THE ROLE OF RESISTANCE TO *VARROA DESTRUCTOR* AND DEFORMED WING VIRUS IN THE EUROPEAN HONEY BEE (*APIS MELLIFERA*)

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I would like to express sincere gratitude to my PhD supervisor, Professor Stephen Martin. firstly, for taking me on as his student, and secondly for all the help, guidance, and patience whilst I undertook this project. This work would not have been possible without his continued support.

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DECLARATION

I have contributed significantly to all the data chapters within this thesis. The data in chapter one has been published in a peer reviewed journal, where I am first author due to my significant contribution to the work. I have conducted the majority of the work detailed in chapters 2-5 and will be listed first author upon publication. I have also included two papers which have recently been submitted to peer reviewed journals, as my contribution was lower in these studies I have included them in the appendices. All funding was provided by the Bee Disease Insurance LTD. The full details of collaborations and my contribution to each chapter is as follows:

Chapter 1: ABC Assay - Method Development and Application to Quantify the Role of Three DWV Master Variants in Overwinter Colony Losses of European Honey Bees

I was heavily involved in the design and conception of this assay, along with Andrea Highfield, Declan C. Schroeder, and Gideon J. Mordecai. The primers were designed by Gideon J. Mordecai and Andrea Highfield. Myself and Gideon Mordecai undertook the initial lab work which involved cloning DWV fragments, quantification of DNA and RNA, RNA synthesis, and testing the primers/standards using RT-qPCR. Andrea Highfield provided the samples from 2007 and HRM data. I then extracted the RNA and performed RT-qPCR on the 2007 samples and compared these to the HRM data. I analysed all the results and created the figures with the help of Declan Schroeder. I am first author on the paper as I was responsible for writing the manuscript, whilst Stephen Martin, Declan Schroeder, and Gideon Mordecai made suggested edits.

Chapter 2: DWV master variant dominance and seasonal prevalence in honey bee colonies from England and Wales

I conceived and designed this study along with Stephen Martin and Martin Smith. I organised the collection of samples, this involved attending a variety of meetings with stakeholders and establishing relationships with participating beekeepers who provided samples for analysis and mortality data. I carried out all the sample processing, RNA extractions, RT-qPCR and analysis of the data. The chapter was written by myself, whilst Stephen Martin made suggested edits.

Chapter 3: DWV infection, mortality and survivorship in pre-wintering *Varroa* susceptible and tolerant honey bee colonies from the USA

This study was conceived and designed by myself along with Stephen Martin and Randy Oliver. Randy Oliver organised the collection of samples. Stephen Martin collected and returned the samples to the UK. The sample processing, RNA extractions, RT-qPCR was conducted in equal parts by myself, Flaviane De Souza, and Christopher Sharples. The remaining data collection, and analysis was conducted by myself. I was responsible for writing the chapter whilst Stephen Martin suggested edits.

Chapter 4: The role of DWV avirulence and superinfection exclusion in wild colonies of honey bees located in the Arnot forest and Ithaca, New York

I was heavily involved in the conception and design of this study along with Stephen Martin, and David Peck. David Peck sourced the Arnot forest colonies. David Peck was also responsible for the establishment of the experimental bee yards and sample collection. The

samples were then shipped to the UK where I was responsible for sample processing, RNA extractions, RT-qPCR, sequencing, and data analysis. The chapter was written by myself whilst Stephen Martin suggested edits.

Chapter 5: DWV recombinant prevalence in DWV positive colonies from England, Wales and the USA

This study was conceived and designed by myself, Declan Schroeder and Andrea Highfield. I was responsible for providing honey bee samples which had previously been collected for chapters two and three. I was responsible for sample processing, RNA extractions, PCR, RT-qPCR, gel electrophoresis, and data analysis. Flaviane De Souza conducted a small proportion of lab work which also contributed to the study. The chapter was written by myself whilst Stephen Martin suggested edits.

Appendices

The appendices contain two additional chapters to which I contributed my time and am listed as second author. These have been provided in the appendices as my contribution is lower in these chapters.

Appendix 1: Effect of *Varroa* on the variation of transmission of DWV strains in honey bee pupae

I was heavily involved in the design and conception of this study along with Stephen Martin. Sample collection was conducted by myself, along with Lucy Chambers, George Hawkins,

and Stephen Martin. Sample processing, RNA extractions, and RT-qPCR were conducted by myself, Lucy Chambers and George Hawkins. I was responsible for showing Lucy Chambers and George Hawkins how to conduct the lab work and subsequent analysis. George Hawkins analysed the date and was responsible for writing the manuscript which is currently under review by the Journal of Invertebrate Pathology.

Appendix 2: Occurrence of Deformed wing virus variants in the stingless *Melipona subnitida* and honey *Apis mellifera* bee populations in North Eastern Brazil

I was heavily involved in the design and conception of this study along Flaviane De Souza, Carlos De Carvalho, and Stephen Martin. Sample collection was conducted by Flaviane De Souza. Sample processing, RNA extractions, and RT-qPCR were conducted by myself and Flavian De Souza who undertook the majority of this work. I was responsible for showing Flaviane De Souza how to conduct the lab work and subsequent analysis. Flaviane De Souza analysed the date and was responsible for writing the manuscript, whilst myself and Stephen Martin made suggested edits. This manuscript is under review by the Journal of General Virology.

ABBREVIATIONS

ABPV	Acute bee paralysis disease
BBKA	British beekeeper's association
DWV	Deformed wing virus
EBV	Egyptian bee virus
ELISA	Enzyme – linked immunosorbent assay
HRM	High resolution melt analysis
JEBV	Japanese Egyptian bee virus
Lp	Leader protein
NGS	Next generation sequencing
ORF	Open reading frame
PCR	Polymerase chain reaction
RdRp	RNA-dependant RNA polymerase
RT-qPCR	Reverse-transcriptase PCR
SIE	Superinfection exclusion

GENERAL ABSTRACT

The European honey bee (*Apis mellifera*) is of global importance as a pollinator. Over the past 30 years an increase in colonies lost during winter has occurred, particularly in the northern hemisphere. These losses are attributed to the ectoparasitic mite *Varroa destructor* that acts as a vector of RNA viruses, most notably Deformed wing virus (DWV). Three master variants of the DWV have been discovered; Type-A, B, and C. The increase in overwinter colony losses are closely linked to DWV. At the same time DWV may also offer protection to colonies via superinfection exclusion (SIE), which may be linked to *Varroa*-tolerant colonies. However, the role of each DWV variant in colony survivorship and mite-tolerance is limited, so the main thrust of the thesis is to address this issue.

Firstly, the detection of all three DWV master variants was only possible via Next Generation Sequencing (NGS), therefore I developed a reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) assay that enabled the detection of all three DWV master variants. This allowed me to re-analyse historic samples, and revealed that type-A and C were involved in the untimely demise of treated colonies.

Secondly, I used the new assay to survey the three DWV master variants in English and Welsh honey bee colonies. DWV type-B was found to dominate colonies regardless of season, whilst overwinter losses remained low. English and Welsh *Varroa* tolerant colonies were also shown to have DWV viral loads similar to colonies which receive *Varroa* treatment throughout the year.

Thirdly, I conducted a similar study as Chapter two except in the USA. Here DWV type-A was dominant and linked to overwinter colony losses. *Varroa* tolerant bees also had DWV loads similar to *Varroa* treated colonies, a result which suggests mite-tolerant traits evolve over time.

Fourthly, DWV populations were assessed in *Varroa*-tolerant colonies from the Arnot forest, USA. This study disproved that avirulent DWV infections were present and supports the importance of other life history traits being involved in the survival of these colonies.

Finally, the role of DWV viral recombinants were investigated on a landscape scale using samples from England and Wales, and the USA. This provided further evidence that viral recombinants are not as fit as parent genomes.

Together these results deepen our understanding of DWV variants and their impact on colony survivorship.

GENERAL INTRODUCTION

The European honey bee

Honey bees belong to the order Hymenoptera, along with sawflies, ants, bees, and wasps. Currently 12 species of honey bee have been described (Lo et al., 2012). However, it is the European honey bee (*Apis mellifera*) which is most commonly domesticated, and as such has a global distribution. Honey bee colonies are comprised of one reproductive female called 'the queen', up-to as many as 80,000 sterile female worker bees, and during high summer contain hundreds of male 'drone' bees. Honey bees are famed for existing as highly organised social groups, headed by the queen whilst sterile female worker bees perform a range of age-related colony task from cooperative brood care, to foraging for food, and guarding the nest entrance. Therefore, honey bee colonies exhibit true eusociality.

Honey bees collect the nectar and pollen of plants; nectar is converted into honey which feeds the colony and pollen into 'bee bread' which is highly nutritious and fed to developing larva. In this way they provide the pollination services required to ensure the seed set of a variety of flora. Typically, wild honey bees nest in the cavities of trees (Seeley & Morse, 1978) whilst managed colonies live in bee hives.

Honey bees have been managed by humans for over 7000 years (Bloch et al., 2010) and are one of the most studied social insect species. Their popularity is part owing to their prolific honey production and bi-products such as wax, but also due to the pollination services they provide. Insect pollination dependant crops make up 35% of foods (Klein et al., 2007) that

our ever growing, human population relies upon (Williams, 1994; Aizen et al., 2008). Crop yield is increased by up to 96% (Potts et al., 2010) when bee pollinators are present in an agricultural setting. Pollination services in the UK for 2010 were estimated to be between £430 – £603 million annually (POST, 2010), with 34% of UK pollination services provided via the European honey bee (Breeze et al., 2011). In the USA major industries rely on European honey bees and they are estimated to provide $14 billion to the USA economy (Morse & Calderone, 2000) making it a species of great importance in terms of food security.

European honey bee (*Apis mellifera*) colony losses

A global decline in European honey bee colonies has been witnessed in the last 30 years, leading to a huge amount of environmental and economic concern (Aizen & Harder, 2009; Gallai et al., 2009). The majority of colony deaths occur in the temperate zone (e.g. Europe, USA & New Zealand) during the winter period when honey bee colonies form the winter cluster. Colony loss data has been collected for many years now in the UK and USA (Figures 1 & 2). However, these losses are still considered to be at an unacceptable level. In the winter of 2016/17 both the UK and USA reported overwinter colony losses (OCL) of 13.2% (British beekeeper's association (BBKA, 2017)) (Figure 1) and 21.1 % (Bee informed partnership, 2017) (Figure 2), respectively. The USA winter losses continued to increase over the winter of 2017/18 to 31%, whereas the UK data has yet to be released.

The cause of these elevated overwinter losses have been linked to the ecto-parasitic mite *Varroa destructor* and its association with several harmful viral pathogens, of which

Deformed Wing Virus (DWV) is evidenced to play an important role (Berthoud et al., 2010; Dainat et al., 2012a & b; Highfield et al., 2009; Martin, Ball, & Carreck, 2010, 2013; Martin et al., 2012).

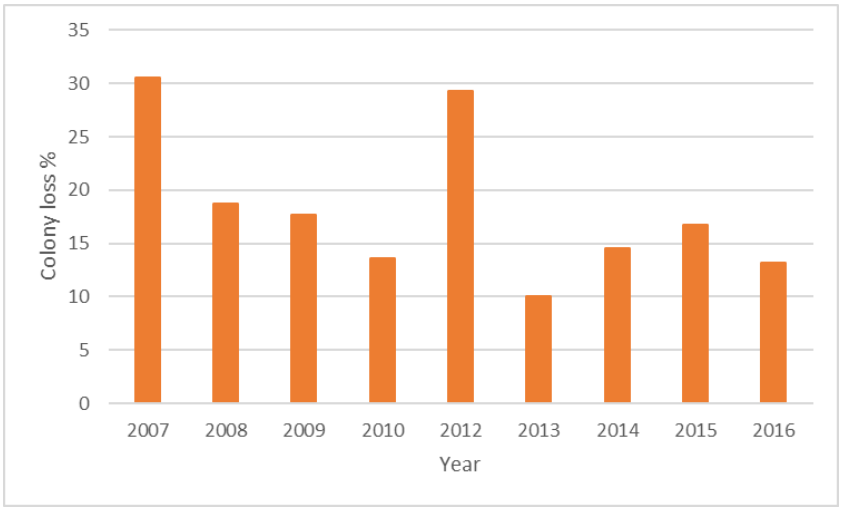

Figure 1. Percentage of overwinter colony losses in the UK, data obtained from the BBKA, (2017).

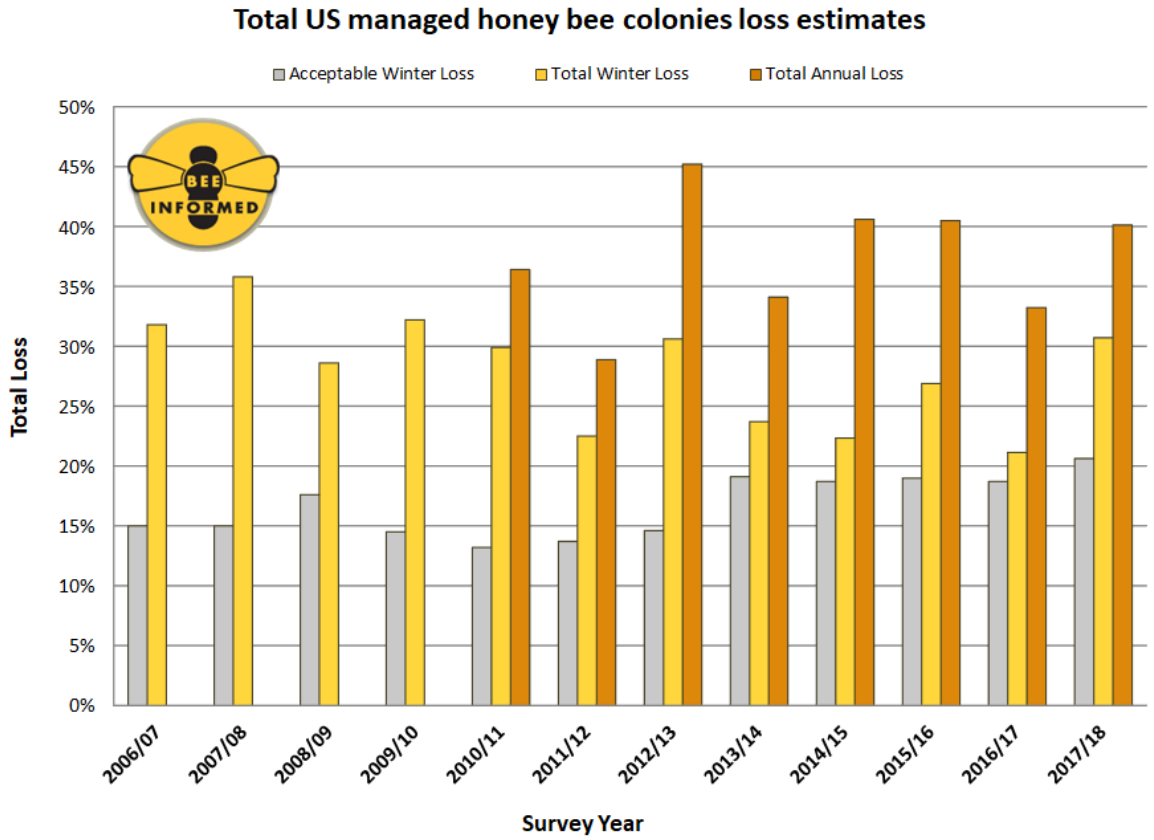

Figure 2. Percentage of total annual losses (orange), total winter losses (yellow) and the suggested acceptable winter losses (grey) of USA honey bee colonies. Figure obtained from beeinformed.org.

Association between Honey bees and *Varroa destructor* mites

There are four defined species of *Varroa*; *V. jacobsoni*, *V. destructor*, *V. rindereri* and *V. underwoodi* (Anderson & Trueman, 2000; Dietemann et al., 2013). *Varroa* was originally described in Java (Oudemans, 1904, cited in Sammataro, Gerson, & Needham, 2000) and confined to South Asia. Here, the *V. jacobson*i mite was originally a parasite of *Apis cerana* (Asian honey bee) but in the late 1950's jumped the species barrier to *A. mellifera* when European honey bees were kept alongside colonies of Asian honey bees (Danka et al., 1995).

Later the *V. jacobsoni* mites infesting colonies of European honey bees were shown to belong to a new species of mite, *V. destructor* (Anderson & Trueman, 2000).

V. destructor (now referred to as *Varroa* throughout the text) is an ectoparasite which feeds solely upon the haemolymph of both adult honey bees (mite phoretic stage) and 5th instar larva and pupa, referred to as sealed brood (mite reproductive stage) since these stages are sealed into the cell by a silk cocoon and wax capping. *Varroa* completes each reproductive cycle inside a sealed brood cell (a hexagonal chamber where eggs and larvae are found). The feeding activities of *Varroa* have negative physiological and behavioural effects at the colony and individual honey bee level. Bees parasitised as pupae are severely affected; They exhibit reduced body weight, haemolymph, and abnormal carbohydrate levels at emergence (Amdam et al., 2004), as well as a reduced lifespan (Dainat et al., 2012a; Benaets et al., 2018). All of which reduce fitness at the individual level by suppression of the honey bee immune system (Berthoud et al., 2010). Other physiological differences occur in adult bees such as degenerated bodies and decreased flight performance in drones (Duay, de Jong, & Engels, 2002). Behavioural differences include; bees foraging earlier (Janmaat & Winston, 2000; Benaets et al., 2018), a reduction in swarming events (Villa et al., 2008) and flight performance (Wells et al., 2016), as well as prolonged absences with a lower return rate to the colony (Kralj & fuchs, 2006) ultimately disrupting normal colony function. Typically, colonies survive for only three to four years when unmanaged for *Varroa* (Korpela et al., 1992).

The *Varroa* mite now has a global distribution with the exception of Australia (Sammataro et al., 2000) and is a novel parasite of *A. mellifera*. As such mite populations can grow exponentially since this new host lacks any traits to control mite population growth (Martin, 1998). A host-parasite equilibrium has evolved as a result of natural selection between *A. cerana* and *Varroa* (Peng et al., 1987). Both behavioural and physiological traits prevent exponential growth of *Varroa* mites within *A. cerana* colonies (Rath, 1999) as mite numbers are maintained at <800 per colony with no adverse effects upon the bees (Martin & Medina, 2004). The worker bees of *A. cerana* have the ability to detect *Varroa* infested cells, even after the brood is sealed. When an infested cell is found workers of *A. cerana* open and remove the mites and brood. This behaviour is termed hygienic or removal behaviour (Peng et al., 1987; Rath & Drescher, 1990) and more recently has been referred to as *Varroa* sensitive behaviour (Carreck, 2011). *Varroa* population growth is further limited in *A. cerana* colonies as *Varroa* females can only reproduce within the drone brood which represents 1-5% of all brood within a colony (Arechavaleta-Velasco & Guzman-Novoa, 2001).

Varroa mites are capable of dispersing from colony to colony and apiary to apiary via the natural drifting of bees and also when bees are foraging (Peck et al., 2016). A colony in the stages of collapse due to *Varroosi*s (negative effects caused by a *Varroa* infestation) is likely to be invaded by drifting honey bees who willingly take advantage of the weaker colony, subsequently a large number of phoretic mites, capable of reproducing, enter the healthy colony. In this way several pathogens are likely to be transmitted from colony to colony (Rosenkranz et al., 2010).

