

Can the frequency of reduced *Varroa destructor* fecundity in honey bee (*Apis mellifera*) pupae be increased by selection?

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Abstract – Crosses were made between queens and drones from 16 different commercial sources of European honeybees to determine if reproductive rates for *Varroa destructor* differed. Worker brood from four different crosses averaged 4.2 mites per cell and were chosen as the high mite reproduction group. Four others averaged 2.4 mites per cell and were chosen for the low mite reproduction group. A second set of crosses within the high and low mite reproduction groups were made and the worker offspring tested for differences in mite fecundity. Worker brood of the high and low mite reproduction lines did not differ significantly in the average number of mites per cell. The proportion of infested cells with non-reproductive mites also was not affected by selection. These results suggest that the frequency of larval or pupal characteristics that we measured in worker honeybees that might influence mite reproductive rates cannot be increased by selection based on average mite fecundity.

Varroa destructor / *Apis mellifera* / genetic crosses / selection / mite fecundity

1. INTRODUCTION

Varroa destructor Anderson and Trueman is an external parasitic mite that is a serious pest of honeybees (*Apis mellifera* L.) and has caused severe losses of colonies worldwide. Mite infestations can be reduced in colonies, at least temporarily, by acaricides. However, the long-term solution to the problem is selecting for mite resistant lines of honeybees.

When mites infest colonies in an apiary, their populations can increase to levels that cause the hives to perish. However, in some colonies the mite populations remain at low levels and the hives show no ill effects. Appar-

ently, there is variation among colonies in their susceptibility to growth of mite populations. One factor that would constrain the growth of mite populations is the reproductive rate of the mites in a given colony (Harbo and Hoopingarner, 1997).

Reproductive rates could vary among colonies for several reasons. Some are related to adult worker bees that remove mites from the bodies of nestmates and thus prevent the mites from entering cells and reproducing (Bucher et al., 1992; Fries et al., 1996). Mite reproductive rates also would be reduced if adult workers detect infested brood cells and remove the pupa before the mites can reproduce (i.e., hygienic

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behavior) (Spivak, 1996). Physiological characteristics of the honeybee larvae or pupae might also afford some resistance. For example, mite reproductive rates might be lowered if there was reduced feeding activity on the larvae or pupae by the foundress mites. Indeed, traits expressed by larvae and pupae might be responsible for the lower fertility rates in the brood of Brazilian honeybees, which show an increased tolerance to *V. destructor* (Ritter and DeJong, 1984).

The effect of various adult behaviors and the length of time that cells remain sealed while bees pupate have been examined for their influence on mite population growth. Of all the traits examined, only non-reproduction of mites was highly correlated with changes in the mite populations (Harbo and Hoopingarner, 1997). Sibling analysis was done to establish the potential heritability of characteristics associated with reducing mite reproductive rates (Harbo and Harris, 1999). While certain traits were found to be heritable, it is important to know whether the frequency of the traits associated with reduced mite fecundity can be increased due to selective breeding. This is the first step in determining the feasibility of breeding honeybees that cause mites to have reduced fecundity.

A second factor that must be considered in selecting honeybee stock where mites have reduced fecundity is determining whether it is the product of adult behaviors or due to the physiology of the larvae or pupae. The lifestage responsible for low mite reproductive rates will influence how the resistance is expressed at the colony level. If the traits were expressed only in the immature stage of the bee, most of the larvae or pupae would need to possess them for mite populations to be reduced at the colony level. This is because only a few of the numerous patriline (workers derived from different fathers) that exist in a colony might express the traits. However, if reduced mite fecundity were due to an adult worker bee behavior, not all adult workers would need to express the trait for the entire colony to benefit from its presence.

Behaviors of adult worker bees that would reduce mite reproduction have been previously examined (Ruttner and Hanel, 1992; Spivak, 1996). However, mite resistance expressed in

the larval stage has not. The purpose of this study was to determine if the frequency of traits expressed in worker honeybee larvae that influence mite reproduction could be increased by selective breeding.

