Journal of Apicultural Research

ISSN: 0021-8839 (Print) 2078-6913 (Online) Journal homepage: http://www.tandfonline.com/loi/tjar20

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To cite this article: Gil Leclercq, Tjeerd Blacquière, Nicolas Gengler & Frédéric Francis (2018): Hygienic removal of freeze-killed brood does not predict Varroa-resistance traits in unselected stocks, Journal of Apicultural Research, DOI: 10.1080/00218839.2018.1426350

To link to this article: https://doi.org/10.1080/00218839.2018.1426350

Published online: 13 Feb 2018.

Journal of Apicultural Research, 2018
https://doi.org/10.1080/00218839.2018.1426350

ORIGINAL RESEARCH ARTICLE

Hygienic removal of freeze-killed brood does not predict *Varroa*-resistance traits in unselected stocks

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(*Received 11 August 2017; accepted 27 December 2017*)

In honey bees (*Apis mellifera* L.), hygienic behavior of workers against *Varroa destructor* provides the colonies with some resistance to this parasite. The removal of freeze-killed brood (FKB) has often been used as a proxy to assess the removal of *Varroa*-infested brood. The question is whether this approximation is reliable enough to estimate the benefits induced by the removal of *Varroa*-infested brood in unselected stocks. For this purpose, we investigated the relation between the removal of FKB and three other variables: (1) the percentage of pupae and workers infested by *V. destructor*; (2) the share of mites in brood compared to phoretic mites; and (3) the reproductive success of mites. To be a reliable estimate, the removal of FKB should correlate with these three variables. Since hygienic behavior is naturally expressed and highly variable in unselected stocks, we chose to use such stocks to get a wide range of FKB removal. There was no correlation between FKB and the three other variables. We conclude that removal of FKB is not a good estimate for hygienic behavior towards *Varroa* mites in unselected stocks.

La eliminación higiénica de la cría congelada no predice caracteres de resistencia ante *Varroa* en las poblaciones no seleccionadas

En las abejas melíferas (*Apis mellifera* L.), el comportamiento higiénico de las obreras contra *Varroa destructor* proporciona a las colmenas resistencia a este parásito. La eliminación de la cría congelada (ECC) se ha utilizado con frecuencia para evaluar la eliminación de la cría infestada por varroa. La pregunta es si esta aproximación es lo suficientemente confiable como para estimar los beneficios inducidos por la eliminación de la cría infestada de varroa en poblaciones no seleccionadas. Para este propósito, hemos investigado la relación entre la eliminación de ECC y otras tres variables: (1) el porcentaje de pupas y obreras infestadas por *V. destructor*; (2) la proporción de ácaros en la cría comparada con ácaros foréticos y (3) el éxito reproductivo de ácaros. Para conseguir una estimación fiable, la eliminación de ECC debería correlacionarse con estas tres variables. Dado que el comportamiento higiénico se expresa naturalmente y es muy variable en las poblaciones no seleccionadas, hemos optado por utilizar dichas poblaciones para obtener un amplio rango de eliminación de ECC. No hubo correlación entre la ECC y las otras tres variables. Se concluye que la eliminación de ECC no es una buena estimación del comportamiento higiénico contra los ácaros de varroa en poblaciones no seleccionadas.

Keywords: *Varroa*-resistance; hygienic behavior; freeze-killed brood; reproductive success

Introduction

The ectoparasitic *Varroa* mite (*Varroa destructor* Anderson & Trueman) is considered as one of the most important threats for apiculture around the world (Rosenkranz, Aumeier, & Ziegelmann, 2010), and plays a central role in the decline of honey bee health, in association with the viruses it vectors (Dainat, Evans, Chen, Gauthier, & Neumann, 2012; Martin et al., 2012). In beekeeping practice, the use of acaricide treatments is recommended to avoid severe colony losses. Over the years, the mite has become partly resistant to some of the chemicals, resulting in a loss of effectiveness. Consequently, substantial efforts have focused on more sustainable solutions, such as the breeding of *Varroa* resistant honey bees (Büchler, Berg, & Le Conte, 2010; Rinderer, Harris, Hunt, & de Guzman, 2010).

Two approaches have been used with the objective to find and breed *Varroa* resistant honey bees: (1) The first is an approach following colonies that are left untreated against *Varroa* mites and kept in isolation. Both, found in nature (Le Conte et al., 2007; Seeley, 2007) as well as experimentally developed honey bee populations (Fries, Imdorf, & Rosenkranz, 2006), have been used (for an overview see Locke, 2016). The presumably non-resistant or non-tolerant colonies had died (Le Conte et al., 2007; Seeley, 2007) or were permitted to die naturally (Fries et al., 2006; Kefuss, Vanpoucke, Bolt, & Kefuss, 2016; Oddie, Dahle, & Neumann, 2017; Panziera, van Langevelde, & Blacquière, 2017). Subsequently, one could breed from the surviving stock. In this case, the mechanisms of resistance or tolerance are unknown. (2) In the second approach, when a trait that confers some resistance or tolerance is known, one can

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increase the expression of this trait through selective breeding. This second approach also includes selection for traits that indirectly confer resistance to the colony (e.g., low mite population growth). In this latter approach, hygienic behavior is a trait that has been commonly used in breeding programs (e.g., *Minnesota Hygienic Bees* in the USA, *Arbeitsgemeinschaft Toleranzzucht* in Germany, *Beebreed* in European countries, *etc.*) (Büchler et al., 2010; Rinderer et al., 2010). In this case one opts for an increased resistance or tolerance through a (to a certain degree-) known mechanism.

