

## Honey bee hygienic behavior and defense against *Varroa jacobsoni*

Marla Spivak

Department of Entomology, 219 Hodson Hall, University of Minnesota, Saint Paul, MN 55108, USA

(Received 17 May 1996; accepted 14 August 1996)

**Summary** — Hygienic and non-hygienic colonies from 'Starline' stock of *Apis mellifera* were tested for their ability to remove pupae infested with *Varroa* mites. The hygienic and non-hygienic lines were selected and bred on the basis of their removal response to freeze-killed brood. A Jenter Box<sup>®</sup> was used to test whether they would remove experimentally infested pupae following methods of Boecking and Drescher (1992). In 1994, the hygienic colonies removed significantly more pupae infested with one mite per cell than the non-hygienic colonies. In 1995, there was no significant difference between the hygienic and non-hygienic colonies when one or two mites were introduced per pupa due to variation in response among hygienic colonies. There was no significant difference between the rate of removal of infested pupae from the Jenter Box and from natural wax comb by the hygienic colonies. The number of mites damaged by grooming ranged from 6.0 to 42.3% among all colonies. The reproductive success of the mites not removed from the cells by the bees was low in both hygienic and non-hygienic colonies.

*Apis mellifera* / *Varroa jacobsoni* / hygienic behavior / grooming / mite resistance / breeding

### INTRODUCTION

The ectoparasitic mite, *Varroa jacobsoni* Oudemans, is the most destructive pest of honey bees in the US and Europe. Because of the risks and disadvantages of using chemical treatments in mite-infested colonies (eg, Lodesani et al, 1992, 1995), it is important to determine if honey bees have any heritable defense mechanisms against the mite which may be readily incorporated into breeding programs.

A balanced host-parasite relationship has evolved between *V jacobsoni* and its

natural host, *Apis cerana*, in Asia. Most female *Varroa* are not able to reproduce successfully in worker brood of *A cerana*, therefore, the reproduction of the mites is limited to the seasonal cycle of drone production within the colony (Boecking and Ritter, 1994; reviewed in Büchler, 1994). In addition, *A cerana* has two behavioral defenses which help maintain the numbers of mites within tolerable limits. These defenses are grooming and removal (hygienic) behaviors.

In grooming, adult bees detect and remove phoretic mites from themselves

(auto-grooming) or from nestmates (allo-grooming) (Peng et al, 1987a). In the process, the legs of the mite may be cut off or the cuticle of the idiosoma may be damaged by the bees' mandibles, causing the damaged mite to fall to the bottom of the colony (Ruttner and Hänel, 1992). Successful grooming of mites has been demonstrated in *A cerana* (Peng et al, 1987a; Büchler et al, 1992; Fries et al, 1996). *A mellifera* of European origin also exhibits grooming behavior but to a lesser extent than *A cerana* (Peng et al, 1987a; Büchler et al, 1992; Büchler, 1994; Fries et al, 1996).

Removal behavior involves the ability of some bees to detect, uncap, and remove infested worker pupae from the cells. *A cerana* efficiently removes infested brood (Peng et al, 1987b; Rath and Drescher, 1990; Rosenkranz et al, 1993). The removal of infested pupae interrupts the reproduction of the fertile mites inside sealed brood cells. In addition, the immature mites are killed which decreases the average number of offspring per mother mite (Rath and Drescher, 1990; Fries et al, 1994).

*A mellifera* of European origin removes infested worker pupae, but to a limited extent compared to *A cerana* (reviewed in Boecking et al, 1993; Boecking and Ritter, 1994; Büchler, 1994). Africanized honey bees in Brazil remove significantly more mite-infested pupae than European bees in the same location (Guerra et al, submitted for publication). Boecking and Drescher (1992) found that the type of comb affected the rate of removal; more infested pupae were removed from plastic comb than from natural wax comb. They also found a positive correlation ( $r = 0.74$ ) between the removal of brood infested with two mites per cell and the removal of freeze-killed brood, a commonly used method to assay bee colonies for hygienic behavior (see *Methods*).

Hygienic behavior is considered the primary mechanism of resistance to at least two diseases of larval and pupal honey bees: American foulbrood caused by the bacterium

*Bacillus larvae* (Rothenbuhler, 1964) and chalkbrood caused by the fungus, *Ascosphaera apis* (Gilliam et al, 1983, 1988). Hygienic bees have the ability to detect, uncap, and remove diseased brood from the nest before the causative organisms reach the sporulating stage (Woodrow and Holst, 1942). Rothenbuhler (1964) postulated that hygienic behavior is controlled by two independently assorting, recessive genes – one for uncapping and one for removing diseased brood from the nest. In a re-evaluation of the two-locus model for hygienic behavior, however, Moritz (1988) found the model to be an oversimplification. He thought a multi-locus model or other more complex patterns of inheritance determined the expression of the phenotype. Rapid hygienic behavior occurs at a relatively low frequency in most honey bee populations thus far studied (Spivak and Gilliam, 1993).

A two-way selection program for hygienic behavior was initiated at the University of Minnesota in 1992 with the original goal of selecting colonies resistant to chalkbrood. Lines of hygienic and non-hygienic colonies were selected on the basis of their removal response to freeze-killed brood, an assay used by previous researchers to select colonies for this purpose (Gilliam et al, 1983, 1988). The present experiment tested whether the hygienic colonies from the breeding program would also remove pupae infested with *Varroa*. A comparison was made between the rate of removal of infested pupae within plastic-based cells and within natural wax comb. In addition, the degree of grooming behavior of the same colonies was determined.

## METHODS

### *Experimental bee colonies*

The hygienic and non-hygienic lines used in the experiment were bred from 'Starline' stock, derived from Italian *A mellifera ligustica*. The

degree of hygienic behavior in the colonies was determined by a freeze-killed brood assay in which the amount of time was recorded for bees to detect, uncap, and remove a 6 x 5.5 cm comb section containing freeze-killed pupae. Each section of pupae, containing approximately 100 pupae per side of the comb, was cut out and frozen at  $-20^{\circ}\text{C}$  for 24 h before it was placed in the colony to be tested. Previous experiments have shown that neither hygienic nor non-hygienic bees removed live pupae from similar size sections of comb which had been cut out and replaced (Spivak and Gilliam, 1993; Spivak, unpublished observations). Therefore, the time taken to remove freeze-killed brood by the colonies was considered a measure of the bees' ability to remove diseased or abnormal brood. Colonies that removed the freeze-killed brood within 48 h in two consecutive trials were considered hygienic; colonies that took longer than 1 week to remove the dead brood in both trials were considered non-hygienic (Taber and Gilliam, 1987; Spivak and Gilliam, 1993).