2. MATERIALS AND METHODS

2.1. Selection of colonies with high and low mite reproduction rates

The first step in determining the heritability of reduced mite reproduction rates in larvae was to establish a distribution of average mite reproduction in unrelated lines of European honeybees. The queen lines at the two tails of the distribution (i.e., highest and lowest mite reproduction rates) were selected for crosses and a second round of testing. Sixteen different commercial sources of European honeybee queens were used to test for variation in mite reproduction in worker brood. Colonies were established and 5–7 daughter queens were grafted from each of the 16 different queen sources, and singly inseminated using brother drones from a different queen source. A total of 16 different lines (hereafter referred to as G1) were generated. Queens were hived separately in 5-frame colonies.

To determine if mite reproduction differed among the G1 crosses, we grafted 50 larvae from each into worker cells in brood frames. The frames that larvae were grafted into were prepared as follows. Laying queens from unrelated European honeybee colonies were placed in caged empty brood combs in their own colony for three days. This enabled the queen to lay eggs over a large section of both sides of the frame. On the fourth day, eight rectangular sections representing 50 cells were marked off on two combs (16 total sections). Larvae from each of the sections were removed and replaced with 50 larvae (1–3 days old) from each G1 cross we created from the single drone inseminations. The frames were then placed in a colony that was highly infested with mites (hereafter referred to as the host colony). The combs with the grafted larvae were placed in the center of the brood nest of the mite-infested colony with the sides containing the larvae facing each other. The procedure described above was repeated three times using a different sister queen from each of the 16 G1 crosses.

Thirteen days after the larvae were grafted, the developing pupae were at the purple eyed or later stage of development and the cells were examined for mites (Harbo and Hoopingarner, 1997). All sealed cells from each rectangular section were examined, and the percentage with mites and the total

number of mites (mature and immature) per cell were recorded. The proportion of cells containing worker pupae and either a dead or a single mite also was recorded. These cells were defined as those where the mites entered the cells but failed to reproduce. In addition, 50 brood cells were selected at random from the frame where the larvae were grafted. The brood cells were not part of any of the rectangular areas that contained larvae we grafted. We also examined 50 cells for mites from a brood frame chosen at random in host colony.

2.2. Selection of queen lines with high and low mite reproduction

The data collected from the procedures described above enabled us to identify eight crosses with either a high or low average number of mites produced per cell. Four crosses were deemed "high mite reproduction" (H_{1-4}) and four were identified as having "low mite reproduction" (L_{1-4} ; see Results). Essentially, we selected queen lines from the two tails of the distribution of reproductive rates from the 16 different queen lines (i.e., the far left tail that represents the queen lines with low reproductive rates and the far right tail that represents the queen lines with high reproductive rates).

Queens were grafted from H_{1-4} and L_{1-4} , and crosses were made within the designated high and low mite reproduction lines. The crosses (hereafter referred to as G2) were made using drones and queens from each of the four lines as follows ($\text{♀} \times \text{♂}$): $H_1 \times H_2$, $H_2 \times H_1$, $H_3 \times H_4$, $H_4 \times H_3$ and $L_1 \times L_2$, $L_2 \times L_1$, $L_3 \times L_4$, $L_4 \times L_3$. Five singly inseminated queens from each cross were hived in separate five-frame colonies. After the queens began to lay, the procedure described above for grafting larvae into cells on frames and placing the frame into a colony infested with mites was repeated. All eight G2 crosses were tested simultaneously by grafting larvae from each line into cells on two frames and placing them into the same mite-infested colony. Thirteen days after the larvae were grafted, the cells were examined for mites as described above. This procedure was repeated three times using different sister queens.

2.3. Statistical analysis

Two separate statistical tests were conducted to determine if mite reproduction differed within a line among the three replications (Sokal and Rohlf, 1995). First, individual one-way analysis of variance (ANOVA) were conducted on the data from each queen line to determine if mite reproduction differed among any of the replications. Next, a stacked one-way ANOVA was conducted using replicate number as the Factor and mites per cell as the Re-

sponse variable (Ryan et al., 1985). If mite reproduction in the three replications did not differ, a second one-way ANOVA was conducted (mites per cell versus G1 cross) to determine if there were differences among the G1 crosses.

