Honey Bees (Hymenoptera: Apidae) With the Trait of Varroa Sensitive Hygiene Remove Brood With All Reproductive Stages of Varroa Mites (Mesostigmata: Varroidae)

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ABSTRACT Varroa sensitive hygiene (VSH) is a trait of honey bees, *Apis mellifera* L. (Hymenoptera: Apidae), which supports resistance to *Varroa destructor* Anderson & Trueman. VSH is the hygienic removal of mite-infested pupa. Bees selectively bred for VSH produce colonies in which the fertility of mites decreases over time. In addition, mite fertility decreases after infested brood is exposed to VSH bees for 1 wk. The purpose of this study was to decide whether the reduction in mite fertility is caused by selective removal of mites that produce offspring. Initially, we monitored changes in a small patch of capped brood during exposure to VSH bees at 2-h intervals through 60 h, which provided a reference for the subsequent experiment. The first test showed that VSH bees uncapped, recapped, and began to remove many pupae in ≈2 h. The approach in the second experiment was to compare the percentage of fertile mites from brood exposed to VSH bees for a 3-h period to the percentage of fertile mites in brood that was protected from hygiene by a screen. There were no significant differences in fertility between mites on pupae that were being removed by the bees and mites on protected pupae. These results suggest that neither egg-laying by foundress mites nor mite offspring are the stimuli that trigger hygienic removal of mite-infested pupae by VSH bees. It may be that hygienic activities such as the uncapping of brood cells inhibits or disrupts reproduction by varroa mites.

KEY WORDS honey bees, varroa, varroa sensitive hygiene, hygiene

Honey bees, *Apis mellifera* L. (Hymenoptera: Apidae), use hygiene (removal of dead or injured brood) as a primary defense against brood diseases, parasites of brood and some nest invaders. Selective breeding can enhance the levels of hygiene in colonies of bees and provide significant resistance to some of these conditions (Rothenbuhler 1964, Spivak and Gilliam 1993, Boecking and Spivak 1999, Spivak and Reuter 2001). Honey bees bred for low growth of varroa populations hygienically remove mite-infested pupae from capped brood (Harbo and Harris 2005, Ibrahim and Spivak 2006), which is varroa sensitive hygiene (VSH) (Harris 2007).

The fertility of varroa mites decreases in colonies beginning several weeks after introduction of VSH queens (Harris and Harbo 1999, 2000). This characteristic initially led to the name of suppression of mite reproduction (SMR) for this trait of honey bees (Harbo and Harris 1999). The fertility of mites also decreases when brood is exposed to VSH bees for 1 wk (Harbo and Harris 2005, 2009; Villa et al. 2009). Preferential hygienic removal of pupae that are infested by mites with offspring may explain the reduced fertility of mites (Harbo and Harris 2005, 2009). However, although the percentage of fertile mites correlated with decreases in infestation (Harbo and Harris 2009), there has been no direct evidence linking hygienic removal of mite-infested pupae to the fertility of the resident foundress mite. Therefore, other aspects of hygiene may reduce fertility of mites. For example, hygienic bees often uncap and then recap brood cells containing mite-infested pupae (Boecking and Drescher 1994, Boecking and Spivak 1999, Aumeier et al. 2000, Boecking et al. 2000, Aumeier and Rosenkranz 2001, Arathi et al. 2006, Villegas and Villa 2006, Harris 2008). This could inhibit reproduction by otherwise fertile mites, making them seem infertile.

Quite often uncapped and chewed pupae can be found on brood combs from hygienic colonies, and many of these pupae are infested with varroa (Corréa-Marques and De Jong 1998, Villegas and Villa 2006, Harris 2008, Villa et al. 2009). The objective of this study was to determine whether fertile mites are preferentially targeted and removed by VSH bees or whether low mite fertility results from other processes related to hygienic behavior. Answers to this question may influence how bee breeders select for the VSH trait. Specifically, the degree of reduction in brood infestation rate after exposure of mite-infested brood to bees could be the major selection criterion if strong

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varroa resistance does not depend on changes in levels of mite fertility that occur after exposure to VSH bees.

