

Changes in Infestation, Cell Cap Condition, and Reproductive Status of *Varroa destructor* (Mesostigmata: Varroidae) in Brood Exposed to Honey Bees With *Varroa* Sensitive Hygiene

JEFFREY W. HARRIS,¹ ROBERT G. DANKA, AND JOSÉ D. VILLA

USDA-ARS, Honey Bee Breeding, Genetics and Physiology Laboratory, 1157 Ben Hur Road, Baton Rouge, LA 70820

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ABSTRACT Honey bees (*Apis mellifera* L.) bred for *Varroa* sensitive hygiene (VSH) selectively remove pupae infested with *Varroa destructor* Anderson & Trueman (Mesostigmata: Varroidae) from capped brood that is inserted into the nest. After 1 wk, remaining brood cells tend to have been uncapped and recapped, and remaining mites are mostly infertile. A primary goal of this experiment was to compare the reproductive status of mites that remained in recapped and normally capped cells after a 1-wk exposure to VSH and control colonies. Differences in distribution of fertile mites in normally capped brood cells between VSH bees and control bees may suggest that the stimulus for hygiene is related to reproduction by mites. Identification of stimuli triggering VSH behavior could be used to develop new bioassays for selective breeding of this important resistance mechanism. Combs of capped brood that were exposed to control bees had 10 times more pupae with fertile mites in normally capped brood as did VSH bees (6.7 and 0.7%, respectively). They also had 3 times more pupae with infertile mites in normally capped brood than did VSH bees (1.4 and 0.5%, respectively). Thus, VSH bees targeted fertile mites by a 3:1 ratio by either removing or uncapping and recapping their host pupae. Biased removal of mite-infested pupae with fertile mites suggested that stimuli triggering VSH behavior were enhanced by the presence of mite offspring within the brood cell. This bias for fertile mites is not seen during experiments of short 3-h duration. The differing results are discussed relative to a behavioral threshold model for hygienic behavior in honey bees in which different experimental protocols may reflect activities of honey bees having different sensitivities to pupae infested by fertile mites. In addition, mortality of mite offspring was significantly higher in recapped cells than in normally capped cells and contributed to decreased reproduction by the mites.

KEY WORDS honey bee, varroa mite, *Varroa* sensitive hygiene

Infestation by *Varroa* mites (*Varroa destructor* Anderson & Trueman [Mesostigmata: Varroidae]) remains the most significant health issue to honey bees (*Apis mellifera* L.) (Rosenkranz et al. 2010). This parasitic mite is usually managed with acaricides, but use of in-hive chemicals leads to chemical residues in honey and wax (Wallner 1999, Martel et al. 2007), development of genetic-based resistance of mites to chemicals (Elzen et al. 2000), and synergistic effects between chemicals that can adversely affect bee health (Johnson et al. 2009). One solution to these problems would be the development and implementation of honey bees with genetic-based resistance to *Varroa* mites. Several behavioral mechanisms that confer tolerance or resistance to *Varroa* mites in honey bees have a genetic basis, and some of these have been enhanced through selective breeding (Boecking and Spivak 1999, Rinderer et al. 2010).

Varroa sensitive hygiene (VSH) is a genetic-based behavioral resistance in which *Varroa*-infested pupae

are removed from capped brood by adult bees (Harbo and Harris 2005, Ibrahim and Spivak 2006). This behavior is similar to generalized hygienic behavior that bees use to remove nest invaders, dead brood, and brood affected by various diseases (reviewed by Spivak 1996, Boecking and Spivak 1999). Removal of a pupa begins with detection of an abnormal condition by hygienic bees. Eventually, the cell cap is perforated by a bee to expose the affected host, and the host is then usually removed from the brood cell (Rothenbuhler 1964, Arathi et al. 2000).

Sometimes *Varroa*-infested pupae that are uncapped are recapped 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). The foundress mite may escape an uncapped brood cell before it is recapped, but she usually remains within the cell (Boecking et al. 2000, Aumeier and Rosenkranz 2001). Brood exposed to VSH bees for 1 wk often have high mean percentages (>30%) of recapped brood cells (Harris 2008, Villa et al. 2009), and some

¹ Corresponding author, e-mail: jeffrey.harris@ars.usda.gov.

colonies may have >90% of all brood recapped. Most of these recapped cells are not infested by *Varroa*, but $\approx 20\%$ of recapped cells can contain a mite (Harris 2008). Given that manual transfer of mites among brood cells reduces subsequent mite reproduction (Kirrane et al. 2011), it may be possible that hygienic uncapping followed by recapping of brood cells by bees could inhibit or alter mite reproduction.