To establish and maintain the lines, queen bees were raised from colonies that displayed the most rapid and the least rapid removal rates. Each daughter queen was inseminated with 4–6  $\mu\text{L}$  of mixed semen drawn from multiple drones from either hygienic or non-hygienic colonies, with the exception of three hygienic queens which were raised in the spring of 1995 and were each inseminated with the semen of a single drone. All colonies were wintered outdoors and then tested again the following spring using the freeze-killed assay. The colonies with single-drone inseminated queens were assayed with freeze-killed brood in August 1995 when all the bees in the colonies were progeny of the introduced queens. Only the most hygienic and least hygienic colonies based on the second freeze-killed brood assays were used in the experiments to test whether the colonies would remove brood infested with *Varroa*.

In 1994, the experiments included four hygienic and three non-hygienic colonies, and in 1995 they included seven hygienic and four non-hygienic colonies. All colonies were treated with two Apistan<sup>®</sup> (flouvalinate) strips per colony the previous fall and were sampled for *Varroa* in the spring. No mites were detected in any of the colonies in the spring of 1994 or 1995 before the experiments began. However, as the season progressed, all colonies became reinfested with *Varroa* due to natural causes (drifting and robbing between colonies from surrounding apiaries). All

colonies were maintained in standard Langstroth equipment and had approximately 8–12 frames of brood when they were tested for removal of *Varroa* mites.

### **Removal of infested pupae**

A Jenter Box<sup>®</sup> (Brushy Mountain Bee Farm, Moravian Fall, NC, USA) was used to test whether the selected hygienic and non-hygienic colonies of bees would remove pupae experimentally infested with *Varroa* mites (following methods of Boecking and Drescher, 1992). This box contains approximately 300 plastic-based worker cells and fits into a standard brood frame. Ninety of the cells within the box have false bottoms fitted with removable plugs which allow one access to individual larvae or pupae through the base of the cell.

The inseminated queens in each experimental colony were confined until they had laid eggs in most of the cells of the Jenter Box (6–24 h). Eight or nine days later, *Varroa* mites were introduced through the plugs in the cells containing fifth-instar larvae. The cells containing these larvae had been sealed with wax within the last 6–8 h, before the fifth instar larvae had spun a cocoon and begun pupation. All mites were collected off adult workers and drones from one highly infested colony located in an apiary over 5 km away. The reproductive status of the mites at the time of collection and introduction was not known; however, care was taken to introduce mites that were fully pigmented. The mites were introduced into the cells using a fine, camel-hair paint brush following the methods of Boecking and Drescher (1992).

In 1994, one *Varroa* mite per cell was introduced into 10–20 cells containing fifth-instar larvae. Another group of cells serving as controls had the plugs removed and replaced with no mite introduction. The infested and control cells were marked on a transparent sheet of plastic (following Infantidis, 1983), and were inspected on days 1, 2, 4, 7, and 10 after infestation to determine if the bees had detected and removed the infested brood. In 1995, the same procedures were followed with the additional treatment of two mites per cell. On the tenth day of the experiment in 1995, or one day before the pupae were due to eclose as adults, all cells containing infested pupae that were not removed by the bees were opened to determine the reproduction of the remaining mites.

To test whether the hygienic colonies removed more infested pupae from the plastic-based cells of the Jenter Box than from natural wax cells, separate trials were conducted in which fifth-instar larvae in wax cells were infested with one or two mites per cell. The wax cappings of recently capped brood cells containing fifth-instar larvae were partially opened using a fine scalpel (following de Ruijter, 1987). After inserting the mite or mites with a paint brush, the cell cappings were carefully closed again. Control cells were opened and closed with no mite introduction. The infested brood cells and the control cells were marked on a transparent plastic sheet and were inspected as before.

### Grooming and mite counts

To determine natural mite mortality, a 'sticky board' (Dewill Inc, Varroa Mite Detector Insert, Elmhurst, IL, USA) covered with mesh screen was placed on the bottom board of the same colonies for a 10 day period in 1994 (in July), and for two 10 day periods in 1995 (July and August). The number of mites that fell to the bottom and adhered to the board was counted under a microscope. Of the total number of fallen mites, the percent that were damaged when adult bees groomed the mites off each other was counted only in 1995. The screen was elevated from the sticky board by a wooden frame to ensure that the bees could not reach any fallen mites. Therefore, all damaged mites collected on the sticky boards would have been damaged by the bees prior to falling on the board.

### Statistical analysis

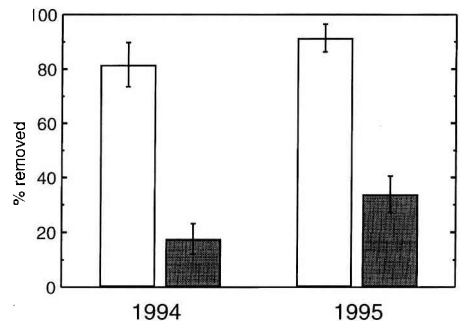
The differences in the results of the freeze-killed brood assays between the hygienic and non-hygienic colonies were analyzed using a Student's *t*-test for each year (Wilkinson, 1990–1992). The mean percentages of mite-infested and control pupae removed from the Jenter Box on day 10 of the experiment were analyzed using a split-plot two-way ANOVA on arcsine-transformed data for each year. The error term for bee type was colony (bee-type), and for the treatment effect was the residual error (SAS Institute, Release 6.10, 1995). The same analyses were used to compare the amount of infested brood removed

from natural wax comb in both years. Paired *t*-tests were used to compare the response of the hygienic colonies to infested brood in the plastic cells of the Jenter Box versus natural wax comb. Separate *t*-tests were used to compare the amount of infested pupae removed by day 10 for each treatment (one mite per cell, two mites per cell, and the controls) (Wilkinson, 1990–1992).

## RESULTS

### Freeze-killed brood assays

The results of the freeze-killed brood assays conducted before the mites were introduced into the colonies in 1994 and 1995 are presented in figure 1. In both years, the hygienic colonies removed significantly more dead brood than the non-hygienic colonies within 48 h ( $P = 0.001$  both years). In 1995, there was no difference between the rate of removal by colonies containing queens inseminated with the sperm of one or of many drones; therefore, the results from all hygienic colonies were pooled for the remainder of the analyses.