Hygienic behavior is described as the workers' detection, uncapping and removal of unhealthy or dead brood (Rothenbuhler, 1964). There are multiple assays to quantify this behavior. Each one consists in using brood that has been artificially infected, infested or killed, and measuring the proportion that has been removed by workers during a given period of time. The detection and the removal by workers are likely triggered by olfactory stimuli from the unhealthy or killed brood (Gramacho & Spivak, 2003; Masterman, Smith, & Spivak, 2000; Parker et al., 2012). An accurate assay has been developed to quantify the hygienic behavior targeting *Varroa* mites. This assay involves the artificial infestation of brood with one mite per cell, and the monitoring of their subsequent removal by workers (Boecking & Drescher, 1991). However, this assay is time-consuming and has therefore often been replaced by the freeze-killed brood (FKB) removal assay. This latter has proven to be more accurate than the pin-killed brood assay (Espinosa-Montano, Guzman-Novoa, Sanchez-Albarran, Montaldo, & Correa-Benitez, 2008; Spivak & Downey, 1998), and was shown to be positively correlated with the removal of brood artificially infested with 2 mites per cell, but not brood with 1 mite per cell (Boecking & Drescher, 1992). Therefore, we researched the question whether FKB removal does accurately predict the outcomes of the hygienic removal of *Varroa*-infested brood in unselected stocks.

In previous studies, the main approach to address this question has been to compare "hygienic" stocks (colonies selected for high FKB removal) with unselected or commercial stocks (e.g., Masterman, Ross, Mesce, & Spivak, 2001; Spivak & Reuter, 1998b, 2001b). However, because different stocks may differ in many more traits, and because hygienic behavior is already naturally expressed and highly variable in unselected stocks (Oldroyd, 1996; Spivak & Reuter, 1998b), we decided to use the variability in FKB removal present in unselected stock.

We tested three hypotheses that should be verified for the FKB removal to be a reliable measure of the hygienic behavior towards *Varroa* mites: (1) The removal of *Varroa*-infested brood is supposed to reduce the infestation (Boecking & Spivak, 1999). Consequently, we tested if the FKB removal was (negatively) correlated with the infestation by *V. destructor*, at the end of the beekeeping season; (2) The hygienic removal of infested pupae is also supposed to increase the phoretic period of the mites that escaped the removal of the infested pupae (Spivak, 1996). Consequently, we tested if the FKB removal could be (positively) correlated with the proportion of mites in the phoretic phase; and (3) To reduce the mite population, the hygienic behavior should mainly concern pupae infested with reproducing mites, and disregard non-reproducing mites (Harbo & Harris, 2009). Consequently, we tested if there was a relationship between the FKB removal and the reproductive success of the mites remaining in our unselected stock.

For the FKB removal assay, there are multiple parameters that can be chosen (i.e., need for repeatability, and optimal period to check for FKB removal). Hence, we discuss the most appropriate settings in the case of unselected stocks, and how these affect our conclusions.

Material and methods

Study colonies

We conducted this study in Wallonia (south of Belgium). In July 2013, we sampled young larvae from backyard beekeepers' colonies all over Wallonia. Their colonies were qualified as "unselected" because they were bred by these beekeepers (no purchased queens from somewhere else), without any purpose to improve any trait by selection. The larvae were grafted into cell-cups containing royal jelly to ensure a safe transportation to our experimental apiaries. Then, we bred queens from these larvae, and allowed them to mate naturally. The mated queens were introduced into colonies that had no open brood to encourage the queen acceptance. After that, the colonies were equally distributed in 3 apiaries and regularly visited. All colonies were treated with Apivar for 6 weeks from the beginning of September.

For this experiment, in July 2014, we randomly chose from our unselected stock 10 colonies for each apiary. Each colony was headed by a queen that had been laying eggs normally for at least the last 2 months. This queen was either the original queen (from the grafted larvae) or its daughter (if the colony had swarmed). All 30 colonies were managed by ourselves in 10 frame-Dadant-Blatt hives using normal beekeeping practices. Queen excluder and honey supers were added when appropriate.

Sampling and data collection

We performed all sampling and field data collection in July 2014. In order to test our three hypotheses, we took three categories of data respectively linked to the FKB removal, the infestation level by the mite (in phoretic phase and reproductive phase), and its reproductive success.

Regarding the FKB removal, we quantified it twice in each colony, with an interval of 1 week, using liquid nitrogen (Spivak & Reuter, 1998a). The number of the

assay (1 or 2) was recorded as a factor called "trial" hereafter. Seven days before each assay, the location of a comb area with fifth instar larvae was recorded on the edge of the frame. In this way, the assay was performed on purple-eyed pupae. The frozen patches (inner diameter of tube = 7.5 cm) contained 115 ± 18 (SD, n = 60 assays) cells of capped brood. Pictures of the tested areas were taken during the assay just after cylinder removal, and 24 and 48 h after freezing. These 2 periods are the 2 levels of a factor called "testing period" hereafter. Therefore, in our design, one trial involves two testing periods.

To assess the infestation by the mite, we took 2 measurements, one for the phoretic phase, and the other for the reproductive phase. In this way, we were able to compute the ratio between the numbers of mites in these two phases. For the phoretic phase, we used the powdered sugar shaking method (Dietemann et al., 2013), with slight modifications. The estimate was based on 3 brood combs to better take into account the spatial variability of the infestation inside the colonies. For each of these brood combs, 61.2 ± 9.6 g (SD, n = 90 samples) of honey bees were collected in a glass jar closed with a 2 mm wire meshed lid. A tablespoon containing approximately 13–14 g of powdered sugar was poured on the bees through the mesh, preventing the bees to fly inside the jar. After that, the jar was weighed (with the bees and sugar inside) to deduce the exact weight of the bees, by subtracting the known weight of the empty jar and the weight of the sugar. Then, the jar was turned upside down over a white tray, and shaken for one minute to knock off the mites. We converted the 3 estimates into a single one by computing the total number of mites for the 3 combs and dividing it by the total weight of bees. The weight of bees was converted into a number of bees, considering an average weight of 110 mg per bee (Bowen-Walker & Gunn, 2001). Although the 110 mg per bee might be a bit arbitrary because bees may have acquired some extra weight by absorbing honey upon opening the hive, it benefits ease of comparison with data from literature as well as with the figures for infestation in brood, which is on a per bee basis too. At the end, the measure for the phoretic phase was the number of mites per 100 bees.

