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Decreased Mite Reproduction to Select *Varroa destructor* (Acari: Varroidae) Resistant Honey Bees (Hymenoptera: Apidae): Limitations and Potential Methodological Improvements

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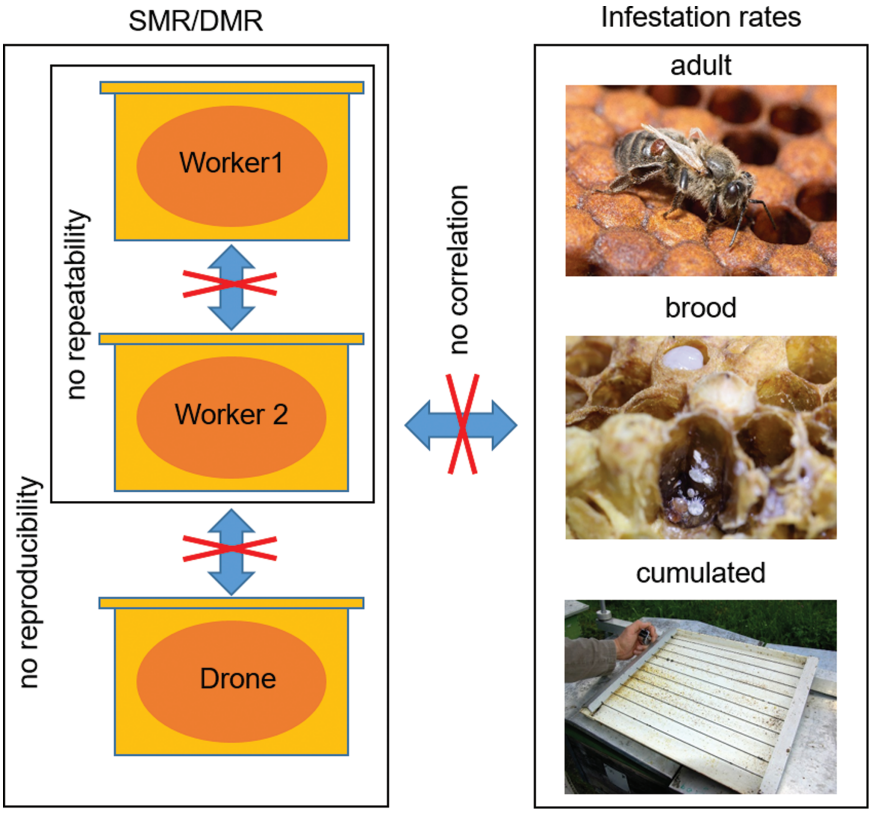
Abstract

The invasive parasitic mite, *Varroa destructor* (Anderson and Trueman), is the major biotic threat to the survival of European honey bees, *Apis mellifera* L. To improve colony survival against *V. destructor*, the selection of resistant lineages against this parasite is considered a sustainable solution. Among selected traits, mite fertility and fecundity, often referred to as suppressed mite reproduction are increasingly used in breeding programmes. However, the current literature leaves some gaps in the assessment of the effectiveness of selecting these traits toward achieving resistance. In the population studied here, we show a low repeatability and reproducibility of mite fertility and fecundity phenotypes, as well as a low correlation of these traits with infestation rates of colonies. Phenotyping reliability could neither be improved by increasing the number of worker brood cells screened, nor by screening drone brood, which is highly attractive for the parasite and available early in the season, theoretically allowing a reduction of generation time and thus an acceleration of genetic progress in selected lineages. Our results provide an evaluation of the potential and limitations of selecting on decreased mite reproduction traits to obtain *V. destructor*-resistant honeybee colonies. To allow for a more precise implementation of such selection and output reporting, we propose a refined nomenclature by introducing the terms of decreased mite reproduction and reduced mite reproduction, depending on the extent of mite reproduction targeted. We also highlight the importance of ensuring accurate phenotyping ahead of initiating long-lasting selection programmes.

Graphical Abstract



Key words: honey bee, resistance, selection, suppressed mite reproduction, varroa mite

Varroa destructor represents the main biotic threat to the survival of European *Apis mellifera* colonies in their natural distribution range and in the region to which this managed pollinator was introduced (Guzman-Novoa et al. 2010, Le Conte et al. 2010, Neumann and Carreck 2010). The natural host of this mite is the Eastern honey bee, *Apis cerana* (Koeniger et al. 1981, Rath 1999). The introduction of *A. mellifera* colonies in the distribution range of *A. cerana* led to the host jumping to *A. mellifera*. The subsequent honey bee trade allowed for the spread of *V. destructor* to Europe and the Americas (Crane 1978, de Jong et al. 1982, Oldroyd 1999, Owen 2017), where *V. destructor* infestations led to the collapse of large numbers of susceptible European *A. mellifera* colonies (hereafter simply designated as *A. mellifera*). This collapse is mainly due to the ability of the invasive mite lineage to infest worker brood, which is present throughout the active season and which leads to the build-up of large populations of parasites to levels damaging to host colonies. In contrast, in the original host, reproduction is limited to the drone brood, which is available only for a short period of several weeks, reducing their ability to reproduce (Boecking et al. 1993, Boot et al. 1997). To reduce *Varroa* mite infestations in susceptible colonies, beekeepers rely on treatments with acaricides, including organic or synthetic compounds (Rosenkranz et al. 2010). The application of such compounds in honey bee colonies can cause negative side effects on workers, drones, and queens (Rinderer et al. 1999, Haarmann et al. 2002, Pettis et al. 2004, Gashout et al. 2018, Tihelka 2018), contaminate hive products (Bogdanov et al. 1998, Wallner 1999, Kast et al. 2019) and lead to the development of resistance in the parasite (Milani 1999, Elzen et al. 2000, González-Cabrera et al. 2018, Almecija et al. 2020). Thus, the development of more sustainable control strategies is desirable. Selecting honey bee lineages resistant to infestation by *V. destructor* is deemed the most sustainable solution to the '*Varroa* problem' (Dietemann et al. 2012). Programmes aiming at breeding *V. destructor*-resistant honey bee lineages focus on the selection of colonies based on traits observed in naturally surviving honey bee populations. These traits are thought to negatively impact the reproduction and hence the population growth of *V. destructor* (Locke et al. 2012, Locke 2016, Mondet et al. 2020a). A common trait used for selection is suppressed mite reproduction (SMR; Büchler et al. 2010, Rinderer et al. 2010, Guichard et al. 2020a). SMR is phenotyped using two methods: the assessment of mite fecundity by counting the number of viable daughters produced (e.g., Martin 1994, 1995, 1997), or of mite infertility, as the lack of mite reproductive success expressed as the percentage of mites producing no viable daughters (Büchler et al. 2020, Eynard et al. 2020, Mondet et al. 2020b). The reduction in mite reproduction by SMR is thought to result from the properties of immature (i.e., brood) or adult hosts (Harbo and Harris 2005, Frey et al. 2013, Conlon et al. 2019). Recently, the denomination SMR was suggested to be specifically reserved to describe brood traits and replaced by mite non-reproduction (MNR) to better distinguish its proximal causes (Mondet et al. 2020a). However, because of the two phenotyping methods implemented, the old (SMR) and newly proposed (MNR) terms do not cover the possibility of selecting decreased reproduction, as opposed to the complete lack of reproduction suggested by the terms 'suppressed' or 'non-reproduction'. Since the terminology is still not optimal, we here propose a more descriptive series of