In addition to the effects of their feeding activities, *Varroa* is an effective vector of honey bee viruses (Berthoud et al., 2010; Martin et al., 2012; Schroeder & Martin, 2012; Francis et al., 2013). Therefore, infested colonies often have viral infections, which have now been evidenced as a major factor in the decline of colonies (Highfield et al., 2009; Martin et al., 2012). Typically, beekeepers employ a number of management techniques to reduce *Varroa* numbers in a colony. These involve the application of *Varroacides* in which mites have been shown to develop resistance (Milani, 1995;1999; Thompson et al., 2002; Sammataro et al., 2005) and biotechnical methods involving various colony manipulations (Rosenkranz et al., 2010). These treatment methods have recently been called into question as they may inhibit natural selection towards *Varroa* resistant colonies (Neumann & Blacquière, 2017).

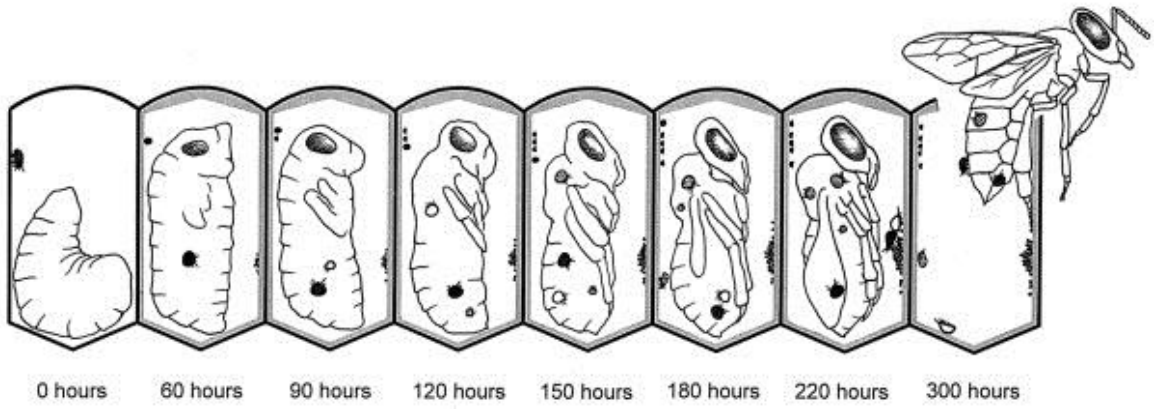

Figure 3. The reproductive life cycle of *V. destructor* inside a honey bee cell (Oldroyd, 1999). The female *Varroa* mite (black) enters the cell of a 5th instar larva prior to capping and lays an unfertilised egg which develops into a male *Varroa* mite (white) and fertilised eggs which develop in to female mites (grey). The female daughter mites then mate with the male who dies and when the bee emerges so do the reproductive females.

Honey bee viruses

Approximately 23 viral pathogens have been identified in honey bees (Chen & Siede, 2007; Runckel et al., 2011; Li et al., 2014). The majority of honey bee viruses are present in a colony at low viral loads and cause asymptomatic disease symptoms. Multiple viral infections can be present in a single honey bee at any one time (Traynor et al., 2016). These are mostly positive-sense RNA viruses belonging to the families *Dicistroviridae* and *Iflaviridae* and are often associated with *Varroa* infestation (Bailey & Ball, 1991; Chen & Siede, 2007; Tentcheva et al., 2004). However new social insect viruses are frequently discovered (Mordecai et al., 2016a & c; Remnant et al., 2017; Brettell et al., 2017) and more recently negative-sense viruses were discovered to infect honey bees (Remnant et al., 2017). The sense of a virus refers to the polarity of nucleic acids (5'-3' positive-sense or 3'-5' negative sense) and determines how they are replicated within a host cell. A prerequisite for viral replication is the requirement of viral mRNA which is translated into the proteins needed to assemble a new virion. Positive sense viruses consist of viral mRNA that is directly translated into viral proteins, whilst negative-sense viruses require RNA dependant RNA polymerase to create positive sense strand mRNA, which in turn are translated into viral proteins (Domingo, 2015).

Of the viruses transmitted by *Varroa* it is the species complex forming the Paralysis viruses (Acute bee paralysis virus [ABPV], Kashmir bee virus [KBV], and Israeli acute paralysis virus [IAPV]) and Deformed wing viruses which are often detected in dying bees (Maori et al., 2007; Highfield et al., 2009; McMahon et al., 2016). Interestingly the viral impact on colony health changes at different times of the year. The Paralysis viruses e.g. ABPV, are more

prevalent during spring (Glenny et al., 2017) and can kill an infected bee in as little as 3-5 days (Maori et al., 2007), whilst Deformed wing virus becomes more prevalent during the late summer when *Varroa* numbers are at their highest (Highfield et al., 2009; Glenny et al., 2017). It is during the winter period when unacceptable numbers of colony losses are witnessed in the temperate zone (Highfield et al., 2009; Genersch et al., 2010; Berthoud et al., 2010), therefore making DWV of major concern and the main focus of this research.

Deformed wing virus

DWV is a single-stranded positive-sense RNA virus of the family *Iflaviridae* (De Miranda & Genersch, 2010). It is regularly detected in social and solitary bee populations across the globe (Genersch et al., 2006) and has recently been discovered in species associated with *A. mellifera* such as ants, wasps (Evison et al., 2012), *Tropilaelaps mercedesae* (Dainat et al., 2009), *V. destructor* (Bowen-Walker et al., 1999; Chen et al., 2005), and Small Hive Beetle *Aethina tumida* (Eyer et al., 2009). DWV can be detected in all life stages (eggs, larvae, pupae) and type (Males, females, queens) of honey bee (Chen et al., 2005; de Miranda & Fries, 2008).

DWV has recently been described as a quasispecies (Mordecai et al., 2016a). A quasispecies exists in a highly mutagenic environment as a number of master variants, each having their own swarm of mutant progeny. This constant process of mutation is due to the nature of RNA viruses which have high error prone replication rates (Palacios et al., 2008; Lauring & Andino, 2010) as a result of the reverse transcription process. A constant process of

mutation drives evolution as clouds of variants can gain a competitive advantage over closely related genomes, thereby allowing them to occupy several biological niches (Domingo et al., 2012). Furthermore, closely related viruses can co-infect a host cell allowing viral recombinants to form and emerge in the viral landscape.

As a quasispecies, DWV exists as three closely related master variants: Variant type-A (Lanzi et al., 2006), B (Ongus et al., 2004), and C (Mordecai et al., 2016a). Variant A is the most commonly detected and has been linked to colony declines (Martin et al., 2012; Schroeder & Martin, 2012; Francis et al., 2013). DWV type-B (previously designated as *Varroa destructor virus-1* (VDV-1)) has been implicated in the long-term survival of a single mite infested honey bee population in the UK (Mordecai et al., 2016b), although other studies suggest that type-B is lethal to honey bees (McMahon et al., 2016; Natsopoulou et al., 2017). The effects of DWV type-C are unknown as it is the most recently described variant (Mordecai et al., 2016a), however type-C was detected in colonies which subsequently died despite having low mite populations (Kevill et al., 2017; Chapter one). Furthermore, the ability of three master variants to infect the same host cell provides the opportunity for viral recombinants of DWV to form. Viral recombinants have been detected for all DWV variants (Mordecai et al., 2016a; Moore et al., 2011, Dalmon et al., 2017) and it is unclear how prevalent these are and their impact upon colony heath.

Deformed wing virus transmission

DWV can be transmitted both vertically and horizontally, with the mode of transmission playing an important factor in virulence and pathogenicity. Both virulence and pathogenicity are used to describe the severity or harmfulness of a disease (Shapiro-Ilian et al., 2005). Virulence describes the ability of a virus to replicate in a host (i.e. increased virulence) and is used to quantify the severity of a virus, allowing for the interaction between the host and pathogen to be assessed (De Miranda et al., 2013). Pathogenicity is a qualitative trait referring to the ability of a virus to cause disease symptoms in the host and is mediated by the virulence of a virus.

Vertical transmission occurs when viruses are transmitted from parent to offspring during reproduction either in sperm or upon the egg surface (Chen et al., 2005; 2006; Yue et al., 2006). Vertical transmission is often associated with less virulent viruses, as the next generation of host is required for the long-term survival and transmission of the virus (Day, 2003; Ebert & Bull, 2003; Fries & Camazine, 2001). Therefore, vertical transmission is associated with reduced virulence and pathogenicity of the virus (the ability of a virus to cause disease symptoms and death of the host) (Shapiro-Ilian et al., 2005).

Horizontal transmission occurs via the feeding activities of honey bees (trophallaxis) with DWV detected in larval food and also pollen (Yue et al., 2007; Chen et al., 2006). The feeding activities of honey bees often lead to a low-level, asymptomatic infection (De Miranda & Genersch., 2010). Horizontal transmission also occurs via the feeding activities of *Varroa*

mites (Ball, 1989; Bowen-Walker et al., 1999) and is evidenced to cause lethal infections in honey bee colonies. This is achieved via the inoculation of DWV directly into the bee haemolymph. The feeding activities of mites in honey bee colonies have transformed a relatively benign virus into one of the most widespread in the world (Highfield et al., 2009; Martin et al., 2012; Schroeder & Martin, 2012).

Deformed wing virus symptoms

Deformed wing virus was given its name due to its regular detection in honey bees which exhibit the deformed wing symptom (Figure 4) however, it is typically present in colonies which have a lack of visible symptoms (Brettell et al., 2017).

Viral infections are classified as overt or covert (De Miranda & Genersch, 2010). Overt infections have obvious disease symptoms, a high level of virus particle production, and are likely to be spread horizontally via feeding *Varroa* mites (Carreck, Ball and Martin, 2010). Symptoms include deformed wings in workers which hinder the worker's ability to fly, as well as stunted growth (Bailey & Ball., 1991). Overt infections are further categorised into acute and chronic infections. Acute infections are short lived and highly productive causing clear symptoms and death of the host. Chronic infections occur over the lifetime of the host in the presence of disease symptoms, such as reduced longevity. The only difference between acute and chronic infection is the duration in which the host survives.

Covert infections are characterised by a lack of symptoms in the presence of low-level viral particle production, which can be detected in more than one life stage of the organism. Covert infections are associated with the vertical transmission of the virus and can become overt in later life stages (Burden et al., 2003). Covert infections of DWV were present in colonies before the spread of *Varroa* as evidenced by Martin et al., (2012).

DWV infection in honey bees, appears to follow a cycle in temperate regions. Covert infections are present in spring and summer during peak brood production but then become overt in autumn when brood production decreases/ceases (Highfield et al., 2009). Elevated winter viral loads are caused by *Varroa* mites becoming phoretic due to the cessation of brood production in the winter months, this is when viral loads become unsustainable causing colony death (Tentcheva et al*.,* 2004, Highfield et al., 2009).

Figure 4. Honey bee with the classic deformed wing symptom. The photograph was taken by the University of Florida.

Varroa as a Vector of Deformed wing virus

Covert DWV infections were detected in *A. mellifera* populations before the global spread of *Varroa*. In the presence of *Varroa,* DWV viral loads are typically high (>10^8 particles per bee) causing overt infections (Highfield et al., 2009; Martin et al., 2013; Martin et al., 2012). A clear association between *Varroa*, DWV and overwinter colony losses have been demonstrated (Highfield et al., 2009; Carreck et al., 2010; Martin et al., 2013; Francis et al., 2013). Pathogenic infections with increased virulence are often associated with the horizontal spread of the virus (De Miranda & Genersch, 2010) and it is suspected that the replication of DWV in *Varroa* can increase virulence (Yue & Genersch, 2005; Gisder, Aumeier & Genersch, 2009) however, this is disputed as DWV particles have only been found in the *Varroa* gut (Santillan-Galicia et al., 2008) and not the tissues.

The combination of DWV and high levels of *Varroa* infestation normally leads to colony death (Korpela et al., 1992). *V. destructor* induced immunosuppression of *A. mellifera* is considered to increase DWV virulence, this is often the case in bee pupae parasitized by *Varroa* which develop symptoms such as deformed wings (Bowen-Walker *et al*., 1999). An increase in virulence of DWV was reported after the wide spread of *Varroa* in the UK (Martin et al., 2013; Carreck, Ball, & Martin., 2010), Hawaii (Martin et al., 2012), and New Zealand (Mondet et al*.,* 2014). This increase in virulence also corresponded with a dramatic decrease in DWV viral diversity (Martin et al., 2012) as *Varroa*-mediated DWV transmission selected for one dominating master variant after a three-year period.

Historical DWV detection in honey bee colonies

DWV was first detected in 1978 and was isolated from diseased adult worker bees of *A. mellifera* collected in Egypt (Bailey et al., 1979). This discovery in Egyptian bees led to DWV first being named Egypt bee virus (EBV). EBV was then discovered in dying colonies in Japan in 1980 (Ball, 1983) this isolate was named Japanese Egypt bee virus (JEBV). However, this virus was later found to be prevalent in honey bee pupa and adults which exhibited the deformed wing symptom and was therefore renamed DWV (Ball, 1997).

Since the discovery of DWV a number of techniques have been developed to identify infections. Early DWV research employed the use of serological techniques such as ELISA (Kulinčević et al., 1990; Bowen-Walker et al., 1999; Shen et al., 2005) which were used to detect the presence of DWV-specific antigens.

Recent technological advancements have led to the use of polymerase chain reaction (PCR) methods, which are now regularly employed in many research areas. PCR relies upon the use of specific primers that act as a starting point for DNA synthesis. This technique is used to amplify DNA or RNA copies of a target gene sequence. There are several PCR methods available. End-point PCR is limited to a negative/positive result whilst quantitative results are required in the field of virology to establish the virulence of a virus, since there is a basic assumption that higher the viral load the greater the virulence. Therefore, real-time quantitative PCR (RT-qPCR) has been useful in identifying DWV as a significant honey bee virus and is widely used (Tentcheva et al., 2004; Genersch, 2005; Berenyi et al., 2007;

Gauthier et al., 2007; Kukielka et al., 2008; Baker & Schroeder, 2008; Highfield et al., 2009; Francis et al., 2013; McMahon et al., 2016).

Next-generation sequencing (NGS) produces a vast amount of data pertaining to viral variants, recombinants, and load. This has led to the identification of new DWV master variants and a range of viral recombinants (Moore et al., 2011; Dalmon et al., 2017; Brettell & Martin, 2017; Mordecai et al., 2016a). The use of NGS has allowed researchers to deepen their understanding of how the DWV complex interacts with and impacts the host.

To date, the majority of research efforts have concentrated upon the presence of DWV or viral load only (Tentcheva et al., 2004; Shen et al., 2005; Gauthier et al., 2007; Kukielka et al., 2008; Highfield et al., 2009; Martin, Ball & Carreck, 2010; Francis et al., 2013). However, due to the discovery of DWV variants and technological advancements, studies are now starting to concentrate upon the presence and role of DWV variants type-A and B (Ryabov et al., 2014; 2017; Mordecai et al, 2016b, McMahon et al., 2016; Natsopoulou et al., 2017), whereas the role and prevalence of type-C is limited (Mordecai et al., 2016a; Kevill et al., 2017, Chapter 1).

Varroa tolerant populations of honey bees

Several *A. mellifera* populations (feral and managed) have developed a tolerance to *Varroa*. These tolerant races and populations of honey bees are of particular interest to researchers and bee breeding programs alike. Mite tolerant populations of *A. mellifera* are found across

the globe: England (Mordecai et al*.,* 2016b), Sweden (Fries, Imdorf & Rosenkranz, 2006; Locke & Fries, 2011), Mexico (Mondragon, Spivak & Vandame, 2005), France (Le Conte et al., 2007) and North America (Seeley, 2007). No one overall cause of tolerance has been identified therefore, much uncertainty exists in the literature about the mechanisms behind tolerant populations.

One common feature of *Varroa* resistance is linked to the ability of honey bees to respond to phoretic mites via grooming. This is an important resistance factor in Africanised honey bee colonies (Aumeier & Rosenkranz, 2001) but also in tolerant colonies of European honey bees (Rinderer et al., 2001). Grooming behaviour is considered when artificially selecting bees in bee breeding programs (Harbo & Harris, 2005; Rinderer et al., 2010), bees which exhibit this trait auto-groom (groom themselves) or elicit allo-grooming (groomed by nest mite) which removes mites from the adult bee (Peng et al., 1987; Fries et al., 1996). However, this trait alone is not completely effective as grooming behaviour may not cause mortality of the mite. Large amounts of undamaged mites were recorded by Fries et al., (1996) whilst conducting studies upon grooming behaviour, suggesting that grooming is more affective when combined with other *Varroa* reducing traits.

Other behavioural responses which suppress mite numbers are also important when considering tolerance. Recently, the uncapping of *Varroa* infected brood cells aided in the long-term survival of *Varroa* tolerant colonies (Oddie et al., 2018). This is also the case for Carniolan bees which were discovered to be able to detect, uncap, and remove pupae infested by *Varroa* (Boecking & Drescher., 1991). Colony size, frequent swarming events, and limited brood production have also been evidenced in long-term surviving populations

of honey bees (Fries & Bommarco, 2007; Locke & Fries, 2011; Loftus et al., 2016). Small colonies have limited brood space. Therefore, mite reproduction is capped by the number of available brood cells. Frequent swarming events interrupt brood production and in-turn halt *Varroa* reproduction. All of these behaviours limit the number of *Varroa* mites present in a colony and reduce the *Varroa*-DWV vectoring cycle, ultimately contributing to tolerance.

Recently, evidence arose which implicated DWV master variant type-B in the survival of long-term *Varroa* infested colonies (Mordecai et al., 2016b). The type-B variant was hypothesised to out-compete lethal type-A. The type-B infected colonies did not succumb to the virus which is considered to offer a protective factor via superinfection exclusion (SIE). SIE has been well documented in other viruses belonging to the *Flaviviridae,* such as hepatitis-C (Tscherne et al., 2007). Mordecai et al. (2016b), speculate that the co-evolution of the honey bee-*Varroa* -DWV cycle has selected for less lethal DWV variant to become dominant in these seemingly resistant colonies. Further research is required to establish the extent to which SIE occurs in other naturally occurring resistant populations of honey bee.

Aims

Human reliance upon honey bees for pollination services and the bi-products they provide makes them one of the most valuable social insect species on earth. The current level of overwinter colony losses is unacceptable (Neumann & Carreck, 2010), with an association between *Varroa* and DWV being evidenced to be a major factor in colony death. The recent identification of three DWV master variants and their differing impacts upon colony health and survivorship is an area which is currently under-researched. Therefore, the overall aim of this Ph.D. is to identify the prevalence and impact of the three DWV master variants in honey bee colonies and to fill the gaps currently present in the literature.

More specifically the aims of this thesis are as follows:

1. To develop a quick, reliable and cost-effective method for the detection of each known DWV master variant (type-A, B and C).

A study detailing the design and implementation of an RT-qPCR assay specific to each DWV master variant. The newly developed 'ABC assay' was used to assess DWV variants present in historic samples which either survived or died unexpectedly during the winter of 2007.

2. To identify the seasonal prevalence, viral load and distribution of the three DWV master variants present in honey bees from *Varroa* tolerant and susceptible colonies, and understand their impact upon colony health in England and Wales.

There are currently no landscape scale comparative studies which address the seasonal prevalence and impact of all three master variants of DWV upon honey bee colony health in the UK. This study aims to address this gap by comparing DWV variant seasonal prevalence and viral loads in colonies of *Varroa* tolerant and susceptible honey bees from England and Wales. The role of DWV master variants in overwinter colony mortality and *Varroa* tolerance is also addressed.