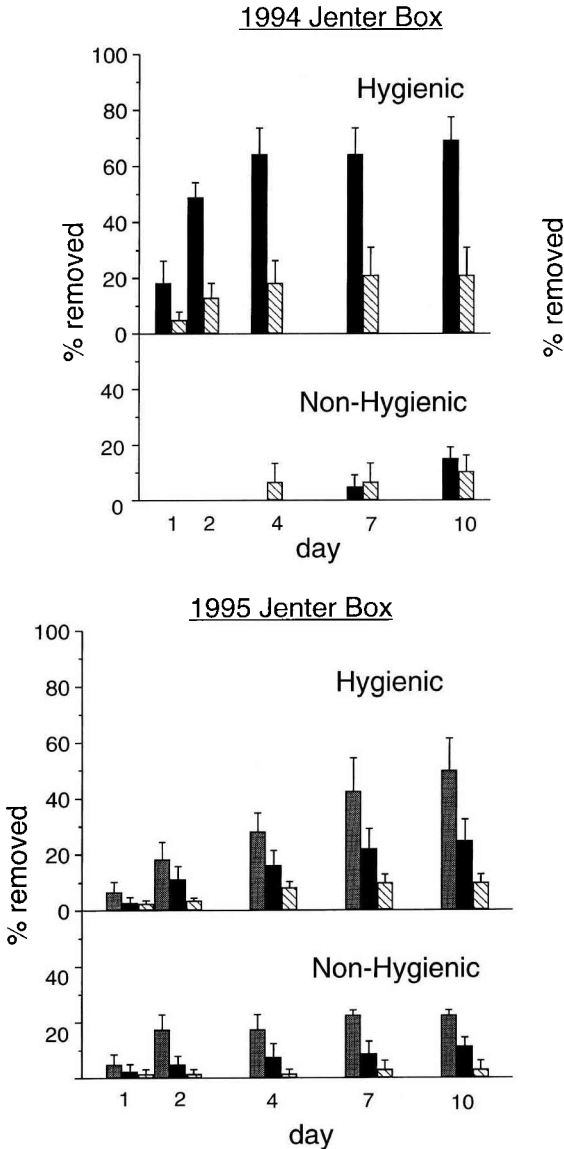


**Fig 1.** The mean ( $\pm$  SE) percent freeze-killed brood removed from the cells within 48 h by four hygienic and three non-hygienic colonies in 1994, and by seven hygienic and four non-hygienic colonies in 1995. Student's *t*-test, 1994:  $t = 6.53$ ,  $df = 5$ ,  $P = 0.001$ ; 1995:  $t = 6.65$ ,  $df = 6$ ,  $P = 0.001$ . Hygienic  $\square$ ; non-hygienic  $\blacksquare$ .

**Removal of mite-infested pupae**

The results of the assay for the ability of the hygienic and non-hygienic colonies to detect, uncap, and remove mite-infested pupae from the cells within the Jenter Box are given

in figure 2. The mean percent infested pupae removed by day 10 and results of statistical analysis are given in table I. In 1994, the effects of bee type (hygienic vs non-hygienic) and of treatment were significant. The four hygienic colonies removed



**Fig 2.** The mean percent removal of mite-infested pupae from the cells of the Jenter Box by the hygienic and non-hygienic colonies in 1994 and in 1995 on days 1, 2, 4, 7, and 10 after the mites were introduced. One or two mites per cell were introduced into 10–20 cells in each colony through the plug at the base of the cell. The controls were cells containing fifth-instar larvae from which the plug was removed and replaced with no mite introduction. Control  $\square$ ; one mite/cell  $\blacksquare$ ; two mites/cell  $\square$ .

**Table I.** Percent (mean  $\pm$  SD) of pupae removed by the bees from the Jenter Box by day 10 after introduction of one or two *Varroa* mites per pupa.

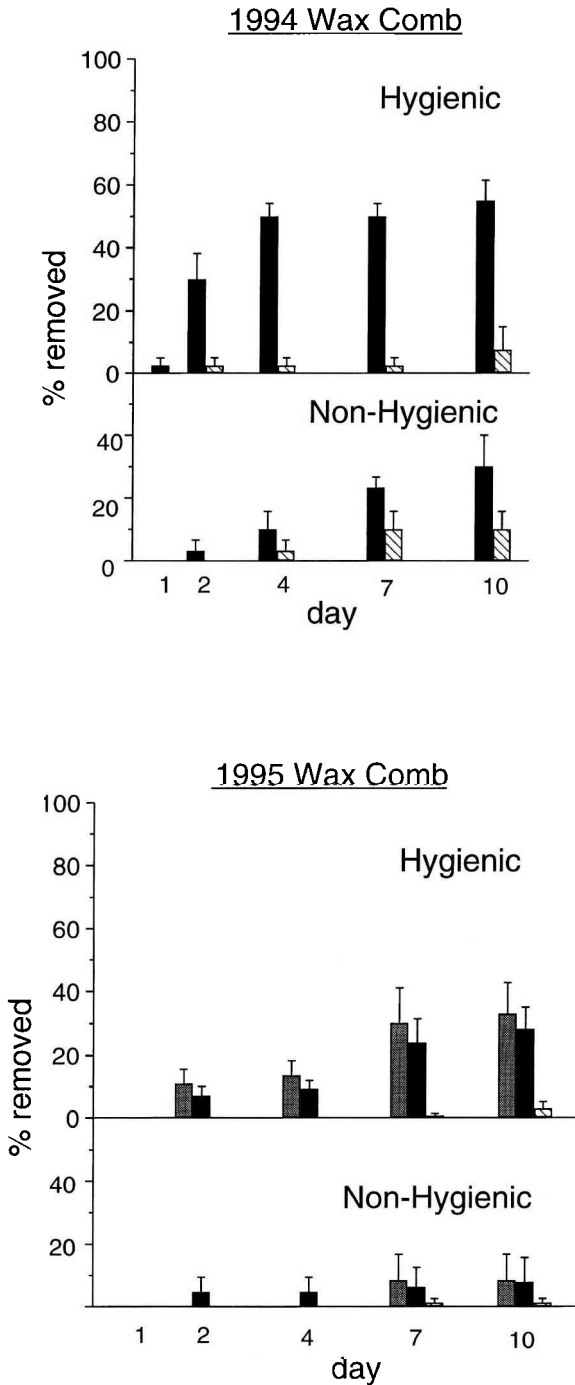
<i>Jenter Box</i> ( <i>plastic cell bases</i> )	1994	1995
<i>Hygienic</i>	<i>n</i> = 4	<i>n</i> = 7
Two mites	–	49.8 $\pm$ 30.49
One mite	69.2 $\pm$ 16.41	24.7 $\pm$ 20.06
Control	21.1 $\pm$ 19.92	9.9 $\pm$ 7.51
<i>Non-hygienic</i>	<i>n</i> = 3	<i>n</i> = 4
Two mites	–	22.5 $\pm$ 3.54
One mite	10.0 $\pm$ 10.00	11.3 $\pm$ 6.29
Control	10.4 $\pm$ 10.02	3.1 $\pm$ 6.25
Bee type	<i>F</i> = 45.87; <i>df</i> = 1.5; <i>P</i> = 0.001	<i>F</i> = 3.96; <i>df</i> = 1.9; <i>P</i> = 0.10
Treatment	<i>F</i> = 6.35; <i>df</i> = 1.5; <i>P</i> = 0.05	<i>F</i> = 9.03; <i>df</i> = 2.16; <i>P</i> = 0.002
Interaction bee type x treatment	<i>F</i> = 4.86; <i>df</i> = 1.5; <i>P</i> = 0.08	<i>F</i> = 0.00; <i>df</i> = 2.16; <i>P</i> = 1.00

Controls refer to cells in which the plug of the Jenter Box was removed and replaced without introducing a mite. The last rows show results of split-plot two-way ANOVA on arcsine-transformed data for each year's data.

