



# Elevated recapping behaviour and reduced *Varroa destructor* reproduction in natural *Varroa* resistant *Apis mellifera* honey bees from the UK

George Peter HAWKINS , Stephen John MARTIN

School of Science, Engineering & Environment, The University of Salford, M5 4WT, Manchester, UK

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**Abstract** – *Varroa destructor* mites remain a major threat to *Apis mellifera* honey bees, yet many populations across the world have naturally evolved survivorship to infestation. Here, we investigated the roles of recapping and mite reproduction in natural *Varroa* resistant (NVR) colonies in the UK. Recapping frequency was higher in NVR colonies and targeted mite-infested cells in which the recapped diameters were larger. Mite reproduction was lower in NVR colonies due to increased offspring mortality, although recapping is unlikely the primary mechanism responsible. In an additional small experiment, infested brood removal was immediately present in naïve colonies, and recapping increased rapidly following initial mite exposure. Targeted recapping behaviour is a common trait in NVR colonies and may provide a useful indicator for mite resistance. In addition, reduced mite reproduction is a key resistance mechanism in NVR colonies in the UK, as also found in Europe, S. Africa, Brazil and Mexico.

*Apis mellifera* / *Varroa destructor* / resistance / recapping / reproduction

## 1. INTRODUCTION

Owing to its vast global distribution that has been facilitated by modern apiculture, the Western honey bee *Apis mellifera* is considered as the most important insect pollinator (Klein et al. 2007). However, along with this expansion, *A. mellifera* has become exposed to a myriad of stressors, including human land-use changes (Otto et al. 2016), pesticides (Goulson et al. 2015) and disease inducing parasites (Brosi et al. 2017), which together contribute towards ongoing colony losses throughout the Northern hemisphere (Brodschneider et al. 2018). The ectoparasitic mite *Varroa destructor* has become a major stressor (Rosenkranz et al. 2010) since switching from its natural host *Apis cerana* (Rath 1999).

Unlike *A. cerana*, *A. mellifera* usually lacks the adaptations to control their mite numbers, which increase beyond a critical threshold (Fries et al. 1994; Martin 1998) and often lead to colony collapse via the transmission of damaging viruses such as deformed wing virus (DWV) (Martin and Brettell 2019). This pandemic has now become almost ubiquitous, with Australia now the only *A. mellifera* inhabited continent to be spared the invasion of *V. destructor* and remains free of DWV (Roberts et al. 2017).

The vast majority of managed *A. mellifera* colonies today owe their survival to beekeeper interventions, usually in the form of chemical-based treatments (Rosenkranz et al. 2010). Despite this, we are becoming aware of an increasing number of *A. mellifera* populations around the world that have naturally evolved resistance to *V. destructor* and consequently survive year on year without needing treatment (Locke 2016). We will refer to these populations herein as ‘Natural *Varroa*

Corresponding author: G. P. Hawkins,  
[g.p.hawkins@edu.salford.ac.uk](mailto:g.p.hawkins@edu.salford.ac.uk)  
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Resistant' (NVR) to distinguish between populations that have been selectively bred for resistance, such as the VSH line from the US (Harris 2007). Precisely how these NVR populations have become adapted to survive *V. destructor* is not clear. Although hypotheses such as DWV genotypes (Mordecai et al. 2016) and frequent swarming (Loftus et al. 2016) have been proposed, the most consistent feature observed in NVR colonies is an impairment of the mites' ability to reproduce, thus controlling rates of mite population growth (Rosenkranz et al. 2010; Locke 2016; Brettell and Martin 2017). The first reports of widespread NVR populations were in 'Africanized' bees, and studies in Mexico (Martin and Medina 2004) and recently Brazil (Martin et al. 2019) both found reduced *V. destructor* reproduction relative to those found in regions where untreated colonies were collapsing (Martin 1994). Following the arrival of *V. destructor* in 1997, the African subspecies *A. m. capensis* and *A. m. scutellata* rapidly developed resistance within 7 years (Allsopp 2006), and reduced *V. destructor* reproduction has again been found in these NVR populations (Nganso et al. 2018; Martin et al. 2019). In addition, the same phenomenon has been observed in allopatric NVR colonies of European origin (Locke and Fries 2011; Locke et al. 2012; Oddie et al. 2017; Brettell and Martin 2017). Although *V. destructor* reproductive success varies by geographical region (Rosenkranz et al. 2010), a disruption in this process is at least in part a host trait (Fries and Bommarco 2007; Locke et al. 2012; Oddie et al. 2017). Such host traits that have been investigated thus far include brood cell size (Calderon et al. 2010; Oddie et al. 2019), post-capping period (Oddie et al. 2018b), smaller colony sizes (Locke and Fries 2011; Loftus et al. 2016), alterations of brood volatile compounds (Frey et al. 2013) and behavioural defences such as mite-infested brood removal (Panziera et al. 2017; Nganso et al. 2018). It is likely that a range of mechanisms lead to mite-resistance (Locke 2016), which is reflected in the fact that studies continue to generate mixed results when attempting to explain it (Aumeier et al. 2000; Panziera et al. 2017; Nganso et al. 2018).