3. To identify DWV master variant prevalence, viral load, and distribution in honey bees from *Varroa* tolerant and susceptible colonies to understand their impact upon colony health in the USA.

During the past decade annual colony losses occuring over the winter period in the USA have varied between 20%-30%, which is one of the highest levels anywhere in the world. The aim of this study was to investigate the prevalence, load, and distribution of the three DWV master variants (A, B, and C), within both *Varroa* susceptible and tolerant colonies from across the USA. This study provides an explanation as to why colony losses are so high in the USA and further explores the role of DWV in *Varroa* tolerant colonies.

4. To assess the role of DWV variants in one of the oldest *Varroa* tolerant populations of honey bees in the world.

The Arnot forest is home to a well-researched population of *Varroa* resistant honey bees. Here, colonies have survived with *Varroa* infestations at population levels similar to those before the arrival of *Varroa*. The mechanism behind the long-term survival of these colonies is still under investigation as current research has failed to pinpoint the precise mechanisms aiding their long-term survival. This has led researchers to suggest that the long-term survival of Arnot colonies is due to a number of life history traits and also the avirulence of mites and the diseases they vector. However, no viral work has ever been conducted.

5. DWV viral recombinant prevalence in DWV positive colonies from England, Wales and USA.

DWV master variants are capable of invading the same host cell, as a result they readily recombine forming a mosaic of novel genomes, however no landscape scale comparative studies have been undertaken.

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Chapter 1: ABC Assay - Method development and application to quantify the role of three DWV master variants in overwinter colony losses of European honey bees

Abstract

Deformed wing virus (DWV) is one of the most prevalent honey bee viral pathogens in the world. Typical of many RNA viruses, DWV is a quasispecies, which is comprised of a large number of different variants, currently consisting of three master variants: Type-A, B, and C. Little is known about the impact of each variant or combinations of variants upon the biology of individual hosts. Therefore, we have developed a new set of master variant-specific DWV primers and a set of standards that allow for the quantification of each of the master variants. Competitive reverse transcriptase polymerase chain reaction (RT-PCR) experimental design confirms that each new DWV primer set is specific to the retrospective master variant. The sensitivity of the ABC assay is dependent on whether DNA or RNA is used as the template and whether other master variants are present in the sample. Comparison of the overall proportions of each master variant within a sample of known diversity, as confirmed by next-generation sequence (NGS) data, validates the efficiency of the ABC assay. The ABC assay was used on archived material from a Devon overwintering colony loss (OCL) 2006–2007 study; further implicating DWV type-A and, for the first time, possibly C in the untimely collapse of honey bee colonies. Moreover, in this study DWV type-B was not associated with OCL. The use of the ABC assay will allow researchers to quickly and cost effectively pre-screen for the presence of DWV master variants in honey bees.

Introduction

RNA viruses have high mutation rates and exist as a diverse population of variants or quasispecies (Domingo & Holland, 1997). Quasispecies are a range of variants, genetically linked through mutation and organised around a master sequence or variant (Biebricher & Eigen, 2006; Lauring & Andino, 2010); they undergo a constant process of mutation, competition, and selection (Domingo et al., 2012). This provides a cloud of variants with an evolutionary advantage, allowing them to occupy several biological niches; however, even a single amino acid change can completely alter the pathogenicity of a virus (Domingo et al., 2012).

Of the many RNA viruses that infect the European honey bee, the Acute bee paralysis virus (ABPV) and Deformed wing virus (DWV) quasispecies complexes are readily detected in asymptomatic bees and often associated with colony losses (Baker & Schroeder, 2008; De Miranda & Genersch, 2010). It is proposed that ABPV, Israeli acute paralysis virus (IAPV), and Kashmir bee virus (KBV) all belong to the same cloud of ABPV variants (Baker & Schroeder, 2008; De Miranda & Genersch, 2010). ABPV follows a classic acute-type infection strategy that rapidly translates into overt symptoms of paralysis and ultimately death for the honey bee. Virulence is highly dependent on the mode of transmission and type of the genetic variant; for example, the differences in pathology among different strains of IAPV found globally is likely due to high levels of standing genetic variation (Chen et al., 2014).

Deformed wing virus (DWV), on the other hand, is a one of an increasing number of emerging RNA viral pathogens that are capable of replicating in a wide range of invertebrate species, such as; bumblebees, wasps, hornets, ants, hoverflies, and solitary bees (Evison et al., 2012; Manley et al., 2015, Brettell, 2017). More importantly, DWV is also vectored by ectoparasitic mites of honey bees (Bowen-walker et al., 1999; Dainat et al., 2009; Eyer et al., 2009). Previous analysis indicates that the DWV population consist of many different variants (Baker & Schroeder, 2008; Martin et a., 2012; Mordecai et al., 2016a). When DWV infection is associated with the mite *Varroa destructor* there is a dramatic increase in viral load (Mordecai et al., 2016a; Lanzi et al., 2006) and a loss in DWV diversity (Martin et al., 2012; Ryabov et al., 2014), which is associated with the death of the honey bee colony (Highfield et al., 2009). Therefore, DWV exists as a quasispecies and is now the most regularly detected virus in honey bees.

Considering DWV's host range and varying impact on different honey bee populations and hosts, it is important to establish the role that each DWV master variant within the various insect communities play. Currently, three master variants of DWV have been identified: Type-A (Lanzi et al., 2006), B (Ongus et al., 2004), and C (Mordecai et al., 2016a). Type-A is the most prevalent and has been linked to colony declines (Martin et al., 2012; Schroder & Martin, 2012; Francis et al., 2013). DWV type-B (previously designated as *Varroa destructor* virus-1 (VDV-1) has been implicated in the protection of the colony against DWV type-A in a mite infested honey bee population in the UK (Mordecai et al., 2016b), although type-B can be pathogenic at the level of the individual honey bee (McMahon et al., 2016). The effects of DWV type-C are still unknown, as it is the most recently-described variant (Mordecai et

al., 2016a). Furthermore, the ability of the three master variants to infect the same host provides the opportunity for viral recombinants of DWV to form and a range of recombinants have been detected for DWV master variants (Mordecai et al., 2016a; Moore et al., 2011; Dalmon et al., 2017).

The most common detection method for DWV is reverse transcriptase polymerase chain reaction (RT-PCR), the majority of primers used are targeting the *RNA dependent RNA polymerase gene* (*RdRp*) (Francis et al., 2013; McMahon et al., 2016; Tentcheva et al., 2004; Highfield et al., 2009; Gauthier et al., 2007; Kukielka et al., 2008). Primers which target other regions in the DWV genome such as the *Lp, Helicase*, *VP1*, and *VP2*, have also been described (Genersch, 2005; Moore et al., 2011; Dalmon et al., 2017). Using several regions allows for the identification of DWV recombinants. With the exception of McMahon et al., (2016) and Moore et al., (2011) current studies do not discriminate between Type-A or B (Tentcheva et al., 2004; Genersch, 2005; Gauthier et al., 2007; Kukielka et al., 2008; Highfield et al., 2009; Carreck et al., 2010; Dainat et al., 2011; Martin et al., 2012; Schroeder & Martin, 2012) and have concentrated upon its prevalence and load, rather than the variants present or only report on one master variant. High resolution melt analysis (HRM) was previously used post RT-PCR to identify variations in nucleic acid sequences by detecting differences in amplicon dissociation curves (Mordecai et al., 2016a; Moore et al., 2011)). Martin et al., (2012) reported the separation of DWV type-A and B via HRM. Unfortunately, DWV type-C fell within the same dissociation curves as DWV type-B (Mordecai et al., 2016a). To our knowledge, no assay has been designed to report on all three known master variants of DWV.

More recent quantitative-PCR (qPCR) methods have been developed, which allows for DWV viral loads to be calculated by either absolute quantification using a DNA (McMahon et al., 2016) or RNA (Highfield et al., 2009) standards or via relative quantification using a ΔC_t (delta critical threshold) value determined relative to a known housekeeping gene (Gauthier et al., 2007; Kukielka et al 2008; Dainat et al., 2009; 2011; Francis et al., 2013). The absolute quantification method is considered the "gold standard" as it provides virus genome equivalent data which, in turn, can also be normalised to housekeeping genes for between-sample comparisons (e.g., Highfield et al., 2009); and it can, in the long run, save both money and time (Leong et al., 2007). Research efforts are now moving towards incorporating next generation sequencing (NGS), which produces a vast amount of data pertaining to viral variants, recombinants, and load (Mordecai et al 2016a; Moore et al., 2011; McMahon et al., 2016; Dalmon et al., 2017; Mordecai et al., 2016c; Brettell et al., 2017). However, this technique is both costly and time-consuming.

As evidenced, a simple DWV presence/absence assay using RT-PCR has limited value as the realisation that the viral load and variant diversity is vital in studying the evolution and impact of DWV master variants upon pollinator health. Therefore, primers which can detect all of the major DWV variants (A, B, and C) are required as a first step to investigate the impact that each DWV master-variant has upon its hosts and how they are evolving or co-evolving. Here we report on a RT-qPCR or ABC assay, based on a new set of primers, to detect all the known DWV master variants (A, B, and C). This will hopefully allow researchers around the globe to investigate the role of the various variants via a common method, thus

allowing the results generated across the various studies to be comparable. We applied the assay to archived material collected during the Highfield et al. overwintering colony loss (OCL) study, which reported that the relative increase in DWV load during the overwintering period was implicated in colony losses. Here we provide evidence that multi-DWV master variant type-A and C are responsible for the decline in colony health. This is the first time in which type-C has been recorded in dying colonies, with the implication that the low levels of DWV type-B were unable to protect the colony (Mordecai et al 2016b).

Methods

Primer design

Primers were designed using the DWV reference sequences for DWV type-A (NC_004830.2), type-B (AY_251269.2) and type-C (CEND01000001.1). The primers were designed in a similar RdRp region used by Highfield et al., (2009) as this is a highly-conserved region of the genome. All three primers use the same forward primer binding site since it has a high identity within, and between, master variants, and three different reverse primers (Table 1). Primers were designed with similar thermodynamic properties to allow each to be used in the same PCR cycling conditions.

Table 1**.** Primers used in this study.

Target	Primer Name	Sequence (5'–3')	Genome Target (NC004830.2)	Size of Product (bp)	Reference
DWV	DWVnew-F1	TACTAGTGCTGGTTTTCCTTT	8653–8673		This study
DWV Type-A	DWVA-R1	CTCATTAACTGTGTCGTTGAT	8808–8788	155	This study
DWV Type-B	DWVB-R1	CTCATTAACTGAGTTGTTGTC	8808–8788	155	This study
DWV Type-C	DWVC-R1	ATAAGTTGCGTGGTTGAC	8805–8788	152	This study
DWV q	DWVq-R1 DWVq-F1	CTGTGTCGTTGATAATTGAATCTC TAGTGCTGGTTTTCCTTTGTC	8656–8676 8800–8777	145	(Highfield et al., 2009)
M13	M13F M13R	GTAAAACGACGGCCA CAGGAAACAGCTATG	Na	361	(Highfield et al., 2009)
Actin	ActinR1	AAGAATTGACCCACCAATCCATAC	Na	120	(Highfield et al., 2009)
	ActinF1	CCTGGAATCGCAGATAGAATGC			

Viral master variant plasmid standards

To create standards for each of the three primers, plasmid vectors containing the target genes (RdRp region) of DWV master variants type-A, B, and C were created (Biomatik, Table 2) and reconstituted as per the manufacturer's guidelines. Heat shock transformation was performed using One Shot® TOP10 chemically-competent *Escherichia coli* following the manufacturer's instructions (Life technologies). Using an aseptic technique, the transformants were selected on LB agar plates, containing kanamycin and left to incubate at 37 °C overnight. A single colony for each master variant was propagated in LB medium containing kanamycin and a Qiaprep® spin mini prep kit (Qiagen) was used per the manufacturer's instructions to extract plasmid DNA. The resultant plasmids were diluted to 56 ng/µL and used in PCRs targeting the M13 regions flanking the multiple cloning sites

(Table 1); sequences were confirmed and used to produce linear strands of DNA which served as templates in PCR assays. M13 PCR products were visualised on 1% agarose gel.

Table 2. DWV master variant (A, B, C) insert used in the plasmids. Primer sequences are underlined and reverse primer sequences are highlighted in bold.

Target	Insert
DWV A	TATCTTGGAATACTAGTGCTGGTTTTCCTTTGTCTTCATTAAAGCCACCTGGAACATCAGGTAA GCGATGGTTGTTTGATATTGAGCTACAAGATTCGGGATGTTATCTCTTGCGTGGAATGCGTCC CGAACTTGAGATTCAATT**ATCAACGACACAGTTAATGAG**GAAAAAGGGA
DWV B	TATCCTGGAATACTAGTGCTGGTTTTCCTTTATCTTCATTAAAACCGCCAGGCTCTTCTGGTAA GCGATGGTTGTTTGATATTGAATTACAAGATTCAGGATGTTATCTTTTGAGAGGGATGAGAC CTGAACTTGAGATACAGTT**GACAACAACTCAGTTAATGAG**GAAGAAGGGA
DWV C	TTTCGTGGAATACTAGTGCTGGTTTTCCTTTATCCTCACTGAAACCAGCTGGAACATCAGGAA AAAGGTGGTTATTTGATATTGAATTGCAAGATTCGGGATGTTATCTTTTACGAGGTATGCGTC CCGAATTAGAAATACAATT**GTCAACCACGCAACTTAT**GAGGAAAAAGGGA

Primer optimisation

Temperature step gradient PCR (48, 50.5, 52.9, 56.1, 58.5, and 61 °C) was performed to assess which annealing temperature provided the most specific result, using the newly-designed primers listed in Table 1. Here, each PCR reaction contained 2 µL plasmid DNA, 10 µL 5× buffer, 5 µL $MgCl_2$, 5 µL dNTPs 2.5 mM, 2 µL DWV-forward primer (10 pmol), 2 µL DWV reverse primer (10 pmol) (A, B, or C, Table 1), 0.2 µL GoTaq® G2 Flexi DNA polymerase (Promega), and 23.8 µL RNAse free H_2O. The results were visualised on 2% agarose gel.

Viral master variant cRNA standards

To synthesise cRNA, 100 ng of gel purified M13 linear PCR product was recovered using a Zymoclean™ gel DNA recovery kit (Zymo research) and quantified using an Agilent 2200 TapeStation. RNA transcription was conducted in vitro using a mMessage mMachine® T7 Kit (Life technologies), following the manufacturer's instructions. Samples were treated with Turbo™ DNase (Life technologies) to remove DNA. cRNA recovery was conducted using an Ambion MEGAClear™ Kit (Life technologies).

Method validation

Quantification of nucleic acids (plasmid DNA and cRNA) for DWV variants A, B, and C was conducted using an Agilent 2200 TapeStation prior to analysis. To test reaction efficiency and create true competition experiments between primer sets for each of the DWV variant targets, a 10-fold dilution series and sample mixes containing each DWV variant in different concentrations were made from the quantified plasmid DNA and cRNA. Analyses were conducted via real-time PCR and real-time RT-PCR, respectively, on a Rotor-Gene 6000 (Qiagen). In addition, competitive real-time qPCR and real-time RT-qPCR was also conducted on each DWV variant for both plasmid DNA and cRNA, respectively, i.e., the amplified C_t value for each variant were compared against a DNA or RNA standard curve. The mixed samples were analysed against a standard curve. Each primer pair was also tested sequentially against all three DWV variants, to ascertain if non-specific amplification occurred. Three master mixes were prepared for each DWV reverse primer (Table 1). The reactions for DNA contained 1 µL DNA, 10 µL SensiFAST™ SYBR® No-Rox (Bioline), 0.75 µL DWV forward primer, 0.75 µL reverse primer (Table 1) and 7.5 µL RNAse free H_2O. Initial

activation occurred at 95 °C for 3 min, followed by 35 cycles of denaturing at 95 °C for 15 sec, annealing at 58.5 °C for primers A and B, and 61.5 °C for primer C for 15 sec, and extension at 72 °C for 15 sec.

RT-PCR was performed on the RNA using a SensiFAST™ SYBR® No-Rox One Step kit (Bioline). Each reaction contained 10 µL SensiFAST™ SYBR® No-ROX One-Step mix (2×), 0.75 µL DWV forward primer, 0.75 µL reverse primer (A, B or C; Table 1), 0.2 µL reverse transcriptase, 0.4 µL RiboSafe RNase Inhibitor, 7.5 µL RNAse free H_2O. The RT step occurred at 45 °C for 10 min and denaturation at 95 °C for 10 min, followed by 35 cycles of denature 95 °C 15 sec, annealing at 58.5 °C for 15 sec and extension at 72 °C for 15 sec. RT negative PCR was also performed on the cRNA to confirm the absence of DNA in the cRNA.

Robustness of the RT-PCR assay was also determined by spiking each target variant with equimolar concentrations at 9.94 ng/µL or 10-fold lower concentrations of competing master variant cRNAs. This was done to determine whether competing RNA could affect the linear range of the assay. The mean C_t values (carried out in triplicate) plus standard deviation was calculated.

For both cRNA and DNA experiments, a melt curve analysis was performed between 72 °C and 90 °C, at 0.1 °C increments, each with a 5 sec hold period. Purity and amplicon size were confirmed on 2% agarose gel. Both methods ensured that no contamination was present in the negative template controls and that one product was amplified per primer set. Each

sample (plasmid DNA and cRNA) was analysed in duplicate. Copy numbers were determined using the following equations:

$$\text{Copy number plasmid DNA} = (\text{Concentration of DNA (ng/µL)} \times 6.022 \times 10^{23}) / (\text{Fragment length base pairs} \times 109 \times 650)$$

$$\text{Copy number RNA} = (\text{Concentration RNA (ng/µL)} \times 6.022 \times 10^{23}) / (\text{Fragment length base pairs} \times 109 \times 325)$$

Application of the RT-qPCR (ABC Assay)

Honey bee samples, which had previously tested positive for DWV (Highfield et al., 2009; Brettell, 2017) and had NGS data available (Mordecai et al., 2016a; Brettell, 2017), were selected for analysis. The 11.6, 6.7, and 21 million reads retained after BLASTn (Basic Local Alignment Search Tool) hits to a DWV reference database for samples GD1 June, GD1 late Oct and OW1a, respectively, were further partitioned by mapping the reads to the three DWV master variant genome (Mordecai et al., 2016a; Brettell et al., 2017). The honey bee samples (GD) were collected throughout 2006–2007 from Devonshire locations, and in 2015 from Hawaii (OW), these samples were archived at −80 °C. A 30 mg sub-sample (representing ~2% of the total biomass) of previously-pooled and ground honey bees were weighed before total RNA was extracted using an RNeasy® mini kit (Qiagen) following the manufacturer's instructions. Quantification of the total RNA was established using a spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific). RNA was diluted to 50 ng/µL to avoid concentration-dependent effects on RT-qPCR efficiency (Evans et al., 2013). The

ABC assay was performed in triplicate for each of the DWV variants and actin. An actin control was deemed necessary to assess levels of degradation due to the long-term storage of samples. Master mixes were made, for each of the DWV primer sets, using a Sensifast™ SYBR® No-Rox One Step Kit (Bioline), and the temperature profile previously detailed. Genome equivalents were calculated per bee using the following equation:

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

HRM assay

High-resolution melt (HRM) analysis was conducted upon the archived Devonshire bee samples as per the protocol described in Martin et al., 2012.

Sequencing

A selection of the PCR or qPCR products generated in method validation and application of the ABC assay were Sanger sequenced (Bowen-Walker et al., 1999) to confirm the specificity of the assay.

