

ORIGINAL RESEARCH ARTICLE

Bees with Varroa Sensitive Hygiene preferentially remove mite infested pupae aged ≤ five days post capping

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Summary

Suppressed Mite Reproduction (SMR) is a trait of honey bees that provides resistance to *Varroa destructor.* The mechanism of resistance in SMR bees is the removal of infested pupae from capped brood, so a better name is VSH bees (acronym for Varroa Sensitive Hygiene). This study compared the removal of infested brood by VSH and control bees to determine whether VSH bees removed infested pupae of different ages at similar rates. A pair of infested combs containing all stages of pupae were transferred into each host colony (six VSH and six control colonies) for 40 hours. VSH bees removed significantly more (55%) infested cells (singly and multiply infested), than controls (13%). They removed significantly more (66%) singly infested pupae aged from one to five days post capping (cohort A) than did controls (16%). The two types did not differ in the removal of singly infested pupae aged five to 10 days post capping (cohort B) (5–22%). Many pupae were found in uncapped cells at the end of the test, and most of the uncapped pupae were infested with mites. None of the uncapped cells contained prepupae, the development stage occurring during the first three days post capping. Thus, removal of infested pupae may be triggered by stimuli in cells with pupae aged 3–5 days post capping.

Abejas con higiene sensible a la varroa retiran preferentemente pupa infestada de menos de cinco días tras la operculación

La supresión de la reproducción del ácaro (SMR) es un rasgo de las abejas que les proporciona resistencia ante *Varroa destructor*. El mecanismo de resistencia en abejas SMR consiste en retirar las pupas infestadas de la cría operculada, por lo que abejas VSH (siglas de higiene sensitiva a la varroa) es un término más adecuado. Este estudio comparó la retirada de cría infestada por abejas VSH y abejas control para determinar si las abejas VSH quitaban pupas infestadas de diferentes edades en proporciones similares. Un par de cuadros infestados que contenían pupas de todas las etapas fueron introducidos en colmenas anfitrionas (seis VSH y seis colmenas control) durante 40 horas. Las abejas VSH retiraron significativamente más pupas de celdas infestadas (55%) (única ó múltiple infestación) que las colmenas control (13%). Las abejas VSH eliminaron considerablemente más (66%) pupas maduras de infestación única de uno a cinco días tras la operculación (cohorte A) que las colmenas control (16%). Los dos tipos de abejas no presentaron diferencias en la retirada de pupas maduras entre los cinco y diez días tras la operculación (cohorte B) (5–22%). Muchas pupas fueron encontradas en celdas desoperculadas al final de la prueba, y la mayor parte de las pupas desoperculadas estaban infestadas por el ácaro. Ninguna de las celdas desoperculadas contuvo pre-pupas, etapa del desarrollo que trascurre durante los primeros tres días tras la operculación. Por lo tanto, la retirada de pupas infestadas se puede activar mediante el estímulo en celdas con pupas de entre 3–5 días tras la operculación.

Keywords: hygiene, varroa mites, resistance

Introduction

Honey bees (*Apis mellifera*) have been selectively bred for resistance to the population growth of *Varroa destructor* (Harbo and Harris, 2001). Selection was based on low percentages of reproductive mites capable of producing a mature daughter (Harris and Harbo, 2000). The primary mechanism of resistance in these Suppressed Mite Reproduction (SMR) bees is the removal of infested pupae from capped brood cells (Ibrahim and Spivak, 2004, 2006; Harbo and Harris, 2005), so it is recommended that the name be changed to VSH bees, which more accurately identifies the mechanism of resistance as Varroa Sensitive Hygiene (VSH).

VSH is probably similar to other forms of hygienic behavior that honey bees direct towards dead brood, brood infected with bacteria or fungi (reviewed by Boecking and Spivak, 1999), or brood infested with eggs or larvae of the small hive beetle (*Aethina tumida*) (Ellis *et al*., 2003, 2004; Neumann and Härtel, 2004) or larvae of the greater wax moth (*Galleria mellonella*) (Corréa-Marques and De Jong, 1998; Villegas and Villa, 2006). As with other forms of hygiene, VSH varies among different populations of honey bees, and various types of bees may have significant resistance to *V. destructor* as a result of their behavior (Boecking and Drescher, 1991, 1992; Spivak, 1996; Boecking and Spivak, 1999; Guzman-Novoa *et al*., 1999; Guerra *et al*., 2000; Flores *et al*., 2001; de Guzman *et al*., 2002).

