Apidologie
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DOI: 10.1007/s13592-013-0195-8

Original article

Varying congruence of hygienic responses to *Varroa destructor* and freeze-killed brood among different types of honeybees

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Received 21 September 2012 – Revised 14 December 2012 – Accepted 11 January 2013

Abstract – Honeybees, *Apis mellifera*, selected for the hygienic removal of freeze-killed brood (FKB), resist several microbial diseases and have some resistance to *Varroa destructor*. Bees with *Varroa*-sensitive hygiene (VSH) have good resistance to *V. destructor*. We determined whether the response to FKB could be used to select for VSH by measuring the responses of different bees (VSH, FKB-selected, F_1 VSH, and unselected control) to combs with FKB and combs with mite-infested brood. All bee types completely removed much FKB (77–88 %) within 24 h. The removal of mite-infested brood after 1 week was much more variable among bee types (VSH, 66 %; F_1 VSH, 51 %; FKB hygienic, 14 %; control, 3 %). There was some relationship between 24-h manipulation of FKB cells (i.e., cell contents at least partially removed) and the removal of mite-infested brood, but this appears to have little practical relevance because of a large inherent variation.

Apis mellifera **/ hygiene /** ***Varroa destructor*** **/ mite resistance / breeding**

1. INTRODUCTION

There is increasing interest by breeders of honeybees (*Apis mellifera* L.) in the USA to incorporate two forms of hygienic behavior into their bees to improve resistance to diseases and mites. The first form, which we call here "freeze-killed brood" (FKB) hygiene, offers excellent protection to the pathogens *Paenibacillus larvae* (White) and *Ascosphaera apis* (Maasen ex Claussen) L.S. Olive & Spiltoir that cause American foulbrood and chalkbrood, respectively (reviewed by Spivak and Gilliam 1998a, b). FKB hygiene also affords moderate protection against the parasitic mite *Varroa destructor* Anderson and Trueman (Spivak and Reuter 2001; Ibrahim et al. 2007), which often is considered to be the primary health threat to honeybees (Rosenkranz et al. 2010). The second form of hygiene, *Varroa*-sensitive hygiene (VSH), affords relatively high resistance to *V. destructor* (reviewed by Rinderer et al. 2010). Bees that have one or the other of these types of hygiene are used successfully for a variety of beekeeping applications.

FKB hygiene and VSH have been selected by breeders in different ways. FKB hygiene often has been selected based on the ability of a colony to remove FKB in a defined period. For example, this method was used to develop Minnesota hygienic bees (Spivak 1996) and is still used for selection during propagation of the stock (Spivak et al. 2009). VSH bees initially resulted from targeted selection of colonies that showed slow population growth of *V. destructor* or high frequency of non-reproduction of mites (Harbo and Hoopingarner 1997). Mite popula-

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Manuscript editor: Stan Schneider

Published online: 05 February 2013

tion growth was not related to the propensity to remove FKB in the population of bees that was initially tested. Recent selection for VSH has been based on the ability of a colony to remove mite-infested brood in a defined period, as first described by Harbo and Harris (2005).

Similarities and differences in function between the two types of hygiene are not well understood. The removal of FKB was correlated with the removal of pupae that were artificially infested with two, but not one, *V. destructor* (Boecking and Drescher 1992). About 2 years after initiating the Minnesota hygienic breeding program, bees selected for high hygiene removed more FKB and usually, but not always, removed more brood artificially infested with *V. destructor* than bees selected for low hygiene (Spivak and Gilliam 1998b). During work to determine how VSH bees (called SMR bees at the time) suppressed mite reproduction, a greater percentage of artificially mite-infested brood was removed by VSH bees (80–85 %) than by Minnesota hygienic bees (62–66 %; Ibrahim and Spivak 2006).

We sought to extend information about the comparative hygienic responses of honeybees against FKB and *V. destructor*. Our primary objective was to compare the removal of FKB and *V. destructor* in contemporary strains of VSH and FKB hygienic bees. Preliminary observations (unpublished) indicated that VSH bees removed more FKB sooner (at 6 and 12 h after freezing) than FKB hygienic and unselected bees, but the removal responses of all types were similar at 24 h after freezing. If this is so, then testing for the removal of FKB at a short time after freezing could serve to screen bees during selection for VSH, which is a trait that is relatively difficult to measure (Villa et al. 2009). The resulting information also is potentially useful for understanding the genetics underlying the different forms of hygiene.