To estimate the infestation in brood, we first looked at the spatial distribution of honey bee pupal stages by randomly opening a few cells of the 3 previous combs. Then, we chose and cut a section of capped brood out of each comb, and we obtained 317.4 ± 46.9 cells per colony (SD, n = 90 samples). These sections were chosen in a way to maximize the diversity of pupal stages sampled in each colony, and to take into account the infestation variability across pupal stages (Dietemann et al., 2013). Each piece of comb was frozen at −20 °C. During winter, we opened all cells, and recorded the percentage of cells infested by *V. destructor*. Then, we computed the ratio between the phoretic phase and reproductive phase measurements (called "P/R ratio" hereafter). This ratio might be influenced by the relative proportion of adult bees and brood within each colony. While we did not measure this proportion, the number of adult bees and capped brood cells sampled in each colony required that all colonies had both a strong adult population and several combs of brood. And, we did not notice any extreme amount of adult bees compared to the amount of brood (or *vice versa*).

To estimate the mite reproductive success, we used the same 3 brood samples for each colony. For each cell, we recorded the pupal stage of the honey bee, and the stages of the mite progeny. These stages were assessed based on their appearance as described in Human et al. (2013) for the honey bee pupae, and in Dietemann et al. (2013) for the mite progeny. The reproductive success of the mites was considered as a binary trait, depending on whether the mother mite was able (1), or not (0), to produce at least one adult female and one adult male before honey bee emergence, given the remaining time. The cells containing bee larvae and white-eyed pupae were disregarded as at this stage the reproductive success cannot be predicted accurately (Corrêa-Marques, Medina, Martin, & De Jong, 2003). The cells which had been infested by multiple mother mites were also disregarded, since the reproductive success was reported to decrease when the number of mother mites per cell increases (Eguaras, Marcangeli, & Fernandez, 1994; Fuchs, 1994; Fuchs & Langenbach, 1989; Martin, 1995). Furthermore, we only found 40 multiple infested cells (in 30 colonies), including 21 cells being in the two most heavily infested colonies. Therefore, their poor occurrence did not allow any reliable comparison among colonies.

Statistical analysis

Data were analyzed using R software (R Core Team, 2012). To know which settings were the most appropriate in the FKB assay, we ran and compared mixed models with the following variables: colony, trial, testing period, and their interactions. We assessed the effect of each of these traits on the FKB removal. The Pearson correlation coefficients, and their significance, were computed between infestation measures and the FKB removal at 24 h, and 48 h. Since the reproductive success is a discrete variable, quantified as 0 or 1, we could not compute such correlation. Consequently, we performed a logistical regression taking into account the variable number of infested pupae per colony used to assess this trait.

Results

Settings of the FKB removal assay

We found that the hygienic behavior towards FKB was expressed very differently across colonies of our

