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ORIGINAL RESEARCH ARTICLE

Repeatability of measurements of removal of mite-infested brood to assess Varroa Sensitive Hygiene

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Varroa Sensitive Hygiene (VSH) is a useful resistance trait that honey bee (*Apis mellifera*) breeders could increase in different populations with cost-effective and reliable tests. We investigated the reliability of a one-week test estimating the changes in infestation of brood introduced into colonies highly selected for VSH and into unselected colonies. We repeated tests on the same 32 colonies up to five times and compared variation within colonies and between colonies of the two types. As expected, selected VSH colonies decreased infestation much more than unselected colonies (−77 vs. −5%, $p < 0.0001$) and also produced final mite populations in the introduced comb with higher infertility (41 vs. 16%, $p = 0.002$). The variance in percentage decrease in infestation of introduced brood within colonies was higher in the unselected group. These variances can be used to estimate the number of repeated tests required to detect true differences between colonies. In an already selected VSH population, potentially useful differences between colonies (*ca.* 45%) can be detected with only one measurement per colony. Multiple measurements of colonies (up to 5 replications) are required to detect similar differences between colonies in less selected and more variable populations.

Repetibilidad de las mediciones de la eliminación de la cría infestada por ácaros para evaluar la higiene sensible a Varroa

La higiene sensible a Varroa (VSH por sus siglas en inglés) es un rasgo de resistencia útil que los criadores de abejas melíferas (*Apis mellifera*) podrían aumentar en diferentes poblaciones con pruebas costo-efectivas y confiables. Se investigó la fiabilidad de una prueba de una semana para estimar los cambios en la infestación de la cría introducida en las colonias seleccionadas para VSH y en colonias no seleccionadas. Repetimos pruebas en las mismas 32 colonias hasta cinco veces y comparamos la variación dentro de las colonias y entre colonias de los dos tipos. Como se esperaba, las colonias VSH seleccionadas disminuyeron la infestación mucho más que las colonias no seleccionadas (−77% frente a −5%, P <0,0001) y también produjeron poblaciones finales de ácaros en el cuadro introducido con mayor infertilidad (41% vs. 16% 0.002). La variación en la disminución porcentual en la infestación de la cría introducida dentro de las colonias fue mayor en el grupo no seleccionado. Estas varianzas pueden usarse para estimar el número de pruebas repetidas requeridas para detectar verdaderas diferencias entre colonias. En una población de VSH ya seleccionada, las diferencias potencialmente útiles entre colonias (aproximadamente el 45%) se pueden detectar con sólo una medición por colonia. Se requieren mediciones múltiples de colonias (hasta 5 repeticiones) para detectar diferencias similares entre colonias en poblaciones menos seleccionadas y más variables.

Keywords: *Varroa destructor*; honey bees; selection; breeding; hygienic behavior

Introduction

Varroa Sensitive Hygiene (VSH) is a behavioral trait of honey bees (*Apis mellifera*) conferring economically useful and heritable resistance to *Varroa destructor*. Colonies with high expression of the trait reduce and therefore tend to maintain mite populations below economic injury levels (Harbo & Hoopingarner, 1997; Villa, Danka, & Harris, 2009; Ward, Danka, & Ward, 2008). When VSH is expressed at lower levels, mite populations increase but at slower rates, thus delaying the need for treatments (Danka et al., 2012; Delaplane, Berry, Skinner, Parkman, & Hood, 2005; Harbo & Harris, 2001; Ibrahim, Reuter, & Spivak, 2007; Rinderer et al., 2014; Ward et al., 2008). Our laboratory has selected and maintained honey bees with this trait at a high level of expression as a resource for stock improvement and for further research. Some degree of the behavior is found in naturally selected populations (Guerra, Goncalves, & De Jong, 2000) and in populations not exposed to mites (Danka, Harris, & Villa, 2010); thus this trait can be found and improved in new populations. Given continued interest in further selection and *de novo* screening for VSH, we have explored faster, simpler and more reliable methods of phenotypic evaluations (Villa et al., 2009).

Several short-term phenotypic measurements correlated with VSH can be used in selection. Initially, suppressed mite reproduction (SMR, i.e., low fertility or low realized fecundity in resident brood) was the primary selection criterion given that such colonies had decreases in mite populations (Harbo and Hoopingarner, 1997). Other selection programs have

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used the removal of frozen brood given that select colonies also preferentially remove mite-infested brood (Ibrahim & Spivak, 2006; Spivak, 1996). More recently, once the primary mechanism for SMR was ascribed to preferential removal of reproductive mites (Harbo and Harris, 2005), we have focused on measuring decreases in brood infestation after introduction of infested brood frames into test colonies. The resulting fertility of mites after introduced brood is exposed to hygienic activities has produced inconsistent results, particularly when brood is exposed to hygienic activities for only hours or a few days (Danka, Harris, Villa, & Dodds, 2013; Harbo & Harris, 2009; Harris, Danka, & Villa, 2010; Villa et al., 2009). Additionally, colonies with desirable levels of the trait greatly reduce infestation in resident or introduced brood to the point that sampling enough infested cells to obtain reliable estimates of mite fertility becomes difficult or impossible.

