Naturally occurring resistance to *Varroa destructor* in the Western honey bee (*Apis mellifera*)

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Chapter 3: Ten years of deformed wing virus (DWV) in Hawaiian honey bees (*Apis mellifera*), the dominant DWV-A variant is potentially being replaced by variants with a DWV-B coding sequence

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Brettell, Schroeder & Martin, 2020a), Kenya (Ongus *et al.,* 2018), Papua new guinea (Roberts *et al.,* 2020), South Africa (de Souza, Allsopp & Martin, 2020), Tunisia (Abdi *et al.,* 2018), Turkey (Tozkar *et al.,* 2015), UK (Kevill *et al.,* 2019), Uruguay (Mendoza *et al.,* 2020), USA (Kevill *et al.,* 2019). The studies used to create this diagram were not required to have used the same primer set as our study.

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Chapter 2: Parallel evolution of *Varroa* resistance in honey bees: A common mechanism across continents?

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Supplementary Table S7. The data, source, location, and colony number for the percentage of infested worker brood cells in resistant colonies of Africanised honey bees between 1996-1999 as shown in figure 1h.

Supplementary Table S8. The data, source, location and colony number for the percentage of infested worker brood cells in resistant colonies of Africanised honey bees between 2018-2019 as shown in figure 1h. * This unpublished data was kindly provided by Dr Luis Medina, Department of Apiculture, Universidad Autonoma de Yucatan, Mexico from an ongoing study, and allows a direct comparison between this 2019 data and the Cabrera 1998, Medina & Martin 1999 data that all came from the same honey bee population.

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Supplementary Table S2. The data, source, location, bee race and colony number for the percentage grooming ability of treated, *Varroa*-susceptible, *Apis mellifera* in Figure 1b. EHB = European honey bees.

DECLARATION

I have provided a significant and major contribution to all the chapters in this thesis. Chapters one, two and three have been all published open access in peer reviewed journals with me as both the lead and corresponding author. I have also in the appendices included several impact articles published in bee keeping journals such as the British Beekeepers Newsletter which is read by over 26,000 members. Funding for all work was provided by Bee Disease Insurance LTD. My contribution to each chapter in the thesis is detailed below

Chapter 1: Spatial distribution of recapping behaviour indicates clustering around *Varroa* infested cells

The idea behind this project was from Stephen Martin. I collected and analysed the data. I was then responsible for writing the bulk of the manuscript with input and edits from S. Martin.

Grindrod, I., & Martin, S. J. (2021). Spatial distribution of recapping behaviour indicates clustering around *Varroa* infested cells. *J. Api. Res., 60*(5), 707-716. doi:10.1080/00218839.2021.1890419.

Chapter 2: Parallel Evolution of *Varroa* Resistance in honey bees: a common mechanism across continents?

I was involved in the conception and design of this project along with S. Martin. I collected and analysed the data. I was then responsible for writing the bulk of the manuscript with input and edits from S. Martin. I dealt with the four sets of reviewer's comments, which included major structural changes to the manuscript with advice and edits form S. Martin.

Grindrod, I., & Martin, S. J. (2021). Parallel evolution of *Varroa* resistance in honey bees: A common mechanism across continents?. *Proc. R. Soc. B., 288*(1956), 20211375. doi:10.1098/rspb.2021.1375.

Chapter 3: Ten years of deformed wing virus (DWV) in Hawaiian honey bees (*Apis mellifera*), the dominant DWV-A variant is potentially being replaced by variants with a DWV-B coding sequence

The idea behind this project was from S. Martin who collected samples of honey bees from Hawaii. I was then taught by Dr. Jess Kevill at the University of Minnesota how to conduct RNA extraction and qPCR to detect DWV-A and DWV-B within the samples using the ABC assay she had developed. I conducted the qPCR and analysed the results and wrote the manuscript with edits from S. Martin and Dr. Kevill.

Grindrod I., Kevill J. L., Villalobos, E. M., Schroeder, D. C., & Martin, S. J. (2021) Ten years of deformed wing virus (DWV) in Hawaiian honey bees (*Apis mellifera*), the dominant DWV-A variant is potentially being replaced by variants with a DWV-B coding sequence. *Viruses, 13*(6), 969. doi:10.3390/v13060969.

Chapter 4: *Varroa* resistance in *Apis cerana*: A review

I designed this project independently and carried out all the research and writing of the manuscript. Small edits were made to the manuscript by S. Martin.

Grindrod I., & Martin, S. (2022). *Varroa* resistance in *Apis cerana*: A review. *Apidologie* (under review)

ABBREVIATIONS

AHB African derived/Africanised honey bees

BBKA British beekeeper's association

BDI Bee diseases insurance Ltd

CHC Cuticular hydrocarbon

DBSCAN Density-based spatial clustering of applications with noise

dsRNA Double stranded RNA

DWV Deformed wing virus

EHB European honey bees

FKB Freeze killed brood assay

NVR Natural/Naturally *Varroa* resistant

PCR Polymerase chain reaction

PKB Pin killed brood assay

RdRp RNA-dependent RNA polymerase

RNA Ribonucleic acid

RT-qPCR Reverse transcription quantitative PCR

VDV-1 *Varroa destructor* virus 1

VSH *Varroa* sensitive hygiene

GENERAL ABSTRACT

The honey bee is an important social insect; it along with other bee species can be regarded as a keystone organism. The pollination services bees provide are invaluable not only in terms of human health and food security, with an estimated worth of €153 billion to food production (Gallai *et al.,* 2009), but also for the health of whole ecosystems (Hung *et al.,* 2018). However, despite their importance, honey bee populations in the modern age face an unprecedented array of stressors (VanEngelsdorp & Meixner, 2009). Arguably one of the most important of these is the combination of the parasitic mite *Varroa destructor* and the virus it vectors, deformed wing virus (DWV) (Rosenkranz *et al.,* 2010). Chemical control methods that have been developed are not only potentially harmful to the bees themselves but are also ultimately unsustainable (Blacquière *et al.,* 2019). Over-time honey bees can develop resistance to the mite and thus survive without treatment and such populations have been observed in number of regions across the globe (Allsopp, 2006; Kruitwagen *et al.,* 2017; Oddie *et al.,* 2018; Martin, 2020; Mullin *et al.,* 2010; Underwood, Traver, & Lopez-Uribe, 2019). Natural *Varroa* resistance is defined as the ability of a population to survive long term without any treatment for *Varroa* within a given environment (Büchler *et al.,* 2010). It is clear that resistant populations have several traits in common that may help them to survive *Varroa* infestations. However, it is also apparent that resistance is not simple and as such the full mechanism behind it is thus far unknown (Büchler, Berg & Le Conte, 2010). Therefore, the main aim of this thesis is to understand the development and maintenance of natural *Varroa* resistance with a view to encouraging the development of resistant populations worldwide.

Firstly, to understand the more recently recognised hygienic behaviour known as recapping, I undertook a study looking at the spatial patterns of recapping behaviour with respect to *Varroa* infestation. This led to the discovery that honey bees recap cells in a clustered pattern associated with infested cells. This suggested that recapping behaviour is a way to prevent mistakes in *Varroa* detection from causing the loss of valuable brood. The uncapping of non-infested cells was commonplace even among resistant colonies indicating that *Varroa* detection is not a fool-proof mechanism. As it is suggested that chemical cues are involved it is perhaps possible that these cues are not easy to locate accurately, for example, if highly volatile, cues may diffuse from their source.

Secondly, I gathered data on the different traits of resistant honey bees. Over the past decades a wealth of information has been collected on the individual traits of resistant honey bees; however, to the author's knowledge, this information had previously not been linked. Therefore, I collected data on each trait and used this to provide evidence for a network detailing how each trait is related and ultimately leads to *Varroa* resistance. As this suite of traits has arisen in separate populations across the globe, it is possible too that this represents a case of parallel evolution.

Thirdly, I undertook a follow up study from the original 2009-2010 study of DWV on Hawaii to see how the DWV strain dominance had altered and to compare this to global prevalence. DWV strain is an important consideration in the development of *Varroa* resistance as the strains are believed to have differing virulence. In areas without DWV, honey bee colonies can tolerate much greater loads of *Varroa* mites indicating DWV is a key part of their lethality and as such may play a large role in the development of resistance by providing additional selective pressure. The study found that in Hawaii, as in numerous

other regions, the prevalence and load of DWV-B had increased to the point at which it actually dominated on one of the islands.

Finally, I conducted a review of all available literature on the resistance traits of *Apis cerena*. My goal was to compare the traits in *A. cerana* to those exhibited in *A. mellifera*. The results of this study indicated that more research is needed on the resistant traits of *A. cerana.* The results of my studies helped to build a framework of how resistance is developed and maintained within honey bee colonies which will help educate future efforts towards the encouragement of resistance in honey bees.

GENERAL INTRODUCTION

The honey bee

Honey bees belong to the genus *Apis* which to date contains ten recognised species. These species can be roughly grouped into three main types: cavity nesting bees, giant bees and dwarf bees (Arias & Sheppard 2005; Raffiudin & Crozier 2007). The two species that are most directly used by humans are of the cavity nesting variety; these are the Western honey bee, *Apis mellifera,* and the Eastern honey bee, *Apis cerena*. *A. cerena* having originated in a tropical climate with mild winters is the least productive species in terms of honey (Le Conte & Navajas, 2008; Pirk *et al.,* 2017). As a result, *A. mellifera* has long been favoured by humans and has been transported across the globe to regions with different endemic species or sub species (Moritz *et al.,* 2005). Originally, the native range of *A. mellifera* was large spanning Europe, the Middle East and Africa (Han *et al.,* 2012); however, it now exists worldwide, in the form of one of the 29 recorded subspecies.

Honey bee colonies are often described as super organisms, made up of thousands of individual members with a complex division of labour. The colonies are comprised of three castes, the queen, the workers and the drones. A typical colony will contain one queen, approximately 2000 drones and up to 50000 workers and a healthy queen can lay a maximum of 1000-2000 eggs per day. Sex determination within colonies is haplodiploid; the queen produces haploid males and diploid females. The males are known as drones that exist to mate with queens at a drone congregation area outside the colony. Queens are polyandrous, they typically mate with twelve drones and store the sperm within their spermatheca to later fertilise eggs (Tarpy *et al.,* 2013). If a queen mates with twelve drones, there will be roughly twelve half-sister groups or patrilines within the colony (Tarpy *et al.,*

2004). The diploid females laid by the queen either become queens or more commonly workers. Members of the worker caste are non-reproductive except under specific circumstances; their primary function is to take care of the colony by caring for brood, collecting food, and maintaining homeostasis of the nest. Labour within the worker caste is determined in an age dependent manner (Toth & Robinson, 2005). As workers age they undergo behavioural and physiological changes and switch from one task to another, this is known as polyethism. The average worker will begin life by caring for brood as a nurse, as the worker ages it will begin to perform other in hive tasks such as hygienic behaviour which is normally seen in bees that are 15-20 days old (Arathi *et al.,* 2000). The final job of a worker is collecting supplies for the colony through foraging. This task is always the last performed by a bee as it is the riskiest and most energy consuming; thus, the foraging life stage consistently lasts about 7 to 10 days (Toth & Robinson, 2005).

Physiological and behavioural differences also apply to the season in which they are active; summer worker bees have a mean lifespan of 30-40 days. They are typically reared between late winter and summer and begin as nurses and then switch to foraging approximately 2-3 weeks later. If bees are active during the most productive summer period their lifespan is reduced further to just 25-30 days (Fukuda & Sekiguchi, 1966). In contrast the longest lived of the worker bees are those reared after summer, the diutinus or “winter” bees.

Diutinus bees and honey bee overwintering

Diutinus bees are required, in colonies in temperate climates, to keep the colony warm over winter and to rebuild the colony once winter ends. Therefore, these bees have be known to survive 10 months without any apparent effects of aging (Remolina *et al.,* 2007). Physiologically they are akin to nurse bees, in particular they have similarly large stores of

the glycolipoprotein vitellogenin, which is believed to act as an antioxidant to delay the aging process and inhibit the onset of foraging behaviour (Doke, Frazier, & Grozinger, 2015; Marco-Antonio *et al.,* 2008; Nelson *et al.,* 2007). Diutinus bees begin to develop when pollen stores begin to diminish, and less brood is being laid. Eventually the colony stops producing brood altogether and clusters within the hive for warmth (Doke *et al.,* 2015). This continues until mid-winter when the diutinus bees begin rearing brood, they cluster around the brood and vibrate their flight muscles to generate heat and keep the nest at ~33 °C.

Brood rearing peaks in spring and this build up often results in swarming in which the old queen leaves the colony accompanied by some of the workers to make a new colony elsewhere (Grozinger *et al.,* 2013). One of her daughters, the first new queen to emerge, will take over as queen of the new colony. After summer, brood rearing begins to slow due to the falling temperature and the growing lack of resources. Rearing stops toward the end of autumn as the colony prepares to over-winter (Mattila *et al.,* 2001). Conversely, in tropical climates the life cycle of a colony is based instead around the wet and dry seasons when conditions become adverse and flowering plants differ in abundance.

Colony losses

In the northern hemisphere colonies are particularly vulnerable over winter. The long lifespans and enlarged fat body stores of diutinus bees are crucial for the colony to survive the winter period (Amdam *et al.,* 2007). The premature deaths of these winter workers can cause a colony to become too cold and die. Many stressors including nutritional deficits, diseases, bad weather, pesticides, and parasites can shorten the extended lifespans of diutinus worker bees (Amdam & Omholt, 2002). Even if the colony survives the cold, the loss of the workforce has a serious impact on the future colony size and structure as it means

reduced brood rearing and a smaller spring population with a skewed division of labour (Perry *et al.,* 2015). As such the weight of a colony in autumn before entering the winter period is an important predictor for winter survival (Doke *et al.,* 2015). Although, colony genotype also appears to be an important factor in winter bee survival. In natural conditions honey bee populations can adapt to their local environment and climate hence the existence of regional subspecies. However, a colony's ability to adapt to the local environment and conditions may be hampered by the mediation of ill effects by the beekeeper and the extensive transporting of bees in migratory bee keeping operations (Simone-Finstrom *et al.,* 2016).

In recent times colony losses, especially over the winter period, have been troublingly high. Some colony losses in early spring are normal but the number of colonies that have been collapsing over the years, especially during the overwinter period, is extensive (VanEngelsdorp & Meixner, 2009). Indeed, historically there have been other periods of high colony losses; however, these were different to the current situation in that the duration was much shorter. Whilst not unprecedented the recent large number of losses is particularly concerning as there is no guarantee of a stable future for honey bees and other species that depend on them (VanEngelsdorp & Meixner, 2009). Importantly, the declining health of honey bees is also an indicator as to the health status of wild populations and other species of bees and pollinators (Manley *et al.,* 2019).

The honey bee immune system

Colonies are also vulnerable over winter as the honey bee immune system, specifically the response to bacterial pathogens, is downregulated in order to conserve energy (Simone-Finstrom *et al.,* 2016). The immune system is important to honey bees as being a eusocial

species with a dense aggregation of colony members they are particularly vulnerable to disease and parasitization. As a result, the immune system of honey bees consists of both individual immune systems within each bee and a social immune system which entails the collective work of the colony (Cremer, *et al.,* 2007; Evans *et al.,* 2006). This two-pronged immune defence is important as honey bees have only a third of the number of immune genes compared to solitary insects (Evans *et al.,* 2006).

The immunity of an individual bee involves physical barriers such as the cuticle and peritrophic membranes of the digestive tract. For instance, in adult bees the fully developed gut epithelium protects against *Paenabacillus larvae*, the bacteria that causes American foulbrood (Yue *et al.,* 2008). Beyond physical barriers there are also cellular and humoral responses which are active against a variety of pathogens such as the bacterial agents of both American (*Paenabacillus larvae*) and European foulbrood (*Melissococcus plutons*) as well as fungi like *Ascosphaera apis* that cause chalkbrood (Li *et al.,* 2018). The innate immunity of honey bees comprises of four signalling cascades, the two nuclear factor-KB (NF-kB) like signalling pathways namely: the Toll, the immune deficiency (IMD), the c-Jun N-terminal kinase (JNK), the Janus kinase (JAK)-signal transducer and the activator of transcription (STAT) pathways (Evans *et al.,* 2006). The Toll and IMD pathways are important in the regulation and transcription of antimicrobial peptides which are active against bacterial and fungal challenges (Gatschenberger *et al.,* 2013). The cellular immune response of bees is less well studied. It largely appears to be the work of haemocytes which carry out the phagocytosis, nodulation, and encapsulation of intruders (Strand & Pech, 1995).

Over 20 viruses are known to affect bees, but the honey bee immune system does not have antibodies (Evans *et al.,* 2006). Instead, some viruses can be destroyed by the small

interfering RNA (siRNA) mediated RNA interference (RNAi) pathway which is stimulated by the replication of the target virus. This pathway is named as such because it uses an enzyme called dicer 2 to cut the replicating virus into pieces known as siRNAs. The siRNAs are then used by the RNA induced silencing complex (RISC) to find and degrade the related viral RNAs (Brutscher *et al.,* 2015). Three of the most common honey bee viruses; deformed wing virus (DWV), black queen cell virus (Al Naggar & Paxton, 2020) and Israeli acute paralysis virus (DeGrandi-Hoffman & Chen, 2015; Galbraith *et al.,* 2015) are known to be targeted by this pathway. In some cases, it may also be possible to artificially kick start these responses via feeding bees double stranded RNA (dsRNA) (Yang *et al.,* 2018). However, some viruses such as black cell queen virus can encode suppressors that inhibit the expression of genes involved in the pathway (Al Naggar & Paxton, 2020). Additionally, viruses that frequently recombine such as DWV may be able to evade RNAi by recombining at sites that are usually targeted by the RNAi machinery (Ryabov *et al.,* 2014).

The social immune system

Outside the individual bee, the social immune system also functions to prevent the spread of virus and other pathogens within the colony. However, unlike the individual immune system the social immune system works to protect the colony which at times can come from sacrificing an individual. Indeed, hygienic behaviour, which is the removal of adults and brood that are infected, dead, or parasitised, is the cornerstone of social immunity among bees (Cremer *et al.,* 2007). In some circumstances highly infected adults remove themselves from a colony of their own volition; however, it is usually the case that they are removed, when dead, by members of the colony known as hygienic bees (Rueppell *et al.,* 2010). Typically, in the literature hygienic behaviour refers to the removal of brood rather than

adults as many diseases are spread through the brood such as American foul brood, for which this behaviour was first described (Rothenbuhler, 1964). Removal helps to contain an infection because it removes the body of the pupae which may contain spores or infective elements. As such this behaviour is most effective if the pupae are expelled before the pathogen reaches the infective stage to prevent transmission occurring during the removal process. A downside to brood removal is the cost of losing valuable brood; however, hygienic behaviour appears to be naturally optimised to prevent loss as bees prioritise the removal of the worst affected pupae, those that are highly infected or have abnormal pheromones as these are going to be the most dysfunctional adults (Bigio *et al.,* 2014; Mondet *et al.,* 2016). In addition to foulbrood, hygienic behaviour has also been observed in response to other pathogens such as the fungal disease chalkbrood (Spivak & Reuter, 2001) and the ecto-parasites of the genus *Varroa*.

Varroa destructor

A number of parasites affect honey bee colonies including the tracheal mite *Acarapis woodi* and the protozoan *Nosema*; however, over the past few decades the mite *Varroa destructor* (Anderson & Trueman, 2000) has become one of the most notorious of these pests. The story of *V. destructor* and its spread worldwide begins with a different species *Varroa jacobsoni* (Oudemans, 1904). Of the four known *Varroa* species *V. destructor*, *V. jacobsoni*, *V. underwoodi* (Delfinado-Baker & Aggarwal, 1987) and *V. rindereri* (Delfinado-Baker & Aggarwal, 1987) *V. jacobsoni* was the first to be discovered, parasitizing Asian honey bees (*Apis cerana*) in Java in the early 1900s (Anderson & Trueman, 2000). Later it was observed more widely throughout Asia, but it rarely caused severe problems for colony survival. This is due to the presence of a degree of balance between the host and the parasite resulting

from a long period of parasite and host co-evolution. However, during the 19th century, the popularity of the western honey bee and the booming international honey bee trade meant that the *Varroa* naïve western honey bee, *Apis mellifera* was brought to Asia and thus into contact with *A. cerana* giving the mite an opportunity to jump host (Anderson & Trueman, 2000; Oldroyd, 1999). The combination of the lower resistance of *A. mellifera* and the uninhibited movement of honey bees meant that *Varroa* quickly became an almost worldwide pest (Anderson & Trueman, 2000). However, contrary to belief at the time it was a completely different species, *V. destructor*, which had begun parasitizing *A. mellifera*. It was not until 2000 that Anderson & Trueman identified the cryptic sister species.

The species *V. destructor* consists of seven haplotypes, two of which are capable of parasitizing *A. mellifera* outside of Asia (Rosenkranz *et al.,* 2010). These two, the Japan, J, and Korea, K, haplotypes vary in terms of mtDNA cytochrome oxidase I (cox I). They are also reproductively isolated suggesting that *V. destructor* underwent at least two independent host shifts (Rosenkranz *et al.,* 2010). The K haplotype, which is currently present worldwide, is thought to have shifted to *A. mellifera* in the 1950s in a region north of the Korean peninsula. Following this it spread from western Russia to Bulgaria (1972), then to Germany (1977) and then finally throughout Europe and the USA. In contrast the host shift of the Japan haplotype is harder to pinpoint. It occurred in the last century in Japan after which it spread to Thailand and Paraguay (1971), Brazil (1972) and North America (1987), a range to which it remains restricted to date (Anderson & Trueman, 2000; Claudia *et al.,* 2003; Muñoz, 2008). It appears that haplotype K may be able to outcompete J as K is now dominant across Brazil and within Japanese apiaries (Ogihara *et al.,* 2020). The other haplotypes of *V. destructor* and the nine known haplotypes of *V. jacobsoni* appear to only be able to reproduce in the drone brood of *A. cerana* (Andino *et al.,* 2016). Andino *et al.* (2016) found

that being on *A. mellifera* caused greater stress to *V. jacobsoni* mites and mites of the other five *V. destructor* haplotypes suggesting why they are unable to reproduce successfully on *A. mellifera*.

Varroa destructor reproduction

A successful reproduction relies on the carrying out of two key phases in the life cycle of *V. destructor* the phoretic phase and the reproductive phase. During the reproductive phase, a female mite lives and produces offspring inside a sealed brood cell. Drone cells are preferred because they offer mites a longer post capping period which means that more offspring can be produced and mate (Fuchs, 1990; Rosenkranz *et al.,* 2010b). Additionally, nurse bees are more attentive to drone brood which provides more opportunity for mites on nurse bees, their preferred adult host, to infest a drone cell (Calderone & Kuenen, 2003; Fuchs, 1990). Mites are attracted toward cells by chemical signals including brood hydrocarbons and brood food constituents such as 2-hydroxyhexanoic acid. The most attractive cells are those of fifth instar larvae. When a mite invades a cell, it moves toward the bottom and hides from hygienic bees within the larval food (Rosenkranz *et al.,* 2010b). Approximately five hours after the cell is capped the larva will have consumed all the larval food which frees the mite. The mite then uses its mouthparts to pierce the larva's integument and feed off of its fat body (Ramsey *et al.,* 2019). Oogenesis is stimulated within the mite and the first egg is laid approximately 70 hours after the cell is capped. The foundress glues this egg to the upper cell wall to prevent it being damaged by movement of the larva as it pupates (Donzé & Guerin, 1994; Steiner *et al.,* 1994). The first egg is unfertilised and always results in a haploid male. Following this, female eggs that have been fertilised are laid in 30-hour intervals (Martin, 1994). In worker brood the mite lays around

three to five eggs and in the drone brood as many as six resulting in approximately 1.3 to 1.45 and 2 to 2.5 mature females per cycle, respectively (Martin, 1995). The offspring feed from a hole created in the cuticle of the pupa and undergo several moults from protonymph to deutonymph and then adult. They become sexually active immediately after the final moult and the male mates with his sisters in the faecal accumulation site (Rosenkranz *et al.,* 2010b). Each female stores the sperm in her spermatheca and once the bee emerges they, along with the mother, leave and attach to adult bees whilst the male remains within the cell and dies (Martin, 2001).

The spermatozoa have to pass through a maturation stage inside the females' genital tract before they can fertilise the female germ cells (Häußermann *et al.,* 2016). Mites that have mated toward the end of their hosts development will need to wait longer before they can invade a cell and reproduce (Häußermann *et al.,* 2016). Typically, the earliest a freshly mated daughter mite can enter a cell is at 3-4 days post mating (Evans & Cook, 2018). During this time, the phoretic phase, the female mites live upon the body of an adult bee, often hiding between the second and third lateral tergites or under the sternites. Mites need to hide as they can be removed or damaged if the bee grooms itself (self-grooming) or is groomed by other bees (allo-grooming) (Pritchard, 2016). Interestingly, and perhaps because of their longer association with *Varroa*, *A. cerana* are much more proficient groomers than *A. mellifera* (Lin *et al.,* 2016). Indeed, if an adult bee suspects it has a mite it can attract other bees to groom it by performing a vibrational dance (Pritchard, 2016). When choosing an adult host, mites prefer nurse bees, which can be distinguished from other workers by the lower composition of (Z)-8-heptadecene on their cuticles (Del Piccolo *et al.,* 2010) (Fernández *et al.,* 1993). Nurses are preferable because they remain within the safety of the hive, provide access to brood and lastly they have an enlarged fat body and

nutrient stores which when fed on has been shown to increase the fertility rate of *Varroa* (Crailsheim, 1986; Fluri *et al.,* 1982; Kuenen & Calderone, 1997; Toth & Robinson, 2005; Xie *et al.,* 2016).

On the other hand, if mites attach to foragers this may favour their transmission to new colonies, particularly if the foragers drift to or rob other colonies (Kuenen & Calderone, 1997). Transmission via drifting and robbing is beneficial for mites as it reduces the inbreeding depression within colonies caused by sibling mating. Inbreeding is also alleviated when multiple foundresses enter a cell, this usually happens during late summer to autumn when the number of *Varroa* mites reaches its peak. Towards the end of autumn there is also a considerable influx of foreign mites into the colony from drifting and robbing bees (Frey & Rosenkranz, 2014). This can have a snowball effect for heavily infested colonies as such colonies are more accepting of drifters (Forfert *et al.,* 2015). Robbing and drifting are also common where hives are kept at high density as the aggregation of similar hives negatively impacts the navigational capacity of bees (Seeley & Smith, 2015).

Negative effects of *Varroa*

Apis mellifera colonies are much more vulnerable to collapse due to mites than are *A. cerana* colonies. The key reason for this is because the mites can only reproduce in the drone brood of *A. cerana* colonies. This severely hampers the population growth of *Varroa* as drones are produced sporadically and only in 100s rather than 10,000s. Reproduction is not possible in the worker brood of *A. cerana* because a protein in the mites' saliva called *Varroa* toxic protein (VTP) kills the worker brood (Zhang & Han, 2018). In *A. mellifera* colonies, worker brood are not susceptible to this protein and thus mites can take advantage of the vast number of worker cells to grow their population dramatically. As a

result, untreated colonies usually die within 1-3 years (Fries *et al.,* 2006; Rosenkranz *et al.,* 2010). In temperate climates, this collapse frequently occurs over winter when the honey bee population is reduced leaving them with a high burden of mites. Consequently, the number of mites in autumn is a critical determinant of whether the colony will survive to spring (van Dooremalen *et al.,* 2012). To prevent winter colony loss, it is recommended that bee keepers reduce the mite burden to below the economic threshold of roughly 2,000 to 3,600 mites (Martin, 2001) before autumn, preferably in summer (van Dooremalen *et al.,* 2012).

A high burden of mites weakens a colony because their feeding negatively impacts honey bee nutrition and immunity by reducing vitellogenin titres as well as protein, carbohydrates and adipose stores (Amdam *et al.,* 2004; Bowen-Walker & Gunn, 2001). Specifically, contents of the mites' saliva act to prevent protein synthesis within the pupae so that the mite has access to an ample supply of free amino acids when it feeds (Aronstein *et al.,* 2012). Consequently, *Varroa* infested pupae emerge as adults with a reduced weight, lifespan, and protein content (De Jong *et al.,* 1982). This is of particular relevance to the diutinus bees whose long-life span relies on an ample vitellogenin and fat supply (Aronstein *et al.,* 2012). Winter workers produced during high mite infestation do not develop the typical features of over wintering bees and have only one third of their expected lifespan (Amdam *et al.,* 2004). The premature death of these workers is highly detrimental as these bees are required to keep the colony warm over winter and to rebuild the colony during the spring (Perry *et al.,* 2015). However, these symptoms are not solely the result of *Varroa* induced nutrient depletion. An arguably more important factor is that *Varroa* vectors a number of honey bee viruses including Israeli acute paralysis virus (Di Prisco *et al.,* 2011), acute paralysis virus (Ball, 1985), sac brood, black queen cell virus, chronic bee paralysis

virus, Kashmir bee virus (Chen *et al.,* 2004; Tentcheva *et al.,* 2004) cloudy wing virus and slow paralysis virus (Carreck *et al.,* 2010; Santillán-Galicia *et al.,* 2010). Possibly the most lethal association it has is with deformed wing virus (DWV) (Brettel & Martin, 2017; Martin *et al.,* 2012; Wilfert *et al.,* 2016).

Deformed wing virus

DWV is an *Iflavirus* of the order Picornavirales. It was first described from samples of deformed western honey bees in Japan in 1986 (Allen & Ball, 1995). It consists of 30 nm icosahedral virion in which there is a single stranded positive sense RNA genome. The genome is roughly 10 kb in size and has a single open reading frame flanked by a long 5'UTR and a highly conserved 3'UTR which function in regulating the replication and translation of the genome (Belsham, 2009). The open reading frame encodes a 2894 amino acid polyprotein which is cleaved to produce non-structural and structural proteins. The genomes functional domains include a helicase, a highly conserved RNA-dependent RNA polymerase (RdRp), two capsid protein domains and a 3C-protease (Lanzi *et al.,* 2006).

The high degree of mutation within the DWV system means that it is thought to exist as a quasispecies (Biebricher & Eigen, 2006; Lauring & Andino, 2010). Under the quasispecies theory, DWV consists of a selection of three master variants, named DWV-A, -B and -C which are surrounded by a 'cloud' of lower fitness genetic variants. DWV-A includes the original classical versions of DWV as well as Kakugo virus and DWV-B includes *Varroa destructor* virus 1 (VDV-1) (Kevill *et al.,* 2017). Each variant in the 'cloud' surrounding these three masters has a frequency that is not determined solely by its fitness but also by the probability of its generation from its neighbouring variants (Biebricher & Eigen, 2006).

Currently of the master variants, DWV-A and DWV-B are prevalent within honey bee colonies whereas the appearance of DWV-C is rare (Kevill *et al.,* 2019).

As expected, DWV was named due to the observation of highly infected individuals with misshapen and unusable wings. Interestingly, since then it has been found that high DWV loads and deformed wings are not mutually exclusive with the symptom sometimes not occurring even in highly infected individuals (Gusachenko *et al.,* 2020; Tehel *et al.,* 2019). Deformed wings are actually only present in a subset of infected individuals (Brettell *et al.,* 2017). The symptom occurs when the virus, seemingly by chance, replicates within the developing wing buds of the pupae (Gusachenko *et al.,* 2020). Disrupted wing development is also not unique to DWV, being a symptom of many other ailments including pupal injuries and hormonal disorders. Thus, the more reliable symptoms of an overt infection are a shortened abdomen and reduced weight on emergence (Mockel *et al.,* 2011).

Deformed wing virus and *Varroa destructor*

Prior to the spread of *Varroa,* overt DWV infections were rare, instead it existed within colonies at very low levels and high strain diversity and rarely caused the death of colonies (Martin *et al.,* 2012). As DWV originated within the honey bee and not the mite, DWV levels in *A. mellifera* colonies were very low for a period following the initial spread of *Varroa*. It was not until the *Varroa* mites themselves became sufficiently infected that DWV was transmitted effectively, and the symptoms and pathology became prevalent (Brettel & Martin, 2017; Le Conte & Mondet, 2017). Infected mites are very efficient DWV vectors as their method of piercing the bee to access its fat body means that DWV is injected straight into the haemolymph. This bypasses the multiple immune barriers including the cuticle and gut allowing it to very quickly replicate to very high levels (Martin *et al.,* 2012). In oral

transmission the gut of the bee is an important immune barrier which effectively hampers the proliferation of DWV passed through contaminated food (Gusachenko *et al.,* 2020). Without *Varroa,* transmission relies on less effective means such as vertical transmission through drone sperm, transovum transmission by adhering to the surface of eggs or horizontal transmission through the consumption of contaminated substances (Amiri *et al.,* 2018; Mockel *et al.,* 2011; Yue & Genersch, 2005).

Initially, *Varroa* was thought to have in some way activated the covert DWV infections within honey bees. However, this proposal has been disputed by the coexistence of *Varroa* and covert DWV in tolerant colonies on the island of Fernando de Noronha (Brettell & Martin, 2017). This island off the Northeast coast of Brazil became home to a small population of Italian honey bees, *Apis mellifera ligustica* circa 1984 (de Mattos *et al.,* 2016). When this population was introduced, mites were accidently brought with them. Since then, the population has remained isolated by strict restrictions on imports to the island. Interestingly, despite never receiving treatments for *Varroa*, no colonies were reported to collapse as a consequence of the mites. Even more curious is they have maintained extremely low levels of DWV; indeed levels that are just at or below the limit of detection by polymerase chain reaction (PCR) methods (Brettell & Martin, 2017). The stable host-parasite equilibrium on Fernando de Noronha may be reliant upon the specific selection of DWV strains that were present before *Varroa* came and the isolation of the population.

Similarly, before *Varroa* DWV loads on the Hawaiian island, Big Island, were very low and the diversity very high. However, unlike Fernando de Noronha, in the two years following the invasion of the mite to this island there was a rapid decrease in viral diversity and concurrent increase in viral loads. At the same time, the islands of Kauai and Maui which

were (and still are) mite-free maintained a very high diversity and very low loads (Martin *et al.,* 2012). Indeed, the *Varroa* mediated route of transmission appears to favour particular strains which, with the reduced competition, can accumulate to greater amounts and dominate populations (Martin *et al.,* 2012; Ryabov *et al.,* 2014).

Why mite vectoring favours particular strains is not clear. It has been suggested that some strains are capable of replication within the mite host. Recently this possibility was confirmed by Gusachenko *et al.,* (2020) but the levels of replication they found were very low. Thus, replication is not likely to be a significant factor in the proliferation of DWV in *Varroa* infested bees (Annoscia *et al.,* 2019). Instead, it seems the high DWV loads in colonies are likely to be the result of some strains having a competitive edge as they are better suited to survive vector transmission and thus replicate without competition within the bee. Vector transmission is difficult as it entails adaptations to survive within two different species. In this case one could speculate that on Fernando de Noronha, the original subset of strains may simply have lacked those which could survive transmission by the mite. Currently, the most successful are the strains of DWV-A which dominate North America (Kevill *et al.,* 2019) and Brazil (de Souza *et al.,* 2019) and strains of DWV-B which is the predominant master variant in the UK (Kevill *et al.,* 2019) and South Africa (de Souza et al., 2020a).

The relative virulence of the master strains may also in part explain their dominance within populations. However, there is a degree of uncertainty about the relative virulence of each master variant as, even at the same load, different variants of DWV can have different effects on a honey bee colony (Barroso-Arévalo *et al.,* 2019). The greater overwinter colony losses in the US would suggest DWV-A to be more virulent than DWV-B but this has been

contradicted by laboratory tests on adult bees (McMahon *et al.,* 2016). Studies on pupae have had equally mixed results, with Tehel *et al.,* (2019) finding that DWV-A and DWV-B have a similar virulence and Norton *et al.,* (2020) finding that DWV-B is less virulent. Interestingly, a number of these studies found that DWV-B replicated to greater amounts than DWV-A in co-infections. If coupled with a lower virulence this could explain why DWV-B is now the dominant strain in the UK, South Africa, and parts of Europe (de Souza *et al.,* 2019; Kevill *et al.,* 2019; Manley *et al.,* 2019; Natsopoulou *et al.,* 2017)

DWV and the death of colonies

DWV mediated colony collapse is usually a result of the reduced productivity of workers and the destabilisation of colony structure. DWV rarely kills pupae outright, instead they emerge later, function sub-optimally and die earlier (Benaets *et al.,* 2017; Koziy *et al.,* 2019). When infected as pupae, adults have a lifespan that is reduced by roughly two thirds; this is particularly detrimental in the case of the diutinus bees as the colony cannot produce enough brood to replace them as the season changes (Martin, 2001). In temperate climates autumn DWV loads, like *Varroa* loads, can predict the likelihood a colony will collapse over winter (Dainat & Neumann, 2013; Natsopoulou *et al.,* 2017). As expected DWV loads in colonies positively correlate with *Varroa* loads thus as the mite population grows to a peak in autumn so does the load of DWV. The enhanced fat bodies of winter bees are also thought to be prime locations for viral replication (Locke *et al.,* 2017). This leads to the higher observed DWV loads in both the colony as a whole and in individual winter workers compared to summer workers (Steinmann *et al.,* 2015). Combined with the dampened immunity of bees in winter this results in a deadly crescendo for the colony (Barroso-Arevalo *et al.,* 2019).

A critical part of the reduced lifespan is that DWV accelerates the bees natural polyethism sequence towards the final stage, foraging. DWV-A, in particular, has been implicated in behavioural maturation and precocious foraging (Pizzorno *et al.,* 2021; Traniello *et al.,* 2020). Precocious foraging, similar to deformed wings, is not unique to DWV infections, it is a natural response of bees to stressors that allows colonies to replace lost foragers and accumulate resources. However, if the stressor is chronic, like a DWV infection, then it becomes pathogenic as the long-term loss of workers and alteration of the work force destabilises the colony (Perry *et al.,* 2015). Additionally, despite foraging earlier, infected bees actually provide less for the colony as they have shorter activity spans, reduced flight capabilities and collect less pollen and nectar (Benaets *et al.,* 2017; Wells *et al.,* 2016). A lack of incoming resources and nurse bees (who have become foragers) may also promote a degree of nutritional stress within colonies. Nurse bees, unlike other workers, can adequately digest pollen and so use pollen to create food for the rest of the colony (Amdam *et al.,* 2009). DWV not only reduces the number of nurses but impacts digestion abilities which could lead to a reduction in the production and quality of the royal jelly and hence cause nutrition stress to the colony (Koziy *et al.,* 2019).

Even bees that are infected as adults undertake foraging at an earlier age, although the reduction in their lifespan is minimal compared to those infected as pupae. Foragers typically do not last long and thus the transition to foraging marks the ending of a bee's life. During this transition many changes that are associated with immune-senescence and aging take place in order to save energy. Specifically, there is a reduction in immunity following the deformation and apoptosis of the specialised blood cells known as haemocytes (Wille & Rutz, 1975). The fat body and hypopharyngeal glands also begin to atrophy causing protein and lipid stores to decline and halting vitellogenin synthesis (Benaets *et al.,* 2017). For the

virus, stimulating early foraging may be beneficial as it may help it to amplify horizontal transmission (Benaets *et al.,* 2017). Infection itself promotes the drifting of bees to other colonies as the localisation of DWV to the honey bee brain affects learning and memory (Fujiyuki *et al.,* 2009; Pizzorno *et al.,* 2021).

Infected individuals also experience a reduction in their ability to fight other pathogens. This is because, in concert with *Varroa*, DWV downregulates elements of the honey bee immune system. Particularly affected are the Toll related genes, the impairment of which leads to a reduction in the level of NF-kB transcripts including dorsal 1a (Nazzi *et al.,* 2012; Ryabov *et al.,* 2014). Dorsal helps regulate the expression of the antimicrobial effectors hymenoptaecin and Defensin-1 (Evans *et al.,* 2006). Interestingly however, both *Varroa* and DWV are required for this reduction in immune capacity to be sufficient as to allow viral replication to increase substantially. Specifically, *Varroa* downregulates immune genes such as autophagic specific gene 18, allowing DWV to replicate without control (Navajas *et al.,* 2008; Nazzi *et al.,* 2012). In turn the DWV mediated NF-kB disruption means that the wound the mother mite creates on the pupae to feed is less able to clot allowing her offspring to feed freely (Nazzi *et al.,* 2012). Conversely, some elements of the bees' immune system are upregulated during DWV infection. Whilst potentially beneficial, this may also be detrimental to the bee as this upregulation is costly in terms of energy which is depleted in infected bees or *Varroa* parasitised bees (Shen *et al.,* 2005). Whilst *Varroa* enables DWV to get a foothold it seems that persistent *Varroa* mite presence is not necessary for DWV to cause an overwinter colony loss (Highfield *et al.,* 2009). It could be said that *Varroa* 'kick starts' the DWV infection as Highfield *et al.* (2009) found that reducing the *Varroa* load before the overwinter period did little to reduce the DWV titres (Highfield *et al.,* 2009). The efficacy of chemical control methods depends heavily on the timing of their application

(Beyer *et al.,* 2018). If the colony is to see the benefits overwinter, mite populations need to be reduced in summer.

Varroa management

Currently, colony survival relies heavily on human intervention such as through the application of chemical controls including acaricides and organic acids. Chemical control methods, however, are not without problems. From a fiscal point of view, they can often be unviable for small time beekeepers, not only because of the cost of the chemicals themselves but also because they contaminate many of the sellable beehive products. The contamination of in-hive products also poisons the bees as colony members unwittingly feed from the toxic pollen and honey stores. This contamination can persist in colonies for a long time as chemicals can impregnate the beeswax, which is recycled by the bees (Mullin *et al.,* 2010). Over time this constant exposure overwhelms the abilities of bees to detoxify the chemicals themselves, leading to acute and sub-lethal effects on their health and behaviour. Sub-lethal effects can occur through the alteration of gene expression in the bees such as the downregulation of vitellogenin production which in turn accelerates immunosenescence and shortens lifespan (Boncristiani *et al.,* 2012). The constant presence of chemicals within the hives also promotes acaricide resistance within *Varroa* thus decreasing the time until the inevitable inapplicability of such chemicals (Beaurepaire *et al.,* 2017). *Varroa* mites, due to their short generation time and high levels of inbreeding, can rapidly develop resistance to them. Whilst the frequently used formamidine, Amitraz, remains largely useable other acaricides such as organophosphates have already been rendered ineffective (Evans & Cook, 2018). Mites do have periods when they outbreed due to overcrowding and it is believed that using acaricides during this period may be more effective at controlling the mite

population (Beaurepaire *et al.,* 2017). Nonetheless controlling mites with acaricides is ultimately unsustainable.

Additionally, chemical control and other human interventions to mediate the effect of *Varroa* infestation remove the selective pressure from the honey bees that is required for them to adapt (Neumann & Blacquière, 2017). This creates a dependence of bee populations on a treatment that is becoming increasingly ineffective (Meixner *et al.,* 2015; Neumann & Blacquière, 2017). If untreated, colonies may be capable of naturally developing resistance to *Varroa*. However, simply stopping treatment is not feasible as it is likely to incur dramatic colony losses. This would be disastrous for many who rely on bees for income and also would not be effective in areas that have a high density of colonies and mite transfer. Also, many domesticated bees have a reduced genetic diversity which may hinder their attempts to adapt (Neumann & Blacquière, 2017). Indeed, since bees were first domesticated circa 2600 BCE they have been selectively bred, whether purposeful or not, for desirable traits such as larger populations, no swarming, earlier and prolonged brood rearing, high honey yield and gentle temperament. Not only has this reduced diversity but many lost traits such as a small population size and swarming were those that enable populations to resist over-infestation and disease (Loftus *et al.,* 2016; Mikheyev *et al.,* 2015). Frequent swarming disrupts *Varroa* population growth as the *Varroa* load is shared and the swarming colony undergoes a brood-less period inhibiting *Varroa* reproduction (Loftus *et al.,* 2016; Rangel & Seeley, 2012).

Honey bee resistance to *Varroa*

In recent years there has been an increasing amount of study on the presence of colonies that are naturally resistant to *Varroa*. This is the ability of a population to survive long term,

more than five years, without any treatment for *Varroa* within a given environment (Büchler *et al.,* 2010). These naturally resistant (NVR) colonies have been reported in many regions including Russia (Rinderer *et al.,* 2001), mainland Europe (Oddie *et al.,* 2018), South Africa (Allsopp, 2006; de Souza *et al.,* 2021) Brazil (Martin *et al.,* 2019), the UK (Hawkins, 2020) and Tunisia (Boecking & Ritter, 1993).

The oldest NVR populations are the African honey bees, African derived honey bees (AHB) and the East Russian primorski bees (de Mattos *et al.,* 2016). Resistance often appears to be achieved following periods of high colony losses. This pattern has been observed in wild populations of bees suggesting they are can eventually adapt to the challenge and a balanced host parasite relationship may evolve in colonies over time (Locke & Fries, 2011; Villa *et al.,* 2008). An exception to this is the Brazilian AHBs, following the invasion of *Varroa* in the early 1970s there were few, if any, documented *Varroa* caused colony losses in the Africanised bees (De Jong *et al.,* 1984; Guerra *et al.,* 2000; Rosenkranz, 1999). In contrast when the mite invaded South Africa circa 1997 the cape bees (*Apis mellifera capensis*) and savannah bees (*Apis mellifera scutellata*) did initially experience a period of enhanced colony losses (Allsopp, 2006; Moretto *et al.,* 1991). Although this period was short lasting only 3-5 years for the cape bee and 6-7 years in the savannah bees (Allsopp, 2006). Similarly, another subspecies of African bee the Tunisian bee (*Apis mellifera intermissa*) was also observed surviving *Varroa* infestation without treatment in the 1990s following *Varroa* invasion some decades previously (Kefuss *et al.,* 2004; Ritter *et al.,* 1990).

The occurrence of resistance within European honey bees has been comparatively rarer and slower than in their AHB and African counterparts. Partly, this is due to the use of acaricides which, unlike in Europe, was not commonplace in Africa or South America. Potentially, like

with Fernando de Noronha, it may also be dependent on the virulence of the DWV strains initially present within the bees. Indeed, it has been suggested that populations in South Africa may have lacked a virulent strain of DWV as colonies had extremely high burdens of mites reaching up to 30,000- 50,000 (Allsopp, 2006). Conversely, in South America virulent strains were present. Thus, their success may be due to the fact that the majority of honey bees were feral and so were not exposed to acaricides or management practices that prevent natural behaviours such as swarming (van Alphen & Fernhout, 2020).

Similarly, many colonies in which resistance was first observed in Europe were either feral, abandoned colonies such as those in observed in Le Mans and Avignon (Le Conte *et al.,* 2007) or the product of "bond" experiments like the Amsterdamse Waterleidingduinen, Tiengemeten and Gotland populations (Blacquière *et al.,* 2019). Bond experiments are negative selection experiments, in which there is no treatment and minimal human interference following the principal of 'live and let die' (Blacquière *et al.,* 2019; Fries *et al.,* 2006). In the case of the Gotland population which began in 1990, 80% of the colonies collapsed during the first three years before populations began to stabilise (Fries *et al.,* 2006). Such fast resistance development is comparative to that of AHBs and African bees. The isolation of the bees could be what helped fast forward the development of resistance by preventing the dilution of resistance alleles from susceptible, treated colonies (Neumann & Blacquière, 2017). The panmicitc mating structure of bees usually prevents local natural selection as the resistance alleles are dispersed into local populations faster than they are acquired by natural selection (van Alphen & Fernhout, 2020). Additionally, it is thought that closed populations (Arnot forest, Gotland, Swindon) encourage the evolution of lower virulence because pathogens and parasites are transmitted vertically (Fries & Camazine, 2001). In this scenario those that kill the host are less able to be transmitted. On the other

hand, whilst isolating colonies during negative selection experiments could speed up the acquisition of resistance it is also likely to come with a reduced genetic diversity. Inbreeding may actually hinder the bees from becoming fully resistant as well as severely impacting the adaptive flexibility of bees in the face of other stressors (Blacquière *et al.,* 2019; Neumann & Blacquière, 2017).

Unfortunately, the negative selection experiments in Europe appear to have been unable to produce fully resistant colonies, due to inbreeding (Gotland) or the dilution of resistance genes. The latter appears to have been a problem for the resistant colonies created at Avignon and Le Mans. Those colonies were created from colonies that had already been surviving without treatment for at least three years using a minimal interference protocol (Le Conte *et al.,* 2007). They were also kept in a similar environment to where they came from (Le Conte *et al.,* 2007). Keeping them in the same environment is beneficial as the stable host-parasite equilibrium may in fact be a balance of genotype-environment interactions which is only effective under the conditions of original location (de Mattos *et al.,* 2016). Despite this attaining full resistance proves to be difficult as the surviving colonies are part of a panmicitc population surrounded by colonies with a low frequency of resistance traits. Thus, it seems pertinent to view resistance as a trait of a whole breeding population rather than a colony or an apiary-based trait.

The traits of *Varroa* resistant bees

In general, negative selection schemes like the bond experiments could be better than positive selection as there is not selection for specific traits (Blacquière *et al.,* 2019; Neumann & Blacquière, 2017). This is important because, at present, the relative importance of the different resistance traits is not clear. As well as this these traits are also

not always easily recognised and selected for. Honey bee resistance to *Varroa* has been an important area of research for many years. The basic principle allowing resistant colonies to survive is that they hinder the growth of the mites' population which in turn decreases the DWV load of the colony. Honey bees can control the mite population growth primarily by reducing the mites' ability to reproduce. For instance, the Fernando de Noronha bees display mite reproductive rates as low as 0.54 (Brettell & Martin, 2017) compared to reproductive rate of 1.4 when unimpeded. All mite populations contain a certain proportion, 5-20%, that are infertile due to missing or immature gametes (Wendling *et al.,* 2014) and more that are reproductively unsuccessful, that is they do not produce viable offspring (Rosenkranz *et al.,* 2010b). However, this proportion is increased in resistant colonies. A reduction in mite reproduction was first described in the 1990s by Harbo and Harris who coined the term suppression of mite reproduction (SMR): selective breeding for bees' apparent suppression of mite reproduction produced the *Varroa* sensitive hygienic stocks (VSH) (Harris, 2007).

Eventually it was discovered that it was the work of adult bees in removing infested brood that drove the phenomenon (Harris, 2007). This removal behaviour was termed *Varroa* sensitive hygiene (VSH) to distinguish it from general hygienic behaviour. *Varroa* infested brood removal follows the same process as hygienic behaviour, the key difference is simply the cue used to detect the malady. Thus, to shift hygienic behaviour in favour of removing *Varroa* infested brood the ability to detect the associated cues is necessary. Although as the basic mechanism of removal already existed it may explain how resistance can develop fairly rapidly (Allsopp, 2006; Perez & Johnson, 2019).

Brood removal plays an important role in resistance. Removing *Varroa* parasitised brood helps to control the mites' population growth and limit the DWV burden of the colony. This is because, whilst brood removal does not necessarily kill the mites, as they usually escape, it does interrupt a single mite's reproduction cycle and its tight synchrony with pupal development (Kather *et al.,* 2015; Kirrane *et al.,* 2011). Emptying the cell's content also destroys any eggs a foundress mite may have produced. After being dislodged the foundress may be able to enter another cell but, due to the asynchrony between her part begun reproduction and the pupa's development, she is likely to have reduced reproductive success and produce inviable female offspring (Kirrane *et al.,* 2011). Thus, at the individual level high levels of brood removal could render a mite as circumstantially non-reproductive, i.e., they have eggs and sperm but get interrupted repeatedly. Indeed Wendling *et al.,* (2014) found that the majority of foundresses that did not lay eggs had full spermathecea. Interestingly, it has been suggested that this asynchrony could be exacerbated by the pupae themselves who may be capable of hindering mite reproduction by altering their own developmental factors that the mites normally use to initiate oogenesis and other stages of reproduction (Frey *et al.,* 2013; Mondet *et al.,* 2016). For example, mutations in the ecdysone pathway could prevent the initiation of vitellogenesis and reproduction in the mite (Conlon *et al.,* 2019).

