The spread and evolution of RNA viruses among honey bees and the wider insect community with particular emphasis on Deformed wing virus (DWV).

Laura Emily BRETTELL

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School of Environment and Life Sciences

University of Salford

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I would like to thank all the people who have helped and supported me throughout this project.

Firstly, I would like to thank Professor Stephen Martin both the opportunity to begin working in this field in the first place back in 2010, and for all the masses of help, support, guidance and encouragement both throughout this PhD and before. Without him I would not be in the position I am now.

I am very grateful to Declan Schroeder and Gideon Mordecai at the Marine Biological Society of the UK for being so helpful in sharing all their virology knowledge and helping me with bioinformatics; starting as a complete novice, this was invaluable.

I would like to thank Ethel Villalobos, Scott Nikaido and the rest of the Bee Lab at the University of Hawaii, for all the field work trips. Their knowledge and connections made for really successful and enjoyable collecting trips. I would like to thank Emilene Correa-Marques and Eliaber Barros Santos for all their help in Brazil and all the beekeepers in Hawaii, Brazil and the UK who provided samples throughout the project.

I have thoroughly enjoyed my three years at the University of Salford and that has been in large part because of the friends I have made and shared an office and lab with, in particular Jess Hall, Maria Morlan Mairal, Paz Aranega Bou and May Rajab. I am thankful for their friendship, support and helpful discussions covering all aspects of my project. I am grateful to everyone else at Salford who listened and provided advice, including Jessica Kevill, Charles Bailey, Rachel Antwis, Ian Goodhead and Jean Boubli.

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Finally, I would like to express my thanks to my family. Their support through this PhD, as always, has been incredible and I am lucky to have them.

DECLARATION

The data chapters, with the exception of chapter 5 have all been published in peer reviewed academic journals, where I am first or joint first author due to my significant contribution. In addition, I have included three other papers, two of which are published and one under review. Since my contributions were lower in these three studies I have decided to place them in appendices. The citations and full descriptions of my individual contributions to all these publications are as follows:

Chapter 1: A comparison of Deformed wing virus in deformed and asymptomatic honey bees.

I was heavily involved in the design of this study. I performed all of the molecular lab work: RNA extractions, RNA quantification and normalisation, RT-qPCR (along with M. V.–R.) and HRM, as well as the analysis of the produced data. I prepared the samples for Sanger sequencing and analysed the resulting data. For the NGS data analysis I collected the Hawaiian samples (along with S. J. M.), I isolated the DWV-like reads from the NGS data (G. J. M. performed the virus assemblies). I calculated the read coverage and produced the graphs which were used as part of Figure 2, and produced the phylogenies of Figure. 3. I drafted the manuscript, doing a significant proportion of the writing and acted as corresponding author.

Citation: **Brettell, L. E**.*, Mordecai, G. J.*, Schroeder, D. C., Jones, I. M., da Silva, J. R., Vicente-Rubiano, M., & Martin, S. J. (2017). A comparison of deformed wing virus in deformed and asymptomatic honey bees. *Insects*, *8*(1), 28.

*Both authors contributed equally to this manuscript

Chapter 2: Oldest *Varroa* tolerant honey bee population provides insight into the origins of the global decline of honey bees.

Along with S. J. M. I designed this study. I used photographs of frames of honey bees and brood to estimate colony size as well as counting numbers of mites infesting adult bees. I used this data along with data from the brood infestation work to calculate the estimated mite loads in the colonies. This data was put into the graph in Figure 2. I carried out all the molecular laboratory work: RNA extractions, RT-PCR, HRM and AGE as well as analysis of the data produced. I was responsible for the majority of the manuscript writing, and acted as corresponding author.

Citation: **Brettell, L. E**., & Martin, S. J. (2017). Oldest Varroa tolerant honey bee population provides insight into the origins of the global decline of honey bees. *Scientific Reports*, *7*.

Chapter 3: Novel RNA virus genome discovered in Ghost ants (*Tapinoma melanocephalum*) from Hawaii.

In this study I collected the samples (along with S. J. M.), and I extracted the RNA. I then used the filtered reads (filtering was done by P. P.) in IVA assemblies (both pooled for the original Milolii virus assembly and individually for each sample). I analysed the data using Blast and used Geneious to map the original reads to the newly assembled genome and to calculate the pairwise similarity. I wrote the article, with proofreading from S. J. M. and acted as corresponding author.

Furthermore, I have added the Supplementary information for this chapter (which was removed from the article prior to publication due to journal specifications). I was responsible for all the work in the Supplementary Information.

Citation: Brettell, L. E., Mordecai, G. J., Pachori, P. & Martin, S. J. (2017) Novel RNA virus genome discovered in Ghost ants (*Tapinoma melanocephalum*) from Hawaii. Genome Announcements, 5:e00669-17.

Chapter 4: Moku virus; a new *Iflavirus* found in wasps, honey bees and *Varroa.*

In this study I compiled all the data from the relevant samples and used the data from the individual wasp, honey bee and *Varroa* samples in a bioinfomatics pipeline (described in the text) to filter and keep all reads which aligned to the Moku virus genome using BLAST and produce *de novo* Moku virus assemblies. I also aligned the reads from each sample to the Moku virus genome and created the genome coverage depth plots in Figure 2. I was also responsible for a significant proportion of the writing and contributed extensively to discussions throughout the work concerning the direction we should take and for naming the virus.

Citation: Mordecai, G. J.*, **Brettell, L. E**.*, Pachori, P., Villalobos, E. M., Martin, S. J., Jones, I. M., & Schroeder, D. C. (2016). Moku virus; a new Iflavirus found in wasps, honey bees and Varroa. *Scientific Reports*, *6*, 34983.

*Both authors contributed equally to this manuscript

Chapter 5: Common apiary pests as potential reservoirs of honey bee – associated viruses in a Hawaiian apiary environment.

With assistance from S. J. M. I designed the study, and collected the samples. I extracted the RNA from all samples. I performed all bioinformatics and data analysis work and wrote the manuscript with assistance from S. J. M.

Appendices

The appendices contain three further manuscripts which I have co-authored and to which I have contributed a smaller amount. Two of these manuscripts have been published in peer reviewed academic journals (The ISME journal and Proceedings of the Royal Society B) and one has recently been submitted. These represent my larger body of work contributing to the field of honey bee virology and answering the aims of this PhD.

Appendix 1: Evidence of Varroa-mediated Deformed Wing virus spillover in Hawaii.

In this study I assisted with field collections and with the RNA extractions, DNAse treatment and RT-PCR work in the laboratory. I helped established the honey bee viral laboratory in the University of Manoa, Hawaii, where the study was conducted and trained several of the authors on the paper. I also contributed to the writing of the manuscript.

Citation: Santamaria, J., Villalobos, E. M., **Brettell, L. E**., Nikaido, S., Graham, J. R. & Martin, S. J. (2017). Evidence of *Varroa*-mediated Deformed Wing virus spillover in Hawaii. (Submitted).

Appendix 2: Superinfection exclusion and the long-term survival of honey bees in Varroa-infested colonies.

Along with S. J. M. I collected the Hawaiian samples used in this study. I also extracted the RNA and performed the initial RT-PCR screening of these samples to detect the presence of DWV prior to NGS library preparations. The second authorship reflects the relative amount of work I contributed to this paper.

Citation: Mordecai, G. J., **Brettell, L. E.**, Martin, S. J., Dixon, D., Jones, I. M., & Schroeder, D. C. (2016). Superinfection exclusion and the long-term survival of honey bees in Varroa-infested colonies. *The ISME Journal*, *10*(5), 1182-1191.

Appendix 3: Covert deformed wing virus infections have long-term deleterious effects on honeybee foraging and survival.

In this study I carried out a large amount of RT-qPCR work, although this was left out of the final publication due to the incompatibility of methods leading to insufficient data being produced. I also contributed a small amount to the writing of the manuscript, but a significant amount of discussion into the findings of the study.

Citation: Benaets, K., Van Geystelen, A., Cardoen, D., De Smet, L., de Graaf, D., Schoofs, L., Larmuseau, M., **Brettell, L.**, Martin, S. & Wenseleers, T. (2017). Covert deformed wing virus infections have long-term deleterious effects on honeybee foraging and survival. *Proceedings of the Royal Society B, Biological Sciences*, *284*(1848), 20162149.

ABBREVIATIONS

ABPV	Acute bee paralysis virus
BQCV	Black queen cell virus
DWV	Deformed wing virus
EBV	Egypt bee virus
ELISA	Enzyme – linked immunosorbent assay
EM	Electron microscopy
HRM	High resolution melt analysis
IAPV	Israeli acute paralysis virus
IRES	Internal ribosomal entry site
JEBV	Japanese Egypt bee virus
KBV	Kashmir bee virus
KV	Kakugo virus
Lp	Leader protein
NGS	Next generation sequencing
RdRp	RNA – dependent RNA polymerase
RT-qPCR	quantitative reverse transcription polymerase chain reaction
SBV	Sacbrood virus
SPV	Slow paralysis virus
UTR	Untranslated region
VDV-1	Varroa destructor virus 1
VP1,2,3	Viral protein 1, 2, 3
VPg	Viral protein genome – linked
VSH	Varroa – sensitive hygiene

GENERAL ABSTRACT

The honey bee is our most significant animal pollinator and is highly valued economically and culturally throughout the world. Unfortunately, over the last 30 years beekeepers across the globe have experienced significantly increased honey bee colony losses, especially in the Northern hemisphere. The spread of RNA viruses, most notably Deformed wing virus (DWV) in association with its vector, the ectoparasitic *Varroa destructor* mite are now widely accepted as a major factor in these colony losses. This has had a devastating effect on beekeepers and farmers alike. It has become apparent that many of these viral pathogens are generalists, able to infect many insect species. Although much work is currently ongoing in this field, there are areas where knowledge is still lacking, which could provide clues to protecting the bees in the long term.

Firstly, I investigated whether there is a unique DWV variant responsible for causing the development of deformed wings by comparing DWV in deformed and asymptomatic bees. This revealed no consistent differences, and greater variation was seen between locations rather than phenotypes, indicating that there is no unique viral variant that induces deformity.

Secondly, I disproved, by studying the oldest known *Varroa*-tolerant honey bee population, the long held theory that *Varroa* feeding activity induces activation of latent DWV, since these bees have long existed with *Varroa* yet still harbour low level, genetically diverse DWV infections, and have had no reported colony losses.

Thirdly, I discovered two new RNA viruses in ants and wasps collected from apiaries, during a honey bee collection trip to Hawaii. Mililolii virus was found to infect the Ghost ant *Tapinoma melanocephalum*. The other, Moku virus, was sequenced in high depth from yellowjacket wasps, *Vespula pensylvanica*, but was also, worryingly, at low levels in both *Varroa* and honey bees, suggesting that it has the potential to infect diverse hosts.

Finally, I detected DWV in a range of species living in Hawaiian apiaries. DWV genetic profiles grouped by species rather than location, suggesting that variants may exist which are better adapted to replicate in different host species.

Together these increase our understanding of the DWV – *Varroa* – honey bee nexus, expand our knowledge of the circulating virosphere within the apiary, and provide new insights into how DWV spreads beyond honey bees and into the wider insect community. Further benefit could now be gained from investigating whether DWV and other viruses detected in different arthropod species are true infections by using negative strand – specific RT-PCR to detect viral replication. Furthermore, it would be of great interest to use experimental infections to discover the nature of any pathogenicity of viruses in non - *Apis* hosts.

GENERAL INTRODUCTION

The honey bee

The European honey bee *Apis mellifera* lies within the order Hymenoptera along with the wasps, ants and sawflies. It is of tremendous importance to mankind both because of man's use of bee products and the pollination services it supplies. Thus the honey bee is one of the most significant and well-studied insects on the planet.

The honey bee exhibits eusocial behaviour, that is, bees live in colonies made up of one (or occasionally two) queens and up to 40,000 sterile female workers who work cooperatively to care for the young and maintain the colony. Colonies nest in cavities and feed on pollen and nectar which they convert into honey and store for food. These traits have made them attractive for beekeepers who keep colonies in nest boxes or hives from which they are able to easily harvest excess honey and wax for human use.

In addition to the production of bee products such as honey, wax and propolis, honey bees are valuable pollinators. They are generalist flower feeders and as such pollinate a number of important agricultural crop plants, such as almonds, fruit, berries and coffee. In addition, they also pollinate plants used for production of biofuels and construction materials (Potts et al., 2016). Because of this, they are considered the most important crop pollinator (Delaplane et al., 2000; McGregor, 1976). The economic value to pollination services in the UK alone is valued at £200 million per year (The British Beekeeping Association, 2016), and is reported as adding ~$40 billion to the global crop value per year (Klein et al., 2007).

Honey bee colony losses

During the past 30 years beekeepers in many parts of the world have experienced elevated colony losses, for example beekeepers in central Europe reported losses of 25% each year

between 1985 and 2005 (Potts et al., 2010), and in the USA annual losses have averaged around 38% from 2010 to 2016 (since they were first recorded) (Beeinformed Partnership, 2017). In temperate climates the majority of colony losses occur over winter as this is when bee numbers are lowest and the colony is at its weakest. Historically in the UK over – wintering colony losses have remained constant at 5-10% per year; however this peaked in the mid-2000s when the British Beekeepers association reported losses spiking at 33.8% (The British Beekeeping Association, 2015). Since then the trend has been towards losses decreasing again; however losses for the year 2014/2015 were still reported at 14.5% which is unsustainable for many beekeepers. These trends have been experienced throughout the world, with the USA reporting even greater annual colony losses of 14% over summer and 43.7% over winter for the same period (Fig. 1) (Beeinformed Partnership, 2017). Furthermore, they report over – winter losses for the recent year (April 2016 – April 2017) as 21.1% (33% total yearly loss), which is an improvement on recent years but still above what is considered by beekeepers as an acceptable level to maintain sufficient productivity and population sizes able to recover from losses (Fig. 1).

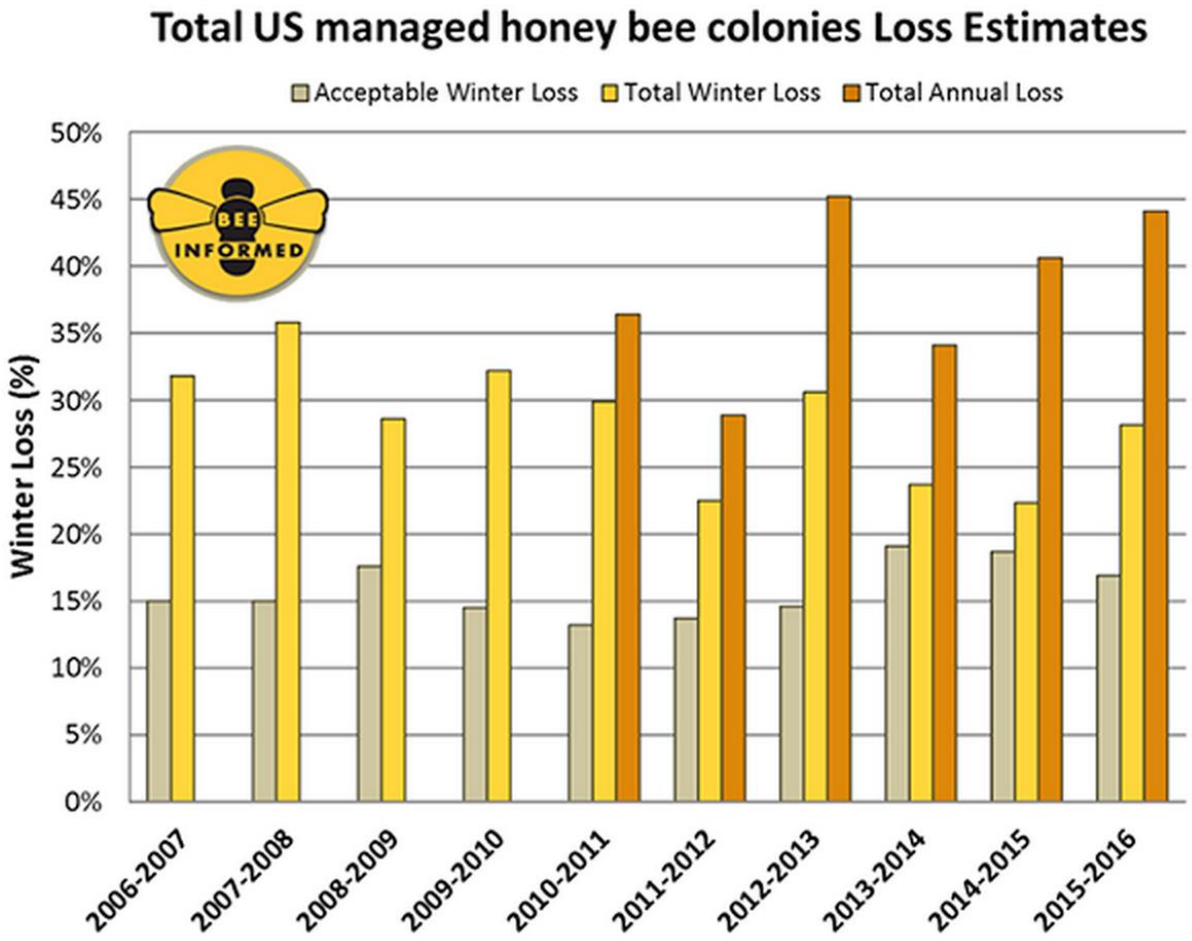

Figure 1. Shows the average acceptable (as described by the surveyed beekeepers) (grey), winter (yellow) and total (orange) annual honey bee colony losses in the USA over the past decade, as calculated by the Bee Informed Partnership (Beeinformed Partnership, 2017).

There has been much speculation about the cause of these losses. Pesticides (Henry et al., 2012), habitat loss and urbanisation (Naug, 2009; Brown & Paxton, 2009) have received much attention as well as a multitude of pathogens (Genersch, 2010). Current opinion however points to the spread of viruses, most notably Deformed wing virus (DWV) as the major contributor. DWV in association with its vector, the parasitic mite *Varroa destructor*, is now considered the most important factor contributing to honey bee declines (Martin et al., 2012). It is important to note, however, that the synergistic effect of multiple factors probably contributes to the overall pattern of colony losses.

Varroa destructor

The ectoparasitic mite *Varroa destructor* (Fig. 2) is a pest of the honey bee which is now found in honey bee colonies throughout the world. The mite was originally mis-classified *Varroa jacobsoni*; a pest of the Asian honey bee *Apis cerana.* It is thought that *V. destructor* jumped the species barrier in the late 1950s (Danka et al., 1995) when *A. mellifera* was moved into East Asia to increase honey production and the colonies were maintained side by side. It was only later, in 2000, when phylogenetic (COI barcoding) and morphometric analysis revealed that there were multiple haplotypes and that the mite that was now persisting in *A. mellifera* was a distinct species, *V. destructor* (Anderson & Trueman, 2000).

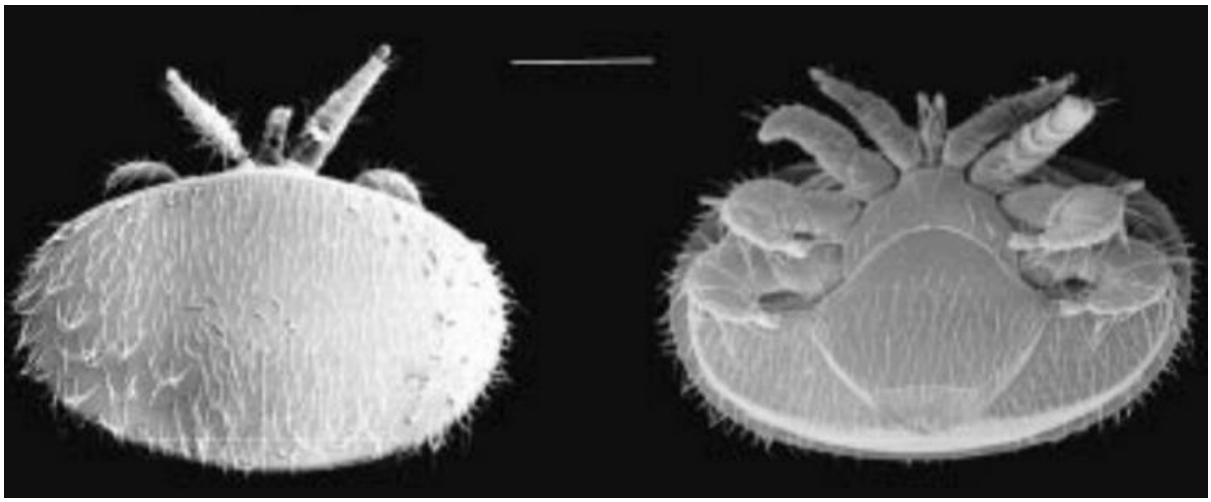

Figure 2. *V. destructor* in dorsal (left) and ventral (right) view. Bar = approx 500 µm (adapted from Anderson and Trueman (2000).

On its native host *A. cerana*, the *Varroa* mite causes relatively little harm. The mites feed on the bees' haemolymph and reproduce inbreeding (brother-sister mating) (Fig. 3) (Rosenkranz et al., 2010). In *A. cerana* colonies, the mites' reproduction almost always occurs in the drone cells, of which colonies often have very little and none at all for long periods (Boot et al., 1997). In *A. mellifera* colonies, however, *Varroa* are able to reproduce in

worker cells (Martin, 1994), although they still preferentially reproduce in drone cells (Oldroyd, 1999). The constant availability of worker cells in a colony means that *Varroa* are able to reproduce constantly and thus reach much higher numbers.

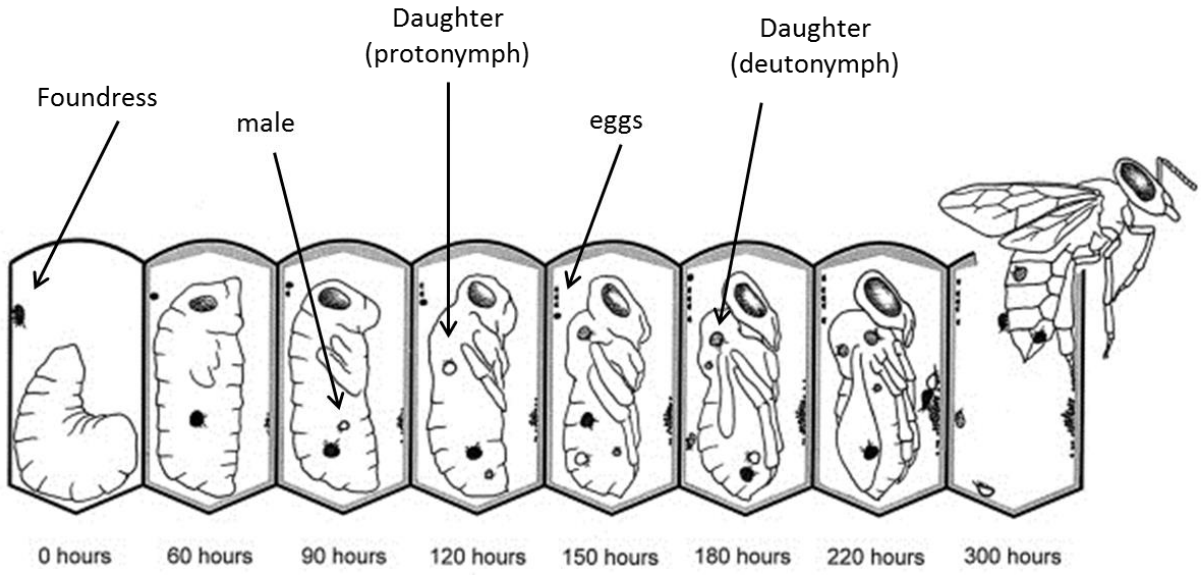

Figure 3. The reproductive cycle of *Varroa* inside the honeybee brood cell (Oldroyd, 1999), labels show *Varroa* life stages.

The first record of *Varroa* infesting *A. mellifera* colonies was in Russia in 1952, with beekeepers in Russia starting to report problems in the 1960s and 70s (Danka et al., 1995), and in China in the 60s (Smirnov, 1978). This initial spread was probably in part to the large amount of migratory beekeeping in Russia at the time i.e. beekeepers transporting colonies between locations depending on availability of floral resources (Danka et al., 1995). The spread continued over the next 20-30 years as global travel became easier and more common (Wilfert et al., 2016), with first reports of *Varroa* infestations in the USA in 1987 (De Guzman et al., 1997), and in the UK in 1992 (Paxton, 1992). There are now only Australia and a few isolated island populations e.g. Colonsay, Scotland (Ryabov et al., 2014), and

Kauai and Maui, in Hawaii, US (Martin et al., 2012) cross the world which remain free of the mite.

Honey bee viruses

Honey bee viruses have long been known to cause disease symptoms and in recent years the magnitude of the threat to honey bee health has increased due to the spread of *Varroa*; the vector of many such viruses. The association between *Varroa* and the viruses it transmits will be discussed at length later in the introduction, in data chapters one and two, and appendices two and three.

Considering the suite of viruses known to infect honey bees, Sacbrood Virus (SBV) was the first described. It was discovered in the 1900s, at which time it was described simply as a filterable agent which could cause sacbrood disease when transferred from a diseased to a susceptible host (White, 1917). There are currently around 18 - 24 viruses known which infect honey bees of all castes and life stages (Allen & Ball, 1996; Remnant et al., 2017; Runckel et al., 2011). The inaccuracy of this number reflects the grouping of some viruses as part of the same families, for example Israeli Acute Paralysis Virus (IAPV), Acute Bee Paralysis Virus (ABPV) and Kashmir Bee Virus (KBV) can be considered part of the same IAPV species group due to their phylogenetic relatedness (Fig. 4).

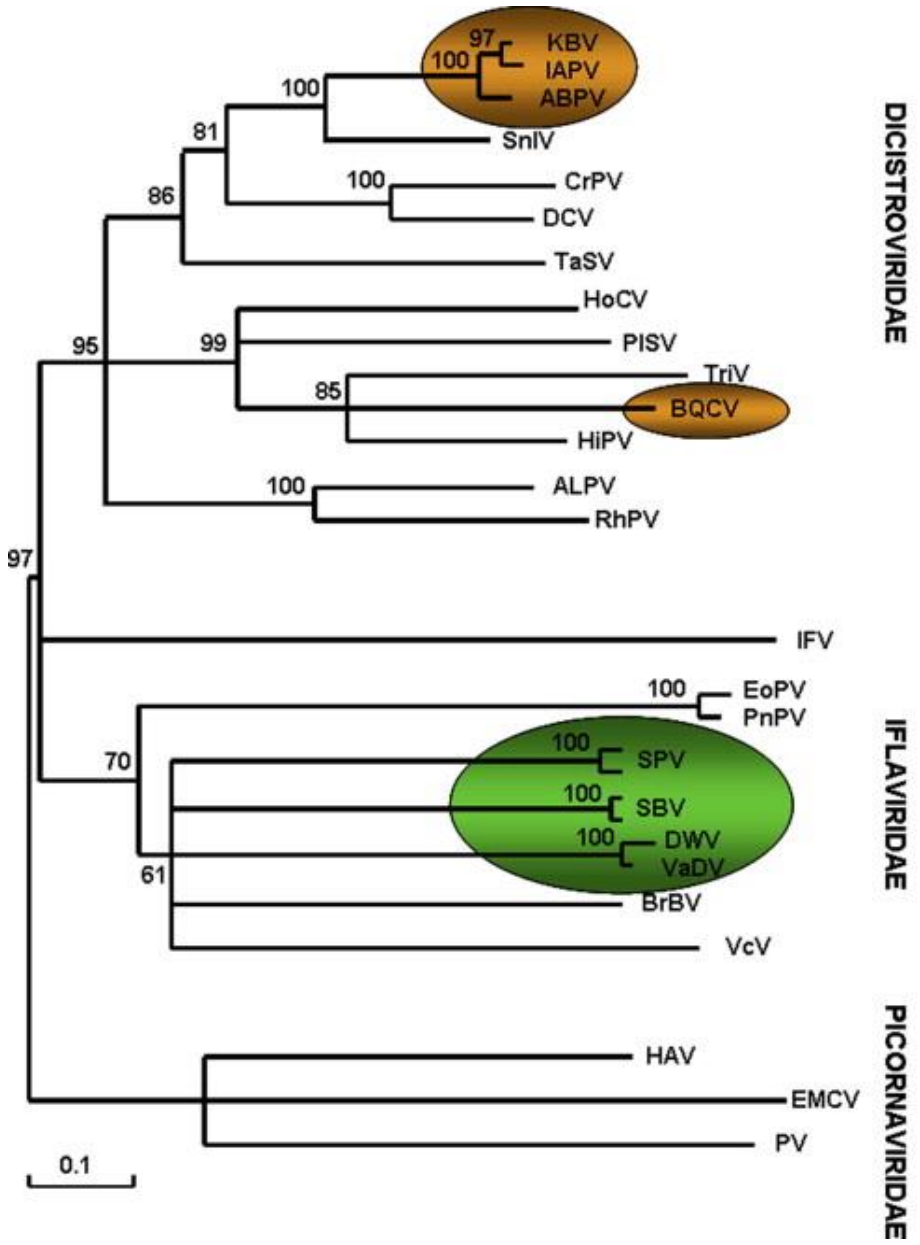

Figure 4. Phylogram showing the common honey bee viruses (KBV, IAPV, ABPV, BQCV, SPV, SBP, DWV and VaDV, which has since been renamed DWV type B) in relation to other members of the *Dicistroviridae*, *Iflaviridae* and *Picornaviridae* families, inferred using conserved amino acid domains in capsid proteins, helicase 3C-protease and RNA-dependent RNA polymerase regions (de Miranda & Genersch, 2010).

Most of the viruses which infect honey bees are single stranded positive sense RNA viruses of around 30µm (Bailey, 1976; Remnant et al., 2017). They commonly exist as covert or inapparent infections and persist at low levels. Furthermore, bees regularly harbour multiple viruses (Traynor et al., 2016) to no ill-effect (Welch et al., 2009). However, occasionally infections can become overt and cause obvious disease symptoms (i. e., the shrivelled wings characteristic of DWV) and/or colony loss.

Some viruses such as those of the IAPV species group are highly virulent and lead to the rapid development of severe disease symptoms (in this case the development of a dark and hairless abdomen, spasms, inability to fly and walking in circles (Maori et al., 2007)), which can act quickly to kill a bee in 3-5 days, whereas other viruses such as SBV employ a different strategy and cause covert infections of long duration. Individual infected larvae can succumb to viral infection and are found dead, still in their unshed skin; however this usually affects few individuals and the colony as a whole generally recovers, with the adults maintaining low viral loads (Blanchard et al., 2014).

Although some viral diseases have obvious distinguishing symptoms e. g. the characteristic blackened queen cells containing dead undeveloped queens of Black queen cell virus (BQCV) (Ball & Bailey, 1991), most infections often show no particular distinguishing symptoms (Allen & Ball, 1996; Gauthier et al., 2007). As such, diagnosis from a beekeeper's perspective can be challenging and can result in the underreporting of virus occurrence.

Deformed Wing Virus

Deformed wing virus, DWV is a typical picorna-like insect virus which lies within the family *Iflaviridae.* It is currently accepted as the most important viral pathogen affecting honey bees across the world (Genersch et al., 2010; Schroeder & Martin, 2012). As such DWV is the main focus of this research.

Symptoms

Overt DWV infection manifests in the individual as bees which emerge from their brood cells with deformed/shrivelled wings (Fig. 5) as well as stunted growth with short abdomens (Allen & Ball, 1996; Koch & Ritter, 1991). These bees are unable to fly and die within 48 hours of emergence (Yang & Cox-Foster, 2007). Infection with DWV has also been reported to affect learning (Iqbal & Mueller, 2007) and aggression (Fujiyuki et al., 2004) and cause bees to have reduced weights at emergence (Khongphinitbunjong et al., 2016). The deformed wing phenotype is often used by beekeepers as a marker for DWV infection in the colony. Although this has been shown to be a good indicator of DWV infection (Dainat & Neumann, 2013) it can lead to an underestimation of viral infection as even in heavily infected colonies, the proportion of bees showing these visible symptoms is very low (Nordström, 2000). Although symptoms generally appear in bees that have the highest viral loads (Chen et al., 2005; Tentcheva et al., 2004), both symptomatic and asymptomatic bees can harbour high viral titres of up to 10^9 genome equivalents per bee (Highfield et al., 2009). The lack of obvious symptoms, however does not mean that these bees are 'healthy'. Larvae injected with DWV show a reduced longevity of 5-10 days (Nazzi et al., 2012), and asymptomatic infected bees have been shown to have reduced flight ability (Wells et al., 2016; Benaets et al., 2017) as well as a reduced lifespan (Martin, 2001). This is arguably the most important effect as it can contribute to over wintering colony loss due to insufficient surviving bees to maintain the colony (Martin, 2001; Dainat et al., 2012).

Figure 5. A honey bee worker showing normal wings (left) (photo courtesy of Ethel Villalobos) alongside a honey bee showing the characteristic deformed wings caused by DWV (right) (de Miranda & Genersch, 2010).

At the colony level, high titres of DWV has been implicated in overwintering colony losses (Dainat et al., 2012; Genersch et al., 2010; Highfield et al., 2009), perhaps in part due to the fact that DWV is not 'rapidly fatal' and thus persists in individuals for a long time, allowing wider spread (Carreck et al., 2002). Although Genersch *et al*. (2010) do also point out that the simple occurrence of DWV doesn't relate to colony loss as often the majority of colonies are infected.

Transmission

DWV is spread via a number of transmission routes. It can be transmitted sexually and vertically through both eggs and sperm (Chen et al., 2006; de Miranda & Fries, 2008; Yue et al., 2006; Yue et al., 2007), as well as horizontally through feeding activity. This has been identified by the detection of DWV in larval food (Yue et al., 2007), as well as pollen, honey and the honey bee gut (Chen et al., 2006), and since, experimentally, through oral infection,

resulting in cells of the gut epithelium becoming infected (Möckel et al., 2011). Significantly, horizontal transmission is also achieved through direct inoculation into the bees' haemolymph via the feeding activity of the *Varroa* mite (Ball, 1989; Bowen-Walker et al., 1999), which occurs on the abdomen of developing pupae and adult bees (Bowen-Walker et al., 1997). It is this association of DWV with *Varroa* that has transformed the virus from a relatively harmless virus which only rarely killed colonies, to one of the most devastating honey bee pathogens on the planet (Martin et al., 2012; Schroeder & Martin, 2012).

Varroa destructor as a vector

Varroa has the ability to vector a number of bee viruses including KBV (Shen et al., 2005a; 2005b), SBV (Chen et al., 2004; Shen et al., 2005a) ABPV (Ball, 1983), IAPV (Di Prisco et al., 2011), and DWV (Bowen-Walker et al., 1999), as well as multiple viruses (Chantawannakul et al., 2006). The effectiveness of this mode of transmission varies considerably between viruses. For highly virulent viruses such as those belonging to the IAPV species group, transmission by *Varroa* results in the virus killing infected pupae before they can emerge from their brood cells. This results not only in the death of the pupa, but also the mite family too as they remain sealed inside the cell. The overall effect on the colony is therefore generally small as the virus is unable to spread among many individuals. Conversely the transmission of less virulent viruses has a much greater effect. When *Varroa* infects the developing pupa with a virus that typically causes covert infections such as DWV, the bees remain able to develop and emerge from their brood cells to live and work in the hive (Fries & Camazine, 2001). This provides the opportunity for not only the infected bee to spread the virus further (via horizontal transmission), but also for the daughter mites, which have fed on the developing bee, to infect new developing pupae. In this way the virus spreads throughout the hive, and then on to neighbouring hives when honey bee foragers rob, or drift into, other hives, sometimes also carrying *Varroa* mites with them (Fries & Camazine, 2001; Forfert et al., 2015).

It is well accepted that the spread of *Varroa* and with it high titres of DWV has been a key factor in honey bee decline (Martin et al., 2012; Mondet et al., 2014); however there is still contention in the literature about the exact nature of the *Varroa* – DWV – honey bee cycle. Shen *et al*. (2005b) suggest from the finding of a positive correlation between the number of (sometimes DWV free) *Varroa* in cells and DWV load in the corresponding bees, that *Varroa* induce DWV activation. However similar experiments carried out by Nordstroem *et al.* (1999) found no correlation between the number of mites infesting a cell and the probability of that bee being infected.

In 2001, Martin *et al.* used a modelling approach to show that with no mite control the increase of *Varroa* within a colony causes DWV to become established, which in turn can lead to colony loss. It was found that keeping mite numbers in a colony below an economic threshold of 2000-3600 per colony gives the colony a higher chance of surviving the winter (Martin, 2001). Keeping mite numbers below <700 should prevent epidemics (Sumpter & Martin, 2004), although even following these guidelines increased losses are felt when compared to *Varroa* free populations.

Currently it is not known why some bees develop the deformed wing phenotype and others do not and there is contention in the literature about whether this is related to *Varroa* – mediated DWV transmission. Wing deformity was initially attributed to the detrimental effects of *Varroa* feeding activity, which certainly also can cause weight loss in developing bees (De Jong et al., 1982). But later, as DWV research progressed, the virus was accepted as the cause. Research by Shen *et al*. (2005b) reports that the deformed wing phenotype only develops when bees acquire their DWV infection through *Varroa* feeding as larvae, however work by Nordström (2000) found bees infected with high viral titres can emerge from cells not infested by *Varroa*, showing that DWV infection can cause deformity without *Varroa*. Furthermore, research has shown viral load to be a critical factor in whether external symptoms develop by the finding of DWV in higher titres in deformed than asymptomatic bees (Bowen-Walker et al., 1999). However, information is still lacking due to experimental difficulty regarding viral titres in larvae that go on to develop deformed wings

i. e., do larvae with high DWV loads go on to develop deformed wings or are the high viral loads at emergence due to increased DWV transmission through mite feeding or other external factors?

The effect of *Varroa* on DWV infection has been reported to influence the characteristics of the circulating DWV toward increased pathogenicity for the bee (Yue & Genersch, 2005). But this could be due to decreasing life spans, resulting in fewer infected adults in a sample (Gauthier et al., 2007). In addition, it should be noted that symptoms of DWV and those directly caused by *Varroa* parasitisation are often confounded due to both the virus and the mite usually being present in a colony at the same time.

There is further contention regarding whether DWV replicates in the mites. Gauthier *et al*. (2007) propose that the occurrence of significantly higher loads of DWV compared to ABPV or SBV in mites suggest viral replication, a theory also supported by both Ongus *et al.* (2004) and Yue and Genersch (2005). However, these studies fail to take into account DWV contamination in mite gut through infected bee material and subsequent work by Erban *et al.* (2015) using proteomic analysis showed that the DWV particles found in the mite were not replicating. The absence of viral replication taking place in *Varroa* would give more weight to the theory that *Varroa* merely acts as a mechanical vector of DWV.

Despite various contentions, it is undeniable that the emergence of *Varroa* as a vector of DWV has had a profound effect on honey bee populations. Ryabov *et al.* (2014) discovered that when a particularly virulent form of DWV is transmitted directly in to the developing bees' haemolymph i. e. by *Varroa*'s feeding activity, it has the ability to replicate to much higher levels than when transmitted orally and also results in a loss of viral diversity. This effect is seen also on a much larger scale in the work of Martin *et al*. (2012) and Mondet *et al*. (2014), who showed that the arrival of *Varroa* to previously *Varroa*-free areas was accompanied by an increase in viral prevalence and load and the reduction of strain diversity, leading to the dominance of a single virulent variant.

History

Deformed Wing Virus (DWV) was first isolated from dead adult Egyptian honey bees in 1978 and as such named Egypt bee virus (EBV) (Bailey et al., 1979). At this time electron microscopy was used to determine that EBV was made up of 30nm diameter isometric particles, dissimilar from any other viruses isolated from bees thus far.

In the early 1980s, a virus was purified from deformed honey bees in colonies in Japan infested with *Varroa* and that had recently been found to be experiencing bee deaths. It showed similarity to ABPV but did not cross react with any known virus antiserum and so was given the name Japanese Egypt bee virus (JEBV) (Ball, 1983). The virus was later detected, and found to predominate over other viruses, in mites, pupae and adult bees from unhealthy colonies containing deformed bees (Ball, 1989). Serological techniques (ELISA, Immunodiffusion) later revealed this virus to be distantly related to EBV and similar to other viruses purified from honeybee samples across Europe, Asia and South Africa (Allen & Ball, 1996; Topolska et al., 1995; Ball, unpublished data) around the same time. As such, the viruses were renamed Deformed Wing Virus (Ball, 1997). In the early phases of *Varroa* establishment, Acute Paralysis Virus (APV) had been identified as being responsible for colony losses (Ball, 1997; Carreck et al., 2002); however after a time lag of approximately 3 years DWV was found to be ubiquitous in *Varroa* infested colonies (Carreck et al., 1999). More recent studies in Hawaii (Martin et al., 2012) and New Zealand (Mondet et al., 2014) have found the same pattern and this is now known to be attributed to the time required for DWV loads to increase and for the sequence variation to reduce, leading to the establishment of a single dominant variant.

In the 1980s large scale colony losses were reported across Europe in areas where *Varroa* had become established (Gomez Pajuelo, 1988; Hartwig, 1994; Martin, 1998). At first the link was made between *Varroa* in isolation and colony losses until investigations started to focus on the role of secondary pathogens, initially Acute Bee Paralysis Virus, ABPV. ABPV had been found in dead bees in regions where *Varroa* was established in the 1970s (Bailey

et al., 1979; Batuev, 1979) and 1980s (Ball & Mladjan, 1990), and Ball (1985) suggested that ABPV, a usually inconsequential virus, could potentially cause colony mortality when in association with *Varroa*. ABPV was subsequently found and linked to an increase in overwintering colony losses in Hungary (Bekesi et al., 1999) and Germany (Ball & Allen, 1988). However these identifications were made using electron microscopy (EM) data and were influenced by previous studies; therefore this finding may not be reliable due to structural similarity of ABPV to other honey bee viruses when viewed with EM (i. e., all are icosahedral in shape and of approximately 30nm diameter).

By the 2000s DWV had become the virus most commonly found in association with *Varroa* (Carreck et al., 2002) and currently is ubiquitous throughout the world. However there are still rare isolated island populations which remain free of *Varroa* and as such maintain only very low levels of DWV which cause little harm (e.g. Australia, Colonsay-Scotland and Molokaii-Hawaii). The spread of DWV worldwide has recently been supported through phylogenetic analysis to be due to large scale movement of honey bee colonies through modern beekeeping practices (Villalobos, 2016; Wilfert et al., 2016), which has transformed DWV from a relatively harmless pathogen present at low titres in low numbers of colonies.

Structure

DWV is typical of insect viruses in that it is as a picorna-like virus with a single (+) stranded RNA genome (Allen & Ball, 1996). It lies within the family *Iflaviridae*, and exists as 30nm icosahedral, non-enveloped particles (Fannon & Ryabov, 2016). The genomic RNA is infectious and serves as a template for translation of the viral polyprotein (Fannon & Ryabov, 2016), which is post-translationally cleaved to form the functional proteins and is transcribed as one open reading frame (Fries et al., 2006). It is made up of structural and non-structural proteins and is flanked by 5’ and 3’ untranslated regions (Fig. 6) (Dalmon et al., 2017). The 5’ terminus is covalently bonded to a small genome linked virus protein (VpG) (Fannon & Ryabov, 2016) and contains an internal ribosomal entry site (IRES), which is used

in directing cap independent translation and also stabilising the 5'end (Belsham, 2009). The coding sequence begins with a highly variable leader protein, Lp which is currently of unknown function (Fannon & Ryabov, 2016), and which precedes three major structural proteins: the capsid proteins VP1, VP2 and VP3 (Lanzi et al., 2006). These are followed by non-structural motifs common to picorna-like insect viruses: RNA helicase which is the most conserved region (Dalmon et al., 2017), chymotrypsin-like 3C protease (responsible for proteolytic processing at various sites characterised by Lanzi *et al*. (2006)) and an RNA-dependent RNA polymerase (Wang et al., 2004; Wu et al., 2002). The 3' UTR is well conserved and like the 5' UTR is involved with the regulation of replication and translation (de Miranda & Genersch, 2010).

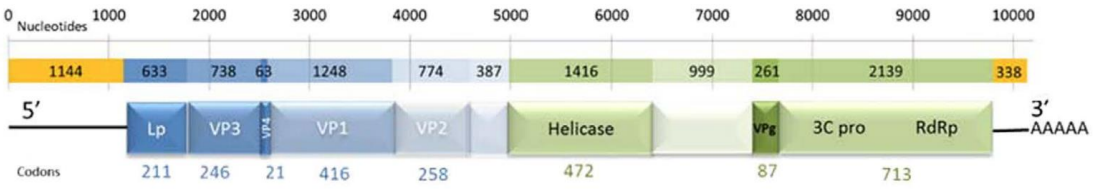

Figure 6. Shows the structure and organisation of the DWV genome as shown by Dalmon *et al*. (2017), with the structural region in blue, the non-structural region in green and untranslated regions in yellow. Nucleotide positions are shown above and length of the amino acid chains (codons) are shown below.