terms (see Glossary). We will here use decreased mite reproduction (DMR) instead of MNR to consider both cases in which zero offspring are produced (MNR) and those with non-null reproduction that is inferior to average published values, which can be designated as reduced mite reproduction (RMR). Because we consider both fertility and fecundity-based decreases in reproduction and wish to reduce the number of initialisms to simplify reading, we will use infertility-based DMR for null and fecundity-based DMR for non-null reproduction as synonyms for these terms.

Glossary:

DMR (a/b): decreased mite reproduction, irrespective of whether the decrease in mite reproduction is partial or complete.

RMR (a/b): reduced mite reproduction, leading to low mite fecundity, i.e., a lower but non-null number of viable daughters produced by foundresses compared to published averages; synonym: fecundity-based DMR.

MNR (a/b): mite non-reproduction leading to mite infertility, i.e., to a complete failure of viable daughter production by a proportion of the foundresses; synonym: infertility-based DMR.

a and **b** can be added to the initialisms to specify whether the effect on mite fecundity or fertility is due specifically to traits of adult workers (a) or brood (b).

DMR has frequently been implemented in selection programmes, showing its popularity amongst honey bee breeders (Guichard et al. 2020a) and leading to an increase in published reports. These reports allowed for first estimates of the effectiveness of selecting this trait toward resistant honey bee lineages (DeGrandi-Hoffman et al. 2002, Büchler et al. 2020, Eynard et al. 2020, Guichard et al. 2020a). Several limitations in using DMR appeared, hindering its practical implementation (Guichard et al. 2020a). Such limitations deserve attention to streamline and improve selection programmes toward delivering the expected outcome, i.e., resistant honey bee lineages. Protocols currently applied for phenotyping DMR differ in sampling method and size as well as in measurement method (Mondet et al. 2020b). It is not known whether the two measurement methods of DMR are equivalent or one is superior to the other for the identification of colonies able to decrease mite reproduction. In theory, fertility-based DMR and fecundity-based DMR could provide distinct colony phenotypes, as some colonies could have a higher proportion of infertile mites, whereas others could only show a general reduction in the number of mite offspring. Phenotyping DMR is also a time-consuming task, as it involves the screening of several hundred worker brood cells in an attempt to reach the recommended 35 infested cells required (Büchler et al. 2017, Mondet et al. 2020b). Such infestation rates are only relatively easily attainable at the end of the summer, when mite populations grow sufficiently to generate high infestation levels. Recording DMR phenotypes late during the colony evaluation process leads to a generation time of two years in colonies selected for this trait in temperate regions, only allowing for slow genetic progress. The literature on DMR also indicates that infertility-based measures have low repeatability and are imprecise (Büchler et al. 2020, Eynard et al. 2020). The poor repeatability has been attributed to varying environmental conditions between measurement times (Büchler et al. 2020, Eynard et al. 2020). An alternative explanation is that the protocols used to measure this trait do not capture accurately enough the colonies' ability to reduce mite reproduction. Whatever its cause, the lack of repeatability reduces the accuracy of phenotyping and, consequently, the perspectives on a successful selection (Guichard et al. 2020a). Whether DMR can lead to resistant populations is also debatable since there is, to this date, contradictory evidence that colonies selected for DMR have low infestation rates (Guichard et al. 2020a).

Here, we aimed at assessing the reliability and improving the phenotyping of DMR in a Swiss population of 100 *A. m. mellifera* colonies originating from a selection programme driven by a local association. The aim of this programme is to provide *V. destructor*-resistant, locally adapted honey bees to the association's members by selecting for hygienic colonies based on the removal rate of pin-killed brood. We first evaluated whether the two currently used methods to measure DMR provide comparable results and then assessed their repeatability in the most comparable situation when environmental variations are most limited, i.e., within colonies within the same week (as opposed to every 10 days for previous short-term measures of the repeatability of infertility-based DMR [Eynard et al. 2020]). We then evaluated the reproducibility of these measurements by assessing DMR from drone brood. In case phenotyping in worker and drone brood provides similar DMR values, measuring this trait in drones, which are produced in spring and are highly attractive for the parasite (Schulz 1984, Fuchs 1990), could allow phenotyping earlier in the season and reduce the generation time from two years to one year. Finally, we verified the correlation between DMR and *V. destructor* infestation rates and whether this correlation is sample size dependent or is improved by phenotyping on drone brood. Based on the repeatability, reproducibility, and link with *V. destructor* infestation rates observed, we assess the potential and limitations of DMR to select *V. destructor*-resistant honey bee colonies and derive recommendations to exploit this trait in the most efficient manner.