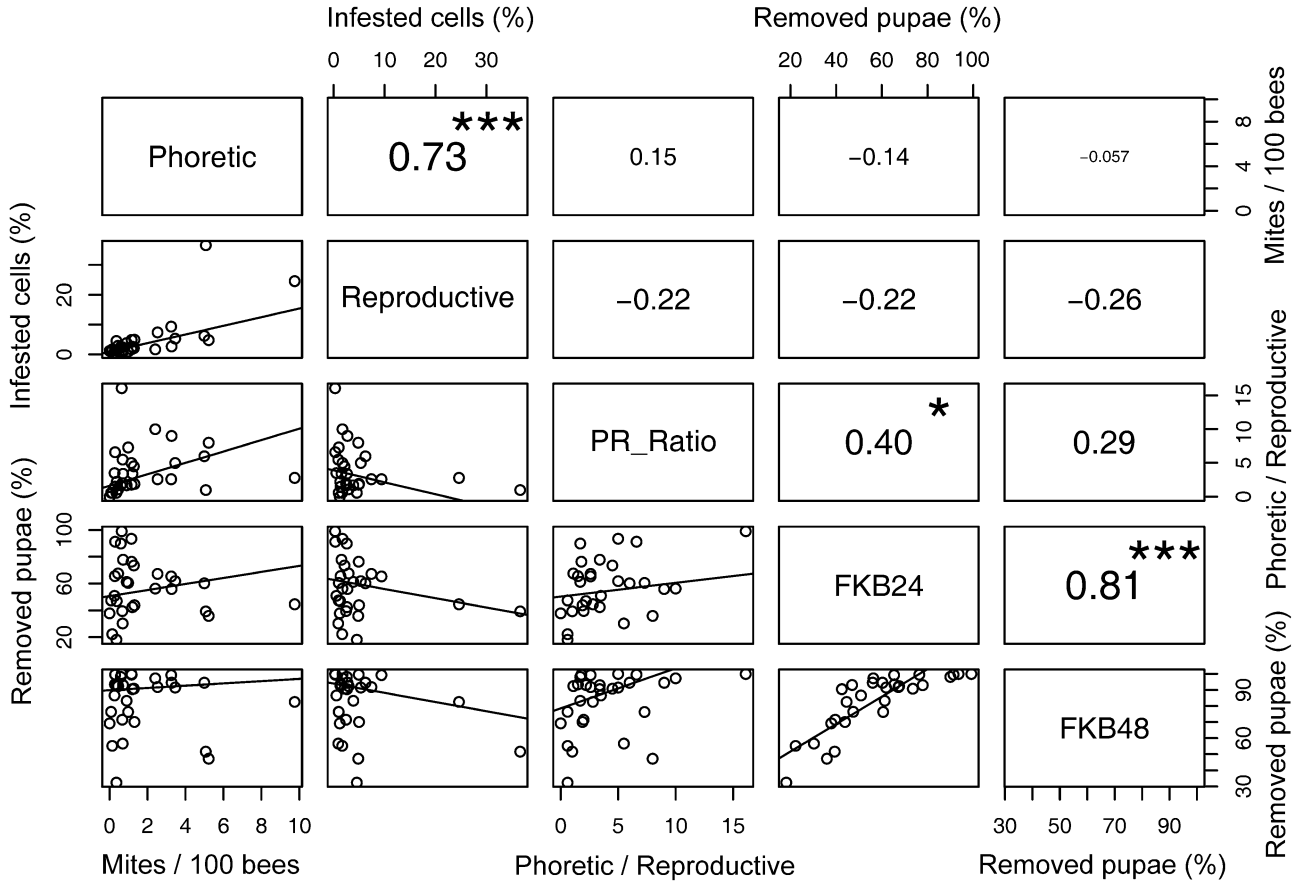

Figure 1. For each combination of traits, we computed the Pearson's correlation coefficients (above diagonal) and we plotted scatterplots with robust regression lines (below diagonal). Robust regressions were performed because they are less impacted by extreme values compared to least squares regression. For this reason, the sign of low-value correlation coefficients might differ from the slope of the regression lines (e.g., negative correlation coefficient associated to a positive slope for the regression line). These complementary results help illustrating the impact of extreme values on the relationships between traits. FKB24 and FKB48 are the freeze-killed brood removal averages for 2 trials, at 24 and 48 h respectively (%). The font size for correlation coefficients (r) is proportional to their own values: $\log_{10}(|r|.100)$.
Notes: The asterisks indicate the statistical significance level of the correlation ("*" for $p \leq 0.05$; "**" for $p \leq 0.01$; "***" for $p \leq 0.001$).

unselected stock ($\chi^2 = 50.1$, df = 1, $p < 0.001$). The FKB removal was also impacted by the length of time (24 or 48 h) given to the colonies to remove it ($\chi^2 = 66.1$, df = 1, $p < 0.001$). Taken all colonies together, and for a given testing period (24 or 48 h), the FKB removal was the same between the two trials, which were performed one week apart ($\chi^2 = 0.00$, df = 1, $p = 1.00$). However, we found differences of FKB removal between trials for the same colonies, i.e., a significant "colony*trial" interaction ($\chi^2 = 17.3$, df = 1, $p < 0.001$). Therefore, hereafter we used the averages for 2 trials for each colony (i.e., one average for two records at 24 h, and one average for two records at 48 h, for each colony). The other interactions were not significant. We also found that the FKB removed after 24 h was strongly correlated with the FKB removed after 48 h ($r = 0.813$, $p < 0.001$; Figure 1).

FKB removal and infestation

A strong correlation ($r = 0.726$, $p < 0.001$) was found between the phoretic mite load per 100 bees and the percentage of infested cells. The infestation was highly variable among colonies, as indicated by high standard deviations (Phoretic: 1.78 ± 2.16 mites/100 bees; Reproductive: 4.77 ± 7.53% of cells had been infested; SD, $n = 30$ colonies), and the scatterplots (Figure 1). Two colonies had much more infested cells (two SD above the mean) than the other 28 (Figure 1). These two colonies were not considered as outliers, but their impact on the relationships between traits was illustrated through the robust regression lines, which are less impacted by extreme values compared to least squares regression (Figure 1). The significance of correlation coefficients was not affected by these two colonies, except for one pair of traits (regarding the second hypothesis), as explained below.

Our first hypothesis, assuming a negative correlation between FKB removal and the infestation, was rejected by the absence of a relationship between these traits. The average FKB removal at 24 (or 48 h) was not correlated to the infestation in phoretic phase and reproductive phase (Figure 1).

We found evidence supporting our second hypothesis, related to the theory that hygienic behavior can extend the phoretic stage of female mites. Indeed, there was a strong and consistent correlation between P/R ratios and average FKB removal in 24 h ($r = 0.395$, $p < 0.05$; Figure 1). However, this correlation dropped to 0.210 ($p = 0.27$) when we removed from the all data set only one colony with high values for both traits, which leads us to conclude that actually also our second hypothesis was not supported by the data.

FKB removal and mite reproductive success

The reproductive success of the mites per colony was defined as the percentage of infested cells in which a mother mite was able to produce at least one adult female and one adult male before honey bee emergence. It amounted to 88.1 ± 21.4%. This was based on 6.46 ± 6.92 infested cells per colony (SD, $n = 30$ colonies). The mites from two colonies had a low reproductive success, compared to the others, but the data that we collected did not allow us to speculate on the reasons that could explain these differences.

Our third hypothesis stated that the FKB removal would explain the values of mite reproductive success per colony, expecting a negative effect in our model. However, as shown on the scatterplot (Figure 2), the logistical regression confirmed that the FKB removal had no significant effect on the reproductive success per colony of *V. destructor* in our model ($z = -1.06$, $p = 0.290$).

