

ORIGINAL RESEARCH ARTICLE

Simplified methods of evaluating colonies for levels of Varroa Sensitive Hygiene (VSH)

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Summary

Varroa Sensitive Hygiene (VSH) is a trait of honey bees, *Apis mellifera*, that supports resistance to *Varroa destructor* mites. Components of VSH were evaluated to identify simple methods for selection of the trait. Mite population growth was measured in colonies with variable levels of VSH in two field trials using 24 and 16 colonies. Mite population growth was significantly lower in VSH and hybrid colonies than in control (i.e., unselected) colonies. In resident brood with mite infestations below 5%, the percentage of uncapped pupal cells did not differ significantly among VSH, hybrid and control colonies, but the percentage of recapped cells was highest in VSH colonies ($P = 0.03$). When brood from more highly infested colonies (9-49% of pupae infested) was introduced for forty hours, VSH colonies reduced infestation more than control colonies ($P < 0.01$) but final mite fertility was similar ($P = 0.12$). When infested brood was exposed in colonies for one week, VSH colonies reduced both mite fertility ($P = 0.05$) and mite infestation ($P = 0.02$). When highly infested brood was exposed to a subset of colonies for two hours, control colonies uncapped no or few cells while uncapping in VSH colonies was variable but on average was much higher. Mite population growth in individual colonies was negatively correlated with reduced infestation after forty hours of brood exposure and with reduced mite fertility after one week. The simpler and shorter-term measures (relative to measuring mite population growth) of uncapping, recapping, and reductions in infestation and mite fertility may facilitate selection of VSH by more bee breeders.

Métodos sencillos para evaluar los niveles de higiene sensible a varroa (VSH) en colonias

Resumen

La higiene sensible a varroa es un carácter de la abeja *Apis mellifera*, que apoya la resistencia al ácaro varroa, *Varroa destructor*. Se han evaluado los componentes de VSH para identificar métodos sencillos para seleccionar dicho carácter. El crecimiento de la población de ácaros varroa fue medido en colonias con niveles variables de VSH en dos ensayos de campo usando 24 y 16 colonias. El crecimiento de la población del ácaro fue significativamente más bajo en VSH y en colonias híbridas que en las colonias control (i.e. no seleccionadas). En cría resistente con infestaciones por debajo del 5%, el porcentaje de celdas de pupas no operculadas no fue significativamente diferente entre las colonias VSH, las híbridas y las control, pero el porcentaje de celdas reoperculadas fue mayor en colonias VSH ($P = 0.03$). Cuando la cría de colonias más infestadas (9-49% de pupas infestadas) se introdujo durante 40 h, las colonias VSH redujeron la infestación más que las colonias control ($P < 0.01$) pero la fertilidad final del ácaro fue similar ($P = 0.12$). Cuando la cría infestada se expuso en colonias durante una semana, las colonias VSH redujeron tanto la fertilidad del ácaro ($P = 0.05$) como la infestación del ácaro ($P = 0.02$). Cuando cría muy infestada fue expuesta a un conjunto de colonias durante 2 h, las colonias control desopercularon muy pocas celdas o ninguna, mientras que la desoperculación fue variable en colonias VSH pero no mucho mayor de media. El crecimiento de la población del ácaro en colonias individuales mostró una correlación negativa con la infestación reducida después de 40 h de exposición de la cría y con la reducción de la fertilidad del ácaro después de una semana. Las medidas más sencillas y de menor duración (relativas a las medidas del crecimiento de la

población de ácaros) de desoperculado, reoperculado y reducción de la infestación y de la fertilidad del ácaro pueden facilitar la selección de VSH para más criadores de abejas.

Keywords: VSH, Varroa Sensitive Hygiene, hygienic behaviour, *Apis mellifera*, *Varroa destructor*

Introduction

Selecting honey bees, *Apis mellifera*, for resistance to *Varroa destructor* mites produced colonies with high expression of the trait 'Varroa Sensitive Hygiene' (VSH; Harbo and Harris, 2005; Ibrahim and Spivak, 2006). VSH describes more precisely the trait formerly known as SMR (suppressed mite reproduction; Harris 2007). VSH is the ability to detect and remove mite infested brood. This hygiene also yields high infertility among mites that remain (Harbo and Harris, 2005; Ibrahim and Spivak, 2006; Harbo and Harris, 2009). Colonies with VSH reduce mite populations during short term experiments (Harbo and Harris, 2001) and often keep mite populations below action thresholds and thus delay the need for acaricide treatments in field colonies (Delaplane *et al.*, 2005; Ibrahim *et al.*, 2007; Ward *et al.*, 2008). The trait is widespread (Boecking and Drescher, 1991; Spivak, 1996; Guerra *et al.* 2000; Vandame *et al.* 2002) and it may be possible to intensify VSH in diverse honey bee populations that are desirable for beekeeping.