As an RNA virus, DWV has a very high mutation rate and a lack of proof reading ability. These factors along with the massive population sizes mean that the virus evolves incredibly quickly and as a group of closely related viruses (de Miranda & Genersch, 2010), referred to a quasispecies (Domingo & Holland, 1997). This quasispecies revolves around three master

variants: type A, the 'classic' DWV first sequenced from bees in Italy and Pensylvania in 2002 (Lanzi et al., 2006), type B, previously known as Varroa destructor virus 1 (VDV-1) since it was sequenced from infected *Varroa* (Ongus et al., 2004), and the newly described type C (Mordecai et al., 2016a). Originally Kakugo virus (KV), a virus found in the brains of aggressive worker bees (Fujiyuki et al., 2004), was classed as a separate distinct virus. Phylogenetic analysis using both the RdRp and Helicase regions later showed KV and DWV to belong to the same group (Chen et al., 2004) (type A). Due to the nucleotide similarity of KV and VDV-1 to DWV (97% and 84% respectively) and their ability to form recombinants (Dalmon et al., 2017; Moore et al., 2011; Mordecai et al., 2016b; Ryabov et al., 2014; Wang et al., 2013), current opinion is that they are all part of the same DWV family (Lanzi et al., 2006; Martin et al., 2012).

Honey bee immune response to DWV transmission by *Varroa*

The increase of DWV load in the *Varroa* – parasitised developing bee, transforming a latent infection to an overt deadly infection (Nazzi et al., 2012), has been put down to a *Varroa*-introduced down-regulation of immune gene transcription (Gregory et al., 2005; Shen et al., 2005), although other studies have shown conflicting results (Kuster et al., 2014).

When *Varroa* feed on the developing pupa, they secrete in their saliva both viral particles (Shen, et al., 2005) and proteins which have been shown to impair the function of haemocytes (Richards, et al., 2011), which would normally aggregate to close the wound (Kanbar & Engels, 2003). Other proteins are also secreted but although the authors speculate about their role in suppression of immunity (Richards et al., 2011), there is currently no evidence to support this.

Mechanisms of honey bee immunity are often poorly understood and candidate immune genes are often inferred from *Anopheles*/*Drosophila* orthologues which as yet are unproven in bees. Moreover, the honey bee genome is known to contain recently diverged and

unannotated immune genes that will be overlooked in many studies. Furthermore, honey bees heavily utilise social immunity which further confuses the issue (Evans et al., 2006). In addition, immunity as a response to bacterial challenge, which the majority of studies discuss, is often different to viral infection that is less well understood. Although the immune response to bacterial challenge through mite feeding is significant (15-30% of mite infested pupae fed on by *Varroa* show bacterial infected wounds), these individuals show no elevation in mortality (Kanbar & Engels, 2003), indicating that the bees are little affected. This is logical given that any increase in the risk of death to the pupa would also harm the mites by removing the food source, and increase the risk of the pupa being removed via hygienic behaviour that would interrupt the mites' reproductive cycle (Martin, 2001).

Immunosuppression has been proposed to be a result of active suppression by *Varroa* (Gregory et al., 2005; Shen et al., 2005), but other studies consider the reduced immune gene transcription occurs due to the combination with bacterial challenge when a mite feeds on a pupa (see above) (Yang & Cox-Foster, 2005). On the other hand, Kuster *et al*. (2014) found that *Varroa* feeding had little effect on immune gene expression. They suggest that because *Varroa* feeding does increase DWV titres, but so does experimental wounding (which also caused decreased immune gene expression), that it may be the mechanical action of feeding rather than immunosuppression which causes *Varroa* to increase DWV levels in the developing bee. This work also showed differences associated with time point and specific immune genes, highlighting that choosing particular time points/genes can result in a different outcome.

Interestingly, Nazzi *et al*. (2012) suggest a downregulation of immune gene expression is due to DWV replication itself. They also used RNAi to demonstrate when NF-kB, an immune gene known to be active in insect antiviral response (Marques & Imler, 2016) is downregulated, DWV loads increase (Kuster et al., 2014). This implies that any other stressor that targets NF-kB results in fewer transcripts available to challenge DWV, which can then take advantage of the depletion of available transcripts and can in turn proliferate.

The matter is further confused by findings from Gregorc *et al*. (2012) who found *Varroa* parasitisation of DWV infected bees to result in an upregulation of immune gene transcription, and Azzami *et al*. (2012) who found that infection with ABPV triggered no cellular or humoral immune response and say that RNAi is likely to be more important in the viral immune response. Indeed it is apparent that RNAi targeting of double stranded viral RNA produced during viral replication is a key factor in insect viral immunity (Ding & Voinnet, 2007; Kingsolver et al., 2013; Marques & Imler, 2016).

Resistance

The continuing economic burden of *Varroa* means that beekeepers, farmers and scientists alike are going to lengths to develop resistance in bees to *Varroa*/DWV. Methods for this include studying populations' natural resistance and exploring potential heritable defence mechanisms.

One such mechanism which continues to be extensively investigated is hygienic behaviour, that is, the physical removal of pupae infested with *Varroa* from their brood cells and the use of grooming behaviour to remove/damage phoretic mites. This serves to interrupt the mites' reproduction, and studies speculate that the removal of infested pupae also results in the colony rearing fewer infected brood (Gauthier et al., 2007). Honey bees have been shown to be able to detect and remove larvae infected with either American foulbrood (Rothenbuhler, 1964) or chalkbrood (Gilliam et al., 1983), thus inhibiting the spread of disease. As such, selective breeding experiments in the US and Europe have been carried out using the bees' ability to remove freeze killed brood in an attempt to breed bees more adapted to removal of *Varroa* infested pupae (Toufaila, et al., 2014; Spivak, 1996). The most detailed studies by the Spivak team found that hygienic bees did remove more mite - infested pupae than non - infested (Spivak & Reuter, 2001), but the results were highly variable and the authors speculate that when a colony is heavily infested the bees may fail to respond to the cues and fail to remove pupae (Spivak, 1996). At one time, it was thought

that an experimental bee line had been successfully developed to selectively remove highly reproductive mites (Harbo & Harris, 2005), although later studies revealed these bees to exhibit the same hygienic traits as other experimental lines and thus were grouped as *Varroa* sensitive hygiene (VSH) bees (Harris, 2007). The continuing variable results and the fact that some of the experimental colonies display lower numbers of viable bee brood (Ibrahim & Spivak, 2006) indicate that this may not be the most optimal target for developing resistance. However, due to the relative ease and low cost of breeding experiments, work continues in this field (Rinderer et al., 2010).

There are several populations across the world that are naturally resistant i.e., not selected by man. The role of viruses in these populations remains unclear as highlighted by Locke *et al*., (2014), when considering the *Varroa* – tolerant bees of Gotland, Sweden. These bees have been shown to exhibit mite – related resistant traits, but also appeared to differ in their tolerance of DWV infection, although this requires further work. There are other groups of bees, for example Africanised bees (Martin & Medina, 2004), which have had their resistance studied in terms of how they deal with *Varroa*, but for which viruses have yet to be considered. Due to the fact that *Varroa* – associated colony loss is generally due to infection with their associated viruses, particularly DWV, it is important to consider viruses when studying resistant colonies. Part of this study will investigate how a *Varroa* – tolerant survives from both a *Varroa* and a DWV point of view.

Honey bee viruses and cross species transmission

The near global distribution of honey bees and their behaviour as generalist pollinators means that they overlap in resources and share overlapping niches with a wide range of other bee species (Steffan-Dewenter & Tscharntke, 2000) and other insects (Chacoff & Aizen, 2006; Sjödin, Bengtsson, & Ekbom, 2008). Cross-species spillover of viruses is well documented in many species (e.g. Parrish et al., 2008; Power & Mitchell, 2004) and it is now understood that a number of viruses once described as honey bee pathogens are actually

general insect viruses (Manley et al., 2015; Singh et al., 2010). Reduction in global biodiversity, and significantly in economically important pollinators, has led to a recent increase in work studying viral spillover between species, especially honey bees and other pollinators.

Honey bees have varied interactions with other species, for example through sharing of floral resources (Heithaus, 1979; Steffan-Dewenter & Tscharntke, 2000), predation and the robbing of honey (Akre & Mayer, 1994; Clapperton et al., 1989), as well as being eaten by ants, wasps and hornets. DWV is of particular concern because of its dramatic spread and the devastation it has caused in honey bee populations and because it is known to infect and replicate in other species (Furst et al., 2014; Manley et al., 2015; Zhang et al., 2012), cause deformed wings (Genersch et al., 2006) and reduce longevity in bumble bees (Furst et al., 2014). Transmission between wild and managed bee species has been shown (McMahon et al., 2015) and directionality from managed honey bees to wild species has been suggested (Tehel et al., 2016; Zhang et al., 2012). Although currently a hot topic, knowledge in this field is lacking and there is much yet to be discovered in terms of the spread and limits of RNA virus transmission between species, and any potential pathogenic effects in non-*Apis* species.

Studying insect viruses

It is worth noting at this point that, before the advent of modern, highly specific molecular techniques, virology studies relied on serological techniques and electron microscopy (EM), which although useful, can be unspecific; serological techniques rely on the production of appropriate antisera by injecting rabbits with viruses purified from bees. Due to their similarity (generally picorna-like, icosahedral ~30nm particles [Bailey et al., 1981]), it is impossible to classify honey bee viruses using their morphology alone (Allen & Ball, 1996; Chen et al., 2004; Allen & Ball, 1995). In addition, these techniques are much less sensitive than current molecular techniques and can only detect the high viral loads sufficient to kill a bee (Allen & Ball, 1996). Enzyme-linked immunosorbent assays (ELISAs) allowed for the

detection of viruses at lower titres with a detection limit of around 10^7 particles per bee (Martin et al., 2013), but the advent of molecular techniques revolutionised virology and now reverse transcription polymerase chain reaction (RT-PCR) can be used which is much more specific and can now reliably detect virus genome equivalents in the 100s per sample range making it the gold standard in virus detection (Genersch, 2005; Yue & Genersch, 2005). The modification of the RT-PCR reaction to include association with Taqman (Chen et al., 2005) or SYBR green (Highfield et al., 2009) chemistries has since allowed accurate quantification of viral load, using a reference of constitutively expressed control genes to normalise for variation in extraction efficiency.

In addition to detecting the presence or absence and quantifying the level of viral RNA in a sample, it is now possible to investigate the strain variation. Since RNA virus particles often exist in very large numbers and considering that their replication mechanisms lack any proofreading activity, replication is very error prone and as such the viruses exist as quasispecies, that is, a population of genetically related sequences (Domingo & Holland, 1997), rather than clonal populations. DWV is typical of this and is a quickly evolving family of viruses (de Miranda & Genersch, 2010) which revolves around three master variants (type A: KV and DWV, type B: VDV-1, and type C) (Martin et al., 2012; Mordecai et al., 2016a). As such, understanding which variant(s) are dominating in samples and the viral population dynamics is key to furthering the understanding of DWV. High Resolution Melt Analysis (HRM) can now be used in addition to qRT-PCR to obtain an idea of the dominant variants present in a sample through the detection of differential melting temperatures of cDNA fragments of different sequences (Martin et al., 2012). Finally, the current increase in availability of Next Generation Sequencing (NGS) technologies is allowing researchers to investigate deeper into the viral sequences present in samples (Grozinger & Robinson, 2015; Liu et al., 2014). The generation of large datasets is now helping to progress the field in new and exciting directions, including aiding the description of novel viruses (Shi et al., 2016; van Aerle & Santos, 2017; Bichaud et al., 2014), but brings its own challenges, namely the issue of storage of such large datasets and reliability and reproducibility owing to the fact that standard methods are not yet described, and that researchers share different levels of descriptions of methods and pipelines used. As such, it is important to archive and describe

datasets in such a way to allow their use for reliable collaborative efforts and future use (Engel et al., 2016)

Aims

Due to the importance of the honey bee as our most significant pollinator, considerable research is currently being carried out in the field of honey bee virology, with the increasing availability and affordability of new technologies regularly leading to new breakthroughs. However, there are gaps in our knowledge which need to be addressed in order to understand how DWV and other viruses are evolving, spreading and affecting honey bees and wider insect communities so that we can better deal with the current threats to bee health, and thus the greater ecosystem and also to try to anticipate future threats. Therefore, the aim of this PhD is to fill knowledge gaps in current bee virology research to understand more fully the DWV – *Varroa* – honey bee cycle, expand our knowledge of the circulating virosphere within the apiary, and provide new insights into how DWV is spreading beyond honey bees and into the wider insect community.

More specifically the aims of this thesis are as follows:

1. To understand why it is that only a small proportion of bees infected by DWV develop the characteristic overt symptom of the deformed, shrivelled wings.

A comparative study used next-generation sequencing (NGS) to examine the DWV genomes present in pairs of deformed and asymptomatic bees to identify whether a unique DWV variant could be responsible for causing the development of deformed wings.

2. To understand how a rare *Varroa*-tolerant population of European honey bees survives despite never having been treated for *Varroa*.

The isolated honey bee population on Fernando de Noronha, Brazil, was studied to gain insights into how they maintain their *Varroa* tolerance. A combination of *Varroa* and honey bee counts was used to assess population sizes, followed by RT-PCR and HRM analysis to investigate whether these bees are infected with DWV and, if so, if their infection provides any clues as to how they are surviving when other populations do not.

3. To expand our knowledge of the circulating virosphere in the apiary, using a number of Hawaiian apiaries.

Various insects collected from the apiary were subjected to NGS (specifically RNAseq due to the dominance of RNA viruses in the honey bee virome), and bioinformatics approaches were used to filter and assemble reads and identify whether as yet undescribed RNA viruses could be circulating in the apiary, which may have the ability to emerge in honey bees.

4. To investigate DWV transmission in the apiary between honey bees and their wider insect communities.

Various common apiary pests were sampled from apiaries known to harbour established *Varroa* populations and high DWV loads in the bees. These were investigated using NGS and bioinformatics approaches to ascertain whether they also carry DWV and, if so, whether the same variants are circling in the bees and the other taxa.

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Chapter 1: A comparison of Deformed wing virus in deformed and asymptomatic honey bees.

Abstract

Deformed wing virus (DWV) in association with *Varroa destructor* is currently attributed to being responsible for colony collapse in the western honey bee (*Apis mellifera*). The appearance of deformed individuals within an infested colony has long been associated with colony losses. However, it is unknown why only a fraction of DWV positive bees develop deformed wings. This study concerns two small studies comparing deformed and non-deformed bees. In Brazil, asymptomatic bees (no wing deformity) that had been parasitised by *Varroa* as pupae had higher DWV loads than non-parasitised bees. However, we found no greater bilateral asymmetry in wing morphology due to DWV titres or parasitisation. As expected, using RT-qPCR, deformed bees were found to contain the highest viral loads. In a separate study, next generation sequencing (NGS) was applied to compare the entire DWV genomes from paired symptomatic and asymptomatic bees from three colonies on two different Hawaiian islands. This revealed no consistent differences between DWV genomes from deformed or asymptomatic bees, with the greatest variation seen between locations, not phenotypes. All samples, except one, were dominated by DWV type A. This small-scale study suggests that there is no unique genetic variant associated with wing deformity; but that many DWV variants have the potential to cause deformity.

Introduction

Honey bees with deformed wings have become a universal sign for the presence of deformed wing virus (DWV) in colonies infested by *Varroa destructor* across the world. DWV is reported as the most important honey bee viral pathogen causing the death of millions of colonies across the northern hemisphere (Tantillo et al., 2015; Martin et al., 2012). However, the proportion of honey bees with deformed wings (i.e., symptomatic bees) in a colony is normally low (<1%) despite a high proportion of asymptomatic honey bees being

infected with high viral titres of DWV (Lanzi et al, 2006). This is, in part, due to symptomatic bees dying as pupa or within 48 h of emerging from their brood cell (Yang & Cox-Foster, 2007). Although normally low, up to 66% of individuals can have wing deformity in a severely infected colony (Nordström et al., 1999), but these levels are rarely seen.

Wing deformity was originally believed to be caused by the removal of the developing bees' haemolymph by the mites' feeding activities (De Jong et al., 1982). Although deformed wings can occur due to insufficient nutrition or fluids (Bowen-Walker et al., 1999), there was a noticeable increase in the number of deformed bees associated with *Varroa* infested colonies, which was later linked to the ability of *Varroa* to transmit DWV to developing honey bees (Bowen-Walker et al., 1999). On the isolated island of Fernando de Noronha *Varroa* mites have been feeding on its honey bees for the past 32 years; unique to this population, DWV has remained a low level covert infection. No bees with deformed wings have ever been recorded on the island (De Jong & Soares, 1997; De Mattos et al., 2016; Brettell & Martin, 2017), indicating that the mites' feeding activity does not directly cause wing deformity. However, although both deformed and asymptomatic bees can have very high viral titres ($x10^9$) (Highfield et al., 2009), deformed bees have consistently higher DWV titres than asymptomatic bees (Tentcheva et al., 2004; Chen et al., 2005). It is not unknown for deformed bees to contain lower than expected DWV titres ($x10^4$–$x10^7$) (Forsgren et al., 2012); this is a very rare occurrence and could be a result of a different, external factor. It was suggested by Gisder *et al*. (2009) that the development of deformed wings was due to viral replication within the mite leading to a higher delivery into the bee and that this may not occur in most mites. Furthermore, DWV appeared to be present in the heads of deformed bees but only present in the thorax and abdomen of asymptomatic bees (Yue & Genersch, 2005). However, subsequent studies have found DWV also in the heads of asymptomatic bees, helping explain changes in their behaviour (Fujiyuki et al., 2004) and effects on their learning (Iqbal & Mueller, 2007). However, there is no specific, proven etiology for the disease and the pathogenesis, and cytopathology of DWV has yet to be directly studied. Alternate causes have been suggested: that deformity may arise as a consequence of the bees' immune response to mite feeding (Yang & Cox-Foster, 2005), or that microbial septicaemia occurs as a result of microorganisms transmitted by *Varroa* (Glinski & Jarosz, 1992), but the weight of evidence especially from the Fernando de

Noronha study (Brettell & Martin, 2017), currently does not support these mechanisms (Gisder et al., 2009; Yue & Genersch, 2005; Koch & Ritter, 1991; Alippi et al., 1995).

DWV is a quickly evolving group of closely related viruses (de Miranda & Genersch, 2010), which is commonly referred to as a quasispecies (Domingo & Holland, 1997). This is made up of three master variants. Martin *et al*. (2012) initially classified DWV as being composed of two master variants, type A which consists of DWV and Kakugo virus (KV) (Lanzi et al., 2006; Fujiyuki et al., 2004) and type B which refers to the Varroa destructor virus-1 (VDV-1) which was first isolated from *Varroa* (Ongus et al., 2004), and was suggested to cause wing deformity (Ongus et al., 2004; Zioni et al., 2011). Recently, type C, a third distinct variant, has been discovered in asymptomatic bees collected in Devon, UK (Mordecai et al., 2016a). Both types A and B are associated with disease symptoms (Zioni et al., 2011; McMahon et al., 2016), and are known to form recombinants (Zioni et al., 2011) but the type A variant is more commonly associated with infestation by *Varroa* and subsequent colony collapse (Martin et al., 2012; Wilfert et al., 2016). Conversely, the dominance of type B in a population has recently been shown to prevent the virulent type A becoming established and causing colony losses (Mordecai et al., 2016b).

Recent work in colonies that have never been exposed to *Varroa* have shown that DWV consists of a wide diversity of variants, but that transmission by *Varroa* causes the amplification of dominant DWV variants and a major reduction in the subsequent virus diversity in the honeybee (Martin et al., 2012; Ryabov et al., 2014). Further experimental manipulations have shown that this reduction in variant diversity occurs within the bee, not the mite (Ryabov et al., 2014). These studies were conducted using asymptomatic bees. The quasispecies theory of viral evolution (Domingo et al., 2012) may help to explain why only a small proportion of the bees become deformed, since a particular DWV variant that is able to reproduce rapidly in both mites and bee pupae may exist within the quasispecies infecting deformed bees, whereas a different variant could dominate in asymptomatic bees. Alternatively, the lack of a dominant variant but high viral diversity in asymptomatic bees could be hypothesised as the reason for the lack of development of the deformed wing phenotype, but given the current data this seems less likely.

The aim of this study was to use RT-qPCR, High resolution melt (HRM) and next generation sequencing (NGS) to determine if a particular variant was associated with wing deformity in honey bees parasitised by the *Varroa* mite.

Materials and Methods

Honeybee Samples

For the RT-qPCR viral quantification and wing deformity study, honey bees were collected from an apiary maintained by Universidade Federal do Recôncavo da Bahia (UFRB), Cruz das Almas (12.67° S, 39.1019° W), Bahia state, Brazil. We confirmed that the honey bees from this population were infected by the type A variant of DWV (Supplementary Figure S1) as were the bees from Hawaii (Martin et al., 2012). For the NGS study, we used previously collected samples of honey bees from Hawaii collected in 2012 (as described in Martin et al., 2012). Three pairs of samples were chosen, each consisting of a single deformed bee and a pool of 30 asymptomatic bees from the same colony. Each colony had been exposed to *Varroa* infestations for different lengths of time (Oahu = 5 years, Big Island East = 4 years, Big Island South = 3 years). The rarity of bees with deformed wings in both populations made it impossible to compare similar numbers of symptomatic and asymptomatic bees. The vast majority of adults with deformed wings contain high DWV loads (Chen et al., 2005, this study), whereas DWV titres in individual asymptomatic bees are more variable (Martin et al., 2013), hence we used a pooled sample for the NGS study to ensure sufficient DWV genomes were present for sequencing.

Effect of Viral Load on Wing Deformity

In January 2015, in Brazil, a frame of an emerging brood was removed from three study colonies. Each emerging bee, along with the cell that it was emerging from, was checked for the presence or absence of *Varroa* mites. A total of 45 parasitised and 45 non-parasitised newly emerged worker bees were collected from the three frames. Only bees seen

emerging from a cell were used. However, no emerging deformed bees were found despite 500 bees emerging from the frames. As such, a visual search of the three study colonies was conducted that resulted in just three deformed bees being located. As the vast majority of deformed bees develop from parasitised pupae (Marcangeli et al., 1992), it is likely that these individuals emerged from infested cells, which is supported by the high DWV titres we detected. All bees were killed by freezing at -20 °C before their forewings were removed and mounted on a glass slide for morphometric analysis. Individual bees were then labelled with a unique label and shipped to the UK in a Dry Vapour Shipper at -186 °C for viral analysis. Each forewing (length and width) was measured using a Leica binocular microscope (x10) magnification fitted with a Leica camera. As these were newly emerged bees, no wing wear was present. As directional asymmetry in wing size in honey bees is well established (Schneider et al., 2003; Mazeed, 2011), we measured both wings of the parasitised and non-parasitised groups and compared the results using a Mann–Whitney U test since not all wing measurement distributions were normally distributed.

For the RT-qPCR analysis, a random subset of ten parasitised and ten non-parasitised newly emerged asymptomatic bees were chosen along with the three deformed bees. Then each of the 23 individual bee samples was ground in liquid Nitrogen to a fine homogeneous powder and 30 mg material used for RNA extraction using the RNeasy mini kit (Qiagen, Venlo, The Netherlands), according to the manufacturer's instructions. Total RNA samples were quantified using a Nanodrop 8000 (Thermo Scientific, Waltham, MA, USA). One microgram of isolated RNA was treated with DNase I (Promega, Madison, WI, USA), followed by Nanodrop quantification to standardise the amounts of total RNA to 25 ng/µL, before storage at -80 °C.

Total RNA was analysed in duplicate for each sample using the one-step SensiFAST SYBR No ROX One-step kit (Bioline, London, UK). RT-qPCR reactions contained 50 ng RNA, 1x SYBR one-step Sensimix, 2.5 mM MgCl2, 5 units of RNase inhibitor, and 7.5 pmol of each primer: DWVQ-F1 and R1 for DWV (primers bind within the RdRp gene) with Actin F1 and R1 as the reference gene (Highfield et al., 2009) (Supplementary Table S1). Reactions were run on a Rotor-Gene Q Thermocycler (Qiagen) with an initial reverse transcription stage at 49 °C for 30 min and a denaturation step of 95 °C for 10 min, followed by 40 cycles of denaturation

for 15 s at 95 °C, annealing for 30 s at 54 °C for DWV, and 58 °C for Actin, and extension for 20 s at 72 °C. The SYBR green signal was measured on the green channel after each extension step. A final dissociation melt curve was performed between 65 °C and 95 °C, at 0.5 °C increments, each with a 10 s hold and acquisition to the green channel. The melt curve was used to ensure that a single targeted product was amplified, and that no contamination was present in the reverse transcription negative controls or in the no-template controls. The threshold cycle (C_t) value was determined for each sample using the QIAGEN Rotor—Gene Q Series Analysis software. All samples were run in duplicate and the average was taken. Those samples which had a standard deviation of ≥1 C_t were re-run to obtain duplicates with standard deviation <1 C_t. Each sample was normalised against Actin, and then presented relative to the asymptomatic non-parasitised bees as $\Delta\Delta C_t$ values. Statistical differences were calculated using a pair wise Mann–Whitney U test when the data were not normally distributed.

Next Generation Sequencing, Assembly and Data Normalisation

RNA was again extracted from 30 mg of material from each of the six Hawaiian samples (three colonies that each contained a single deformed bee and 30 asymptomatic bees) using the RNeasy mini kit (see above). Total RNA was used for a cDNA amplification step using oligo dT priming followed by sequencing. Illumina sequencing (Hi-Seq 100 bp paired end reads) was carried out by The Earlham Institute, Norwich. A Bioinformatics pipeline designed to accommodate the large amount of variation found within DWV, first described in Mordecai *et al*. (2016b) was applied. This involved using reads which mapped to a custom BLAST database of DWV master variants type A (NC_004830.2 and Kakugo virus NC_005876.1), B (AY251269.2) and C (CEND01000001.1) database using an e value of 10e−05 to assemble DWV-like contigs using VICUNA which was specifically developed to deal with highly variable data. Read data were uploaded to the NCBI Sequence Read Archive under study number SRP095247.

Viral contigs were imported into Geneious (Version 7.04, created by Biomatters, Aukland, New Zealand) and the “Map to Reference tool” was used to align the contigs against the

DWV type A (NC_004830.2), B (AY251269.2) and C (ERS657949) reference genomes. These contigs were used to assess the breadth of genome coverage as well as to phylogenetically analyse the dominant variants in each sample. The phylogenetic trees were created within Geneious (Version 7.04, created by Biomatters) using a Tamura-Nei Genetic Distance model and a neighbor joining tree building method. In order to ensure that the contigs produced truly represented the viral populations, Geneious competitive alignments were performed in which the raw sequencing read files in FASTA format were competitively aligned against DWV types A, B and C reference genomes (allowing for 5% mismatches and no gaps with reads with multiple best matches being discarded) to produce coverage graphs for reads corresponding to each type.

Results

Viral Quantification and Wing Deformity in Honey Bees

All 23 individual bees from Brazil tested positive for DWV using RT-qPCR. HRM analysis and Sanger sequencing (carried out by Source Bioscience, Warrington, in the forward direction using the DWVQ_f1 primer (Highfield et al., 2009), indicated that all bees were dominated by the DWV type A (Supplementary Figures S1 and S2). The highest loads were consistently detected in the three bees with wing deformities (Fig. 1b). This was followed by asymptomatic bees that had been parasitised by *Varroa* mites as pupae. The lowest DWV loads were detected in asymptomatic bees that had developed free from *Varroa* (non-parasitised bees). Due to the low viral load in the non-parasitised bees, primer dimer was also amplified along with the DWV RdRp diagnostic fragment and this would have led to an overestimate in viral load, so the actual amount may be lower than shown. Despite these significant differences in DWV load between the three groups, their wing morphology did not follow the same trend. The bees with deformed wings had the highest viral load, as expected. However, no significant differences in wing length (Left wing, $U = 668$, $Z = 1.21$, $p = 0.22$: Right wing, $U = 709$, $Z = -1.53$, $p = 0.12$) or wing width (Left wing, $U = 694$, $Z = 0.91$, $p = 0.36$: Right wing, $U = 871$, $Z = 0.28$, $p = 0.78$) were found (Fig. 1c,d) between the asymptomatic, non-parasitised and parasitised groups of bees. In both groups, directional

asymmetry was detected in wing length but not wing width. That is, the bees' left wing was significantly longer than their right wing in both the non-parasitised (U = 493, Z = 3.71, *p* = 0.0002) and *Varroa*-parasitised (U = 402, Z = −3.52, *p* = 0.0004) groups (Fig.1c).

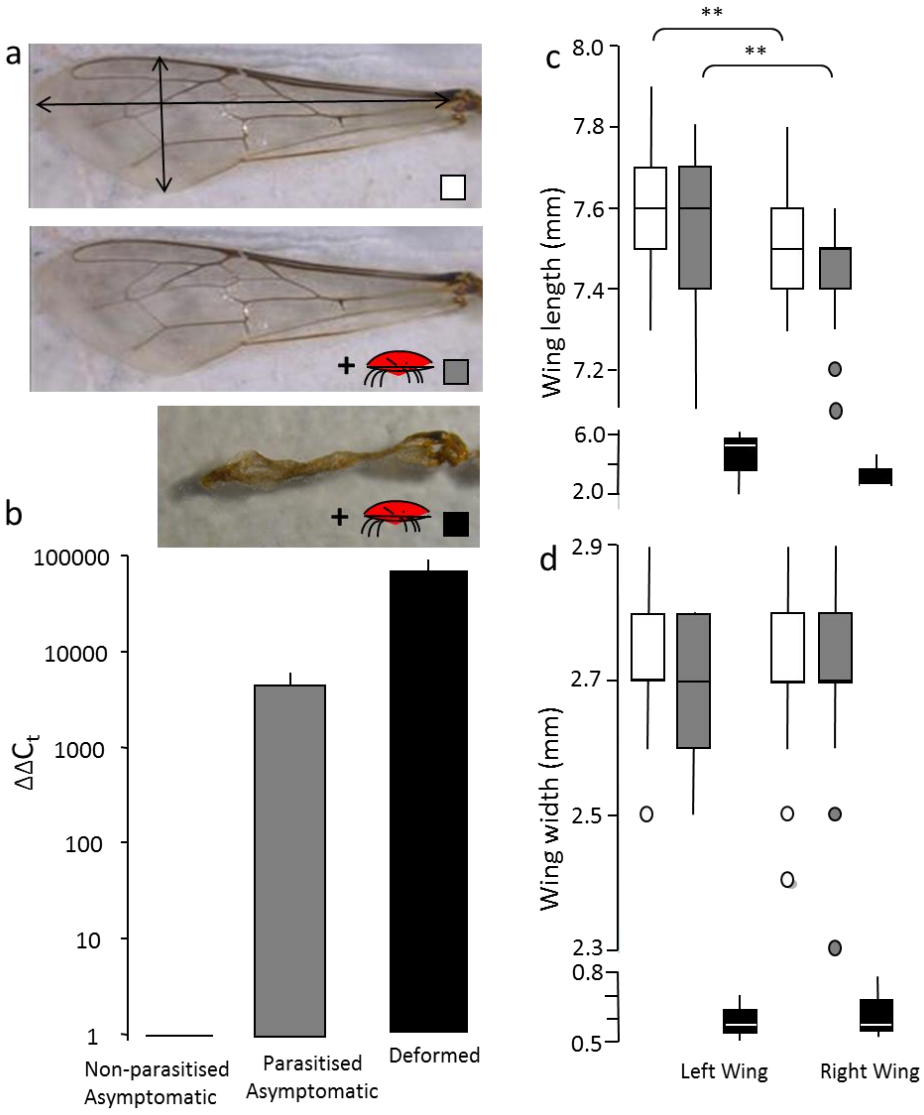

Figure 1. a) Shows images of a normal forewing from a non-parasitised, parasitised asymptomatic, and a deformed honey bee. b) Deformed wing virus (DWV) load of non-parasitised, parasitised asymptomatic, and deformed bees quantified by DWVQ RT-qPCR. Delta delta Cycle threshold (C_t) values normalised against an actin control gene (Highfield et al., 2009) and relative to the non-parasitised asymptomatic bees, shown on a log scale c) wing length and d) width of 45 non-parasitised (clear box blots), 45 parasitised (grey box plots) and three bees with deformed wings (black box plots). Note the broken axis to deal with the large size differences between deformed and normal forewings. ** $p <$ 0.001 between right and left wings.

Next Generation Illumina Sequencing

Each of the six samples from Hawaii contained sufficient DWV reads to assemble into contigs that together covered the entire length of the genome (Fig. 2). Five of the six samples were dominated by type A reads that yielded full genome coverage. However, a single asymptomatic sample from Big Island East was found to be dominated by type B reads that yielded full genome coverage of the type B genome (Fig. 2). The breadth of type B coverage is less in the samples from Oahu where *Varroa* has been present for a long time where the type A variant dominates. The read depth coverage graphs for all samples showed a strong 3' bias which can be attributed to the inherent 3' bias of reverse transcription produced using oligo dT priming in the library preparation (Brooks et al., 1995). The plots confirmed that the asymptomatic sample from Big Island East was the only one that contained type B read coverage across the entire genome and that all others contained type B coverage at the 3' end only (Fig. 2). The samples from Oahu did contain low amounts of type B reads; however there was insufficient read depth for the assembler to produce contigs. The asymptomatic sample from Oahu contained a small number of DWV type C reads (n = 359). Although type C was not the dominant variant, coverage was sufficiently high to assemble contigs spanning the majority of the genome. Very low numbers of type C reads were found in other samples by counting reads unambiguously mapping to type C and were used when normalising DWV variants to actin (Fig. 2). However the read depth was insufficient to generate type C contigs in any other sample. However, it is impossible to rule out that the DWV type C identified in this study is a result of contamination via barcode shifting originating from samples dominated by type C run on the same flow cell lane (see Mordecai et al., 2016a), as evidenced by the divergent 3' bias in the type C read density for HB_S67 (Fig. 2); we suggest the small number of sequence differences in the HB_S67 assembled contigs are significantly distinct compared to those in Mordecai *et al*. (2016a) (Supplementary Table S2). However, the presence of small amounts of type C in one sample is interesting but does not influence the findings of this study.

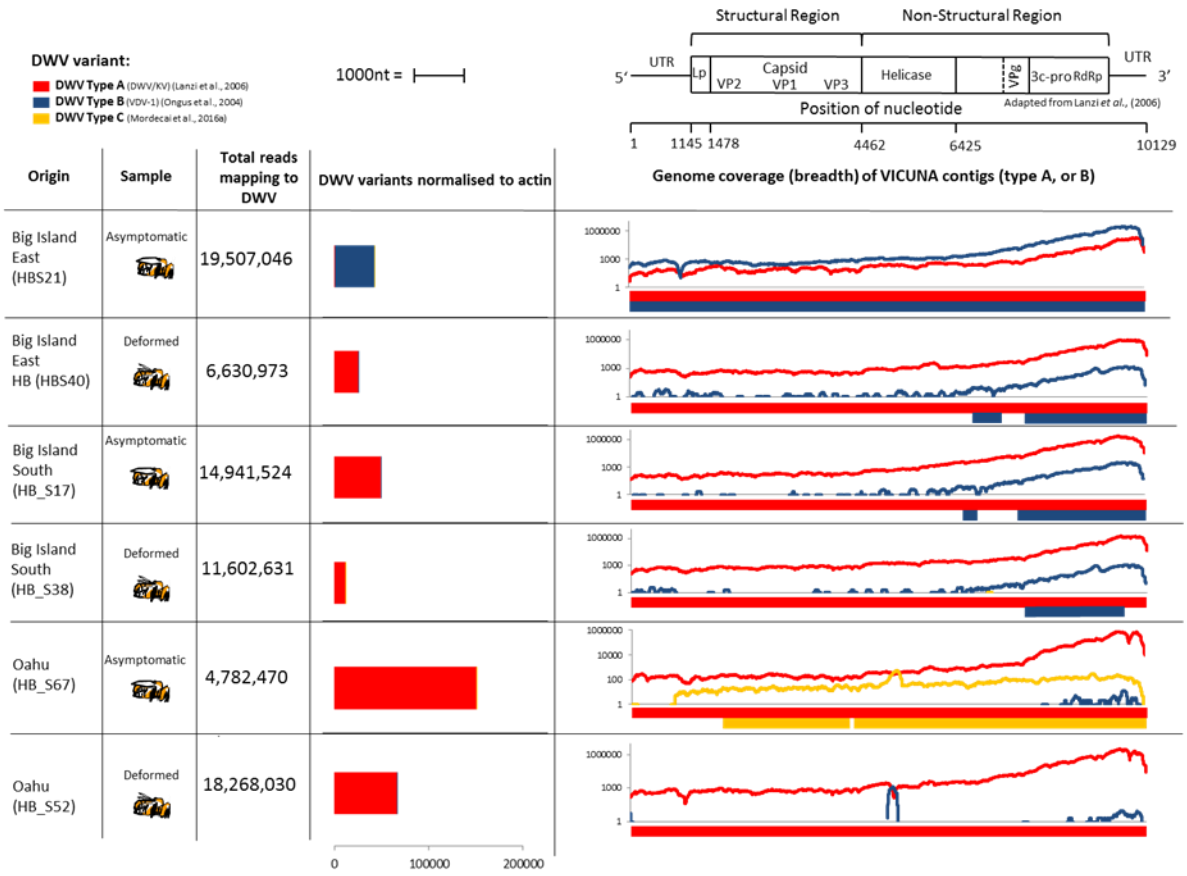

Figure 2. Genome coverage from the Illumina Hi-Seq data for the Hawaii colonies including a map of the DWV genome adapted from Lanzi *et al*. (2006). DWV type A, B and C genomes (in red, blue and yellow respectively) were assembled from the Illumina next generation sequencing (NGS) data from honeybees from Hawaii. DWV load was normalised to actin. Breadth of genome coverage by Vicuna contigs is shown against the DWV genome for type A, B and C variants, as well as individual competitive alignment read depth coverage plots.

Using the NGS data, both types A and B DWV Vicuna contigs were aligned across the entire genome to look for differences that correlated with deformity. Despite this unprecedented level of detail, there were no regions of the genome where all three deformed or asymptomatic samples grouped together. Neighbor-joining trees were created to examine the phylogeny of DWV variants sequenced and assembled using three

regions of the DWV genome: a 4360 bp region spanning the majority of the non-structural block including Helicase and 3C protease, and the majority of the RdRp gene (Fig. 3a); and two further regions, both 145 bp in length, that represent a portion of the RdRp gene (Fig. 3b and Supplementary Figure S3a) and the Capsid region (Fig. 3c and Supplementary Figure S3b). The DWV variants are split into the three master sequences; types A, B and C. The phylogenies show that within the type A clade, deformed and asymptomatic samples from the same site never share the same dominant variant of DWV. The low amount of genetic diversity within the type B clade can be attributed to the low viral load.

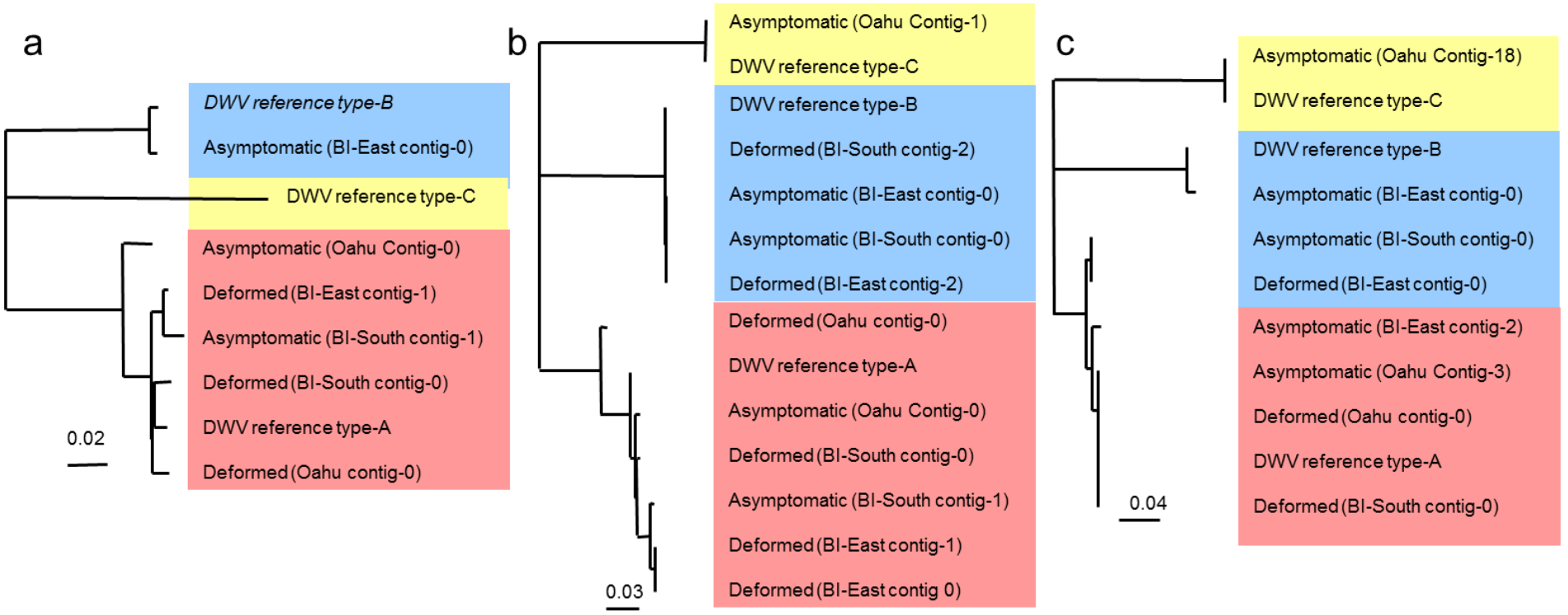

Figure 3. Phylogeny (neighbor joining) of type A, B and C Vicuna contigs covering the a) 4360 bp region spanning the majority of the non-structural block; b) RdRp region (Highfield et al., 2009) and c) Capsid region. DWV type A, B and C sequences are highlighted in red, blue and yellow respectively. The low diversity of type B sequences can be attributed to a low viral load. At no gene location do the deformed and asymptomatic form groups. BI = Big Island.

Although, within each location, the sequences differed for deformed and asymptomatic samples, the variation between colonies is always greater than that within each colony i.e., deformed vs. asymptomatic. Interestingly, the asymptomatic Big Island (East) sample produced an RdRp contig which contained elements of both A and B variants (Supplementary Figure S3a, HB_S21 contig 1) indicating a possible recombination between variants. This was removed prior to creating the phylogeny (Fig. 3b). Another possible A–B recombinant was also observed in the deformed Oahu sample within the Helicase gene (Fig. 2). However, we could not confirm the precise recombination junction site due to the lack of specific reads covering this region.

Discussion

Although it is well known that DWV can cause wing deformity in infected individuals, the reason for those symptoms affecting only a small proportion of infected individuals remains poorly understood. Morphometric analysis carried out in this pilot study revealed wings to be either deformed or not (asymptomatic) with no intermediate phenotype. Despite a significant increase in DWV type A load detected using RT-qPCR, no significant differences in wing length were seen between bees that had been parasitised as pupae and those which had not. Furthermore, directional asymmetry is common in honey bees (Schneider et al., 2003; Mazeed, 2011; Smith et al., 1997, this study), and flies (Klingenberg et al., 1998), and is commonly regarded as a sensitive indicator of developmental perturbation (Smith et al., 1997). However, directional asymmetry was not affected by an increased DWV load as might be expected.

The *Varroa* mites' ability to act as a vector and host of DWV (Yue & Genersch 2005; Bowen-Walker & Gunn, 1998), by providing an alternative transmission route; directly inoculating virus particles into the haemolymph, helps explain the higher DWV load in parasitised bees relative to non-parasitised bees (Yang & Cox-Foster, 2007; Ryabov et al., 2014). The presence of DWV in the non-parasitised group indicates that an active non-mite transmission route must also be present, most likely via horizontal transmission (the

brood food) (Yue & Genersch, 2005) and/or by vertical transmission from queen to egg (Yue et al., 2007). *Varroa* may indirectly impact this "non-mite" transmitted DWV population by increasing the amount of DWV circulating within the honey bee population. A previous study by Teixeira *et al*. (2008) found DWV in approximately 20% of adult bees' abdomens in Brazil, while in 2015, 100% of individuals were positive for DWV [this study]. An increase in Brazilian bees with deformed wings has not been reported, despite colonies hosting mite populations of up to 3500 (Medina et al., 2002). Mites entering brood cells normally have very low DWV loads relative to those leaving cells 12 days later (Martin et al., 2013) potentially as a result of viral replication within the mite (Gisder et al., 2009). However, DWV is continually passed between the bee and mite during regular bouts of feeding (Bowen-Walker & Gunn, 1998) and potentially replicates in both. The host in which predominant amplification occurs remains unclear. A recent study demonstrated the absence of non-structural and high abundance of structural proteins in *Varroa*, suggesting that DWV proteins accumulated in the gut after feeding and not as a result of viral replication in the mite (Erban et al., 2015). However, studies using FISH (Fluorescence in situ hybridization) probes or immunohistochemical techniques may help resolve this uncertainty. At the present time, it is difficult to say whether the high viral load in bees is a symptom of being parasitised by a mite carrying a high viral load, or if the mites have a high viral load because of a high level of viral replication in the bee on which the mite is feeding. As a result, there is no clear explanation for why only a small proportion of parasitised bees develop deformed wings. A study by Bowen-Walker *et al*. (1999) found that when transferring mites from pupae which developed deformed wings to new host pupae, the majority but not all of the new pupae went on to develop deformity. Further experiments are required to repeat this work.