Discussion

In our unselected stocks, we found that FKB assays were reproducible in the overall population (no difference between trials), and since there were for some colonies differences between two replications, we used the average between the two trials for the calculations. The testing period (24 h or 48 h) chosen in the assays was decisive for the data distribution, and the forthcoming results (Figure 1). For example, the correlation of FKB removal with the P/R ratio was significant at 24 h, but not at 48 h. Even if FKB removals at the two testing periods were correlated (Figure 1), the FKB removal at 48 h narrows the variability across colonies, and probably tends us to consider colonies more "hygienic" than they truly are. Spivak (see Wilson-Rich, Spivak, Fefferman, & Starks, 2009) discriminates between fast and slow hygienic bees, with all variation between these, which stresses the relevance of the rate of removal, and the consequences of the choice of the timing of observations in the FKB assays. Older studies which used liquid nitrogen in the FKB assays checked for removal at 48 h (e.g., de Guzman et al., 2002; Kavinseksan, Wongsiri, Rinderer, & de Guzman, 2004), and also more recent ones used this period as a reference (e.g., Bigio, Al Toufailia, & Ratnieks, 2014; Bigio, Schürch, & Ratnieks, 2013). Nonetheless, our results show that a period of 24 h is more suitable to assess the hygienic behavior, to preserve the discriminatory power of the assay, even in unselected stocks. In stocks selected for fast hygienic behavior, much shorter time to check may

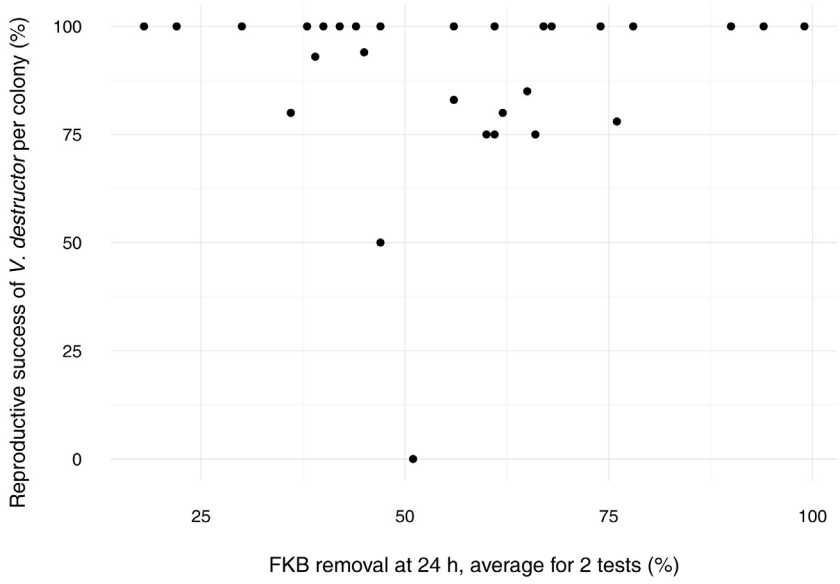

Figure 2. Reproductive success of *V. destructor* in relation to FKB removal. Each point represents a colony. The reproductive success of *V. destructor* is considered as the percentage of mother mites able to produce at least one adult female and one adult male before honey bee emergence.

be advisable. Summarizing, we support the current recommendation that FKB assays should be performed at least twice in each colony (Spivak & Downey, 1998), but with the first checking for removal the latest after 24 h, even in unselected stocks.

Using these settings, we tested three hypotheses that had to be confirmed for the FKB removal to be a reliable approximation of the benefits induced by the removal of *Varroa*-infested brood in unselected stocks. The situation in our unselected stocks is summarized as: (1) We found no significant correlation between the mite load and the FKB removal at 24 h (nor at 48 h). In future studies, other periods of time should probably also be tested; (2) FKB removal appeared to be correlated to the phoretic/reproductive ratio, (which would support the theory of an extended phoretic phase), but when tested after removing one colony the correlation was lost. In our study, this leads us to conclude that the criteria to adopt hypothesis two are not met, but further studies are needed to deepen the relationship between FKB and the P/R ratio; (3) The ability of a colony to remove FKB did not affect significantly the reproductive success of the mites in the colonies.

Taken together, we conclude that the FKB removal assay might not be a good criterion for screening or selection of colonies that display *Varroa*-resistance traits, at least in unselected stocks. The FKB removal has been shown to be significantly correlated with the removal of *Varroa*-infested brood, but only when two mother mites were infesting the pupae (Boecking & Drescher, 1992). However, in natural conditions, pupae infested by more than one mite become frequent only in highly infested colonies (Martin, 1995). The stimulus from the FKB may trigger a form of hygienic behavior that is not efficient to target low mite infested pupae (Danka, Harris, Villa, & Dodds, 2013; Harbo & Harris, 2009). To remove *Varroa*-infested brood, workers probably rely on their ability to discriminate different cues and thresholds linked to brood health (Masterman et al., 2001). Consequently, the choice of the assay and stimulus could lead to different results regarding this behavior (Espinosa-Montano et al., 2008). Unfortunately, nowadays there is no assay that has been designed to quantify easily the hygienic behavior involved in the removal of *Varroa*-infested brood, and that could be used routinely in early stages of breeding programs.

Besides the fact that the stimulus might be inappropriate, the FKB removal assay could occasionally remain useful to screen for *Varroa* resistant colonies. Indeed, some colonies qualified as "highly hygienic" (i.e., > 95% of FKB removed in 24 h) were reported to be able to negatively affect the mite population growth over nearly one year (Al Toufailia, Amiri, Scandian, Kryger, & Ratnieks, 2014). In two previous studies, no correlation was found between the FKB removal and the decrease in the mite population, but these studies did not include "highly hygienic" colonies (Harbo & Hoopingarner, 1997; Mondragón, Spivak, & Vandame, 2005). And of course a decrease in mite population growth can be caused by bee traits that do not have any relationship with hygienic behavior. However, selection based on a binary variable (namely, >95% of FKB removed in 24 h; yes or no) does not allow the quantification of differences among these "highly hygienic" colonies, which sets some limitations to the improvement of the hygienic behavior.

