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Article

Descriptive analysis of the varroa non-reproduction trait in honey bee colonies and association with other traits related to varroa resistance

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Abstract: In the current context of worldwide honey bee colony losses, among which the varroa mite plays a major role, hope to improve honey bee health lies in part in the breeding of varroa resistant colonies. To do so, methods used to evaluate varroa resistance need better understanding. Repeatability and correlations between traits such as Mite Non-Reproduction (MNR), Varroa Sensitive Hygiene (VSH) and hygienic behaviour are poorly known, due to practical limitations and to their underlying complexity. We investigate (i) the variability, (ii) repeatability of the MNR score and (iii) its correlation with other resistance traits. To reduce the inherent variability of MNR scores, we propose to apply an Empirical Bayes correction. On the short-term (ten days) MNR had a modest repeatability of 0.4 whereas on the long-term (a month) it had a low repeatability of 0.2, similar to other resistance traits. Within our dataset there was no correlation between MNR and VSH. Although MNR is amongst the most popular varroa resistance estimates in field studies, its underlying complex mechanism is not fully understood. Its lack of correlation with better described resistance traits and low repeatability suggest that MNR need to be interpreted cautiously, especially when used for selection.

Keywords: *Apis mellifera; Varroa destructor*; Mite non reproduction (MNR); Suppressed mite reproduction (SMR); Varroa sensitive hygiene (VSH); hygienic behaviour

1. Introduction

Today there is a common consensus that while the origin of worldwide *Apis mellifera* colony losses is multifactorial, the parasite *Varroa destructor* contributes significantly to the weakening of honey bee populations [1,2]. The varroa mite is a honey bee parasite that affects bees by feeding on them while also transmitting potent viruses [3–5]. Currently, *A. mellifera* colonies, with only a few documented exceptions, are dependent on human intervention to survive mite infestations [1,6,7]. However, methods for controlling mite levels by means of acaricide treatments are becoming less effective due to the ability of varroa to become resistant to certain molecules [8]. Furthermore, beekeepers struggle with the possibility that these chemicals can contaminate bee products such as honey or wax [9].

In the 1990s, *A. mellifera* colonies that do not require acaricide treatments to survive *V. destructor* infestation were discovered [10,11]. Since then, the existence of resistant *A. mellifera* populations has been confirmed in different regions of the world [12]. This has led to the hope, in

the scientific community as well as among beekeepers, that an attractive sustainable long-term solution to counter the mite is the selection of honey bee populations that can naturally survive the parasite without the need for acaricide and regular human intervention [13]. In an *A. mellifera* colony the varroa mite population size typically increases exponentially during the season due to the varroa foundresses being able to produce several fertile daughters during several reproductive cycles [14]. Contrary to the colonies that need treatment, surviving ones can support varroa mite infestation without a reduction of the longevity of the colony (tolerance) or can resist *V. destructor* by maintaining low varroa levels on their own (resistance) [15]. The selection and conservation of these colonies is however still time consuming and a difficult task. To date the success of breeding programs worldwide is low [16] and the commercial availability of resistant honey bee stock is rare. This is partly due to the difficulty to unambiguously identify and understand the resistant traits before being able to use them in dedicated selection programs.

One of the important traits identified was termed Suppressed Mite Reproduction (SMR) [17]. It was first observed as a reduced reproductive output of foundresses in, for example, Africanised honey bees [18,19]. Recently renamed Mite Non reproduction (MNR) [20], this trait depends on multiple mite-or bee related factors. Firstly, the mites might already have a reduced fecundity when entering the brood cell [21,22]. Secondly, the brood itself can have an influence on the reproductive success of the varroa mite. Milani et al. [23] could show that molecules found in the brood cells can reduce the number of offspring produced by a varroa foundress. Lastly, the adult bees themselves can also reduce the reproductive success of the varroa mite by a behaviour termed Varroa Sensitive Hygiene (VSH) as well as by a recapping of infested cells. Bees that express VSH can detect and remove varroa infested brood before the foundress can produce fertile daughter mites [24–27]. Recapping, a less costly option for the bees, is expressed by the uncapping of infested brood by the nurse bees followed by a subsequent recapping of the cell without harming the developing pupae [28–30]. So far the outcome of selecting MNR colonies ranges from successfully identifying and selecting MNR colonies [12,31–33] to seeing no effect in the survival of honey bee colonies when looking at their MNR trait [34–37]. While it is possible to pin this on differences in the survival mechanisms used by distinct honey bee populations [12], it is also possible that this is at least partially due to methodological biases. Although there have been recommendations concerning how to perform the MNR measurement, they have changed over time [17,38] and different authors seem to use different research protocols which makes it difficult to correctly compare their findings internationally.

We believe that the MNR measurement is indispensable to continue comprehensive research on bee resistance to the varroa mite in the future as well as contributing to a successful and pertinent selection of resistant honey bees. However, we are in high need of a standard protocol to be used worldwide. Therefore, we aim in this study to validate an MNR protocol and point out the constraints and opportunities of this method to encourage its use in the future.

2. Materials and Methods

This study investigates (i) the variability of the MNR score obtained using the reference protocol, (ii) the repeatability of the MNR trait in comparison with other resistance traits, (iii) and the correlation between MNR and other resistance traits. For this purpose, a total of 317 honey bee colonies were used. All colonies were located in the facilities of ITSAP and INRAE (Avignon, France); they were closely monitored for colony dynamics, varroa infestation and varroa resistance behaviours from 2016 to 2019. Colonies were managed according to local beekeeping practices, including a yearly transhumance to the lavender fields between July and August. In order to infer the impact of varroa infestation and to be able to measure correlated varroa resistance related traits, the colonies were left untreated for varroatosis during the course of the experiment.