Two recent studies have identified another trait that is appearing consistently in NVR

populations. Oddie et al. (2018a) compared four NVR honey bee populations with local populations receiving treatment across mainland Europe. They found that all four NVR populations showed an increased frequency of 'recapping' behaviour relative to the four treated populations. Furthermore, they found that recapping was strongly biased towards mite-infested brood cells (Oddie et al. 2018a), as did Martin et al. (2019) in Brazilian and South African honey bee populations. Martin et al. (2019) additionally found extremely low levels of recapping in mite-naïve populations (those that have never been exposed to *V. destructor*) relative to all other infested populations. A 'recapped cell' is where an adult bee has pierced a hole into a sealed brood cell cap that has been subsequently re-sealed without removing the brood (Boecking and Spivak 1999). This trait has previously been associated with infested brood removal behaviour; however, since all the study populations also displayed reduced mite reproduction, Oddie et al. (2018a) proposed for the first time that recapping is a previously overlooked and independent trait that directly reduces mite reproductive success in the targeted cells. This conclusion was based on a controlled experiment that has since not been supported by the later study (Martin et al. 2019). Instead, Martin et al. (2019) support the idea that recapping is associated with infested brood removal behaviour and that recapped cells are evidence for failed instances of brood removal. They added that brood removal behaviour on the other hand, when executed successfully, disrupts the mites' reproductive cycles and leads to increased levels of nonlaying foundresses.

The aim of this study was to investigate the roles of these traits in naturally evolved resistance to *V. destructor* among the UK honey bee population. We compared NVR and treated colonies by measuring recapping frequencies in infested and non-infested brood cells and mite reproductive success in recapped and undisturbed cells. In addition, we tested levels of brood removal behaviour by conducting artificial mite infestation experiments on a small number of NVR, treated and naïve colonies.

## 2. MATERIALS AND METHODS

### 2.1. UK survey: recapping and mite reproduction

#### 2.1.1. Source colonies

Worker brood combs were collected from volunteer beekeepers across North West England, North Wales, the Midlands (England) and Southern England, from July to September 2017–2019. ‘NVR’ colonies were classified as those that have been surviving *V. destructor* infestation without treatment regimens for over 10 years (from Gwynedd [Hudson and Hudson 2020], Swindon area [Mordecai et al. 2016], Pershore and Bruton) or at least 5 years (from Reading, Salford and Wigan), without the implementation of targeted selection for resistance. In contrast, the ‘treated’ colonies are those that receive at least annual mite treatment regimens (from Manchester, Anglesey, Sutton-Coldfield and Warwick). A total of 42 colonies (26 NVR and 16 treated) were used to assess recapping rates; of these, 36 colonies provided sufficient mites to assess *V. destructor* reproduction (the unsuitable colonies were all NVR, with either very low infestation levels or early stage brood). In addition, four mite-naïve colonies were sourced in mid-June from the Isle of Man (an island that has always been free of *V. destructor*) and moved to the mainland where the artificially infested brood removal experiments were conducted (see below). These colonies were also assessed for recapping prior to mite introduction and 1 month later following mite introduction. A detailed breakdown of all colonies sampled is given in Supplementary Table S1. All brood samples were freeze-killed within a few hours of collection and stored at minus 20°C prior to examination.