Hygienic behavior is a multi-step process involving several bees (Rothenbuhler, 1964; Arathi *et al*., 2000). Generally, the affected brood is detected and the cell cap is removed so that the sick or dead brood can be eliminated from the brood cell. However, removal of the host from uncapped cells is not always the final outcome. Often uncapped cells are re-sealed without the host pupa being injured (Boecking and Spivak, 1999; Aumeier and Rosenkranz, 2001; Aumeier *et al*., 2000; Boecking *et al.,* 2000; Arathi *et al*., 2006; Villegas and Villa, 2006). In the case of *V. destructor*, sometimes the foundress mite escapes or is removed by bees before an uncapped pupa is re-sealed by the bees (Boecking *et al.,* 2000; Aumeier and Rosenkranz, 2001).

The removal of pupae that are infested by V. destructor from capped brood can be estimated from naturally infested combs that are exposed to bees for a week by comparing the prevalence of mites in capped brood cells that are sampled on a comb before and after exposure to the bees. The infestation rate in the final sample will be much lower than in the initial sample if hygienic bees preferentially remove mite infested pupae from capped brood cells. Although this procedure estimates the net loss of infested cells, it may not reveal changes in brood cells that occur during shorter periods. For example, uncapped brood cells containing whole or partially eaten pupae are often scattered throughout the capped brood area on a comb that is removed from a hygienic colony (Corrêa-Marques and De Jong, 1998; Villegas and Villa, 2006). These uncapped pupae represent snapshots of different processes that were occurring when the comb was taken from the bees. Some uncapped cells may be in the initial stages of detection, while others may be being resealed a day or so after being inspected by hygienic bees (Villegas and Villa, 2006).

The current study is an attempt to learn more about the uncapping and removal of mite infested pupae by VSH bees after a much shorter period than that used in previous studies. The specific objectives of this study were: 1. to compare the removal of mite infested brood after a very short period (40 hours) by VSH bees and commercial controls; 2. to decide which stages of infested pupae are most likely to be removed by VSH bees, and; 3. to describe the contents of uncapped cells that are found on combs at the end of the test. The 40-hour duration of the test was chosen for convenience, as it allowed combs to be put into colonies in the afternoon (e.g. 3:00 pm) of the first day and examined in the morning (7:00 am) at the end of the test.

Materials and Methods

Lines of bees

The experiment was conducted at the USDA, ARS Honey Bee Breeding, and Genetics and Physiology Laboratory in the autumn of 2005. The VSH bees were derived over many years of a program that initially focused on breeding from colonies that kept mite populations low in standardized field tests (Harbo and Hoopingarner, 1997). The most important characteristic of the selected lines of bees was a low percentage of reproductive mites that could produce a mature daughter, which was measured six to eight weeks after establishing uniform colonies (Harbo and Harris, 1999). This characteristic responded well to selection, and colonies with high levels of the VSH trait are now characterized by high percentages of infertile mites that do not lay eggs (Harris and Harbo, 1999; Harbo and Harris, 2005). VSH queens in this study were produced by instrumentally inseminating queens of various VSH lines with semen from three to five drones from other VSH lines. Queens used in control colonies were either Italian purchased from a commercial queen producer, or daughters of commercial queens that were instrumentally inseminated with semen from drones of other Italian queens.

Removal of mite-infested brood by VSH and control bees

Hygienic responses of VSH and control bees towards mite infested pupae were investigated by transferring two combs of capped brood from a mite infested source colony (12 different sources) into each of 12 host colonies (six VSH and six control) and monitoring hygienic responses that occurred during a 40-hour period. Each host received a pair of combs from a single source. One comb of each pair contained predominantly young capped brood with all stages from prepupae (Pre) to purple eyed pupae with white bodies (PRW), whilst the second consisted mainly of older brood stages from purple eyed pupae with tanned joints (PRT) to teneral adult bees (Ten). All combs were in standard Langstroth deep frames. Combs were chosen only if the area of capped brood on each side exceeded 50 % of the total area on a side.

Each host colony consisted of six or seven combs of adult bees within a single deep 10 frame Langstroth hive body. Most colonies had three or four combs of capped brood just prior to the experiment. Two combs of capped brood were removed from each colony before transferring the pair of mite infested

combs into the brood nest. This was done in order to avoid drastic changes in the existing brood: bee ratio within each colony. The queen in each colony remained free running during the test. For logistical reasons, four to five host colonies were tested at two to three day intervals until sampling of the last colonies was finished (eight days to complete the experiment).