Ultimately, frequent interruption also makes it more likely that the daughters a foundress produces, if any, will be infertile as they will have less chance to copulate, due to either a missing male or reduced time within the cell (Harbo & Harris, 1999). If offspring manage to mate it may be very close to the emergence of the bee meaning the females will have to remain in the phoretic period for longer, increasing the time between reproductive cycles (Häußermann *et al.,* 2016; Rinderer *et al.,* 2001). Typically, a female mite undergoes two to

three reproductive cycles after which she runs out of eggs. If the interruption is persistent the mother mite will run out of eggs and sperm potentially without contributing to the next generation. She will then be infertile to the fullest extent and may have only produced infertile daughters (Kirrane *et al.,* 2011). Viewing this from a population perspective, there would be fewer mites contributing to the next generation thus meaning a slower population growth and a reduced proportion of new fertile mites compared to old eggless mites and young unfertilised mites (Harris *et al.,* 2010). Mite infertility appears to be increased in resistant populations including South African bees, Africanised bees in Mexico and the Gotland bees in which respectively 61% (Allsopp, 2006), 44% (Medina *et al.,* 2002) and 52% (Locke & Fries, 2011) of mites were infertile. However, measuring mite non-reproduction comes with a large amount of variation and so it is difficult to get an accurate result particularly with small sample sizes (Eynard *et al.,* 2020).

Therefore, in a colony that displays a high level of brood removal, a large proportion of the mites will be unable to reproduce and there will be a marked reduction in the population growth of and thus the number of mites. This in turn reduces the DWV load as there are fewer vectors to spread the disease plus the heavily infected pupae are also removed. African and Africanised honey bees (AHB) are well documented as having exceptional removal abilities such as the cape bee which removes 54% of mite infested brood (Martin *et al.,* 2019) and Brazilian AHBs which remove 56% (Guerra *et al.,* 2000). In contrast the brood removal capabilities of NVR-EHBs appear somewhat lower with 32 to 41% infested brood removed by the Fernando de Noronha bees (Guerra *et al.,* 2000). However, this can be partially accounted for by the fact that NVR-EHBs have been less well studied and often in smaller sample sizes. There is also lot of variability in the measurement of brood removal due to differing measurement methodologies. Moreover, bioassays that measure hygienic

behaviour toward dead brood are often used to infer a colonies ability to remove *Varroa* infested brood.

These assays are the pin killed brood (PKB) assay and the freeze killed brood (FKB) assay. The PKB method is popular for screening colonies for further testing as it is the simplest and most convenient method. However, it also happens to be one of the most problematic (Newton & Ostasiewski, 1986). It involves killing a section of brood by stabbing them through the cell capping with a pin and recording the proportion removed after a set time. Results for PKB are inconsistent as the pin size is not standardised and so the damage done to the pupae, haemolymph leakage and cue intensities vary greatly within and between studies (Leclercq *et al.,* 2018). The Gotland population was deemed to have low hygienic abilities using the PKB method and so their ability to remove *Varroa* has thus far gone un-studied.

In contrast one of the most well-known methods, the FKB method is less variable and kills the brood whilst keeping cells intact. This assay, originally described by Taber (1982), involves freezing patches of brood with liquid nitrogen (freezer used in original method) to kill the brood. The patch of brood is then returned to the hive and the proportion of dead pupae that have been detected and removed after 24 hours is recorded (Spivak & Downey, 1998). This method is popular as it is fairly quick, keeps cells intact and does not involve pathogens (Leclercq *et al.,* 2018). One of the most well-known breeding lines, the Minnesota hygiene (HYG) line was selected based on the removal of freeze killed brood (Spivak, 1996). FKB is often used as a proxy for hygienic behaviour towards *Varroa*. However, it has been observed that there is no correlation between FKB and *Varroa* removal (Boecking & Drescher, 1992) and that bees selected for high FKB, often have low

Varroa removal abilities (Danka *et al.,* 2013). This is because in FKB pupae are killed which is likely to release different cues than the infestation by *Varroa* in which pupae usually survive (Spivak, 1996). The pupae are also killed simultaneously and in the same location which is likely to create a high concentration of cues making it easier to detect than say one mite infested cell surrounded by a number of normal cells.

The most accurate way to measure the ability of bees to remove *Varroa* infested cells is to monitor the brood removal of artificially or naturally infested cells (Leclercq *et al.,* 2018). To infest cells, the capping is carefully peeled back with a razor to introduce the mites and then resealed with warm wax (Martin *et al.,* 2019). Thus, it is an incredibly time consuming and labour-intensive method compared to FKB and PKB. To keep cell caps intact artificial infestation can also be accomplished using a Jenter comb in which the cells can be opened at the bottom to insert mites. However, the choice of comb used, wax or plastic, and the method of inserting the mite, top or bottom of cell, can contribute to variation in the results achieved (Leclercq *et al.,* 2018). In contrast, natural infestation involves less fiddly work but it is difficult to locate infested cells to monitor and the sample size can be restrained by the low infestation rates in resistant colonies (Vandame *et al.,* 2002).

Recently, it has been suggested that recapping may be a good proxy for brood removal (Martin *et al.,* 2019). It occurs in high levels in resistant colonies along with brood removal (Hawkins, 2020) and positively correlates with the level of *Varroa* infestation (Beaurepaire *et al.,* 2019). Recapping is a behaviour in which a cell capping, or more frequently just a part of it, is removed to allow bees to check for the source of infestation before resealing them, if non-infested (Martin *et al.,* 2019). As such recapping is an alternative ending to hygienic behaviour that prevents the removal of healthy brood. Crucially, the hygienic response to

Varroa infestation follows three key steps, which are undertaken by different bees with different sensory acuities (Scannapieco *et al.,* 2016). One bee will act as the initial detector which isolates and partially uncaps suspicious cells. A second bee will investigate the suspicious cells and if they are triggered, will fully uncap, and remove the contents. Otherwise, a third bee will recap the partially uncapped cells (Scannapieco *et al.,* 2016). As such, mistakes can often be made in which healthy cells are uncapped by highly sensitive bees that detect cues from another cell and that infested cells are recapped by bees with a very low sensitivity. Indeed, it is thought that the 'recappers' could be the non-hygienic members of the colony as in all colonies there are several patrilines and thus it is likely some of the bees in the colony may not be hygienic (Gramacho & Spivak, 2003). Thus, it is possible for sensitive 'uncappers' to be present in a colony as their mistakes can be remedied by less sensitive 'recapper' bees. Mistakes appear to be quite common even in resistant colonies as there is a high level of recapping of healthy, non-infested cells. Why so many non-infested cells are mistakenly uncapped and then recapped is not clear, but it may be due to the diffusion of the chemical cues from infested cells to nearby non-infested cells. Because of this it is thought that hygienic behaviour may be controlled by two separate cues, one that initiates creation of a hole in the cap and a second that triggers brood removal or in its absence recapping.

The cues involved in detecting *Varroa* infestation

The precise identity and origin of chemical cues involved in *Varroa* infested brood removal have not yet been clarified. Considering the mite is the problem, it would be logical to suggest that it is the source, but it does not appear to be this simple. Mites can camouflage themselves very effectively using cuticular hydrocarbons from the bee, mimicking their

odour profiles. Hence the cue is highly unlikely to come from the chemical profile of the mite itself (Kather *et al.,* 2015). Additionally, worker bees do not uncap newly infested cells suggesting the cue is something that may take some time to be produced or to reach a detectable concentration rather than a scent from the mite (Harris, 2007). Instead, it has been suggested that the cue could be a chemical produced during the ovulation of the mite as cells containing a greater number of offspring were found to have a greater probability of being uncapped (Kim *et al.,* 2018). However, it could also suggest that the brood is the source of the signals for hygienic behaviour (Wagoner *et al.,* 2018). A greater number of mite offspring in the cell would put a greater pressure on or cause more damage to the brood inside and hence could cause the release of a high concentration of stress related cues.

Indeed, DWV and *Varroa* stress causes changes to the cuticular composition of pupae and adults which appear to be detectable by other colony members (Baracchi *et al.,* 2012; Wagoner *et al.,* 2020). These cuticular hydrocarbons (CHCs) have been suggested to be the initiators of hygienic behaviour (Mondet *et al.,* 2016; Nazzi *et al.,* 2004; Salvy *et al.,* 2001; Schoning *et al.,* 2012; Wagoner *et al.,* 2019; Wagoner *et al.,* 2020). Two chemicals associated with *Varroa* and DWV stressed brood (Z)-6-pentadecene (Z6-C15) and (Z)-10-tritriacontene (Z10-C33) have been found to elicit hygienic behaviour (Wagoner *et al.,* 2020). Being of high and low volatility, respectively, these may act as the primary (pentadecene) and secondary cues (tritriacontene) discussed earlier (Wagoner *et al.,* 2020).

However, another study by Mondet *et al.* (2021) isolated six non-CHC cues consisting of four ketones and two acetates from infested cells. The difference may have come from their decision to look at cells during pupal development whereas Nazzi *et al.,* (2004) utilised those

from the first post capping phase. Despite the difference both studies suggest that the cue is likely to be made up of a mixture of molecules. This could provide the dual step mechanism described but also could build some redundancy into the signal. Moreover, as the cues potentially come from stressed brood, it may be that brood removal is somewhat reliant upon the ability of the brood to produce these signals for the adults to detect (Wagoner *et al.,* 2018). The presence of a brood effect on hygienic behaviour has been observed in *A. mellifera* colonies (Wagoner *et al.,* 2018). Using a cross fostering system Wagoner *et al.,* (2018) found that hygienic brood was more likely to be removed than non-hygienic brood no matter which colony type (hygienic or non-hygienic) it was fostered in.

Interestingly, the enhanced hygienic behaviour of *A. cerana* compared to *A. mellifera* may also be because of a brood effect. *A. cerana* pupae are more susceptible to *Varroa* and other damage inflicting stressors (Lin *et al.,* 2016). The heightened susceptibility of pupae to damage may mean that they produce a greater cue in response and are thus more likely to be removed (Page *et al.,* 2016). In terms of *Varroa*, the toxic saliva means that the mites often cannot reproduce in worker brood at all (Zhang & Han, 2018). In hygienic or resistant *A. mellifera* colonies a greater reaction of pupae to damage from the mite may explain the amplified removal response. Whether the damage relates more to the feeding hole created by the mites, nutritional stress from mite feeding or the transmission of disease is not clear. Although, pupae parasitised by mites with a highly virulent form of DWV have been found to produce an odour that was more distinct than pupae parasitised by mites with a less virulent form (Schoning *et al.,* 2012).

As well as the capacity to create cues, another important factor to consider is the ability of worker bees to detect the cues. Olfactory senses are vital for hygienic behaviour; the

olfactory sensitivity of an individual relies on the sensitivity of their antennae (Mondet *et al.,* 2016). Within a colony, it appears that the individual workers vary in their olfactory sensitivity as do the workers that carry out the different stages of hygienic behaviour (Scannapieco *et al.,* 2016). Individuals with a greater olfactory sensitivity initiate the behaviour by perforating and removing the cappings of cells and those with a lower sensitivity complete the behaviour by removing the brood (Gramacho & Spivak, 2003). It seems that all bees have the potential to detect and remove brood but that some have a lower threshold for response (Gramacho & Spivak, 2003). This optimises the behaviour by utilising the most sensitive individuals for the most sensitive stage rather than having them waste time on other stages of the behaviour that could be done by any bee. Hygienic colonies may therefore be more efficient because they have a greater proportion of highly sensitive individuals.

Bees bred for VSH behaviour have different expression patterns of olfactory and metabolic genes on their antenna compared to non-VSH bees (Mondet *et al.,* 2015). Hygienic behaviour appears to be controlled by a limited gene set and so differences in hygienic behaviour are caused by an alteration of gene expression. It is thought that exposure to *Varroa* mites may be one cause for an alteration of gene expression (Boutin *et al.,* 2015). The presence of *Varroa* may act as a trigger hygienic or recapping behaviour by sensitising the bees to the cues for detection. This sensitisation may be facilitated by the altering of gene expression patterns as exposure to *Varroa* mites has been found to upregulate olfactory genes. However, the positive affect of this may be negated by its partner, DWV, which accumulates in the basal regions of antennal epithelium disrupting sensory abilities (Kim *et al.,* 2019; Mondet *et al.,* 2015). Highly infected colonies may be less able to remove *Varroa* causing a snowballing effect.

Aims

Solutions to the *Varroa* and DWV crisis are likely to involve enhancing the natural adaptation of bees by selecting for mite resistant traits. Therefore, the overall aim of this Ph.D. is to fully illustrate the inner workings of honey bee resistance to the *Varroa* mite with a view to determine beekeeper friendly ways of identifying and selecting for mite resistant traits.

Specifically, the aims of this thesis are as follows:

1. To explore the pattern of recapping behaviour in honey bees.

A study looking at how bees undertake recapping behaviour in order to ascertain why it is a prominent feature of resistant honey bee colonies. In particular the study highlights the clustered spatial patterns involved in recapping behaviour.

2. To identify and connect the key traits of resistant honey bees with a view to understanding how resistance has developed and how it can be encouraged in future populations.

Whilst there is a wealth of information on the various traits of resistant honey bees, to the author's knowledge, there is no study that has yet connected each trait to fully illustrate the dynamics of a resistant population. Thus, this study aims to bring together this information in order to show how resistance is established and maintained.

3. To investigate how DWV variant dominance has changed in Hawaii over the past decade and how this relates to changes taking place across the globe.

Recently in a number of regions the master variant DWV-B has been outcompeting DWV-A and reaching dominance. This study shows how the DWV population on the Hawaiian islands of Oahu and Big Island have so far mirrored other regions.

4. To review the hygienic capabilities of *Apis cerana*

This review analyses the studies done on the resistance of *Apis cerana* to *Varroa* and highlights where there are gaps in our knowledge which could be reinforced with future research

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Chapter 1: Spatial distribution of recapping behaviour indicates clustering around *Varroa* infested cells.

Abstract

Varroa destructor is arguably the most important threat to *Apis mellifera* honey bees. Despite the recentness of the invasion of *Varroa*, *A. mellifera* colonies naturally resistant to the mite are being observed in a growing number of populations across Europe, South Africa, and Brazil. Appearing in concert with this resistance is an increase in the ability of workers to detect mite-infested cells, which is closely associated with the recapping of such cells. However, many non-infested cells are also uncapped and then recapped which would appear to be a waste of time and energy. In this study we looked at the spatial patterns of recapping and its association with *Varroa* infestation to understand in what way the uncapping of non-infested cells occurs. We found that recapping occurred in clusters consisting of infested cells and their surrounding non-infested cells. This helped explain our finding that a significant positive correlation existed between levels of recapped infested and non-infested cells. Furthermore, we found that bees responded to an artificial increase in the mite infestation level by increasing their recapping behaviour. We confirmed that the recapped area of non-infested cells was significantly smaller, relative to the holes made in the infested cells. Given these findings we propose that recapping behaviour is stimulated either by a diffuse signal emanating from the infested cell or that cursory checks are conducted in the vicinity of an infested cell.

Introduction

The Western honey bee, *Apis mellifera,* is a highly abundant and important pollinator (Hung *et al.,* 2018). However, populations are currently experiencing pressure from multiple

stressors both natural and man-made. The increasing global trade of honey bees has led to the spread of devastating pests and pathogens, one of the most prolific being the ectoparasitic mite, *Varroa destructor* commonly referred to as *Varroa* (Rosenkranz *et al.,* 2010). *Varroa* feeds on the fat body of the adult and pupal stages of bees (Ramsey *et al.,* 2019), depleting them of nutrients and transmitting viruses (Martin, 2001). A virus they are commonly associated with is the *Iflavirus*, deformed wing virus (DWV) (Highfield *et al.,* 2009; Martin *et al.,* 2012; Wilfert *et al.,* 2016). Bees infected with DWV as pupae emerge as smaller adults with severely shortened lifespans and reduced productivity (Mockel *et al.,* 2011). If a colony has a high mite burden then DWV viral loads will be high, which will ultimately lead to an unbalanced workforce and colony collapse (Martin, 2001).

A key part of colony health is the social immune system which is comprised of innate behaviours directed towards protecting the colony as a whole (Cremer *et al.,* 2007). Hygienic behaviour is an integral part of this, in which bees detect, uncap and remove dead or diseased brood (Spivak & Gilliam, 1998). It is effective against many brood diseases such as the American foulbrood bacteria (Rothenbuhler, 1964; Woodrow & Holst, 1942) and the fungal disease chalkbrood (Gilliam *et al.,* 1983; Spivak & Reuter, 2001). Hygienic behaviour also acts as a defence against *Varroa* in its original host, *Apis cerana* (Rath & Drescher, 1990). Indeed, it is thought that different subsets of worker bees within a colony can be more sensitive to the presence of *Varroa* and thus detect and remove mite-infested pupae (Scannapieco *et al.,* 2016). However, compared to *A. mellifera, Varroa* is a relatively new parasite having only jumped species during the first half of the 20th century (Oldroyd, 1999). Furthermore, the addition of miticides and other chemicals used to control the *Varroa* population reduce the selective pressure that allows the bees to adapt to this new challenge (Neumann & Blacquière, 2017).

Despite this, naturally *Varroa*-resistant (NVR) colonies are being observed in an increasing number of regions including Africa, Latin America (Martin *et al.,* 2019), mainland Europe (Oddie *et al.,* 2018) and the UK (Hawkins, 2020). NVR colonies are those who have survived without treatment for more than five years and have similar traits. Typically, each case of resistance appears to have been preceded by an initial period of high colony losses. This suggests that resistance takes time to develop and the ability to resist the mite may initially be found in only a small part of the population. A key factor associated with the majority of incidences of resistance is the reduction of the reproductive success of the mite (Locke *et al.,* 2012; Mondet *et al.,* 2020). Reduced reproductive success seems likely to be caused by the interruption of the mites' reproductive cycle when infested brood cells are emptied or recapped (Harbo & Harris, 2005; Kirrane *et al.,* 2011). However, there has also been some indication that the brood themselves are able to negatively impact *Varroa* mites' reproductive ability (Broeckx *et al.,* 2019; Conlon *et al.,* 2019; Frey *et al.,* 2013).

A behaviour that is becoming of particular interest is 'recapping' in which workers repeatedly create and reseal holes in the cell capping of worker pupa (Oddie *et al.,* 2018). Recapping appears to be an innate behaviour of bees frequently seen in association with wax moth larva (Galleriinae) that burrow through the capped cells (Villegas & Villa, 2006). However, it is apparent that it can be co-opted for defence against *Varroa*, since recapping rates are the lowest in *Varroa* naïve colonies and highest in NVR populations (Hawkins, 2020; Martin *et al.,* 2019). Precisely why they make these holes, some of which are only 1mm in size, is unknown but it may be to improve the detection of olfactory cues/signals that trigger hygienic behaviour. At present, many researchers seem to agree that the cues come from the brood and that these cues are likely to consist of cuticular hydrocarbons

(CHC) (Mondet *et al.,* 2016; Nazzi *et al.,* 2004; Salvy *et al.,* 2001; Schoning *et al.,* 2012; Wagoner *et al.,* 2019; Wagoner *et al.,* 2020).

Indeed, *Varroa* and DWV have been found to cause changes in the expression of components of the CHC profile, which in turn elicit a hygienic response (Baracchi *et al.,* 2012; Wagoner *et al.,* 2019). In particular the CHCs, (Z)-6-pentadecene and (Z)-10-tritriacontene are associated with *Varroa* and DWV stressed brood respectively (Wagoner *et al.,* 2020). The ability to pinpoint the source of such cues may be aided by creating a small hole in the thick wax capping. If no cue or secondary cue is detected after creating the hole, it can be easily resealed (Martin *et al.,* 2019). In this context recapping would be highly beneficial for colonies to prevent the loss of erroneously uncapped, healthy brood whilst maximising the surveillance of suspicious cells. Importantly, the different potential stages of hygienic behaviour (uncapping, removal, and recapping) are undertaken by different bees within the colony (Scannapieco *et al.,* 2016). The presence of highly sensitive 'uncappers' is thought to be offset by 'recapper' bees with a lower level of sensitivity. This lower sensitivity may explain why infested cells are often recapped instead of being immediately removed (Martin *et al.,* 2019). Recapping correlates with the removal of infested cells and so may be considered a good proxy for removal behaviour (Martin *et al.,* 2019). To measure removal behaviour, one would normally be required to artificially infest brood cells and then check for removal at a later date. Checking for recapping is comparatively easier and less time consuming.

It appears that all *A. mellifera* honey bee colonies have the ability to detect mite-infested cells as both susceptible and NVR colonies locate and recap a greater number of infested cells than non-infested cells (Oddie *et al.,* 2018; Martin *et al.,* 2019). However, NVR

populations that are thought to be more sensitive to mites actually recap a greater proportion of non-infested cells than susceptible populations (Hawkins, 2020; Martin *et al.,* 2019). This uncapping and recapping of non-infested brood would appear to be an unnecessary expenditure of energy, especially since it occurs several times during the development of worker pupae (personal observation, and personal communication Marla Spivak). Recapped non-infested brood cells also appear clustered together alongside recapped infested cells (personal observation). Initial mapping of this clustering alluded to the possibility of their being a common pattern associated with the behaviour. Despite this there has, to the author's knowledge, been no research into the spatial patterns of recapping behaviour or detection strategies.

Therefore, the aims of this study are to investigate in what way the uncapping and recapping of non-infested cells occurs and to determine if there is spatial pattern associated with recapping behaviour. Specifically, we tested the hypothesis that the recapping of non-infested brood cells is triggered by the proximity of infested brood and that the spatial distribution of recapped cells is not random. We predicted that 1) all bees (*Varroa* naïve, susceptible or NVR) have the ability to detect mite infested cells, 2) recapping would occur in a clustered pattern, 3) the clusters would contain recapped infested cells, 4) infested cells would have larger recap sizes than non-infested cells, 5) the predilection of bees to recap non-infested cells would correlate with their ability to recap infested cells and 6) NVR bees would recap more infested cells than susceptible bees.

Methods

Direct effect of *Varroa* on recapping of non-infested cells

The initial study conducted in 2019 involved testing the effect of *Varroa* on recapping rates of nearby cells. We used four *Varroa* naïve colonies from the Isle of Man, UK and five hygienic colonies with low (<0.5%) levels of mite infestation in brood, due to previous acaricide treatment, from the University of Minnesota research apiary, USA. Firstly, we measured the recapping levels in one frame from each colony based on opening 150 cells for each Isle of Mann colony (*n* = 600) and an average of 230 cells for each Minnesota colony (*n* = 1131), 150 cells is the minimum sample size required to provide an accurate result (Hawkins, 2020). Recapping was measured following the protocol outlined in previous studies (Boecking & Spivak, 1999; Harris *et al.,* 2012). The cap of each cell was carefully peeled back using fine forceps to check for signs of recapping, which can be seen when the silk cocoon has been removed and filled in with a matte disc of wax particles (Martin *et al.,* 2019). We then inserted 120 live *Varroa* mites, 30 per colony, into newly capped worker brood of four Isle of Mann colonies and 250 mites, 50 per colony, into five Minnesota colonies. Mites for this artificial infestation were sourced from live *A. mellifera* drone pupae from Anglesey for the Isle of Man colonies and from a single untreated colony in the Minnesota University apiary for the USA colonies. After a period of 10 days the infested cells and the cells adjacent to the infested cells were checked for recapping. A Wilcoxon signed-rank test was conducted to compare the recapping values before *Varroa* introduction and afterward. (UK) or 50 (USA)

Spatial distribution of recapped cells

For the spatial analysis, frames containing worker sealed brood that had been capped for between four and ten days were removed from a mixture of three NVR and three susceptible colonies from across England and Wales during August 2019 and stored at -20°C. Susceptible colonies were those that received acaricide treatment at least once per year. NVR colonies were those that beekeepers stated had been surviving without acaricides treatment for at least three years. Additional data from three frames of NVR colonies from Hawaii that were created from feral, untreated populations were collected in November 2019. The Hawaiian bees were caught in the forest and maintained treatment free for several years (Martin, 2020). In total 17 frames were used, six from three UK NVR colonies, three from three Hawaiian NVR colonies and eight from four UK susceptible colonies.

NVR and susceptible colonies were chosen so that there would be a greater variation in infestation rates which may affect any spatial patterning. The two groups also allowed for the comparison of recapping ability between NVR and susceptible colonies. Each frame was examined under a x16 binocular microscope using a bright cold light source. Individual cell caps were checked for recapping in line with the aforementioned method. If recapped, the diameter of the recapping (matte wax circle) was recorded to the nearest mm, then the brood was removed to determine if the cell was infested or not. Infestation was based upon observation of mites, mite frass or mite exuviae in the cell. The data were transferred into an Excel spreadsheet which was designed to spatially represent a honey comb. To achieve this pairs of cells in each row were merged and each alternate row was offset by one cell.

Data analysis

For each frame, coordinates of the recapped cells were generated in Excel and imported into R version 3.6.2 (R Core Team, 2019). A distance matrix was generated from the coordinates, with each data point representing the centre of a recapped cell. The distance matrix was then analysed using the cluster detection algorithm DBSCAN (Density-based spatial clustering of applications with noise) (Ester *et al.,* 1996; Hahsler *et al.,* 2019) (for code see Fig S1). DBSCAN searches spatial data points for clusters of a user defined minimum size (MinPts) within a user defined maximum search radius (eps). The minimum cluster size is the smallest number of points (recapped cells) that DBSCAN will consider a cluster. The search radius is the area in which DBSCAN will look for a recapped cell from the starting cell. Potential values of these parameters were first decided from observation of patterns in brood combs. In this case radii needed to be in multiples of 5 mm to allow the measure from one cell centre (data point) to another (cells are approximately 5mm). For example, with a maximum of 10 mm the scan will look for recapped cells within a 2-cell radius. If recapped cells are in this radius, the search moves to that cell (or cells). This continues until a recapped cell cannot be found within the radius. All the cells the program has searched are recorded as a cluster if the number is above the minimum cluster size. If it is below this size then no cluster is reported, and it moves on to the next search. Cells that do not fit the requirement, i.e., do not have at least 2 other recapped cells within a 10 mm radius are considered outside of the clusters. The investigated parameters were radius sizes of 5 mm, 10 mm, and 15 mm with minimum cluster sizes of 2, 3, 4 and 5 cells. Each permutation (5 mm with 2, 10 mm with 2, etc) was run in DBSCAN, which provided a visual output. The final parameters were decided based on whether the clusters could be considered realistic given the DBSCAN output and the natural spacing of cells. After

preliminary runs a search radius of 10 mm (eps = 10) and a minimum cluster size of three cells (MinPts = 3) were chosen, as the two key DBSCAN variables. The resulting clusters were manually transferred onto the Excel spreadsheet. Two frames from one colony (Colony name Wal 11) were excluded from spatial analysis and table 1 as they contained too few recapped cells i.e., no clusters.

To address our second and third predictions, the number of clusters per frame; total number of cells per cluster; number of infested cells per cluster; number of non-infested cells per cluster; and the recapping values of infested and non-infested cells within clusters were tabulated (Table 1). Statistical analyses were conducted using Minitab ® version 18 (Software, 2017). In addition to Wal 11, another frame (Colony name Rhona, frame 2, side 2) was removed from comparisons of mean recap sizes of infested and non-infested cells and the number of infested and non-infested clusters per frame because it contained no recapped infested cells. To address the prediction that the clusters would contain recapped infested cells Mann Whitney U-tests were used to compare the numbers of non-infested (*n* = 31) and infested clusters (*n* = 61) and the sizes of these non-infested and infested clusters across the 14 remaining frames. The comparison of cluster sizes was repeated with clusters greater than 50 cells removed from infested (*n* = 49) and non-infested clusters (*n* = 30). An infested cluster is defined as one that contained at least one recapped infested cell. In line with the fourth prediction, a Mann Whitney U-test was used assess whether there was a significant difference in the size of the recapped areas of infested (*n* = 504) and non-infested cells (*n* = 3141). A Mann Whitney U test was also used to compare the proportion of infested (*n* = 546) and non-infested cells recapped (*n* = 3383) across all 15 frames in Table 1.

Relationship between the recapping of infested and non-infested cells

To address our fifth and sixth predictions we pooled our UK (*n* = 12) and Hawaiian data (*n* = 3) with recapping data from Martin *et al.* (2019) (*n* = 44), Hawkins (2020) (*n* = 40), Oddie *et al*. (2018) (*n* = 57) and from unpublished data provided by Marla Spivak (*n* = 5). In total there were 159 data points, 106 from resistant colonies and 53 from susceptible colonies. The data come from a variety of locations; this range was chosen to provide a good variation in data. The Oddie *et al.* (2018) data are from NVR and susceptible populations in Avignon and Sarthe, France. Martin *et al.* (2019) includes data on NVR populations from South Africa (*Apis mellifera scutellata* and *Apis mellifera capensis*) and Brazil (Africanised honey bees). Marla Spivak's unpublished data are from bees of the Minnesota hygiene line. Hawkins (2020) includes NVR and susceptible colonies from the UK.

A Spearman's Rho test was used to determine whether there was a correlation between the percentage of infested cells recapped and the percentage of non-infested cells recapped and the strength of such correlation. A scatter diagram was created to illustrate the relationship. Spearman's Rho tests were also used to assess whether this correlation was present in data if separated by colony type (susceptible and NVR). A Mann Whitney U-test was used to determine whether there was a significant difference in the percentage of infested cells and non-infested cells recapped by NVR colonies (*n* = 106) and susceptible colonies (*n* = 53).

Results

Direct effect of *Varroa* on recapping of non-infested cells

The addition of mites consistently and significantly (W = 0, critical value for W at *n* = 8 (*p* < .05) is 3) increased the level of recapping of non-infested cells on that frame in both the

USA and UK colonies (Fig. 1.). In one Isle of Man colony, zero recapping was recorded irrespective of the mites' presence or not.

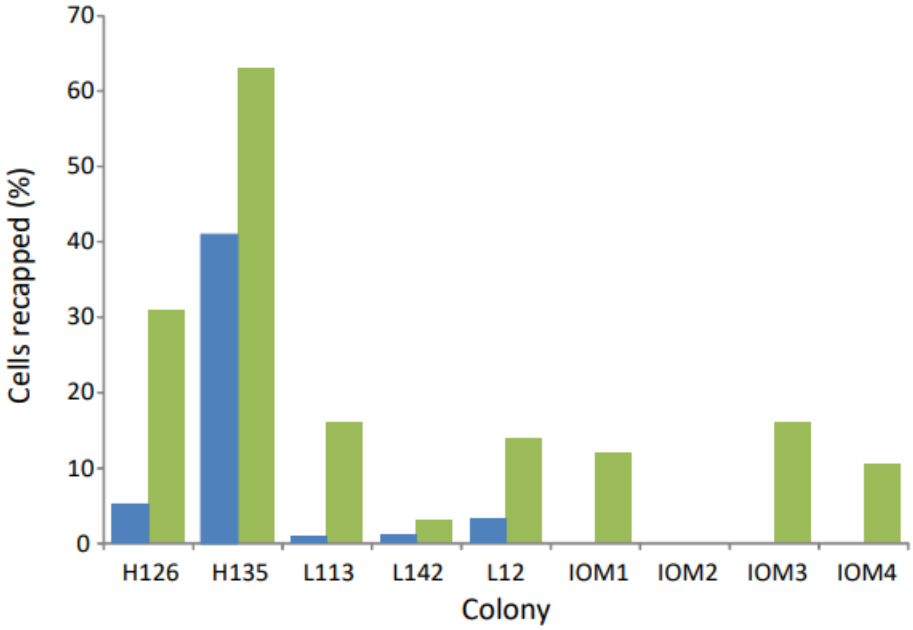

Figure 1. Recapping rates of non-infested cells in the five USA effectively *Varroa* free colonies (Before mite introduction colonies had brood infestation levels of 0% except for colonies H126 and L142 which had 0.4% and 0.3% respectively), along with three *Varroa* naïve Isle of Man colonies. The recapping levels before mite introduction (blue) and after mite introduction (green).

Spatial distribution of recapped cells

A total of 8450 cells were mapped across 15 frames: six from three UK NVR colonies, three from three Hawaiian NVR colonies and six from three UK susceptible colonies. The DBSCAN algorithm found that recapped cells form clusters associated with infested cells (Fig. 2A, Table 1). High levels of recapping, typically due to higher infestation levels, resulted in

fewer, larger clusters. The clearer cluster patterns were seen when sealed brood infestation levels were below 10% and when efficient targeting of the infested cells occurred (Fig. 2B).

The total number of clusters was 92 of which 61 contained at least one infested cell. The percentage of infested and non-infested cells located within clusters was 85% and 88% respectively. This indicates that the majority of recapped cells occur within clusters of three cells or more rather than as single points. Furthermore, clusters containing infested cells were significantly greater in size than those comprised of just non-infested cells ($U=604$, $p = 0.002$). This finding remains significant even when all clusters greater than 50 cells are removed ($U = 604$, $p = 0.048$). Additionally, the number of infested clusters per frame was significantly greater than the number of non-infested clusters ($U = 52.5$, $p = 0.038$). The size of the recapped area of the infested cells, median 3.1 mm (IQR 1.2), was significantly greater than those found on non-infested cells, median 2.1 mm (IQR 0.3), ($U = 57.5$, $p = 0.024$). Including all the cells of the frames in Table 1 a significantly greater percentage of the infested cells were recapped than the non-infested cells ($U = 50$, $p = 0.01$).

Relationship between the recapping of infested and non-infested cells

When data from this study were combined with data from all previous studies we found a significant positive correlation between the percentage of infested cells recapped and the number of non-infested cells recapped ($r_s = 0.754$, $p < 0.0001$) (Fig. 3). This correlation was stronger for susceptible colonies ($r_s = 0.818$, $p < 0.001$) than NVR colonies ($r_s = 0.677$, $p < 0.001$). NVR colonies also recapped a significantly greater percentage of infested cells 58% versus 32% ($U = 1563$, $p < 0.0001$) and non-infested cells 27% vs 16% ($U= 1891$, $p = 0.0024$) than susceptible colonies.

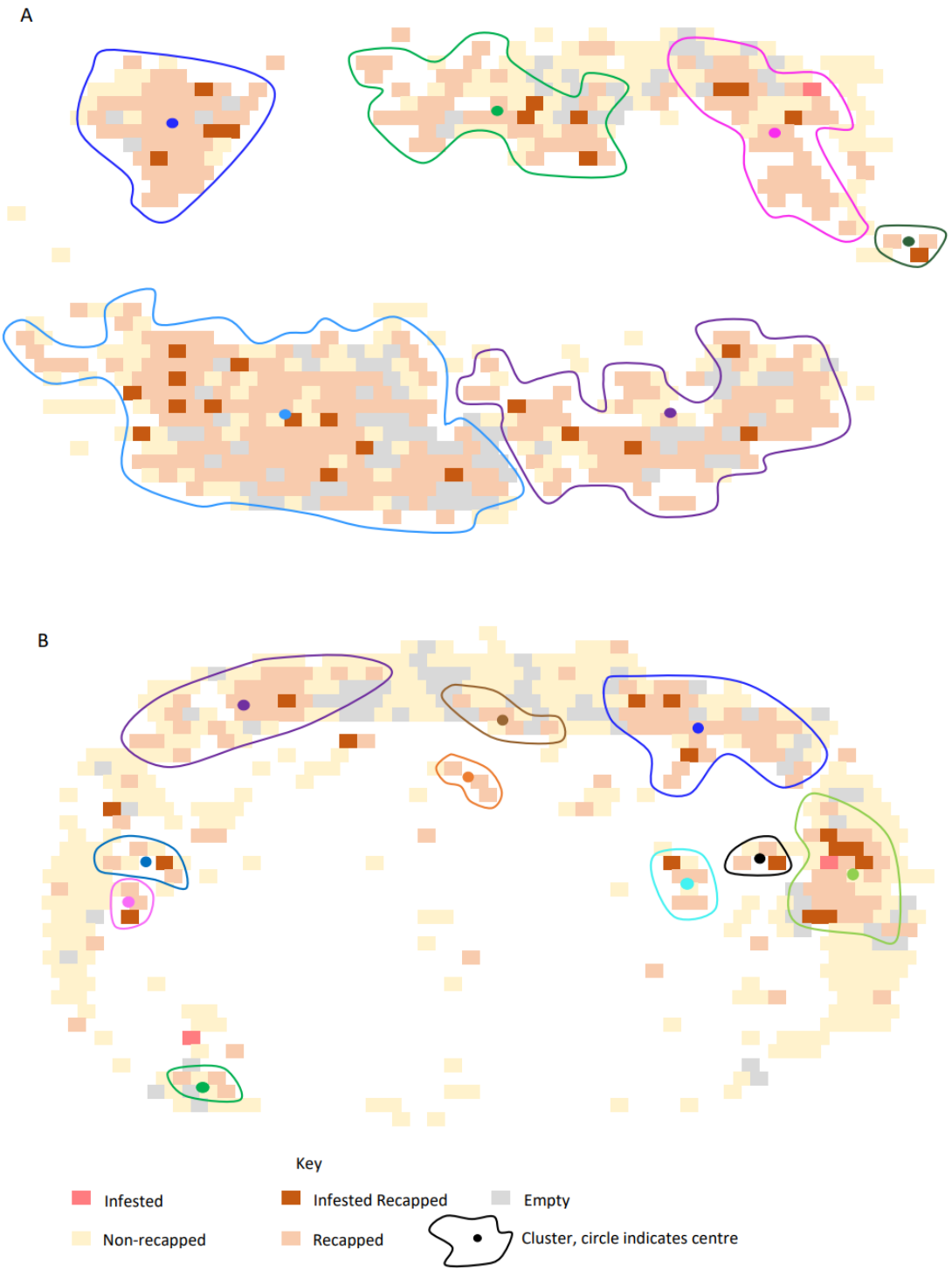
A
B
Key
Infested
Infested Recapped
Empty
Non-recapped
Recapped
Cluster, circle indicates centre

Figure 2. Excel generated maps of cells on two separate brood frames with the clusters predicted by the DBSCAN algorithm manually added. Both frames have an approximately 5% infestation level but a A) high (63%) and B) lower (29%) level of recapping.

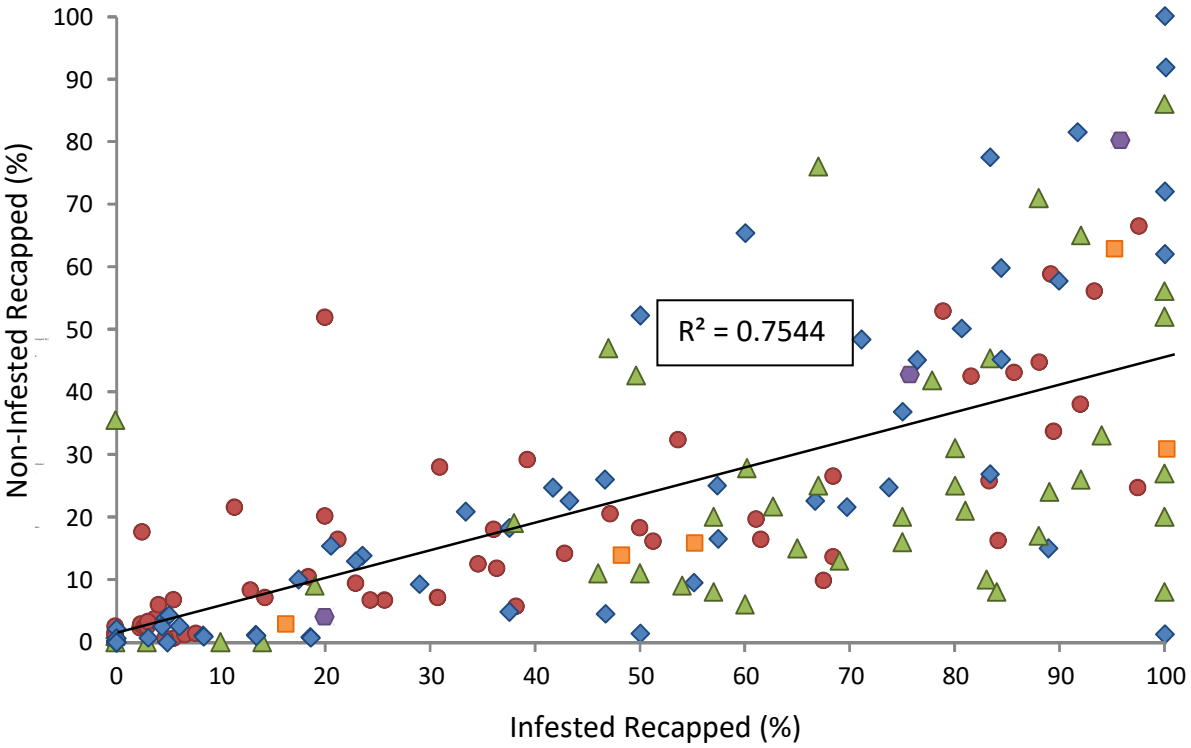

Figure 3. The colony level relationship between the percentages of infested recapped cells against percentages of non- infested recapped cells across several studies. Red circles = Europe (Oddie *et al.* 2018 *n* = 57), green triangles = Brazil/Africa (Martin *et al.,* 2019 *n* = 44), blue diamonds = UK (Hawkins, 2020, *n* = 40) and this study *n* = 12, orange squares = Minnesota (M. Spivak unpublished data *n* = 5), and purple hexagons = Hawaii (this study, *n* = 3).

Table 1. Summary of frame and recapping data alongside the subsequent DBSCAN generated cluster data for each frame. If the second side of the frame has been analysed, the prefix 's2' was used. The images of each frame and their predicted clusters are provided in the supplementary data (Fig. S2).

Colony class & code		Sample size (cells)	Mite infestation level (%)	Recap level (%)	Mean recap size (mm)		Number of DBSCAN predicted clusters		Mean ±SD cluster size (cells)	Mean ±SD No. infected cells per cluster	Percentage of recapped cells in clusters %	
					Non-Infested	Infested	Non-Infested	Infested			Non-infested recapped	Infested recapped
NVR	R6	650	8	57	2.50	3.48	2	3	72 ± 129	7 ± 13	97	97
	R6s2	555	5	65	2.32	3.07	0	6	59 ± 50	5 ± 4	97	100
	R2	431	4	31	2.23	2.77	3	7	15 ± 14	2 ± 2	83	88
	R2s2	298	1	14	2.08	2.44	3	0	7 ± 2	N/A	51	0
	R65	169	1	88	3.39	4.75	3	1	36 ± 37	1 ± 1	97	100
	R65s2	301	1	96	1.86	2.31	6	1	40 ± 79	1 ± 2	99	100
	HD	593	12	48	1.60	2.29	0	2	139 ± 188	27 ± 33	98	96
	JF	208	22	8	1.89	1.61	1	1	4 ± 1	2 ± 2	50	25
	UH60	302	8	82	2.06	3.11	0	1	246	22	100	100
Susceptible	B1.3	884	34	34	1.93	4.05	1	13	21 ± 43	10 ± 21	97	97
	B1.3s2	815	38	26	2.65	3.54	0	5	35 ± 36	21 ± 21	80	87
	B1.4	746	3	49	2.15	1.00	6	6	28 ± 65	2 ± 2	92	100
	B1.4s2	489	5	44	3.17	5.00	4	6	17 ± 13	2 ± 3	78	95
	M2	955	3	46	1.86	2.50	4	6	42 ± 105	3 ± 6	98	93
	M2 s2	1054	6	47	2.80	3.76	0	4	122 ± 208	11 ± 18	100	100

Discussion

Ultimately this study shows that the uncapping and recapping of non-infested cells is being driven by the presence of mite infested cells. We found, in agreement with our initial predictions, that *Varroa* naïve, susceptible and NVR bees all have the ability to detect mite infested cells (Fig. 1, Fig. 3), that the recapping of non-infested cells occurs in clusters associated with infested cells (Fig. 2) and that the recapping of non-infested cells increases alongside the recapping of infested cells (Fig. 3). These findings are important as they suggest firstly that all colonies have the ability to detect and thus potentially to remove mite infested brood. Secondly that whether a cell is checked for *Varroa* is influenced by the infestation status of its surrounding cells. We also found that NVR colonies recapped a greater percentage of infested and non-infested cells than susceptible colonies which could suggest that NVR bees have an enhanced sensitivity to cues and/or a heightened ability to recognise potential areas of infestation based on the location of known infested cells.

Cursory checking of the cells surrounding infested cells may explain why we found recapping to occur in clusters. This could reflect the natural clustered brood infestation pattern that has been observed in *Varroa* (Fuchs, 1988; Kim *et al.,* 2018). Bees may be more likely to check around an infested cell if *Varroa* are more likely to infest in a clustered fashion (Kim *et al.* 2018). The clustering of infestation patterns has been disputed by some researchers (e.g., Salvy *et al.,* 1999); however, this may be because it varies depending on the severity of infestation (Kim *et al.,* 2018). Additionally, the pattern-based checking of cells may explain why the recapped areas of non-infested cells are significantly smaller than those of infested cells. Cells that are being checked on this pattern basis may only be opened slightly as, should the cell be non-infested, the hole is easier to repair and requires less wax. On the

other hand, it is also plausible that the smaller holes are created because these cells carry a weak chemical stimulus that has drifted from an infested cell. This diffusion of cues from an infested cell to its surrounding non-infested cells could also explain why recapping occurs in clusters. However, it is important to note that the explanations of cue diffusion and cursory checking are not mutually exclusive and so may operate alongside one another. In contrast to cursory checking, cue diffusion would appear to be an unintended consequence of the infestation signalling system. Cues that are volatile escape the cell and attract a hygienic worker, but this volatility may also mean that they drift over neighbouring cells resulting in the cells appearing suspicious. If each cell that was tainted in such a way was emptied then a lot of healthy brood would be wasted. Therefore, bees may create small holes in the caps of suspicious cells which could enhance the diffusion of cues out of the cell, if it is infested, increasing the accuracy in pinpointing the source.

Interestingly it may be that the accuracy of this system is reinforced through the use of both low and high volatility cues (Wagoner *et al.,* 2019). A cue such as (Z)-6-pentadecene which has a relatively high volatility compared to other hygienic cues like oleic acid would elicit attention through the cap and direct a bee towards the infested cell (Nazzi *et al.,* 2004). Once the bee bites into the infested cell a second, less volatile cue such as heptacosene or tritriacontane, which is normally stifled by the cap, may become detectable allowing confirmation of the infestation (Wagoner *et al.,* 2019). If a non-infested cell is opened then no secondary cue will be present meaning the cell can be resealed. This secondary cue would not diffuse and so could increase the accuracy of brood removal and reduce the chances of healthy brood being removed. However, if the cue was only of a low volatility it may be insufficient as to direct the attention of bees toward the infestation. This system is not just proposed for *Varroa* infestation but also for more general hygienic behaviour.

McAfee *et al.* (2018) suggest hygienic behaviour is triggered by the blend of the volatile food begging cue beta-ocimene and the death pheromone oleic acid. The ability of beta-ocimene to illicit workers attention could be co-opted to direct them towards an infested cell which they will then bite into and gain access to the non-volatile cue, oleic acid. The secondary signal oleic acid would trigger the enlargement of the cell and removal of the pupa. In its absence, the small hole can easily be resealed without harm to the pupa. For *Varroa* infestation this combination is unlikely as infested pupae usually do not die and thus do not emit oleic acid. However, as the ability to remove *Varroa* infested brood stems from hygienic behaviour then the same dual cue process may occur but with different cues (Nazzi *et al.,* 2004; Wagoner *et al.,* 2019). It seems that the second cue in this process is fairly prone to error as in NVR colonies a high number of infested cells are erroneously recapped. In speculation, this may be due to the lower olfactory sensitivities of 'recapper' bees in comparison to 'uncapper' bees (Gramacho & Spivak, 2003). Although it is also important to note that evaluating recapping provides a snap shot in time and so we can only speculate on the fate of recapped infested cells. It may be that they will be uncapped and removed at a later time. Indeed, cells can be uncapped and recapped many times during the sealed stage.

The 'uncapper' bees are those that take part in the initial detection and opening of suspicious cell caps (Gramacho & Spivak, 2003). As these bees start the behaviour it seems reasonable to assume that the higher recapping rates of NVR colonies may be because their 'uncapper' bees have a higher sensitivity to cues or are present in a greater number than in susceptible colonies. Exposure to *Varroa* may allow individual bees to learn to recognise the cues involved in infestation (Gronenberg *et al.,* 2014). This could explain why *Varroa* naïve colonies and colonies with very low infestation levels had low levels of recapping until after substantial exposure to *Varroa*. Repeated exposure may increase the numbers of sensitive

bees, enhance their sensitivity or lower the bees' threshold of response to cells that carry cue traces (Masterman *et al.,* 2001; Mondet *et al.,* 2015). Indeed, the positive correlation between recapping of infested cells and non-infested cells suggests that individuals in colonies that are more able to detect *Varroa* are also more likely to investigate non-infested cells. Experience dependent behaviour like this has been observed in another eusocial insect species, the clonal ant *Platythyrea punctata* (Westhus *et al.,* 2014). Adult ants that had more frequently encountered fungus-exposed (*Metarhizium robertsii*) larvae groomed exposed larvae for longer and more effectively (removed more fungal conidiospores). Similarly recapping, a form of social hygiene like grooming is enhanced (in frequency rather than duration) after naïve bees are exposed to *Varroa* (Fig. 3). Whilst this explanation may be undermined by the presence of non-infested clusters it is important to note that one cannot exclude the possibility that these non-infested clusters at some point contained an infested cell that was removed.

Encouraging the prevalence of resistance traits appears to be a sustainable solution to the *Varroa* problem. However, the complexity of linking genetic traits to observable phenotypes confounds screening and breeding efforts (Beaurepaire *et al.,* 2019; Mondet *et al.,* 2020). The recapping trait has been observed in NVR colonies and is an example of the way colonies are adapting to the *Varroa* threat (Martin *et al.,* 2019; Oddie *et al.,* 2018). Recapping may provide a useful marker for resistance (Martin *et al.,* 2019). Indeed, we found that the recapping of non-infested and infested cells was positively correlated suggesting recapping (of both cell types) is a trait of more hygienic colonies or those with more sensitive 'uncappers'. Additionally, both potential explanations for recapping non-infested cells, i.e., checking areas around infested cells or a diffuse signal, could suggest the influence of experience on the performance of social hygiene be it through learning patterns

(Gould, 1986), becoming sensitive to cues (Masterman *et al.,* 2001; Mondet *et al.,* 2015) or a combination of both. Hygienic behaviour has been shown to have a genetic basis (Boecking *et al.,* 2000; Harbo & Harris, 1999). However, it is thought that the underlying gene set is somewhat limited and behavioural differences may rely on changes in regulation patterns (Boutin *et al.,* 2015). Indeed, Mondet *et al.* (2015) found that olfactory genes were upregulated in the antenna of bees that could detect mites. It may thus be worth exploring whether the environment can influence the behaviour. For example, whether bees can become sensitised to (or learn) certain cue odours overtime, priming them for recapping and brood removal.

