Low Sperm Counts and Reduced Fecundity of Mites in Colonies of Honey Bees (Hymenoptera: Apidae) Resistant to *Varroa jacobsoni* (Mesostigmata: Varroidae)

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ABSTRACT A breeding program for honey bees, *Apis mellifera* L., resistant to varroa mites, *Varroa jacobsoni* Oudemans, selected for bees that maintain low populations of mites with decreased reproduction. This study examines the causes for this suppressed mite reproduction. Nonreproductive mites were classified into 3 groups as follows: (1) mites that were alive but did not lay eggs, (2) mites that began laying eggs later than normal, and (3) mites that died before laying eggs (usually <2% of mites in worker brood). Mites that did not lay any eggs had significantly fewer spermatozoa (4 ± 8 [mean $\pm$ SD] $n = 33$) in their seminal receptacle than normally reproductive mites (27 ± 14, $n = 45$). About half of the mites that produced no progeny had no spermatozoa in their seminal receptacles. Hence, the failure to produce progeny was related to fertilization of the female mite. Reproductive mites produced fewer progeny in colonies that had a higher proportion of nonreproductive mites. Therefore, both absolute infertility and reduced fecundity may be associated with the same host-related factors.

KEY WORDS *Apis mellifera*, *Varroa jacobsoni*, mite fecundity, suppression of mite reproduction

Varroa jacobsoni OUDEMANS is an ectoparasitic mite that feeds on immature (last instars, prepupae, and pupae) and adult stages of honey bees, *Apis mellifera* L., and the eastern hive bee, *Apis cerana* F. Mites reproduce only within capped brood cells.

Our approach to controlling varroa mites is to select for bees that suppress mite reproduction. Bee hosts of particular genotypes can suppress the reproduction of mites in a colony of bees (Camazine 1986, Thrybom and Fries 1991, Stürmer and Rosenkranz 1994, Guzman-Novoa et al. 1996). We showed that nonreproduction of mites is a heritable characteristic of honey bees (unpublished data). Selection has increased the percentage of nonreproductive mites from 10 to 25% normally found in European bees to values ranging between 45 and 100% (Harbo and Harris 1998).

Stürmer and Rosenkranz (1994) demonstrated that the duration of starvation before placement into a brood cell affects whether a mite lays eggs. Mites starved for long periods are less likely to lay eggs. The number of progeny, (fecundity) however, produced by mites that lay eggs was not affected by the duration of starvation. Hence, they concluded that reproduction in varroa mites is triggered by an on–off mechanism. Several other researchers have reported that fecundity of reproductive mites is independent of the frequency of nonreproductive mites in a colony (Rehm and Ritter 1989, Rosenkranz and Engels 1994, Guzman-Novoa et al. 1996, Martin 1995). Our data, however, suggest that there is a relationship between fecundity and the percentage of nonreproductive mites.

The concept of all-or-none control of reproduction in varroa mites agrees with the observation that mites do not reproduce if they are put into a cell 24 h after capping, whereas mites that enter a cell before capping usually lay eggs (Beetsma and Zonneveld 1992, Steiner et al. 1994). Mites that begin feeding on the host before it spins a cocoon are stimulated to lay eggs, whereas those that begin to feed after the cocoon is spun are not stimulated to lay eggs (Steiner et al. 1994). This discrete period in the life of the host bee suggests a possible role of juvenile hormone as a trigger for varroa mite reproduction. Although exogenous application of juvenile hormone to bee larvae increases reproduction of varroa mites (Hänel 1983, 1986; Hänel and Koeniger 1986), endogenous juvenile hormone titers are similar in bee larvae from races and species of bees that differ dramatically in their abilities to support mite reproduction (Rosenkranz et al. 1989, 1990, 1993).

A mite that is stimulated to reproduce lays her 1st egg 70 h after the brood cell is capped, and she will lay each additional egg at 30-h intervals (Steiner et al. 1994, 1995). Although as many as 5 eggs can be laid in worker cell, a mite is doing well if just 1 of her daughters matures before the bee becomes an adult (Ifantidis 1983, Schulz 1984, de Ruijter 1987).