The initial infestation rate (the sum of multiply and singly infested cells) was found by sampling 200–300 capped brood cells in straight line transects on each side (at least 50 cells per comb side) for each comb of a pair. The initial infestation averaged 26 ± 8 % (mean ± SD) for all 12 pairs of combs that were used in this test (range, 11–43 %). The infestation rate after the test was estimated in the same way. The ratio of the number of infested pupae to the number of uninfested pupae was compared between the initial and final samples to calculate the percentage decrease of all mite infested pupae from each pair of combs.

Removal of mite-infested pupae at different stages of development

The percentage decrease in mite infested pupae was also estimated for each of two mutually exclusive cohorts of pupae. Each cohort was a subset of the larger pool of pupae that were examined to determine the infestation rate for pairs of combs at the beginning and end of the test. Only singly infested cells were considered in the study of cohorts. Cohorts were defined by body color and eye pigmentation, associated with the metamorphic development of worker honey bees (Jay 1962, 1963; Martin 1994). The developmental stages within the two cohorts were chosen so that the interval spanned by the two groups was about the same and so that the oldest stage in the second cohort would not have emerged during the 40 hour testing period. The oldest stage of pupa that was considered in cohort B at the start of the test had to be about 10 days post capping. To provide a bit of a cushion, the oldest stage in cohort B was assigned to the pupal stage with even tan coloration and white wing pads (TW), which under ideal conditions occurs at eight to ten days post capping. At the start of the test, the cohorts were divided evenly for the nine day period that spans the beginning of the prepupal (Pre) stage to the end of the TW stage, which gives each cohort about a 4.5 day period. Thus, at the start of the test, the young cohort (A) consisted of prepupae (Pre), pupae with non-pigmented eyes and white bodies (WE), and pupae with lightly pink eyes and white bodies (PKE). The older cohort (B) consisted of pupae with purple eyes and white body without any tanning (PRW), pupae with purple eyes and tanned joints of the antennae and legs (PRT), and pupae with evenly tan body coloration and white wing pads (TW).

The final infestation rate for each cohort was determined by examining pupae with morphological characteristics expected after 40 hours of development from the start of the test. For example, after 40 hours (1.7 days), cohort A included prepupae (Pre), pupae with non-pigmented eyes and white bodies (WE), pupae with lightly pink eyes and white bodies (PKE), and pupae with purple eyes and white bodies with no tanning of joints (PRW). Similarly, at the end of the test, cohort B included pupae with purple eyes and tanned appendages (PRT), pupae with an evenly tan body and white wing pads (TW), pupae with tan body and grey wing pads (TG), pupae with blackened head and thorax (BLK), and teneral adult bees (Ten).

Contents of uncapped cells

All cells with uncapped pupae were examined for evidence of infestation by *V. destructor* (adult mites, mite offspring, or mite feces).

Statistical Analyses

Differences in the percentage decrease in: 1. the overall number of mite-infested cells and; 2. the number of infested cells within each cohort (A and B) were compared between the two types of bees (VSH and control) using Analysis of Variance with type of bee as a fixed effect (SAS Institute 2000). The same model was used to analyze the numbers of uncapped cells and the percentage of uncapped cells infested by *V. destructor.*

Results

Removal of mite-infested brood by VSH and control bees

The percentage decrease in the proportion of infested cells (singly and multiply infested) after a 40 hour exposure to bees was significantly higher for combs with VSH bees than for those with controls ($F=13.6$; $df=1, 10$; $P=0.004$) (Table 1). Pairs of combs kept with VSH bees lost 55% of all mite infested cells, while combs with control bees lost 13% of all infested cells (Table 1). The differences between the two types of bee was primarily caused by significant differences in the decrease in proportion of singly infested cells ($F=10.8$; $df=1, 10$; $P=0.008$). The two types of bee did not differ in the decrease of the proportion of multiply infested cells ($F=4.2$; $df=1, 10$; $P=0.07$).

Removal of mite-infested pupae of different stages

The percentage decrease in the proportion of singly infested cells within cohort A after 40 hours was significantly higher in VSH bees than in the controls ($F=12.4$; $df=1, 10$; $P=0.006$). Combs with VSH bees lost 66% of infested cells in cohort A, whilst those with controls lost 16% of the infested cells in cohort A (Table 2). The two types of bee did not differ in the percentage decrease (5–22%) in singly infested cells for cohort B after 40 hours ($F=0.9$; $df=1, 10$; $P=0.36$) (Table 2).