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Supplementary information

Supplementary figures S1-S2o

#generate distance matrix

read.csv("location of file\\filename.csv ", header = FALSE)

p <- as.matrix(read.csv("location of file\\filename.csv", header = FALSE))

Importing the data from location on the computer

```
## Converting the data from a frame to a data matrix and setting it as object p

# Apply the DBSCAN algorithm

install.packages("dbscan")

install.packages("tidyverse")

install.packages("factoextra")

## install the R packages required to run DBSCAN

library(dbscan)

library(tidyverse)

library(factoextra)

## Call the installed packages

o <- dist(p, method = "euclidean", diag = TRUE, upper = TRUE)

db <- dbscan::dbscan(o, eps = 10, minPts = 3)

fviz_cluster(db, p, stand = FALSE, ellipse = FALSE, geom = "point")

## Create distance matrix for data and set as object o

## Input distance matrix, o, into DBSCAN

## Plot the resulting clusters
```

Fig. S1. R script for generating distance matrix and running DBSCAN

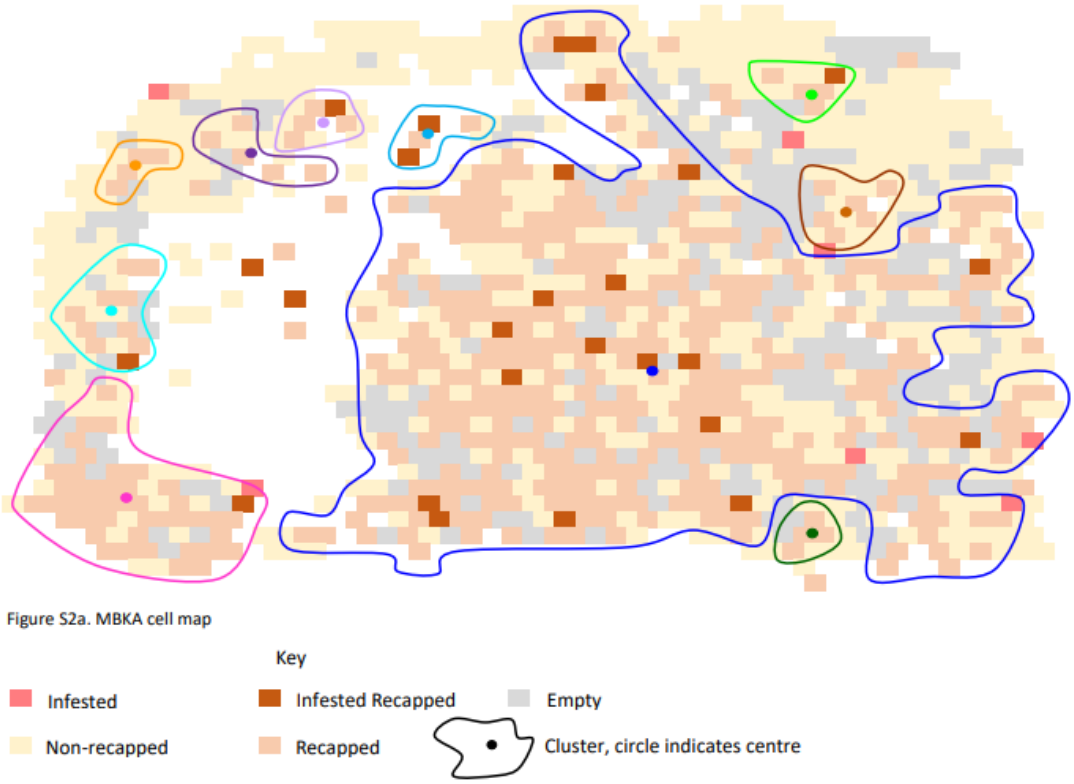

Fig. S2a. Excel generated map of cells on brood frame MBKA with the clusters predicted by the DBSCAN algorithm manually added.

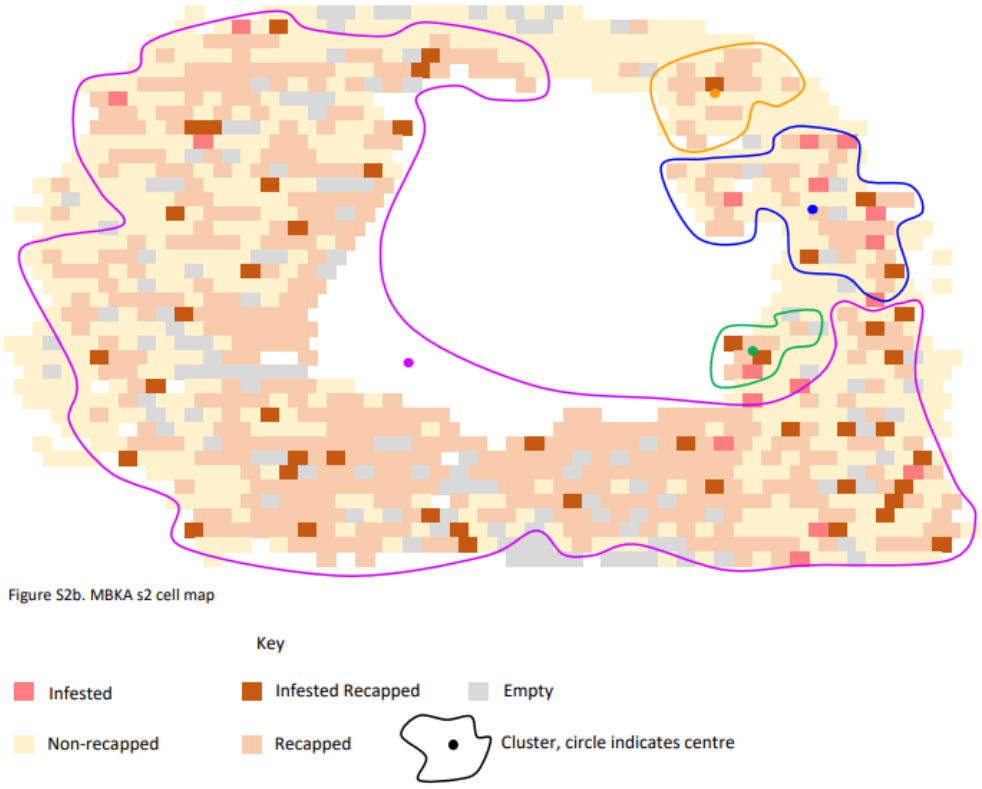

Fig. S2b. Excel generated map of cells on brood frame MBKA s2 with the clusters predicted by the DBSCAN algorithm manually added.

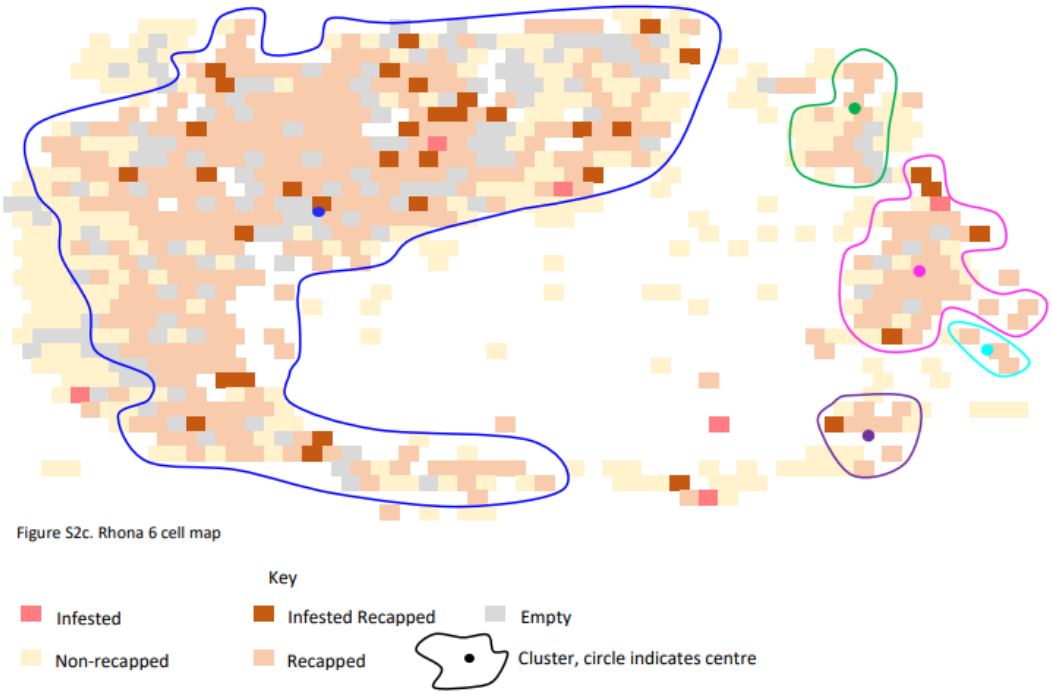

Figure S2c. Rhona 6 cell map

Key

Infested

Infested Recapped

Empty

Non-recapped

Recapped

Cluster, circle indicates centre

Fig. S2c. Excel generated map of cells on brood frame Rhona 6 with the clusters predicted by the DBSCAN algorithm manually added.

Fig. S2d. Excel generated map of cells on brood frame Rhona 6 s2 with the clusters predicted by the DBSCAN algorithm manually added.

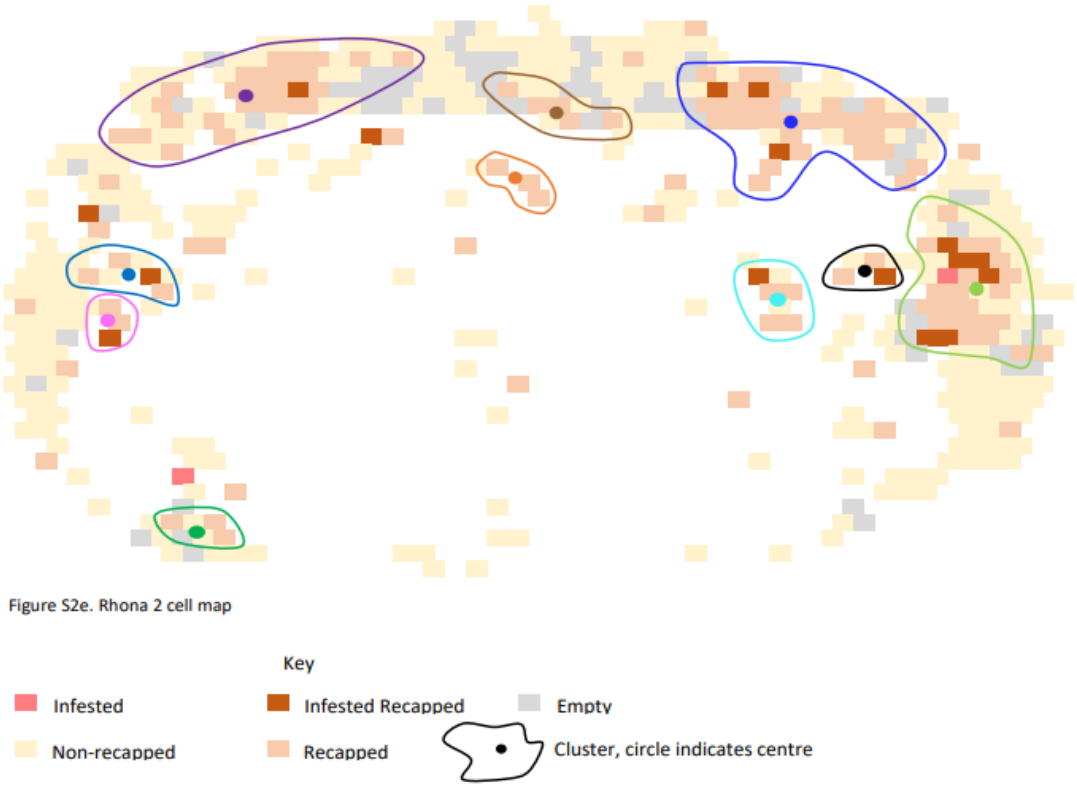

Fig. S2e. Excel generated map of cells on brood frame Rhona 2 with the clusters predicted by the DBSCAN algorithm manually added.

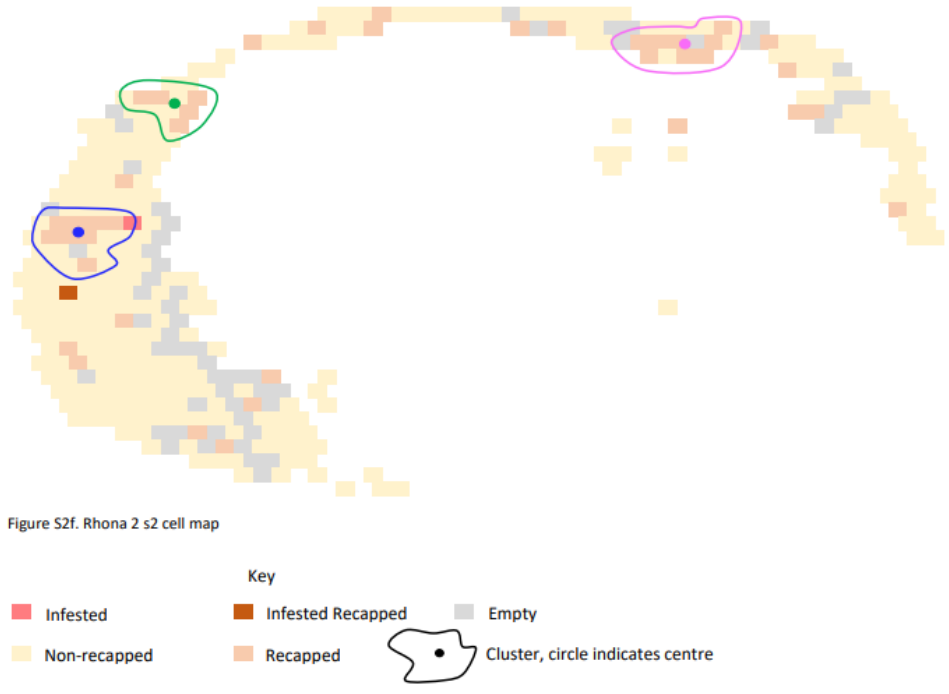

Fig. S2f. Excel generated map of cells on brood frame Rhona 2 s2 with the clusters predicted by the DBSCAN algorithm manually added.

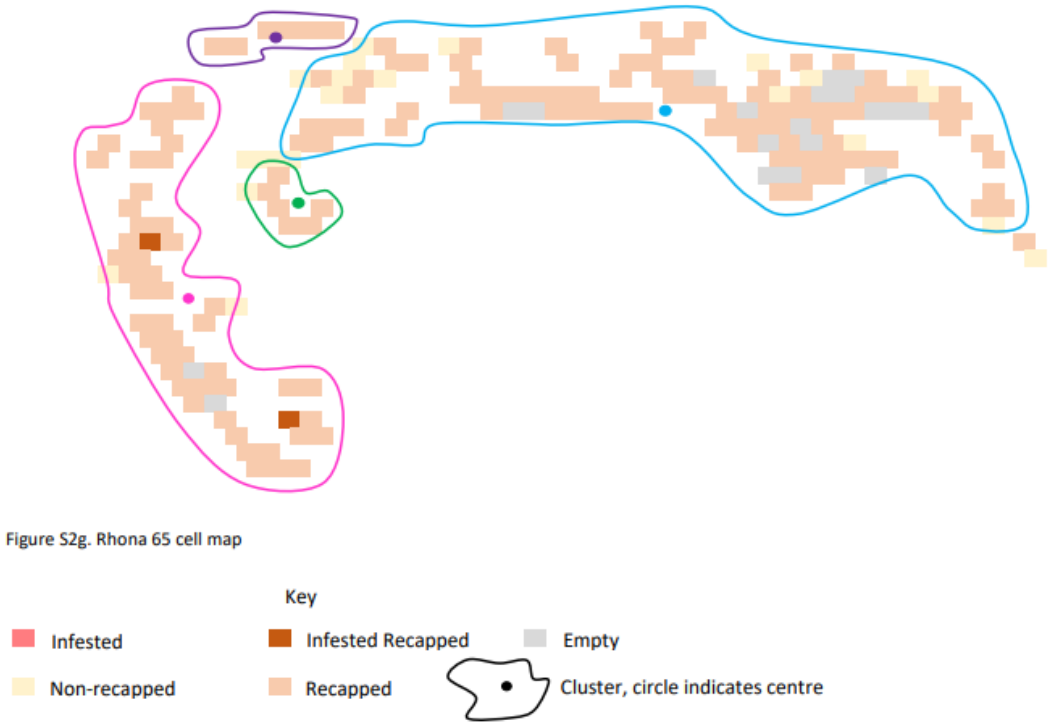

Fig. S2g. Excel generated map of cells on brood frame Rhona 65 with the clusters predicted by the DBSCAN algorithm manually added.

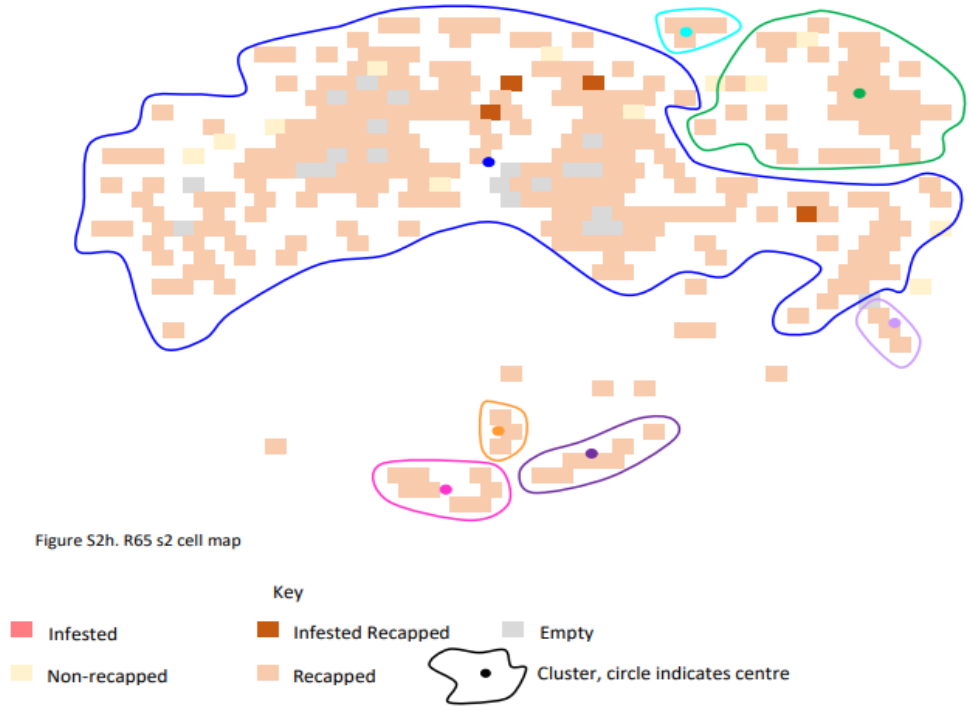

Fig. S2h. Excel generated map of cells on brood frame Rhona 65 s2 with the clusters predicted by the DBSCAN algorithm manually added.

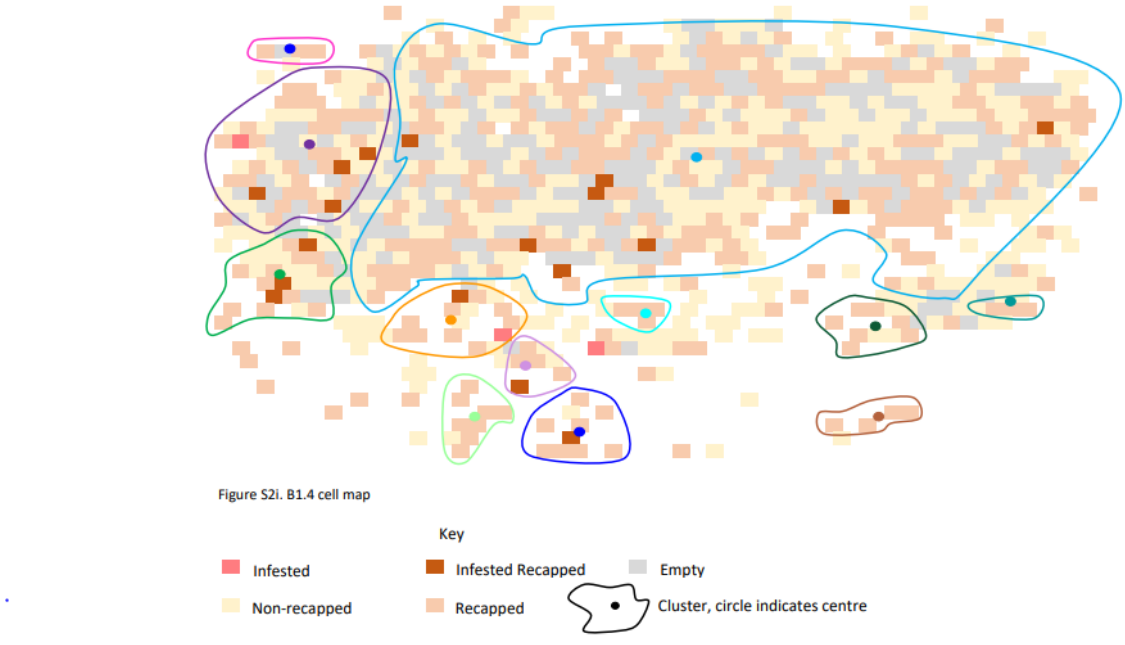

Fig. S2i. Excel generated map of cells on brood frame B1.4 with the clusters predicted by the DBSCAN algorithm manually added.

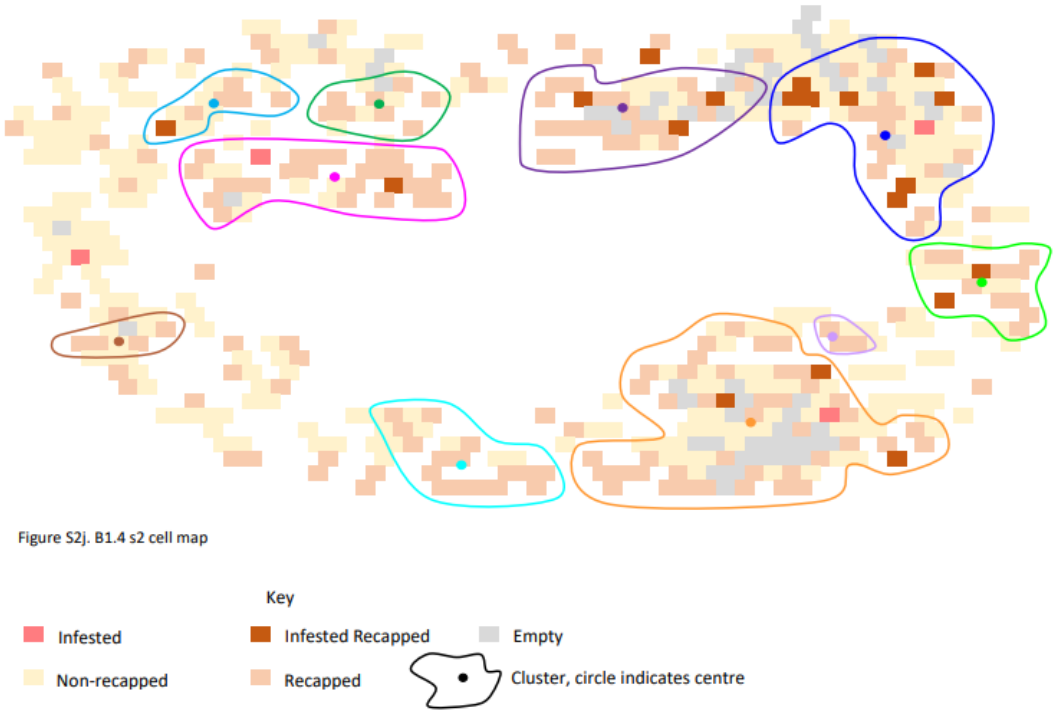

Fig. S2j. Excel generated map of cells on brood frame B1.4 s2 with the clusters predicted by the DBSCAN algorithm manually added.

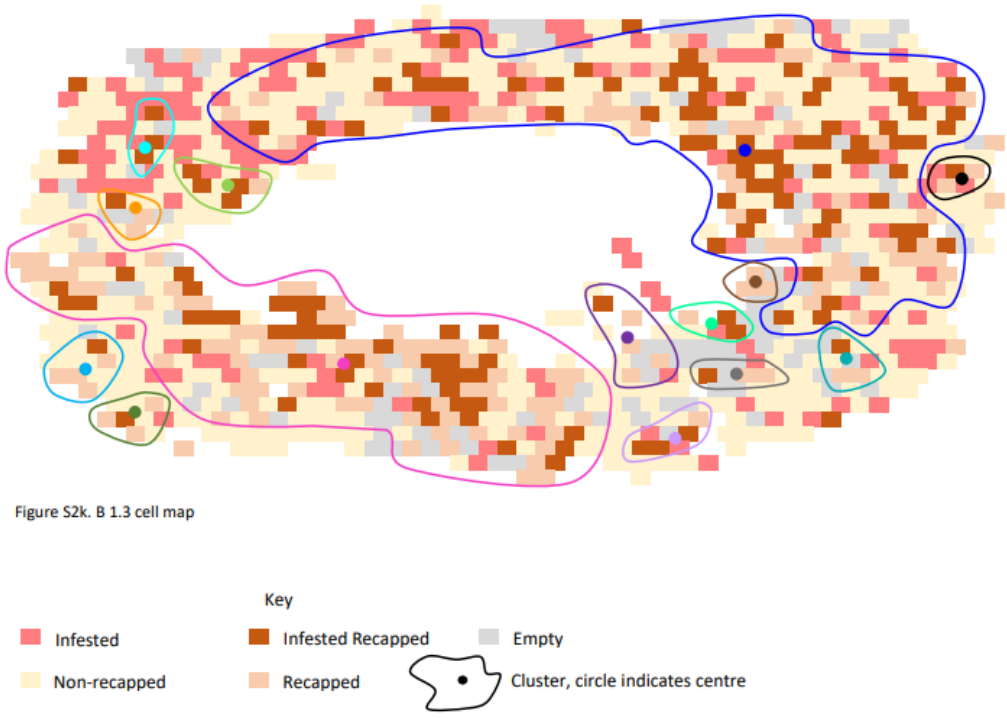

Fig. S2k. Excel generated map of cells on brood frame B1.3 with the clusters predicted by the DBSCAN algorithm manually added.

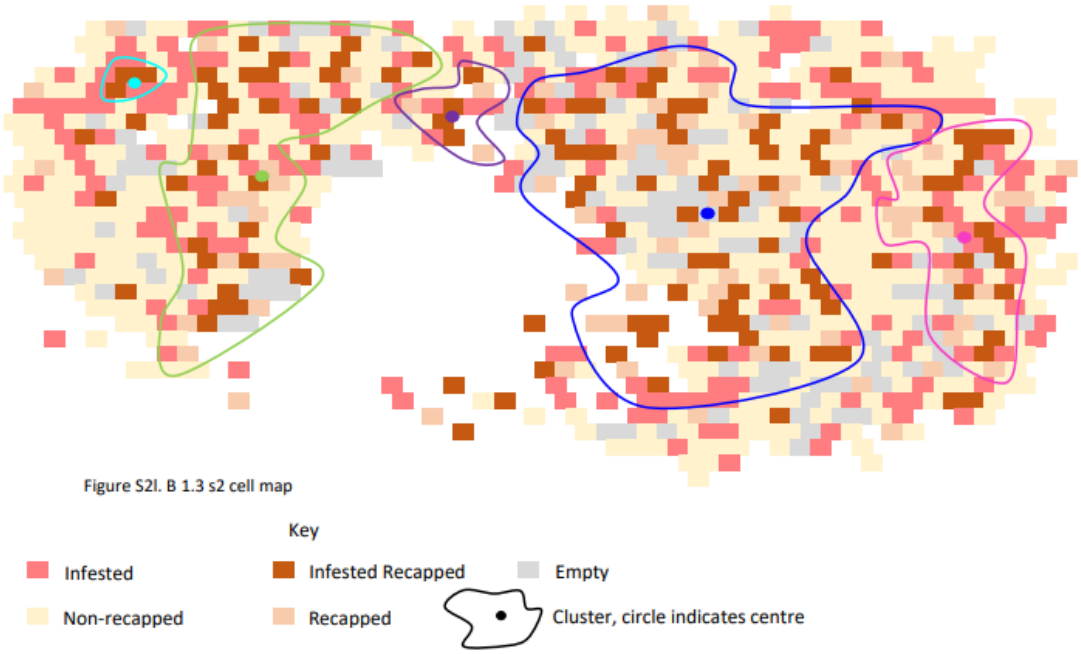

Fig. S2l. Excel generated map of cells on brood frame B1.3 s2 with the clusters predicted by the DBSCAN algorithm manually added.

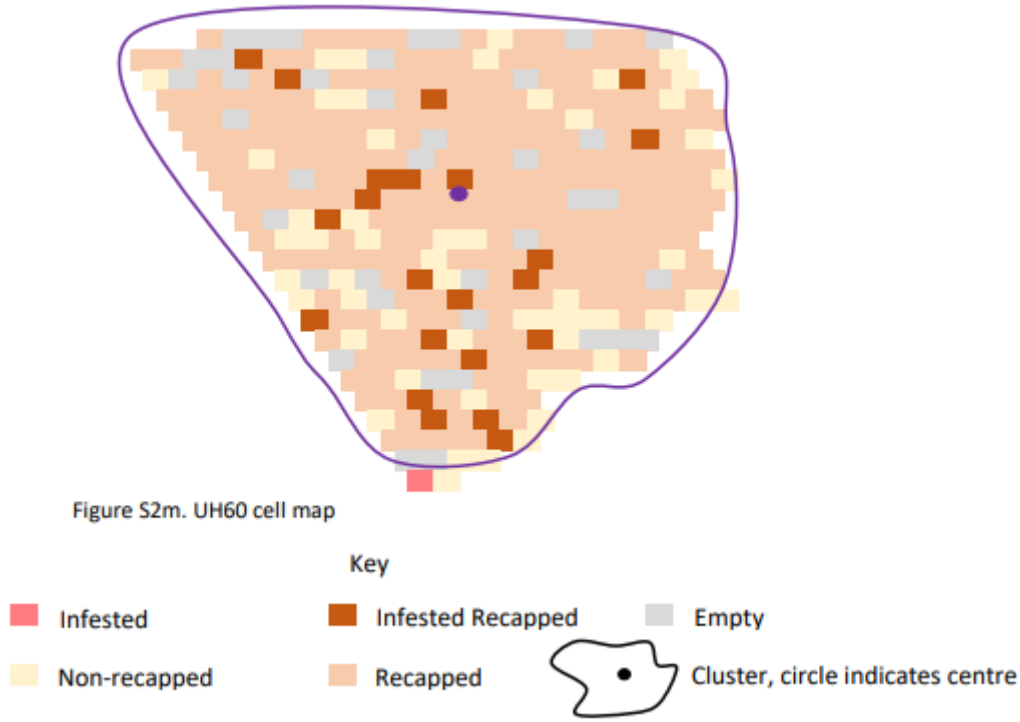

Fig. S2m. Excel generated map of cells on brood frame UH60 with the clusters predicted by the DBSCAN algorithm manually added.

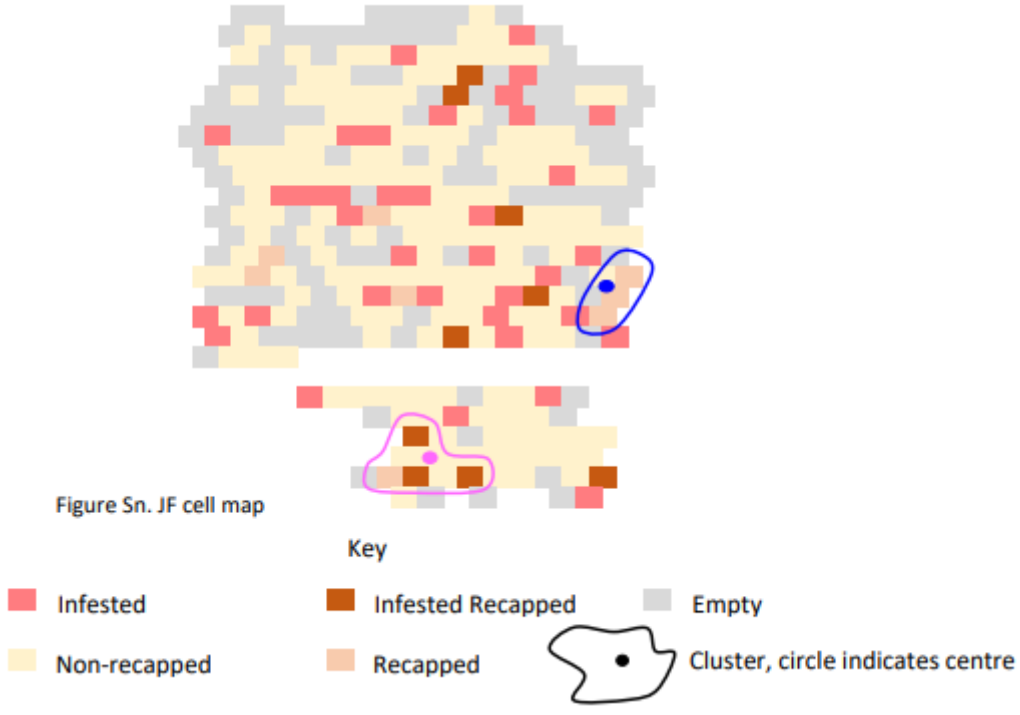

Fig. S2n. Excel generated map of cells on brood frame JF with the clusters predicted by the DBSCAN algorithm manually added..

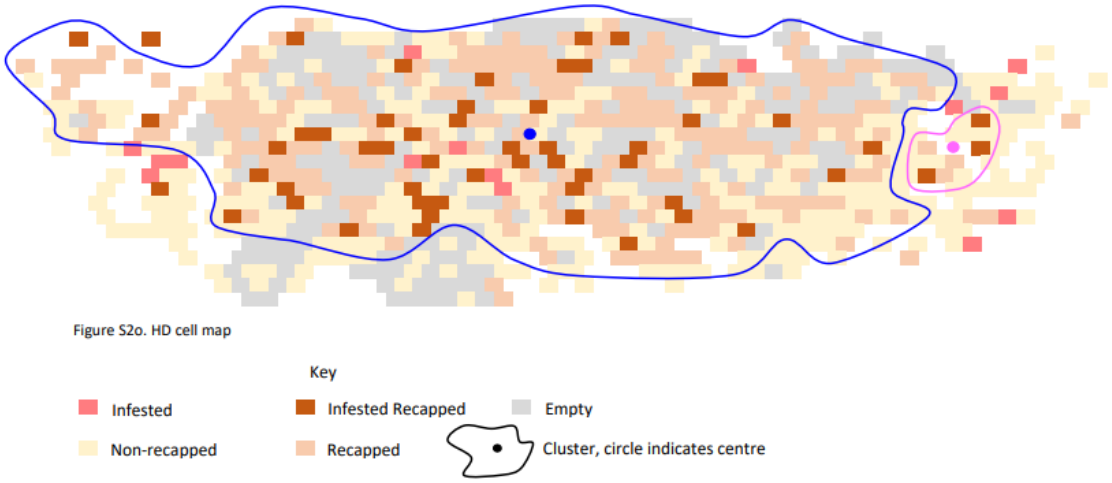

Fig. S2o. Excel generated map of cells on brood frame HD with the clusters predicted by the DBSCAN algorithm manually added.

Chapter 2: Parallel evolution of *Varroa* resistance in honey bees: A common mechanism across continents?

Abstract

The near-globally distributed ecto-parasitic mite of the *Apis mellifera* honey bee, *Varroa destructor*, has formed a lethal association with deformed wing virus, a once rare and benign RNA virus. In concert the two have killed millions of wild and managed colonies, particularly across the northern hemisphere, forcing the need for regular acaricide application to ensure colony survival. However, despite the short association (in evolutionary terms), a small but increasing number of *A. mellifera* populations across the globe have been surviving many years without any mite control methods. This long-term survival, or *Varroa* resistance, is consistently associated with the same suite of traits, recapping, brood removal and reduced mite reproduction, irrespective of location. Here we conduct an analysis of data extracted from 60 papers to illustrate how these traits connect together to explain decades of mite resistance data. We have potentially a unified understanding of natural *Varroa* resistance that will help the global industry achieve widespread miticide-free beekeeping and indicate how different honey bee populations across four continents have resolved a recent threat using the same suite of behaviours.

Introduction

Throughout the world the western honey bee, *Apis mellifera*, is an irreplaceable species particularly in terms of their pollination services that contribute to food security and wider ecosystem health (Gallai *et al.*, 2009; Hung *et al.,* 2018). Despite the huge reliance on and commercialisation of honey bees their populations have for many years suffered high losses particularly over the winter period (Gray *et al.,* 2019; Potts *et al.,* 2010). Whilst it is apparent

that numerous stressors such as intensive agriculture and diseases are owing to this decline, it is well established that during the past 70 years the synergy between deformed wing virus (DWV) and its vector *Varroa destructor* has become a critical global threat to honey bee health (Nazzi & Le Conte, 2016).

After *Varroa* jumped the species barrier circa the 1950s, from its native host *Apis cerana* (Asian honey bee) onto *A. mellifera*, it spread globally along with DWV (Martin & Brettell, 2019; Oldroyd, 1999; Wilfert *et al.,* 2016). Currently only Australia and a few small, isolated islands are free of both DWV and *Varroa* (Roberts *et al.,* 2017; Shutler *et al.,* 2014). As *A. mellifera* was completely naïve to the mite *Varroa* typically increased uncontrollably, which coupled with a new viral transmission route (during mite feeding) led to the catastrophic collapse of both managed and feral populations across the globe (Eliash & Mikheyev, 2020). As a result, particularly in the northern hemisphere, the constant use of acaricides is necessary for beekeeping to survive (Boecking & Genersch, 2008). However, whilst acaricides help reduce the *Varroa* and DWV burden, they also remove the selective pressure from *A. mellifera* hampering any adaptation to the parasite (Büchler *et al.,* 2010; Fries & Bommarco, 2007; Fries *et al.,* 2006; Neumann & Blacquière, 2017; Råberg *et al.,* 2009; Traynor *et al.,* 2020). Only three *Varroa*-infested *A. mellifera* populations exist without DWV and hence have never been treated with acaricides. These exits in the highlands of Papua New Guinea, the Solomon Islands (Roberts *et al.,* 2020) and on the island of Fernando de Noronha, Brazil (Brettell & Martin, 2017). Although the mechanism is unknown, natural *Varroa* resistance arose quickly, caused no colony losses, and resulted in high levels of infertile mites in the Fernando de Noronha population (Brettell & Martin, 2017).

In the presence of DWV and absence of treatment, *A. mellifera* populations are able to gradually develop *Varroa* resistance, typically after an initial period of colony losses (Locke, 2016). Resistance is the ability of a population to survive long term without any treatment for *Varroa* within a given environment (Büchler *et al.,* 2010). Thus, we do not view resistance as a fixed trait but the product of adaptive traits and adaptation to the local environment (Le Conte *et al.,* 2020; Traynor *et al.,* 2020) in terms of the surrounding managed and feral colonies. *Varroa* resistant colonies first appeared in Africa (Allsopp, 2006; Nganso *et al.,* 2018) and Africanised honey bees (African x European hybrid) in South America (Moretto *et al.,* 1991) and were associated with widespread lack of control due to acaricide cost and the general resilience of the bee populations. These populations, unlike in developed countries, are not frequently treated or medicated against a range of pathogens and pests (Pirk *et al.,* 2017). Despite this a small but increasing number of beekeepers in Europe (Oddie *et al.,* 2018), the UK (Kruitwagen *et al.,* 2017; Mullin *et al.,* 2010) and the USA (Martin, 2020; Underwood *et al.,* 2019) have stopped all regular acaricide treatment and often establish their managed colonies from feral swarms (Hudson & Shan, 2020; Martin, 2020).

Independently, each *Varroa* resistant honey bee population previously studied across seven countries have all developed the same traits to control the mite. These are: 1- brood removal; in which *Varroa* infested pupae are removed, 2- recapping; where holes are created allowing direct access to the pupa and then resealed and 3- mite infertility; where female mites are unable to produce viable (mated) female offspring.

Unlike many maladies the *Varroa*-DWV association is a new problem especially in evolutionary terms, since *Varroa* has only been in *A. mellifera* populations between 15-70

years depending on the location (Oldroyd, 1999). However, three studies (Hawkins & Martin, 2021; Martin *et al.,* 2019; Oddie *et al.,* 2018) using the same methods found two traits (increased recapping and mite infertility) in *Varroa* resistant populations in South Africa, Brazil, France, UK, Norway and Sweden, countries with different environmental conditions (tropical to subarctic). This indicates that *Varroa* resistance has arisen in multiple locations, irrespective of honey bee variety or environment, especially since recapping behaviour is rarely seen in *Varroa* naïve populations in Australia, Isle of Man and Isle of Colonsay, UK (Hawkins & Martin, 2021; Martin *et al.,* 2019).

This study's aim is to bring together data from 60 publications ranging from the beginning of research into *Varroa resistance* four decades ago to the present day combined with the recent breakthrough study (Oddie *et al.,* 2018) to compare the expression of brood removal, recapping and mite infertility in resistant colonies and susceptible colonies. Then to construct a potential framework that links these three traits and use modelling to explore various aspects of the framework.

Method

Data collection

We searched published literature using Scopus, Web of Science and Google Scholar to collect data on the three key traits namely brood removal, recapping, and *Varroa* non-reproduction in worker brood from susceptible and resistant *A. mellifera* populations. We define resistant populations as those that have survived five or more years without any form of mite-treatment, although many populations studied have survived untreated more than 10 years and some for decades. Despite the many studies used to collate the data the methods employed are all basically the same. Furthermore, a study was only included if a

minimum sample size of 50 cells were recorded as this is above the minimum number of 35 cells required to get an accurate result for brood removal and mite reproduction (Buchler *et al*., 2020; Eynard *et al.,* 2020). A sample size of 100 cells would be ideal however, to increase the data available for this study 50 cells was deemed appropriate (Buchler *et al*., 2020) Additionally, studies were only included if they used natural comb and only included cells infested with a single foundress because both of these factors can affect brood removal and mite reproduction (Boecking & Drescher, 1992; Martin, & Kemp, 1997).

We extracted information from 60 key data rich papers (see supplementary data). Where possible single colony data were extracted. For example, all recapping data (*n* = 163) came from single colonies; for brood removal nine of the 86 data points are colony averages; and for mite infertility 75 of the 99 data points are colony averages, due to sample size limitations (see supplementary data for all source data and studies). No susceptible colonies are known from where Africanised and African bees occur hence comparisons with resistant colonies in these locations are not possible. Almost all the data collected concerns the Korean 'K' haplotype of *Varroa* (see supplementary data for more information).

Brood removal

We used the standard bee search string ("*Apis mellifera*" OR "honeybee" OR "honey bee") AND ("removal" OR "brood removal" OR "hygienic behaviour" OR "VSH" OR "*Varroa* sensitive hygiene" OR "*Varroa* specific hygiene") AND "*Varroa*". We looked for studies that measured the removal of brood that had been artificially or naturally infested (one study (Vandame *et al.,* 2002)) with *Varroa*. Studies using artificial infestation all had to follow the same basic protocol outlined in (Martin *et al.,* 2019). In brief, a frame of freshly capped brood is taken from a colony and mites are inserted carefully into the capped cells

containing recently capped cells. After around 10 days in the colony the frame is inspected, and the number of infested cells removed is recorded.

Recapping

We used the standard bee string AND ("Re-capping" OR "Recapping") AND "*Varroa*". To be included, studies had to have measured the recapping of *Varroa* infested cells following the correct protocol outlined in Boecking & Spivak (1999) and Harris *et al.,* (2012).

Mite infertility

We used the standard bee search string AND ("*Varroa*" OR "*Varroa* mite" OR "mite") AND ("reproduction" OR "non-reproduction" OR "fertility" OR "infertility"). Here we define infertility as the inability to produce a viable (mated) female offspring and so we collected data following this definition. Importantly, some data used were collected from papers that utilised the definition of no egg laying. The justification for this, is that non-egg laying also falls within the definition, and at worst provides an underestimate of the reduced reproductive rate of mites. To calculate the effect of brood removal on offspring production by *Varroa*, a simple equation was formulated:

$$(1-a) \times b = c$$

Where: a = proportion of infested cells removed

b = maximum number of viable offspring produced pre cycle

c = average number of viable female offspring produced pre reproductive cycle

Data analysis

The sample sizes (in cells) were used to calculate weighted averages for each of the traits for resistant and susceptible populations. Statistical analyses were conducted in Minitab® version 18 on unweighted data (Software, 2017). Mann Whitney U tests were used to compare the removal abilities, recapping abilities and infertile mite proportions of resistant and susceptible populations. Statistical significance for all tests was $p < 0.05$.

The effect of brood removal on mite and honey bee population growth was modelled using the BEEHAVE model (Becher *et al.,* 2014). Increasing worker pupal mortality rates were used to simulate brood removal (as dead brood is removed in the simulation). The mortality was independent of mite infestation as the effect of DWV was removed from the equation for simplicity since within the BEEHAVE model DWV also affects pupa mortality confounding the observation of the effect of brood removal. This simplification was deemed acceptable as the result would only provide an underrepresentation. In actuality, as bees target infested cells it would likely take less removal to achieve the same outcome.

Framework construction

After collecting and analysing the data we constructed a hypothetical framework to explain how many of the various traits are connected. Data from this study or findings from related studies were used to justify the proposed link between each trait.

Results

Honey bee behaviour

Recapping behaviour is the resealing of holes made in the cap that covers the developing worker pupa; holes allow better access to the signal(s) that trigger hygienic behaviour

(Grindrod & Martin, 2021; Martin *et al.,* 2019). We collected data from 163 colonies from five studies that took place across seven countries (see Fig. 1c, page 116). This showed that in resistant colonies significantly more infested cells are recapped than in susceptible colonies (55% vs 33%) ($U = 1280$, $p < 0.00001$).

Brood removal is a trait of honey bees where diseased or dead pupae are removed. It defends the colony against the spread of several diseases including chalkbrood, American foul brood and *Varroa* infestation. Data from mite-infestation experiments from 403 colonies (86 data points) across 10 studies conducted in seven countries demonstrate that resistant colonies are significantly better at removing mite-infested brood than susceptible colonies (38% vs 22%; $U = 341.5$, $p < 0.0001$) (Fig. 1b). When separated into populations both Africanised bees and their African relatives (*A. m scutellata* and *A. m capensis*) have significantly greater removal abilities than susceptible colonies in Europe ($U = 83$, $p < 0.0001$ and $U = 207.5$, $p = 0.002$).

***Varroa* reproduction**

Using the equation '(1-a) x b = c' (see methods), which generates a linear relationship between brood removal and reproductive output (Fig. 1d). The removal of 38% and 22% infested brood in resistant or susceptible colonies (Fig. 1b) predicts 0.87 (resistant) and 1.09 (susceptible) viable female offspring are produced per reproductive cycle when no removal allows 1.4 viable female offspring to be produced (Martin, 1994). If a maximum value of 1.6 (56) is used, values of 0.99 (resistant) and 1.25 (susceptible) are obtained. These values are independent from the total number of reproductive cycles performed, which varies between two and three (Martin, 2001; Martin & Kemp, 1997; Rosenkranz *et al.,* 2010). The decrease in reproductive output increases the proportion of infertile mites (see discussion

for details). Data from 786 colonies (99 data points) across 40 studies in 14 countries showed that resistant populations had significantly greater proportions of infertile mites than susceptible colonies (45% vs 17%; U= 28, $p < .0001$) (Fig. 1e).

Colony level effects

The BEEHAVE model predicted that removing greater than 40% of infested pupae results in negative mite population growth (Fig. 1f). Additionally, it predicted that, irrespective of infestation status, if the brood removal rate were to exceed 40% in spring, 55% in summer or 60% in winter, the colony would collapse (Fig. S2). However, resistant colonies now typically only have worker brood infestation rates of around 4% (Fig. 1h).

Decreasing worker-brood infestation levels

In the Africanised colonies, which are all resistant, average worker-brood infestation rates have fallen from 20% during 1996-1998 to 4% in 2018-2019 (Fig. 1h). Additional preliminary data from UK resistant colonies (n = 44) collected by the authors and Hawkins (2020) found that brood infestation averaged at 6% and was not significantly different to Africanised colonies in 2018/19 (U = 460, p = 0.052).

Framework

Using the data and analyses presented above we constructed a framework to link them together to explain how *Varroa* resistance may develop in *A. mellifera* (Fig. 1a-j). Our interpretation centres on the idea that an existing trait, hygienic behaviour, when adapted to detecting and removing mite-infested pupae, can explain all other traits. Given the data and the models used as well as the findings of other studies, we believe our framework to

be the most plausible interpretation of the results we have presented here. Further justifications for the framework are presented in the discussion.

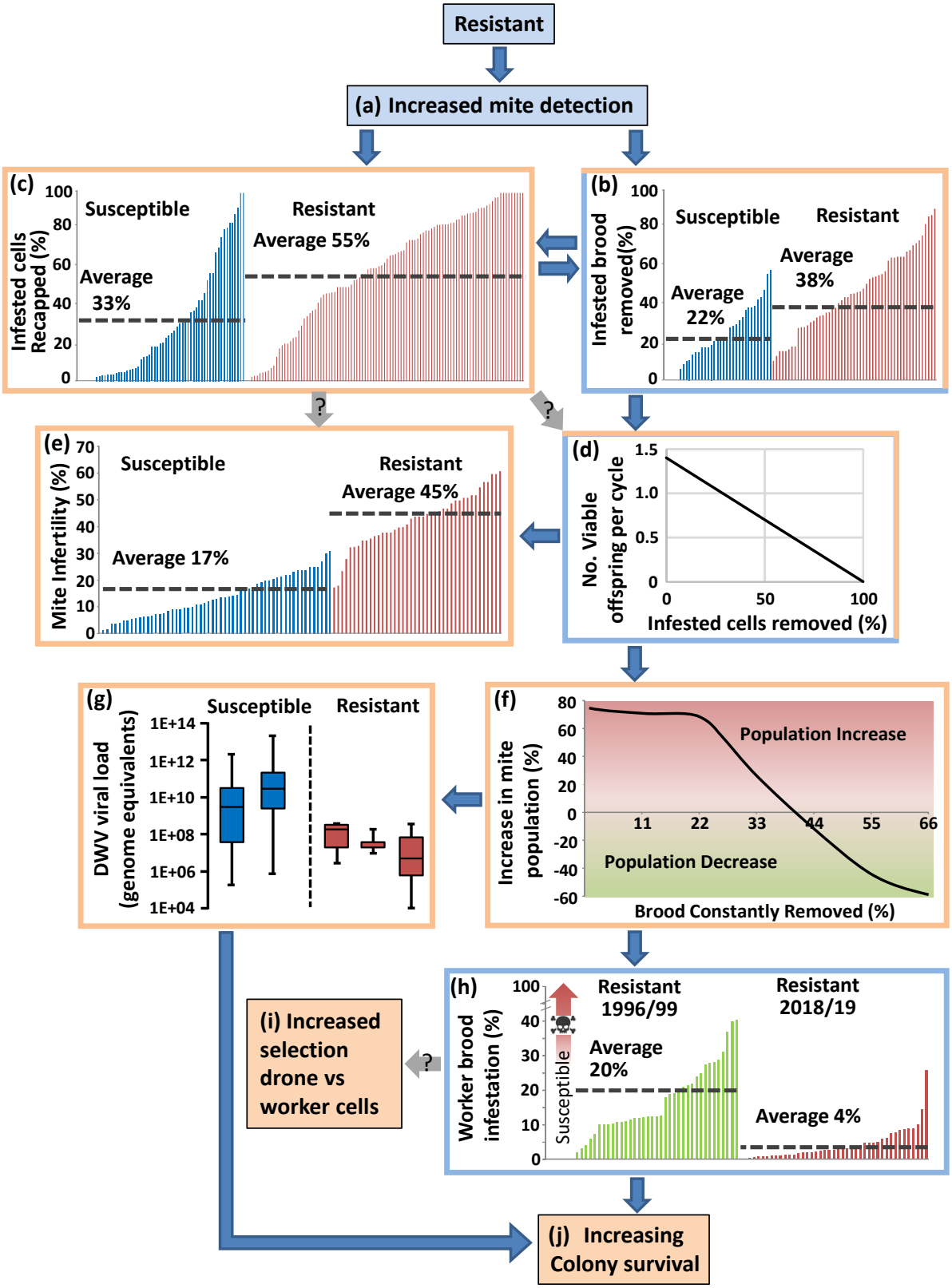

Figure 1 (a-j). A proposed framework for the development of *Varroa* resistance. Boxes in blue or with a blue border are "causes" of the "effects" that are indicated by boxes in orange or with orange borders. All source data for each chart is available in the supplementary data (Tables S1-S8 and Figure S1). Grey arrows with a question mark indicate possible links suggested in the literature. In box h, the red arrow indicates that in untreated, susceptible colonies *Varroa* infestations continuously rise until colony death. deformed wing virus (DWV) data in box g is adapted from (de Souza *et al.,* 2021) and discussed below.

Discussion

The proposed framework attempts to explain how *Varroa* resistance may develop in honey bee (*A. mellifera*) populations. The framework suggests that resistance is a sequence of events that generate the key traits (increased recapping, brood removal and mite infertility) rather than a single trait (Locke, 2016; Mondet, *et al.,* 2020). Here we found that the enhanced expression of these three key traits is common amongst resistant populations. This independent occurrence of the key traits within colonies across the world could be an example of parallel evolution (Oddie *et al.,* 2018) because whilst the recapping and removal behaviours pre-date *Varroa*, they have been co-opted to control *Varroa*. Recapping is rare trait in mite-naïve colonies but occurs at low and high levels in susceptible and resistant colonies respectively (Grindrod & Martin, 2021; Martin *et al.,* 2019). Similarly, other traits such as brood suppression of mite reproduction (Conlon *et al.,* 2019), or DWV tolerance (Locke *et al.,* 2021; Thaduri *et al.,* 2019) may compliment those within the framework. There is also likely to be a mite element to resistance which could be illuminated by further studies into the co-evolution of *A. mellifera* and *Varroa* (Beaurepaire *et al.,* 2019; Moro *et al.,* 2021).