Although VSH bees actively remove mite-infested pupae (Harris 2007), it is not clear whether they primarily target pupae with mites that are reproducing. Reduction of brood infestation after a 1-wk exposure of capped brood to colonies with increasing levels of VSH correlated with a reduction in fertility of mites (Harbo and Harris 2005, 2009). These studies concluded that VSH bees targeted pupae with fertile mites and ignored pupae with infertile mites. However, these studies did not examine caps of brood cells for signs of manipulation by the bees, and it was impossible to know whether mite-infested pupae that remained in combs, especially those with infertile mites, had been uncapped and recapped by hygienic bees. A subsequent study that used a 3-h assay found that VSH bees did not initially target mite-infested pupae that were infested with fertile mites (Harris et al. 2010). In that study, pupae with fertile or infertile mites were targeted at rates that were similar to their relative frequencies in brood that was protected from hygiene by a screen.

The purpose of the current study was to compare the frequencies of fertile and infertile mites in brood cells that were exposed to VSH bees or to relatively nonhygienic control bees for 1 wk, with emphasis on whether brood cells with mite-infested pupae had been uncapped and recapped or whether they remained with a normal cap, which would suggest that they were not manipulated by the bees. If VSH bees uncapped and recapped or removed predominantly pupae with fertile mites, the difference in frequency of fertile mites in normally capped brood between VSH and control bees should be greater than the difference in frequency of infertile mites in normally capped brood between the two types of bees. By focusing on fertility of mites in nonmanipulated brood cells, we avoid the possible confounding effect of mite reproduction being inhibited or altered by hygienic manipulation. The results of this study may suggest a probable source of semiochemicals that trigger *Varroa* sensitive hygiene. For example, if hygienic removal of mite-infested pupae is biased to fertile mites, cues used by hygienic bees to find mite-infested pupae are directly or indirectly associated with mite offspring. The effects of uncapping and recapping on the number and mortality of mite offspring also were examined.

Materials and Methods

Source of Bees. This experiment was conducted at the USDA-ARS Honey Bee Breeding, Genetics and Physiology Laboratory in Baton Rouge, LA. VSH queens were produced by instrumentally inseminat-

ing queens from various VSH lines with semen from five to eight drones from other VSH lines. Italian queens used in control colonies were either naturally mated from a commercial queen producer or instrumentally inseminated and crossed to five to eight drones from other Italian queens. All colonies were established at least 2 mo before being tested.

Measurements. *Varroa* sensitive hygiene was investigated by transferring one comb of mite-infested capped brood (39 different brood sources) into each of 53 host colonies (30 VSH and 23 control colonies) and then monitoring changes in brood infestation that occurred after 1 wk. Each infested comb was a standard Langstroth size and had primarily prepupae. Each test comb had a minimum of 0.0451612 m^2 (70 in.²) of capped brood, and 10–25% of brood cells were infested by *Varroa*. The 53 host colonies were tested in three blocks at three different times.

Colonies had five to eight combs covered with adult bees in a single deep Langstroth hive body that had seven to 10 combs. Most colonies had three to five combs of capped brood. A comb containing mostly capped prepupae was removed from each colony before transferring the mite-infested comb into the nest. This removal was done to avoid large changes in the existing brood-to-bee ratio within each colony.

The initial infestation for each transferred comb was found by counting the number of mite-infested prepupae in 181 ± 10 capped brood cells (mean \pm SE) that were sampled in straight line transects on each side of the comb. Normal versus recapped cells were discriminated by examining the integrity of the silk and noting the presence of the granular wax patches of holes that were made by hygienic bees to inspect brood cells (Boecking et al. 2000). Pupae in normally capped brood cells were not manipulated by hygienic bees, and silk lined the entire undersurface of the cap (Fig. 1). Pupae from recapped cells had been uncapped at least once and then resealed, and the silk lining did not completely cover the underside of the cap (Fig. 1). Combs were rejected from testing if >1% of the prepupae had been recapped to avoid confounding hygienic activity of the donor colony with that of the colony being tested.