Another potential issue regarding the methodology is the assessment of the reproductive success of the *Varroa* mites in the colonies. Because of the relatively low infestation of most colonies, the number of infested pupae used to estimate this trait was rather low (6.46 ± 6.92 cells per colony; SD, n = 30 colonies). Dietemann et al. (2013) mentioned an arbitrary number of 30 infested pupae for statistical significance, which is a challenge in low infested colonies. Harbo and Harris (2005) chose to reach 18 infested cells, by examining up to 800 cells. Other studies avoided this sample size problem by pooling brood samples from different colonies, and then comparing the reproductive success between different stocks, but losing information at the colony level (Locke, Le Conte, Crauser, & Fries, 2012).

Finally, we should still consider another possible explanation to our results: it might be that without having been selected up to a high degree, FKB removal does not significantly correlate with the infestation level and the reproductive success of the mite. In natural populations which survived the *Varroa* mite without treatment, the removal of FKB (Mondragón et al., 2005) or pin-killed brood (Locke & Fries, 2011) was not correlated with the resistance to the mite. Furthermore, as demonstrated by Ibrahim and Spivak (2006), and recently confirmed by Danka et al. (2013), selection based on low reproductive success of the mite can lead to colonies that are highly efficient at detecting and removing FKB. However, it does not work the other way around. Indeed, the mites in their tested FKB-hygienic lines were not characterized by a low reproductive success. To date, the relationship between these traits remains predominantly unexplained, and the real benefits of the hygienic behavior against *V. destructor* still need to be clearly demonstrated. A recent study (Panziera et al., 2017) investigating the hygienic removal of brood artificially infested with *Varroa* mites in two *Varroa* surviving honey bee populations, demonstrated increased removal in colonies of one of the populations. However, there was no difference in pin-killed or freeze killed brood removal between these populations (Tjeerd Blacquière, unpublished results).

In conclusion, the assessment of the hygienic behavior through FKB assays had previously been shown to be reliable to select for resistance against multiple brood diseases, such as American foulbrood and chalkbrood (Spivak & Reuter, 2001a). However, regarding *V. destructor*, our results suggest that the benefits of this assay should be nuanced. In unselected stocks, we could not show any significant correlation of the outcome of the

test with the infestation nor with the reproductive success of the mite. Methodological issues have been one of the main sources of contradictory results regarding this trait in the literature. Therefore, we would like to underline the need for an appropriate standard method to quantify the form of hygienic behavior that is directly involved in the removal of *Varroa*-infested pupae.

Author contributions

GL, NG and FF designed the research. GL carried out the experiments and analyzed the data. GL and TB wrote the article. All authors read and approved the final manuscript.

Acknowledgements

Our technicians, Vianney Janssens and Thomas Rafalowicz (University of Liège) provided a great help in the experimental apiaries and in the lab. Yves Brostaux (University of Liège) gave us multiple statistical advices. Dennis vanEngelsdorp (University of Maryland, USA) and Delphine Panziera (Wageningen Plant Research) provided very useful comments on the manuscript. We also address special thanks to Walloon beekeepers who collaborated in this project.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the Walloon Region (*Service Public de Wallonie*), as part of the *Selapis* project [grant number D31-1280], [grant number D31-1328].

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References

Al Toufailia, H.M., Amiri, E., Scandian, L., Kryger, P., & Ratnieks, F.L.W. (2014). Towards integrated control of *Varroa*: Effect of variation in hygienic behaviour among honey bee colonies on mite population increase and deformed wing virus incidence. *Journal of Apicultural Research, 53*(5), 555–562. doi:10.3896/ibra.1.53.5.10

Bigio, G., Al Toufailia, H., & Ratnieks, F.L.W. (2014). Honey bee hygienic behaviour does not incur a cost via removal of healthy brood. *Journal of Evolutionary Biology, 27*(1), 226–230. doi:10.1111/jeb.12288

Bigio, G., Schürch, R., & Ratnieks, F.L.W. (2013). Hygienic behavior in honey bees (*Hymenoptera*: *Apidae*): Effects of brood, food, and time of the year. *Journal of Economic Entomology, 106*(6), 2280–2285. doi:10.1603/ec13076

Boecking, O., & Drescher, W. (1991). Response of *Apis mellifera* L colonies infested with *Varroa jacobsoni* Oud. *Apidologie, 22*(3), 237–241. doi:10.1051/apido:19910308

Boecking, O., & Drescher, W. (1992). The removal response of *Apis mellifera* L. colonies to brood in wax and plastic cells after artificial and natural infestation with *Varroa jacobsoni* Oud. and to freeze-killed brood. *Experimental and Applied Acarology, 16*(4), 321–329. doi:10.1007/bf01218574

Boecking, O., & Spivak, M. (1999). Behavioral defenses of honey bees against *Varroa jacobsoni* Oud. *Apidologie, 30*(2–3), 141–158. doi:10.1051/apido:19990205

Bowen-Walker, P.L., & Gunn, A. (2001). The effect of the ectoparasitic mite, *Varroa destructor* on adult worker honey bee (*Apis mellifera*) emergence weights, water, protein, carbohydrate, and lipid levels. *Entomologia Experimentalis et Applicata, 101*(3), 207–217. doi:10.1046/j.1570-7458.2001.00905.x

Büchler, R., Berg, S., & Le Conte, Y. (2010). Breeding for resistance to *Varroa destructor* in Europe. *Apidologie, 41*(3), 393–408. doi:10.1051/apido/2010011

Corrêa-Marques, M.H., Medina, L.M., Martin, S.J., & De Jong, D. (2003). Comparing data on the reproduction of *Varroa destructor*. *Genetics and Molecular Research: GMR, 2*(1), 1–6.