The approach was to compare fertility between mites from pupae that were being chewed after a short exposure to VSH bees and mites from pupae on the same comb that were protected from the bees. A short exposure allowed hygienic bees time to find and begin removing mite-infested pupae but not enough time to completely remove pupae or mite offspring. Thus, the reproductive status of the infesting mite on each pupa being targeted for removal by bees could be evaluated.

Materials and Methods

Lines of Bees. VSH bees have been selectively bred over many years at our laboratory (Harbo and Hoopingarner 1997). VSH queens in this study were produced by instrumentally inseminating queens from various VSH lines with semen from 8 to 10 drones from other VSH lines. Queens for control colonies were either Italian stock purchased from a commercial queen producer, or daughters of commercial Italian queens that were instrumentally inseminated with semen from 8 to 10 drones from other Italian queens. Hybrid VSH colonies were either from naturally mated daughter queens of pure VSH mothers, or VSH queens instrumentally inseminated with 8 to 10 drones from commercial Italian queens.

Time Course of VSH. To find an exposure period long enough to allow VSH bees to chew targeted pupae but short enough to avoid their complete removal, we monitored the hygienic activity directed to a small patch of naturally infested brood at 2-h intervals through 60 h. An entire comb (Langstroth deep) of capped brood in the prepupal stage was placed into a pure VSH colony, but only a section of 306 capped brood cells was monitored. Another section containing 253 capped prepupae on the same comb was protected from hygienic behavior by a push-in wire screen (≈10 by 15 cm with 2-mm mesh).

The exposed brood was photographed at the beginning and thereafter at 2-h intervals. All digital color photographs were sized and cropped to the same scale. Sizing the images was made easier by four reference pins (with large white heads) that had been pressed into comb at the corners of the rectangular section of brood. All photos were made at the same distance from the camera and with the same lighting. Photographs were converted to black and white images in bitmap format on a computer. The baseline photograph was subtracted from each subsequent photograph using Scion Imaging software for Windows (Scion Corporation, Frederick, MD; www.scioncorp.com). The difference between any two photographs resulted in a new image in which brood cells that were similar between them were converted to a neutral gray color. Removal of a capped pupa (and sometimes the uncapping of a pupa) resulted in a white color in the new image. Each difference image was printed and served as a map for finding manipulated pupae when conducting microscopic exams of uncapped pupae. All chewed pupae were carefully removed from brood cells, and the presence or absence of varroa mites and mite offspring was recorded. All other uncapped pupae were not touched but were examined for the presence of mites, mite offspring, or mite feces. The comb was returned to the VSH colony after each examination, which typically lasted ≈15 min.

At the end of 60 h, all capped pupae from the exposed and protected sections were examined for the presence of varroa mites and mite offspring. In addition, all cell caps were carefully examined for integrity of the silk and the presence of granular wax patches of holes that were made by hygienic bees when they inspected the brood (Boecking et al. 2000). Caps were categorized as normal (silk lining of cap was complete) or recapped (an obvious hole patched with granular wax). The infestation rate, percentage of fertile mites, and the percentage of recapped cells were determined for the exposed and protected brood at the end.

Mite Reproduction and Chewed Pupae after 3-h Exposures to VSH Bees. Hygienic responses to mite-infested brood were compared between well established control ($n = 12$), hybrid VSH ($n = 7$), and pure VSH ($n = 8$) colonies at the end of August through September 2008 in Baton Rouge, LA. All colonies were reduced to four to five combs of bees and one to two combs capped brood (Langstroth deep frames) before the experiment began. A colony was tested by inserting a naturally mite-infested comb into the center of the nest and examining the contents of uncapped pupae after 3 h. We chose an exposure time slightly greater than in the time course experiment to be a compromise between opposing factors. A longer period allowed hygienic bees to manipulate more mite-infested brood, but it probably permitted a few pupae to be completely removed before the contents of their brood cells could be examined.

In total, 52 combs were tested in 27 colonies. Most colonies (25) were tested twice, but two were tested only once because their queens superseded before a second test. Trials for each colony were separated by at least 1 wk. The combs of mite-infested brood were harvested from 20 unrelated source colonies.

Each comb had 2,170 ± 130 capped white- to pink-eyed pupae (mean ± SE, $n = 52$), as estimated using a grid to measure area and converting using 24 brood cells per square inch. Half of these were protected from hygiene by placing a 2-mm mesh screen over them and stapling it to the wooden frame. Any uncapped pupae were removed from the exposed brood before a comb was transferred into a colony.