The final infestation was estimated for each comb 1 wk later by examining another sample of 221 ± 14 capped pupae (mean \pm SE). Sampling was restricted to pupae with purple eyes and tanned joints of the antennae and legs or to pupae with an evenly tanned body and white wing pads. These stages were the cohort of bees that had been sampled at the beginning of the test 1 wk prior (Jay 1962, 1963). Removal of mite-infested pupae by hygienic bees was estimated with the percentage change in brood infestation, calculated by dividing the difference between final and initial infestations by the initial infestation.

Cell caps of all mite-infested pupae were examined and categorized as normal or recapped (Fig. 1). The reproductive success of the foundress from each singly infested cell was evaluated by counting total offspring and the number of living offspring. A mite was defined

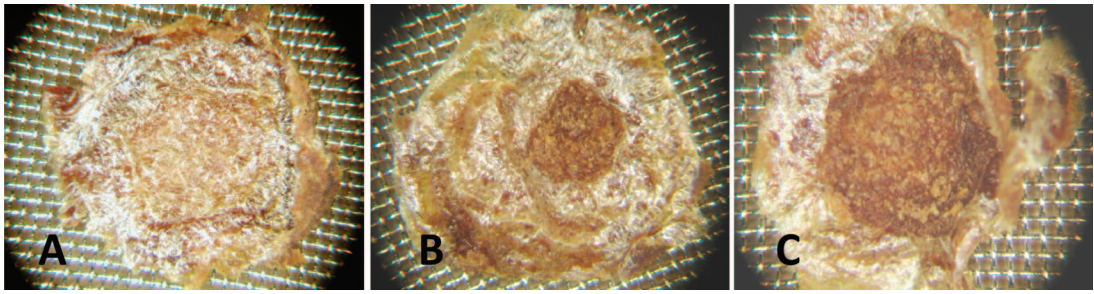


Fig. 1. Underside of different types of cell caps from brood exposed to hygienic colonies of honey bees. The entire surface of normal cell caps (A) is lined with silk that is spun by bee larvae before pupation. Brood cells may be punctured or uncapped by hygienic bees, and the holes can be subsequently recapped with wax that will not be lined with silk. The diameter of repaired holes can vary from relatively small (B) to larger holes nearly the size of the entire brood cell diameter (C). (Online figure in color.)

as fertile if she had one or more offspring (even if only a male).

Statistical Analyses. The initial and final mite infestations of brood, percentage of mite-infested pupae that were removed, frequencies of remaining fertile and infertile mites per hundred sampled pupae, and the frequencies of fertile and infertile mites found in normally capped and recapped cells per hundred sampled pupae were compared between the two types of bees using an analysis of variance (ANOVA) with a mixed model (Proc Mixed, SAS Institute 2000). The model included type of bee, block and type of bee \times block as fixed effects. A random effect for source of the mite-infested brood within a block was included in the model. All variables were either a percentage or a ratio, and separate analyses were conducted for non-transformed values and arcsine-transformed values of each variable (Zar 1984). For all variables, the analysis of the nontransformed data resulted in F-tests that were very similar in magnitude to those resulting from analysis of the transformed data; therefore, the analyses of nontransformed data are presented here. Degrees of freedom were adjusted using the Kenward-Roger method.

The total number of offspring and the number of live offspring per fertile mite were compared between the two types of honey bees (VSH and control) and the two types of brood cells (normally capped and recapped) by using an ANOVA for count data (Proc

Glimmix, SAS Institute 2000). For each analysis, the model included fixed effects for type of bees, type of cell cap, and the type of bee \times type of cell cap interaction. Random effects included the block of colonies being tested (three levels) and source of mite-infested brood (39 levels). Degrees of freedom were adjusted using the Kenward-Roger method.

Results

The infestation of brood added to VSH and to control colonies was similar (17.3 ± 2 and 17.5 ± 2 mite-infested pupae per hundred pupae, respectively; $F = 0.01$; $df = 1, 41.4$; $P = 0.94$). After 1 wk, the infestation of introduced brood was significantly lower in VSH colonies than in control bees (5.2 ± 1 versus 12.7 ± 1 mite-infested pupae per hundred pupae) ($F = 30.3$; $df = 1, 42.2$; $P < 0.0001$). Thus, the 1-wk reduction of *Varroa* infestation was 3 times greater by VSH bees ($67 \pm 5\%$) than by control bees ($23 \pm 5\%$) ($F = 43.55$; $df = 1, 43.3$; $P < 0.0001$). The block and type of bee \times block interaction were not significant for the initial or final brood infestations, or for the percentage of mite-infested pupae removed (all $P > 0.05$).