Dainat, B., Evans, J.D., Chen, Y.P., Gauthier, L., & Neumann, P. (2012). Dead or alive: Deformed wing virus and *Varroa destructor* reduce the life span of winter honey bees. *Applied and Environmental Microbiology, 78*(4), 981–987. doi:10.1128/AEM.06537-11

Danka, R.G., Harris, J.W., Villa, J.D., & Dodds, G.E. (2013). Varying congruence of hygienic responses to *Varroa destructor* and freeze-killed brood among different types of honey bees. *Apidologie, 44*(4), 447–457. doi:10.1007/s13592-013-0195-8

de Guzman, L.I., Rinderer, T.E., Stelzer, J.A., Beaman, L., Delatte, G., & Harper, C. (2002). Hygienic behavior by honey bees from far-eastern Russia. *American Bee Journal, 142*(1), 58–60.

Dietemann, V., Nazzi, F., Martin, S., Anderson, D., Locke, B., Delaplane, K.S., … Ellis, J.D.. (2013). Standard methods for *Varroa* research. In V. Dietemann, J.D. Ellis, & P. Neumann (Eds.), *The* COLOSS BEEBOOK, *Volume II: Standard methods for* Apis mellifera *pest and pathogen research. Journal of Apicultural Research*, *52*(1). doi: 10.3896/IBRA.1.52.1.09

Eguaras, M., Marcangeli, J., & Fernandez, N. (1994). Influence of "parasitic intensity" on *Varroa jacobsoni* Oud. reproduction. *Journal of Apicultural Research, 33*(3), 155–159. doi:10.1080/00218839.1994.11100863

Espinosa-Montano, L.G., Guzman-Novoa, E., Sanchez-Albarran, A., Montaldo, H.H., & Correa-Benitez, A. (2008). Comparative study of three assays to evaluate hygienic behavior in honey bee (*Apis mellifera* L.) colonies. *Veterinaria Mexico, 39*(1), 39–54.

Fries, I., Imdorf, A., & Rosenkranz, P. (2006). Survival of mite infested (*Varroa destructor*) honey bee (*Apis mellifera*) colonies in a Nordic climate. *Apidologie, 37*(5), 564–570. doi:10.1051/apido:2006031

Fuchs, S. (1994). Non-reproducing *Varroa jacobsoni* Oud. in honey bee worker cells – status of mites or effect of brood cells? *Experimental & Applied Acarology, 18*(5), 309–317. doi:10.1007/bf00132320

Fuchs, S., & Langenbach, K. (1989). Multiple infestation of *Apis mellifera* L. brood cells and reproduction in *Varroa jacobsoni* Oud. *Apidologie, 20*(3), 257–266. doi:10.1051/apido:19890308

Gramacho, K., & Spivak, M. (2003). Differences in olfactory sensitivity and behavioral responses among honey bees bred for hygienic behavior. *Behavioral Ecology and Sociobiology, 54*(5), 472–479. doi:10.1007/s00265-003-0643-y

Harbo, J.R., & Harris, J.W. (2005). Suppressed mite reproduction explained by the behaviour of adult bees. *Journal of Apicultural Research, 44*(1), 21–23. doi:10.1080/00218839.2005.11101141

Harbo, J.R., & Harris, J.W. (2009). Responses to *Varroa* by honey bees with different levels of *Varroa* Sensitive

Hygiene. *Journal of Apicultural Research, 48*(3), 156–161. doi:10.3896/ibra.1.48.3.02

Harbo, J.R., & Hoopingarner, R.A. (1997). Honey bees (*Hymenoptera*: *Apidae*) in the United States that express resistance to *Varroa jacobsoni* (*Mesostigmata*: *Varroidae*). *Journal of Economic Entomology, 90*(4), 893–898. doi:10.1093/jee/90.4.893

Human, H., Brodschneider, R., Dietemann, V., Dively, G., Ellis, J.D., Forsgren, E., … Zheng, H.-Q.. (2013). Miscellaneous standard methods for *Apis mellifera* research. In V. Dietemann, J.D. Ellis, & P. Neumann (Eds.), *The* COLOSS BEEBOOK, Volume I: *standard methods for* Apis mellifera *research. Journal of Apicultural Research*, *52*(4). doi: 10.3896/IBRA.1.52.4.10

Ibrahim, A., & Spivak, M. (2006). The relationship between hygienic behavior and suppression of mite reproduction as honey bee (*Apis mellifera*) mechanisms of resistance to *Varroa destructor*. *Apidologie, 37*(1), 31–40. doi:10.1051/apido:2005052

Kavinseksan, B., Wongsiri, S., Rinderer, T.E., & de Guzman, L.I. (2004). Comparison of the hygienic behavior of ARS Russian and commercial honey bees in Thailand. *American Bee Journal, 144*(11), 870–872.

Kefuss, J., Vanpoucke, J., Bolt, M., & Kefuss, C. (2016). Selection for resistance to *Varroa destructor* under commercial beekeeping conditions. *Journal of Apicultural Research, 54*(5), 563–576. doi:10.1080/00218839.2016.1160709

Le Conte, Y., de Vaublanc, G., Crauser, D., Jeanne, F., Rousselle, J.-C., & Bécard, J.-M. (2007). Honey bee colonies that have survived *Varroa destructor*. *Apidologie, 38*(6), 566–572. doi:10.1051/apido:2007040

Locke, B. (2016). Natural *Varroa* mite-surviving *Apis mellifera* honey bee populations. *Apidologie, 47*(3), 467–482. doi:10.1007/s13592-015-0412-8