#### 2.1.2. Assessing recapping and mite reproduction

Brood combs were examined using a  $\times 16$  binocular microscope and bright cold light source. Cell caps were carefully opened with fine forceps and inverted to reveal the underside of the cap; if the cell had been recapped, the glossy layer of

spun cocoon could be clearly identified as having been pierced and refilled with duller wax, whereas if the cell was undisturbed, the layer of spun cocoon remained fully intact. The size of the recapped area ranged from  $<1$  mm in diameter to the entire cap (approximately 5 mm); therefore, each instance of recapping was estimated to the nearest mm. Cells containing mites were classified as infested.

The brood were removed and categorised by developmental stage according to Martin (1994), and all adult and offspring mites were also removed and examined where possible. The *V. destructor* reproductive success was measured by reconstructing the mite families according to standard methods (Dietemann et al. 2013). For a brood cell to be considered as successfully reproductive, an adult male was required to be present alongside at least one female offspring of the correct age; these could be either adult females (evidenced by exuviate) or female deutonymphs, depending on the developmental stage of the brood (Dietemann et al. 2013). Only brood at the yellow thorax stage (190-h post-capping) and older were considered in this measurement. Since we were working with frozen brood combs, offspring were considered as dead (offspring mortality) if they were clearly dead at the time of freezing (e.g., by being desiccated), too underdeveloped for their reproductive phase to reach adulthood or missing entirely (likely due to death at a very early stage); this was in contrast to nonlaying foundresses, where no eggs were laid at all.

### 2.2. Mite detection and subsequent brood removal experiments

A small controlled brood removal experiment was additionally conducted in September 2019 at the Salford University research apiary and at a single apiary in Sutton-Coldfield, England. Four mite-naïve colonies from the Isle of Man were held at the Salford University apiary alongside three NVR colonies sourced from Gwynedd, North Wales in August 2019. Three colonies classified as ‘susceptible’ (had not been treated for two years and were showing signs of damage, such as heavy infestation

and wing deformity) were used at their own apiary in Sutton Coldfield.

Brood removal was assessed for each group (NVR  $n = 3$ , susceptible  $n = 3$ , naïve  $n = 4$ ) using artificial mite introductions. Three separate trials were conducted on all 10 colonies, the first using live mites, the second using dead mites and the third marked unmanipulated cells to be used as controls (to control for cells that had been infested naturally). Live adult foundresses were harvested from highly infested drone brood combs from a 'treated' apiary in Anglesey prior to administering the colonies' mite treatment. Dead foundresses were freeze-killed and sourced from various locations from the UK survey. For each of the three trials, a single frame of recently capped worker brood was sourced from each of the 10 receiver colonies containing cells that had been capped but prior to cocoon spinning (<24-h post-capping) that were selected for introductions or controls. Under a  $\times 16$  binocular microscope, fine forceps were used to create a small incision at one side of the cell cap, and a fine-tipped paintbrush was used to insert a single live or dead foundress into the cell and reseal the cap. The artificially infested cells, or unmanipulated control cells, of each brood comb were marked on an acetate sheet, and the frames were returned immediately to their source colonies. Rather than using sham manipulated cells, the acceptance rate (cells that were repaired by the adult bees rather than immediately removed) for each colony was checked after 24 h to control for experimenter manipulation. The overall brood removal was then measured after 10 days.

In the first trial, 20–30 live mites were introduced into each of the 10 receiver colonies of which 18–30 per colony were accepted (281 total); in the second trial, 15–20 dead mites were introduced into each of the 10 receiver colonies of which 13–20 per colony were accepted (181 total); in the third trial, 20–30 control cells per colony were marked (275 total). In addition, tests for hygienic behaviour (dead brood removal) were also administered on 9 colonies (NVR  $n = 3$ , susceptible  $n = 3$ , naïve  $n = 3$ ) by freeze-killing sections of worker brood and measuring removal rates after 24 and 48 h. Individual colony data for all trials are given in Supplementary Table S2.