Contents of uncapped cells

Pairs of combs with VSH bees had significantly more uncapped cells than pairs with control bees ($F=14.9$; $df=1, 10$; $P=0.003$). About 68 ± 32 (mean ± SD) uncapped cells were found on combs that were with VSH bees (n=6 pairs), whilst 13 ± 15 uncapped cells were found on combs with control bees (n=6 pairs) during the test. None of the uncapped cells contained prepupae (Pre), and the most commonly encountered stages of pupae in uncapped cells were WE, PKE and PRW.

The two types of bee did not differ in the percentage of uncapped pupae that were infested by *V. destructor* ($F=1.2$; $df=1, 8$; $P=0.29$). About 75 ± 16 % (mean ± SD) of uncapped cells from combs housed in control colonies (n=4 pairs) were infested by mites (only four pairs of combs were included because there were no uncapped cells from the other two pairs of combs). Similarly, 62 ± 20 % of uncapped cells from combs kept with VSH bees (n=6 pairs) were infested by mites.

No eggs or larvae of the small hive beetle (*Aethina tumida*) were encountered in this study. About 2–5% of uncapped cells had larvae of the greater wax moth (*Galleria mellonella*), but the prevalence of these larvae in uncapped cells was not significantly different between combs that were kept with the two types of bee.

Table 1. Percentage decrease in the numbers of infested cells (sum of singly and multiply infested) after pairs of mite infested brood combs were given to two types of colonies of honey bees. Each pair of combs consisted of a frame of young capped brood and a frame of older capped brood from the same source colony. Each pair was placed in the center of the brood nest of a host colony for 40 hours. All data are means ± SD for pairs of combs. Combs kept with VSH bees lost significantly more mite infested pupae than those kept with controls (α=0.05). The percentages of singly- or multiply-infested cells were not significantly different between controls and VSH bees at the start or end of the test.

Type of Bees (no. of pairs)	Time (hrs)	% Singly Infested Cells	% Multiply Infested Cells	Total Cells Examined	% Decrease in Infested Cells
control (n=6)	0	20 ± 6	6 ± 5	257 ± 31	13 ± 25
	40	18 ± 8	4 ± 2	244 ± 86	
VSH (n=6)	0	21 ± 4	6 ± 2	238 ± 42	55 ± 12
	40	12 ± 3	2 ± 1	292 ± 50	

Table 2. Changes in the number of infested brood cells for two cohorts of immature host bees. At the start of the test, the first cohort (A) included Pre, WE and PKE stages; the second (B) included PRW, PRT and TW stages. Only singly infested cells were analyzed. Combs with VSH bees lost significantly more infested brood cells from cohort A than did controls, but the two types of bees did not differ in reduction of infested pupae from the older cohort B (α=0.05). All data are means ± SD for pairs of combs.

		Cohort A			Cohort B		
Type of Bees (no. of pairs)	Time (hrs)	% Infested Cells	No. Cells	% Decrease	% Infested Cells	No. Cells	% Decrease
control (n=6)	0	24 ± 9	76 ± 37	16 ± 31	20 ± 8	90 ± 19	5 ± 33
	40	20 ± 9	132 ± 46		19 ± 10	104 ± 48	
VSH (n=6)	0	26 ± 9	87 ± 43	66 ± 16	19 ± 6	88 ± 18	22 ± 28
	40	11 ± 5	143 ± 57		14 ± 3	144 ± 27	

Discussion

These results suggest that VSH bees hygienically removed more mite-infested pupae from capped brood cells than did the commercial controls. Hygienic removal of mite infested brood was inferred because the overall infestation rate dropped by 55 % in combs kept with VSH bees and 13 % in combs kept with control bees (Table 1). The reduction in the number of infested pupae in combs housed with VSH bees is similar to the results of previous studies (Harbo and Harris, 2005; Ibrahim and Spivak, 2006).

VSH bees showed a stage preference in removal of mite-infested pupae. They removed a significantly greater proportion of mite infested pupae from a younger cohort (ca. one to five days post capping at the start) of host pupae than did control bees (Table 2). The two types of bee did however, remove a similar and smaller proportion of mite infested cells from an older cohort of pupae (*ca.* five to 10 days post capping at the start) (Table 2). It thus appears that stimuli eliciting the maximal hygienic removal of mite infested brood by VSH bees were associated with hosts aged less than or equal to five days post capping.