Results

PCR and RT-PCR sensitivities

The sequencing results of the three DWV variant plasmids confirmed that they could be used in the PCR assays and for cRNA synthesis for the eventual use in the RT-PCR assays (Table 2). The optimum annealing temperature (as defined by the tightest PCR band of the expected size produced on an agarose gel without the presence of other fainter non-specific multiple sized bands) for the three primer sets was determined to be 58.5 °C when using plasmid DNA and cRNA as a template. Interestingly, when honey bee total RNA was used as a template the annealing temperature needed to be increased to 61.5 °C, especially when amplifying type-C.

Competitive PCR

Competitive PCR confirmed primer sets were specific to each DWV variant and amplification of non-targets did not occur (Figure 1). This was further confirmed through RT-PCR melt curve analysis (Figure S1A, supplementary information) and sequencing (Figure S1B, supplementary information).

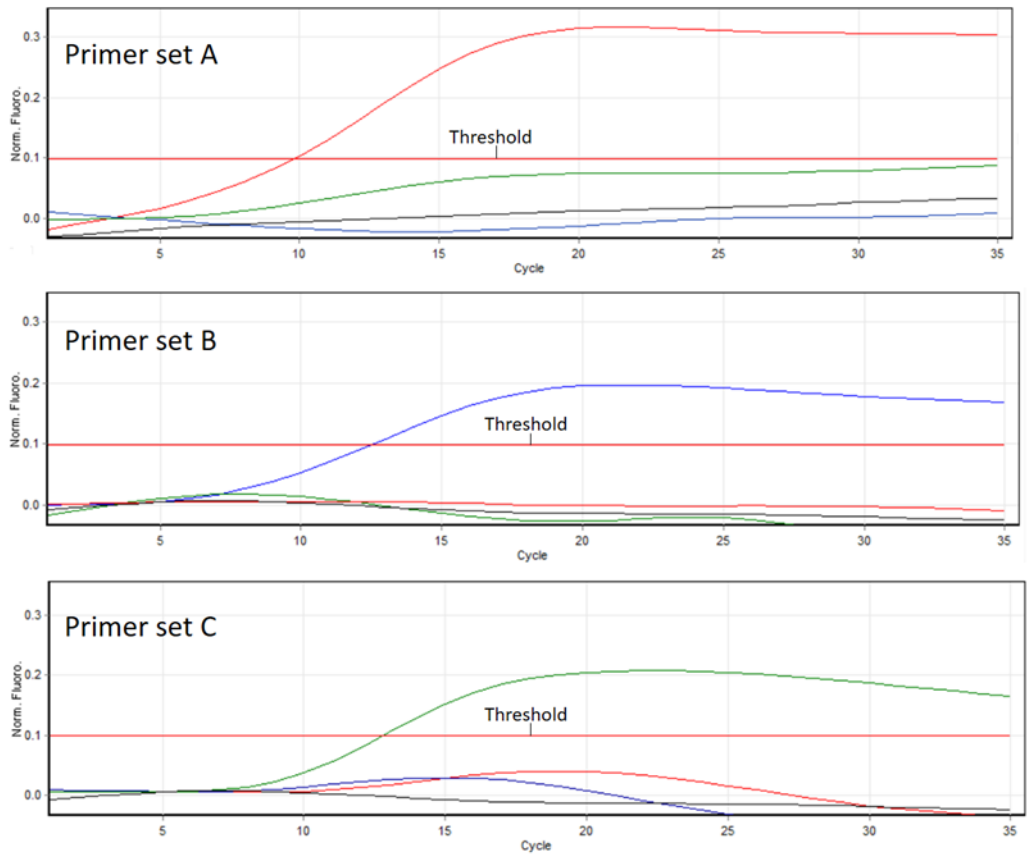

Figure 1**.** Amplification plot of competitive PCR. Red line = type-A, blue line = type-B, green line = type-C, and black line = no template control. All primer sets amplified the target DWV variant, and non-targets are represented by coloured lines under 0.1 fluorescence.

PCR and RT-PCR efficiencies

The performance of each primer set targeting the DWV RdRp region in a dilution series reveals that all the reactions were between 99% and 100% efficient (Figure 2). Interestingly, the RT-PCR (R^2 = 1, −3.3 slope) was marginally more efficient than the PCR (R^2 = 0.99, −3.5 slope), however, the sensitivity of the reactions differs greatly within and between the RNA and DNA based assays. The cRNA standards (10^9 to 10^3 copies) detected in the C_t range spanning five to 25 cycles, while the PCRs starting with plasmid DNA as the template (10^8 to

10^4 copies) fell within the C_t range of between 8 and 25 C_t values (Figure 2). Primer set A was more sensitive and quantification can be carried between 4.5 and 25 C_t cycles whereas, for primer sets B and C, the C_t range is between 0 and 25 C_t cycles. The maximum number of cycles achievable for both RT-PCR and PCR were 35 cycles (=C_t 30) before non-specific and background cross-contamination could be detected. PCRs containing less than 10^3 and 10^4 copies of cRNA and plasmid DNA, respectively, fell outside of the range of quantification.

Both RT-PCR and PCR assays within and across different dilutions were highly reproducible (Figure 3). The mean Ct value for primer set A, B, and C detecting 10^9 copies of plasmid DNA being 8 (± 0.49), 8 (± 0.20) and 7 (± 0.70), respectively. This was different for the cRNA which had a mean C_t for primer sets A, B, and C of 3 (± 0.85), 9 (± 0.97), and 5 (± 0.91), respectively. Variation of <1 C_t from the average C_t value was witnessed for both RNA and DNA, therefore, reactions containing both RNA and DNA are highly reproducible (Figure 3).

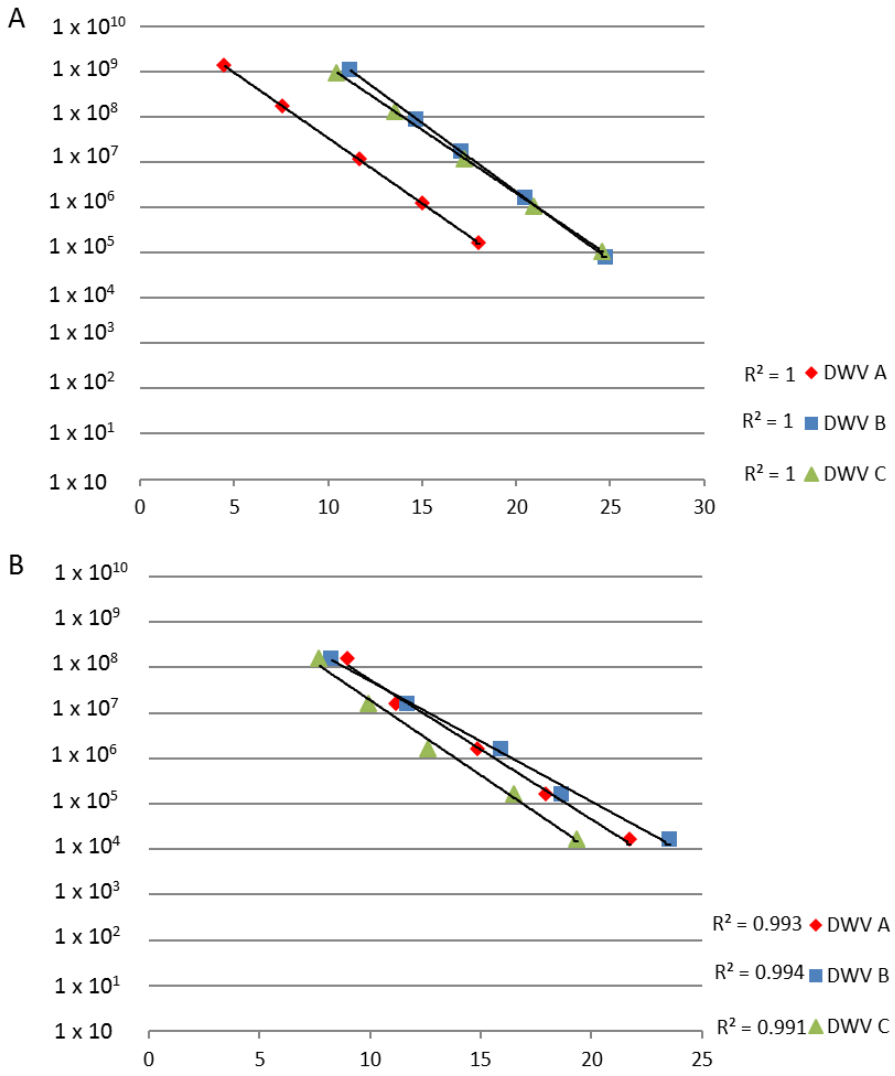

Figure 2**.** Ten-fold dilution series for cRNA (A) and plasmid DNA (B) using the RT-PCR and PCR assays; the standard deviation bars are not shown for clarity.

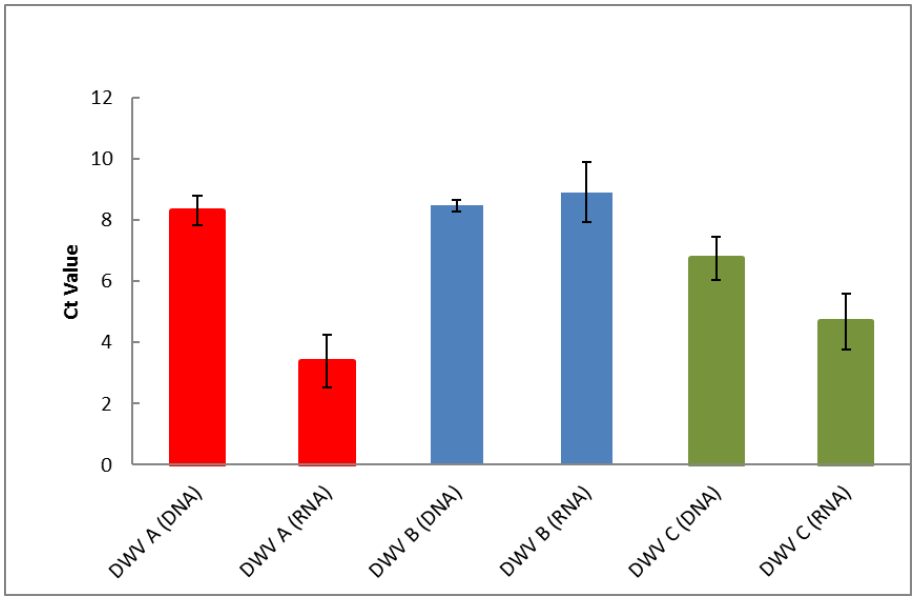

Figure 3**.** Reproducibility of RT-PCR and PCR assays for each DWV primer set. Bars display the mean C_t of the average data calculated from the dilution series. Error bars show the deviation from the mean C_t between serial dilutions. DNA from plasmid, RNA refers to cRNA, DNA copy number = 10^9, RNA copy number = 10^{10}.

Robustness of the RT-qPCR (ABC assay)

C_t values varied from the standard curve (triangles, Figure 4) when samples contained multiple master variants (circles, Figure 4). A 3.3 C_t value deviation is the equivalent of a 10-fold increase/decrease in copy number, the deviation in this assay for primer set A is equal to 1.2 C_t's, B = 0.87, and C = 0.61. Therefore, multiple variants present in the sample can affect the reaction stability but by no more than a three-fold increase/decrease in copy number.

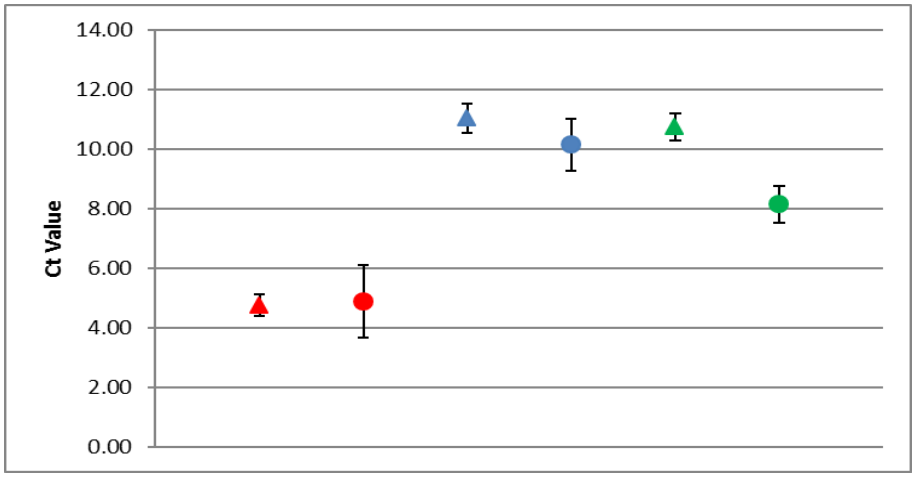

Figure 4. ABC assay. Red = DWV type-A, blue = DWV type-B, and green = DWV type-C. Triangles represent DWV target only, and circles represent DWV samples containing multiple variants. Error bars represent the standard deviation from the mean C_t.

Detection of DWV in honey bees using the ABC assay

The results of RT-qPCR (ABC assay) revealed that all three master DWV variants were detected in honey bee samples (Figure 5). To further confirm the specificity of the new primers, the ABC assay data was compared to NGS data obtained for the same samples (Figure 5). Both assays detected the same DWV variants; a further confirmation of the specificity of ABC assay. The number of reads and viral loads differ between the results of the NGS and ABC assay, respectively, however, the presence of similar variants in each sample, and also that the sample was dominated by the same master variant, was consistent across all samples.

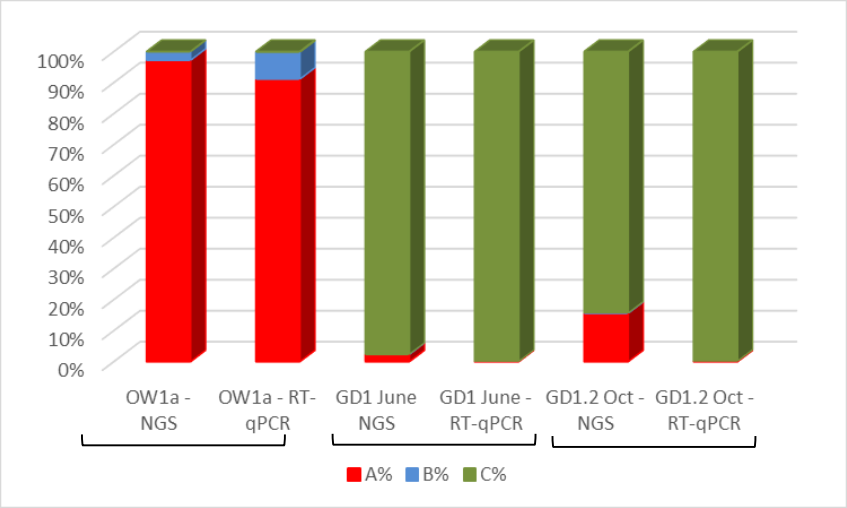

Figure 5. Comparison of ABC assay and NGS data for honey bees. Red = DWV variant A, blue = DWV variant B, and green = DVW variant C.

DWV master variants implicated in OCL

A study carried by Highfield et al., (2009) resulted in the collection of asymptomatic worker honey bees from three separate hives per apiary from a total five apiaries (n = 15 colonies), all known to have a history of *Varroa* mite infestation (Highfield et al., 2009). They were sampled over a year (bimonthly between May and October 2006, monthly between November 2006 and March 2007, and bimonthly in April 2007). All colonies with *Varroa* mites underwent control treatments to ensure that mite populations remained low throughout the study. Despite this, multiple virus infections were detected, yet a significant correlation was observed only between DWV viral load and overwintering colony losses (OCL). Our current dataset based on HRM analysis (Figure 6 and Figure S2 shown in supplementary information) reveal that multiple infections with DWV master variants coupled with consistently high viral loads (>10^7 DWV genome equivalents per honey bee) for several consecutive winter months played a causative role in OCL (Highfield et al., 2009)

observed in colonies GD1, GD3, PW2, and DM1 (Figure 6). Conversely, multiple DWV master variant infections were less frequent and at relatively lower levels ($<10^7$ DWV genome equivalents per honey bee) during the winter months in the colonies that survived (Highfield et al., 2009) the winter (Figure 6).

The ABC assay was applied to select samples, focusing on months which represent the summer (July), autumn to winter transition (October) and winter months (January). The first spring bees (April) were also screen for the colonies that survived the overwintering period (Figure 6 and Table 3). DWV master variants type-A and C were detected in the majority of samples screened (Table 3), type-B was infrequent and up to three orders of magnitude fewer in viral load when compared to the other two master variants, especially in the colonies that suffered OCL. In addition, of the colonies which collapsed, type-C was the dominant variant in the final month before collapsing, reaching 10- to 100-fold more that the next highest variant. The ABC assay was only two orders of magnitude more sensitive that the HRM assay.

DWV type-A was consistently present in all the samples tested. Interestingly, of the colonies that collapsed, GD1 and GD3 reported mid-winter highs (January) of $>10^{10}$ DWV genome equivalents per honey bee (Table 3). This is in contrast to summer (July 06) samples which revealed through NGS (Figure 5) and the ABC assay (Table 3), respectively, to have been present in only a tiny proportion of type-A (0.002087% of the total 10^7 DWV infection in GD1, July 06) (Table 3). We note that it is at this time that DWV type-C starts to dominate the DWV variant landscape (Table 3). The role of DWV type-C is less clear in GD3.

Nonetheless, both DWV variants type-A and C dominated over type-B in the colonies which experienced OCL.

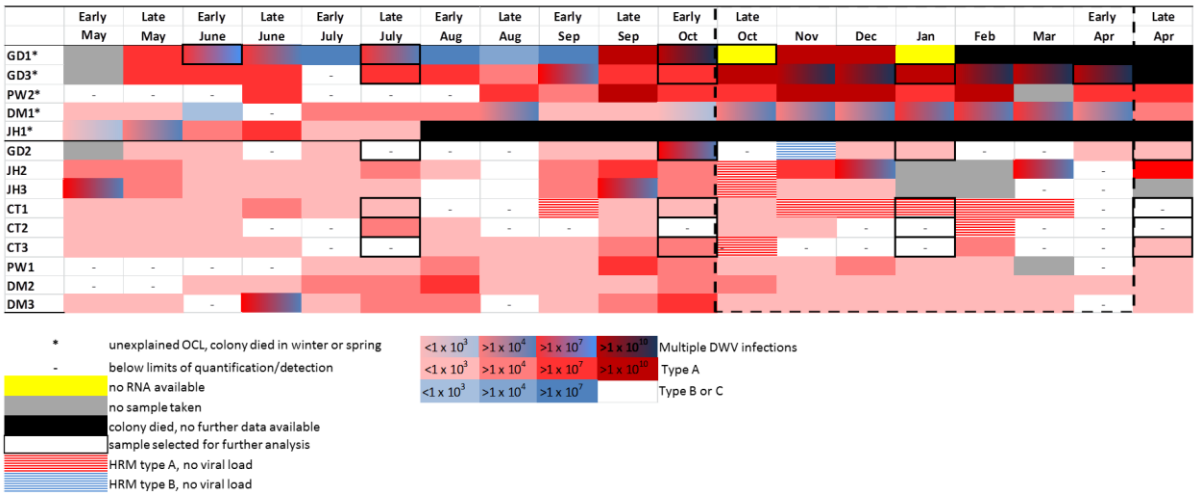

Figure 6. DWV load plus variant data as revealed by HRM for bee colonies sampled in Devon, in the southwest of England (Shute, Honiton, Plymouth, Ashburton, and Newton Abbot), over a year (bimonthly between May and October 2006, monthly between November 2006 and March 2007, and bimonthly in April 2007) as described in the Highfield et al., (2009) study. The DWV variants are displayed by colours: red = DWV type-A, blue = DWV type-B or C, and the shade of the colour relates to the viral load; light colours represent low viral loads, dark colours represent high viral load.