This study examines potential causes of the suppression of mite reproduction by honey bees. Colonies that suppress mite reproduction have normally repro-

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ductive mites in the 1st reproductive cycle after the mites enter the colony. This delayed effect may be caused by the previous experience of a mite in a brood cell or by the genotype of adult bees. In several experiments we monitored the frequency of nonreproductive mites through time. We compared the number of spermatozoa stored by nonreproductive and reproductive female mites to see if fertilization of the mite could explain their differences. We also examined the relationship between the total number of progeny produced by reproductive mites (fecundity) and the proportion of nonreproductive mites.

Materials and Methods

Except for experiment 1, all experiments were field tests that examined mite reproduction in colonies of bees. Colonies were established with the same protocol for all field tests by subdividing a large mixture of mite-infested bees into equal test populations (Harbo 1986). Uniformity of bee populations among colonies in a test was achieved by producing 2 cages of bees for each colony. About 500 g of bees was scooped from the large population into the 1st cage for each colony (each cage 14 by 22 by 16 cm). These were set aside and another series of cages was filled with bees from the same source. The cages had been initially weighed, and the weight of bees was recorded for each cage. Cages in the 1st series were each paired with a cage from the 2nd series to achieve maximum uniformity among the pairs for each experiment.

For installation of the caged bees, 2 cages of bees, a caged queen, and 5 combs were placed into a standard Langstroth hive that could contain 10 frames. The worker bees were immediately released from their cages, but the hives remained closed (with screen over entrances). Entrances were opened after dark on the following day; queens were released after an additional 24 h. Colonies were given only combs with worker-sized cells for brood and honey storage.

With this method of establishing colonies, both mites and bees were pooled from a heterogeneous group of source colonies to establish colonies with equalized populations of bees and mites. During the 1st 6 wk, the bee population of a test colony shifted from a heterogeneous mix to the progeny of the test queen.

Percentage of Nonreproductive Mites. Measurements of the percentage of nonreproductive mites per colony were made 2 or 3 times during each of the 3 experiments. A reproductive mite was one that could produce at least 1 mature daughter before the host bee emerged from its cell. We examined pupae at the purple-eyed stage or older to determine the reproductive success by mites in cells that had been infested with a single mite. At this stage of host development, it is too late for mite eggs to mature to adult females. Mites were considered nonreproductive when they had no progeny beyond protonymph by the time bee pupae had matured to the tan body stage. We counted 20–30 infested cells from each colony during each evaluation period. Nonreproduction was divided into 3 categories as follows: (1) mites that are alive in the cell but do not lay eggs, (2) mites with progeny that are too young to mature and mate before the host bee emerges from its cell, and (3) mites that die in the cell before laying eggs.

Fecundity of Reproductive Mites. The number of progeny produced by reproductive mites from singly infested cells was recorded during each evaluation period. Only mites from pupae that had purple eyes or mites on bees in older developmental stages were counted. This analysis did not include nonreproductive mites, which includes mites that produced progeny too late to mature.

Experiment 1: Counts of Stored Sperm. We compared the number of spermatozoa that were stored by reproductive and nonreproductive female mites at 2 different times. In 1996, the average number of sperm was compared for 3 groups of mites as follows: (1) normally reproductive mites, (2) mites that had laid no eggs, and (3) mites that had female progeny too young to mate and mature before the host bee emerged from its cell. In 1997, only the first 2 groups were compared.

For each test year, mites were captured from colonies that had high levels of nonreproductive mites (4 different colonies per year). These colonies were unrelated and had variable populations of bees. Only foundress mites from singly infested cells were evaluated. In 1996 we sampled 27 reproductive mites (6 or 7 per colony); 14 mites that had laid no eggs (2–6 per colony) and 13 mites with progeny too young to mature (1–8 per colony). In 1997 we sampled 18 reproductive mites (4 or 5 per colony) and 19 mites that had laid no eggs (4 or 5 per colony).