**Table II.** Percent (mean  $\pm$  SD) of pupae removed by the bees from natural comb by day 10 after introduction of one or two mites per pupa through an opening made in the edge of the cell capping.

<i>Wax comb</i>	1994	1995
<i>Hygienic</i>	<i>n</i> = 4	<i>n</i> = 7
Two mites	–	33.0 $\pm$ 25.63
One mite	55.0 $\pm$ 12.91	28.2 $\pm$ 18.15
Control	7.5 $\pm$ 15.00	2.9 $\pm$ 5.67
<i>Non-hygienic</i>	<i>n</i> = 3	<i>n</i> = 4
Two mites	–	8.5 $\pm$ 12.02
One mite	30.0 $\pm$ 17.32	7.8 $\pm$ 15.63
Control	10.0 $\pm$ 10.00	1.3 $\pm$ 2.5
Bee type	<i>F</i> = 1.62; <i>df</i> = 1.5; <i>P</i> = 0.26	<i>F</i> = 3.61; <i>df</i> = 1.9; <i>P</i> = 0.09
Treatment	<i>F</i> = 43.84; <i>df</i> = 1.5; <i>P</i> = 0.001	<i>F</i> = 8.00; <i>df</i> = 2.16; <i>P</i> = 0.004
Interaction bee type x treatment	<i>F</i> = 6.90; <i>df</i> = 1.5; <i>P</i> = 0.05	<i>F</i> = 0.60; <i>df</i> = 2.16; <i>P</i> = 0.56

Controls refer to cells in which the plug of the Jenter Box was removed and replaced without introducing a mite. The last rows show results of split-plot two-way ANOVA on arcsine-transformed data for each year's data.



**Fig 3.** The mean percent removal of mite-infested pupae from the natural wax cells by the hygienic and non-hygienic colonies in 1994 and in 1995 on days 1, 2, 4, 7, and 10 after the mites were introduced. One or two mites per cell were introduced into 10–20 cells in each colony through an opening made in the wax capping over the cell. The controls were cells containing fifth-instar larvae from which the wax capping was opened and closed with no mite introduction. Control ▨; 1 mite/cell ■; 2 mites/cell □.

**Table III.** The fate of the mites that were introduced into the hygienic and non-hygienic colonies in 1995.

1995	Hygienic		Non-hygienic	
	Jenter Box	Natural comb	Jenter Box	Natural comb
No of mites introduced				
One mite/cell	138	134	50	56
a) % mites removed with pupae	25.4	29.9	10.0	8.9
b) % mites not found	7.3	6.7	8.0	8.9
No of mites recovered	93	85	41	46
c) % mites with offspring	17.2	27.1	7.3	37.0
d) % mite with no offspring	74.2	60.0	87.8	47.8
e) % mites dead	8.6	12.9	4.9	15.2

Only cases where one mite per cell was introduced are tabulated. All counts were made on day 10, or one day before the pupae eclosed. Of the total number of mites introduced into either the Jenter Box or natural comb, the percentage of mites is recorded that a) were removed by the bees along with the pupa; b) were not found in the cell with the pupae. In addition, of the mites that were recovered, the percentage of the mites is recorded that c) had at least one female offspring within the cell; d) remained alive within the cell but did not reproduce; and e) were found dead within the cell with the pupa.

significantly more pupae infested with one mite per cell by day 10 than the three non-hygienic colonies and the controls from both bee types. The same assay in 1995 yielded different results. There was no significant effect of bee type; the seven hygienic colonies did not remove significantly more infested pupae than the non-hygienic colonies. However, there was a significant treatment effect; significantly more pupae that were infested with two mites per cell were removed than cells infested with one mite per cell and the control cells (Tukeys test for mean separation:  $P < 0.05$ ). The bees removed the infested pupae at any time during the 10 day interval (fig 2), and

thus the detection of infested pupa did not appear to be limited to any particular stage in the development of the immature bee or in the reproductive stages of the mite.

There was considerable variation in the amount of infested brood removed by the seven hygienic colonies in 1995. Four of the hygienic colonies, one of which contained a single-drone inseminated queen, removed  $< 15\%$  of the infested pupae when one mite per cell was introduced. The remaining three removed  $45.5 \pm 6.46\%$  and  $69.6 \pm 26.69\%$  of the pupae when one and two mites per cell were introduced, respectively.

The removal of infested brood from natural wax comb by the hygienic and non-



hygienic colonies is shown in table II and figure 3. In both years, there was a high degree of variability in the response of the hygienic colonies and no statistical difference between the hygienic and non-hygienic colonies was found. There was a significant treatment effect in 1994 between the removal of brood infested with one mite per cell and the controls, and in 1995 between the removal of brood infested with two mites per cell and the controls (Tukey's mean separation  $< 0.05$ ).

The hygienic colonies did not remove significantly more infested or control pupae from the plastic-based cells of the Jenter Box than from natural wax comb by day 10 (all paired *t*-tests  $> 0.05$ ).

### **Mite reproductive success**

The reproductive success of the mites introduced into cells in the Jenter Box and natural wax comb in 1995 is shown in table III. Only the cells into which one mite was introduced were counted, as the mites' reproductive success decreases as more mites infest each cell (Moosbeckhofer et al, 1988; Fuchs and Langenbach, 1989; Eguaras et al, 1994; Fuchs, 1994). Fewer mites repro-

duced on pupae within cells of the Jenter Box than in natural wax cells in both the hygienic and non-hygienic colonies. Of the mites that successfully reproduced on pupae in wax cells, a higher percentage was observed in the non-hygienic colonies (37.0%) than in the hygienic colonies (27.1%).

When an introduced mite did not reproduce, it often deposited feces on the pupae rather than in the normal location on the cell wall. In the hygienic colonies, 87.1% of the non-reproductive mites introduced into the Jenter Box cells, and 50.1% introduced into natural wax cells deposited feces on the pupae. In the non-hygienic colonies, 82.1 and 86.4% of non-reproductive mites deposited feces on the pupae in cells of the Jenter Box and natural wax, respectively. Only in two cases (hygienic and non-hygienic colonies combined) did a successfully reproducing mite deposit feces on the pupae; both of these occurred within the Jenter Box.