After 3 h, the condition of each uncapped pupa (chewed or not) and the presence of mites and mite offspring in their brood cells were recorded during microscopic examination of the comb. A sample of 200 capped pupae from the protected brood was examined along straight-line transects for mites and mite offspring. Another 200 capped pupae from the exposed brood were examined to verify that the infestation rate of exposed brood was similar to the protected brood.

The percentage of all mite-infested pupae in exposed brood that was uncapped by the bees was estimated by dividing the total number of mite-infested pupae that were uncapped by the total mite-infested pupae estimated to be in exposed brood at the start. The number of mite-infested pupae in exposed brood at the start was estimated by multiplying the infestation rate of the protected brood by the number of pupae in the exposed brood. This calculation assumes that the infestation rate of the exposed brood before exposure to bees was the same as the infestation of protected brood found at the end.

The percentage of all mite-infested pupae in the exposed brood that were chewed by bees was estimated by dividing the total number of chewed, mite-infested pupae by the total number of mite-infested pupae estimated to be in the exposed brood.

The percentage of fertile mites was estimated for 1) all mite-infested pupae that were uncapped, 2) only those mite-infested pupae that were uncapped and chewed, and 3) all mite-infested pupae found in the sample of 200 pupae from the protected patch of brood. A mite was considered fertile if she had produced any offspring, even if only a male.

Statistical Analyses. All variables were compared among the three types of bees using an analysis of variance (ANOVA) with a mixed model having source of mite-infested brood ($n = 20$) and host colony being tested ($n = 27$) as random effects and type of bees ($n = 3$) as a fixed effect (PROC MIXED, SAS Institute 2000). Degrees of freedom were adjusted using the Kenward–Roger method. All percentage data were arcsine transformed (Zar 1984) before analysis, but the nontransformed data are reported.

The percentage of fertile mites from protected brood was compared with that from chewed pupae by using a paired *t*-test (PROC TTEST, SAS Institute 2000). This analysis was restricted to combs that had 10 or more chewed pupae, which eliminated 17 of 52 combs from the analysis. The paired *t*-test analysis was conducted on the entire data set of 35 remaining combs (ignoring type of bee), and separate paired *t*-tests were conducted within each of the three types of bees.

Results

Time Course of VSH. Direct examination and photography revealed short-term dynamics of VSH activity. VSH bees uncapped and chewed relatively few prepupae during the first 20 h of observation. Maximal manipulation of brood occurred at ≈30–42 h (Fig. 1) when most pupae were white- or pink-eyed (Jay 1963). We examined 39 pupae that were being chewed and removed from the section of exposed brood; three were prepupae, 10 were white-eyed, 10 were pink-eyed, and 16 were white-bodied pupae that were partially removed (headless). Thirty-two of the 39 pupae were infested with single foundress varroa, and 29 of these mites (81%) were fertile. Thirty-five of 37 mites (95%) found in the protected section of brood were fertile. Removal of infested brood by VSH bees thus was not biased toward fertile mites.

Uncapping and recapping of pupal cells occurred extensively and rapidly. Fifty-two cells were observed to be uncapped and recapped during 60 h. One cycle of uncapping and recapping was seen for 33 cells, but uncapping and recapping occurred at least twice for 10 other cells and at least three times for the other nine cells. Six of the 52 recapped pupae were infested with single varroa. Two others were infested with larvae of *Galleria mellonella* (L.). Overall, 99% of the 261 cells remaining in the exposed section had been recapped by the end of the test. In comparison, 7% of the 253 pupae in the protected section had been recapped (i.e., before being protected). In a previous study, VSH bees recapped 38% of all brood during a 1-wk exposure of mite-infested brood to the bees, which was significantly higher than a recapping rate of 17% for commercial controls (Villa et al. 2009). We have seen higher recapping rates (>60%) for VSH colonies, but unselected control colonies almost never exceed 25% recapping rates (unpublished data).

Removal of pupae also was sometimes rapid. Six pupae were completely removed within 2 h between observations. One was infested, as evidenced by varroa feces on the cell wall. After 60 h, hygienic activity had decreased varroa infestation to 5% in the remaining brood of the exposed section, whereas infestation was 15% in the protected brood.