Each mite was glued dorsal side down onto a microscope slide to anchor it for dissection of the reproductive tract. The ventral genital plate was pried off by lifting it from the opening of the oviduct. The internal structures were bathed with mite saline (40 mM NaCl, 20 mM KCl, 15 mM $MgCl_2$, 1 mM $CaCl_2$, and 1.7 mM 1,4 piperazine diethanesulfonic acid, pH = 6.80) (Steiner et al. 1994) during dissection. The gastric cecae, rectum, and Malpighian tubules were separated from the reproductive tract (lyrate organs, ovary, and seminal receptacle), and the seminal receptacle was transferred to a separate slide. Gentle pressure was applied to the cover slip to rupture the seminal receptacle so that the sperm could be dispersed for counting. Magnification of 125x or 312x was used to count the sperm.

Statistical Analyses. Data from the 2 yr were analyzed separately. A high number of mites with zero sperm created a problem with normality. Therefore, the sperm counts were ranked (SAS Institute 1997) and the analysis of variance (ANOVA) was performed on the rank variable. In both tests, variation caused by different source colonies was treated as a random effect, and variation caused by differences between the types of mites was treated as a fixed effect.

Experiment 2: Field Test Using Queens Inseminated With a Single Drone. Ten honey bee colonies that showed reduced reproduction of varroa mites

were chosen as sources of queens and drones to be used in crosses for this experiment. Each of 26 queens was mated to a single drone. The 26 test colonies were formed on 7 May 1996. Colonies began with 733 ± 8 g (mean ± SD) of bees, 184 mites, and no brood. The queens were released on 9 May. The percentage of nonreproductive mites and the average number of progeny per reproductive mite were determined for each colony on 19 July and again on 28 August.

Experiment 3: Field Test Using Queens Inseminated With a Mixture of Semen From Many Drones. We inseminated 28 queens for this experiment. The queens were unrelated to each other and to the 7 queens used to produce the drones for the sire lines. Semen for a sire line was collected from at least 40 drone progeny from 1 queen. Semen was diluted 1:1 with saline, mixed, and used to inseminate 4 randomly chosen queens for each sire line (Harbo 1990).

Each test colony began with 1,002 ± 8 g of bees and 784 mites. Queens were released on 15 May 1997. Measurements of percentage of nonreproduction and average number of progeny per reproductive mite were made on 2 June, 23 June, and 24 July.

Experiment 4: Field Test Using Supersister Queens. In this experiment 25 supersister queens were inseminated and established into test colonies. These queens were daughters of the best queen from experiment 2 (its colony had high suppression of mite reproduction [90%] and the lowest mite population at the end of the test). Six different colonies were used as sources of drones. Each queen was inseminated with either a mixture of semen from a single source colony ($n = 17$), or semen from a single drone ($n = 8$).

Colonies began with 753 ± 8 g of bees and 433 mites on 19 June 1997. Queens were released on 21 June. The percentage of nonreproductive mites and the average number of progeny per reproductive mite were determined on 8 July, 13 August, and 6 October.

Statistical Analyses. The data from experiments 2 and 4 were combined for the analysis of fecundity. Measurements of fecundity at the 9th or 10th wk and the 15th or 16th wk were pooled between experiments to form the data set. Repeated measures ANOVA by using Proc Mixed (SAS Institute 1997) evaluated the effects of time and nonreproduction on fecundity. The year of the experiment (1996 or 1997) was treated as a block variable. For experiment 3, repeated measures analysis (Proc Mixed) evaluated the effects of time and nonreproduction on fecundity, and the sire group (1–7) was treated as a block variable.

