Investigating naturally evolved *Varroa destructor* resistance in *Apis mellifera* honey bees: host behavioural traits and parasite reproductive biology

George Peter Hawkins

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School of Science, Engineering & Environment

The University of Salford

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yet there was no difference between recapped and undisturbed cells (GLMM: χ^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 resistant (rho=0.26, p=0.3) or susceptible (rho=-0.02, p=0.9) colonies.

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were artificially inserted into cells, along with the recapping data for remaining unremoved infested pupae. Also, the amount of dead freeze-killed brood removed in both the 11 *A. m. capensis* and ten AHB colonies is given, along with basic recapping data for the ten AHB colonies. All key results are given in bold.

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DECLARATIONS

At the time of writing (February 2020), Chapter 2 has been published in the peer reviewed academic journal Apidologie in December 2019 (Appendix 3) and Chapter 3 was submitted in January 2020 to the same journal and is currently under review. Appendix 1 was published in the BBKA news in March 2019 and Appendix 2 is due for publication in the BBKA news and The Welsh Beekeeper in April 2020.

Chapter 1: General Introduction

Researched and written by G. P. H. with minor revisions from S. J. M.

Chapter 2: *Varroa destructor* reproduction and cell recapping in mite-resistant *Apis mellifera* populations

S. J. M. conceived and designed the study; L. E. B. performed the study in Australia; G. P. H., N. R. and M. H. A. assisted with the study in South Africa and M. E. C. assisted with the field work in Brazil. S. J. M. analysed the data and drafted the manuscript with input from G. P. H., N. R. and M. E. C. The manuscript was edited by S. J. M., G. P. H., L. E. B and M. H. A.

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Chapter 3: Elevated recapping behaviour and reduced *Varroa destructor* reproduction in mite-resistant *Apis mellifera* honey bees from the UK

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.

Chapter 4: General Discussion

Researched and written by G. P. H. with minor revisions from S. J. M.

Appendix 1: Changing the guard at Salford Uni Honey Bee Research Team

Written by S. J. M. and G. P. H.

Appendix 2: Investigating naturally evolved *Varroa* resistance in the UK and beyond: BDI research at Salford University

Written by G. P. H. with minor revisions from S. J. M.

GENERAL ABSTRACT

The ectoparasitic mite *Varroa destructor* remains a major threat to *Apis mellifera* honey bees amidst ongoing colony losses throughout the Northern Hemisphere. While the vast majority of colonies still require artificial treatments to control their mite populations, an increasing number are evolving mite-resistance and are thus surviving without intervention. Here we investigated reduced mite reproductive success (a well-established resistance mechanism) and two host behavioural traits, recapping and infested brood removal, to ascertain their roles in mite-resistance across the UK, South Africa, Brazil and Australia. Both behaviours involve adult workers detecting and opening mite-infested brood cells, followed by either resealing the cell (recapping) or removing the brood (brood removal). In line with a previous study from mainland Europe, we found that recapping was significantly higher in resistant populations when compared to susceptible (those requiring treatment) and was strongly targeted to mite-infested cells. We additionally found that recapping was virtually absent in mite-naïve (those that have never been exposed to the mites) colonies and increased rapidly following initial exposure. We also found that mite reproductive success was significantly lower in resistant populations, however in contrast to a previous hypothesis, our data suggests that recapping did not cause the failed mite reproduction and is instead involved in the detection process of brood removal behaviour. Brood removal was highest in the long-term resistant *A. m. capensis* however it was also present in naïve colonies and susceptible colonies that had ceased treatment, suggesting that brood removal and recapping are innate social immune responses to *V. destructor,* as well as other parasites. Recapping is a promising trait that could be used as a proxy for both mite-resistance and evidence of brood removal behaviour, and reduced mite reproductive success is a key resistance mechanism in *A. mellifera* populations around the world.

CHAPTER 1

General Introduction

The western honey bee *Apis mellifera* is a eusocial insect that lives in colonies of tens of thousands of individuals, the majority of which are workers (non-reproductive females) alongside a single queen (a reproductive female) and a relatively small number of drones (reproductive males). The species has existed for at least 1.6 million years (Engel, 1998) and has naturally evolved into 27 locally adapted subspecies across Africa and Europe (Ruttner, 1988). In common with many other species around the world, the global spread of modern humans *Homo sapiens* has drastically altered their distribution and ecology. One of the most remarkable anthropological changes to the ecology of planet Earth is the domestication of animals for livestock (for example, the total biomass of both mammalian and avian livestock now far outweighs that of their wild counterparts [Bar-On et al., 2018]). The domestication of bees for the harvest of their honey, or 'apiculture', has been practised for at least 4000 years (Crane, 1999; Bloch et al., 2010). Today, the industry has grown to additionally provide industrial agricultural pollination services and a multitude of hive products, and consequently *A. mellifera* now inhabits every continent except Antarctica (Ruttner, 1988). Modern beekeeping practises such as densely packed apiaries (Seeley & Smith, 2015), global import/export of queens for breeding (Lodesani & Costa, 2003) and replacing harvested honey with artificial nutrition (Wheeler & Robinson, 2014) have considerably altered the biology and ecology of managed stocks of *A. mellifera* in comparison to their naturally-evolved ancestors.

Two important side-effects have arisen from the domestication and expansion of *A. mellifera*. Firstly, with such a vast distribution this single species has become an extremely important pollinator (both inadvertently and intentionally), providing an essential ecosystem service to both wild ecosystems and global arable agriculture. Pollinating insects are responsible for the reproduction of 85% of wild flowering plants (Ollerton et al., 2011) and 75% of food crops (Klein et al., 2007), with an estimated global value of US$215 billion in 2005 (Gallai et al., 2009), and *A. mellifera* contributes to this service more so than any other single species (Klein et al., 2007; Breeze et al., 2011). Secondly, *A. mellifera* has now become exposed to a myriad of novel stressors affecting their

health both at the individual and colony level, which include pesticides (Goulson et al., 2015), land-use changes (Winfree et al., 2009; Otto et al., 2016), beekeeping practises (Neumann & Blacquière, 2017) and disease/disorder inducing parasites (Brosi et al., 2017). These stressors are associated with the long-term colony decline across the Northern Hemisphere, with a total of 59% of colonies in North America (vanEngelsdorp, 2009) and 25% in Europe (Potts et al., 2010) lost since the 1950s. In the short term, annual overwintering colony losses are variable between regions, for example recent data from 27 European countries plus Algeria, Israel and Mexico, reported an overall winter colony loss of 20.9% between 2016-2017 (Brodschneider et al., 2018), with 12 countries experiencing significantly higher losses than the previous year, 11 remaining stable and 3 significantly lower (Brodschneider et al., 2016). In the US, 26.9% were lost over winter between 2015-2016 which was one of the lowest figures in the past decade, however 59% of the respondent beekeepers still deemed their losses higher than acceptable (Kulhanek et al., 2017). Given the ecological and economic importance of *A. mellifera*, which has direct implications for the health of humanity (Chaplin-Kramer et al., 2014; Ellis et al., 2015; Smith et al., 2015) and wildlife (Ollerton, 2011) alike, these trends are a serious cause for concern.

Parasites, pathogens and their associated diseases or disorders are major drivers of these colony losses. Today, *A. mellifera* is exposed to a range of damaging viruses (e.g. Israeli acute paralysis virus, Kashmir bee virus, Sacbrood virus), bacteria (e.g. *Paenibacillus* larvae inducing American foulbrood), microsporidia (e.g. *Nosema ceranae* inducing Nosemosis), fungi (e.g. *Ascosphaera apis,* inducing Chalkbrood) and arthropods (e.g. small hive beetle *Aethina tumida*) (Brosi et al., 2017). The ectoparasitic mite *Varroa destructor* is the most destructive of these parasites (Boecking & Genersch, 2008; Neumann & Carreck, 2010; Rosenkranz et al., 2010) via the transmission of several viruses, the most significant of which being Deformed Wing Virus (DWV) (Martin & Brettell, 2019). The mite *V. destructor* originally existed (and remains) in a stable host-parasite relationship with the Asian honey bee *Apis cerana* (Rath, 1999). However, following transport of *A. mellifera* to east Asia and Russia (Oldroyd, 1999), reports of the host-switch began in the 1950s (Ruttner & Ritter, 1980) and the spread has since become almost ubiquitous, with Australia now the only honey bee inhabited continent remaining to be spared the invasion of *V.*

destructor (Roberts et al., 2017). Prior to *V. destructor* exposure, DWV and *A. mellifera* also had a stable relationship; the virus was transmitted between the bees via sexual reproduction and oral ingestion, at low viral loads, and remained asymptomatic (Martin & Brettell, 2019). However, the nature of DWV changes drastically when vectored by *V. destructor*; viral loads increase exponentially and highly virulent genotypes (types A, B or C) become dominant (Martin et al., 2012; Ryabov et al., 2014; Mordecai et al., 2016b), often inducing symptomatic infections (characterised by crippled wings) and reducing the longevity of colonies to the point of collapse (Martin & Brettell, 2019).

Owing to the widespread threat of *V. destructor* and DWV, the vast majority of *A. mellifera* colonies today owe their survival to beekeeper interventions, namely in the form of chemical, biotechnical and/or biological treatments used to control their mite populations (Rosenkranz et al., 2010). Despite this, multiple *A. mellifera* populations exist around the world that are naturally evolving resistance to *V. destructor*; that is, they are surviving *V. destructor* infestation year after year without needing any form of treatment. The first reports of widespread mite-resistance came from Brazil in 1956, when an African subspecies *Apis melliferra scutellata* was selectively bred with local honey bees of European origin to improve genetic stock (Kent, 1988) and to select for desirable beekeeping traits (Francoy et al., 2009). These hybrids, or 'Africanized' honey bees (AHB), were initially kept under controlled conditions, however in 1957 they were accidentally released and subsequently spread rapidly throughout the Americas (Winston, 1992). Curiously, AHB displayed resistance to *V. destructor* that the sympatric European-origin honey bees (EHB) did not (Martin & Medina, 2004). This ability to evolve mite-resistance was later demonstrated again in South Africa following the arrival of *V. destructor* in 1997; colony losses were fewer than expected, and the African subspecies *Apis mellifera capensis* and *A. m. scutellata* rapidly developed mite-resistance after 3-5 years and 5-7 years respectively (Allsopp, 2006). *V. destructor* has continued to spread throughout Africa as far as Ghana and Kenya, with no reports from beekeepers regarding any negative impacts (Frazier et al., 2010). In addition, an increasing number of allopatric EHB populations are independently evolving mite-resistance, with reports from Europe (Fries et al., 2006; Le Conte et al., 2007; Mordecai et al., 2016a; Oddie et al., 2017; McMullan, 2018), the US (Seeley, 2007) and Fernando de Noronha, Brazil (de Mattos et al.,

2016). Beekeepers have also been selectively breeding *A. mellifera* lines for decades in both Europe (Büchler et al., 2010) and the US (Rinderer et al., 2010) in an attempt to replicate and spread *V. destructor* resistance, however the efficacy of these selected lines when tested in field operations has been mixed (Spivak & Reuter, 2001; Rinderer et al., 2014; Danka et al., 2016).

Despite extensive research efforts, precisely how these populations have become adapted to survive *V. destructor* remains unclear. Potential resistance mechanisms that have been investigated include environmental conditions (such as climate and resource availability), host population dynamics (such as smaller colony sizes, variation in brood availability, and increased swarming or absconding), host behavioural defences (grooming and hygienic behaviour) and varying virulence of *V. destructor* haplotypes (Rosenkranz et al., 2010; Locke, 2016). Viral resistance to DWV has also been suggested (Locke et al., 2014) and more recently DWV genotypes have been proposed to be involved; DWV type A has been suggested to be responsible for colony death while type B is avirulent and leads to resistance (Mordecai et al., 2016a; Kevill et al., 2019), although this hypothesis has been challenged (McMahon et al., 2016; Natsopoulou et al., 2017). Despite this, the most consistent feature observed within resistant colonies is an impairment in the mites' ability to reproduce, which in turn controls rates of mite population growth. This decrease in *V. destructor* reproductive success has been reported in both AHB and EHB across Europe (Locke & Fries, 2011; Locke et al., 2012; Oddie et al., 2017), Latin America (Rozenkranz & Engels, 1994; Martin & Medina, 2004; Calderon et al., 2012; Brettell & Martin, 2017) and Africa (Strauss et al., 2016; Nganso et al., 2018). Again however, the mechanisms behind this reduction are still unclear. Although *V. destructor* reproductive success varies by geographical region (Rosenkranz et al., 2010), the phenomenon is at least in part a host trait (Fries & Bommarco, 2007; Locke et al., 2012; Oddie et al., 2017). Possible host traits that have been investigated thus far include: a reduced post-capping duration (Büchler & Drescher, 1990; Oddie et al., 2018b), smaller brood cell sizes (Oddie et al., 2019), alterations of brood volatile compounds that are responsible for triggering mite oogenesis (Frey et al., 2013) and behavioural defences such as mite-infested brood removal (Panziera et al., 2017; Nganso et al., 2018).

A recent study by Oddie et al. (2018) has identified another trait that has

appeared consistently at higher levels in resistant populations. ‘Recapping’ is a behaviour performed by adult bees, whereby a sealed brood cell cap is opened and subsequently resealed without removing the brood. This behaviour has previously been thought to be associated to infested brood removal behaviour (Boecking & Spivak, 1999), however Oddie et al. (2018) proposed for the first time that it is a previously overlooked, independent resistance mechanism. They studied four populations across mainland Europe and found that recapping frequencies were consistently higher in resistant populations when compared to local susceptible (colonies receiving treatment) controls. They also found that recapping was strongly targeted towards mite-infested brood cells, and since each resistant population also displayed a reduced *V. destructor* reproductive success, they suggested that the act of opening and recapping the cell is responsible for the impaired mite reproduction within it. They then supported this conclusion using a controlled experiment, where brood cell caps that were artificially opened and subsequently recapped contained a reduction in mite reproduction. If this hypothesis is correct, then recapping is a previously overlooked, independent and cost-effective resistance mechanism that is responsible for the reduction in mite reproduction in resistant colonies.

The aim of this thesis is therefore to expand on these recent findings into the roles of recapping, reduced *V. destructor* reproduction and infested brood removal in naturally evolved, long-term mite-resistant *A. mellifera* populations. We aimed to develop our understanding as to whether these behaviours are indeed adaptive rather than detrimental towards these resistant colonies’ survival. Chapter 1 investigates recapping levels in mite-naïve populations (those that have never been exposed to *V. destructor*) from Australia and Scotland, and recapping, mite reproduction and infested brood removal in long-term mite-resistant populations from Brazil and South Africa. Chapter 2 consists of a UK-wide survey, comparing recapping and mite reproduction levels between resistant and susceptible colonies. In addition, a controlled experiment was conducted to investigate brood removal in a small number of resistant, susceptible and naïve colonies. Developing our understanding of these resistance traits could provide important insights into promoting treatment-free, sustainable control strategies for one of the most significant threats to modern apiculture.

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CHAPTER 2

Varroa destructor reproduction and cell recapping in mite-resistant *Apis mellifera* populations

Abstract

Globalization has facilitated the spread of emerging pests such as the *Varroa destructor* mite, resulting in the near global distribution of the pest. In South African and Brazilian honey bees, mite-resistant colonies appeared within a decade; in Europe, mite-resistant colonies are rare, but several of these exhibited high levels of 'recapping' behaviour. We studied recapping in *Varroa*-naïve (UK/Australia) and *Varroa*-resistant (South Africa and Brazil) populations, and found very low and very high levels respectively, with the resistant populations targeting mite-infested cells. Furthermore, 54% of artificially infested *A. m. capensis* worker cells were removed after 10 days, and 83% of the remaining infested cells were recapped. Such targeted recapping of drone cells did not occur. We propose that cell opening is a fundamental trait in mite-resistant populations, and that recapping is an accurate proxy for this behaviour.

Introduction

During the past 70 years, the ectoparasitic '*Varroa*' mite (*Varroa destructor*) has spread worldwide and has become the greatest threat to apiculture, killing large numbers of managed *Apis mellifera* honey bee colonies (Rosenkranz et al. 2010) and decimating feral and wild populations (Wenner et al. 2009). Many beekeepers originally advocated breeding from stock that survived, but in the vast majority of cases their colonies ultimately died, since any pre-existing defence adaptations were either not sufficiently developed or were overwhelmed by the massive number of mites initially circulating in the population. As such, countries across the Northern Hemisphere, and those (e.g. Argentina, New Zealand) which had imported Northern Hemisphere honey bees that subsequently became infested by *Varroa*, were forced to use miticides to control mite numbers and to protect their bee populations.

By contrast, and although the evolution of defence mechanisms can occur rapidly (<100yrs) but is rarely seen occurring simultaneously in allopatric populations (Thompson 1998), in South Africa and Brazil their honey bees quickly became resistant to *Varroa* (Rosenkranz 1999). That is, they did not receive nor require the administering of any mite-control methods to ensure their long-term survival, and no population-wide loss of colonies occurred.

The western honey bee *A. mellifera,* which consists of approximately 30 geographical subspecies, originated in Africa and appears to have expanded twice into Eurasia, followed by a more recent anthropogenic expansion into the Americas (including Brazil, see below), Asia and Australasia (Whitfield et al. 2006). African honey bees are resilient to many of the pathogens and parasites that often plague (and need to be controlled) in other parts of the world, as evidenced by the limited pest management practiced in Africa (Pirk et al. 2015). The *Varroa* mite arrived in Africa in 1997 to the Cape Region of South Africa (Allsopp 2006). This was initially followed by some colony losses; however, these were short-lived, with mite-resistance appearing after 3-5 years in the Cape honey bee (*A. m. capensis*) and 6-7 years in the Savanna honey bee (*A. m. scutellata*) (Allsopp 2006). This pattern of short-lived colony loss prior to the appearance of mite-resistance is frequently mentioned in other mite-resistant populations (e.g. Fries et al. 2006; Mordecai et al. 2016; Oddie et al. 2017).

The Africanized honey bee (AHB) is a hybrid between *A. m. scutellata* from South Africa and East Africa, and various European races, e.g. *A. m. iberiensis* and *A. m. ligustica*. In 1957, 26 swarms of *A. m. scutellata* spread northwards throughout Brazil from Rio Claro, hybridizing with European races to form the AHB which reached the USA in 1990 (Winston 1992). In 1971, during this expansion, the *Varroa* mite arrived in Brazil (Moretto et al. 1991) and spread rapidly throughout both the AHB and European honey bees. The subsequent establishment of AHB throughout the tropical and sub-tropical regions of South America was due, in part, to AHBs' natural resistance to *Varroa* (Rosenkranz 1999). In both AHB (Camazine 1986; Medina et al. 2002; Mondragon et al. 2006) and *A. m. scutellata* (Martin and Kryger 2002; Nganso et al. 2018), poor mite reproduction limits their population growth, although the mechanism(s) by which this occurs has remained unknown.

Targeted selective bee-breeding programs to combat *Varroa* have been ongoing for decades in both America (Rinderer et al. 2010) and Europe (Buchler et al. 2010). Selection for traits such as hygienic behaviour (based on the removal of killed sealed brood) is being used by beekeepers to help reduce their mite-treatment regime, and the *Varroa* Sensitive Hygiene (VSH) line (developed from Suppression of Mite Reproduction lines) that targets the removal of living mite infested brood (e.g. Harris 2008) is undergoing further selection in Hawaii to make it more suitable for use in beekeeping operations. Meanwhile, naturally selected mite-resistant populations are being maintained without any mite control measures across a vast range of environments, i.e. that exist across Africa and South and Central America.

Recently, low rates of mite reproduction similar to those found in African and AHB were reported in four European mite-resistant populations (Oddie at al. 2017), which raised the possibility that a similar mechanism had arisen in these geographically distinct populations. Oddie et al. (2018) then linked the low mite reproduction in these European populations with a high incidence of 'recapping' behaviour, when a cell containing a developing pupa has its silk/wax cap partially removed by the worker bees and then resealed with wax, without the removal of the pupa. Although recapping (even of mite-infested cells) is not a new phenomenon, e.g. both hygienic and non-hygienic colonies recapped around 90% of artificially created holes in the cell caps (Spivak and Gilliam 1993), its

importance may have previously been overlooked.