We quantified variation in removal rates within both selected and unselected colonies by multiple measurements of responses to introductions of infested brood for one week. Estimates of variability within colonies permit estimation of how the detection of significant differences between colony means varies with the number of replications (Zar, 1981). Calculations like these can be used to decide which colonies are worthwhile to include in a selection program with already high levels of VSH or to initiate selection for VSH in unselected populations with highly variable levels of the behavior.

Materials and methods

We started the experiments with 16 colonies selected for VSH and 16 unselected colonies. VSH colonies came from our breeding population and were headed by queens inseminated with mixed semen from other VSH colonies. Unselected colonies came from a single US commercial source of Italian stock that had not been selected for varroa resistance directly. Workers were all progeny of the queens at the time of the first test. Colonies were housed in single or double brood chambers of Langstroth dimensions.

From August to November 2012, we tested colonies in five repeated tests, initiating between one and five introductions of brood into different colonies a day depending on availability of brood of the right age and infestation level: mostly sealed larvae, prepupae or early white-eyed pupae with between 9 and 29% of cells infested. We followed the procedures described previously for tests lasting one week (Villa et al., 2009). This involves measuring the infestation in 150 cells on one comb and then introducing it into a test colony for one week. After the 1 week exposure to potential hygienic removal, the final infestation is measured in 200 cells of a matching age for the initial cohort (i.e., purple-eyed and tanned pupae). We also classified the reproductive status of each mite as fertile (with at least one progeny) or infertile. Due to queen losses or serious weakening in some colonies, not all colonies could be tested all times. Data were collected on a total of 131 colony test combinations out of a possible total of 160 (32 colonies potentially tested five times each).

The percentage change in brood infestation [100 × (percentage final infestation – percentage initial infestation)/percentage initial infestation] and the infertility of remaining mites (100 × number of infertile mites/number of sampled mites) were compared between the two groups of colonies by analysis of variance using bee type (VSH vs. unselected) as a fixed effect and test (one to five) as a random block. For percentage change in brood infestation, the variance within colonies of each type was calculated separately due to higher variance in the unselected group. The average variance within colonies of each type was used to calculate the relationship between sample sizes (number of tests per colony) and the least significant differences between means (LSD). This 95% confidence interval of differences between means (with $\alpha = 0.05$) has power $(1 - \beta) = 0.50$. Other combinations of α and β depending on need for precision can be used to calculate relationships between sample sizes and minimum detectable differences between means:

$n = 2\sigma^2 \ (Z_{\beta} + Z_{\alpha/2})^2/(\text{difference between means})^2$ (Zar, 1981).

Results

The reduction in brood infestation after one week was significantly greater in VSH colonies than in unselected colonies (−77% ± 3.6 vs. −5% ± 3.8, $F = 220$, $df = 1{,}30$, $p < 0.0001$). Over all tests, the average change in brood infestation of individual colonies did not overlap between the two groups (Figure 1).

The infertility of mites that remained in brood one week after introduction into test colonies was also significantly greater in VSH colonies than in unselected colonies (41% ± 4.1 vs. 16% ± 4.1, $F = 18.7$ $df = 1{,}30$, $p = 0.0002$). Higher removal of mites by VSH reduced the number of mites available for which we could determine infertility levels in 200 cells evaluated at the end of the one-week test. The number of sampled mites in brood introduced into VSH colonies averaged 8.4, while the number in unselected colonies was 32.6. This makes the estimation of infertility in VSH colonies imprecise unless larger numbers of cells are sampled in search for mites. Despite these imprecise measurements, there was a significant negative relationship between infertility (*y*, expressed as percentage infertile mites) and removal (*x*, as percentage change in infestation). The linear regression formula is $y = -0.29\ x + 16.7$ ($R^2 = 0.21$, $p > b < 0.0001$). When observations of fewer than 5 mites per colony are deleted, the relationship becomes stronger ($R^2 = 0.37$).

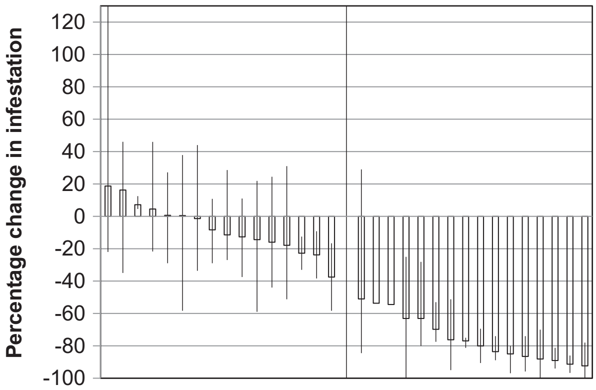

Figure 1. Changes in mite infestation of brood introduced for one week into 16 colonies each of VSH (left of vertical line) and of unselected Italian (right) bees.
Note: Columns indicate the average from up to five repeated tests for a colony, lines indicate the range between the lowest and highest removal rate measured in each colony. Due to queen losses, two VSH colonies only had one measurement (no range indicated). Increases in infestation mostly in unselected colonies are likely the result of sampling error evident when there is very low removal.