Material and Methods

Honey Bee Colonies

The experiment lasted for over two years. In 2019, the colonies ($N = 40$) used were kept on a single apiary and headed by four groups of sister *A. m. mellifera* queens obtained from the Swiss beekeepers association mellifera.ch (www.mellifera.ch). The tested colonies belonged to four lineages, two with low and two with high values for hygienic behavior toward pin-killed brood and for *V. destructor* infestation rates. We assumed that using colonies at either end of the spectrum of a divergent selection would provide a wide range of DMR values if the latter were functionally linked to the hygienic behavior or infestation rate. In 2020, the colonies ($N = 60$) headed by daughters of four queens (one for each group) were selected from among those tested in 2019. These colonies were divided into two groups of 30, of which one was relocated to another apiary. In both years, the virgin queens to head the experimental colonies were brought to a mating apiary, where they were allowed to mate with drones from unrelated colonies headed by sister *A. m. mellifera* queens selected by mellifera.ch beekeepers.

To promote drone brood production to allow for DMR phenotyping in this type of brood, the colonies were repeatedly fed sugar water and the queens were caged on a comb with drone-sized cells approximately three weeks before the intended collection date. Drone and worker combs for DMR phenotyping were collected in August of each year, just ahead of acaricidal treatments, when mite infestations are at their highest (Ritter et al. 1984, Imdorf et al. 2003) to maximize the number of infested cells available for phenotyping. Depending on the amount of worker brood on the combs, up to three combs were collected to ensure that enough infested cells would be found. Worker and drone brood combs were collected within five days (occasionally within the same

day and up to seven days apart, depending on queen laying dynamics). All combs were stored at –20°C for subsequent dissection during the winter. Colonies were treated with formic acid (Formivar 70%) according to manufacturer instructions at the end of each sampling season before exceeding the threshold of 5 mites/100 adult workers to guarantee colony survival and testing in the following year.

DMR Phenotyping

The DMR value of a colony is obtained from the mite family composition infesting the honey bee worker brood. The infertility-based method is expressed as the percentage of nonreproductive foundress mites (Büchler et al. 2020, Eynard et al. 2020, Mondet et al. 2020b). Nonreproductive mites did not produce at least one viable daughter, i.e., a mature daughter along with a mature male who could mate with the latter. The fecundity-based method measures the number of viable mated daughters produced by a foundress mite (Martin 1994, 1995, 1997), which can only be accurately established in cells containing a host close to emergence (Dietemann et al. 2013). However, to ensure that enough data were available to measure fecundity-based DMR, we also considered cells with younger host developmental stages. The earliest host stage that can be considered is that at which the presence of the male mite can be ascertained as it becomes easily distinguishable from the female offspring, i.e., from the purple eyes stage onwards, seven days post-capping (Büchler et al. 2017).

Capped brood cells containing pupae aged seven to 12 d postcapping were opened until 35 singly infested cells were found (Büchler et al. 2017). The number 35 was estimated as a minimal sample size to ensure reliable measurement of the phenotype (Mondet et al. 2020b). Only worker pupae infested by a single foundress mite were considered, as attribution of offspring to their respective mothers and therefore quantification of the rate of nonreproduction per foundress is impossible in case of multiple infestations. In the relevant cells, we then determined the number of daughters expected to be mature and mated at host emergence. These parameters are easy to determine when the host is close to emergence but require extrapolation in cells containing hosts at earlier developmental stages. Extrapolation about whether individual daughters or sons would be mature at host emergence or at daughter sexual maturity, respectively, was determined by taking the models of Martin (1995, 1997) as references. Daughters will not reach maturity before emergence if they are younger than the typical developmental stage for a given host age and will not be mated if the son is too young or missing when daughters are sexually mature (see Table 1).

To the best of our knowledge, no detailed protocol is available to assess DMR in drone brood, despite the description of the phenotyping strategy (e.g., Broeckx et al. 2019, Elmi et al. 2021); we thus adapted the timing of worker DMR phenotyping to drones. Since, also in drone cells, mature male and female mite offspring only occur from the pink eye pupal stadium, we chose cells containing drone pupae at and beyond this stage, i.e., at nine to 15 d post-capping (Fig. 1) for phenotyping. Mite family composition in drone brood was more challenging to determine due to the longer development time compared to worker brood (Jay 1963). With more time for the oldest daughter mite to reach the same degree of cuticular sclerotization, and hence color, as the foundress mite, it becomes impossible to distinguish them. When mother and daughters are indistinguishable, it is impossible to identify with certainty cells infested by multiple foundresses. In these cases, the number of daughters per foundress for the corresponding pupal stage (Martin 1997), the number of exuviae (one per adult daughter), and the number of males (one per foundress [Rosenkranz et al. 2010]) found in a cell were used as an indication of multiple infestation. If the number of adult daughters surpassed by more than one the expected number for a given pupal stage (e.g., five adult daughters instead of three for a drone aged 12 d post-capping [see Martin 1997]), the drone had likely been infested by several foundresses and the cell was dismissed.

Table 1. Maximal number of days postcapping of worker and drone brood, which allows the various *Varroa destructor* daughter mite developmental stages to reach maturity before host emergence

	Number of days post-capping and host stage description	
Varroa destructor developmental stage	Worker Martin 1997	Drone This study
Female deutonymph	11 Black head Gray thorax	13 Black antennae Gray thorax
Female protonymph	8 Purple eyes Yellow thorax	10 Purple eyes Yellow thorax
Female adult	No restriction	No restriction
Male deutonymph	8 Purple eyes Yellow thorax	9 Pink/Purple eyes White body
Male adult	No restriction	No restriction

The description of the host developmental stage on these days is also given. For adult mite stages, 'no restriction' indicates that host stages are not limiting to enable emergence of mature mite offspring.

Since not every offspring produced on both drone or worker brood reaches adulthood, the number of viable and mated offspring determined as described above was multiplied by the theoretical probability of surviving until emergence (Martin 1994, 1995, 1997).