Selection for resistance to *V. destructor* that resulted in VSH was based largely on measuring mite population growth in colonies started from a common pool of worker bees but having queens of different genetic backgrounds (Harbo and Hoopingartner, 1997; Harbo and Harris, 1999, 2001; Delaplane *et al.*, 2005). This method offers precision, but is labour intensive and time consuming (e.g., two months minimum). Simplified or faster methods of evaluating VSH would facilitate selection, and some methods show promise. Selection for general hygiene (expressed as removal of freeze-killed brood) yielded moderate levels of VSH (Ibrahim and Spivak, 2006). Responses to mite infested brood introduced into VSH colonies (Harbo and Harris, 2005; Harris, 2007, 2008; Harbo and Harris, 2009) typically include reductions in mite infestation and in mite fertility (Harbo and Harris, 2005; Ibrahim and Spivak, 2006). The proportion of sealed brood cells that are uncapped in field colonies may be an indicator of VSH (Correa-Marques and De Jong, 1998; Villegas and Villa, 2006; Harris, 2008).

Field trials attempting to relate mite population growth to some potentially VSH related variables have yielded inconsistent results. Mite infertility was a significant factor in the original test (Harbo and Hoopingartner, 1997), in one of two tests with Africanized bees (Arechavaleta-Velasco and Guzman-Novoa, 2001; Mondragon *et al.*, 2005) but not in other tests with European bees (Lodesani *et al.*, 2002; Villa *et al.*, unpublished observations). General hygiene was related in some tests (Ibrahim *et al.*, 2007) but not others

(Arechavaleta-Velasco and Guzman-Novoa, 2001; Mondragon *et al.*, 2005). The association of mite population growth with other variables potentially related to VSH has not been established.

We investigated simplified techniques to identify VSH by evaluating growth of mite populations in standardized tests and then in the same colonies measuring components of VSH. Our goal was to find either improved methods for scientific breeding that would shorten generation time, or simpler approaches that queen breeders could use for easier selection.

Materials and methods

Field trials to estimate mite population growth

Mite population growth was measured in two field trials with 24 and 16 colonies in Baton Rouge, Louisiana, USA. Colonies had a wide range of levels of VSH. Colonies with the highest level of VSH were from our research and breeding population. The lowest level of VSH came from control colonies maintained by us because of their low levels of VSH (Trial 1) or from unselected commercial colonies (Trial 2). Colonies in each trial were initiated by subdividing a mix of ca. 30 kg of mite infested worker bees into uniform packages and introducing test queens (Harbo and Hoopingartner, 1997; Harbo and Harris, 1999). Colonies in Trial 1 were established in May 2007 with 8 VSH (VSH × VSH), 8 hybrid (VSH × Control), and 8 control (Control × Control) queens produced with instrumental insemination. Trial 2 was established in June 2007 with 8 instrumentally-inseminated VSH × VSH queens and 8 naturally mated commercial Italian queens (referred to as control colonies). Initial mite populations were determined by taking a subsample of ca. 50 g of workers, washing mites from bees with 70% ethanol, and calculating the mite population in each colony as weight of bees in package × (mites in subsample/weight of bees in subsample). After ca. 10 weeks, the following measurements were taken in each colony: hive weight with and without adult worker bees, infestation of adult workers based on a sample of ca. 150 g workers, area of sealed brood and mite infestation of 200 capped worker brood cells. The final total adult mite population in each colony was calculated as [total bee weight × (mites in sample / bee weight in sample)] + [total sealed brood cells × (mites / 200 brood cells)]. Growth of worker bee and mite populations in each colony was calculated as the ratio of final to initial populations.