Table 3. ABC assay data alongside its respective HRM analysis on a reduced dataset (Figure 6). DWV variant type and viral loads (darker shades indicate relatively high viral loads) are displayed for each sample.

Colony ID	Collection Date	DWV Type A	DWV Type B	DWV Type C	HRM Data ^
GD1 *	Late July 06	2.62×10^{7}	-	1.26×10^{12}	
	Early Oct 06	1.09×10^{11}	1.73×10^{8}	2.68×10^{12}	
	Jan-07	$2.65 \times 10^{1°}$	1.54×10^{7}	1.38×10^{11}	
GD2	Late July 06	9.75×10^{6}	-	5.84×10^{7}	-
	Early Oct 06	2.13×10^{8}	-	1.22×10^{7}	
	Jan-07	2.12×10^{7}	-	2.51×10^{5}	
	Late April 07	2.61×10^{8}	-	-	
GD3 *	Late July 06	3.61×10^{6}	-	-	
	Early Oct 06	4.41×10^{8}	7.45×10^{4}	4.69×10^{4}	
	Jan-07	2.82×10^{11}	4.99×10^{6}	9.50×10^{5}	
	Early April 07	4.74×10^{6}	-	6.79×10^{8}	
CT1	Late July 06	1.19×10^{6}	-	6.34×10^{5}	
	Early Oct 06	3.56×10^{7}	2.07×10^{6}	-	
	Jan-07	1.89×10^{8}	6.64×10^{5}	-	-
	Late April 07	4.23×10^{6}	9.82×10^{5}	-	-
CT2	Late July 06	6.71×10^{7}	-	1.24×10^{6}	-
	Early Oct 06	1.23×10^{7}	-	6.64×10^{6}	-
	Jan-07	8.69×10^{7}	-	4.23×10^{7}	-
	Late April 07	4.02×10^{6}	-	1.03×10^{6}	-
CT3	Late July 06	9.53×10^{6}	-	3.82×10^{7}	-
	Early Oct 06	1.74×10^{8}	-	-	
	Jan-07	2.20×10^{7}	-	-	-
	Late April 07	1.50×10^{6}	-	-	

*	unexplained OCL, colony died in winter or spring
^	taken from Figure 6
-	below limits of quantification/detection
	no RNA available
	HRM type A, no viral load

Discussion

The PCR assay that forms the basis of the ABC assay was shown to be specific for the detection of each DWV variant with no observed cross-reactivity, as evidenced via competitive PCR (Figure 1). We also go on to show the importance of using RNA, as opposed to DNA, in standards in RT-PCR-based assays when quantifying RNA viruses (Figures 2 & 3).

As expected, the PCR efficiencies were the same for both PCR and RT-PCR assays, confirming the doubling effect of the product during each PCR cycle. Deviations were nonetheless observed in C_t values (Figure 4) when multiple DWV master variants were present in a sample. These deviations are considered to be caused by the forward primers being targeted by all the master variants in the reaction. In addition, as RNA viruses exist as a constantly mutating cloud of similar variants it is impossible to predict the role of unknown variants upon the RT-PCR assay. Nonetheless, the accuracy and robustness of the RT-PCR assay appears to be within a 0.5- to two-fold level (Figure 4).

A comparison of the ABC assay with the NGS data confirms the efficacy of the assay. Discrepancies were observed between the precise amounts of DWV variant genome equivalents present; however, both methods yielded similar results, i.e., showing one dominant master variant over the less dominant master variant. These discrepancies are considered to be caused, firstly, by the NGS data obtained from oligo dT priming with a 3' amplification bias. Given that the RdRp target is in this 3' region (Dalmon et al., 2017), the inefficiency of the RT step could account for minor differences. Secondly, as we compared

the output of the top hit BLAST (Basic Local Alignment Search Tool) analysis from all the reads across the whole genome, samples confirmed to be free or with low-level recombinants, whereas our ABC assay is reporting only on the RdRp region. Thirdly, different methodologies will have different sensitivities. Nassirpour et al., (2014) conducted a comparative study between NGS and RT-qPCR and found minimum agreement between NGS and RT-qPCR platforms. They conclude that RT-PCR is more sensitive due to the use of targeted specific primer pairs.

The HRM analysis revealed multiple DWV variants present in the Devonshire bees. The HRM technique can only separate DWV type-A from type-B & C master variant mix, with type-B and C sharing similar melting temperatures (Martin et al., 2012). However, analysis using the ABC assay revealed that type-C was the most prevalent variant for DWV melt curves falling within the type-B or C melt temperature range (<77.5 °C) (Martin et al., 2012). Type-B was infrequent and was present at lower levels independent on whether the colony collapsed or not. This result supports the superinfection exclusion hypothesis proposed by Mordecai et al., (2016b), who suggest that type-B is less virulent than other master variants of the virus. Here we hypothesize that it was not in sufficiently high enough numbers to protect colonies from the more virulent type-A, and possibly C, variants. This is the first time that type-C has been shown to be present, although not exclusively so, in colonies which collapsed during the winter period, and highlights the importance of variant specific assays for DWV detection. Given the sample size of both the Swindon (Mordecai et al., 2016b) and Devon (Highfield et al., 2009) studies, further validation of the virulence of type-C and putative protective nature of type-B (through superinfection exclusion) is still required.

Nonetheless, this study clearly shows how the ability to track variants throughout the season, at various sampling points, provides the opportunity for researchers to assess the putative role of DWV viral competition and evolution in honey bees. The HRM data revealed that DWV infection is variable between sampling points, as expected with RNA viruses which are under a constant state of competition (Domingo & Holland, 1997).

The development of the DWV ABC assay provides an opportunity for a consensus approach to DWV research efforts. Standard curves are considered laborious to develop (Klein, 2002) and primer design can be troublesome, as efficiency and specificity must be equal to avoid poor reactions (Diffenbach et al., 1999). Past studies focused on the effects of multiple viruses upon colony health (Leong et al., 2007; Highfield et al., 2009; Berthoud et al., 2010; Genersch et al., 2010; Dainat et al., 2011; Ball et al., 2012; Nassirpour et al., 2014; Thompson et al., 2014; Mordecai et al., 2016c) that have revealed the role of DWV as a major factor in colony losses. Now, research efforts must focus on the effects of DWV variants. Currently, the knowledge about variant diversity upon honey bee health is limited, in particular for the type-C variant. Using three distinct primer sets provides an opportunity to study the effects of DWV variant diversity upon honey bee health for each of the known master variants of DWV. The ABC assay allows for a quick, reliable, robust, and quantitative method of three known master variants of DWV, providing an insight into total DWV infection in honey bees.

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Supplementary information

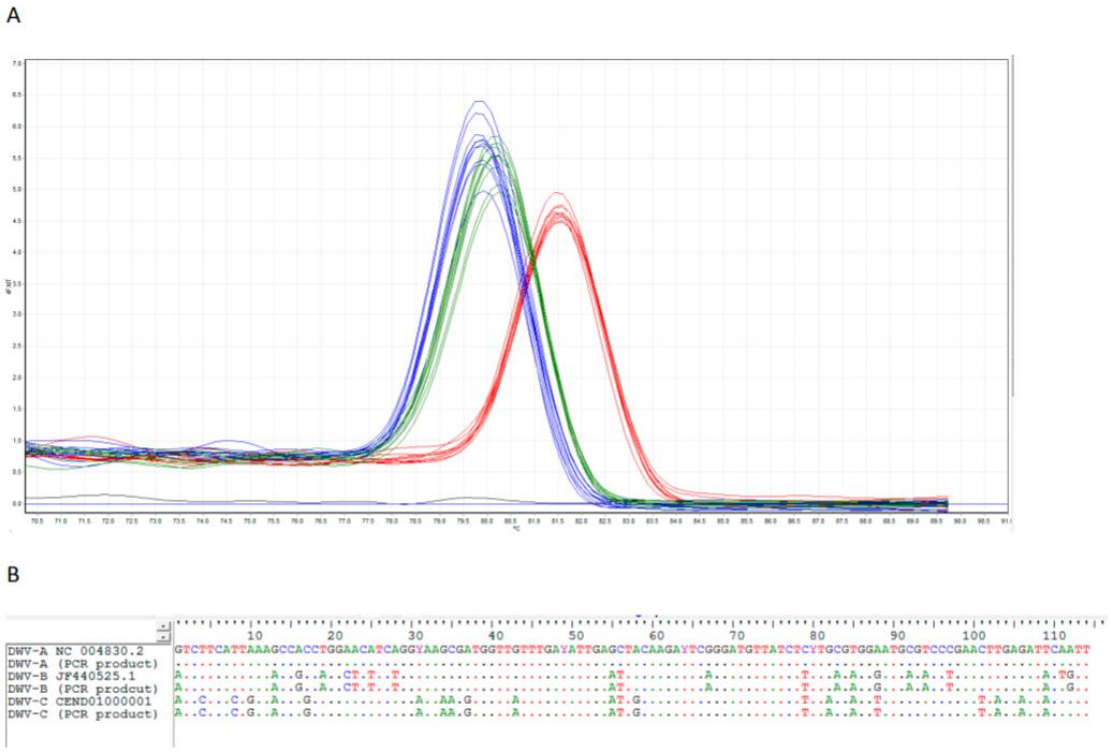

Figure S1: Validation of specificity of target amplification through A) Melt curve analysis and B) sequencing of resultant PCR product.

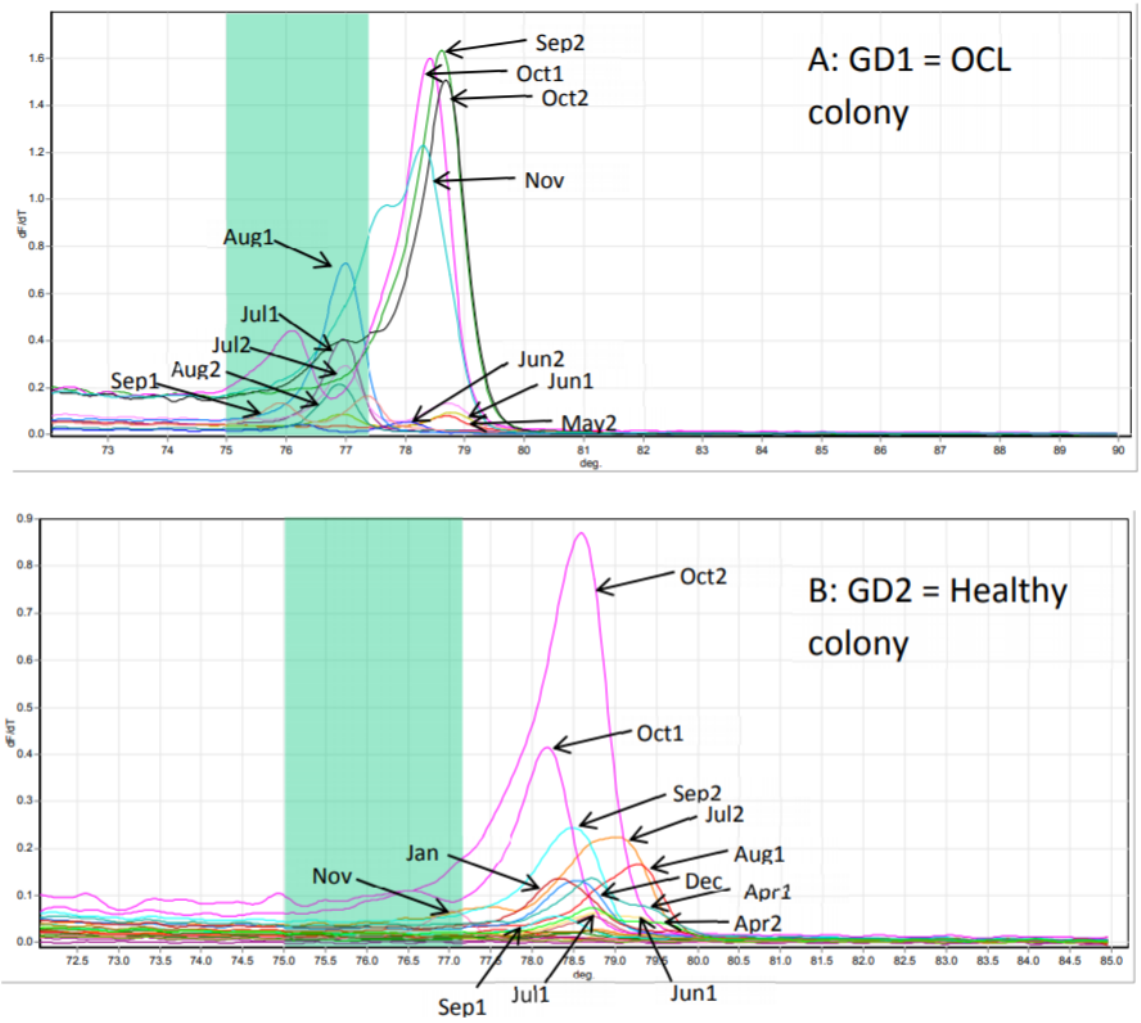

Figure S2. HRM melt curves of monthly samples from colonies that A) GD1 = collapsed during the over-wintering period, i.e. over-winter colony loss (OCL) and B) GD2 = survived the following year i.e. healthy colony. Shaded area indicates the melt position of the type-B or C variants in the months May to Sept in the OCL colony.

Chapter 2: DWV master variant dominance and seasonal prevalence in honey bee colonies from England and Wales

Abstract

Strong evidence supports an association between *Varroa* and Deformed wing virus (DWV) as the causal agent behind high overwinter mortalities of honey bee colonies in temperate regions. DWV persists as three master variants; Type-A, B, and C. Each variant may have differing degrees of virulence and their role in overwintering colony losses remains unknown. The aim of this study is to investigate the seasonal prevalence, viral load, and distribution of the three DWV master variants within *Varroa* susceptible and tolerant honey bee colonies from England and Wales. The viral data was compared to overwinter colony mortality to address the impact of DWV variants upon colony health and survivorship. Honey samples were screened using the ABC assay detailed in Chapter one. The analysis revealed that DWV type-B is now the most prevalent and dominant DWV master variant in English and Welsh colonies regardless of *Varroa* control or location. Type-C was rarely detected and the once commonly detected type-A variant has now been almost totally replaced by type-B. Reported overwinter colony losses were low in the colonies sampled therefore yearly overwinter monitoring of DWV infection is required to further evidence if type-B dominance is a factor in reduced colony loss.

Introduction

Typically, the northern hemisphere suffers from high overwinter losses of honey bee colonies each year (Lee et al., 2015: Van Engelsdorp et al., 2011). It is now well evidenced that the cause of these overwinter losses is attributed to the ectoparasitic mite, *Varroa destructor* (Anderson & Trueman, 2000), as high *Varroa* infestation rates (>2000 mites per colony) cause the collapse of colonies (Martin, 2001). The first report of *Varroa* in Western Europe was made in 1970 (Griffiths & Bowman, 1981) and then in England in 1992 (Rosenkranz et al., 2010). Before the arrival of *Varroa* UK colony losses were consistently below 12%, whilst post *Varroa* UK losses increased to 30% (BBKA, 2017). An increase in colony losses due to the presence of *Varroa* has also been reported elsewhere in the temperate zone (Kraus & Page, 1995; Dahle, 2010; Genersch et al., 2010; Guzman-Novoa et al., 2010; Topolska et al., 2010; Le Conte et al., 2010; Van Dooremalen et al., 2012).

Varroa mites reproduce in the brood of honey bees and feed upon the haemolymph (Webster & Delaplane, 2001) and fat bodies (Ramsey & Van Engelsdorp, 2016) of honey bee larva and adults. The mites feeding activities have several deleterious physiological and behavioural effects upon the host, including; deformed wings, stunted growth (Bailey & Ball, 1997), reduced foraging activity (Janmaat & Winston, 2000) and colony related tasks. However, it is *Varroa's* ability to transmit harmful viral pathogens especially Deformed wing virus (DWV) which is the cause of increased colony mortality (De Miranda & Genersch, 2010; De Miranda, Cordoni & Budge, 2010; Genersch & Aubert 2010; Martin et al., 2012). Of all the known 23 honey bee viruses (McMenamin & Genersch, 2015) DWV has been repeatedly shown to play a major role in overwinter colony losses of honey bees (Berthoud et al., 2010;

Dainat, et al., 2012a; 2012b; Highfield et al., 2009; Martin et al., 2010, 2012; 2013; Cornman et al., 2012; Nazzi et al., 2012; Francis et al., 2013). Furthermore, DWV is not a major driver behind colony losses in countries free from *Varroa* (Roberts, Anderson, & Durr, 2017) or where DWV infections are comprised of avirulent variants (Brettell & Martin, 2017). DWV viral loads are reduced alongside an increase in colony survivorship in studies where *Varroa* mites are removed (Martin et al., 2010), and beekeepers usually manage for the mite to enhance colony survivorship. Several authors (Nazzi et al., 2012; De Prisco et al., 2016) comment on the association between *Varroa*, DWV, and the down regulation of immune genes coinciding with increased DWV replication. Clearly demonstrating an association between *Varroa*, DWV, and honey bees, hereby termed the *Varroa*-DWV vectoring cycle.

DWV was discovered in symptomatic honey bees from Japan in the early 1980's (Ball &Bailey, 1991) and the first full genome sequenced in 2006 (Lanzi et al., 2006). A study into DWV among the Hawaiian honey bee population (Martin et al., 2012) revealed that diverse DWV variants persisted prior to the arrival of *Varroa.* However, the establishment of *Varroa* selected the classic type-A variant which coincided with a dramatic reduction in wild honey bee colonies. Three DWV master variants have since been described; Type-A which is lethal (Martin et al., 2012; Schroeder & Martin, 2012; Francis et al., 2013); type-B which may help provide protection to the colony from more lethal variants (Mordecai et al., 2016a) but has been shown to be lethal to inoculated adult honey bees (McManhon et al., 2017) and; type-C (Mordecai et al., 2016b) which was detected in *Varroa* treated colonies prior to the overwinter period (Kevill et al., 2017; chapter 1).

The seasonal role and prevalence of all three DWV master variants on a landscape scale is currently an under researched area. Further evidence is required to establish virulence and dominance of these viruses, which in turn can be used as a predictor for overwinter colony losses. Therefore, the aim of this study is to investigate the seasonal prevalence, viral load, and role of the three DWV master variants in colonies of honey bee from England and Wales. Samples include both managed *Varroa* treated/untreated bees and free-living feral colonies collected in 2016. Established feral colonies which were sampled in 2010 (Thompson et al., 2014) were also screened to provide a historic insight into how DWV variant prevalence may change over time.

Given the apparent association between *Varroa* and DWV and the recent implication of type-B offering a protective factor to colonies (Mordecai et al., 2016) via superinfection exclusion (SIE), it is important to compare both *Varroa* tolerant and susceptible colonies to assess the role of DWV upon overwinter colony survivorship. Typically, *Varroa* untreated colonies die after four years of infestation (Korpela et al., 1992), therefore tolerant colonies in this study have survived with *Varroa* for greater than four years and are considered tolerant to effects of *Varroa* and DWV. This reason for tolerance is yet to be explained and the presence of DWV variants and ability to be replicated (as indicted by viral load) in tolerant bees may explain resistance.