2. MATERIALS AND METHODS

Bees of four types were tested in summer 2011 at our laboratory. VSH breeder colonies maintained by us were used as grafting sources to create queens that produced colonies of two types. Queens which produced VSH colonies were instrumentally inseminated using semen from drones from the same group of breeders. Queens that produced F_1 VSH colonies were naturally mated to non-VSH drones in areas away from concentrations of resistant colonies. FKB hygienic queens were reared from five breeder queens obtained from a commercial producer of Minnesota hygienic stock (Hull Apiaries, Battle Lake, MN) and then naturally mated in mating apiaries of the producer near Monroe, LA. Control queens were purchased from two commercial sources (Wooten's Golden Queens, Palo Cedro, CA, and C. F. Koehnen and Sons, Inc., Glenn, CA) whose bees in previous tests showed relatively little hygienic removal of mite-infested brood. Twenty test queens of each type were established in colonies in early May 2011. Colonies of all types were distributed approximately equally among two apiaries. Hygienic activity was evaluated beginning 8 weeks after the colonies were established to ensure that all bees were from the new test queen. All colonies began the test with at least five deep combs which were at least two thirds covered with adult bees. Colonies in which queens superseded were not used further.

The level of general hygiene of each colony was assessed by measuring the removal of FKB at intervals during 48 h. We used a standard protocol of killing a patch of sealed brood by freezing the cell contents with liquid nitrogen (Spivak and Reuter 1998). One end of a PVC cylinder [7.6 cm (3 in.) diameter, 10 cm (4 in.) long] was pushed into the patch down to the midrib of the comb. Patches contained 143±15 (SD, n=70 colonies) cells of brood that had sealed larvae, prepupae, or young pupae (white- to purple-eyed with no cuticular tanning). Liquid nitrogen (300 ml) was poured into the cylinder to kill the brood. After about 20–30 min, when the nitrogen evaporated, the brood thawed, and the cylinder was removed, the patch of brood was photographed and the comb was returned to the center of the colony. The patch was photographed again at 6, 12, 24, and 48 h after freezing. Each colony was tested again after 2 weeks. Photographs were examined to determine the number of dead bees that were uncapped or partially removed and the number that were completely removed at each time after freezing.

These yielded the following two variables: the percentage of FKB removed and the percentage of FKB manipulated (i.e., the sum of brood uncapped, partially removed, or completely removed) at each time.

The level of hygiene against *V. destructor* of each colony was assessed by measuring the change in mite infestation in an infested comb exposed to the colony for 1 week. Mite infestation first was measured in a brood comb obtained from a mite-infested donor colony that was not in the test. We measured infestation in 197±12 cells of brood that had sealed larvae, prepupae, or white-eyed pupae. Initial infestation was 16.8±4.9 % in the 61 combs of brood used to test colonies that remained with original queens; initial infestations were not different among the bee types (F=0.23, df=3, 57, P=0.876). The comb then was inserted into the broodnest of a test colony and allowed to remain for 1 week. The comb was retrieved and the final infestation was measured in 199±6 cells containing purple-eyed, tan-bodied pupae; these were bees of the same age cohort as those initially measured (Jay 1962). Changes presumably are related to the hygienic removal of infested brood. The percentage of removal of infested brood was calculated as ([initial infestation−final infestation]/initial infestation)×100. Infestation was greater in the final measure in 15 of 61 colonies tested. We assumed that this was due to sampling error and so used the apparent mite gain in analyses rather than adjusting the response to zero so as not to bias sampling error in one direction. We also measured the rate of recapping of the cells. Recapping occurs when a cell is opened and then subsequently sealed without the cell contents being removed. It is observable because the cell cap has had the silk cocoon removed from all or part of the inner surface.

After measuring hygienic response to freeze-killed brood and before measuring hygienic response to *V. destructor*, we examined late-stage sealed brood (purple-eyed pupae and older) in resident comb. We recorded the percentage of infested cells and the percentage of recapped cells. We determined whether the mites in singly infested cells were alive and were fertile (had at least one progeny); if so, their fecundity (the number of progeny per fertile mite) was recorded. Sampling continued until we had observed 100–820 cells and found 0–44 mites; in bee types with lower infestations, we found fewer mites despite observing more cells (Table I). Recapping was determined in 100–720 brood cells.