2.1. MNR measurements

Mite Non Reproduction (MNR) was measured following the COLOSS protocol [38]. A brood frame was randomly taken from each colony, and brood with worker pupae (purple stage or older) infested by a single varroa foundress were dissected. The reproductive status, reproducing (R) or non-reproducing (NR), of the varroa foundress within each single-infested cell was inferred: mites that produced at least one viable daughter were considered as reproducing (R), while the others were considered as non-reproducing (NR) mites (i.e. they could not produce any viable daughters by the time the adult bee would leave the cell). Three different NR cases can be identified: foundresses that had not reproduced at all, foundresses that had produced only female offspring and foundresses that had started to produce offspring too late for their daughter to reach maturity before the bee would emerge from its cell. This measurement is ideally performed after the end of the honey production period, when varroa infestation is expected to peak. MNR was measured, at least once during the beekeeping season, for 231 colonies.

In order to evaluate the repeatability of the MNR assay, repeated measures of MNR were performed, with two different time intervals: in the first set of measures (short-term repeatability), MNR was tested every 10 days for 30 days, thus three times at the end of the beekeeping season for 31 colonies; in the second set of measures (long-term repeatability), MNR was measured between one and five times across the beekeeping season, for 55 colonies.

2.2. Variance of MNR measurements

MNR measurement protocol can lead to up to 20% theoretical error in MNR scores [3]. To validate this theory and estimate the variance of MNR score on field data, we measured MNR from 60 to 101 cells infested by a single varroa foundress for 39 colonies. First, the MNR score was estimated from the first 35 dissected cells, as done usually over the normal course of the experiment ('First MNR'). Second, a total MNR score was estimated based on all the dissected cells ('Total MNR'). Finally, for all the colonies 10, 35 and 50 cells were resampled randomly 100 times from the dissected cells to estimate MNR scores; 32 of these colonies had enough dissected cells to allow for a random resampling of 80 cells to estimate MNR scores.

2.3. Other measures of varroa resistance and colony dynamics

2.3.1. Hygienic behaviour

Hygienic behaviour was tested using the pin-test protocol [39,40]. This test consists in piercing 50 brood cells with a pin and counting the number of such pinned cells that are cleaned or under ongoing cleaning after six hours. Hygienic test (HYG) was measured two to five times during the beekeeping season, on 139 colonies.

2.3.2. Varroa Sensitive Hygiene

Varroa Sensitive Hygiene (VSH) was measured at the end of beekeeping season by artificial infestation [39] on 26 colonies. For each colony tested, 30 freshly capped brood cells were artificially infested by one varroa foundress. To do so, each cell was carefully uncapped with a scalpel, a vigorous varroa mite (from varroa-donor colonies) was placed inside the cell, which was then recapped and the frame returned into the tested colony for seven days. VSH was calculated as the proportion of artificially infested cells that are uncapped and cleaned (emptied) on the 8^{th} day after artificial infestation.

2.4. Colony and mite monitoring

Colony dynamics was monitored using the ColEval method [41]. The number of bees, the number of open and capped brood cells, and the quantity of honey and pollen were estimated on average once a month across the whole beekeeping season.

Varroa mite infestation and dynamic were measured in two different ways. First of all, varroa infestation in the brood was measured simultaneously to MNR scoring as this measure produces estimation of brood infestation. Secondly, phoretic varroa load was measured using the detergent method [39]; the number of varroa mites on adult bees was estimated and expressed as number of mites per 100 bees. Phoretic varroa load was measured multiple times during the beekeeping season (March to October), on the day ColEval was performed.

2.5. Statistical analysis

All statistical analyses and visualisations were executed using R 3.6.2 [42].

2.5.1. MNR variance

MNR score minimum, mean and maximum were estimated for the resampling of 10, 35, 50 and 80 cells infested by a single varroa foundress as well as for the first MNR score (corresponding to the first 35 cells dissected) and the total MNR score. Pooled standard deviation, for samples of identical sizes, for the resampled colonies was estimated as follows

$$\bar{s} = \sqrt{\frac{s_1^2 + s_2^2 + \ldots + s_k^2}{k}} \quad (1)$$

where s_i is the sample standard deviation over k colonies.

Finally, the Coefficient of Variation (CV), for re-sampled colonies, was estimated as

$$CV_k = \frac{SD_k}{Mean_k} \quad (2)$$

The Coefficient of Variation (CV) is commonly used to assess the precision of an estimate as it is a ratio of deviation to the mean. This estimate was pooled across the k colonies, as for the standard deviation in equation (1). Exponential regressions were fitted on the average CV for the MNR score. CV were predicted, using the package *car* [43], for a number of dissected cells infested by a single varroa foundress going from one to 150 in order to infer the minimum necessary number of cells infested by a single varroa foundress dissected to obtain CV of 10% and of 5%, respectively.

2.5.2. MNR score correction

Bias in MNR score is highly dependent on the number of cells infested by a single varroa foundress that one can find on the selected brood frame. The COLOSS protocol recommends the dissection of a minimum of 35 such cells. Leaning towards the application of this protocol we managed to score MNR on 35 cells or more (up to 49 cells) in 81 % of the tested colonies. However, due to low infestation level and/or low amount of capped brood in some colonies, the MNR score was measured on between 34 and as little as one cell infested by a single varroa foundress for 19% of the frames (n = 82). To avoid discarding such data points we applied an Empirical Bayes correction, as proposed by [3].

$$\widehat{MNR} = \frac{nr}{c} \sim Beta(\alpha, \beta) \quad (3)$$

with c the number of dissected cells infested by a single varroa foundress, nr the number of non-reproductive mites and $\frac{nr}{c}$ being an estimator of MNR. A Beta distribution is fitted to available

observations, using the package *MASS* [44]. Parameters alpha and beta were estimated by the L-BFGS-B method based on MNR scores available ranging but not including 0 and 1 (416 observations on 229 colonies). Observed values are corrected as follows

$$EB\ MNR = \frac{\alpha + nr}{\alpha + \beta + c} \quad (4)$$

Consequently, we estimated Spearman rank correlation between raw MNR scores and Empirical Bayes MNR scores, EB_MNR. Such a correction was applied throughout the rest of the analysis.