## 2.3. Statistical analyses

All statistical analyses were conducted in RStudio (version 1.2.5019). Three generalised linear mixed models (GLMMs) were fitted to the data using the lmer package, each with a binomial distribution and logit link function, to measure significance in recapping, mite reproduction and brood removal. Therefore, the response variables for each model were recapping, Varroa reproduction and brood removal in a binomial format. Fixed explanatory variables included status (NVR, treated/susceptible or naïve), region (North-West England, North Wales, Midlands and Southern England), brood age (according to Martin 1994), infested (whether the cells contained mites), sampling month and year, recapping and test (live mites, dead mites or controls for brood removal experiments). For each model, colonies were considered as the statistical individual, and colony ID was used as a random factor. Additional models were conducted by editing the response variables to test specifically for nonlaying foundresses, offspring mortality and larger recapped diameters (>2.5 mm). Adjusted mean proportions and pairwise comparisons were calculated using the emmeans package, and figures were visualised using Microsoft Excel. Spearman rank tests were used to assess correlations between proportions of infested cells recapped and total mite reproductive success, and pairwise Kolmogorov-Smirnov tests were used to compare the frequency distributions of the recapped diameters.

## 3. RESULTS

### 3.1. UK survey

#### 3.1.1. Recapping

A total of 14,802 worker brood cells were examined from 42 colonies of which 1,639 contained mites and 4,293 were recapped. Brood infestation levels ranged from 0.3% to 22.7% in NVR colonies and 2.9% to 58.2% in treated colonies. Proportions of total examined cells recapped, and infested cells recapped, were both highly variable across both NVR and treated

colonies (individual colony data is given in Supplementary Table S1). Recapping probability was significantly higher in NVR colonies ( $x^2 = 11.543$ ,  $p < 0.001$ ) as was infested cells ( $x^2 = 322.25$ ,  $p < 0.001$ ), varied significantly between sampling region ( $x^2 = 32.76$ ,  $p < 0.001$ ) and brood developmental stages ( $x^2 = 417.61$ ,  $p < 0.001$ ) (Table 1, Figure 1). The four mite-naïve colonies originally displayed 0–0.7% (mean 0.2%) total recapping, which later increased to 0–16% (mean 9.6%) 1 month following mite introduction (Supplementary Table S1). In NVR colonies, the frequency distributions of the recapped diameters (<1–5 mm) were significantly different between infested and non-infested cells ( $D = 1$ ,  $p = 0.007$ , with larger diameters more frequently infested); however, a similar pattern was not seen in treated colonies ( $D = 0.8$ ,  $p = 0.079$ ) (Supplementary Figure S1).

### 3.1.2. Mite reproduction

Of the 1,639 mite-infested worker brood cells, 1,068 mite families from 36 colonies were suitable to assess for reproductive success. Proportions of successfully reproducing brood cells per colony were highly variable (individual colony data is given in Supplementary Table S1). Probability of successful mite reproduction was significantly lower in NVR colonies ( $x^2 = 10.301$ ,  $p = 0.001$ ) due to offspring mortality (GLMM:  $x^2 = 8.2562$ ,  $p = 0.004$ ) rather than nonlaying foundresses (GLMM:  $x^2 = 0.3255$ ,  $p = 0.6$ ); however, there was no difference in reproductive success between recapped and undisturbed cells ( $x^2 = 0.0796$ ,  $p = 0.778$ ) (Table 1, Figure 2a), including when only considering the larger recapped diameters (>2.5 mm) ( $x^2 = 1.5067$ ,  $p = 0.219$ ) and offspring mortality (GLMM:  $x^2 = 0.4549$ ,  $p = 0.5$ ). In addition, no significant correlations were found between proportions of successful mite reproduction and infested cells recapped for both NVR ( $\rho = 0.26$ ,  $p = 0.3$ ) and treated ( $\rho = -0.02$ ,  $p = 0.9$ ) colonies (Figure 2b). Again, these correlations remained insignificant when considering only the larger recapped cells (NVR:  $\rho = 0.29$ ,  $p = 0.3$ ; treated:  $\rho = 0.03$ ,  $p = 0.9$ ). Probability of successful mite reproduction also varied between brood

developmental stages ( $x^2 = 8.5947$ ,  $p = 0.003$ ) and marginally between sampling years ( $x^2 = 6.0582$ ,  $p = 0.014$ ) (Table 1).