We hypothesised that a specific variant within the quasispecies causes deformity through either an increased ability of specific sequences to replicate in the bee, or potential tissue tropism of certain variants. Analysis of NGS data showed there to be no consistent differences between deformed and asymptomatic bees in terms of the dominant DWV consensus genomes. In addition, inter-colony variation was always larger than intra-

colony variation i.e., between deformed and asymptomatic bees. However, as previously reported (Lanzi et al., 2006; Berényi et al., 2007), the DWV infection of deformed bees was always dominated by the type A master variant. This suggests that a "deformed phenotype variant" of type A is unlikely since it is also present in asymptomatic bees. We suggest that the alteration of the DWV variant landscape (e.g., by *Varroa*), which differs from that already present in the hive may result in disease progression. Recombinants of types A and B have previously been proposed to result in a virulent infection (Ryabov et al., 2014), however this was not seen here and has not since been shown in other populations. We did find recombinants between types A and B; however they were not dominant in the samples. Their low load, and resulting low genetic diversity, as observed in the phylogeny, indicates a low level of replication and thus virulence. Although no clear link between DWV genomes and deformity was detected, Vicuna consensus contigs within the RdRp segment were consistently different in each pair of samples from each location (Fig. 3b and Supplementary Figure S3a), suggesting that many variants have the potential to cause wing deformities. The Fernando de Noronha study (De Mattos et al., 2016) has helped support ideas that wing deformity is not caused by the direct effects of mite feeding or haemolymph extraction, and this study failed to find any unique DWV variant linked with deformity. So currently, the only consistent factor associated with deformed wings is the high DWV load, but it remains unclear if the high load causes deformity or results from another factor that initially causes the deformity. As such, future work is needed on a larger scale with the investigation of additional considerations to ascertain the influence of other factors on the development of deformity.

Conclusions

These two pilot studies aimed to ascertain whether there was a specific DWV variant within the replicating quasispecies which was associated with the development of the deformed wing phenotype. Using a combination of NGS, RT-qPCR and HRM, we confirmed that DWV type A dominated in all samples although types B and C, as well as A/B and A/C recombinants were also found to be replicating at lower levels. Significantly, there was no clustering between deformed samples and asymptomatic samples,

indicating that no unique DWV variant is associated with wing deformity. Furthermore, we found that neither DWV load nor dominant variant correlated with wing asymmetry which might have been expected given the fact that wing asymmetry is often used as an indicator of developmental perturbation. This study indicates that no specific genomic pattern of DWV can be used in predicting wing deformities in honey bees.

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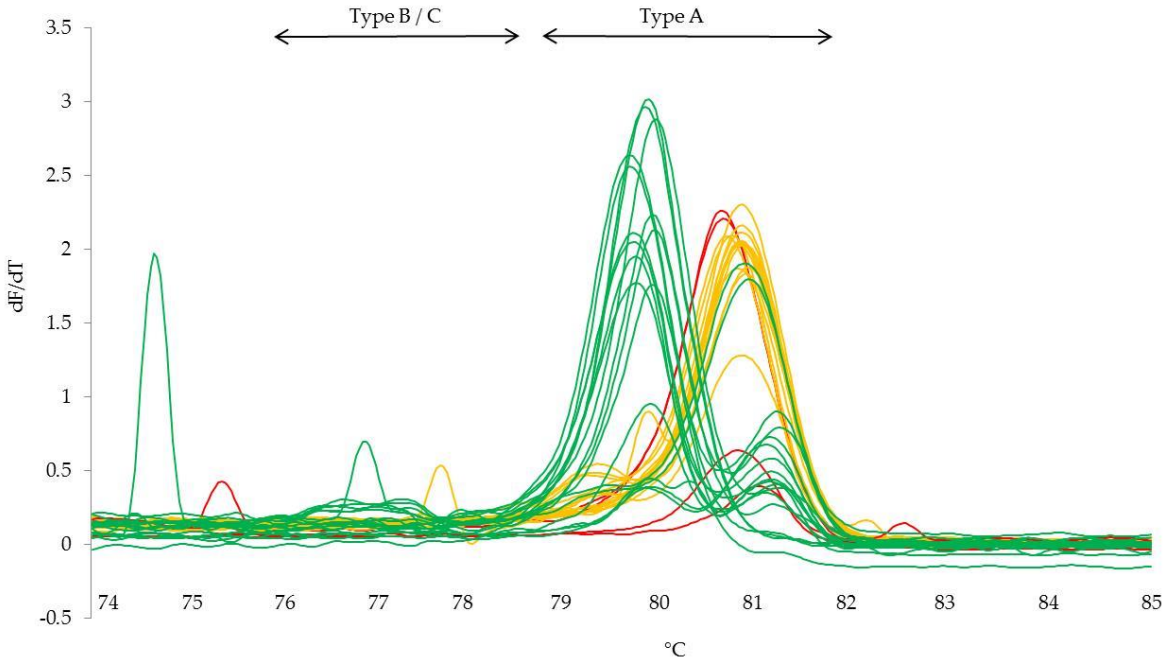
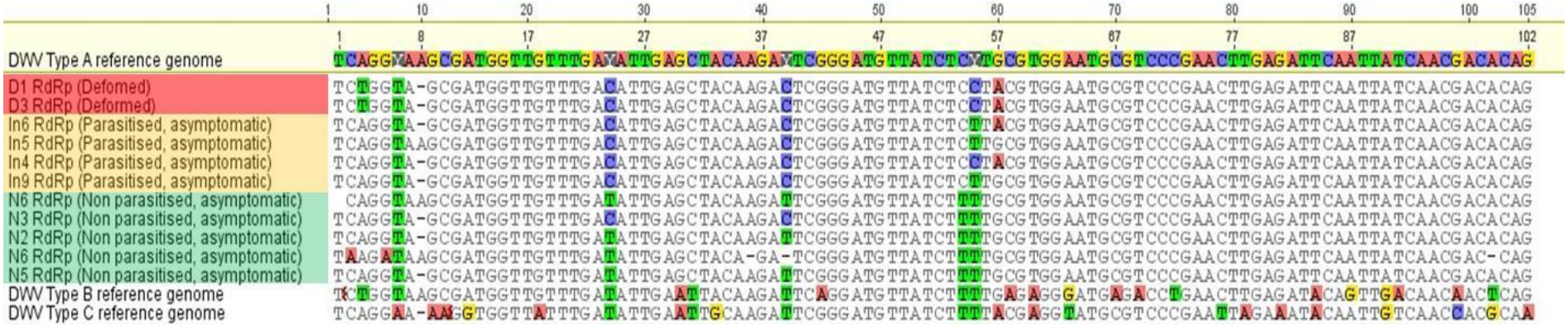
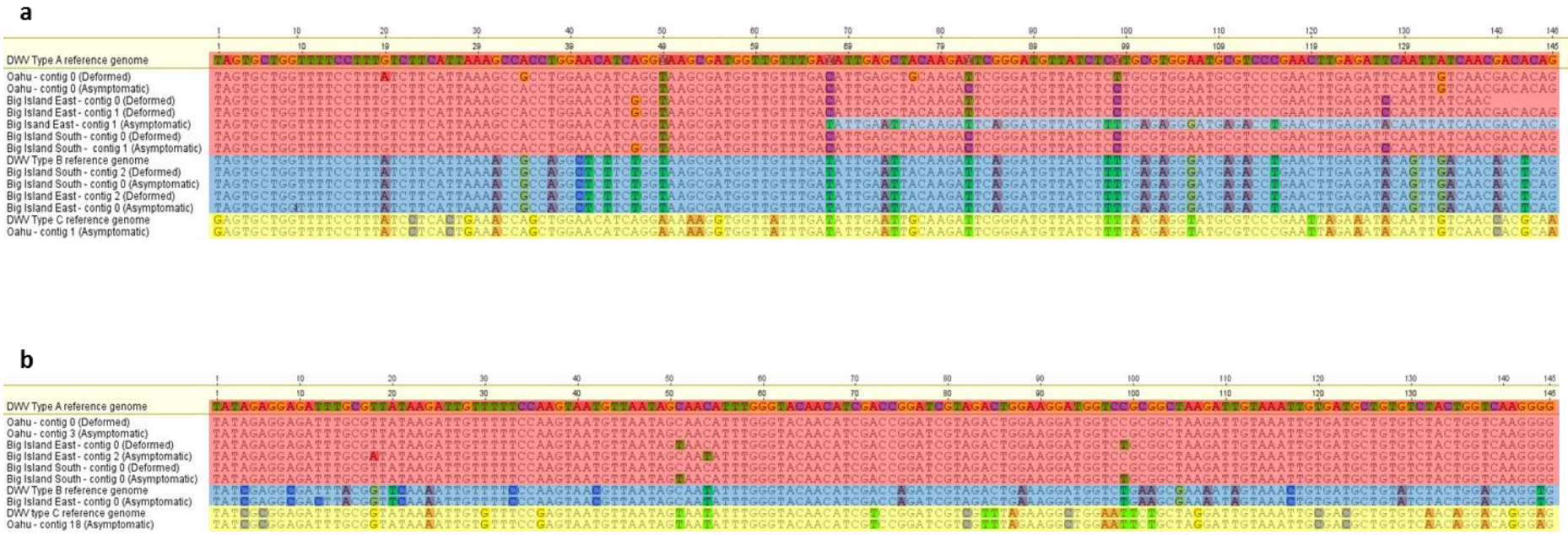
Supplementary Information



Supplementary Figure S1: High Resolution melt plot of DWV RdRp RT-PCR products amplified from Brazilian deformed bees (red) and asymptomatic bees from cells infested with (yellow) and without *Varroa* (green). All melt peaks are in the predicted DWV type A variant region (78.5 °C – 82 °C (Mordecai et al., 2016b).



Supplementary Figure S2: Multiple sequence alignment (Geneious 8.1.7, Biomatters) of sequenced RdRp RT-PCR products amplified from Brazilian bee samples and used for HRM analysis (Supplementary Figure S1) mapped to DWV type A. Deformed bee samples are highlighted red, asymptomatic bees from cells infested with *Varroa* are yellow and without *Varroa* are green, as in Supplementary Figure S1. Nucleotides that differ to the reference sequence are highlighted. Sequences are also included for the Type B and Type C genomes for comparison.



Supplementary Figure S3: Multiple sequence alignment of DWV Type A (NC_004830.2), B (AY251269.2) and C (ERS657949) reference sequences with contigs assembled by Vicuna in the a) RdRp region and b) Capsid encoding region of the DWV genome. Nucleotides that differ to the DWV type A reference sequence are highlighted. DWV type A, B and C sequences are highlighted in red, blue and yellow respectively; Table S1: The primers used for RT-qPCR in this study; Supplementary Table S2: Degree of similarity calculated using a global alignment between the DWV type C genome previously described and the type C contigs assembled from sample HB_S67 in this stud y.

Supplementary Table S1. The primers used for RT-qPCR in this study, taken from Highfield *et al.* (2009).

Target	Primer name	Sequence (5'-3')
DWV (RdRp region)	DWVQ_F1	TAGTGCTGGTTTTCCTTTGTC
	DWVQ_R1	CTGTGTCGTTGATAATTGAATCTC
Actin	Actin_F1	CCTGCAATCGCAGATAGAATGC
	Actin_R1	AAGAATTGACCCACCAATCCATAC

Supplementary Table S2. Degree of similarity calculated using a global alignment between the DWV type C genome previously described (Mordecai et al., 2016a) and the type C contigs assembled from sample HB_S67 in this study using VICUNA.

Contig #	Similarity
dg-29	99.52%
dg-10	100%
dg-8	100%
dg-14	99.33%
dg-7	100%
dg-5	100%
dg-18	100%
dg-0	99.38%
dg-3	99.72%
dg-1	99.91%

generated by VICUNA.

Chapter 2: Oldest *Varroa* tolerant honey bee population provides insight into the origins of the global decline of honey bees.

Abstract

The ecto-parasitic mite *Varroa destructor* has transformed the previously inconsequential Deformed Wing Virus (DWV) into the most important honey bee viral pathogen responsible for the death of millions of colonies worldwide. Naturally, DWV persists as a low level covert infection transmitted between nest-mates. It has long been speculated that *Varroa* via immunosuppression of the bees, activate a covert infection into an overt one. Here we show that despite *Varroa* feeding on a population of 20-40 colonies for over 30 years on the remote island of Fernando de Noronha, Brazil no such activation has occurred and DWV loads have remained at borderline levels of detection. This supports the alternative theory that for a new vector borne viral transmission cycle to start, an outbreak of an overt infection must first occur within the host. Therefore, we predict that this honey bee population is a ticking time-bomb, protected by its isolated position and small population size. This unique association between mite and bee persists due to the evolution of low *Varroa* reproduction rates. So the population is not adapted to tolerate *Varroa* and DWV, rather the viral quasispecies has simply not yet evolved the necessary mutations to produce a virulent variant.

Introduction

The ecto-parasitic *Varroa destructor* mite in combination with its associated viruses, most notably Deformed Wing Virus (DWV) has been associated with the death of millions of European honey bee colonies (*Apis mellifera*) across the world (Schroeder & Martin, 2012; Genersch, 2010). The *Varroa* mite, initially a pest of the Asian honey bee (*Apis cerana*) jumped the species barrier in the 1950s and subsequently spread around the world facilitated by modern beekeeping practices (Wilfert et al., 2016; Villalobos, 2016), where it

has changed the viral landscape in honey bee populations by transforming DWV from a relatively harmless pathogen present in low prevalence and titre to a deadly virus now present in large amounts in virtually every *Varroa* infested colony in the world (Schroeder & Martin, 2012; Martin et al., 2012; Carreck et al., 2010).

In the absence of DWV, a very large mite population are required to kill a mature honey bee colony (Martin, 1998) due to haemolymph removal during feeding. Since healthy colonies in South Africa have been reported to regularly maintain mite populations of 30,000-50,000 (Allsopp, 2006). Whereas, in the presence of DWV the number of mites needed to kill a mature colony becomes vastly reduced to just a couple of thousand mites (Carreck et al., 2010; Martin, 2001; Delaplane & Hood, 1997). This is due to the premature death of both developing honey bee brood (Bowen-Walker et al., 1999) and adult bees (Dainat et al., 2012) that become infected with DWV via the feeding activities of *Varroa* (Bowen-Walker et al., 1999). This causes the colony to enter a downward spiral of bee losses, ultimately resulting in the collapse of the colony. However, colony losses only start to occur several years after the mites' arrival. During this period both DWV prevalence and load increase accompanied with a loss of viral diversity (Martin et al., 2012; Ryabov et al., 2014). However, the mite's initial role in DWV transmission and amplification in the bee remains conjecture. The two main theories are; the mite's feeding activity activates covert virus within the host (Denholm, 1999; Gregory, et al., 2005; Shen et al., 2005; Yang & Cox-Foster, 2005) or the mite has to encounter and feed on a bee (adult or brood) which is suffering from an overt infection. This allows the feeding mite to become infected with sufficient DWV particles (including virulent variants) that allows the infection to be passed to another bee, thus establishing a new viral transmission route (Schroeder & Martin, 2012). These two theories still persist as it is exceedingly difficult for studies to separate out the effects of the bee, mite or virus, since bees or mites cannot be easily reared in large numbers on artificial media to ensure they are guaranteed 100% viral free.

It is now well established that *Varroa* has transformed DWV from an inconsequential virus to the most important honey bee viral pathogen but the nature of the interaction is still the

subject of much contention. Under laboratory conditions the feeding activity of *Varroa* has been shown to increase the viral load in the developing bee (Nazzi et al., 2012), which numerous studies have attributed to a *Varroa*-induced down-regulation of host immune gene transcription (Shen et al., 2005; Gregory et al., 2005). Yang and Cox-Foster (2005) suggested this is a response to bacterial infections introduced at the mites' feeding site, however Nazzi et al. (2012), put it down to DWV replication itself. Conversely other studies found honey bee viral infections to have little or no effect (Kuster et al., 2014; Azzami et al., 2012), or even cause an up-regulation of immune genes (Gregorc et al., 2012). Part of this confusion may lie in the fact that many studies focus on immune genes known to be active against bacterial infections, rather than antiviral immunity, which is more complex (e.g. involving virus-derived small interfering RNAs and piwi-interacting RNAs) and currently is poorly understood (Evans et al., 2006; Marques & Imler, 2016).

Prior to the global spread of *Varroa*, DWV was associated with death in a very small number of honey bee colonies. The virus was detected in dead adult bees from Britain, South Africa (Allen & Ball, 1996) and Belize (Brenda Ball personal communication), but these events were very rare considering the large numbers of dead bees sent to Rothamsted Research centre for viral testing by Bailey & Ball between the 1960s and 1990s. In each case where DWV was detected the viral loads were very high ($>10^8$ particles/bee) i.e. an overt infection, as this is the detection limit of the ELISA methods used to detect honey bee viral pathogens. Furthermore, a study in Hawaii of 341 colonies discovered one *Varroa* free colony that had overt DWV titres (Martin et al., 2012) the following year this remote colony was dead. Therefore, DWV can emerge as an overt infection in the absence of *Varroa*, but this is a rare event.

The initial aim of the study was to discover how the oldest *Varroa* tolerant European honey bee (*Apis mellifera ligustica*) population in the world has survived with *Varroa* for the past 32 years. This bee population was established in 1984 on the remote Brazilian island of Fernando de Noronha (De Jong & Soares, 1997) where the 20-40 managed colonies have never been treated for *Varroa* and no unexpected colony deaths have ever been reported.

This honey bee population has maintained a consistently high *Varroa* infestation level (De Jong & Soares, 1997; De Mattos et al., 2016) with seemingly no effect on the honey bees. This provides a perfect opportunity to study mite tolerance mechanisms and impact of DWV, if present. So specifically, we investigated the presence or influence of DWV and how the stable *Varroa* population is maintained.

Methods

Mite & honey bee samples

The island of Fernando de Noronha in North-eastern Brazil (S 3° 50' 47.5''; W 32°25' 40.8''), lies 350km from the mainland and has a tropical climate. All honeybee colonies are maintained in Langstroth hives in various combinations of one or two brood boxes and one or two supers as their size dictates. No queen excluders are used and colonies are allowed to swarm and re-queen themselves naturally. During the past 32 years the island population has been confirmed using mitochondrial DNA and isozyme analysis (De Jong & Soares, 1997) as belonging to *Apis mellifera ligustica*. This is further supported by their very mild temperament and yellow colour.

During the first visit (1-7 July, 2015), 13 of the 20 managed colonies across the three apiaries on island were sampled. From each colony 40-100 adult bees were collected from the brood comb of each colony. These were used to roughly estimate the *Varroa* infestation level and determine the presence or absence of DWV (see below). In addition, from 10 colonies approximately 25 drone (if present) and 50 worker sealed brood cells were opened to determine the proportion of *Varroa* infested cells. The aim of the second visit (18-23 May, 2016) was to obtain further samples for DWV analysis and detailed data on *Varroa*'s ability to reproduce in both drone and worker cells. Therefore, six colonies in the apiary maintained by Lidia Albuquerque were chosen at random. For each, a photograph of every brood frame was taken to allow an estimation of colony size using methods from Calis *et al*.

(1999) and Martin (1998). Approximately 100 bees from a brood frame were collected for viral analysis and determining the proportion of phoretic mites. Lastly a single frame that contained sealed worker brood older than 7 days, where possible, was removed for the *Varroa* reproductive study.

Detection of DWV

All adult honeybees were killed by freezing, and transport to the laboratory using a Dry Vapour Shipper that maintains the samples at -186°C, prior to long term storage at -80°C.

Pools of 20 adult honeybees were taken per colony and searched for mites. Where found, these were removed and stored separately. Pooled bees and two pools of mites, one removed from sealed brood and the other removed from adult hive bees were crushed to a fine homogenous powder in liquid Nitrogen using a sterile pestle and mortar. 30mg of powder was used for RNA extraction. Remaining bee powder was stored at -80°C. Total RNA was extracted using the RNeasy mini kit (Qiagen) following manufacturers' instructions eluting in 30µl RNase free water followed by quantitation using a Nanodrop 8000 (Thermo Scientific). Samples were then standardised to 50ng/µl. cDNA was synthesised from 200ng RNA using the Quantitect Reverse Transcription kit (Qiagen) following manufacturers' conditions. The resulting cDNA was then used for PCR and HRM analysis which was performed using the SensiFAST HRM kit (Bioline). Reactions contained 1× HRM mix, 7.5pmol each primer (DWVQ R1 and F1 (Highfield et al., 2009)) and 4ul cDNA in a final volume of 20µl. Reactions were run on a Rotor-gene Q Thermocycler (Qiagen) using an initial denaturation of 95°C for ten min, followed by 40 cycles of 95°C for 15 sec, 54°C for 10 sec and 72°C for ten sec. A HRM curve was produced by a final dissociation step rising from 65°C to 95°C with 0.1°C increments acquiring to the HRM channel. HRM PCR products were run on a 2% agarose gel stained with 0.001% GelRed to confirm the correct sized band had been amplified. Fragments were excised from the gels using a sterile scalpel and PCR products cleaned up using the Zymoclean gel DNA recovery kit (Zymo) followed by Sanger sequencing using the DWVQ forward primer by Source Bioscience (Rochdale). Electropherograms were inspected using FinchTV and converted to FASTA files which were aligned to DWV types A

(NC_004830.1), B (AY251269.2) and C (ERS657949) using Geneious v8.1.2 (Biomatters). qRT-PCR reactions were also carried out to amplify the actin control gene in all samples using the SensiFAST SYBR No Rox One-step kit (Bioline). Reactions contained 50ng RNA which had been DNase treated using a RQ1 Dnase 1 kit (Promega), 7.5 pmol each primer (actin f1 and r1 (Highfield et al., 2009)), 1x SYBR onestep sensimix, 5 units of RNase inhibitor and 0.2µl Reverse transcriptase. DWV loads calculated from RT-PCR and HRM data relative to actin expression (qRT-PCR) were then calculated as ΔCts and compared to a known DWV positive newly emerged bee which had developed free of *Varroa*.

Varroa reproduction measurements

Each frame had all sealed drones cells (if present) and between 128-236 worker cells aged Pink-eyed or older (140 hours post-capping) carefully opened under a x5 binocular microscope using watch-maker forceps. If a cell was infested by *Varroa* the entire cell contents were removed onto a microscope slide using a fine 'wetted' paint brush. The development stage of each bee and any mites present were determined using the ontogenic development charts (Martin, 1994; Dietemann et al., 2013; Martin, 1995). In addition, the female deutonymphs were classified into 4 stages (small & medium mobile; large immobile; moulting) using Figure 11 in Dietemann *et al*. (2013) as a guide. Moulted skins of the male and females were used to confirm the presence of a new adult male or female within the cell. The moulted skins and development age of each mite offspring allows the reconstruction of each mite family so mortality rates and other reproductive behaviours can be determined (Martin, 1994; 1995). Cells containing a single mite family were analysed separately from all cells invaded by two or more mites. Several key reproductive factors, egg number, development times, mortality rates and number of viable adult female offspring produced are calculated and compared with previous studies conducted in European (Martin, 1994; Rosenkranz & Engels, 1994; Guzman-Nova et al., 1996) and Africanised honey bees (Medina & Martin, 1999; Medina et al., 2002; Martin & Kryger, 2002, Boot et al., 1997) that critically used the same methodology.

Results

DWV analysis

Surprisingly DWV was detected in honey bees on Fernando de Noronha using both highly sensitive High resolution melt (HRM) analysis, Agarose Gel Electrophoresis and Sanger sequencing of purified gene fragments in five out of twelve colonies sampled in July 2015, and four out of six sampled in May 2016. qRT-PCR of actin gene fragments showed all samples to contain intact RNA (C_t = 18.15, S.D. = 2.45) (Supplementary Table S1). DWV loads were at the borderline of detection limits (Fig. 1a, Supplementary Table S1), far below the level where accurate quantification was possible and considerably lower than the positive control, which was a DWV positive, newly emerged bee which had never been parasitised by *Varroa,* which is considerably lower than what occurs in a mite parasitised bee. Replicate samples did not always provide consistent results due to the very low DWV loads present i.e. at the limits of detection. Therefore, the specific numbers of DWV infected colonies should not be given much weight. The variation in HRM profiles produced using all bee and mite DWV positive samples (Fig. 1b) indicate that the DWV sequences present are variable and no one dominant variant exists in this population, a scenario typical of *Varroa* free honey bee populations (Martin et al., 2012; Ryabov et al., 2014). During the sampling and reproductive studies which involved opening over 75% of all managed hives on the island no bees with deformed wings were observed.

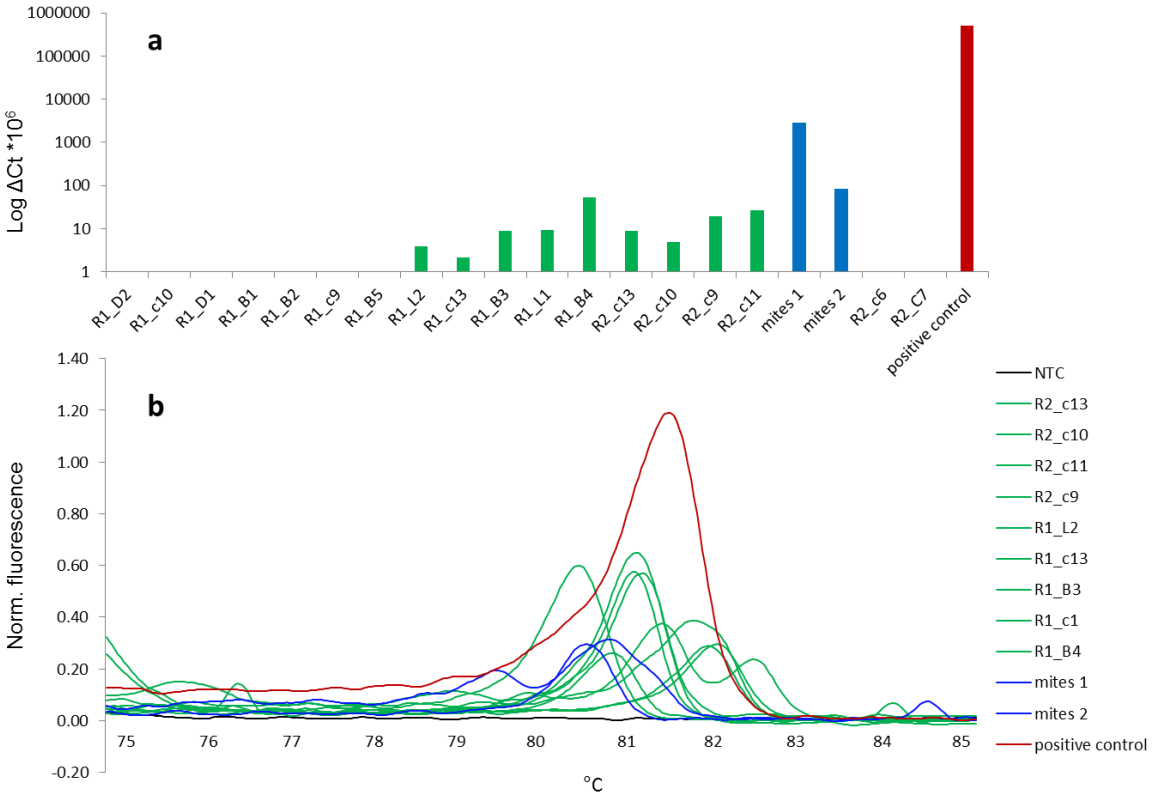


Figure 1. RT-PCR HRM results of all DWV positive samples using primers developed by Highfield *et al*. (2009) with a known DWV (type A) positive asymptomatic honey bee sample (red) and a no template control (black). a) DWV levels in each colony relative to an actin control shown as ΔCt $*10^6$ values to enable visualisation of low level samples b) HRM profiles generated indicate diverse DWV genotypes are present in the population.

The resulting fragments from HRM analysis were subjected to Sanger sequencing which confirmed that DWV had been amplified from all positive samples (Fig. 2). Variation was seen between samples but the dominant variant found in both bees and mites was closest to the type A variant (Fig. 2).

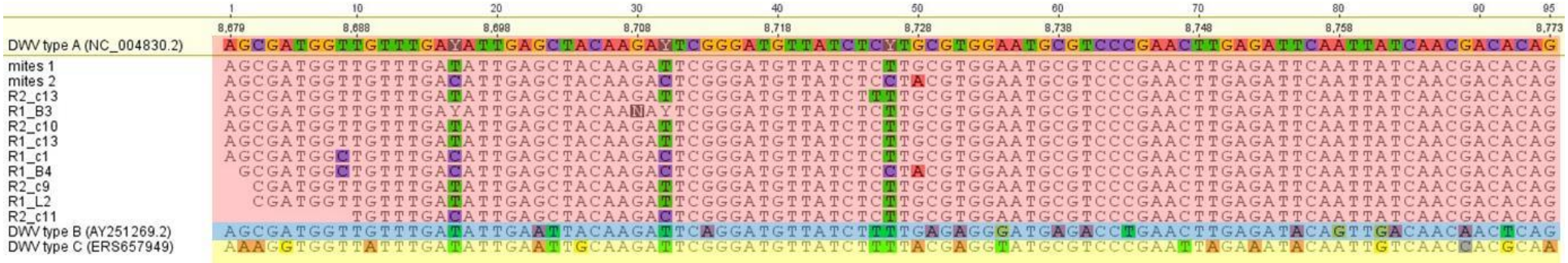


Figure 2. Geneious alignment of a 95bp fragment amplified from all positive honey bee and mite samples aligned to DWV type A (pink). DWV types B (blue) and C (yellow) are also shown.

Honey bee and *Varroa* populations

A total of 276 drone and 921 worker sealed brood cells from six colonies were opened. Of these, 106 drone and 201 worker sealed brood cells were infested with one or more *Varroa* mites. The infestation levels of sealed brood and adult workers were variable; both between colonies and month of collection (Fig. 3a) as previously found (De Mattos et al., 2016). All colonies were infested, with adult bee infestation levels much lower (1-2%) than found in the worker (10-20%) or drone (23-38%) brood cells. In May 2016, the six study colonies contained an average of 8400 (±2865 SD; range 4684-11839) sealed brood cells, 13894 (±4560 SD; range 7655-19982) adult bees and 1749 (±1565 SD; range 290-4647) mites per colony (Fig. 3b).

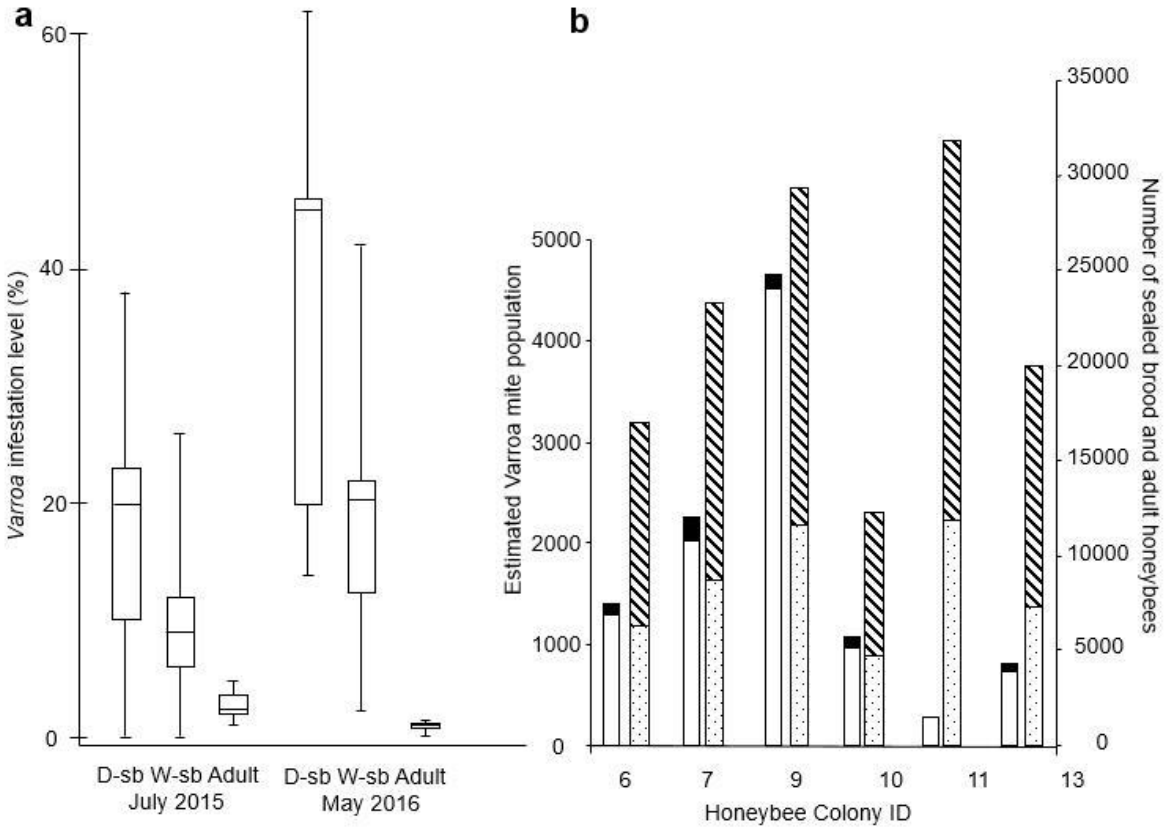


Figure 3. a) *Varroa* Infestation levels of drone (D-sb) sealed brood, worker (W-sb) sealed brood and adult workers sampled at July 2015 & May 2016 and b) the calculated mite population in sealed brood (clear bar) and on adult bees (black bar) alongside the number of sealed brood (dotted bar) and adult bees (striped bar) in each of the six colonies studied in 2016.

Varroa reproduction

The developmental times of the mite offspring based on the age of bee pupa are all indistinguishable to that found in previous studies (Martin, 1994; Donze & Guerin, 1994) (Supplementary Fig. 1). Furthermore, the average number of eggs laid in worker cells (4.9) and drone cells (5.3) is typical for *V. destructor* (Rosenkranz et al., 2010). However, the highest non-reproduction rates and high offspring mortality rates combined to produce the lowest number of viable female offspring produced per mite (0.54 in worker & 1.6 in drone

sealed brood) ever recorded (Supplementary Table S2). These numbers fell to 0.39 and 1.0 in worker and drone cells respectively that were invaded by two of more mother mites (Supplementary Table S2).

Discussion

The viral analysis indicates that DWV is present among the 'Fernando de Noronha' honey bee and mite population, but the levels are very low and genotype (strain) diversity is high. Sequencing of HRM products detected predominantly DWV type A despite the HRM analysis of the same samples often indicating multiple variants. This apparent paradox arises due to the much higher sensitivity of HRM compared to sequencing of samples containing very low amounts of virus. We know from previous experience that even using high-depth NGS methods it is very difficult to detect multiple variants that we know to be present using HRM analysis in honey bee samples from *Varroa* free areas with low level DWV infections. It should also be noted that although the sequenced RdRp region is closest to type A master variant, the dominant DWV genomes could be recombinants and contain structural genes more similar to types B or C. This pattern of DWV, i.e. low amounts and diverse genotypes, has also been seen in *Varroa* free honey bee populations on Colansay Island, Scotland (Ryabov et al., 2014) and Hawaii, USA (Martin et al., 2012). However, this is the first honey bee population where DWV is associated with a *Varroa* infested honey bee population for a long period of time (32 years) and no virulent strain has appeared. This confirms for the first time that the feeding activity of *Varroa* cannot activate DWV replication, either by immunosuppression or by any other mechanism such as the injection of proteins during mite feeding (Denholm, 1994). This population has lived with *Varroa* since its establishment in 1984 (De Jong & Soares, 1997), and so must have also acquired the original DWV infection (containing no virulent variants) at that time, either through the imported queens, the Africanized workers originally used to initiate the colonies or the *Varroa* mites which arrived with them, as no bees have been moved on to the island since 1984. If *Varroa*-induced viral replication were taking place viral loads would be much higher and killing the bees.

We propose the explanation for this population's survival may be mere probability. The mechanism underlying the *Varroa* induced transformation of DWV infection in bees from a genetically variable and low titre inconsequential virus to a deadly virus dominated by a virulent genotype has until now been unknown. The data from this study, along with data from Ryabov *et al*. (2014), which showed that the decrease in viral variation occurred in the bee rather than the mite, suggests that in order for a virulent variant to become established it must first become an overt infection within a bee (pupae or adult). This then allows *Varroa* to transmit sufficient amounts of this virulent variant throughout the population. Although RNA viruses exhibit high mutation rates and no proof reading activity leading to extremely fast evolution of their quasispecies (Doming & Holland, 1997) amplification of a lethal DWV variant, which goes on to kill a colony is a rare occurrence. Under natural conditions, without *Varroa* to spread the virus, the overt infection would likely go unnoticed as the colony would quickly die without spreading the virus further. Pre-*Varroa*, isolated colony deaths associated with overt infections of DWV were reported in Hawaii (Martin et al., 2012), UK and South Africa (Allen & Ball, 1996) and Belize (Brenda Ball, personal communication). It has now been shown (Ryabov et al., 2014) that the appearance of a virulent DWV-variant occurs within the bee, not the mite, which appears to be acting only as a mechanical vector. However, the conditions required for the sudden amplification of the type A variant within the bee remains unknown. Although once present in the honey bee population it is vectored very efficiently by the mites.

So, we propose that the honey bee population in Fernando de Noronha has thus far evaded the catastrophic consequences of DWV and *Varroa* because the incredibly small and isolated population size (ca. 20-40 colonies) has meant that there hasn't yet been sufficient time for a virulent variant to have become established in a colony. The estimated mite populations in the colonies would no-doubt result in the rapid death of the colonies if a virulent genotype of DWV was to emerge, since up to 42% of the worker brood can be infested by *Varroa*, levels never observed in healthy hives of European honey bees. Moreover it may just be a matter of time before an overt outbreak of a virulent variant appears that has the capability to spell disaster for the bees of Fernando de Noronha. It also explains why when in 1997 six queens were transferred from Fernando de Noronha to

Germany to head colonies and study whether heritable hygienic behaviour is responsible for their *Varroa* tolerance (Correa-Marques et al., 2002). Although no difference in hygienic abilities compared to the local population were found indicating no genetic basis for the tolerance is present. These colonies all died during the winter or early spring (Peter Rosenkranz, personal communication) since the bees and mites would for the first time be exposed to the virulent DWV strains (Martin et al., 2012; McMahon et al., 2016) circulating in the local bee population.

The second insight from this study is the co-evolution of the honey bee and *Varroa* mites on Fernando de Noronha, free of any influence from DWV. Again this is a globally unique situation. Over the past 25 years (De Jong & Soares, 1997; De Mattos et al., 2016; this study) the initial high infestation rates on adult bees has fallen from 25% to just a few percent (this study). However, the average infestation rates of the worker (18%-1996, 20%-2012 & 20%-2016) and drone (38%-1996, 45%-2012 & the 38%-2016) sealed brood has remained remarkably high and stable. It is estimated that currently mite populations range from 290 to 4684 per colony, which for some colonies is well above the economic threshold of 1000-2000. It was originally suggested that the Japanese haplotype of *Varroa* was less 'virulent' than the Korean haplotype (Solignac et al., 2005). This was proposed as the reason for *Varroa* tolerance among the Fernando de Noronha population (Strapazzon et al., 2009; Locke, 2016) and in Africanized bees (Anderson & Trueman, 2000). However, the Korean haplotype is now found in Africanized bees without any loss of tolerance (Garrido et al., 2003). This study found that the number of eggs laid and developmental timing in both worker (and drone brood) are indistinguishable from those of the Korean haplotype. However, adult mite and offspring mortality are higher than reported in other studies (Supplemental Table S2). This results in only 0.54 viable i.e. mated, female offspring being produced per reproductive cycle, which is one of the lowest values ever recorded. As the total number of reproductive cycles is around 2 to 3 (Fries et al., 1994; Martin & Kemp, 1997) the mite population is unable to significantly increase within worker cells relying on the more limited drone cells. Furthermore, both in this study and all previous studies a large drop in reproductive success occurs when increasing numbers of mothers invade a cell (Supplemental Table S2, Supplemental Figure 2 (De Mattos et al., 2016)) which can

potentially led to a stable mite population when mite reproductive success in worker brood is low (Martin & Medina, 2004), as found in this population. It must be noted, however, that to our knowledge , no studies have as yet been carried out comparing the role of Korean to Japan haplotypes of Varroa in terms of their association with DWV, and although we believe unlikely, it could be that DWV has the ability to replicate in, or be vectored more effectively by, the Korean than the Japan haplotype.

Currently known *Varroa* tolerant populations are surviving due to increased swarming (Fries et al., 2006; Loftus et al., 2016) or superinfection exclusion (Mordecai et al., 2016). However, on Fernando de Noronha the mite and bee populations are both able to persist without any severe effects, however, the dark spectra of DWV lurks in the background, ready to decimate this small unique island population.

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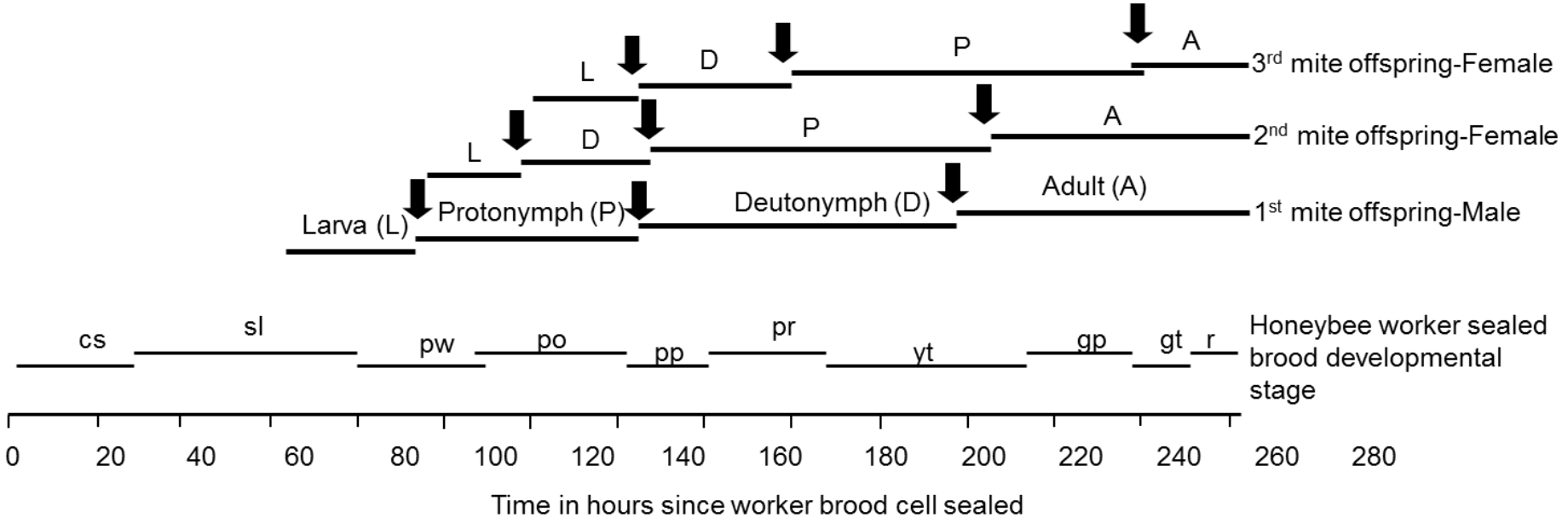
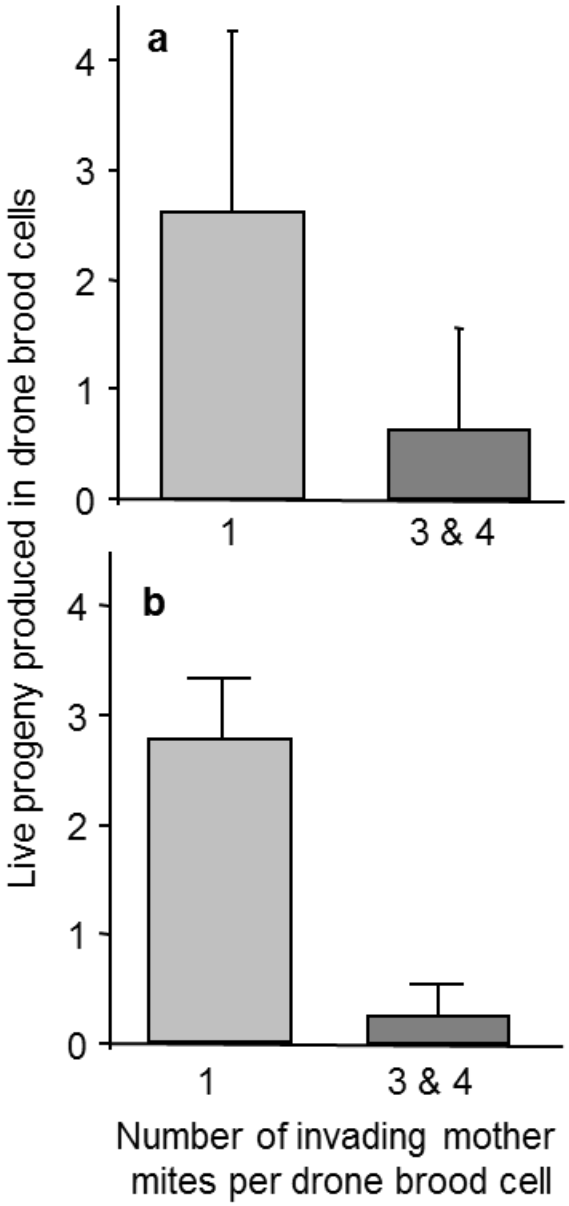
Supplementary Information



Supplementary Figure 1. The development and timing of *Varroa* in relation to the development and timing of the honey bee inside the sealed brood cell adapted from Martin (1994). Honey bee development stages: cs=cocoon spinning, sl= stretched larva, pw=pupa with white eyes, po=pale eyes, pp=pink eyes, pr=purple eyes, yt=yellow thorax, gp=grey wing pads, gt=grey thorax, r=resting adult.