We measured DMR twice to evaluate its repeatability. The brood areas in which cells were screened to measure DMR were not overlapping and thus constituted biological replicates, thereafter designed as Worker1 and Worker2. Depending on the number of infested cells available per comb, one up to two combs were sampled per replicate. DMR was also measured once in drone brood to assess trait variability between castes. Worker1, Worker2, and Drone measurements were performed by a single trained evaluator to minimize measurement errors.

The mean fecundity and infertility values per measurement per colony were calculated in R (R Core Team 2018) by means of a specifically written script (Script S1).

Colony *V. destructor* Infestation Rate Evaluation

The real infestation rate of a colony can only be measured precisely in a destructive manner, which is not compatible with breeding goals (Dietemann et al. 2013). Breeders thus rely on estimations collected using several nondestructive methods (Ellis et al. 1988, Branco et al. 2006, Lee et al. 2010, Dietemann et al. 2013). To cover the range of methods available and to determine whether they provide comparable results, we estimated infestation rates in three different ways: 1) with the cumulated weekly or bi-weekly natural mite fall on the hive bottom board throughout the season (Dietemann et al. 2013); 2) with the adult infestation rate expressed in 'mite per bee' units and derived from a soapy water wash of 300 bees per colony in August (Dietemann et al. 2013); 3) with brood infestation rates derived from the ratio of infested (including multiple infestations) to noninfested cells found during DMR evaluation.

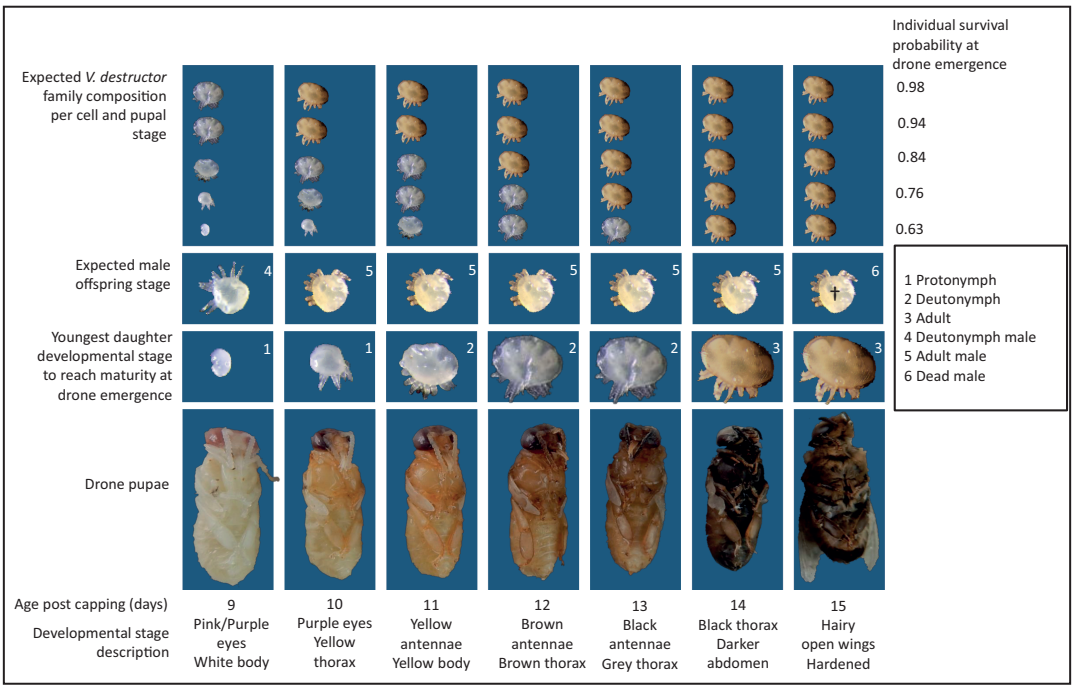


Fig. 1. Drone pupal stage determination criteria as described by Jay (1962) and corresponding family composition according to Martin (1997). The survival probability of each offspring at drone emergence is also indicated.

Data Analysis

We first compared infertility and fecundity-based DMR measures. Second, we assessed the repeatability of DMR traits. Third, we compared the DMR values of worker and drone brood. The DMR values acquired from drone brood were compared to each value acquired from worker samples separately, as one could be more representative than the other, given the spatial heterogeneity of infestations on combs (Fuchs 1985, Wendel 1989). Finally, we compared DMR values with infestation levels. In this last step, we also calculated DMR values on the pooled Worker1 and Worker2 samples to determine whether the sample size affected the strength of the correlation with the infestation level.

Between-group comparisons of the different measures, e.g., DMR and infestation rates from Worker1, Worker2, and Drone samples, were performed using a Kruskal–Wallis rank-sum test, and differences between samples were assessed by pairwise Wilcoxon tests (Bonferroni-adjusted). Correlations between traits or measures (repeatability of fertility and fecundity-based DMR, correlation between DMR obtained from worker and drone brood, correlation between DMR and infestation rates) were, after a Shapiro-Wilke's test indicating the nonnormal distribution of several variables, expressed using Kendall's tau b rank correlation coefficients after correction for the year–apiary combination to account for environmental effects (Guichard et al. 2021). To allow for comparisons with other publications employing Pearson's coefficients, the latter were also calculated and included in the Supp Material (online only). Rank correlations were calculated for all brood samples, including those in which fewer than the intended 35 singly infested cells were found, and only for samples where 35 singly infested cells were found, to determine the effect of sample size on DMR phenotyping. All analyses were performed with R (R Core Team 2018).

Results

Of the initial 100 colonies, 17 requeened or swarmed during the experimental period, so that data could be obtained from 83 of them. Since some colonies lacked brood at the sampling date, the three measures could not be performed on all of them. For Worker1, 82 brood samples could be taken; in 23 of them (28%), less than 35 singly infested worker brood cells were found after opening all the cells available. For Worker2 and Drone brood samples, this occurred in 27 out of 79 samples (34%) and in 10 out of 80 samples (13%), respectively. In 76 colonies, all *V. destructor* infestation parameters could be measured.