### 3.2. Brood removal experiments

Acceptance rates were high for both the live mite (98.5%) and dead mite (97.8%) trials. Brood removal rates after 10 days were highly variable, ranging between 6.7–70% in the live mite trial, 5–35% in the dead mite trial and 3.3–36.7% in the control trial. Brood removal probability was significantly higher for the live mite tests ( $x^2 = 36.009$ ,  $p < 0.001$ ) whereas no overall difference was found between NVR ( $n = 3$ ), susceptible ( $n = 3$ ) and naïve ( $n = 4$ ) colonies ( $x^2 = 2.5113$ ,  $p = 0.285$ ) (Table 1, Figure 3). Pairwise comparisons revealed that live mite removals for susceptible bees were significantly higher than naïve, whereas NVR colonies did not differ from either group (Figure 3). The freeze-killed hygienic tests (NVR  $n = 3$ ; susceptible  $n = 3$ ; naïve  $n = 3$ ) generally resulted in low rates of dead brood removal, ranging from 3.9% to 35.9% after 24 h and 4.7% to 46.6% after 48 h except for one naïve colony that removed 88%. Individual colony data for all trials are given in Supplementary Table S2.

## 4. DISCUSSION

Our data have shown that in UK honey bees, both recapping behaviour and reduced *V. destructor* reproductive success are traits involved in naturally evolved, long-term mite resistance. Recapping was strongly targeted towards mite-infested brood cells, and the frequency was higher in NVR populations (Figure 1), while mite reproductive success was lower in NVR populations (Figure 2a) due to increased offspring mortality or underdevelopment, particularly in male offspring. However, in contrast to a recent hypothesis (Oddie et al. 2018a), recapping appeared not to be the primary mechanism responsible for the failed reproduction (Figure 2b) and instead could be a trait involved in the detection and removal of infested brood (Boecking and Spivak 1999; Martin et al. 2019). Recapping and brood removal were consistently increased in response to the artificial infestation of live mites, with the highest removals observed in susceptible,

**Table 1.** Significance of individual explanatory variables from GLMM models

| Response variable   | Explanatory variable | <i>n</i> (colonies) | DF | $\chi^2$ | <i>p</i> value |
|---------------------|----------------------|---------------------|----|----------|----------------|
| Recapping           | Status               | 42                  | 1  | 11.543   | <0.001***      |
|                     | Region               |                     | 3  | 32.76    | 3.619e-07***   |
|                     | Brood age            |                     | 1  | 417.61   | <2.2e-16***    |
|                     | Infested             |                     | 1  | 322.25   | <2.2e-16***    |
|                     | Month                |                     | 2  | 1.3529   | 0.508          |
|                     | Year                 |                     | 2  | 0.1311   | 0.937          |
| Varroa reproduction | Status               | 36                  | 1  | 10.301   | 0.001**        |
|                     | Region               |                     | 3  | 2.7821   | 0.427          |
|                     | Brood age            |                     | 1  | 8.5947   | 0.003**        |
|                     | Recapping            |                     | 1  | 0.0796   | 0.778          |
|                     | Month                |                     | 2  | 3.5091   | 0.173          |
|                     | Year                 |                     | 1  | 6.0582   | 0.014*         |
| Brood removal       | Test                 | 10                  | 2  | 36.009   | 1.516e-08***   |
|                     | Status               |                     | 2  | 2.5113   | 0.285          |

Response variables with binomial distribution were used to describe whether the cell had been recapped (recapping), whether the foundress mite within an infested cell had reproduced successfully (Varroa reproduction) and whether marked brood had been removed (brood removal). Explanatory variables describe the colonies' resistance level (status), sampling location from the UK (region), brood developmental stages (brood age; from Martin, 1994), whether the cell contained mites (infested), sampling month and year and the artificial infestation categories (test). Colonies were considered as the statistical individual with colony ID as a random effect

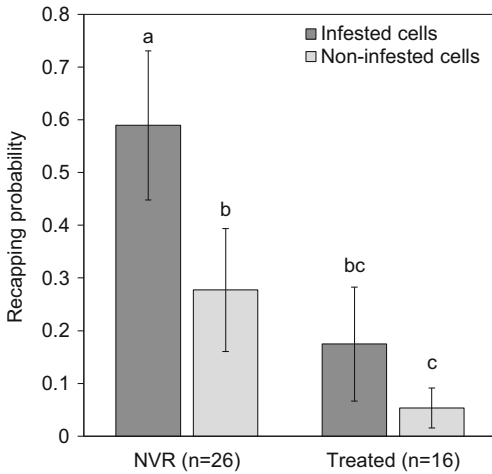
Significance codes = \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$

followed by NVR and finally naïve colonies, although our sample sizes are small, and this trait is known to be highly variable even within an NVR population, ranging from 15% to 89% in *A. m. capensis* bees in S. Africa (Martin et al. 2019).