Supplementary Figure 2. Comparison of the number of live progeny produced in drone brood per invading mother in a) this study and b) a previous study (De Mattos et al., 2016), calculated using the method used in De Mattos *et al*. (2016). This indicates the importance of using the same method when comparing mite reproductive data across studies.

Supplementary Table S1. Presents the mean DWV and actin Ct values for each colony tested as well as the two pooled *Varroa* samples and a positive control of an asymptomatic newly emerged worker bee which had been parasitised by *Varroa* during development

collection	sample	Ct (DWV)	Ct (actin)
Jul-15	R1_c10	neg	19.48
Jul-15	R1_D1	neg	13.71
Jul-15	R1_B1	neg	18.12
Jul-15	R1_B2	neg	16.82
Jul-15	R1_L2	34.43	16.49
Jul-15	R1_c13	35.24	16.43
Jul-15	R1_B3	34.11	17.34
Jul-15	R1_c1	33.29	16.61
Jul-15	R1_B4	31.3	17.11
Jul-15	R1_c9	neg	15.35
Jul-15	R1_B5	neg	18.01
May-16	R2_c6	neg	17.87
May-16	R2_C7	neg	18.48
May-16	R2_c13	34.42	17.67
May-16	R2_c10	35.76	18.12
May-16	R2_c9	34.12	18.44
May-16	R2_c11	34.4	19.22
May-16	*Varroa* 1	33.83	25.38
May-16	*Varroa* 2	36.55	23.04
	positive control	17.59	19.32

Supplementary Table S2. Presents the various reproductive categories for the J type mites in this study compared to other studies using a similar methodology. po=pale eyed pupa and gp=grey wing pads, referring to host honey bee life stages. *Percentages will not add up to 100 since they are considering different sub-sets of data for each calculation. †=recalculated from Martin (1994) and Rosenkranz & Engels (1994).

	Worker sealed brood				**Drone sealed brood**			
Category*	This study	EHB †	African (Martin & Kryger, 2002)	AHB (Medina et al., 2002)	This study	EHB †	African (Martin & Kryger, 2002)	*A.cerana* (Boot et al., 1997)
Dead Mothers (all)	11%	2	6	2	18%	5	6	
Non-reproductive >po	19%	10	13	12	10%	3	2	0-2
Viable mothers >po	70%				65%			
Only males >po	6%	9	15	11	20%	14	20	0-2
Viable offspring (>gp) Live male + female	40%	63	51	43	53%	63	59	94-99
Mean # of eggs laid	4.9	4.9	4.5	4.38	5.3	5.5	4.9	
No of viable females per mother >gp	0.54	0.9	0.9	0.6-0.7	1.6	1.9-2.1	2.2	4.6
No of viable females from multiple infested cells	0.39	1.0			1.0	1.6		

Chapter 3: Novel RNA virus genome discovered in Ghost ants (*Tapinoma melanocephalum*) from Hawaii.

Abstract

Here we report the full genome sequence of Milolii virus, a novel single stranded (positive sense) RNA virus discovered from *Tapinoma melanocephalum* ants in Hawaii. The genome is 10,475 nucleotides long encoding a polyprotein of 3304 amino acids.

Introduction

The ghost ant (*Tapinoma melanocephalum* Fabricius.) is a widely distributed invasive pest, probably of African or Asian origin (Wheeler, 1910), now found in tropical and subtropical climates as well as in glass houses in temperate regions (Smith, 1965). The species was first recorded in Hawaii in 1899 (Forel, 1899), and soon became a common household pest (Clagg, 1957).

The Ghost ant is now a major pest across the Pacific islands (O'Connor, 2004) and Florida (Klotz et al., 1995) being listed on the Global Invasive Species Database (2015). They are highly adaptable feeding on waste food (Smith, 1965) and in hospitals where they pose an additional threat of carrying pathogenic bacteria (Moreira et al., 2005).

The combination of an invasive species with an emerging disease poses a potentially major risk to biodiversity (Strauss et al., 2012) since many RNA viral pathogens have wide host ranges that can belong to different but overlapping food webs. This provides vast potential for viral pathogens to spread via different networks to new hosts (McMahon et al., 2015; Mordecai et al., 2016).

Methods

RNA was extracted from two pooled samples of 20 to 25 *T. melanocephalum* ants collected from inside two honey bee (*Apis mellifera*) hives from an apiary in Milolii, Big Island, Hawaii in December 2012, using the RNeasy mini kit (Qiagen). Oligo dT priming was used to create cDNA libraries which were then sequenced using the Illumina HiSeq 2000 at The Earlham Institute, Norwich, England (formerly The Genome Analysis Centre) to produce 100bp paired end reads. QC filtering was carried out using FastQC. Kontamination, a pipeline developed by TGAC to remove host reads was applied using the related reference *Linepithema humile* ant genome due the unavailability of a *T. melanocephalum* genome. The resulting reads were pooled and IVA (Hunt et al., 2015) was used to generate *de novo* assembled contigs, which resulted in the assembly of the Milolii virus genome.

Results and Discussion

The genome is 10,475 nucleotides long with a 3' polyadenylated tail end and a 9,930 nucleotide open reading frame encoding a polyprotein of 3,304 amino acids. A Blastx search of the protein coding sequence against the NCBI protein database revealed conserved RNA helicase and RNA dependent RNA polymerase (RdRp) domains typical of single stranded (positive) RNA viruses. The protein coding sequence showed closest matches (29-30%) to the hypothetical protein of Hubei tetragnatha maxillosa virus 5 (Shi et al., 2015), and the P1 protein of Acrythosiphon pisum virus (van der Wilk et al., 1997), both invertebrate viruses. The coverage across the protein coding sequences were 56% and 60% respectively. For each sample total reads (FASTA format) were mapped against the Milolii virus genome in Geneious (Biomatters). Both showed full length genome coverage with depths ranging from approximately 1000-1,000,000x (data not shown), with 28.12% and 69.65% of total reads aligning to Milolii virus. Furthermore, full length genomes were assembled individually from each of the two samples and Geneious nucleotide alignments revealed the sequences to share 99.9% pairwise nucleotide similarity.

Nucleotide Sequence Accession Number: MF155030

Due to the strict requirements of the journal, additional information on the discovery of Milolii virus was removed from the manuscript. In order to provide a more comprehensive description for this thesis, the information has been included as Supplementary Information.

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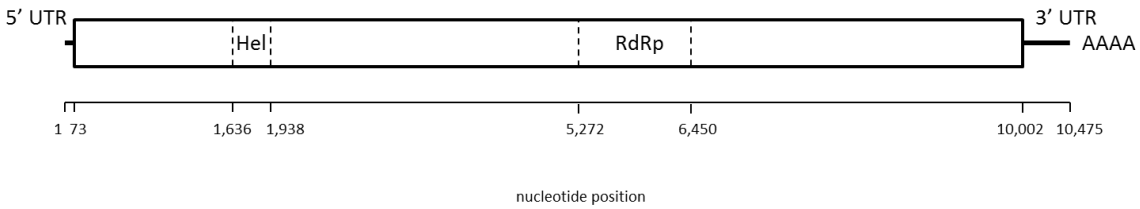
Supplementary Information

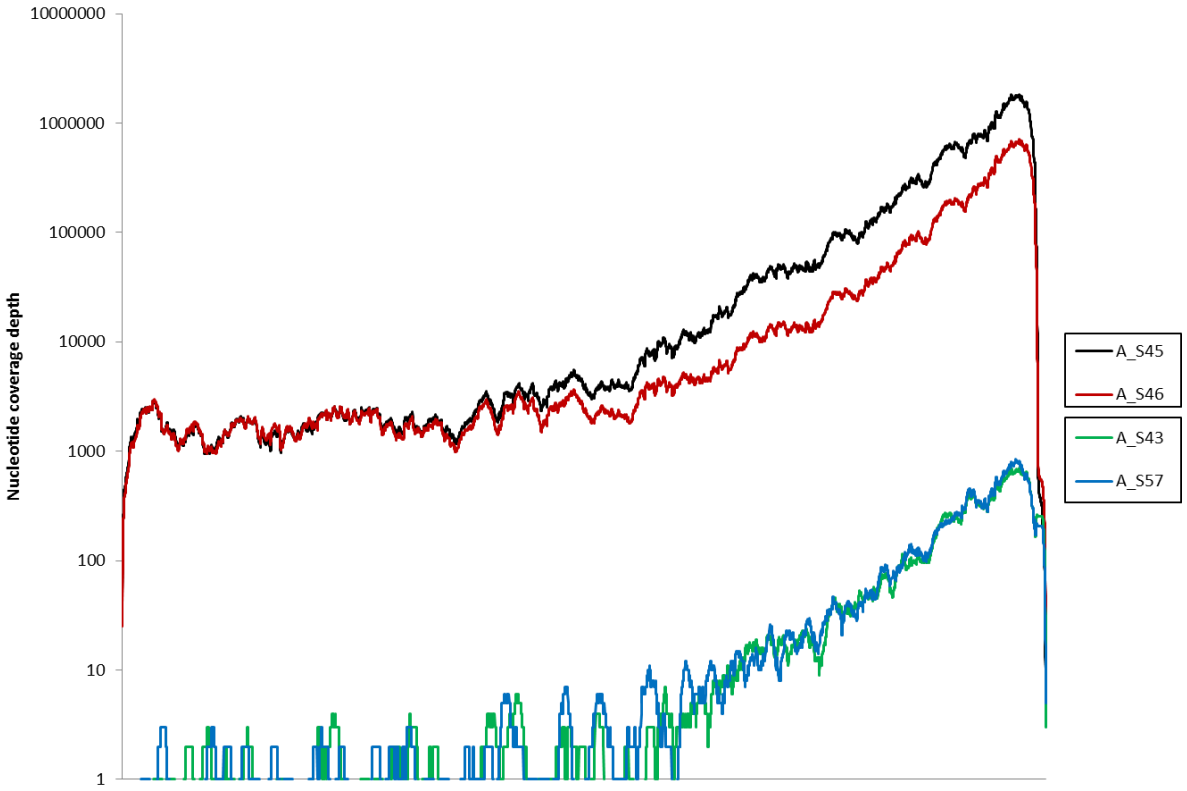
When investigating the assembled contig from pooled ghost ant samples, it was initially viewed in Geneious and the open reading frame (ORF) was identified using the 'Find ORF' tool. Due to the genetic dissimilarity from other known viruses, a BLASTn search was insufficient to provide any information. Thus a BLASTx search using the protein coding sequence was carried out against the NCBI non-redundant protein database, which revealed the presence the conserved Helicase (pfam 00910) and RdRp (pfam 00680) domains. The resulting genome organisation with nucleotide positions is shown in Supplementary Figure S1.



Supplementary Figure S1. Genome arrangement of Milolii virus, showing ORF as found with Geneious and nucleotide positions of conserved Helicase and RdRp regions as identified by BLASTx.

Milolii virus was initially assembled *de novo* using pooled sequence data from ghost ant samples A_S45 and A_S46, and then subsequently from each sample individually. In order to investigate genome coverage of Milolii virus from the individual samples, genome coverage plots using read 1 FASTA files which had been filtered to remove host reads using the related host *Linepithema humile,* were created using the Geneious ‘Map to Reference’ tool (Supplementary Figure S2). This showed both samples to have high coverage across the length of the genome, with plots showing the characteristic 3’ bias typical of the preparation method (Brooks et al., 1995).

The high Milolii virus read counts led me to speculate that this virus could be a more general ant virus. So, two samples of big headed ants, *Pheidole megacephala*, one collected from an apiary in Big Island East (A_S43), and other from an apiary in Oahu East (A_S57) were also analysed. Insufficient reads were found by BLAST to be able to assemble any contigs, but genome coverage plots were created using read 1 FASTA files filtered to remove host reads (using the related host (*Solenopsis invicta*), which showed a small amount of coverage primarily at the 3’ end (Supplementary Figure S2). Although suggesting the virus could be present at low levels in these samples, there was insufficient data to confirm this.



Supplementary Figure S2. Genome coverage graph showing nucleotide coverage across the Milolii virus genome, for the ghost ants A_S45 and A_S46 and also for two big headed ant samples A_S43 and A_S57.

Supplementary Reference

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Chapter 4: Moku virus; a new *Iflavirus* found in wasps, honey bees and *Varroa*.

Abstract

There is an increasing global trend of emerging infectious diseases (EIDs) affecting a wide range of species, including honey bees. The global epidemic of the single stranded RNA Deformed wing virus (DWV), driven by the spread of *Varroa destructor* has been well documented. However, DWV is just one of many insect RNA viruses which infect a wide range of hosts. Here we report the full genome sequence of a novel *Iflavirus* named Moku virus (MV), discovered in the social wasp *Vespula pensylvanica* collected in Hawaii. The novel genome is 10,056 nucleotides long and encodes a polyprotein of 3050 amino acids. Phylogenetic analysis showed that MV is most closely related to Slow bee paralysis virus (SBPV), which is highly virulent in honey bees but rarely detected. Worryingly, MV sequences were also detected in honey bees and *Varroa* from the same location, suggesting that MV can also infect other hymenopteran and Acari hosts.

Introduction

Emerging and re-emerging diseases affecting a diverse range of organisms pose an ongoing threat to global health and food security. The transmission of DWV around the world in conjunction with *Varroa* is a well-studied example of an emerging insect pathogen (Martin et al., 2012; Wilfert et al., 2016). The spread of *Varroa* from Asia to the rest of the world was mirrored by the spread of DWV throughout the European bee populations (Wilfert et al., 2016) and introduced a new transmission route for the virus, leading to selection of a virulent strain, DWV type A, which replicates to high levels and results in colony collapse (Martin et al., 2012).

RNA viruses, such as Deformed wing virus, are of particular interest due to their lack of host specificity and capacity to jump between hosts (Singh et al., 2010; Manley et al., 2015; Moya et al., 2004; Levitt et al., 2013). To enable this generalist infection strategy with little host specificity, Iflaviruses, such as DWV, exist as a cloud of variants known as a quasispecies (Mordecai et al., 2016a). The level of diversity or 'cloud size' within a viral quasispecies has been correlated with the host range size the virus (Schneider & Roossinck, 2001). Predicting virus emergence before epidemic spread allows mitigating action to be considered but is not always possible. As a general rule, the larger the reservoir species population size, the more viruses it can harbour, and as a consequence viruses with higher virulence arise more frequently (Calisher et al., 2006). The large population sizes and high densities of many insect populations provide a perfect environment for emerging viruses to arise and transmit freely. Social insects epitomise this, making up just 2% of all insect species, but more than half of the total insect biomass (Wilson, 1990). Transmission between hosts is facilitated by interactions between insect species, including predation and sharing of resources (Singh et al., 2010).

It is becoming apparent that insects which interact with honey bees can act as viral reservoirs and infect honey bees via spillover events (Singh et al., 2010; Villalobos, 2016; Schroeder & Martin, 2012). In addition, the introduction of invasive species through anthropogenic processes offers the opportunity for new hosts, with their own assortment of viromes, to be introduced to previously isolated populations such as those in the Hawaiian archipelago.

The predatory social wasp, *Vespula pensylvanica* is a common species native to the western half of temperate North America. In the Hawaiian archipelago, it was first recorded in Kauai in 1919, but was not recorded on Maui and the Big Island until 1978 (Gambino, 1992) and has since flourished becoming a serious pest. *V. pensylvanica* is a general predator that feeds on a wide range of arthropods including the honey bee, *Apis mellifera* (Gambino, 1992). The ecological role of *V. pensylvanica* as an invasive species with widespread geographic distribution, abundant numbers in some areas and generalist feeding

preferences including its use of floral resources (Hanna et al., 2013), make it important to investigate whether or not it could act as a reservoir for a new or emerging honey bee virus.

Here we report a novel RNA virus, genetically dissimilar to any other virus at the nucleotide level, detected in *V. pensylvanica*. We named the novel virus "Moku", which means Island in Hawaiian.

Methods

RNA was extracted from eight asymptomatic *V. pensylvanica* individuals collected from managed honey bee apiaries on Big Island, Hawaii in 2012. Bees were sampled from the frames inside the hive, so will likely be mostly nurses with some foragers and newly emerged bees. Samples W_S23-27 and HB_S11-12 were collected from the North of Big Island, and samples HB_S13, V_S32 and W_S28-30 were from the East. 30 honey bees were pooled for RNA extraction. The *Varroa* samples were a pool of 10 mites taken from drone brood. cDNA libraries were prepared using oligo dT priming followed by Illumina 2x100bp Hiseq sequencing by the Earlham Institute (Norwich).

QC was done using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) to confirm the quality of the raw read data. An in-house contamination-screening pipeline called Kontaminant (http://www.tgac.ac.uk/kontaminant/) was used to check for any contamination in the raw reads. The wasp libraries showed less than 5% of host mRNA. Even with very low host contamination, kmer filtering was performed to remove any host RNA. There was no viral mapping/filtering done, so we carried out a metagenomic study to assemble all the non-host RNA.

From a total of 8 wasp individuals, around 116 million reads (115,842,147 total reads before filtering) were assembled together in a single assembly run using MetaCortex (Unpublished, developed by Richard Leggett in TGAC). MetaCortex is a recently developed variant of Cortex (Baker et al., 2013; Iqbal et al., 2012) based on de Bruijn graphs, which are constructed by dividing reads into smaller, overlapping sequences called kmers. Contigs were aligned (blastx) against a refseq protein database (NCBI) to identify putative viruses.

One contig in particular was translated within Geneious (Biomatters) and aligned with other *Iflavirus* amino acid sequences obtained from Genbank. The alignment was carried out using the Muscle aligner with 8 iterations. The phylogenetic tree was built by the Geneious tree builder using a neighbour joining method and the Jukes-Cantor genetic distance model based on the conserved RdRP region of picorna-like viruses (Koonin et al., 1993). Finally, Geneious was used to map reads against the putative virus contig and Vicuna (Yang et al., 2012) was used to assemble reads from each individual separately using a pipeline adapted from assembling DWV (Mordecai et al., 2016a).

Individual reads were aligned against the novel Moku virus genome to create coverage plots for each Illumina sample (Fig. 2a). From these reads a consensus of the RdRp region was obtained for MV in *Varroa* and honey bees by keeping bases that match at least 90% of the sequences. The Moku virus genome was annotated based on an amino acid alignment with the SBPV and DWV genomes (de Miranda et al., 2010; Lanzi et al., 2006). Regions were identified by protease sites based on the DWV and SBPV genomes and homologous protein domains identified by BLAST. Reads from *Varroa* and honey bees were pulled out and made into a consensus and aligned with the MV genome from the wasps to confirm that they were indeed MV (Supplementary Figures S1 and S2).

The insect viromes (Fig. 3b) were created by aligning individual Illumina reads using BLAST against a custom virus database which included the Moku virus genome, Slow bee paralysis virus, and all three variants of DWV (Mordecai et al., 2016a). The top hits were counted for

each viral species. BLAST hits of individual reads that did not cover the whole read were excluded from the analysis.

Results and Discussion

A blastx search against the NCBI protein database revealed the polyprotein of MV shared 46% amino acid similarity to slow bee paralysis virus (SBPV) and phylogenetic analysis confirmed that Moku virus (MV) lies within the genus *Iflavirus* (Fig. 1). The genome is 10,056 nucleotides long (accession number KU645789) with a poly-A tail at the 3' end and full genome coverage was observed in all the 8 wasp samples (Fig. 2a). The genome contains a 9153 nucleotide open reading frame encoding for 3,050 amino acids (Fig. 2b). In addition, partial genome-wide coverage was observed in *A. mellifera* and *Varroa destructor* collected on Big Island (Fig. 2a and Supplementary Figure S1).

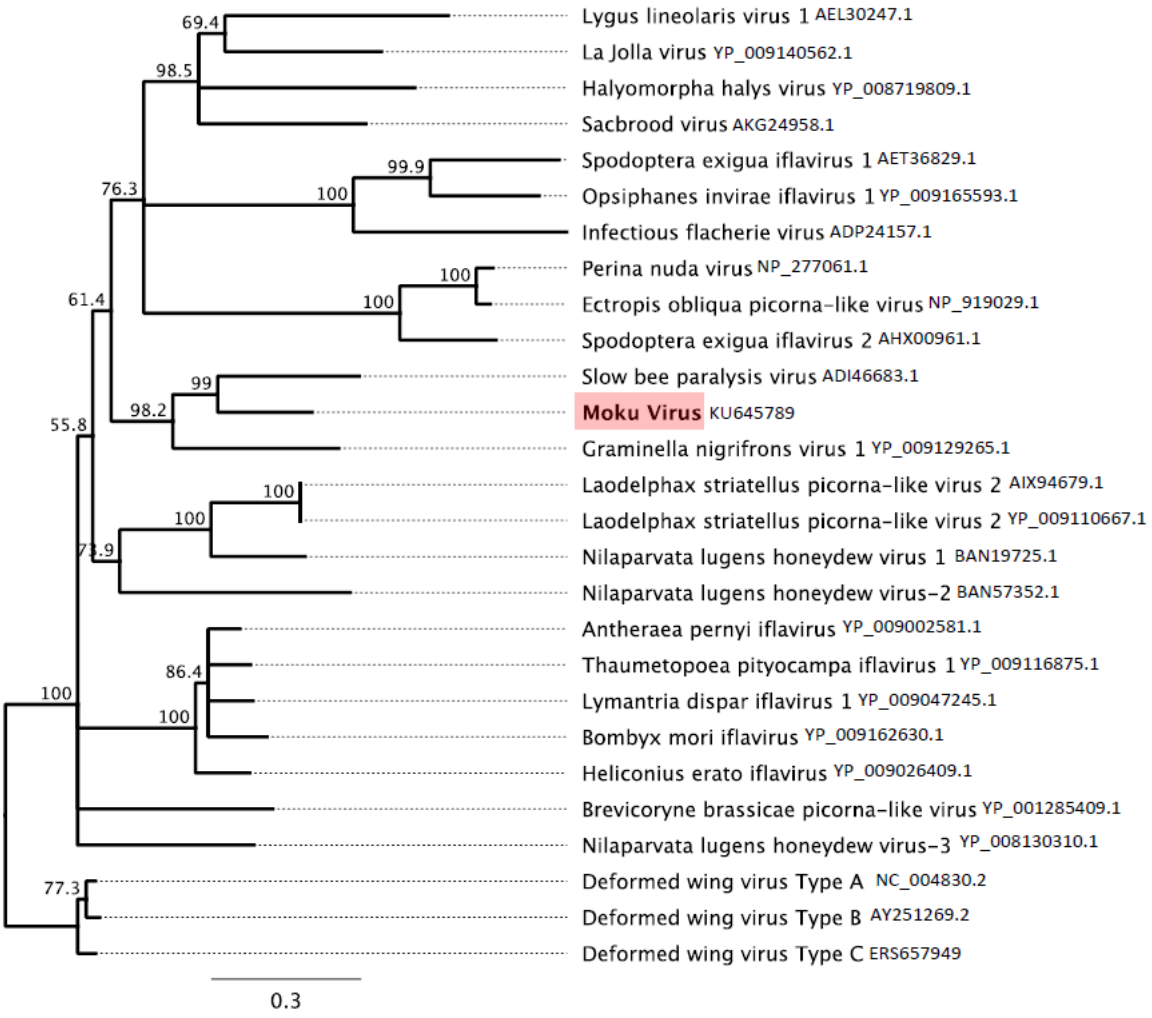

Figure 1. Neighbour joining tree using the amino acid sequences of a conserved region of the RdRP (Koonin et al., 1993). Values show the consensus support (%).

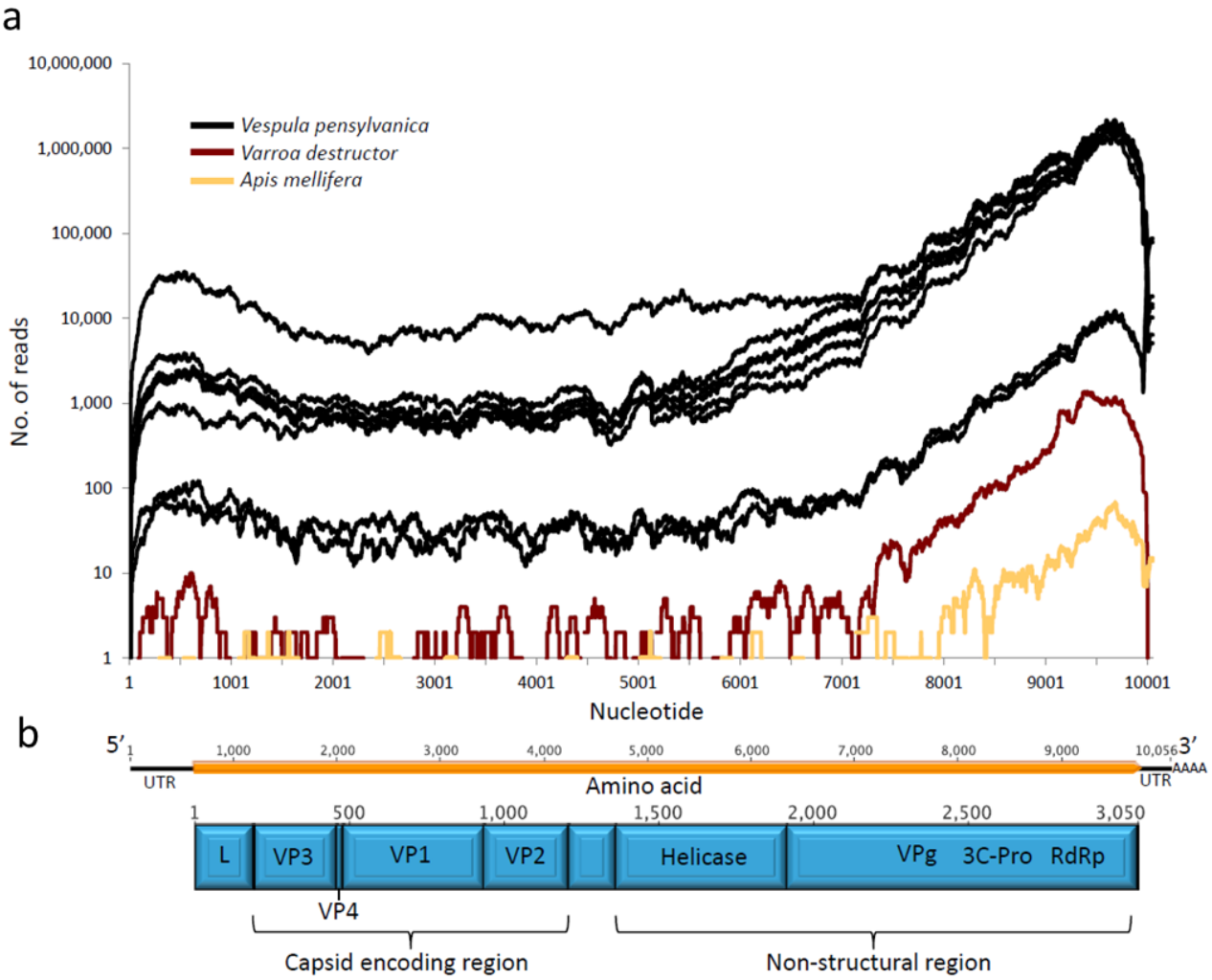

Figure 2**.** a) Moku virus genome coverage from Illumina Hi-seq data for samples collected in Hawaii. *V. pensylvanica* are shown in black, *Varroa* in red and honey bees in yellow. Three different honey bee Illumina runs were pooled together for the honey bee data. b) Organisation of the 10,056 nucleotides Moku virus genome (black line) coding for a 3050-aa polyprotein (orange box) and the predicted polyprotein coding regions are shown in blue.

Further genome annotation was carried out by comparing the 3C protease sites with the previously annotated SBPV and DWV genomes (de Miranda et al., 2010; Lanzi et al., 2006). The 3,050-aa polyprotein contained conserved domains typical of the iflaviruses, including capsid (cd00205, pfam08762, pfam00073), helicase (pfam00910), RNA dependent RNA polymerase (pfam 00680) and 3C protease (pfam00548) domains (Fig. 2b). These were arranged in the canonical *Iflavirus* genome structure; structural proteins in the N-terminal region and non-structural proteins in the C-terminal region (RNA helicase, protease, RdRp) (Fig. 2b). No conserved domain was recognised for the leader protein, the most variable region of the *Iflavirus* genome (Lanzi et al., 2006). Assemblies of MV from each individual wasp using Vicuna (Yang et al., 2012) showed that all wasp samples shared at least 98% nucleotide identity suggesting that MV is relatively invariant, at least in the wasps sampled. However, greater variation occurred between species. For example, when the RdRp, helicase and VP3 consensus regions of the MV genome from the honey bees and *Varroa* were aligned against the wasp MV genome, we observed roughly 2 nucleotide substitutions per 100 nucleotide base pairs for the RdRp region for both the honey bees and the *Varroa* (Supplementary Figure S1a), and in *Varroa* one of these substitutions resulted in an amino acid change. Background genomic variation (likely due to mutations created during genome replication) can also be seen in the reads that represents the honey bee and *Varroa* MV quasispecies (Supplementary Figure S2). This group of viruses exist as a cloud of mutants (Mordecai et al., 2016a), therefore contigs generated by *de novo* assembly represent a consensus of the most dominant sequence. Although consensus sequences of MV were similar and belong to the same master variant, a cloud of mutants around the main consensus can be seen in individual reads (Fig. S2).

Since we were unable to assemble a full-length MV genome from both the honey bee and *Varroa* samples, pairwise comparison between these genomes was not possible. Taken together, genomic variants of MV were observed in both the honey bee and *Varroa* samples. The amino acid identities between MV variants were highly conserved (Supplementary Figure S1), therefore, these variants belong to the same master MV variant. MV is likely similar to DWV (Mordecai et al., 2016a), i.e. it exists as a quasispecies with variation around one or more master variant(s). Further screening and sequencing of MV in

different hosts and geographic locations is likely to reveal further genetic variation within the virus quasispecies.

Advancements in NGS technologies have enabled an exponential increase in RNA virus discovery (Liu et al., 2015) yet only a few new honey bee viruses have been discovered since the pioneering work of Bailey & Ball (1991). Recent discoveries include a Macula-like virus in honey bees and *Varroa* (de Miranda et al., 2015) and the replication of a plant virus (Tobacco ringspot Virus) in honeybees (Li et al., 2014). NGS technologies have shown that viral loads in insects can be high; for example, we have previously shown that DWV reads made up 46.3% of all Illumina reads in *Varroa* and 9.7% in honey bees (Mordecai et al., 2016). Similarly, here we show that in *V. pensylvanica*, virus reads can make up to 91.5% of total Illumina reads (average of 54.6%) (Fig. 3a). Due to the quasispecies nature of the iflaviruses (Mordecai et al., 2016a), Moku virus was named after the location of its discovery rather than the host or disease symptoms (of which there is no particular phenotype recorded to date). It is likely that MV is able to replicate in several hosts, potentially with several master variants each of which differs in pathogenicity depending on the host. However, the high viral load (up to 99.87% of total virus reads in wasps, Figs. 3b and c) and full genome coverage (Figure 2A) observed for MV in *V. pensylvanica* suggests it is likely to be MV's native host. Use of "Moku" as a name also conforms to the International Committee on Taxonomy of Viruses (ICTV) preference not to use host species names to assign virus species (Adams et al., 2013). Many insect RNA viruses discovered by NGS data (Liu et al., 2015) do not result in overt symptoms, preventing the use of disease symptoms for virus classification.

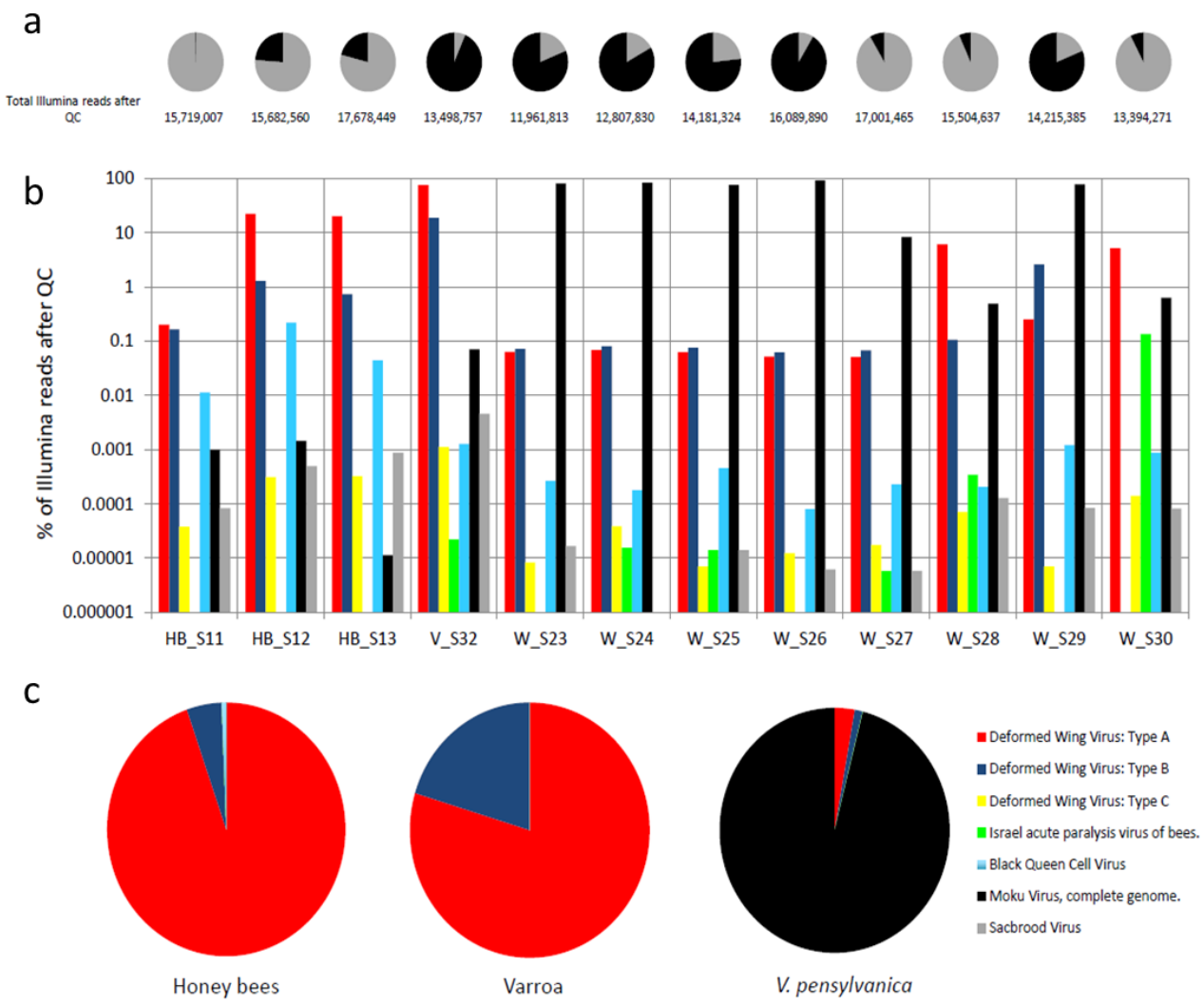

Figure 3. a) Proportion of total Illumina Hi-Seq reads which were attributed to viruses by BLAST labelled with the total number of Illumina reads after QC. b) Illumina Hi-Seq Virome for each sample (W=*V. pensylvanica*, V= *Varroa*, HB= honey bee) showing the number of top BLAST hits against a custom virus database. Note a logarithmic scale has been used to display the vast differences between viruses. c) Pie charts showing samples grouped per species showing the proportion of viral hits determined by BLAST.

Moku virus is most closely related to SBPV (Fig. 1). SBPV is highly virulent in honey bees but has only been found in the UK, Fiji and Western Samoa despite the numerous surveys across the world (de Miranda et al., 2010). More recent evidence suggests that SBPV's natural host is the bumble bee (*Bombus* spp.) rather than honey bees (McMahon et al., 2015). Similarly, wasps could act as the reservoir for Moku virus, which commonly circulates in the Vespine host, but with the potential to be highly virulent in honey bees, as with SBPV. This is worthy of regular monitoring as invasive species such as the *V. pensylvanica* in Hawaii and *Vespa velutina* (Asian hornet) in Europe could act as a new transmission route and source of emerging viruses in honey bees.

Detection of MV in *Varroa* is of concern, as *Varroa* is known to facilitate the spread and amplification of some RNA viruses (Martin et al., 2012). Our data suggests a possible transmission route of MV from *Vespula pensylvanica* to honey bees (or vice versa as *V. pensylvanica* is known to predate on honey bees) and then onto *Varroa*. Once in *Varroa*, transmission at epidemic proportions within honey bee populations is a possible outcome (Martin et al., 2012). However, DWV still dominates the virome of honey bees with only low levels of MV detected. This suggests that currently, honey bees and *Varroa* are not the likely origin of MV, however, negative strand RT-PCR tests (Manley et al., 2015) must now be used to reveal replication efficiency of MV in different hosts and tissue types. As well as preying on arthropods, *V. pensylvanica* supplements its diet by feeding on floral nectar (Hanna et al., 2013), a shared resource, which could be a possible route of transmission of MV from wasp to honey bee. However, the mechanism of transmission is yet to be determined; further screening for MV is required to determine the full host range and indeed, epidemiology of this virus.

The dominance of the DWV type A master-variant in honey bees and *Varroa* is the result of the arrival of *Varroa* on Big Island, which facilitated the spread of this variant (Martin et al., 2012). Reads identified as Moku virus, DWV type C, Sacbrood virus and Black queen cell virus were only present at low levels compared to the high number of DWV type A reads,

and to a lesser extent DWV type B reads (Fig.3). Only a very small number of reads (0-200 range) were attributed to DWV type C (Mordecai et al., 2016a).

A recent study demonstrated *in vitro* that DWV can cause premature death of adult honey bees (McMahon et al., 2016). The honey bee harbours a lethal cocktail of RNA viruses, which dependent on circumstance (environmental conditions, anthropogenic stressors or the introduction of a new vector) can result in the most severe of outcomes. In addition, insects often found associated with honey bees also carry highly virulent RNA viruses. This is evident by the presence of Israeli acute paralysis virus (IAPV) in *V. pensylvanica* (Fig. 3). In one wasp sample, IAPV reads make up 1.7% of the total virus reads sequenced; despite no IAPV reads being detected in the honey bee samples from the same location on the same day. This suggests that IAPV is replicating in the vespine host. 'Honey bee' RNA viruses are generalists, capable of infecting a variety of insect hosts (Singh et al., 2010; Manley et al., 2015) and they can be readily transmitted between Hymenopteran insects such as bees and now wasps.

The pathogenicity of Moku virus in wasps and honey bees remains unknown. The high viral load of MV in wasps suggests that *V. pensylvanica* is its natural host. Interestingly, two of the wasp samples (W_S28 and W_S30) contained several orders of magnitude less MV reads than the other wasp samples (Fig. 3). These two samples were instead dominated by DWV (type A & B), suggesting that there is a possible competitive interaction between MV and DWV, plausibly for sites of replication. Therefore, it is possible that the relatively high viral loads of DWV in honey bees and *Varroa* effectively exclude Moku virus from replicating to higher levels. Competitive exclusion has previously been suggested between iflaviruses where a persistent DWV infection *in vitro* was suggested to restrict the replication of a related virus (Carillo-Tripp et al., 2016) as well as *in vivo* where one variant of DWV prevented superinfection by another (Mordecai et al., 2016b). The detection of Moku virus in wasps, *Varroa* and honey bees suggests that cross-species transmission of RNA viruses is a threat to pollinator health worldwide. This is further supported by the recent discovery of

a plant virus replicating in honey bees, demonstrating the host range of RNA viruses can even cross kingdoms (Li et al., 2014).

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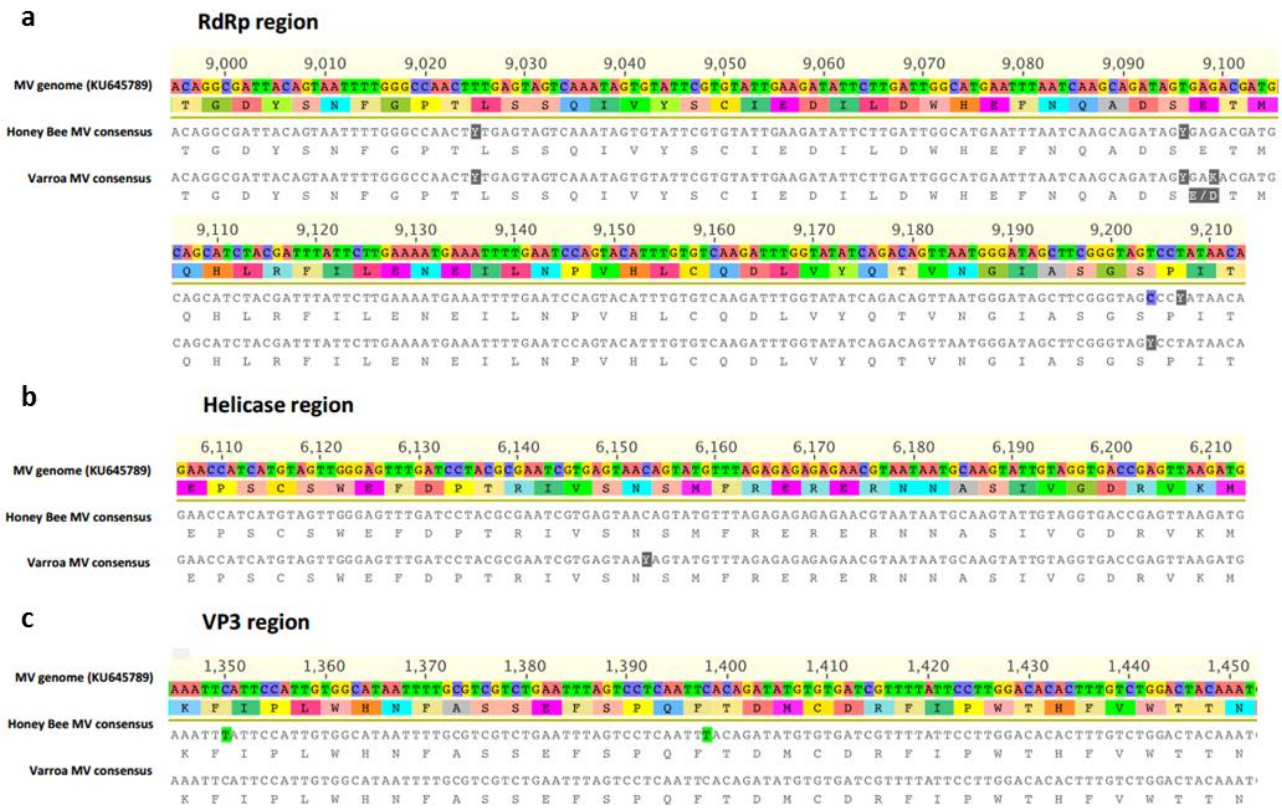
Supplementary Information



Supplementary Figure S1**.** Multiple sequence alignment of three MV genomic regions (a: RdRp, b: Helicase, c: VP3) from honey bees and *Varroa* aligned against the wasp MV genome (KU645789). Substitutions against the MV genome are highlighted.

Supplementary Figure S2. Honey bee (a) and *Varroa* (b) and Wasp (c) Illumina reads aligned to the MV genome in the RdRp region showing individual reads and nucleotide substitutions which make up the quasispecies ‘cloud of variants’.

Chapter 5: Common apiary pests as a potential source of honey bee – associated viruses in a Hawaiian apiary environment.

Abstract

Deformed wing virus and other viruses once thought to be 'honey bee viruses' are now understood to be widespread generalists capable of infecting diverse hosts. Given that honey bees live amongst, and share resources with, a range of other often closely related arthropods, and that these viruses are often fast to evolve and genetically variable, the potential for virus spillover and emergence in the apiary environment is of great concern, both for honey bees and the wider invertebrate community. RNAseq data generated from several common apiary pests revealed DWV to be the most widespread virus in the apiary, with full genomes assembled from ants, wasps, small hive beetles, honey bees and *Varroa*. Comparing proportions of DWV types A, B and C, genome coverage and read counts revealed samples to group by host species, indicating variants/recombinants may be present in the quasispecies which are particularly adapted to replicate in different host species. However, phylogenetic analysis revealed genetically similar DWV sequences to be circulating in the apiary suggesting frequent inter – species transmission events. Species groupings also applied when considering other viruses, many of which were widespread in the apiary. In the case of the wasps, samples grouped further by geographic location, with location potentially influencing viral load. These findings suggest that common apiary pests have the potential to act as reservoirs of honey bee – associated viruses and highlight the importance of considering the wider community in the apiary when considering honey bee health.

Introduction

Across the globe emerging infectious diseases (EIDs) pose a significant threat to biodiversity and health (Daszak, Cunningham, & Hyatt, 2000), as demonstrated by recent large-scale

declines of amphibians (Blaustein & Johnson, 2010) and honey bees (Schroeder & Martin, 2012), caused by pathogenic fungi and viruses respectively.