2.5.3. Repeatability of mite resistance at different scales

Repeatability, considered as an estimation of the likelihood of obtaining multiple times the same result upon multiple evaluation of EB_MNR in a given colony, was estimated for short-term (multiple EB_MNR score within a month) and long-term (multiple EB_MNR score within a year) EB_MNR scores, as well as for hygienic behaviour (HYG) as:

$$R = \frac{V_g}{V_p} = \frac{V_g}{V_g + V_e} \quad (5)$$

where V_g is the genetic variance, V_e is the environmental variance and V_P is the phenotypic variance. In our case V_g was the colony variance and V_e the residual variance estimated from a linear mixed-effect model.

2.5.4. Correlations between EB_MNR and other resistance traits

The cleaning of infested brood cells (VSH), was tested with an exact Binomial test with p-values adjusted for multiple testing by the Bonferroni correction. The correlation between VSH and EB_MNR was estimated using a Spearman rank correlation.

2.5.5. Effects on resistance traits

The effects of variables linked to scoring period (year, month), location (apiary, county), beekeepers groups and queen origin, experimentation (observer), colony dynamics and varroa infestation were tested for EB_MNR and HYG. For EB_MNR nine qualitative and nine quantitative variables were tested using 446 observations on 231 colonies and for HYG five quantitative and seven qualitative variables using 375 observations on 175 colonies. When testing EB_MNR and HYG, in order to obtain a complete data set, missing data for the qualitative variables were set as 'unknown' and missing data for the quantitative variables were imputed using the FAMD imputation function (with two principal components) from the *missMDA* package [45]. Quantitative variables were set as fixed effects while qualitative variables (scoring period, location, beekeepers and queen information and observer) were set as random to control for structure in the population in the model. Fitting qualitative variables as random effects also allows for comparison between groups effects within these qualitative variables in cases were a large number of groups are represented each by a small number of data points, these groups not representing the full grouping possibilities in the population.

We performed a backward model reduction on the two data sets (function step from *lmerTest* package in R [46]) as suggested by Zuur et al. [47] first selecting random effects, for EB_SMR and HYG, from a full model and then selecting fixed effects. Such model selection is based on Akaike Information Criterion (AIC). The effects of the selected variables were estimated by a mixed-effect model analysis. Significance of the fixed effects was inferred using p-values from the model and significance of the random effects was inferred using confidence intervals, computed with the confint function (*lme4* package in R [48]).

3. Results

3.1. Variance of the MNR score

Variance of the MNR score was estimated by 100 resampling events, for 10, 35, 50 (n = 39) and for 80 cells (n = 32) infested by a single varroa foundress. The MNR score based on the first 35 cells, equivalent to the scoring in the field throughout the experiment, ranged from 0.17 to 0.69, with a mean of 0.39 and a standard deviation of 0.14.

The CV across colonies for the 100 resampling events are 41.2% when MNR score is measured on 10 cells, 18.2% when measured on 35 cells, 12.7% when measured on 50 cells and 6.0% when measured on 80 cells (Table 1 and Figure 1).

Table 1. Summary table containing the minimum, mean, maximum, standard deviation and coefficient of variation for the first (MNR_first) and the total MNR (MNR_tot). For the 10 (MNR_10), 35 (MNR_35), 50 (MNR_50) and 80 (MNR_80) resampling events minimum, mean, maximum, as well as pooled standard deviation and pooled coefficient of variation with their 95% confidence interval are reported.

	Minimum	Mean	Maximum	Standard deviation	Coefficient of variation
MNR_first (35 cells)	0.171	0.394	0.686	0.141	0.357
MNR_tot	0.165	0.373	0.680	0.123	0.331
MNR_10	0.000	0.370	1.000	0.142 [0.138; 0.145]	0.420 [0.380; 0.460]
MNR_35	0.000	0.374	0.886	0.062 [0.060; 0.064]	0.180 [0.164; 0.197]
MNR_50	0.080	0.373	0.820	0.044 [0.041; 0.046]	0.128 [0.115; 0.141]
MNR_80	0.125	0.356	0.725	0.020 [0.017; 0.022]	0.060 [0.051; 0.070]