These results are similar to other studies in which the maximal removal of varroa mites was reached a few days after transferring mites into brood cells that contained young prepupae (Boecking and Drescher, 1992; Guerra *et al*., 2000; Aumeier *et al*., 2000; Aumeier and Rosenkranz, 2001). Other studies showed maximal removal of mite-infested pupae only after host pupae were four to seven days post capping (Corrêa-Marques and De Jong, 1998; Boot *et al*., 1999; Vandame *et al*., 2002).

It appears that honey bees do not immediately respond to newly infested cells, which implies that hygienic bees may not respond to foundress mites. Aumeier and Rosenkranz (2001) found that odors and movements of invading foundress mite are not the major stimuli for hygienic removal of mite infested brood by Africanized and Carniolan bees. These results were contrary to a previous study in which odors associated with mites removed from colonies of *Apis mellifera* were shown to increase the hygienic responses of bees in colonies of *Apis cerana* (Rosenkranz *et al*., 1993). Results from the two studies may not be contradictory if the sensitivity to mite related odors is much higher in *A. cerana* than in the two races of *A. mellifera.*

Various hypotheses have been proposed to explain why hygienic bees respond to infested cells only after a few days of natural cell invasion or the artificial introduction of mites. These explanations suggest that the stimuli triggering hygiene may be derived from time dependent processes such as the development of stress responses in infested pupae such as pheromone release or increased movement, the development of bacterial and / or viral diseases vectored by mites to the host pupa, and the development of the mite family, such as odors associated with offspring, or fecal accumulation reaching a detectable size (Boot *et al*., 1999; Boecking and Spivak, 1999; Vandame *et al*., 2002; Nazzi *et al*., 2004). VSH bees seem to preferentially remove mite infested pupae with foundress mites that produce families (Harbo and Harris, 2005), which supports the ideas that Varroa Sensitive Hygiene is related to development of the mite family. The current study cannot eliminate other possibilities, but whatever the stimulus, it must be present in a cell from one to five days post capping. Since no prepupae were found in uncapped cells, this time range can probably be narrowed to an interval of three to five days post capping, which corresponds to oviposition of the first few mite eggs (Martin, 1994).

The rate of mite infestation for uncapped cells often exceeds the natural infestation rate for the comb, which indicates that hygienic bees target mite infested cells (Corrêa-Marques and De Jong 1998; Villegas and Villa, 2006). In this study, VSH bees uncapped significantly more brood cells (410 cells from six pairs of combs) than control bees (76 cells from six pairs of combs). The mite infestation rate for uncapped cells for both types of bees exceeded the initial infestation rate (26%) measured using random transects, indicating that hygienic bees in both types of colonies preferred to uncap cells that were infested by mites. The mite infestation of uncapped cells was not significantly different between the two types of bee. About 75% (57) of the uncapped cells from control colonies (n=4 pairs) were infested, whilst 58% (254) of the uncapped cells from VSH colonies (n=6 pairs) were infested.

The major differences between VSH bees and the controls were the greater number of pupae uncapped and removed by the VSH bees. In another study, VSH bees also uncapped and removed significantly more mite infested pupae than Minnesota hygienic bees (Ibrahim and Spivak, 2006), which had been selectively bred for hygienic removal of freeze killed brood (Spivak, 1996). These results suggest that VSH bees may have a greater olfactory sensitivity to the cues that elicit removal of mite infested pupae from brood cells, than do the Minnesota hygienic bees (Spivak *et al*., 2003), or they respond faster and direct hygiene to more infested cells in a given time.

An alternative explanation is that the physiological sensitivity to odors is similar between VSH and control bees, but the two types of bee differ in the density of mites tolerated by the colony. In the case of Africanized bees, Vandame *et al*. (2002) suggested that the infestation rate of brood may need to reach a critical threshold before hygienic behavior is initiated by bees resistant to *V. destructor*. At very low mite densities, no differences in hygienic behavior or the growth rate of mite populations were found between resistant (hygienic) Africanized bees and non-resistant European honey bees (Vandame *et al*., 2000). VSH bees may, therefore, have a lower behavioral threshold to mite density, which is not related to a greater olfactory sensitivity to odors.

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