EIDs often occur as a consequence of human-mediated translocations of infected hosts and/or parasites, and due to the close proximity of wild and domesticated hosts (Daszak et al., 2000). As such the honey bee, which over the last century has been spread across the globe by humans for honey production and pollination services (Wilfert et al., 2016), and which shares complex communities with a wide range of insect taxa (Potts et al., 2003; Steffan-Dewenter & Tscharntke, 2000; 2002; Thomson, 2004), is a prime candidate to cause the spread of EIDs into new insect hosts. Pollinators and other insects with which they share environments are of particular interest due to their value in terms of economy (including pollination services) and biodiversity and are currently already experiencing a number of pressures e.g. from habitat loss and pesticides (Potts et al., 2010; Brown et al., 2016; Vanbergen, 2013). The combination of multiple pressures can have the additional effect of decreasing immunity and thus potentially increasing the susceptibility to new pathogens (Cornman et al., 2012), although further studies are needed in this area (Collison et al., 2016).

Most host switching results in a dead end or a limited low-level outbreak, however, on rare occasions the transmission can result in sustained outbreaks or major epidemics. This can happen when there is sufficient increased exposure, or the evolution of new variants in the original or new host allows successful replication and efficient spread between members of the new host species (Parrish et al., 2008). These events can have disastrous consequences e.g. the emergence in humans of HIV-1 originating from old world primates has caused the death of 35 million people (WHO, 2017), and continues to cause death as a result of AIDS even in developed countries where treatment is cost – free for the patient (Croxford et al., 2017). Furthermore, continued Hendra spillover from pteropid bats into both humans and horses causes acute respiratory and neurological disease (Field, 2016; Murray et al., 1995).

Deformed wing virus (DWV) is now well known to be one of the major factors responsible for honey bee colony losses across the world (Ryabov et al., 2014; Schroeder & Martin, 2012; Wilfert et al., 2016). The virus came to dominate the virome of honey bee populations due to its spread and amplification by the ectoparasitic mite *Varroa destructor* (referred to as just *Varroa* from now on) (Martin et al., 2012; Mondet et al., 2014). Additionally, the new transmission route served to reduce viral genotypic diversity and select for the amplification of virulent strains (Martin et al., 2012; Ryabov et al., 2014). Although initially described as a honey bee virus since it was first described from this host and due to its association with large-scale colony losses, it has since become apparent that DWV is a generalist insect virus capable of infecting a range of taxa (Zhang et al., 2012; Levitt et al., 2013; McMahon et al., 2015; Singh et al., 2010). However, the extent of the generality is still the subject of contention (Eyer et al., 2009) and little is yet known about the extent of the pathogenicity in non-*Apis* hosts (Furst et al., 2014; Genersch et al., 2006; Gisder & Genersch, 2016), partly because the typical wing deformities associated with DWV infection in honey bees are not optimal for diagnosis because this is a non-specific symptom can be caused by other factors (Manley et al., 2015), including the lack of fluids (Bowen-Walker et al., 1999). Furthermore, with non-managed species it is more challenging to assess information about pathogenic effects as overt virulent infections resulting in mortality would likely go unnoticed.

A number of recent studies have discussed the possibility that viral pathogens circulating in managed pollinators may be driving infections in wild species e.g. (McMahon et al., 2015) and it is well accepted that the study of these circulating RNA viruses is of great importance due to the catastrophic effects they have had on honey bees and their ability to spread between phylogenetically related insect hosts (Manley et al., 2015). Thus, we aimed to conduct a pilot study using next generation sequencing to investigate the arthropod community living in managed Hawaiian apiaries.

The invasive pests investigated were *Varroa* mites, small hive beetles, yellowjacket wasps and two species of ant. *Varroa* are the most significant pest of the European honey bee, now ubiquitous in colonies across the world, where they survive as parasites feeding on

haemolymph. They were first recorded in Hawaii in 2007 on Oahu, and then in 2009 on Big Island, where, after a failed eradication attempt, they spread through the island's managed and feral populations (Martin et al., 2012). Small hive beetles (*Aethina tumida*) also live in the hive feeding on honey, nectar, bee larvae and both dead and newly emerged adult bees (Neumann et al., 2016). Heavy infestations can kill a healthy colony but usually this species cause colony loss only when they are able to take advantage of already weak colonies. They complete part of their life cycle in the soil and thus disperse very easily and are almost impossible to eradicate (Neumann et al., 2016). The species originates from Africa, but in recent decades has spread around the world, having been first recorded in Hawaii (Big Island and Oahu) in 2010 (Robson, 2012). The yellowjacket wasp (*Vespula pensylvanica*) was first recorded in Hawaii on Kauai in 1919 and since the late 1970s has been established throughout the archipelago (Nakahara, 1980; Gambino, 1992). It is a generalist predator which often feeds on honey bees (Gambino, 1992), robs honey from weak colonies and also can also share floral resources with bees (Hanna, Foote & Kremen, 2013). This study concerned the two ant pests: big headed ants (*Pheidole megacephala*), which was first discovered in Hawaii in 1825 (Banko & Banko, 1976), and ghost ants (*Tapinoma melanocephalum*), first discovered in Hawaii in 1899 (Forel, 1899). The generalist foraging and nesting behaviour of both species has led to them becoming widespread pests in the home, in agriculture and in the hive (Banko & Banko, 1976; Clagg, 1957). Both species are a nuisance to beekeepers in Hawaii where they are commonly found nesting in hives (Ethel Villalobos, personal communication).

These pest species were targeted as they would come into the most intimate and frequent contact with honey bees known by our previous work to harbour high DWV loads (Martin et al., 2012; Mordecai et al., 2016a). We hypothesised that the frequency of interactions would result in common DWV genotypes circling in the apiary environment due to repeated viral transmission events between species. Furthermore, we aimed to investigate whether other viruses commonly found in honey bees are to be found in apiary pests, and if so, whether particular viruses are associated with particular hosts. This is a first attempt to construct a virome map of the network of viral interactions between a range of species.

Methods

In November and December 2012 managed apiaries on the islands of Oahu and Big Island, Hawaii were visited and samples of asymptomatic honey bees and common apiary pests were collected. *Varroa* populations had been established at all locations sampled for at least three to five years, and honey bee populations were known to harbour high DWV levels (Martin et al., 2012). Pools of 50-100 honey bees (*Apis mellifera*) were collected from individual colonies and where found yellowjacket wasps (*Vespula pensylvanica*), ghost ants (*Tapinoma melanocephalum*) and big headed ants (*Pheidole megacephala*) were collected from inside brood boxes or at the hive entrance and kept on ice for transportation to the laboratory where they were stored at -80°C. Additionally small hive beetles (*Aethina tumida*) had been collected earlier in the year from colonies in an advanced stage of collapse. These had also been stored at -80°C.

Pools of 30 honey bees were taken and checked for the presence of *Varroa* mites and, where found these were removed. The pools were then ground in liquid Nitrogen using a sterile pestle and mortar to produce a fine homogenous powder, 30mg of which was used for RNA extraction. Small hive beetles were crushed in pools of six using a mini pestle and 1.5ml eppendorf tube, yellowjacket wasps were crushed this way but individually and all ants were crushed in pools of 20-40 due to their very small size. RNA extractions proceeded for all samples in the same way using the RNeasy mini kit (Qiagen) following manufacturers' instructions. RNA was then treated with DNase I (Promega) followed by the production of cDNA libraries using oligo (Kleijn et al.) priming. Resulting libraries were then run on the HiSeq 2000 (The Earlham Institute, Norwich).

Initially, quality control of generated reads was performed using FASTQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Then a pipeline initially described by Mordecai *et al*. (2016a) was applied to identify DWV-like reads in individual samples. Briefly, this involved taking all reads which passed QC and using a nucleotide BLAST against a custom database (e value of 10e -05) containing DWV types A, B and C (type A:

NC_004830.2 and NC_005876.1 Kakugo virus, type B: AY251269.2(VDV-1) and type C: CEND01000001.1). Custom perl, sed and awk scripts were used to parse the data, take the top BLAST hit, remove empty lines and remove reads which didn't map to the database. BLAST top hit analysis was used to quantify the numbers of reads belonging to each of the three master variants. These were used to produce pie charts showing the proportions of type A, B and C reads in each sample.

The DWV-like reads were balanced and then used to generate *de novo* assemblies using IVA, an assembler specifically designed to accommodate highly variable sequence data (Hunt et al., 2015). Previously our work had involved assembling viral sequences using VICUNA (Yang et al., 2012). However due to the better performance of IVA in terms of ease of use and generation of comparable assembled contigs, IVA was used for all subsequent assemblies.

Geneious (Version 7.04, Biomatters) was used to visualise contigs and to map all reads passing QC (read 1, in fasta format) to a 500bp fragment of the DWV type A RdRp gene (nucleotide positions 8629-9128, DWV accession number NC_004830.2), and those reads spanning the whole 500bp were kept and trimmed. This was repeated mapping to type B and type C (accession numbers AY251269.2 and CEND01000001.1, respectively), to ensure all contigs spanning the 500bp region of interest were present, representing the entire population. Trimmed reads were aligned using ClustalW and Geneious tree builder was used to create a neighbor – joining phylogeny using a Tamura-Nei Genetic Distance model.

Competitive alignment plots were created using the 'Map to Reference' tool in Geneious, using DWV types A, B and C (NC_004830.2, AY251269.2 and CEND01000001.1) reference genomes, discarding all ambiguous reads.

To investigate the presence of other honey bee – associated viruses circulating in the apiary community, a custom BLAST database was created using ten commonly found viruses

(Supplementary Table S1), and BLASTn was used to search for reads belonging to each virus using a cut-off of 10e -05. Numbers of reads belonging to each virus were calculated and the data was transformed (square root) to produce histograms in Microsoft Excel.

Additionally, Kraken (Version 1.1.11, Center for Computational Biology, John Hopkins University) was used to investigate entire RNA viromes and to compare two methods of viral read detection (comparing with BLASTn). Data from one of each species was picked at random, with the exception of ants, where both species were pooled. The same read 1 fasta files which were used for BLAST analysis were searched against a custom Kraken database comprising the NCBI viral genomes database with the addition of Lake Sinai virus (NC_032433.1), Moku virus (NC_031338.1) and Mililii virus (MF155030.1), which were not in the standard virus database at the time of downloading (12/06/2017).

Results

Deformed wing virus

DWV was detected in all honey bees (n=3), small hive beetles (n=5), yellowjacket wasps (n=5), big headed ants (n=2) and ghost ants (n=2) sampled. Numbers of DWV reads, calculated as all reads identified using BLAST, were highly variable and ranged from 17,502 in the beetle sample B_S74 (0.10% of reads) to 31,996,646 in the *Varroa* sample V_S48 (91.25% of reads). BLAST Top Hit Analysis suggested the proportions of reads belonging to each of the master variants grouped by species (Fig. 1). Type A dominated in most honey bees, *Varroa* and both ant species, wasps usually contained relatively similar proportions of types A and B, and beetles were all dominated by by type C.

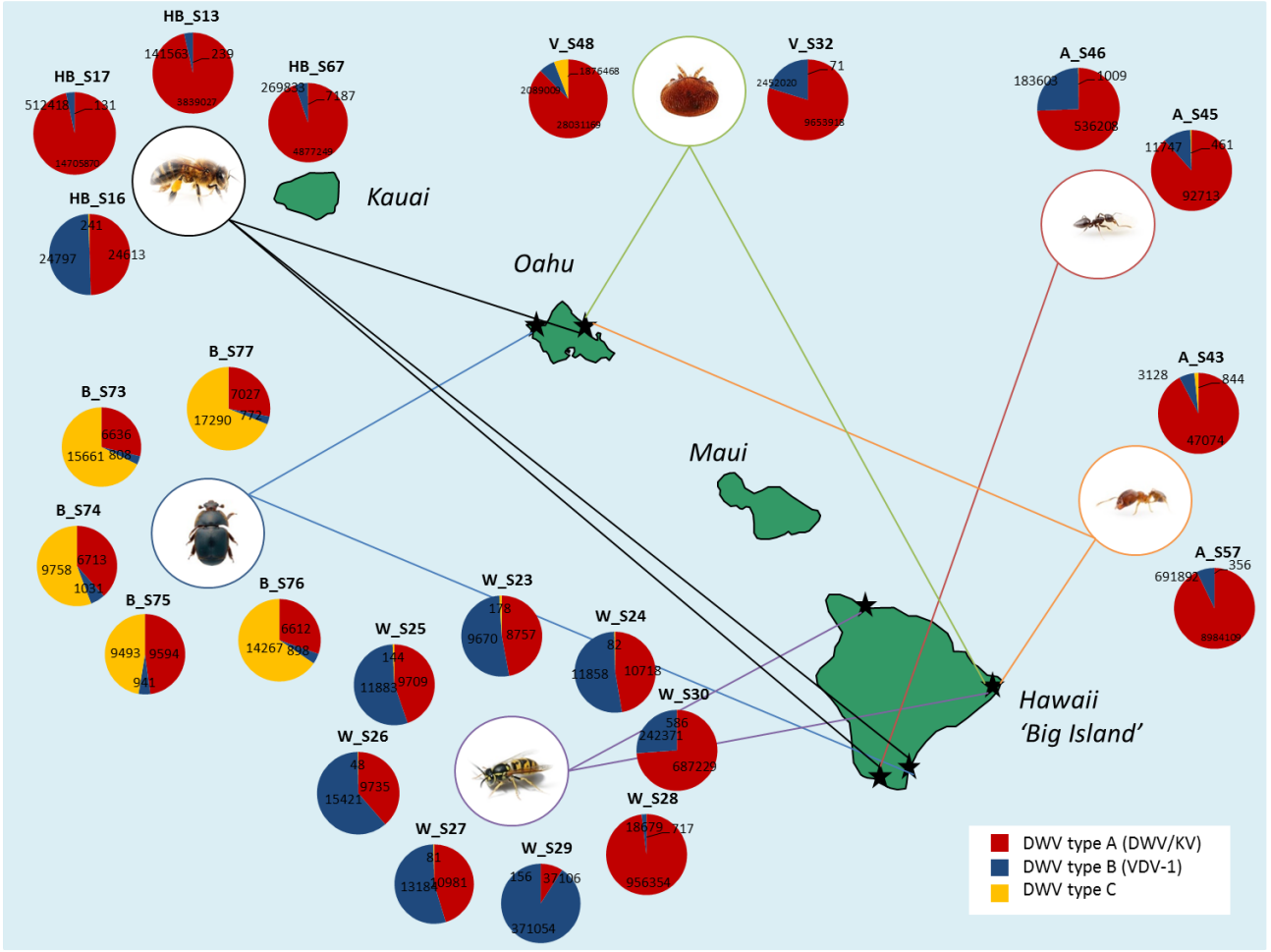
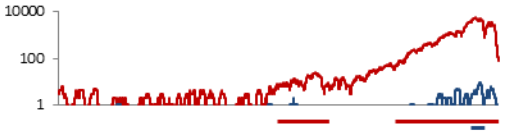
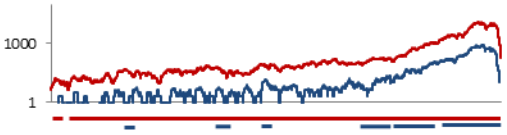
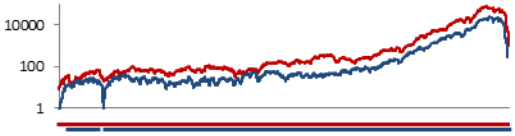
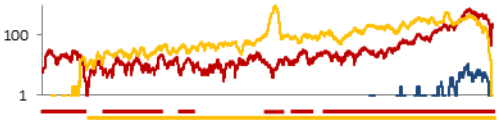
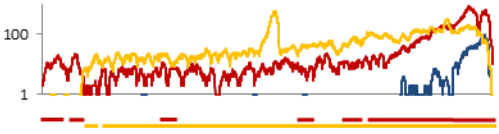
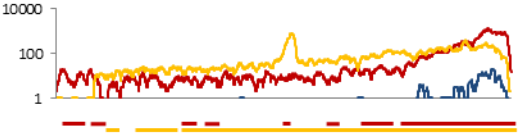
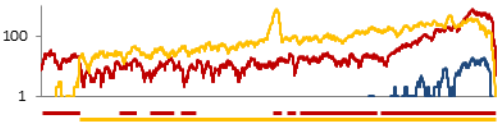
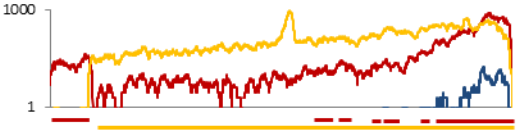
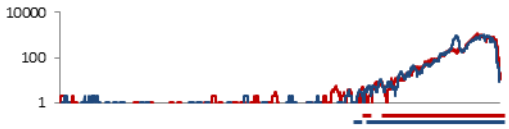
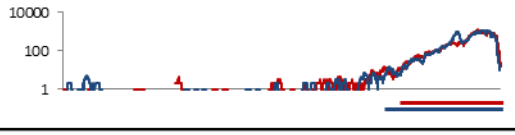
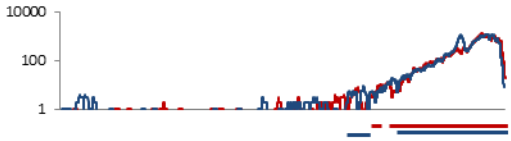
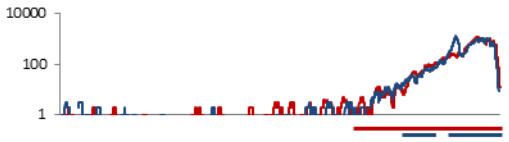
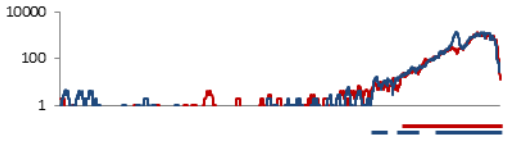
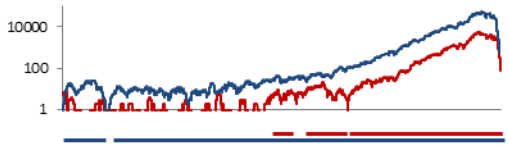
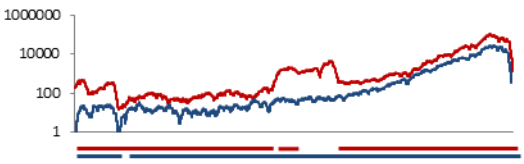


Figure 1. Shows the approximate locations of the apiaries where samples from each species were collected. The pie charts show the proportions of DWV types A, B and C in each sample annotated with numbers of reads.

Reads identified by BLAST as belonging to the DWV quasispecies were then assembled *de novo* for each sample using IVA and generated contigs were mapped to the DWV type A genome (NC_004830.2) which revealed full length coverage was achieved for most samples (Fig. 2). Competitive alignments all showed the typical 3' bias resulting from the oligo (dT) priming method of cDNA synthesis in library preparation (Brooks, Sheflin, & Spaulding, 1995). Similarly, the samples containing type C all showed a spike in the Helicase region caused by the presence of a poly-(A) region of the genome. The competitive alignment results were all consistent with the contig alignments, with the exception of sample HB_S67 for which no type C contigs were produced due to the low coverage depth (Fig. 2).

Sample name	species	location	DWV genome coverage
A_S43	*Pheidole megacephala*	Big Island, East	
A_S57	*Pheidole megacephala*	Oahu, East	
A_S45	*Tapinoma melanocephalum*	Big Island, South 2	
A_S46	*Tapinoma melanocephalum*	Big Island, South 2	
B_S73	*Aethina tumida*	Oahu, West	
B_S74	*Aethina tumida*	Big Island, South 1	
B_S75	*Aethina tumida*	Big Island, South 1	
B_S76	*Aethina tumida*	Big Island, South 1	
B_S77	*Aethina tumida*	Big Island, South 1	

Sample name	species	location	DWV genome coverage
W_S23	*Vespula pensylvanica*	Big Island, North	
W_S24	*Vespula pensylvanica*	Big Island, North	
W_S25	*Vespula pensylvanica*	Big Island, North	
W_S26	*Vespula pensylvanica*	Big Island, North	
W_S27	*Vespula pensylvanica*	Big Island, North	
W_S28	*Vespula pensylvanica*	Big Island, East	
W_S29	*Vespula pensylvanica*	Big Island, East	
W_S30	*Vespula pensylvanica*	Big Island, South 1	

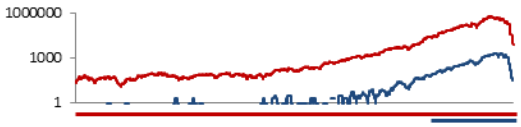
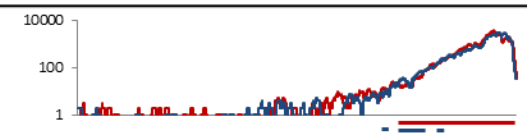
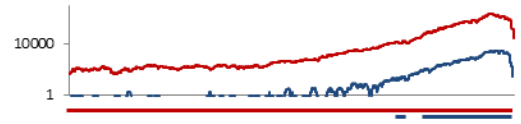
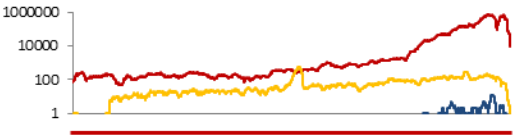
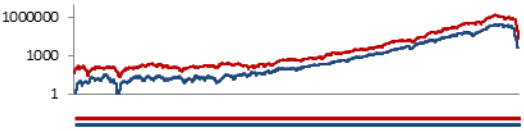
Sample name	species	location	DWV genome coverage
HB_S13	*Apis mellifera*	Big Island, East	
HB_S16	*Apis mellifera*	Big Island, South 1	
HB_S17	*Apis mellifera*	Big Island, South 2	
HB_S67	*Apis mellifera*	Oahu, East	
V_S32	*Varroa destructor*	Big Island, East	
V_S48	*Varroa destructor*	Oahu, East	

Figure 2: Genome coverage for all samples in this study along with species name and locations from which they were collected. DWV coverage is shown as competitive alignments plots, underneath which mapped contigs are represented by bars. The DWV genome organisation, adapted by Lanzi *et al*. (2006), and Wood *et al*. (2014) is shown above for reference and type A is shown in red, type B in blue and type C in yellow.

Competitive alignments and mapped contigs in Figure 2 show three of the four ant samples were dominated by type A sequences, although sample A_S46 also contained full length type B coverage. Interestingly the competitive alignment plots showed all beetle samples were all dominated by a type A/C recombinant which was also present in the honey bee sample HB_S67 although it did not dominate. The wasp samples grouped by location, with the Big Island North samples showing coverage only towards the 3' end (both types A and B), but those samples from Big Island East showing full length coverage of types A, B or both. The competitive alignment produced from one sample from Big Island East, W_S30 appears to show 3 recombination breakpoints (one in the 5' UTR, and one at either end of the helicase gene) which aren't present in any other samples. With the exception of HB_S16, all bee and Varroa samples showed full length type A genomes, with the *Varroa* sample V_S32 also containing full length type B.

A nighbor – joining phylogeny created using a 500bp fragment of the RdRp gene assembled from all samples showed that the majority of samples contained phylogenetically similar type A sequences (Fig. 3). There appear to be minor species/location groupings, with three contigs assembled from wasp samples from Big Island, North forming a distinct type A clade. These same samples also contained the only other type B contigs which were assembled for this 500bp region. The type C sequences present were almost identical to one another and were assembled from beetles from Big Island, South and along with one from a honey bee sample from Oahu East.

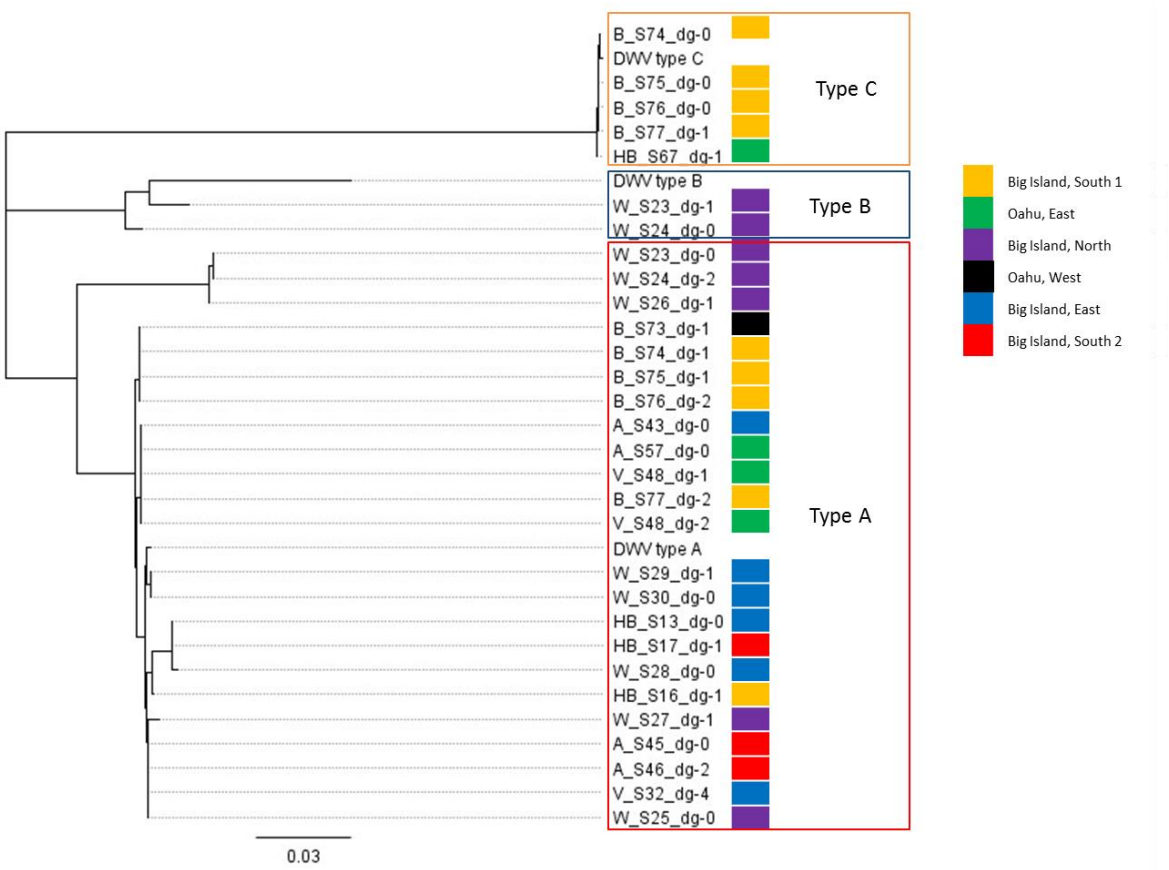


Figure 3. A neighbor - joining phylogeny of trimmed DWV contigs spanning a 500bp region of the RdRp gene, showing distinct type A, B and C clades. Honey bee sample names are prefixed with HB, *Varroa* = V, wasp = W, ant = A and beetle = B. Samples are colour coded by location.

Non – DWV viruses

In addition to generally grouping by family in terms of DWV types A, B and C proportions and coverage, families also grouped together when considering the other viruses they harboured. Using BLAST to look for ten viruses commonly found in honey bees revealed that honey bees (with the exception of sample HB_S16), *Varroa* and big headed ants, *P. megacephala* were all dominated by DWV; ghost ants, *T. melanocephalum* were dominated by Milolii virus; wasps were dominated by Moku virus; and beetles contained relatively few

virus reads (Fig. 4, Supplementary Table S2). The wasp samples further separated into two groups, with samples W_SS23 – W_S27 containing high numbers of Moku virus reads and also consistent amounts of SBPV, whereas W_S28 – W_S30 showed increased DWV, more variable Moku virus and no notable SBPV. The honey bees were the only samples to contain any notable BQCV.

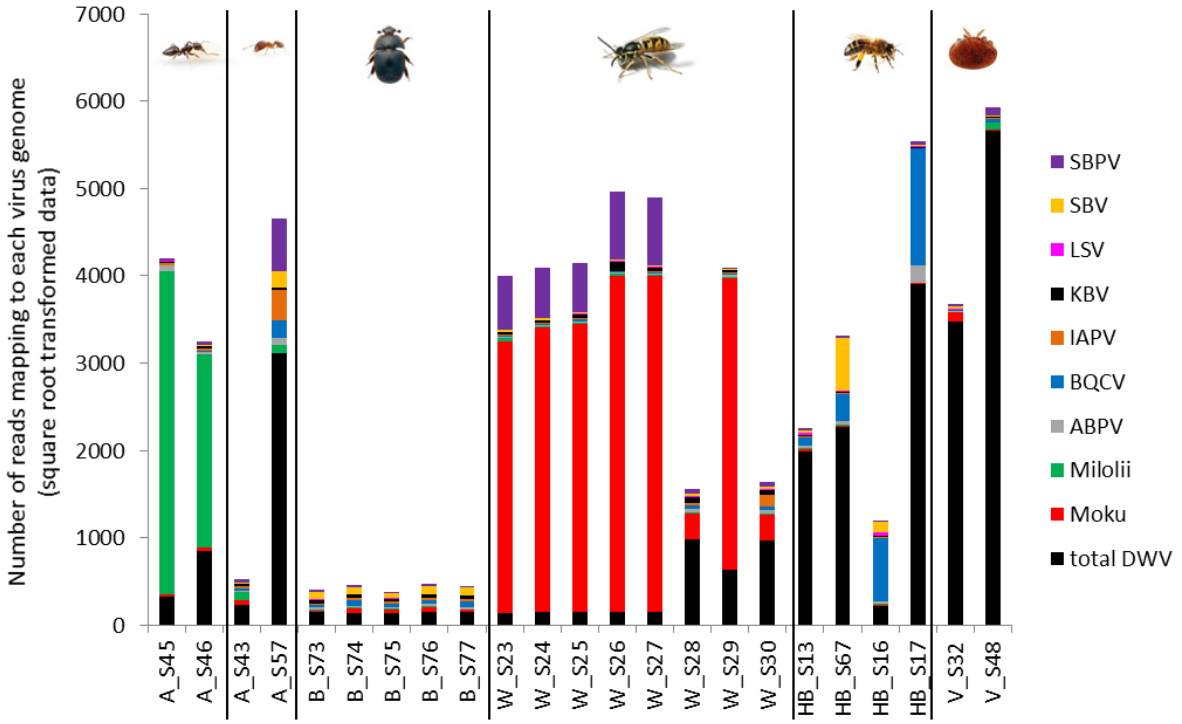


Figure 4. Histograms represent square root transformed read counts mapping to each of ten commonly found insect viruses. Samples are grouped by family: ghost ants, big headed ants, small hive beetles, yellowjacket wasps, honey bees and *Varroa*.

Sequences belonging to common viruses were also identified using Kraken for one sample per family to validate BLAST results. Although results were similar when comparing relative read numbers for each virus between samples, BLAST gave consistently higher read numbers owing to reduced stringency (Fig. 5). As such, BLAST was chosen to identify reads

in each sample individually (Fig. 4) so as to ensure reads more distantly related within the quasispecies were not discarded. This was evidenced when considering SBPV reads. BLAST showed all samples to contain SBPV reads; in particular the wasp samples showed considerable read numbers. But Kraken analysis resulted in no reads whatsoever. In particular sample W_S24 contained 330,002 reads with BLAST but no reads with Kraken. This difference may be attributed to the SBPV – like reads being too variable and insufficient for Kraken to identify, yet similar enough for the BLAST search to hit.

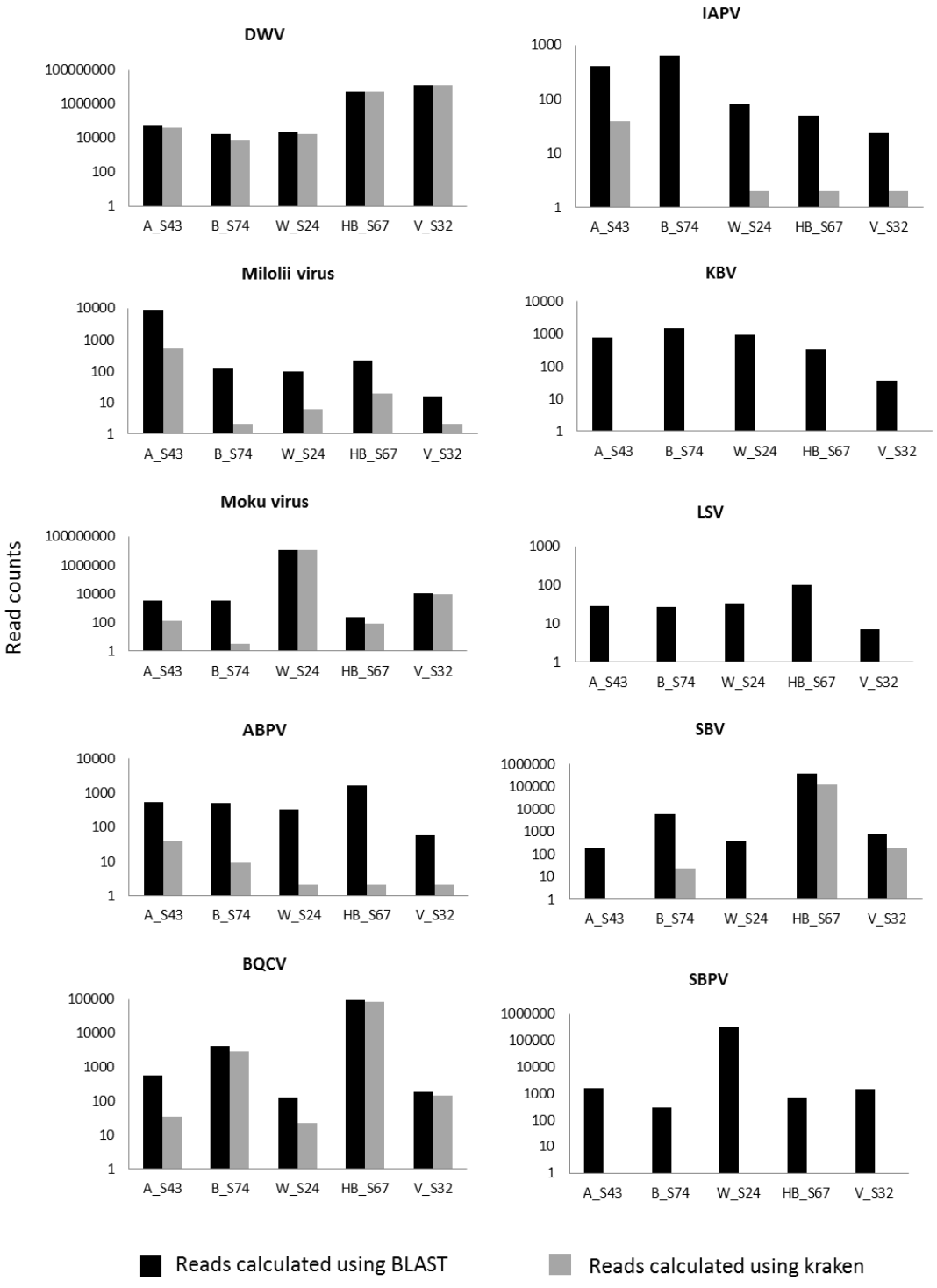


Figure 5. Bar charts show numbers of reads (log scale) mapping to each of ten common insect viruses as calculated by BLAST analysis (black) and by kraken (grey).

Discussion

This study revealed common apiary pests have the potential to act as reservoirs of a number of honey bee – associated viruses. Although recent studies have highlighted the ability of a number of viruses initially described as honey bee pathogens to infect a range of taxa (Furst et al., 2014; Gisder & Genersch, 2016; Levitt et al., 2013; Singh et al., 2010), replicate (Eyer et al., 2009; Radzevičiūtė et al., 2017), and in some cases cause pathogenic effects (Genersch et al., 2006), this is the first study to assess the viral burden of taxonomically diverse common apiary pests in a subtropical environment.

Of ten common RNA viruses surveyed, DWV was the most common virus in the honey bees and *Varroa*, as expected following our previous work (Martin et al., 2012). Consistently with other studies (Radzevičiūtė et al., 2017), DWV was the most prevalent virus in the apiary with full genome coverage achieved from samples of each species. Comparable genome coverage profiles and proportions of DWV types A, B and C present were seen between samples of the same species, with wasps separating further by location. Interestingly, beetles sampled from across two locations were the only ones to be dominated by DWV type C, albeit with low read counts.

Although investigating viral reads by RNAseq is not an optimal method to assess whether the samples are harbouring true infections, the often high read counts, full genome coverage and assembled full genomes suggest that the DWV is replicating in at least some samples from each species. Furthermore, species – specific patterns of genome coverage are seen e.g. the presence of an A/C recombinant in all beetles, and the dominance of a recombinant(s) in the wasp sample W_S30 that was not seen in any other sample. This suggests there are specific variants/recombinants present in the quasispecies that are able to successfully replicate in different species. The beetle samples from Big Island South were collected earlier in the year than the honey bee sample from the same location (HB_S16), from colonies in an advanced stage of collapse due to an extremely heavy infestation by the

beetles. Therefore it is possible that their recombinant A/C infections were acquired from the honey bees at that time.

The impact from potential contamination must not be ruled out, either from the guts through contaminated hive materials; from the lab during sample preparation; or during the Illumina run through barcode switching. The latter is of particular concern in studies such as this with DWV reads numbers being so variable across our samples. For example, in the *Varroa* sample V_S48 up to 91.25% of total reads belonged to DWV, whereas as little as 0.10% of reads in the beetle sample B_S74 belong to DWV. However, the species, and location specific DWV profiles shown by the genome coverage plots suggest any contamination is minimal and would have had little if any effect on the results.

The phylogeny (neighbor – joining) of a conserved region of the RdRp gene revealed the majority of samples were dominated by genetically similar type A variants, with only three of the wasp samples from Big Island North harbouring genomes from a separate distinct type A clade. The genetic similarity between sequences present in all species suggest common variants circling as a result of frequent inter – species transmission events. The nature of these transmission events remains unclear, with trophylaxis (between small hive beetles) (Eyer et al., 2009), faecal – oral routes and predation on bees and consumption of contaminated hive materials, pollen and nectar all being implicated as routes by which viruses can spread both between and within species (Singh et al., 2010).

The read count data for the wasps appears to suggest that location can have an effect on viral load. Although read count data is not a reliable method of viral quantification, it is apparent that the amounts of DWV and SBPV are comparable from wasps from Big Island North (W_S23 – W_S27), whereas the wasps from Big Island East (W_S28 – W_S30) show much more variable amounts. Unfortunately, there were no other samples from Big Island North so we are unable to speculate upon any differences between the two locations.

This is the first study of arthropods in the wider apiary community to consider the newly described Moku (Mordecai et al., 2016b) and Milolii (Brettell et al., 2017) viruses. Although these viruses only dominated in the samples from which they were first described, low numbers of reads were also found in other *Varroa*, beetle, wasp and ant samples. The wider RNA virus detection study revealed that all of the common honey bee – associated viruses tested for were present at some level in the Hawaiian apiaries, although IAPV, KBV, ABPV and LSV were only present at very low levels (as detected through both BLAST and Kraken) and therefore true infections cannot be confirmed in any sample. The comparison of BLAST and kraken data also provided additional information, primarily where SBPV was concerned. The fact that SBPV was not found at all by kraken, but was found consistently using BLAST led us to hypothesise that this is due to genotype(s) of SBPV circulating which are too genetically dissimilar from the SBPV genome (NC_014137.1) to be identified as SBPV using Kraken. Furthermore, the fact that wasps from Big island North all contained comparable levels of SBPV (using BLAST), suggests there may be variants present which are adapted to replicate in wasps.

In conclusion, although this pilot study has limitations, namely unbalanced sampling design, we have shown that several common honey bee – associated RNA viruses are common in taxonomically diverse apiary pests. We showed that DWV was the most prevalent virus and that DWV infections grouped between species in terms of dominant variants and recombinants, but that common variants (predominantly type A) were circling between all species, suggesting repeated transmission events between species. Within the wasps, DWV further separated by location. Species also grouped in terms of which other honey bee – associated viruses they harboured, i. e., particular viruses are associated with particular hosts. Therefore, this study highlights the necessity to consider the wider arthropod community as potential reservoirs of viral pathogens in the apiary environment.

Supplementary Information

Supplementary Table S1: Viruses commonly found in bees used for BLAST analysis along with accession numbers.

Virus	**Accession number**
Deformed wing virus – type A	NC_004830.2
Deformed wing virus – type A, Kakugo virus	NC_005876.1
Deformed wing virus – type B, Varroa destructor virus 1	AY251269.2
Deformed wing virus – type C	ERS657949
Mililii virus	MF155030.1
Moku virus	NC_031338.1
Acute bee paralysis virus (ABPV)	NC_002548.1
Black queen cell virus (BQCV)	NC_003784.1
Israeli acute paralysis virus (IAPV)	NC_009025.1
Kashmir bee virus (KBV)	NC_004807.1
Lake Sinai virus (LSV)	NC_032433.1
Sacbrood virus (SBV)	NC_002066.1
Slow bee paralysis virus (SBPV)	NC_014137.1

Supplementary Table S2. Raw read count data showing numbers of reads (from read 1, after QC) mapping to each of ten common viruses. Heat map is calculated for each sample separately, and sample names are coloured to signify location. Red = Big Island South 2, blue = Big Island East, green = Oahu East, black = Oahu West, orange = Big Island South 1, purple = Big Island North. Honey bee sample names are prefixed with HB, *Varroa* = V, wasp = W, ant = A and beetle = B. Samples are colour coded by location.

sample	total reads	total DWV	Moku	Milolii	ABPV	BQCV	IAPV	KBV	LSV	SBV	SBPV
A_S45	19669974	104921	763	1.4E+07	3375	88	517	217	48	73	410
A_S46	17461610	720820	1193	4926727	733	84	993	363	90	192	942
A_S43	18268406	51046	3244	8694	527	579	414	786	28	182	1534
A_S57	19795762	9676357	20	7501	7949	40832	117344	582	18	36740	360438
B_S73	18581324	23105	305	160	506	551	537	1585	51	7620	277
B_S74	16891197	17502	3382	122	508	4258	635	1537	26	6060	293
B_S75	16609397	20028	1401	65	310	2247	694	589	31	3306	204
B_S76	17083341	21777	3973	100	404	1715	720	2068	23	8056	373
B_S77	17683443	25089	242	78	315	5090	547	1477	25	8573	240
W_S23	11961813	18605	9705139	1003	229	165	120	1262	20	480	385689
W_S24	12807830	22658	1.1E+07	97	317	124	84	921	34	374	330002
W_S25	14181324	21736	1.1E+07	157	530	316	198	2105	48	496	319108
W_S26	16089890	25204	1.5E+07	1383	110	74	45	11723	18	536	592927
W_S27	17001465	24246	1.5E+07	73	303	161	102	2049	50	536	605077
W_S28	15504637	975750	82330	175	2387	953	737	4222	300	774	3577
W_S29	14215385	408316	1.1E+07	44	528	348	132	769	59	122	141
W_S30	13394271	930186	89865	217	2013	1104	18752	2472	269	456	4130
HB_S13	17678449	3980829	268	241	686	8727	99	454	997	724	708
HB_S67	13765791	5154269	217	211	1639	96874	49	322	102	365756	724
HB_S16	12183541	49651	217	101	479	535525	46	349	1521	15618	108
HB_S17	23505172	15218419	146	93	38717	1783406	23	142	161	251	1565
V_S32	13498757	12106009	9926	16	57	188	24	37	7	723	1420
V_S48	35064635	31996646	406	6006	41	1345	145	72	3	331	8517

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GENERAL DISCUSSION

In recent decades our most important pollinator, the honey bee (*Apis mellifera*) has experienced losses across the globe, especially in the Northern Hemisphere. Although many factors have contributed to this decline, emerging infectious diseases are now understood to be the main driver, the most significant of which has been, and remains to be, deformed wing virus (DWV) in association with its vector, the parasitic mite *Varroa destructor* (Schroeder & Martin, 2012). The spread of this parasite and pathogen have had a devastating effect on honey bees (Genersch et al., 2010; Martin et al., 2012) and although there is much ongoing research in the field there are still clear gaps in our knowledge which, if filled, could provide clues to protecting honey bees, other pollinators and associated members of the ecosystem. This PhD aimed to go some way to filling these gaps.

Within this complex field there also remain sources of great contention such as the subject of virulence: DWV type A was initially found to be the typical more virulent form of DWV responsible for colony losses (Martin et al., 2012), but this has since become more complicated with the discovery of virulent A/B recombinants (Moore et al., 2011; Ryabov et al., 2014) and a recent study reporting type B as the more virulent variant (McMahon et al., 2016). Furthermore, a recently described third master variant, type C (Mordecai et al., 2016a), has been discovered about which we still know very little.

Another source of contention is the role of *Varroa* in the 'honey bee – *Varroa* – DWV' triangle of associations. It is well known that *Varroa* effectively vectors DWV and other RNA viruses, but beyond that its role is disputed, with some studies suggesting that it amplifies (Gisder et al., 2009) or activates (Shen et al., 2005) DWV replication. But others dispute this and suggest its role is merely the mechanical transferring of viral particles between honey bee hosts (Erban et al., 2015). Throughout this PhD I also aimed to provide some clarity to these discussions.