A multivariate ANOVA (Proc GLM) tested for significant changes in the percentages of mites in the 4 classes (normally reproductive, alive but no progeny, alive with delayed oviposition, and dead) through time in all 3 experiments (SAS Institute 1997). Because the sum of these percentages must equal 100, they cannot be analyzed independently. We overcame this problem by using the log-ratio transformation to create a new set of 3 variables to be used in the multivariate analysis (Attchison 1982). These variables were Y1 = log (percentage alive but no progeny/percentage normally reproductive), Y2 = log (percentage alive with delayed oviposition/percentage normally reproductive) and Y3 = (percentage dead/percentage normally reproductive). Only 3 log-ratio variables are needed to adequately analyze a data set with 4 categories. All 3 experiments were doubly multivariate because the multiple variable set was measured repeatedly on each colony through time. The analysis for experiment 3 included modeling terms for the effects of the 7 different sire groups.

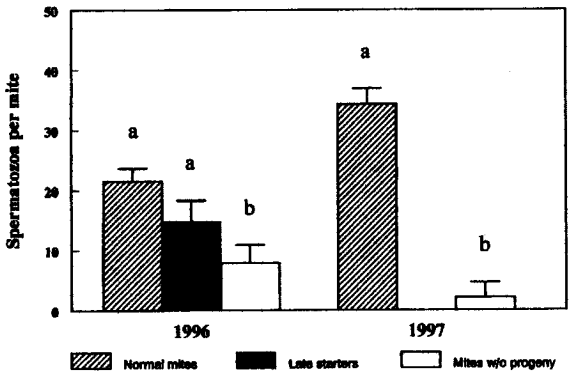

Fig. 1. Differences in numbers of spermatozoa in the seminal receptacle of female mites. Three classes of mites were compared in 1996: (1) mites that produced progeny that could mature (normal mites), (2) mites that laid no eggs (mites without eggs), and (3) mites that were nonreproductive because of delayed oviposition (late starters). Only mites in classes 1 and 2 were compared in 1997. Each bar represents the average number (mean ± SE) of sperm in the seminal receptacle for 13–27 varroa mites. For each year, bars with the same letter are not significantly different (Tukey mean separation, $\alpha = 0.05$).

Results and Discussion

Stored Sperm in Mites. In both years, mites that had not laid eggs had fewer sperm than normally reproductive mites (Fig. 1). Sperm counts were significantly different among 3 types of mites in 1996 ($F = 7.89$; df = 2, 51; $P < 0.002$). In 1997, mites without eggs had only 1/10 as many sperm as normally reproductive mites ($F = 127.19$; df = 1, 35; $P < 0.001$).

The significantly low sperm counts found in mites that had not laid eggs in our experiments suggests problems with mating or a failure of the prosperm to mature in the reproductive tract of the female mites. Prosperm are transferred from male mites to the female mites during mating. The prosperm change morphologically into mature spermatozoa after they migrate from the solenostomes down the rami and into the seminal receptacle within the female mite's sperm-access system (Alberti and Hänel 1986). Our data cannot distinguish between nonmating and the failure of sperm maturation. Many prosperm were present in the seminal receptacle of several mites that did not have mature sperm; this supports the hypothesis that sperm maturation was abnormal. Many mites that did not lay eggs, however, had no form of sperm (prosperm, intermediate fusiform sperm, or mature

sperm) within the seminal receptacle, suggesting either nonmating or depletion of sperm. Counts of zero sperm were found in 55% (18 of 33) of the mites that had not laid eggs. Some spermatozoa were found in the remaining mites, but the numbers were lower than in normally reproductive mites. Normally, multiple matings are needed to fill the seminal receptacle of a varroa mite (Donzé et al. 1996).

If the lack of progeny is related to mating failure, suppression of mite reproduction can be traced to a prior residence of the mite in a brood cell where mating should have occurred. In 1 scenario, some factor related to resistant host larvae reduces either the availability of male mites for mating with female mite progeny or the fertility of male mites. Female mites can mate just after their final molt (Akimov and Yastrebstov 1984), and mating between a mother mite and her son is rarely successful (de Ruijter 1987, Donzé et al. 1996, Martin et al. 1997). Martin et al. (1997) report that an unmated adult female cannot lay eggs when she enters a brood cell to reproduce, and we did not find any females that laid eggs when they had no spermatozoa in their seminal receptacle.