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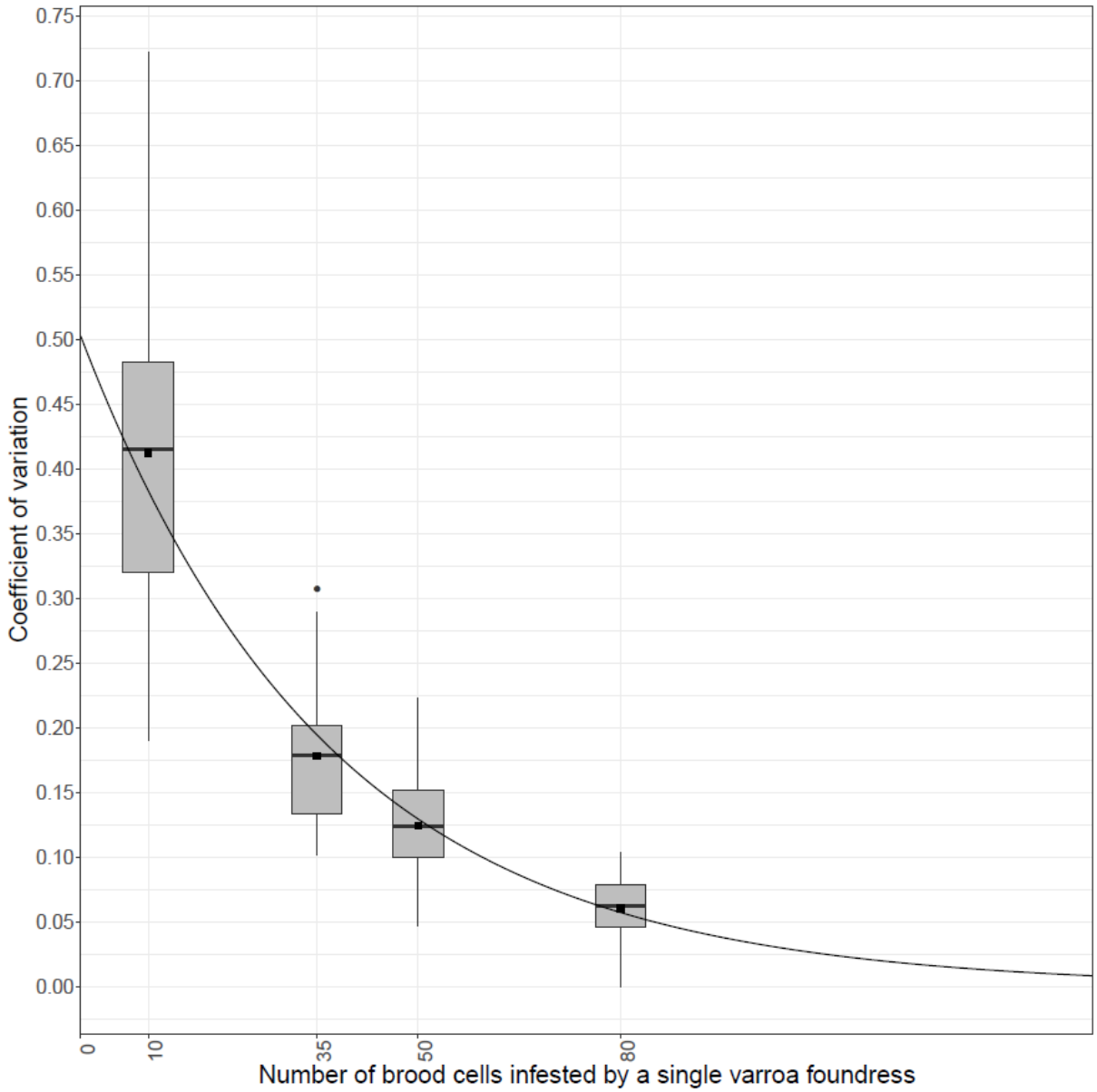

Figure 1. Boxplot of the coefficients of variation for the resampling events with 10, 35, 50 and 80 cells infested by a single varroa foundress. The black square is the mean coefficient of variation (CV) value and the black line represents the exponential regression curve fitted to the data and allows the prediction of the minimum number of cells infested by a single varroa foundress necessary to reach 10% (60 cells) and 5% (85 cells) variation for MNR.

It is possible to predict the theoretical number of cells to be dissected to reach a specific variation by fitting an exponential regression to the average CV. If the aim is to reach a maximum of 10% variation, meaning that if the same colony is resampled multiple times raw MNR scores will only vary by 10%, we inferred that at least 60 cells infested by a single varroa foundress were necessary to be dissected. To reach 5% of variation the dissection of at least 85 cells infested by a single varroa foundress was necessary (Figure 1).

3.2. MNR score correction

In order to avoid bias in MNR score due to a variable number of cells infested by a single varroa foundress dissected per brood frame, especially if estimates based on a small number of dissected cells, an Empirical Bayes correction was applied. Overall, raw MNR scores range from 0 to 1, when Empirical Bayes MNR scores (EB_MNR) range from 0.08 to 0.79. Both raw MNR and

EB_MNR have a mean of 0.41, which is expected when applying Empirical Bayes correction, as it is equivalent to a shrinkage of the data, therefore reducing the dispersion of the data but not affecting its mean. Spearman rank correlation between EB_MNR and raw MNR score was 0.99 (p-value $<10^{-16}$) (Figure 2). Thereafter we use EB_MNR scores.