The aim of this study was to investigate mite-naïve populations from Scotland, Isle of Man and Australia, and well-established mite-resistant populations from Brazil (AHB) and South Africa (*A. m. scutellata* and *A. m. capensis*), to identify whether recapping is a reliable proxy for mite-resistance, and whether it is associated with reduced mite reproduction. We then focused on *A. m. capensis*, which was found to have the highest targeted recapping of mite-infested cells.

Methods

Varroa-naïve colonies

During 2018, *Varroa*-naïve brood samples were obtained from three colonies, each from a different apiary, from across the Island of Colonsay, Scotland, UK; and three colonies from a single apiary belonging to Western Sydney University, Hawkesbury Campus, NSW, Australia. Four additional *Varroa*-naïve colonies were sampled in 2019 from the Isle of Man, UK.

Mite-resistant AHB colonies, Brazil

The AHB were located at Cruz das Almas, Bahia State, NE Brazil. Recapping and mite reproduction were studied in February 2018, using six colonies (minimum of 300 worker cells per colony). Recapping rates and mite infestation data were collected from an additional ten colonies (150-200 worker cells per colony) which were used in a freeze-kill brood removal test.

Mite-resistant South African colonies

Four *A. m. scutellata* colonies were studied in July 2018 and again in March 2019, while 20 *A. m. capensis* colonies were studied in July 2018 (n=3) and in March 2019 (n=17) (Table 1, S1). As only one *A. m. scutellata* and two *A. m. capensis* colonies contained drone brood, in addition ten *A. m. capensis* colonies with drone brood were sampled. All colonies are maintained within 20km of Stellenbosch, Western Cape, South Africa, with the four *A. m. scutellata* colonies having been moved 800km from their natural distribution into the area for research purposes. As no mite-susceptible colonies are present in either Brazil or South Africa, no direct comparisons with treated colonies from the same region are possible,

although as African bees are the ancestral population both resistant and susceptible populations all originated from Africa (Whitfield et al. 2006).

Cell recapping

From each colony a single frame containing mainly purple eyed pupae (e.g. 180-190hrs post-capping) or older worker or drone brood was removed, and on average 300 cells per colony (Table S1) were examined for recapping and mite reproduction. To determine whether a cell was recapped, fine forceps were used to carefully cut around the edge of the cap, which was then inverted to allow the underside to be inspected under a binocular microscope (x16). If the silk cocoon spun during the first 30hrs of the sealed stage (Martin 1994) had been partially removed and replaced by wax it was classified as recapped. The diameter of the recapped area for worker brood was estimated to the nearest mm.

Mite reproduction in worker brood

After the recapping status of the cell had been determined, the pupa was carefully removed and aged according to standard methods (Dietemann et al. 2013). If the cell was infested then all mites, offspring and shed skins (exuviate) were removed and the mite family reconstructed using the method and developmental chart of Martin (1994). Only the 497 infested cells containing yellow-thorax pupae or older (>190hrs post-capping) were used in the reproductive calculations (Table S2). The number of mated adult female offspring were counted, that is the cell must also contain a living adult male (evidenced by the exuviate), accompanied by daughters at the correct developmental stage. Only the number of foundresses per cell were determined in the drone brood. Two methods were used to calculate the average number of mated female offspring produced during one reproductive cycle in each of the three study populations, since not all samples are at the same developmental stage, therefore any mite mortality from the sampling point to bee emergence will not be accounted for. Therefore, firstly we counted the total number of mated female offspring and divided it by the number of invading foundresses. Secondly, we counted the number of females that would be mated and were assumed to mature prior to bee emergence, but accounted for a further in-cell mortality, by multiplying the first daughter by 0.94, the second daughter by 0.38 and third daughter by 0.13. These mortality values were based on a study of

over 1000 mite families (Martin 1994). The results of both calculations are provided (Table S2) and are similar.

Artificial infestation of A. m. capensis cells with Varroa

Since the pattern of *Varroa* infestation within and among frames is non-random (Fuchs 1989), we studied the bees' ability to detect and remove mite-infested pupae by artificially infesting cells with mites (Boecking 1992; Rosenkranz et al. 1993) rather than comparing relative changes in infestation levels over a period of time (Harris 2007). Therefore, a total of 390 mother mites harvested from *A. m. capensis* drone brood cells containing stretched larvae were inserted into *A. m. capensis* worker cells that were less than one day post-capping, as indicated by the lack of a completed cocoon (Martin 1994). Of these mites, 325 were alive and 65 had died since being collected the previous day. Dead mites were also used for artificial infestation experiments, as comparing rates of detection using living vs dead mites may help indicate cues used to detect infested cells, e.g. movement. A frame containing cells capped within the past 24hrs (evidenced by the larvae spinning their cocoons) were removed from each of 11 *A. m. capensis* colonies over a period of several days. Freshly sealed cells were opened and a mother mite inserted using a fine paint brush before resealing the cell and recording its position on an acetate sheet. After returning the frames to their colonies, an inspection after 24hrs revealed any cells removed by the bees as a result of the manipulation. Five days later (six days post-capping), the number of pupae removed from the artificially infested cells were recorded. After 10 days (11 days post-capping), each frame was removed and any remaining artificially infested cells were inspected for mites, and the state of the cell cap (recapped or not) was recorded (Table S3). We also inspected the cap condition of a similar number of neighbouring non-infested control cells. Insufficient mites were available to conduct artificial infestation experiments in AHB.

Test for hygienic behaviour using freeze-killed brood

In ten AHB and 11 *A. m. capensis* colonies, their ability to remove freeze-killed pupae (classic hygienic behaviour) was studied to compare with their recapping rates. An area of purple-eyed pupae or older (>7 days post-capping) was freeze-killed using liquid nitrogen, and then the number of removed pupae determined

after 24hrs and 48hrs (Table S3). The 48hrs data was not analysed since several of the colonies in both populations had removed 100% of the killed brood by 48hrs.

Data analysis

All data sets were non-normally distributed, thus statistical tests included: Chi-squared, Kolmogorov-Smirnov, Mann-Whitley U, Wilcoxon signed-rank, and Spearman's rank correlation. The test statistic indicates which test was used if not mentioned in the text.

Results

Cell recapping and mite reproductive behaviour in worker cells

Based on 497 infested cells we confirmed that the average number of mated female offspring produced per mother foundress during a single reproductive cycle (*Wr*) was between 0.8 and 0.9 in the three mite-resistant populations (Table 1, Table *S2*), with only 54-55% of the invading mothers reproducing, and producing between 1 to 3 mated female offspring each. Across the ten *Varroa*-naïve colonies from three different populations, only 0.5% (median) of the worker sealed brood cells were recapped (Table 1, Table S1, Figure S1). In contrast, the median recapping rates in the *Varroa*-resistant AHB, *A. m. scutellata* and *A. m. capensis* were 35%, 20% and 27% respectively, although the average infestation rate was <10% in every mite-resistant population. The recapping rate of infested cells was always significantly higher than for non-infested cells (AHB U=17, z=2.46, p=.014; *A. m. scutellata* U=0 z=3.31 p<.001; and *A. m. capensis* U=10.5, z=4.41 p<.0001) (Table S1, Figure S1). Combining the data from the three populations, we found the estimated number of viable offspring in recapped (0.82) and undisturbed (0.76) cells were similar (Table S2). The frequency distribution of the size of the recapped area (diameter of the opening which had been resealed) of non-infested cells in all four populations followed the same negative trend (Figure 1a-d). In contrast, infested cells all had significantly different size distributions (AHB, D= .2051, p=0.002; *A. m. scutellata* D= 0.3406, p<0.0001 and *A. m. capensis*, D=1, p=.037) due to the recapped area being larger i.e. larger than 3 mm, if the cell was infested in all three populations (Figure 1e, f). Furthermore, all recapped cells in the *Varroa*-naïve populations were small ranging from 1 to 3 mm. Recapped cells

Table 1. Meta-data from the four groups of honey bees studied. The number of colonies (# col) and cells studied along with the number of cells recapped and infested with *Varroa* are presented, along with the group percentages, either in worker (W) or drone (D) cells. For individual colony data see Table S1. *Wr* = number of viable female offspring produced per mother mite during one reproductive cycle. This is based on 'n' mothers from 12 AHB, four *A. m. scutellata* and 20 *A. m. capensis* colonies. All cells contained yellow-thorax pupae (190hrs post-capping) or older (see Table S2 for more mite reproductive details).

Population	Country	# col	# cells opened	# cells recapped	# cells infested	% cells recapped	% cells infested (range)	% inf. cells recapped	% non-inf. cells recapped	*Wr* (n=mothers)
Naïve	UK/Aus	6	5846	87	0	1.5	-	-	1.5	-
AHB-W	Brazil	12	3417	1402	224	41	7 (0-26)	71	39	0.8 (143)
scutellata-W	S Africa	8	3235	620	294	19	9 (3-21)	64	15	0.9 (183)
scutellata-D	S Africa	1	103	2	59	2	59	3	0	-
capensis-W	S Africa	20	5599	1452	190	26	3 (0-14)	74	24	0.9 (171)
capensis-D	S Africa	12	1183	251	330	21	32 (9-92)	17	23	-

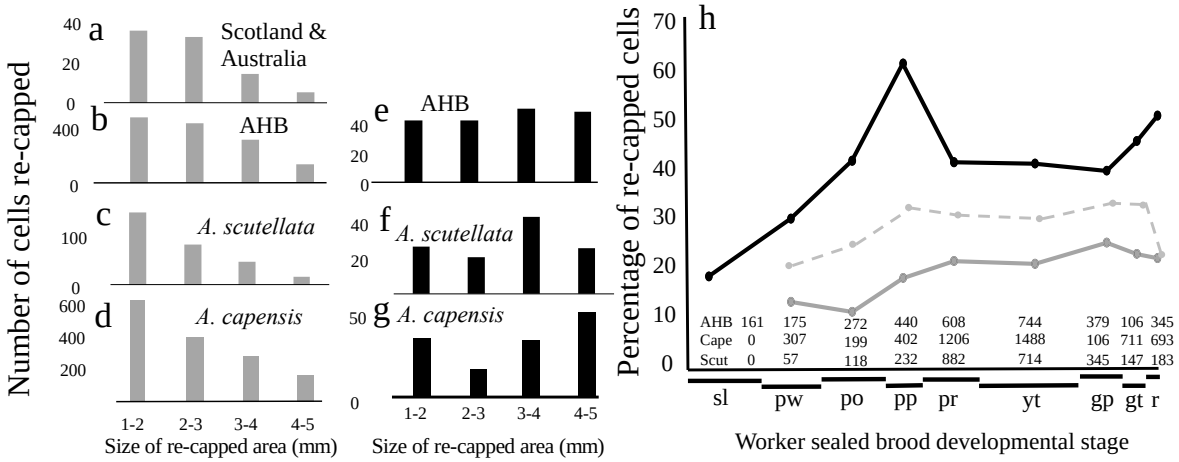

Figure 1. Frequency distributions of the recapped areas and recapping across brood developmental stages. a-g) indicates the frequency distributions in the diameter of the recapped area in non-infested cells (grey, a-d) and *Varroa*-infested cells (black, e-g) in the four study groups, h) indicates the percentage of recapped cells at each stage of pupal development in AHB (black line), *A. m. scutellata* (solid grey line) and *A. m. capensis* (dashed grey line). The pupal developmental stages following (Martin 1994) are sl = stretched larvae, pw = white-eyed, po = pale-eyed, pp = pink-eyed, pr = purple-eyed, yt = yellow thorax, gp = grey pad, and gt/r = grey thorax/resting and total number of cells opened per stage is also given.

appeared from the early stages of pupal development and their proportion increased steadily as the pupae developed (Figure 1h). We noticed that some infested cells had been recapped more than once, as they contained two or three distinct holes cut out of the silk cap.

Cell recapping and mite infestation in A. m. capensis drone cells

The average infestation level of drone brood was 31% ± 27 (Table 1; S1), with cells typically containing multiple foundresses. A total of 330 infested cells (single and multiple infested cells) were invaded by 569 foundress mites. This varied considerably by site; only 5% ± 9 of drone cells were recapped in nine colonies in

the Stellenbosch area (Table S1-R* colonies), whereas three colonies from the Pniel (Table S1-PA* colonies) region in the next valley recapped 65% of all drone cells. However, the proportion of infested (66%) and non-infested (65%) cells recapped were similar. Therefore, a pattern of targeting mite-infested drone cells was not seen (Table 1; Figure S1). The single drone frame from *A. m. scutellata* showed the same pattern, of high infestation (57%), low recapping (2%) and multiple foundresses in cells (Table 1; S1).

Detection and removal of artificially mite infested cells in A. m. capensis

Of the 392 *A. m. capensis* worker pupae artificially infested with mites (326 alive and 66 dead), only 3% were removed within 24hrs, most likely due to the experimental opening and resealing of the cell (manipulation). After 10 days, we found 21 (5%) cells containing no mites or evidence of mites i.e. mite faecal droppings on the cell wall, which must have escaped during the uncapping/recapping process, and a further 30 (8%) mites had become sealed into the cell wall and died during the spinning of the pupal cocoon. The mites lost due to manipulation, the recapping process or being sealed into the cell wall were removed prior to the analysis of removal behaviour. Across the 11 colonies, 32% of the infested cells had been removed after six days, and this increased to 54% after 10 days (Table S3). The percentages of dead (47%) and alive (46%) mites removed after 10 days were not significantly different ($U=49.5$, $p = .75$). Of the remaining 152 artificially infested cells, 83% had been recapped, while only 27% of mite-free 'control' cells were recapped (Table S3). Again, typically larger recapped areas were found in infested cells relative to neighbouring non-infested control cells (Figure 2); however, there were no significant differences in sizes of the recapped area between both non-infested controls and cells containing dead mites trapped in the walls, and between infested cells that contained living or dead mites (Figure 2).

Test for hygienic behaviour using freeze-killed brood

Among ten AHB colonies tested for both classic hygienic and recapping behaviours, a Spearman's rank correlation found no significant correlation (r_s = .03, $p = .93$) between the two behaviours after 24hrs (Table S3), with 19-98% of

the dead brood having been removed in 24hrs while the recapping rates ranged from 4-50%. A similar result (r_S = .356, p = .282) was also found across the 11 *A. m. capensis* colonies (Table S3), with 48-100% of freeze-killed brood removed while the range of recapping rates was 12-66%. However, there was a weakly significant positive correlation (r_S = .67, p = .024) between the removal rate of freeze-killed brood and the proportion of artificially infested cells removed (Table S3).

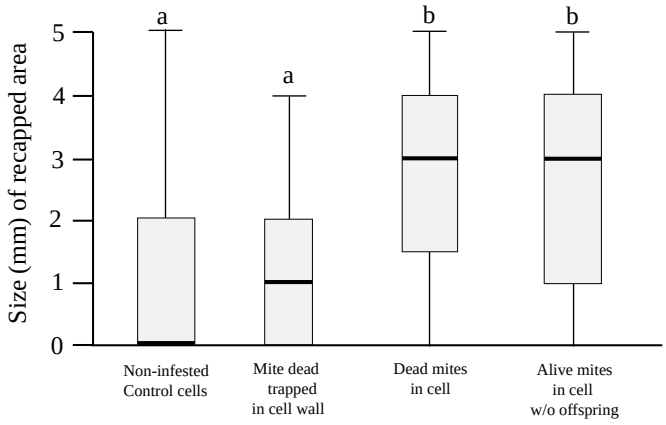

Figure 2. Box plots showing the size of the recapped area of *A. m. capensis* at 11 days post-capping among four groups of cells (11 colonies).
Kolmogorov-Smirnov analysis showed significantly different frequency distributions of recapped areas between the four groups of brood cells ($D_{(499, 3)}$ = 69.4, p<.0001). Pairwise comparisons between the four groups showed there was no significant difference between the mites trapped in cell walls and non-infested control cells (D =0.678, p=.41), nor between the cells in which dead and live mites were found (D= 0.839, p=.36). However, there were significant differences between the cells containing mites (dead or alive) and those cells that were either mite-free or contained mites trapped in the cell wall (D>11, p always <.005).

Discussion

Although we observed the recapping of brood cells in all colonies, the recapping rates were lowest in *Varroa*-naïve, and the highest were consistently found in worker brood of mite-resistant populations from Brazil (AHB) and South Africa (*A. m. scutellata* and *A. m. capensis*), as well as Europe (Oddie et al 2018). The key behaviour in all these mite-resistant populations appears to be the bees' ability to detect mite-infested cells, as indicated by consistently higher recapping rates of infested cells relative to non-infested cells, particularly since infestation rates are typically below 10% (Figure 3; Figure S1). These low infestation rates are most likely explained by the reduced mite reproduction, which may be linked to instances of recapping and brood removal. The initial detection of a possible infested cell leads to the opening of a small hole in the cell cap that could allow better access to any volatile or non-volatile cues, i.e. on the pupae, within the sealed cell (see below). If a non-infested cell is opened in error, the hole is recapped and the disturbed area remains small (1-2 mm), but if infested, the hole is enlarged to 3-4 mm to gain better access (Figure 1). A second trigger, or lack thereof, causes the infested cell to either be recapped or the pupa to be cannibalized (Figure 4). This idea is in line with previous studies (Gramacho 1999; Arathi et al. 2006) that found the initial step of detecting diseased brood does not necessarily lead to brood removal, with repeated uncapping and recapping prior to brood removal. The removal of pupae artificially infested with mites was 54% in *A. m. capensis* (this study), 33% in *A. m. scutellata* (Cheruiyot et al 2018), 10-25% in AHB (Aumeier et al. 2000), and up to 40% in a single mite-resistant population in the Netherlands (Panziera et al 2017). All values are well below the 99% removal of artificially infested worker cells in the mite's original host, *A. cerana* (Rath and Drescher 1990).