Comparison Between DMR Measures

The DMR measures based on mite infertility and fecundity showed a strong and significant rank correlation with each other within single worker (Worker1: $\tau = -0.85$, $P < 0.001$, $N = 82$; Worker2: $\tau = -0.88$, $P < 0.001$, $N = 79$) and drone samples ($\tau = -0.77$, $P < 0.001$, $N = 80$).

Repeatability of DMR Phenotyping

When all available data were included in the analysis (i.e., including the colonies with fewer than 35 singly infested cells), neither when measured as mite infertility ($\tau = 0.03$, $P = 0.71$, $N = 78$) nor as mite fecundity ($\tau = 0.02$, $P = 0.76$, $N = 78$) did DMR values acquired from the two worker samples within colonies correlate with each other. Rank correlation coefficients were slightly higher but still not significantly different from zero when only measures obtained from samples with 35 singly infested cells were used. This was the case for both the infertility ($\tau = 0.09$, $P = 0.38$, $N = 49$) and fecundity-based DMR values ($\tau = 0.10$, $P = 0.30$, $N = 49$).

Comparing DMR Values Acquired from Worker and Drone Brood

The average percentage of nonreproductive mites was significantly different between the three groups (Kruskal–Wallis rank-sum test, $P < 2.2e\text{-}16$), with lower mean values in the two worker brood (20–22%) compared to drone brood measures (36%) but with no significant difference between the former two (Pairwise Wilcoxon

rank-sum tests: Drone vs Worker1: P = 1.3e-15, Drone vs Worker2: P = 2.3e-10, Worker1 vs Worker2: P = 0.31, p_{adjust}: Bonferroni; Fig. 2). The pattern for mite fecundity was the same, significantly differing between groups (Kruskal–Wallis rank-sum test, P < 2.2e-16) with on average 1.1 viable offspring produced per foundress mite in both worker brood samples, and significantly more, 2.3 on average, on male brood (pairwise Wilcoxon rank-sum tests: Drone vs Worker1: P < 2e-16, Drone vs Worker2: P < 2e-16, Worker1 vs Worker2: P = 0.31, p_{adjust}: Bonferroni; Fig. 2).

Neither DMR values obtained from Worker1 nor from Worker2 samples correlated significantly with those acquired from drone brood (Table 2). This was the case for both the mite infertility and fecundity DMR values. Including only measurements for which 35 singly infested cells were found increased the rank correlation coefficients, but these remained not significantly different from zero.

Association Between DMR Values and *V. destructor* Infestation Levels

Varroa destructor infestation rates obtained from brood samples, adult samples, and cumulated natural mite fall correlated moderately with each other, with rank correlation coefficients (τ) significantly different from zero, ranging from 0.30 to 0.43 (Table 3). The distributions of the two latter infestation phenotypes are given in Supp Fig. 1 (online only).

Average infestation rates significantly varied between groups (Kruskal–Wallis rank-sum test, P < 2.2e-16) and were significantly higher in drone brood, at 35%, compared to worker brood: 5.1% for Worker1 and 5.6% for Worker2 (pairwise Wilcoxon rank-sum tests: Drone vs Worker1: P < 2e-16, Drone vs Worker 2: P < 2e-16, Worker1 vs Worker2: P = 0.93, p_{adjust}: Bonferroni; Fig. 3). Rank correlations between the DMR values, either obtained on all data or on samples with 35 singly infested cells, and the three infestation rate measures were low for both worker and drone brood, with τ ranging from −0.27 to 0.16 for infertility-based DMR and from −0.18 to 0.21 for fecundity-based DMR and rarely significantly differing from zero (Table 4). Considering the rank correlations including all data available, the highest negative rank correlations were obtained between infertility-based DMR for drone brood and the infestation level of drone brood (τ = −0.27), the brood infestation level of Worker2 (τ = −0.23) and the adult infestation (τ = −0.20; Table 4). The fecundity-based DMR including all data had its highest positive rank correlation with the infestation level of drone brood (τ = 0.17; Table 4). When including only samples with 35 singly infested cells, the highest rank correlations were obtained between infertility and fecundity-based DMR measured in drone brood and infestation level of drone brood (τ = −0.26 and τ = 0.21, respectively; Table 4).

When summing Worker1 and Worker2 observations to a single observation (Worker1 + Worker2), no significant rank correlations with the infestation parameters were obtained (Table 5). This was the case when all data were included and when only samples with 35 singly infested cells were kept.

Discussion

The aim of this study was to assess the limitations and potential of DMR in selecting *V. destructor*-resistant honey bee lineages in a population representative of the native *A. m. mellifera* bred by beekeepers in Switzerland. Our results showed that the infertility and fecundity-based methods to measure DMR provided equivalent values, but that neither method was repeatable, even in the same colony when measured within a week by a single trained evaluator, i.e., under conditions as standardized as possible in a realistic field setting. The outputs of the two types of DMR measures did not correlate when acquired from worker or drone brood and correlated poorly with the three mite infestation rate parameters tested. Increasing the number of cells screened for DMR phenotyping did not improve this correlation, and considering more colonies by including those with fewer than the recommended 35 singly infested cells only marginally worsened the results.

When two sets of 35 singly infested cells could be obtained from the experimental colonies, the DMR measures based on mite infertility and fecundity correlated highly with each other for each colony, suggesting that both can be used to capture the same phenomenon. However, given that counting the number of potentially mated daughters likely to reach maturity is not more time-consuming than determining the presence of at least one such individual, we recommend implementing the former, more informative method. This measure, indeed, also allows the selection of colonies in which mites have a low fecundity toward the aim of decreasing colony infestation. The downside of this method is an increased analysis time (corrections for offspring mortality, extrapolation of mature daughters), but automated data analysis methods can be implemented to minimize this added cost. We provide such a tool in Supp Material (online only).