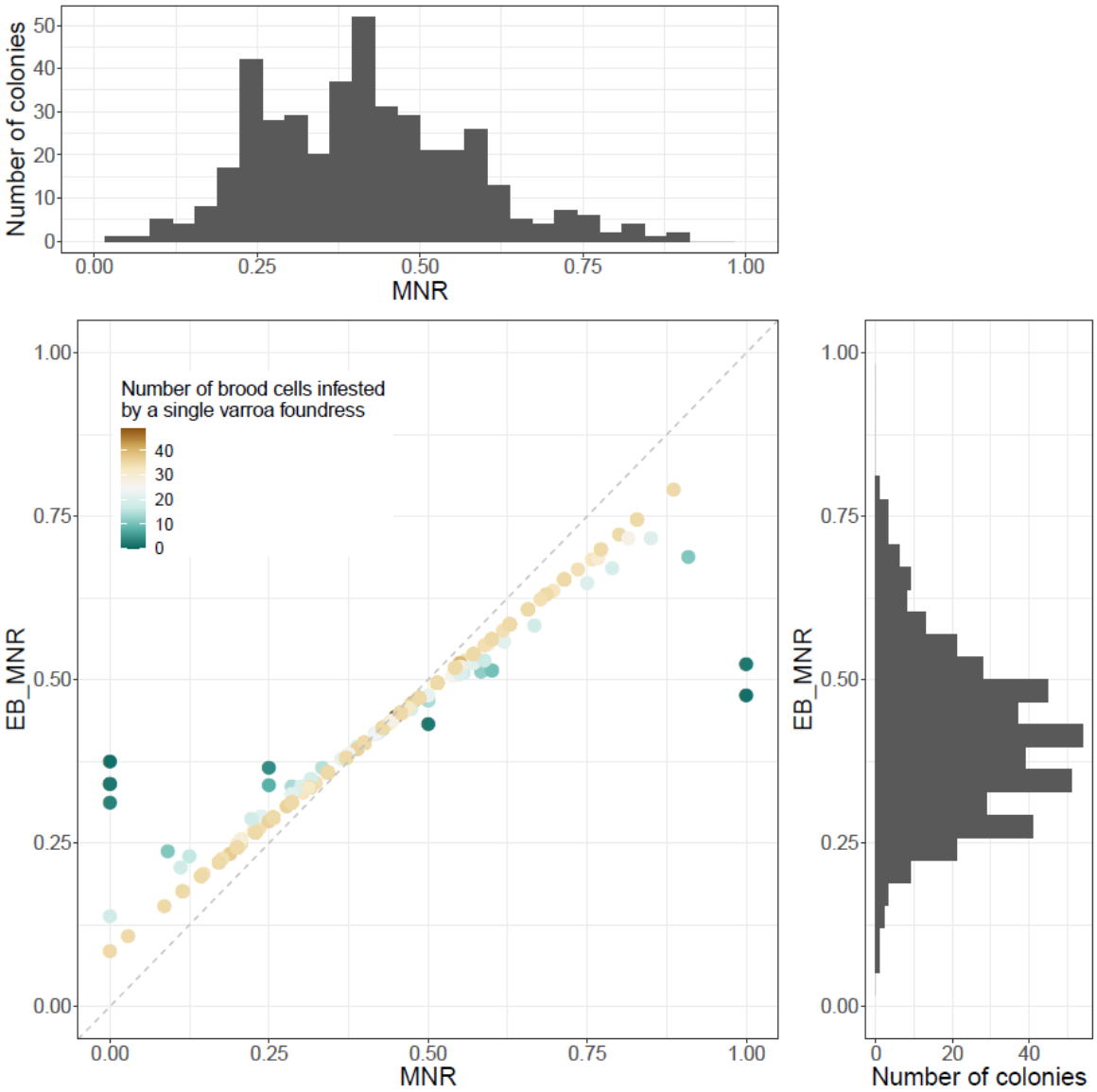

Figure 2. Scatterplot of the raw MNR score and the Empirical Bayes EB_MNR score. Data points are coloured according to the number of cells infested by a single varroa foundress dissected, dark green being close to zero to brown being up to more than 40. The histogram on the top represents the distribution, in number of colonies, of raw MNR score and the histogram on the right, the distribution of EB_MNR score.

3.3. Repeatability of mite resistance traits

Repeatability is a measure of the likelihood of obtaining multiple times the same result. The higher the repeatability the more likely we are to obtain multiple times the same value, in our case the same EB_MNR score.

3.3.1. EB_MNR repeatability

EB_MNR short-term repeatability, as estimated by three measures once every 10 days, was 0.43 (standard error = 0.11, for 93 EB_MNR scores on 31 colonies). Intra colony variance could be

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clustered, using k-means, in three groups having average variances of EB_MNR score of 0.034 ('high variability', two colonies), 0.011 ('average variability', 13 colonies), 0.003 ('low variability', 16 colonies) (Figure 3). Long-term EB_MNR repeatability, over multiple measures within a year, was 0.17 (standard error=0.09, for 148 EB_MNR scores on 55 colonies).