As resistance is a population level trait rather than a single colony trait, a resistant colony becomes vulnerable if moved out of its population and could collapse if a sudden influx of mites occurs due to excessive (40-60%) brood removal (Fig. S2). This may explain why resistant colonies moved out of their population typically do not survive ((Büchler *et al.,* 2015); SJM personal observation).

Honey bee behaviour

The framework begins with the increased detection of *Varroa*-infested cells, an ability that has been linked to resistant bees by numerous studies (Gramacho & Spivak, 2003; Martin *et al.,* 2019; Masterman *et al.,* 2001; Mondet *et al.,* 2015; Mondet *et al.,* 2021) (Fig. 1a). Unlike most brood diseases *Varroa*-DWV is a chronic condition that does not kill the developing host pupae but shortens its lifespan as an adult (Benaets *et al.,* 2017; Dainat *et al.,* 2011; Martin, 2001). Bees already have a well-developed hygienic behaviour response but it typically deals with diseases that cause dead brood (Spivak & Gilliam, 1993). Despite this clear evidence exists for the detection of infested cells, directly from six mite insertion experiments and one natural infestation experiment (Fig. 1b) and indirectly from the behaviour known as recapping (Fig. 1c).

Given that on average resistant colonies remove and recap significantly greater proportions of infested cells than susceptible colonies (Fig. 1b and 1c) indicates that increased detection of infested cells causes these traits to increase. Additionally, recapping has been shown to be positively correlated to brood removal (Martin *et al.,* 2019; Oddie *et al.,* 2018) further suggesting a common trigger. Increased recapping may occur because more sensitive adults (Gramacho & Spivak, 2003; Masterman *et al.,* 2001; Mondet *et al.,* 2015) investigate sealed brood around infested cells either due to a diffuse signal emanating from infested cells or

increased cursory checking near infested cells (Grindrod & Martin, 2021; Martin *et al.,* 2019).

Typically, hygienic behaviour tests use the freeze-killed brood method (Spivak & Gilliam, 1998) and this does not correlate with removal of mite-infested brood (Boecking & Drescher, 1992; Danka *et al.,* 2013; Hawkins, 2020; Leclercq *et al.,* 2018; Leclercq *et al.,* 2017; Martin *et al.,* 2019; Oddie *et al.,* 2017). However, this does not negate the contribution of hygienic behaviour to mite resistance, since the cues are different (living vs dead pupae) (Mondet *et al.,* 2020) and freezing kills a lot of brood at the same time in the same location, thus generating an abnormally high concentration of cues. Therefore, if colonies perform exceptionally well (remove > 95% dead brood within 24 hours) they may remove a reasonable amount (average of 66%) of *Varroa* infested brood and have high recapping rates (Leclerq *et al.,* 2018).

It is unclear whether the cues involved are emanating from the mites or pupae (Gramacho & Spivak, 2003; Masterman *et al.,* 2001; Mondet *et al.,* 2016; Wagoner *et al.,* 2019; Wagoner, *et al.,* 2018) or both (Mondet *et al.,* 2021) since parasitisation by *Varroa* and DWV infection causes changes to the chemical profile of pupae (Baracchi *et al.,* 2012; Salvy *et al.,* 2001; Schoning *et al.,* 2012; Wagoner *et al.,* 2019; Wagoner *et al.,* 2020; Wagoner *et al.,* 2018). Six compounds (four ketones and two acetates) have been detected on both infested pupae and mites and although all adult workers can detect these compounds only workers from resistant colonies can distinguish the mix of six compounds from healthy brood (Mondet *et al.,* 2021). Other studies (Nazzi *et al.,* 2004; Wagoner *et al.,* 2020) have detected different compounds that could also stimulate a hygienic response. The general consensus is that multiple chemical cues are involved in hygienic behaviour, which may prevent the loss of

healthy brood if a cell is wrongly opened the subsequent lack of the secondary cue could trigger resealing or "recapping" (Grindrod & Martin, 2021). Indeed, recapping of both non-infested and infested cells is consistently elevated in all resistant populations (Martin *et al.,* 2019). The hole made in the cell cap is generally less than 1mm in non-infested cells, but significantly larger (up to 5mm) in infested cells (Hawkins, 2020; Martin *et al.,* 2019), which may increase the detection of less volatile cues such as those described (Mondet *et al.,* 2021).

***Varroa* reproduction**

In our framework we link increased removal of mite-infested to reduced reproductive output and thus increased mite infertility (Fig. 1b, d & e). Previous studies have also suggested links between increased brood removal, potentially recapping (Oddie *et al.,* 2018; Oddie *et al.,* 2021), and reduced mite reproductive success (Kirrane *et al.,* 2011). In agreement, we found that resistant colonies had a significantly greater percentage of infertile mites (Fig. 1e). A simple explanation is that disrupting the very uniform sequence of mite-reproduction leads to foundress-mites producing fewer offspring and depleting their finite supply of 18–30 eggs (Akimov & Yastrebtsov, 1984; Alberti & Hänel, 1986; Mikityuk, 1979; Ruijter, 1987) and limited supply of spermatozoa (Alberti & Hänel, 1986; Donzé *et al.,* 1996). Infertile mites have fewer spermatozoa (Harris & Harbo, 1999), and the number of laid eggs steadily declines in mites preforming more than two reproductive cycles (Ruijter, 1987). Using the simple equation (Fig. 1d) the estimated reproductive values for resistant and susceptible colonies of between 0.87-0.99 and 1.09-1.25 respectively were similar to actual values from resistant and susceptible colonies (Martin *et al.,* 2019; Medina & Martin, 1999; Oddie *et al.,* 2018). Whatever the reason, the reproductive asynchrony caused by the

removal of infested pupa causes less mites to contribute to the next generation, thus population growth slows and there is a reduced proportion of new fertile mites compared to older infertile mites (Harris, Danka, & Villa, 2010; Kirrane *et al.,* 2011). In addition to brood removal, reductions in mite fertility may be the result of similar interruptions by recapping (Oddie *et al.,* 2021) and/or brood effects (Conlon *et al.,* 2019) but more data are needed.

Colony level effects

Reduced fertility we then linked to reduced population growth because our BEEHAVE model predicted that infested brood removal above 40% caused negative mite population growth (Fig.1f). Thus, in our framework the detection and removal via cannibalisation of infested worker-brood leads to reduced mite population growth, a commonly occurring outcome in surviving populations (Mondet *et al.,* 2020). Additionally, because brood removal varies within a population (Fig. 1b) the BEEHAVE model helps explain the fluctuating mite populations observed in long term studies of resistant colonies (Medina & Martin, 1999; Mondragón *et al.,* 2006; Souza, 2019). Other studies also found an association between increased mite infertility and a reduced mite burden (Kefuss *et al.,* 2015; Locke *et al.,* 2012; Nganso *et al.,* 2018; Oddie *et al.,* 2017; Strauss *et al.,* 2015) again suggesting it may link brood removal and population growth.

Furthermore, reduced mite burden also reduces the number of viral vectors (Le Conte *et al.,* 2020) causing lower viral titres (Fig. 1g. (de Souza *et al*., 2021; de Souza *et al.,* 2019; Kevill *et al.,* 2019; Ryabov *et al.,* 2017)) and a reduced number of deformed bees (Dainat & Neumann, 2013; Francis *et al.,* 2013; Gusachenko *et al.,* 2020). One study found that removal above 95% of freeze killed pupae lowered mite population growth and significantly

lower DWV titres in workers than colonies below 95% removal (Toufailia *et al.,* 2014). However, cannibalism of infested pupae allows DWV prevalence to remain high (Posada-Florez *et al.,* 2021) even in resistant populations (Kevill *et al.,* 2017), but titres fall since oral (natural) viral transmission is much less infective than via vector transmission (Gusachenko *et al.,* 2020; Posada-Florez *et al.,* 2021).

Decreasing worker-brood infestation levels

In non-resistant untreated colonies mite populations increase until colony collapse with increasing brood infestation levels from 30% to 100% at colony collapse (Martin *et al.,* 2010), whereas in resistant colonies worker brood infestation rate is maintained below 20% (Fig. 1h). Interestingly, we found that worker brood infestation has fallen significantly ($U = 123$, $p < 0.0001$) from 20% to just 4% over the past two decades in resistant colonies in South America (Fig. 1h), currently the only location with long-term data.

We speculate that this is because mites are increasingly waiting for drone brood, which is not targeted by hygienic behaviour in either *A. mellifera* or *A. cerana* (Harris, 2008). Furthermore, the proportion of mites on adult bees decreased when drone brood was plentiful and increased when it was scarce (Medina *et al.,* 2002). Similarly, in resistant colonies from Uruguay the ratio of the mites' distribution between worker and drone cells was much greater (1:12.6) than in susceptible colonies (1: 5.7) (Mendoza *et al.,* 2020). Heavily infested drone brood has also been observed in resistant populations in Mexico, Brazil, and South Africa (30% (Martin *et al.,* 2019)) however, much of the evidence is anecdotal and needs studying further.

In fact, the evolutionary reason why *V. jacobsoni* avoids worker brood in its natural host *A. cerana* remains unclear. It is well established in *A. cerana* that *V. jacobsoni* rarely

reproduces in worker brood (Anderson, 1994; Boot *et al.,* 1997; Koeniger & Koeniger, 1983; Tewarson, Singh, & Engels, 1992), and the drone pupa dies if infested by multiple mite families and becomes entombed within the cell rather than removed (Rath, 1999). When *V. destructor* mites are artificially inserted into incubated *A. cerana* worker brood 30-50% of the pupae die (Page *et al.,* 2016), potentially due a saliva toxin protein from *V. destructor*, but no mortality occurs in *A. mellifera* (Page *et al.,* 2016; Zhang & Han, 2018). This implies that hygienic behaviour in *A. cerana* relies on detecting dead brood making the ability of detecting living infested pupa and mites (Mondet *et al.,* 2021) in *A. mellifera* even more unique. However, further studies in *A. cerana* are required to differentiate between or link together: 1- the detection and removal of living mite-infested brood, 2- social apoptosis and removal of dead brood, 3- any co-evolution by *Varroa* or worker brood that prevents mite reproduction

Finally, in a small resistant *A. mellifera* population on the remote Fernando de Noronha Island, Brazil, adult mite infestation levels fell from 26% in 1991 to 1-2% in 2016. However, worker and drone brood infestation levels have stabilised around 20% and 40% respectively (Fig. S3) (Brettell & Martin, 2017; de Mattos *et al.,* 2016) despite very high infertility rates (Brettell & Martin, 2017). This may be explained by the very rare absence of DWV from this population that allows high brood infestation levels to persist without the negative impacts of DWV. Confirmatory studies from the other two DWV-free *Varroa* infested populations (Roberts *et al.,* 2020) are needed.

Reduced colony losses

The final link in our framework is that reduced mite and virus burden will lead to enhanced colony survival (Martin, 2001). Indeed, the reduction of mite burden and associated

enhanced survival is the primary function of acaricides. Enhanced survival is hard to measure as susceptible colonies are usually treated with acaricides. However, the annual loss rates of treated colonies are higher than resistant populations in Le mans and Avignon, France (Le Conte *et al.,* 2007). Additionally, over 100 beekeepers across a 2,500 km² region of North Wales, UK have maintained 499 colonies treatment free for 11 years (Hudson & Shan, 2020) and in Swindon (UK) a small beekeeper group have kept treatment free colonies since 1995 (Hoskins, 2014) and neither group has reported increased losses. In South Africa, after an initial period of high losses, annual colony losses stabilised at around 5% between 1998 and 2004, which is similar to pre-*Varroa* levels (Allsopp, 2006). Also, in Algeria, Tunisia and Morocco initial colony losses were high, although short lived (Fazier *et al.,* 2010). Across most of Africa (Allsopp, 2006; Dietemann *et al.,* 2009; Fazier *et al.,* 2010; Muli *et al.,* 2014; Nganso *et al.,* 2017) and in Africanised colonies throughout Latin America no widespread losses were reported where lack of acaracide use, due to cost and availability, may have helped resistance develop. Instead, widespread colony losses occurred in the Northern hemisphere as *Varroa* spread from Asia throughout Europe and into the Americas, where acaracides were quickly adopted.

Variability of data

A substantial issue when it comes to measuring resistance traits is the inherent variability within colonies and thus across populations. Within a colony, traits themselves are not static and fluctuate with the changing season along with the associated availability of worker and drone brood and the infestation levels (Bienefeld *et al.,* 1995; Eynard *et al.,* 2020; Kulinčević *et al.,* 1988; Marcangeli *et al.,* 1992; Mondet *et al.,* 2020b; Moretto *et al.,* 1997; Moro *et al.,* 2021; Otten & Fuchs, 1990). Variability is also likely due to temporal changes in the

composition of the different hygienic workers. To elaborate, the three main stages of brood removal: the initial detection and opening of the cell cap, the full uncapping of the cell and finally removing or cannibalising the pupae or recapping the cell (Palacio *et al.,* 2010) are conducted by bees of different ages and sensory acuity, a division of labour further affected by genetic, neural, social and environmental conditions (Goode *et al.,* 2006; Gramacho & Spivak, 2003; Page & Robinson, 1991; Scannapieco *et al.,* 2016; Spivak *et al.,* 2003). For example, an imbalance of "uncapper" vs. "recapper" bees may cause many brood cells to be left open (Gramacho & Spivak, 2003). Consequently, it can be very hard to accurately measure resistance associated traits (Buchler *et al.,* 2020; Eynard *et al.,* 2020; Mondet *et al.,* 2020b) resulting in a high degree of variability within colonies and across colony level data sets (Fig. 1b, c and e). Ultimately, variability severely affects selection programmes [reviewed in Guichard *et al.,* 2020], whereas, in natural selection-based experiments such as bond experiments (Fries *et al.,* 2006), black box experiments (Blacquière *et al.,* 2019; Neumann & Blacquière, 2017) assumptions on the importance of traits are not made.

Conclusion

This study shows that the resistance traits of recapping, brood removal and mite infertility are expressed at significantly higher levels in resistant colonies than susceptible ones, and we present a framework to potentially explain how these common traits shared by resistant colonies can link together.

Although, many local sub-species exist, *A. mellifera* remains a single species and environmental conditions within the colony i.e., those that *Varroa* are subject to, remain remarkably constant irrespective of location, which has aided its semi-domestication and

global distribution. Natural bee-driven resistance to *Varroa* is a sustainable, long-term solution, prevents the constant usage of acaricides, and will not weaken bees to any other maladies should they arise and may provide an example of parallel evolution with the same three traits arising in populations in several different continents.

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Supplementary data

Supplementary Tables S1-8 & Supplementary Figures S1-3

Supplementary Table S1. The data, source, location, and the number of colonies for the percentage of infested worker brood removed in **susceptible colonies** shown in figure 1.

EHB = European honey bees

Susceptible			
Author	Location	No. of colonies	Data
Boecking & Drescher, 1992	Germany	1	0.0
Boecking & Drescher, 1992	Germany	1	0.0
Guerra *et al.,* 2000	Brazil, Bees imported from USA	1	0.0
Vandame *et al.,* 2002	Mexico EHB	1	5.7
Vandame *et al.,* 2002	Mexico EHB	1	8.0
Moro *et al.,* 2021	The Netherlands	6	9.7
Vandame *et al.,* 2002	Mexico EHB	1	10.4
Martin & Cook, 1996	UK	1	13.0
Guerra *et al.,* 2000	Brazil, Bees imported from USA	1	14.3
Boecking & Drescher, 1992	Germany	1	14.3
Guerra *et al.,* 2000	Brazil, Bees imported from USA	1	16.6
Guerra *et al.,* 2000	Brazil, Bees imported from USA	1	16.6
Boecking *et al.,* 2000	Brazil, Bees imported from USA	77	16.7
Boecking & Drescher, 1992	Germany	1	18.2
Guerra *et al.,* 2000	Brazil, Bees imported from USA	1	20.0
Guerra *et al.,* 2000	Brazil, Bees imported from USA	1	20.0
Boecking *et al.,* 2000	Germany	76	21.2
Guerra *et al.,* 2000	Brazil, Bees imported from USA	1	22.2
Guerra *et al.,* 2000	Brazil, Bees imported from USA	1	22.2
Guerra *et al.,* 2000	Brazil, Bees imported from USA	1	27.2
Panziera *et al.,* 2017	The Netherlands	5	28.0

Boecking *et al.,* 2000	Germany	55	29.0
Lobb & Martin, 1997	UK	1	31.0
Boecking *et al.,* 2000	Germany	92	32.4
Guerra *et al.,* 2000	Brazil, Bees imported from USA	1	36.4
Boecking & Drescher, 1992	Germany	1	37.5
Guerra *et al.,* 2000	Brazil, Bees imported from USA	1	37.5
Guerra *et al.,* 2000	Brazil, Bees imported from USA	1	38.5
Guerra *et al.,* 2000	Brazil, Bees imported from USA	1	41.6
Boecking & Drescher, 1992	Germany	1	42.9
Guerra *et al.,* 2000	Brazil, Bees imported from USA	1	46.6
Guerra *et al.,* 2000	Brazil, Bees imported from USA	1	55.0
Boecking & Drescher, 1992	Germany	1	57.1

Supplementary Table S2. The data, source, location, and the number of colonies for the percentage of infested worker brood removed in **resistant colonies** shown in figure 1. EHB = European honey bees

Resistant			
Author	Location	No. of colonies	Data
Boecking & Ritter, 1993	Tunisia	1	10.0
Guerra *et al.,* 2000	Brazil	1	12.5
Martin *et al.,* 2019	South Africa *A. capensis*	1	15.0
Boecking & Ritter, 1993	Tunisia	1	15.0
Boecking & Ritter, 1993	Tunisia	1	15.0
Panziera *et al.,* 2017	The Netherlands	5	16.0
Moro *et al.,* 2021	The Netherlands	5	17.4
Boecking & Ritter, 1993	Tunisia	1	17.5
Martin *et al.,* 2019	South Africa A. *capensis*	1	27.0
Boecking & Ritter, 1993	Tunisia	1	27.5
Boecking & Ritter, 1993	Tunisia	1	27.5
Vandame *et al.,* 2002	Mexico	1	28.4
Boecking & Ritter, 1993	Tunisia	1	30.0
Guerra *et al.,* 2000	Fernando de Noronha EHB	1	30.8
Boecking & Ritter, 1993	Tunisia	1	32.5
Guerra *et al.,* 2000	Fernando de Noronha EHB	1	33.3
Vandame *et al.,* 2002	Mexico	1	33.7
Martin *et al.,* 2019	South Africa	1	35.0
Vandame *et al.,* 2002	Mexico	1	35.3
Boecking & Ritter, 1993	Tunisia	1	37.5
Guerra *et al.,* 2000	Brazil	1	37.5
Guerra *et al.,* 2000	Brazil	1	40.0
Guerra *et al.,* 2000	Fernando de Noronha EHB	1	41.4

Martin *et al.,* 2019	South Africa *A. capensis*	1	43.0
Panziera *et al.,* 2017	Europe	1	43.0
Guerra *et al.,* 2000	Brazil	5	44.7
Martin *et al.,* 2019	South Africa *A. capensis*	1	45.0
Guerra *et al.,* 2000	Brazil	1	45.5
Martin *et al.,* 2019	South Africa *A. capensis*	1	46.0
Boecking & Ritter, 1993	Tunisia	1	47.5
Guerra *et al.,* 2000	Brazil	1	50.0
Boecking & Ritter, 1993	Tunisia	1	52.5
Guerra *et al.,* 2000	Brazil	1	53.3
Guerra *et al.,* 2000	Brazil	1	53.8
Guerra *et al.,* 2000	Brazil	1	54.5
Boecking & Ritter, 1993	Tunisia	1	55.0
Guerra *et al.,* 2000	Brazil	1	56.5
Guerra *et al.,* 2000	Brazil	1	62.0
Guerra *et al.,* 2000	Brazil	1	63.6
Guerra *et al.,* 2000	Brazil	1	63.6
Martin *et al.,* 2019	South Africa *A. capensis*	1	64.0
Martin *et al.,* 2019	South Africa *A. capensis*	1	64.0
Guerra *et al.,* 2000	Brazil	1	64.0
Guerra *et al.,* 2000	Brazil	1	66.7
Boecking & Ritter, 1993	Tunisia	1	67.5
Guerra *et al.,* 2000	Brazil	1	70.0
Guerra *et al.,* 2000	Brazil	1	71.0
Boecking & Ritter, 1993	Tunisia	1	72.5
Boecking & Ritter, 1993	Tunisia	1	75.0
Martin *et al.,* 2019	South Africa *A. capensis*	1	81.0
Martin *et al.,* 2019	South Africa *A. capensis*	1	85.0
Guerra *et al.,* 2000	Brazil	1	85.7
Martin *et al.,* 2019	South Africa *A. capensis*	1	89.0

Supplementary Table S3. The data, source, location, and the number of colonies for the percentage of infested worker brood recapped in **susceptible colonies** shown in figure 1.

Susceptible			
Author	Location	No. of colonies	Data
Oddie *et al.,* 2018	France	1	0.0
Oddie *et al.,* 2018	France	1	0.0
Oddie *et al.,* 2018	France	1	0.0
Oddie *et al.,* 2018	France	1	0.0
Oddie *et al.,* 2018	France	1	0.0

Hawkins & Martin, 2021	UK	1	0.0
Oddie *et al.,* 2018	Norway	1	0.0
Oddie *et al.,* 2018	Norway	1	0.0
Oddie *et al.,* 2018	France	1	2.4
Oddie *et al.,* 2018	France	1	2.6
Oddie *et al.,* 2018	France	1	3.0
Oddie *et al.,* 2018	France	1	3.1
Oddie *et al.,* 2018	Norway	1	3.6
Oddie *et al.,* 2018	France	1	3.8
Oddie *et al.,* 2018	Norway	1	3.8
Oddie *et al.,* 2018	France	1	4.2
Oddie *et al.,* 2018	France	1	4.8
Hawkins & Martin, 2021	UK	1	4.9
Oddie *et al.,* 2018	Norway	1	4.9
Oddie *et al.,* 2018	France	1	5.6
Hawkins & Martin, 2021	UK	1	6.0
Oddie *et al.,* 2018	France	1	6.7
Oddie *et al.,* 2018	Sweden	1	7.0
Oddie *et al.,* 2018	France	1	7.7
Oddie *et al.,* 2018	France	1	11.4
Oddie *et al.,* 2018	France	1	12.9
Hawkins & Martin, 2021	UK	1	13.3
Oddie *et al.,* 2018	France	1	14.3
Oddie *et al.,* 2018	Norway	1	18.2
Oddie *et al.,* 2018	France	1	18.4
Hawkins & Martin, 2021	UK	1	18.5
Oddie *et al.,* 2018	France	1	20.0
Hawkins & Martin, 2021	UK	1	20.5
Hawkins & Martin, 2021	UK	1	22.9
Oddie *et al.,* 2018	France	1	24.3
Oddie *et al.,* 2018	France	1	25.7
Oddie *et al.,* 2018	Norway	1	26.7
Hawkins & Martin, 2021	UK	1	28.9
Oddie *et al.,* 2018	France	1	30.8
Oddie *et al.,* 2018	France	1	31.0
Moro *et al.,* 2021	The Netherlands	6	32.3
Oddie *et al.,* 2018	Sweden	1	33.0
Oddie *et al.,* 2018	France	1	36.4
Hawkins & Martin, 2021	UK	1	37.5
Oddie *et al.,* 2018	France	1	39.3
Oddie *et al.,* 2018	France	1	42.9
Grindrod & Martin, 2021	UK	1	43.1
Oddie *et al.,* 2018	Sweden	1	47.0
Oddie *et al.,* 2018	France	1	53.7
Hawkins & Martin, 2021	UK	1	57.3
Hawkins & Martin, 2021	UK	1	57.4

Oddie *et al.,* 2018	France	1	68.4
Hawkins & Martin, 2021	UK	1	71.1
Grindrod & Martin, 2021	UK	1	76.3
Hawkins & Martin, 2021	UK	1	80.6
Oddie *et al.,* 2018	France	1	81.6
Grindrod & Martin, 2021	UK	1	84.1
Oddie *et al.,* 2018	France	1	84.2
Hawkins & Martin, 2021	UK	1	88.9
Oddie *et al.,* 2018	France	1	92.0
Hawkins & Martin, 2021	UK	1	100.0
Hawkins & Martin, 2021	UK	1	100.0

Supplementary Table S4. The data, source, location, and the number of colonies for the percentage of infested worker brood recapped in **resistant colonies** shown in figure 1.

Resistant			
Author	Location	No. of colonies	Data
Martin *et al.,* 2019	Brazil	1	0.0
Hawkins & Martin, 2021	UK	1	0.0
Oddie *et al.,* 2018	France	1	2.4
Oddie *et al.,* 2018	France	1	2.8
Hawkins & Martin, 2021	UK	1	3.0
Hawkins & Martin, 2021	UK	1	4.3
Hawkins & Martin, 2021	UK	1	4.7
Hawkins & Martin, 2021	UK	1	5.0
Oddie *et al.,* 2018	France	1	5.6
Oddie *et al.,* 2018	Sweden	1	7.0
Hawkins & Martin, 2021	UK	1	8.3
Oddie *et al.,* 2018	Sweden	1	13.0
Hawkins & Martin, 2021	UK	1	17.4
Martin *et al.,* 2019	South Africa *A. capensis*	1	19.0
Oddie *et al.,* 2018	France	1	20.0
Grindrod & Martin, 2021	Hawaii	1	20.0
Oddie *et al.,* 2018	France	1	21.3
Oddie *et al.,* 2018	France	1	22.9
Hawkins & Martin, 2021	UK	1	23.5
Oddie *et al.,* 2018	Norway	1	26.9
Moro *et al.,* 2021	The Netherlands	5	29.6
Hawkins & Martin, 2021	UK	1	33.3
Oddie *et al.,* 2018	France	1	34.6
Oddie *et al.,* 2018	France	1	36.1
Hawkins & Martin, 2021	UK	1	37.5
Oddie *et al.,* 2018	France	1	38.2

Hawkins & Martin, 2021	UK	1	41.7
Oddie *et al.,* 2018	Norway	1	44.4
Martin *et al.,* 2019	South Africa *A. scutellata*	1	46.0
Hawkins & Martin, 2021	UK	1	46.6
Hawkins & Martin, 2021	UK	1	46.7
Oddie *et al.,* 2018	France	1	47.2
Oddie *et al.,* 2018	Norway	1	47.4
Martin *et al.,* 2019	Brazil	1	50.0
Martin *et al.,* 2019	Brazil	1	50.0
Oddie *et al.,* 2018	France	1	50.0
Hawkins & Martin, 2021	UK	1	50.0
Hawkins & Martin, 2021	UK	1	50.0
Oddie *et al.,* 2018	France	1	51.3
Oddie *et al.,* 2018	Norway	1	53.6
Martin *et al.,* 2019	South Africa *A. capensis*	1	54.0
Oddie *et al.,* 2018	Norway	1	59.5
Hawkins & Martin, 2021	UK	1	55.1
Martin *et al.,* 2019	South Africa *A. scutellata*	1	57.0
Martin *et al.,* 2019	South Africa *A. scutellata*	1	57.0
Martin *et al.,* 2019	South Africa *A. scutellata*	1	60.0
Martin *et al.,* 2019	Brazil	1	60.0
Hawkins & Martin, 2021	UK	1	60.0
Oddie *et al.,* 2018	France	1	61.1
Oddie *et al.,* 2018	France	1	61.5
Martin *et al.,* 2019	Brazil	1	63.0
Martin *et al.,* 2019	South Africa *A. scutellata*	1	65.0
Hawkins & Martin, 2021	UK	1	66.7
Martin *et al.,* 2019	South Africa *A. capensis*	1	67.0
Oddie *et al.,* 2018	France	1	67.5
Oddie *et al.,* 2018	France	1	68.4
Martin *et al.,* 2019	South Africa A. *scutellata*	1	69.0
Grindrod & Martin, 2021	UK	1	69.5
Hawkins & Martin, 2021	UK	1	73.7
Martin *et al.,* 2019	South Africa *A. capensis*	1	75.0
Martin *et al.,* 2019	Brazil	1	75.0
Hawkins & Martin, 2021	UK	1	75.0
Grindrod & Martin, 2021	Hawaii	1	75.7
Martin *et al.,* 2019	Brazil	1	78.0
Oddie *et al.,* 2018	France	1	78.9
Martin *et al.,* 2019	South Africa *A. scutellata*	1	80.0
Martin *et al.,* 2019	South Africa *A. scutellata*	1	80.0
Martin *et al.,* 2019	South Africa *A. capensis*	1	81.0
Oddie *et al.,* 2018	Norway	1	82.0
Martin *et al.,* 2019	South Africa *A. capensis*	1	83.0
Martin *et al.,* 2019	South Africa *A. capensis*	1	83.0
Oddie *et al.,* 2018	France	1	83.3

Hawkins & Martin, 2021	UK	1	83.3
Hawkins & Martin, 2021	UK	1	83.3
Martin *et al.,* 2019	South Africa *A. capensis*	1	84.0
Grindrod & Martin, 2021	UK	1	84.2
Oddie *et al.,* 2018	France	1	85.7
Oddie *et al.,* 2018	Sweden	1	86.0
Martin *et al.,* 2019	South Africa *A. capensis*	1	88.0
Oddie *et al.,* 2018	France	1	88.1
Martin *et al.,* 2019	South Africa *A. capensis*	1	89.0
Oddie *et al.,* 2018	France	1	89.2
Oddie *et al.,* 2018	France	1	89.5
Hawkins & Martin, 2021	UK	1	89.9
Hawkins & Martin, 2021	UK	1	91.7
Martin *et al.,* 2019	South Africa *A. capensis*	1	92.0
Martin *et al.,* 2019	South Africa *A. capensis*	1	92.0
Oddie *et al.,* 2018	France	1	93.3
Martin *et al.,* 2019	Brazil	1	94.0
Grindrod & Martin, 2021	Hawaii	1	95.7
Oddie *et al.,* 2018	France	1	97.5
Oddie *et al.,* 2018	France	1	97.6
Martin *et al.,* 2019	Brazil	1	100.0
Martin *et al.,* 2019	Brazil	1	100.0
Martin *et al.,* 2019	South Africa *A. capensis*	1	100.0
Martin *et al.,* 2019	South Africa *A. capensis*	1	100.0
Martin *et al.,* 2019	South Africa *A. capensis*	1	100.0
Martin *et al.,* 2019	South Africa *A. capensis*	1	100.0
Hawkins & Martin, 2021	UK	1	100.0
Hawkins & Martin, 2021	UK	1	100.0
Grindrod & Martin, 2021	UK	1	100.0

Supplementary Table S5. The data, source, location, how infertility was measured and the number of colonies for the percentage of infertile foundresses in worker brood cells in **susceptible colonies** shown in figure 1e. * >1 indicates where more than one colony was used but the exact number could not be ascertained from the paper.

Susceptible				
Author	Location	Infertility measure	No. of colonies	Data
Moosbeckhofer *et al.,* 1988	Austria	No Female Offspring	1	1.4
Moosbeckhofer *et al.,* 1988	Austria	No Female Offspring	1	1.7
Moosbeckhofer *et al.,* 1988	Austria	No Female Offspring	1	3.6
Moosbeckhofer *et al.,* 1988	Austria	No Female Offspring	1	3.7
Moosbeckhofer *et al.,* 1988	Austria	No Female Offspring	1	4.1
Moosbeckhofer *et al.,* 1988	Austria	No Female Offspring	1	4.9
Kulinčević *et al.,* 1988	Yugoslavia	No Offspring	16	5.0
Fries & Rosenkranz, 1996	Sweden	No Offspring	6	5.6
Kulinčević *et al.,* 1988	Yugoslavia	No Offspring	16	5.9
Rosenkranz, 1999	Brazil, bees imported from Europe	No Offspring	>1*	6.0
Kulinčević *et al.,* 1988	Yugoslavia	No Offspring	16	6.3
Moosbeckhofer *et al.,* 1988	Austria	No Female Offspring	1	6.4
Kulinčević *et al.,* 1988	Yugoslavia	No Offspring	14	6.8
Kulinčević *et al.,* 1988	Yugoslavia	No Offspring	14	7.2
Kulinčević *et al.,* 1988	Yugoslavia	No Offspring	14	7.3
Kulinčević *et al.,* 1988	Yugoslavia	No Offspring	16	7.6
Moosbeckhofer *et al.,* 1988	Austria	No Female Offspring	1	8.6
Rosenkranz & Engels, 1994	Brazil, Bees imported from Germany	No Female Offspring	1	9.0
Kulinčević *et al.,* 1988	Yugoslavia	No Offspring	16	9.0
Kulinčević *et al.,* 1988	Yugoslavia	No Offspring	16	9.1
Harris & Harbo, 1999	USA	No viable female offspring	28	9.6
Harris & Harbo, 1999	USA	No viable female offspring	28	9.6
Locke *et al.,* 2012	France	No viable female offspring	8	10.0
Harris & Harbo, 1999	USA	No viable female offspring	28	10.9
Kulinčević *et al.,* 1988	Yugoslavia	No offspring	14	11.0
Alattal *et al.,* 2017	Saudi Arabia	No offspring	4	11.6
Rosenkranz *et al.,* 1988	Germany	No offspring	2	12.0

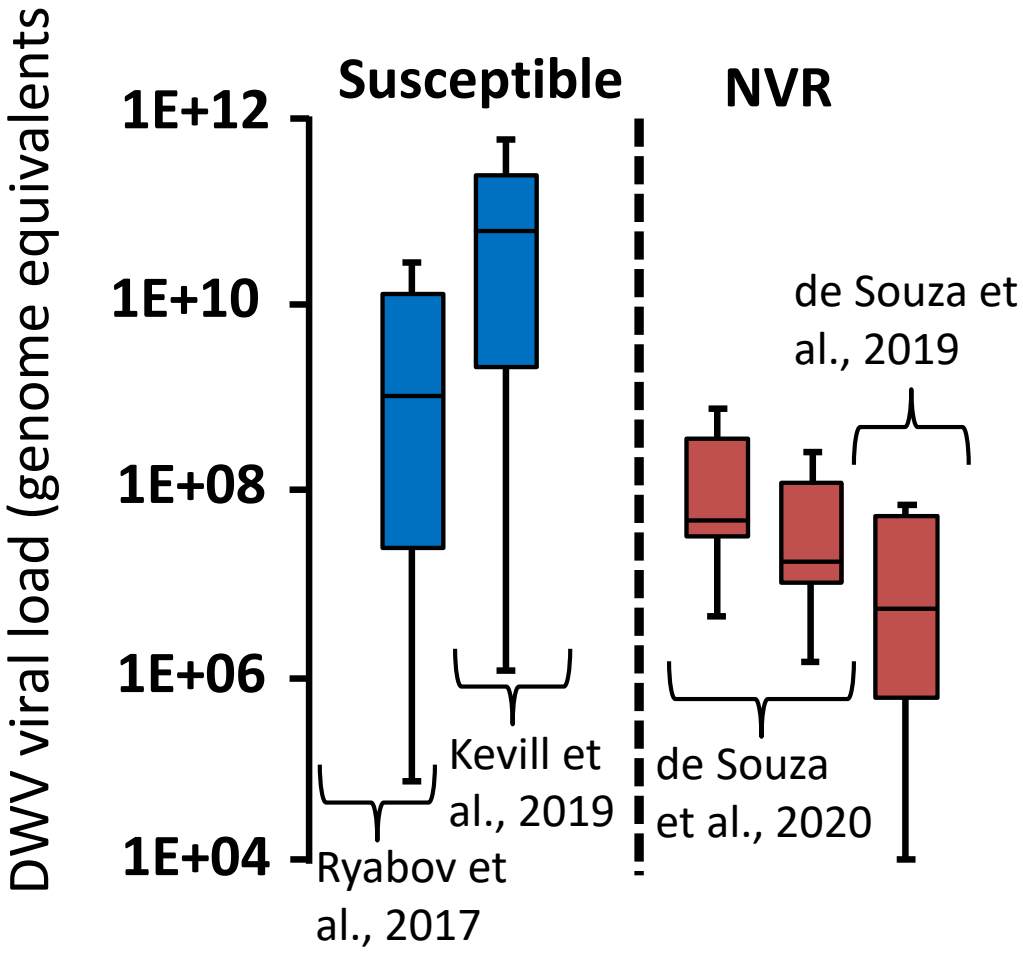
Ropstorf, 1989	Germany	No offspring	33	12.7
Bienefeld *et al.,* 1995	The Netherlands	No offspring	>1*	13.0
Moosbeckhofer *et al.,* 1988	Austria	No female offspring	1	13.6
Ghamdi & Hoopingarner, 2003	USA	No female offspring	10	13.7
Garrido *et al.,* 2003	Germany	No offspring	10	14.0
Moosbeckhofer *et al.,* 1988	Austria	No female offspring	1	14.2
Ghamdi & Hoopingarner, 2003	USA	No female offspring	10	14.5
Boot *et al.,* 1995	The Netherlands	No female offspring	1	16.0
Rosenkranz *et al.,* 1988	Brazil, bees imported from Germany	No offspring	2	17.0
Aumeier *et al.,* 1996	Brazil, Bees imported from USA	No offspring	>1*	17.0
Ghamdi & Hoopingarner, 2003	USA	No female offspring	10	17.0
Ghamdi & Hoopingarner, 2003	USA	No female offspring	10	18.7
Rosenkranz & Engels, 1994	Brazil, Bees imported from Germany	No female offspring	1	19.4
Rosenkranz, 1999	Brazil, bees imported from Europe	No offspring	>1*	20.0
Boot *et al.,* 1995	The Netherlands	No female offspring	1	20.0
Ruijter, 1987	The Netherlands	No offspring	>1*	20.6
Boot *et al.,* 1995	The Netherlands	No female offspring	1	21.0
Fuchs, 1994	Germany	No offspring	>1*	21.6
Locke & Fries, 2011	Sweden	No viable female offspring	23	22.0
Ritter & Jong, 1984	Germany	No offspring	6	22.0
Hawkins & Martin, 2021	UK	No viable female offspring	1	23.3
Infantidis, 1984	Greece	No female offspring	>1*	23.7
Kulinčević *et al.,* 1988	Yugoslavia	No offspring	14	24.0
Ritter & Jong, 1984	Germany	No offspring	13	24.0
Hawkins & Martin, 2021	UK	No viable female offspring	1	25.0
Camazine, 1986	Brazil, Bees imported from USA	No offspring	3	25.0
Martin, 1994	UK	No viable female offspring	8	25.0
Moro *et al.,* 2021	The Netherlands	No viable female offspring	6	27.2
Hawkins & Martin, 2021	UK	No viable female offspring	1	30.0

Hawkins & Martin, 2021	UK	No viable female offspring	1	31.0

Supplementary Table S6. The data, source, location, how infertility was measured and the number of colonies for the percentage of infertile foundresses in worker brood cells in **resistant colonies** shown in figure 1e. * >1 indicates where more than one colony was used but the exact number could not be ascertained from the paper.

Resistant				
Author	Location	Infertility measure	No. of colonies	Data
Moro *et al.,* 2021	The Netherlands	No viable female offspring	5	17.4
Garrido *et al.,* 2003	Brazil	No offspring	10	18
Calderon *et al.,* 2007	Costa Rica	No offspring	10	23.5
Carneiro *et al.,* 2007	Brazil	No viable female offspring	>1*	28.0
Hawkins & Martin, 2021	UK	No viable female offspring	1	32.4
Hawkins & Martin, 2021	UK	No viable female offspring	1	32.6
Medina *et al.,* 2002	Mexico	No viable female offspring	10	33.0
Medina *et al.,* 2002	Mexico	No viable female offspring	10	35.0
Martin & Kryger, 2002	South Africa *A. scutellata*	No viable female offspring	6	35.0
Ropstorf, 1989	Germany	No offspring	33	35.7
Hawkins & Martin, 2021	UK	No viable female offspring	1	36.6
Rosenkranz, 1999	Brazil	No offspring	>1*	37.0
Medina *et al.,* 2002	Mexico	No viable female offspring	10	38.0
Medina *et al.,* 2002	Mexico	No viable female offspring	10	38.0
Medina *et al.,* 2002	Mexico	No viable female offspring	10	38.0
Medina *et al.,* 2002	Mexico	No viable female offspring	10	39.0
Gebremedhn *et al.,* 2019	Ethiopia	No offspring	24	39.9

Medina *et al.,* 2002	Mexico	No viable female offspring	10	40.0
Locke *et al.,* 2012	France	No viable female offspring	16	41.0
Rosenkranz & Engels, 1994	Brazil	No female offspring	3	43.2
Moretto, 1995	Brazil	No offspring	>1*	44.0
Moretto *et al.,* 1997	Brazil	No offspring	5	44.0
Martin *et al.,* 2019	South Africa A. *capensis*	No viable female offspring	10	44.0
Medina *et al.,* 2002	Mexico	No viable female offspring	10	45.0
Martin *et al.,* 2019	Brazil	No viable female offspring	1	45.0
Martin *et al.,* 2019	South Africa A. *scutellata*	No viable female offspring	1	45.0
Nganso *et al.,* 2018	South Africa	No viable female offspring	7	46.0
Rosenkranz, 1999	Brazil	No offspring	>1*	47.0
Moretto, 1988	Brazil	No offspring	>1*	47.0
Aumeier *et al.,* 1996	Brazil	No offspring	>1*	49.0
Medina *et al.,* 2002	Mexico	No viable female offspring	10	50.0
Medina *et al.,* 2002	Mexico	No viable female offspring	10	50.0
Camazine, 1986	Brazil	No offspring	3	51.0
Rosenkranz *et al.,* 1988	Brazil	No offspring	3	51.0
Medina *et al.,* 2002	Mexico	No viable female offspring	10	52.0
Locke & Fries, 2011	Sweden	No viable female offspring	23	52.0
Medina *et al.,* 2002	Mexico	No offspring	10	55.0
Quiñonéz *et al.,* 1996	Brazil	No offspring	8	57.0
Ritter & Jong, 1984	Brazil	No offspring	5	57.0
Corrêa-Marques *et al.,* 2003	Mexico	No viable female offspring	>1*	60.0
Medina & Martin, 1999	Mexico	No viable female offspring	10	60.0
Allsopp, 2006	South Africa	No viable female offspring	33	61.0



Supplementary Figure S1. Data sources for figure 1g adapted from de Souza, Allsopp, & Martin, 2021, Ryabov *et al.,* 2017, Kevill *et al.,* 2019 and de Souza *et al.,* 2019

Supplementary Table S7. The data, source, location and the number of colonies for the percentage of infested worker brood cells in **resistant colonies** of Africanised honey bees between 1996-1999 as shown in figure 1h.

AHB 1996 – 1999			
Author	Location	No. of colonies	Data
Corrêa-Marques & De Jong, 1998	Brazil	1	2.0
Corrêa-Marques & De Jong, 1998	Brazil	1	3.0
Corrêa-Marques & De Jong, 1998	Brazil	1	4.0
Corrêa-Marques & De Jong, 1998	Brazil	1	6.0

Medina & Martin, 1999	Mexico	10	7.4
Corrêa-Marques & De Jong, 1998	Brazil	1	10.0
Corrêa-Marques & De Jong, 1998	Brazil	1	10.0
Corrêa-Marques & De Jong, 1998	Brazil	1	10.0
Medina & Martin, 1999	Mexico	10	10.3
Medina & Martin, 1999	Mexico	10	10.8
Medina & Martin, 1999	Mexico	10	10.9
Cabrera, 1998	Mexico	15	11.1
Vandame *et al.,* 2000	Mexico	10	11.6
Medina & Martin, 1999	Mexico	10	12.1
Cabrera, 1998	Mexico	15	12.1
Medina & Martin, 1999	Mexico	10	12.3
Cabrera, 1998	Mexico	15	12.4
Cabrera, 1998	Mexico	15	12.4
Cabrera, 1998	Mexico	15	12.5
Cabrera, 1998	Mexico	15	12.8
Medina *et al.,* 2002	Mexico	10	18.1
Medina & Martin, 1999	Mexico	10	18.9
Medina & Martin, 1999	Mexico	10	19.2
Medina & Martin, 1999	Mexico	10	19.8
Corrêa-Marques & De Jong, 1998	Brazil	1	21.0
Medina & Martin, 1999	Mexico	10	21.6
Cabrera, 1998	Mexico	15	22.1
Cabrera, 1998	Mexico	15	24.2
Corrêa-Marques & De Jong, 1998	Brazil	1	25.0
Cabrera, 1998	Mexico	15	27.5
Corrêa-Marques & De Jong, 1998	Brazil	1	28.0
Medina & Martin, 1999	Mexico	10	28.4
Guzman-Novoa *et al.,* 1996	Mexico	6	29.0
Medina & Martin, 1999	Mexico	10	31.4
Cabrera, 1998	Mexico	15	37.2
Cabrera, 1998	Mexico	15	40.3
Cabrera, 1998	Mexico	15	40.7

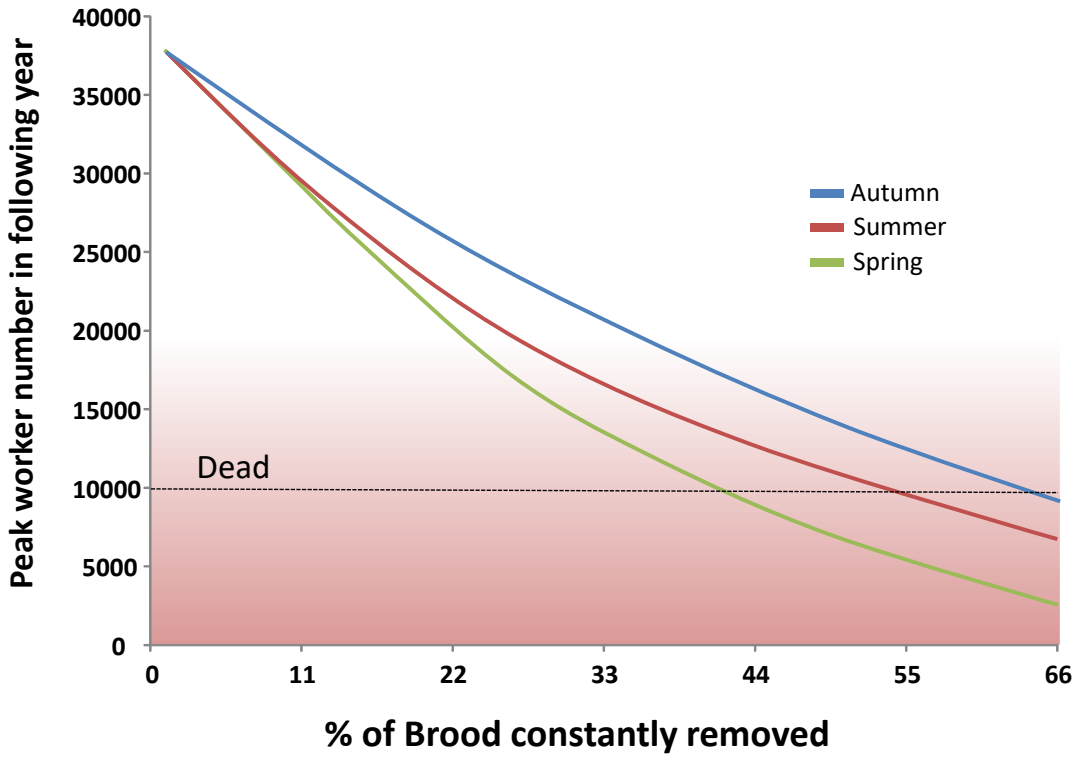
Supplementary Table S8. The data, source, location, and the number of colonies for the percentage of infested worker brood cells in **resistant colonies** of Africanised honey bees between 2018-2019 as shown in figure 1h. * These unpublished data were kindly provided by Dr Luis Medina, Department of Apiculture, Universidad Autonoma de Yucatan, Mexico from an ongoing study, and allows a direct comparison between this 2019 data and the Cabrera 1998, Medina & Martin 1999 data that all came from the same honey bee population.

AHB 2018 – 2019			
Author	Location	No. of colonies	Data
Martin *et al.,* 2019	Brazil	1	0.0
Martin *et al.,* 2019	Brazil	1	0.0
Souza, 2019	Brazil	1	0.3
Medina, 2019*	Mexico	1	0.5
Medina, 2019*	Mexico	1	0.7
Medina, 2019*	Mexico	1	0.8
Martin *et al.,* 2019	Brazil	1	0.9
Medina, 2019*	Mexico	1	1.0
Medina, 2019*	Mexico	1	1.0
Martin *et al.,* 2019	Brazil	1	1.1
Martin *et al.,* 2019	Brazil	1	1.2
Souza, 2019	Brazil	1	1.2
Medina, 2019*	Mexico	1	1.3
Souza, 2019	Brazil	1	1.6
Martin *et al.,* 2019	Brazil	1	1.9
Martin *et al.,* 2019	Brazil	1	2.0
Medina, 2019*	Mexico	1	2.0
Medina, 2019*	Mexico	1	2.1

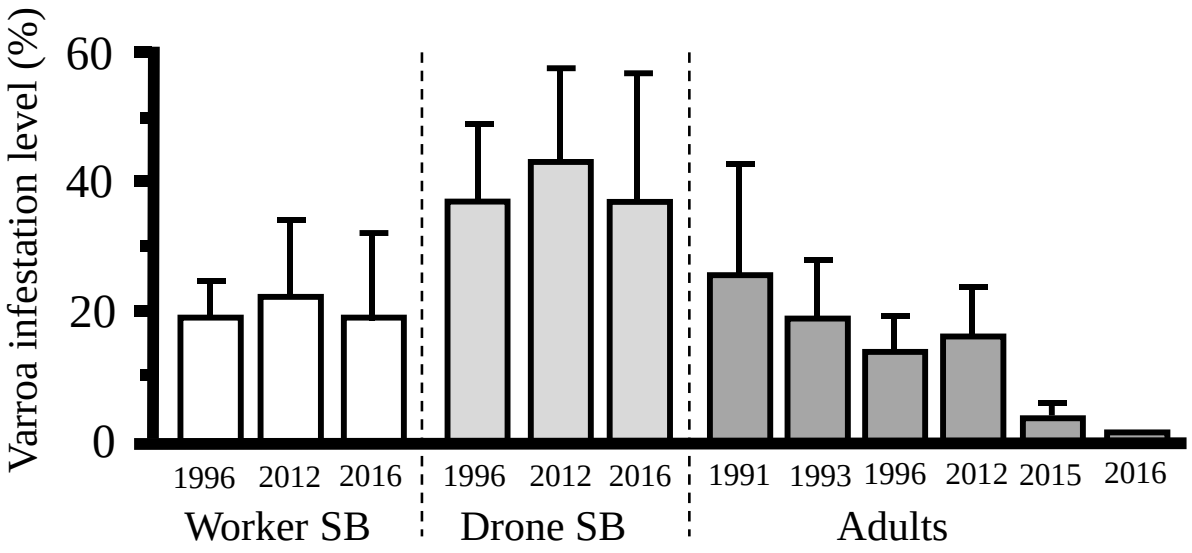
Souza, 2019	Brazil	1	2.5
Medina, 2019*	Mexico	1	2.6
Medina, 2019*	Mexico	1	2.6
Medina, 2019*	Mexico	1	2.7
Medina, 2019*	Mexico	1	2.8
Medina, 2019*	Mexico	1	3.0
Martin *et al.,* 2019	Brazil	1	3.0
Souza, 2019	Brazil	1	3.6
Martin *et al.,* 2019	Brazil	1	3.8
Martin *et al.,* 2019	Brazil	1	4.1
Medina, 2019*	Mexico	1	4.7
Souza, 2019	Brazil	1	4.7
Martin *et al.,* 2019	Brazil	1	4.8
Medina, 2019*	Mexico	1	5.0
Souza, 2019	Brazil	1	6.0
Souza, 2019	Brazil	1	6.1
Souza, 2019	Brazil	1	7.5
Souza, 2019	Brazil	1	7.7
Souza, 2019	Brazil	1	8.5
Souza, 2019	Brazil	1	8.7
Souza, 2019	Brazil	1	8.9
Souza, 2019	Brazil	1	9.0
Souza, 2019	Brazil	1	10.2
Medina, 2019*	Mexico	1	14.5
Martin *et al.,* 2019	Brazil	1	26.0

Almost all, or all of the data collected concerns the Korean 'K' haplotype of *Varroa*. A very small number of the pre 1990 studies from Brazil potentially involved the Japanese 'J' haplotype; however, by 1996 J type was very rare in Brazil (Garrido *et al.,* 2003).