Comparing our biological replicates (Worker1 and Worker2) showed that neither the infertility nor the fecundity-based DMR

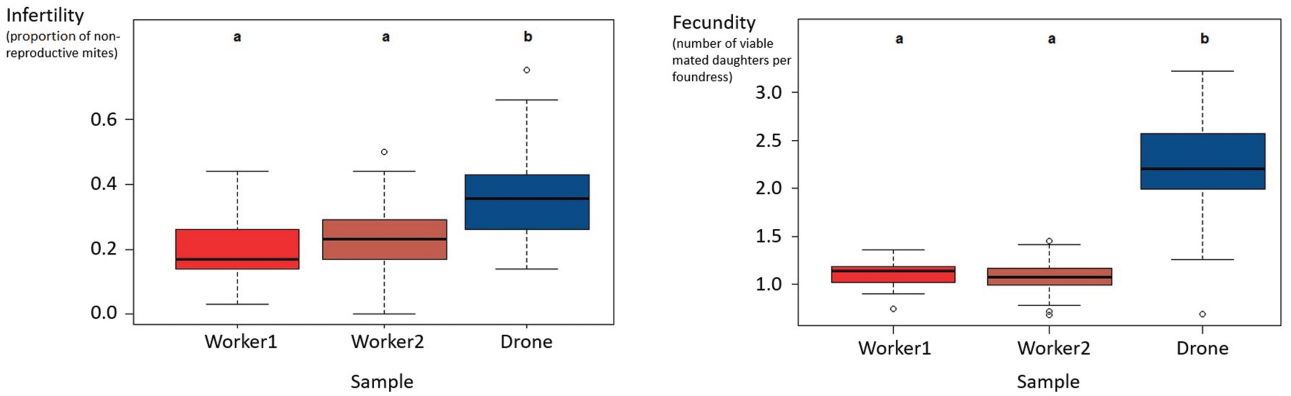


Fig. 2. Average infertility and fecundity-based DMR values for the three brood samples (Worker1, Worker2, and Drone). Box plots represent minimum value, first quartile, median, third quartile, and maximum values. Dots indicate points located more than 1.5 times above or below the interquartile range. Different letters indicate significant (P < 0.001) differences between groups following a pairwise Wilcoxon test.

measures proved repeatable when measured in a context of low environmental variation that was as standardized as possible, i.e., in the same colony within the same week and by a single trained evaluator. Restricting the sample to the recommended 35 singly infested cells marginally improved the rank correlation coefficients, but these were not significantly different from zero. Given that not all traits followed a normal distribution, we calculated Kendall's tau instead of Pearson's correlation coefficients, as commonly applied in the literature (e.g., Büchler et al. 2020, Hoppe et al. 2020). However, both methods provided similar results (Tables 2–5 and Supp Tables 1–4 [online only]). Our repeatability estimate was based on a correlation between two spatially distinct measures across the colonies tested. As such, the correlation was sensitive to the range of values obtained in these colonies. This range covered approximately 50% of the possible spectrum (0–100%) for the infertility-based measure and about 50% of the spectrum for the fecundity-based measure (Fig. 2), which spans between zero and three viable mated females per foundress for worker brood and between zero and five for drone brood (Martin 1997). In populations showing a wider range of values for the infertility-based measure, variation in sampling errors is likely lower than the variation in phenotype compared to populations with a low phenotypic range. Unbiased phenotyping may therefore be easier to obtain in populations with a high phenotypic range. In order to estimate how sampling errors affect phenotyping, a better knowledge of evaluator's consistency, as is performed for honey bee colony strength estimation (Dainat et al. 2020, Hernandez et al. 2020), is required. However, this is challenging, given that a single brood cell can only be opened once, which could be circumvented by the development of corresponding digital tools.

Even though our divergent selection for mite infestation and hygienic behavior aimed to favor phenotypic divergence for DMR, phenotyping a high number of colonies with various genetic backgrounds may increase the chances of identifying colonies with a higher level of DMR. This was done during a study covering various European countries and including 414 colonies, which revealed a large variation in DMR across the continent: the mean infertility score was 33%, while 16% of the tested colonies showed values over 50% (Mondet et al. 2020b). In another study, colonies preselected for DMR showed significantly higher infertility values (Mondet et al. 2020b), suggesting a benefit in selecting this trait. Our results, however, indicate that it cannot be taken for granted that DMR phenotyping, as currently applied, accurately captures a

Table 2. Correlations for infertility and fecundity compared between worker samples (Worker1 and Worker2) and Drone sample, with either all data included or only samples with 35 singly infested cells

				Drone
Infertility	All data included	Worker1	τ	0.03
			N	79
			P	0.66
		Worker2	τ	0.03
			N	77
			P	0.68
	35 singly infested cells/sample	Worker1	τ	0.06
			N	50
			P	0.57
		Worker2	τ	0.17
			N	44
			P	0.12
Fecundity	All data included	Worker1	τ	0.02
			N	79
			P	0.80
		Worker2	τ	–0.01
			N	77
			P	0.94
	35 singly infested cells/sample	Worker1	τ	0.07
			N	50
			P	0.47
		Worker2	τ	0.13
			N	44
			P	0.22

Kendall's tau *b* coefficients (τ) are given, as well as *P*-values (*P*) and sample size (*N*) associated with each correlation.

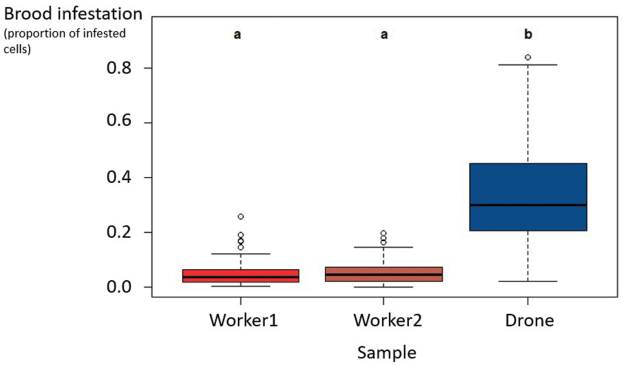


Fig. 3. Brood infestation rates based on dissection data for the three brood samples. Letters indicate significant ($P < 0.05$) differences between groups following pairwise Wilcoxon tests.