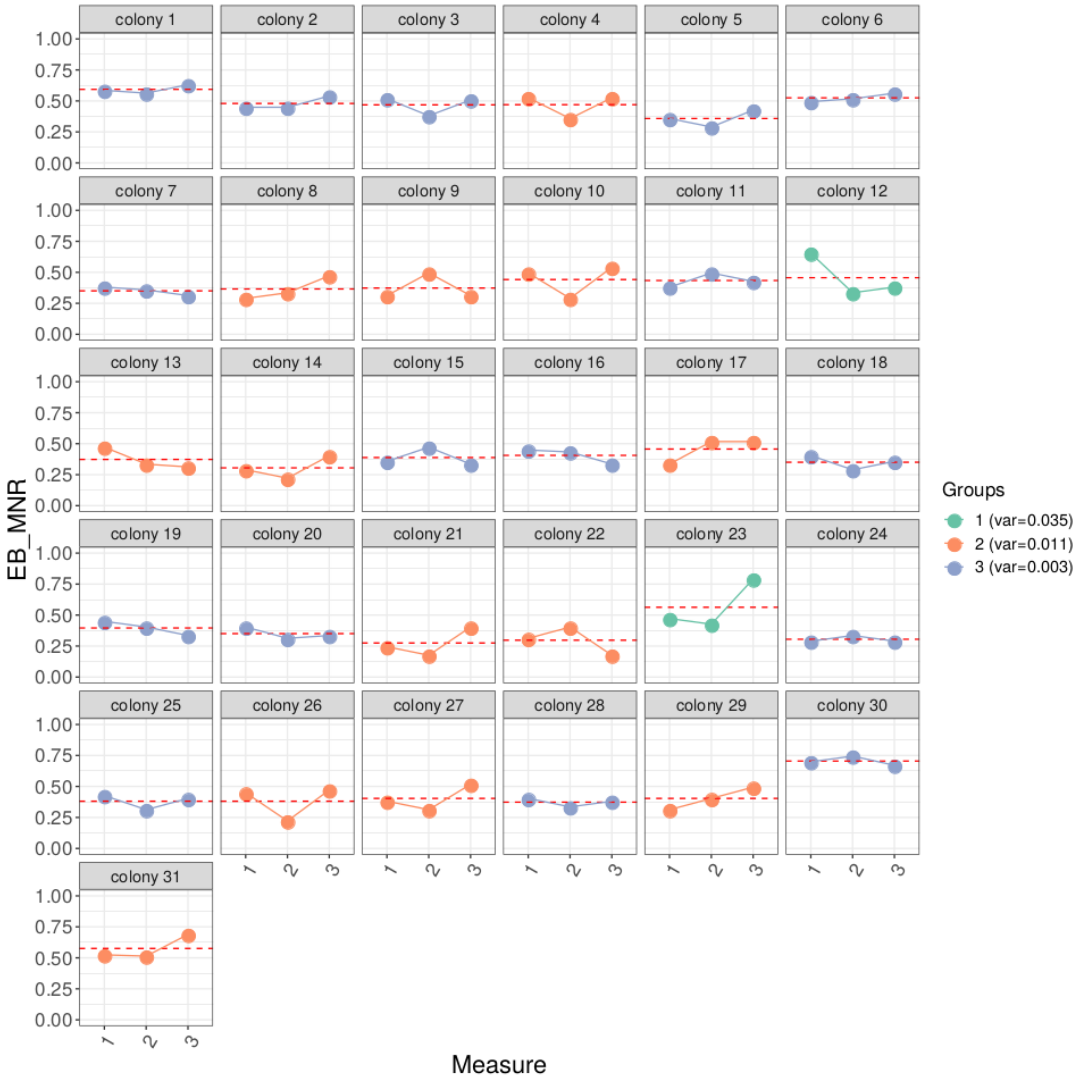

Figure 3. Short-term repeatability of MNR trait. EB_MNR score measured three times, once every 10 days, for each of the 31 colonies. The red dotted line represents the colony EB_MNR averaged across the three measures. The colour informs on the belonging to one of the three variance groups, in green the high variance group (two colonies), in orange the medium variance group (13 colonies) and in blue the low variance group (16 colonies).

3.3.2. Hygienic behaviour repeatability

Hygienic behaviour ranged between 0 and 1, with a mean of 0.74. Hygienic behaviour measured multiple times within a year had a repeatability of 0.21 (standard error = 0.07, 339 measures of hygienic behaviour on 139 colonies).

3.4. Correlations between mite resistance traits

VSH, measured by artificial infestation on 26 colonies, ranged between 0.10 to 0.97 cleaning rate, with a mean of 0.40 and a median 0.36. Three colonies had a significantly low VSH, meaning that less brood cells were cleaned than expected under the null hypothesis of 0.40 cleaning (the

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mean VSH score of the tested colonies), whereas four colonies had a significantly higher VSH, meaning that more brood cells were cleaned than expected under the null hypothesis (Figure 4).