Events After 3-h Exposure to VSH Bees. The percentage of fertile mites in brood being removed by VSH bees (73 ± 5%) did not differ from—and clearly was not greater than—that in protected brood (84 ± 5%) on the same combs ($t = 1.33$, df = 12, $P = 0.21$). Lack of bias also was found for hybrid bees (89 ± 3% for chewed versus 88 ± 3% for protected brood; $t = 0.41$, df = 10, $P = 0.69$), control bees (82 ± 10% for chewed versus 88 ± 3% for protected brood; $t = 0.69$, df = 10, $P = 0.51$), and for all bee types combined (81 ± 4% for chewed versus 86 ± 2% for protected brood; $t = 1.26$, df = 34, $P = 0.22$).

In addition to analysis of exposed versus protected brood on the same comb, we compared the fertility of mites from various brood cells among the three types of bees. We found no differences between bee types regarding mite fertility in all uncapped cells or on pupae being chewed (Table 1). Mite fertility in the protected brood also did not differ among the three types. These data also suggest that there was no bias of hygiene toward pupae with fertile mites by VSH bees.

The degree of hygiene significantly differed between bee types. VSH bees and hybrid bees showed greater hygienic activity against infested brood than control bees did (Table 1). VSH and hybrid bees uncapped and chewed mite-infested pupae at rates 3.4- to 5-fold greater than control bees. VSH bees uncapped more of all pupae (infested or not) than control bees did within 3 h; hybrid bees uncapped an intermediate amount.