Combs exposed to VSH bees had significantly fewer fertile mites than did those exposed to control bees. Combs from control bees had a 10 times greater frequency of fertile mites in normally capped brood than did VSH bees (Table 1). They also had twice the

Table 1. Percentage (least squares mean \pm SE) of pupae infested with fertile or infertile mites after combs were exposed to two types of bees during a 1-wk assay of hygienic behavior

Variable ^a	VSH colonies (n = 30)	Control colonies (n = 23)	F	NDF ^b	DDF ^c	P
Pupae with a fertile mite	2.3 \pm 0.8	10.2 \pm 0.9	49.7	1	42.6	<0.0001
In cells with normal caps	0.7 \pm 0.7	6.7 \pm 0.8	32.3	1	47	<0.0001
In recapped cells	1.7 \pm 0.4	3.3 \pm 0.4	7.3	1	42.1	0.010
Pupae with an infertile mite	2.9 \pm 0.6	2.4 \pm 0.6	0.39	1	39.3	0.54
In cells with normal caps	0.5 \pm 0.3	1.4 \pm 0.3	6.8	1	43.2	0.012
In recapped cells	2.4 \pm 0.6	1.1 \pm 0.6	3.18	1	40.1	0.082

Varroa-infested pupae were subdivided into two categories based on the condition of the cell cap. Normally capped cells were not manipulated by hygienic bees, while recapped cells had been uncapped at least once by hygienic bees and subsequently recapped.

^a Neither the block term nor the type of bee \times block interaction term were significant for any variable ($\alpha = 0.05$).

^b Numerator degrees of freedom in F-test for type of bee.

^c Denominator degrees of freedom in F-test for type of bee.

Table 2. Number of offspring (least squares means \pm SE) from fertile mites that had been exposed to VSH or control bees for 1 wk

Variable	VSH bees ($n = 30$ colonies)		Control bees ($n = 23$ colonies)	
	Normal cap	Recapped	Normal cap	Recapped
Total offspring per mite	3.28 \pm 0.29 ($n = 44$)	3.20 \pm 0.18 ($n = 109$)	3.42 \pm 0.12 ($n = 293$)	3.29 \pm 0.15 ($n = 168$)
Live offspring per mite	2.89 \pm 0.38 ($n = 44$)	2.18 \pm 0.22 ($n = 109$)	3.35 \pm 0.26 ($n = 293$)	3.16 \pm 0.27 ($n = 168$)

The number of total offspring was not significantly different between the two types of bees or between the two types of brood cells. However, the number of live offspring was significantly lower for VSH bees and recapped brood cells (see Results).

frequency of fertile mites in recapped brood cells as did VSH bees (Table 1). Although the overall frequency of infertile mites was not significantly different between the two types of bees (Table 1), combs from control bees had three times higher frequencies of infertile mites in normally capped brood than did those from VSH bees (Table 1). Combs from VSH bees tended to have higher frequencies of infertile mites in recapped cells than did control bees, but the difference was not statistically significant (Table 1).

The mean number of offspring per fertile mite was not significantly different between brood introduced into colonies of the two types of bees ($F = 0.30$; $df = 1, 565$; $P = 0.59$) (Table 2). Fertile mites from control bees had 3.35 ± 0.10 offspring, whereas those from VSH bees had 3.24 ± 0.17 offspring. There were also no differences in total offspring for fertile mites in normally capped versus recapped brood cells ($F = 0.28$; $df = 1, 565$; $P = 0.60$) (Table 2). Fertile mites in normally capped cells had 3.35 ± 0.16 offspring, and those in recapped brood cells had 3.25 ± 0.11 offspring. The type of bee \times type of cell interaction term was not significant ($F = 0.01$; $df = 1, 565$; $P = 0.92$).