Martin et al. (1997) reported that nonmating of varroa mites was caused by premature death of male mites. There is evidence that leg and body movements of the host bee can kill or injure male mites and that some male mites die from never finding the feeding site (Donzé and Guerin 1994). We did not see dead male mites on host bees that were purple-eyed or at an older developmental stage in any of our experiments. In many colonies of bees from experiment 3, we noticed a lack of mature males and a high incidence of protonymphs or male deutonypmhs on host pupae that normally have mature male mites. Perhaps nonmating in our experiments was caused by a failure to produce male mites or the failure of male mites to mature in time to mate with their sisters.

Mite Fecundity and Percentage of Nonreproductive Mites. In experiments 2 and 4, the mean number of progeny produced by reproductive mites (or fecundity) was lower in colonies with a higher percentage of nonreproductive mites (% NR) in the colony ($F = 10.38$; df = 1, 46; $P < 0.003$). Fecundity of reproductive mites was not significantly affected by other factors related to time ($F = 0.02$; df = 1, 46; $P > 0.89$), and the interaction term (% NR x time) was not significant ($F = 0.59$; df = 1, 46; $P > 0.45$).

These results suggest that even reproductive mites may be affected by factors in bees that suppress mite reproduction. Therefore, activation of oogenesis and eventual oviposition in varroa mites was not controlled strictly by an on–off switch.

Although the number of progeny produced by reproductive mites can be related to the level of suppression of mite reproduction, other factors affect the fecundity of mites. In experiment 3, fecundity of the reproductive mites significantly decreased through time ($F = 4.17$; df = 2, 27; $P < 0.03$) for all 7 sire groups. The overall average fecundity for the 7 sire groups on 2 June, 23 June, and 24 July was 3.903 ± 0.05, 3.707 ± 0.06, and 3.427 ± 0.06, respectively (mean ± SE). The

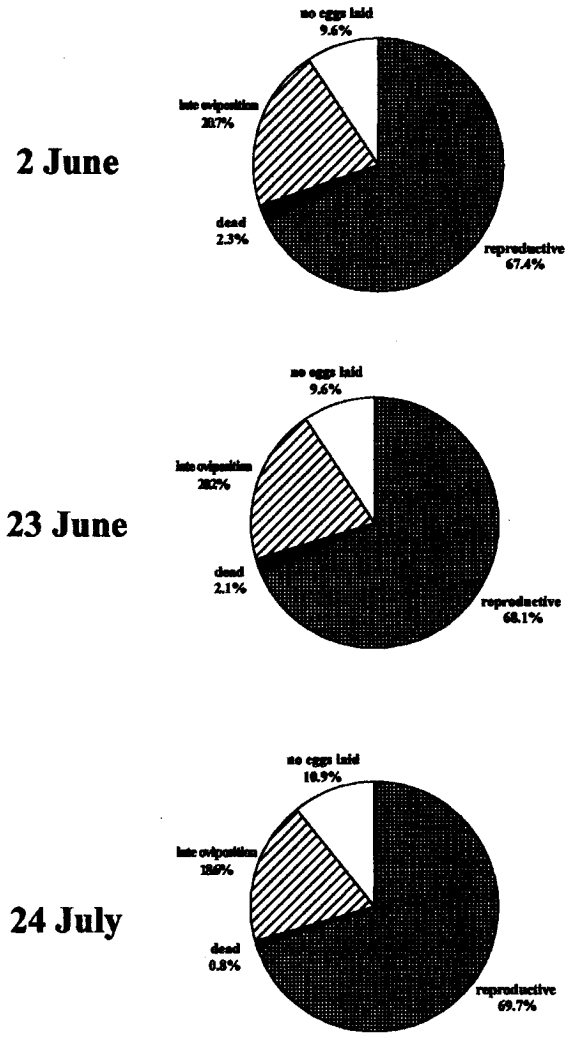

Fig. 2. Changes in the percentages of mites in the 4 categories (normally reproductive, live without progeny, live with delayed oviposition, dead) between 3 time periods for experiment 3. The frequency of nonreproduction did not change in these colonies through time ($\alpha = 0.05$).