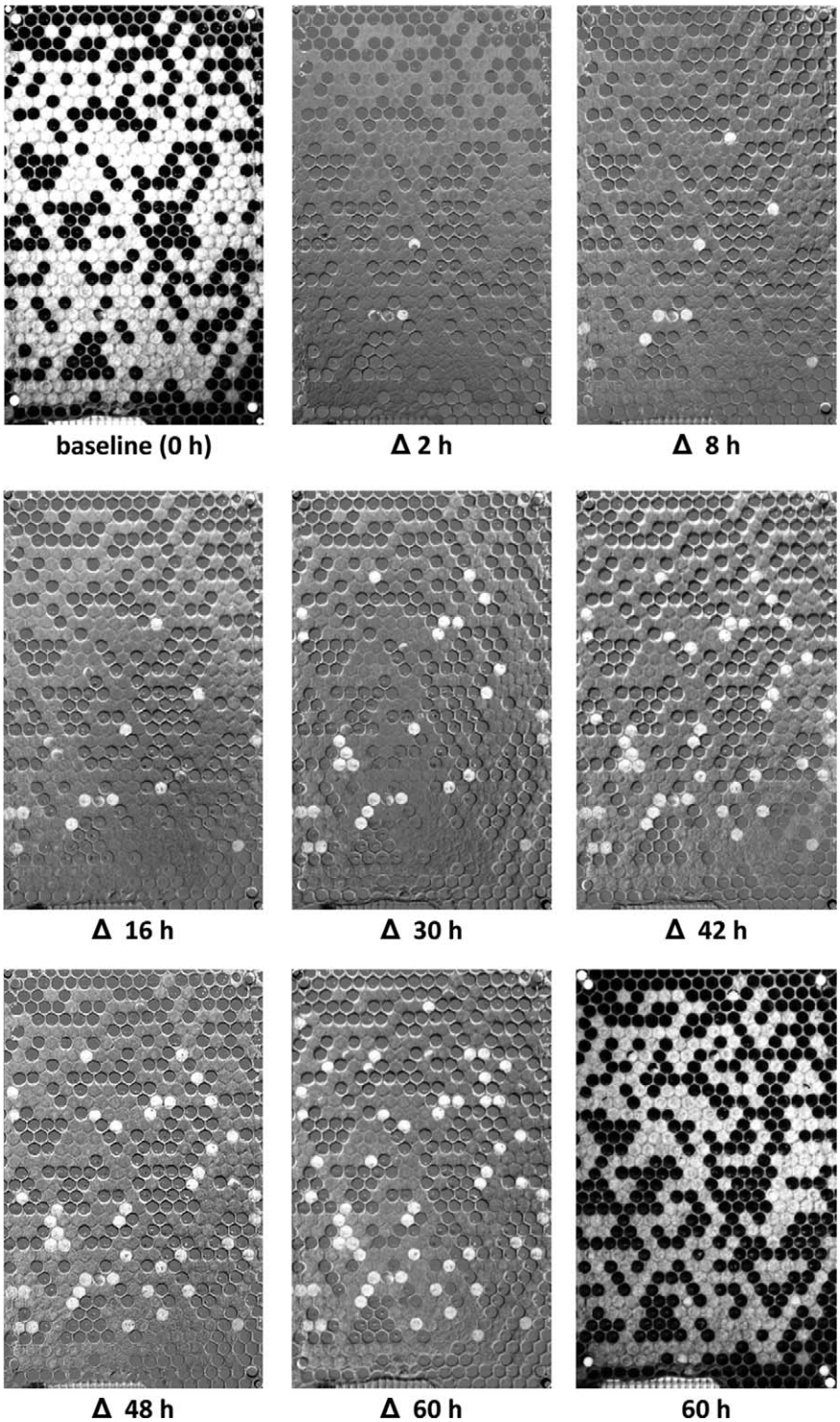

Fig. 1. Photographic series of capped brood that was exposed to VSH bees through 60 h. The brood began in the prepupal stage (≈1–2 d postcapping) and ended as pink- or purple-eyed pupae (≈4–5 d postcapping). Subtraction of the baseline photograph from the photograph at each interval resulted in an image in which cells that had no change in status between the two images are depicted as neutral gray, but cells that were uncapped are depicted as white. The manipulations of 97 pupal cells were photographed throughout the test: 52 cells were uncapped and recapped, 39 had chewed pupae that were removed by us during microscopic exams, and six had pupae that were removed by the bees.

Table 1. Hygienic responses to varroa-infested brood by three types of bees during a 3-h period

Factor	Variable	Control bees ($n = 23$)[a]	Hybrid VSH bees ($n = 13$)	Pure VSH bees ($n = 16$)	F	NDF	DDF	P
Fertility of varroa mites	% fertile mites from protected pupae	89 ± 2 (211)[b]	86 ± 3 (155)	84 ± 2 (213)	2.11	2	44	0.13
	% fertile mites from all uncapped pupae (chewed and not chewed)	75 ± 6 (161)	82 ± 6 (204)	70 ± 6 (487)	1.56	2	18.2	0.24
	% fertile mites from chewed pupae	80 ± 7 (80)	87 ± 7 (136)	74 ± 6 (257)	1.46	2	17.2	0.26
Measures of hygiene	% mite-infested pupae that were chewed	2 ± 2a	8 ± 2b	10 ± 2b	9.08	2	20.4	0.002
	% mite-infested pupae that were uncapped	5 ± 3a	17 ± 4b	20 ± 4b	12.10	2	19.6	<0.001
	% pupae (infested or not) that were uncapped	1 ± 1a	3 ± 1a,b	6 ± 1b	6.89	2	23	0.005
Comb infestations	% infested pupae in protected brood	13 ± 2	15 ± 2	15 ± 2	0.75	2	33.3	0.49
	% infested pupae in exposed brood	13 ± 2	13 ± 2	13 ± 3	0.06	2	27.9	0.94

Despite similar infestations of combs, pure and hybrid VSH bees uncapped and chewed significantly more mite-infested pupae than did controls. The fertility of mites from chewed pupae was not significantly higher than protected pupae (see Results), which suggests that hygienic removal of mite-infested pupae is not dependent on the presence of mite offspring. All data reported as mean ± SE.

NDF, numerator degrees of freedom; DDF, denominator degrees of freedom.

[a] Sample size is the number of combs that were tested for each type of bee. For example, 23 combs were tested in 12 control colonies, 13 combs in seven hybrid VSH colonies, and 16 combs in eight pure VSH colonies (all but two colonies were tested twice).

[b] Total number of pupae from all combs exposed to the type of bees.

Discussion

The fertility of mites on pupae that were being removed was not significantly different from, and clearly was not greater than, fertility of mites on pupae that were protected from the hygienic activity of VSH bees. Thus, the reproductive status of mites did not dictate the removal of mite-infested pupae by these bees. This suggests that the process of varroa sensitive hygiene is more complex than suggested by previous studies in which removal of mite-infested pupae was correlated with an apparent reduction in the population of fertile mites after a 1-wk exposure (Harbo and Harris 2005, 2009).