High and low mite reproduction lines were selected from the G1 crosses for making G2 crosses. The selections were based upon the average number of mites per cell and the percentage of cells with non-reproductive mites.

The same type of data analyses described above for the G1 lines was used to determine if there were differences in the average number of mites per cell among the three replications for each G2 line (mites per cell versus replicate). If the means did not differ, the data for each line were pooled and a single mean for each high and low mite reproduction line was estimated. A one-way ANOVA was conducted with the G2 lines to determine if any were significantly different in the average number of mites per cell (mites per cell versus G2 cross). A similar ANOVA was conducted with the four low mite reproduction lines. If there was no difference among the average number of mites per cell within the high and low mite reproduction lines, we pooled all values for the number of mites per cell for the four G2 lines of each type. We then estimated the average number of mites per cell for all high and low mite reproduction lines. A t-test was conducted to determine if the average number of mites per cell differed between the high and low mite reproduction lines. An ANOVA was conducted to determine if the number of mites per cell differed among the high and low lines and cells examined at random on the frame where the larvae were grafted and on a frame chosen at random in the host colony.

The average percentage of non-reproductive mites and 95% confidence intervals were estimated using pooled data from the three replications with the high and low mite reproduction (G2) lines, cells chosen at random on the same frame where the larvae were grafted and in the host colony at large.

3. RESULTS

3.1. Selection of colonies with high and low mite reproduction rates

Data from the three replicates within each G1 line did not differ significantly within any of the lines ($F = 1.96$, $P = 0.14$, d.f. = 2, 260; individual ANOVA for each queen line had $P > 0.05$). Hence, we pooled the data from all three replications for each queen line and calculated

a single mean for the number of mites per cell. We also estimated the percentage of infested cells, and the percentage of infested cells with only one mite (i.e., the percentage of infested cells with non-reproductive mites) for each G1 line. The one-way ANOVA for mites per cell versus G1 line indicated no significant difference among any of the crosses ($F = 1.39$, $P = 0.14$, d.f. = 17, 245). There also was no significant difference in the average number of mites per cell among the G1 crosses and either the cells chosen at random on the same frame where the larvae were grafted or in the colony at large.

The average number of mites per cell among the G1 crosses ranged between 2.4–4.4 (Tab. I). The G1 lines deemed as “high mite reproduction” ranged between 3.7 ± 0.7 – 4.4 ± 0.6 mites per cell and 5.5–28.6% of infested cells with non-reproductive mites. The “low mite reproduction” lines ranged between 2.4 ± 0.5 – 2.8 ± 0.5 mites per cell and 23.1–40.0% of infested

cells with non-reproductive mites. The lines represent the left and right tails of the distribution of mites per cell among all G1 crosses. There is no overlap in the 95% confidence intervals between the means of the high and low lines, so they were used for making the G2 crosses.

3.2. Mite reproduction in queen lines selected for high and low mite reproduction

As in the G1 crosses, the three replications with the G2 crosses did not differ significantly within any of the crosses for the average number of mites per cell ($F = 0.43$, $P = 0.65$, d.f. = 2, 40 for the high mite reproduction lines and $F = 0.44$, $P = 0.65$, d.f. = 2, 33 for the low mite reproduction lines). The data from the three replications with each cross were pooled, and the average number of mites per cell was calculated for each line. A second ANOVA indicated that the individual high mite reproduction

Table I. Proportion of cells with mites and the average number of mites per cell in European honeybees. Worker larvae from each queen line were grafted into cells on a frame that was placed in a colony infested with *Varroa destructor*. ‘Random’ represent cells examined for mites on the frame where the larvae were grafted. ‘At large’ represents cells examined for mites on frames other than where the larvae were grafted. Data presented below are means from three replications.