interaction term (% NR x time) was not significant ($F = 0.38$; df = 2, 27; $P > 0.68$). Fecundity in this group was not related to the nonreproductive mites in the colony ($F = 0.36$; df = 1, 27; $P > 0.55$), probably because the numbers of nonreproductive mites were relatively low (Fig. 2), and therefore it was difficult to detect differences. We suspect that seasonal variation in the diet of bees could affect mite fecundity; however, Stürmer and Rosenkranz (1994) report that the number of progeny produced by activated mites is not affected by starvation of the mites or by changes in brood rearing activities of bees. Mite fecundity is independent of the time that mites spend on adult bees (Wendel and Rosenkranz 1990, Boot et al. 1995).

Changes in Percentages of Nonreproductive Mites Through Time. In experiments 2 and 4, the percentages of mites in the 4 categories (normally reproductive, alive but no progeny, alive with delayed ovipo-

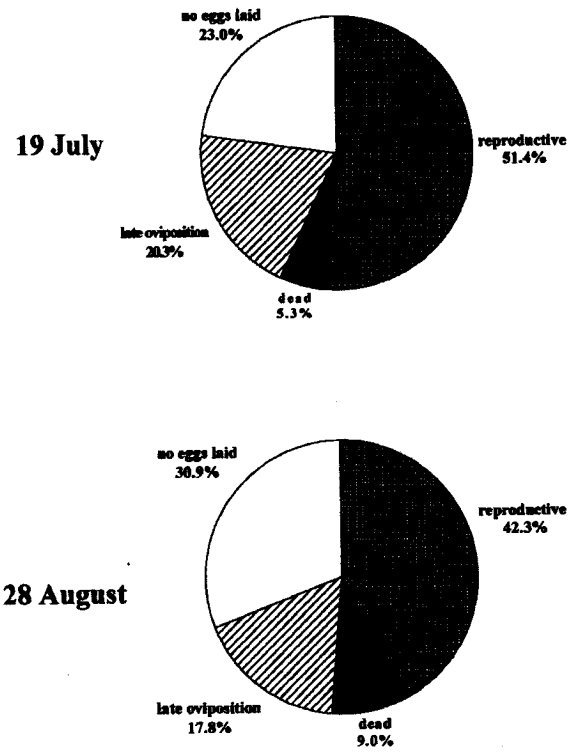

Fig. 3. Changes in the percentages of mites in the 4 categories (normally reproductive, live without progeny, live with delayed oviposition, and dead) between the 2 time periods for experiment 2. The changes in these distributions were significantly different ($\alpha = 0.05$, MANOVA). The percentage of mites that were living but produced no progeny significantly increased in August.

sition, and dead) changed significantly with time ([Wilks lambda = 0.66; $F = 3.10$; df = 3, 19; $P < 0.05$; Fig. 3] and [Wilks lambda = 0.133; $F = 19.59$; df = 6, 18; $P < 0.0002$, Fig. 4], respectively). For experiment 2, repeated measures analysis of each variable (independent of the other 2) indicated that the transformed variable Y1 (log [percentage mites that produced no eggs/percentage normally reproductive mites]) changed significantly with time, but variables Y2 and Y3 did not ($\alpha = 0.05$). This suggests that the major change in mite reproduction was an increase in the category of living mites that had no progeny (Fig. 3). For experiment 4, similar analyses showed that all 3 variables (Y1, Y2, and Y3) changed significantly with time ($\alpha = 0.05$). These results indicate that the percentages of mites in all 4 categories changed significantly with time (Fig. 4). Experiment 3 did not contain colonies that suppressed mite reproduction at high levels, and this may explain why the percentages of mites in the 4 categories did not change through time (Wilks lambda = 0.64; $F = 1.50$; df = 6, 16; $P > 0.23$; Fig. 2).

Therefore, colonies that suppressed mite reproduction were the colonies that expressed the greatest changes in mite reproduction with time. Changes in the category of living mites that produced no progeny may be linked to the nonmating of progeny mites from previous brood cycles. Martin et al. (1997) concluded that unmated varroa mites never lay eggs. Some other species of mites also require mating to produce haploid male progeny (Nelson-Rees et al. 1980).