Two recent studies have assumed that genetical derived host-factors within the brood prevent the initiation of mite oogenesis, which accounts for the increase in non-reproduction of mites in resistant colonies. For example, Broechx et al (2019) suggested brood pheromones fall to a level that prevents the mites reproducing, whereas Conlon et al. (2019) suggested an ecdysone gene was linked to mite-resistance, since low ecdysone levels may prevent mite oogenesis, hence increasing non-reproduction in mites. However, the greater proportion of

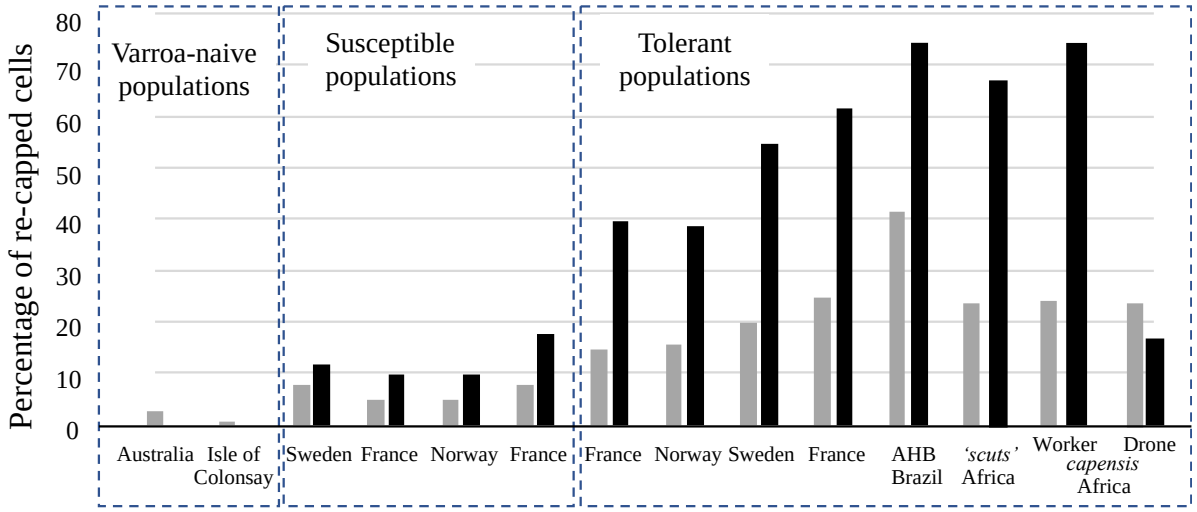

Figure 3. Recapping levels of non-infested (grey) and *Varroa*-infested (black) worker cells. In susceptible populations, levels are consistently lower than those recorded in mite-resistant populations, except for the '*capensis* drone brood'. The European data is taken from Oddie et al. (2018), while the Australia, Colonsay, AHB and Africa data are from the current study. '*scuts*' = *A. m. scutellata; capensis* = *A. m. capensis*. For individual colony data see Figure S1.

non-reproducing mites found in mite-resistant colonies (Martin et al. 1997; Broechx et al. 2019) can simply be explained by the behavioural trait of increased disruption of mite reproduction. That is the removal of infested pupae reduces the number of successful mite reproductive cycles, increasing the proportion of non-reproductive mites in subsequent reproductive attempts. The latter may in part account for the lower reproductive values (*Wr*) found in mite-resistant colonies, since a consistent 50% removal rate will result in 12.5%-25% of mites never reproducing due to having been disturbed by the removal of pupae before mating. Since the number of mite reproductive cycles is estimated between 2-3 (Martin and Kemp 1997), these mites may still invade cells and attempt to reproduce but produce either no offspring or only males (Martin et al. 1997), both categories common in African (Martin and Kryger 2002), AHB (Medina et al 2002) and this study. This would also explain why reproducing and non-reproducing mites could

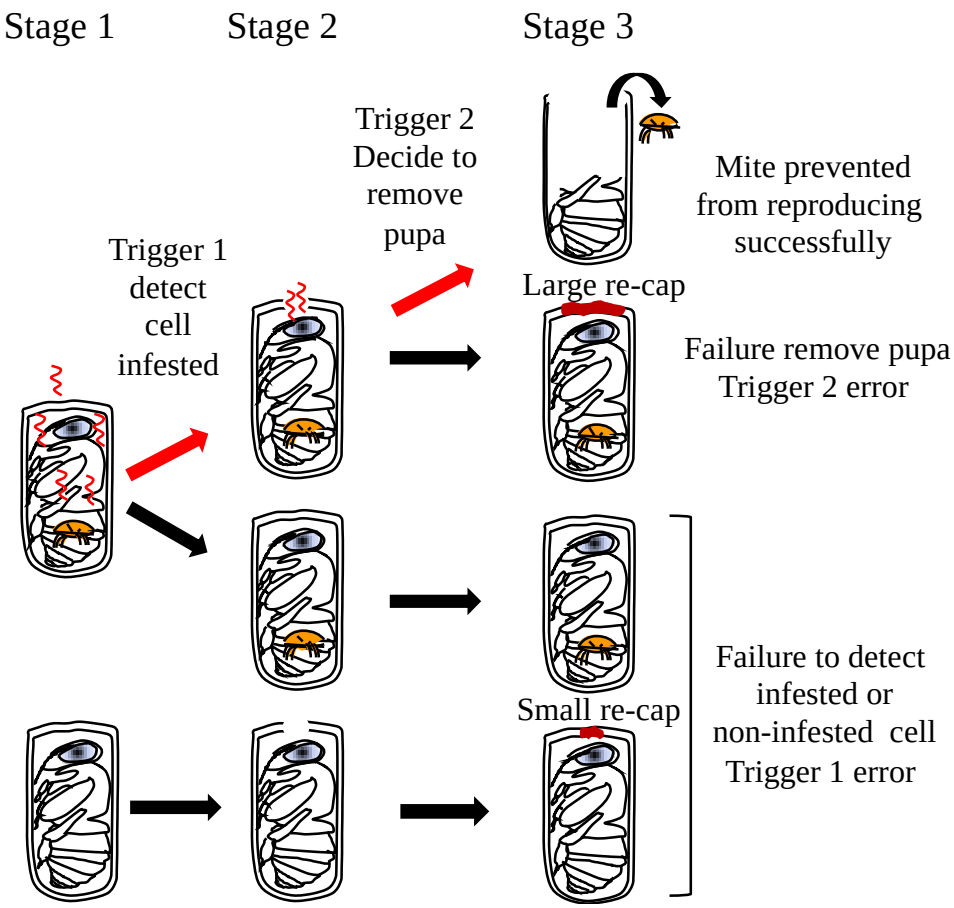

Figure 4. Process of detection of infested cells and subsequent removal of the pupa, and where errors are generated. Stage 1, a mite-infested cell produces a stimulus detectable through the cell cap (Trigger 1). Stage 2, a small hole is made in the cap to allow a more detailed inspection. At this point a second trigger may be detected. Stage 3, if a mite is present and is detected the pupa is cannibalized and *Varroa* prevented from reproducing, if the mite is missed (or no mite is present) the cell is recapped. The red arrows indicate the ideal situation and black arrows indicate observed errors due to the failure of one or both of the theorised behavioural triggers. The red wavy lines indicate the putative density of the mites' odour.

not be distinguished using DNA micro-satellites (Broechx et al. 2019), since they do not represent two distinct genotypes, just that non-reproducing mites have either run out of sperm or eggs.

Mite reproductivity in recapped and undisturbed infested cells were similar in this study (Table S2), which was also found in previous studies (Harris et al. 2012; Oddie et al. 2018). Mondet et al. (2016) and Oddie et al. (2018) have suggested that the bees are more likely to ignore cells containing non-reproducing mites (selection bias), while preferentially removing cells containing reproducing mites or with more offspring. However, we found no evidence for this in *A. m. capensis* since the removal rate of cells containing live (reproducing) and dead (non-reproducing) mites were similar, and Panziera et al. (2017) also found no relationship between mite reproductive success and brood removal. When looking at actual numbers rather than averages (Table S2), the majority of cells studied were recapped and contained successfully reproducing mites, reflecting the high recapping rates of infested cells in resistant colonies and the relative success of reproducing *Varroa* females (even in resistant colonies where failed reproduction is more common).

In both AHB (Mondragon et al. 2006) and African honey bees (*A. m. capensis* and *A. m. scutellata*) (Table 1; S1), drone brood frequently becomes heavily infested, which may impact on the honey bee colony and population reproductive success. This can lead to density dependent control of the mite population via the drone brood (Martin and Medina 2004), which occurs in *A. cerana*, either via a reduction in mite offspring survivorship in multiple infested brood (Martin 1995) or increased failure for the bee to emerge (Rath 1999). Recent studies have found that mite feeding causes *A. cerana* worker brood to die (Page et al. 2016) via injection of a toxic mite salivary protein into the pupae during feeding (Zhang et al. 2018), which could explain the high removal rates previously seen (Rath 1999). However, this salivary protein has no effect on *A. mellifera* pupa (Zhang et al. 2018).

As only around 27% (Figure 3) of non-infested worker cells are recapped, this suggests that initial detection of the mite is made without disturbing the cap. Whilst the ability to detect mite infested cells is high in almost all mite-resistant colonies (Figure S1 and Oddie et al. 2018), it is the trigger to remove the infested pupa that remains error prone since only around 50% of infested cells that are

opened are subsequently removed in *A. mellifera*, and a high proportion of those cells are recapped. The triggers for the initial detection and subsequent decision to cannibalise the pupa are both currently unknown (Figure 4), although this study indicates that olfaction could be a key factor for the initial detection as several previous studies have proposed (e.g. Rosenkranz et al 1993; Mondet et al 2015, Scannapieco et al 2017), since dead mites elicited a similar recapping behaviour as live mites, as also reported in *A. cerana* (Rath and Drescher 1990). This suggests that motion or associated changes in the pupa (odour or temperature) may not be important as previously suggested by Aumeier and Rosenkranz (2001) and Wagoner et al. (2018). Nor would the level of oleic acid, which is known to trigger hygienic behaviour (McAfee et al. 2018), be important, unless also produced by the living mites. Furthermore, as mites sealed into the cell wall by the pupal cocoon did not elicit any increased recapping response relative to non-infested cells (Figure 2), a volatile odour is a likely candidate. For example, Nazzi et al. (2004) found pentadecane ($C_{15}H_{30}$) was present only in the air of infested cells and the application of Z-(6)-pentadecene increased hygienic behaviour, whereas Z-(7)-pentadecene, Z-(8)-heptadecene and pentadecane had no effect. Longer cuticular hydrocarbons are unlikely to be the odour cue due to their lack of volatility, and the pupa's profile is mimicked precisely by the mite (Kather et al. 2015). Rath and Drescher (1990) also found that dead mites washed in ethanol were still removed at a high rate in *A. cerana*. However, Wagoner et al. (2019) suggested that two long cuticular hydrocarbons (heptacosene [$C_{27}H_{54}$] and tritriacontane [$C_{33}H_{66}$]) removed from the surface of the pupa were associated with the uncapping of infested worker brood. In addition, Mondet et al. (2016) suggested changes in the brood pheromone that consists of ten ethyl and methyl esters can be detected between infested and non-infested brood, although this was found using discriminate analysis that is error prone if the sample to variable ratio is not high (Martin and Drijfhout 2009; Mitteroecker and Bookstein 2011 [Figure 5]).

Why recapping behaviour exists even in the *Varroa*-naïve populations is unknown, but when non-infested brood are not removed, any cost to colony fitness is minimal. We observed in African honey bee brood invaded by the lesser wax moth (*Achroia grisella*), cells were frequently recapped rather than the pupae being removed. Likewise, 57% of the uncapped cells in a colony heavily infested

with the greater wax moth (*Galleria mellonella)* were recapped within 24 hours of uncapping (Villegas and Villa 2006). Interestingly, the three *A. m. capensis* colonies at Pniel were unique in recapping high numbers of drone cells. These were all survivor colonies from an American Foulbrood (*Paenibacillus larvae*) outbreak. Therefore, making a small hole in the cell cap may be a general response to allow more detailed investigation of the developing pupa (which may account for the low-level presence of this trait in *Varroa*-naïve populations). After the arrival of the mites, this behaviour appears to have been co-opted and selected for as part of a defence mechanism against *Varroa*, hence the recapping rate is elevated in all infested colonies (Figure 3, S1), reaching the highest levels in mite-resistant colonies. Throughout Brazil and Africa, beekeeping pest management is minimal and so selective pressures for such traits have always been high. The constant management of a wide range of brood pests and pathogens throughout the Northern Hemisphere removes much of this selective pressure. In this and in previous studies (Oddie et al. 2018), the ability to detect mites (Figure S1) and remove infested brood (Table S3) is highly variable. No doubt colony composition plays a role since recapping occurred most in mixed colonies rather than in highly hygienic, or highly non-hygienic colonies (Arathi et al. 2006).

Mondragon et al. (2005) suggested hygienic behaviour towards freeze-killed brood may not correlate closely with hygienic behaviour towards *Varroa* mites. We found no correlation between recapping levels and removal of freeze-killed brood. We did, however, find a weak positive correlation between the ability of a colony to remove freeze-killed brood and the removal of artificial mite-infested cells, which is similar to data from Spivak (1996), where colonies selected for their ability to remove freeze-killed brood removed significantly more artificially mite-infested cells than ‘non-hygienic’ colonies in one year but not another. Perez and Johnson (2019) indicate that task specialization, e.g. hygienic behaviour, can be used to predict specialization in other related tasks, which may help explain the weak link between response to freeze-killed brood and removal of living mite-infested brood.

It appears that resistance towards *Varroa* mites in both *A. cerana* and *A. mellifera* is following a similar path, that of targeting mites invading worker cells and not drone cells, which will eventually lead to the combined effect of lower mite

reproductive success in worker brood and density-dependent control in drone brood. As the ability of the bees to detect mites within worker cells, evidenced by increased recapping, has arisen naturally in *A. mellifera* in five different countries, this may prove to be an excellent proxy for mite-resistance. The challenge will be selecting for these traits (the abilities to initially detect and subsequently remove infested pupae) while moving away from a regime using insecticides, especially in large commercial beekeeping operations.

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Supplementary Information

Table S1. Individual colony data from all of the worker and drone brood studied. Includes the number of cells opened, number of recapped and infested cells along with the numbers that were recapped. These values have been standardized by presenting the percentages, derived by the simple calculations presented. These include the *Varroa*-naïve colonies from Scotland (Scot), Isle of Man (IoM) and Australia (Aus); Africanized bees (AHB) from Brazil; *A. m. scutellata* (scuts) and *A. m. capensis* (cape) honey bees.

Population	Colony Code	# cells opened	# infested cells	% cells infested	# cells infested recapped	# cells non-inf. recapped	# cells recapped	% cells recapped	% infested cells recapped	% cells non-infested recapped
		(a)	(b)	(c)	(d)	(e)	(f)			
Calculation				=b/a			=d+e	=f/a	=d/b	=e/(a-b)
WORKERS										
Naïve	Scot-1	1300	-	-	-	3	3	0.2	-	0.2
Naïve	Scot-2	811	-	-	-	2	2	0.2	-	0.2
Naïve	Scot-3	1737	-	-	-	17	17	1.0	-	1.0
Naïve	Aus-1	907	-	-	-	8	8	0.9	-	0.9
Naïve	Aus-2	564	-	-	-	48	48	8.5	-	8.5
Naïve	Aus-3	527	-	-	-	9	9	1.7	-	1.7
Naïve	IoM-1	150	-	-	-	0	0	0	-	0
Naïve	IoM-2	151	-	-	-	1	1	0.7	-	0.7
Naïve	IoM-3	150	-	-	-	0	0	0	-	0
Naïve	IoM-4	155	-	-	-	0	0	0	-	0
AHB	A9	600	18	3	14	245	259	43	78	42
AHB	A12	413	5	1.2	3	114	117	28	60	28
AHB	A5	580	151	26	95	88	183	32	63	21
AHB	A11	443	18	4.1	17	140	157	35	94	33
AHB	A15	448	9	2	9	378	387	86	100	86

AHB	A8	315	15	4.8	15	150	165	52	100	50
AHB	A6	106	4	3.8	3	21	24	23	75	21
AHB	L7	104	0	0	0	13	13	13	-	13
AHB	L1	108	0	0	0	9	9	8	-	8
AHB	L2	87	1	1.1	0	30	30	35	0	35
AHB	L5	106	1	0.9	1	11	12	11	50	10
AHB	L10	107	2	1.9	1	45	46	43	50	43
scuts	S1-18	416	35	8.4	28	118	146	35	80	31
scuts	S2-18	631	39	6.2	27	74	101	16	69	13
scuts	S3-18	857	63	7.4	36	65	101	12	57	8
scuts	S4-18	644	92	14.3	60	81	141	22	65	15
scuts	2019/1	117	24	20.5	11	10	21	18	46	11
scuts	2019/2	154	21	13.6	12	27	39	25	57	20
scuts	2019/3	184	5	2.7	4	44	48	26	80	25
scuts	2019/4	232	15	6.5	9	14	23	10	60	6
cape	C1-18	558	0	-	-	109	109	20	-	20
cape	C2-18	516	0	-	-	50	50	10	-	10
cape	C3-18	266	2	0.8	2	22	24	9	100	8
cape	LV-1	246	26	10.6	21	47	68	28	81	21
cape	LV-2	193	18	9.3	15	79	94	49	83	45
cape	LV-3	274	2	0.7	2	74	76	28	100	27
cape	LV-4	259	13	5	12	159	171	66	92	65
cape	LV-5	324	4	1.2	3	52	55	17	75	16
cape	LV-6	217	9	4.1	8	50	58	27	89	24
cape	LV-7	122	1	0.8	1	24	25	21	100	20
cape	LV-8	265	6	2.3	4	65	69	26	67	25
cape	LV-9	182	13	7.1	7	15	22	12	54	9
cape	LV-10	229	5	2.2	5	120	125	55	100	54
cape	LV-11	253	0	0	-	93	93	37	-	37
cape	LV-12	199	27	13.6	5	15	20	10	19	9
cape	RH-1	323	8	2.5	7	53	60	19	88	17
cape	RD-2	201	24	11.9	20	18	38	19	83	10
cape	RD-3	467	13	2.8	12	118	130	28	92	26
cape	RD-4	244	0	0	-	129	129	53	-	53

cape	RV-6	261	19	7.3	16	20	36	14	84	8
Pop	Colony Code	# cells opened	# inf cells (Varroa mothers)	% cells infested	# cells infested recapped	# cells non-infested recapped	# cells recapped	% cells recapped	% infested cells recapped	% cells non-infested recapped
DRONES										
scuts	2019/4	103	59 (119)	57	2	0	2	2	3	0
cape	RD-2	97	9	9	0	4	4	4		5
cape	RH-2	89	9	10	-	-	0	-		
cape	RH-3	103	7 (8)	7	1	0	1	0	14	0
cape	RV-6	134	38 (49)	28	0	1	1	1	0	1
cape	RO-1	90	48 (70)	53	18	8	26	29	38	19
cape	RG-1	26	24 (62)	92	-	-	0	-		
cape	RG-2	100	67 (177)	67	7	0	7	7	10	0
cape	RG-3	106	33 (38)	31	-	-	0	0	0	0
cape	RG-4	112	48 (89)	43	0	1	1	1	0	2
cape	PA-1	111	12 (12)	11	8	75	83	75	67	76
cape	PA-2	114	19 (24)	17	9	45	54	47	47	47
cape	PA-3	101	16 (22)	16	14	60	74	73	88	71

Table S2. Mite reproduction data across the three mite-resistant honey bee populations. Includes the reproductive fate (%) of *V. destructor* along with the percentage of various fates that determine the overall production of viable (fertilized) females produced per invading mother mite. Only mites from worker cells sealed for 190hrs or longer were used. * is the percentage of mated female offspring taking into account any future mortality in brood younger than grey pads, see methods for more details.

Varroa mite category	AHB			*A. m. scutellata*			*A. m. capensis*			Total	
	Total cells (%)	Cap	Recap	Total cells (%)	Cap	Recap	Total cells (%)	Cap	Recap	Cap	Recap
Foundress dead	9 (6)	3	6	5 (3)	2	3	9 (5)	2	7	7	16
Fertilized female offspring	79 (55)	8	71	100 (55)	37	63	96 (56)	28	68	73 (52)	202 (57)
Unfertilized females due to male death	20 (14)	5	15	26 (13)	10	16	29 (17)	6	23	21	54
Unfertilized female due to missing males	4 (3)	1	3	10 (6)	2	8	7 (4)	2	5	5	16
Only male produced	14 (10)	6	8	30 (16)	13	17	20 (12)	4	16	23	41
No offspring produced	17 (12)	7	10	12 (7)	2	10	10 (6)	2	8	11	28
Total cells studied	143			183			171			140	357
Mated female	0.8 0.8*			0.9 0.8*			0.9 0.8*			0.76*	0.82*

Table S3. Brood removal data for mite-resistant *A. m. capensis* and AHB colonies. Includes the 11 *A. m. capensis* colonies into which mites were artificially inserted into cells, along with the recapping data for remaining unremoved infested pupae. Also, the amount of dead freeze-killed brood removed in both the 11 *A. m. capensis* and ten AHB colonies is given, along with basic recapping data for the ten AHB colonies. All key results are given in bold.