Table 1. Least squares means (\pm s.e.) of mite population growth and of components of VSH measured in two trials using VSH, hybrid and control colonies (Trial 1) or VSH and control colonies (Trial 2). ANOVAs for each variable include genetic type and trial as fixed effects; interactions were not significant. Results for the effects of genetic type are indicated. Within a variable, values followed by different letters differ at $P < 0.05$. ¹These variables had non-normal distributions, were transformed to \sqrt{x} for analysis and converted back to original scale for presentation.

Variable	VSH	Hybrid	Control	Effect of Type		
	(n = 14-16)	(n = 6-7)	(n = 14-16)	df	F	P > F
Mite population growth (Final / Initial)	0.40 \pm 0.14 b	0.55 \pm 0.23 ab	1.07 \pm 0.14 a	2, 35	5.98	0.0058
VSH components in sealed brood of test colonies at end of field trial:						
Uncapped cells (per thousand) ¹	0.55 \pm 0.011	0.70 \pm 0.031	0.29 \pm 0.011	2, 35	3.74	0.2428
Recapped cells (%) ¹	38 \pm 0.3 a	19 \pm 0.8 ab	17 \pm 0.3 b	2, 35	3.74	0.0337
VSH components in brood introduced to test colonies for different time periods:						
Forty hour reduction in infestation (%)	61 \pm 8 a	24 \pm 14 b	8 \pm 8 b	2, 30	10.10	0.0004
Mite fertility after forty hours (%)	78 \pm 4	75 \pm 6	87 \pm 4	2, 30	2.28	0.1193
One week reduction in infestation (%)	68 \pm 10 a	34 \pm 16 ab	29 \pm 10 b	2, 29	4.25	0.0241
Mite fertility after one week (%)	64 \pm 5 a	82 \pm 9 ab	83 \pm 5 b	2, 29	3.31	0.0506

Field measures of VSH related variables

The number of uncapped brood cells, the diameter of the hole in each uncapped cell and the life stage of the pupa in each uncapped cell were recorded in the field for each colony at the time that final brood areas were measured in each trial. Large uncappings (more than half the diameter of the cell) in cells with white-, pink- and purple-eyed pupae (Jay, 1962) were used for analysis because those are the ages most commonly targeted by VSH (Harris, 2007). The uncapping rate for each colony was calculated as the number of uncapped cells per total number of sealed cells. Bees sometimes recap cells that were previously uncapped; recapped cells have circular areas covered by wax but lacking the silk lining of a cocoon (Boecking and Spivak, 1999). The percentage of recapped cells was obtained by microscopic examination (5-10X) of 100 capped cells.

Short-term measures of VSH components

At the end of each trial of mite population growth, each colony was tested by introducing a comb with infested brood from a pool of unrelated infested colonies (mean sealed brood area = 490 cm²; mean infestation of introduced combs = 21%; range = 9-49%). In one test, white- to pink-eyed pupae were left in test colonies for *ca.* forty hours. In a second test, prepupal brood was introduced and exposed for one week. Prior to introducing the comb into a test colony, its area of sealed brood and the infestation of 100 (Trial 1) or 200 cells (Trial 2) were measured. After introduction into test colonies for the required duration, 200 pupae (development at purple eyes with early tanning of the cuticle) were examined microscopically for infestation and fertility (i.e., production of any offspring) of mites. The relative decrease in the infestation of an introduced comb was estimated as (initial percentage infestation – final percentage

infestation) / initial percentage infestation before exposure.

In April 2008, a group of 12 surviving colonies that showed a broad range of mite population growth in the field trials were used for two hour exposures of brood from highly infested (10-35% of pupae infested) colonies. A section of one side of a comb was protected from hygienic activity by covering it with a 2 mm wire cage (*ca.* 15 x 10 cm). Immediately after retrieval, all uncapped pupal cells were counted in the field. Combs were then examined microscopically to assess the infestation in all uncapped cells and in two transects of 50 cells (one inside and one outside the wire cage). The specificity of uncapping was estimated as the number of adult mites in uncapped cells / estimated number of adult mites in the uncaged brood area. Each colony was tested three times.