Firstly, on the topic of virulence, I considered why some bees show the deformed wing phenotype. The appearance of bees showing the characteristic shrivelled, deformed wings attributed to DWV is commonly used by beekeepers to diagnose overt DWV infections. Although previous studies have shown that deformed bees have consistently high viral loads (Chen et al., 2005), these levels can also be found in asymptomatic bees (or rather, bees with no obvious deformity) (Highfield et al., 2009). So, it is not known why only a small proportion of infected bees develop the symptom. I aimed to address this by investigating whether there is any unique DWV variant responsible for causing the development of deformed wings by comparing the DWV genomes present in deformed and asymptomatic bees using a combination of quantitative RT-PCR and next generation sequencing approaches (Chapter One). This revealed no consistent differences between the dominant DWV genotypes present in deformed and asymptomatic bees, even when comparing sequence data across the entire genome. In fact, greater variation was seen between locations rather than phenotypes, indicating that there is no unique variant responsible for inducing deformity. Rather, many DWV variants have the potential to cause deformed wings. This study has since been backed up by findings using a much larger sample size (Christopher Moffat – personal communication), who found, again using phylogenetic approaches, that deformed and asymptomatic bees were infected by a range of shared DWV genotypes, and no genotypes were unique to deformed bees. The DWV genotypes found in Chapter One belonged to the type A master variant, whereas Moffat *et al* (unpublished), found both type A and type B. Furthermore, the DWV variants purified from a mixture of deformed and asymptomatic bees by Benaets *et al* (2017, Appendix Three) were all type B. This demonstrates quite clearly that no unique variant is responsible for causing deformity. The factor(s) which are responsible warrant further investigation.

There are a number of factors which could be involved in the development of deformity, for example *Varroa* parasitisation. Although it is well known that the majority of deformed bees develop from parasitised brood (Marcangeli et al., 1992; Möckel et al., 2011), as previously mentioned, the effect of *Varroa* – parasitisation in itself is still not fully understood. Although we now know that *Varroa* feeding activity, at least in the Japan haplotype of *Varroa* infesting the bees of Fernando de Noronha (Chapter Two), but presumably also in

the Korean haplotype, does not induce DWV activation in a pupa, the effect it does have is still unknown.

Additionally, the role of the immune response warrants further investigation. It may be proposed that some individuals will vary in their immune response to DWV infection, which could lead to varying outcomes. This could be due to genetic differences; although nest-mates will have the same mother, brood from different patrilines may see differing effects. Individuals may also vary in terms of the other pathogens they carry. Although the sociality of honey bees and the environment in the hive means it is likely that nest – mates will share a common suite of viruses (Naug & Camazine, 2002), there is always the possibility of subtle, yet important differences. Furthermore, although there is now strong evidence that unique DWV variants do not induce deformity, due to the impossibility of sampling a pupa before deformity begins to develop (i.e. to ascertain which variant(s) are circulating at the onset of wing development), this cannot be entirely ruled out.

Another area to consider when investigating virulence is those symptoms which cause the colony to weaken but are less easy to spot. DWV has been found to affect learning (Iqbal & Mueller, 2007), cause stunted growth (Allen & Ball, 1996), cause aggression (Fujiyuki et al., 2004) and reduce longevity (Nazzi et al., 2012; Dainat et al., 2012). But due to the general nature of these symptoms, it can be difficult to attribute a specific symptom to DWV at any one time. To investigate the impact of how covert, or overt – chronic, DWV infections cause colony loss, experimental injections were used to test for potential effects on foraging behaviour. We showed that individuals injected with low doses of DWV caused bees to forage at a premature age, reduced their time as foragers and decreased longevity (Appendix Three). These effects can weaken the colony in a number of ways: by reducing the numbers of nurse bees; reducing the number of individuals in a hive overall; and reducing the efficiency of foraging trips.

Secondly, a direction in which researchers are looking to provide insights into how bees may be able to better cope with the burden of *Varroa* and the associated viruses it transmits is to look to naturally resistant/tolerant populations.

When investigating these populations, and *Varroa* – virus – honey bee associations more generally, it is important to consider both at the *Varroa* part of the story and the virus side. Both parasite and pathogens have the ability individually to kill bees, although colony loss is usually due to a combination of the two (or often multiple) factors (Shen et al., 2005; Le Conte et al., 2010; Martin et al., 2012). Heavy *Varroa* infestations can weaken the colony by feeding on large proportions of the bees (and thus weakening pupae through depletion of haemolymph), and heavy infestations can even kill a colony if numbers get sufficiently high (Martin, 1998). Mite infestation levels in a colony vary greatly and, whilst they are usually kept under control by beekeepers using chemical treatments, they are also affected by mite life histories and reproduction levels (Locke, 2016). For example, mite reproduction may be reduced by frequent swarming, causing disruption to the brood cycle (Loftus et al., 2016).

DWV on the other hand, was responsible for rare colony killing in a time before *Varroa* (Ball, unpublished data) and now in the era of ubiquitous *Varroa* infestations it is now able to kill colonies even when *Varroa* numbers are kept low (Highfield et al., 2009). As such, when investigating resistant populations it is important to consider both sides of the coin. The constraints of the PhD were such that I decided to concentrate on the virus side of the story, but it is important to be aware when interpreting any results which do not investigate the *Varroa* aspect, that in reality it may be more complex.

Studying resistant/tolerant populations, it has become apparent that there is no one way in which bees defend themselves but rather different mechanisms have been described in different populations, for example, increased grooming behaviour (Büchler et al., 2010); small colony sizes and frequent swarming (Loftus et al., 2016); increased tolerance to DWV infection (Locke et al., 2014); and superinfection exclusion (Mordecai et al., 2016b). The

latter study, which I was involved with during my PhD (Appendix Two) identified superinfection exclusion to be the mechanism by which a population of bees is surviving in Swindon, UK despite harbouring high mite and DWV loads. In this population, type B has come to dominate and, in doing so, has prevented more virulent forms of type A from becoming established and killing the bees. To investigate whether this mechanism was widespread, I studied the oldest known *Varroa*-tolerant population of European honey bees on the island of Fernando de Noronha, 400 miles off the Brazilian coast. This small population has survived healthily for over 32 years despite never having experienced any *Varroa* control (De Jong and Soares, 1997; De Mattos et al., 2016; Locke, 2016). I found that, while DWV (type A) is present on the island, levels remain very low and genotypes remain diverse. This led me to hypothesise that at some point a virulent variant will emerge and, when it does, the high mite loads in the population will serve to effectively transmit the virulent variants, which will result in dramatic colony losses (Chapter Two).

The Fernando de Noronha study also resulted in my being able to disprove the long-held theory that *Varroa* feeding activity induces activation of latent DWV within a bee (Shen et al., 2005; Yang & Cox-Foster 2005; 2007), since these bees have existed with *Varroa* for many years and still harbour low level, genetically diverse DWV infections, just as we found in *Varroa* free honey bee populations in Hawaii (Martin et al., 2012), and has been found in the UK (Ryabov et al., 2014).

When considering honey bee health, and pollinator health more generally, it is important, as with all ecological questions to consider a more comprehensive picture. Although DWV is currently the most important viral pathogen of honey bees, there are a suite of other viruses known to infect bees that must not be overlooked, including those which undoubtedly exist but have not yet been identified. A recent study by Olival *et al*. (2017) suggested there could be vast numbers of as yet undescribed mammalian viruses, so one can only imagine how many unknown invertebrate viruses are present with the potential to cause catastrophe for pollinators, either directly or indirectly via the disruption of the ecosystem. This is being evidenced by the rapid increase in numbers of new viruses being

discovered using cutting edge NGS approaches (e. g. Shi et al., 2016; Liu et al., 2015). During this PhD I discovered two new RNA viruses circulating within the apiary. The first was Mililolii virus, found to infect the Ghost ant *Tapinoma melanocephalum* (Chapter Three). The second was Moku virus, which was sequenced in very high depth from the social wasp *Vespula pensylvanica*, but was also worryingly found at low levels in both *Varroa* and honey bees, suggesting that this virus has the potential to infect hosts from diverse taxa (Chapter Four).

Finally, to further expand the scope of my studies, I investigated the viral burdens of a taxonomically diverse group of common pest species found in Hawaiian apiaries with established *Varroa* populations and high DWV loads in the bees (Chapter Five). DWV was found in every sample tested, as expected in bees and *Varroa*, but also in all samples of ants, wasps and small hive beetles. NGS data analysis showed that DWV profiles grouped by host species, with samples from the same host species showing comparable proportions of DWV types A, B and C, and in the case of small hive beetles each sample contained the same A/C recombinant. The consistency of results between samples of the same species suggest that variants may exist within the quasispecies framework, where different variants are better adapted to successfully replicate in different hosts. The wasp samples showed further grouping, with all samples from one location showing consistent results and those from a second location being much more variable, suggesting that, within species, location can have a role in determining the nature of viral infections. A phylogenetic approach using a 500bp region of the conserved RdRp gene revealed genetically similar genotypes were circulating in all species sampled from the apiaries suggesting potential frequent inter- and potentially intra- species transmission events, similar to the findings of Singh *et al*. (2010). Although our study did not investigate the nature of these events, the intimacy of the hive environment clearly provides ample opportunity for species to come in to frequent contact, whereby viruses suitably adapted could well spread easily. For example, ants, wasps and small hive beetles all feed on bees, and scavenge other hive products (Banko & Banko, 1976; Clagg, 1957; Gambino et al., 1992; Neumann et al., 2016), so could well acquire virus particles orally from infected honey bee tissues or contaminated hive material (Evison et al.,2012; Sébastien et al., 2015).

I also surveyed the non – *Apis* arthropods for the presence of nine other invertebrate viruses, including those described in Chapters Four and Five. This again revealed there to be species – specific signatures in viral infections, and also again differences were seen between the wasps from each of the two locations sampled. This study showed the importance of considering the wider apiary environment and networks of interactions when considering viruses infecting bees. As shown by this study, and that of others (e.g. Sébastien et al., 2015), the prevalence of honey bee – associated viruses in non – *Apis* hosts suggests that apiary pests could potentially serve as reservoirs of honey bee pathogens. This is particularly important if variants exist which are capable of successfully infecting different species or if a sufficiently diverse quasispecies is persisting in non – *Apis* hosts. However we manage honey bees, there could be perpetual sources of pathogens inside the hive ready to spill back into the honey bees.

In addition to the findings of Chapter Five, we also found that, when comparing DWV prevalence in non – *Apis* insects between Hawaiian Islands with and without established *Varroa* populations, clear differences emerged (Appendix One). That is, the wasps and solitary bees (*Polistes* spp. and *Ceratina smaragdula*) were significantly more likely to be infected with DWV where *Varroa* was present and DWV load and prevalence were high in the honey bees than areas with no *Varroa* and low DWV. This is despite the fact that these wasps and solitary bees' only association with honey bees is via the shared resource of flowers. It is likely that transmission is directional from honey bees to the wasps and solitary bees and involves the shared floral resources. This provides more information on the link between the viruses infecting different arthropod hosts with overlapping host ranges and shows that inter – species transmission of RNA is both common and relevant to not only the health of honey bees but the wider apiary community and beyond.

Together these results have served to fill gaps in our knowledge of the 'DWV – *Varroa* – honey bee' associations, expand our knowledge of the circulating virosphere within the

apiary, and provide new insights into how DWV is spreading beyond honey bees and into the wider insect community. The study is, however, not without its limitations. To concentrate on the viruses that infect honey bees and other arthropods, it has been necessary to exclude from the study any considerable discussions around, or investigations into, immune responses. Undoubtedly the immune response plays a significant role in the outcome of viral infection (Nazzi et al., 2012; Marques & Imler, 2016; Ryabov et al., 2016; Di Prisco et al., 2011; Doublet et al., 2017) and as such would likely have contributed to the outcomes of this study. However, viral immunity in invertebrates is a complex, vast and still poorly understood area (Siva – Jothy et al., 2005; Vilcinskas, 2013; Kingsolver et al., 2013; Bronkhorst, & van Rij, 2014) and to give it the depth and consideration due would have been far beyond the scope of this PhD. As such I decided to focus only on the viruses themselves.

Conclusions and Future Work

I have shown that the DWV quasispecies is complex and shares an intricate relationship with its hosts, both honey bees and beyond. I found type A in high levels in deformed bees, but also in asymptomatic bees, demonstrating that there is no one unique variant associated with deformity. Type A was present in honey bee colonies harbouring well established mite populations, both at high levels in areas which have seen large scale honey bee losses due to *Varroa* and DWV (Hawaii), but also [genotypically variable] type A was found at very low levels in a *Varroa* tolerant population (Fernando de Noronha). Type B was also found in high levels in colonies with high mite levels; and in a *Varroa* – tolerant population where it prevented more virulent type A variants from becoming established. Through studying the bees of Fernando de Noronha, I also disproved the commonly held theory that *Varroa* feeding causes the activation of latent DWV within a bee.

I discovered two new RNA viruses, Milolii virus and Moku virus in common apiary pest species (*T. melanocephalum* and *V. pensylvanica*). The finding of two new viruses at high levels in the apiary was of particular concern when it came to Moku virus because it was

found also, albeit at low levels, in *Varroa* and honey bees. The broader study of honey bee – associated viruses in the apiary environment revealed taxonomically diverse common apiary pests to be potential reservoirs of invertebrate viral pathogens. Furthermore, it appears that there are variants of at least DWV, but perhaps more RNA viruses, which are able to successfully replicate in non – *Apis* hosts. Future work should elucidate, through laboratory and field studies, the extent to which DWV and other viruses are successfully replicating and, then, pathogenic to non – *Apis* hosts. Furthermore, this PhD has provided more information about the distribution of DWV recombinants. It has become increasingly obvious that recombinants are both varied and widespread both in honey bees and in other species. Interestingly, this is evidenced by the case of the small hive beetles which all contained a type A/C recombinant; particular recombinants are potentially better suited to infecting different host species. The widespread nature of a diverse range of recombinants must therefore be considered when embarking upon molecular studies using primers for just one part of the genome.

Furthermore, there are potentially many more invertebrate viruses to be discovered which may have the potential to spell disaster for honey bees and their wider arthropod communities, as evidenced by the recent finds of widespread Lake Sinai Virus in some honey bee populations (Cornman et al., 2012). Further studies are needed which cover both a wide range of hosts and of viruses to aim to pre-empt future disease emergence and measure the actual impact of the pathogen on its host.

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Appendix 1: Evidence of *Varroa*-mediated Deformed Wing virus spillover in Hawaii.

Santamaria, J.[1], Villalobos, E. M.[1], Brettell, L.E.[2], Nikaido, S.[1], Graham, J. R.[1] and Martin, S. J.[2]

[1] University of Hawaii at Mānoa, United States, 3050 Maile Way, Honolulu, HI 96822

[2] The University of Salford, Manchester, UK M5 4WT

Abstract

Varroa destructor, a parasitic mite of honey bees, is also a vector for viral diseases. The mite displays high host specificity and requires access to colonies of *Apis spp*. to complete its lifecycle. In contrast, Deformed wing virus (DWV), one of the many viruses transmitted by *V. destructor*, appears to have a much broader host range. Previous studies have detected DWV in a variety of insect groups that are not directly parasitized by the mite. In this study, we take advantage of the discrete distribution of the *Varroa* mite in the Hawaiian archipelago to compare DWV prevalence on non-*Apis* flower visitors, and test whether *Varroa* presence is linked to a "viral spillover". We selected two islands with different viral landscapes: Oahu, where *V. destructor* has been present since 2007, and Maui, where the mite is absent. We sampled individuals of *Apis mellifera*, *Ceratina smaragdula*, *Polistes aurifer*, and *Polistes exclamens* to assess and compare DWV prevalence in the Hymenoptera community of the two islands. The results indicated that, as expected, honey bee colonies on Oahu have much higher incidence of DWV compared to Maui. Correspondingly, DWV was detected in the non-*Apis* Hymenoptera collected from Oahu, but was absent in the species examined on Maui. The study sites selected shared a similar geography, climate, and insect fauna, but differed in the presence of the *Varroa* mite, suggesting an indirect, but significant, increase on DWV prevalence in the Hymenoptera community on mite-infected islands.

Keywords:
Varroa mite, deformed wing virus, pollinator health, viral transmission, viral spillover, *Apis mellifera*

Abbreviations:
Deformed Wing Virus (DWV)

1. Introduction

In the last two decades, emerging diseases have caused extensive damage to crops and livestock (Morens and Fauci, 2013; Voyles et al., 2014,). Pathogens have been repeatedly shown to jump between species (Levitt et al., 2013; Li et al., 2011; Malmstrom and Alexander, 2016) and Deformed wing virus (Iflaviridae; DWV) affecting honey bees is no exception (Villalobos, 2016). Recent molecular studies have shown that DWV may have co-evolved with the European honey bee (*Apis mellifera*), and the original virus may have been a low prevalence pathogen with many variants and low virulence (Martin et al, 2012; Wilfert et al., 2016). Upon contact with the Asian honey bee (*Apis cerana*), a new mite vector, *Varroa destructor*, jumped species from *A. cerana* to *A. mellifera* and with this new transmission route the prevalence and virulence of DWV in *A. mellifera* was amplified (Wilfert et al., 2016; Martin et al., 2012). Recent studies by Yanez *et al*. (2015) on sympatric colonies of the Asian honey bee, *Apis cerana*, and *A. mellifera* indicated that there are a large number of shared strains of DWV circulating in the Asian and the European honey bee populations; however the virus is more prevalent in the European honey bee colonies, suggesting a more efficient transmission route via the mite and/or greater susceptibility of *A. mellifera* to infection to the virus or the vector. A similar situation has been reported for the native Japanese honey bee *Apis cerana japonica*, which shares DWV infections with sympatric *A. mellifera* but at a much lower prevalence (Kojima et al., 2011).

While DWV evolved in close association with *Apis* bees, it also appears capable of infecting a broad range of non-*Apis* hosts (Genersch et al., 2006; Li et al., 2011; Melathopoulos et al., 2017). So far, DWV has been detected in 22 insect genera across Europe, North and South America (Levitt et al., 2013; Singh et al., 2010, Reynaldi et al.,

2013, Guzman-Novoa et al., 2016), including social and non-social bees, wasps, ants, and a myriad of other insect groups. Although not much is known about the impacts of the virus in these host species (Tehel et al., 2016), the discovery of DWV among a wide range of species has created concerns about a possible "viral spillover" from honey bee colonies to other insect species, especially economically important pollinators such as bumble bees (Graystock et al. 2016). Work on viral spillover has been conducted, so far, in regions where *V. destructor* is present and DWV is prevalent in the honey bee population (Budge et al., 2015; Traynor et al., 2016, Tehel et al., 2016). In fact, the presence of *V. destructor* in honey bee colonies has been linked to increased viral loads, virulence, and prevalence of DWV in honey bee populations (Martin et al., 2012). The fragmented distribution of the *Varroa* mite on the Hawaiian archipelago makes for ideal study sites in which to examine pollinator communities with or without *Varroa* mites in the ecosystem. In this study, we sampled local honey bees and non-*Apis* Hymenoptera species on the *Varroa*-positive island of Oahu and the *Varroa*-negative island of Maui. The selected study sites shared similar geography, floral resources, and insect communities; however, Oahu's honey bees have been in contact with *V. destructor* since 2007, and have high DWV prevalence and increased viral loads. In contrast, Maui remains mite free to this date, and the honey bee populations on that island have a much lower incidence of DWV (Martin et al., 2012). The non-*Apis* Hymenoptera species selected as representatives of the community were: *Ceratina smaragdula, Polistes aurifer*, and *Polistes exclamens. C. smaragdula*, commonly known as the small carpenter bee, is a mostly solitary bee abundant in garden environments in Hawaii, sharing nectar and pollen resources with honey bees. *Polistes* spp. are common social wasps that visits flowers occasionally to feed on nectar.

Re-emerging viral diseases such as DWV represent one of the major threats to honey bee health, and the "spillover" of pathogens to wild bees and other insects may also contribute to the current global pollinator decline (Fürst et al., 2014, Genersch et al., 2006; Graystock et al., 2013a; 2013b; Manley et al., 2015, Tehel et al., 2016). Here we carry out a preliminary comparison of the incidence of DWV on non-*Api*s insects in areas with and without *V. destructor*.

2. Methods

2.1 Specimen collection

We selected three species within two different Hymenoptera genera as representatives of the local community of flower visitors: the introduced small carpenter bee *Ceratina smaragdula* (Apidae) which was first recorded in Hawaii in 1999 (Magnacca and King, 2013), and introduced paper wasps *Polistes aurifer* and *Polistes exclamans* (Vespidae) first recorded in Hawaii in the 19th century and in 1952 respectively (Beggs et al., 2011). All samples were collected from five sites on Oahu (*Varroa*-positive island), and four sites on Maui, (*Varroa*-negative island). Collection sites on both islands consisted on a mix of agricultural fields, parks, gardens, and beach edge vegetation strips. The selected insect species are all relatively abundant and can be found in urban and agricultural environments, where they overlap in resource use with *A. mellifera. Polistes* wasps collected on Oahu are *P. aurifer* and the specimens from Maui are *P. exclamans*. Consequently the comparisons between the paper wasps were at the genus level. Samples were collected from August 2014 to November 2015. Insects were collected while they were foraging in fields or flower patches, via a hand-held net. Paper wasps samples were also collected from around their nests. Each insect was stored individually and kept on ice in the field until transferred to a -80 °C freezer for long term storage.

2.2 RNA Extraction & Reverse Transcription PCR

Each individual was transferred to a nuclease free 1.5ml centrifuge tube, which was submerged in liquid nitrogen before the sample was crushed using a sterile mini pestle. Total RNA was then extracted from the resulting powder using the RNeasy Mini Kit (Qiagen) following manufacturer's conditions and resuspended in 30 µl of RNase-free water. RNA concentration was determined using a Nanodrop 2000c (Thermo Scientific) and samples were diluted to 25ng/µl. Reverse Transcription-PCR (RT-PCR) protocols adapted from Martin et al (2012) were carried out to determine whether samples contained DWV. Endogenous control reactions were also carried out to ensure RNA was intact. RT-PCR reactions contained 50ng RNA, 1x OneStep RT-PCR Buffer (QIAGEN), 400 µM each dNTP, 10units

RNase Inhibitor (Applied Biosystems) and 0.6µM each primer. DWVQ_F1 and DWVQ_R1 primers (Highfield et al 2009) were used to amplify a conserved region of the RNA dependant RNA polymerase (RdRp) gene. For the endogenous controls actin primers were used (Highfield et al 2009) using an aliquot of the same RNA as the template. Reactions were run using a T100 Thermocycler (Bio-Rad) starting with reverse transcription at 50°C for 30 minutes, followed by an initial denaturation step at 94°C for 30 seconds. This was followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for DWVQ primers (58°C for actin) for 30 seconds, extension at 72°C for 1 minute, and a final extension step at 72°C for 10 minutes. Agarose gel electrophoresis was used to determine the results. RT-PCR products were ran on a 2% agarose gel stained with SYBR Safe DNA gel stain (Invitrogen) with a 100bp TrackIt ladder (Invitrogen), and visualised under ultraviolet light.

2.3 Statistical Analysis

To compare the DWV prevalence between species across islands, data were arranged across a contingency table and Fisher's exact test was used to test for heterogeneity in the data.

3. Results

We established via RT-PCR that DWV was present in the honey bee population on both islands; however, virus prevalence was significantly higher ($p<0.0001$, Fisher's exact test) among *Apis mellifera* from Oahu (83%, n=58) compared to individuals from Maui (7%, n=29) (Fig.1). The RT-PCR results for the non-*Apis* insects showed a distinct dichotomy based on island; DWV was found on both of our non-*Apis* study species on Oahu, while the virus was completely absent from both of the non-*Apis* Maui samples (Fig. 1). Within the Oahu samples, the prevalence of DWV in *Ceratina smaragdula* and *Polistes aurifer* was 27% (n=61) and 45% (n=20) respectively (Fig.1).

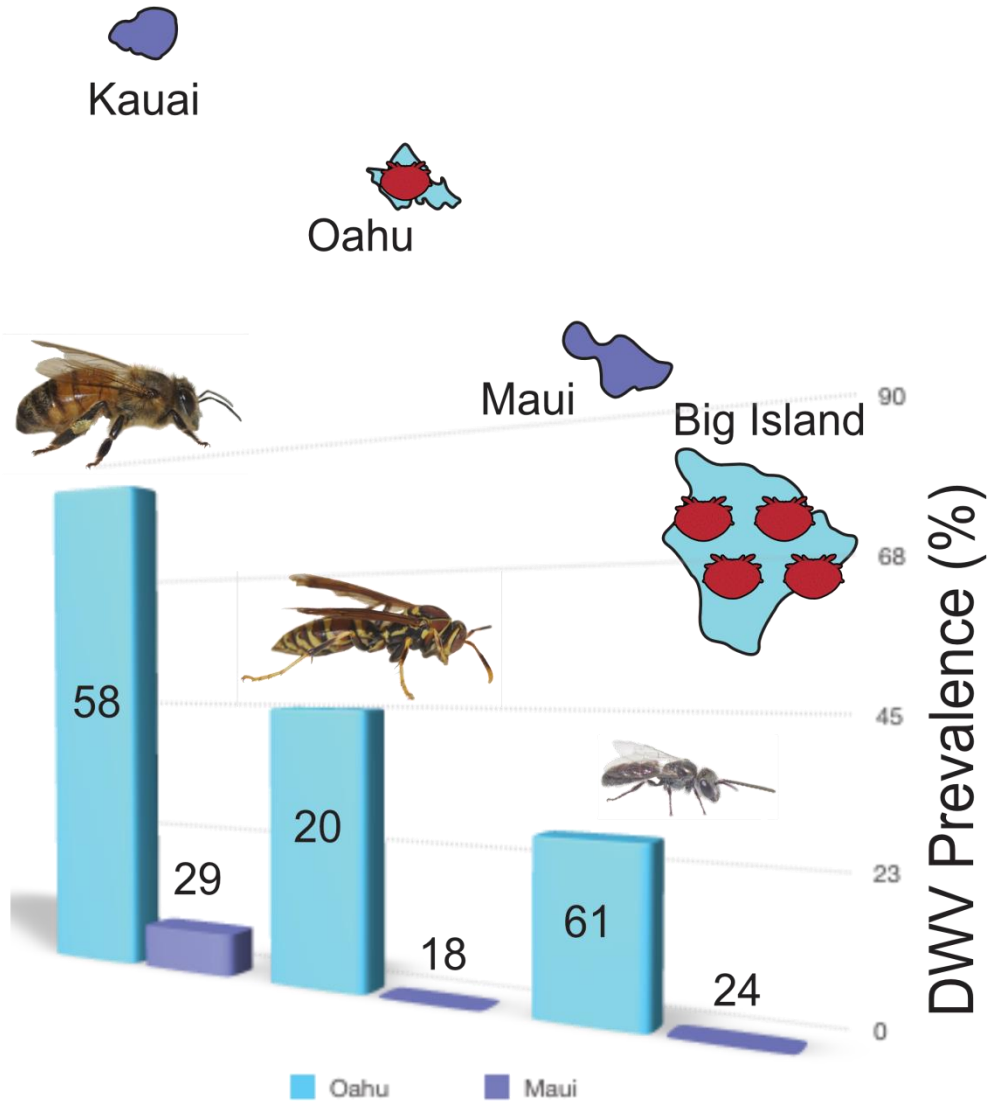

Fig 1. –Comparison of DWV prevalence in several species of Hymenoptera on Oahu (blue purple), an island where the *Varroa* mite is well established and DVW prevalence and viral load among honey bees is very high, and the island of Maui (red purple) were the absence of the mite means the prevalence and load of DWV is very low in honey bees. The numbers within each bar represent the sample size for each group. Map shows the current distribution of *V. destructor* in the Hawaiian archipelago using mite icons and the same color-codes as the histogram bars.

4. Discussion

We confirmed, as expected, that the presence of the *Varroa* mite on Oahu was associated with greatly increased prevalence of DWV in honey bees (Table 1). In this study, eight out of 10 forager honey bees collected on Oahu were positive for DWV, compared to a DWV detection rate of 0.7 out 10 bees in Maui. The low prevalence of DWV in Maui's bees concurs with a previous survey by Martin et al. (2012) in which four out of 33 Maui colonies tested positive for DWV. The detection of DWV on *Varroa*-negative islands also agrees with the theory that this virus arrived in Hawaii along with the European honey bee prior to the global spread of the mite, and that it remains present in the *Varroa*-negative honey bee population as a low prevalence pathogen (Martin et al., 2012, Wilfert et al., 2016; Ryabov et al., 2014).

According to the review by Tehel et al. (2016), 17 species of bee, including one species in the genus *Ceratina*, have been described as positive for DWV. In our study, detection of DWV in *C. smaragdula* was associated only with *Varroa*-positive areas, where one out of four small carpenter bees sampled tested positive for the virus. Singh et al (2010) reported DWV infection in *Ceratina dupla*, where two out of three individuals sampled were positive. DWV has also been detected in several wasp species, including yellow jackets (*Vespula* spp.) (Levitt et al., 2013) and several *Polistes* spp (Singh et al., 2010). Our study shows that, as with the small carpenter bees, the presence of DWV in paper wasps was limited to the samples from Oahu where 45% of the *P. aurifer* specimens collected were positive for DWV.

Our results suggest possible DWV spillover from honey bees to flower visitors that is indirectly linked to *Varroa* presence in the region. However, there are still large gaps in our knowledge with regard to cross species transfer of DWV, in particular: the routes of virus transmission, the range of species that are susceptible, and the potential impact, if any, of the virus on non-*Apis* hosts. Tehel et al. (2016) argue that simple PCR detection of DWV at a single location does not provide enough information to make inferences about viral spillover from honey bees to the rest of the insect community. We agree with the authors about the need for studies that include multiple locations, samples from a wide range of insect

species, and identification of DWV strains and confirmation of viral replication. Nevertheless, the absence of the mite on Maui provided us with the opportunity to completely exclude the effects of *Varroa* parasitism from one site, while comparing the prevalence of the DWV on two geographically close regions. Consequently, the information collected in this study can be considered preliminary evidence in support of directionality of transfer of DWV from honey bees to other insect species, as mediated by the presence of *Varroa*.

One of the proposed routes of DWV transmission involves ingestion of contaminated hive products such as, pollen and honey, and/or consumption of larvae, pupae, or adult bees (Singh et al., 2010; Chen et al., 2006; Genersch et al., 2006; Möckel et al., 2010). Insects that rob colony resources, or those that feed directly on live or dead bees, may take in viral particles with the food they ingest. This mechanism of infection has been suggested for yellow jackets, possibly ants, and for hive parasites (Evinson et al., 2012; Sébastien et al., 2015). This transmission route, however, is not a likely explanation for our study species. Small carpenter bees feed solely on flowers, and, although carnivorous, *Polistes* exclusively hunt caterpillars to feed their young and do not rob honey bee colonies. A more likely transmission route in our study is through the flowers shared by the insects. Floral resources have been identified as a potential contact point between species and viable DWV particles have been found in pollen (Mazzei et al, 2014, Singh, 2011). Both honey bees and *C. smaragdula* were found foraging on the same common garden herbs, crops, and ornamentals – such as *Scaevola sericea* (Naupaka) and *Heliotropium foertherianum* - on both Oahu and Maui (pers. obs.). Bees require pollen and nectar to rear their young, and it is possible that either of those resources could have been contaminated with DWV. *Polistes* spp. are active foragers that move quickly among the vegetation looking for caterpillar prey, but they occasionally pause to feed on nectar from a variety of flowers during a foraging bout (pers. obs.). Consequently, shared floral use could also be an alternative route of viral transmission in predatory or parasitic wasps.

The honey bee colonies on Maui, as well as those on other *Varroa*-negative Hawaiian islands, showed a much lower DWV prevalence (Martin et al., 2012), thus the number of infected individuals, and the viral titer of the infected bees foraging in that community, is expected to be much lower. In contrast, forager honey bees on Oahu are more likely to be

DWV positive and to carry an elevated viral load, which could translate into a higher rate of floral contamination on this island and a higher prevalence of DWV in non-*Apis* flower visitors (Fig. 1).

The pathogenicity of DWV and the relationship between the virus, the mite, and the honey bee continue to be the focus of much research in honey bee pathology (Ryabov et al., 2014; Möckel et al., 2010; Di Prisco et al., 2016). The known DWV strains (Type A, B, and C), and recombinants thereof, may be linked to differences in DWV virulence in honey bee colonies (Martin et al., 2012; McMahon et al., 2016; Zioni et al., 2011; Ryabov et al., 2014); however, there is no evidence that strains may be specifically linked to wing deformities on bees. Rather it appears that viral loads of DWV play a significant role in the expression of this phenotype in honey bees (Brettell et al., 2017). By comparison to the existing work on honey bees, our understanding about DWV transmissibility and its effect on the fitness of non-*Apis* bees, and other insects, is much more limited. Based on the summary presented by Tehel et al. (2016), 17 species of non-*Apis* bees carry DWV, 7 species show evidence of DWV replication (via a negative RNA strand), and the pathogenicity of DWV has been confirmed for two species of bumble bee, *Bombus terrestris* (Furst et al., 2014) and *Bombus pascuorum* (Genersch et al., 2006). DWV replication in non-bee species has also been reported; Levitt et al. (2012) found evidence of RNA replication in paper wasps, *Vespula spp.,* Eyer et al. (2009) reported negative RNA strands in the small hive beetle, *Aethina tumida,* and recently Radzevičiūtė et al. (2017) found evidence of replication of a number of RNA viruses in a number of bee species. Research on alternative commercial pollinators such as the alkali bee and the alfalfa leafcutter bee have shown that food stores, eggs, and larvae may be infected with numerous viruses shared with honey bees, including DWV, IAPV, and BQCV (Singh, 2011). However, quantifying the impact of DWV infection on non-*Apis* insects can be difficult since for many species we only have access to the non-symptomatic adults. In depth research is needed on examine fitness impacts to non-*Apis* bees of these viruses and to survey wild hosts that could become reservoirs of DWV, leading to a complex web of infections.

5. Conclusion

1-In this study, a higher rate of DWV detection in non-*Apis* insects was associated to *Varroa*-positive areas.

2-Across-species transmission of DWV in our study was likely the result of shared flower use by honey bees and non-*Apis* insects.

3-The prevalence of DWV in *C. smaragdula* and *P. aurifer* in Hawaii is comparable to that of other species of bee and wasp from the mainland US, where the mite has been present for about 30 years.

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Appendix 2: Superinfection exclusion and the long-term survival of honey bees in Varroa-infested colonies.

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ORIGINAL ARTICLE

Superinfection exclusion and the long-term survival of honey bees in Varroa-infested colonies

Gideon J Mordecai[1,2], Laura E Brettell[3], Stephen J Martin[3], David Dixon[1], Ian M Jones[2] and Declan C Schroeder[1]

[1]*Viral Ecology, Marine Biological Association, Plymouth, UK;* [2]*School of Biological Sciences, University of Reading, Reading, UK and* [3]*School of Environment and Life Sciences, The University of Salford, Manchester, UK*

Over the past 50 years, many millions of European honey bee (*Apis mellifera*) colonies have died as the ectoparasitic mite, *Varroa destructor,* has spread around the world. Subsequent studies have indicated that the mite's association with a group of RNA viral pathogens (Deformed Wing Virus, DWV) correlates with colony death. Here, we propose a phenomenon known as superinfection exclusion that provides an explanation of how certain *A. mellifera* populations have survived, despite *Varroa* infestation and high DWV loads. Next-generation sequencing has shown that a non-lethal DWV variant 'type B' has become established in these colonies and that the lethal 'type A' DWV variant fails to persist in the bee population. We propose that this novel stable host-pathogen relationship prevents the accumulation of lethal variants, suggesting that this interaction could be exploited for the development of an effective treatment that minimises colony losses in the future.
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Introduction

The recent global decline of the European honey bee (*Apis mellifera*) populations (Ratnieks and Carreck, 2010; Schroeder and Martin, 2012) is of grave concern because of their role as pollinators which contribute an estimated $225 billion to the global economy (Gallai *et al.*, 2009). For over half a century, the global spread of the ectoparasitic mite, *Varroa destructor,* has resulted in the death of many millions of managed and feral honey bee colonies (Martin *et al.*, 2012; Schroeder and Martin, 2012; Thompson *et al.*, 2014). The mite has introduced a new viral transmission route that has dramatically altered the viral landscape (Martin *et al.*, 2012). This has resulted in a massive loss of diversity in Deformed Wing Virus (DWV) (Martin *et al.*, 2012), the pathogen now linked with the collapse of honey bee colonies (Highfield *et al.*, 2009; Di Prisco *et al.*, 2011). However, prior to *Varroa* spread, DWV stably co-existed with honey bees (Martin *et al.*, 2012) albeit at viral loads many orders of magnitude lower than is now observed (Martin *et al.*, 2012; Mondet *et al.*, 2014). For example, the recent arrival of *Varroa* into the Hawaiian honey bee population was accompanied by a million fold increase in the viral load of DWV, loss of DWV diversity and the predominance of a single highly virulent DWV variant (type A) (Martin *et al.*, 2012). These landscape scale changes have also been demonstrated at the individual honey bee level within the UK honey bee population. For example, Ryabov *et al.* (2014) demonstrated the dominance of a single variant of DWV when a mixture of viral strains were injected into developing pupae leading to a rapid loss of DWV diversity and million fold increase in viral loads.

DWV is a rapidly evolving group of closely related variants (de Miranda and Genersch, 2010), commonly referred to as a quasispecies (Domingo and Holland, 1997; Lauring and Andino, 2010). Within the DWV quasispecies, there are several master variants, each with its own swarm of variants. Each variant can form potential recombinants with other variants, within a swarm and between master variants. Kakugo virus is a variant of the DWV type A that differs from the master sequence (Lanzi *et al.*, 2006) by 6% in the non-structural coding region (Fujiyuki *et al.*, 2006; Baker and Schroeder, 2008), whereas Varroa destructor Virus-1 (VDV-1) (Ongus *et al.*, 2004) is genetically dissimilar to DWV type A (84% genome identity) and is referred to as DWV type B (Martin *et al.*, 2012). Notably, both DWV type A and B master variants are able to replicate within mites and honey bees, and both have been detected in honey bees in the absence of *Varroa* (Yue and Genersch, 2005; Zioni *et al.*, 2011; Martin *et al.*, 2012). Recombinants between the variants have been

Correspondence: D Schroeder, Viral Ecology, Marine Biological Association, Citadel Hill, Plymouth PL1 2PB, UK.
E-mail: dsch@mba.ac.uk
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reported (Moore *et al.*, 2011; Zioni *et al.*, 2011; Ryabov *et al.*, 2014) and a novel recombinant between DWV type A and a new DWV master variant, type C, has also been recently discovered (Mordecai *et al.*, 2015), suggesting that they are part of the same quasispecies and share a recent common ancestor. DWV type A has been detected in honey bee populations around the world and in the presence of *Varroa* leads to colony death (Di Prisco *et al.*, 2011; Martin *et al.*, 2012), whereas there are no known instances of type B being linked to colony death. The role of the new type C in overwintering colony losses is currently unclear (Highfield *et al.*, 2009; Mordecai *et al.*, 2015).

In the early 1990s, *Varroa* swept across the UK and was followed by widespread colony deaths 1–3 years later. To ensure the long-term survival of their honey bee colonies, beekeepers in *Varroa*-infested countries manage *Varroa* populations (Sumpter and Martin, 2004), largely through chemical methods. Nonetheless, there are reports of rare isolated untreated *A. mellifera* colonies of European origin thriving despite *Varroa* infestation, including cases on an island in Brazil (DeJong and Soares, 1997) and in small forest patches in France (Conte *et al.*, 2007) and New York, USA (Seeley, 2007). The survival of these colonies is well documented and not questioned, however, the mechanism by which tolerance to *Varroa* and its association with DWV is maintained remains elusive. In the UK, a small number of beekeepers opted not to control their mite populations and, in most cases, lost their bees. However, one UK beekeeper, Ron Hoskins, initiated a closed breeding programme from colonies that survived the initial *Varroa* infestation and this isolated population of up to 40 colonies persists in Swindon, central England, without chemical control of *Varroa* (http://www.swindonhoneybeeconservation.org.uk/). The aim of this study was to assess the viral landscape in this apiary thereby determining whether the colonies remained disease-free owing to an absence of DWV. We show here that the Swindon apiary is dominated by an avirulent DWV type B master variant with the concomitant absence of the virulent DWV type A master variant. Taken together, these data suggest that a phenomenon known as superinfection exclusion (SIE) (Salaman, 1933; Labrie *et al.*, 2010) is a plausible explanation for why this isolated UK honey bee population has survived, despite *Varroa* infestation and high DWV loads.

Material and methods

Sample collection

Pooled asymptomatic honey bees were collected from sites in Hawaii and from the Swindon Apiary. A time series of three hives in Swindon was taken (10 time points over a year, 4 of which were used for Illumina sequencing per hive (Illumina, Inc., San Diego, CA, USA)). Varroa samples were collected from the same three hives in the Swindon apiary alongside the honey bee samples.

DWV detection assay

RNA extractions, RT-PCR and High Resolution Melt (HRM) analysis were carried out according to a previous study (Martin *et al.*, 2012). In brief, total RNA was extracted from whole worker honey bees using an RNeasy mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. RT-PCR via oligo dT priming using previously designed DWV primers (Highfield *et al.*, 2009) and subsequent HRM was carried out using Sensifast No-Rox One Step Kit (Bioline, London, UK). RNA was diluted to $\sim 100\ ng\ \mu l^{-1}$ and 1 µl of template RNA was added per reaction. The DWV load per worker honey bee was calculated according to the method developed by Highfield *et al.* (2009). The amount of RNA used for each RT-PCR reaction was normalised per bee and the DWV load per bee was calculated through a DWV cRNA standard curve conversion ($y = -3.695x + 32.744$).

Bioinformatics pipeline

Illumina Hi-seq (2 × 100 bp) pair-end sequencing was carried out by The Genome Analysis Centre (TGAC) and the University of Exeter. Total RNA was sequenced after a cDNA synthesis step with no amplification step necessary. Varroa RNA was pooled for three of the time points (January, April and May) prior to Illumina sequencing. A bioinformatics pipeline (Figure 1) was developed to accommodate the large amount of variation found within the DWV species complex. Firstly, the quality of the raw reads was verified using FastQC (Babraham Bioinformatics, Cambridge, UK). Samples were then converted from fastq to fasta using the fastq_to_fasta script which is part of the FASTX-toolkit (Hannonlab, http://hannonlab.cshl.edu/fastx_toolkit/). To isolate the reads sequenced from the DWV complex from the host and other contaminating sequences the BLASTn tool was used (Altschul *et al.*, 1990). The reads were searched against a custom BLAST database containing the DWV, VDV-1 and Kakugo virus genomes, with an e value of 10e-05. BLAST was carried out against Read 1 of the Illumina data. The ncbiblastparser perl script (http://www.bioinformatics-made-simple.com/2012/07/ncbi-blast-parser-extract-query-and.html) was then used to parse and read the top hit of the BLAST output. Next, 'sed' and 'awk' scripts were used to delete empty lines and the reads, which contained 'nohits'. The corresponding BLAST hits were then pulled out from the Read 2 raw reads using QIIME. The paired reads were then balanced using a custom script written in R version 3.2.0 (R Core Team, 2015), which deletes any read in the Read 1 BLAST top hit file that did not have a pair in the corresponding read 2 BLAST top hit file and

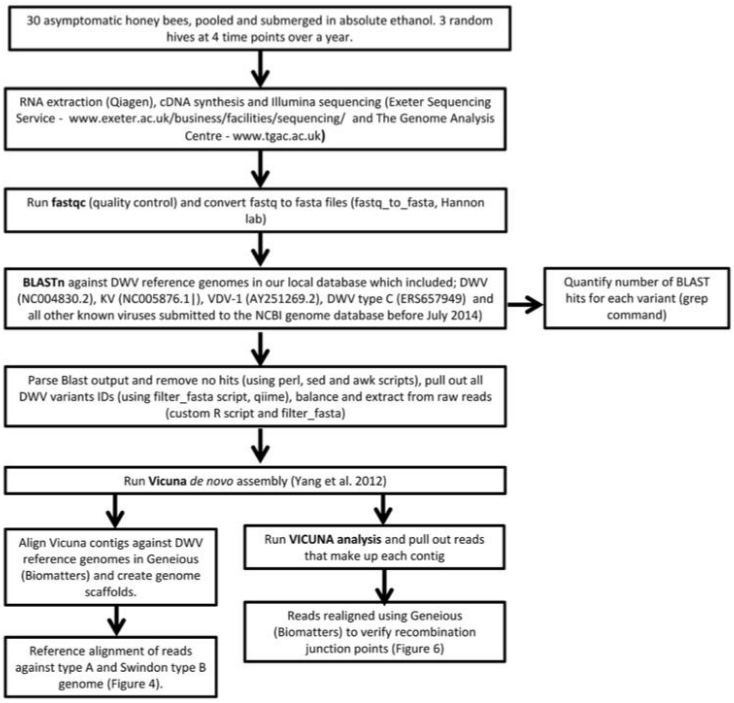

Figure 1 Bioinformatics pipeline leading to the application of the VICUNA *de novo* assembler.

vice versa. The balanced DWV family reads were then assembled using the VICUNA assembler, which was developed to generate consensus assemblies from genetically heterogeneous populations, specifically RNA viruses (Yang *et al.*, 2012).