This is the first time in which a UK wide initiative has been established and funded by beekeepers to assess the role of DWV master variant prevalence and viral load during spring

and late summer in free living and managed *Varroa* tolerant and susceptible honey bee colonies.

Methods

Sample collection

The beekeepers of 35 apiaries located over a broad geographical range across England and Wales were contacted and requested that they identify up to five colonies in their apiary to be sampled. Each beekeeper was asked to collect a sample of up to 50 worker bees from a frame of brood. The first samples were collected upon the initial inspection in April (spring) and the second samples from the same colonies between mid-August to mid-September (late summer). All samples were stored in 100% ethanol at 4^{o}C, Forsgren et al., (2017) shown that RNA yield of bees stored under these conditions was similar to that of live bees stored at 15^{o}C, therefore RNA yield was not considered to be affected. A health questionnaire was sent to beekeepers who provided samples and was to be completed in the spring of 2017. The health questionnaire asked for the number of colonies lost during the overwintering period, the details surrounding any losses, and if any *Varroa* treatments where given to the colonies during the beekeeping season thus allowing the survival of the surveyed apiaries to be compared with the results of the DWV analysis. Unexpected overwinter losses are defined as a colony which died during the overwinter period which had no visible factors leading to colony death, such as lack of food, high mite drop etc. In total complete data sets from spring and late summer were obtained from 28 apiaries, with additional spring or summer only samples (Table 1), as well as 25 completed health

questionnaires. Untreated samples were selected from colonies which had survived for four or more winters as these were considered to exhibit a true tolerance to *Varroa*.

Table 1. Number of apiaries involved in sampling and the number of samples received.

No samples per apiary	5	4	3	2	1	Total No of Apiaries
Spring (all samples)	15	10	2	0	2	29
Late summer (all samples)	15	8	4	2	1	30
Treated spring	11	9	2	0	0	22
Treated late summer	9	8	4	2	1	24
Untreated spring	4	1	0	0	2	7
Untreated late summer	6	0	0	0	0	6

In addition, 16 historic samples of adult worker bees collected from feral colonies across England, in spring 2010 and had since been stored at -80^{o}C (Thompson et al., 2014) were donated by C. Thompson. These samples had all previously tested positive for DWV but the variants were unknown.

Sample processing

Each sample was manually checked for *Varroa* mites and any visual deformities to the bee, which were both removed when found. Pools of 20 asymptomatic whole worker bees were homogenised in liquid nitrogen using a mortar and pestle. The homogenate was stored at -80^{o}C until RNA extraction was conducted. RNA was extracted from 30mg of honey bee tissue from the homogenised pool of 20 bees using an RNeasy Mini prep kit (Qiagen). RNA

was then quantified on Nanodrop 2000 (Thermo Fisher Scientific) and standardised to 50ng/µl per sample.

RT-qPCR

RT-PCR was performed on the 1 µl total RNA (50 ng/µl) extracted from a pool of 20 adult asymptomatic honey bees using a SensiFAST™ SYBR® No-Rox One Step kit (Bioline). Each reaction contained 10 µL SensiFAST™ SYBR® No-ROX One-Step mix (2×), 0.75 µL DWV forward primer, 0.75 µL reverse primer (A, B or C; Table 1), 0.2 µL reverse transcriptase, 0.4 µL RiboSafe RNase Inhibitor, 7.5 µL RNAse free H2O. The Reverse transcriptase step occurred at 45 °C for 10 min and denaturation at 95 °C for 10 min, followed by 35 cycles of denature 95 °C 15 secs, annealing at 58.5 °C for 15 secs and extension at 72 °C for 15 secs. A melt curve analysis was performed between 72 °C and 90 °C, at 0.1 °C increments, each with a 5 sec hold period. RT-qPCRs also contained a negative template control (NTC) where 1 µl RNAse free water was used instead of total RNA. Both the amplification plot and melt curve were used to assess if contamination occurred in the controls. RT-qPCRs were repeated if contamination was found present in the NTC.

Samples were analysed in triplicate, any samples which had a 3 Ct value deviation between triplicates were removed from the analysis, as 3 Ct values are the equivalent to a ten-fold increase or decrease in viral load. A total of 258 colonies were screened for each DWV variant.

Analysis of the results

The samples were grouped into the historic (2010) feral colonies, and the more recent 2016 samples into *Varroa* susceptible and tolerant colonies. The *Varroa* tolerant colonies have persisted without *Varroa* control for more than four years and as such are considered to have developed a mechanism for resistance, such as SIE (Mordecai et al., 2016a).

The copy number of each DWV variant and viral load were calculated and converted into the genome equivalent per asymptomatic honey bee per colony, for each sample point (spring and late summer). The average DWV viral load per asymptomatic bee was then calculated for each apiary, i.e. a single location.

The following equation was used to calculate the copy number as per Kevill et al., (2017);

$$\text{Copy number RNA} = (\text{Concentration RNA (ng/µL)} \times 6.022 \times 10^{23}) / (\text{Fragment length base pairs} \times 109 \times 325)$$

The genome equivalent was calculated as follows as per Kevill et al., (2017);

$$\text{Genome equivalents} = (\text{average copy number}) \times (\text{RNA dilution factor}) \times (\text{elution volume of RNA}) \times (\text{proportion of bee material})$$

The viral load of each DWV variant were converted into percentages for individual colonies and the apiary averages. These are represented by a single pie chart, the size of which reflects the viral load which were plotted onto a map of England and Wales, although the map is not an exact geographical representation as to protect beekeeper identity. As the viral load data was not normally distributed across all the samples, the statistical analysis was conducted using non-parametric tests. A Kruskal-Wallis test was used to compare the 2010 and 2016 viral load data per DWV variant, as well as the viral load per DWV variant in colonies that survived or died in 2016, as the data were independent. Post-hoc analysis was conducted using a Dunn's test of multiple pairwise comparisons (Dunn, 1961) and allowed for the identification of groups which differed significantly when using the Kruskal-Wallis test. The 2016 apiary viral load data per DWV variant was compared using a Wilcoxon signed rank test, which is the non-parametric equivalent to a paired T-test and is used on dependant data. The significance threshold was set at $p<0.05$ for all tests and when multiple comparisons were made the significance levels were adjusted using a Bonferroni correction (significance threshold / number of comparisons). The overall aim of the statistical analysis was to identify differences in DWV load per year, season, and variant between *Varroa* tolerant and susceptible colonies.

Results

A comparison of DWV variant load and distribution in 2010 and 2016

RT-qPCR data of feral bees collected in 2010 reveals that colonies are either DWV type-A dominated (>50% of the DWV viral load) (n = 5) or DWV type-B dominated (n = 10) (Figure 1), whereas the 2016 colonies were mostly type-B dominated. Only two colonies from 2010 tested below the quantifiable limit (e.g. outside the standard range at >30 Ct values) for type-A, three for type-B, and ten for type-C. Significant differences in DWV load for each variant was found between the 2010 and 2016 samples (Kruskal-Wallis; n = 217, H = 46.13, df = 3, p = <0.001) post hoc analysis using a Dunn's test revealed that viral load for each DWV variant differed significantly from 2010 and 2016 (p = <0.001 for all pairwise comparisons). This result can be attributed to the differences in Type-A and B viral load between 2016 and 2010 (Figure 2).

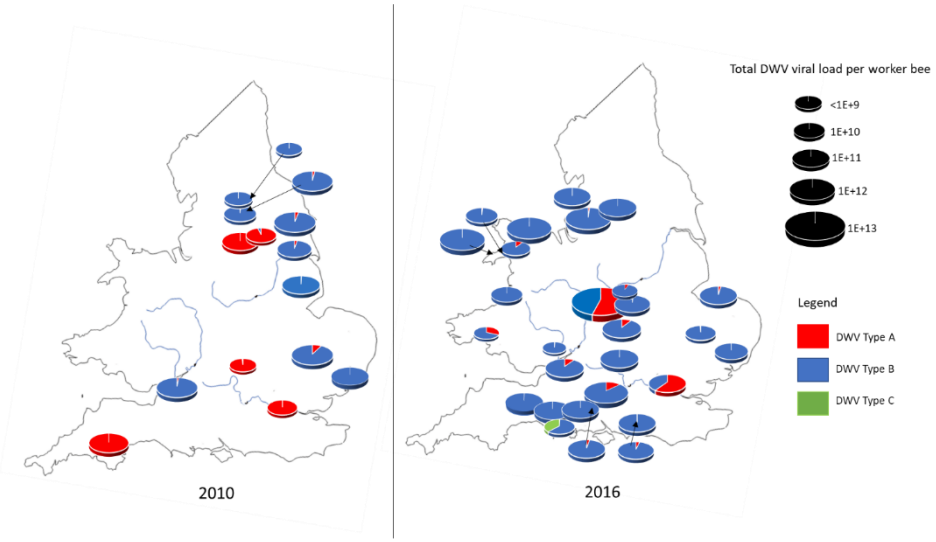

Figure 1. DWV map of honey bee colonies collected in spring 2010 and 2016. The size of the pie chart is indicative of viral load, and colour pertains to DWV variant (Type-A = red, B = blue, C = green).

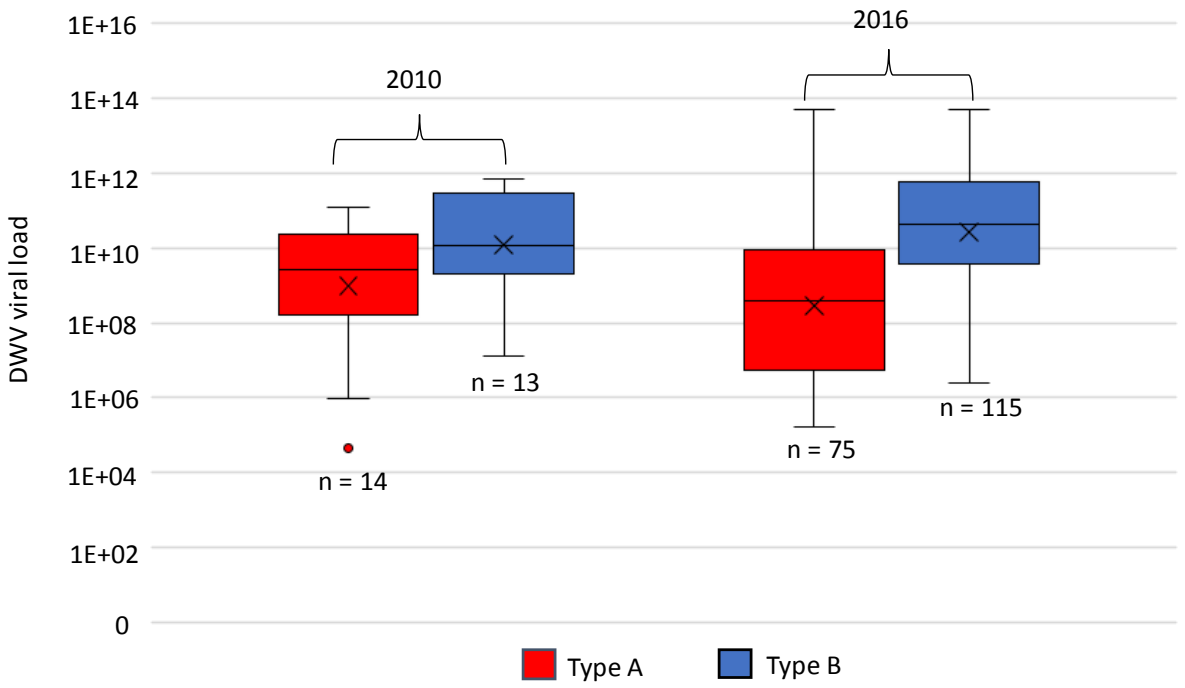

Figure 2. A comparison of DWV load and variant in honey bee colonies in 2010 and 2016. X = mean, bold black line represents the median.

Varroa tolerant honey bee samples from 2016 were compared to the feral bees of 2010 (Figure 3) and show a reduction in type-A load and an increase in type-B load between 2010 and 2016. A statistical difference was recorded in viral load between the two time points for type-A and type-B (Kruskal-Wallis - n = 70, H = 23.21, df = 3, p = <0.001), further post-hoc analysis confirmed that viral loads for DWV type-A and B differed significantly from 2010 to 2016 (Dunn's test p = <0.001 for all pairwise comparisons) (Figure 3). Type-C was quantifiable in one colony in 2010 and ten in 2016, when detected loads are often low (1E+08).

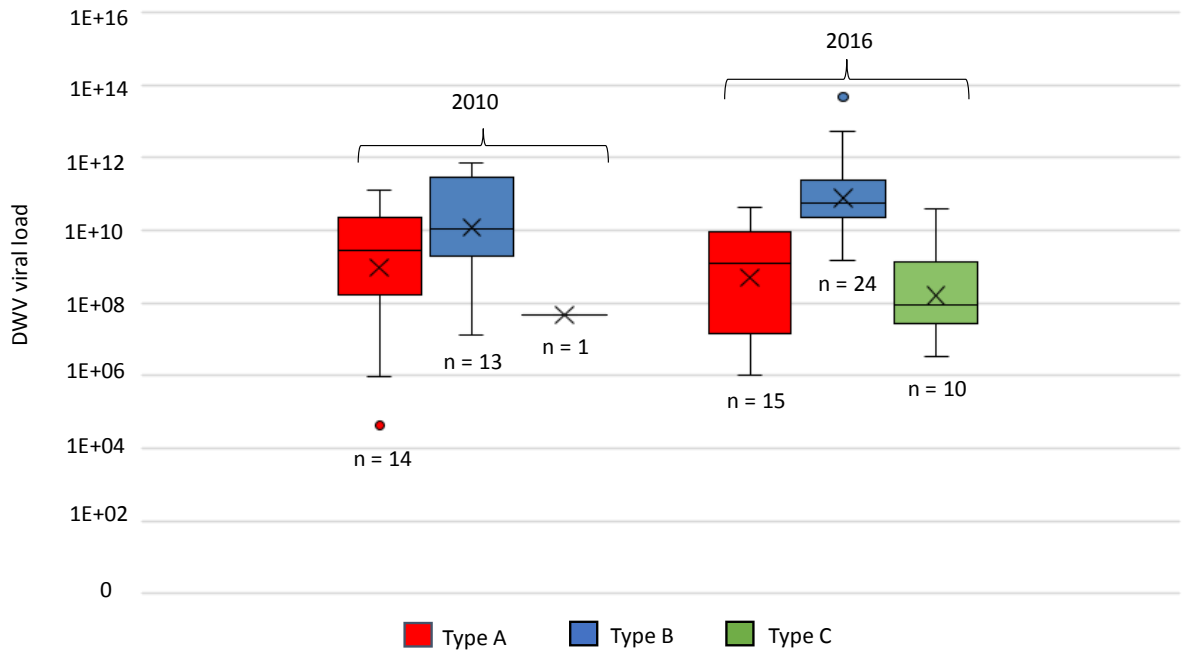

Figure 3. Comparison of DWV load and variant in feral colonies from 2010 and *Varroa* tolerant colonies from 2016. DWV type-A = red, type-B = blue, type-C = green, X = mean, bold black line represents the median.

A comparison of apiary level DWV variant load and prevalence between spring and late summer 2016

Apiary level analysis of the RT-qPCR data of the 2016 samples reveals that across both sampling points (spring and late summer) every apiary (spring n =29, late summer n = 30) tested positive for type-B and 99% tested positive for type-A. England and Wales are dominated by DWV type-B (Figure 4). DWV type-A was often below the quantifiable level of detection (<1E+02) and was dominant in only two apiaries in spring and two different apiaries in summer (Figure 4). Although DWV type-C was detected in 14 apiaries during spring and 12 in late summer the proportional amount of virus was low <0.31% and <0.70% in spring and late summer, respectively. In only one apiary did type-C infection represent a

high proportion (38%) of the DWV load (Figure 4). This occurred during the spring period and was localised as 50% of colonies screened in this apiary testing positive for type-C, however this was not maintained at the apiary level since in late summer type-B became dominant (Figure 4).

The 2016 apiary level viral load data (Figure 5) reveals a pattern between the three DWV variants throughout the year i.e. from spring to late summer with type-B>type-A>type-C. No significant differences occurred between DWV variant viral load for spring and late summer, (Wilcoxon signed rank - Type-A; W = 87, n = 20, p = 0.50; Type-B; W = 76.5, n = 18, p = 0.69; Type-C; W = 82.5, n = 18, p = 0.89).

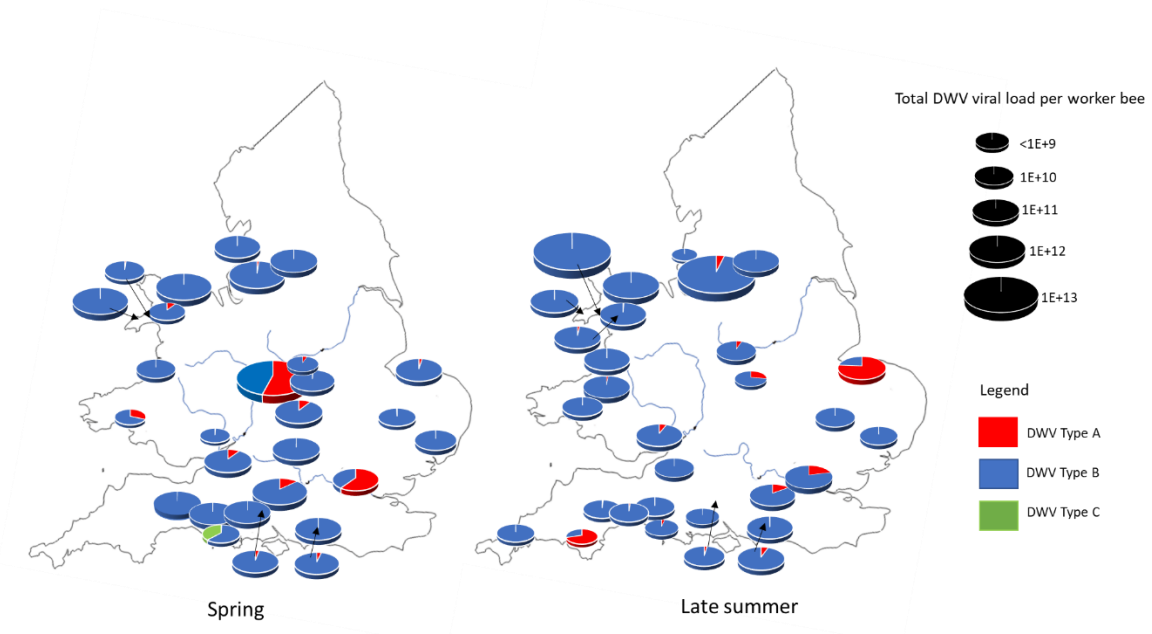

Figure 4. A map of England and Wales showing the average DWV load and variant per apiary in 2016.