Statistical analyses

The fixed effects of genetic type and trial on mite population growth and VSH related variables were evaluated with analysis of variance (ANOVA). Other factors (initial brood infestation as covariates and the interaction between trial and type) had no significant effect and were excluded from the models. Counts of uncapped and recapped cells in each colony were transformed (\sqrt{x}) to correct for lack of normality and are reported in the original scale after analyses. For measures of final mite fertility, the number of mites in the sample for each colony was used as a weighting factor in the ANOVA.

Uncapping and specificity towards mite infested cells in brood combs introduced for two hours initially was analyzed as a random block design with test as a random block. In a subsequent analysis, values from the three tests were averaged for each colony and analyzed as a single factor ANOVA.

Because the average mite population growth differed between the

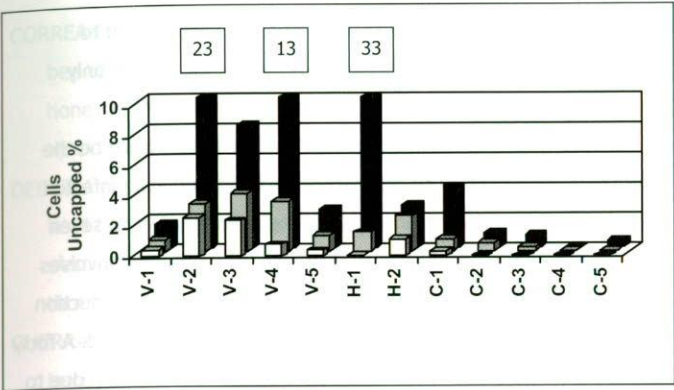


Fig. 1. Percentage of pupal cells which were uncapped after infested brood was exposed for two hours in 12 colonies that varied in mite population growth the previous season. Each colony was tested three times and results were placed in ascending order for each colony in the graph. Numerical values are indicated for the three highest observations for colonies V2, V4 and H1 which appear truncated at 10%.

two trials, the mite population growth in each colony was indexed to the maximum and minimum growth rates in the respective trial $[(x - \text{minimum}) / (\text{maximum} - \text{minimum})]$. This places the growth rate of each colony on a common scale from 0 to 1. Pearson's correlation coefficients were used to measure the association between the index of mite population growth in each colony and individual VSH related variables, and between all pairs of VSH related variables.

Results

Mite population growth

Overall, mite populations in VSH colonies decreased by 60% while those in control colonies increased slightly (Table 1, $P < 0.01$). Growth of mite and bee populations varied between the two trials. In Trial 1 (May to July), worker bee populations in individual colonies

increased two to four fold, while average mite populations decreased to half in pure VSH colonies, did not change in hybrid colonies and increased by half in susceptible control colonies. In Trial 2 (June to August), bee populations decreased or increased only moderately, while mite populations decreased by 75% in VSH bees and by 42% in control bees.

Field or simplified VSH related variables

Field counts of uncapped cells in resident brood at the end of each trial were very low (less than one per thousand) and did not differ between groups ($P = 0.24$). The percentages of recapped cells were highly variable but differed between genetic groups in both trials ($P = 0.03$). Averaged over both trials, 38% of cells in VSH colonies and 18% of cells in hybrid and control colonies were recapped. Mite infestations were low in all colonies when uncapping and recapping were measured [mean (range) of 1.7% (0-5.5%) and 2.9% (0.7 -7.3%) in Trials 1 and 2, respectively].

Short term measurements of VSH related components

When infested brood was placed in colonies for forty hours, the reduction in infestation differed significantly between genetic groups in both trials. Brood infestation decreased by 61% in VSH, 24% in hybrid and 8% in control colonies ($P < 0.01$). The percentage of fertile mites after a forty hour exposure of brood in colonies did not differ significantly between groups ($P = 0.12$).

When infested brood was exposed to test colonies for one week, the relative decreases in infestation differed between genetic groups: 68% in VSH; 34% in hybrid; and 29% in control colonies ($P = 0.02$). This longer period of exposure produced differences in the final fertility of mites: 64% in VSH; 82% in hybrid, and 83% in control colonies ($P = 0.05$).

Table 2. Correlation (Pearson's rho and significance) between mite population growth (indexed to growth in each trial) and combinations of VSH variables in individual colonies ($n = 33$ -39 for different pairs of variables) in two trials. Associations with $P > 0.10$ are indicated as ns. ¹Mite population growth in each colony was scaled to an index between 0 and 1 represented by the lowest and highest growth, respectively, in the trial.