VICUNA contigs greater than 300 bp in length were imported into Geneious (Version 7.04, created by Biomatters) and the 'Map to Reference tool' was used to align the contigs with the type A and B reference genomes. For several of the samples, the VICUNA assembly yielded full-length contigs that covered the whole genome, whereas others yielded only several smaller contigs (Supplementary Table S1). The ends of the contigs were then edited to remove discernable assembly or sequencing artefacts. Assembled DWV contigs were uploaded to the European Nucleotide Archive under the Study accession PRJEB8112. VICUNA contigs from hive 6 January 2013 were used to assemble a type B variant genome (accession number ERS754547).

The identity of the type B genome was compared with the VDV reference genome using the mVista tool (Supplementary Figure S1) (Frazer *et al.*, 2004), and the phylogeny of the Swindon variant was determined from a neighbour-joining tree of the polyprotein encoding region of the DWV genome (Lanzi *et al.*, 2006). In addition, genome scaffolding was carried out to produce full-length genomes representing the unique recombinant present in Swindon. SimPlot software was used to visualise the recombination event (Lole *et al.*, 1999).

To investigate the genome coverage of each DWV variant in Swindon, reads were grouped per hive (Varroa samples were all grouped together) and competitively aligned against the type A reference genome (NC004830.2) and the Swindon type B genome using the Geneious map to reference tool. The maximum percentage of mismatches per read accepted was 5% and no gaps per read were allowed.

To examine individual reads that make up the consensus sequence of each contig, the VICUNA analysis tool was used. To view the reads in Geneious, the VICUNA analysis output was modified by using a sed script to keep just the sequence reads. These were then converted from a tabular format into a fasta format using the python script 'tab2fasta.py' and then visualised using Geneious. To quantify the number of reads with sequences similar to either DWV variant (type A or B), the Illumina reads were searched against a viral database using BLAST and the number of top hits attributed to each reference genome was quantified. Finally, genome coverage was calculated using the Lander/Waterman equation (read length × number of reads/genome length),

which estimates the depth of sequencing across the genome (Sims *et al.*, 2014).

Results and Discussion

Using a combination of RT-qPCR, HRM (Martin *et al.*, 2012) and Illumina (2 × 100 bp) Hi-seq sequencing (Figure 1), we investigated the DWV viral load and diversity in this small honey bee population in Swindon and their associated *Varroa* mites. Three hives were chosen at random and pools of 30 asymptomatic worker bees were sampled from inside the colony on 10 occasions at roughly monthly intervals between October 2012 and October 2013. RT-qPCR on an RNA-dependent RNA polymerase (*RdRp*) gene fragment for all 30 samples collected confirmed the persistence of high DWV loads (10^7 to 10^8 copies per bee) during the entire study period in all three hives (Supplementary Table S2). Both the DWV load and prevalence found within this study suggest that DWV presence alone cannot explain colony losses as proposed in previous Hawaiian (Martin *et al.*, 2012) and Devon, UK, studies (Highfield *et al.*, 2009).

To explore other factors that might contribute to this discovery, we exploited the known nucleotide polymorphisms in the *RdRp* gene fragment among the known DWV master variants (A, B and C) (Martin *et al.*, 2012; Mordecai *et al.*, 2015). HRM indicated the dominance of the type B or C master variant (Figure 2a), as these have similar melting temperatures (Martin *et al.*, 2012; Mordecai *et al.*, 2015). Only a single honey bee sample out of 30 tested contained both DWV type A and B/C, suggesting that although a colony can be exposed to type A, it fails to establish and neither persists nor accumulates. In contrast to the bee samples, the *Varroa* samples contained a greater mix of both DWV type A and B/C (Figure 2b), although type B/C remained the most prevalent. This prevalence of type B/C over A contrasts to what a previous study showed in Hawaii where the type A master variant dominated (Martin *et al.*, 2012) and suggests that type B/C may be an avirulent form of DWV. However, given that HRM analysis only detects limited genomic change (within the *RdRp* gene fragment), the possibility of recombination outside the RdRp region cannot be excluded. Both Mordecai *et al.* (2015) and Ryabov *et al.* (2014) showed that certain recombinants of the master variants A–C and A–B, respectively, could be more lethal than the type A master variant.

DWV type B master variant dominance was, however, confirmed by Illumina sequencing (Figures 3–5). As a proportion of the total sequenced Illumina reads, DWV hits accounted for an average of 46.3% of reads in the *Varroa* samples and 9.7% of reads in the honey bees. The average DWV genome

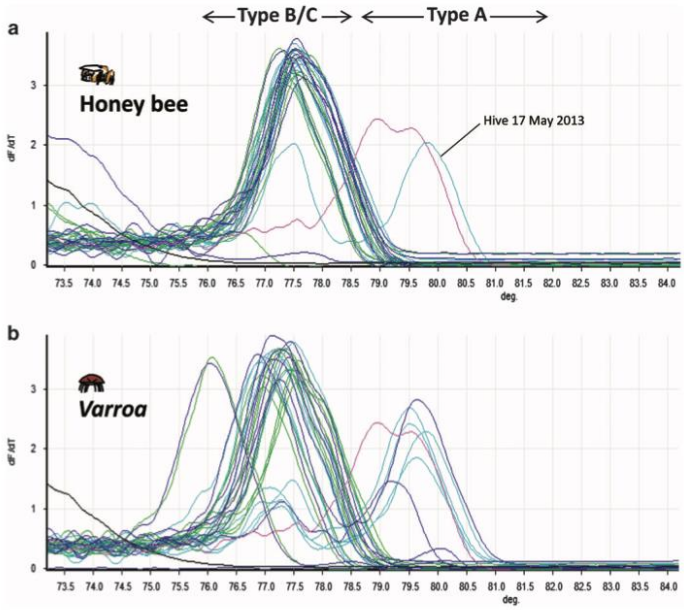

Figure 2 HRM curve analysis for DWV RdRp RT-qPCR region for three hives in the UK colonies (hive 6–blue, hive 17–cyan, hive 19–green). (**a**) Honey bees and (**b**) Varroa mites distinguishing between DWV type A and B/C variants. Deformed winged symptomatic bees were used as a positive control (pink line). A no template negative control was also run (black line).

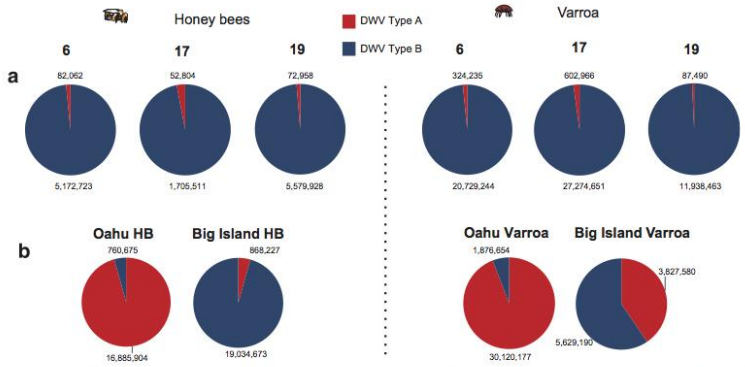

Figure 3 Proportions of DWV subgroups within colonies sequenced using Illumina Hi-seq. (**a**) Swindon samples collapsed into their respective hives 6, 17 and 19. (**b**) The Hawaiian samples from Oahu and Big Islands. A BLASTn algorithm against a custom DWV quasispecies database was used and the numbers indicate of hits to each DWV variant.

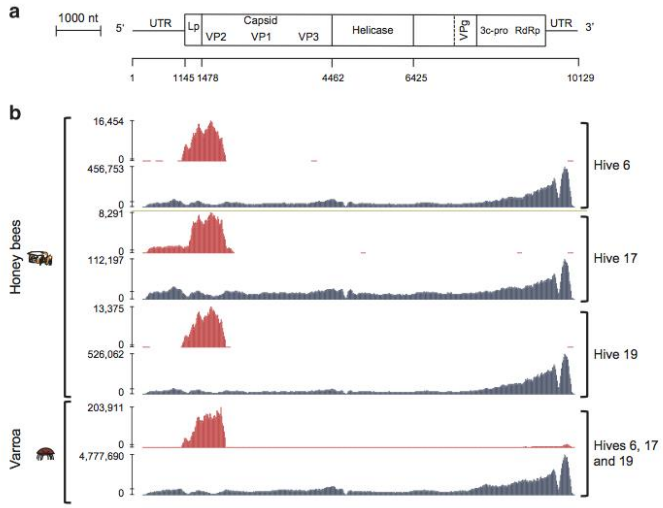

Figure 4 Genome coverage from the Illumina Hi-seq data for the Swindon colonies. (**a**) Map of the DWV genome adapted from Lanzi *et al* (2006). (**b**) DWV type A and B genomes (in red and blue, respectively) assembled from the Illumina NGS data from honey bees and mites from the Swindon apiary (hives 6, 17 and 19). *De novo* assembled VICUNA contigs that makeup these genomes for each hive were deposited in European Nucleotide Archive (ENA) under accession numbers ERS636096 to ERS636117.

coverage for the honey bee samples was 22 484X, while the *Varroa* samples had an average DWV coverage of 599 558X. VICUNA assembly produced 6410 contigs across the 18 samples (Supplementary Table S1). Sample 'Hive 6 January 2013' was used to assemble the 'Swindon' DWV type B variant (Supplementary Table S3), which was found to be 99.5% identical to the type B reference genome (VDV-1) (Supplementary Figure S1). Figure 4 also shows that the type B DWV coverage was high, with over 15 million reads aligned from the honey bee samples compared with 241 000 reads aligned to the type A reference. Similarly, in the *Varroa* samples, 71.5 million reads aligned to type B compared with just over 1 million for type A. Type B reads aligned across the whole genome, whereas full genome coverage of type A was restricted to the *Varroa* samples. No reads unique to the Devon DWV type C genome could be found, whether in the honey bee or *Varroa* samples. In all, the honey bee- and *Varroa*-associated virome of the isolated UK study colonies was predominantly DWV type B (Figure 5), indicating that alternate DWV master variant competitive outcomes are possible.

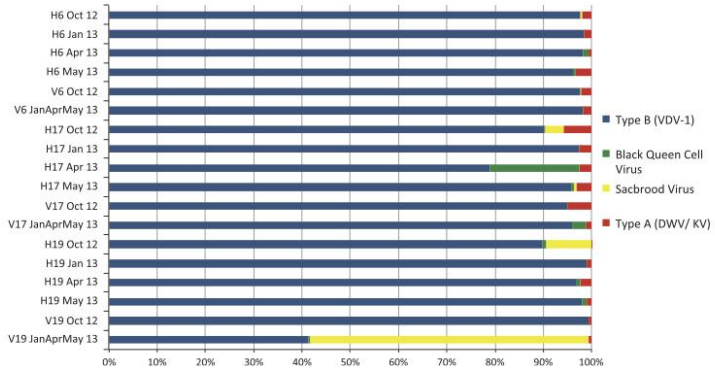

Figure 5 Virome of the Swindon apiary. Illumina reads were searched against a viral database (Figure 1) using BLASTn and the proportion of top hits associated with honey bee viruses was counted. DWV type B dominated the monthly samples in both the honey bee samples (H6, H17, H19) and the Varroa samples (V6, V17, V19).

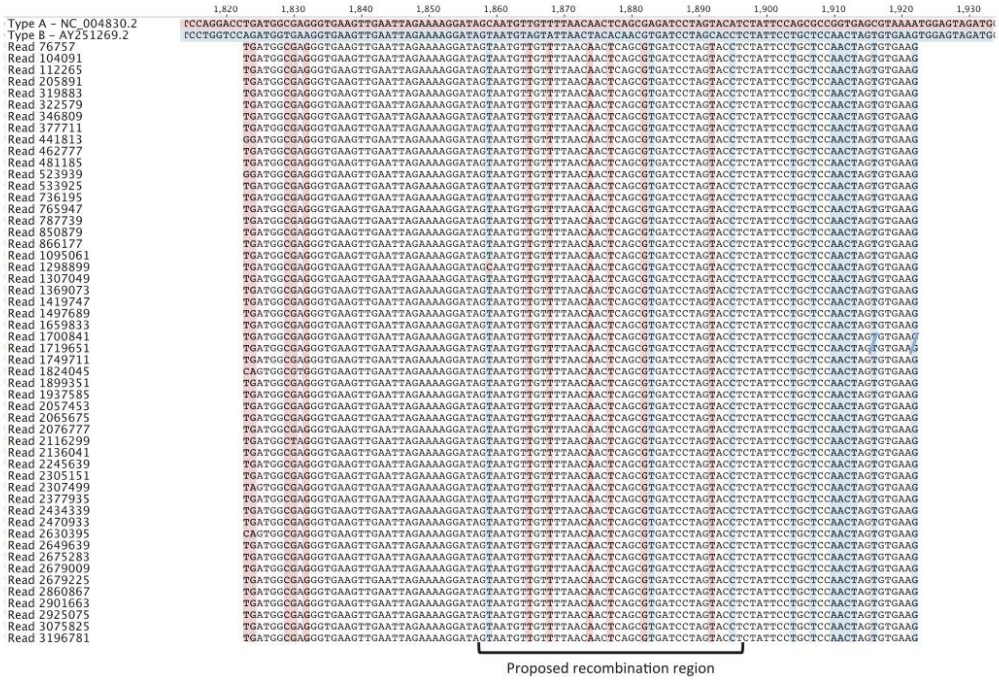

Figure 6 A multiple sequence alignment of the reads covering the recombination junction in the DWV recombinant from H19, April 2013. Output from VICUNA analysis was converted into a suitable format and imported into Geneious to visualise the reads over the type A–B recombination junction point. The DWV type A and B reference sequences are shown at the top and highlighted red and blue, respectively. Base pair substitutions common to either DWV type A and B variants are highlighted in each 100 bp Illumina read. In this example, 52 out of a total of 2464 reads is shown that covers the proposed recombination region.

De novo and reference assembly of the DWV variant genomes suggested that recombination has taken place with type A possibly being recombined out, as evidenced by the presence of DWV recombinants within the honey bee samples (Figures 4, 6 and 7). Full genome scaffolds of each recombinant were made using the VICUNA contigs. These were aligned with type A and B genomes and

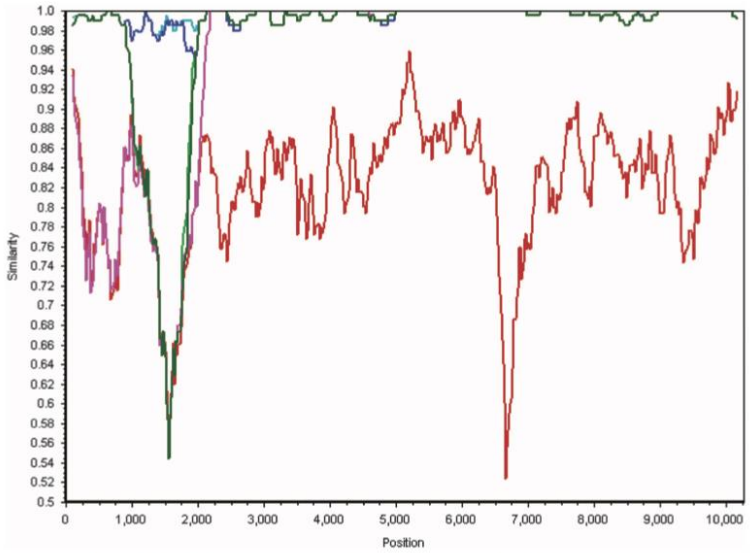

Figure 7 Simplot analyses of the different genomes present in the Swindon samples. Nucleotide similarities of various variants are compared with the type B (VDV) reference genome (AY251269.2). The type A (DWV) reference genome (NC004830.2) is shown in red. A selection of DWV genome scaffolds containing recombination in the 5′ end of the genome are shown; neon and dark green (type B-A-B recombinant from January 2013 Hives 17 and 6, respectively), cyan (Swindon type B genome from Hive 6 January 2013), dark blue (Swindon type B genome from Hive 17 January 2013) and pink (type A- B recombinant, H17 April 2013). A sliding window of 200 nt was used, moving in a step of 20 nt.

Simplot (Figure 7) revealed that the recombination junction in the Swindon samples differed from that previously reported. Moore *et al.* (2011) showed that a recombination junction occurred in the 5′ untranslated region of the genome whereas the Swindon DWV type A-B recombinant junction found here occurs in the structural region of the open reading frame (Figures 4 and 7). Although full genome coverage was not achieved in both honey bee and *Varroa* samples by *de novo* assembly for type A, interestingly, reference alignment of DWV reads from the Swindon *Varroa* mites shows that the whole genome of type A is present at low levels (Figure 4), although HRM analysis indicated that the type A (master or any recombinants thereof) is rapidly removed in the following 5 months (see Figure 2, hive 17). A low number of type A reads (1.68% according to BLAST analysis) present in the UK study population (Figure 3) were uniquely associated with novel recombinants (Figures 4, 6 and 7, Supplementary Table S4) in which the majority of the genome were type B but contained a region of type A sequence at the 5′ end of the genome (the UTR and leader protein, Figure 4). The number of reads within the region of recombination for each of the hives was counted to compare the depth of coverage between the two variants (Supplementary Table S4). As this is a direct comparison of the same region of the genome, that is, the 3′ end, which is caused by bias in reverse transcription oligo dT priming (Figure 4), the 3′ bias is not relevant. In all hives, the number of type B reads exceeded the number of type A (recombinant) reads by an order of between 4.5 (Hive 19) and 36.4 (hive 17). Therefore, the dominance of type B master variant in this UK study population appears to be correlated with a level of colony protection as it appears to exclude type A or C (and any virulent recombinants thereof).

To compare this discovery of type B dominance in this study with respect to the previous Hawaiian study (Martin *et al.*, 2012), a small number of honey bee and *Varroa* Hawaiian samples with a known *Varroa* history were also subject to Illumina (2 × 100bp) Hi-seq sequencing. The same analytical VICUNA pipeline as that used for the UK samples (Figure 1) resulted in 212 contigs being assembled (Supplementary Table S1). On Oahu, where *Varroa* had established and caused widespread colony death, a colony analysed by Hi-seq (173 567X coverage) revealed that type A dominated (Figure 3b) confirming HRM data from another 28 colonies from Oahu, which also had predominantly type A (Martin *et al.*, 2012). However, in the colony from Big Island where *Varroa* had been present for less than 2 years and widespread colony collapse was yet to occur, type B dominated the sequence reads (195 760X coverage). In contrast, the *Varroa* sample from the

same colony on Big Island contained a nearly equal mix of type A and B (93 014X coverage), whereas *Varroa* from Oahu (314 713X coverage) was dominated by type A (Figure 3b). A switch in dominance between type A and B in the Big Island honey bees suggests active competition between the two DWV variants consistent with the suggested 1–3-year time lag for DWV variants adapted to mite transmission to undergo selection (Martin *et al.*, 2012). As in Swindon, no significant matches to type C could be found in the honey bee or *Varroa* samples on either island. The time lag of the B to A switch in Big Island corresponds to the period when the mite becomes established but before colonies start dying. The normal outcome of this variant competition is the dominance of type A as evidenced by its transmission around the world (Berényi *et al.*, 2007). In the *Varroa*-resistant Swindon apiary, once established, the avirulent type B variant appears to prevent type A from becoming dominant. Crucially, in Swindon, the *Varroa* mites contained a proportion of type A reads (representing the whole type A genome) which were not detected in the honey bees suggesting that effective transmission of type A from parasite to host was prevented (Figure 4).

SIE has been well documented in viruses related to DWV, for example, Tscherne *et al.* (2007) used cell lines to show that infection by one genotype of hepatitis C virus prevented infection by others. SIE best explains the phenomenon of why, despite high DWV load and *Varroa* infestation, the isolated UK colonies do not collapse. We speculate that co-evolution of the honey bee-*Varroa* mite-DWV system has selected for a new stable equilibrium where both the *Varroa* and an avirulent type B variant of DWV protect the honey bee, and thus the colony, from the virulent type A (Figure 8). Further work to validate this and determine the mechanism of the viral exclusion is required. For example, to demonstrate whether type B can protect against type A or C at the cellular and individual honey bee-level using assays similar to those described by Ryabov *et al.* (2014). If true, this would be the first report of SIE acting on the *Iflavirus* pathogens of bees. Ironically, it may be the presence of the mite population that is protecting the colony as *Varroa* may be providing the opportunity for constant re-introduction of type B into the population via horizontal transmission. In addition, although recombinants were present in both honey bee and *Varroa* samples, it is unclear whether these originate in the honey bees, *Varroa* or both.

It also remains unclear under what conditions type B can prevail or whether similar mechanisms of protection operate in the Brazilian, USA and French populations. Although the mechanism for exclusion seen in the Swindon apiary is unclear, a unique recombinant between type A and B was found (Figure 6) suggesting that the full-length type A genome (Figure 4) is actively suppressed. This is the counterpart of recombination causing acute infections as described by Moore *et al.* (2011) and Ryabov *et al.* (2014). Other candidate mechanisms have previously been identified in different viruses at various stages of the viral life cycle, including blocking of virus entry to the cell at the level of receptor interaction or occupation of sites for RNA replication (Lee *et al.*, 2005). Alternatively, the dominance of type B in the Swindon samples could be because of the induction of a differential immune response from the host such as RNAi (Hunter *et al.*, 2010).

Studies on honey bee pathogens have suggested that natural selection favours the survival and transmission of DWV over viruses of the Acute Bee Paralysis Complex (ABPV, KBV and IAPV), which have a higher virulence (de Miranda and Genersch,

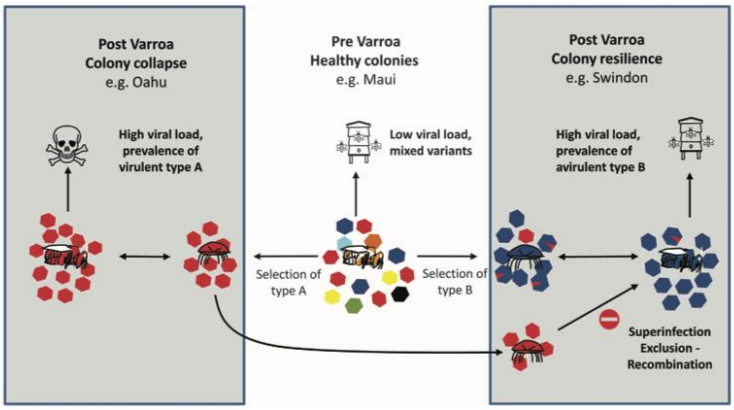

Figure 8 New honey bee-Varroa mite-DWV equilibrium. Type A DWV is represented in red and type B in blue. In Varroa-free hives, DWV exists as a cloud of variants present at low levels. In diseased hives such as Oahu, the type A is present in a Varroa-mediated transmission cycle. Whereas in Swindon, transmission of type B between bees and Varroa prevents the incursion of the type A variant into honey bees and consequently the hive survives.

2010; Schroeder and Martin, 2012). In this scenario, virus survival requires that the pupae live long enough to enable *Varroa* maturation and allow onward virus transmission. For example, the acute virulence of ABPV kills both adults and pupae quickly, ending the transmission cycle as mites associated with the pupae do not survive (Schroeder and Martin, 2012). The same reasoning can be applied to the DWV quasispecies where a particular host-variant dynamic dictates stable transmission or prevalence. Therefore, the Swindon UK population in question could have evolved to favour DWV type B persistence as a result of husbandry practices that have selected for a new stable non-pathogenic equilibrium. However, this phenomenon is not peculiar to Swindon as a recent study in South Africa found only DWV type B in four study apiaries, with no type A detected in either mites or honey bees (Strauss *et al*., 2013). This raises the possibility that SIE may be operating on a wider scale in some geographical locations.

On the basis of our study, we hypothesise that within the swarm of DWV, owing to SIE, different viral variants are competing with two discernible outcomes. Either the disease-causing variants dominates, which can lead to colony collapse (Martin *et al*., 2012), or an avirulent variant can prevail, reaching high viral loads which excludes the virulent variants. In the Swindon apiary, an evolutionary stable state has been reached in which disease symptoms are minimal and colonies survive. The data show that the dominance of type B in this isolated UK apiary has been stable only over a year of sampling, but anecdotal evidence suggests that the viral makeup of the bees at the Swindon Honey bee Conservation Trust has been stable for some time longer.

The discovery of a potential SIE mechanism in honey bees gives those wishing to limit or eradicate the sources of honey bee colony decline the possibility of active intervention. For example, in the citrus industry, where SIE is used to reduce crop losses by inoculating plants with a benign variant of *Citrus tristeza* virus to protect against infection by a more pathogenic form (Lee and Keremane, 2013). Accordingly, the direct introduction of DWV type B could provide a form of biocontrol against further collapse of European honey bee colonies in the face of *Varroa* infestation.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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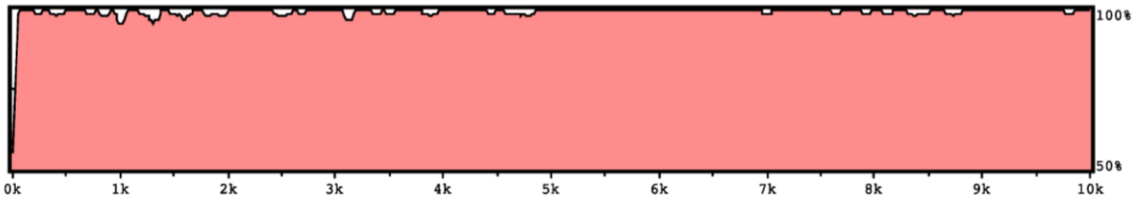
Supplementary Information accompanies this paper on The ISME Journal website (http://www.nature.com/ismej)

Supplementary Information



Supplementary Figure S1. Plot showing the percentage identity across the whole genome of the genome scaffold from Hive 6 January 2013 (ERS754547) compared to the type B VDV reference genome (AY251269.2). The two genomes are 99.5% identical.

Table S1 Contigs assembled from Hi-seq Illumina pair end reads for the Swindon samples

Sample name	Number of contigs	Maximum contig length	Average length of contigs	Number of reads used in all contigs
H6_Oct_12	230	4300	267	1,977,833
H6_Jan_13	253	7757	261	1,556,582
H6_Apr_13	241	5039	263	2,521,732
H6_May_13	372	8734	249	2,347,253
V6_Oct_12	405	3891	247	1,287,116
V6_JanAprMay_13	764	4982	256	35,802,604
H17_Oct_12	22	8622	711	136,123
H17_Jan_13	53	5488	424	701,666
H17_Apr_13	238	4274	267	1,905,055
H17_May_13	18	3752	594	3289
V17_Oct_12	654	5086	243	16,283,045
V17_JanAprMay_13	902	10,449	251	30,620,382
H19_Oct_12	52	3331	416	508,140
H19_Jan_13	198	2642	270	1,314,217
H19_Apr_13	388	10,253	247	3,671,238
H19_May_13	362	5108	248	3,342,658
V19_Oct_12	834	4374	253	14,793,194
V19_JanAprMay_13	424	6179	248	4,579,032
Oahu HB	41	6255	590	38,364,999
Oahu Varroa	69	6276	429	34,234,151
Big Island HB	62	10509	598	41,880,850
Big Island Varroa	40	12237	806	19,036,992

Table S2 DWV load per worker honey bee collected over the sampling period of October 2012 to October 2013 from the Swindon, UK, Apairy.

Hive	Sampling Date	mg sample	RNA (ng/ul)	Elution vol	Dilution in qPCR	RNA per qPCR (ng)	Ct from qPCR	DWV Ct cDNA standard curve conversion	RNA (ng) per bee (bee = 100 mg)	DWV per bee
H17	Oct-12	50	42.4	30	N/A	42.4	20	3.45E+05	2544	2.07E+07
	Dec-12	50	31.5	30	N/A	31.5	17.45	4.14E+05	1890	2.48E+07
	Jan-13	50	152.7	30	N/A	152.7	19.05	3.71E+05	9162	2.22E+07
	Apr-13	50	120.8	30	N/A	120.8	17	4.26E+05	7248	2.56E+07
	May-13	50	443.3	30	1 in 4	110.8	16.56	4.38E+05	26598	1.05E+08
	Jun-13	50	941.3	30	1 in 9	104.6	16.58	4.37E+05	56478	2.36E+08
	Jul-13	50	413.8	30	1 in 4	103.45	19.25	3.65E+05	24828	8.76E+07
	Aug-13	50	93.5	30	N/A	93.5	24.36	2.27E+05	5610	1.36E+07
	Sep-13	50	238.9	30	1 in 2	119.45	18.19	3.94E+05	14334	4.73E+07
	Oct-13	50	274.9	30	1 in 2	137.45	16.48	4.40E+05	16494	5.28E+07
H19	Oct-12	50	390.9	30	1 in 3	130.3	18.7	3.80E+05	23454	6.84E+07
	Dec-12	50	142.4	30	N/A	142.4	16.37	4.43E+05	8544	2.66E+07
	Jan-13	50	92.9	30	N/A	92.9	16.18	4.48E+05	5574	2.69E+07
	Apr-13	50	51.8	30	N/A	51.8	17.15	4.22E+05	3108	2.53E+07
	May-13	50	119.1	30	N/A	119.1	17.08	4.24E+05	7146	2.54E+07
	Jun-13	50	476.5	30	1 in 4	119.1125	18.9	3.75E+05	28587	8.99E+07
	Jul-13	50	614.3	30	1 in 6	102.4	26.41	1.71E+05	36858	6.17E+07
	Aug-13	50	125.2	30	N/A	125.2	22.71	2.72E+05	7512	1.63E+07
	Sep-13	50	212.4	30	1 in 2	106.2	26.37	1.73E+05	12744	2.07E+07
	Oct-13	50	437.4	30	1 in 4	109.4	21.84	2.95E+05	6561	1.77E+07
H6	Dec-12	50	13.3	30	N/A	13.3	18.94	3.74E+05	798	2.24E+07
	Jan-13	50	108.2	30	N/A	108.2	19.38	3.62E+05	6492	2.17E+07
	Apr-13	50	311.8	30	1 in 3	103.9	18.97	3.73E+05	18708	6.71E+07
	May-13	50	543.6	30	1 in 5	108.7	18.41	3.88E+05	32616	1.16E+08
	Jun-13	50	87.0	30	N/A	87.0	17.68	4.08E+05	5220	2.45E+07
	Jul-13	50	1014.0	30	1 in 10	101.4	17.76	4.06E+05	60840	2.43E+08
	Aug-13	50	197.7	30	N/A	197.7	20.82	3.23E+05	11862	1.94E+07
	Sep-13	50	108.1	30	N/A	108.1	21.63	3.01E+05	6486	1.80E+07
	Oct-13	50	311.7	30	1 in 3	103.9	28.02	1.28E+05	18702	2.30E+07

Table S3 Swindon DWV type B genome assembly

Genome name	Number of contigs used to create genome scaffold	Average length of contigs	Total number of reads that make up genome	Percentage (%) of reads used in genome scaffold of total reads assembled by Vicuna
H6_Jan_13 Type B genome	2	5245	1,310,540	84.2

Table S4 Sequence depth comparison for the recombination region**.** The start and end region represent the nucleotide where the recombinant type A sequence began and end for each hive on the DWV reference genome (gi|71480055) for type A variants and on the type B genome scaffold from Swindon, hive 6 (ERS754547).

Hive	Variant	Region Start	Region End	Number of reads
H6	Type A	762	1885	98,505
H6	Type B	748	1858	470,062
H17	Type A	0	2094	10,221
H17	Type B	26	2067	372,801
H19	Type A	852	1887	78,580
H19	Type B	838	1860	361,345

Appendix 3: Covert deformed wing virus infections have long-term deleterious effects on honeybee foraging and survival.

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Author for correspondence:
Tom Wenseleers
e-mail: tom.wenseleers@kuleuven.be

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Covert deformed wing virus infections have long-term deleterious effects on honeybee foraging and survival

Kristof Benaets[1], Anneleen Van Geystelen[1], Dries Cardoen[1], Lina De Smet[2], Dirk C. de Graaf[2], Liliane Schoofs[3], Maarten H. D. Larmuseau[1,4,5], Laura E. Brettell[6], Stephen J. Martin[6] and Tom Wenseleers[1]

[1]Department of Biology, KU Leuven, Laboratory of Socio-ecology and Social Evolution, Leuven, Belgium
[2]Department of Biochemistry and Microbiology, UGent, Laboratory of Molecular Entomology and Bee Pathology, Gent, Belgium
[3]Department of Biology, KU Leuven, Research group of Functional Genomics and Proteomics, Leuven, Belgium
[4]Laboratory of Forensic Genetics and Molecular Archaeology, UZ Leuven, Leuven, Belgium
[5]Department of Imaging and Pathology, KU Leuven, Forensic Medicine, Leuven, Belgium
[6]School of Environment and Life Sciences, The University of Salford, Manchester, UK

TW, 0000-0002-1434-861X

Several studies have suggested that covert stressors can contribute to bee colony declines. Here we provide a novel case study and show using radiofrequency identification tracking technology that covert deformed wing virus (DWV) infections in adult honeybee workers seriously impact long-term foraging and survival under natural foraging conditions. In particular, our experiments show that adult workers injected with low doses of DWV experienced increased mortality rates, that DWV caused workers to start foraging at a premature age, and that the virus reduced the workers' total activity span as foragers. Altogether, these results demonstrate that covert DWV infections have strongly deleterious effects on honeybee foraging and survival. These results are consistent with previous studies that suggested DWV to be an important contributor to the ongoing bee declines in Europe and the USA. Overall, our study underlines the strong impact that covert pathogen infections can have on individual and group-level performance in bees.

1. Introduction

Over the past decades, serious declines in both wild and managed bee pollinators have been recorded in many parts of the world [1–5], thereby threatening the ecosystem services they provide. The underlying cause of the recent wave of honeybee colony losses has been subject to much debate and the current consensus is that multiple stressors likely contribute to these declines, including malnutrition owing to a lack of flower diversity, exposure to agrochemicals or the spread of emerging pathogens and parasites [2,5–10]. Pinpointing these stressors, however, can be hard, especially when their effect becomes obvious only over extended periods of time, such as following exposure to sublethal doses of pesticides [9,11] or after contracting some seemingly harmless 'covert' pathogen infections [12,13].

Among pathogens, recent studies have suggested that deformed wing virus (DWV) represents an important long-term stressor, as it has been statistically associated with both winter mortality and colony collapse in many studies [14–24]. DWV has a near global distribution and is the most widespread of the currently described viruses that infect honeybees, often affecting between 50% and 75% of all honeybee hives [25,26]. In addition, the virus can spill over to other bees [27,28], thereby posing an additional threat. DWV is named after the characteristic wing deformities that can arise when honeybees are infected in

the larval or pupal stage via the ectoparasitic mite vector *Varroa destructor* [29]. In this case, virus infections result in bloated abdomens, miscolouring and shortened lifespans [29]. Typically, however, infections take on a more 'covert' form, resulting in no visible morphological symptoms, especially when infection occurs in the adult stage or when mites carry only low virus titres. Nevertheless, the fact that colonies with covert infections can suffer from weakness, depopulation and sudden collapse [22] and that the presence of the virus has been linked with both winter mortality and colony collapse [14–24] suggests that DWV exerts a significant amount of long-term stress.

Indeed, several recent studies using controlled artificial infection of adult honeybee workers have shown a number of important effects of DWV, including the impairment of sensory responsiveness, associative olfactory learning and memory formation [30] (possibly linked to replication of the virus in the mushroom bodies and antennal lobes [31]), an accelerated pace of behavioural transition through their age-linked task allocation [32] and reduced lifespans of infected adult honeybees [25]. Furthermore, two studies in which natural variation in infection levels of DWV was combined with experimentally manipulated variation in infection levels with the microsporidian parasite *Nosema* showed that DWV-infected bees displayed shortened flight distances and flight durations in flight mill experiments [33], but no differences in orientation flight behaviour in a harmonic radar tracking set-up [34], whereas the reverse pattern was seen for *Nosema* [33,34]. As yet, however, effects of DWV on honeybee flight behaviour and foraging patterns have not yet been investigated using controlled infection set-ups.

The aim of this study, therefore, was to determine the impact of DWV as a long-term stressor in honeybees, and test experimentally if inoculation of adult bees with the virus negatively affected honeybee foraging behaviour and performance. To this end, we used passively powered radiofrequency identification (RFID) transponder tags [9,11,35–40] as a key technology that enabled us to non-invasively monitor the long-term out-hive activity of honeybee workers that were or were not experimentally infected with the virus. Tracking out-hive activity is key in studies of the impact of pathogens on honeybee health, as the worker foraging force is responsible for all resource acquisition, and the foraging range, worker activity and the magnitude of resource influx are vital to colony growth and survival [41,42]. Previously, RFID technology has been successfully used to study sublethal effects of nutritional stress and pesticides on honeybee and bumblebee foraging behaviour [9,11,35–40]. Nevertheless, applications to the study of pathogen-induced stress on honeybee foraging behaviour are still rare, and are currently limited to one study on Israeli acute paralysis virus (IAPV) [43], which documented virus-induced differences in homing ability, but without taking full advantage of the technology to study the long-term impact on foraging behaviour, and one study that showed adverse effects of a microsporidian gut parasite, *Nosema apis*, on honeybee foraging and survival [44]. In addition, another tracking technology—harmonic radar—was recently used by one group to show that the emerging pathogen *Nosema ceranae* caused impaired homing behaviour in honeybees [45] and that *Nosema* infection also affected honeybee orientation flight behaviour [34]. The tracking method, however, was unable to reveal any correlation between DWV infection levels and orientation flight characteristic [34].

2. Material and methods

(a) Radiofrequency identification tracking set-up

Three three-frame observation hives with *Apis mellifera carnica* honeybees were installed at the laboratory's apiary in Leuven, Belgium to serve as host colonies for RFID-tagged bees that were or were not experimentally infected with DWV (400 of each treatment condition per host colony, see below). Each host colony contained two frames of brood, one frame with stored pollen and honey, a queen and around 3000 host colony workers (figure 1). The colonies were placed indoors at room temperature and were connected to the outside via a single entrance tunnel to allow free foraging. The end of the tunnel was outfitted with two iID® MAJA 4.1 RFID reader modules placed in series and connected to a MAJA 4.1 host computer (Microsensys, Germany) to record and log the timing of all RFID-tagged honeybees leaving or entering the hive. By setting up the reader modules in a serial set-up, successive signals from both readers gave information regarding the direction of movement of the detected bees. The readers were separated from each other by a 4 cm wooden tunnel block to prevent interference between the readers (figure 1).

(b) Introduction of control and experimentally infected bees

In each of the host colonies, we introduced 400 DWV-negative control bees and 400 DWV infection-positive honeybees. This was done by allowing bees to emerge from a single donor colony that based on a prior screen was confirmed to be free of DWV as well as of any of the major known honeybee viruses or pathogens [46], injecting newly eclosed workers with appropriate treatment solutions, and introducing these bees into one of three host colonies (see electronic supplementary material Methods for details). All colonies in our apiary, including the donor and host colonies, were treated with Thymovar for *Varroa* control according to the manufacturer's recommendations. The fact that only a single donor colony was used in our experiments was linked with the difficulty of finding a host colony that was free of the major known honeybee pathogens, but our experimental design partly compensated for this by incorporating replication across different host environments. Bees were allowed to emerge by placing brood frames of the donor colony in a MIR-253 incubator (Sanyo, Belgium) at 34°C and 60% humidity, after which newly eclosed workers were collected daily. Subsequently, 400 newly eclosed workers per treatment condition and host colony were injected with 3 µl of the appropriate treatment solution, using a 5 µl 26 s gauge Hamilton syringe inserted into the apical part of the thorax. Immediately afterwards, each of these bees were outfitted with a mic3® 64-bit read-only RFID transponder (Microsensys, Germany) by gluing the tag to the bee's thorax using Kombi Turbo two-component glue (Bison, The Netherlands). The tags measured 2.0 × 1.7 × 0.5 mm, weighed less than 5 mg and transmitted at 13.56 MHz. The RFID codes of all experimentally manipulated workers, together with the treatment condition, host colony and time of introduction, were added to a transponder information database by reading each code using a iID® PENmini USB pen (Microsensys, Germany). Up to 50 tagged individuals subjected to one of the two treatment conditions were kept in separate 15 × 10 × 7 cm cages kept at 34°C and 60% humidity, and contained a 10 × 8 cm piece of honey-filled comb and water, to allow the bees to settle down before introducing them into the host colonies (figure 1). Before introduction, the cages were placed on top of the observation hives, separated only by a wire mesh, for a 30 min period to increase acceptance rates [47]. Each of the 400 workers per host colony and treatment condition were introduced over the course of a period of 5 days, and foraging

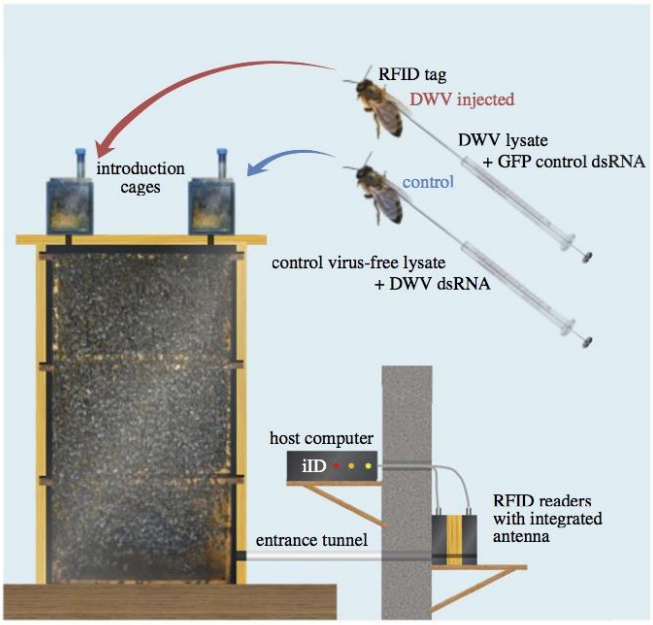

Figure 1. Experimental set-up. Observation hives were installed indoors with two RFID readers at the hive entrance to detect and log RFID-tagged bees entering or leaving the hive. The two RFID readers modules, connected to the host computer, were placed in series to determine the walking direction of detected bees. Tagged bees which were or were not experimentally infected with deformed wing virus were introduced into the host colony via separate introduction cages shown at the top ($n = 400$ bees per treatment and host colony). (Online version in colour.)

behaviour was monitored up to 40 days after introduction, in August–September 2012 for replicate host colonies A and B and September–October 2012 for host colony C.

(c) Controlled infection

In order to obtain two groups of adult age-matched bees that were or were not infected with DWV, we injected bees with lysate of honeybees that were either infected with DWV (and none of the other common honeybee viruses) or with that of honeybees that were confirmed to be virus-free (for details, see electronic supplementary material). To reduce the likelihood of horizontal transmission from the DWV infected to the group of uninfected bees [48], we also added a double-stranded RNA treatment to our DWV-negative lysate [49,50] to try to keep those bees DWV-free, and added a control GFP-dsRNA treatment in the DWV positive lysate to control for the possible effects of foreign dsRNA injection. The amount of DWV injected was estimated at 1.2×10^4–4.6×10^5 DWV copies per bee, and was aimed at mimicking infection loads reported for bees with covert infections (1.4×10^3–2.4×10^9 copies per bee, [51]). Based on Illumina ultra-high throughput sequencing, the DWV strain used for inoculation was determined to belong to the type B DWV master variant [52], which has recently been found to be an emergent, slightly more virulent strain of the DWV virus [25] that is currently also the most common strain in Britain [25] (for details, see the electronic supplementary material).

To validate the effects of the two treatment solutions, 150 additional eclosed workers were injected with each treatment solution, paint-marked and introduced into a fourth observation. Every 4 days, 20 individuals of each treatment were sampled from this colony and subjected to MLPA analysis to determine their DWV infection state [46]. These analyses confirmed the establishment of stable DWV infections in the DWV-lysate-inoculated bees,

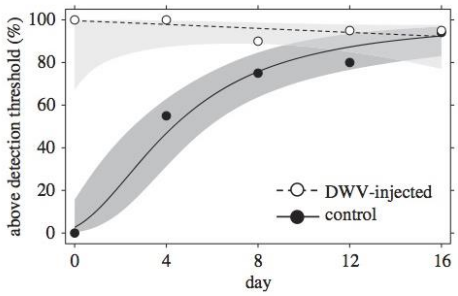

Figure 2. Treatment validation results. Evolution of the proportion of infected individuals over time in control and DWV treatment groups, based on MLPA analysis [46] of four daily sample sets of 20 individuals each from both treatments (fits and 95% CIs based on binomial GLM with treatment, $\log(x + 1)$ tranformed duration after introduction into the host colony and their interaction included as independent variables).

even if there was evidence that some control bees became infected during the later stages of the experiment as well (figure 2), though possibly at lower levels (see electronic supplementary material for a full discussion of the methods and results of these analyses).

(d) Data analysis

Raw RFID tracking data were analysed with the Track-A-Forager Java application [53], which filters out rapid-succession scans of the same scanner, labels ingoing and outgoing flights by tagged workers, and corrects occasional errors in the data, including the possible occurrence of missed scans (for details see electronic

supplementary material). To compare the foraging behaviour of DWV-infected and control bees, we quantified the number of trips performed by each individual, trip duration and the proportion of the introduced workers of each treatment group which survived up to foraging age, to gauge differential early-life mortality. In addition, we measured the age at onset of foraging, defined as the age of each individual at their first reconstructed trip, the foragers' life expectancy, measured as the age of each individual at their last scan, and forager activity span, i.e. the time difference in days between the first and last registered scan of each bee. We should note that our experiment could not distinguish between a DWV-induced reduction in direct mortality and a DWV-induced mortality owing to indirect effects, e.g. caused by a decrease in homing ability or an increase in the susceptibility to predation or other environmental stressors. Details of all statistical analyses performed are given in electronic supplemental material and the R script included on the Dryad repository.