Comparisons of the different bee types for all variables related to hygiene against FKB used analysis of variance (ANOVA; Proc Mixed in SAS 9.3 using the Kenward–Roger method to adjust the degrees of freedom; SAS Institute Inc. 2009). Separate analyses were conducted for the percentages of FKB removed and FKB manipulated at each of the four intervals after freezing during each of two replicates. The ANOVA model included fixed terms for type of bee (four levels), replicate (two levels), and the type of bee×replicate. The model also included random terms for apiary, type of bee×apiary, colony (type of bee×apiary), and apiary×replicate (type of bee). An effect of colony size was considered because colonies of different bee types varied in size (control, 11.7±4.6 combs of bees; FKB hygienic, 8.7±2.7; F_1 VSH, 9.9±2.9; VSH, 6.7±3.2; F=5.67, df=3, 57, P=0.002). When included as a covariate, colony size did not influence the removal of FKB at any time, but was related to the manipulation of FKB at 12 h (F=4.00, df=1, 53, P=0.051) and 48 h (F=4.72, df=1, 53, P=0.034) after freezing. Larger colonies tended to manipulate less FKB than smaller colonies. It appears that this unexpected trend occurred because colony size was confounded with bee type, especially because control colonies were larger but less hygienic and VSH colonies were smaller but more hygienic. Given the apparent large effect of bee type and the relatively small effect of colony size, we chose to remove colony size from the ANOVA model.

Response to mite-infested brood used a mixed model having type of bee (four levels) as a fixed effect and random effects for apiary (two levels) and type of bee×apiary. Initially, a random term for the source of mite-infested brood was included for all variables, but all variance estimates for this effect were zero and the term was removed. Colony size initially was included as a covariate and was found not to influence the response to *V. destructor*.

Relationships between the removal of mite-infested brood and the removal or manipulation of FKB were examined using analysis of covariance (ANCOVA). The average removal of FKB for the two replicates and the removal of FKB for each replicate

Table I. Sample sizes related to metrics of *V. destructor* populations in resident brood.

Variable	Overall	Control	FKB hygienic	F_1 VSH	VSH
No. of cells checked for infestation	378±194	237±196	310±146	481±164	503±113
No. of infested cells	17±8	26±8	20±12	13±10	5±8
No. of singly infested cells	14±9	21±3	17±9	11±8	5±7
No. of cells checked for recapping	369±180	227±155[a]	320±151	468±144	486±129

Number of colonies per group were: control=17, FKB hygienic=15, F_1 VSH=16, and VSH=14. Values are the mean±SD per colony

[a] Recapping data available for 16 control colonies

were independently tested as covariates. The model for each ANCOVA had the removal of mite-infested brood as the dependent variable, and all models initially included fixed effects for the type of bee, covariate, and the type of bee×covariate. The interaction term was used to test for parallel slopes among the different types of bees and then was dropped from the model because it was not significant. The model also included random terms for apiary and the type of bee×apiary. The relationship between the removal of mite-infested brood and recapping also was analyzed using this method. Pearson's correlation analysis (Proc Corr) was used as an additional test of relationships.

Variables in the form of percentages were analyzed both untransformed and after being arcsine-transformed. Metrics related to resident mite populations were analyzed when weighted for the number of cells inspected to obtain data. Because neither transformation nor weighting affected the results, we present information from original data.

3. RESULTS

The removal of FKB did not differ between the four types of bees at 6, 12, 24, or 48 h after the brood was killed (Table II and Figure 1). Removal by all 71 colonies averaged 29 % at 6 h, 57 % at 12 h, 83 % at 24 h, and 95 % at 48 h. The manipulation of FKB (i.e., cells uncapped, partially removed, or completely removed) differed between bee types at 12 and 24 h after freezing, but not at 6 or 48 h (Figure 1). FKB cells were manipulated more by VSH bees than by control bees, while manipulation by F_1 VSH and FKB hygienic bees generally was intermediate.