Subspp./Test	Colony code											
A. m. capensis												
MITE INSERTIONS	LV-7	LV-4	LV-11	LV-1	LV-2	LV-5	LV-6	LV-3	LV-9	LV-8	LV-12	Totals(avg.)
(a) # mites initial inserted	32	61	33	31	29	38	30	38	39	30	31	392
(b) # mites escaped	2	5	3	1	0	2	2	0	2	3	1	21
(c) # mite's dead in wall	0	3	0	4	0	8	2	5	4	2	2	30
(d) # pupae/mites removed after 24h	1	6	4	0	1	0	0	0	0	0	1	13
e=a-(b+c+d) **actual start number**	**29**	**47**	**26**	**26**	**28**	**28**	**26**	**33**	**33**	**25**	**27**	**328**
# mites removed after 6 days	10	17	8	7	6	16	20	6	3	ns	2	95

# mites removed after 10 days	13	30	22	9	12	25	21	15	9	16	4	176
% mites removed after 6 days	35	36	31	27	21	57	77	18	9	ns	7	(32)
% mites removed after 10 days	**45**	**64**	**85**	**35**	**43**	**89**	**81**	**46**	**27**	**64**	**15**	**(54)**
# infested cells remaining	16	17	4	17	16	3	5	18	24	9	23	152
# infested cells undisturbed	1	1	0	7	2	2	0	8	1	0	4	26
# infested cells recapped	15	16	4	10	14	1	5	10	23	9	19	128
% infested cells recapped	**94**	**94**	**100**	**59**	**88**	**33**	**100**	**56**	**96**	**100**	**82**	**(83)**
# of control cells opened	122	259	253	246	193	324	217	274	182	265	199	2534
% non-infested control cells recapped	**20**	**65**	**37**	**21**	**45**	**16**	**24**	**27**	**9**	**25**	**9**	**(27)**
FREEZE-KILL TEST												
# start cells	75	72	71	75	75	69	74	74	73	66	75	799
# cells remaining 24 h	5	14	11	8	30	2	1	28	38	0	30	167
# cells remaining 48 h	0	0	0	0	0	0	0	11	2	0	15	28

% removed after 24 h	**93**	**81**	**85**	**89**	**60**	**97**	**99**	**62**	**48**	**100**	**60**	**(79)**
AHB												
FREEZE-KILL TEST	HB1	HB2	HB12	HB11	HB5	HB7	HA5	HB6	HB9	HB10		
# start cells	205	325	203	362	234	156	229	163	153	146		2176
# cells remaining 24 h	104	45	5	38	44	56	186	29	31	42		580
# cells remaining 48 h	31	0	0	0	38	48	128	21	29	19		314
% removed after 24 h	**49**	**86**	**98**	**90**	**81**	**64**	**19**	**82**	**80**	**71**		**(72)**
Recapping rate of colony												Totals
# cells inspected	99	57	92	65	94	91	87	82	57	61		785
# cells recapped	9	30	4	46	12	13	22	24	56	46		262
% cells recapped	9	53	4	71	13	14	25	29	98	75		33

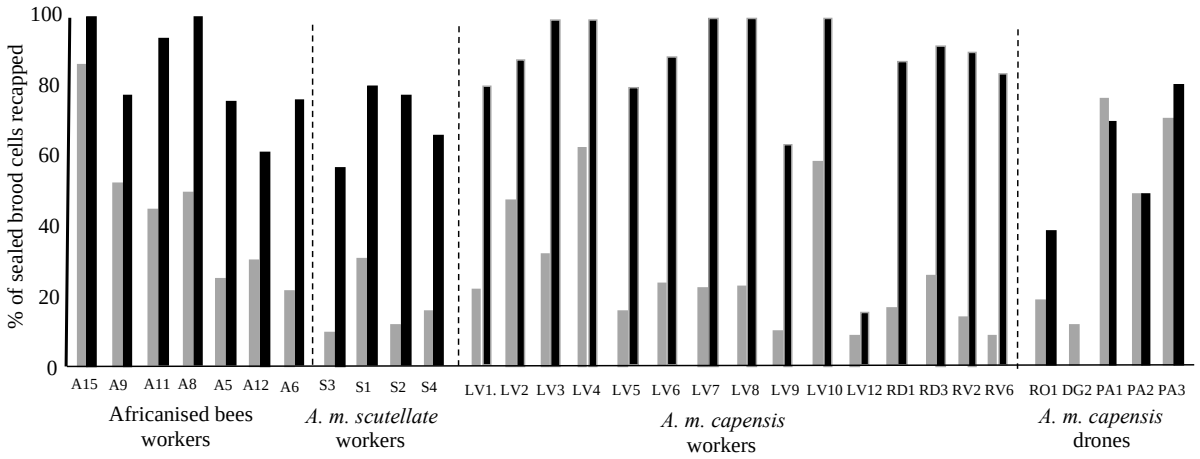

Figure S1. The proportions of non-infested (grey) and mite-infested (black) worker brood cells recapped in colonies of AHB, *A. m. scutellata* and *A. m. capensis*. Only AHB colonies with infestation rates greater than 2% are shown. Also *A. m capensis* drone brood with more than 2% of the cells recapped are shown. See Table S1 for the raw data.

CHAPTER 3

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

Abstract

The ectoparasitic mite *Varroa destructor* remains a major threat to *Apis mellifera* honey bees yet many populations across the world are evolving long-term mite-resistance. Here we investigated the roles of recapping, mite reproduction and infested brood removal in mite-resistance in the UK. Recapping frequency was higher in resistant colonies and targeted mite-infested cells, in which the recapped diameters were larger. Mite reproduction was lower in resistant colonies due to increased offspring mortality, although recapping is unlikely the primary mechanism responsible. Infested brood removal was immediately present in naïve colonies and recapping increased rapidly following initial mite exposure. Recapping and brood removal thus appear to be innate immune responses to *V. destructor* as well as other parasites, and reduced mite reproduction is a key resistance mechanism in the UK, as also found in Europe, S. Africa, Brazil and Mexico.

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 & Brettell, 2019). This global pandemic has now become almost ubiquitous, with Australia now the only *A. mellifera* inhabited continent to be spared the invasion of *V. destructor* (Roberts et al., 2017).

The vast majority of managed *A. mellfiera* 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). Precisely how these 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 resistant colonies is an impairment of the mites' ability to reproduce, thus controlling rates of mite population growth (Rosenkranz et al., 2010; Locke, 2016; Brettell & Martin, 2017). The first reports of widespread mite-resistance were in 'Africanized' bees, and studies in Mexico (Martin & 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 populations (Nganso et al., 2018; Martin et al., 2019). In addition, the same phenomenon has been observed in allopatric mite-resistant colonies of European origin (Locke & Fries, 2011; Locke et al., 2012; Oddie et al., 2017; Brettell & 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 & 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 & 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 resistant populations. Oddie et al. (2018a) compared four resistant honey bee populations with local susceptible (those receiving treatment) populations across mainland Europe. They found that all four resistant populations showed an increased frequency of ‘recapping’ behaviour relative to the four susceptible 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 resealed without removing the brood (Boecking & 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 non-laying foundresses.

The aim of this study was to investigate the roles of these traits in resistance to *V. destructor* among the UK honey bee population. We compared resistant and susceptible 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 resistant, susceptible and naïve colonies.

Methods

Recapping and mite reproduction source colonies

Worker brood combs were collected from volunteer beekeepers across North West England, North Wales, the Midlands (England) and Southern England, from July-September 2017-2019. 'Resistant' colonies were classified as those that have been surviving *V. destructor* infestation without treatment regimens for over 10 years (from Gwynedd, Swindon, Pershore and Bruton) or at least 5 years (from Reading, Salford and Wigan). In contrast, the 'susceptible' colonies are those that receive at least annual mite treatment regimens (from Manchester, Anglesey, Sutton Coldfield and Warwick). A total of 42 colonies (26 resistant and 16 susceptible) were used to assess recapping rates; of these, 36 colonies provided sufficient mites to assess *V. destructor* reproduction (the unsuitable colonies were all resistant, with either very low infestation levels or too early stage brood). In addition, four mite-naïve colonies sourced for artificially infested brood removal experiments (see below) were also assessed for recapping prior to mite introduction, and one month later following mite introduction. A detailed breakdown of all colonies sampled is given in Table S1. All brood samples were freeze-killed within a few hours of collection and stored at minus 20°C prior to examination.

Assessing recapping and mite reproduction

Brood combs were examined using a x16 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 <1mm in diameter to the entire cap (approximately 5mm), 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 (190hrs post-capping) and older were considered in this measurement.

Mite detection and subsequent brood removal experiments

Controlled brood removal experiments were conducted in September 2019 at the Salford University research apiary and at a single apiary in Sutton Coldfield, England. Four naïve colonies were sourced from the Isle of Man and established at the Salford University apiary in June 2019, and three resistant colonies were sourced from Gwynedd, North Wales and established at the same apiary in August 2019. Three susceptible colonies that 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 (resistant 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 (this was

due to limited availability of suitable brood for insertions). Live adult foundresses were harvested from highly infested brood combs from a susceptible apiary prior to administering the colonies' mite treatment; mites infesting larval stage drone brood were preferred as they were at the correct reproductive phase and highly infested. 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 (<24hrs post-capping) that were selected for introductions or controls. Under a x16 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 24hrs 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 (resistant n=3, susceptible n=3, naïve n=3) by freeze-killing sections of worker brood and measuring removal rates after 24 and 48hrs. Individual colony data for all trials are given in Table S2.

Statistical analyses

All statistical analyses were conducted in RStudio (version 1.2.5019). Three Generalised Linear Mixed Models (GLMM) 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 (resistant, 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 non-laying foundresses, offspring mortality, and larger recapped diameters (>2.5mm). 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.

Results

Recapping

A total of 14,802 worker brood cells were examined from 42 colonies, of which 1639 contained mites and 4293 were recapped. Brood infestation levels ranged from 0.3-22.7% in resistant colonies and 2.9-58.2% in susceptible. Proportions of total examined cells recapped, and infested cells recapped, were both highly variable across both resistant and susceptible colonies (individual colony data is given in Table S1). Recapping probability was significantly higher in resistant colonies (χ^2=11.543, p<0.001) and infested cells (χ^2=322.25, p<0.001), and varied significantly between sampling region (χ^2=32.76, p<0.001) and brood developmental stages (χ^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%) one month following mite introduction (Table S1). In resistant colonies, the frequency distributions of the recapped diameters (<1mm-5mm) were significantly different between infested and non-infested cells (D=1, p=0.007; larger diameters more common in infested), however a similar pattern was not seen in susceptible colonies (D=0.8, p=0.079) (Figure S1).

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

Response Variable	Explanatory Variable	n (colonies)	DF	χ^2	P-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

Significance codes = $*$ p<0.05, $**$ p<0.01, $***$ p<0.001.

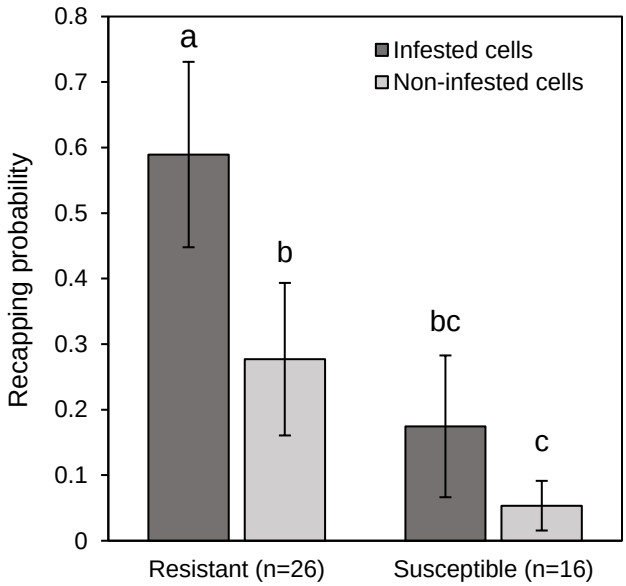

Figure 1. Adjusted mean proportions (+/- SE) of recapped worker brood cells in mite-resistant and mite-susceptible colonies from around the UK.
Recapping probability was significantly higher in resistant colonies (GLMM: χ^2=11.543, p<0.001) and in mite-infested cells (GLMM: χ^2=322.25, p<0.001). Groups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

Mite reproduction

Of the 1639 mite-infested worker brood cells, 1068 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 Table S1). Probability of successful mite reproduction was significantly lower in resistant colonies (χ^2=10.301, p=0.001) due to offspring mortality (GLMM: χ^2=8.2562, p=0.004) rather than non-laying foundresses (GLMM: χ^2=0.3255, p=0.6); however, there was no difference in reproductive success between recapped and undisturbed cells (χ^2=0.0796, p=0.778) (Table 1, Figure 2a), including when only considering the larger recapped diameters (>2.5mm) (χ^2=1.5067, p=0.219) and offspring mortality (GLMM: χ^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 resistant (rho=0.26, p=0.3) and susceptible (rho=-0.02, p=0.9) colonies (Figure 2b). Again, these correlations remained insignificant when considering only the larger recapped cells (resistant: rho=0.29, p=0.3; susceptible: rho=0.03, p=0.9). Probability of successful mite reproduction also varied between brood developmental stages (χ^2=8.5947, p=0.003) and marginally between sampling years (χ^2=6.0582, p=0.014) (Table 1).

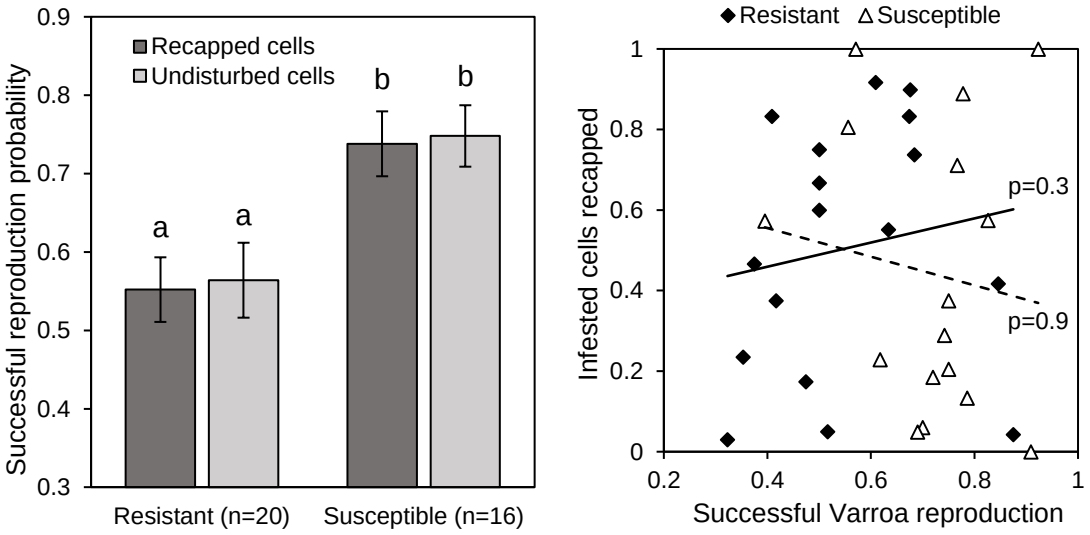

Figure 2. (a) Adjusted mean proportions (+/- SE) of infested brood cells containing successful mite reproduction; (b) scatter graph depicting proportions of successful mite reproduction and infested cells recapped per colony. (a) Successful mite reproduction probability was significantly lower in resistant colonies (GLMM: χ^2=10.301, p=0.001) yet there was no difference between recapped and undisturbed cells (GLMM: χ^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 resistant (rho=0.26, p=0.3) or susceptible (rho=-0.02, p=0.9) colonies.

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 (χ^2=36.009, p<0.001) whereas no overall difference was found between resistant (n=3), susceptible (n=3) or naïve (n=4) colonies (χ^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 resistant colonies did not differ from either group (Figure 3). The freeze-killed hygienic tests (resistant n=3; susceptible n=3; naïve n=3) generally resulted in low rates of dead brood removal, ranging from 3.9-35.9% after 24hrs, and 4.7-46.6% after 48hrs except for one naïve colony that removed 88%. Individual colony data for all trials are given in Table S2.

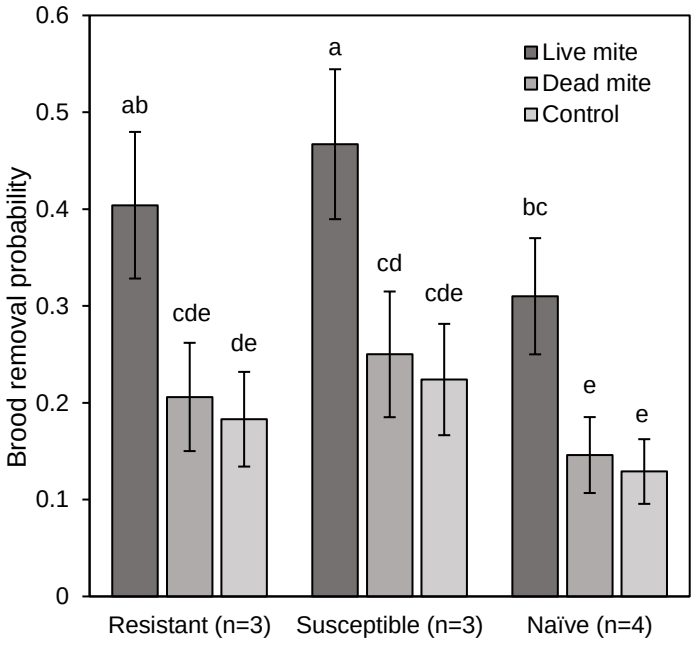

Figure 3. Adjusted mean proportions (+/- SE) of worker brood removal from artificial mite introduction experiments. Brood removal probability was significantly higher in the live mite tests (GLMM: χ^2=36.009, p<0.001) whereas there was no overall difference between resistant, susceptible and naïve colonies (GLMM: χ^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.

Discussion

Our data has shown that in UK honey bees both recapping behaviour and reduced *V. destructor* reproductive success are traits involved in long-term mite-resistance. Recapping was strongly targeted toward mite-infested brood cells and the frequency was higher in resistant populations (Figure 1), while mite reproductive success was lower in resistant populations (Figure 2a) due to increased offspring mortality/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, and instead could be a trait involved in the detection and removal of infested brood (Boecking & 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, followed by resistant and finally naïve colonies, although our sample sizes are small and this trait is known to be highly variable even within a resistant 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 adult bees are more likely to detect infested brood cells that have successful mite reproduction is controversial (Nazzi & Le Conte, 2016; Leclercq et al., 2017; 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 resistant populations, as opposed to non-laying 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 resistant colonies. Overall, 34% of undisturbed infested cells in this study failed to reproduce successfully, while 36% failed in recapped cells; conversely, 42% failed in resistant and 28% failed in susceptible. 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 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% 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 & Spivak, 1999; Martin et al., 2019). When brood removal is executed successfully it disrupts the surviving foundresses' reproductive cycles, increasing the instances of non-laying mites circulating in the population (Boecking & Spivak, 1999; Kirrane et al., 2011; Martin et al., 2019). This mechanism is a candidate in resistant populations whereby non-laying, or laying male only, foundresses account for much of the failed mite reproduction (Martin & Kryger, 2002; Locke et al., 2012); however, it is unlikely to explain the stark difference in offspring mortality observed in this study and other resistant populations (Medina et al., 2002; Ibrahim & Spivak, 2006; Locke & Fries, 2011). 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 & 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 resistant, susceptible and naïve colonies by using artificial mite introductions. 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 resistant and naïve bees. Nevertheless, brood removal across all groups was significantly increased in

response to live mite introductions. 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. 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 resistant 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 pre-adapted to detect and remove mite-infested cells, as their live mite removals were well within the ranges of both resistant and susceptible populations in this study and previously (Boecking & Ritter, 1993; Aumeier et al., 2000; Boecking et al., 2000; Panziera et al., 2017; Cheruiyot et al., 2018), and were significantly higher than their controls (Figure 3). 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). Given the high variability of brood removal behaviour in general, our relatively small data set and potential variability from the time factor (as the trials were conducted separately due to limited suitable brood per colony), more work is needed to draw firm conclusions on the role of this trait in mite-resistance in the UK.

Recapping and brood removal are both traits that are closely associated to *V. destructor* and, according to the small amount of data collected on the naïve colonies, appear to be innate social immune responses to mite infestation. Although mite-targeted recapping is a feature consistently appearing at high levels in resistant 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 & 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. Another important consideration that could explain the difference between the expression of these traits is the effects of long-term mite control measures; when mite populations are repeatedly decimated by acaricides, this may act as a force of artificial selection that reduces the frequency of these natural immune responses in treated colonies, that may increase once more when treatment is ceased. This could explain why recapping was not significantly targeted to infested cells in the susceptible colonies (Figure 1). Additionally, if other mechanisms lead to reduced mite reproduction and ultimately resistance through natural mite population control, the behaviour may also be selected out or remain highly variable in many resistant colonies (for example, the resistant colonies from Gwynedd). 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 & Hudson, 2016) and beyond (Medina et al., 2002; Locke & Fries, 2011; Brettell & Martin, 2017). 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. mellfiera* 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.

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Supplementary Information

Table S1. Individual colony data from the UK-wide survey of recapping and *V. destructor* reproductive success.