Furthermore, in Brazil fertility was not found to be congruent with haplotype as first suggested (Garrido *et al.,* 2003), and the decrease in worker brood infestation rates between 1996 to 2018 (see results) occurred across Latin America were all infested with the K haplotype. We also included three data points from Fernando de Noronha, in the brood removal data that have the J haplotype. Mite reproduction in both J and K have been found to be the same (Brettel & Martin, 2017) further supporting that haplotype is not associated with *Varroa* resistance.



Supplementary Figure S2. BEEHAVE model results indicating the relationship between peak worker population in the following year and the effect of different levels of consistent brood removal.



Supplementary Figure S3. The changes over time in the *Varroa* infestation levels within the isolated resistant European honey bees on Fernando de Noronha Island, Brazil since 1991 adults and 1996 Worker and Drone sealed brood with whiskers showing the range. This indicates high but stable brood infestations but a continuously declining level of infestation in adult worker bees. Data sources, 1991-1996 (De Jong & Soares, 1997); 2012 (de Mattos *et al.,* 2016); 2015-2016 (Brettell & Martin, 2017).

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Chapter 3: Ten years of deformed wing virus (DWV) in Hawaiian honey bees (*Apis mellifera*), the dominant DWV-A variant is potentially being replaced by variants with a DWV-B coding sequence

Abstract

The combination of deformed wing virus (DWV) and *Varroa destructor* is arguably one of the greatest threats currently facing western honey bees, *Apis mellifera*. *Varroa*'s association with DWV has decreased viral diversity and increased loads of DWV within honey bee populations. Nowhere has this been better studied than in Hawaii, where the arrival of *Varroa* progressively led to the dominance of the single master variant (DWV-A) on both mite-infested Hawaiian islands of Oahu and Big Island. Now, exactly 10 years following the original study, we find that the DWV population has changed once again, with variants containing the RdRp coding sequence pertaining to the master variant B beginning to co-dominate alongside variants with the DWV-A RdRp sequence on the mite-infested islands of Oahu and Big Island. In speculation, based on other studies, it appears this could represent a stage in the journey towards the complete dominance of DWV-B, a variant that appears better adapted to be transmitted within honey bee colonies.

Introduction

Western honey bees (*Apis mellifera*) and the pollination services they provide are important both economically and environmentally (Hung *et al.,* 2018). However, concerns for the health of honey bee populations have been mounting over the years as they face a whole host of threats, including pollution, pests, and parasites (Dainat *et al.,* 2012; Potts *et al.,* 2010; van Engelsdorp *et al.,* 2009). No single threat can be isolated as the leading factor but the bee-mite-virus tripartite relationship is an integral part of this struggle. The ectoparasite

mite *Varroa destructor*, first became a problem around the 1940s when it jumped species from Eastern (*Apis cerana*) to Western honey bees and was traded across the globe (Oldroyd, 1999). Being naïve to this new threat, *A. mellifera* populations were easily overwhelmed and collapsed. Whilst *Varroa* can directly weaken honey bee adults and pupae, their true lethality lies in their ability to vector the deformed wing virus (DWV).

Prior to the spread of *Varroa*, DWV, originally known as the Egyptian bee virus, was known only from a few rare cases (Allen & Ball, 1996). Indeed, despite its long co-existence with honey bees, it was only isolated in 1986 (Bailey & Ball, 1991). This is largely because, without *Varroa*, DWV was limited to less effective oral and sexual transmission routes, and as a consequence, it existed at low viral loads as a covert and usually symptomless infection (Gusachenko *et al.,* 2020; Martin *et al.,* 2012). DWV only became a major problem for honey bees after *Varroa* arrived and, through its feeding habits, introduced a new, highly effective transmission mechanism (Gusachenko *et al.,* 2020). This direct injection of DWV causes emerging adults to have a shortened abdomen, a reduced lifespan (Mockel, Gisder & Genersch, 2011), precocious foraging (Benaets *et al.,* 2017; Traniello *et al.,* 2020) and if the virus happens to replicate in the wing buds of the pupae, deformed wings (Gusachenko *et al.,* 2020). If infection rates are high, the reduced longevity quickly leads to an imbalanced workforce and a collapsing of the colony, particularly during the winter period for bees in the northern hemisphere. Precocious foraging, which DWV can stimulate, accelerates the behavioral and physiological maturation of worker bees, further reducing their lifespan (Traniello *et al.,* 2020).

Accordingly, in areas without DWV, such as Papua New Guinea, Solomon Islands (Roberts *et al.,* 2020), colonies are able to tolerate *Varroa* without suffering colony losses. Similarly, in

areas absent of *Varroa*, colonies do not succumb to DWV infections, as genome equivalents are very low and highly diverse (Martin *et al.,* 2012). A pivotal study in Hawaii found that prior to the spread of *Varroa*, DWV infections consisted of a diverse array of variants, and post *Varroa*, this diversity was drastically reduced (Martin *et al.,* 2012), a finding that was independently found in the UK honey bees (Ryabov *et al.,* 2014). This variant called DWV-A is one of the three highly successful variants, known as master variants, which make up the DWV quasispecies (Biebricher & Eigen, 2006). DWV-A includes the classical versions of DWV and Kakugo virus. The other two master variants are DWV-B, previously known as *Varroa destructor* virus 1 (VDV-1), and DWV-C, which is the rarest of the three (Kevill *et al.,* 2019). Within quasispecies, the master variants exist surrounded by a 'cloud' of less successful variants that are generated due to the rapid mutation of the RNA genome (Biebricher & Eigen, 2006).

The transmission pathway introduced by *Varroa* has altered the dynamics of the quasispecies by favoring particular variants that can survive within the bee (Biebricher & Eigen, 2006; Kevill *et al.,* 2019; Mordecai *et al.,* 2016; Ryabov *et al.,* 2014) and now can replicate within mites' salivary glands (Gisder & Genersch, 2021), be efficiently transmitted by mite feeding (Ryabov *et al.,* 2014), and replicate to high levels within the bee (Ryabov *et al.,* 2014). Originally only the master variant DWV-A was detected and this was associated with the death of infested colonies; later another dominant variant DWV-B appeared (Ryabov *et al.,* 2017). Large scale surveys and longitudinal studies are showing that where DWV and *Varroa* are present, DWV-A and DWV-B seemingly vie for dominance, with a pattern of the increasing dominance of DWV-B (Kevill *et al.,* 2019; Manley *et al.,* 2019). This change could possibly be explained by several factors firstly the potentially lower virulence of DWV-B compared to DWV-A (Norton *et al.,* 2020) and secondly that DWV-B can, unlike

DWV-A, replicate within the mite (Gisder & Genersch, 2021) and finally that DWV-B can replicate to higher titers within pupae (Dubois *et al.,* 2019; Tehel *et al.,* 2019). Furthermore, co-infection with more than one DWV variant has led to the identification of DWV recombinant genomes (Dalmon *et al.,* 2017; Fei *et al.,* 2019; Moore *et al.,* 2011; Mordecai *et al.,* 2016). To date, several recombinants have been detected in honey bees, between DWV-A and DWV-B (Dalmon *et al.,* 2017; Fei *et al.,* 2019; Moore *et al.,* 2011) and also DWV-A and DWV-C (Mordecai *et al.,* 2016). The most commonly detected recombinant breakpoints have been located in the 5' UTR (Dalmon *et al.,* 2017), Lp, Vp1, Vp2, Vp3, helicase (Moore *et al.,* 2011), and more recently, a recombinant between DWV-A and an unknown variant in the Vpg and RNA dependent RNA polymerase (RdRp) coding sequences (Fei *et al.,* 2019).

In 2012, DWV-B was first detected in samples from *Varroa*-infested Hawaiian Islands (Brettel & Martin, 2017) and again in 2016 (Brettel *et al.,* 2020a). Therefore, 10 years on from the original Hawaiian study that sampled 239 colonies detecting primarily DWV-A (Martin *et al.,* 2012), we returned to resample three island populations. Here, we investigate how DWV has changed in respect to prevalence and load of DWV-A and -B RdRp coding sequence, a highly conserved region of the genome, and then compare any changes to the current global status of DWV. During the past 10 years, the *Varroa* status of the Hawaiian Islands has remained the same with Maui and Kauai been mite-free while *Varroa* is ubiquitous on Oahu and Big Island, where colonies are treated with miticides regularly, although a small number of beekeepers are maintaining increasing numbers of colonies without treating (Martin, 2020).

Methods

Sample Collection

Samples were collected during November 2019, 10 years after the original collection date in the field (Nov 2009 and 2010), and stored on ice before being transferred into ethanol for storage at −20 °C. Samples of at least 30 adult bees were collected from both the *Varroa* infested islands of Oahu (*n* = 41 colonies, *n* = 6 apiaries, *n* = 11 feral colonies), Big Island (*n* = 43 colonies, *n* = 9 apiaries, *n* = 1 feral colony), and the *Varroa*-free island of Kauai (*n* = 22 colonies, *n* = 4 apiaries, *n* = 2 feral colonies). Two of the 11 feral samples on Oahu, T4 and UH127, only 29 bees were collected from each colony.

In addition, two sets of five pupal samples were taken from two colonies on Oahu from an apiary that showed the signs of natural mite resistance. All samples were transported directly too and processed one to two months later at the University of Minnesota.

Sample processing

For each sample: 30 asymptomatic bees were dabbed lightly with tissue to remove residual ethanol and individually inspected for *Varroa*, and if present, the mite was removed. This was to prevent contamination of the samples with viral RNA from *Varroa* and to standardize the test. The bees were frozen using liquid nitrogen and homogenised in a mill mixer (Ritesch) for 30 s. The Oahu pupal samples were also inspected for *Varroa* and if present, any mites were removed. The pupae were individually dried, frozen using liquid nitrogen and crushed in an Eppendorf tube using a sterile pipette tip. The bee material was then stored at −80 °C until RNA extraction. An empty open Eppendorf tube served as a blank for any aerial contamination during the crushing process.

RNA extraction and quantification

RNA was extracted from the 50 mg of each sample using the MagMAX mirVana total RNA isolation kit with the MagMAX express 96 on program AM1830_DW (Applied Biosystems). Following the manufacturer's protocol, 302.1 µL of lysis binding mix (300 µL of lysis buffer and 2.1 µL of 2-Mercaptoethanol) was added to each sample and the samples were vortexed for 15 s before being put into the 5× g for 5 minutes at 2000 rpm. The manufacturer's protocol was modified slightly, thus 150 µL of the lysate was put into each well of the processing plate rather than 100 µL. To each sample on this plate, 20 µL of binding mix (10 µL RNA beads and 10 µL enhancer) was added and the plate shook for 5 min using the plate shaker Lab Line™ at 950 rpm.

In total, RNA was extracted from 116 samples, 9 blanks from the crushing stage and 2 negatives to check for contamination during the extraction process. RNA was quantified using the Nanodrop 2000 (Thermo Fisher Scientific) and standardised to 50 ng/µL per sample using RNase free water before storage at −80 °C.

RT -qPCR

To quantify the viral load of each DWV master variant, RT-qPCR was performed on the 116 samples using the ABC assay method (Kevill *et al.,* 2017). The samples were screened for the DWV master variants A, B, and C, using primers targeting the RdRp region and, therefore, this assay can only provide insight into the presence of each of the DWV master variants and associated recombinants at the time of sampling. It cannot report on the prevalence of any DWV recombinant but rather provides an overview of whether there was a shift from DWV-A and its associated recombinants and DWV-B and its associated recombinants using a conserved region of the viral genome.

Reactions were performed on a quant studio 3 (Applied Biosystems/Thermo Fisher Scientific, USA), using a powerup SYBER® Green RNA-to-Ct 1-Step kit™ from applied Biosystems. The 50 ng/µL samples were run singly alongside a 10-fold dilution series run in triplicate. The 10-fold dilution series was made using a standard specific to each DWV master variant, the concentration of which was determined using the Nanodrop 2000 (Thermo Fisher Scientific) before dilution. Reactions contained 1 µL of the 50 ng/µL RNA sample and 9 µL of master mix. The master mix was comprised of 0.08 µL reverse transcriptase, 1 µL DWV forward primer and 1 µL DWV reverse primer (Type A, B or C), 5 µL PCR mix, and 1.92 µL H2O. A negative control consisting of 1 µL H2O and 9 µL master mix was included on each PCR plate. An actin control was not deemed necessary as the samples had not undergone long-term storage. The reactions were run on the quant studio 3, the reverse transcription stage occurred at 45 °C for 10 min and denaturation at 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at 58 °C (types A and B) or 61 °C (type C) for 15 s and extension at 72 °C for 15 s. The final stage was a dissociation melt curve at 70 to 95 °C, this was to check for any contamination.

Analyzing the results

DWV-C was not detected in the screened samples; therefore, results were analyzed for DWV- A and -B only. The average viral copy number was calculated by the quantstudio software. The average viral copy number was used to calculate the quantity DWV genome equivalent per bee. This was obtained using the formula:

Genome equivalent = (average copy number) × (RNA dilution factor) × (elution volume of RNA) × (proportion of bee material)

The dilution factor can be calculated by dividing the RNA concentration of the original sample (before it was diluted) by 50 (the concentration it was diluted to). This original concentration was determined after RNA extraction using the nanodrop. The elution volume of RNA was 50 µL, and the proportion of bee material used was ¼ of a bee per sample, thus we need to multiply by 4 to obtain the genome equivalents of one bee.

The maximum number of cycles for this assay was 35 cycles (equating to a critical threshold value = 30). Above 35 cycles, non-specific and background cross-contamination could be detected leading to inaccurate results. Additionally, samples containing less than 100 copies of RNA were out of the range of accurate quantification (Kevill *et al.,* 2017). As a result, samples with PCR values less than 100 copies or with a critical threshold value of 30 or above were not included in further analysis. As the data did not follow a normal distribution, even after log10 transformation, the median and interquartile range of DWV-A and DWV-B genome equivalents was determined for each island. For the apiaries, the percentage of DWV-A RdRp and DWV-B RdRp was calculated using the genome equivalents. The percentages of colonies were then averaged to obtain the average for the apiary. The median and interquartile range of pupal samples were determined separately from the adult bees of Oahu Island due to high variability. The medians were used to calculate the percentage of DWV-A and DWV-B on each island.

A Mann–Whitney U test was used to compare the viral loads (genome equivalents) on Oahu and Big Island. Kauai samples were excluded from this analysis as there were only four samples with quantifiable levels of DWV. Fisher's exact probability tests were conducted to compare the prevalence of detectable and quantifiable amounts of DWV-A and DWV-B between the islands. The level of significance for all tests was $p < 0.05$.

Treated vs. untreated colonies

Out of the 41 colonies on Oahu, 15 were from managed apiaries that used *Varroa* treatment, and 15 were from managed apiaries that chose not to treat for *Varroa* mites. The remaining 11 colonies were feral colonies that did not receive treatment. The colonies were divided into the three groups to compare the differences in DWV-A and DWV-B load between them. The genome equivalents were log10 transformed and then tested for normality using the Ryan-Joiner normality test and histogram plots. The data were normally distributed and thus two, one-way ANOVA tests with were used to look for significant differences in the mean viral loads of the three groups. In the event the ANOVA test returned a significant result a Tukey's Kramer test was used to look for significant differences between pairs of groups. This post-hoc test was selected due to unequal sample sizes between the groups with the feral group having a lower sample size than the other groups.

Results

Prevalence and viral titre

On the *Varroa*-free island of Kauai, DWV-A and -B were detected in 36% (8/22 colonies) and 59% (13/22 colonies) of colonies, respectively. However, the viral genome equivalents were only just quantifiable in four colonies, and these were low (105 to 106) (Figure 1, Table 1). In contrast, on the *Varroa*-infested islands of Oahu and Big Island, median DWV genome equivalents were several orders of magnitude greater ($\times 10^9$). The levels of DWV-A on Oahu were not significantly different from the levels of DWV-A on Big Island ($U = 809.5$, $p = 0.78$), this was also the case for DWV-B ($U = 692$, $p = 0.30$).

Additionally, DWV-A and –B were detected in 100% of mite-infested colonies sampled on both islands (Oahu *n* = 41, Big Island *n* = 43) that was significantly greater than the number of colonies with detectable DWV-A (both $p < 0.01$) and DWV-B on Kauai (both $p < 0.01$). DWV-A and -B were also detected above the quantifiable threshold in 100% of colonies on Oahu and over 90% of colonies on Big Island (90.7% DWV-A 39/43 colonies, 95.3% DWV-B 41/43 colonies). The differences in the number of colonies with quantifiable DWV-A and DWV-B between Oahu and Big Island were not significant (DWV-A: $p = 0.12$ and DWV-B $p = 0.49$). However, both Oahu and Big Island had significantly more quantifiable cases of DWV-A (both $p < 0.01$) and DWV-B (both $p < 0.01$) than Kauai.

The island genome equivalents of DWV-A vs. -B were not significantly different on Oahu ($U = 793$, $p = 0.35$) or Big Island ($U = 713$, $p = 0.41$), with DWV-A making up 46% and 59% of median genome equivalents on Oahu and Big Island, respectively. All of the Oahu pupal samples had quantifiable levels of DWV-B, but only 60% had quantifiable amounts of DWV-A, and 9 of the 10 samples were dominated by DWV-B (Table 1). Conversely, on Kauai, DWV-A and B co-infection were rarer, occurring in only 18% of colonies, and where coinfection occurred, only one variant was dominant whilst the other was below the quantifiable limit. For colony level data, see supplementary Tables S1–S3. All reported negative samples tested were negative of any DWV variant.

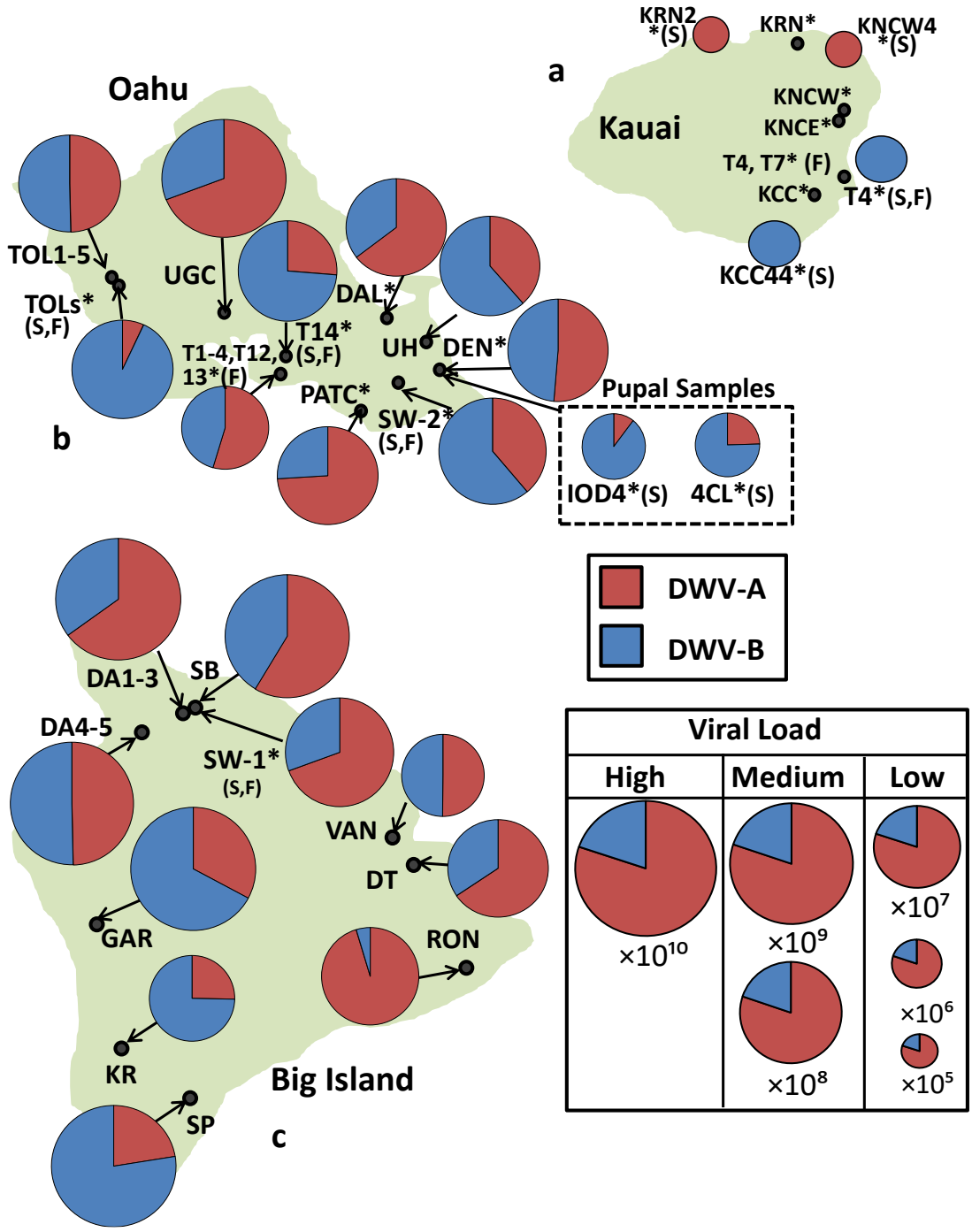

Figure 1. (a–c). Islands showing proportions of DWV-A RdRp (red) and DWV-B RdRp (blue) in each apiary (* = A colony that is not chemically treated for *Varroa*, S = Sample(s) came from a single colony, F = feral). The size of each pie chart is relative to the median total DWV genome equivalents per apiary.

Table 1. Island median DWV genome equivalent and interquartile range (standard range for Kauai) and the year *Varroa* was first detected on each island.

Island	DWV-A	IQR	DWV-B	IQR
Kauai Varroa-free	7.53×10^5 (n = 2)	2.07×10^5	4.39×10^6 (n = 2)	6.21×10^6
Oahu Infested since 2007	1.03×10^9 (n = 41)	1.69×10^6	7.10×10^8 (n = 41)	1.31×10^6
Oahu—Pupae	1.44×10^6 (n = 6)	5.13×10^9	1.01×10^7 (n = 10)	7.54×10^6
Big island Infested since 2009	1.61×10^9 (n = 41)	1.18×10^{10}	1.42×10^9 (n = 39)	2.32×10^{10}

Treated vs. untreated colonies

All the colonies in each group, managed treated (n = 15), managed not-treated (n = 15), and feral (n = 11) had quantifiable amounts of DWV-A and DWV-B (supplementary Figure S1). The one-way ANOVA for DWV-B revealed that the genome equivalents were not significantly different between the three groups (F(2, 38) = [1.216], p = 0.31). In contrast, the one-way ANOVA for DWV-A found that there was a significant difference in the mean load of DWV-A genome equivalents between at least two of the groups (F(2, 38) = [3.454], p = 0.042). However, the follow up Tukey's Kramer test did not find a significant difference between any of the pairs with all q values being below the critical value of 3.449 for 5% significant level, 3 groups, and degree of freedom of the denominator of 38. These q values were 3.324 (feral v managed untreated), 3.277 (feral v managed treated) and 0.0516 (managed untreated v managed treated).

Discussion

In the original 2010 Hawaii study (Martin *et al.,* 2012), the islands with *Varroa*, Oahu and Big Island, were entirely made up of the same DWV-A sequence. Our results indicate a large proportion of RdRp sequences now contain those that match the DWV-B variant. This suggests that the Hawaiian Islands of Oahu and Big Island are transitioning from DWV-A to DWV-B dominance, mirroring that observed in the UK, USA, Europe, South Africa (Figure 2) (Brettell *et al.,* 2019; de Souza *et al.,* 2021; Kevill *et al.,* 2017; Kevill *et al.,* 2019; Kevill *et al.,* 2021; Manley *et al.,* 2019; Natsopoulou *et al.,* 2017). However, to confirm this would require future studies analyzing the full genome sequence of past and present samples from each island. Due to roughly a 100-fold increase in sensitivity of the PCR method (Kevill *et al.,* 2017), the viral genome equivalents in this study are not directly comparable to the original study. However, the relative ratios show that on Big Island and Oahu DWV-A is no longer solely dominant and that DWV load on Kauai remains very low with a significantly lower prevalence of infected colonies compared to the two *Varroa* infested islands. In fact, on both Big Island and Oahu, the proportions of DWV-A and DWV-B are close to co-dominance, with DWV-A variants making up 59% and 46% of median genome equivalents on Big Island and Oahu, respectively. Additionally, at the colony level, 59% of colonies on Oahu are dominated by DWV-A and 56% on Big Island.

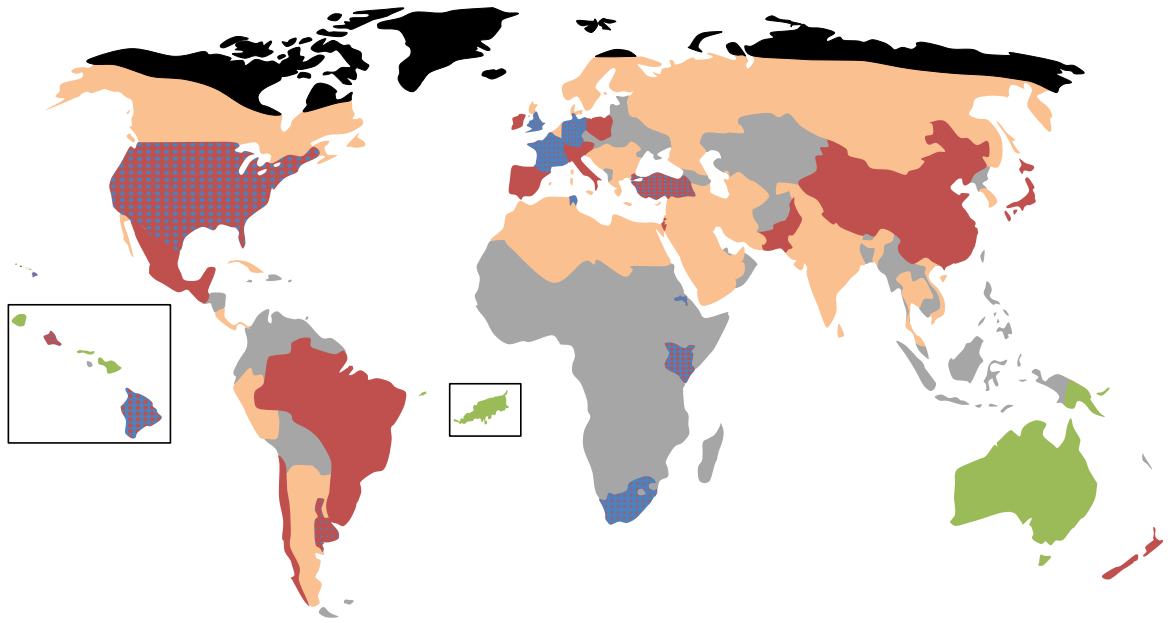

Figure 2. Global distribution of DWV in *Apis mellifera*. Red = DWV-A, blue = DWV-B, orange = DWV present but dominant strain unknown, grey = no data available, green = DWV absent or present at very low genome equivalents, Black = *Apis mellifera* absent. Blue dots on a red background indicate that DWV-A is dominant, but DWV-B is present conversely red dots on a blue background indicate that DWV-B is dominant, but DWV-A is present. The map was constructed by combining global level DWV data (Beaurepaire *et al.,* 2020; Wilfert *et al.,* 2016) with more detailed country level info as follows: Argentina (Buenos Aires and Sante Fe) (Brasesco *et al.,* 2020), Australia (Roberts *et al.,* 2017), Brazil (de Souza *et al.,* 2019), Chile (Riveros *et al.,* 2019), China (Diao *et al.,* 2019), Cuba (Luis *et al.,* 2020), Ethiopia (Tigray) (Gebremedhn *et al.,* 2020), Fernando de Noronha (Brettel & Martin, 2017), France (Manley *et al.,* 2019), Germany (Natsopoulou *et al.,* 2017), Hawaii (This study, Brettell *et al.,* 2020), Kenya (Ongus *et al.,* 2018), Papua new guinea (Roberts *et al.,* 2020) , South Africa (de Souza *et al.,* 2021), Tunisia (Abdi *et al.,* 2018), Turkey (Tozkar *et al.,* 2015), UK (Kevill *et al.,* 2019), Uruguay (Mendoza et al 2020), USA (Kevill *et al.,* 2019). The studies used to create this diagram were not required to have used the same primer set as our study.

Intriguingly, the majority of change on Oahu appears to have occurred within the last three years, with samples from 2015 to 2016 consisting of mostly DWV-A (99% of reads) (Brettell *et al.,* 2020). This is interesting because given the changing from DWV-A to DWV-B dominance over time in other countries, one would expect the island which had hosted *Varroa* the longest, Oahu, to become dominated by DWV-B and to do so first. Whereas, it appears Big Island has become dominated more rapidly, with one study finding DWV-B domination in 2012 (96% of RNAseq reads) (Brettell *et al.,* 2020) and another in 2016 (>99% of RNAseq reads) (Brettell *et al.,* 2019) (Figure 3). However, whilst striking, these results should be interpreted with caution as coming from just one and two samples, respectively, they may not be fully representative of the island at the time. In addition, it is fair to say that the change from DWV-A and DWV-B is not necessarily universal because, in South America, which was invaded by the mite some 50 years ago, DWV-A still prevails as the dominant variant (Figure 3) (de Souza *et al.,* 2019; Mendoza et al 2020; Riveros *et al.,* 2019). In fact, de Souza *et al.,* (2019) only detected DWV-B in three of their 27 honey bee samples from Brazil. Whereas, in South Africa, DWV-B appeared to dominate from the mite's introduction in 1997 or shortly afterward (de Souza *et al.,* 2021). The median viral genome equivalent of DWV-A is similar on Oahu and Big Island but the median viral genome equivalent of DWV-B on Oahu is half the value on Big Island (Table 1.). A potential key difference between the colonies sampled was that the majority of the Big Island colonies were acaricide treated, whereas on Oahu, the colonies were a mix of treated, not treated, and feral (also not treated) colonies. All colony types had similar levels of DWV-B however the levels of DWV-A did vary between the three groups with feral colonies having the lowest loads (Figure S1). However, whilst an initial ANOVA indicated a significant difference between at least two of the groups the post hoc tests did not find a significant difference

between the means of the three groups. This was unexpected because other studies using the same methodology have found a reduced DWV burden in resistant, not treated, managed populations in South Africa and Brazil (de Souza *et al.,* 2021). Arguably the lack of significance could be due to the low number of samples used and may be worth future investigation.

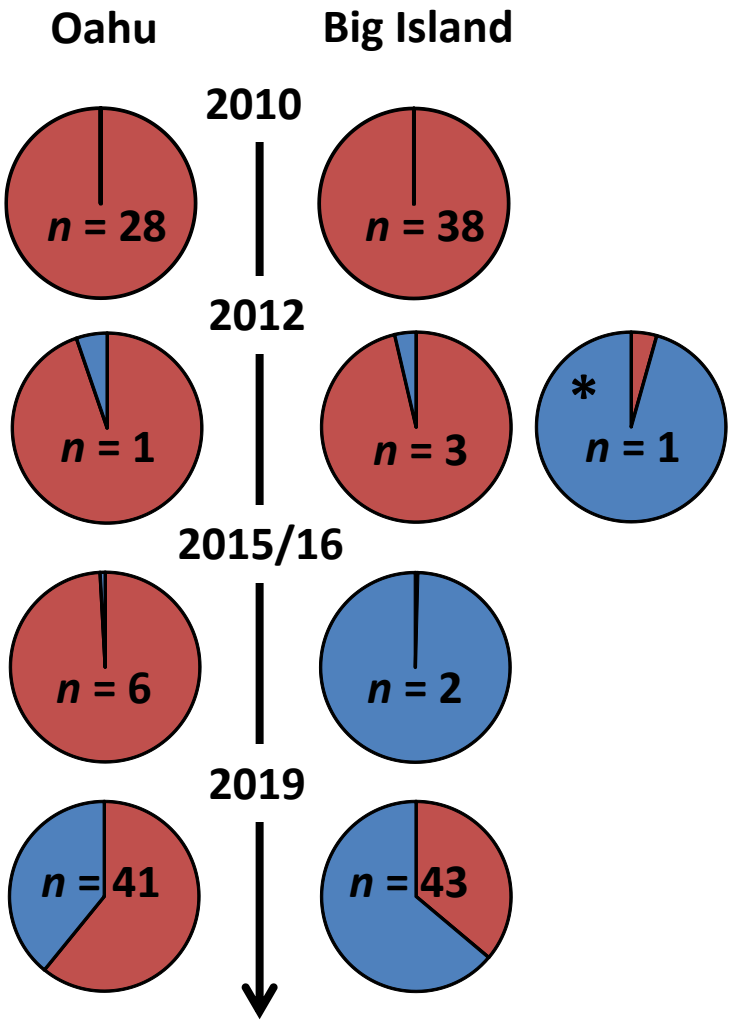

Figure 3. Changing proportions of DWV-A (red) and DWV-B (blue) on Big Island and Oahu over time. Sample sizes of the studies are given within the pie charts. Data for 2010 is from (Martin *et al.,* 2012), 2012 (Brettell *et al.,* 2019), 2012 * (Mordecai *et al.,* 2016), 2015/16 (Brettell *et al.,* 2020) and 2019 (this study). 2012 and 2012 * could not be combined due to

the different methodologies used. N.B. Pie chart sizes do not convey DWV genome equivalents.

As expected, given the inefficiency of bee-to-bee routes of transmission (Gusachenko *et al.,* 2020), the number of DWV genome equivalents on the *Varroa*-free island Kauai are still very low. Indeed, only four colonies had sufficient genome equivalents that were quantifiable. Additionally, in contrast to the original study, which detected DWV in 13% of colonies on *Varroa*-free islands, we detected DWV in the majority of colonies on Kauai 77%. This result is attributed to the increased sensitivity of the methods used.

Recombinants have been found to be prevalent in samples from Oahu and Big Island (Brettell *et al.,* 2020). Considering the high incidence of co-infection, we found it is entirely possible that our samples from Big Island and Oahu could contain recombinants. However, as the RT-qPCR used in this study focused upon the RdRp region, we can only speculate on this possibility. Although the RdRp region is conserved and not known to be a common site for recombination relative to other regions of the genome (Brettel *et al.,* 2020; Dalmon *et al.,* 2017; McMahon *et al.,* 2016).

Ultimately, this study has shown that since 2010 when DWV-B was not detected, the viral load and prevalence of DWV-B have increased to the point at which DWV-B now dominates colonies found on Big Island and co-dominates with DWV-A on Oahu. Thus far, this increase in DWV-B fits with what has been observed in numerous other regions (Figure 2) (de Souza *et al.,* 2021; Kevill *et al.,* 2019; Manley *et al.,* 2019). We know that DWV-B replicates to greater titres than DWV-A when injected into pupae (Dubois *et al.,* 2019; Tehel *et al.,* 2019) whilst being equally (Tehel *et al.,* 2019) or less virulent (Norton *et al.,* 2020). Furthermore, evidence suggests that DWV-B is able to replicate in *Varroa* mites, whereas DWV-A is not

(Gisder & Genersch, 2021; Posada-Florez *et al.,* 2019). These findings help explain the field observations where DWV-B consistently occurs at higher titers than DWV-A (Kevill *et al.,* 2019). The enhanced replication combined with a reduction in pupal virulence will give DWV-B the competitive edge during co-infection with DWV-A (Posada-Florez *et al.,* 2019) since the 10–20% mortality of pupal infected with DWV-A prevents the vector (mites) from reproducing, hence breaking the transmission cycle. This may be negated by the fact that DWV-B is more virulent than DWV-A to caged adult bees (McMahon *et al.,* 2016); however, it seems unlikely as, especially in cases of high infestation, where irrespective of DWV variant colonies still collapse.

Additionally, it is curious, given the advantageous replicative abilities of DWV-B, why DWV-A initially gained dominance after *Varroa* spread to Oahu and Big Island. The reasons for this are at this point unclear; however, it has been shown that the rise of the near clonal master-variant (now called DWV-A) occurred within the pupae not the mite (Ryabov *et al.,* 2014). Once this occurred, either DWV-A was selected again in the pupae or more likely transmitted directly by *Varroa*. Perhaps the initial dominance is dependent on the variants present before *Varroa*. Between 1998 and 2009 of 484 mite and honey bee samples from 32 geographic regions testing positive for DWV, 83% were DWV-A, and the few DWV-B samples all originated from Europe (Wilfert *et al.,* 2016). Thus, perhaps DWV-B would have the chance to dominate if mites were to infest the island of Kauai.

Nonetheless, at this point, it is difficult to speculate on the future as there are still many gaps in our knowledge of the current prevalence of DWV-A and B worldwide that need to be filled (Figure 2). Indeed, it is not clear whether the two variants will continue to co-exist in Hawaii or whether DWV-B will eventually dominate Oahu and Big Island.

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Supplementary information

Supplementary tables S1-S4 & Supplementary figure S1

Supplementary Table S1. Kauai samples, NEG/UD = Negative/Undetected, BL = Below the quantifiable threshold

								Average copy no.		Genome quivalent	
Sample ID	Collection date	Crush date	RNA conc.	Extraction date	Dilution factor	Type	Treatment	DWV-A RdRp	WV-B RdR	DWV-A RdRp	DWV-B RdRp
KCC44	04/12/2019	09/01/2020	624.0	24/11/2020	12.48	Managed	No	BL	2782.58	BL	2782.58
T7	15/11/2018	08/01/2020	233.7	24/11/2020	4.67	Feral	No	NEG/UD	BL	NEG/UD	BL
T4	29/08/2018	08/01/2020	283.3	24/11/2020	5.67	Feral	No	BL	649.58	BL	649.58
KRN4	04/12/2019	09/01/2020	693.1	24/11/2020	13.86	Managed	No	BL	NEG/UD	BL	NEG/UD
KRN5	04/12/2019	09/01/2020	502.4	24/11/2020	10.05	Managed	No	NEG/UD	NEG/UD	NEG/UD	NEG/UD
KNCW4	04/12/2019	10/01/2020	614.9	24/11/2020	12.30	Managed	No	264.16	NEG/UD	264.16	NEG/UD
KNLEW2	04/12/2019	10/01/2020	326.9	24/11/2020	6.54	Managed	No	NEG/UD	BL	NEG/UD	BL
KRN2	04/12/2019	10/01/2020	673.4	24/11/2020	13.47	Managed	No	317.90	BL	317.90	BL
KNCE5	04/12/2019	10/01/2020	916	24/11/2020	18.32	Managed	No	BL	BL	NEG/UD	BL
KNCW1 124-19	04/12/2019	10/01/2020	734.9	24/11/2020	14.70	Managed	No	NEG/UD	BL	NEG/UD	BL
KCC 73P	04/12/2019	10/01/2020	530.8	24/11/2020	10.62	Managed	No	NEG/UD	NEG/UD	NEG/UD	NEG/UD
KCC41	04/12/2019	13/01/2020	523.9	24/11/2020	10.48	Managed	No	BL	NEG/UD	BL	NEG/UD
KCC60	04/12/2019	13/01/2020	669.8	24/11/2020	13.40	Managed	No	NEG/UD	BL	NEG/UD	BL
KNCE4	04/12/2019	13/01/2020	653.3	24/11/2020	13.07	Managed	No	NEG/UD	BL	NEG/UD	BL
KNCE1	04/12/2019	13/01/2020	723.4	24/11/2020	14.47	Managed	No	NEG/UD	NEG/UD	NEG/UD	NEG/UD
KCC4	04/12/2019	13/01/2020	661	24/11/2020	13.22	Managed	No	NEG/UD	NEG/UD	NEG/UD	NEG/UD
KNCW3	04/12/2019	13/01/2020	1071	24/11/2020	21.42	Managed	No	NEG/UD	BL	NEG/UD	BL
KRN3	12/04/2019	13/01/2020	566.1	24/11/2020	11.32	Managed	No	NEG/UD	NEG/UD	NEG/UD	NEG/UD
KRN1	12/04/2019	13/01/2020	553.1	24/11/2020	11.06	Managed	No	BL	NEG/UD	BL	NEG/UD
KNCE3	12/04/2019	13/01/2020	419.4	24/11/2020	8.39	Managed	No	NEG/UD	BL	NEG/UD	BL
KNCE2	12/04/2019	13/01/2020	341.3	24/11/2020	6.83	Managed	No	NEG/UD	BL	NEG/UD	BL
KNCW5	12/04/2019	13/01/2020	685	24/11/2020	13.70	Managed	No	NEG/UD	BL	NEG/UD	BL

Supplementary Table S2. Oahu samples, NEG/UD = Negative/Undetected, BL = Below the quantifiable threshold

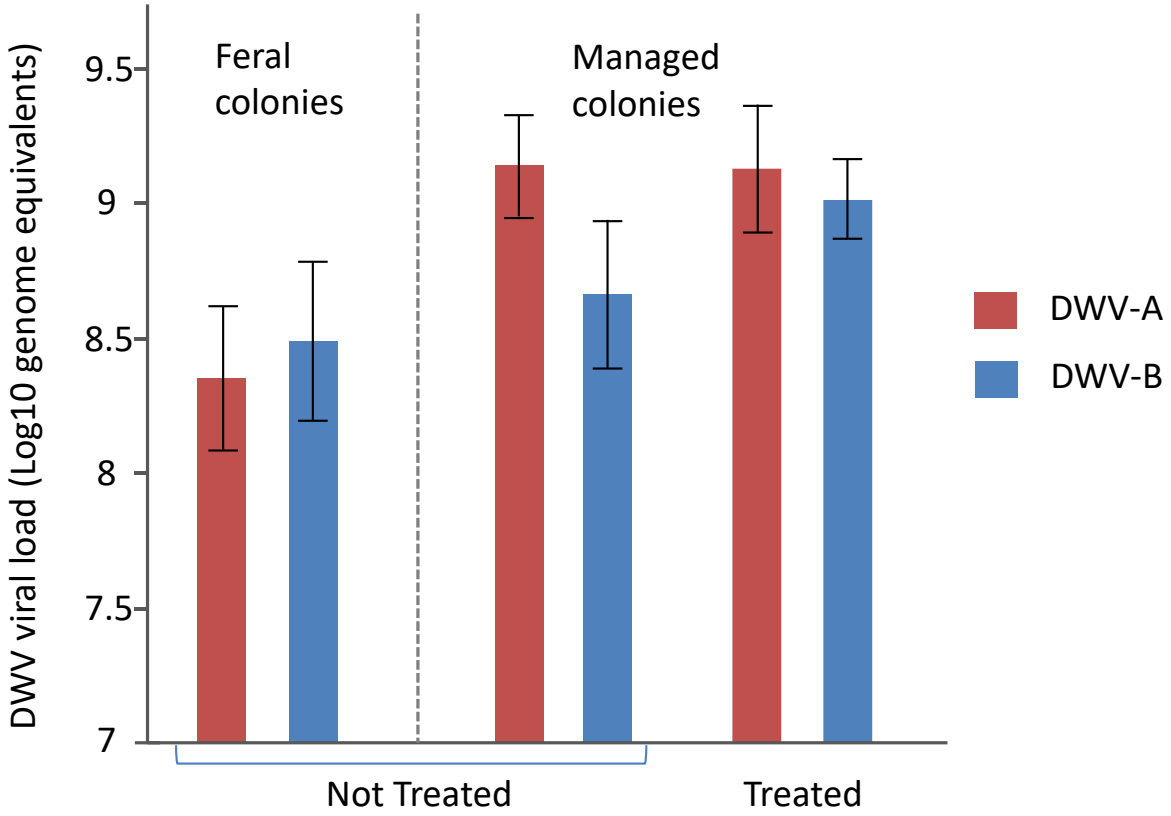
								Average copy no.		Genome equivalent	
Sample ID	Collection date	Crush date	RNA conc.	Extraction date	Dilution factor	Type	Treatment	DWV-A RdRp	DWV-B RdRp	DWV-A RdRp	DWV-B RdRp
SW-2	28/11/2019	21/01/2020	303.1	23/01/2020	6.06	Feral	No	3.93E+06	6.20E+06	4.77E+09	7.52E+09
TOL4	12/03/2019	09/01/2020	586.4	24/11/2020	11.73	Managed	Yes	2.05E+04	8.76E+04	4.81E+07	2.06E+08
TOL1	12/03/2019	09/01/2020	444.4	24/11/2020	8.89	Managed	Yes	2.79E+06	1.48E+05	4.97E+09	2.63E+08
TOL2	12/03/2019	10/01/2020	575.8	24/11/2020	11.52	Managed	Yes	6.07E+05	4.73E+05	1.40E+09	1.09E+09
TOL3	12/03/2019	10/01/2020	725.2	24/11/2020	14.50	Managed	Yes	7.35E+05	6.77E+05	2.13E+09	1.96E+09
TOL5	12/03/2019	13/01/2020	496.9	24/11/2020	9.94	Managed	Yes	1.78E+05	5.07E+05	3.53E+08	1.01E+09
TOLs	03/12/2019	08/01/2020	155.3	24/11/2020	3.11	Feral	No	4.24E+05	5.71E+06	2.63E+08	3.55E+09
UH42	29/11/2019	08/01/2020	460.3	24/11/2020	9.21	Managed	Yes	7.50E+04	2.48E+05	1.38E+08	4.56E+08
UH132	29/11/2019	08/01/2020	693.6	24/11/2020	13.87	Managed	Yes	3.34E+05	5.64E+05	9.28E+08	1.56E+09
UH127	29/11/2019	08/01/2020	754.7	24/11/2020	15.09	Managed	Yes	2.89E+06	2.36E+06	8.72E+09	7.12E+09
UH140	29/11/2019	09/01/2020	639.8	24/11/2020	12.80	Managed	Yes	1.09E+04	5.42E+04	2.80E+07	1.39E+08
UH107	29/11/2019	09/01/2020	700.3	24/11/2020	14.01	Managed	Yes	2.10E+06	1.38E+06	5.89E+09	3.87E+09
T14	16/06/2019	09/01/2020	433.7	24/11/2020	8.67	Feral	No	8.72E+05	2.45E+06	1.51E+09	4.25E+09
OPATCR	01/11/2019	21/01/2020	646.1	23/01/2020	12.92	Managed	No	4.22E+06	6.14E+06	1.09E+10	1.59E+10
OPATC	01/11/2019	21/01/2020	456.8	23/01/2020	9.14	Managed	No	4.34E+05	2.53E+03	7.93E+08	4.62E+06
OPATC SCOT	01/11/2019	21/01/2020	352.8	23/01/2020	7.06	Managed	No	1.23E+06	5.05E+05	1.73E+09	7.13E+08
PATC	20/11/2019	16/01/2020	396.3	23/01/2020	7.93	Managed	No	6.47E+05	1.82E+04	1.03E+09	2.89E+07
OPATCM	20/11/2019	22/01/2020	581.6	23/01/2020	11.63	Managed	No	1.73E+05	1.07E+05	4.02E+08	2.50E+08
OUGCP20	01/11/2019	21/01/2020	515.9	23/01/2020	10.32	Managed	Yes	5.38E+06	2.67E+05	1.11E+10	5.50E+08
UGUPC5	20/11/2019	16/01/2020	443.6	23/01/2020	8.87	Managed	Yes	1.76E+07	1.41E+06	3.12E+10	2.50E+09
OUGCPC3	21/11/2019	21/01/2020	746.2	23/01/2020	14.92	Managed	Yes	3.44E+06	2.46E+06	1.03E+10	7.36E+09
OUGCWhite	20/11/2019	22/01/2020	511.5	23/01/2020	10.23	Managed	Yes	1.29E+06	1.51E+06	2.63E+09	3.10E+09
OUGCJ	20/11/2019	16/01/2020	630.5	23/01/2020	12.61	Managed	Yes	8.35E+04	7.24E+04	2.11E+08	1.83E+08
ODAL1	01/11/2019	21/01/2020	747.4	23/01/2020	14.95	Managed	No	4.73E+04	6.79E+03	1.41E+08	2.03E+07
ODAL4	21/11/2019	21/01/2020	689.8	23/01/2020	13.80	Managed	No	8.51E+03	2.03E+04	2.35E+07	5.59E+07
ODAL2	21/11/2019	22/01/2020	549.8	23/01/2020	11.00	Managed	No	3.59E+06	3.21E+04	7.90E+09	7.07E+07
ODAL5	21/11/2019	22/01/2020	629.0	23/01/2020	12.58	Managed	No	1.42E+06	4.19E+05	3.58E+09	1.06E+09
ODAL3	21/11/2019	22/01/2020	584.1	23/01/2020	11.68	Managed	No	7.82E+05	1.88E+06	1.83E+09	4.40E+09
ODEN6AA4	21/11/2019	21/01/2020	715.3	23/01/2020	14.31	Managed	No	1.90E+06	3.46E+05	5.45E+09	9.89E+08
ODEN5E3	21/11/2019	21/01/2020	713.1	23/01/2020	14.26	Managed	No	9.06E+05	1.35E+06	2.58E+09	3.84E+09
ODEN8C2	21/11/2019	22/01/2020	583.4	23/01/2020	11.67	Managed	No	1.65E+06	2.74E+06	3.85E+09	6.39E+09
ODEN7AI	21/11/2019	22/01/2020	750.8	23/01/2020	15.02	Managed	No	1.82E+06	1.40E+06	5.46E+09	4.19E+09
ODEN6A2	21/11/2019	22/01/2020	803.9	23/01/2020	16.08	Managed	No	1.34E+05	2.21E+05	4.30E+08	7.10E+08
4CI 3	29/11/2019	22/01/2020	729.3	23/01/2020	14.59	Managed	No	1.20E+07	9.07E+04	3.50E+10	2.65E+08
4CI 4	29/11/2019	22/01/2020	820.9	23/01/2020	16.42	Managed	No	562.22	4849.26	1.85E+06	1.59E+07
4CI 5	29/11/2019	22/01/2020	499.7	23/01/2020	9.99	Managed	No	512.31	3420.49	1.02E+06	6.84E+06
4CI 1	29/11/2019	22/01/2020	450.9	23/01/2020	9.02	Managed	No	BL	625.15	BL	1.13E+06
4CI 2	29/11/2019	22/01/2020	725	23/01/2020	14.50	Managed	No	NEG/UD	3191.33	NEG/UD	9.25E+06
IOD4 1	29/11/2019	22/01/2020	745.0	23/01/2020	14.90	Managed	No	2.30E+06	4.22E+06	6.84E+09	1.26E+10
IOD4 2	29/11/2019	22/01/2020	786.3	23/01/2020	15.73	Managed	No	261.25	2630.45	8.22E+05	8.27E+06
IOD4 3	29/11/2019	22/01/2020	824.6	23/01/2020	16.49	Managed	No	BL	3385.47	BL	1.12E+07
IOD4 4	29/11/2019	22/01/2020	411.8	23/01/2020	8.24	Managed	No	259.37	2887.09	4.27E+05	4.76E+06
IOD4 5	29/11/2019	22/01/2020	833.2	23/01/2020	16.66	Managed	No	BL	3277.73	BL	1.09E+07
T13	08/10/2018	08/01/2020	295.4	24/11/2020	5.908	Feral	No	1.58E+05	7.40E+04	1.87E+08	8.74E+07
T2	15/06/2018	08/01/2020	355.5	24/11/2020	7.11	Feral	No	7.84E+04	3.16E+04	1.12E+08	4.50E+07
T1	11/01/2018	08/01/2020	438.1	24/11/2020	8.762	Feral	No	5.21E+03	1.14E+05	9.13E+06	2.00E+08
T3	13/07/2018	08/01/2020	366.8	24/11/2020	7.336	Feral	No	3.20E+03	2.26E+03	4.70E+06	3.31E+06
T12	18/05/2018	08/01/2020	393.3	24/11/2020	7.866	Feral	No	9.11E+04	2.92E+05	1.43E+08	4.60E+08
T12	15/06/2018	08/01/2020	472.2	24/11/2020	9.444	Feral	No	3.39E+05	9.00E+04	6.41E+08	1.70E+08
T4	09/07/2018	08/01/2020	536.9	24/11/2020	10.738	Feral	No	2.56E+05	2.29E+05	5.50E+08	4.92E+08
T4	10/08/2018	08/01/2020	356.6	24/11/2020	7.132	Feral	No	6.08E+05	1.70E+05	8.67E+08	2.42E+08

