data were in general agreement (Pearson's r=0.840; t=1.77; P=0.331), non-reproduction values were averaged and used for subsequent analyses (Table 4.1). As expected, final mite concentration and mite non-reproduction were inversely correlated (Pearson's r = -0.718; P=0.029).

Mean JH titers per Colony

No significant differences in JH titer were found among the queen-types used (df = 2,79; F = 0.75; P = 0.304). Also, no significant differences in JH titer were found among the nine colonies (df= 8,79; F=0.75; P=0.681). Regression analyses determined that the relationship between mean JH titers and final mite concentration was best described with a polynomial equation (df = 1,8; R^2 = 0.546; P = 0.023) (Figure 4.1). A significant relationship was also found between mean JH titer and the levels of mite non-reproduction when described as an exponential curve (df = 1,8; R^2 = 0.478; P = 0.039) (Figure 4.2).

Variance of JH titers

As with mean JH titers, the relationship between JH variance and final mite concentration is best described as a polynomial equation (df = 1,8; R^2 = 0.548; P = 0.023) (Figure 4.3), and an exponential curve best describes the relationship between JH

Table 4.1 *Varroa* concentrations and percent non-reproduction (means) in honey bee colonies with Russian, SMR, and Italian queens

Queen Type	Final Mite Concentration	Mean Mite Non-reproduction (%)
Russian 1	0.114	23.27
Russian 2	0.916	11.20
Russian 3	0.447	27.50
SMR 1	0.711	20.00
SMR 2	0.232	42.50
SMR 3	0.313	27.50
Italian 1	0.042	55.43
Italian 2	0.527	24.29
Italian 3	0.169	25.00

Nine colonies with varying mite concentrations and mite reproduction used for juvenile hormone assays. (SMR=suppression of mite reproduction).

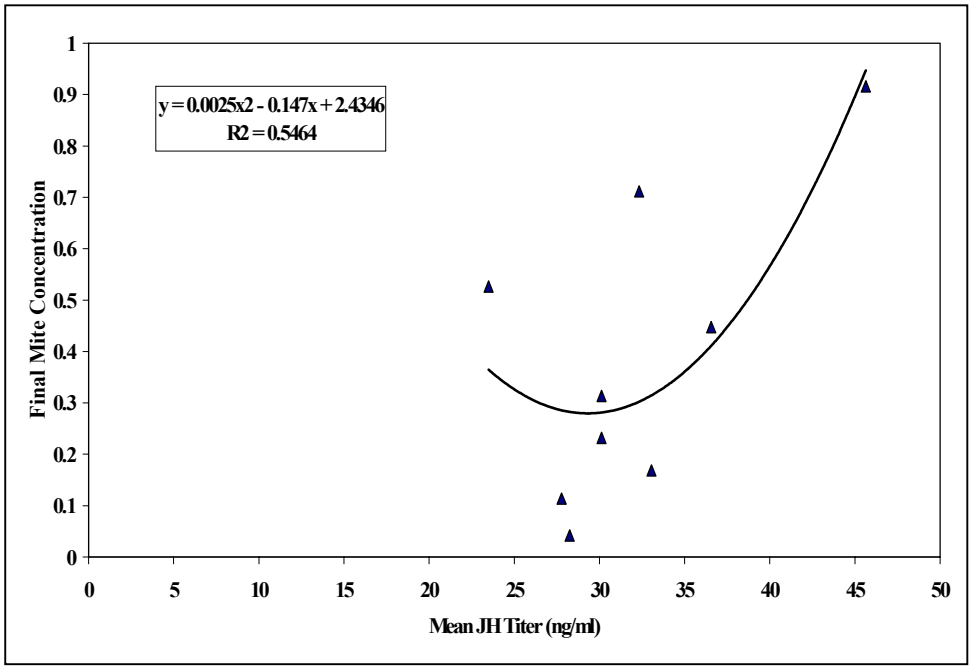

Figure 4.1 Regression analyses between final mite concentration and mean JH titers in nine honey bee colonies
A second-order polynomial best describes the relationship (P=0.023).