Status	Region	Location	Apiary	Colony	Month	Year	Cells	Infested (%)	Recapped (%)			Varroa Reproduction	
									Total	Infested	Non-inf.	Cells	Success (%)
Resistant	N. West	Salford	Sal.Uni	Sal.Uni.1	July	2018	1127	10.5	28	46.6	25.9	24	37.5
		Wigan	Michele	Mich.1	Aug.	2019	574	0.3	52.1	50	52.1	1	100
	N. Wales	Gwynedd	Shan	Tree17	Aug.	2017	241	6.2	7.1	46.7	4.4		N/A
				Tree18.1	July	2018	244	1.6	2	50	1.3		N/A
				Tree18.2	July	2018	278	1.1	2.2	0	2.2		N/A
				Tree18.3	July	2018	265	0.4	1.5	100	1.1		N/A
				Fedw.2	Sept.	2018	200	21.5	4	4.7	3.8	2	100
				Fedw.3	Sept.	2018	203	22.7	3	4.3	2.5	8	87.5
				NW1	Sept.	2019	300	11.0	1	3	0.7	31	32.3
				NW2	Aug.	2019	302	5.6	14.2	23.5	13.7	17	35.3
				NW3	Sept.	2019	305	7.9	19.7	37.5	18.1	24	41.7
			D.Heaf	D.Heaf.1	Aug.	2019	314	12.7	4.5	5	4.4	31	51.6
				D.Heaf.2	Aug.	2019	269	11.2	33.1	83.3	26.8	22	40.9
				D.Heaf.3	Aug.	2019	222	8.6	28.8	73.7	24.6	19	68.4
	Midlands	Pershore	Per.Col	Rho.2	Aug.	2019	302	2.6	37.7	75	36.7	8	50
				Rho.6	Aug.	2019	275	3.6	65.1	60	65.3	8	50
				Rho.65	Aug.	2019	300	1.7	100	100	100	5	60
			Smallh.	Rho.S73	Aug.	2019	304	7.9	82.2	91.7	81.4	23	60.9
	South	Swindon	C.Park	C.Park.1	July	2018	524	18.9	63.7	89.9	57.6	74	67.6
			Ross.F	Ross.F.1	July	2018	524	11.5	26.5	41.7	24.6	13	84.6
			Ross.G	Ross.G.1	July	2018	834	15.1	78.3	83.3	77.4	92	67.4
		Reading	J.White	JW.Black	July	2018	497	1.2	20.9	33.3	20.8		N/A
				JW.Blue	July	2018	501	19.6	18.4	55.1	9.4	82	63.4
				JW.Yellow	July	2018	883	2.7	1.1	8.3	0.9		N/A
		Bruton	Joe.B	Joe.B.1	Aug.	2019	275	8.4	10.5	17.4	9.9	19	47.4
				Joe.B.2	Aug.	2019	153	3.9	24.2	66.7	22.4	6	50
Susceptible	N. West	Manchester	MDBKA	MDBKA.1	Aug.	2019	240	3.3	5.8	37.5	4.7	8	75
				MDBKA.2	Aug.	2019	316	4.1	73.1	100	71.9	13	92.3
				MDBKA.3	Aug.	2019	311	2.9	17	88.9	14.9	9	77.8
	N. Wales	Anglesey	Wally	Wally.2	Aug.	2019	321	5	0.6	0	0.7	11	90.9
				Wally.4	Aug.	2019	300	12.7	11.7	28.9	9.2	31	74.2

				Wally.9	Aug.	2019	242	20.7	3.3	6	2.6	50	70
				Wally.11	Aug.	2019	177	58.2	2.8	4.9	0	100	69
				Wally.12	Aug.	2019	303	8.9	2.3	18.5	0.7	25	72
				Wally.15	Aug.	2019	305	4.9	1.6	13.3	1	14	78.6
	Midlands	Warwick	M.West	M.West.1	Sept.	2018	593	33.7	17	20.5	15.3	88	75
		Sutton.Cold	Road	Bern.1.1	Aug.	2019	327	9.5	52.9	80.6	50	18	55.6
				Bern.1.3	Aug.	2019	201	41.3	57.7	71.1	48.3	60	76.7
				Bern.1.4	Aug.	2019	303	4.6	63.7	100	61.9	14	57.1
			L.Aston	Bern.2.1	Aug.	2019	300	25	33	57.3	24.9	38	39.5
				Bern.2.2	Aug.	2019	300	11.7	14	22.9	12.8	34	61.8
				Bern.2.3	Aug.	2019	115	47	35.7	57.4	16.4	46	82.6
Naïve (preVarroa)	Isle of Man	Salford	Sal.Uni	IoM.1	June	2019	150	0	0	0	0	N/A	
				IoM.2	June	2019	151	0	0.7	0	0	N/A	
				IoM.3	June	2019	150	0	0	0	0	N/A	
				IoM.4	June	2019	155	0	0	0	0	N/A	
Naïve (postVarroa)	Isle of Man	Salford	Sal.Uni	IoM.1	Sept.	2019	150	0	12	0	12	N/A	
				IoM.2	Sept.	2019	151	0.7	0	0	0	N/A	
				IoM.3	Sept.	2019	150	0.7	16	0	16.1	N/A	
				IoM.4	Sept.	2019	154	0.6	10.4	0	10.5	N/A	

Table S2. Individual colony data from the controlled brood removal experiments

Status	Colony	Live mites			Dead mites			Control		Hygienic test (FKB assay)		
		Inserted	Accepted (24hrs)	% removed (10 days)	Inserted	Accepted (24hrs)	% removed (10 days)	Marked	% removed (10 days)	Inserted	% removed (24hrs)	% removed (48hrs)
Resistant	NW1	30	30	20	20	20	10	30	3.3	105	10.5	16.2
	NW2	25	24	70.8	20	20	35	30	20	134	11.2	26.9
	NW3	30	30	33.3	20	20	25	30	36.7	118	8.5	46.6
Susceptible	Bern.2.1	30	30	60	15	15	26.7	20	25	108	12.0	38
	Bern.2.2	30	29	37.9	15	15	6.7	20	10	102	29.4	43.1
	Bern.2.3	30	30	56.7	15	13	30.8	25	24	157	10.8	10.8
Naïve	IoM.1	30	30	33.3	20	20	5	30	10	117	35.9	88
	IoM.2	30	30	36.7	20	18	22.2	30	13.3	127	3.9	4.7
	IoM.3	30	30	6.7	20	20	15	30	20	75	9.3	12
	IoM.4	20	18	38.9	20	20	25	30	16.7		N/A	

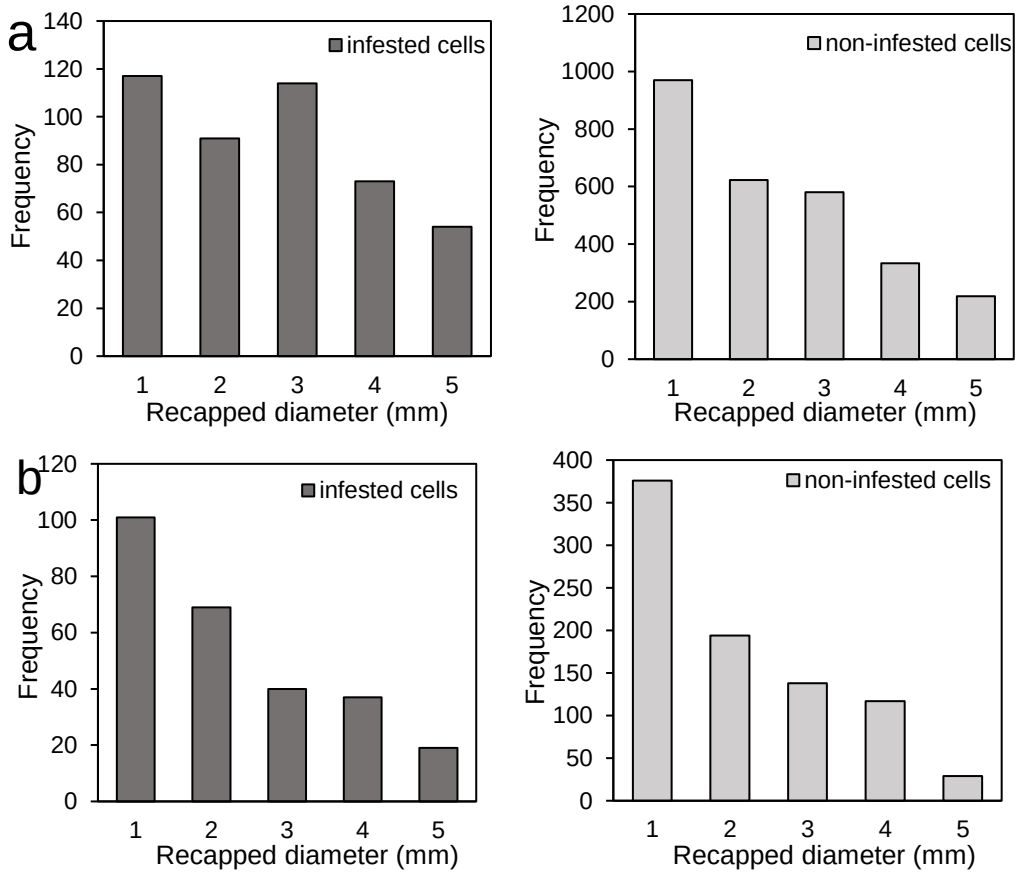

Figure S1. Frequency distributions of the recapped diameters in resistant (a) and susceptible (b) colonies. In resistant colonies, the diameters were significantly larger in infested cells (D=1, p=0.007), whereas no difference was found in susceptible (D=0.8, p=0.079).

CHAPTER 4

General Discussion

We have now found the same phenomenon across Brazil, South Africa (Chapter 2) and the UK (Chapter 3), as well as mainland Europe (Oddie et al., 2018a). That is, *V. destructor* resistant *A. mellifera* populations show an increased frequency of recapped brood cells that are strongly targeted towards mite-infested cells (Figure 1) and the recapped diameters are significantly larger when detecting infested cells. We have also found in Australia, Scotland and the Isle of Man that recapping is virtually absent in mite-naïve colonies, and at least in the Isle of Man colonies, recapping rapidly increases following initial exposure to the mites. We hypothesise that recapped brood cells are evidence of the ability of adult bees to detect cells that are infested with mites, with the intention of the infested brood then being removed from the colony. However, while this trait shows that the adult bees have successfully responded to an initial stimulus that does not require the opening of the cell, a second stimulus that triggers brood removal has subsequently failed and the cell is recapped (Chapter 2). This error could be explained by varying colony composition, with the tasks of initial detection and subsequent brood removal performed by specialised bees (Arathi & Spivak, 2001). Recapping could become more likely if these roles become imbalanced within a colony, i.e. with a higher proportion of initial detectors or recappers as opposed to removers (Arathi & Spivak, 2006). Another possibility is that the health of the brood itself is being investigated, which may not always be compromised by mite-infestation (for example, varying titres or genotypes of DWV), thus not triggering removal (Schoning et al., 2012). Of these possibilities, it currently appears that colony composition is the most likely to have the greatest effect on removal errors, given the strength of evidence in support of this phenomenon (Arathi & Spivak, 2001; 2006). Although recapped infested cells individually may constitute failed instances of brood removal, the presence of the trait may nevertheless show that brood removal is still being performed at the colony level.

We have additionally found that reduced mite reproductive success is a key trait leading to resistance in Brazil, South Africa and the UK (Chapters 2 & 3), supporting previous research from mainland Europe (Oddie et al., 2018a), Latin America (Rosenkranz & Engels, 1994; Martin & Medina, 2004) and Africa (Strauss

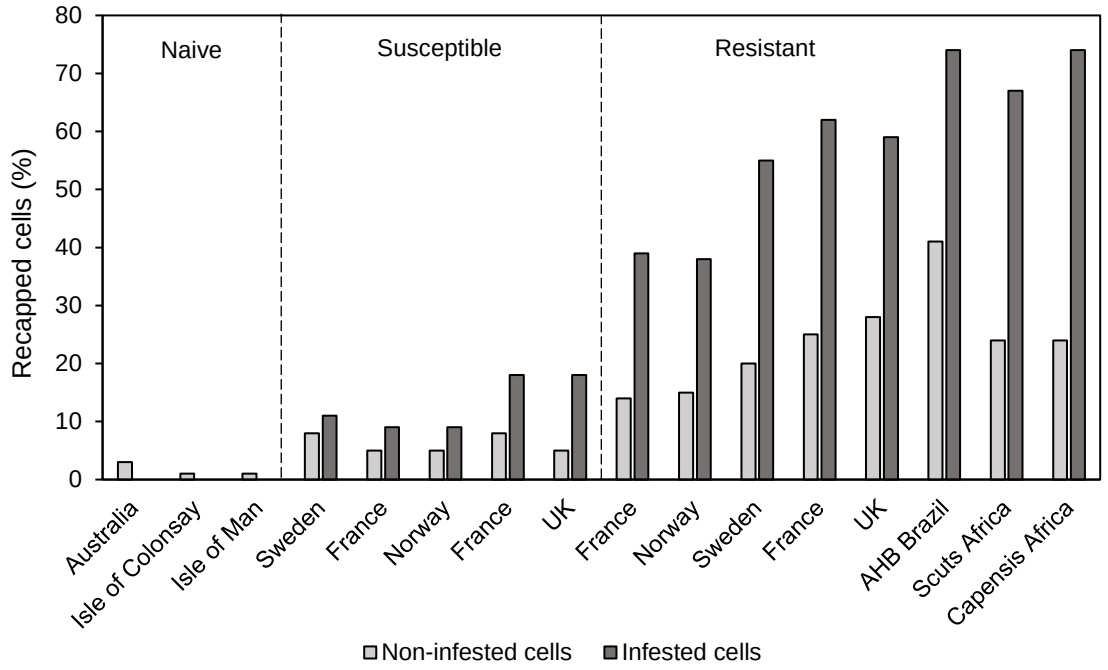

Figure 1. Recapping levels of naïve, susceptible and resistant populations. Data combined from Chapter 2 (Australia, Colonsay, AHB and Africa), Chapter 3 (Isle of Man and UK) and Oddie et al. (2018a) (Sweden, France and Norway).

et al., 2016; Nganso et al., 2018). Although Oddie et al. (2018a) argued that recapping is a previously overlooked trait that directly leads to failed reproduction in the recapped cells, our studies do not support this hypothesis. However, since we hypothesise that recapping is evidence, or a 'proxy', for brood removal behaviour, when this trait is executed successfully it disrupts the foundresses' breeding cycles and can account for an increased proportion of the non-laying mites in the population. Although brood removal is a resistance trait and likely contributes in part to the decrease in mite reproductive success, much of the decrease in mite reproduction in our studies and others (Medina et al., 2002; Ibrahim & Spivak, 2006; Locke & Fries, 2011) are attributed to offspring mortality, particularly males, regardless of whether the cell had been detected or not. It is therefore likely that other mechanism(s) are involved, since currently observed infested brood removal rates cannot entirely account for the ~45% associated drop in reproduction seen in mite-resistant populations. It is well known that AHB

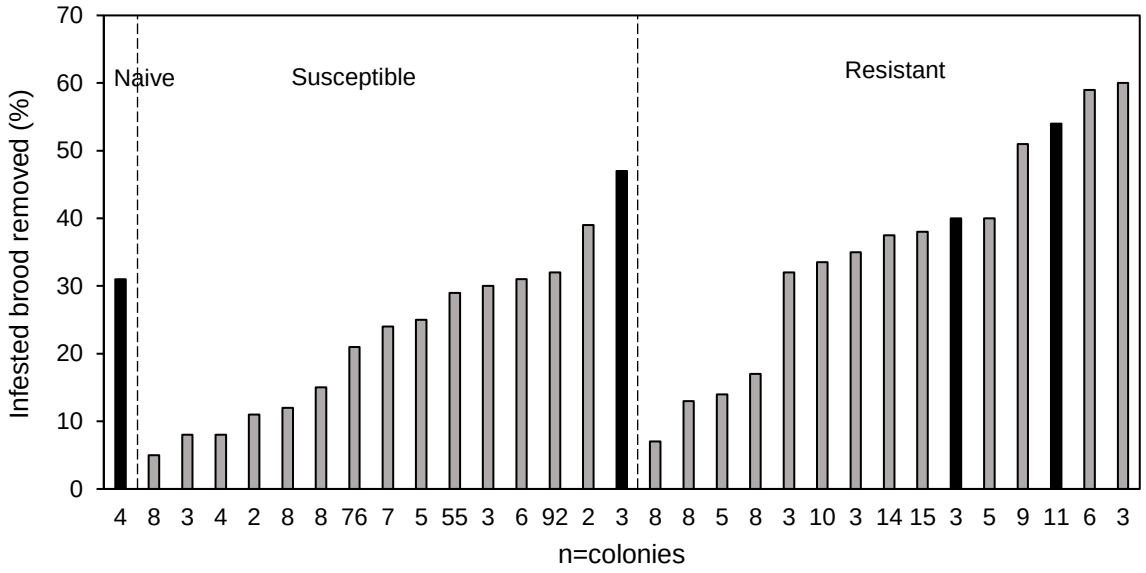

Figure 2. Mite-infested brood removal levels of naïve, susceptible and resistant populations. Brood removal is significantly higher in resistant populations when compared to susceptible (Mann Whitney U: W=175.5, p=0.019). Black bars indicate data from Chapters 2 and 3, grey bars indicate meta-data from: Boecking & Drescher (1992), Boecking & Ritter (1993), Spivak (1996), Aumeier et al. (2000), Boecking et al. (2000), Guerra et al. (2000), Vandame et al. (2002), Panziera et al. (2017), Cheruiyot et al. (2018) and Wagoner et al. (2018). All studies used artificial mite infestations using a single live foundress.

and African subspecies have a reduced post-capping period, ranging from 8-48hrs (Moritz & Hanel, 1984), and this has also recently been suggested in a European population (Oddie et al., 2018b). By directly dissecting the mites' spermathecea, Donze et al. (1996) showed that 48hrs is required for sufficient mating to inseminate the young females, and in a normal breeding cycle this occurs between 230-280hrs post-capping. It may be that within a reduced post-capping duration, the standard methods of reconstructing the mite family may count 'fertilised' females that have not had enough time for sufficient mating. However, Rosenkranz et al. (2010) suggest that a reduced post-capping period would simply select for earlier initial egg-laying, and since the first egg is laid after 60hrs post-capping (Infantidis, 1983), enough time would be available to cover the reduction

seen in AHB and African subspecies. Furthermore, since we argue that the opening of the cell itself does not directly affect the mite reproduction within it, the varying lengths of time they are open for (which may also be affected by a reduced post-capping period) are unlikely to have a significant effect.

We have additionally investigated levels of infested brood removal behaviour in *A. m. capensis* from South Africa, and a small number of resistant, susceptible and naïve colonies from the UK. The resistant *A. m. capensis* displayed the highest overall removals at 54%, followed by untreated susceptible (47%), resistant (40%) and naïve (31%) colonies from the UK. It appears that *A. mellifera* may be pre-adapted to detect and remove *V. destructor* infested cells, as the naïve colonies' removals are comparable to both resistant and susceptible in previous studies (Figure 2). The high levels of brood removal and recapping observed in the untreated susceptible colonies in Chapter 3 suggest that ceasing treatment could lead to an increase in the behaviours. However, these colonies were harbouring heavy mite infestations and many individuals displayed wing deformity, therefore in these instances the behaviours have not likely saved them from collapse, possibility due an overwhelming income of mites which the removal behaviour of the colony could not keep pace with. Our data therefore suggests that recapping and brood removal are innate immune responses to *V. destructor*, and since brood removal is also performed at a significantly lower level in treated colonies ($W=175.5$, $p=0.019$, Figure 2), these behaviours may be selected out of treated colonies via the use of artificial mite control measures. This then raises an important issue: if *A. mellifera* is pre-adapted to detect and remove mite infested cells, yet the majority of colonies will collapse without treatment, the behaviour may not be the most important host trait leading to resistance. This could also explain the mixed survivorship of selected lines in field trials that have been specifically bred for this trait (Spivak & Reuter, 2001; Rinderer et al., 2014; Danka et al., 2016). Since colony composition likely plays an important role in the expression of these behaviours, a rapid increase in the mite population may outpace the colonies' response to produce more specialised workers. However, it is important to note that the trait is highly variable across all colony types and our sample size is small; therefore, more work is needed on brood removal behaviour in the UK and elsewhere, in resistant, treated susceptible, untreated susceptible and naïve colonies, to draw a firm conclusion on its role in mite-resistance.

Our studies have provided additional insight into the mechanisms underlying naturally evolved *V. destructor* resistance in *A. mellifera* populations from Europe, Latin America and Africa. Recapping is a promising trait that could be used as a proxy for both mite-resistance and evidence of brood removal behaviour, although the trait is still highly variable across all colony types, so understanding what causes this variability is an important question for future research. Identifying the detection stimuli triggering infested cell detection and brood removal could help explain the variability in recapping, improve screening methods for individual colony levels of brood removal, and improve the assays used for artificial selection of the behaviour. In addition, mite offspring mortality is a key mechanism underlying long-term colony survival and is most likely a trait independent of brood removal behaviour, therefore understanding the mechanisms behind this will no doubt provide additional value. It is becoming increasingly likely that a range of traits and conditions are required to ultimately lead to a stable host-parasite relationship between *A. mellifera* and *V. destructor* (Rosenkranz et al., 2010; Locke, 2016) and developing our understanding of these remains important for promoting long-term, treatment-free survival of *A. mellifera* populations around the world.