### **Grooming behavior**

The percentage of mites that were damaged as a result of grooming in 1995 ranged from 6.0 to 42.3% in the seven hygienic colonies, and from 14.9 to 31.4% in the four non-

**Table IV.** Number of mites (mean  $\pm$  SD) that fell from natural causes and were collected on the sticky boards.

Year	Colony type	10 day natural mite fall		Chalkbrood mummies
		July	August	
1994	Hygienic	32.5 $\pm$ 16.84	—	0
	Non-hygienic	291.7 $\pm$ 468.83	—	1 395.3 $\pm$ 1 371.18
1995	Hygienic	19.7 $\pm$ 12.45	73.7 $\pm$ 53.68	0
	Non-hygienic	12.0 $\pm$ 12.36	80.3 $\pm$ 42.55	0

Mites that fell from natural causes were collected for one 10-day interval in July 1994 and for two 10-day intervals in July and August 1995. The number of chalkbrood mummies that were collected on the sticky boards during the 10-day experimental intervals was also counted.

hygienic colonies. The differences between the colony types were not significant ( $t$ -test:  $T = 0.591$ ;  $df = 9$ ;  $P = 0.57$ ). Of the three colonies headed by single-drone inseminated queens, one displayed the lowest number of damaged mites, and another the highest number. Most of the damage was to the mites' legs, and less often to the idiosoma.

### ***Colony infestations***

The numbers of mites that fell from natural mortality to the sticky boards during the experiment are given in table IV. In 1994, few mites but large numbers of chalkbrood mummies were collected on the sticky boards in two of the non-hygienic colonies. The third non-hygienic colony was highly infested with mites, but had a lower chalkbrood infestation, resulting in large standard deviations around the means (table IV). Inspection of the brood in these non-hygienic colonies indicated that the mites died along with the pupae that were infested with chalkbrood. Many of the chalkbrood mummies and dead mites remained within sealed cells. However, the bees removed some of the chalkbrood mummies from the cells, and the mummies which were not carried from the nest fell and adhered to sticky boards. No chalkbrood mummies were found in the hygienic colonies.

In 1995, sticky boards were placed in the colonies for 10 day intervals once in July and again in August to monitor natural mite fall. No chalkbrood infection was observed in either the hygienic or non-hygienic colonies, and no mummies were collected on the sticky boards. The number of mites increased dramatically in one of the single drone-inseminated hygienic colonies between July and August; concomitantly, the number of adult bees in the colony increased abnormally. It was suspected that an infested swarm entered this colony. The marked inseminated queen, however, was

not superseded. Over three times as many mites fell to the bottom boards in August than in July in the hygienic colonies (excluding the hygienic colony mentioned above). Over six times as many mites fell in August than in July in the non-hygienic colonies.

### **DISCUSSION**

Boecking and Drescher (1992) found a correlation between the removal of freeze-killed brood and removal of pupae experimentally infested with two mites per cell. They suggested that using the freeze-killed brood assay could facilitate the selection of colonies that would remove brood infested with *Varroa*. In the present study, colonies were selected first on the basis of their removal of freeze-killed brood and were tested subsequently for their removal of infested pupae. In 1994, the four hygienic colonies that removed freeze-killed brood within 48 h removed an average of 69.2% of the pupae infested with just one mite by day 10. In 1995, however, of the seven hygienic colonies that consistently removed freeze-killed brood within 48 h, only three removed an average of 45.5% of the pupae infested with one mite by day 10; the remaining four removed an average of 9.2% by day 10. (Preliminary data from 1996 indicated that some of the same hygienic colonies, headed by the same queens, that removed low numbers of infested pupae in 1995 removed significantly higher numbers in 1996. These results will be published at a later date.) The cues the bees used to detect and remove frozen pupae are not necessarily the same as those used to detect and remove mite-infested pupae. Because the non-hygienic colonies generally did not detect and remove significant amounts of infested pupae, the results of this experiment indicate that the freeze-killed brood assay is a useful screening procedure in

selecting colonies for their ability to remove pupae infested with *Varroa*.

Hygienic behavior is genetically determined. Previous studies, however, have shown that there is high degree of variability in the expression of the behavior. For example, lack of incoming nectar has been shown to reduce the hygienic response (Momot and Rothenbuhler, 1971). Weakened colonies (those with small populations) also display a reduced hygienic response (Boecking and Drescher, 1993; Spivak and Gilliam, 1993). The tests in 1994 and 1995 were conducted in July and August when all experimental colonies were collecting large amounts of nectar and pollen and were strong and populous.

Research on *A. cerana* indicated that mites introduced from foreign colonies (either intra- or inter-specific colonies) were removed more rapidly by the bees than mites collected from their own colony, but this was not the case with *A. mellifera* (Rosenkranz et al, 1993). In the present experiment, all mites were collected from one 'foreign' colony of *A. mellifera* each year, which controlled for the possibility that the mites could have acquired distinctive odors from the colonies in which they developed.

Boecking and Drescher (1992) reported that the type of comb influenced the removal response of the colonies. Pupae infested with mites in the Jenter Box with plastic cell bases, and in fully plastic ('ANP') comb were removed more quickly than were pupae in natural wax comb. The same trend was evident in the present study, but no statistically significant differences were detected between the removal of infested pupae from the Jenter Box and wax comb in either year.

The hygienic colonies removed more pupae from control cells than the non-hygienic colonies in both years (table 1), although this trend was not statistically different. The removal and replacement of the cell plug in the Jenter Box may have dis-

rupted the larva, providing a stimulus for the hygienic bees to uncap, inspect, and possibly remove the larva. Also, some pupae in controls cells may have been removed by the hygienic bees if they were infested naturally (Boecking and Drescher, 1991).

It is not known how the bees determine that a particular larva or pupa is infested with *Varroa* (Boecking and Drescher, 1992; Boecking and Drescher, 1994). Moreover, it is unclear how the type of comb (plastic or wax) might influence the bees' ability to detect mites within a wax-capped cell. It has been speculated that the bees use mechanical and olfactory cues to detect mites under the wax cell capping of the pupa. For example, Tewarson et al (1992) reported that *A. cerana indica* bees release the mites from infested cells without removing the pupae in some cases. These researchers speculated that a foreign object, such as a mite under a capped cell, may alter the behavior of the larva or pupa, which may be signaled by sound. Workers cue into the sound and open the cell. If the object has an alien scent, the pupa may be eaten or removed. If there is no alien scent, the cell may be recapped allowing the pupa to emerge normally (Tewarson et al, 1992). Rosenkranz et al (1993) demonstrated that the removal of mites by *A. cerana* depends on the alien scent adhering to the mite, but the response of *A. mellifera* to the odor of mites collected from different colonies was very low. Preliminary experiments indicated that hygienic colonies did not detect and remove an appreciable amount of pupae inoculated with frozen mites, mites preserved in alcohol, or dead mites collected from hive debris (Boecking and Drescher, 1994; M Spivak, unpublished data) which implies that the odor of the mite itself may not be an important cue to *A. mellifera*.