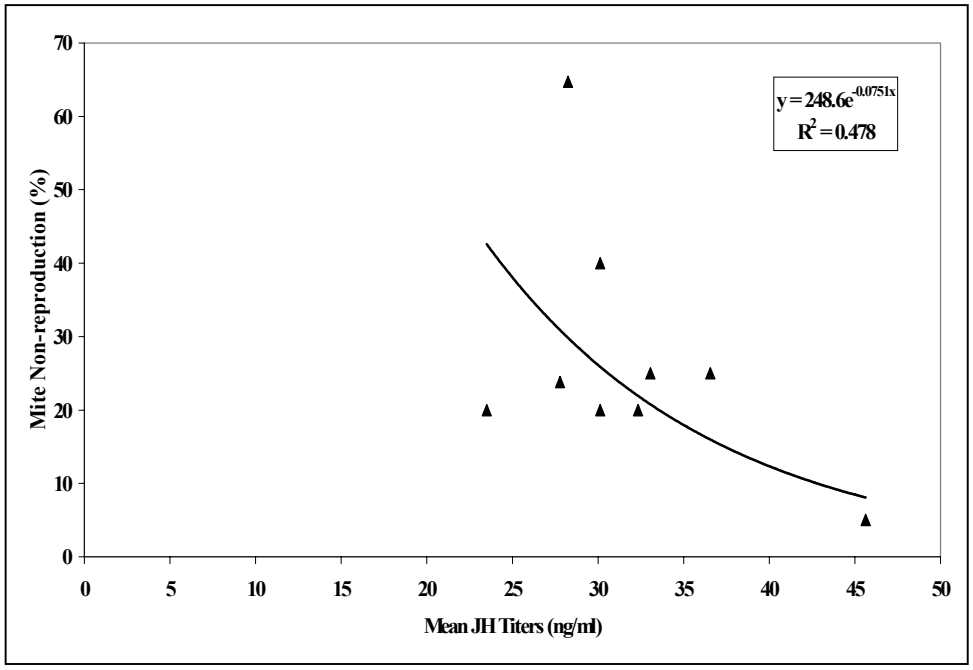

Figure 4.2 Regression analyses between mite non-reproduction and mean JH titers in nine honey bee colonies

The relationship is best described with an exponential curve (P=0.039).

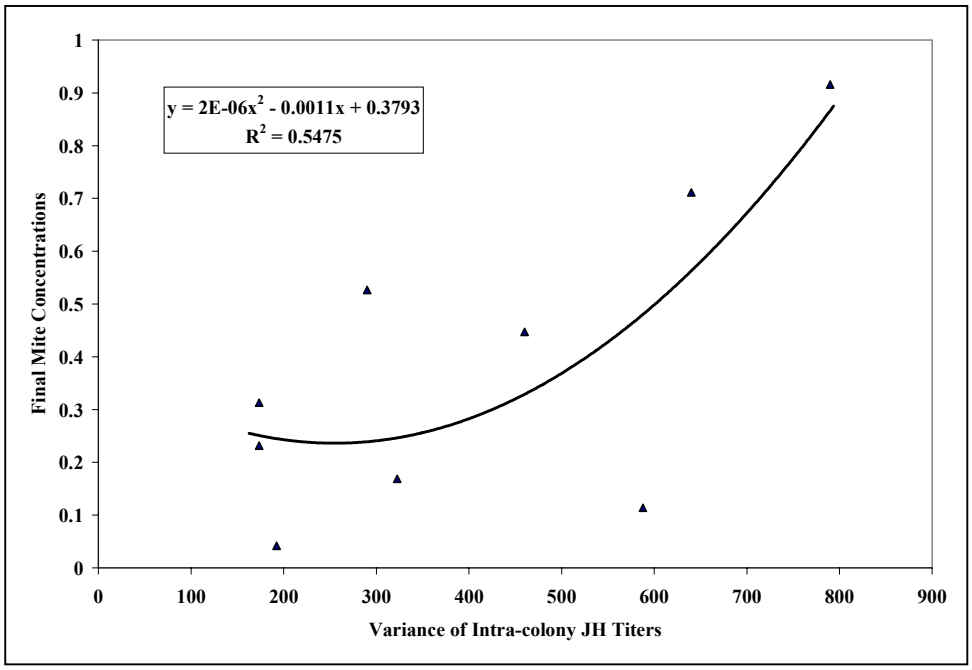

Figure 4.3 Regression analyses between final mite concentrations and variance of JH titers in nine honey bee colonies

A second-order polynomial equation best describes the relationship (P=0.023).

variance and mite non-reproduction (df=1,8; R^2=0.539; P=0.024) (Figure 4.4).

Discussion

Mean JH Titers per Colony

The hypothesis of JH influence on *Varroa* reproduction might yet be valid. Although we found no significance differences in JH titers among colonies, the small size of this study (n = 9 colonies) made finding significance difficult.