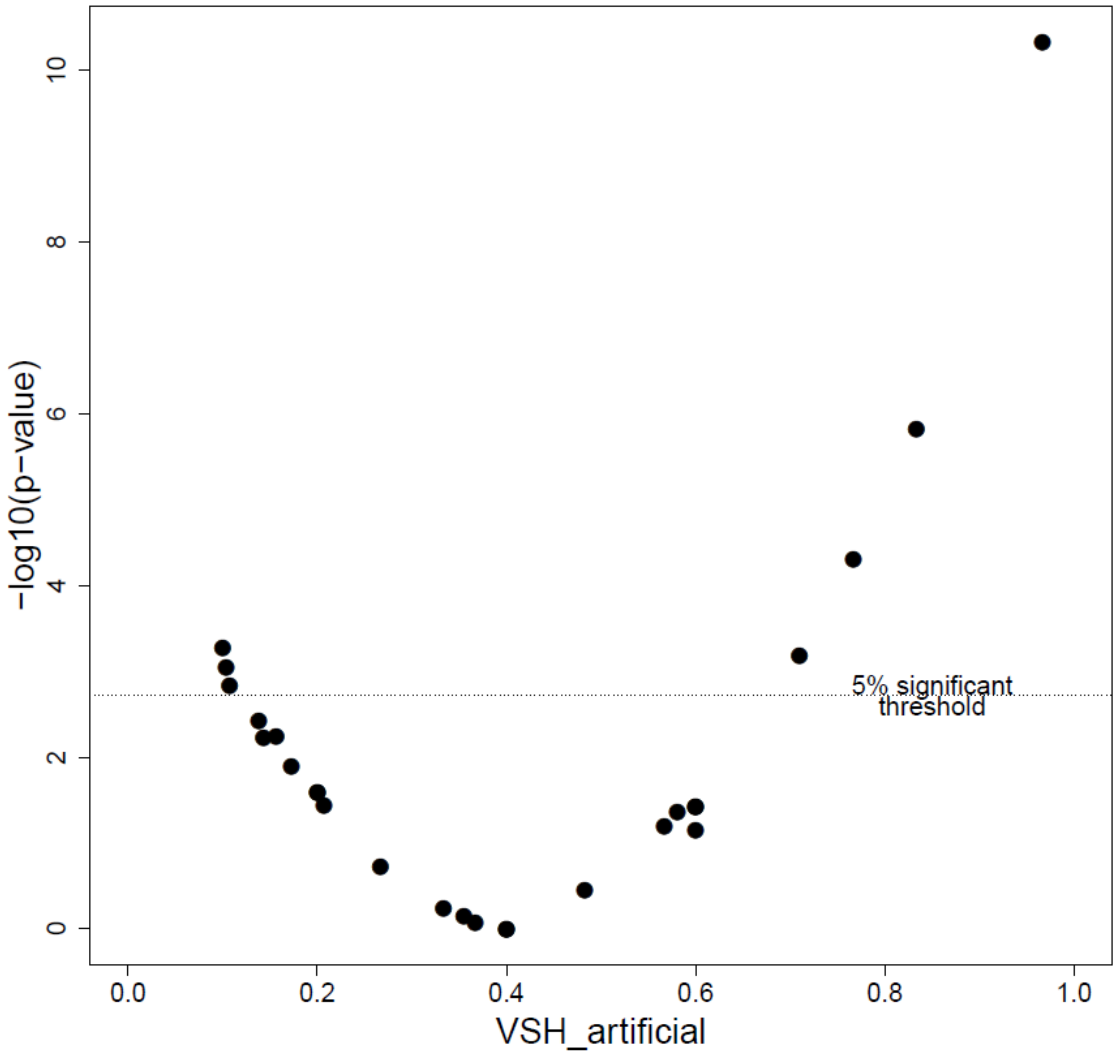

Figure 4. Scatterplot of the -log10(p-values) for VSH. The dotted line represents the 5% significance threshold, in -log10, after Bonferroni correction for multiple tests. Points above the threshold line are colonies with significantly lower (to the left) or higher (to the right) cleaning rate than expected under the null hypothesis.

Finally, we estimated the Spearman rank correlation between EB_MNR and VSH, as both measures were made in the same conditions (date, location …). There were no correlations between EB_MNR and VSH (25 colonies, Spearman rank correlation = 0.22, p-value = 0.29) (Supplementary Figure 1).

3.5. Effects on resistance traits

3.5.1. EB_MNR

Variables retained as random effects in the best model were: scoring period, breeders group, and the observer while variables retained as fixed effects were: the amount of honey in the hive and the recapping behaviour of the colony. Overall EB_MNR was significantly influenced by the random effects: scoring period and breeders group, with the breeders group INRAE, having 'survivor' colonies kept for at least three years [49], presenting the highest effect and showing the

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highest EB_MNR values. The trait EB_MNR was also significantly influenced by both fixed effect: amount of honey in the hive (negative effect) and recapping behaviour of the colony (positive effect) (Table 2), meaning that little honey and high recapping behaviour was associated with high EB_MNR score.

Table 2. Summary table of the best model for EB_MNR. Standard deviations and 95% confidence intervals (to determine significance) are presented for the selected random effects. Coefficients, standard errors, degrees of freedom and p-values are presented for the selected fixed effects.

Random effects	**Standard deviation**	**95% Confidence interval**		
Colony	0.044	[0.024; 0.058]		
Breeders group	0.034	[0.004; 0.054]		
Scoring period	0.036	[0.014; 0.057]		
Observer	0.030	[0.000; 0.050]		
Residuals	0.090	[0.083; 0.099]		
Fixed effects	**Coefficient**	**Standard error**	**Degree of freedom**	**p-value**
Intercept	0.395	0.018	19.560	$<10^{-15}$
Number recapped cells	0.015	0.005	420.554	0.007
In hive honey	-0.022	0.006	257.714	0.001

3.5.2. Hygienic behaviour

Variables retained as random effects in the best model were: queen's genetic origin and the testing apiary while the only variable retained as fixed effects was: the phoretic varroa infestation level, being the only one linked to varroa infestation level in the colony available for HYG measure. Overall HYG was significantly influenced by the testing apiary and the varroa infestation. The fixed effect linked to varroa infestation was negative (Table 3), meaning the higher the varroa infestation the lower the HYG score.

Table 3. Summary table of the best model for HYG. Standard deviations and 95% confidence intervals (to determine significance) are presented for the selected random effects. Coefficients, standard errors, degrees of freedom and p-values are presented for the selected fixed effects.

Random effects	**Standard deviation**	**95% Confidence interval**		
Colony	0.075	[0.000; 0.116]		
Queen's genetic origin	0.069	[0.000; 0.107]		
Testing apiary	0.087	[0.032; 0.139]		
Residuals	0.210	[0.190; 0.230]		
Fixed effects	**Coefficient**	**Standard error**	**Degree of freedom**	**p-value**
Intercept	0.731	0.034	8.0.38	$<10^{-8}$
Phoretic varroa infestation	-0.042	0.012	305.427	$5*10^{-4}$

4. Discussion

In this study we aim to describe a more pertinent MNR protocol as the current protocols lead to a large variation in MNR scores. This variation can be lessened by increasing the number of dissected cells or if this does not lie in the scope of the study, by applying an Empirical Bayes correction. MNR short-term repeatability was larger than long-term repeatability, the latter being similar to the repeatability of hygienic behaviour. Finally, no correlation between MNR and VSH could be observed in our dataset. This in-depth analysis of Mite Non-Reproduction (MNR) highlights several points that need to be considered when using this trait for experiments and breeding efforts.

The current state of art for MNR measure advises on a minimum of 35 cells infested by a single varroa foundress to be dissected and for which mite reproduction should be analysed per colony [38]. When using such guidelines, we reported a 18.2% variation for raw MNR. This suggests that estimates relying on 35 cells or less can potentially be unreliable. For instance, when the goal of an experiment is to sample for colonies with an MNR of at least 60%, while relying on a 35 cells estimate, only colonies with an MNR of at least 78% are guaranteed to fit the criteria. Low precision observed for the MNR score supports theoretical findings from Traynor et al. [3], where it was shown that 35 cells is the minimum target to get relatively reliable MNR scores. This needs to be considered especially for scientific studies where case rankings have to be performed with extreme care. If the interest lies in exclusively examining high values, as can be done for breeding efforts, sampling 35 or less cells may be sufficient to select colonies as shown in [21]. In fact, one needs to stay careful in the threshold used for selection and tighten it when a smaller number of cells are dissected to estimate MNR.