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APPENDIX 1: BBKA News article 2019

Changing the Guard at Salford Uni Honey Bee Research Team

By Prof Stephen Martin, Salford University

Steve Martin gives us the low-down on the current research team and projects at Salford University.

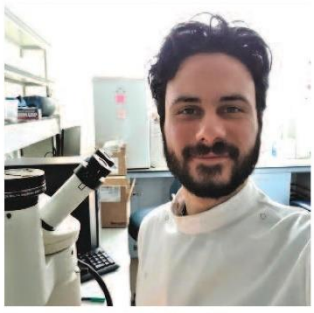
I am currently travelling across China at 300km/h on a new bullet train to give an invited talk at Zhejiang University; £16 for business class for the 1.5 hour ride of over 350km! Anyway, it is with great pleasure that I can announce that the BDI and beekeeper funded PhD student, Jess Kevill, is now Dr Kevill having passed her PhD in early October and she has already taken up a post-doctoral research position at the University of Minnesota working in Declan Schroeder's virology department. This university is also home to the top USA female bee scientist, Marla Spivak, with her very large and productive honey bee research team. This will allow Jess to have a foot in both the viral and honey bee camps allowing her to continue her DWV honey bee research.

We would like to take the opportunity to thank all the associations involved in the ReViVe (Rolling out the evolution of resistance to varroa and DWV) project for their time and patience during the past three-years. During that time Jess and colleagues developed a new molecular method by which to detect the three known strains if DWV. This new method was then used to analyse a large number of colonies from across the UK and USA, sampling both varroa-tolerant and susceptible colonies. The data obtained from these colonies provided an insight into continuing emergence of DWV and raises the question how this changing viral landscape will impact colony survival. We are currently writing up two major scientific papers that will cover all the key discovers of Jess's three years of hard work and once these are published, I will communicate the key findings to the beekeeping community via *BBKA News* and various talks.

Brazilian PhD sandwich student, Flaviane S. de Souza, enjoying the novelty of snow in Manchester.

The success of Jess's project has helped to secure another three-year PhD student, this time funded by BDI Ltd with some internal funds from the University of Salford. George Hawkins was our number one undergraduate from our department in 2018 and I look forward to working with him alongside two MSc students funded by the BBKA. The first student, Natasha Reece, was awarded a Santander travel fund so we both travelled to South Africa and worked on the African savannah honey bee (*Apis scutellata*) and the cape honey bee (*A. capeinis*) both species being tolerant to the varroa mite. In addition, Natasha started work looking for parallels between the African and UK honey bees that help explain long-term mite tolerance in some UK honey bee populations. A scientific paper is already under review showing a behavioural link between varroa-tolerant honey bees in South Africa, Brazil and Europe. Again, once published we will pass on the key findings to beekeepers via *BBKA News*. The last news is that Flaviane de Souza, a Brazilian PhD sandwich student who has been working in the team for a year, will return to the Brazil at the end of the year. Flaviane is a wizard in the molecular laboratory and so has been an immense help to both Jess and me completing various DWV analyses and projects. Again her work has generated a scientific paper on the presence of DWV in Brazilian stingless bees. Flaviane will be greatly missed by everyone, and we all wish her well for the future. I will now hand over to George to introduce himself to the beekeeping community:

My name is George Hawkins and I am a new PhD student working under the supervision of Professor Stephen Martin at the University of Salford. Bee Diseases Insurance (BDI) Ltd has kindly provided funding, in partnership with Salford University, to continue investigating the mechanisms behind naturally evolved honey bee tolerance to varroa mites. We hope to apply the information we gather to develop practical methods for beekeepers to use which will promote and spread varroa tolerance in their colonies, thus reducing the need for chemical treatments and improving overall colony health.



George Hawkins, begins his PhD.

My first taste of science and wildlife research came in 2014 when I spent time volunteering in South Africa and Costa Rica, which inspired me to head back into education and begin a degree studying Wildlife Conservation with Zoo Biology, also at the University of Salford. My involvement in honey bee research began in my final year when I took on a dissertation project with Professor Martin studying deformed wing virus (DWV), a pathogen now considered as one of the greatest threats to honey bee health worldwide. Given that this is such an important and interesting area of research, when I was offered the opportunity to continue the research as a PhD student I was eager to accept.

As I am sure many readers know all too well, varroa mites have become a highly prevalent and damaging pest since spreading from their natural host, the Asian honey bee. The greatest threat comes not from the mites alone, but rather their efficient transmission of viruses such as DWV, which have been strongly and consistently associated with colony collapses across the Northern hemisphere. Although the majority of managed honey bee colonies today only survive varroa infestation through the use of chemical treatments, a number of colonies around the world have become tolerant and survive year on year without needing treatment. Previously we looked at whether the differing strains of DWV might be responsible for this, and an increasingly complex picture is emerging. A key trait within these tolerant colonies is a reduction in the mites' ability to reproduce, which limits the mite population to a safe level. However, the mechanisms responsible for this are not well understood. Defence behaviours such as varroa sensitive hygiene, where infected brood are discarded from the colony, and grooming, where the bees remove the parasites from each other, may contribute towards it but they cannot explain it entirely.

Recent studies have provided new insights which we will pursue. They have found a strong association linking varroa tolerance with 'recapping' behaviour, where workers open and reseal brood cell caps without removing the brood. Our initial aim is therefore to confirm that this phenomenon is also present in varroa tolerant honey bees in the UK. If this is the case, we will then attempt to discover precisely how this behaviour disrupts the mites' reproduction, and what implications it presents to the biology of the virus. Finally, and crucially, we will then develop methods for beekeepers to assess their recapping levels and mite reproduction. This information can then be used to guide further action, such as reducing treatments or selective breeding, with the long-term aim of eradicating the need for such treatments to combat varroa.

I am immensely grateful to BDI Ltd, Professor Martin and the University of Salford for this exciting opportunity, and I will make every effort to produce the highest-quality outcome worthy of the time and funding invested into it. Honey bees are a fascinating and important species both for humankind and the natural world, and in conjunction with the beekeeping community I hope to contribute towards ensuring a healthy and lasting future for them. I will be attending the BBKA Spring Convention and am looking forward to working with the British beekeeping community.

What is the GB Non-native Species Secretariat?

By Lucy Cornwell, GB Non-Native Species Secretariat

The GB Non-native Species Secretariat (NNSS) was established in 2006 to help coordinate work on non-native species across Government in GB, and it reports to a coordinating GB Board chaired by Defra.
We are a small team of four and our work is extremely varied, but covers three main areas:

- Policy and Ministerial support. The NNSS works very closely with Defra, the Devolved Administrations and their agencies.
- Risk analysis. We have developed a mechanism to produce and validate risk-assessments for invasive non-native species which is unique in the EU. We are also developing risk management procedures to aid prioritisation of action on invasive species.
- Communications. The Secretariat is a hub for communication between Government, interested stakeholders and the general public. As part of this we maintain the NNSS website and run annual forums for local and national stakeholders. We run an annual Invasive Species Week and two ongoing awareness-raising campaigns on biosecurity. These are:
  - Be Plant Wise preventing the spread of non-native garden plants.
  - Check Clean Dry preventing the spread of non-native freshwater species by anglers and recreational water users.

Why do we do it?

There are nearly 2,000 non-native species established in GB with another ten to twelve new ones becoming established every year. Luckily most are harmless, but about 10% to 15% become invasive causing harmful impacts on the environment, economy or our way of life. They cost the British economy over £1.7 billion a year, are one of the key drivers of global biodiversity loss and some can even pose a risk to human health. Our work helps to reduce the future impact of invasive non-native species by preventing more species being introduced and becoming established, and by better managing existing problem species.

Asian hornet

We are closely involved with the Asian hornet response, and support the alert system led by the Centre for Ecology and Hydrology by providing a backup on incoming reports. We notify our stakeholders of new outbreaks and provide posters and ID sheets to support awareness raising. Suspected Asian hornet sightings can be reported online at https://www.brc.ac.uk/irecord/, on the Hornet Watch app for smartphones, or by sending a photo to the alert email address: alertnonnative@ceh.ac.uk. Contact nnss@apha.gov.uk for free posters and ID sheets.

Invasive Species Week 2019

Last year over 300 organisations worked together to raise awareness of invasive non-native species during Invasive Species Week. This year Invasive Species Week will run from 13-19 May. You can get involved by visiting www.nonnativespecies.org/invasivespeciesweek or looking up #invasivesweek on social media. Contact nnss@apha.gov.uk to be added to our mailing list for updates.
Find out more about invasive non-native species and how you can help to stop the spread at www.nonnativespecies.org.

APPENDIX 2: BBKA News and The Welsh Beekeeper article 2020

Investigating naturally evolved *Varroa* resistance in the UK and beyond: BDI research at Salford University.

George P. Hawkins

Professor Stephen Martin and I from the University of Salford, and in partnership with Bee Diseases Insurance Ltd (BDI), have travelled across the UK and South Africa to study the curious cases of 'mite-resistant' honeybee colonies that are surviving long-term *Varroa* infestations without any need for treatment. It's becoming more of a debate in the beekeeping world as to whether *Varroa* treatments are still the best way to go, with more beekeepers around the UK revealing that they do not need to use them. Many researchers from the scientific community also argue that *Varroa* control measures prevent the bees from developing their own adaptations to deal with the mites themselves. This begs the important questions- why do some colonies survive without treatment while the vast majority perish within a few years? Precisely how do they survive- is there one mechanism or many? Is it a change in the bees, the mites, their environment, or a combination of these? Furthermore, what will these answers mean for the future of apiculture?

Mite resistance and Varroa reproduction

The first reports of widespread *Varroa* resistance in Western honeybees came in 1956 from 'Africanized' bees in Brazil, a selectively bred hybrid between European and African subspecies. Later in 1997, *Varroa* arrived in South Africa yet the local Savannah and Cape honeybees rapidly evolved mite-resistance within a decade. In addition, an increasing number of European-origin honeybee populations are independently evolving mite-resistance, with reports from mainland Europe, the US, North Africa and the small Brazilian Island of Fernando de Norohna. Decades of research has been employed to figure out precisely how this has occurred, and although the answer is still largely unclear, there is a phenomenon that has been consistently found in resistant colonies- the mites are often unable to reproduce properly. *Varroa* mites reproduce within sealed brood cells while feeding on the developing pupae. The mother mites, or 'foundresses', enter both worker and drone brood cells and lay a series of offspring, many of which reach adulthood

before the adult bee emerges. The single male offspring, which is always laid first, will complete his only task in life by mating with up to three (in worker cells) of his adult sisters before dying in the cell. These fertilised female offspring and their foundress will then leave the cell with the adult bee to start the cycle again. This process however is often less than successful. Many foundresses do not lay any eggs at all, while others start laying too late for their offspring to reach adulthood. In many other cases the male offspring dies too soon to fertilise his sisters or is missing entirely, and the female offspring can also die early before emergence. What we do know is that in resistant colonies there are many more of these instances of poor mite reproduction, which prevent the mite populations from increasing out of control. What we don't know however, is what causes them.

Is recapping behaviour the answer?

A number of traits have been investigated over the years in an attempt to explain this, although a recent study (Oddie et al., 2018) from a team of researchers in mainland Europe had potentially identified a new one. They compared four populations of resistant colonies with local susceptible (ordinary colonies that receive at least annual mite treatments) across France, Sweden and Norway. As well as finding reduced mite reproduction in all the resistant populations, they also found higher frequencies of 'recapping' behaviour. Recapping is where adult bees open sealed brood cell caps and then reseal them without removing the brood, and in the resistant colonies this behaviour was strongly targeted towards brood cells containing mites. Recapping has historically been associated with the detection process involved in infested brood removal behaviour (a form of hygienic behaviour), where adult bees detect cells containing mites and remove the brood from the colony altogether. However, the team then conducted an experiment which implied for the first time that the act of opening and closing the cell cap (recapping) was sufficient to impair the reproduction of the mites inside, and that recapping is an important and previously overlooked mechanism that could explain the reduction of mite reproduction in resistant colonies.

Our first study in South Africa

Our research has now expanded on this idea. In our first study we looked at recapping levels in *Varroa*-naïve colonies (those that have never been exposed to

the mites) from Australia and Scotland, and in long-term mite-resistant bees from Brazil and South Africa. Building on the study from Europe, we found that recapping was virtually absent in the naïve colonies and was at the highest levels observed so far in the resistant colonies. We spent six weeks at a government research institution in Stellenbosch, South Africa, to extend the research season by studying recapping, mite reproduction and brood removal behaviour in the resistant Cape honeybee. We collected brood samples and carefully examined them under a microscope to look for evidence of recapping and to see how well the mites were reproducing. The recapping patterns fitted the studies from Europe and Brazil, and the mite reproduction levels were also typically low for resistant bees, although we began to suspect that recapping was not the cause of the reduced mite reproduction. This is because the mite reproduction was equal between recapped and untouched cells- many recapped cells contained successfully reproducing mites while many untouched cells contained poor reproduction. Instead, we suspect that recapping is indeed associated to brood removal behaviour, as we also found high levels of this behaviour in the Cape bees and we directly observed the traits in action using observation hives. This study has now been published open-access in the journal Apidologie (Martin et al., 2019) so is free for anyone to download at no cost.

Our second study in the UK

Our second study looked at the same phenomenon here in the UK, as we have been building up a network of beekeepers in addition to those identified by previous BDI student Jess Kevill, that own mite-resistant colonies across England and North Wales. We again found the same pattern, that resistant colonies displayed higher recapping levels that were strongly targeted towards mite-infested cells, and lower levels of successful mite reproduction. Again however, it appeared unlikely that recapping was directly connected to the mite reproduction. We additionally set up an apiary at Salford University dedicated to our research, consisting of a collection of healthy naïve colonies sourced from the Isle of Man and resistant colonies from North Wales. In conjunction with an apiary of susceptible colonies, we conducted a small experiment to investigate brood removal behaviour by inserting live *Varroa* foundresses into freshly sealed brood cells. Interestingly, once we had introduced the mites into the naïve colonies their

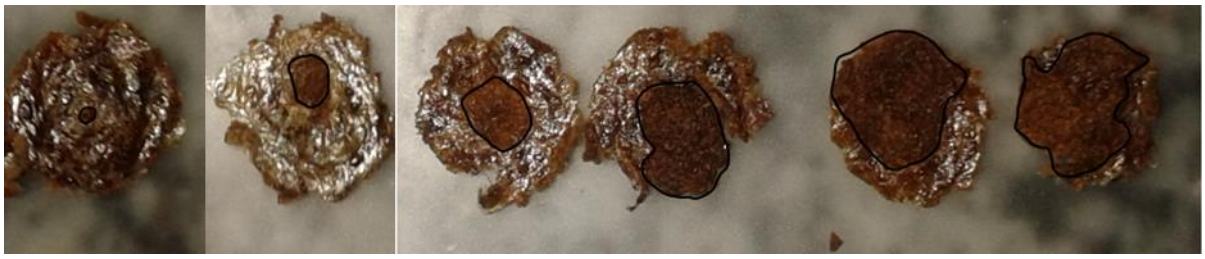
recapping levels increased rapidly, and their brood removal response was immediately comparable to both resistant and susceptible colonies in previous research. In addition, the susceptible colonies had not been treated for two years prior to the experiment and their recapping and brood removal levels were the highest we found. These findings suggest that recapping and brood removal behaviours are inbuilt social immune responses to *Varroa* that may be reduced by using treatment, although given our small sample size more work will be needed in future to confirm this. This study has been submitted for publication to Apidologie and is currently under review.

Conclusions and outlook

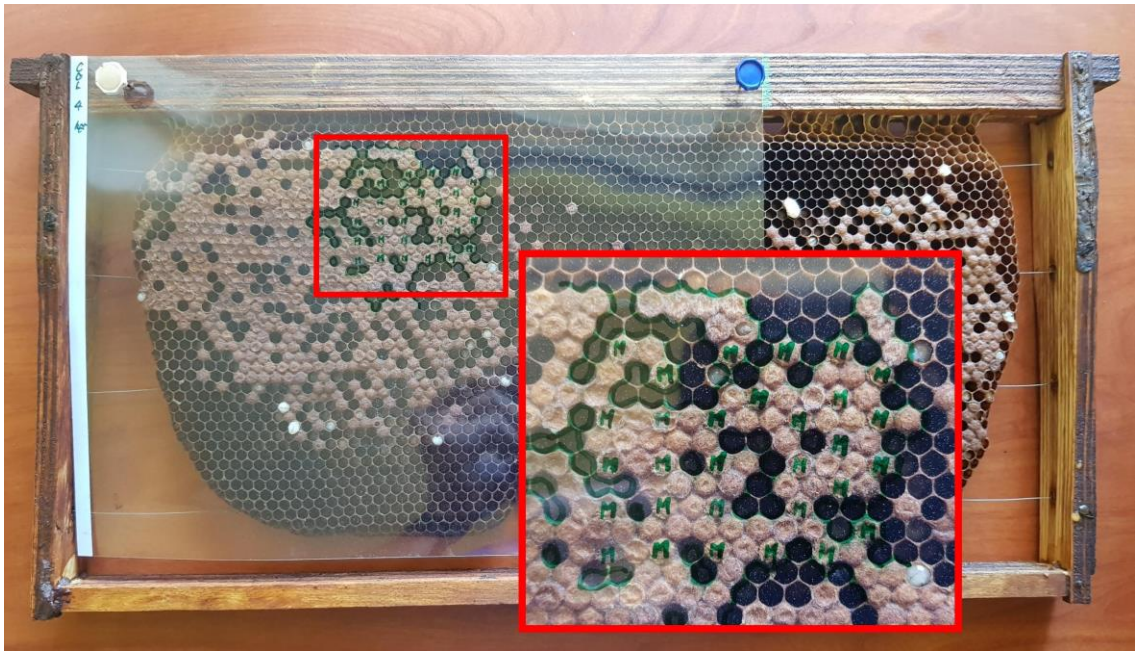
Given the results of these studies, recapping remains a promising trait that could be used as an indicator for mite-resistance and brood removal behaviour, although the levels of the behaviour are highly variable across all colony types and understanding why will be an important direction for future research. Additionally, reduced mite reproduction appears to be another key mechanism leading to resistance in the UK and elsewhere and understanding why this happens is another important challenge. Brood removal behaviour is known to contribute to this somewhat, as the behaviour disrupts the mites' reproductive cycles, however it cannot account for the common cases of offspring death we found within the undetected cells. Given the complexity of honeybee colonies and their wider ecology, it is likely that a range of traits and conditions are ultimately required to lead to a stable host-parasite relationship between western honeybees and *Varroa* and developing our understanding of these remains important for promoting treatment-free apiculture. We would like to thank BDI and BBKA for help in funding this research and the many volunteer beekeepers around the UK who kindly offered their time and brood samples- without you this research would not have been possible!

Article images

Another day at the office: our study site in Stellenbosch, South Africa.