Although total offspring per fertile mite was similar, the mean number of live offspring per fertile mite was significantly lower for brood exposed to VSH bees than for brood exposed to control bees ($F = 9.31$; $df = 1, 48.69$; $P = 0.0037$) (Table 2). Fertile mites from combs exposed to VSH bees had 2.51 ± 0.25 live offspring, and those from combs exposed to control bees had 3.25 ± 0.24 live offspring. In addition, the mean number of live offspring per fertile mite was significantly lower in recapped cells than in normally capped cells ($F = 5.74$; $df = 1, 414.4$; $P = 0.0171$) (Table 2). Fertile mites in normally capped brood cells had 3.11 ± 0.27 live offspring, and those in recapped brood cells had 2.63 ± 0.21 live offspring. The interaction of type of bee and type of cell was not significant ($F = 2.48$; $df = 1, 423.2$; $P = 0.1158$).

Discussion

The greatly reduced frequency of pupae with fertile mites after exposure to VSH bees suggests a strong relationship between hygiene and mite reproduction and was similar to previous findings (Harbo and Harris 2005, 2009). Previous studies did not compare the fertilities of mites in normally capped and in recapped brood cells, and reduced fertility was attributed to selective removal of pupae with fertile mites. However, inhibition of mite reproduction caused by uncapping and recapping of infested pupae could have

contributed to reduced fertility. In the current study, the frequency of pupae with remaining fertile mites in normally capped brood cells for control bees was 10 times that found for VSH bees (Table 1). In addition, control bees had three times the frequency of pupae with infertile mites in normally capped brood cells as did VSH bees (Table 1). In combination, these results suggest that VSH bees target pupae with fertile mites (by either removing or by uncapping and recapping) over those with infertile mites by a 3:1 ratio. In addition, optimal hygienic removal of mite-infested pupae occurs in developmental stages of bees in which the first mite offspring are produced (Harris 2007). For these reasons, we hypothesize that most hygienic removal of mite-infested pupae during 1-wk experiments occurred after offspring were produced.

Hygienic bees have increased olfactory sensitivities to odors associated with diseased brood (Masterman et al. 2000, 2001; Gramacho and Spivak 2003; Spivak et al. 2003). Odors triggering hygienic removal of *Varroa*-infested brood have not been fully described, but odors related to stress of infested pupae, pathogens, and resultant diseases vectored to host pupae, or to the mites and their offspring have been suggested as probable cues (Boecking and Spivak 1999, Aumeier and Rosenkranz 2001, Salvy et al. 2001, Martin et al. 2002, Vandame et al. 2002, Nazzi et al. 2004, Schöning et al. 2012). The biased removal of fertile mites in this study suggests that some stimuli triggering VSH behavior are related to the presence of offspring within the brood cell. These odors could originate directly from the offspring (feces or cuticular hydrocarbons) or from increased damage (feeding wounds or viral transmission) to a host pupae caused by a family of mites. Identification of the specific semiochemical triggers could foster development of new laboratory bioassays for VSH behavior that might improve selection of this important resistance mechanism. For example, breeder queens could be selected based upon electroantennogram responses or response thresholds of their worker daughters to specific odors known to trigger hygienic removal of mite-infested pupae.

Reduction in the frequency of fertile mites observed in normally capped cells could have occurred by complete hygienic removal of pupae with fertile mites, or by a change in the cell cap from "normal" to "recapped" when a pupa with a fertile mite was uncapped and then recapped. If cells with fertile mites had been recapped frequently by VSH bees, then fertility in those cells could have been higher for VSH bees compared with control bees. On the contrary, the frequency of fertile mites in recapped cells was sig-

nificantly lower for VSH bees than for control bees (Table 1). However, the net change in frequency of fertile mites in normally capped cells does not identify the most likely sequence for removal. For example, there could have been a significant transition of pupae with fertile mites from normally capped cells to recapped cells, followed by subsequent removal of the pupae with fertile mites from recapped cells.

If mite reproduction was inhibited by uncapping and recapping of brood cells by VSH bees, fertile mites that would have produced offspring without hygienic manipulation were reclassified as infertile and in recapped brood cells. Thus, the frequency of infertile mites in recapped cells could have been higher for VSH bees compared with control bees. The frequency of infertile mites in recapped cells was not significantly different between the two types of bees (Table 1), which suggests that uncapping and recapping of brood cells was not a major cause of infertility of mites exposed to VSH bees. However, it is possible that there was a significant production of infertile mites (from potentially fertile mites) in recapped cells before large numbers of them were removed by VSH bees. This mechanism does not explain the asymmetry in reduction of fertile and infertile mites from normally capped cells, unless hygienic VSH bees can detect fertile mites that have not laid eggs (but are capable of reproduction) more frequently than infertile mites that can never lay eggs.