Queen line	Sample size	% Cells infested	% Cells with 1 mite ^a	Average number of mites per cell
1	48	23.4	33.3	3.1
2	31	36.6	18.2	4.1 - h ^b
3	67	21.5	28.6	3.1
4	69	21.0	28.6	4.0 - h
5	57	32.1	5.5	4.4 - h
6	38	19.0	28.6	2.9
7	53	25.0	23.1	2.5 - l
8	50	10.2	40.0	2.4
9	43	19.0	12.5	3.6
10	22	19.0	0.0	3.5
11	57	19.3	9.0	3.7 - h
12	44	23.3	40.0	2.8 - l
13	30	21.4	16.7	3.0
14	26	24.0	16.7	2.8
15	29	28.8	28.6	2.4 - l
16	18	18.0	33.3	2.5 - l
Random	80	26.0	7.0	3.6
At large	33	22.7	10.0	2.3

^a Cells with one mite were characterized as those with non-reproductive mites.

^b h = lines selected as having high mite reproduction rates and l = lines selected as having low mite reproduction rates.

lines did not differ significantly from each other ($F = 2.25$, $P = 0.10$, d.f. = 3, 39). An ANOVA followed by a Tukey's-W procedure indicated that there was no significant difference among the four low mite reproduction lines ($F = 3.5$, $P = 0.03$, d.f. = 3, 31; range of means = 1.1–2.3; Tukey's-W critical value = 1.3).

A second analysis based upon the percentage of cells with non-reproductive mites was conducted using data from worker offspring resulting from crosses between queens and drones from parent lines 5 and 11 (high lines) and 12 and 16 (low lines). Colony-5 was crossed with Colony-2 that had 5.5% and 18.2% of infested worker cells with non-reproductive mites respectively. Worker offspring from the crosses had 80% of infested cells containing a single mite. Colony-11 was crossed with Colony-4 (9.0% and 28.6% non-reproductive mites in infested worker cells, respectively) and the worker offspring had 78% of infested cells with one mite. Reciprocal crosses of queens and drones from Colonies 12 and 16 (40% and 33.3% of infested cells with non-reproductive mites, respectively) produced worker offspring that had an average of 43% of infested cells containing non-reproductive mites. There was no significant difference in the average percentage of non-reproductive mites among the G2 lines from the crosses described above ($F = 1.14$; d.f. = 3, 40; $P > 0.05$).

Data from high and low lines were pooled, and overall averages for number of mites for all high mite reproduction lines and for all low mite reproduction lines were calculated. A

two-sample t-test indicated no significant difference between the high and low mite reproduction lines in the number of mites produced per cell ($t = -1.47$, d.f. = 79, $P = 0.15$). The average number of mites per cell was 1.7 ± 0.2 ($n = 41$) for the high mite reproduction lines and 2.2 ± 0.3 ($n = 39$) for the low (Tab. II). Cells examined at random on the frames where the larvae were grafted averaged 2.6 ± 0.3 mites per cell ($n = 40$), which was significantly greater than the colony at large (1.5 ± 0.2 , $n = 21$). Mites entered cells but did not reproduce in an average of $65.0 \pm 14.2\%$ of the cells with larvae from the high mite reproduction lines and $58.0 \pm 16.1\%$ in the low lines. An average of $39.0 \pm 14.9\%$ of the cells examined at random on the frames where the larvae were grafted had non-reproductive mites, while cells in the colony at large had $76.2 \pm 19.4\%$ of infested cells containing a single mite. The average number of mites per cell did not differ significantly among high and low mite reproduction lines and cells selected at random on the same frames and in the colony at large (Tab. II).

4. DISCUSSION

Bees from the high and low lines did not differ for any of the variables measured. This is important because we applied strong selection using carefully controlled methods and were unable to detect a response to selection. These results suggest that selection for properties of the larvae that might reduce their attractiveness to mites or decrease the mite reproductive

Table II. The average number of *Varroa destructor* per cell and cells containing one mite in lines of honeybees selected for high and low mite reproduction. Larvae from each line were grafted into cells and placed in a common hive environment. 'Random' represents cells sampled for mites on the same frame where the larvae were grafted but were not from either the high or low mite reproduction lines. 'At large' represents cells examined for mites on frames in the common hive environment other than where the larvae were grafted.