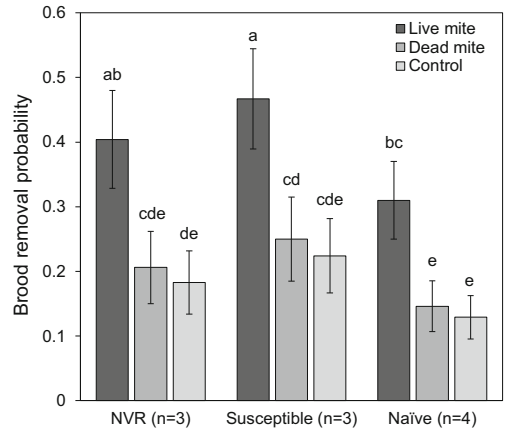
As reported in previous studies (Harris et al. 2012; Oddie et al. 2018a; Martin et al. 2019), mite reproductive success did not differ between recapped and undisturbed cells (Figure 2a), which suggests that recapping itself was not responsible for the failed mite reproduction. Oddie et al. (2018a) proposed in response to this that the adult bees are less likely to detect infested cells that already have low mite reproduction and instead detect and recap the cells that are reproducing successfully; this action then impairs the mite reproduction in the detected cells, thus balancing the reproductive success in recapped and undisturbed cells. However, the evidence for whether NVR bees are more likely to detect infested brood cells that have successful mite reproduction is mixed (Mondet et al. 2016; Panziera et al. 2017), and if

recapping were a primary mechanism, then comparing the proportions of infested cells recapped with total mite reproductive success at the colony level should produce a negative correlation, yet no such correlation existed (Figure 2b). Offspring mortality/underdevelopment was the primary cause of mite reproductive failure in this study as it was significantly higher in NVR populations, as opposed to nonlaying foundresses which were not. In contrast to Harris et al. (2012), offspring mortality alone also could not be explained by recapping, including when considering only the larger recapped diameters that were more common in the infested cells of the NVR colonies. Overall, 34% of undisturbed infested cells in this study failed to reproduce successfully, while 36% failed in recapped cells; conversely, 42% failed in NVR, and 28% failed in treated. If recapping did affect mite reproduction directly, then it was overshadowed by other mechanism(s).

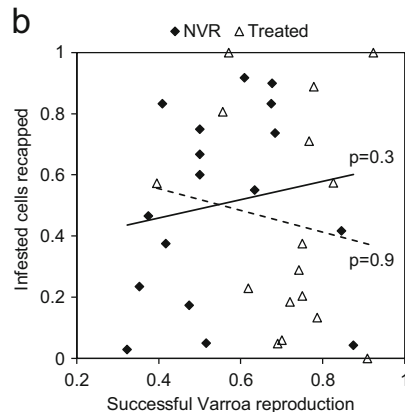
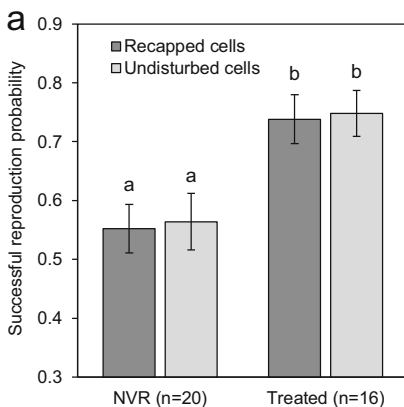
Nevertheless, there is little doubt that recapping is associated to *V. destructor*, due to the strong



**Figure 1.** Adjusted mean proportions ( $\pm$  SE) of recapped worker brood cells in natural *Varroa* resistant (NVR) and treated colonies from around the UK. Recapping probability was significantly higher in NVR colonies (GLMM:  $\chi^2 = 11.543$ ,  $p < 0.001$ ) and in mite-infested cells (GLMM:  $\chi^2 = 322.25$ ,  $p < 0.001$ ). Groups that do not share a letter indicate significant differences from pairwise comparisons (GLMM:  $p < 0.05$ ). 'n' = number of colonies per group.