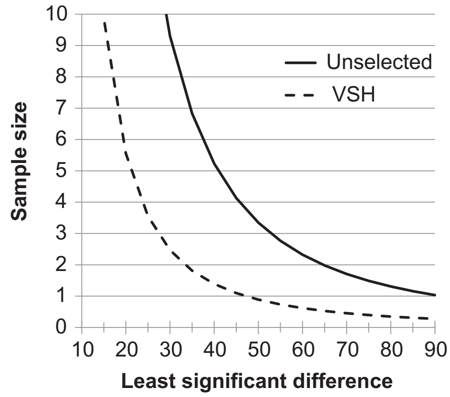

Figure 2. Relationship between number (sample size) and the least significant differences (LSD) between the mean decrease in infestation of brood exposed in colonies for one week.
Note: Differences larger than the LSD for a given number of replications would need to be observed to determine that means are different (α = 0.05).

Discussion

After the discovery that bees with suppressed mite reproduction (SMR) actually had VSH, a number of studies have compared hygienic removal of varroa-infested brood by VSH bees and unselected bees (Danka, Harris, & Villa, 2011; Danka et al., 2010, 2013; Harbo & Harris, 2005; Ibrahim & Spivak, 2006; Villa et al., 2009). Colonies selected for high levels of VSH will reduce the infestation of introduced brood by more than half within one week while many unselected colonies only slightly reduce the level of infestation. Net increases in the estimated infestation of comb in unselected test colonies are likely the result of sampling error using 150 and 200 cells as estimates of initial and final infestation, respectively. These estimation errors are more evident when removal rates are low.

The relationship between rising infertility and decreasing mite infestation previously was found in a population of colonies with the complete range of expression of VSH (Harbo & Harris, 2009). While removal and infertility are related, selection for VSH using infertility is most efficient when mite infestation in brood is relatively high in all test colonies. This can be achieved by placing test queens in infested colonies, allowing for offspring of the queen to replace original workers and soon thereafter evaluating brood before strong VSH activities have reduced the mite population in the most desirable colonies.

Measuring removal of mite-infested brood is more appropriate for situations where the best test colonies have already reduced mite sample sizes in either introduced or resident brood, making estimates of infertility imprecise. However, the variability in removal of introduced mite-infested brood between tests of the same colony that we measured here needs to be considered in any screening process. Variability is higher in unselected colonies than in VSH colonies (average variance: 1104 vs. 300 respectively, with corresponding standard deviations of 33 vs. 17). Given the higher variability between tests of the same colony for the unselected group, the LSD (95% confidence interval of the difference between mean percentage reduction in infestation) between colonies is larger for these colonies than for colonies with high levels of VSH (Figure 2). With 5 replications per colony, differences of 40% between colonies from an unselected population and of 20% from a VSH-like group should be considered statistically valid. With fewer replications, the LSD becomes larger and only extreme colonies would be detected. For unselected colonies, with only one measurement per colony the LSD would be 90% which is close to the largest differences measured between all colonies. In contrast, for VSH colonies the LSD with one measurement per colony would be 45%, allowing for statistical separation between the most extreme colonies in the VSH group. In situations in which some introgression of VSH has occurred, colonies with intermediate means and variances would require intermediate sample sizes between the two lines in Figure 2.

Choosing an approach to select for bees hygienic towards *V. destructor* should consider the characteristics of the initial population of colonies, available techniques and resources, and need for precision. The simplest and fastest techniques allow screening of larger groups of unselected colonies (e.g., testing for removal of freeze-killed brood as a correlate of responses to mites may yield initial progress in selection from populations that are highly variable (Spivak, 1996)), but may not be as useful for populations that manipulate more than 70% of frozen brood in 24 h (Danka et al., 2013). In populations like the latter, more involved techniques may have greater chances of reliably obtaining useful colonies (e.g., measuring responses to mite infested brood (Danka et al., 2010, 2011, 2013; Harbo & Harris, 2005; 2009; Villa et al., 2009)), but our current results should be used to guide the need for replicated measurements on colonies. Our results also indicate that replications of measurements of removal are necessary to find good colonies in unselected populations with generally low levels of response and correspondingly high variability in responses. In such cases, using single drone inseminated queens and standardized testing protocols of colonies may yield the most precise results (e.g., the discovery of a few colonies with high mite infertility and decreases in mite population that were the beginning of a selection program for a high level of VSH (Harbo & Hoopingarner, 1997). More technically involved approaches, such as genetic markers for the trait, have potential but require validation and optimization.

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Disclosure statement

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