Responses toward brood infested by *V. destructor* varied by type of bee (Table II and Figure 2). A greater percentage of infested pupae was removed after 1 week by VSH and F_1 VSH colonies (mean, 59 %) than by FKB hygienic and control bees (mean, 9 %). The percentage of recapped cells on the infested combs was less in control colonies (17 %) than for the other three bee types (mean, 50 %).

ANCOVA evaluations of the relationships between hygiene against brood infested with *V. destructor* and hygiene against FKB found no interactions between bee type and the covariate (FKB hygiene), so the analyses proceeded on data from all bee types. The removal of mite-infested brood was not significantly related with the removal of FKB at 6, 12, 24, or 48 h after brood was frozen (Table III). The removal of mite-infested brood was positively related with the percentage of FKB cells that was manipulated (i.e., uncapped, or partially or wholly removed) at 6, 12, and 24 h after freezing (Table III). However, the biological relevance and practical value of this relationship appear very limited (see Section 4). The removal of mite-infested brood and the recapping of sealed cells were not related.

Two of five variables related to *V. destructor* infestation in resident brood combs varied according to the type of bee (Table II and Figure 3). The percentage of mite-infested brood cells ranged from 1.3 % in colonies of VSH bees to 17.7 % in colonies of control bees (Figure 3a). Infestation in FKB

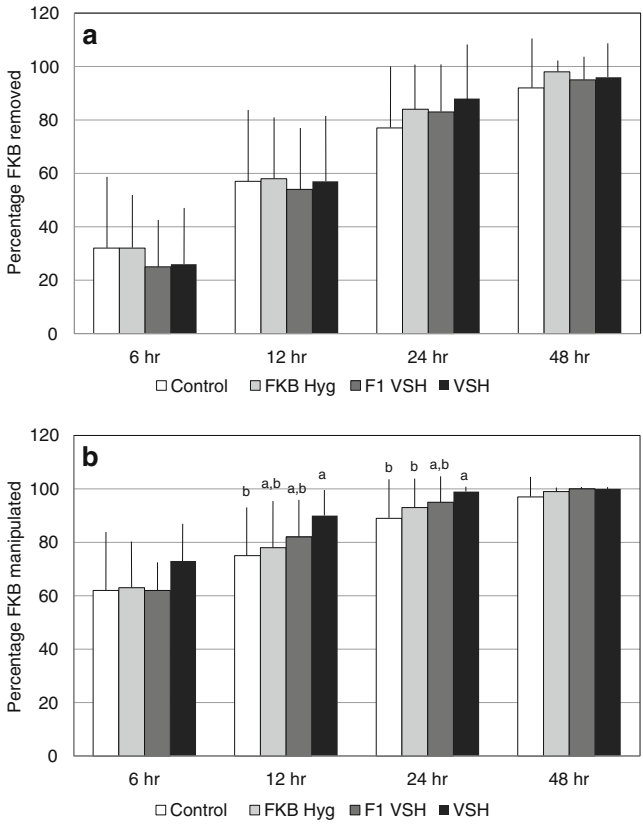

Figure 1. Responses of four types of honeybees to FKB at four times after brood was killed. **a** Mean percentage of FKB removed. **b** Mean percentage of FKB manipulated, i.e., either uncapped, partially removed, or completely removed. *Error bars* are 1 SD. *Means with different letters* differ at $P \leq 0.05$.

hygienic colonies was statistically similar to those in controls and F_1 VSH, and F_1 VSH were similar to FKB hygienic and VSH. The percentage of recapped cells in resident brood was greater in VSH colonies (63 %) than in colonies of the other three bee types (mean, 42 %; Figure 3e). There were no differences between bee types for the percentage of infertile foundress mites (mean, 19 %; Figure 3b), the percentage of dead foundress mites (mean, 4 %; Figure 3c), or mite fecundity (mean, 3.1 offspring per foundress mite; Figure 3d).