Perhaps the gradual increase in the percentage of nonreproductive mites occurs as the initial reproductive mites are lost (natural mortality) and are replaced by their infertile progeny. Because reproductive and nonreproductive mites from 1 brood cycle tend to remain in the same reproductive state in their next cycle (Martin et al. 1997), the gradual decrease in the percentage of reproducing mites may reflect the natural mortality of the original mite population.

In experiment 4 we noticed for the 1st time a high proportion of dead mites (Fig. 4). A survey of old data showed 43 dead mites in 2,500 foundress mites (1.71%)

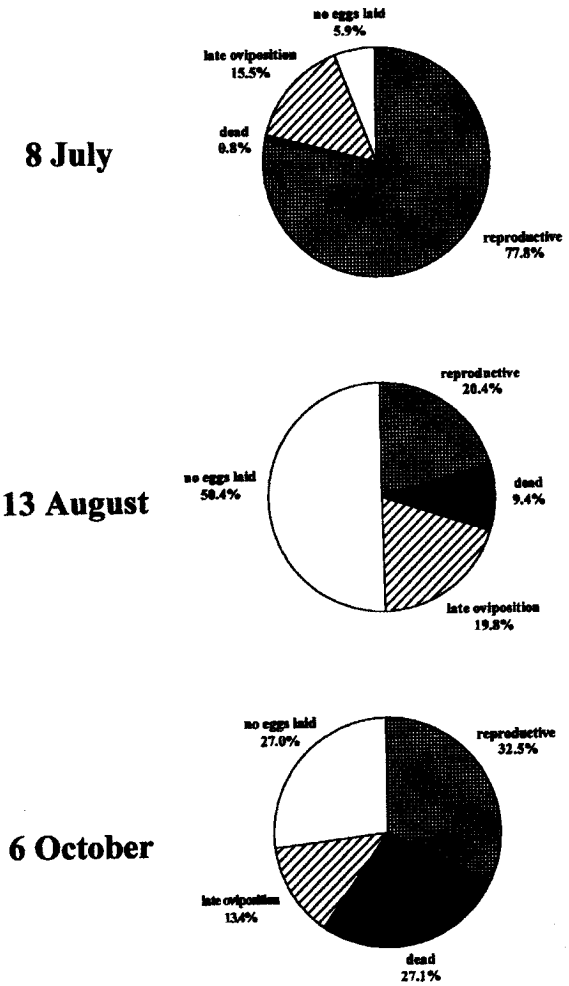

Fig. 4. Changes in the percentages of mites in the 4 categories (normally reproductive, live without progeny, dead, live with delayed oviposition) between 3 time periods for experiment 4. The frequencies of mites in all 4 categories changed through time ($\alpha = 0.05$, MANOVA). The large number of dead mites were entombed beneath the cocoon that is spun by the last-instar bee (see text).

in capped brood cells. However, in the August evaluation of experiment 4, the percentage of dead mites averaged 9.4, and by October the percentage of dead mites averaged 27.1 (range, 3.6–100%). Most of the dead mites for August and October were killed by becoming trapped against the cell wall, beneath the cocoon that was spun by the last instar larva. We call this *entrapped by the cocoon*. In August, 7.5% of all mites were entrapped by the cocoon and 1.8% were dead and free in the cell. In October, 23.7% were entrapped by the cocoon and 3.4% were found dead and free in the cell.

Reasons for mites becoming entrapped by the cocoon are speculative, but we noticed residues of brood food beneath the cocoons of bee larvae (with and without mites) from colonies that had high levels of entrapped mites. Perhaps mites are not released from the brood food before the bee larva begins spinning its cocoon. Excess brood food, however, cannot explain all cases of being entrapped by the cocoon because trapped mites are found in cells without food residues. If being entrapped by the cocoon is a genetic character of host bees, it could represent a powerful selection criteria for enhancing resistance to varroa mites in honey bees.