Variable	Uncapped cells	Recapped cells	Forty hour reduction in infestation	Mite fert. after forty hours	One week reduction in infestation	Mite fert. after one week
Mite population growth ¹	-0.20 ns	-0.22 ns	-0.50 0.002	+0.22 ns	-0.29 0.094	+0.38 0.030
Uncapped cells	-	+0.39 0.013	+0.29 0.099	-0.21 ns	-0.00 ns	-0.07 ns
Recapped cells		-	+0.24 ns	-0.14 ns	+0.29 ns	-0.33 0.057
Forty hour reduction in infestation			-	-0.32 0.064	+0.39 0.027	-0.38 0.030
Mite fertility after forty hours				-	-0.08 ns	+0.23 ns
One week reduction in infestation					-	-0.23 ns

A two hour exposure of infested comb to 12 colonies having a broad range of mite population growth produced uncapping more than 100 fold higher than measured earlier in the field in the resident brood of each colony. Control colonies exhibited consistently low levels of uncapping in three repeated tests. VSH and hybrid colonies were highly variable at uncapping (Fig. 1). When averaged across three tests, uncapping rates were greater in VSH colonies (5%) than in control colonies (0.6%) ($P = 0.03$). Similarly, the average specificity towards mite infested cells in brood combs introduced into each colony (mites in uncapped cells / estimated mites in brood) was greater in VSH colonies (13%) than in control colonies (2%) ($P = 0.03$).

Correlations between mite population growth and VSH related variables

Two VSH related variables were strongly correlated with mite population growth: reduction in brood infestation after unrelated brood was introduced in colonies for forty hours and final fertility of mites after brood was placed in colonies for one week (Table 2). A relationship also was suggested between the one week reduction in infestation and reduced mite population growth. Only 3 out of 15 possible pairings of VSH-related variables were significantly correlated. Numbers of uncapped cells and recapped cells in resident comb were significantly correlated. Reduction in infestation after forty hours correlated with reduction in infestation and with final fertility after brood was introduced for one week.

Discussion

The measurement of mite population growth in colonies begun from a common pool of infested bees was laborious but clearly separated genetic sources in both trials. The results provided a standard against which to evaluate a variety of techniques to identify VSH. The three simplest techniques varied in their usefulness and reliability. The first, estimating the proportion of uncapped pupal cells in resident comb, is not recommended because of its variability and very low rate of expression when mite infestations are low (Villegas and Villa, 2006; Harris, 2008). The second, measuring the percentages of recapped pupal cells, is more useful because it can be found at very high levels even at low infestations. The high variability among colonies of the same type, however, suggests that it may be limited for use only as an initial screening tool. For example, while instantaneous recapping rates in control colonies were below 54%, those in VSH colonies ranged from about 2% to 100 %. The third technique, estimating uncapping rates in an infested comb introduced with test bees for a few hours, has the advantage of not needing any microscopic examination, but does require a supply of infested brood of an age targeted by VSH (i.e., white- to purple- eyed pupae with no tanning of

cuticle; Harris, 2008). These last two techniques would tend to identify colonies with high levels of VSH, but not those with only moderate or low levels of the behaviour.

An advanced step for bee breeders and scientists would be the more complicated but more reliable method of introducing infested comb into test colonies for a period of approximately two to seven days and measuring changes in infestation. This technique involves microscopic evaluations of infestations before and after introduction into test colonies, and also requires a supply of infested brood. A forty hour exposure of brood reliably detects changes in infestation due to removal of infested brood. After a longer, one week exposure, VSH activity is strongly indicated by reductions in infestation and in mite fertility. The reliability of the measures of reduction in infestation after forty hours and mite fertility after one week is underscored by their correlation with mite population growth.

We have documented variables of potential use in measuring VSH that ranged from simple but less reliable to complex and more reliable. Selection programs targeting VSH will have to gauge the best fit for their own resources and goals. The simpler techniques could be applied in an initial screening of a broad population of colonies. The higher reliability obtained from evaluating changes in introduced infested brood would need to be justified to commit the use of resources, labour and time. All of these methods are, however, less involved than measuring changes in mite population growth and provide useful options for selection and breeding.

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