4. DISCUSSION

There was varying congruence of the hygienic responses to *V. destructor*-infested brood and FKB among the four types of bees, i.e., the relationship of these responses within a bee type was inconsistent between bee types. In general, there was a high hygienic response to FKB: an average of 77–88 % of dead brood was removed within 24 h by each type. About 50 % of FKB hygienic and VSH colonies and 25 % of control and F_1 VSH colonies could be classified as useful breeders based on having removed ≥95 % of FKB within 24 h (e.g., Spivak et al. 2009). This is notable because of the varying breeding approaches represented among the bee types. FKB (i.e., Minnesota) hygienic bees have been selected intensively for the removal of FKB, and their performance here was in accordance with other recent observations (Spivak et al. 2009). VSH bees have never been selected using FKB

Table II. ANOVA results for effects related to the removal and manipulation of FKB, removal of brood infested with *V. destructor*, and population metrics for *V. destructor* in resident brood.

Variable	Effect	*F*	*df*	*P*
Removal of FKB				
6 h	Type of bee	0.99	3, 65	0.401
	Replicate	8.06	1, 62	0.006
	Type of bee×rep	1.78	3, 62	0.161
12 h	Type of bee	0.19	3, 65	0.902
	Replicate	7.44	1, 62	0.008
	Type of bee×rep	2.53	3, 62	0.066
24 h	Type of bee	1.22	3, 66	0.308
	Replicate	1.55	1, 63	0.218
	Type of bee×rep	1.58	3, 63	0.158
48 h	Type of bee	1.00	3, 38	0.403
	Replicate	1.06	1, 5	0.349
	Type of bee×rep	0.51	3, 5	0.694
Manipulation of FKB				
6 h	Type of bee	1.90	3, 66	0.138
	Replicate	15.54	1, 63	<0.001
	Type of bee×rep	2.37	3, 63	0.079
12 h	Type of bee	3.45	3, 66	0.021
	Replicate	10.01	1, 63	0.002
	Type of bee×rep	2.55	3, 63	0.064
24 hr	Type of bee	3.53	3, 65	0.020
	Replicate	1.02	1, 63	0.317
	Type of bee×rep	1.32	3, 63	0.276
48 h	Type of bee	1.59	3, 65	0.202
	Replicate	0.01	1, 5	0.940
	Type of bee×rep	0.33	3, 5	0.802
Removal of brood infested with *V. destructor*				
Removal of mite-infested brood	Type of bee	15.05	3, 56	<0.001
Percentage of recapped cells on comb tested for the removal of *V. destructor*	Type of bee	7.44	3, 56	<0.001
Mite population factors in resident brood				
Percentage of infested brood	Type of bee	12.78	3, 54	<0.001
Percentage of recapped cells	Type of bee	6.31	3, 4	0.055
Percentage of infertile foundresses	Type of bee	1.47	3, 3	0.379
Percentage of dead foundresses	Type of bee	0.33	3, 3	0.806
Fecundity per single foundress	Type of bee	2.63	3, 4	0.181

assays. There has been some recent selection for FKB hygiene in the control stocks we used (Spivak 2011). F_1 VSH colonies were phenotypically intermediate between VSH and control bees

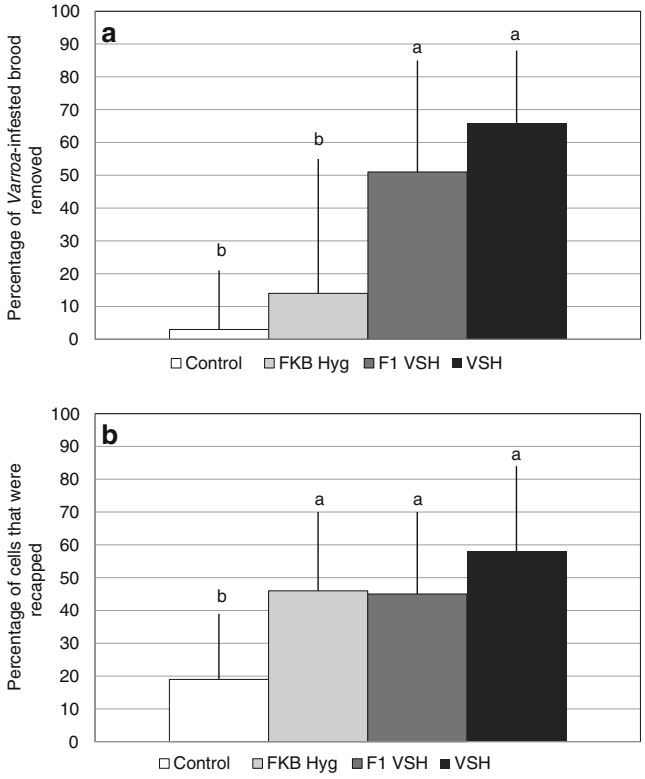

Figure 2. Responses of four types of honeybees to combs of mite-infested brood introduced for one week. **a** Mean percentage of infested brood removed. **b** Mean percentage of recapped brood cells. *Error bars* are 1 SD. *Means with different letters* differ at $P \leq 0.05$.