Queen line	Sample size	% cells with 1 mite \pm S.E.	Mites per cell \pm S.E.
High mite reproduction	43	65.1 ± 14.2	1.7 ± 0.2 ab
Low mite reproduction	36	55.6 ± 16.2	2.2 ± 0.3 ab
Random	41	39.0 ± 14.9	2.6 ± 0.3 a
At large	21	76.2 ± 19.4	1.5 ± 0.2 b

Averages of the number of mites per cell followed by the same letter are not significantly different as determined by an analysis of variance ($F = 3.5$, d.f. = 3, 137, $P = 0.02$) and a Tukey's-W procedure.

capacity is unlikely to succeed. The bees used in the study were derived from a highly diverse population, so it is unlikely that our results were due to lack of sufficient genetic variation for these traits. Another selection program probably could not significantly increase the genetic variation of the foundation population.

The assay we employed has been used successfully to determine differences in the attractiveness of mites to larvae from European and Africanized sources (Guzman-Novoa et al., 1996) and has been used in previous studies that have attempted to detect differences in mite reproductive capacity (Guzman-Novoa et al., 1999). A limitation to the assay though, is that we selected lines based primarily upon high and low mite reproduction expressed as the number of adult mites per infested worker cell. When counting adult mites, we could not determine if, for example, the adult females that emerged were a foundress mite and her daughters or foundresses that did not reproduce because the cell was multiply infested. Indeed mite reproductive rates are reduced in cells that harbor more than one foundress (Fuchs and Langenbach, 1989; Eguaras et al., 1994; Martin, 1994; Medina and Martin, 1999). We also could not determine if offspring were mated. However, we also examined the inheritance of non-reproduction of mites in worker cells. We did not find a trend that indicated the frequency of the trait could be increased by selection. When lines where mites had low rates of non-reproduction were crossed, the frequency of non-reproduction actually increased to almost twice the rate of crosses between lines with high rates of non-reproduction. Crosses between lines where mites had high rates of non-reproduction did not increase the frequency of the trait. Furthermore, the average number of mites per cell in progeny from our G2 crosses and the percentages of non-reproductive mites in infested cells did not differ from that in random cells on the same frame where the larvae were grafted or the host colony at large. Since our lines created from selection were no different in mite reproduction rates than the host colony that was chosen at random, and crosses within lines where mites had either high or low mite fecundity did not result in progeny with significantly different mite

reproductive rates, we conclude that selection based upon non-reproduction and low numbers of mites per cell has no effect on mite fecundity.

An underlying question always present in studies of mite reproductive rates is whether they are influenced by the host or due to a mite population that has low virulence. Colony conditions and characteristics of the mite alone or in combination can be responsible for the limited growth rate of the mite population and it is often difficult to separate the two. In our study, all grafted larvae were exposed to a common hive environment and the same mite population. Consequently, differences in mite reproductive rates, if present, would have been attributed to differences in the larvae among the lines we created rather than differences in mite populations or colony conditions.

The average number of mites per cell and the percentage with non-reproductive mites were higher in cells from randomly selected frames in the host colony compared with those from the frames where the larvae were grafted particularly in the replications with the G2 crosses. The frames where we grafted larvae were placed in the center of the colony's brood nest, while those selected at random in the colony at large were at least one frame away. The reproductive rates of mites are affected by temperature (LeConte et al., 1990). There is less variation in broodnest temperature on frames in the central broodnest compared with those further away (Simpson, 1961; Kronenberg and Heller, 1978; DeGrandi-Hoffman et al., 1993). Wide variation in temperatures is unfavorable for the development of mites and could have caused the lower number of mites per cell in those sampled at large compared with cells selected at random on the frame in the center of the broodnest. The percentage of cells with non-reproductive mites differed greatly between the trials with the G1 and G2 lines indicating a high degree of variability in this trait that might be due to colony conditions and the virulence of the mite population.