The result also suggests that high VSH activity can be produced by selective breeding for removal of mite-infested pupae by bees without regard to any measure of mite fertility. Although reduction in brood infestation requires a comparison of two brood infestation estimates (before and after exposure to the bees), this measurement correlates well with the reduced growth of mite populations from field trials (Villa et al. 2009). An advantage to using the reduction of brood infestation rate as a selection criterion is that foreign brood of known infestation rate can be prescreened to guarantee an adequate infestation level (15–20%) for testing. Measurements of mite fertility can be difficult in well established VSH colonies because the brood infestation rate can decrease to low levels (e.g., <5%), which makes finding enough mites ($n = 30$) to estimate the percentage of infertile mites difficult.

Because VSH bees do not target pupae with only fertile mites, other events related to highly hygienic VSH bees may reduce the fertility of mites when brood combs are exposed for longer periods. For example, it may be that reproduction by otherwise fertile mites is disrupted by the extensive uncapping of brood cells and that some uncapped cells later are recapped with nonlaying mites inside them. Furthermore, mites which already had produced offspring may escape as host pupae are being removed by bees but then reinvade other uncapped cells where if sampled they are classified as being infertile. Under these circumstances the frequency of uncapping could be correlated to both the removal of mite-infested pupae and the reduced fertility of mites. In the previous studies that concluded that VSH bees target fertile mites (Harbo and Harris 2005, 2009), cells caps were not examined for uncapping and recapping. Varroa mites remaining in capped brood in those tests may not have been simply ignored because they were infertile but rather may have been infertile (or apparently infertile) because hygienic manipulation of their host cells inhibited their reproduction.

Another possible explanation is that removal of mite-infested pupae was less selective in the current study because the placement of a highly infested comb (e.g., 300 mites in 2,100 capped pupae) into colonies was an excessive stimulus for at least the short (2- to 3-h) term. Perhaps VSH bees become more selective toward fertile mites as the stimulus strength or infestation of brood decreases during a longer exposure. This could explain why fertility measured in mites after a 40-h exposure to VSH bees did not correlate with their level of varroa resistance of the bees, but fertility of mites after a 1-wk exposure did (Villa et al. 2009).

We cannot rule out that VSH bees expressed a bias toward removing fertile pupae during experiments in 2005, but that the current VSH lines no longer have this bias. This is possible because recent selection for VSH has emphasized short-term removal of mite-infested pupae rather than a low percentage of fertile mites or low growth of mite populations after longer field trials. Regardless, because the current bees seem to remove mite-infested pupae without discrimination of mite fertility, the stimulus for

varroa sensitive hygiene most likely comes from the host pupa. Odors and movements of adult varroa mites do not elicit hygiene (Aumeier and Rosenkranz 2001). Cuticular hydrocarbons of pupae change in the presence of varroa mites, and these changes may signal ill health to hygienic bees (Salvy et al. 2001, Martin et al. 2002). Alternatively, behavioral stress responses by infested pupae or diseases vectored to them by mites may induce their hygienic removal (Boecking and Spivak 1999, Boot et al. 1999, Vandame et al. 2002, Nazzi et al. 2004).

Although the percentages of mite-infested pupae that were uncapped varied among the three types of bees, half of all mite-infested pupae that were uncapped after a 3-h period were chewed or partially removed from brood cells (Table 1), regardless of the bee stock. This may suggest a common mechanism of detection and removal by hygienic bees among the different types of bees. However, there were notable differences in hygienic responses between hybrid and pure VSH bees. Hybrid and pure VSH bees uncapped about one fifth of all mite-infested pupae (and chewed half of these), but the pure VSH bees uncapped twice as many pupae as the hybrid VSH bees (Table 1). Thus, uncapping of brood was less selective in the pure VSH stock. There may be negative effects on pupae when their brood cells are uncapped, so our future breeding will focus upon improving selective removal of mite-infested pupae. Ideally, the best VSH colonies would find all mite-infested pupae in an infested comb without uncapping any pupae that were not infested.

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