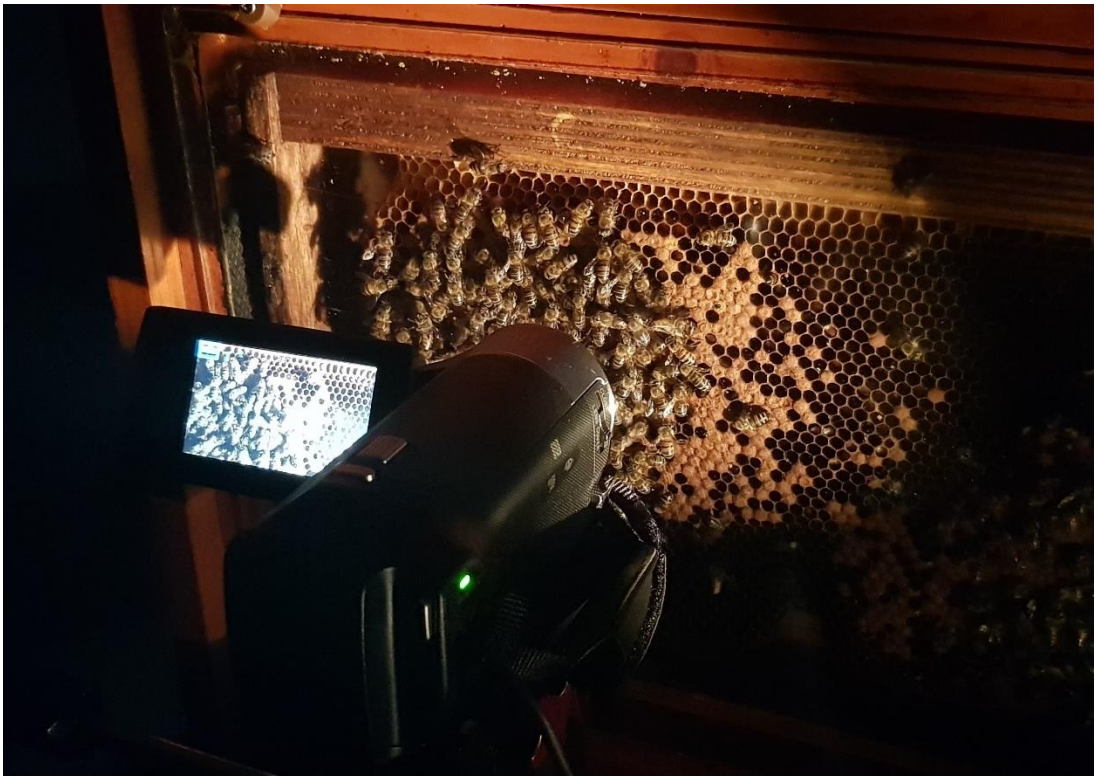


Evidence for recapping behaviour: the shiny silk cocoon on the underside of a brood cell cap has been opened and repaired with wax.

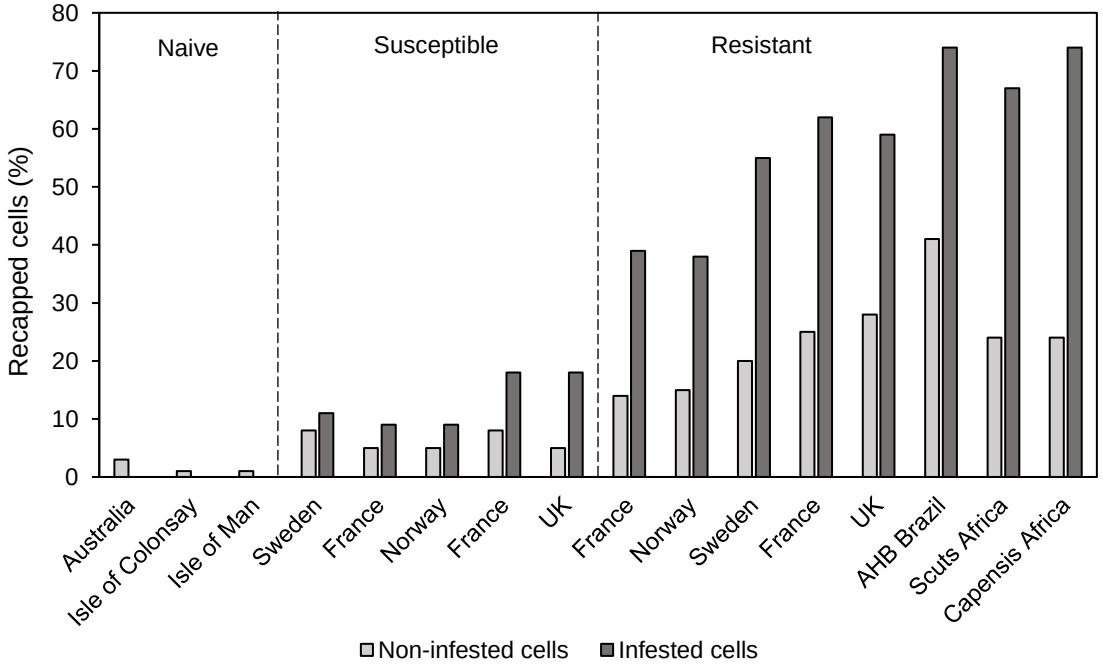


Measuring brood removal behaviour: recently sealed brood cells are carefully opened with sharp tweezers before inserting a live *Varroa* mite. The cells are then repaired by the adult bees, and brood removal rates are measured 10 days later.

Setting up our observation hive in Stellenbosch, South Africa (author on the right).



Observation hives used to see recapping and brood removal behaviours in action.



Recapping frequency is consistently higher in resistant colonies and is targeted towards *Varroa* infested brood cells.

APPENDIX 3: Chapter 2 Apidologie journal article 2019

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Original article

Varroa destructor reproduction and cell re-capping in mite-resistant *Apis mellifera* populations

Stephen J. Martin[1], George P. Hawkins[1], Laura E. Brettell[2], Natasha Reece[1], Maria E. Correia-Oliveira[3], Michael H. Allsopp[4]

[1]School of Environment and Life Sciences, The University of Salford, M5 4WT, Manchester, UK
[2]Hawkesbury Institute for the Environment, Western Sydney University, Locked bag 1797, Penrith, Richmond, NSW 2751, Australia
[3]Universidade Federal do Recôncavo da Bahia, Rua Rui Barbosa 710, Cruz das Almas, Bahia 44380-000, Brazil
[4]ARC-Plant Protection Research Institute, P/Bag X5017, Stellenbosch 7599, South Africa

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Abstract – Globalization has facilitated the spread of emerging pests such as the *Varroa destructor* mite, resulting in the near global distribution of the pest. In South African and Brazilian honey bees, mite-resistant colonies appeared within a decade; in Europe, mite-resistant colonies are rare, but several of these exhibited high levels of "re-capping" behavior. We studied re-capping in Varroa-naïve (UK/Australia) and Varroa-resistant (South Africa and Brazil) populations and found very low and very high levels, respectively, with the resistant populations targeting mite-infested cells. Furthermore, 54% of artificially infested *A. m. capensis* worker cells were removed after 10 days and 83% of the remaining infested cells were re-capped. Such targeted re-capping of drone cells did not occur. We propose that cell opening is a fundamental trait in mite-resistant populations and that re-capping is an accurate proxy for this behavior.

re-capping / hygienic / tolerance / resistance / *Varroa*

1. INTRODUCTION

During the past 70 years, the ectoparasitic "Varroa" mite (*Varroa destructor*) has spread worldwide and has become the greatest threat for apiculture, killing large numbers of managed *Apis mellifera* honey bee colonies (Rosenkranz et al. 48), while decimating feral and wild populations (Wenner et al. 56). Many beekeepers originally advocated breeding from stock that survived, but in the vast majority of cases, their colonies ultimately died, since any preexisting defense adaptations were either not sufficiently developed or were overwhelmed by the massive number of mites initially circulating in the population. As such, countries across the Northern Hemisphere, and those (e.g., Argentina, New Zealand) which had imported Northern Hemisphere honey bees that subsequently became infested by Varroa, were forced to use miticides to control mite numbers and to protect their bee populations.

By contrast, and although the evolution of defense mechanisms can occur rapidly (< 100 years) but is rarely seen occurring simultaneously in allopatric populations (Thompson 52), in South Africa and Brazil, their honey bees quickly became resistant to Varroa

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Corresponding author: S. Martin,
s.j.martin@salford.ac.uk
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(Rosenkranz 46). That is, they did not receive nor require the administering of any mite-control methods to ensure their long-term survival, and no population-wide loss of colonies occurred.

The Western honey bee *A. mellifera*, which consists of approximately 30 geographical subspecies, originated in Africa and appears to have expanded twice into Eurasia, followed by a more recent anthropogenic expansion into the Americas (including Brazil, see below), Asia, and Australasia (Whitfield et al. 57). African honey bees are resilient to many of the pathogens and parasites that often plague (and need to be controlled) in other parts of the world, as evidenced by the limited pest management practiced in Africa (Pirk et al. 42). The Varroa mite arrived in Africa in 1997 to the Cape Region of South Africa (Allsopp 1). This was initially followed by some colony losses; however, these were short-lived, with mite resistance appearing after 3–5 years in the Cape honey bee (*A. m. capensis*) and 6–7 years in the Savanna honey bee (*A. m. scutellata*) (Allsopp 1). This pattern of short-lived colony loss prior to the appearance of mite resistance is frequently mentioned in other mite-resistant populations (e.g., Fries et al. 12; Mordecai et al. 33; Oddie et al. 37).

The Africanized honey bee (AHB) is a hybrid between *A. m. scutellata* from South Africa and East Africa, and various European races, e.g., *A. m. iberiensis* and *A. m. ligustica*. In 1957, 26 swarms of *A. m. scutellata* spread northwards throughout Brazil from Rio Claro, hybridizing with European races to form the AHB which reached the USA in 1990 (Winston 58). In 1971, during this expansion, the Varroa mite arrived in Brazil (Moretto et al. 34) and spread rapidly throughout both the AHB and European honey bees. The subsequent establishment of AHB throughout the tropical and subtropical regions of South America was due, in part, to AHBs' natural resistance to Varroa (Rosenkranz 46). In both AHB (Camazine 8; Medina et al. 27; Mondragon et al. 32) and *A. m. scutellata* (Martin and Kryger 23; Nganso et al. 36), poor mite reproduction limits their population growth, although the mechanism(s) by which this occurs has remained unknown.

Targeted selective bee-breeding programs to combat Varroa have been ongoing for decades in both America (Rinderer et al. 45) and Europe (Büchler et al. 7). Selection for traits such as hygienic behavior (based on the removal of killed sealed brood) is being used by beekeepers to help reduce their mite treatment regime, and the Varroa Sensitive Hygiene (VSH) line (developed from Suppression of Mite Reproduction lines) that targets the removal of living mite infested brood (e.g., Harris 16) is undergoing further selection in Hawaii to make it suitable for use in beekeeper operations. Meanwhile, naturally selected mite-resistant populations are being maintained without any mite control measures across a vast range of environments, i.e., that exists across Africa and South and Central America.

Recently, low rates of mite reproduction similar to those found in African and AHB were reported in four European mite-resistant populations (Oddie et al. 37), which raised the possibility that a similar mechanism had arisen in these geographically distinct populations. Oddie et al. (38) then linked the low mite reproduction in these European populations with a high incidence of "re-capping" behavior, when a cell containing a developing pupa has its silk/wax cap partially removed by the worker bees and then resealed with wax, without the removal of the pupa. Although re-capping (even of mite-infested cells) is not a new phenomenon, e.g., both hygienic and non-hygienic colonies re-capped around 90% of artificially created holes in the cell caps (Spivak and Gilliam 51), its importance may have previously been overlooked.

The aim of this study was to investigate mite-naïve populations from Scotland, Isle of Man and Australia, and well-established mite-resistant populations from Brazil (AHB) and South Africa (*A. m. scutellata* and *A. m. capensis*), to identify whether re-capping is a reliable proxy for mite-resistance, and whether it is associated with reduced mite reproduction. We then focused on *A. m. capensis*, which was found to have the highest targeted re-capping of mite-infested cells.

2. METHODS

2.1. Varroa-mite naïve colonies

During 2018, Varroa-naïve brood samples were obtained from three colonies, each from a different apiary, from across the Island of Colonsay, Scotland, UK, and three colonies from a single apiary belonging to Western Sydney University, Hawkesbury Campus, NSW, Australia. Four additional Varroa-naïve colonies were sampled in 2019 from the Isle of Man, UK.

2.2. Mite-resistant AHB colonies, Brazil

The AHB were located at Cruz das Almas, Bahia State, NE Brazil. Re-capping and mite reproduction were studied in February 2018, using six colonies (minimum of 300 worker cells per colony). Re-capping rates and mite infestation data were collected from an additional ten colonies (150–200 worker cells per colony) which were used in a freeze-kill brood removal test.

2.3. Mite-resistant South African colonies

Four *A. m. scutellata* colonies were studied in July 2018 and again in March 2019, while 20 *A. m. capensis* colonies were studied in July 2018 ($n = 3$) and in March 2019 ($n = 17$) (Tables I and S1). As only one *A. m. scutellata* and two *A. m. capensis* colonies contained drone brood, in addition, ten *A. m. capensis* colonies with drone brood were sampled. All colonies are maintained within 20 km of Stellenbosch, Western Cape, South Africa, with the four *A. m. scutellata* colonies having been moved 800 km from their natural distribution into the area for research purposes. As no mite-susceptible colonies are present in either Brazil or South Africa, no direct comparisons with treated colonies from the same region are possible, although as African bees are the ancestral population both resistant and susceptible populations all originated from Africa (Whitfield et al. 57).

2.4. Cell re-capping

From each colony, a single frame containing mainly purple eyed pupae (e.g., 180–190 hpc) or older worker or drone brood was removed, and on average, 300 cells per colony (Table S1) were examined for re-capping and mite reproduction. To determine whether a cell was re-capped, fine forceps were used to carefully cut around the edge of the cap, which was then inverted to allow the underside to be inspected under a binocular microscope (× 16). If the silk cocoon spun during the first 30 h of the sealed stage (Martin 19) had been partially removed and replaced by wax, it was classified as re-capped. The diameter of the re-capped area for worker brood was estimated to the nearest mm.

2.5. Mite reproduction in worker brood

After the re-capping status of the cell had been determined, the pupa was carefully removed and aged according to standard methods (Dietemann et al. 11). If the cell was infested, then all mites, offspring, and shed skins (exuviate) were removed and the mite family reconstructed using the method and developmental chart of Martin (19). Only the 497 infested cells containing yellow-thorax pupae or older, i.e., over 190 h since the cell had been capped, were used in the reproductive calculations (Table S2). The number of mated adult female offspring were counted, that is the cell must also contain a living adult male (evidenced by the exuviate), accompanied by daughters at the correct developmental stage. Only the number of foundresses per cell was determined in the drone brood. Two methods were used to calculate the average number of mated female offspring produced during one reproductive cycle in each of the three study populations, since not all samples are at the same developmental stage; therefore, any mite mortality from the sampling point to bee emergence will not be accounted for. Therefore, firstly, we counted the total number of mated female offspring and divided it by the number of invading foundresses. Secondly, we counted the number of females that would be mated and were assumed to mature prior to bee emergence, but accounted for a further in-cell mortality in cells younger than grey pads, by multiplying the first daughter by 0.94, the second daughter by 0.38, and third daughter by 0.13. These mortality values were based on a study of over 1000 mite families (Martin 19). The

Table I. Meta-data from the four groups of honey bees studied. The number of colonies (# col) and cells studied along with the number of cells re-capped and infested with Varroa are presented, along with the group percentages, either in worker (W) or drone (D) cells. For individual colony data, see Table S1. Wr = number of viable female offspring produced per mother mite during one reproductive cycle. This is based on "*n*" mothers from 12 AHB, four *A. m. scutellata* and 20 *A. m. capensis* colonies. All cells contained yellow-thorax pupae (190 h post-capping) or older (see Table S2 for more mite reproductive details)

Population	Country	# col	# cells studied	# cells re-cap	# cells infested	% cells re-cap	% cells infested (range)	% infested cells re-cap	% uninfested cells re-cap	*Wr* (*n* mothers)
Naïve	UK/Oz	10	6452	88	0	1.4	–	–	1.4	–
AHB-W	Brazil	12	3417	1402	224	41	7 (0-26)	71	39	0.8 (143)
Scutellata-W	S Africa	8	3235	620	294	19	9 (3-21)	64	15	0.9 (183)
Scutellata-D	S Africa	1	103	2	59	2	59	3	0	-
Capensis-W	S Africa	20	5599	1452	190	26	3 (0-14)	74	24	0.9 (171)
Capensis-D	S Africa	12	1183	251	330	21	32 (9-92)	17	23	–

results of both calculations are provided (Table S2) and are similar.

2.6. Artificial infestation of *A. m. capensis* cells with Varroa

Since the pattern of Varroa infestation within and among frames is non-random (Fuchs 13), we studied the bees' ability to detect and remove mite-infested pupae by artificially infesting cells with mites (Boecking 5; Rosenkranz et al. 47) rather than comparing relative changes in infestation levels over a period of time (Harris 15). Therefore, a total of 390 mother mites harvested from *A. m. capensis* drone brood cells containing a stretched larva were inserted into *A. m. capensis* worker cells that were less than 1 day post-capping, as indicated by the lack of a completed cocoon (Martin 19). Of these mites, 325 were alive and 65 had died since been collected the previous day. Dead mites were also used for artificial infestation experiments, as comparing rates of detection using living vs dead mites may help indicate cues used to detect infested cells, e.g., movement. A frame containing cells capped within the past 24 h were as evidence by the larvae spinning their cocoons, were removed from each of 11 *A. m. capensis* colonies over a period of several days. Freshly sealed cells were opened, and a mother mite inserted using a fine paint brush before re-sealing the cell and recording its position on an acetate sheet. After returning the frames to their colonies, an inspection after 24 h revealed any cells removed by the bees as a result of the manipulation. Five days later (6 days post-capping), the number of pupae removed from the artificially infested cells were recorded. After 10 days (11 days post-capping), each frame was removed and any remaining artificially infested cells were inspected for mites, and the state of the cell cap (i.e., re-capped or not) was recorded (Table S3). We also inspected the cap condition of a similar number of neighboring non-infested control cells. Insufficient mites were available to conduct artificial infestation experiments in AHB.

2.7. Test for hygienic behavior using

freeze-killed brood.

In ten AHB and 11 *A. m. capensis* colonies, their ability to remove freeze-killed pupae (classic hygienic behavior) was studied to compare with their re-capping rates. An area of purple-eyed pupae or older (> 7 days post-capping) was freeze-killed using liquid nitrogen, and then the number of removed pupae determined after 24 h and 48 h (Table S3). The 48 h data was not analyzed since several of the colonies in both populations had removed 100% of the killed brood by 48 h.

2.8. Data analysis

All datasets were non-normally distributed, thus statistical tests included chi-squared, Kolmogorov-Simirnov, Mann-Whitley *U*, Wilcoxon signed-rank, and Spearman's rank correlation. The test statistic indicates which test was used if not mentioned in the text.

3. RESULTS

3.1. Cell re-capping and mite reproductive behavior in worker cells

Based on 497 infested cells, we confirmed that the average number of mated female offspring produced per mother foundress during a single reproductive cycle (*Wr*) was between 0.8 and 0.9 in the three mite-resistant populations (Table I, Table S2), with only 54–55% of the invading mothers reproducing, and producing between 1 and 3 mated female offspring each. Across the ten Varroa-naïve colonies from three different populations, only 0.5% (median) of the worker sealed brood cells were re-capped (Table I, Table S1, Figure S1). In contrast, the median re-capping rates in the Varroa-resistant AHB, *A. m. scutellata* and *A. m. capensis* were 35%, 20% and 27%, respectively, although the average infestation rate was < 10% in every mite-resistant population. The re-capping rate of infested cells was always significantly higher than for non-infested cells (AHB $U = 17$, $z = 2.46$, $p = 0.014$; *A. m. scutellata* $U = 0$, $z = 3.31$, $p < 0.001$; and *A. m. capensis* $U = 10.5$, $z = 4.41$, $p < 0.0001$) (Table S1, Figure S1). Combining the data from the three populations, we found that the estimated number of viable offspring in re-capped (0.82) and undisturbed (0.76) cells were similar (Table S2). The frequency distribution of the size of the re-capped area (diameter of the opening which had been resealed) of non-infested cells in all four populations followed the same negative trend (Figure 1a–d). In contrast, infested cells all had significantly different size distributions (AHB, $D = 0.2051$, $p = 0.002$; *A. m. scutellata* $D = 0.3406$, $p < 0.0001$ and *A. m. capensis*, $D = 1$, $p = 0.037$) due to the re-capped area being larger, i.e., larger than 3 mm, if the cell was infested in all three populations (Figure 1e, f). Furthermore, all re-capped cells in the Varroa-naïve populations were small ranging from 1 to 3 mm. Re-capped cells appeared from the early stages of pupal development and their proportion increased steadily as the pupae developed (Figure 1h). We noticed that some infested cells had been re-capped more than once, as they contained two or three distinct holes cut out of the silk cap.

3.2. Cell re-capping and mite infestation in *A.m. capensis* drone cells

The average infestation level of drone brood was 31 ± 27% (Table I; S1), with cells typically containing multiple foundresses. A total of 330 infested cells (single and multiple infested cells) were invaded by 569 foundress mites. This varied considerably by site; only 5 ± 9% of drone cells were re-capped in nine colonies in the Stellenbosch area (Table S1-R* colonies), whereas three colonies from the Pniel (Table S1-PA* colonies) region in the next valley re-capped 65% of all drone cells. However, the proportion of infested (66%) and non-infested (65%) cells re-capped were similar. Therefore, a pattern of targeting mite-infested drone cells was not seen (Table I; Figure S1). The single drone frame from *A. m. scutellata* showed the same pattern, of high infestation (57%), low re-capping (2%) and multiple foundresses in cells (Tables I and S1).