Supplementary Table S3. Big Island samples, NEG/UD = Negative/Undetected, BL = Below the quantifiable threshold

								Average copy no.		Genome quivalent	
Sample ID	Collection date	Crush date	RNA conc.	Extraction date	Dilution factor	Type	Treatment	DWV-A RdRp	DWV-B RdRp	DWV-A RdRp	DWV-B RdRp
SP1	25/11/2019	15/01/2020	699.7	23/01/2020	13.99	Managed	Yes	1.78E+06	2.38E+04	4.97E+09	6.65E+07
SP2	25/11/2019	15/01/2020	745.1	24/11/2020	14.90	Managed	Yes	2.10E+06	3.08E+07	6.26E+09	9.19E+10
SP3	25/11/2019	09/01/2020	565.3	24/11/2020	11.31	Managed	Yes	4.67E+04	3.11E+06	1.06E+08	7.03E+09
SP4	29/11/2019	09/01/2020	709.2	24/11/2020	14.18	Managed	Yes	3.76E+06	9.52E+07	1.07E+10	2.70E+11
SP5	25/11/2019	14/01/2020	592.9	24/11/2020	11.86	Managed	Yes	8.10E+05	4.51E+07	1.92E+09	1.07E+11
SB4	24/11/2019	15/01/2020	844.8	23/01/2020	16.90	Managed	Yes	9.64E+06	6.66E+06	3.26E+10	2.25E+10
SB3	24/11/2019	15/01/2020	672.6	23/01/2020	13.45	Managed	Yes	1.06E+04	8.58E+03	2.85E+07	2.31E+07
SB2	24/11/2019	16/01/2020	910.8	23/01/2020	18.22	Managed	Yes	3.23E+06	1.49E+06	1.18E+10	5.44E+09
SB5	24/11/2019	16/01/2020	882.0	23/01/2020	17.64	Managed	Yes	4.24E+04	2.47E+03	1.50E+08	8.72E+06
SB1	24/11/2019	09/01/2020	796.2	24/11/2020	15.92	Managed	Yes	4.74E+06	2.54E+07	1.51E+10	8.10E+10
GAR1	23/11/2019	22/01/2020	228.0	23/01/2020	4.56	Managed	Yes	4.87E+04	1.55E+06	4.44E+07	1.42E+09
GAR4	23/11/2019	22/01/2020	457.3	23/01/2020	9.15	Managed	Yes	5.57E+05	4.96E+05	1.02E+09	9.06E+08
GAR2	23/11/2019	14/01/2020	543.5	24/11/2020	10.87	Managed	Yes	6.87E+06	3.19E+07	1.49E+10	6.93E+10
GAR3	23/11/2019	16/01/2020	509.2	23/01/2020	10.18	Managed	Yes	6.80E+06	1.77E+06	1.38E+10	3.61E+09
GAR5	23/11/2019	14/01/2020	318.9	24/11/2020	6.38	Managed	Yes	9.29E+06	7.23E+07	1.19E+10	9.22E+10
KR3	23/11/2019	21/01/2020	719.8	23/01/2020	14.40	Managed	Yes	3.32E+03	6.60E+04	9.57E+06	1.90E+08
KR5	23/11/2019	14/01/2020	503.7	24/11/2020	10.07	Managed	Yes	2.82E+05	6.47E+06	5.69E+08	1.30E+10
KR7	23/11/2019	16/01/2020	480.5	23/01/2020	9.61	Managed	Yes	1.05E+06	5.37E+06	2.01E+09	1.03E+10
KR4	23/11/2019	14/01/2020	526.2	24/11/2020	10.52	Managed	Yes	BL	BL	BL	BL
KR2	23/11/2019	14/01/2020	675.0	24/11/2020	13.50	Managed	Yes	473.29	71110.55	1.28E+06	1.92E+08
KR6	23/11/2019	15/01/2020	550.5	23/01/2020	11.01	Managed	Yes	1.63E+06	8.62E+03	3.59E+09	1.90E+07
KR1	23/11/2019	14/01/2020	437.4	24/11/2020	8.75	Managed	Yes	1.65E+04	4.59E+04	2.88E+07	8.03E+07
RON1	26/11/2019	15/01/2020	985.9	23/01/2020	19.72	Managed	Yes	4.77E+07	9.42E+06	1.88E+11	3.72E+10
RON5	26/11/2019	14/01/2020	394.7	24/11/2020	7.89	Managed	Yes	1445.87	BL	2.28E+06	BL
RON2	26/11/2019	14/01/2020	649.7	24/11/2020	12.99	Managed	Yes	1.07E+07	5.51E+03	2.78E+10	1.43E+07
RON4	26/11/2019	14/01/2020	864.2	24/11/2020	17.28	Managed	Yes	5.63E+04	2.87E+03	1.94E+08	9.91E+06
RON3	26/11/2019	16/01/2020	657.9	23/01/2020	13.16	Managed	Yes	4.41E+05	9.53E+03	1.16E+09	2.51E+07
DA1	24/11/2019	15/01/2020	659.0	23/01/2020	13.18	Managed	Yes	2.83E+07	9.33E+06	7.45E+10	2.46E+10
DA2	24/11/2019	15/01/2020	700.6	24/11/2020	14.01	Managed	Yes	BL	352279.44	BL	9.87E+08
DA3	24/11/2019	21/01/2020	640.1	23/01/2020	12.80	Managed	Yes	7.20E+05	6.51E+03	1.84E+09	1.67E+07
DA5	24/11/2019	16/01/2020	553.4	23/01/2020	11.07	Managed	Yes	1.55E+03	6.09E+06	3.43E+06	1.35E+10
DA4	24/11/2019	15/01/2020	385.0	23/01/2020	7.70	Managed	Yes	2.39E+07	1.67E+05	3.67E+10	2.57E+08
DT1	25/11/2019	15/01/2020	797.8	23/01/2020	15.96	Managed	Yes	3.14E+03	6.81E+06	1.00E+07	2.17E+10
DT2	25/11/2019	14/01/2020	684.8	24/11/2020	13.70	Managed	Yes	7.83E+06	2.64E+06	2.14E+10	7.24E+09
DT3	25/11/2019	15/01/2020	705.7	23/01/2020	14.11	Managed	Yes	4.27E+05	8.41E+04	1.20E+09	2.37E+08
DT4	25/11/2019	15/01/2020	970.5	24/11/2020	19.41	Managed	Yes	931.92	6043.39	2.22E+06	9.99E+05
DT5	25/11/2019	09/01/2020	860.6	24/11/2020	17.21	Managed	Yes	645.84	BL	3.62E+06	BL
VAN LW1	26/11/2019	15/01/2020	663.1	23/01/2020	13.26	Managed	Yes	3.57E+06	9.05E+06	9.47E+09	2.40E+10
VAN17	26/11/2019	15/01/2020	619.4	24/11/2020	12.39	Managed	Yes	283.38	1170.97	7.02E+05	2.90E+06
VAN8	26/11/2019	14/01/2020	777	24/11/2020	15.54	Managed	Yes	174.69	BL	5.43E+05	BL
VAN L25	26/11/2019	15/01/2020	810	24/11/2020	16.20	Managed	Yes	121.06	3263.12	3.92E+05	1.06E+07
VAN4	26/11/2019	15/01/2020	1067.3	24/11/2020	21.35	Managed	Yes	3.77E+05	2.81E+03	1.61E+09	1.20E+07
SW1	24/11/2019	16/01/2020	578.4	23/01/2020	11.57	Feral	No	2.49E+07	1.10E+07	5.77E+10	2.54E+10

Supplementary Table S4. DWV world map references

Country/Region	Source reference
Argentina – Buenos Aires and Santa Fe province	(Brasesco *et al.,* 2020)
Australia	(Roberts *et al.,* 2017)
Brazil	(de Souza *et al.,* 2019)
Chile	(Riveros *et al.,* 2019)
China	(Diao *et al.,* 2019)
Cuba	(Luis *et al.,* 2020)
Ethiopia – Tigray	(Gebremedhn *et al.,* 2020)
Fernando de Noronha	(Brettell & Martin, 2017)
France	(Manley *et al.,* 2019)
Germany	(Natsopoulou *et al.,* 2017)
Hawaii	This study, (Brettell *et al.,* 2020)
Kenya	(Ongus *et al.,* 2018)
Other	(Beaurepaire *et al.,* 2020)
Other	(Wilfert *et al.,* 2016)
Papua New Guinea	(Roberts *et al.,* 2020)
South Africa	(de Souza *et al.,* 2020)
Tunisia	(Abdi *et al.,* 2018)
Turkey	(Tozkar *et al.,* 2015)
UK	(Kevill *et al.,* 2019)
Uruguay	(Mendoza *et al.,* 2020)
USA	(Kevill *et al.,* 2019)



Supplementary Figure S1. Average DWV-A and –B loads in colonies of different treatment type from Oahu with bars showing the standard error.

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Chapter 4: *Varroa* resistance in *Apis cerana*: A review

Abstract

Varroa is a major world-wide pest to Western honey bees (*Apis mellifera*), causing huge ongoing losses of colonies every year. Conversely, the Eastern honey bee (*Apis cerana*) is less vulnerable to the mite having existed alongside it over a long evolutionary period. Research conducted during the 1980s and 1990s, shortly after *Varroa* had spread across the globe, concluded that the Eastern honey bee was less vulnerable because it displayed higher levels of grooming behaviour, brood removal behaviour and mite infertility than its Western counterpart. However, this review on these *Varroa* resistance traits in *A. cerana* indicates that there is surprisingly little evidence for these conclusions. This review explores this evidence and discusses the potential flaws in the studies and the gaps that still remain in our knowledge of *Varroa* resistance traits in *A. cerana.*

Introduction

Varroa is a genus of ectoparasitic mite which parasitises honey bee colonies across the world. Female *Varroa* mites live on the body of adult honey bees and reproduce in the brood cells alongside the developing honey bee pupae. *Varroa* garnered attention approximately 70 years ago when the now infamous species *Varroa destructor* species jumped from its original host, the Eastern honey bee (*Apis cerana*) to the Western honey bee (*Apis mellifera*) (Oldroyd, 1999). This jump was actually a collection of independent species jumps that were made possible because *A. mellifera* populations were moved into regions in the range of native *A. cerana* (Roberts *et al.,* 2015; Rosenkranz *et al.,* 2010). Once the parasite switched host it was accidentally traded worldwide with its Western honey bee hosts, excluding Australia and a few small islands (Roberts *et al.,* 2017; Shutler *et al.,* 2014),

causing widespread colony losses particularly in regions within the northern hemisphere. To date *Varroa* is still a major pest in the northern hemisphere and financial burden to Western honey bee apiculture (Rosenkranz *et al.,* 2010). Eastern honey bees, on the other hand, suffer fewer negative effects of the parasitisation and generally lack the need for human intervention (Lin *et al.,* 2016). Indeed, *A. cerana* can be described as resistant to the mite, which is defined here as the ability of a *Varroa* infested colony to survive long term (approximately 5 years), without control methods administered by humans, within a given environment (Grindrod & Martin, 2021).

Over the decades since *Varroa* spread outside Asia, *Varroa* resistance has been increasingly observed in western honey bees, firstly within Africa (Allsopp, 2006; Nganso *et al.,* 2018) and South America (Moretto *et al.,* 1991) and then Europe and the USA (Oddie *et al.,* 2018; Martin *et al.,* 2019; Hawkins & Martin, 2021; Grindrod & Martin, 2021). Research on these populations suggests that *Varroa* resistance is the product of a number of resistance traits that regulate the populations of mites within the colony. These traits include brood removal behaviour, which is the removal of dead or diseased or *Varroa*-infested pupae, grooming behaviour in which bees remove mites from themselves or other individuals, recapping behaviour in which infested cells are opened and resealed and finally mite infertility where mites are incapable of producing viable offspring. Three of these traits brood removal, grooming and mite infertility are the same as those previously reported in *A. cerana*. The fourth behaviour, recapping, has not been studied in *A. cerana* as it was more recently discovered by Oddie *et al.,* (2017). Data, from *A. cerana*, on all four of these traits would therefore benefit comparison and be a valuable asset in understanding *Varroa* resistance in *A. mellifera.* Recently, an evidence-driven framework was constructed to suggest how these traits may interlink and allow for the development of resistance in *A. mellifera* (Grindrod &

Martin, 2021). However, it is not possible to see if the same occurs in *A. cerana* as there exists relatively little published data and the data that does exist are from studies with small sample sizes and outdated knowledge, including the absence of the identification of *Varroa destructor* (Anderson & Trueman, 2000).

Henceforth, our understanding of the relationship between *Varroa* and *A. cerana* and consequently resistance traits is limited and often based on assumptions, which are then used to make further assumptions about *A. mellifera*. With the continual advancement of *Varroa* research methodologies it seems prudent that the relationship between *Varroa* and *A. cerana* is re-evaluated. In this review the major areas of research into *Varroa* resistance traits, grooming, brood removal, and mite infertility, in *A. cerana* are outlined and discussed to identify gaps and provide suggestions for future research.

Grooming

Grooming behaviour is often included in the suite of behaviours used in defence against *Varroa.* It entails adult bees either removing mites from themselves (auto-grooming) or from other adult bees (allo-grooming) using their legs and mandibles (Pritchard, 2016). The removal and possible injury of the mites is thought to control the size of the phoretic mite population and thus the overall colony infestation (Moosbeckhofer, 1992). However, grooming behaviour is difficult to measure accurately as it relies either on indirect measurements such as mite damage or from direct observations. Despite this a single study by Peng *et al.,* (1987) (cited almost 300 times in web of science, accessed 17/02/22) appears to have led to the acceptance of *A. cerana* as the superior groomer over *A. mellifera* and from this the assumption that grooming is a considerable factor in *Varroa* resistance. Certainly, at first glance the results are very enticing as the 99.6% removal of mites by *A.*

cerana vastly overshadows the 0.3% removal by *A. mellifera* seemingly solving the mystery of why *A. cerana* are more resistant in one shot. There are, however, a number of reasons to be highly sceptical of the results.

The issue of mite source

Firstly Peng *et al.,* (1987) used mites from *A. mellifera* colonies on *A. cerana* adults. This is likely to have unintentionally exaggerated the results because *A. cerana* respond much more strongly to mites sourced from *A. mellifera* colonies compared to their own species (Büchler *et al.,* 1992; Fries *et al.,* 1996; Rath 1991a; Rosenkranz *et al.,* 1993). This heightened response is possibly due to the mites being of another species (*V. destructor*) and/or the mites having mimicked the original hosts cuticular hydrocarbons (Kather *et al.,* 2015; Le Conte *et al.,* 2016; Martin *et al.,* 2001) which are distinct from the new *A. cerana* hosts (Rahman *et al.,* 2016). The new *A. cerana* hosts can rapidly detect these cuticular hydrocarbons as foreign to their own (Fries *et al.,* 1996; Rath 1991a). An undisclosed proportion of these mites were also gravid females which have rounded bodies that can make them more vulnerable to removal via grooming (Delfinado-Baker *et al.,* 1992; Rath 1999), since gravid females never naturally occur outside the protection of sealed honey bee brood cells.

Limitations to direct observation methods

The results may also have been spuriously elevated because, to assess grooming ability, Peng *et al.,* (1987) attempted to directly observe the adult bees undertaking the behaviour. Naturally, this approach is prone to inaccuracy because it is difficult to follow individual mites and to be sure of their fate (Fries *et al.,* 1996). As a result, the authors considered both the movement of mites from one bee to another and the disappearance of mites to

the observer as a successful removal. It would be interesting to ascertain the removal ability without the data generated by the movement of mites from one bee to another however the raw data from this study is not provided. Indeed, the potential inflation of the results was highlighted nearer the time in a review by Boecking *et al.,* (1993). They also indicated that a constant removal rate as high as 99% would mean that *A. cerana* colonies would be devoid of mites during periods when drone brood is absent, which is not the case. Nonetheless, despite these shortfalls the Peng *et al.,* (1987) article is still highly cited with 19 citations in 2021 (web of science, accessed 17/02/22).

Since its publication only three other studies have sought to repeat or re-evaluate these results (Table 1). Büchler *et al.,* (1992) also utilised the direct observation method and used a mix of phoretic and brood mites for *A. mellifera* colonies. They did improve the methodology by using phoretic *A. cerana* mites for the *A. cerana* colonies, however they chose to source these mites from different *A. cerana* colonies. This may still affect the results because *Varroa* mites can mimic the colonies cuticular hydrocarbon profile down to the level of each colony (Kather *et al.,* 2015). Despite this their results appear more realistic, in terms of the earlier criticism by Boecking *et al.,* (1993), with 75% removal rather than 99% for *A. cerana.* They also found a much greater result for *A. mellifera* at 48% removal but both figures need to be interpreted with care as, in comparison to the other two studies, they were based on very small sample sizes of 36 and 25 mites respectively.

Mite damage as a proxy for grooming ability

To avoid the issues with direct observation experiments Fries *et al.,* (1996) used mite damage as a proxy for grooming success. During grooming, mites can endure damage to their idosima and legs caused by the bee's mandibles (Rosenkranz *et al.,* 1997; Ruttner &

Hänel, 1992). Using this as a proxy allowed the experiment to be conducted in a normal, full-size hive compared to the smaller observation hives used in previous studies. Fries *et al.,* (1996) found that, over a six-hour period, 29.6% of introduced mites were damaged by *A. cerana* and 12.3% by *A. mellifera*. The result for *A. cerana* may again have been impacted by the use of *A. mellifera* mites although the difference between the species is notably smaller than the results of both Peng *et al.,* (1987) and Büchler *et al.,* (1992). The smaller difference could be the result of using an indirect method, however this is not supported by Rath (1991a). They recorded the number mites, sourced from *A. mellifera*, that died and were injured when introduced to adult bees in a cage experiment. Furthermore, Peng *et al.,* (1987) also found a large difference between the two species, 61.7% of introduced mites died on *A. cerana* in 48 hours, whereas only 2.8% died on *A. mellifera.* Of those that died they found that 83% from *A. cerana* had injuries whilst none of the dead mites from *A. mellifera* showed any sign of injury.

The uncertainty caused by using a proxy

Measuring grooming indirectly brings its own level of uncertainty to the results because grooming is not the single cause of damage to mites. Mites may also be damaged when infested brood cells are cleaned out (Boecking & Drescher, 1991) or by other hive predators such as ants (Bienefeld *et al.,* 1999; Davis *et al.,* 2007) or wax moth (Szabo & Walker, 1995). Care also needs to be taken when observing damage to the idosima to prevent regular dorsal dimples, a developmental defect, from being confused for grooming induced damage (Davis, 2009; Rosenkranz *et al.,* 1997). Also, as with the observation methods, Fries *et al.,* (1996) noted that the artificial introduction of mites into a colony substantially increased the initial mite drop. Additionally, the presence of emerging brood increases the mite fall

and mite damage (Hoffman, 1995; Lobb & Martin, 1997; Martin & Kemp, 1997; Rosenkranz *et al.,* 1997) thus adding to the variability of measurements. It is also difficult to conclude whether the damage occurred pre or post mortem; for example Fries *et al.,* (1996) found that in an *A. mellifera* colony, 26.4% of naturally fallen dead mites (killed by freezing the combs) had damage but only 9.1% of naturally fallen live mites were damaged, suggesting that either bees injure dead mites or that the injury caused by bees leads to the death of mites.

Table 1. Details of previous studies conducted on the grooming behaviour in *A. cerana.*

Author	*Varroa* source	*A. cerana* Grooming (%)	A. mellifera Grooming (%)	Observation time	Hive type	How grooming is assessed?
Peng *et al.,* (1987)	*A. mellifera* – brood and phoretic	99.6 (*n*=270)	0.3 (*n*=270)	Up to 2 hours	Observation hive	Direct observation
Büchler *et al.,* (1992)	*A. cerana* phoretic, *A. mellifera* - phoretic and brood	75 (*n*=36)	48 (*n*=25)	10 minutes	Observation hive	Direct observation
Fries *et al.,* (1996)	A. mellifera phoretic	29.6 (*n*=115)	12.3 (*n*=65)	6 hours	Full size Langstroth hives	No. of damaged mites
Rath (1991a)	A. mellifera phoretic	61.7*	2.8*	48 hours	Cage experiment	No. of dead mites

* Sample size could not be ascertained

Summary

It is widely believed that *A. cerana* perform grooming to a high extent and that this behaviour plays a large role in controlling the *Varroa* mite population. However, this belief is based largely on a single study by Peng *et al.,* (1987) that may have elevated results due to flaws in the methodology. In addition to discrepancies in methodology grooming is high variable both within and between colonies due to the season (Büchler *et al.,* 1993;

Mondragón *et al.,* 2005; Moosbeckhofer, 1997; Russo et al 2020), environmental conditions (Currie, & Tahmasbi, 2008), presence of emerging brood (Hoffman, 1995; Lobb & Martin, 1997; Martin & Kemp, 1997; Rosenkranz *et al.,* 1997) and levels of brood removal behaviour (Kirrane *et al.,* 2018). This means many measurements are required to increase the accuracy of results. Thus, the existing four studies do not provide enough data to accurately suggest the role that grooming plays in resistance in *A. cerana*.

Indeed, despite the larger number of studies on grooming in *A. mellifera* the results have been highly variable. In some instances, resistant colonies have been found to groom to a significantly more (Mendoza *et al.,* 2020) and some studies found a negative correlation between mite damage and infestation rate (Arechavaleta-Velasco, & Guzmán-Novoa, 2001; Mondragón *et al.,* 2005; Moosbeckhofer, 1992; Ruttner, & Hänel, 1992). Conversely, many others have found the opposite with no significant difference between resistant and susceptible populations suggesting that grooming does not significantly contribute to resistant behaviour (Aumeier, 2001; Kovačić *et al.,* 2018; Kruitwagen *et al.,* 2017; Locke & Fries, 2011; Nganso *et al.,* 2017; Oddie *et al.,* 2018). Certainly, when the impact of reduced mite fertility is considered, grooming behaviour is not necessary to explain *Varroa* resistance in *A. mellifera* (Locke & Fries, 2011; Oddie *et al.,* 2017) or in *A. cerana* in which there is complete infertility of mites in the worker brood (Fries *et al.,*, 1994). This is why grooming was not included in the framework proposed by Grindrod & Martin (2021).

Brood removal

Brood removal is the archetypal hygienic behaviour in which adult bees uncap and remove dead, diseased or parasitised pupae. Whilst it is used in response to *Varroa* infestation it was first described as a response to American foulbrood (Rothenbuhler, 1964) and then to

chalkbrood (Gilliam, Taber III, & Richardson, 1983). Such brood diseases usually result in the death of the pupae and in turn the release of potent death pheromones such as oleic acid (McAfee *et al.,* 2018). As a consequence of this and the hazards involved in inoculating pupae with diseases hygienic behaviour has typically been measured using methods that cause the death of the pupae including freeze killed brood (FKB) and pin killed brood (PKB) methods (Spivak & Downey, 1998).

The results and limitations of freeze killed brood (FKB) methodology

A. cerana respond well to FKB, they remove fairly high levels of FKB, 82% in 24 hours (Rath & Drescher, 1990) and also remove it faster than *A. mellifera* (Lin *et al.,* 2016; Shakeel *et al.,* 2020). However, *A. cerana* colonies can be highly susceptible to the brood diseases sac brood (Abrol, 2000; Ai *et al.,* 2012; Hassanyar *et al.,* 2019; Ma, 2014; Vung *et al.,* 2020) and American foul brood (Chen, *et al.,* 2000) which suggests the hygienic response may not be uniformly high across populations. Additionally, whilst FKB and PKB can offer some insight into the general hygienic capabilities of a colony they have so far failed to correlate with the results of *Varroa* infested brood removal (Boecking & Drescher, 1992; Danka *et al.,* 2013; Grindrod & Martin, 2021; Leclerq *et al.,* 2018; Martin *et al.,* 2019). Arguably this is because *Varroa* rarely kills the developing brood and thus the cues used by workers to detect a *Varroa* infestation are different (Mondet *et al.,* 2021; Spivak, 1996).

Artificial mite infestation experiments

Ultimately due to their observed natural resistance to the mite *A. cerana* are generally believed to express a higher level of brood removal behaviour than *A. mellifera.* However, despite a plethora of anecdotal evidence, this literature search only found three studies that measured the ability of *A. cerana* to remove cells artificially or naturally infested with

Varroa. The first of these papers by Rath & Drescher (1990) found very high removal rates, 97.4% and 91.9%, of *A. cerana* worker cells artificially infested with live and dead ethanol washed mites respectively, which indicated the ethanol wash had little, if any, effect. The mites used were again sourced from *A. mellifera* colonies which, as with grooming, may artificially increase the removal response (Boot *et al.,* 1999; Rosenkranz *et al.,* 1993) due to different chemical profiles. One could argue that the scent of previous hosts would be negated by the ethanol wash of the dead mites; however even when washed with ethanol, cuticular hydrocarbons, potentially from the original host, are very likely to remain on the mite (da Silva Cunha *et al.,* 2021). Boot *et al.,* (1999) found that *A. cerana* however, removed 84% of mite (sourced from *A. mellifera*) infested worker brood cells over 10 days, which whilst lower is still a high result for the removal of *Varroa* brood in comparison to resistant western honey bees (Grindrod & Martin, 2021). Although, in a separate experiment comparing both species Boot et al., (1999) showed that *A. mellifera* and *A. cerana* removed a similar percentage of *Varroa* infested cells over four days, 32% (*n*=104) and 29% (*n*=131) respectively.

Additionally, the results of Rath & Drescher (1990) may also be somewhat artificially inflated as the results include the cells in which mites had disappeared from as well as fully emptied cells. Whilst workers do seem to be able to remove dead mites and re-seal cells (Rosenkranz *et al.,* 1993) live mites pose more of a challenge to remove and can also exit cells of their own volition whilst the cell is left open. This uncertainty means that the “disappearance” of live and dead mites should ideally be reported as a separate statistic as exemplified by Rosenkranz *et al.,* (1993) and Boot *et al.,* (1999) (Table 2). A proportion of live mites also “disappear” in *A. mellifera* colonies; specifically 13% (*n*=450) in Italian honey bee colonies and 7% (*n*=454) in Russian honey bee colonies (De Guzman *et al.,* 2016).

Observations of natural mite infestation

Boot *et al.,* (1999) noted, albeit without numerical evidence, a low removal response in naturally infested colonies, but this may be because of the low infestation rates and thus low levels of stimulus. Conversely, low natural responses may also be the result of the fact that *A. cerana* mites avoid reproducing in worker brood and thus do not produce the cues necessary to be detected (Mondet *et al.,* 2021). This may also explain why, unlike Rath & Drescher (1990) who found an immediate high removal response, Boot *et al.,* (1999) noticed that the removal response of *A. mellifera* mites was delayed by a couple of days, as time may be needed for reproduction to produce the cues. Accordingly, in *A. mellifera,* peak removal has been shown to occur roughly 3-5 days post capping (Harris, 2007, De Guzman *et al.,* 2016). Although, if a low removal response is due to a lack of reproduction then it is not easy to explain why Rosenkranz *et al.,* (1993) found a low removal response of 8% in *A. cerana* when mites were transferred within the same colony (intracolonial) but a high removal response of 50% with mites from a different *A. cerana* colony (intercolonial). Mites from another *A. cerana* colony would be likely to avoid reproducing in worker brood to the same degree.

Table 2. Details on the studies conducted on *Varroa* infested worker brood removal behaviour in *A. cerana.*

Study	Emptied cells (%)	Cells resealed without mite (%)	*n*	No. Colonies	Control cells removed (%)	*n*	Observation time (days)	Mite source/ status
Rath & Drescher (1990)	97%*		105	Not Stated	13%	107	5	A. mellifera brood
	92% *		148	Not Stated	12%	149	5	Dead *A. mellifera* mites (ethanol washed)
Boot *et al.,* (1999)	84%	7%	127	10	4%	122	10	A. mellifera phoretic
	29%	27%	131	10	-	-	4	A. mellifera phoretic
	0%	0%	13	10	-	-	4	*A. cerana* (natural infestation)
Rosenkranz *et al.,* (1993a)	8%	40%	26	5	10%	62	5	*A. cerana* (intracolonial transfer)
	50%	20%	74	5	-		5	*A. cerana* (intercolonial transfer)
	62%	30%	29	5	-		5	A. mellifera
	40%	5%	46	5	-		5	Dead *A. cerana* mites (ethanol washed)

*Cells resealed without mite was not treated as a separate statistic and raw data were unobtainable

The social apoptosis phenomenon

Indeed, other studies have suggested that brood removal may be stimulated by damage to the pupae rather than scents from the mite. Page *et al.,* (2016) and Lin *et al.,* (2018) discovered that the worker pupae of *A. cerana* in Thailand and China were more susceptible to wounding and infestation by *V. destructor* of the Korean haplotype than *A. mellifera* pupae. The increased susceptibility meant that *A. cerana* pupae were more likely to be developmentally delayed and die, which would simultaneously prevent successful mite reproduction and provide a signal to worker bees for removal (Lin *et al.,* 2018). As a result, they termed the phenomenon social apoptosis. In support of this Zhang *et al.,* (2018) discovered a protein in the saliva of mites called *Varroa* toxic protein, or VTP, that was extremely toxic to *A. cerana* worker brood but not *A. mellifera*. However, whilst these results are promising they seemingly lack support from previous mite reproduction studies in which an enhanced death rate of worker brood was not observed, or at least not recorded (Koeniger, & Koeniger, 1983; Koeniger *et al.,* 1981; Rath 1991a; Rosenkranz *et al.,* 1993b). Although, in the majority of these previous infertility studies *V. jacobsoni* was the infesting mite and may differ to *Varroa destructor* in terms of the impact of wounding towards its host. It may also be different with other haplotypes of *V. destructor* (Lin *et al.,* 2018) again highlighting the need for more research.

Summary

The removal of *Varroa* infested brood is thought to be the cornerstone for resistance in honey bee populations. Unfortunately, however, there remains a lot missing in our understanding of brood removal behaviour in *A. cerana*. Firstly, as with grooming, there is very little data concerning removal of artificially infested cells which combined with the high

variability of the behaviour means we do not have a reliable indicator of its relevance to resistance. Additionally of the data collected, the methodology varies with live mites often being sourced from *A. mellifera* colonies and sometimes from *A. cerana* and also sometimes dead mites are used. There are additional data on the removal of FKB, however as *Varroa* does not usually kill the brood FKB ability does not tend to correlate with the ability to remove infested brood. As a final note, there is a distinct lack of clarity concerning the phenomenon entitled social apoptosis, in particular its prevalence and whether it occurs with both mite species and all the haplotypes.

Mite infertility

The definition of mite infertility can include *Varroa* females producing no eggs at all or *Varroa* females failing to produce viable, i.e., fully matured and mated, female daughters. The former definition, also known as strict or complete infertility, was used in the studies conducted in the 80s and 90s and thus applies to the data reviewed here. Infertility was first reported as a characteristic of *Varroa* mites on *A. cerana* worker brood in Sri Lanka and Java (Koeniger *et al.,* 1981; Koeniger *et al.,* 1983) and has since been reported in Vietnam (Boot *et al.,* 1997), Papua New Guinea, Java, Irian Jaya (Anderson *et al.,* 1994), India (Rosenkranz *et al.,* 1993b; Twearson *et al.,* 1992). De Jong (1988) did note some rare incidences of reproduction in worker brood cells in South Korea.

The potential causes of *Varroa* infertility in worker brood

Whilst this infertility is fairly well-documented the exact cause remains elusive. Research by Grindrod & Martin (2021) on *A. mellifera* has suggested that a cause of infertility is simply the disruption of reproduction due to brood removal. They suggest that continual high levels of targeted brood removal could cause mites to avoid worker brood in favour of

drone brood that is not removed in *A. mellifera* (Grindrod & Martin, 2021) or *A. cerana* (Harris, 2008). In speculation this may have occurred in *A. cerana* with the resultant separate evolution of *Varroa* reproduction and worker pupal development leading to a loss of synchrony in the cycles and thus infertility of the mites. This loss of synchrony could include the loss of specific oogenesis triggers from the pupal host which are normally acquired by the mite when feeding. Although, these triggers could also be lost via selective pressure from mites.

Alternatively, infertility may be a factor of the mites not the pupae. This was suggested by the work of Boot *et al.,* (1999) that showed that mites from an *A. cerana* colony will not reproduce in the worker brood of another *A. cerana* or *A. mellifera* colony if transferred but that mites from an *A. mellifera* colony will. Rath (1991b) also found it was possible to get *Varroa* mites from *A. mellifera* to reproduce on *A. cerana* worker brood in a lab setting. Boot *et al.,* (1999) propose that the loss of fertility was the result of *A. cerana* removing reproducing mites more frequently and thus inadvertently selecting for non-reproducing mites. Indeed, a bias toward the removal of reproductive mites is possible because mite reproduction may be required to produce a stimulus that the bees can detect (Mondet *et al.,* 2021). However, due to the time period of the study, the suggestion of Boot *et al.,* (1999) overlooks the possibility of differences relating to the *Varroa* species. It would be beneficial to understand the differences, if any, between *Varroa* species and their ability to reproduce on different species and castes.

In the two decades following it, the observations of Boot *et al.,* (1999) and Rath (1991b) have only been repeated once by Li *et al.* (2019) who investigated the reproductive capabilities of *Varroa destructor* of the Korea and China haplotypes in China which parasitise

A. mellifera and *A. cerana* respectively. They found that whilst *Varroa* of the Korea haplotype could reproduce in worker brood of both honey bee species, albeit at a higher fecundity in *A. mellifera*, those of the China haplotype were completely sterile in *Apis mellifera* colonies.

The fertility of *Varroa jacobsoni* parasitising *Apis mellifera*

In the absence of competition with *V. destructor*, in Papua New Guinea (PNG), Roberts *et al.,* (2015) found that in that it is possible for *V. jacobsoni* to over time develop the ability to reproduce in both the drone and worker brood of *A. mellifera* colonies. Initially, these attempts to reproduce were directed at the drone brood and were largely unsuccessful (Anderson *et al.,* 1994). However, by 2008 *V. jacobsoni* was reproducing in high numbers on both drone and worker brood in PNG (Anderson, 2008) and later Roberts *et al.,* (2015) discovered that there had been two independent host shifts of *V. jacobsoni* onto *A. mellifera*. Thus, it does appear that the infertility of V*. jacobsoni* on *A. cerana* is a product of their relationship that may be reversible if the barriers to reproduction are removed. In speculation if these barriers are created in *A. mellifera* populations then perhaps the same infertility of mites in worker brood can be established. Although it is important to note that in PNG deformed wing virus is also absent which will alter the relationship between the mite and the honey bee host (Roberts *et al.,* 2020).

Summary

Mite infertility is the most strongly supported trait in *A. cerana;* however, there is no consensus on its origin or how it is maintained. Mite infertility in worker brood appears to play a large role in resistance and is believed to be the main reason why *A. cerana* do not succumb to the mite. Given that some studies have found that it is possible to get *V.*

destructor to reproduce on *A. cerana* it seems worthwhile to explore this relationship further as it may allude to the cause of the infertility of *V. jacobsoni*. For example, whether it is the result of selection by removing reproducing mites and/or the lack of cues in the pupal feed.

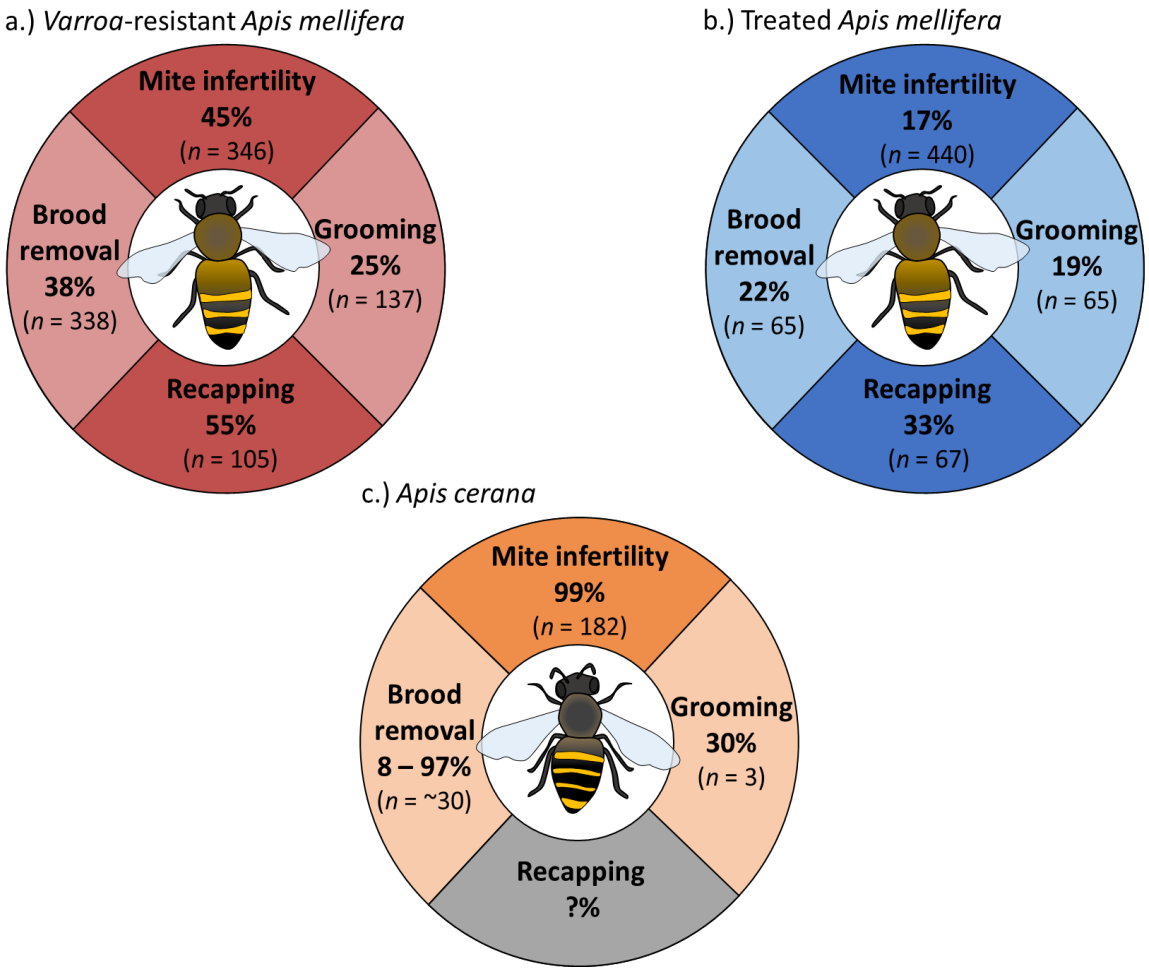

Figure 1. Summary of resistance traits displayed in a.) *Varroa* resistant *A. mellifera*, b.) *Varroa* Treated *A. mellifera* and c.) *A. cerana. n* = the number of colonies studied. Data for mite infertility, brood removal and recapping in a.) and b.) is taken from Grindrod & Martin, (2021) for studies used to calculate grooming in a.) and b.) see supplementary data. Data for

c.) comes from this study. All grooming averages are based on results using the mite damage proxy.

Conclusion

Here the main areas of *Varroa-A. cerana* research have been presented and gaps in the research have been highlighted and discussed. Ultimately, what has become clear is that our assumptions about the ability of *A. cerana* to perform the resistance traits grooming and brood removal are based on only a small number of decades old studies, often using small sample sizes. This is problematic because there is considerable natural variation in the displaying of resistance traits between colonies. Some variation exists naturally within and between populations, but it is amplified by many other factors including the seasons, environmental conditions, mite infestation levels and the methodology used.

Additionally given its more recent discovery there are also no published data on the presence of recapping behaviour in *A. cerana* colonies. This leaves gaps in our understanding of the relationship between *A. cerana* and *Varroa* and it is difficult to relate this to the trajectory of *A. mellifera* and *Varroa*'s relationship. Thus, there is a need to complete new research to ascertain the level of grooming, infested brood removal, recapping and mite infertility displayed in *A. cerana* populations across different regions. Those data could provide important evidence to either support or rebuke the framework of *Varroa* resistance acquisition presented in Grindrod & Martin (2021).

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Supplementary data

Supplementary Table 1. The data, source, location, bee race and the number of colonies for the percentage grooming ability of *Varroa*-resistant *Apis mellifera* shown in Figure 1a. EHB = European honey bees, AHB = Africanised honey bees

Author	Bee race	location	No. of colonies	Data
Vandame *et al.,* (2002)	EHB	Mexico	3	9.4
Kruitwagen *et al.,* (2017)	EHB	The Netherlands	3	10.1
Kruitwagen *et al.,* (2017)	EHB	The Netherlands	4	10.4
Vandame *et al.,* (2002)	AHB	Mexico	3	14.9
Mendoza *et al.,* (2020)	AHB	Uruguay	21	15.0
Boecking & Ritter, (1993)	A. m. intermissa	Tunisia	15	19.3
Zaitoun *et al.,* (2001)	A. m. syriaca	Jordon	8	22.8
Russo *et al.,* (2020)	EHB	Argentina	22	25.0
Guzman-Novoa *et al.,* (2012)	AHB	Mexico	7	26.2
Guzman-Novoa *et al.,* (2012)	EHB – Russian line	Canada	8	30.3
Locke & Fries, (2011)	EHB	Sweden	14	31.0
Locke and fries, (2011)	EHB	Sweden	7	36.0
Oddie *et al.,* (2017)	EHB	Norway	22	39.5

Supplementary Table 2. The data, source, location, bee race and the number of colonies for the percentage grooming ability of treated, *Varroa*-susceptible, *Apis mellifera* in Figure 1b. EHB = European honey bees.

Author	Bee race	location	No. of colonies	Data
Mendoza *et al.,* (2020)	EHB	Uruguay	17	6
Russo *et al.,* (2020)	EHB	Argentina	11	9
Kruitwagen *et al.,* (2017)	EHB	The Netherlands	5	9.7
Guzman-Novoa *et al.,* (2012)	EHB– bees imported from Hawaii	Mexico	7	16.3
Guzman-Novoa *et al.,* (2012)	EHB	Canada	8	23.8
Oddie *et al.,* (2017)	EHB	Norway	10	37.9
Locke & fries, (2011)	EHB	Sweden	7	46

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General discussion

Varroa and DWV form a pest-pathogen complex that cannot be eradicated. Combined, they cause huge financial losses and colony losses particularly in the northern hemisphere and commercial bee farms (Rosenkranz *et al*., 2010). The impact of *Varroa* and DWV has clearly indicated that our current methods of bee keeping, such as densely packed apiaries and chemical controls, are not only encouraging the spread of disease but also weakening the ability of bees to tolerate or adapt to new stressors. Indeed, chemical controls are typically used to control mite infestations and prevent colony losses, however the use of these controls prevents bees from adapting to the mite as the selective pressure is removed (Neumann, & Blacquière, 2017). The chemicals can also linger in the hive environment leading to contaminated and unsellable hive goods, chemical-resistant mites, and sub-lethal poisoning of the bees (Mullin *et al.,* 2010). It is therefore paramount that we focus our energy on developing sustainable ways to control the mite so that bees retain their health and genetic viability to cope with the next *Varroa* scale pandemic. Considering this, natural *Varroa* resistance which is the ability of colonies to survive long-term without treatment would be an ideal solution. Natural *Varroa* resistance has been commonplace in African and Africanised bees in South Africa and South America since the 1990s. However, despite the continual research into resistance and the traits it encompasses and many breeding programmes, resistance so far remains uncommon in European honey bees (Guichard *et al.,* 2020; Mondet *et al.,* 2020). To date, there is still no agreement on how resistance traits link together and also their relative contribution to controlling the mite population. Therefore, the focus of this PhD has been to explore naturally occurring resistance in *Apis mellifera* populations, specifically the role of different traits and deformed wing virus in resistance,

with the view to understand how it develops and thus how it could be encouraged in European honey bee populations.

Through this PhD a workable framework of resistance traits has been developed that may help pave the path toward solving this quandary. The framework (Chapter 2) hypothesises that naturally occurring *Varroa* resistance is the result of the interaction between recapping, brood removal and mite infertility with brood removal being the key connecting trait. Brood removal is the quintessential hygienic trait that bees use to control the large number of brood diseases they face. However, brood removal in the case of *Varroa* is more nuanced as the bees face the unique challenge that the pupae are not killed by the mite. Other brood diseases usually entail the death of the pupae and thus a large release of death associated pheromones. To face *Varroa* bees must learn to detect a new chemical signal or signals, that according to a recent study by Mondet *et al.* (2021) may be produced by the reproduction of *Varroa*. An increased ability of worker bees to detect mite infested cells marks the beginning of the progression towards resistance within the framework. The increased ability to detect mites can be inferred from the increased removal of mite infested brood as well as the increase in recapping of infested brood. Certainly, both of these three traits were on average significantly higher in resistant colonies compared to susceptible colonies, across multiple regions.

Recapping, however, has only been explored in-depth recently because of the work by Oddie *et al.,* (2017). The trait involves the opening, inspection, and then resealing of both infested and non-infested cells. In the resistance framework increased recapping is presented as a by-product of increased brood removal rather than a solo trait that directly leads to resistance. Its main function appears to allow workers to check and be certain of

the infestation status of a cell before removing it, thus preventing the unnecessary loss of healthy brood. This precautionary step is necessary because it is evident, given the high recapping of non-infested cells in resistant colonies, that the detection of mite infested cells is fairly inaccurate. In speculation this inaccuracy may stem from difficulties in detecting the signals emanating from the infested cell, perhaps due to the inability of cues to penetrate the wax capping or the diffusion of volatile cues from their point of origin. Indeed, when the spatial pattern of recapping is presented (chapter 1), it can be seen that these recapped, non-infested cells form clusters around recapped infested cells thus suggesting the difficulty in precisely locating the cell of origin.

Given that the infested cells have supposedly been checked before resealing it seems contrary that they would be recapped. However, there are numerous possible explanations for this. Firstly, errors may occur in the checking process. The chemical signals that bees detect are thought to contain a mix of different chemicals, if one is missing then perhaps the cell will not be removed (Nazzi *et al.,* 2004; Wagoner *et al.,* 2019). Additionally, each stage in the recapping or removal process (opening, checking and then removing or resealing) is carried out by a different bee (Scannapieco *et al.,* 2016). These bees are believed to differ in sensitivity with “recappers” having the lowest sensitivity they thus may inadvertently recap an open cell that is infested (Gramacho & Spivak, 2003). Cells can in fact be uncapped and recapped numerous times during the sealed period thus a recapping event does not mean that this cell would never been removed. Moreover, brood removal above a certain point, even if the brood is infested, can be detrimental to the growth and survival of the colony (chapter 2; supplementary figure 2). It is possible that there is a biologically enforced upper limit to the removal capabilities of bees which would also explain why even resistant colonies can be overwhelmed if enough mites are suddenly introduced.

Recapping may itself be an effective way to control the mite as the opening of the cell can potentially disrupt the reproductive cycle of the mite (Oddie *et al.,* 2018; Oddie *et al.,* 2021). Certainly, the disruption of the reproductive cycle due to brood removal and the consequent reduced reproductive success or mite infertility is a key part of resistance (framework, chapter 2). When a cell is emptied during brood removal the mite offspring die and the foundress mite is displaced. She may then infest a second cell; however, because her reproduction was part begun it is more likely that any offspring she produces will be unable to mature or mate due to the delayed egg laying or a missing male (Kirrane *et al.,* 2011). Over time consistent removal and disruption could lead to the depletion of the female mites' limited supply of spermatozoa (Alberti & Hänel, 1986; Donzé et al., 1996; Harris & Harbo, 1999) and eggs (Akimov & Yastrebtsov, 1984; Alberti & Hänel, 1986; Mikityuk, 1979; de Ruijter, 1987). Assuming an optimum of 1.4 viable offspring per cycle (Martin, 1994), it was calculated that in resistant colonies, with an average removal of 38%, mites are able to produce 0.87 viable offspring per cycle (chapter 2). A value which is similar to those measured in resistant colonies (Martin *et al.,* 2019; Medina & Martin, 1999; Oddie *et al.,* 2018). A low rate of offspring production predicts a reduction in the population growth and thus a reduced ratio of new fertile mites to older infertile mites (Harris, Danka, & Villa, 2010; Kirrane *et al.,* 2011). Fewer mites in the colony equates to fewer vectors of DWV and thus fewer infected individuals and a lower overall DWV load, ultimately enhancing the colonies survival. Therefore, it can be surmised that the key function of resistance traits in the hypothetical framework function is to reduce the mite numbers in the colony and in turn the DWV levels.

In the absence of DWV, or with covert DWV infections, colonies have a higher tolerance for mites (Roberts, Anderson, & Durr, 2017; Roberts *et al.,* 2020) and can support higher mite

loads than resistant colonies, without succumbing (Brettell & Martin, 2017; Martin, 1998). DWV thus appears to be an important driving force for the upper limit of *Varroa* mites that a colony can survive with and thus the lower limit of expression for the resistance traits, removal, recapping and mite non-reproduction, needed to keep a colony alive. However, DWV infections are not uniform as DWV is a quasispecies that encompasses an indeterminate number of variants which can be categorised as belonging to one of the four master variants DWV-A, DWV-B, DWV-C and DWV-D. Although, the most recently isolated variant DWV-D has thus far only been detected in historical samples (de Miranda *et al.,* 2022). Different DWV variants have different characteristics which may alter the dynamics of the *Varroa*-DWV-honey bee relationship; for instance, the two most prevalent master variants, DWV-A and DWV-B, have been shown to differ in their replicative ability and virulence, both on the individual level and to the colony as a whole (Dubois *et al.,* 2019; Gisder *et al.,* 2018; McMahon *et al.,* 2016; Norton *et al.,* 2020; Tehel *et al.,* 2019). DWV-B is thought to be less virulent at the colony level and has been shown to replicate to higher levels within infected pupae which is suggested to be why it has been increasing in prevalence and is dominating over type A in South Africa, the UK and parts of Europe (Chapter 3, Figure 2) (Brettell *et al.,* 2020; de Souza *et al.,* 2021; Kevill *et al.,* 2017; Kevill *et al.,* 2019; Kevill *et al.,* 2021; Manley *et al.,* 2019; Natsopoulou *et al.,* 2017). DWV-B is also increasing in prevalence in the US mainland (Kevill *et al.,* 2019; Ryabov *et al.,* 2017) and, as shown in chapter 3, has reached near co-dominance with DWV-A on the Hawaiian Islands Oahu and Big Island.

The closed populations of the Hawaiian Islands make them prime locations for monitoring the evolution of the DWV quasispecies. In particular the mix of *Varroa* naïve islands Kauai, Molokai, and Maui, and *Varroa* infested islands, Oahu and Big Island allowed for the

momentous discovery that *Varroa* infestation caused a decrease in DWV variant diversity and an increase in viral load leading to the domination of one master variant, DWV-A (Martin *et al.,* 2012). Chapter 3 follows on from this pivotal study showing that, a decade later, the viral landscape has changed again to a split dominance between DWV-A and DWV-B variants mirroring the changes seen across Europe, the UK and south Africa (Brettell et al., 2020; de Souza *et al.,* 2021; Kevill *et al.,* 2017; Kevill *et al.,* 2019; Kevill *et al.,* 2021; Manley *et al.,* 2019; Natsopoulou *et al.,* 2017). The methodology used in chapter 3 means that the proportion of recombinants compared to pure DWV-B variants cannot be ascertained but, it is still a clear increase in presence of variants containing DWV-B RNA dependent RNA polymerase (RdRp) coding regions. DWV-B may be gaining dominance because of its superior replicative ability (Dubois *et al.,* 2019; Tehel *et al.,* 2019) and lower (Norton *et al.,* 2020) or equal virulence (Tehel *et al.,* 2019). Indeed DWV-B outcompeting the potentially more virulent DWV-A may provide some protection to colonies (Mordecai *et al.,* 2016; Posada-Florez *et al.,* 2019) which may further explain why its increase has coincided with the increase in resistant colonies in Europe, South Africa, and the UK. That being said, it is difficult to disentangle the relative virulence of variants as the enhanced survival of resistant colonies may also be the result of genetic variation in viral tolerance (Locke *et al.,* 2021; Thaduri *et al.,* 2019). Additionally, the change in dominance is not universal as DWV-A still dominates in Brazil where colonies have been resistant for many decades (de Souza *et al.,* 2019). It would be beneficial for future research to complete the world map of DWV variants (figure 2 chapter 3). This would help aid understanding of DWVs progression as well as its possible relationship to resistance.