Because mean JH titer (and mean JH variance) per colony was a slightly better predictor of final mite concentration than mite non-reproduction, we can speculate that JH titer does not affect mite populations in the strict terms of non-reproduction as it is measured in this assay. It could be that there is simply a critical JH requirement for *Varroa* embryogenesis and that the earlier this requirement is satisfied, the more viable progeny can be produced per cell. It might take longer for mites feeding on pupae with lower hormone levels to acquire the critical JH; thus they would have a reduced fecundity, but still might be reproductive. A reduced effective reproduction rate (the number of viable females produced per foundress mite (Corrêa-Marques et al., 2003)) would not be reflected by the non-reproduction assay used in this study. Of the non-reproducing mites in this study, the high percentage that had laid eggs but had no progeny beyond the protonymph stage (54.7 %) supports this theory. It would be helpful to know the relationship between JH titers and time of oviposition in individual cells, though this would be difficult, if not impossible, to determine.

Variance of JH Titers

According to our results, mite concentrations—and to a lesser degree, non-reproduction—is correlated to the variability of intra-colony JH titers. As titer variance increases, mite concentrations rise and non-reproduction falls. We can only speculate that increased variance denotes greater genotypic variation (Robinson et al., 1989; Elekonich et al., 2003) and that from some colonies, we collected hemolymph from more sub-populations than in others. That homogeneity appears to be a factor, calls into question

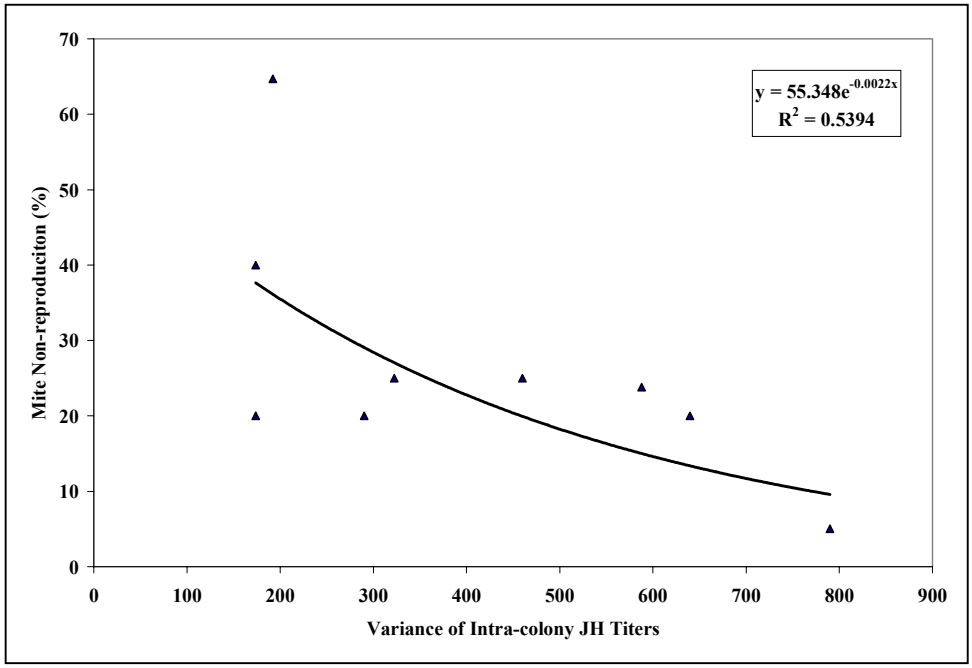

Figure 4.4 Regression analyses between mite non-reproduction and variance of JH titers in nine honey bee colonies

The relationship is best described as an exponential curve (P=0.024).

the genetics of the queens used. Though the number of colonies tested is too small to draw conclusions—and given that all queens used were open-mated to drones of unknown origin—we contend that some of the "resistant" queens used were more closely related than others to the P_1 (pure parent) generation. This might have led to more consistent JH titers in some colonies. Because the data show that the more consistent the JH values, the better the colony coped with *Varroa,* it is possible that there is a relationship between juvenile hormone levels and degree of hybridization in resistant queens.

Finally, many studies support the theory of a host-influenced factor that affects *Varroa* reproduction, whether it is during the phoretic or capped stages of association. Because there has been limited research in temperate climates with JH and mite reproduction, and because JH is such a labile compound, we feel that a more substantial study would help to clarify the relationship.

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Vita

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