The results from our study are similar to previous reports where there were no differences among colonies for the attractiveness of brood to mites or for the effect of brood on mite reproduction (Arechavaleta-Velasco and Guzman-Novoa, 2001). Our results also are similar to

studies that found no significant difference in mite reproduction between high and low lines of infested colonies in Mexico (Arechavelata, unpublished data cited in Guzman-Novoa et al., 1999). The attractiveness of brood to mites also does not appear to be based on the genotype of the brood (Bienefeld et al., 1998). Apparently, seasonal and environmental conditions that an adult female mite is exposed to before or during reproduction (i.e., a colony effect) more strongly determine fecundity than the genotype of the brood (Otton and Fuchs, 1990; Fuchs, 1994). In contrast, Harris and Harbo (1999) reported reduced mite fecundity and low sperm counts in colonies with low mite reproductive rates, and have created lines where mite reproduction is suppressed. Whether characteristics of the adult or immature bees are responsible for reducing mite reproduction has not been determined (Harris and Harbo, 2000). Our study indicates that attributes present in the larval or pre-pupal stage probably do not contribute to a reduction in mite reproduction.

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Résumé – La fréquence de la réduction de la fertilité de *Varroa destructor* chez les nymphes d’abeilles (*Apis mellifera*) peut-elle être augmentée par la sélection ? Une solution durable au problème de la varroose est la sélection de lignées d’abeilles résistantes. Un faible taux de reproduction de l’acarien *Varroa destructor* chez certaines lignées d’abeilles limiterait la population d’acariens et constituerait pour celles-ci un moyen de devenir résistantes. Le but de cette étude était de déterminer s’il est possible d’augmenter par la sélection la fréquence de la réduction du taux de fertilité des acariens chez les larves et les nymphes d’abeilles. Nous avons sélectionné quatre lignées d’abeilles chez lesquelles *V. destructor* avait un taux de reproduction élevé (lignées H) et quatre autres lignées où il avait un taux de reproduction faible (lignées L) (Tab. I) et nous avons opéré des croisements entre ces lignées. Si la

fréquence de la réduction du taux de fertilité peut être augmentée par la sélection, la descendance des croisements devrait avoir un taux de reproduction significativement plus faible que celle des lignées H. Pourtant ce ne fut pas le cas. Toutes les lignées issues des croisements entre lignées H et lignées L ont eu des taux de reproduction des acariens similaires (Tab. II). En outre la proportion des cellules infestées par des acariens ne se reproduisant pas n’a pas été affectée par la sélection. Nous en concluons qu’il est impossible d’accroître par la sélection la fréquence des caractéristiques larvaires ou nymphales que nous avons mesurées chez les ouvrières d’abeilles et qui sont susceptibles d’influencer les taux de reproduction de *V. Destructor*.

Varroa destructor / *Apis mellifera* / croisements / sélection / fécondité de l’acarien

Zusammenfassung – Kann die Häufigkeit von Puppen mit der Eigenschaft einer begrenzenden Fertilität von *Varroa destructor* durch Zucht erhöht werden? Eine dauerhafte Lösung für die Probleme mit *Varroa destructor* ist die Selektion von Linien der Honigbienen, die gegen die Milbe resistent sind. Eine Möglichkeit für eine Resistenz wäre eine geringe Reproduktionsrate der Milben bei bestimmten Linien der Bienen, durch die das Populationswachstum begrenzt würde. Unsere Untersuchung wurde mit dem Ziel durchgeführt zu bestimmen, ob die Häufigkeit von verringerten Reproduktionsraten bei Arbeiterinnen und Puppen durch Selektion gezielt erhöht werden kann. Wir suchten 4 Linien von Honigbienen mit hoher und 4 Linien mit geringer Reproduktionsrate aus (Tab. I) und kreuzten innerhalb der Linien. Wenn die Anzahl mit geringen Reproduktionsraten durch die Selektion erhöht werden können, müssten unsere “niedrig-Linien” signifikant weniger Milbenvermehrung aufweisen als unsere “hoch-Linien”. Leider war das nicht der Fall. Alle Linien hatten nach der Selektion gleiche Reproduktionsraten (Tab. II). Demnach fehlt noch immer der Nachweis, dass die Eigenschaft von Larven oder Puppen, die eine geringe Milbenfertilität bewirken, selektiert werden kann.

Varroa destructor / *Apis mellifera* / Linienkreuzung / Selektion / Milbenfertilität

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