DWV plays a major role in the *Varroa* honey bee relationship and resistance, however research into resistance traits of honey bees to *Varroa* began 70 years ago sometime before

DWV was first isolated. That research was initiated when the mite first jumped species and spread outside Asia but the history of resistance to the mite began long before this with the Eastern honey bee. The relationship between *Varroa* and *Apis cerana* spans a long evolutionary period and could henceforth provide insights into the development of resistance and thus the further development or rejection of the framework. However, whilst *A. cerana* are often assumed to be highly hygienic with exceptional brood removal and grooming abilities there has been only three studies on *Varroa* infested brood removal and four on grooming behaviour (Chapter 4). The results of those studies do seem encouraging with levels of removal way above the calculated average of 38% for resistant colonies (Chapter 2) and grooming reaching a high of 99.6% (Peng *et al.,* 1987). However, the limited amount of data they provide is not sufficient to mitigate the high variability in the measurement and expression of these traits (Büchler *et al.,* 2020; Guichard *et al.,* 2020).

Additionally, the data from three of the grooming studies (Fries *et al.,* 1996; Peng *et al.,* 1987; Rath, 1991a) and two of the removal studies (Boot *et al.,* 1999; Rath & Drescher, 1990) may have been unintentionally inflated by of the use of mites from *A. mellifera* colonies in *A. cerana* colonies. This might have biased the results firstly because the mites sourced from *A. mellifera* are more likely to be *Varroa destructor* and not *Varroa joacobsoni* which is normally found on *A. cerana.* Secondly, because the colony specific scent on the mites may have elicited a stronger response from the *A. cerana* hosts (Büchler, Drescher, & Tornier, 1992; Boot *et al.,* 1999; Fries *et al.,* 1996; Rath 1991a; Rosenkranz, Tewarson, Singh, & Engels, 1993). Importantly, one of the most influential studies on grooming in *A. cerana* by Peng *et al.,* (1987) suffers from this problem yet is still regularly cited to date and seemingly provides the basis for the assumption that grooming is a predominant feature in resistance. On the other hand, the effect may be less pronounced in brood removal studies

where the cues being detected seem to be the product of mite reproduction rather than the scent of the mite (Mondet *et al*., 2021). The infertility of *A. cerana* mites may explain why the removal response is low in naturally infested *A. cerana* colonies.

Mite infertility in *A. cerana* is the most supported trait with eight studies over ten separate regions. Those studies all came to a similar conclusion that mite reproduction, with a few rare exceptions (De Jong, 1988; Yoshida, & Kittaka, 2000), is strictly limited to the drone brood of *A. cerana*. However, the cause of the infertility has not been successfully addressed. Certainly, it is difficult to isolate the key cause of infertility in mites in *A. cerana* because the fertility was likely lost over a distant period of evolutionary time and barriers to reproduction that once existed may no longer be observable in the present day. It has been suggested, albeit without empirical evidence, that the infertility may be due to the loss of needed nutritional cues from the pupae. Indeed, the reproductive cycle of *Varroa* is tightly linked to the pupa's development so much so that stages such as oogenesis may be stimulated by factors received from feeding on the pupa. Conversely, Boot *et al.* (1999) and Rath (1991b) suggest that infertility may instead be a factor of the mites because they found that mites from *A. mellifera* can reproduce on *A. cerana* worker brood. Boot *et al.* (1999) proposed that the infertility trait of the mites was accidently selected for by the biased removal of reproducing mites by *A. cerana*. In contrast more recent work has suggested that brood removal in *A. cerana* is predominantly triggered by the death or injury of the worker pupae due to a toxic protein in the mite's saliva. Although, a high death rate of infested *A. cerana* pupa was not reported in any of the previous infertility or brood removal studies, or by Boot *et al.* (1999) and Rath (1991b) who both successfully got *Varroa destructor* to reproduce on *A. cerana* worker brood. Ultimately this and other queries highlight the necessity for research to revisit the relationship between *Varroa* and *A. cerana*. A

reassessment of mite infertility and brood removal would be invaluable in assessing whether resistance in *A. mellifera* is following the same trend, for instance whether high removal behaviour does lead to a decrease in fertility in worker brood and hence pressure mites toward drone brood (Chapter 2).

Conclusion

Overall, I aimed to improve the current understanding of the natural resistance of *Apis mellifera* to *Varroa destructor*. Towards this aim I have been able to provide evidence that the more recently popularised hygienic trait, recapping, occurs in a distinct spatial pattern associated with infested cells. The clustered pattern suggests that the presence of infested cells drives the recapping behaviour, and that the detection of such cells may not be entirely accurate thus requiring a mechanism to check the cells and prevent the loss of healthy brood. Further research is required to ascertain whether recapping is just for this purpose or whether it too impacts on the reproductive capabilities of the *Varroa* mites. Building on this first finding I was able to join together the three hygienic traits recapping, brood removal and infertility to create and provide support for a framework of how *A. mellifera* have begun to develop resistance to the mite. This framework, whilst hypothetical, will be a useful stepping stone for further research in natural resistance. Indeed, it has already been useful in helping to inform beekeepers on encouraging resistance in their own colonies (appendix 1-8). To provide more support or amend the framework more data on the resistance traits from *A. mellifera* and *A. cerana* is needed. I discovered that *Varroa* resistance in *A. cerana* has been woefully under studied and hopefully the highlighting of this absence of data will push future efforts into examining the resistance traits. I predict that *A. cerana* will have followed the same path to resistance as *A. mellifera* but the characteristics, particularly

infertility, may present differently due to the longer evolutionary relationship between *Varroa* and *A. cerana.*

Finally, I also found evidence of a shift from DWV-A dominance to DWV-A and DWV-B co-dominance in Hawaii. This will help add to our understanding of the prevalence of different DWV variants and how this has changed over time. Certainly, the results of chapter 3 support other work showing that DWV-B dominance is increasing across the globe. DWV-B is thought to be less virulent at the colony level and thus may provide a colony with some protection. However, the exact ramifications of an increase in type B dominance are not yet clear and should be the subject of future research efforts.

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IMPACT ACTIVITIES & ARTICLES

All the research I have conducted is aimed at helping beekeepers who fund this research into reducing or stopping treatments for *Varroa,* hence I have been encouraged by my funders and supervisor to produce a series of beekeeper articles, participate in public talks, and produce science communication materials to inform beekeepers on how *Varroa* resistance develops and how they could measure, monitor and encourage key *Varroa* resistance traits within their own bees thus reducing or eliminating the need for treatments.

Activities: List of presentations, workshops, and interviews

Ormskirk beekeepers' association: 30/06/20

BDI annual general meeting: 10/09/20

Wimbledon beekeepers' association: 01/03/21

Canterbury beekeepers' association: 07/04/21

Hays county bee keepers' association: 16/06/21

Coloss conference 2021 survivors' workshop: 06/10/21

BBC Radio 4 segment Inside Science interview: 05/08/21

Shropshire bee keepers' association: 9/02/22

Measuring recapping workshop: 11/06/22

Published works: List of published articles and videos

Martin, S. J. & Grindrod, I. Natural *Varroa*-resistant honey bees: Biology, testing, and propagation. (2020). *BBKA news special issue series.* ISSN: 2513-9517

Grindrod, I., & Martin, S. J. Natural *Varroa* resistant bees in the UK. (2021). *Bee craft, 103*(1), 9-11. ISSN: 0005-7703.

Instructional Video: Measuring recapping and infested brood removal

Grindrod, I. (2021). Honey bees are becoming resistant to *Varroa*. *The British Bee Journal published in conjunction with BBKA news, 7*, 1-3.

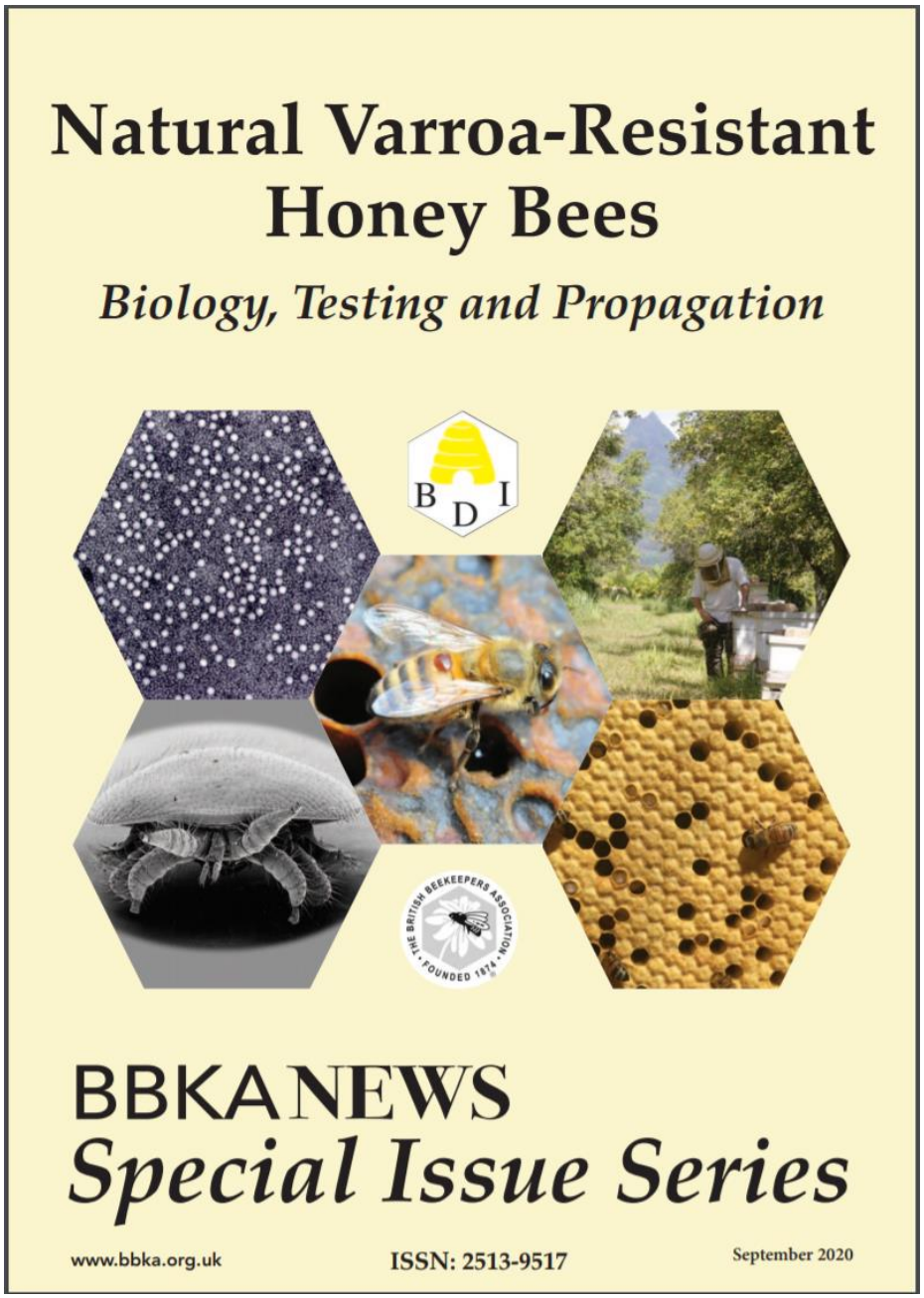
Webb, G., Grindrod, I., & Martin, S. J. (2021). *Varroa*-resistance: A team update. *BBKA news incorporating the British Bee Journal,* 331-332.

Article for BBC Radio 4 segment Inside Science

BBKA spring conference poster

Natural *Varroa*-resistant honey bees: Biology, testing, and propagation. *BBKA news special issue series*

The bulk of the text of this article was written by Stephen Martin, I created the graphs and provided feedback and edits.



Foreword

Bee Diseases Insurance Ltd (BDI) and the British Beekeepers' Association (BBKA) have, for a long time, funded research into varroa, so I am delighted to introduce this new *Special Issue* dedicated to this one topic.

The management of our colonies for this pest is one of the ways that beekeeping has evolved in the recent past. The arrival of varroa in the UK contributed to a steep decline in the number of beekeepers and colonies at the time. Since then, a variety of ways of managing colonies for varroa have evolved and learning these have become an integral part of every beekeeping groups' beginner courses.

Early treatments focussed on invasive chemical compounds, and while these still have a place, the emphasis is now on biological methods of control wherever possible. As this *Special Issue* shows, the latest research also looks at ways in which bees and the mites can co-exist.

Professor Stephen Martin and his team have been studying the varroa mite for many years and he is a world leader in this research field. As British beekeepers we should be proud of the level of financial support we have provided for this research, demonstrating, as it does, that our various groups can make a significant difference towards understanding the threats that face these important food producers and pollinators.

This *Special Issue* shows how the research can be applied by an interested beekeeper. It explains the practical methods by which they can assess the level of natural resistance to varroa that their colonies have. I commend you to try some of the procedures that are outlined in this booklet.

Martin Smith, BDI President,
BBKA Past President

Authors' Foreword

The honey bee and natural selection have provided a lasting solution to the varroa problem. Our aim is understanding this and helping inform beekeepers so they, in turn, can help their bees.

The format of this *Special Issue* is to provide all the necessary background information, so you can understand the logic behind the advice.

Prof Stephen Martin and Isobel Grindrod,
Salford University, UK.

In this issue...

How varroa kills a colony	3
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OTHER TOPICS WITHIN THE *BBKA News Special Issues Series*:
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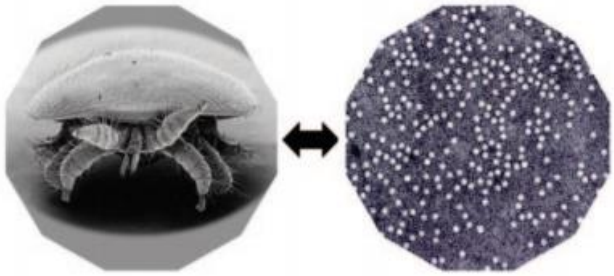
General Husbandry. April 2017, reprinted April 2018.
Honey Bee Anatomy. Nov 2016; reprinted: Feb 2017.
In the Apiary. Jan 2017; reprinted: Feb 2017.

How Varroa kills a Colony



- Understanding how a varroa infestation kills a colony leads to a better understanding of how mite-resistance can arise. As ever the 'devil is in the detail'.
- The two components can be treated as either side of the same coin.

Varroa reproduction

Varroa remains the number one pest

Every beekeeper knows that varroa remains a major concern and has caused the death of millions of colonies as it spread around the world. Beekeeping survived only by controlling varroa populations using a wide variety of methods; see *BBKA News Special Issue on Integrated Pest Management*. This was critical, since allowing the mites to propagate freely would have overwhelmed the majority of managed colonies, as it did in devastating feral populations.

The current situation

Widespread, regular and sustained varroa treatments by the majority of beekeepers has reduced the numbers of varroa in the environment. This allows colonies, especially feral ones, to start evolving the ability to deal with the mites and provides an opportunity for beekeepers to start helping their bees to help themselves. This is important because the downside of long-term treatment is that it prevents, or masks, natural selection from acting to produce natural varroa-resistant (NVR) populations.

WARNING

Just stopping varroa treatment will, in the majority of cases, cause your colonies to die, so an informed approach is required (see pages 9–14).

Female varroa mites

Varroa is a brood parasite that lives exclusively on honey bees, *Apis cerana* and *Apis mellifera*. Only the adult female mites exist outside of the sealed brood cells and they are just referred to as 'mites'.

Female varroa mites are highly adapted to living on honey bees. Features include:

- Their crab shape, which allows them to fit between the segments of the bee's abdomen.
- They can chemically mimic either the odour of the adult bee or pupa and can change between the two odours in 3–4 hours.
- Retractable suckers, claws and hairs allow them to move quickly and securely among bees.
- They have peritremes (snorkels) that allow breathing while submerged in brood food.
- Their thick hard skin prevents damage and water loss.
- They have specialised piercing mouthparts to pierce the bees' tough inter-segmental skin for feeding on the bees' fat bodies, which lie just under the skin.

These adaptions evolved over millions of years, hence it is impossible to eradicate this pest, despite the efforts of beekeepers.

Varroa's lifecycle

The lifecycle of the female consists of two distinct phases:

- The phoretic phase.
- The reproductive phase.

The phoretic stage

- This is when the female mite lives on the adult bees.
- Most of its life is spent living on the adult bees.
- Typically, mites live on the bee's underside feeding regularly and are almost impossible to see.
- Only at high infestation levels do mites appear on top of the bees (Figure 1).
- On their native host, *Apis cerana*, mites survive around a year, since reproduction occurs only in drone brood, which typically appears annually.

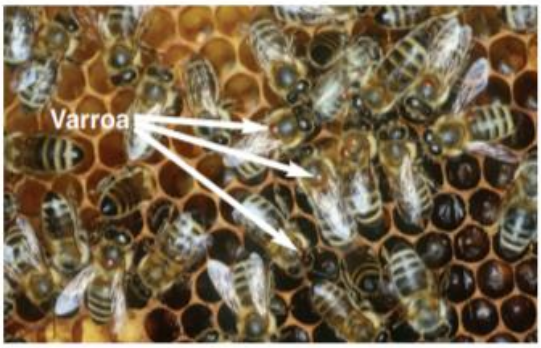


Figure 1. A heavily infested colony. Mites are seen on top of the bees, but the bees cannot detect them since a mites' odour is the same as a bee.

The reproductive stage

- Occurs in the worker and drone sealed brood (Figure 2).
- If present, drone brood which is the mite's natural host, is always preferred.
- In *A. mellifera* mites also use worker brood, which is a major cause of all the varroa problems.
- During the past thirty years, the timings and sequence of reproductive events (Figure 3) have not changed.
- It starts when the mite leaves the bee and invades a brood cell during the day before it is sealed.

Figure 2. A worker pupa infested with a mite family, with a darker mother on the thorax, the lighter, just matured, female offspring on the abdomen, and smaller mature male between the thorax and abdomen. The white object beneath the pupa is a skin of the final moult, proving the lighter female offspring is mature.

- The mite hides in the brood food, breathing via its peritremes and is released when the mature larva consumes the remaining brood food.
- The first mite egg is laid around sixty hours after the cell is capped and is always a male. This is followed every thirty hours by a female egg.
- Typically, mites lay lay 4–5 eggs in worker brood and 5–6 eggs in drone brood cells.
- Each egg develops through a series of stages (Figure 3) and after their final moult, they mate with their brother, within the safety of the cell.
- Mature female offspring and their mother emerge with the bee, leaving the male and immature offspring to perish in the hive.

Varroa population dynamics

Due to the shorter developmental time of the worker bees' sealed stage (11–12 days), relative to a drone's (14–15 days), drone brood produces at least twice as many new mature female mites as does worker brood. However, drone brood is present for only a short period annually, so the worker brood is where the real problem and solution lies.

- Figure 3 indicates, on average, 1.45 new adult female offspring are produced in each reproductive cycle.
- First offspring show a 94% survival rate, second offspring show 38% and the third offspring, 13%. So, in 100 cells there will be 1.45 survivors, or 1.45 new female mites per cell.
- This value of 1.45 is under optimal conditions.
- However, 10%–20% of males die for many reasons before being able to mate.
- Unmated females cannot successfully reproduce.
- Therefore, only around 1.2 new viable (mated) female mites are produced in one reproductive cycle.
- Each mated mite undergoes between two and three reproductive cycles, before running out of eggs.
- 1.2 mites x 2 reproductive cycles = 2.4 mites.
- 1.2 mites x 3 reproductive cycles = 3.6 mites.
- Thus, the growth rate lies between 2.4 and 3.6 in worker brood, under optimal conditions e.g. singly infested cells, no disturbance, etc.
- This explains the rapid build-up of mites in a colony during the summer months (Figure 4).
- The peak brood infestation occurs in autumn when the mite population reaches it maximum and the brood nest is contracting.
- This peak in mite numbers is when the vital winter bees are being produced.

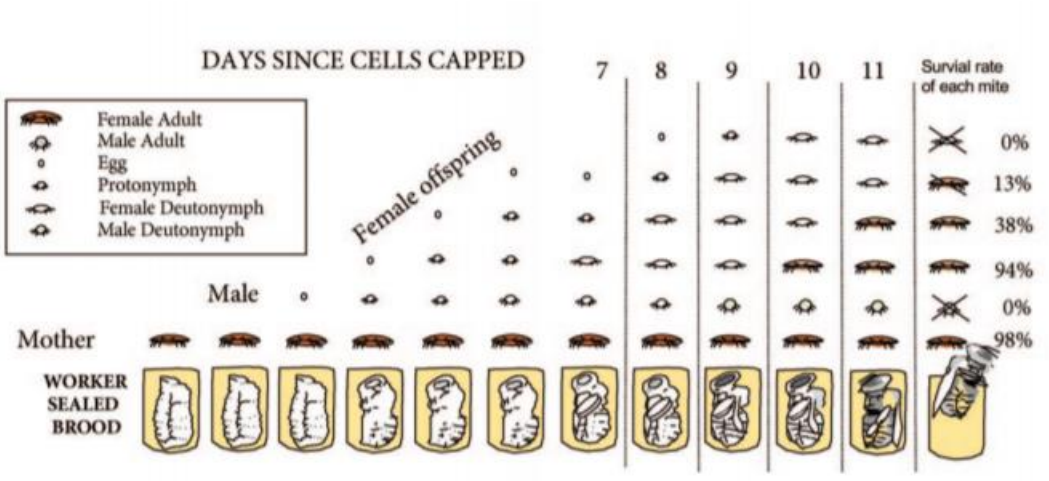


Figure 3. The daily pattern of reproductive events of a single invading mother in an A. mellifera worker sealed brood. A total of five eggs are laid and the first is always a male followed by four females. The different stages of development (see inset box) appear at different times. The key average, final survival rate at bee emergence is presented as a percentage. For example, 94% of the first females mature successfully but only 13% of the third females.

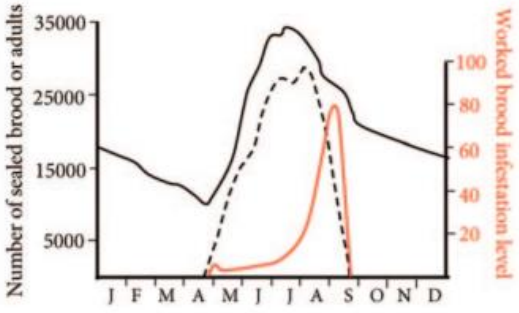


Figure 4. Typical numbers of adult bees (solid black line), worker sealed brood (dashed black line) and pattern of mite growth (red line) in a colony under UK conditions.

- Those infested with mites and deformed wing virus (DWV) will die before the end of the year and cause the colony to enter a downward spiral that ends in the death of the colony.
- In the UK, mite populations need to be controlled below a peak of 2,000 to prevent this from happening.

Deformed Wing Virus

Discovery of DWV

Prior to the global spread of varroa, deformed wing virus (DWV) was a little-known RNA virus (Figure 5; top) described in 1982 by Brenda Ball at Rothamsted UK. It was first isolated from dead Japanese honey bees and named due to the deformed wings of some of the infected bees (Figure 5; bottom). However, the vast majority of DWV-infected bees have normal wings. Pre-varroa, DWV was detected in dead colonies from Belize, South Africa and England.

Pre-varroa colonies infected with DWV

DWV is a rare viral pathogen that rarely kills colonies in the absence of varroa. If detected, it is always at very low levels, and then only in a few colonies or bees. It is naturally transmitted via food e.g. an oral route and is benign, since at such low levels it does not impact the bee's health.

Post-varroa colonies infected with DWV

After the global spread of varroa, DWV has become the most prevalent honey bee virus, with a minimum of 55% of all colonies or apiaries infected across the 32 countries surveyed. The vast majority of DWV-infected bees, even those with high viral loads, look healthy. DWV has now also been detected in 62 other insects species and three mite species.

The role of varroa

The mite has accidentally introduced a totally new bee-to-bee viral transmission cycle for DWV. When mites feed on a bee pupa, it becomes infected with a small amount of DWV. Over just three days this grows rapidly in the pupa or adult, from a few hundred viral particles to billions of them. The success of DWV lies in its ability not to kill the pupae, despite these high viral loads. This allows the mites to reproduce successfully. However, the subsequent adult life expectancy of infected pupae is shortened by two-thirds, whereas, if an adult becomes infected later, it transitions into a forager sooner and has a slightly shorter life expectancy. DWV appears to be able to replicate within varroa but has no known impact on the health of the mites.

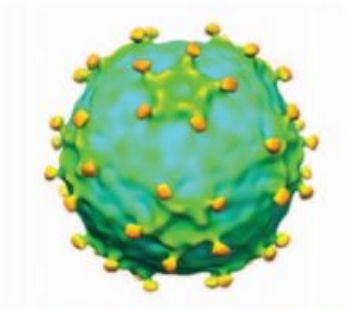


Figure 5. Top: An actual image of DWV, showing its external structure and regions used to attach itself to host cells, courtesy of S. Hafenstein and L. Organtini. Bottom: The classic deformed wings associated with DWV and with high levels of varroa infestation, by E. Villalobos.

Varroa-DWV-honey bee relationship (Figure 6)

Stage 1:

- No DWV, or tiny natural amounts of DWV, are circulating among the bees.
- An estimated 80,000 viral-free mites are required to kill a colony by feeding only.
- 'Healthy colonies' containing 30,000–50,000 mites in South Africa and 26,000 in the UK were seen in the first two years after the mites' arrival, before DWV became established.
- Over time the bees and mites adapt to each other; for examples of DWV-free, but varroa infested populations; see Box 1 (also on p6).

Stage 2:

- The DWV in a naturally infected bee, develops into a serious (overt) infection resulting in large amounts of DWV circulating in the bee's body (illustrated in red).
- The reason why this happens is unknown, but it is a rare event and occurs in either the bees or pupae, but not the mites.
- The mite and her subsequent offspring now become infected with DWV during feeding.

Stage 3:

- A new devasting viral transmission cycle begins.
- DWV-infected mites transmit the virus to both developing honey bee pupae and adults.
- Pupae infected with DWV have their expected adult lifespan reduced by 66% (two-thirds) irrespective of the time of year.

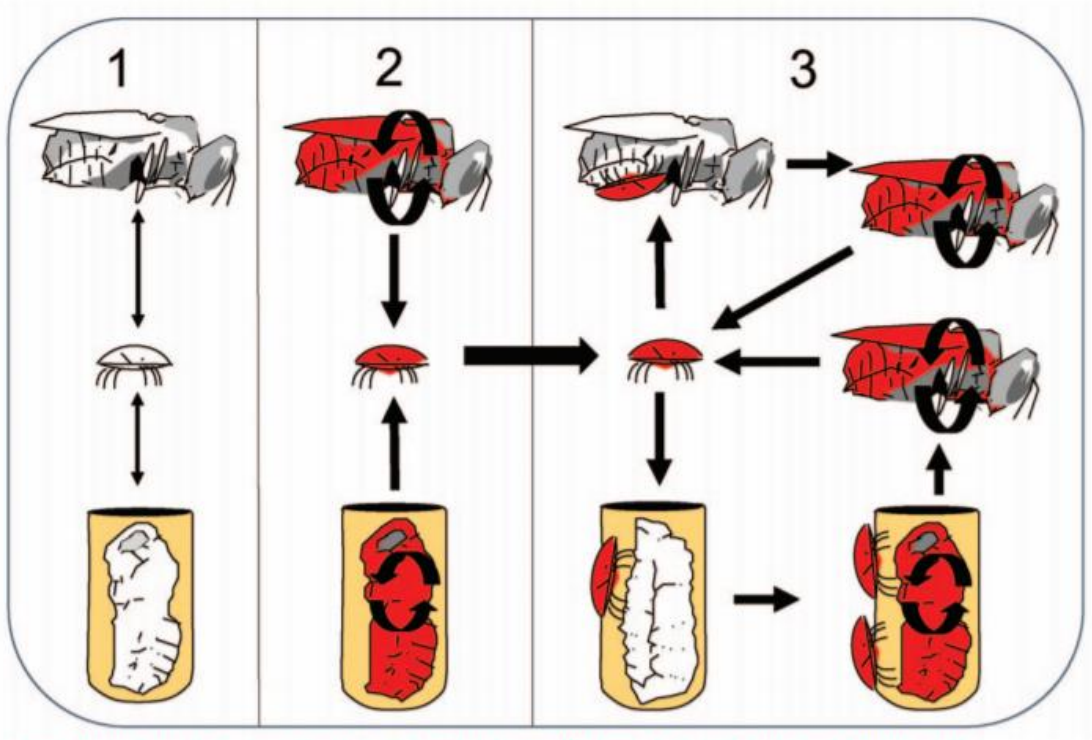


Figure 6. Illustrates the three different stages in the development of a varroa-DWV-honey bee infection. Red indicates a DWV infection.

- Adult honey bees becoming infected, suffer a reduction in lifespan of only a few days.
- Infected over-wintering adult honey bees die in early winter causing the colony to spiral down to a point where it becomes non-viable in spring and dies.

Box 1. Two DWV-free varroa-infested populations provide unique insight into impact of DWV

Case: 1 Fernando de Noronha, Brazil

- A remote tropical island 350 km from Brazil.
- Longest surviving population of untreated European honey bees.
- In 1984, *A. m. ligustica* colonies were established on the island and now consist of 20–50 managed hives, plus a small feral population.
- Some varroa were present in the original colonies.
- No varroa treatments have ever being required and no varroa-related deaths have occurred.

Photo by Stephen Martin.

- DWV levels in the bees were negative or so low they were at the limit of detection. This is precisely as found in mite-free populations, on the Island of Colansay, Scotland and Hawaiian Islands of Maui and Kauai.
- For over 35 years no virulent strain of DWV has appeared in this population. This is helped by the small number of colonies and ban on imports.
- Over 35 years, mite-infestation of adult bees has decreased, but brood infestation remained stable in worker and drone brood at around 20% and 40% respectively.
- The mites' ability to produce new viable females is only 0.54 per reproductive cycle, the lowest in any NVR population, due to only 40% of mites producing mated female offspring.
- The importance of mite detection and removal is unknown but expected to be high.

Case 2: Papua New Guinea

- After some initial losses beekeepers have not treated for over ten years.
- DWV was not detected in any colonies/bees.
- The varroa resistance mechanism is currently unknown.
- The Solomon Islands' honey bees are also free of DWV but detailed data on bees are lacking.

Hygienic Behaviour

Role in disease control

The term 'Hygienic Behaviour' was coined in the 1950s by Walter Rothenbuhler during research into American foulbrood (AFB).

Hygienic behaviour involves the detection and removal of diseased and infected brood from their cells by workers.

Honey bees are unique among social insects in that brood cells are re-used rather than rebuilt. Therefore, hygienic behaviour of sealed brood is an important aspect of a honey bee's lifestyle.

Hygienic behaviour is the primary mechanism of resistance towards AFB, chalkbrood, wax moth and now, varroa. Therefore, hygienic behaviour is an important trait providing multiple benefits to maintaining colony health. In managed colonies their ability to detect and remove sealed brood killed by freezing or picking with a pin improves over time, and it can be improved by selecting for this behaviour (Figure 7). The lower, typically unselected, proportion may be due to treatment strategies e.g., varroa control, AFB destruction policy or re-queening to improve desirable traits. These are all actions that may lessen a colony's natural resistance, but modern beekeeping is almost impossible without recourse to many of these actions.

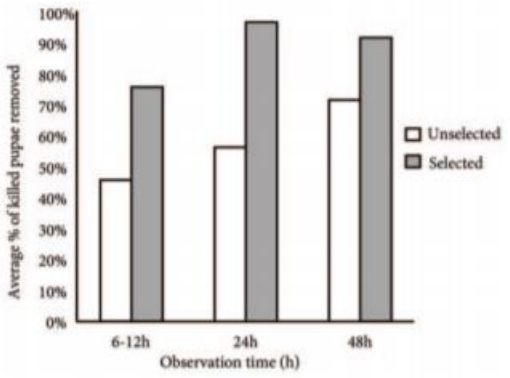


Figure 7. Average levels of killed brood removal over time of 334 unselected (clear bars) and 212 selected (grey bars) managed colonies from the analysis of 21 studies over the past thirty years.

The mechanics of hygienic behaviour

Hygienic behaviour towards sealed brood, irrespective of the malady, is the same basic response (Figure 8). This is:

- Detect -> uncap -> remove or recap.

The only critical difference is that the signal or cue of each malady needs to be learnt before becoming hardwired into the bees' genetics, especially for any new pest, like varroa.

The signal or cue could be:

- A chemical odour.
- A physical property i.e. temperature change, movement, or lack of, within the cell.
- Or both.

Currently there is a major research effort to discover the signal or cue used by bees to detect varroa-infested cells.

- A signal actively conveys information, e.g., when a pupa informs a bee it is injured.
- A cue is passive; it provides the observer with information, e.g., learning the odour of a mite, and it looks like this is occurring.

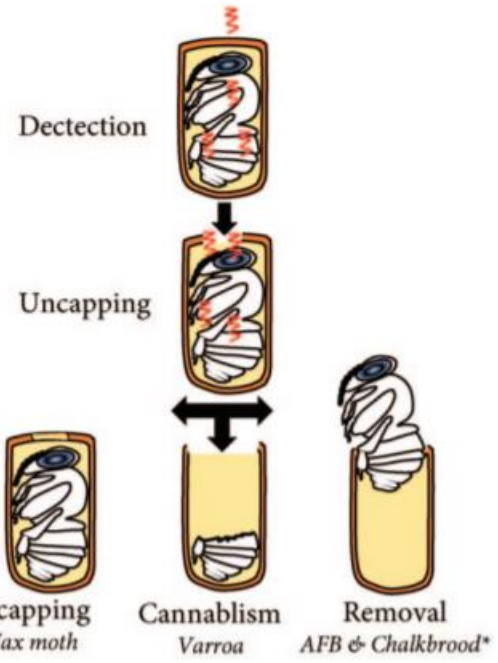


Figure 8. Hygienic behaviour towards sealed brood, where a cue is detected. This leads to the cell being partially uncapped. The hole is then either: recapped by a different group of bees; or enlarged allowing the bee to be removed, as in AFB or advanced stages of chalkbrood; or cannibalised as in varroa or early stages of chalkbrood.

Response of adult bees to signals or cues

Largely based on the work of Marla Spivak's group, we know hygienic behaviour is a complex process. The ability of bees to detect signals or cues vary. For example:

- Low-stimulus threshold = high sensitivity.
- High-stimulus threshold = low sensitivity.

High sensitivity bees detected and removed chalkbrood-infected pupae in the early stages of infection, while colonies with a low sensitivity only detected strong stimuli i.e. only when chalkbrood mummies had formed.

These thresholds are dynamic and change with:

- The age of the honey bee.
- Environmental conditions, e.g. nectar flows.
- Tasks being performed by the honey bee.

When responding to dead or live pupae the average age of the bees performing each task were as follows:

- Bees detecting and uncapping dead pupae were between 15 to 17.5 days old.
- Bees removing dead pupae were 17.5 days old.
- Bees detecting and uncapping live, mite-infested pupae were 11 days old.
- Nectar flows can increase hygienic behaviour in older bees.

This system is further complicated by the various tasks being conducted by different bees and no bee has been seen doing more than two tasks:

- Some bees detect and start uncapping.
- Different bees uncap and remove.
- Another group recap the cells.

It was found that those bees that detect, have a higher sensitivity compared to those that remove. Sensitivity to an odour can be artificially increased by injecting the neurotransmitter octopamine into the bee's brain. Furthermore, Yves Le Conte's team found the antennae of bees that could detect mite-infested brood, expressed more genes related to detecting chemicals and were more activated than in bees unable to detect mite-infested cells. Recappers may have a lower sensitivity, hence do not detect the problem. This would explain why cells can be repeatedly uncapped and recapped during the pupal development.

Detectors, removers and recappers

For a colony to be highly hygienic it must contain the correct proportion of detectors, removers and recappers. The detectors need to be sensitive to the correct stimulus and start the uncapping process. Recappers are required to correct any mistakes made by detectors, to avoid the costly removal of healthy pupae. We can see the consequences when imbalances between the various groups appear.

Bald brood

This situation arises when areas of sealed brood are uncapped, and the cells typically contain healthy-looking pupae with white or purple eyes. Such cells normally disappear due to being recapped. Bald brood can also be a result of wax moth larvae tunnelling just below the capping.

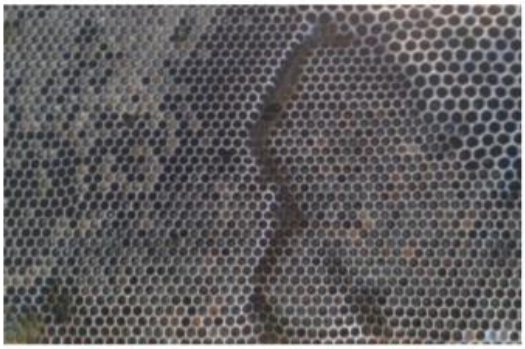


Figure 9. The evidence that a wax moth larva has travelled through the comb. Typically, it leaves a straight line behind in its wake. Photo by Stephen Martin.

The bees respond by removing the entire cap, which is later recapped, since the pupae are unharmed. When wax moths are present, uncapping and recapping occurs in straight lines (Figure 9) and the moth's faecal pellets are in the brood cells. Incidences of bald brood have increased since the arrival of varroa, although it lacks any regular pattern (Figure 10) which may be due to imbalances in the numbers of bee's detecting and recaping in the colony, but further research is needed to evaluate this.

Figure 10. The typical bald brood pattern seen in varroa-infested colonies, showing some apparently healthy white-eyed pupae and a couple of partly-cannibalised pupae. Photo by E.Villalobos.

Is hygienic behaviour learnt or genetic?

Rothenbuhler determined hygienic behaviour towards AFB is now genetic rather than a learnt behaviour. Likewise, chalkbrood daughters of hygienic stock retained their hygienic behaviour even when mated with unselected drones. This appears to be the case with natural varroa-resistant (NVR) bees, but needs to be scientifically tested.

Measuring hygienic behaviour

- Pin-killed pupae is less labour-intensive but leaves haemolymph from the pierced brood on the caps.
- The technique of freeze-killing pupae is used widely among scientists as it is considered a more conservative test. The average values are similar if pins or freezing is used.
- Inserting varroa into cells (see page 14) is time-consuming, needs practise and some equipment.

Tests using artificially killed brood should never be used in isolation to determine if a colony is resistant to any disease, but only as a first selection step to be followed up with challenging the colony with the actual disease, e.g. inserting mites into cells.

"For a colony to be highly hygienic it must contain the correct proportion of detectors, removers and recappers."

Natural Varroa-Resistant (NVR) Populations

Although you may think natural varroa-resistant (NVR) populations are uncommon, in fact they occur throughout many areas of the world (Figure 11). The traits found in *Apis cerana*, the varroa mite's natural host, are their higher efficiency of detection and removal of mite-infested worker cells, so that mites only reproduce in drone brood. These traits are also found in NVR populations, although as yet, not as well developed.

Figure 11. Regions of the world (in yellow) where A. mellifera NVR populations are known to exist. The Apis cerana distribution is shown in green and blue indicates islands where DWV is absent.
See Box 1 on page 6.

Africanised bees (AHB)

This was the first population to become mite-resistant. AHB are a man-made hybrid between African *A. m. scutellata*, and European races. In 1957 they escaped and spread throughout all sub-tropical and tropical regions of Latin America. When varroa arrived in the 1970s, it was the Africanised bees' natural resistance towards varroa that helped them in replacing the remaining European honey bees.

African bees

In South Africa it took 3–5 years for Cape honey bees, *A. m. capensis*, and 6–7 years for Savanna honey bees, *A. m. scutellata*, to develop varroa-resistance. This pattern of short-lived colony losses prior to the appearance of mite-resistance is typical in almost all NVR populations.

Fernando de Noronha Island population

For details on this unique population see Box 1, page 6.

Two French populations

Near Le Mans and Avignon are two independent NVR populations, discovered in the early 1990s, and not treated since. Both have been studied intensively and originally consisted of a small number of colonies that grew to over thirty colonies in both locations.

Gotland Island, Sweden population

This was created by placing 150 mite-infested colonies, representing several strains, on Gotland in 1999. Colonies were then left, unmanaged and free to swarm. After five years, seven colonies remained. Thereafter, numbers increased to 20–30 by 2006. The colonies became smaller and swarmed frequently.

Arnot forest, USA populations

This is a small population of around ten wild colonies studied by Tom Seeley. This well studied population has been through a genetic bottleneck after the arrival of varroa. Now, the number of colonies are similar to that before varroa arrived. Unlike all the other studied populations, this one is unique in not knowing the actual resistance mechanism, although small colony size and frequent swarming are believed to play important roles.

UK populations

A small but increasing number of beekeepers are successfully keeping treatment-free bees in the UK. The majority remain quiet, enjoying their beekeeping and they are found across the England, Wales and, no doubt, Scotland (Figure 12). Two of the more prominent groups who have shared their different experiences are:

- The Swindon Honey Bee Conservation Group, which is 25 years treatment-free. http://www.swindonhoneybeeconservation.org.uk
- Shan and Clive Hudson who are eleven years treatment-free. http://Beemonitor.org; *BBKA News* 227: 229–232; 2020.

The UK's largest treatment-free area consists of almost 500 colonies kept by around 100 beekeepers in Gwynedd, North Wales. Typically, in this area, beekeepers do not move their colonies, but either catch swarms from wild colonies or buy locally and monitor their mite populations, at least in the early stages.

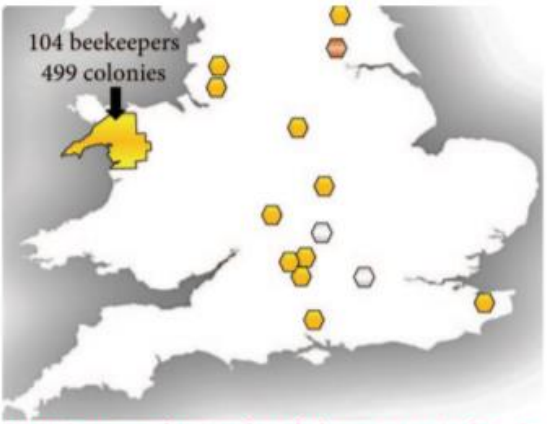
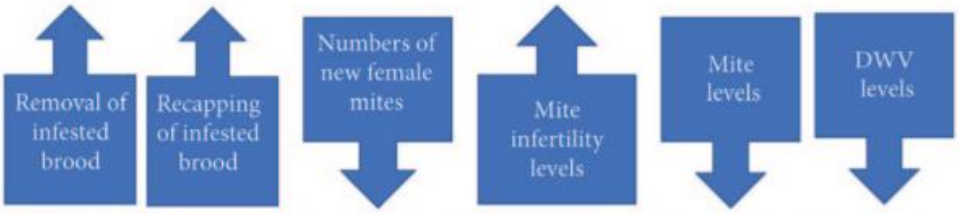


Fig. 12. Approximate locations of some beekeepers not treating for 3–10+ years. Key: standards of NVR indicated are: Gold = Over 10 years treatment free; Silver = 5-10 years treatment free; Bronze = 3-5 years treatment free.

Mechanism Behind NVR Populations



The different directions of a group of traits or outcomes that underlie the natural varroa resistance (NVR) mechanism in honey bees.

Basic NVR mechanism

- Full understanding of the NVR mechanism is far from complete, but sufficient information exists to help beekeepers to start selecting or testing what they already have.
- Across all the studies (see page 9) the bees have appeared to solve the mite problem by using the same mechanism.
- The one exception is the small Arnot forest population.
- Typically, NVR populations all have common characteristics, which are:
  - Poor mite reproduction.
  - Increased mite detection and removal.
  - Increased recapping rates.

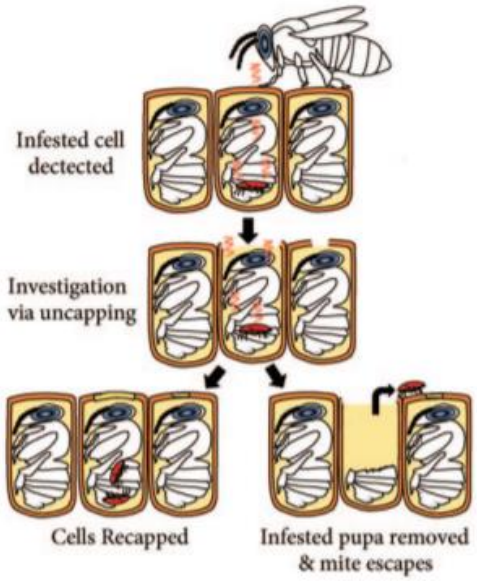


Figure 13. The three key hygienic behavioural stages involved in natural varroa resistance (NVR).

- The process illustrated in Figure 13 is typical for any sealed brood hygienic behaviour and follows the same order of events:
  - Detection.
  - Uncapping.
  - Removal or recapping.

Detection

- All worker honey bees can detect some mite-infested cells to some extent. The cue may be chemical, but it remains the focus of much investigation.

Uncapping

- Once detected the same bee makes a small hole in the cell cap.
- This allows direct access to the pupa and a better ability to detect the original odour.
- If the cell is mite-infested the hole is typically enlarged. If an error has been made, the hole in the cell cap remains small.

Removal

We think at some point during the uncapping, a second cue is required to cause the hole to be enlarged by the original or a new group of bees.

- Once the cap is fully opened, the removal of the infested pupa, typically via cannibalism, can proceed.

Recapping

- If an error is made, the cell is recapped using wax by a different group of bees.
- Recapping avoids the costly removal of healthy pupae.
- Cells are often opened and recapped several times during the development of the pupae.

The entire process is highly variable both between colonies and potentially over time in a single colony. Therefore, data are always collected from many colonies and average values used.

Consistent features in NVR populations

The two main behaviour traits that are consistently elevated in NVR populations are:

- Increased removal of mite-infested brood.
- Increased levels of recapped brood.

BOX 2. Common Acronyms

- MN-HB = Minnesota Hygienic Bees

Since 1994 Marla Spivak's team have selected bees that quickly removed freeze-killed brood. After three to four years, they also removed 60% of mite-infested cells, so in a commercial setting less mite control in needed.

- SMR= Suppression of mite reproduction

USDA Baton Rouge laboratory started breeding varroa-resistant lines, based on lowest mite reproductive success. SMR was changed to VSH to reflect the possible mechanism.

- VSH = Varroa sensitive hygiene

Originally referred to the selected line of bees at USDA, but now widely used for the trait of detection and removal of mite-infested brood.

- NVR = Natural varroa resistance

A new acronym that distinguishes naturally evolved from artificially VSH bred lines.

Increased removal of mite-infested worker brood

Background

- Detection and removal of mite-infested brood is the key mechanism behind NVR.
- **However, the mother mite is rarely killed or damaged during this process.**
- In *A. mellifera* NVR populations the situation is similar to that seen in *A. cerana*. This behaviour is very young in evolutionary terms, so in *A. mellifera* it remains highly variable.

Mite removal rates

- In 1994, just after the mites arrived into the UK, only 1% of infested brood was removed.
- Studies on varroa-naïve colonies indicated that a range of between 0% and 30% of artificially-infested worker cells with mites can be removed.
- Data from many studies indicate, in most susceptible populations, 30% or less of mite-infested pupae are removed, whereas in NVR colonies it is generally above 30% (Figure14).

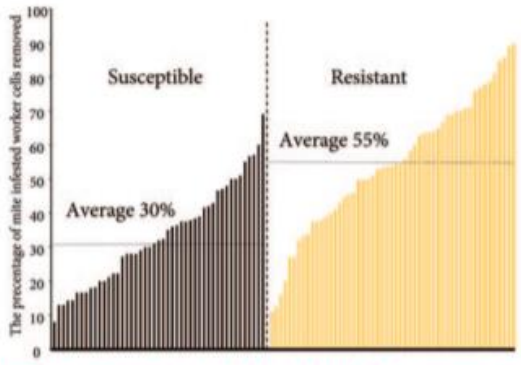


Figure 14. Percentage of artificially mite-infested brood cells removed in different colonies of Apis mellifera, indicating that, generally, more infested cells are removed by resistant populations relative to susceptible populations.

- The best population from South Africa has an average of 60% removal, but ranges between 11% to 89% depending on the colony. All colonies remain healthy one year later.

How does the removal of infested pupae lead to reduced mite reproduction?

- Infested pupa removal interrupts the mite's reproductive cycle, causing the loss of eggs/offspring of which the mite has a limited supply. Each female mite can produce only 20–25 eggs in her lifetime.
- Persistent interruptions mean mites quickly run out of eggs, becoming infertile.
- Therefore, you would expect to see an increase in the proportion of infertile mites in NVR populations. However, this trait is difficult to measure accurately, since there are many potential causes of infertility.

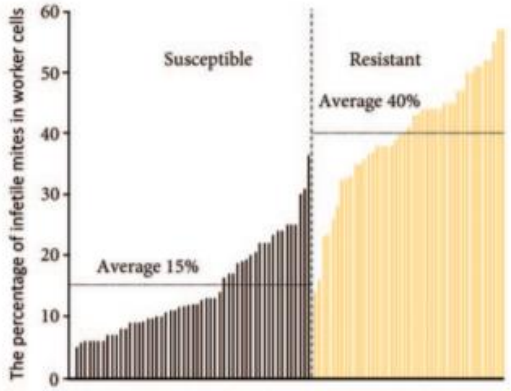


Figure 15. The percentage of infertile mites in susceptible and NVR populations in different colonies of Apis mellifera, indicating an average higher mite infertility in resistant (NVR) populations relative to susceptible populations.

- ***More importantly, the increased interruptions of the mites' reproductive cycles reduces the average number of viable female offspring produced per reproductive cycle.***
- The number of new viable female mite offspring produced per reproductive cycle derived from studies in the UK, USA, Norway, Brazil, Mexico and Africa are:
  - Susceptible populations = 0.9 to 1.4
  - NVR populations. = 0.6 to 0.8
- The reproductive value per cycle is ***not*** affected by the number of cycles, but the lifetime number of females produced ***is*** affected by the number of reproductive cycles.

Table 1. Comparison of the percentage of mite removal between a typical treated population and a NVR population*

% mite-infested cells removed		No. of times mite interrupted			No.of viable female offspring produced/cycle
		0	1	2	
0%		100	0	0	1.4
30%	Suspectable	49%	42%	9%	1.0
50%	Resistant	25%	50%	25%	0.7

**Each mother mite undergoes two reproductive cycles. In a typical treated population 30% of mites are removed, shown in Figure 14. By contrast, in the NVR population 50% of mites are removed (for simplicity). With no removal, a mite can produce 1.4 viable female offspring as shown in Figure 3.*

- This is demonstrated by consistently preventing 50% of the mite population from reproducing. It both reduces the average reproductive success of the mite population and increases the number of infertile mites in the population.
- The effect of infested pupal removal on the reproductive rate is linear. That is, the more mite-infested pupae that are removed, the greater the reduction in mite population.

Warning

If an NVR colony is moved outside its population, it typically dies with high mite levels. Modelling work and personal experience indicate that this occurs if there is a large influx of mites from a nearby collapsing colony. Then, the efficient removal of infested brood rates means that when infestation rates exceed 40-60% the colony dies, due the persistent removal of large amounts of infested brood.

Increased recapping in NVR populations

What is recapping?

- Recapping is where a circular hole is cut by a bee into the wax cap enclosing the pupae. It can vary in diameter from below 1mm to the removal of the entire cap.
- During this process the silk cocoon spun by the mature honey bee larvae is removed. Thus, when the hole is recapped with wax, it is possible to detect it by looking at the underside of the cap, where the matt texture of the recapped area contrasts against the shiny/reflective nature of the silk cocoon (Figure 16).
- If the entire cap has been replaced, then there is complete absence of the silk cocoon.
- It is currently impossible to detect recapped cells without opening up the cell and looking on the underside.