Questions arose especially for colonies with a small number of cells infested by a single varroa foundress. These colonies have the potential to be particularly interesting, as the low varroa load could point to a resistance to infestation. Two approaches can be used to deal with such data: one can set a cut-off for the number of cells infested by a single varroa foundress necessary to validate the subsequent MNR score or one can apply an Empirical Bayes correction to the raw MNR scores leading to shrinkage in MNR score distribution. The first strategy is likely to be preferred by professional beekeepers due to its simplicity whereas the second might be preferred for research purposes as it allows avoiding the application of arbitrary thresholds. However, both lead to the rejection of the colonies with extreme MNR scores, one by removal, the second by fading due to the shrinkage. This means that potentially resistant colonies are not being taken into account because they cannot be properly evaluated due to a low varroa load. One solution could be to artificially control the in hive varroa infestation. However, this could cause bias in the MNR scoring procedure. Up to date there has been no strategy developed to counter this bias, we recommend future studies to focus on estimating sampling bias by dissecting an extensive number of cells, ideally full frames without applying cut-off or by carefully combining MNR trait with other traits of the colony. However, the development of such index relies on a thorough understanding of the different traits to combine, their interactions and still has to be developed and generalised.

The results presented here also highlight that short-term repeatability (ten days' time intervals) of MNR was modest whereas long-term repeatability (within years) measurements was relatively low, although comparable to that of hygienic behaviour [50,51], a main trait used as a selection criterion in several breeding programs. Measuring hygienic behaviour has successfully led to the selection of hygienic honey bees [52] meaning that nevertheless there is potential for MNR to be used for selection if its heritability is high enough, which still has to be confirmed.

The low repeatability may be partially explained by the variance of estimates described above (i.e. values potentially may be up to 18% too high or too low when using 35 cells), meaning that the resistance score of a colony could be much more congruent than what we see in our dataset. A shortcoming of our study is the absence of consideration for environmental variables. However, it is known that environmental variables such as temperature and humidity can potentially affect resistant traits [53,54] with temperature being negatively and humidity being positively correlated

to the infestation growth rate of the mite [55]. Resource availability is also known to influence hygienic behaviour [56,57], as well as task repartition in the colony, which may also affect the MNR trait. For instance, during a period with strong nectar flow as experienced by our colonies in July, there may be trade-offs between brood care and foraging and the expression of the VSH trait. Evaluation at the end of August can thus be influenced by the low proportion of brood in the colony versus stored honey (Tison et al. personal communication). Furthermore, resistance traits can be biased by the horizontal transmission of varroa mites by the drifting of bees or the robbing of hives [58–60], especially if the amount of transferred mites differs between colonies of the same apiary [16]. Additionally, colony and mite dynamics are highly changing through time and may influence mite reproduction and therefore MNR results. In our study, we accounted for colony dynamics, colony management and location of the hives, which all did not significantly impact MNR. Neither did mite infestation in the brood cells and on adult bees affect the MNR trait. This correlates with the observation of different authors, stating that the link between MNR and mite infestation levels is not universal [3,34,61,62].

All of the above demonstrate that MNR is a complex mechanism combining multi-factorial effects, such as the adult bee behaviour, the brood and mite physiology and bee and mite genetics. There is a high need for further analysis to disentangle potential environmental effects on MNR mechanism. We can also assert that experimental design is one of the major limitations to draw solid conclusions. The lack of balance in the experimental design can lead to spurious effect estimation and interpretations. Controlling the experimental design is somehow difficult in the field but should be evaluated upstream at the research facilities. Lastly, even though we could observe significant differences between VSH across our colonies and to the contrary of what has been previously found by Harbo and Harris [24], no correlation between MNR and VSH (Varroa Sensitive Hygiene) were observed in our data set. However, it is known that varroa resistance mechanisms can differ between populations and that resistance traits are not always informative of resistance to varroa infestation in unselected populations [63]. It thus seems legitimate to genetically distinguish each population and look into its unique resistance behaviour [64]. Moreover, it has been shown that selection for resistance traits are potentially challenging because of parasite adaptation. As observed earlier [65] it is possible that even though some colonies harbour significantly higher VSH scores selecting them might not lead to a perennial selection for many generations. We could expect the same to be valid for MNR making the use of this resistance trait hard in practice.

5. Conclusion

In conclusion, the MNR measurement remains one of the few measurements for varroa resistance in honey bee populations, which can be achieved in the field on a relatively large scale. Although time consuming and tedious to implement, it also gives a lot of different information which can help us better understand the control mechanisms that bees use to counteract the varroa mite. However, the results here highlight the need for a precise protocol using enough single infested cells (> 35), performed multiple times over a short period of time to provide solid estimates. The weak points should be taken into consideration when designing an experiment and a combination of different measurements to correctly assess honey bee resistance like mite infestation levels (inside and outside the brood cells) and genetic analysis could be additionally taken into account when analysing the varroa resistance of a colony. Up to date very few breeding programs aiming for resistant honey bees have produced commercially available colonies. We believe that, when using the MNR measurement with a new awareness of its weaknesses and strengths, it could be an important tool for successful future selection programs of resistant honey bees.

Supplementary Materials: The following are available online, Figure S1: Scatterplot of VSH for EB_MNR values for the 26 colonies used to estimate correlation between these traits.

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