Table 3. Correlation between *Varroa destructor* infestation estimation methods. Kendall's tau b coefficients (τ) are given as well as associated *P*-values (*P*)

		Adult infestation	Cumulative mite fall	Worker1 brood infestation	Worker2 brood infestation
Cumulative mite fall	τ	0.33			
	P	1.1e-05			
Worker1 brood infestation	τ	0.43	0.43		
	P	1.8e-08	2.2e-08		
Worker2 brood infestation	τ	0.31	0.36	0.39	
	P	7.0e-05	2.7e-06	3.7e-07	
Drone brood infestation	τ	0.36	0.30	0.42	0.43
	P	3.2e-06	1.1e-04	3.1e-08	2.1e-08

Data from all colonies ($N = 83$) were used, including those with less than 35 singly infested cells for DMR evaluation. All correlation values significantly differed from zero ($P < 0.05$).

Table 4. Correlations of the infertility and fecundity-based DMR obtained from workers and drone samples with *Varroa destructor* infestation rates, with either all data included or only samples with 35 singly infested cells

				Adult infestation	Cumulative	Worker1	Worker2	Drone
					Mite fall	Brood infestation	Brood infestation	Brood infestation
Infertility	All data included	Worker1	τ	−0.14	−0.07	−0.07	−0.11	0.03
			N	81	80	82	79	79
			P	0.07	0.37	0.35	0.15	0.69
		Worker2	τ	**0.16**	0.09	0.02	0.07	0.08
			N	**78**	78	78	79	77
			P	**0.04**	0.23	0.78	0.40	0.31
		Drone	τ	**−0.20**	−0.06	−0.14	**−0.23**	**−0.27**
			N	**79**	78	79	**78**	**80**
			P	**0.01**	0.43	0.08	**3.2e-02**	**5.4e-04**
	35 singly infested cells/sample	Worker1	τ	−0.18	−0.03	−0.12	−0.12	0.08
			N	58	58	59	49	50
			P	0.06	0.72	0.18	0.25	0.44
		Worker2	τ	0.18	0.07	0.00	0.06	0.11
			N	52	52	49	53	44
			P	0.06	0.46	1.00	0.56	0.30
		Drone	τ	**−0.18**	−0.03	−0.11	−0.19	**−0.26**
			N	**69**	68	50	44	**70**
			P	**0.03**	0.69	0.28	0.08	**1.7e-03**
Fecundity	All data included	Worker1	τ	0.14	0.05	0.09	0.09	0.01
			N	81	80	82	79	79
			P	0.07	0.51	0.22	0.25	0.95
		Worker2	τ	−0.15	−0.10	0.00	−0.08	−0.09
			N	78	78	78	79	77
			P	0.05	0.20	1.00	0.28	0.23
		Drone	τ	**0.16**	0.08	0.10	**0.15**	**0.17**
			N	**79**	78	79	**78**	**80**
			P	**0.04**	0.33	0.19	**0.05**	**0.02**
	35 singly infested cells/sample	Worker1	τ	0.16	0.03	0.14	0.11	−0.05
			N	58	58	59	49	50
			P	0.08	0.73	0.12	0.26	0.60
		Worker2	τ	−0.18	−0.07	0.02	−0.07	−0.17
			N	52	52	49	53	44
			P	0.05	0.45	0.84	0.47	0.10
		Drone	τ	0.16	0.06	0.10	0.14	**0.21**
			N	69	68	50	44	**70**
			P	0.05	0.40	0.30	0.19	**0.01**

Infestations rate estimates of adult workers, brood, and colony (as mite fall) were used. Kendall's tau b coefficients (τ) are given, as well as *P*-values (*P*) and sample size (*N*) associated with each correlation. Correlations which significantly ($P < 0.05$) differed from zero are indicated in bold.

colony's aptitude to influence mite reproduction. A truly and generally nonrepeatable phenotype would jeopardize the success of selection programmes and may require a change in the evaluation method. This would be facilitated by a better understanding of the proximal resistance mechanisms of *A. mellifera* against *V. destructor*, especially regarding the timing of resistance trait expression (Guichard et al. 2020a). The reliability of phenotyping should thus be addressed before launching large-scale DMR-based selection programmes.

Comparing DMR phenotypes acquired on worker and drone brood showed higher values for both fecundity and infertility-based measures on the latter (Fig. 3). This is expected, given that *V. destructor* fecundity is higher in drone than in worker brood in *A. mellifera* (Martin 1997). However, the values acquired from the two types of brood did not correlate, showing that the methods cannot be used interchangeably. The question arising from this observation is, which is the 'right' DMR value corresponding to the colony's true ability to decrease mite reproduction? The better phenotype is the one showing a better correlation with the infestation rate, since it is assumed that lower mite reproduction leads to lower infestation rates (Guichard et al. 2020a). Because it is not clear which of the various infestation rate parameters that can be measured is the best to use, we tested two parameters representing the situation at the time of the DMR measures (adult and brood infestation rates) and a cumulative one (mite fall over the season) representing the overall colony infestation. These parameters correlated significantly but moderately with each other, again indicating that they cannot be used interchangeably. Correlations between brood infestation levels and DMR in the expected direction (i.e., negative for fertility-based and positive for fecundity-based DMR) were only significant for DMR acquired from drone brood, whereas infestation rate of adults also significantly correlated with infertility-based DMR recorded from one worker brood sample (Worker2). Despite the slightly higher coefficients of rank correlation between drone-acquired DMR and infestation rates, these coefficients were low and did not bring convincing evidence that phenotyping DMR on drone brood provides a better alternative to measure the ability of the colonies to decrease infestation rates. Replacing phenotyping on worker