**Figure 3.** Adjusted mean proportions ( $\pm$  SE) of worker brood removal from artificial mite introduction experiments. Brood removal probability was significantly higher in the live mite tests (GLMM:  $\chi^2 = 36.009$ ,  $p < 0.001$ ) whereas there was no overall difference between NVR, susceptible and naïve colonies (GLMM:  $\chi^2 = 2.5113$ ,  $p = 0.285$ ). Groups that do not share a letter indicate significant differences from pairwise comparisons (GLMM:  $p < 0.05$ ). Live mite tests introduced a single live foundress per cell; dead mite tests introduced a single dead foundress per cell; control tests marked unmanipulated cells. 'n' = number of colonies per group.



**Figure 2.** (a) Adjusted mean proportions ( $\pm$  SE) of infested brood cells containing successful mite reproduction; (b) scatter graph depicting proportions of successful mite reproduction and infested cells recapped per colony. (a) Successful mite reproduction probability was significantly lower in natural *Varroa* resistant (NVR) colonies (GLMM:  $\chi^2 = 10.301$ ,  $p = 0.001$ ), yet there was no difference between recapped and undisturbed cells (GLMM:  $\chi^2 = 0.0796$ ,  $p = 0.778$ ); groups that do not share a letter indicate significant differences from pairwise comparisons (GLMM:  $p < 0.05$ ); 'n' = number of colonies per group. (b) Proportions of infested cells recapped did not correlate to successful mite reproduction for NVR ( $\rho = 0.26$ ,  $p = 0.3$ ) or treated ( $\rho = -0.02$ ,  $p = 0.9$ ) colonies.

targeting towards mite-infested brood cells (Figure 1; Oddie et al. 2018a; Martin et al. 2019) and the near absence of the trait in mite-naïve populations (Martin et al. 2019) that increased rapidly in our study following initial exposure (an increase from 0.2–9.6% total average within one month). Yet, rather than directly impairing mite reproduction, recapping is instead evidence of differing stimuli that trigger initial detection (cell opening) followed by either brood removal or recapping (Boecking and Spivak 1999; Martin et al. 2019). When brood removal is executed successfully, it disrupts the surviving foundresses' reproductive cycles, increasing the instances of nonlaying mites circulating in the population (Boecking and Spivak 1999; Kirrane et al. 2011; Martin et al. 2019). This mechanism is a candidate in NVR populations whereby nonlaying, or laying male only, foundresses account for much of the failed mite reproduction (Martin and Kryger 2002; Locke et al. 2012); however, it is unlikely to explain the difference in offspring mortality observed in this study and other NVR (Medina et al. 2002; Locke and Fries 2011) and selectively bred (Ibrahim and Spivak 2006) populations. Kirrane et al. (2011) found that mite reproductive cycles that are disrupted by brood removal can lead to increased offspring mortality/underdevelopment in their next cycle; however, when reproductive mites were disrupted at pink-eyed pupae stage (as opposed to prepupae), the more common stage for brood removal behaviour to be performed (Harris 2007), 92% laid no eggs in their next cycle. Furthermore, Ibrahim and Spivak (2006) showed that failed mite reproduction, which was almost exclusively offspring mortality/underdevelopment, had a significant 'brood effect'; i.e., the adult bees were not required for the impairment in reproduction to take place. Again, it appears that other mechanism(s) are involved. For example, the possible alteration of brood volatiles could delay (rather than prevent entirely) mite oogenesis (Frey et al. 2013), leaving younger offspring underdeveloped and more vulnerable to damage from late stage pupal movements or moulting (Locke 2016).