in response to FKB; this parallels the apparently additive genetics underlying the hygienic response of VSH-based bees toward *V. destructor* (Harbo and Harris 2001).

Table III. Results of partial correlations (ANCOVA) between the removal of *V. destructor* and hygiene against FKB.

Variable		*F*	*df*	*P*
Percentage of FKB removed	6 h	0.11	1, 52	0.740
	12 h	2.42	1, 52	0.126
	24 h	3.07	1, 52	0.086
	48 h	0.54	1, 52	0.464
Percentage of FKB manipulated	6 h	4.59	1, 52	0.037
	12 h	5.80	1, 52	0.020
	24 h	10.47	1, 52	0.002
	48 h	2.30	1, 52	0.135

The removal of mite-infested brood was much more variable among the bee types than the removal of FKB. VSH had a high response, as expected given their selection history. Neither FKB hygienic nor control bees have been selected for response to *V. destructor*.

A regression analysis approach indicated a relationship between the removal of mites and the percentage of FKB that was manipulated at 6, 12, and 24 h. However, the relationship appears to have limited biological or practical relevance with regard to selecting for mite resistance because of inherent variation. For example, the relationship was strongest at 24 h after freezing ($F=10.47$, $df=1, 52$, $P=0.002$). An examination of the data (Figure 4) suggests that 10–15 % of colonies were relatively poor at both manipulating FKB and