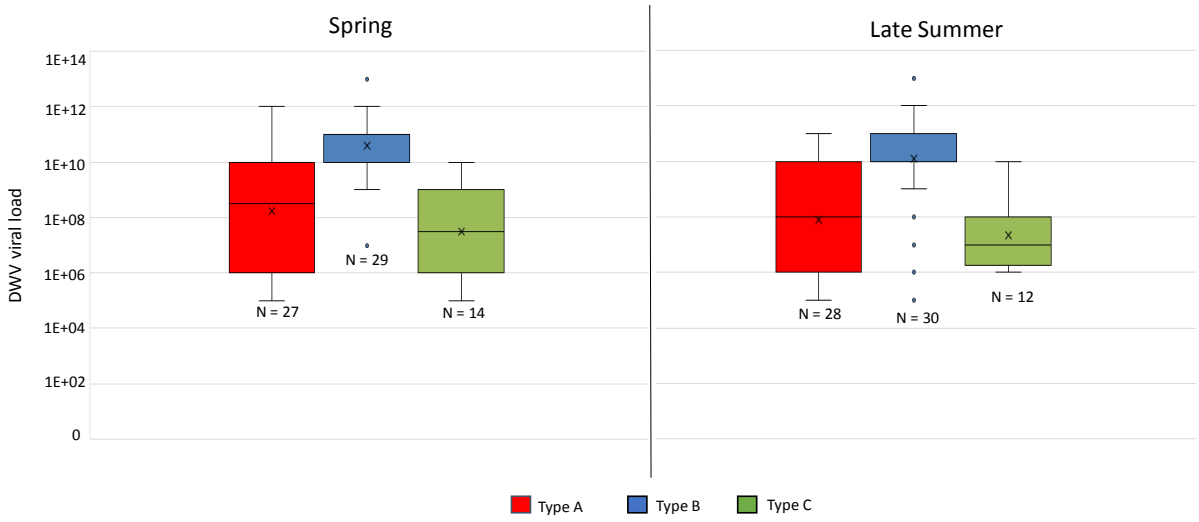

Figure 5. A comparison of the average DWV variant load per apiary in 2016. X = mean. DWV type-A = red, type-B = blue, type-C = green, X = mean, bold black line represents the median.

Colony Level DWV viral load, within apiary variation, and associated colony mortality

Colony level analysis of the RT-qPCR data of the 2016 samples reveals that across both sampling points (spring and late summer) every colony (n = 258) tested positive for DWV type-B and 99% tested positive for type-A (Figure 6). Only 16% (n = 258) of the samples had a >10% proportional load of DWV type-A, whereas type-B was detected at >50% proportional load in 88% (n = 258) of colonies screened. Type-C was detected in 58% (n = 258) of colonies although it was only quantifiable in 16% (n =258), quantifiable samples had type-C viral loads which rarely exceed 1E+07.

Type-B dominance was maintained across the season, with proportional viral loads exceeding 50% in 85% of colonies in spring and 89% in summer (Figure 6). Type-A dominated nine non-corresponding colonies in both spring and summer, whilst type-C

dominated two colonies in spring and one in summer (Figure 6). Samples which have a corresponding spring and summer data are displayed in Figure 7, here competition between type-A and B can be seen in nine colonies and also between B and C in two colonies. Despite competition between variants type-B infection remained dominant throughout the season.

The health questionnaire indicated that mortality was recorded in 9 out of 100 (9%) colonies for both sampling points; three colonies died in spring; one from robbing and two of no apparent cause. Six colonies died during the overwinter period; two winter deaths were attributed to weather, four were recorded as unexpected overwinter colony losses (Table S1, Supplementary information). A comparison of DWV loads at the end of summer was made between known surviving (n = 93) colonies and those which died of no apparent cause (n = 4). No statistical difference in DWV load or variant was recorded in colonies which died and survived (Kruskal-Wallis - n = 97, $H = 0.76$, df = 1, $p = 0.38$). Furthermore, viral loads in dead colonies did not exceed those of surviving colonies therefore, these deaths could not be attributed to DWV infection alone.

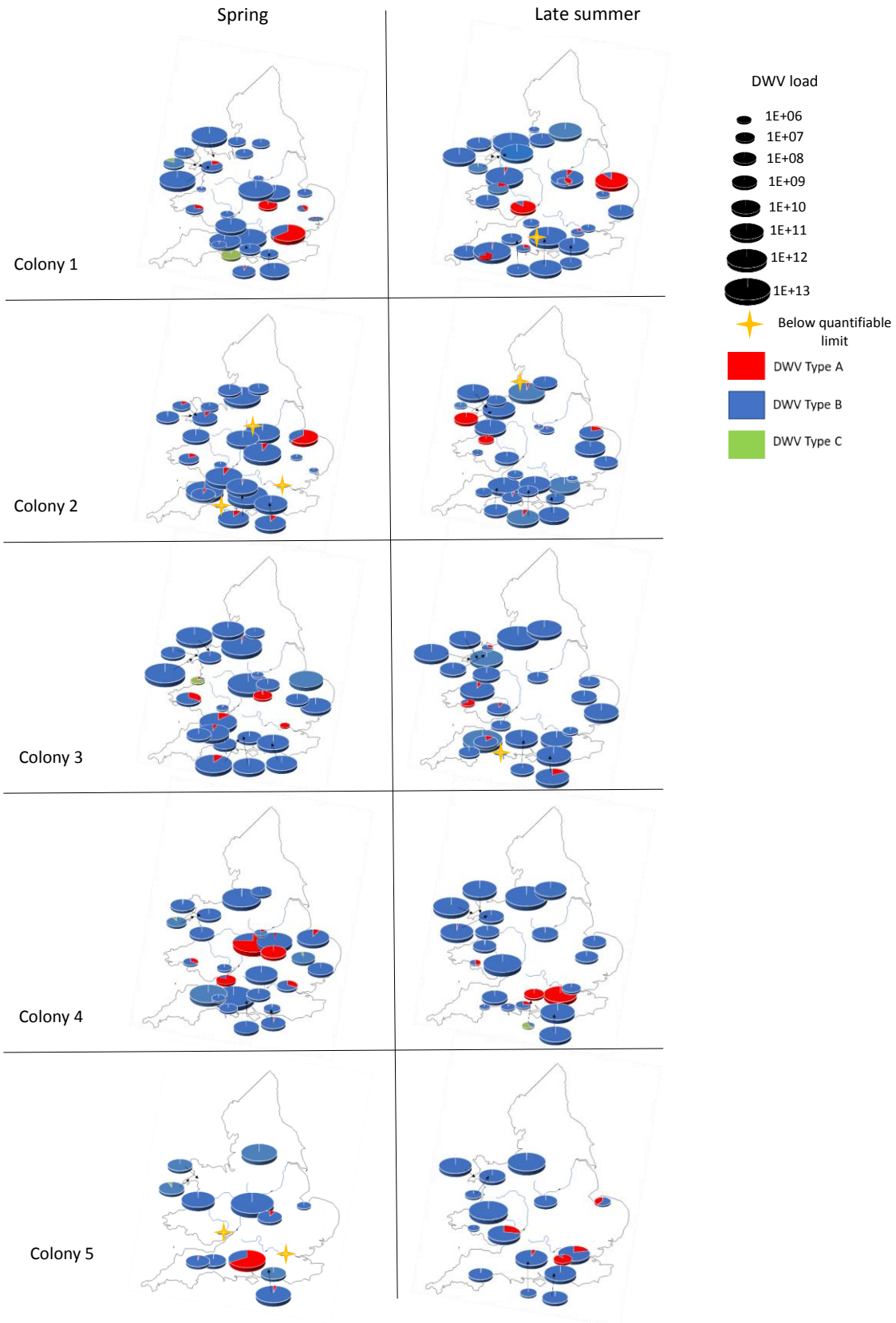

Figure 6. Colony level DWV load and variant maps for spring (left) and summer (right) in England and Wales 2016.

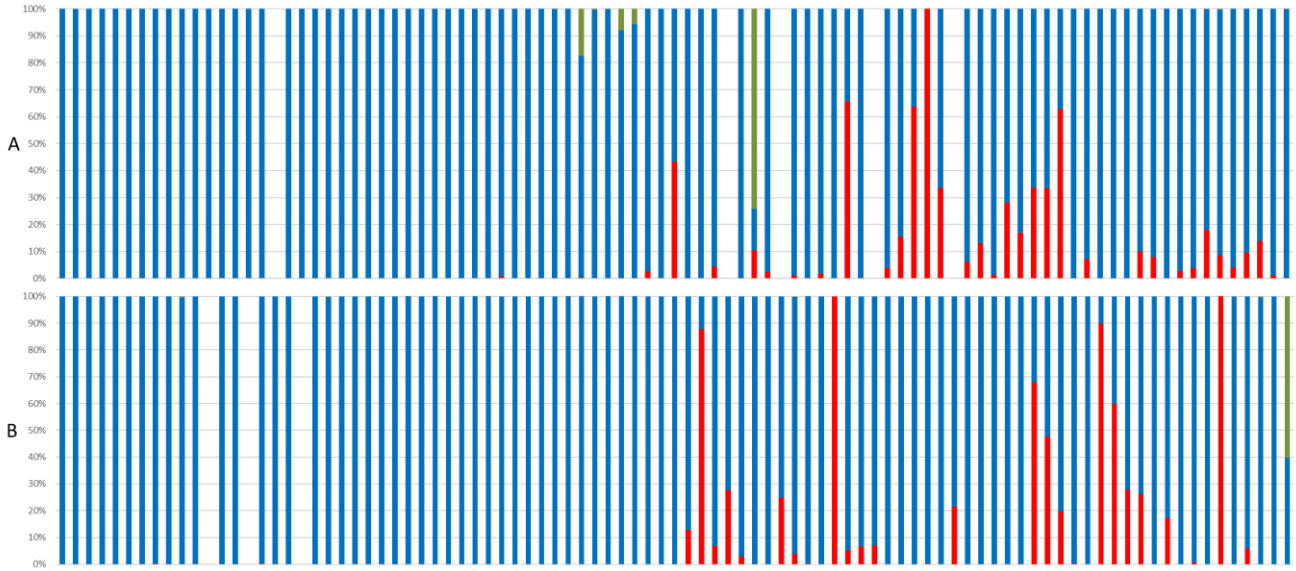

Figure 7. DWV variant proportional amounts (%) per corresponding colony for spring (A) and summer (B). Type-A = red, type-B = blue, type-C = green. Blank spaces indicate samples which fell below the quantifiable limit of detection.

Varroa susceptible versus tolerant colonies

A comparison of mean DWV infection for each variant remains the same in spring and late summer (type-A = 1E+08, type-B = 1E+10, type-C = 1E+08) for the susceptible bees, whilst the untreated bees had a 1.3-fold, 0.5-fold and 0.8-fold reduction in mean load for type-A, B and C (respectively) from spring to late summer (Figure 8).

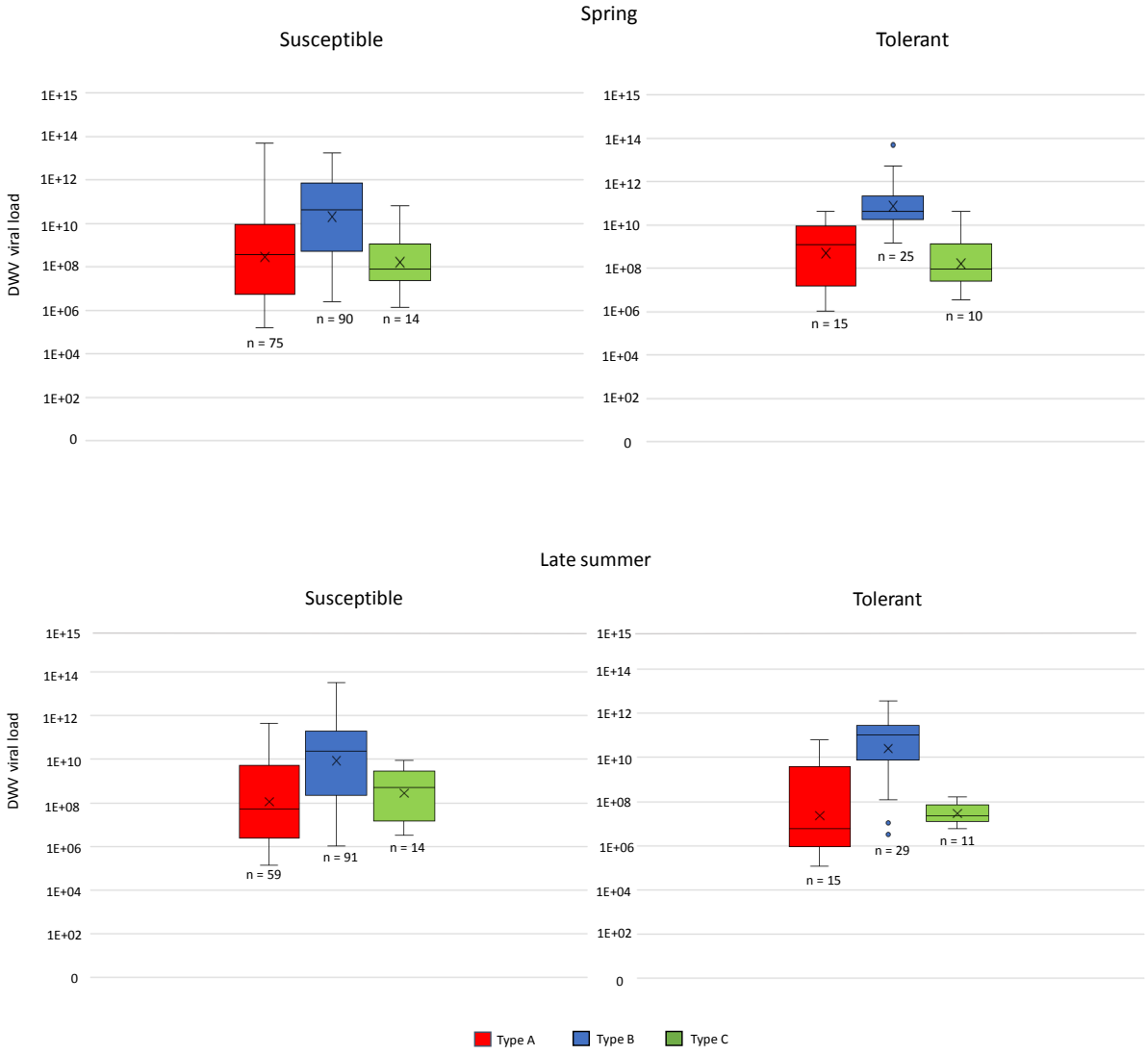

Figure 8. Average DWV master variant viral load in *Varroa* susceptible and tolerant colonies during spring and late summer. DWV type-A = red, type-B = blue, type-C = green, X = mean.

Discussion

The DWV type-B variant is currently widespread and dominant in UK honey bee colonies, a situation which has been maintained in the viral landscape from 2010 to 2016 (Figure 1). In 2016 Mordecai et al, found type-B dominating a *Varroa* resistant population of honey bees, leading to the suggestion that this variant was involved is the long-term survival of the colonies via SIE. SIE occurs when a virus has the ability to successfully outcompete and exclude other viruses and has been well documented in hepatitis C (Tscherne et al., 2007). The results presented here-within suggest DWV type-B SIE is not an isolated phenomenon and has occurred in honey bee colonies for at least six years. An increase in the prevalence of type-B over a six-year period has also been reported in USA honey bee colonies, where type-B was rarely detected in 2010 but was regularly detected in 2016 (Ryabov et al., 2017).

Both the colony and apiary level analysis shows DWV type-B to be the dominant variant. Colonies were shown to successfully enter the beekeeping season with high type-B viral loads per bee (>1E+10) which were maintained into late summer. In addition, very few over winter losses were recorded (9%) and colonies that died of no apparent reason (n = 4) had mid-high type-B viral loads (1E+7-1E+11), with a mid-range (1E+06-1E+09) type-A co-infection present in the majority of colonies. Chapter one implicates DWV type-A and C co-infections in colonies which died unexpectedly in the 2007 overwinter period, however these variants were rare in the 2016 data. In addition, high type-B loads (>1E+10) were detected in 63% of the surviving colonies (Table S1, Supplementary information) therefore it is difficult to attribute the deaths of honey bees in this study solely to DWV. A clearer result may have been produced if samples were taken from later on in the season, however this

was not possible as participants did not wish to disturb the honey bees whilst they formed the winter cluster. Therefore, the exact DWV composition prior to colony death is unknown. To make a more robust seasonal study it would be best to sample colonies throughout the season at bi-monthly intervals (such as Highfield et al., 2009). Furthermore, as co-infection allows for DWV recombinants to form and viral recombinants between type-A and B have been shown to prevail in *Varroa* infested honey bee colonies in a previous study (Moore et al., 2011), it is hypothesised that viral recombinants are present in the honey bee colonies screened. Despite these limitations, the data clearly shows that DWV type-B dominance is maintained throughout the season in surviving colonies.

DWV infection in *Varroa* susceptible and tolerant colonies differed slightly however the results were not significant (Figure 8). The *Varroa* susceptible colonies receive *Varroa* control therefore it was expected that viral loads in these colonies would be low, due to the lack of mites, however this is not the case. Recent evidence by Locke et al., (2017) shows that DWV infection is maintained after the removal of mites and may explain this result. The tolerant bees are considered to have developed some resistance as these colonies have survived with *Varroa* for longer than four years and comparable DWV loads between the two groups suggests that a mechanism for tolerance has evolved.

The mechanism of *Varroa* resistance remains unclear in the tolerant bees, however a slight reduction in DWV load from spring to late summer was seen for all three DWV variants and was greater for the type-A variant (1.3-fold reduction in type-A and a 0.5, 0.8 reduction in B and C viral loads between spring and late summer). This reduction in mean viral load was

not witnessed in the susceptible bees. Over-all this result suggests that long-term *Varroa* untreated bees may have evolved traits that aid in resistance as has recently been evidenced in long-term *Varroa* resistant colonies (Oddie et al., 2018).

Evolved resistance in the *Varroa* tolerant bees could be due to a trade-off in DWV virulence (ability to replicate) and pathogenicity (ability to cause disease symptoms) (Shapiro-Ilan et al., 2005) caused by the *Varroa*-DWV vectoring cycle, as both the vector and the virus require a living host to ensure their survival. This study indicates low pathogenicity of DWV type-B in the presence of high viral loads, as evidenced by the large number of surviving colonies and the low number of overwinter colony losses reported here-within and also by the BBKA in their 2016 overwinter loss survey (13.2%). However, this is a complex scenario in which further research needs to be conducted.

Furthermore, whilst there has been an upward trend in overwinter colony loss since the arrival of *Varroa*, and UK overwinter colony losses are still considered high (10%-30.5% in the last 9 years, [Carreck & Ashton, 2011; BBKA, 2017]), the British beekeepers association (BBKA) reported a reduction in overwinter colony mortality from 16.7% in 2015/2016 to 13.2% for 2016/2017. The sample years in this study were years where overwinter colony losses were the 2nd and 3rd lowest on record (Figure 9) since the arrival of *Varroa* and the data shows that both 2010 and 2016 colonies are dominated by DWV type-B. Type-B was found to be low (<1E+07) in colonies which died from DWV type-A and C infection in 2007 (Kevill et al., 2017, Chapter 1), this was also the year in which colony losses were the highest (Figure 9). Whilst my results show a high number of colonies surviving with type-B

dominated DWV infections, others have shown type-B to be lethal (McMahon et al., 2017 & Natsopoulou et al., 2017). High DWV type-B (>1E+12) loads were shown to kill inoculated caged bees (McMahon et al., 2017), and Natsopoulou et al, (2017) attributed a low type-B spring-time occurrence to a loss of winter worker bees. Therefore, it is hypothesised that type-B viral loads must exceed the threshold for host tolerance in all adult bees in order to kill a colony, however further studies of this nature are required to ascertain when a type-B infection becomes lethal to honey bee colonies.