Bees may have a threshold response to the cues elicited by the mite or by the infested brood. If the colony is highly infested, the bees may cease to respond to

the cues and not remove the infested pupae which could explain why some hygienic colonies did not remove higher numbers of infested pupae in 1995. Also, it may not be advantageous for the bees to remove all infested worker pupae which could substantially reduce the adult population of the colony.

The mite counts in this study only estimate how many mites were in the phoretic stage, and do not include the number of mites reproducing within brood cells. The number of mites which fell to the sticky boards during the experiment may indicate mortality of newly emerged mites (Boot et al, 1995). To determine to what extent hygienic behavior actually reduces the mite load in a colony, it would be necessary to compare mite levels and mite reproductive success in colonies that were not manipulated and into which no mites were experimentally introduced.

Other factors in combination with hygienic behavior contribute to overall defense against *Varroa*. Grooming behavior is another defense, but there is no reason to expect that bees with genetic tendency to groom mites from adult bees would also be hygienic. The results in 1995 demonstrated that there was no difference between the hygienic and non-hygienic lines in the number of mites that were damaged by grooming. However, the hygienic colony that damaged the highest number of mites (42.3%) was also one that removed a relatively high number of infested pupae (52.6 and 58.8% when one and two mites per cell were introduced, respectively). It is possible, therefore, to select colonies that display both behaviors in breeding programs for bees that display defenses against *Varroa*.

Another factor which contributes to colony resistance to *Varroa* is the apparent inability of the mites to reproduce on worker pupae in *A. cerana* and some *A. mellifera* colonies (Camazine 1986; Ruttner and Hänel, 1992; Anderson, 1994; Fuchs, 1994;

Rosenkranz and Engels, 1994). In the present experiment, a low percentage of introduced mites reproduced successfully on the worker pupae, but this low fertility most likely was not due to colony resistance. Rather, the low reproductive success may have resulted from the handling of the mites during the experimental procedure, or the introduced mites may not have been reproductively mature. For example, previous studies have shown that young mother mites may not always reproduce in the first cell they enter, but may do so in subsequent cells (de Ruijter, 1987). Young mites that were not allowed sufficient time in the phoretic stage on adult bees before they were introduced may have delayed oviposition and lay fewer eggs (Beetsma and Zonneveld 1992; but see Boot et al, 1995). Also, the fertility of the mites decreased when they were introduced 24 h or more after the larval cell was sealed (Beetsma and Zonneveld, 1992). In all cases in the present experiment, however, care was taken to introduce the mites within 24 h of the cells being sealed; thus, the timing of mite introduction probably did not contribute to the observed lack of fertility.

It was noted that mites which did not reproduce on worker pupae deposited feces on the pupa itself rather than in the normal location on the upper cell wall. The placement of feces on the upper cell wall apparently serves as a 'rendezvous site' for the mother and her immature offspring within the cell (Donzé and Guerin, 1994) and so plays an important role in the life-cycle of the mite. In most cases, the non-reproductive mites deposited feces on the abdomen of the pupae (see also Ruttner et al, 1984, p 47), but in other cases, the feces was found scattered over the entire body of the pupa. The placement of the feces is probably a symptom of the mite not being ready or able to reproduce. However, this phenomenon warrants further investigation.

In conclusion, many factors seem to regulate the expression of hygienic behavior of honey bees and the removal of pupae infested with *Varroa*. Hygienic behavior has the potential to limit the population growth of *Varroa* in three ways: 1) the immature mites are killed when the pupa is removed, which decreases the average number of offspring per reproducing mite; 2) the phoretic period of adult female mites is extended of those mites that survive removal of the pupae; and 3) the mortality of the adult mites increases if they are damaged by the adult bees through grooming when they escape through the opened cell. However, the extent to which the behavior actually reduces the mite load within a colony remains to be studied.

## ACKNOWLEDGMENTS

I thank O Boecking for demonstrating his methods for introducing mites into the Jenter Box to me, and for his valuable comments on the experiments and manuscript. M Gilliam, G Richardson, and I Burns also provided helpful suggestions on the manuscript. Thanks to J Breyfogle, G Reuter, R Melton, and T Bruce for their help collecting data. The research was funded by beekeeping associations from Minnesota, Wisconsin, North Dakota, South Dakota and California, with matching funds from the Minnesota Agricultural Utilization Research Institute (AURI PRO-130) and the Almond Board of California. Special thanks to the Minnesota Honey Producers Association for their generous and continued support of this project.

**Résumé — Comportement hygiénique de l'abeille mellifère (*Apis mellifera*) et défense contre *Varroa jacobsoni*.** On a testé la capacité de colonies présentant un comportement hygiénique (colonies «hyg») et de colonies ne le présentant pas (colonies «non-hyg»), issues de la souche «Starline» d'*Apis mellifera*, à éliminer les nymphes infestées par l'acarien *Varroa*

*jacobsoni*. Les lignées «hyg» et «non-hyg» ont été sélectionnées et élevées sur la base de leur réponse à éliminer du couvain tué par le froid. Une boîte Jenter® a été utilisée pour tester l'élimination des nymphes infestées expérimentalement selon les méthodes de Boecking et Drescher (1992). Deux supports ont été utilisés : la boîte Jenter®, qui comporte des cellules avec une base en plastique, et le rayon en cire naturelle. On a comparé le taux d'élimination des nymphes infestées en fonction du support. On a aussi déterminé le succès reproducteur des varroas, le degré de comportement de toilettage et l'estimation de la charge des varroas dans les colonies. En 1994, au 10<sup>e</sup> jour après l'infestation, les quatre colonies «hyg» avaient éliminé significativement plus de nymphes infestées par un acarien/cellule ( $69,2 \pm 16,41$ ) que les colonies «non-hyg» ( $10,0 \pm 10,0$ ) et que les témoins des deux groupes. En 1995, les sept colonies «hyg» n'ont pas éliminé significativement plus de nymphes infestées ( $24,7 \pm 20,06$ ) que les quatre colonies «non-hyg» ( $11,3 \pm 6,29$ ), quand un varroa était introduit par cellule. Quatre de ces colonies «hyg» ont éliminé  $\leq 15$  % des nymphes infestées et les trois restantes  $45,5 \pm 6,46$ . Néanmoins, il y a eu plus de nymphes éliminées que dans les témoins quand il y avait deux acariens par cellule. Au 10<sup>e</sup> jour, chez les colonies «hyg», il n'y a pas eu de différence significative entre les deux types de supports dans l'élimination des nymphes infestées et des nymphes témoins (tous les tests *t* par paires  $> 0,05$ ). Le succès reproducteur des varroas, qui n'étaient pas éliminés avec les nymphes par les abeilles, a été bas. En 1995, sur l'ensemble des varroas introduits, le pourcentage de varroas avec une descendance a été de 17,2 % (cellules de la boîte Jenter) et de 27,1 % (cellules en cire naturelle) chez les colonies «hyg» et, respectivement, de 7,3 % et 37,0 % chez les colonies «non-hyg» (tableau III). Quand un varroa introduit ne se reproduisait pas, il