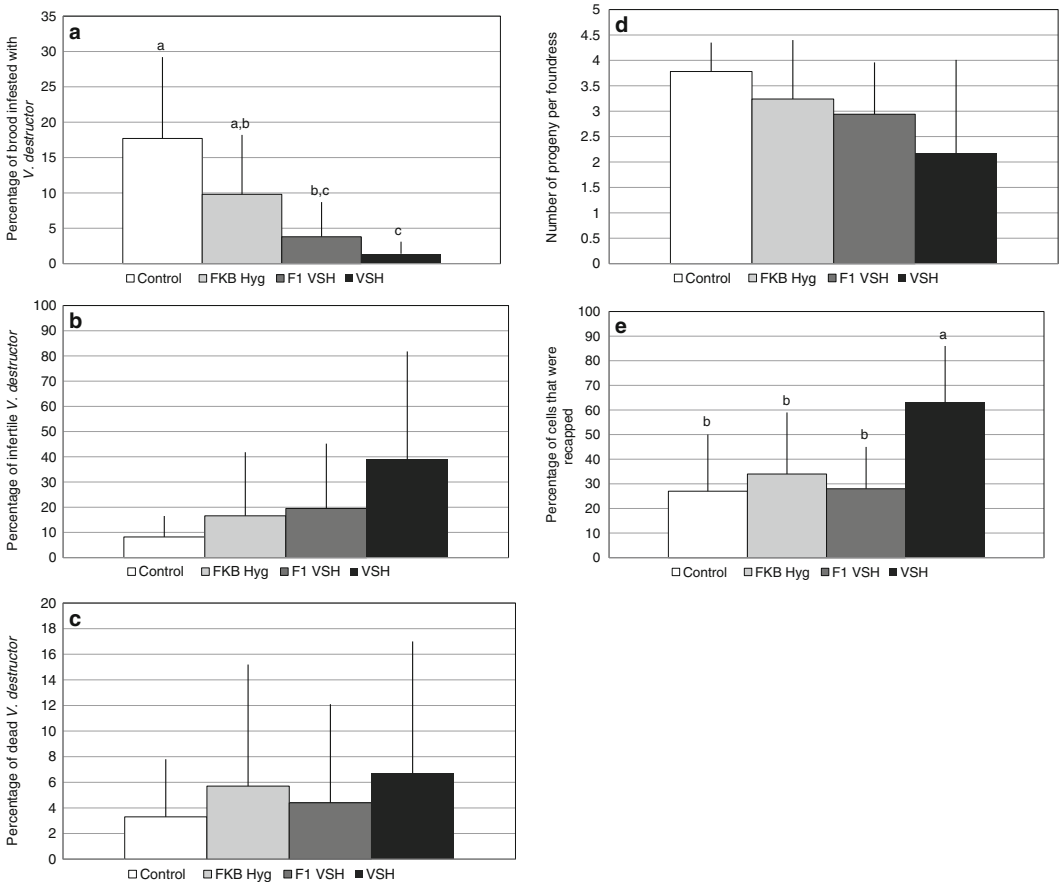

Figure 3. Variables characterizing populations of *V. destructor* in resident combs of colonies of four types of honeybees. **a** Percentage of cells infested with *V. destructor*. **b** Percentage of cells of late-stage pupae with an infertile foundress mite. **c** Percentage of cells of late-stage pupae with a dead foundress mite. **d** Number of progeny per foundress mite. **e** Percentage of cells of late-stage pupae that were recapped. *Error bars* are 1 SD. *Means with different letters* differ at $P \leq 0.05$.

removing *V. destructor*, but at any higher values of 24-h manipulation, colonies varied greatly in their removal of mite-infested brood. Testing for the manipulation of FKB at 24 h thus apparently has little power to reliably discover colonies that are exceptional removers of *V. destructor*. Pearson's correlation analysis supports this interpretation in that $R^2 = 0.11–0.25$ for the significant correlations, i.e., less than one fourth of the response to *V. destructor* is explained by the manipulation of FKB. Our results do not match those of an earlier study that found a relationship between 48-h removal of FKB and the level of VSH in commercial bees (Strange and Calderone 2009). In the earlier study, however, VSH [measured in that study as the percentage of non-reproductive (infertile or dead) mites] was low relative to the more highly selected VSH bees we used (8–15 versus 46 %).

The recapping frequency of cells is of interest as a possible selection measurement for VSH activity because it is relatively easy to measure. The removal of mite-infested brood was not strongly associated with recapping in either resident brood ($F = 2.24$, $df = 1, 51$, $P = 0.141$) or introduced brood ($F = 2.69$, $df = 1, 51$, $P = 0.107$) according to

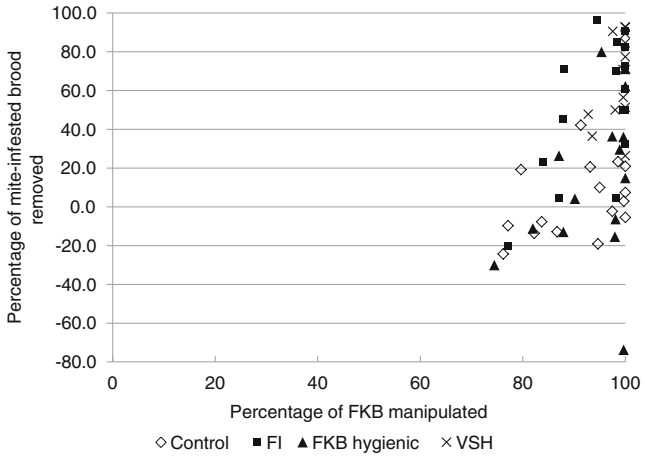

Figure 4. Removal of brood infested with *V. destructor* versus manipulation of FKB at 24 h after freezing for 61 colonies of four bee types. Manipulated cells include those in which brood was uncapped, partially removed, or completely removed. ANCOVA indicated that this relationship was significant (F=10.47, df=1, 52, P=0.002).