Locke, B., & Fries, I. (2011). Characteristics of honey bee colonies (*Apis mellifera*) in Sweden surviving *Varroa destructor* infestation. *Apidologie, 42*(4), 533–542. doi:10.1007/s13592-011-0029-5

Locke, B., Le Conte, Y., Crauser, D., & Fries, I. (2012). Host adaptations reduce the reproductive success of *Varroa destructor* in two distinct European honey bee populations. *Ecology and Evolution, 2*(6), 1144–1150. doi:10.1002/ece3.248

Martin, S.J. (1995). Reproduction of *Varroa jacobsoni* in cells of *Apis mellifera* containing one or more mother mites and the distribution of these cells. *Journal of Apicultural Research, 34*(4), 187–196. doi:10.1080/00218839.1995.11100904

Martin, S.J., Highfield, A.C., Brettell, L., Villalobos, E.M., Budge, G.E., Powell, M., … Schroeder, D.C. (2012). Global honey bee viral landscape altered by a parasitic mite. *Science, 336* (6086), 1304–1306. doi:10.1126/science.1220941

Masterman, R., Ross, R., Mesce, K., & Spivak, M. (2001). Olfactory and behavioral response thresholds to odors of diseased brood differ between hygienic and non-hygienic honey bees (*Apis mellifera* L.). *Journal of Comparative Physiology A, 187*(6), 441–452. doi:10.1007/s003590100216

Masterman, R., Smith, B.H., & Spivak, M. (2000). Brood odor discrimination abilities in hygienic honey bees (*Apis mellifera* L.) using proboscis extension reflex conditioning. *Journal of Insect Behavior, 13*(1), 87–101. doi:10.1023/a:1007767626594

Mondragón, L., Spivak, M., & Vandame, R. (2005). A multifactorial study of the resistance of honey bees *Apis mellifera* to the mite *Varroa destructor* over one year in Mexico. *Apidologie, 36*(3), 345–358. doi:10.1051/apido:2005022

Oddie, M.A.Y., Dahle, B., & Neumann, P. (2017). Norwegian honey bees surviving *Varroa destructor* mite infestations by means of natural selection. *PeerJ, 5*, e3956. doi:10.7717/peerj.3956

Oldroyd, B. (1996). Evaluation of Australian commercial honey bees for hygienic behaviour, a critical character for tolerance to chalkbrood. *Australian Journal of Experimental Agriculture, 36*(5), 625–629. doi:10.1071/EA9960625

Panziera, D., van Langevelde, F., & Blacquière, T. (2017). *Varroa* Sensitive Hygiene contributes to naturally selected *Varroa*-resistance in honey bees. *Journal of Apicultural Research, 56* (5), 635–642. doi:10.1080/00218839.2017.1351860

Parker, R., Guarna, M.M., Melathopoulos, A.P., Moon, K.-M., White, R., Huxter, E., … Foster, L.J. (2012). Correlation of proteome-wide changes with social immunity behaviors provides insight into resistance to the parasitic mite, *Varroa destructor*, in the honey bee (*Apis mellifera*). *Genome Biology, 13*(9), R81. doi:10.1186/gb-2012-13-9-r81

R Core Team. (2012). *R: A language and environment for statistical computing*. Vienna: R Foundation for Statistical Computing. Retrieved from http://www.R-project.org/

Rinderer, T.E., Harris, J.W., Hunt, G.J., & de Guzman, L.I. (2010). Breeding for resistance to *Varroa destructor* in North America. *Apidologie, 41*(3), 409–424. doi:10.1051/apido/2010015

Rosenkranz, P., Aumeier, P., & Ziegelmann, B. (2010). Biology and control of *Varroa destructor*. *Journal of Invertebrate Pathology, 103*(SUPPL. 1), S96–S119. doi:10.1016/j.jip.2009.07.016

Rothenbuhler, W.C. (1964). Behaviour genetics of nest cleaning in honey bees. I. Responses of four inbred lines to disease-killed brood. *Animal Behaviour, 12*(4), 578–583. doi:10.1016/0003-3472(64)90082-X

Seeley, T.D. (2007). Honey bees of the Arnot Forest: A population of feral colonies persisting with *Varroa destructor* in the northeastern United States. *Apidologie, 38*(1), 19–29. doi:10.1051/apido:2006055

Spivak, M. (1996). Honey bee hygienic behavior and defense against *Varroa jacobsoni*. *Apidologie, 27*(4), 245–260. doi:10.1051/apido:19960407

Spivak, M., & Downey, D.L. (1998). Field assays for hygienic behavior in honey bees (*Hymenoptera*: *Apidae*). *Journal of Economic Entomology, 91*(1), 64–70. doi:10.1093/jee/91.1.64

Spivak, M., & Reuter, G.S. (1998a). Honey bee hygienic behavior. *American Bee Journal, 138*(4), 283–286.

Spivak, M., & Reuter, G.S. (1998b). Performance of hygienic honey bee colonies in a commercial apiary. *Apidologie, 29* (3), 291–302. doi:10.1051/apido:19980308

Spivak, M., & Reuter, G.S. (2001a). Resistance to American foulbrood disease by honey bee colonies *Apis mellifera* bred for hygienic behavior. *Apidologie, 32*(6), 555–565. doi:10.1051/apido:2001103

Spivak, M., & Reuter, G.S. (2001b). *Varroa destructor* infestation in untreated honey bee (*Hymenoptera*: *Apidae*) colonies selected for hygienic behavior. *Journal of Economic Entomology, 94*(2), 326–331. doi:10.1603/0022-0493-94.2.326

Wilson-Rich, N., Spivak, M., Fefferman, N.H., & Starks, P.T. (2009). Genetic, individual, and group facilitation of disease resistance in insect societies. *Annual Review of Entomology, 54*, 405–423. doi:10.1146/annurev.ento.53.103106.093301