Selective manipulation (either removal or uncapping and recapping) of pupae with fertile mites by VSH bees in this study was different from that in a previous experiment in which brood was exposed to VSH bees for only 3 h (Harris et al. 2010). In that study, VSH bees uncapped and removed pupae with fertile and infertile mites at rates similar to the frequencies of both types of mites from a portion of the brood that was protected from hygiene by a screen (Harris et al. 2010). One possible explanation for these different outcomes is that the durations of the two experiments provided different perspectives of a cumulative and complex colony-level behavior. Differences in apparent bias for hygienic activity toward pupae with fertile mites could be explained by a behavioral threshold model for *Varroa* sensitive hygiene (Beshers and Fewell 2001, Gramacho and Spivak 2003). The model assumes that behavioral thresholds for initiating hygienic removal of *Varroa*-infested pupae vary among genotypically different workers in a VSH colony. Perhaps the most sensitive workers—those with the lowest thresholds—detected and uncapped pupae infested with either fertile or infertile mites without a bias, whereas workers with higher thresholds only detected and removed pupae with fertile mites. These less sensitive workers may have either ignored or recapped pupae with infertile mites. When infestations are high, or when experiments have a relatively long duration, workers with a wide range of sensitivities may be stimulated to remove mite-infested pupae. However, the 3-h experiment may have been only long enough to reveal the nonbiased activities of the

most sensitive workers that initiated uncapping and removal of mite-infested pupae.

The total number of offspring for fertile mites did not vary between normal and recapped cells, indicating that offspring were not removed before cells were recapped. Perhaps removal of mite offspring only occurs when the host pupae is being chewed by hygienic bees (as in Harris et al. 2010). These results also imply that either uncapping occurred after fertile mites had laid their full complements of eggs, or uncapping and recapping of brood cells did not interrupt egg-laying in these mites.

The current study showed that hygiene by VSH bees increased mortality of mite offspring in brood cells that were recapped by the bees. This result implied that the average mortality of mite offspring (Ifantidis et al. 1999, Mondragon et al. 2006) may be regularly higher in colonies of hygienic bees. Removal of the cell cap could alter the temperature and humidity within the brood cell and thereby impact the health of immature mites (Bruce et al. 1997). Inspection of a brood cell by hygienic bees may simply lead to young mites being injured or killed by physical contact. The relatively long duration of the current experiment allowed multiple cycles of uncapping and recapping of mite-infested brood (Villegas and Villa 2006, Harris et al. 2010), which would be expected to exacerbate these deleterious effects. In particular, male offspring may be vulnerable to attack by hygienic bees because the eggs producing males are often located near the cell cap (Donzé and Guerin 1994, Donzé et al. 1998). Death of male offspring in recapped brood cells could help explain the phenomenon of unmated mites seen in VSH bees (Harris and Harbo 1999). Unmated mites do not lay eggs, and death of male offspring is a major cause of nonmating (Martin et al. 1997). We did not quantify the sex of dead offspring so the level to which recapping may have contributed to poor mating success is unclear.

Honey bees bred for highest expression of VSH can affect mite reproduction in several ways. This and previous studies suggest that hygienic removal of mite-infested pupae is the primary mechanism that reduces reproduction by *Varroa* mites. The question of biased removal of pupae infested with fertile mites by VSH bees was supported by results of the current study. Even if mite-infested pupae are not removed from brood cells by VSH bees, reproductive potential can be reduced by increased mortality of offspring in brood cells that are initially uncapped and then recapped by the bees. In addition, previous research showed that a genetically based factor from the brood produced by VSH queens reduces mite reproduction (Harbo and Harris 2001, Ibrahim and Spivak 2006). It is also possible that hygienic activity reduces reproduction when mites subsequently invade other brood cells (Kirrane et al. 2011). Finally, a poorly understood factor of VSH bees, which could be caused by mortality of male offspring during hygiene, leads to increased frequencies of nonmating in the *Varroa* population after several months (Harris and Harbo 1999). These multiple effects on mite reproduction provide

significant *Varroa* resistance even when pure VSH lines are outcrossed to nonresistant drones and used in commercial beekeeping operations (Harbo and Harris 2001, Ward et al. 2008, Danka et al. 2011).

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