déposait souvent des fécès sur les nymphes plutôt que sur la paroi de la cellule comme il le fait normalement. Le pourcentage de varroas lésés par le toilettage, calculé d'après le nombre de varroas qui tombaient pendant l'expérience sur les planchers collants, a varié entre 6,0 et 42,3 % selon les colonies. Il n'y a pas eu de différence entre les colonies «hyg» et les «non-hyg» ( $p > 0,05$ ). Il n'y a pas eu de différence significative entre le nombre de varroas récoltés sur les planchers collants pendant l'expérience (tableau IV). D'autres expériences sont nécessaires pour déterminer dans quelles mesure le comportement hygiénique diminue la charge des acariens dans une colonie.

#### ***Apis mellifera* / *Varroa jacobsoni* / comportement hygiénique / sélection**

**Zusammenfassung — Hygienisches Verhalten der Honigbiene und Abwehr von *Varroa jacobsoni*.** Hygienische und nicht-hygienische Völker der Linie 'Starline' (*Apis mellifera*) wurden darauf untersucht, wie häufig sie von *Varroa* befallene Puppen entfernen. Die Zucht der hygienischen und nicht-hygienischen Stämme erfolgte auf Grundlage der Entfernung von durch Tiefgefrieren abgetöteter Brut. Um das Entfernen künstlich infizierter Puppen zu beobachten, wurde eine Jenterwabe entsprechend der Methode von Boecking und Drescher (1992) benutzt. Die Rate der Entfernung von befallenen Puppen aus den Plastikzellen der Jenterwabe wurde mit der aus Zellen von natürlichen Wachswaben verglichen. Der Fortpflanzungserfolg der Milben und der Grad des Putzverhaltens wurde bestimmt. Zusätzlich wurden Schätzungen über die Anzahl der Milben im Volk durchgeführt. Im Jahr 1994 entfernten die vier hygienischen Völker bis zum 10. Tag nach der Infektion signifikant häufiger von mehr als einer Milbe befallene Puppen ( $69,2\% \pm 16,41$ ) als die 3 nicht-hygi-

schien Völker ( $10,00 \pm 10,00$ ) und als die Kontrollvölker für beide Gruppen. Jedoch entfernten die 7 hygienischen Völker 1995 nicht signifikant mehr befallene Puppen als die 4 nicht-hygienischen Völker, wenn nur eine Milbe pro Zelle eingesetzt wurde (hygienisch  $24,7 \pm 20,6$ ; nicht-hygienisch  $11,3 \pm 6,29$ ). Vier dieser hygienischen Völker entfernten  $\leq 15\%$  der befallenen Puppen und die restlichen 3 Völker  $45,5\% \pm 6,46$ . Es wurden jedoch gegenüber den Kontrollen signifikant mehr Puppen entfernt, die mit 2 Milben infiziert waren. In den hygienischen Völkern wurden bis zum 10. Tag nicht signifikant mehr befallene bzw. Kontrollpuppen aus den Plastikzellen der Jenterwabe entfernt als aus Zellen der natürlichen Wachswaben (alle gepaarten  $t$ -Teste  $> 0,05\%$ ). Der Reproduktionserfolg der Milben, die nicht mit den Puppen entfernt wurden, war niedrig. 1995 betrug der Prozentsatz reproduzierender Milben, bezogen auf die insgesamt eingesetzten Milben in den hygienischen Völkern, 17,2% (Jenterwabe) und 27,1% (Wachswabe). In den nicht-hygienischen Völkern waren es 7,3% (Jenterwabe) und 37,0% (Wachswabe). Wenn eine eingesetzte Milbe keine Nachkommen hatte, setzte sie häufig die Fäkalien auf der Puppe statt wie normalerweise an der Zellwand ab. Der Prozentsatz der durch Putzen verletzte Milben wurde aus der Anzahl der Milben bestimmt, die während des Experiments auf die klebrigen Bodeneinlagen fielen. Er schwankte zwischen 6,0% und 42,3% bei allen Völkern. Zwischen dem Prozentsatz der verletzten Milben der hygienischen und der nicht-hygienischen Völker bestand kein Unterschied ( $P > 0,05\%$ ). Es gab während der Versuche keine signifikanten Unterschiede in der Anzahl der Milben, die auf der Bodeneinlage gesammelt wurden. Für die Bestimmung des Grades, in dem hygienisches Verhalten die Milbenanzahl in einem Volk reduziert, sind weitere Versuche notwendig.