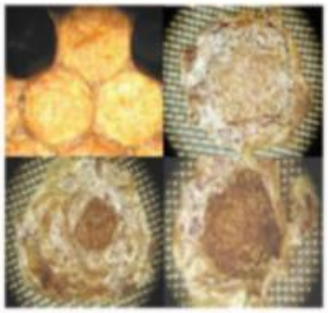


Figure 16. The upper side of a recapped worker cell (upper left) compared to the underside of an uncapped (upper right) with two recapped cells of different sizes (lower panels). In good light the contrast between the silk cocoon and matt wax that refills the central hole becomes clear. Photo by N. Reece

History of recapping

- Uncapping and subsequent recapping of worker cells was first noted in the 1940s during studies into AFB resistance.
- More recently, studies into the removal of artificially killed pupae (hygienic behaviour) found some cells had been recapped. This indicated that recapped cells can result from errors made by some bees in detecting a problem.
- Under natural conditions recapping prevents the accidental removal of valuable healthy brood, due to the error-prone nature of hygienic behaviour.
- In 1998, researchers in Brazil found that Africanised bees selectively uncapped cells containing varroa, but its real significance was missed.
- In 2018 a young Swedish researcher, Melisa Oddie, and colleagues realised that recapping levels were consistently elevated in all four European NVR populations (see page 9), relative to nearby treated colonies.
- Since then, elevated recapping levels have been found in NVR populations in the UK and Brazil, and in Africa, *A. m. scutellata* and *A. m. capensis*.
- Recapping in varroa-free colonies occurs, but at very low levels (Figure 17).

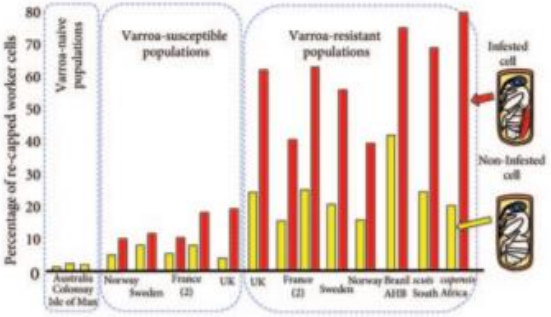


Figure 17. Percentage of uninfested (yellow) and mite-infested (red) cells recapped in naïve, susceptible and NVR populations around the world.

Why is recapping important?

- Higher recapping levels of mite-infested cells relative to uninfested cells indicate that all bees have the ability to detected mite-infested cells.
- This ability is greatly elevated in NVR populations.
- Increased recapping does not cause lower mite reproductive values, since the same low mite reproductive rates are found in recapped and untouched infested cells.
- Recapping reflects errors in the process of mite-detection that leads to the removal of the infested pupa.
- Elevated recapping levels in a population is currently the best indication of an NVR population.
- Targeted recapping of drone brood does not occur. The reason(s) why is unknown, but it mirrors the situation in *A. cerana* where mites are prevented from reproducing in worker brood only.

Pattern of recapping

- The more mite-infested cells recapped, the greater the number of uninfested cells recapped (Figure 17).
- Recapping occurs in clusters around infested cells (Figure 18). The three possible explanations are:
  - The chemical cue to detect infested cells is diffused.
  - The stimulus to detect infested cells is diffused.
  - On finding an infested cell, bees check surrounding cells.

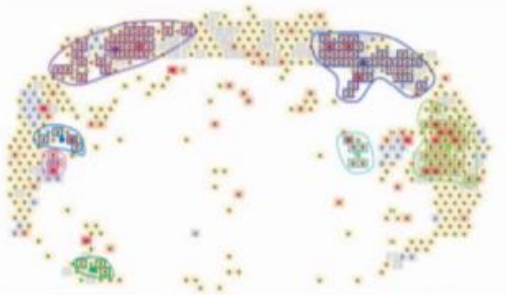


Figure 18. Shows how recapping (pink) occurs in cluster typically around infested cells (red). Different coloured lines indicate separate clusters.

Practical Guide to Measuring Recapping Rates, Mite Removal Rates and Mite Reproduction

The methods are presented in order of importance, from the easiest to the most difficult to do.

Measuring recapping rates

Equipment

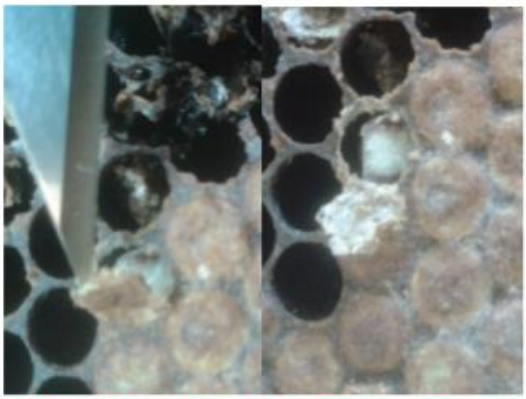
- Newspaper or covering for the work surface.
- A magnifying lamp, good illumination is essential.
- Fine forceps or tweezers.
- A needle, razor blade or scalpel.
- A small clear dish; a petri dish is best.

Various tools for uncapping a brood cell. All photos by Stephen Martin.

The procedure

- Collect a frame or section of sealed worker brood containing pupae with dark eyes; easily checked by uncapping a few cells in the field with your hive tool. Avoid using pupae with white eyes or younger. This could lead to an ambiguous result.
- If a brood section is used it must contain at least 100 intact sealed cells.
- Place the frame/section on your worksurface. Place it on a small object, so the frame does not lie horizontally, as this will prevent deforming the cell caps on the underside of the frame/section.
- Place the light directly over the frame so both of your hands are free to work underneath the magnifying light and you are in a position that you can easily see the enlarged sealed brood.
- Using the needle/scalpel or tip of the fine forceps, carefully cut around five edges of the cap and carefully flip it over to reveal its underside.

Magnifying lens with inbuilt light.



Scalpel used to cut round cell (left) and cell cap inverted to see underside.

- Now determine if it has been recapped or not. You will want to gently alter the position of the cap to see the contrast between the silky cocoon and matt recapped area.
- All this needs to be done under a magnifying lens so you can see all the details. If a binocular microscope is available this can really help.
- Remember, the size of the recapped area varies. The smaller it is the harder it is to determine if the cell has been recapped or not.
- To increase the accuracy of your data, determine if the cells is infested with a mite of not. Note if a mite is seen during the time you were uncapping the cell. If not, the pupa can be removed carefully using the forceps and placed on the petri dish. Check the pupa and cell for any signs of the mites, e.g., offspring, or white faecal dots on the cell wall.
- Remove any adult mites before starting on the next cell to avoid double counting.
- Repeat until around 100 cells have been studied.
- Record your data for your colony. Repeat on more colonies to improve accuracy; between five to ten colonies should give reliable results.

Example of a data table

Beekeeper details, years since last treated	Colony i.d. & date	Colony i.d. & date
# infested cells recapped		
# uninfested cells recapped		
# infested cells untouched		
# uninfested cells untouched		

E-mail to s.j.martin@salford.ac.uk, or post to Prof S. Martin, SEE, University of Salford, Manchester, M5 4WT.

Measuring mite removal rates

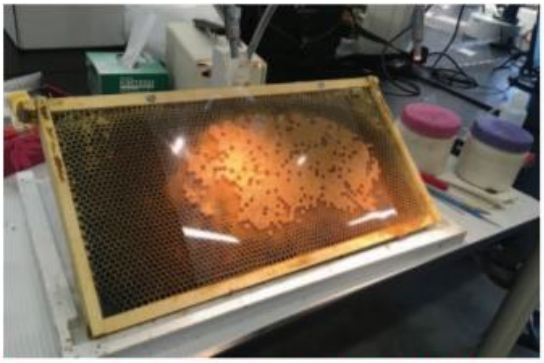
First master opening cells to investigate recapping. Then, the more adventurous may want to try this, but it requires a lot of work and planning.

Equipment

- All the basics for measuring recapping.
- A fine paint brush 0 or 00 size.
- Acetate sheets, poster pin and a permanent fine black marker pen.
- Source of live mites. 'Fresh' dead mites may also work.

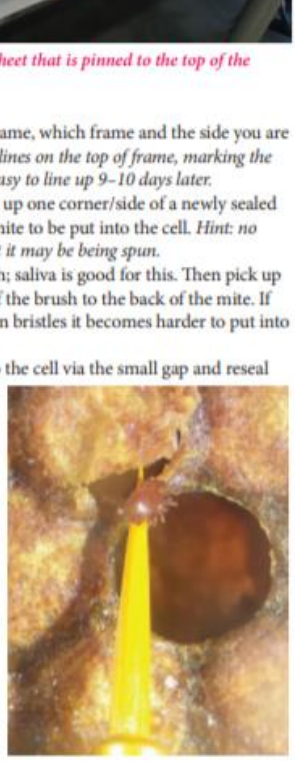
The procedure

- Remove an entire frame of worker brood whose cells have just (within hours) been capped over.
- Place under the magnifying light as described previously.
- Place the mites in a petri-dish or container with a couple of pupae, giving the mites something to sit on.
- Attach the acetate sheet to the frame using two pins and write the date and colony number in permanent ink on top of the sheet.



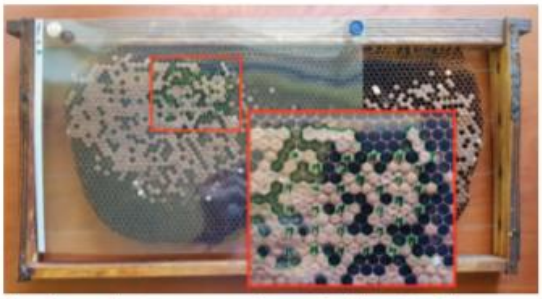
Sealed brood covered by acetate sheet that is pinned to the top of the frame. Photo by H. Urbina.

- Mark clearly on top of the frame, which frame and the side you are working on. *Hint: draw two lines on the top of frame, marking the position of the sheet, so it is easy to line up 9–10 days later.*
- Using the needle/scalpel, lift up one corner/side of a newly sealed cell, just enough to allow a mite to be put into the cell. *Hint: no cocoon should be present, but it may be being spun.*
- Wet the end of the fine brush; saliva is good for this. Then pick up a mite by attaching the tip of the brush to the back of the mite. If you pick up the mite between bristles it becomes harder to put into the cell.
- Carefully place the mite into the cell via the small gap and reseal using the brush. *Hint: all this requires practise.*
- Place the acetate over the frame and mark the cell's position with the permanent marker.
- Repeat until 25–30 cells contain mites.
- You can also do the same number of controls, where the corner is lifted up and resealed, but no mite inserted. Also mark the position on the acetate sheet.
- When completed, remove the acetate sheet and replace the frame into the colony.
- The next day, in the field remove the control frame, brush off the bees, then replace the acetate sheet and mark all the cells removed. This indicates human error when opening and resealing the cells.
- Nine or ten days after the mites were inserted, remove the test frame and, using the acetate sheet, record all empty cells. This can be done in the field, but if the frame is placed on the worktop, any remaining manipulated cells can be opened. So recapping and the presence of the any mites can also be recorded.



Right: Mite is being placed into recently capped cell via the small hole. Photo S. Martin.

A sheet on acetate is placed over the frame and the cell's position marked. Photo by G. Hawkins



Brood removal rates are measured by counting the empty cells.

Example of a data table

Beekeeper details, years since last treated mites initial inserted	Colony i.d. & date	Colony i.d. & date
# pupae removed next day		
# removed after 9–10 days		
# remaining infested cells recapped		
# control cells		
# controls cells removed next day		
# controls removed after 9–10 days		
# remaining control cells recapped		

E-mail to s.j.martin@salford.ac.uk, or post to Prof S. Martin, SEE, University of Salford, Manchester, M5 4WT.

Left: Developmental stages of female (upper) and male (low) mites. Right: Various stages in the base of a cell. Both photos Stephen Martin.

Measuring Mite Reproduction

- This requires much practise, time, patience, access to a good quality binocular (x 10) microscope and to a cold light source that allows you to see clearly into the bottom of the cells.
- The amount of expertise in determining the age of the pupae, the various mite stages and available time determines the level of the data one can collect. This is, broadly, categorised as:
  - Basic: have mites produced any offspring?
  - Intermediate: is the adult male present along with mature female offspring using presence of moulted mite skins?
  - Advanced: reconstruct entire mite families, determine mortality, reproductive potential etc.

For those potentially interested you must read the relevant sections in this free to download instruction guide to standard procedures involving varroa studies, since its far beyond the scope of this booklet. https://www.tandfonline.com/doi/pdf/10.3896/IBRA.1.52.1.09?needAccess=true

Propagation

- Use queens from proven local NVR populations.
- Better to use splits or swarms from local NVR populations.
- Collect wild swarms and test recapping rates.
- There is a 'halo' effect, with beekeepers benefiting from nearby NVR populations.
- Test and promote your colonies with high recapping rates.
- Try not to move colonies outside of an NVR area.
- Continue to treat colonies with very high mite levels, especially if recapping rates are low.
- Change slowly and methodically.
- Form and work as small local groups of like-minded beekeepers.
- Run workshops to share knowledge and equipment.
- Enjoy and remember to share your results with the wider community of beekeepers and scientists.

Acknowledgements

Our thanks go especially to all the beekeepers who helped over the decades with different aspects of this research, and to the bee research community who have added so much to everyone's understanding of bee health. The information in this *Special Issue* has only been made possible by the hard work of my PhD, MSc and UG Salford research students, all of whom have been, in part, funded by British beekeepers, either through BDI or the BBKA. Many continue to conduct research into various aspects of bee health all over the world, which will continue to benefit beekeepers over the next decades. Special thanks to friends who have helped with some images, especially Hector Morales and Ethel Villalobos.

Further reading, free to download:

Oddie M, Büchler R, Dahle B *et al.* Rapid parallel evolution overcomes global honey bee parasite. *Sci Rep* 2018; 8: 7704. https://doi.org/10.1038/s41598-018-26001-7

Martin SJ, Hawkins GP, Brettell LE *et al. Varroa destructor* reproduction and cell re-capping in mite-resistant *Apis mellifera* populations. *Apidologie* 2019; 51(3):369-381. https://doi.org/10.1007/s13592-019-00721-9

The Salford Team

From top left to bottom right: George Hawkins MSc, Georgi Webb UG, Izzy Grindrod PhD, Stephen Martin, Natasha Reece MSc, [Dr. Flaviane de Souza, Dr. Jess Kevill &Dr. Laura Brettell]

Natural *Varroa* resistant bees in the UK. *Bee craft*

I wrote this article and created the diagrams included, the article was edited by S. Martin.

Naturally varroa-resistant bees in the UK

Isobel Grindrod and Stephen Martin
School of Science, Engineering and Environment, University of Salford

Images courtesy Stephen Martin, Richard Rickitt

As all beekeepers are aware, *Varroa destructor* is a major threat to the majority of honey bee colonies. The key problem is that when varroa feeds on the fat body of either the adult or pupa bee it transmits deformed wing virus (DWV), an RNA virus (as is covid-19). The virus shortens the lifespan of adults that are infected as pupae by around two thirds; it also shortens the lifespan of those infected as adults but by a smaller amount. This shortening of the lifespan leads to a loss of workers which can cause the colony to die, typically during the long winter in the UK.

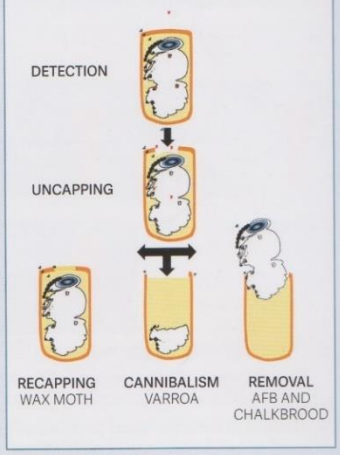
We now know that, in the absence of DWV, varroa-infested colonies can survive indefinitely without requiring treatment as the mites become unable to produce many new offspring, although the mechanism behind this is unknown. Varroa-infested colonies that are free of DWV are very rare; populations have been found on the remote Brazilian island of Fernando de Noronha, Papua New Guinea and the Solomon Islands. However, in all other populations the eradication of DWV is impossible and the solution to the varroa-DWV problem needs to be found elsewhere.

Hygienic behaviour

Fortuitously, the bees already have an existing behaviour called hygienic behaviour. This is a natural response to disease that is seen in many social insects (bees, ants, wasps) including our bees. Honey bees conduct hygienic behaviour in response to many diseases and pests such as chalkbrood, European foulbrood and wax moth infestation. Hygienic behaviour entails the detection and uncapping of infected cells and the removal of the contents.

The good news is that despite varroa being a novel parasite for *Apis mellifera*, the bees have already begun using hygienic behaviour to combat this threat. Hygienic behaviour or brood removal has several steps: mite detection, the creation of a hole in the cap and then resealing the cell (recapping) or removing the contents.

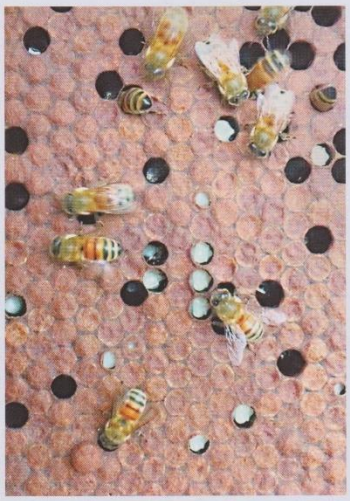
Diagram 1 Stages of hygienic behaviour showing the different outcomes depending on the disease. (Reproduced from the BBKA special issue on Natural Varroa Resistance).



Mite detection is thought to occur via an odour coming from within the cell. Creating a hole may allow this smell to be more easily detected or may allow access to a second cue (eg odour) which, if present, could drive the emptying of the cell. Interestingly, each of the steps is thought to be undertaken by a different group of bees. Each group may have differing sensitivities to odour, explaining why errors in uncapping and recapping may occur. For example, non-infested cells are often uncapped perhaps by overly sensitive bees and infested cells are falsely recapped by less sensitive bees. Additionally, each group of bees may differ in numbers within each colony, which may help explain the incidence of bald brood (see photo, right), which can occur when there are more 'uncapper' bees than 'recapper' bees. Bald brood is when many of the sealed brood have their entire cell caps removed. Typically, the uncapped sealed brood are at the white-eyed to purple-eyed developmental stage and, if left, disappear. We now know that the majority of these uncapped cells are eventually recapped and develop normally. Often beekeepers see bald brood as a bad thing and requeen their colonies, which we are now realising may not be the best thing to do.

Natural varroa resistance

This hygienic behaviour is at the very heart of the evolution of naturally varroa-resistant (NVR) populations. NVR was first detected in populations of Africanised bees as they spread throughout South America. It was their ability to develop varroa resistance that helped them to spread quickly and take over from the susceptible European honey bees. Later, NVR populations appeared in South Africa after a few years of colony losses during which time the more resilient populations adapted quickly to the mite.



Typical bald brood in a colony with the opened cells containing pupae with white eyes in this case. Some cells in the centre contain half-eaten pupae, probably infested by varroa.

In both Africa and Latin America, chemical varroa-control methods were not commonly used, often because of the cost and the remote location of beekeeping communities. Chemical control halts the development of resistance by removing the selective pressure (the mite) from the bee populations.

Don't suddenly stop treating

However, it is not advisable for beekeepers to suddenly stop using chemical control methods because this would cause the majority, if not all, of their colonies to die. This is because the bees need time alongside the mites to learn how to deal with them, and we know that even NVR colonies can collapse if a large number of mites overwhelm the colony.

For NVR to be successfully encouraged, colonies need first to be screened for their potential to be able to control the mite without chemical aid. Additionally, any change in regimen needs to be phased to prevent a sudden massive mite build-up. Hence, if you reduce the number of treatments applied you must increase the amount of monitoring. It is also imperative that you do not allow any of your colonies to collapse and therefore treatment needs to be applied if mite populations begin increasing again.

NVR traits

Despite these problems, there have been increasing reports of varroa-resistant populations across Europe and the UK. All NVR populations appear to have the same traits in common: enhanced mite detection, increased recapping behaviour, increased brood removal and reduced mite reproduction. These traits all connect together and lead to a reduced mite burden, reduced DWV load and enhanced colony survival. Importantly, as these traits are all shared between NVR (resistant) populations it indicates that bees have adopted the same solution for dealing with varroa irrespective of location, type of beekeeping, colour or subspecies of bee. This is great news since you can still keep your favourite type or colour of bee and end up with an NVR population.

The NVR mechanism relies on the bees being more able to detect mite-infested pupae; this is indicated by their increased ability to remove mites. A side effect of the removal behaviour is increased rates of recapping of both infested and non-infested cells. Measuring the recapping rates in a colony is the easiest way to get an indicator of how NVR your colony may be.

In Diagram 3, the recapping rates of infested cells are consistently higher

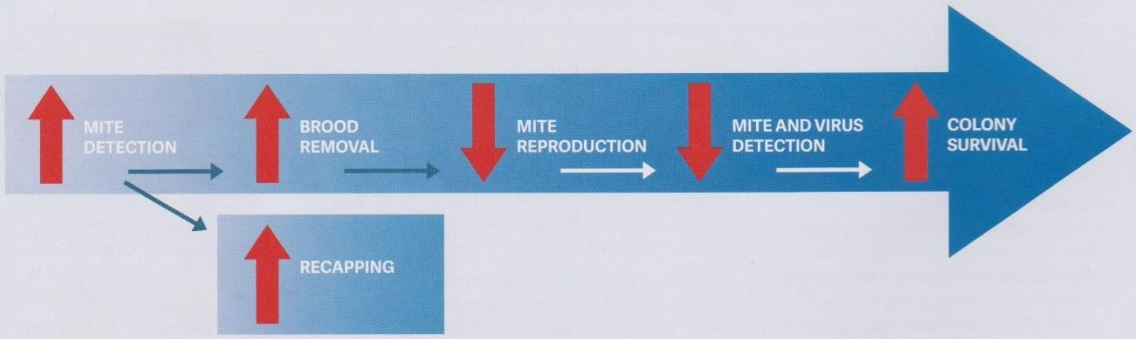


Diagram 2
How increased detection leads to increases (up arrows) or decreases (down arrows) for each measurable trait

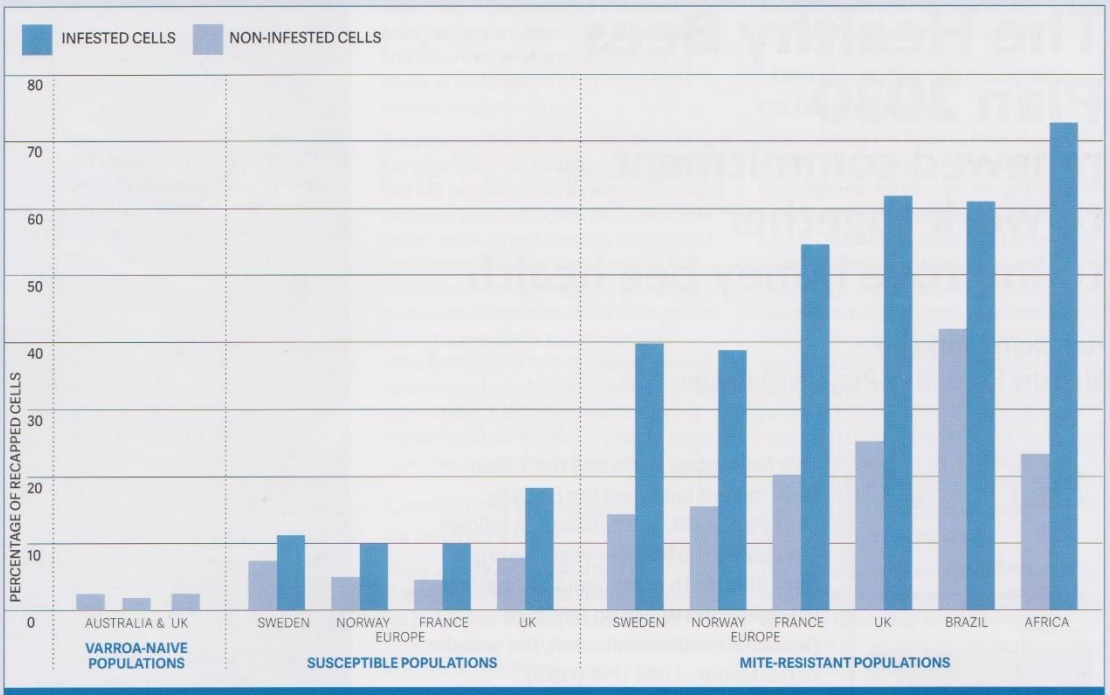
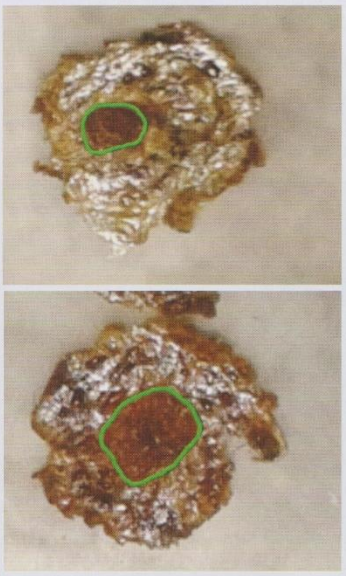


Diagram 3 A chart comparing the recapping rates across several countries and an indication that all colonies can detect mites since the black bars (infested cells) are higher than grey bars (non-infested cells)

than the recapping rate of non-infested cells, irrespective of the location or whether the bees are susceptible or have NVR characteristics. This indicates that all honey bee colonies have the ability to detect varroa; it's simply that NVR colonies are much better at doing it. Beekeepers should be looking for recapping rates of greater than 40% for infested cells, but colonies above 30% are well on the way to becoming NVR.

This increased brood removal leads to varroa resistance because brood removal inhibits the reproduction and thus population growth of the mites. This is because, when an infested cell is emptied, all of the mite offspring die. The mother mite does escape because of chemical camouflage, but her limited egg supply is reduced. The mother survives and tries again to reproduce in another cell but, when the rate of removal is high enough, this impediment to reproduction can drastically reduce overall mite reproductive success and subsequent population growth. Over time this can lead to a build-up of infertile mites as they have used up their eggs in failed reproductive attempts. Ultimately the reduced mite population and increased removal means that the DWV load of the colony is also reduced because there are fewer vectors (the mites) to spread the disease and fewer infected pupae (because they are removed).



The underside of two recapped cells shows the contrast between the silky cocoon and matt waxy region where the hole has been resealed. The green line indicates the boundary of the recapped area for clarity.

A combination of a reduced DWV and mite burden results in enhanced survival because there are fewer weakened colony members.

Encouraging NVR

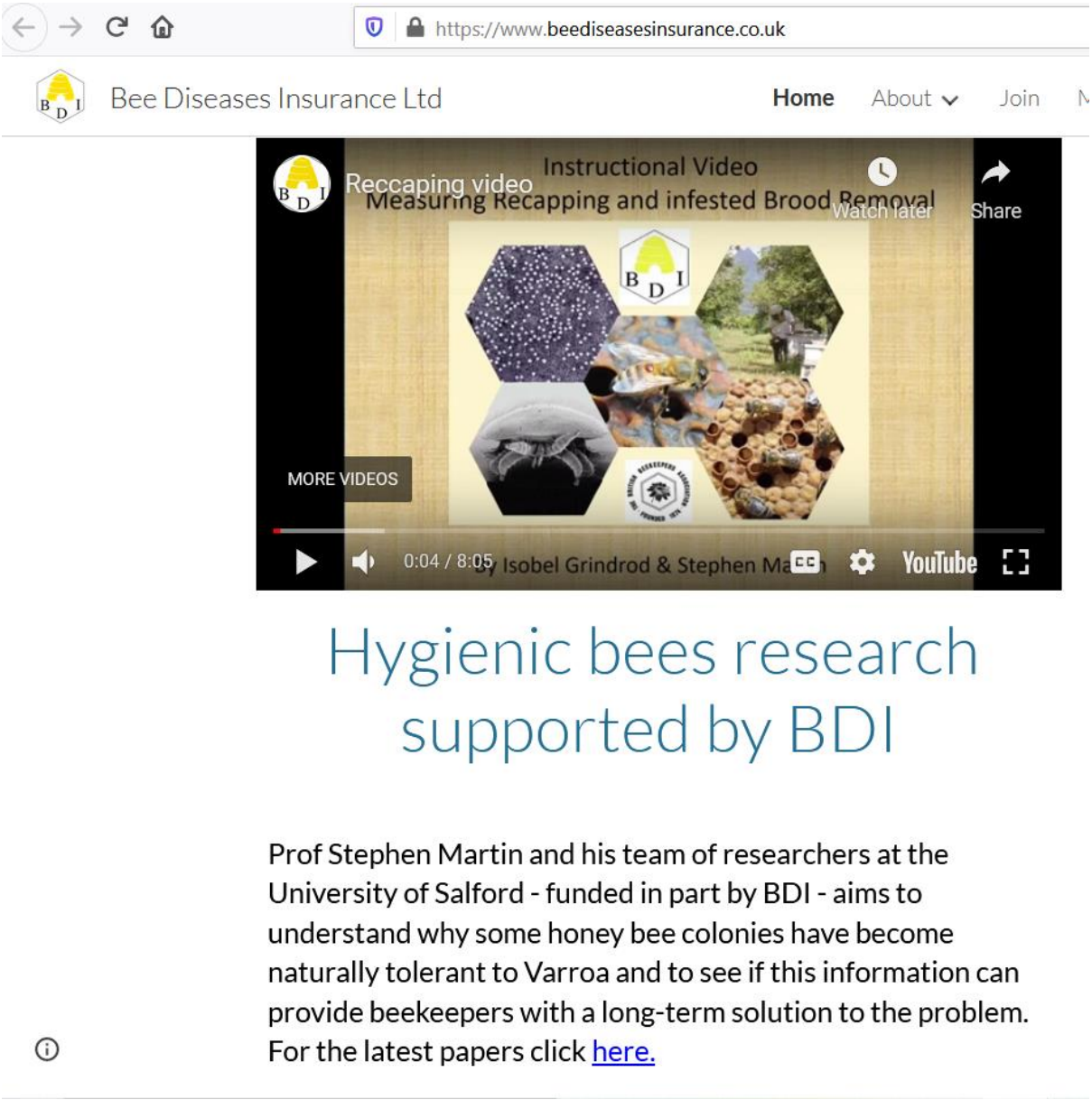
This may all sound too good to be true, but already in the UK there are numerous beekeepers who have been keeping their bees treatment-free, several for more than a decade. The largest area is in North Wales, where over 100 beekeepers have been maintaining around 500 colonies treatment-free for more than ten years. They started by collecting wild swarms from the surrounding woods where they had observed feral colonies surviving for long periods of time.

We have written a small BBKA Special Issue Series booklet entitled *Natural Varroa Resistant Honey Bees: Biology, Testing and Propagation* that starts to show how to look for NVR and the science behind it. It has been written for beekeepers in a way that makes the problem and how to test for and propagate NVR easy to understand. It costs £4 and is available from the BBKA shop.

So, despite all the current problems and gloomy outlook for our bees, the future has never been so good. We thank the beekeepers who helped with our research and the funding from the Bee Diseases Insurance and the BBKA without which this research would be impossible.

Instructional Video: Measuring recapping and infested brood removal

S. Martin was responsible for the general direction of this video, I participated by appearing within the video to demonstrate the procedures. I also edited the video.



Honey bees are becoming resistant to Varroa. The British Bee Journal published in conjunction with BBKA news

I independently wrote this article and created the diagrams included. Some feedback on the readability for the general beekeeper was given by Rhona Toft an associate and member of the beekeeping community.

B|B|J | The British Bee Journal
Published in conjunction with BBKA News
Volume 7 ♦ December 2021

In this issue:
Honey bees are becoming resistant to Varroa. 1

This research is part-funded by Bee Diseases Insurance Ltd.

Honey bees are becoming resistant to *Varroa*

By Isobel Grindrod, University of Salford, Manchester.

***Varroa* has been the scourge of Western honey bee (*Apis mellifera*) colonies since the mites first started spreading around the world, approximately seventy years ago.** They reached the UK in the early 1990s. The mites themselves are not too harmful, but as they feed on honey bee adults and pupae they spread harmful viruses. Arguably, the most important virus associated with *Varroa* is deformed wing virus (DWV) which, as its name suggests, can cause a very small number of bees that are infected as pupae to develop into adults with misshapen wings (Figure 1).

The most deleterious impact, however, is on the adult lifespan of the worker bee, which is reduced by up to two-thirds if the bee is infected with DWV as a pupa. The reduction in lifespan means that workers die faster and the colony struggles to replace them, which further weakens the colony. In temperate climates, such as the UK, this is particularly problematic over winter as the normally long-lived winter bees die out prematurely in late winter or early spring. Small colonies, at this critical time of year, may struggle to build up in the spring or even to survive. As a result, beekeepers often have no choice but to use various methods, including acaricides (miticides), to control the mite. Unfortunately, chemical controls cannot be relied on indefinitely as the *Varroa* mite is capable of developing resistance to them.

There is, however, good evidence now from a range of studies conducted in many countries, including the UK, that honey bees can develop a natural resistance to the mite. Resistance means that they can withstand a *Varroa* infestation without treatment for decades. Resistance first appeared among the Africanised bees of

Figure 1. DWV-afflicted adult bee with crippled wings and a shortened abdomen.

South America and within the Cape bee, *Apis mellifera capensis*, and savannah honey bee, *Apis mellifera scutellata*, subspecies in Africa. In both Africa and South America, acaricides are not frequently used due to the cost and availability, which is believed to be one key factor influencing why resistance has developed so rapidly there, but other factors, such as low colony densities, level of management and natural robustness against other pests or pathogens also play roles.

Acaricides are designed to reduce the number of mites in a colony and, as a consequence, they prevent the bees from adapting as they are no longer under the selective pressure of the mite. Despite the heavy use of acaricides within the northern hemisphere, resistant populations have arisen in multiple regions including the UK, France, Sweden, Norway and the Netherlands. Additionally, an increasing number of beekeepers in Europe, including the UK, and in the USA are stopping using treatments, and there have been increasing reports of resistant populations.

Key traits of *Varroa*-resistant populations

Intriguingly, resistant populations from all different regions, appear to have the same three key traits in common. These traits are: increased brood removal in which pupae infested with *Varroa* are removed from their cells; increased recapping where holes are created to access the pupa and then resealed, and finally decreased mite fertility since adult female mites are unable to produce as many female offspring as usual. For a long time, these traits have been researched independently, but in our recent paper (Grindrod & Martin, 2021), using data extracted from sixty previously published scientific studies, we suggested that these three key traits in fact link together to create resistance, Figure 2.

Our proposed sequence in Figure 2 begins with an increase in the ability of bees to detect mites within the worker brood cells. A female 'mother' mite enters a worker brood cell to reproduce just before it is sealed. Within the cell she feeds on the developing pupae and lays a succession of eggs. The first egg is a male whose only role is to mate with his following sister offspring once they are mature. The male dies when the adult bee emerges while all the new adult females, including the mother, leave attached to emerging adult bees. Recent studies have shown that bees have evolved the ability to detect the presence of mites in sealed cells through the cell capping.

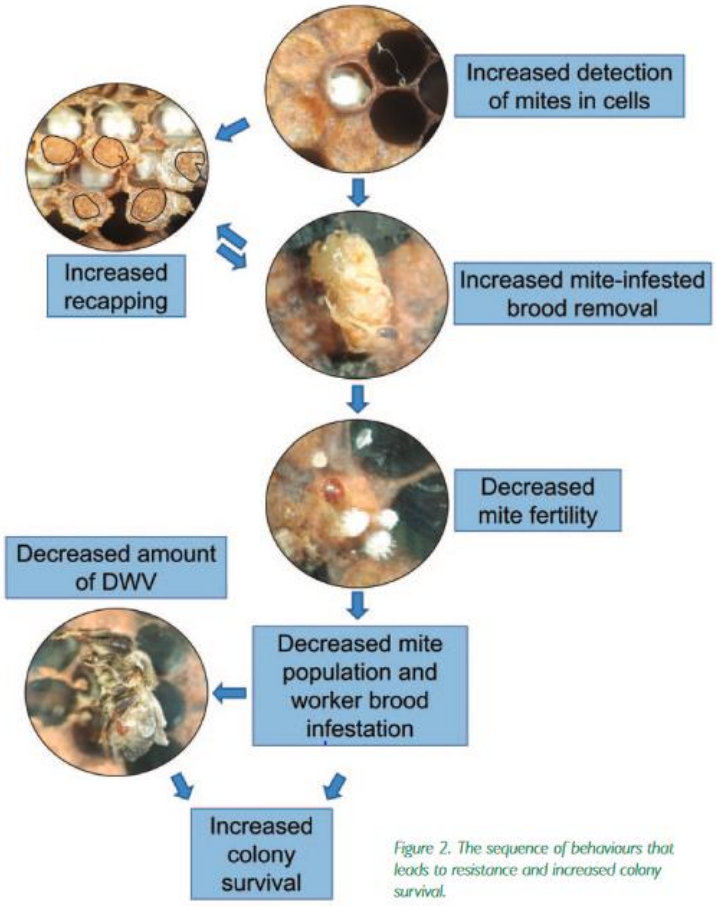


Figure 2. The sequence of behaviours that leads to resistance and increased colony survival.

Currently it is uncertain exactly what they are detecting, but numerous studies have indicated the possibility of a mixture of chemicals coming from the cell that the bees can smell. Indeed, other diseases such as chalkbrood and the foulbroods cause the death of the pupa which releases a strong scent, and these pupae can sometimes be removed via the bees' hygienic behaviour. In the case of *Varroa*, the host pupa rarely dies, thus it is likely that the scents involved are different to chalkbrood and foulbrood. However, the removal response is the same sequence of hygienic behaviours.

With the increased ability to detect *Varroa*-infested cells comes an increase in both the recapping of cells (Figure 3) and removal of infested pupae. While recapping is measured as a trait separate to removal, it is an alternative ending to the same hygienic behaviour sequence that initiates brood removal. This sequence begins with the detection of a suspicious cell, possibly due to an abnormal scent arising from inside. A hole is then created in the cap of this cell, which may allow the scent mixture to be more easily analysed by the bees. At this stage a decision, possibly based on the composition of the scent mixture, is made to either remove the pupa or to recap the cell. To complicate the situation, it appears that the uncapping stage and the decision to remove are conducted by different workers. Recapping is thought to be a cautionary response to prevent the loss of healthy brood in the event that the infestation status cannot be confirmed. It appears that bees often err on the side of caution when responding to suspicious cells. This is because brood removal can be very costly for a colony. For example, computer modelling showed that if a colony consistently removes over 40% of its brood, it will collapse the following year.

Additionally, the bees need to be cautious because many cells containing healthy pupae are suspected in error. This may be because the smells drift from infested cells

Figure 3. The underside of a recapped cell, showing the matte wax (circled) which has replaced the hole created by another worker bee.

making it difficult to pinpoint the exact source. Indeed, the recapping of both infested and healthy cells is increased in resistant colonies. This is probably because workers in resistant colonies have an increased ability to detect scents, even trace scents that have drifted. Importantly, while recapping of infested pupae is significantly greater in resistant colonies, the removal of infested cells is significantly greater too. Thus, it appears that they are more able to detect and correctly identify infested cells.

Increased brood removal (Figure 4) is an important stepping-stone towards resistance as it leads to a decrease in mite fertility. By removing an infested pupa, the offspring of the mother mite who invaded the cell, do not survive. The mother mite escapes, but she has already wasted some of her limited supply of eggs and sperm on a failed attempt at reproduction. Additionally, the disruption can cause her to have less reproductive success in the next cell if the mother invades it immediately, as her reproductive cycle is 'out of sync'. Consequently, the percentage of fertile mites is significantly lower in resistant colonies than in susceptible colonies. Computer modelling also showed us that the greater the removal of brood, the slower the mite population growth. Other studies have also confirmed this link between decreased mite fertility and reduced mite burden. A reduced mite burden is beneficial as it lessens the spread of DWV between adult bees and pupae. As a result, DWV amounts (the viral load the colony carries) are, on average, lower in resistant colonies than susceptible colonies. However, while reduced, DWV still persists even in resistant colonies as it can still spread via the surface of eggs, contaminated food, or the cannibalisation of infested brood.

Another effect that stems from brood removal is a reduction in the infestation of worker brood. We found that in the resistant Africanised honey bee colonies in South America, the worker brood infestation has fallen from around 20% to 4% over the past twenty years. We speculate that this means that mites may be beginning to wait for drone brood, which is not targeted by hygienic behaviour. Another study in Uruguay found that a greater proportion of mites were infesting drone brood than worker brood in resistant colonies compared to susceptible colonies. This avoidance of worker brood may be similar to that found in the resistant Asian honey bee, *Apis cerana*. In other resistant populations, average worker brood infestation is typically is 4–20% across many countries, including the UK.

Ultimately, reduced mite and virus burden will lead to enhanced colony survival. In areas where resistance has arisen, including

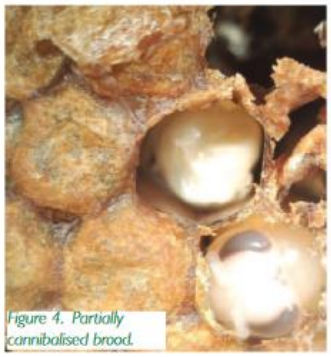


Figure 4. Partially cannibalised brood.

South Africa, Algeria, Tunisia and Morocco, it was preceded by a period of high colony losses which then stabilised with time. For example, in South Africa where widespread resistance has arisen, annual losses have stabilised at 5% between 1998 and 2004 which is similar to losses before *Varroa*. In the northern hemisphere this is harder to measure due to the use of acaricides. However, there are some promising data from beekeeper groups in the UK. In North Wales a group has kept 499 colonies treatment-free for eleven years and in Swindon a smaller group has been keeping treatment-free colonies since 1995. Additionally, in Le Mans and Avignon in France, the loss rates per year are greater in treated than in treatment-free colonies. These results are encouraging as natural resistance to *Varroa* is a sustainable and long-term solution to the mite problem. It also removes the reliance we currently have on acaricides and will not weaken honey bees in the face of other stressors. ■

More information

For information on how to monitor and encourage resistance traits in your honey bee colonies see the *BBKA News Special Issue Natural Varroa -Resistant Honey Bees*, and the associated video on the Bee Diseases Insurance Ltd (BDI) website https://www.beediseasesinsurance.co.uk/research/hygienic-bees-bdi-prof-stephen-martin

For more details you can download for free our scientific publication (Grindrod and Martin, 2021) by typing 'https://doi.org/10.1098/rspb.2021.1375' into your search engine.

Acknowledgements

Finally, I would like to thank all the beekeepers for their help and BDI Ltd. that is funding my PhD, and Prof. Stephen Martin for my supervision.

Varroa-resistance: A team update. BBKA news incorporating the British Bee Journal

This article was written jointly with a section by each author. All authors were also involved in the editing process.

Varroa-Resistance: A Team Update

By Georgiana Webb, Isobel Grindrod and Stephen Martin, University of Salford

Catch up on the latest developments at Salford with news from Georgiana Webb (Georgi) a new MPhil student part-funded by the BBKA; Isobel Grindrod (Izzy) and Stephen Martin. Varroas' days could be numbered.

Georgi's news

During my time at the University of Salford, I completed a degree in Wildlife Conservation with Zoo Biology, gaining a 1st class. My final year dissertation topic revealed a passion I never knew I had: 'bee-research'. I was previously unaware of the many problems that honey bees encounter. My dissertation was entitled 'Selection for Hygienic Behaviour in Honey bees (*Apis mellifera*): A Meta-Analysis', and I focused on honey bees' normal hygienic behaviour of removing dead brood. The conclusions from data I gathered from 21 scientific research papers was that selectively-bred hygienic colonies are superior to non-selected colonies when performing hygienic behaviour. This suggested that hygienic behaviour can be selected for and it was likely to be due to the earlier detection and removal of dead pupae by hygienic bees. I was hooked, and applied to do a two-year MPhil degree on honey bees that started February 2021.

This year, I have been very busy in the laboratory measuring the recapping rates of many colonies, both experimental and control, in our current 'queen-swap' experiment. Recapping is an important behaviour that appears to be linked to decreased mite reproduction and increased colony survival.[1] The queen-swap experiment is designed to understand if the recapping trait is genetic or a learnt behaviour; we hope to have an answer this winter. It is important as it will help beekeepers to understand if mite-resistant colonies need to be split or if propagation of locally-mated queens will be sufficient to ensure that recapping and thus mite-resistant traits can be passed on.

I am also saving mite offspring and pupae that are both infested and non-infested for future chemical analysis. This analysis aims to identify where the signal for bees to perform hygienic behaviour emanates from; it could be the mites, the pupae or even an entirely different source. It will also allow us to see which key compounds we can detect in the UK honey bee population. To date, several compounds have been identified by groups in the USA, Italy and France, with the French team having the most compelling data. Finally, I am working with a new graduate, Alex Vatentine, who, last winter, conducted a survey into the treatment habits of British beekeepers. Together we are writing a scientific publication as the data, Alex collected is very interesting. We hope to publish the results this winter if all goes well.

I have been fortunate enough, despite COVID, to have already had several beekeeping experiences, finding queens and even witnessing a swarm; we subsequently collected the bees off a nearby branch and carefully transferred them to a hive. I believe if you question everything, you can often discover topics that may need more consideration and therefore further research and I think this is important because we do not know all of the answers. Therefore, I hope to contribute to the vital research on mite-resistant honey bees and helping beekeepers reduce or eventually stop mite-treatments while completing my Master's degree.

Izzy and Georgi getting ready to collect their first swarm.

An important update from Izzy

I started my three-year PhD in October 2019 and am funded by Bee Diseases Insurance Ltd (BDI) with all my studies focused on trying to understand *Varroa*-resistant honey bees. I am pleased to say that after almost two years of hard work, Stephen and I have completed a major part of my research programme. This comprises three key parts of work that have resulted in:

- The *BBKA News Special Issue* on *Natural Varroa-Resistant Honey Bees: Biology, Testing and Propagation.*[2]
- An eight-minute instructional video showing beekeepers how to measure recapping and mite removal behaviours.[3]
- A major high-impact scientific publication bringing together data from over sixty previous studies conducted over the past

forty years to propose a simple framework that explains how *Varroa*-resistance arises in the *A. mellifera* population across many continents.[4] This paper is free to download and print.

This mix of scientific and outreach work is designed to help both beekeepers and scientists understand, measure and propagate *Varroa*-resistant honey bees in the UK. For the first time we have a simple framework that indicates how mite-resistance may have evolved in honey bees. Over time, knowledge gaps will be filled and theories will be tested. The framework should allow the beekeeper to see how the various traits, often long-associated with mite-resistant colonies, link together. Although, the basic mechanism is shown in the *BBKA News Special Issue*,[2] anyone interested in the full details should read the paper.[4] Originally the paper was concise and somewhat difficult to understand. However, after several rounds of reviewer comments it has become a much more detailed and involved piece of writing. I have had to develop a thick-skin dealing with some reviewer comments, but in the end, it was all worth it. My next task is to continue working with the queen-swap experiment for the rest of the year.

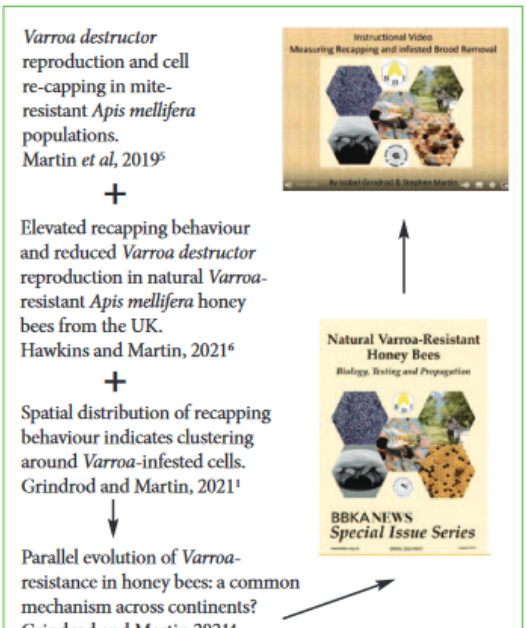
A brief overview from Stephen

I have been in *Varroa* research for many decades now and this is the first time I can see a path to the end of the *Varroa* problem for UK beekeepers. A small number of beekeepers have already had over a decade of mite-free treatment. Also, an increasing number are switching to reduced or even no treatments to control the mite populations and instead allowing their bees to adapt to the mite. This is possible in the UK since we do not typically move our colonies long-distances or keep large colony numbers.

I originally studied mite-resistance of Africanised honey bees in Mexico back in the 1990s. At that time, we understood that this trait was restricted to just Africanised bees. However, as time went by, other isolated populations started to appear in many countries. Despite these populations being studied, and lots of ideas were proposed, no clear evidence was forthcoming. The breakthrough came when a Scandinavian PhD student, Melissa Oddie found that 'recapping behaviour' was elevated consistently in five mite-resistant populations throughout Europe, relative to five nearby non-resistant populations. This was my 'light-bulb moment' since this was the first consistent behavioural data I had seen linked with resistance. I quickly was able to confirm Melissa's original findings during trips to Brazil and South Africa,[4] (see Martin *et al.*, 2019 for the full story[5]). My BDI/BBKA-funded postgraduate student, George Hawkins, then confirmed the link between increased recapping and resistance in the UK.[6] Despite some initial scepticism by bee scientists, Izzy got to work, first explaining the potential reasons behind the increase in 'recapping of non-infested cells'[1] and then progressed to her '*magnum opus*', bringing together forty years of past *Varroa*-resistance research to provide the first comprehensive mechanism of mite-resistance in honey bees.[4] 'The bottom line is that any type of honey bee population e.g. strain, colour etc, kept in any environment, by whatever method the beekeeper choses, is capable of developing *Varroa*-resistance if given the chance.

Before we start to advise the best way to achieve this, our aim is to have a sound and detailed understanding of the mechanism of mite-resistance, as this allows all the advice we give to be evidence-based. The work my team is doing is going a long way to achieving that goal. For the first time in decades, I am confident that we will see *Varroa* treatments eventually phased out in the UK. Feral populations have a major role to play in this because they are typically the first colonies to become resistant since the selective forces are greatest in these colonies.

The team at Salford will continue to focus on helping beekeepers in their fight against the mite, and to that end we will try to ensure



A diagram indicating how the key publications link together along with data from around the world to produce the evidenced-based *BBKA News Special Issue* on *Varroa*-resistance and then the instructional video. Unusually, we produced the *Special Issue* in parallel with the final research paper, and due to the importance of the topic for beekeepers we published the *Special Issue* before the paper since academic publishing can be a long process.

the studies we publish are all open access publications, allowing any beekeeper, to download for free, read and make up their own mind. In the near future we aim to publish the treatment survey Alex conducted earlier this year, finish the queen-swap experiment and continue our work on mite-resistance both in the UK and elsewhere. Finally, I must thank all BDI/BBKA beekeepers, as it is their funding that helps this research to be conducted and the next generation of bee scientists to emerge.

References

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3. Instructional video available at https://www.youtube.com/watch?v=Hfa9C1xvtec&t=3s and the BDI website www.beediseasesinsurance.co.uk
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Article for BBC radio 4 segment inside science

This article was written by Victoria Gill of the BBC based on an interview I gave for her segment inside science. The Interview can be found on the BBC at https://www.bbc.co.uk/programmes/m000yfkv.

'Resistance increasing'

Another study published this week, however, suggests bees around the world are developing the ability to "clear out" a particularly damaging parasite - varroa, a mite that lives and feeds on honeybees and larvae.

Varroa mites (red spot) on a honeybee

Bees already have complex organised hygienic behaviours, such as removing infected broods of larvae from the hive.

And now, data published **in the Royal Society journal Proceedings B**, from 40 years of research into colonies that survive infestations, without any chemical treatment, reveals they are evolving to "repurpose" that behaviour against varroa.

"We're seeing this resistance increasing around the world," Isobel Grindrod, from the University of Salford, said.

"And we're also seeing an increase recently in bee-keepers not having to treat [the mites] with chemical treatments."

"Pressure" from the mites was driving healthy bees to adapt, she said.

"Their adaptability is really important, and that's why we need to maintain healthy bee colonies - to keep that adaptability - because there will be other, new diseases and pressures in the future."

Hear more about bees' battles with parasites and pesticides on **BBC Inside Science on Radio 4 and BBC Sounds**

BBKA spring conference poster

I designed this poster for the BBKA conference in April 2022. Feedback and edits were given by S. Martin.