Table 5. Correlation of the infertility and fecundity-based DMR for the Worker1 + Worker2 sample pool with *Varroa destructor* infestation rates, with either all data included or only samples with 35 singly infested cells found in each sample (=70 singly infested cells for Worker1 + Worker2)

			Adult infestation	Cumulative mite fall	Worker1 brood infestation	Worker2 brood infestation	Drone brood infestation	Worker1+Worker2 brood infestation
Infertility	All data included	Worker1 + Worker2 τ	0.01	0.05	0.06	−0.03	0.01	−0.07
		N	82	81	82	80	80	83
		P	0.89	0.53	0.49	0.77	0.89	0.38
	35 singly infested cells/sample	Worker1 + Worker2 τ	−0.03	0.06	−0.09	−0.03	0.09	−0.04
		N	48	48	49	49	41	49
		P	0.80	0.53	0.38	0.79	0.39	0.66
Fecundity	All data included	Worker1 + Worker2 τ	−0.02	−0.04	−0.05	0.02	−0.04	0.05
		N	82	81	82	80	80	83
		P	0.79	0.58	0.58	0.85	0.68	0.52
	35 singly infested cells/sample	Worker1 + Worker2 τ	0.02	−0.07	0.11	0.02	−0.11	0.06
		N	48	48	49	49	41	49
		P	0.82	0.50	0.28	0.80	0.31	0.58

Infestations rate estimates of adult workers, brood, and colony (as mite fall) were used. Kendall's tau b coefficients (τ) are given, as well as *P*-values (*P*) and sample sizes (*N*) associated with each correlation.

brood by phenotyping on drones to decrease generation time and foster genetic progress thus does not appear as a promising option based on these results, but this should be confirmed in other populations and on more colonies.

The impact of sampling size on DMR phenotyping in our population could be derived from the comparison of correlations obtained with the complete data set with those obtained from the data set including only the sample with 35 singly infested cells. Using all samples, only adult infestation, one of the worker brood, and the drone brood infestation rates correlated significantly, albeit moderately, with infertility-based DMR values acquired in drone brood. Adult infestation also slightly correlated with infertility in the Worker2 sample. Except for the latter, the same correlations remained significant when fecundity-based DMR was considered (Table 4). When only samples for which 35 singly infested cells were available were used, the pattern was similar, except that there was no correlation of DMR measures with worker brood infestation rates (Table 4). Given the small absolute difference between DMR values calculated on all data and on samples with more than 35 singly infested cells, reaching this threshold was not a critical point to ensure reliable phenotyping in our population. This is not in line with the findings from Eynard et al. 2020, which could be due to the different methodologies (resampling vs different samples compared here) and population sizes investigated.

Correlations were similar when all data were included and when only samples with 35 singly infested cells were used. These results regarding the impact of the number of singly infested cells are possibly explained by the fact that, in small datasets, the sample size and accuracy of phenotypes (expected to be higher in the case of 35 singly infested cells [Mondet et al. 2020b]) have conflicting effects on estimated correlations. However, the quality of the phenotyping was not improved by increasing the number of cells screened. Pooling our Worker1 and Worker2 samples to reach 70 cells to phenotype DMR even lowered the correlations with *V. destructor* infestation rates. If a further increase in the number of cells screened is required to improve phenotyping, this trait would become very challenging to measure and would be unlikely to be applicable in the field. Indeed, we already maximized the infestation rate of brood in our experimental colonies by performing DMR phenotyping at the end of the season, just before the acaricide treatments needed to reduce infestation rates (Supp Fig. 1 [online ony]) and ensure colony survival for subsequent selection. Despite this, we could not reach the recommended number of 35 singly infested cells (Büchler et al. 2017, Mondet et al. 2020b) in 28 and 34% of Worker1 and Worker2 brood samples, respectively, and in 13% of the Drone brood samples. An alternative could be to infest colonies with mites from highly infested donor colonies, but besides requiring the maintenance of highly infested colonies, this method biases infestation levels and does not exclude donor colony effects or sampling method effects on the later-measured DMR.

In none of the cases did the overall infestation rate of the colony (mite fall over the season) correlate strongly with DMR values ($|\tau_{max}| = 0.10$). This result indicates that a snapshot measure of DMR may not capture the colonies' true ability to reduce mite population growth. The lack of a correlation between DMR and infestation rates might be due to the influence of multiple environmental factors on infestation rates, as shown by the low heritability of this trait (Guichard et al. 2020b, Hoppe et al. 2020). Factors such as an influx of mites due to drift or reinvasion of foreign mites (Frey et al. 2011) may mask the relationship between phenotypes and genetic values (Guichard et al. 2020a). Therefore, the effects of selecting DMR on colony *V. destructor* infestation level are likely to be moderate (see

Eynard et al. 2020) because it does not impact the influx of foreign mites (Guichard et al. 2020a).

Our results suggest that the current DMR protocol does not allow accurate capturing of the phenotype, at least not in all honey bee populations. It may, however, be possible to identify colonies with high DMR abilities despite the apparent imprecision of the phenotyping method. In the absence of extreme differences in DMR values within a population, this imprecision makes programmes based on diverging selection unlikely to lead to genetic progress. Given the amount of time required for DMR phenotyping (Guichard et al. 2020a), it should therefore be verified whether this trait can be routinely implemented in a selection programme. A better understanding of the association between trait expression variability, measure repeatability, and measurement errors is required to improve phenotyping precision and effectiveness. Besides sampling size and methodological issues, DMR may also not be a strong driver of *V. destructor* population dynamics on its own and could act in concert with other resistance traits to influence mite population dynamics negatively and promote colony survival (Guichard et al. 2020a, Mondet et al. 2020a). Overall, a better ability to phenotype DMR and other resistance mechanisms and better knowledge of the role and interactions of resistance traits leading to colony survival against *V. destructor* infestations are mandatory to improve selection strategies in resistance breeding programmes.

Supplementary Data

Supplementary data are available at *Journal of Economic Entomology* online.

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