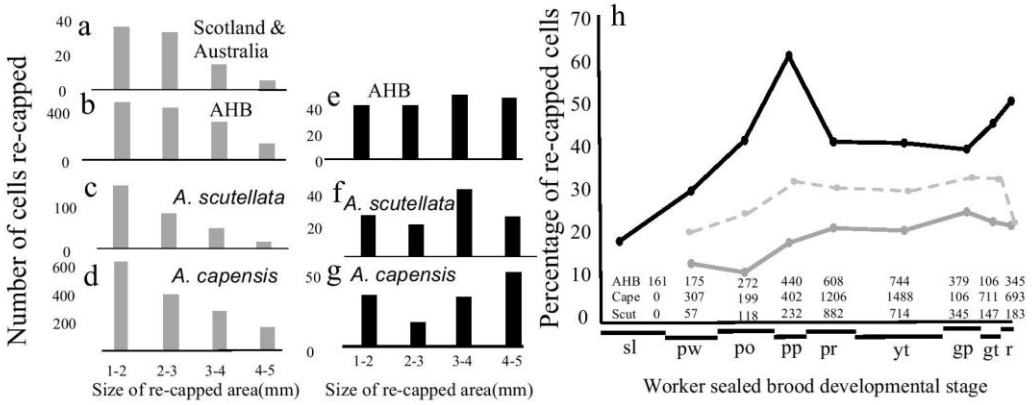

Figure 1. a–g Frequency distributions in the diameter of the re-capped area in non-infested cells (grey, **a–d**) and Varroa-infested cells (black, **e–g**) in the four study groups. **h** Percentage of re-capped cells at each stage of pupal development in AHB (black line), *A. m. scutellata* (solid grey line), and *A. m. capensis* (dashed grey line). The pupal developmental stages following (Martin 19) are sl = stretched larvae, pw = white-eyed, po = pale-eyed, pp = pink-eyed, pr = purple-eyed, yt = yellow thorax, gp = grey pad, and gt/r = grey thorax/resting and total number of cells opened per stage is also given.

3.3. Detection and removal of artificially mite infested cells in *A. m. capensis*

Of the 392 *A. m. capensis* worker pupae artificially infested with mites (326 alive and 66 dead), only 3% were removed within 24 h, most likely due to the experimental opening and resealing of the cell (manipulation). After 10 days, we found 21 (5%) cells containing no mites or evidence of mites, i.e., mite fecal droppings on the cell wall, which must have escaped during the uncapping/re-capping process, and a further 30 (8%) mites had become sealed into the cell wall and died during the spinning of the pupal cocoon. The mites lost due to manipulation, the re-capping process or being sealed into the cell wall were removed prior to the analysis of removal behavior. Across the 11 colonies, 32% of the infested cells had been removed after six days, and this increased to 54% after 10 days (Table S3). The percentage of dead (47%) and alive (46%) mites removed after 10 days were not significantly different ($U = 49.5$, $p = 0.75$). Of the remaining 152 artificially infested cells, 83% had been re-capped, while only 27% of mite free cells 'control' cells were recapped (Table S3). Again, typically larger re-capped areas were found in infested cells relative to neighboring non-infested control cells (Figure 2); however, there were no significant differences in sizes of the re-capped area both between non-infested controls and cells containing dead mites trapped in the walls, and between infested cells that contained living or dead mites (Figure 2).

3.4. Test for hygienic behavior using freeze-killed brood

Among ten AHB colonies tested for both classic hygienic and re-capping behavior, a Spearman's rank correlation found no significant correlation ($r_s = 0.03$, $p = 0.93$) between the two behaviors after 24 h (Table S3), with 19–98% of the dead brood having been removed in 24 h while the re-capping rates ranged from 4-50%. A similar result ($r_s = 0.356$, $p = 0.282$) was also found across the 11 *A. m. capensis* colonies (Table S3), with 48–100% of freeze-killed brood removed while the range of re-capping rates was 12–66%. However, there was a weakly significant positive correlation ($r_s = 0.67$, $p = 0.024$) between the removal rate of freeze-killed brood and the proportion of artificially infested cells removed (Table S3).

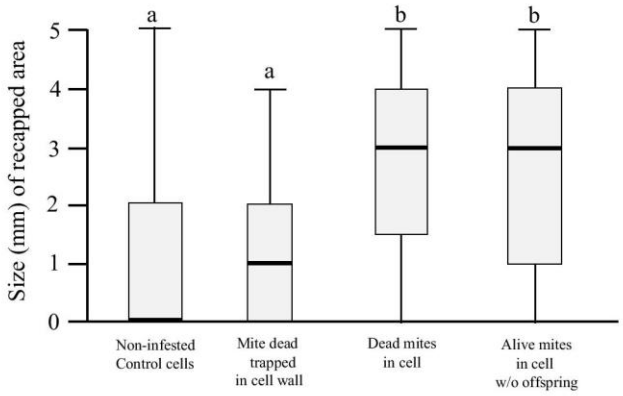

Figure 2. Box plots showing the size of the re-capped area of *A. m. capensis* at 11 days post-capping among four groups of cells (11 colonies). Kolmogorov-Simirnov analysis showed different frequency distributions of re-capped areas between the four groups of brood cells ($D_{(499,\ 3)} = 69.4$, $p < 0.0001$). Pairwise comparisons between the four groups showed that there was no significant difference between the mites trapped in cell walls and non-infested control cells ($D = 0.678$, $p = 0.41$), nor between the cells in which dead and live mites were found ($D = 0.839$, $p = 0.36$). However, there were significant differences between the cells containing mites (dead or alive) and those cells that were either mite-free or contained mites trapped in the cell wall ($D > 11$, p always < 0.005).

4. DISCUSSION

Although we observed the re-capping of brood cells in all colonies, the re-capping rates were lowest in Varroa-naïve, and the highest were consistently found in worker brood of mite-resistant populations from Brazil (AHB) and South Africa (*A. m. scutellata* and *A. m. capensis*), as well as Europe (Oddie et al. 38). The key behavior in all these mite-resistant populations appears to be the bees' ability to detect mite-infested cells, as indicated by consistently higher re-capping rates of infested cells relative to non-infested cells, particularly since infestation rates are typically below 10% (Figure 3; Figure S1). The initial detection of a possible infested cell leads to the opening of a small hole in the cell cap that could allow better access to any volatile or non-volatile cues, i.e., on the pupae, within the sealed cell (see below). If a non-infested cell is opened in error, the hole is re-capped and the disturbed area remains small (1–2 mm), but if infested, the hole is enlarged to 3–4 mm to gain better access (Figure 1). A second trigger, or lack thereof, causes the infested cell to either be re-capped or the pupa to be cannibalized (Figure 4). This idea is in line with previous studies (Gramacho 14; Arathi et al. 3) that found the initial step of detecting diseased brood does not necessarily lead to brood removal, with repeated uncapping and recapping prior to brood removal. The removal of pupae artificially infested with mites was 54% in *A. m. capensis* (this study), 33% in *A. m. scutellata* (Cheruiyot et al. 9), 10–25% in AHB (Aumeier et al. 4), and up to 40% in a single mite-resistant population in the Netherlands (Panziera et al. 40). All values are well below the 99% removal of artificially infested worker cells in the mite's original host, *A. cerana* (Rath and Drescher 44).

Two recent studies have assumed that genetically derived host factors within the brood prevent the initiation of mite oogenesis, which accounts for the increase in non-reproduction of mites in resistant colonies. For example, Broeckx et al. (6) suggested brood pheromones fall to a level that prevents the mites reproducing, whereas Conlon et al. (10) suggested an ecdysone gene was linked to mite-resistance, since low ecdysone levels may prevent mite oogenesis, hence increasing non-reproduction in mites. However, the greater proportion of

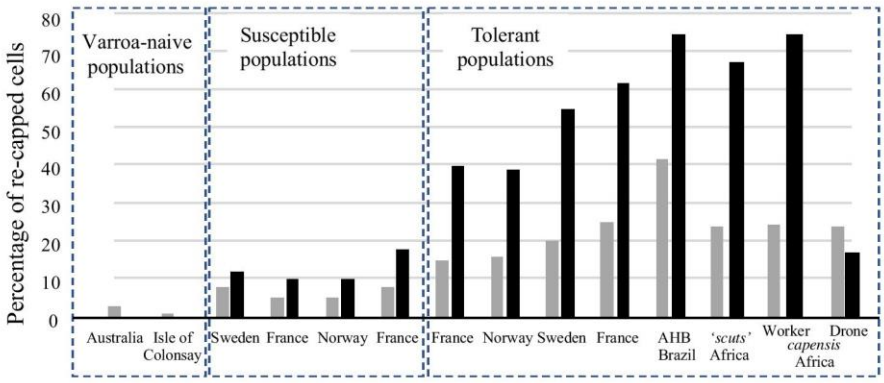

Figure 3. Re-capping levels of non-infested (grey) and Varroa-infested (black) worker cells, showing that in susceptible populations levels are consistently lower than those recorded in mite-resistant populations, except for the "*capensis* drone brood." The European data is taken from Oddie et al. (38), while the Australia, Colonsay, AHB, and Africa data are from the current study. "*scuts*" = *A. m. scutellata*; *capensis* = *A. m. capensis*. For individual colony data, see Figure S1.

non-reproducing mites found in mite-resistant colonies (Martin et al. 25; Broeckx et al. 6) can simply be explained by the behavioral trait of increased disruption of mite reproduction. That is the removal of infested pupae reduces the number of successful mite reproductive cycles, increasing the proportion of non-reproductive mites in subsequent reproductive attempts. The latter may in part account for the lower reproductive values (*Wr*) found in mite-resistant colonies, since a consistent 50% removal rate will result in 12.5–25% of mites never reproducing due to having been disturbed by the removal of pupae before mating. Since the number of mite reproductive cycles is estimated between 2 and 3 (Martin and Kemp 22), these mites may still invade cells and attempt to reproduce but produce either no offspring or only males (Martin et al. 25), both categories common in African (Martin and Kryger 23), AHB (Medina et al. 27) and this study. This would also explain why reproducing and non-reproducing mites could not be distinguished using DNA microsatellites (Broeckx et al. 6), since they do not represent two distinct genotypes, just that non-reproducing mites have either run out of sperm or eggs.

Mite reproductivity in re-capped and undisturbed infested cells was similar in this study (Table S2), which was also found in previous studies (Harris et al. 17; Oddie et al. 38). Mondet et al. (30) and Oddie et al. (38) have suggested that the bees are more likely to ignore cells containing non-reproducing mites (selection bias), while preferentially removing cells containing reproducing mites or with more offspring. However, we found no evidence for this in *A. m. capensis* since the removal rate of cells containing live (reproducing) and dead (non-reproducing) mites were similar, and Panziera et al. (40) also found no relationship between mite reproductive success and brood removal.

In both AHB (Mondragon et al. 32) and African honey bees (*A. m. capensis* and *A. m. scutellata*) (Tables I and S1), drone brood frequently becomes heavily infested, which may impact on the honey bee colony and population reproductive success. This can lead to density dependent control of the mite population via the drone brood (Martin and Medina 24), which occurs in *A. cerana*, either via a reduction in mite offspring survivorship in multiple infested brood (Martin 20) or increased failure for the bee to emerge (Rath 43). Recent studies have found that mite feeding causes *A. cerana* worker brood to die (Page et al. 39) via injection of a toxic mite salivary protein into the pupae during feeding (Zhang and Han 59), which could explain the high removal rates previously seen (Rath 43).

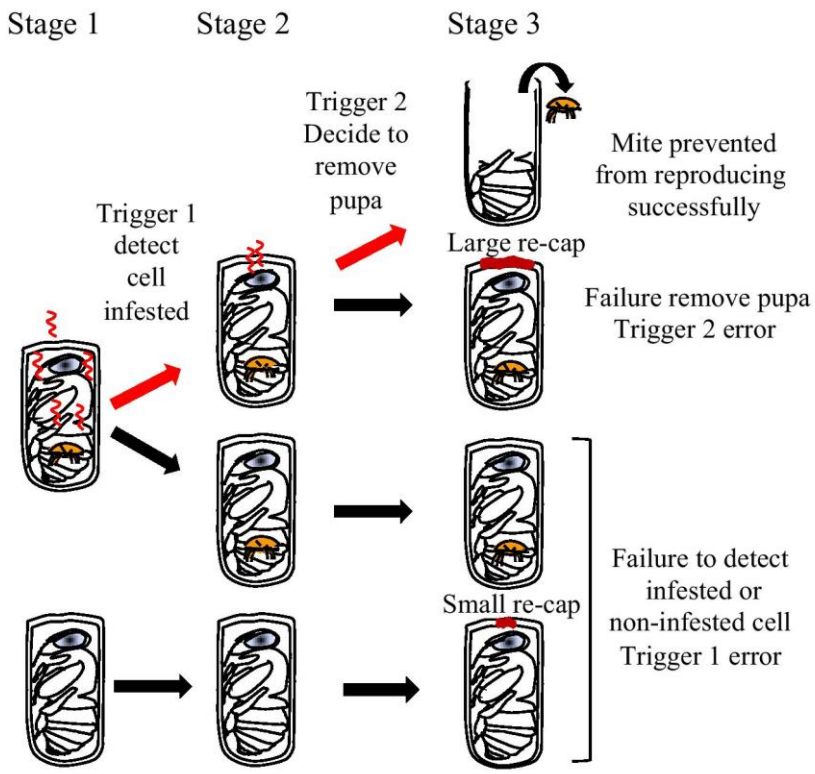

Figure 4. Process of detection of infested cells and subsequent removal of the pupa, and where errors are generated. Stage 1, a mite infested cell produces a stimulus detectable through the cell cap (Trigger 1). Stage 2, a small hole is made in the cap to allow a more detailed inspection. At this point a second trigger may be detected. Stage 3, if a mite is present and is detected the pupa is cannibalized and Varroa prevented from reproducing, if the mite is missed (or no mite is present) the cell is re-capped. The red arrows indicate the ideal situation and black arrows indicate observed errors due to the failure of one or both of the theorized behavioral triggers. The red wavy lines indicate the putative density of the mites' odor.

However, this salivary protein has no effect on *A. mellifera* pupa (Zhang and Han 59).

As only around 27% (Figure 3) of non-infested worker cells are re-capped, this suggests that initial detection of the mite is made without disturbing the cap. While the ability to detect mite infested cells is high in almost all mite resistant colonies (Figure S1 and Oddie et al. 38), it is the trigger to remove the infested pupa that remains error prone since only around 50% of infested cells that are opened are subsequently removed in *A. mellifera*, and a high proportion of those cells are re-capped. The triggers for the initial detection and subsequent decision to cannibalize the pupa are both currently unknown (Figure 4), although this study indicates that olfaction could be a key factor for the initial detection as several previous studies have proposed (e.g., Rosenkranz et al. 47; Mondet et al. 29; Scannapieco et al. 49), since dead mites elicited a similar re-capping behavior as live mites, as also reported in *A. cerana* (Rath and Drescher 44). This suggests that motion or associated changes in the pupa (odour or temperature) may not be important as previously suggested by Aumeier and Rosenkranz (3) and Wagoner et al. (54). Nor would the level of oleic acid, which is known to trigger hygienic behavior (McAfee et al. 26), be important, unless also produced by the living mites. Furthermore, as mites sealed into the cell wall by the pupal cocoon

did not elicit any increased re-capping response relative to non-infested cells (Figure 2), a volatile odour is a likely candidate. For example, Nazzi et al. (35) found pentadecane ($C_{15}H_{30}$) was present only in the air of infested cells and the application of Z-(6)-pentadecene increased hygienic behavior, whereas Z-(7)-pentadecene, Z-(8)-heptadecene and pentadecane had no effect. Longer cuticular hydrocarbons are unlikely to be the odour cue due to their lack of volatility, and the pupa's profile is mimicked precisely by the mite (Kather et al. 18). Rath and Drescher (44) also found that dead mites washed in ethanol were still removed at a high rate in *A. cerana*. However, Wagoner et al. (55) suggested that two long cuticular hydrocarbons (heptacosene [$C_{27}H_{54}$] and tritriacontane [$C_{33}H_{66}$]) removed from the surface of the pupa were associated with the uncapping of infested worker brood. In addition, Mondet et al. (30) suggested changes in the brood pheromone that consists of ten ethyl and methyl esters can be detected between infested and non-infested brood, although this was found using discriminate analysis that is error prone if the sample to variable ratio is not high (Martin and Drijfhout 21; Mitteroecker and Bookstein 28 [Figure 5]).

Why re-capping behavior exists even in the Varroa-naïve populations is unknown, but when non-infested brood are not removed, any cost to colony fitness is minimal. We observed in African honey bee brood invaded by the lesser wax moth (*Achroia grisella*), cells were frequently re-capped rather than the pupae being removed. Likewise, 57% of the uncapped cells in a colony heavily infested with the greater wax moth (*Galleria mellonella)* were re-capped within 24 h of uncapping (Villegas and Villa 53). Interestingly, the three *A. m. capensis* colonies at Pniel were unique in re-capping high numbers of drone cells. These were all survivor colonies from an American Foulbrood (*Paenibacillus larvae*) outbreak. Therefore, making a small hole in the cell cap may be a general response to allow more detailed investigation of the developing pupa (which may account for the low-level presence of this trait in Varroa-naïve populations). After the arrival of the mites, this behavior appears to have been co-opted and selected for as part of a defense mechanism against Varroa; hence, the re-capping rate is elevated in all infested colonies (Figures 3 and S1), reaching the highest levels in mite-resistant colonies. Throughout Brazil and Africa, beekeeping pest management is minimal and so selective pressures for such traits have always been high. The constant management of a wide range of brood pest and pathogens throughout the Northern Hemisphere removes much of this selective pressure. In this and in previous studies (Oddie et al. 38), the ability to detect mites (Figure S1) and remove infested brood (Table S3) is highly variable. No doubt colony composition plays a role since recapping occurred most in mixed colonies rather than in highly hygienic or highly non-hygienic colonies (Arathi et al. 2).

Mondragon et al. (31) suggested hygienic behavior towards freeze-killed brood may not correlate closely with hygienic behavior towards Varroa mites. We found no correlation between re-capping levels and removal of freeze-killed brood. We did, however, find a weak positive correlation between the ability of a colony to remove freeze-killed brood and the removal of artificial mite-infested cells, which is similar to data from Spivak (50), where colonies selected for their ability to remove freeze-killed brood removed significantly more artificially mite-infested cells than "non-hygienic" colonies in 1 year but not another. Perez and Johnson (41) indicate that task specialization, e.g., hygienic behavior can be used to predict specialization in other related tasks, which may help explain the weak link, between response to freeze-killed brood and removal of living mite infested brood.

It appears that resistance towards Varroa mites in both *A. cerana* and *A. mellifera* is following a similar path, that of targeting mites invading worker cells and not drone cells, which will eventually lead to the combined effect of lower mite reproductive success in worker brood and density-dependent control in drone brood. As the ability of the bees to detect mites within worker cells, evidenced by increased re-capping, has arisen naturally in *A. mellifera* in five different countries, this may prove to be an excellent proxy for mite resistance. The challenge will be selecting for these traits (the abilities to initially detect and

subsequently remove infested pupae) while moving away from a regime using insecticides, especially in large commercial beekeeping operations.

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AUTHOR CONTRIBUTIONS

S.J.M. conceived and designed the study; L. E. B. performed the study in Australia; G. P. H., N. R. and M. H. A. assisted with the study in South Africa and M. E. C. assisted with the field work in Brazil. S. J. M. analyzed the data drafted the manuscript with input from G. P. H., N. R. and M. E. C. The manuscript was edited by S. J. K., G. P. H., L. E. B and M. H. A.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest The authors declare that they have no conflict of interest.

Reproduction de *Varroa destructor* et réoperculation dans les populations d'*Apis mellifera* résistantes aux acariens.

réoperculation / hygiène / tolérance / résistance / *Varroa.*

***Varroa destructor* Reproduktion und Brutzellen-"Recapping" bei varroaresistenten *Apis mellifera* Populationen.**

Recapping / Hygiene/ Toleranz / Resistenz / *Varroa*

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