3. Results

Visual analysis of foraging activity over the course of our experiment revealed clear disparities in the age at onset of foraging, forager life expectancy and forager activity span between the DWV-infected and control bees (figure 3). In addition, bees that survived to foraging age showed a clear deviation from the 50 : 50 ratio at which they were first introduced (figure 3), with control bees evidently having much better chances to survive to foraging age than DWV-infected ones. To thoroughly examine each of these effects as well as to look for other possible effects of DWV on foraging behaviour, we conducted a number of detailed statistical analyses. In particular, we tested for significant effects of DWV on the probability that bees would survive to foraging age, the onset of foraging, forager life expectancy and activity span as well as the number and trip duration of foraging trips carried out by individuals that survived to foraging age.

(a) Effect of deformed wing virus on the likelihood that bees would survive to foraging age

The biased representation of control bees among bees that started to forage relative to the 50 : 50 ratio at which they were first introduced (figure 3) indicates that the DWV-inoculated bees experienced greater mortality early on in their life. This is confirmed by the fact that a significantly greater proportion of the DWV-inoculated bees died before making it to foraging age than control bees in each of the three host colonies (277/400 versus 244/400, 324/400 versus 266/400 and 279/400 versus 175/400; binomial colony × treatment full factorial GLM, $z = 2.44$ and $p = 0.015$, $z = 4.61$ and $p = 4 \times 10^{-6}$, $z = 7.33$ and $p = 2 \times 10^{-13}$; figure 4*a*). We should note that this mortality also includes baseline mortality linked to occasional rejection of tagged bees by the host colony. To control for this baseline mortality, we also calculated the relative odds that bees would survive to foraging age. This showed that DWV-inoculated bees on average had 2.1 (s.e. 0.19) times lower odds to make it to foraging age than control bees.

(b) Effect of deformed wing virus on age at onset of foraging, life expectancy and activity span

We also found significant differences between the DWV-infected and non-infected treatments in the observed age at onset of foraging. In particular, bees that were inoculated with DWV and which survived until foraging age starting to forage 2.31 days earlier (s.e. 0.73), on average, than bees inoculated with the control solution (figure 4*b*; full factorial colony × treatment ANOVA, main effect of treatment: $p = 7 \times 10^{-9}$; LR $\chi^2_1 = 33.63$, sample sizes as shown in figure 3*a*). In addition, infected foragers had a life expectancy that was reduced by 4.74 days on average (s.e. 0.20) compared with control bees (figure 4*c*; ANOVA, main effect of treatment: $p = 0.01$; LR $\chi^2_1 = 28.7$) and a total activity span that was reduced by 2.60 days (s.e. 0.79) compared with control foragers (figure 4*d*; ANOVA, main effect of treatment: $p = 0.0003$; LR $\chi^2_1 = 13.26$; sample sizes for both are as given in figure 3*a*). Hence, DWV-infected bees had a reduced life expectancy and activity span also after they started to forage, even though the onset of foraging occurred 2.3 days earlier in the DWV-infected group than in the control group. Overall, the onset of foraging (at 12–17 days) was somewhat earlier and forager life expectancy shorter (11–26 days) than that observed in mature hives (onset at *ca* three weeks and forager life expectancy of *ca* six weeks [47,54]). Nevertheless, both figures were comparable to those found in other studies that used a comparable observation hive set-up (e.g. onset of foraging at *ca* 10 days and forager life expectancy of 14–40 days [55]). This discrepancy may be linked with stress induced by the RFID tags or the injection itself, as stress in honeybees is known to induce precocious foraging and shorten worker lifespan [32,56]. Even so, we expect our conclusions to be robust as our treatment effects are all measured relative to the control under identical conditions.

(c) Effect of deformed wing virus on number of foraging trips or trip duration

Although DWV infection could in principle also have affected the number of foraging trips and the duration of the trips carried out by individuals that survived to foraging age [33], our statistical analyses revealed that there was no strong evidence for this. For example, the effect of treatment on the number of trips performed was inconsistent and different across the replicate host colonies used (quasi-Poisson GLM, effects of treatment in colonies A, B and C: A. $z = 7.55$ and $p = 1 \times 10^{-7}$, B. $z = -2.49$ and $p = 0.04$; C. $z = -1.38$, $p = 0.42$). On average, across all three colonies, workers made 5.46 trips per bee per day (s.e. 0.50) in the DWV-inoculated group versus 5.85 trips per bee per day (s.e. 0.80) in the control (sample sizes as in figure 3*a*), which are figures consistent with those given in other studies [47,54]. Nevertheless, given that DWV infection strongly reduced the chances for workers to survive to foraging age and that DWV infection reduced the life expectancy and total activity span of foragers (cf. results above), it is clear that DWV still had a strongly negative overall effect on the net number of trips and amount of foraging performed. Similarly, the effect on trip duration was not consistent across host colonies, as DWV-inoculated bees made significantly longer trips than control bees in colony A (gamma GLMM, Tukey post-hoc test, 1.86 h versus 1.22 h, s.e. 0.17 and 0.08, $p = 0.0004$), whereas there was no significant effect on trip duration in colony C (2.89 h versus 2.50, s.e. 0.41 and 0.47, $p = 0.90$), and an opposite trend in colony B than in colony A (1.62 h versus 1.99 h, s.e. 0.12 and 0.27, $p = 0.39$). Inclusion of time or worker age as explicit linear or polynomial terms, either in interaction with treatment or not, did not

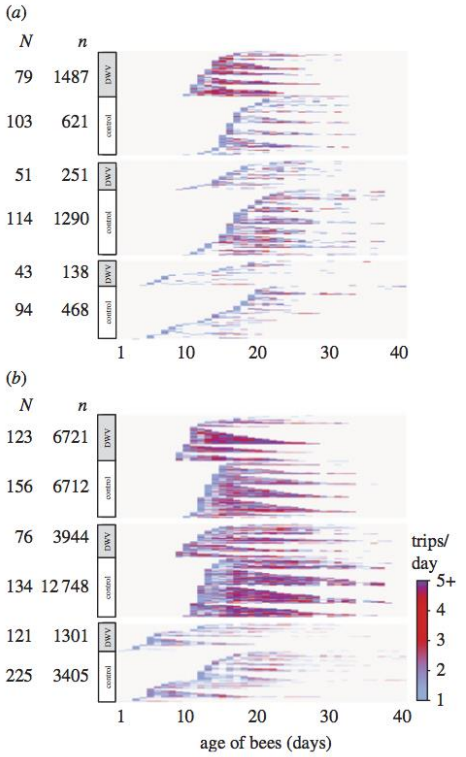

Figure 3. Total foraging activity of DWV-infected and control bees. The daily foraging activity over the course of the experiment is shown based on both the reconstructed foraging trips (*a*) and the unfiltered RFID scans (*b*) for colonies A (top), B (middle) and C (bottom) (N = total number of unique bees detected per treatment and host colony, n = total number of reconstructed foraging trips or scan events across all tracked bees). Individuals, represented as rows in the diagram, are sorted by treatment, age at onset of foraging and total activity span. The overrepresentation of control bees among observed foragers demonstrates that control workers had higher odds to survive to foraging age, and visual analysis of the data also indicate disparities between DWV and control bees in the age at onset of foraging, forager life expectancy and activity span (cf. figure 4). (Online version in colour.)

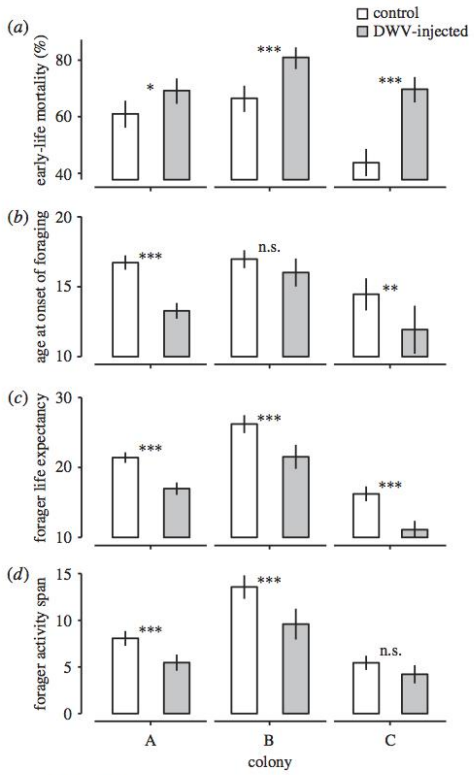

Figure 4. Effect of DWV infection on honeybee foraging and survival. Out of the 400 bees that were tagged per treatment and host colony, a significantly smaller proportion survived to foraging age (being detected at least once by the RFID scanners) in the DWV injected group than in the control group (panel *a*, binomial full factorial GLM, overall $p < 2 \times 10^{-16}$, LR $\chi^2_1 = 71.89$; means and 95% C.I.s are shown and significance levels per colony based on Tukey post-hoc Wald z tests shown by asterisks). In addition, honeybees that were artificially infected with DWV and which survived to foraging age started to forage significantly earlier than uninfected control bees (panel *b*, two-way full factorial ANOVA, $p = 7 \times 10^{-19}$, LR $\chi^2_1 = 33.63$; means and 95% C.I.s are shown and significance levels per colony based on Tukey post-hoc Wald z tests shown by asterisks; total number of tracked bees as in figure 3*a*), and DWV-infected foragers had a significantly reduced life expectancy (defined as age at last detection, panel *c*; ANOVA, $p = 0.01$, LR $\chi^2_1 = 28.7$) and activity span (defined as age at last foraging trip minus age at first foraging trip, panel *d*; ANOVA, $p = 0.0003$, LR $\chi^2_1 = 13.26$) (means and 95% C.I.s are shown and significance levels per colony based on Tukey post-hoc Wald z tests shown by asterisks; total number of tracked bees and foraging trips as in figure 3*a*).

improve the explanatory power of any of the fitted models, and hence such analyses were not further pursued.

4. Discussion

Overall, our results demonstrate that DWV infections have strongly deleterious effects on adult honey bees, with both mortality rates, and—to a lesser extent—foraging behaviour being clearly affected. In particular, DWV-infected bees started to forage at an earlier age and showed reduced lifespans and total activity spans than control bees. Finally, next-gen sequencing demonstrated that the DWV strain we used for inoculation belonged to the type B DWB master variant [52], which has recently been found to be an emergent, more virulent strain of the DWV virus [25] that currently appears to be the most common strain in Britain [25]. The fact that our DWV lysate was prepared from a randomly selected sample of bees with overt DWV infection symptoms suggest that this strain is now also common in Continental Europe.

Our finding that DWV-inoculated workers started foraging at an earlier age and experienced so-called precocious foraging was in line with expectation, as previous studies have also found that unhealthy or stressed honeybee workers start to perform risky foraging tasks at an earlier age compared with healthy individuals. For example, *Nosema* [13,57,58], sacbrood virus [59] and *Varroa* [60,61] have all been found to induce

precocious foraging in honeybees. From an ultimate perspective, diseased or health compromised workers have been suggested to benefit from starting to foraging earlier as a way to protect other individuals inside the nest from getting infected [58] or to make the most of their reduced lifetime [56]. In addition, it would be possible that the disease agent itself benefited from an earlier onset of foraging if this promoted its horizontal transmission to other host colonies [62]. Indeed, in the case of DWV, a direct influence on the behaviour of its host is not unlikely, given that DWV particles have previously been found in the mushroom bodies—a key higher brain centre of these insects [31] (but see [34] for a study where no behavioural effects were found). Irrespective of these possible adaptive causes, it is clear that precocious foraging would have a major effect on colony well-being, as premature foraging partially depletes the nurse bee population [63] and disrupts various activities inside the hive [64–66], and rapid behavioural maturation has been shown to strongly accelerate the failure of stressed honeybee colonies [63].

The strong evidence we found for a DWV-induced effect on mortality patterns and long-term survival was more unexpected. Traditionally, secondary DWV infections in adult workers are regarded as 'covert' and largely asymptomatic [29,67,68], but this proposition is clearly challenged by our findings, which document very clear and significant long-term effects of the virus. Although increased mortality has been documented in bees that display overt DWV infection symptoms and crippled wings [69–71], similar mortality in bees that acquired the virus in the adult stage has been demonstrated only recently in experimentally caged and non-foraging bees [25]. Our results now show that this mortality effect continues after the onset of foraging, and that the virus therefore acts as a long-term stressor on honeybee health and survival. DWV-induced mortality could have several causes. Given that DWV have been shown to occur in the honeybee brain, including in the mushroom bodies [31,62,72], which are involved in learning and memory, and that DWV infections have been shown to induce learning deficits [30], it is possible that increased mortality is caused by impaired orientation capabilities or predator avoidance or that it makes them more susceptible to other environmental stressors. Indeed, the DWV Kakugo strain has earlier been found to be associated with increased aggression and risk-taking behaviour [73,74]. Alternatively, it is possible that DWV directly results in increased mortality, e.g. owing to costly upregulation of the host's immune system [75]. However, given the well-documented effect on direct, early-life mortality, both in our study and that of McMahon *et al.* [25], we consider a direct mortality effect most likely. Furthermore, and regardless of the underlying causes, it is clear that the early disappearance of DWV-infected bees and their significantly shorter activity spans would have strongly deleterious effects on the total amount of pollen and nectar foraging performed by infected colonies. Additionally, a shorter activity span of workers would also cause fewer workers to engage in discovering novel food patches, thereby impacting the flow of information and causing further synergistic costs to global colony health [47].

The fact that in our RFID data, DWV inoculation did not affect trip duration or the number of trips performed by DWV-infected foragers went against the conclusions of [33], who concluded that DWV infection but not *Nosema ceranae* reduced average trip duration. As there was significant variation in the impact of the virus across our three replicate host environments, however, it is possible that the same effect would still have been found with a larger number of replicate donor and/or host colonies. Given that our donor bees all came from a single, rare uninfected colony, we had *a priori* not expected any large variation in the impact of the virus. Possible reasons for this variation could be linked with seasonal factors, variation in the genetic compatibility with the host colonies, or subtle differences in the performance or health of the host colonies, such as the possible presence of *Nosema* among the host workers, which we did not explicitly look at, but which is known to cause precocious foraging and affect longevity, activity and out-of-hive performance of honeybees [13,45,58,76,77]. Alternatively, it is possible that the variation in DWV impact is linked to some of the control bees having become infected during the later stages of our experiment, which our treatment validation results suggest may have been the case (though likely at lower levels, figure 2), and that the speed at which this occurred differed across host colonies. These results also suggest that a single dsRNA injection was not sufficient to fully protect bees for extended periods of time, and that continued oral administration would have to be used for effective long-term control via RNA interference [49,50,78]. Despite this variation in the effect of the virus, however, it was clear that overall, DWV had a strongly deleterious effect across all three colonies, with significant effects on early-life mortality (figure 4*a*), forager life expectancy (figure 4*c*) and forager activity span (figure 4*d*) in three replicate host colony environments, and significant effects on the onset of foraging in two out of three host colonies (figure 4*b*). These findings are consistent with studies showing that DWV is among the most important predictors implicated in honeybee colony declines in both Europe [14,16,17,24] and the USA [18] and hence an important contributory factor to the current pollination crisis.

Overall, our results highlight the impact of long-term stressors on bee health and survival, thereby reinforcing the conclusions of several recent tracking studies that have studied stress-induced changes in bees caused by either pathogens [33,43–45], nutritional stress [36] or pesticide exposure [9,11,35,37–40]. We hope that in the future, these approaches may continue to be used to further our understanding of the factors involved in the ongoing pollinator declines [1–5] and how they interact with each other in exerting long-term stress [2,5–10].

Data accessibility. All raw data files and code used in analyses are available in Dryad: http://dx.doi.org/10.5061/dryad.fm0r1. Illumina reads and the full genome sequence of the DWV inoculate are available from the Sequence Read Archive and GenBank (accession nos. PRJNA336281 and KX783225).

Authors' contributions. K.B., D.C., D.C.d.G., L.S. and T.W. conceived and designed the work; K.B., D.C. and L.D.S. performed the experiments; K.B., A.V.G. and T.W. analysed the data; L.D., D.C.d.G., L.D.S., L.B., S.J.M. and T.W. contributed reagents, materials, or analysis tools and K.B., M.H.D.L., L.B., S.J.M. and T.W. wrote the paper. All authors gave final approval for publication.

Competing interests. We have no competing interests.

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SUPPLEMENTAL METHODS

Controlled infection

In order to obtain two groups of adult age-matched bees that were or were not infected with DWV, we first prepared two treatment solutions, which upon injection were designed to increase or keep down DWV virus titers. This required preparing lysates of honeybees that were infected with DWV but none of the other common honeybee viruses, as well as of honeybees that were confirmed to be virus-free. To this end, we produced a lysate of a pool of 10 bees, which based on multiplex ligation-dependent probe amplification (MLPA) analysis and the use of specific PCR primers were confirmed to be entirely virus and pathogen-free or infected only with DWV (see below) [1]. These pools of bees were obtained following an initial screening of 10 individuals each from a set of 10 *A. mellifera carnica* colonies available in our apiary in Leuven, as well as based on the screening of individuals with overt symptoms of DWV-infection (i.e. displaying crippled wings). This screening also allowed the selection of the previously mentioned virus and pathogen-free donor colony. DWV lysate and virus-free control lysate were extracted from the selected set of DWV-infected and virus-free bees by homogenizing the mass equivalent of five bees (around 500 mg) of the selected samples (stored at -80°C) after immersion in liquid nitrogen, and mixing the homogenate with 5 ml phosphate-buffered saline (PBS, pH 7.4). Subsequently, the samples were centrifuged at 3000 r.p.m. for 30 min at 4°C and the supernatant was stored in aliquots at -80°C for future use [2].

The artificial inoculation with a DWV lysate that was used in this study has previously been shown to be effective in short-term, individual-based setups [2]. In our present study, however, the bees stayed in close contact with each other in the observation hive, throughout their adult lives, so that the virus could still be transmitted horizontally during the experiment. To try to mitigate the effects of horizontal transmission from the DWV infected to the group of uninfected bees [3] we therefore opted to also add a double-stranded RNA treatment to our DWV-negative lysate [4, 5]. In particular, DWV-specific dsRNA was added to the control lysate to try to keep these bees DWV-free and prevent DWV cross-infection inside the host colony, whereas control GFP-dsRNA was added to the DWV lysate to control for the possible effects of foreign dsRNA injection on honeybee physiology. DWV and GFP-dsRNA were provided by Beeologics (Israel) and were dissolved in nuclease free water at 3 mg/ml. Prior to injection, both DWV and control lysates were diluted 1:1000 (cf. Iqbal and Mueller [2] in insect saline buffer (ISB, containing 150 mM NaCl, 10 mM KCl, 4 mM CaCl2, 2 mM MgCl2, and 10 mM HEPES; pH 7.0), after which dsRNA was added at a dosage of 5 µg per bee to obtain the final treatment solutions. Given that bees with overt symptoms have been reported to contain between 1.8×10^{10} and 6.9×10^{11} DWV genome equivalents per bee [6], we estimate that our protocol resulted in the injection of between 1.2×10^{4} and 4.6×10^{5} DWV copies per bee, which is well within the infection loads reported for bees with covert infections ($1.4 \times 10^{3} - 2.4 \times 10^{9}$ copies per bee, [6]). Hence, our DWV inoculation treatment adequately mimicked covert DWV infection levels.

RFID data analysis

Raw RFID tracking data were analyzed with the Track-A-Forager Java application, which filters out rapid-succession scans of the same scanner, labels ingoing and outgoing flights by tagged workers, and corrects occasional errors in the data, including the possible occurrence of missed scans [7]. The

setup options were adjusted based on the foraging type and entrance/exit system that was used in the experimental setup, namely 'natural foraging' and 'joined two scanners'. In the data filtering phase, the default time constraint settings were applied to filter out rapid-succession scans of the same scanner, determine 'IN' and 'OUT' events of tagged foragers and reconstruct forager trips. The output of the application generated the complete list of all the scans of each RFID tag at each scanner with their corresponding time stamp, the reconstructed trips per tag with their corresponding time stamp and the durations of reconstructed trips with the age of each individual at the time of the trip. The resulting data was then joined with the transponder information database consisting of the unique RFID tag code, the treatment, colony and date of introduction of each individual.

To test for differences in the proportion of workers of each treatment group that survived to foraging age, we used a binomial GLM in which 'colony', 'treatment' and their interaction effect were included as fixed factors. ANOVAs with 'colony', 'treatment' and their interaction effect included as fixed factors were used to assay the difference in the age at onset of foraging, life expectancy and activity span. To assess the number of reconstructed trips made by individuals of each group we used a quasipoisson generalized linear model (GLM) with 'colony', 'treatment' and their interaction effects included as fixed factors and 'activity span' included as a covariate. Finally, the duration of the reconstructed trips was analysed with a gamma distributed generalized linear mixed model (GLMM) with the unique RFID tag specified as a random factor and 'colony' and 'treatment' plus their interaction effect specified as fixed factors. All statistical analyses were carried out using *R* v. 3.2.2 and the *lme4* 1.1-9 (for generalized mixed models) and *effects* 2.3-0 packages (to produce effect plots).

MLPA-based screening for viral infections and treatment validation

Multiplex ligation-dependent probe amplification (MLPA) was used to identify virus-free donor colony candidates, select honeybee samples that were best suited for preparing the lysates for the two treatments and to validate the effects of these treatments. MLPA was performed as described in De Smet *et al.* [1], using MLPA probes and RT-primers designed for six virus targets, covering the ten most common honeybee viruses: chronic bee paralysis virus (CBPV), deformed wing virus (DWV, including A, B, i.e. VDV-1, and C type virus, [8]), acute bee paralysis virus (ABPV)/Kashmir bee virus (KBV)/Israeli acute paralysis virus (IAPV), black queen cell virus (BQCV), slow bee paralysis virus (SBPV) and sacbrood virus (SBV) (Table S1). The amplified MLPA products were analyzed by electrophoresis on a 4% high resolution agarose gel with a specific MLPA ladder.

Table S1. Primers and half-probes used for detecting the positive strand of different honeybee viruses and virus species complexes through RT-MLPA. Adapted from De Smet *et al. [1]*.

VIRUS	FUNCTION	SEQUENCE (5'-3')	SIZE (bp)
CBPV	(-)cDNA	GCCCCGATCATATAAGCAAA	88
	(+)MLPA-LPO	gggttccctaagggttggaCCGTAGCTGTTTCTGCTGCGGT	
	(+)MLPA-RPO	P-ACTCAGCTCAGCTCGACGCTAGAtctagattggatcttgctggcac	
DWV	(-)cDNA	TCACATTGATCCCAATAATCAGA	95
(A+B+C type)	(+)MLPA-LPO	gggttccctaagggttggaTGACCGATTCTTTATGAGCGAGCTCT	
	(+)MLPA-RPO	P-TACGTGCGAGTCGTACTCCTGTGACAtctagattggatcttgctggcac	
ABPV	(-)cDNA (ABPV)	CAATGTGGTCAATGAGTACGG	104
KBV	(-)cDNA (KBV&IAPV)	TCAATGTTGTCAATGAGAACGG	
IAPV	(+)MLPA-LPO	gggttccctaagggttggaCTCACTTCATCGGCTCGGAGCATGGATGAT	
	(+)MLPA-RPO	P-ACGCACAGTATTATTCAGTTTTTACAACGCCCtctagattggatcttgctggcac	
BQCV	(-)cDNA	CGGGCCTCGGATAATTAGA	122
	(+)MLPA-LPO	gggttccctaagggttggaCTTCATGTTGGAGACCAGGTTTGTTTGCCGACTTACGGAA	
	(+)MLPA-RPO	P-TGTCGTTAAACTCTAGGCTTTCCGGATGGCTTCTTCATGGtctagattggatcttgctggcac	
SBPV	(-)cDNA	CGCAAACACGACGAATTTTA	131
	(+)MLPA-LPO	gggttccctaagggttggaCGTTCAATGGTCGAGATAGAAGCCACAGTAGAAGTATTACGCGCT	
	(+)MLPA-RPO	P-TCTTGTGTTTTGGCTTATGGGCGTGGGCCTGATCTTCATTCAGCtctagattggatcttgctggcac	
SBV	(-)cDNA	TGGACATTTCGGTGTAGTGG	140
	(+)MLPA-LPO	gggttccctaagggttggaCGTTGATCCAATGGTCAGTGGACTCTTATACCGATTTGTTTAATGGTTGG	
	(+)MLPA-RPO	P-GTTTCTGGTATGTTTGTTGACAAGAACGTCCACCTTCAGCCATTCAGCtctagattggatcttgctggcac	

Samples to derive DWV lysates from were selected for further use if they tested DWV positive but negative for the other tested viruses. To identify suitable honeybee samples, RNA was first extracted from collected bees with and without deformed wings and screened them for pathogens using MLPA. 20 sets of ten adult bees from different colonies without and where possible with overt symptoms of DWV were pooled and whole bodies were homogenized in liquid nitrogen. 80-100 mg of the homogenized tissues were then mixed with 1 ml Qiazol reagent in MagNA Lyser Green Beads sample tubes (Roche, Belgium) for RNA-extraction with the RNA lipid tissue mini kit (Qiagen, Germany). Leftover tissue was kept and stored at -80°C for lysate preparation. Pooled tissues in Qiazol were further disrupted and homogenized on a MagNa Lyser Instrument (Roche, Belgium) for 30 sec at 6500 Hz. The total RNA was isolated according to the kit's instructions and eluted in a final volume of 50 µl. The quality and concentrations of RNA was checked using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Belgium).

To validate the effects of the two treatment solutions, 150 additional eclosed workers were injected with each treatment solution, paint-marked and introduced into a fourth observation hive without the RFID setup. Every four days, 20 individuals of each treatment were sampled from this colony and frozen in liquid nitrogen for storage at -80°C. Subsequently, RNA extraction and MLPA analysis was carried out as described above. We should note that this method produces binary data and scores bees as containing DWV titers that are above or below the MLPA detection threshold. Several quantitative qPCR-based approaches were also tried, but unfortunately failed – likely due to a mismatch in the primer sites.

Confirmation of disease-free status of donor colony using specific PCR primers

To test that our DWV-free donor colony identified before was also free of most other common honeybee pathogens, we collected three sets of ten bees each, which were each homogenised in 5 ml PBS in MagNA Lyser Green Beads sample tubes (Roche, Belgium) on a MagNa Lyser (30 sex at

6500 Hz), extracted their DNA from 120 µl supernatant using the DNeasy Blood & Tissue Kit (Qiagen), and used the specific PCR primer sets given in Ravoet *et al.* [9, 10] to detect the possible presence of for the microsporidian parasites *Nosema apis* and *N. ceranae*, the fungal parasite *Ascosphaera*, the Mollicute pathogen *Spiroplasma* spp. and the Trypanosomatid parasites *Lotmaria passim* and *Crithidia mellificae*. None of the PCR reactions resulted in a PCR product of the expected size, thereby demonstrating that our DWV-free donor colony was not only free of DWV, but that it was also free of most other major known honeybee pathogens (although evidently we cannot exclude their presence at a low rate in a small subset of the workers). We should note that the presence of pathogens in any of the host colonies was not determined, as with n=3 host colonies, the statistical power to relate the presence of particular pathogens to variation in the impact of DWV would have been too low, and that such differences would also have been intrinsically confounded with genetic and environmental differences between those colonies.

Characterization of experimental inocula via ultra-deep sequencing

Five bees that were inoculated with DWV lysate, collected 12 days post injection, were subjected to ultra-deep sequencing to determine the DWV strain type the bees were injected with as well as to confirm the MLPA and PCR-based results showing that the bees were free of any other major known honeybee pathogens. For these analyses, total RNA was conducted as described above with an additional on-column DNase digestion with the RNase-free DNase set (Qiagen, Germany) to remove DNA contamination. The quality and concentration of the RNA samples was assessed using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Belgium). Subsequently, the libraries were subjected to 90 bp pair-end RNA sequencing on the Illumina Hiseq2000 platform at BGI, using random hexamer primed cDNA synthesis and a 200 bp short insert library. Adaptors, contamination and low-quality reads were removed by BGI and quality control was performed using FastQC [11]. This produced between between 9 and 10 million filtered, high-quality reads per sample, and a total of 88 936 091 reads from our DWV inoculated bees.

To determine that only a single strain of DWV was present in our inoculated bees, and that no other major pathogens were present, we used Bowtie v. 2.2.6 [12] to align the pooled 88.9 million reads to the reference genome sequences of a set of major honeybee pathogens, which included deformed wing virus types A, B and C (accession nrs. NC_004830.2, AY251269.2 and CEND01000001), acute bee paralysis virus (NC_002548.1), aphid lethal paralysis virus (NC_004365.1), black queen cell virus (NC_003784.1), chronic bee paralysis virus-1 (NC_010711.1), chronic bee paralysis virus-2 (NC_010712.1), Israeli acute paralysis virus (NC_009025.1), Kashmir bee virus (NC_004807.1), sacbrood virus (NC_002066.1), the Trypanosomatid parasite *Lotmaria passim* (GCA_000635995.1), the microsporidian parasites *Nosema apis* (GCA_000447185.1) and *Nosema ceranae* (GCA_000182985.1) and the Mollicute pathogen *Spiroplasma apis* (GCA_000500935.1) and *Spiroplasma melliferum* (GCA_000236085.3) as well as the *Apis mellifera* genome, in order to filter out host RNA (using the latest version 4.5 NCBI assembly). Out of the obtained 88.9 million sequence fragments, 78% (69.2 million) did not map onto the host genome and out of these non-host fragments, 73% (50.7 million) mapped onto one of the included pathogens. Out of all the pathogen-mapped fragments, 99.91% mapped to deformed wing virus type B, whereas the remaining reads (0.09%) mapped onto DWV type A. Nevertheless, the latter had such low counts that they were likely caused by either sequence mismatches between our inoculate and the reference DWV type B strain,

sequencing error, or mapping mistakes. In addition, no reads mapped onto any of the other pathogens, thereby confirming our MLPA and PCR results that the donor colony was indeed free of any of the major known honeybee pathogens.

The interpretation that only a single strain of DWV was present in the inoculate was confirmed based on a de-novo assembly of all the fragments that mapped onto any of the RNA viruses, obtained using the Vicuna viral assembler version 1.3, which was developed to characterize possibly heterogeneous virus populations [13]. As expected if only a single DWV strain was present, the Vicuna assembly returned only one single 5941 nt long contig that mapped with very high fidelity (99.0% high sequence similarity) to the 5' end of the DWV type B reference strain. As coverage across the complete DWV genome was highly variable, however, the Vicuna pipeline was not able to recover the full DWV genome. To obtain the full genome sequence, and given the very high sequence similarity of the obtained fragment with the type B reference strain, we therefore used Bowtie v. 2.2.6 [12] instead to map all virus-mapping reads to the DWV type B reference genome (using option –very-sensitive-local, to allow for a maximum number of sequence mismatches), after which *samtools mpileup* and *bcftools call* was used to obtain the consensus sequence of the position-sorted BAM alignment. The resulting full DWV genome of our inoculated strain (accession number KX783225) was 10112 nts long and contained a 2893 AA long polyprotein, which had sequence similarities of 99.28% at the nucleotide level (73/10112 nts substituted) and 99.76% at the AA level (7/2893 AAs substituted) with the DWV type B reference strain (accession number AY251269.2), as well as an identical length, but greater sequence divergence with reference types A or C (Fig. S1). There was no evidence of our strain being a recombinant between strain types [14]. The DWV type B strain was formerly known as *Varroa destructor virus*-1, but is now classified into the deformed wing virus complex as one of three master variants [8], and has recently been found to be an emergent, more virulent strain of the DWV virus [14], which currently appears to be the most common deformed wing virus strain in Britain. As our inoculate was prepared from bees with overt DWV symptoms from a randomly selected hive, our results suggest that this strain is now also common in Continental Europe. Overall per-nucleotide coverage, calculated using *bedtools coverage*, was 902k on average, with a range of 19 to 15 million. Coverage, together with the tentative positions of polyprotein cleavage sites and their resulting products and other genomic features, annotated following Lanzi *et al.* [15], and the overall structure of the virus, based on what is known from other Picornaviridae, are shown in Fig. S2.

Treatment validation results

MLPA analysis of 4-daily sets of samples of 20 individuals per treatment shows that, as expected, there was a strong main effect of treatment on DWV infection rates (binomial GLM, $p = 0.0006$, $z = 3.4$) (Fig. 2), but that DWV infection rates also increased and that the difference in infection rates between the two treatment groups decreased over the course of the experiment (main effect of log(x+1) transformed duration after introduction into the host colony: $p = 3.4E\text{-}6$, $z = 4.6$; interaction effect of log(time+1) and treatment: $p = 0.003$, $z = -3.0$). Based on the calculated 95% confidence limits, however, the difference in infection rate between the two treatments remained statistically significant until the 12th day of the experiment (Fig. 2).

These treatment results imply that in the beginning of our experiment, infection rates strongly differed between the two treatment groups, but that the control workers also may have horizontally

acquired new DWV infections at the later stages of our experiment or perhaps fed on DWV infected pollen or nectar leading to a sizeable virus load in the gut. It is important to note, however, that MLPA is a qualitative technique that only signals the presence or absence of virus particles in the analyzed samples but does not yield any information on the virus titers. Hence, it is possible, and indeed likely, that the DWV titers in control bees that became infected over the later stages of our experiment were still significantly lower than those in the group which were experimentally infected with DWV, even if we did not succeed in formally demonstrating this using a qPCR-based approach. Nevertheless, the fact that we find statistically significant behavioural effects between our two treatment groups up until late into our experiment, when the proportion of DWV positive bees in the control group approached that in the DWV inoculated group, only strengthens our conclusion that DWV has strongly deleterious effects on honeybee foraging behaviour. That is, the total effect could in fact have been even greater if the control group had remained completely uninfected for the full duration of our experiment, and our estimates of the effects on foraging behaviour and mortality should therefore be interpreted as minimum estimates.

That the dsRNA RNAi treatment [4] in the control bees was not fully effective at keeping bees virus-free may have several causes. First and foremost, our setup combined a mix of uninfected and artificially inoculated workers, and this led to an unusually high potential for horizontal transmission inside the colony. Second, in our experiment we only treated the bees one single time, using a single dsRNA injection, to ensure a standardized approach with equal amounts of dsRNA provided to each bee. In real-life applications, by contrast, dsRNA could be administered orally over extended periods in the food [4, 5, 16], and colonies are treated pro-actively, so that colonies may be able to fight off the virus at a much earlier stage than in our experiments. Hence, the potential of dsRNA treatments to combat DWV infections and help to mitigate its associated costs still deserves further study.

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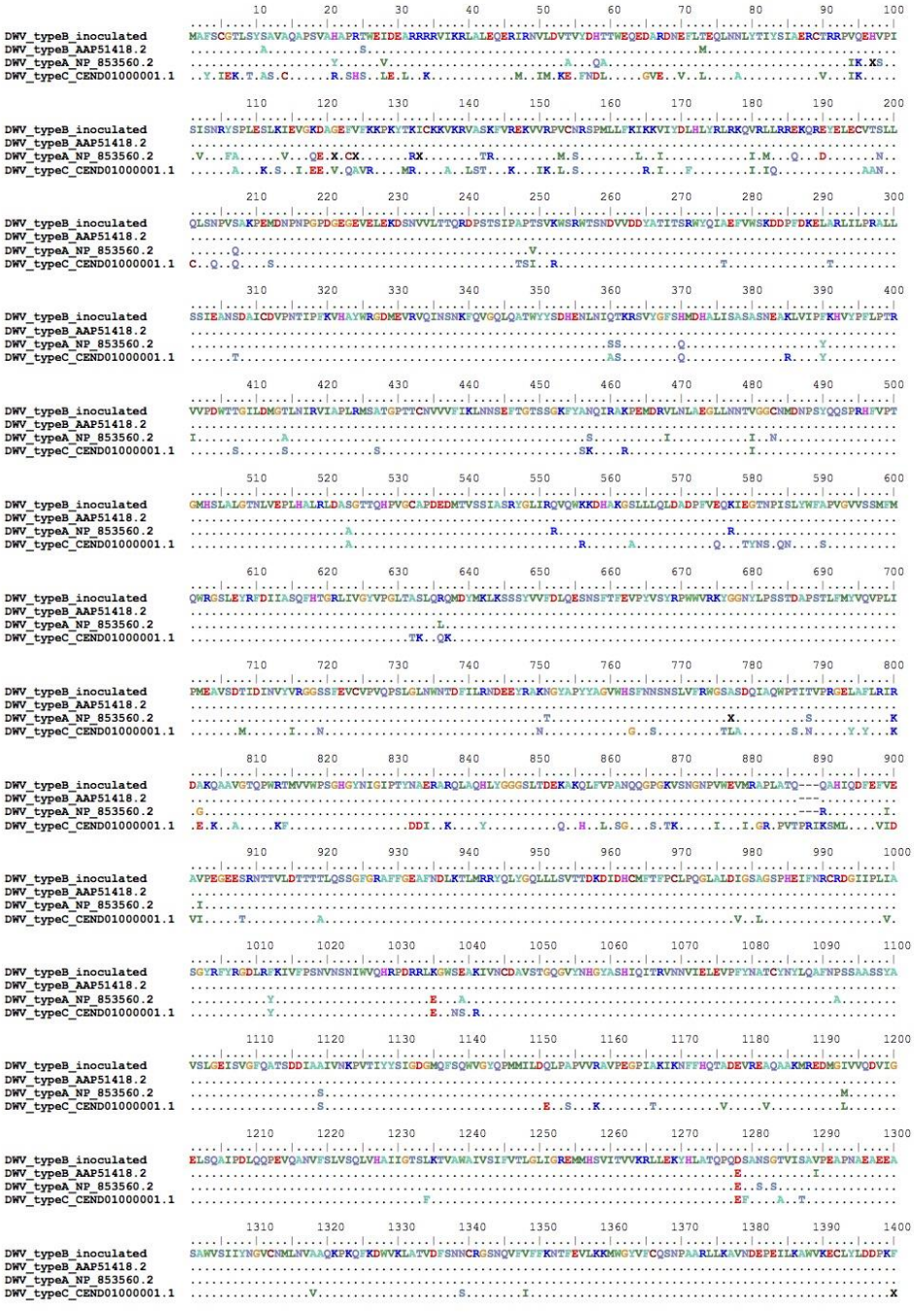
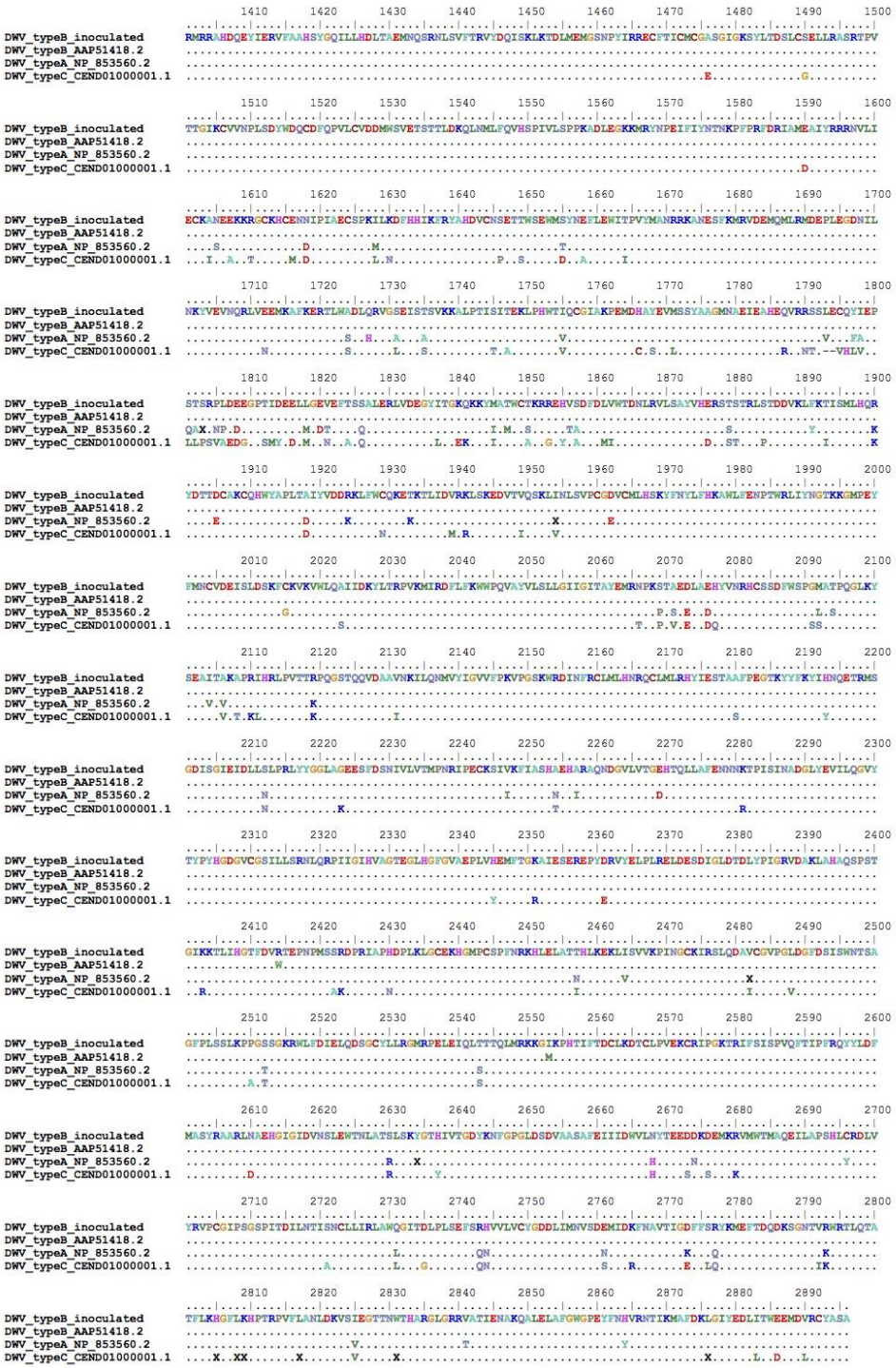


Fig. S1. Amino-acid sequence of the polyprotein encoded by the DWV type B strain that we used to inoculate our bees and comparison to the reference DWV master variants of type B, A and C.



(Fig. S1 continued)

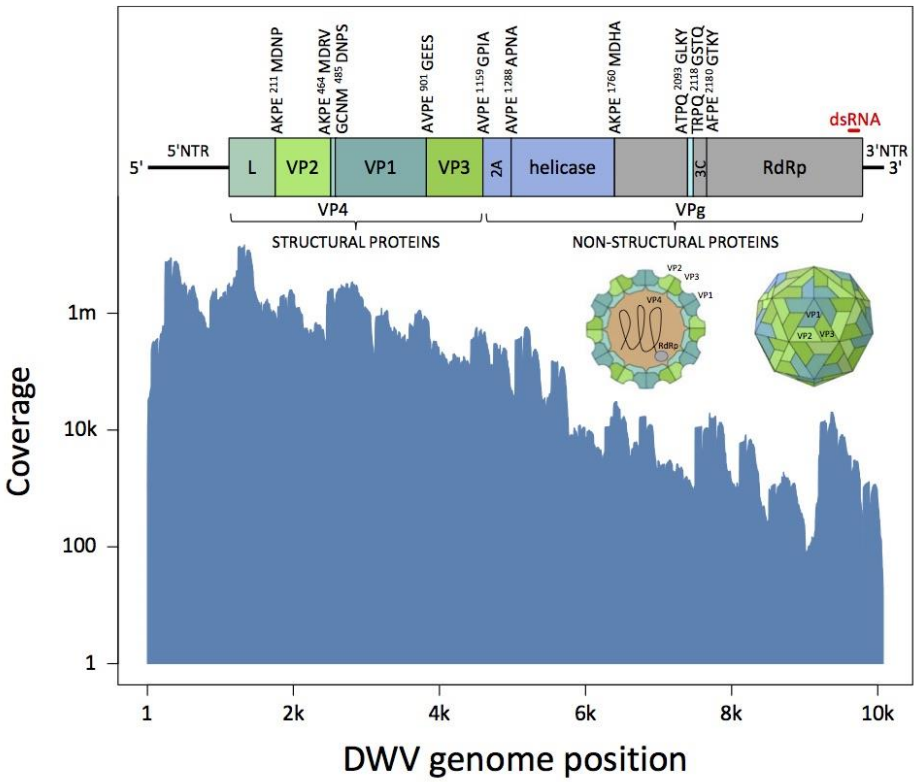


Fig. S2. Fragment coverage over the DWV genome reached in our Bowtie mapping shown together with inferred cleavage sites in the virus's polyprotein and the resulting products, and other genomic features (5'NTR and 3'NTR = non-translated regions, L = L-protein, VP1 to VP4 = capsid proteins, 2A = 2A-like protease site, VPg = VPg protein, 3C = 3C-protease, RdRp = RNA-dependent RNA polymerase, dsRNA = position of dsRNA fragment used in our RNA interference protocol [4], cf. [15]).