We additionally tested for differences in brood removal behaviour between NVR, susceptible and naïve colonies by using artificial mite introductions. It is important to note here that our sample size is small (three colonies per group); therefore, these

data should be treated as preliminary, and more work is needed to support these findings. Brood removal in the unmanipulated control trials was generally higher than expected, likely due to the heavy mite infestation rates of the susceptible brood and the presence of chalkbrood found in the NVR and naïve bees. Despite the presence of these additional diseases, brood removal across all groups was still significantly increased in response to live mite introductions, suggesting that all groups were specifically detecting and removing cells infested with live adult mites. Interestingly, the susceptible colonies displayed the highest overall average (Figure 3); these colonies had not received treatment for 2 years prior to the experiment and were harbouring heavy mite loads (up to 47% brood infestation) and showing symptomatic infections of DWV that could have been influencing their removal behaviour (Schöning et al. 2012). In addition, 88% of the artificially infested brood cells that had not been removed had been recapped. It appears that despite both behaviours being performed at high levels, in this instance, they have not sufficed to save these colonies from potentially irreversible damage. A similar phenomenon may be present when NVR colonies become overwhelmed with mites and cannot survive when moved outside of their local area (Correa-Marques et al. 2002). Another surprising finding was that the mite-naïve colonies appeared to be preadapted to detect and remove mite infested cells, as their live mite removals were significantly higher than their controls (Figure 3) and within the ranges of both NVR and susceptible populations in this study and previously (Boecking and Ritter 1993; Aumeier et al. 2000; Boecking et al. 2000; Panziera et al. 2017; Cheruiyot et al. 2018). In contrast to *A. m. capensis* (Martin et al. 2019), the European bees in this study did not detect and remove brood that had been artificially infested with dead mites (Figure 3), which could either be attributed to differing detection stimuli across these subspecies or the fact that the dead mites in this study were freeze-killed rather than dying naturally on the day prior to insertion (Martin et al. 2019).

Recapping and brood removal are both traits that are closely associated to *V. destructor* and appear to be innate immune responses to mite infestation, as well as other diseases (Rothenbuhler 1964; Gilliam et al. 1983). Although mite-targeted recapping is a



feature consistently appearing at high levels in NVR colonies (Oddie et al. 2018a; Martin et al. 2019), it appears not to be the primary mechanism impairing mite reproduction as previously hypothesised (Harris et al. 2010; Oddie et al. 2018a). Recapping instead likely provides evidence for infested brood removal behaviour (Boecking and Spivak 1999; Martin et al. 2019), a trait that no doubt contributes to resistance (Locke 2016; Panziera et al. 2017), although again it appears that the failed mite reproduction in this population is largely independent from this behaviour. Finding the primary mechanisms behind reduced mite reproduction, with an emphasis on offspring mortality, appears particularly important in understanding mite resistance in the UK (Hudson and Hudson 2020) and beyond (Medina et al. 2002; Locke and Fries 2011; Brettell and Martin 2017). In addition, evidence for mite-targeted recapping could provide a useful indicator for naturally evolved resistance; in order to achieve this in practise at the colony level, more work is needed to understand the high variability in recapping levels within both NVR and treated groups. Given the complexity of eusocial insect colonies and their pests, pathogens and wider ecology, a mosaic of traits and conditions are likely required to ultimately lead to the stable host-parasite relationship between *A. mellifera* and *V. destructor* (Rosenkranz et al. 2010; Locke 2016), and continuing to develop our understanding of these will provide insight to inform the development of sustainable apiculture.

## SUPPLEMENTARY INFORMATION

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**Availability of data and material** Summaries of individual colony data are given in the supplementary information files. The raw data, including excel files and R code,

are available from the corresponding author on reasonable request.

## AUTHORS CONTRIBUTION

S. J. M. and G. P. H. conceived this research and collected the data; G. P. H. analysed and interpreted the data; G. P. H. wrote the paper and S. J. M. provided revisions; both authors read and approved the final manuscript.

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## DECLARATIONS

**Ethics** Ethical approval was not required for this study as the experimental work was conducted with unregulated invertebrate species.

**Consent to participate and publication** All volunteer beekeepers consented for their colony data to be included in this study.

**Conflict of interest** The authors declare that they have no potential conflict of interest in relation to the study in this paper.

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**Comportement de réoperculation élevée et reproduction réduite de *Varroa destructor* chez les abeilles**

domestiques *Apis mellifera* naturellement résistantes à *Varroa* au Royaume-Uni.

*Apis mellifera* / *Varroa destructor* / résistance / réoperculation / reproduction.

Gesteigertes Recapping-Verhalten und verminderte Fortpflanzung von *Varroa destructor* bei natürlicherweise varroaresistenten *Apis mellifera* Honigbienen im Vereinigten Königreich.

*Apis mellifera* / *Varroa destructor* / Resistenz / Recapping / Fortpflanzung.

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