ANCOVA. Yet, a plot of the data of recapping in resident brood (Figure 5) suggests some possibility of finding colonies that have high response against *V. destructor* when the recapping frequency is high (approximately >70 %) and low response against *V. destructor* when the recapping frequency is low (approximately <10 %). Villa et al. (2009) suggested that low recapping rates could be used to cull colonies that have low VSH activity, and the current data support that suggestion. Unfortunately, even combining recapping with the manipulation of FKB does not facilitate easy selection for the removal of mite-infested brood. For example, among the 26 colonies which manipulated 100 % of brood within 24 h, the 12 that had higher than average recapping (i.e., >38 % recapped cells) removed 47 % of mite-infested brood,

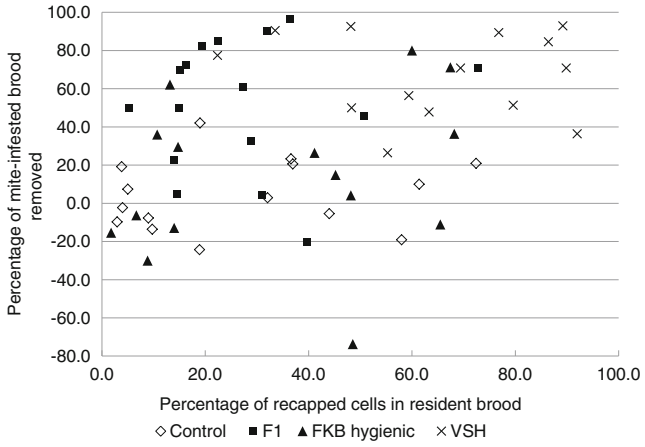

Figure 5. Removal of brood infested with *V. destructor* versus recapping frequency in resident brood for 61 colonies of four bee types. ANCOVA indicated that this relationship was not significant (F=2.24, df=1, 51, P=0.141).

while those with below average recapping removed 52 % of mite-infested brood.

The lack of a strong phenotypic association between FKB hygiene and VSH is reflected in genotypic evidence to date. Current understanding is that about six quantitative trait loci (QTL) relate to components of FKB hygiene (Lapidge et al. 2002; Oxley et al. 2010) and two QTL relate to the uncapping or removal for VSH (Tsuruda et al. 2012). Each of these studies identified QTL on chromosome 9, but the regions do not appear to be the same. Furthermore, there is no apparent overlap in peptide signatures between VSH and FKB hygiene (Parker et al. 2012). This information suggests that VSH bees may respond to *Varroa*-related stimuli that are somewhat distinct from—and likely in addition to—stimuli that regulate a more generalized response to dead brood.

Our results support an earlier finding that a greater percentage of mite-infested pupae are removed by VSH bees than by Minnesota hygienic bees (Ibrahim and Spivak 2006). We cannot say whether all strains of these two types of bees will behave similarly as we tested FKB hygienic from only one breeding source of Minnesota hygienic bees and tested very highly selected VSH. As was noted by Ibrahim and Spivak (2006), it is likely that the somewhat different responses to *V. destructor* are based on the different selection criteria. VSH bees are selected directly for activity affecting *V. destructor*, while Minnesota hygienic bees are selected for response to the more general situation of dead brood (via FKB). While it is clear that FKB hygiene confers some ability to remove *Varroa*, the response toward mites can be somewhat inconsistent (Spivak 1996; Spivak and Gilliam 1998b) and apparently stimulus-dependent (more hygiene against two versus one mite; Boecking and Drescher 1992).

Screening for the removal of FKB as a means to select for resistance to *V. destructor* has been recommended based on prior observations that the hygienic responses toward FKB and *V. destructor* are somewhat related (Boecking and Drescher 1992; Spivak 1996). Our results using a variety of bee types having different selection histories do not support this recommendation; many colonies that had good hygiene against FKB had poor hygiene against *V. destructor*. A simple, effective way to select for strong VSH-based resistance remains elusive.

ACKNOWLEDGMENTS

David Dodge, Victor Rainey, and Daniel Winfrey (USDA-ARS) provided excellent technical assistance. Amy Weeks (Beeline Apiary) created the Minnesota hygienic bees in cooperation with Jeff Hull (Hull Apiaries). Debbie Boykin (USDA-ARS) gave statistical advice.

Adéquation variable des réponses 'hygiéniques' vis-à-vis de *Varroa destructor* et de couvain congelé parmi différents types d'abeilles

Apis mellifera / **hygiène** / ***Varroa destructor*** / **résistance aux acariens** / **élevage**

Unterschiedliche Übereinstimmung der hygienischen Antwort auf *Varroa destructor* und gefriergetötete Brut bei unterschiedlichen Typen von Honigbienen

Apis mellifera / **Hygiene** / ***Varroa destructor*** / **Milbenresistenz/Züchtung**

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