## REFERENCES

- Anderson DL (1994) Non-reproduction of *Varroa jacobsoni* in *Apis mellifera* colonies in Papua New Guinea and Indonesia. *Apidologie* 25, 412-421
- Beetsma J, Zonneveld K (1992) Observations on the initiation and stimulation of oviposition of the *Varroa* mite. *Exp & Appl Acarol* 16, 303-312
- Boecking O, Drescher W (1991) Response of *Apis mellifera* L colonies to brood infested with *Varroa jacobsoni* Oud. *Apidologie* 22, 237-241
- Boecking O, Drescher W (1992) The removal response of *Apis mellifera* L colonies to brood in wax and plastic cells after artificial and natural infestation with *Varroa jacobsoni* Oud and to freeze-killed brood. *Exp & Appl Acarol* 16, 321-329
- Boecking O, Drescher W (1993) Preliminary data on the response of *Apis mellifera* to brood infested with *Varroa jacobsoni* and the effect of this resistance mechanism. In: *Asian Apiculture* (LJ Conner, T Rinderer, HA Sylvester, S Wongsiri) Wicwas Pres, Cheshire, UK, 454-462
- Boecking O, Drescher W (1994) Rating of signals that trigger *Apis mellifera* L bees to remove mite-infested brood. *Apidologie* 25, 460
- Boecking O, Ritter W (1994) Current status of behavioral tolerance of the honey bee *Apis mellifera* to the mite *Varroa jacobsoni*. *Am Bee J* 134, 689-694
- Boecking O, Rath W, Drescher W (1993) Grooming and removal behavior – Strategies of *Apis mellifera* and *Apis cerana* bees against *Varroa jacobsoni*. *Am Bee J* 133, 117-119
- Boot WJ, Calis JNM, Beetsma J (1995) Does time spent on adult bees affect reproductive success of *Varroa* mites? *Entomol Exp Appl* 75, 1-7
- Büchler R (1994) *Varroa* tolerance in honey bees – occurrence, characters and breeding. *Bee World* 75, 54-70
- Büchler R, Drescher W, Tournier I (1992) Grooming behaviour of *Apis cerana*, *Apis mellifera* and *Apis dorsata*, reacting to *Varroa jacobsoni* and *Tropilaelaps clareae*. *Exp & Appl Acarol* 16, 313-319
- Camazine S (1986) Differential reproduction of the mite, *Varroa jacobsoni* (Mesostigmata: Varroidae), on Africanized and European honey bees (Hymenoptera: Apidae). *Ann Entomol Soc Am* 79, 801-803
- de Ruijter A (1987) Reproduction of *Varroa jacobsoni* during successive cycles of the honeybee. *Apidologie* 18, 321-326
- Donzè G, Guerini PM (1994) Behavioral attributes and parental care of *Varroa* mites parasitizing honeybee brood. *Behav Ecol Sociobiol* 34, 305-319
- Eguaras M, Marcangeli J, Fernandez NA (1994) Influence of 'parasitic intensity' on *Varroa jacobsoni* Oud reproduction. *J Apic Res* 33, 155-159
- Fries I, Camazine S, Sneyd J (1994) Population dynamics of *Varroa jacobsoni*: a model and a review. *Bee World* 75, 5-28
- Fries, I, Huazhen, W, Wei, S, Jin, SC (1996) Grooming behavior and damaged mites (*Varroa jacobsoni*) in *Apis cerana cerana* and *Apis mellifera ligustica*. *Apidologie* 27, 3-11
- Fuchs S (1994) Non-reproducing *Varroa jacobsoni* Oud in honey bee worker cells – status of mites or effect of brood cells? *Exp & Appl Acarol* 18, 309-317
- Fuchs S, Langenbach K (1989) Multiple infestation of *Apis mellifera* L brood cells and reproduction in *Varroa jacobsoni* Oud. *Apidologie* 20, 257-266
- Gilliam M, Taber S III, Richardson GV (1983) Hygienic behavior of honey bees in relation to chalkbrood disease. *Apidologie* 14, 29-39
- Gilliam M, Taber S III, Lorenz BJ, Prest DB (1988) Factors affecting development of chalkbrood disease in colonies of honey bees, *Apis mellifera*, fed pollen contaminated with *Ascosphaera apis*. *J Invertebr Pathol* 52, 314-325
- Infantidis MD (1983) Ontogenesis of the mite *Varroa jacobsoni* in worker and drone honeybee brood cells. *J Apic Res* 22, 200-206
- Lodesani M, Pellacani A, Bergomi S, Carpana E, Rabitti T, Lasagni P (1992) Residue determination from some products used against *Varroa* infestation in bees. *Apidologie* 23, 257-272
- Lodesani M, Colombo M, Spreafico M (1995) Ineffectiveness of Apistan treatment against the mite *Varroa jacobsoni* Oud in several districts of Lombardy (Italy). *Apidologie* 26, 67-72
- Momot JP, Rothenbühler WC (1971) Behaviour genetics of nest cleaning in honeybees. VI. Interactions of and genotype of bees, and nectar flow. *J Apic Res* 10, 11-21
- Moosbeckhofer R, Fabsicz M, Kohlich A (1988) Untersuchungen über die Abhängigkeit der Nachkommensrate von *Varroa jacobsoni* Oud vom Befallsgrad der Bienenvölker. *Apidologie* 19, 181-207
- Moritz RFA (1988) A reevaluation of the two-locus model for hygienic behavior in honeybees (*Apis mellifera*). *J Hered* 79, 257-262
- Peng YS, Fang Y, Xu S, Ge L (1987a) The resistance mechanism of the Asian honey bee, *Apis cerana* Fabr, to an ectoparasitic mite *Varroa jacobsoni* Oudemans. *J Invertebr Pathol* 49, 54-60
- Peng YS, Fang Y, Xu S, Ge L, Nasr ME (1987b) Response of foster Asian honey bee (*Apis cerana* Fabr) colonies to the brood of European honey bee (*Apis mellifera* L) infested with parasitic mite *Varroa jacobsoni* Oudemans. *J Invertebr Pathol* 49, 259-264
- Rath W, Drescher W (1990) Response of *Apis cerana* Fabr towards brood infested with *Varroa jacobsoni* Oud and infestation rate of colonies in Thailand. *Apidologie* 21, 311-321
- Rosenkranz P, Engels W (1994) Infertility of *Varroa jacobsoni* females after invasion into *Apis mellifera*

- worker brood as a tolerance factor against varroasis. *Apidologie* 25, 402-411
- Rosenkranz P, Tewarson NC, Singh A, Engels W (1993) Differential hygienic behaviour towards *Varroa jacobsoni* in capped worker brood of *Apis cerana* depends on alien scent adhering to the mites. *J Apic Res* 32, 89-93
- Rothenbuhler W (1964) Behavior genetics of nest cleaning behavior in honeybees. I. Response of four inbred lines to disease killed brood. *Animal Behav* 12, 578-583
- Ruttner F, Marx H, Marx G (1984) Beobachtungen über eine mögliche Anpassung von *Varroa jacobsoni* an *Apis mellifera* L in Uruguay. *Apidologie* 15, 43-62
- Ruttner F, Hänel H (1992) Active defense against *Varroa* mites in Carniolan strains of honey bees. *Apidologie* 23, 173-187
- SAS Institute Inc (1995) Release 6.10, Cary, NC, SAS Institute, Inc
- Spivak M, Gilliam M (1993) Facultative expression of hygienic behaviour of honey bees in relation to disease resistance. *J Apic Res* 32, 147-157
- Taber S, Gilliam M (1987) Breeding honey bees for resistance to diseases. *Korean J Apic* 2, 15-20
- Tewarson NC, Singh A, Engels W (1992) Reproduction of *Varroa jacobsoni* in colonies of *Apis cerana indica* under natural and experimental conditions. *Apidologie* 23, 161-171
- Wilkinson L (1990-1992) SYSTAT: The System for Statistics. Evanston, IL, SYSTAT, Inc
- Woodrow AW, Holst EC (1942) Removal of diseased brood in colonies infected with American foulbrood. *Am Bee J* 83, 22-23