Nonreproduction of Mites and Mite Population Growth. The growth of a mite population can be described by the following equation $P_2 = P_1\ (rs)^n$, where P_1 is the initial mite population, P_2 is the final mite population, r is the rate of increase in the brood cell (which is the average number of adult female mites that leave 1 cell after a brood cycle divided by the number of foundress mites), s is the probability that an adult mite will survive outside the brood, and n is the average number of reproductive cycles per mite during an evaluation period, which is dependent on the duration of each reproductive cycle (t) (Harbo 1996). The percentage of nonreproducing mites (% NR) in a colony of bees is inversely correlated to the rate of increase (r) (Fig. 5). A high % NR reflects a low r, and a low r retards growth of the mite population (P_2/P_1).

The linear relationship between %NR and r (Fig. 5) was obtained by measuring the %NR (as described in this article) and the rate of increase (r) from 78 different colonies of bees in 1997 and 1998. We define r as the sum of mature daughters and surviving foundress mites that exit the cell divided by the number of foundress mites that entered these cells. We estimated r by observing mites in the brood cells. Mite-infested cells were examined when bees were at the tan body stage (about 16 or 17 d after egg laying). In each cell we counted the number of foundress mites and the number of female progeny that were deutonymphs or adults. Each adult daughter and immobile female deutonymph had a 100% chance of entering into the next cycle, whereas each mobile female deutonymph had only a 39% chance of reaching adulthood and entering the next cycle (Fuchs and Lagenbach 1988).

Our experiments are part of a breeding program designed to select for resistance to varroa mites. Our major focus is the selection of bees that produce the highest levels of nonreproducing mites. We have shown that the percentage of nonreproducing mites is a heritable characteristic of bees (unpublished results), and it is negatively correlated with mite population growth. There are other characteristics of bees that should impart resistance to varroa mites. These characteristics include hygienic behavior (Spivak 1996), grooming (Peng et al. 1987, Moretto et al. 1993, Fries et al. 1996), a reduced postcapping time (Moritz and Hänel 1984, Moritz 1985, Harbo 1992, Moritz and Jordan 1992, Bienefeld 1996), and a decrease in the proportion of mites that are found in the brood (Harbo 1996). Increased hygienic behavior and reduced postcapping times reduce the value of r. Increased grooming behavior reduces the survival of the mites outside the brood cell (s). The proportion of mites in the brood is a measure of the length of the reproductive cycle (phoretic period + 12.5 d postcapping period) (Otten 1991, Harbo 1996). Variation in the proportion of mites in brood may be explained by variation in the chemical signals that attract varroa mites to the last instar (Trouiller et al. 1993). A lower proportion of mites in the brood translates into a longer reproductive cycle (t) and fewer generations (n) during a field test. All of these effects translate into a reduced mite population at the end of a field trial. Probably the most resistant honey bees will incorporate several resistant characteristics into 1 stock.

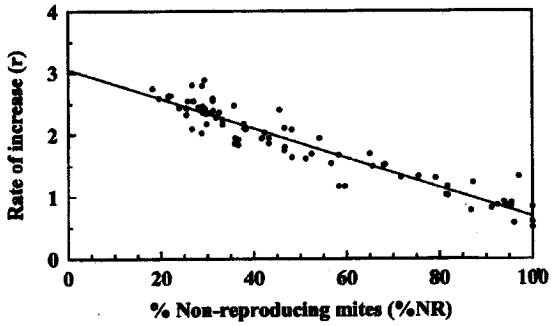

Fig. 5. The relationship between overall percentage of nonreproductive mites (% NR) in a colony and the average rate of increase (r) from each brood cell. The rate of increase (r) is defined as the number of adult females that leave a brood cell divided by the number of foundress mites that entered the cell. Both variables are measures of mite reproduction, but r can be related directly to mite population growth (see text). The equation for the line is $r = 3.053 - (0.0237 \times [\%\text{NR}])$ and the regression coefficient is $R^2 = 0.89$ ($n = 78$ colonies).

Acknowledgments

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