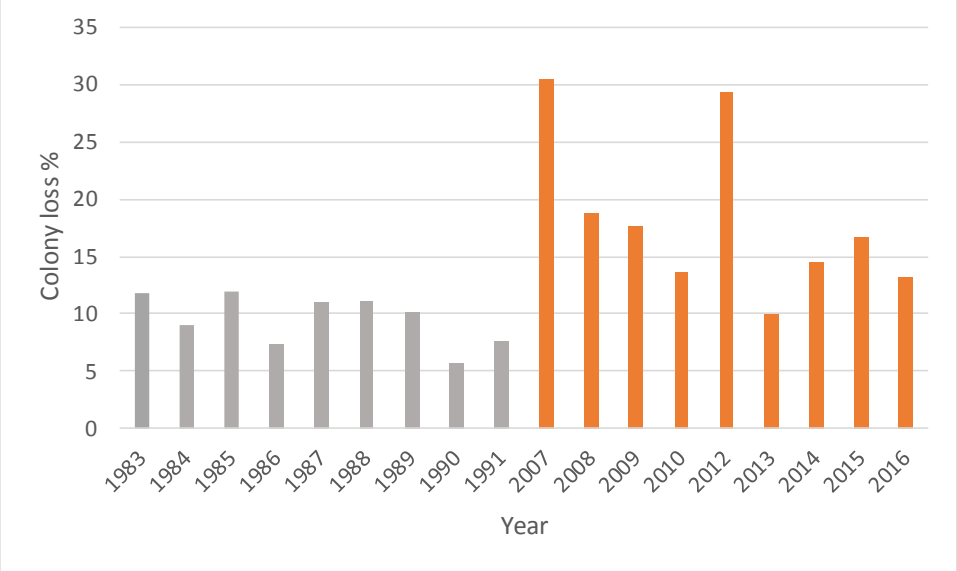

Figure 9. UK colony loss data. Grey bars show pre *Varroa* total colony loss and orange bars show overwinter losses from 2007 onwards. Data from the BBKA and David Aston.

Conclusion

This is the first time in which colonies across England and Wales have been tested for each variant of DWV and shows DWV type-B to be dominant throughout the season. Type-B's prevalence and dominance appear to be advantageous to honey bee colonies due to the low number of colony losses reported, however the situation will require monitoring over years. In addition, *Varroa* tolerant bees have been shown to have similar viral loads as colonies which undergo *Varroa* controls. The exact cause of the maintained DWV loads remains unknown and future work is required to establish the behavioural or physiological traits behind this result.

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Supplementary information

Table S1. The DWV master variants and total viral load in colonies which survived or died.

Samples were collected in late summer 2016 from England and Wales

Survivorship	ID	A	B	C	Total
Dead – Unexpected	LH1	3.91E+08	1.60E+09	BL	1.99E+09
Dead – Unexpected	AB3.2	3.69E+06	1.70E+11	NEG	1.70E+11
Dead – Unexpected	AB4.2	BL	3.31E+10	BL	3.31E+10
Dead – Unexpected	NLBK1#2	BL	4.84E+07	NA	4.84E+07
Dead - Weather	TREE2#2	1.01E+10	3.33E+06	6.65E+06	1.01E+10
Dead - Weather	TREE3#2	BL	3.74E+11	1.36E+07	3.74E+11
Alive	LH2	1.76E+08	BL	BL	1.76E+08
Alive	LH5	1.41E+10	1.97E+11	BL	2.12E+11
Alive	LH6	2.81E+08	2.66E+10	BL	2.68E+10
Alive	LH8	1.03E+09	1.03E+12	BL	1.04E+12
Alive	BTG	BL	1.14E+10	BL	1.14E+10
Alive	NE2	1.39E+07	2.00E+07	NA	3.39E+07
Alive	NE1	1.64E+06	2.09E+07	NEG	2.26E+07
Alive	TT5	2.52E+06	9.62E+06	NEG	1.21E+07
Alive	TT52	9.75E+08	6.85E+11	BL	6.86E+11
Alive	TT7	1.43E+09	4.88E+11	NA	4.89E+11
Alive	TT9	1.99E+11	5.05E+07	NA	1.99E+11
Alive	TT6	3.35E+08	4.06E+07	NEG	3.75E+08
Alive	IP5.2	BL	6.53E+10	NEG	6.53E+10
Alive	IP7.2	BL	2.45E+10	BL	2.45E+10
Alive	IP4.2	BL	1.03E+11	NEG	1.03E+11
Alive	SW9	6.71E+05	8.89E+09	BL	8.89E+09
Alive	SW3.1	2.58E+06	6.12E+10	BL	6.12E+10
Alive	SW2.1	1.96E+09	1.33E+10	BL	1.53E+10
Alive	SW5	BL	5.61E+10	BL	5.61E+10

Alive	CB88.2	BL	9.06E+07	BL	9.06E+07
Alive	CB3.2	BL	1.32E+11	NA	1.32E+11
Alive	CB12.2	BL	2.12E+10	NEG	2.12E+10
Alive	CB8.2	BL	2.09E+09	BL	2.09E+09
Alive	DFBKA19#2	1.29E+10	1.80E+09	NEG	1.47E+10
Alive	DFBK8.2	2.40E+06	9.12E+10	NA	9.12E+10
Alive	DFBK9.2	4.77E+09	6.50E+10	BL	6.97E+10
Alive	DFBK7.2	2.69E+09	2.04E+12	NA	2.04E+12
Alive	DFBK6.2	1.44E+11	3.76E+11	BL	5.20E+11
Alive	AB2.2	1.07E+11	3.52E+12	BL	3.62E+12
Alive	AB5.2	BL	3.15E+10	1.16E+08	3.16E+10
Alive	163.2	BL	2.84E+10	BL	2.84E+10
Alive	161.2	1.30E+09	8.34E+11	7.52E+06	8.36E+11
Alive	153.2	BL	2.04E+12	NEG	2.04E+12
Alive	WD2.2	1.40E+07	4.26E+07	NEG	5.67E+07
Alive	WDL2	6.11E+07	1.47E+09	3.23E+06	1.54E+09
Alive	WD12.2	BL	BL	BL	BL
Alive	WD7.2	2.01E+06	3.31E+07	NA	3.51E+07
Alive	98.2	BL	1.79E+12	NA	1.79E+12
Alive	A1.2	BL	1.92E+11	NA	1.92E+11
Alive	R1.2	2.20E+07	6.10E+11	BL	6.10E+11
Alive	20.2	9.08E+09	BL	BL	9.08E+09
Alive	66.2	1.17E+10	2.14E+11	BL	2.26E+11
Alive	fH1	BL	BL	BL	BL
Alive	SKH1#2	8.02E+06	4.09E+09	NEG	4.10E+09
Alive	NLBK6#2	BL	7.40E+06	NA	7.40E+06
Alive	NLBK8#2	5.42E+05	2.19E+08	NA	2.19E+08
Alive	NLBK9#2	1.35E+05	2.57E+09	BL	2.57E+09
Alive	NLBK10#2	1.18E+11	4.32E+11	NA	5.50E+11
Alive	BP1#2	BL	1.08E+06	BL	1.08E+06
Alive	BP2#2	BL	BL	BL	BL
Alive	WS4.2	2.63E+06	4.85E+11	1.49E+09	4.87E+11
Alive	WS9.2	BL	6.87E+09	BL	6.87E+09
Alive	WS8.2	1.09E+06	2.14E+11	NEG	2.14E+11
Alive	WS6.2	BL	1.87E+11	NEG	1.87E+11
Alive	WS1.2	2.05E+07	1.79E+11	NA	1.79E+11
Alive	ER1.2	BL	1.85E+11	BL	1.85E+11
Alive	ER2.2	BL	5.58E+10	BL	5.58E+10
Alive	ER8.2	1.29E+06	2.06E+11	2.70E+08	2.06E+11
Alive	ER4.2	BL	6.60E+11	BL	6.60E+11
Alive	ECH10.2	BL	7.63E+10	BL	7.63E+10
Alive	ECH2.2	BL	2.16E+06	NEG	2.16E+06
Alive	ECH4.2	2.71E+07	1.28E+07	BL	4.00E+07
Alive	ECH5.2	BL	8.80E+06	BL	8.80E+06
Alive	ECH7.2	1.84E+06	2.03E+06	NA	3.87E+06
Alive	W1Y#2	4.45E+11	5.06E+10	NEG	4.96E+11
Alive	W4A#2	5.35E+09	2.20E+10	BL	2.73E+10
Alive	W4#2	2.15E+06	1.06E+09	NA	1.06E+09

Alive	W3#2	1.66E+07	6.72E+10	BL	6.72E+10
Alive	WN2#2	2.89E+07	1.95E+07	NEG	4.84E+07
Alive	C6	4.92E+06	3.02E+12	1.03E+09	3.02E+12
Alive	C8	BL	7.42E+09	BL	7.42E+09
Alive	C2	2.13E+06	5.49E+06	NA	7.62E+06
Alive	fEDW1#2	3.73E+05	4.16E+10	BL	4.16E+10
Alive	FEDW4#2	BL	3.92E+11	5.50E+07	3.92E+11
Alive	fEDW2#2	9.13E+05	7.28E+10	BL	7.28E+10
Alive	fEDW3#2	4.00E+09	2.68E+11	BL	2.72E+11
Alive	fEDW5#2	BL	1.19E+08	BL	1.19E+08
Alive	TREE1#2	1.15E+07	1.00E+11	NA	1.00E+11
Alive	TREE5#2	BL	1.43E+10	BL	1.43E+10
Alive	TREE4#2	BL	5.99E+10	5.80E+06	5.99E+10
Alive	WILLOW2	2.68E+06	1.76E+11	NA	1.76E+11
Alive	WILD2	1.08E+10	1.79E+11	BL	1.90E+11
Alive	garage 2	BL	9.46E+10	BL	9.46E+10
Alive	TRUNK2	BL	1.10E+07	BL	1.10E+07
Alive	ASTER2	BL	1.34E+07	2.03E+07	3.37E+07
Alive	W10H2	BL	2.73E+09	1.31E+07	2.74E+09
Alive	W14H2	BL	2.15E+11	BL	2.15E+11
Alive	W5H2	BL	2.45E+11	BL	2.45E+11
Alive	W12H.2	6.20E+06	2.79E+11	6.61E+07	2.79E+11
Alive	LB3#2	2.20E+06	2.24E+11	2.31E+07	2.24E+11
Alive	LB5#2	BL	4.91E+06	BL	4.91E+06
Alive	LB2.2	2.22E+05	2.32E+11	9.44E+07	2.32E+11
Alive	LB1#2	BL	3.53E+12	1.63E+08	3.53E+12
Alive	LB4#2	BL	3.27E+11	6.91E+07	3.27E+11

Chapter 3: DWV infection, mortality and survivorship in pre-wintering *Varroa* susceptible and tolerant honey bee colonies from the USA

Abstract

During the past decade annual colony losses during the winter period in the USA have varied between 22%-35%, which is one of the highest levels anywhere in the world. It is suspected that the association between *Varroa* and Deformed wing virus (DWV) is a factor in colony losses. The aim of this study was to investigate the prevalence, load and distribution of the three DWV master variants (A, B and C), within both *Varroa* susceptible and tolerant colonies from across the USA. The samples were collected in October-November 2016 and represent the pre-wintering population of USA honey bees when *Varroa* levels are at their maximum. Samples were screened using the ABC assay (Chapter one). This revealed that DWV type-A, was the most prevalent and dominant DWV master variant in US colonies in 2016. Of the apiaries screened 63% were type-A dominated, type-B and C dominated 23% and 1%, respectively. Type-B appears to be an emerging virus in the USA honey bee population. Mortality data obtained for 45 colonies revealed that 18 colonies died during the winter period; of which 89% were dominated by type-A rather than type-B, the remaining 11% had DWV loads below the quantifiable limit. Due to the dominance of type-B in England and Wales (Chapter two), I predict that this variant will eventually replace type-A and may lead to the long-term reduction in USA over-wintering colonies losses. No one master variant of DWV was found to be associated with mite treatment history i.e. if they were treated (*Varroa* susceptible) or untreated (*Varroa* tolerant), indicating that other factors are contributing to the evolution of mite tolerance.

Introduction

Over the past decade the USA has continued to experience high annual levels of overwintering mortality of honey bee colonies (Hayes et al., 2008, VanEngelsdorp et al., 2010; 2011; 2012; Spleen et al., 2013; Steinhauer et al., 2014; Seitz et al., 2015; Kulhanek et al., 2017). These losses are typically greater than those in the UK, Europe and the rest of the temperate zone (Neumann & Carreck, 2010). Overall winter colony losses in the USA have been recorded in the range of 22% - 35% in the last decade (Bee informed partnership, 2017), whilst European winter losses range between 12% - 21% (Van der Zee et al., 2012; Brodschneider et al., 2016; 2018), with UK winter losses for the same period between 10% - 30% (BBKA, 2016). The sustained level of colony losses is of grave concern in the USA since many major industries rely on honey bees for the pollination of crops, e.g. almonds, soft fruit and canola (oil seed rape), with honey bees estimated to provide $14 billion to the USA economy (Morse & Calderone, 2000). In order to sustain beekeeping operations, colony losses are recovered by beekeeping practices (e.g. splitting colonies, buying packages), although a decline of 5.8% in the total number of colonies maintained was witnessed in the USA between 2007-2008 (NASS, 2009 from Ellis et al., 2010) suggesting that bee populations are not always recoverable by conventional means.

To help understand the reasons behind annual losses the Bee informed partnership conducts a yearly survey, where beekeepers are asked to complete and return a detailed questionnaire. These results are then collated and presented in annual reports, which inform the wider beekeeping community. The bee informed partnership is comprised of researchers, advisors and stakeholders, who rely on honey bees. The reasons given for the

loss of a colony in the USA are many, with hobbyist beekeepers mentioning queen failure, starvation, and poor weather. Whereas commercial beekeepers state 'colony collapse disorder' (CCD), *Varroa* infestation, and pesticides (Hayes et al., 2008; van Engelsdorp et al., 2010; 2011; 2012; Ellis et al., 2010; Spleen et al., 2013; Steinhauer et al., 2014; Lee et al., 2015). These differences, along with changing perceptions of the beekeeper provide difficultly in assessing the true causes of colony losses, particularly during the overwinter period when traditionally it is rare to inspect colonies. The European scientific community commonly report pathogens, especially RNA viruses in the presence of *Varroa* to be a major factor in overwintering losses (Berthoud et al., 2010; Dainat, et al., 2012; Highfield et al., 2009; Martin et al., 2010; 2013; Nazzi et al., 2012; Francis et al., 2013), with studies in the USA also providing evidence that pathogens are linked to weakened colonies and colony loss (Cornman et al., 2012; Martin et al., 2012; Traynor et al., 2016; Gleeny et al., 2017).

In temperate regions, all the available evidence suggests that DWV is the main RNA virus associated with *Varroa* and the presence of *Varroa* leads to elevated DWV viral loads (Bowen – Walker et al., 1999; Gisder et al., 2009). DWV has been strongly associated with *Varroa* and the death of honey bee colonies during the overwinter period as evidenced by a range of studies in many countries (e.g. Berthoud et al., 2010; Dainat, et al., 2012; Highfield et al., 2009; Martin et al., 2010; 2012a 2013; Nazzi et al., 2012; Francis et al., 2013). A key issue with DWV is that it is largely a hidden problem since almost all members of a colony are frequently infected and continue to appear healthy but their life spans are reduced (Dainat et al., 2012, Benaets et al., 2018), this is known as a chronic infection (De Miranda & Genersch, 2010). The characteristic deformed wing symptom associated with DWV is a poor

indicator of colony health, as only a small proportion of heavily infected individuals exhibit this symptom (Nordstrom, 2000), which often leads to the assumption that other causes are behind overwinter losses. Very few studies have emerged out of the USA which have concentrated on DWV as a driver for loss and often studies comment upon pathogen webs, and the interactions between *Varroa* and several viruses (Cornman et al., 2012; Runckel et al., 2011; Traynor et al., 2016; Cavigli et al., 2016; Gleeny et al., 2017). DWV was found to be abundant in USA colonies and follows the same seasonal pattern as the rest of the northern temperate zone (Traynor et al., 2016; Cavigli et al., 2016 Gleeny et el., 2017), however these studies failed to identify one cause behind colony loss and suggest that co-infection with a number of pathogens are behind weak colonies which subsequently die. Strong evidence exists in support of DWV being a major driver behind overwinter losses, it is important to establish the role of DWV master variants upon colony health, especially due to the seasonality of the disease and the fact that the USA has elevated yearly winter losses of honey bees.

DWV exists as three known master variants; Type-A, B, and C. Briefly, type-A has been evidenced as lethal (Martin et al., 2012a); there is contention around the impact of type-B, as at a colony level bees can survived with high viral loads (Mordecai et al., 2016; Chapter two) but at the individual bee level has been evidenced as more lethal that type-A (McMahon et al., 2017), type-C was shown to be rare in England and Wales (Chapter two) but was detected in mite-treated colonies which died in 2007 (Kevill et al., 2017; Chapter one). Of the three known master variants, type-A was mainly present in the USA until the recent discovery of type-B by Ryabov et al., (2017). Therefore, the aim of this study is to

measure the prevalence, and load of all three DWV variants (Type-A, B, and C) from across the USA. DWV variant prevalence and load may explain why the USA suffers from periodically high overwinter losses of honey bees. This data will be used to assess associations between DWV variants, overwinter colony mortality and mite treatment strategy, since samples include managed colonies that are treated annually for *Varroa* (susceptible) and colonies that have survived for five years or more untreated (tolerant colonies). The colonies which have survived for longer than five years may be exhibiting *Varroa* tolerance, as untreated colonies typically succumb to effects of feeding mites at four – five years untreated (Korpela et al., 1992).

Methods

Sample collection

Randy Oliver organised the collection of three honey bee samples containing 30-50 adult worker bees, per apiary from 78 apiaries across the USA, in the Autumn of 2016 (November & October). The geographical distribution of apiaries sampled spanned over 34 states, across the USA. Eleven of these apiaries were commercial beekeeping operations, the remaining 67 apiaries were hobbyist beekeepers. As the cause of over winter colony death is perceived differently in returned overwinter surveys between hobbyist and commercial beekeepers (Hayes et al., 2008; van Engelsdorp et al., 2010; 2011; 2012; Ellis et al., 2010; Spleen et al., 2013; Steinhauer et al., 2014; Lee et al., 2015) and DWV is largely a hidden problem it was considered appropriate to assess differences in DWV variants and load between the two groups to ascertain if differences in beekeeping operations (commercial or hobbyist) can influence DWV viral infections.

Both *Varroa*-tolerant and susceptible colonies were also targeted to allow for a comparison between DWV variants and *Varroa* treatment history and also to assess if resistance is occurring in the *Varroa* tolerant bees. Beekeepers provided details of if a colony was untreated or treated for *Varroa* alongside their samples. The cut-off point at which an untreated colony is deemed *Varroa*-tolerant was determined to be five years in this study. The rationale for this was based upon the fact that untreated colonies typically succumb to the effects of *Varroa* before this point (Korpela et al., 1992). The majority of colonies in this study were treated yearly and therefore are suspected to not have evolved traits seen in tolerant bees which have been left untreated for *Varroa* (Locke & Fries, 2011; Fries & Bommarco, 2007; Le Conte et al., 2007; Seeley, 2007; Loftus et al., 2017; 2017; Oddie et al., 2018) and as such are considered non-resistant to the effects of *Varroa*.

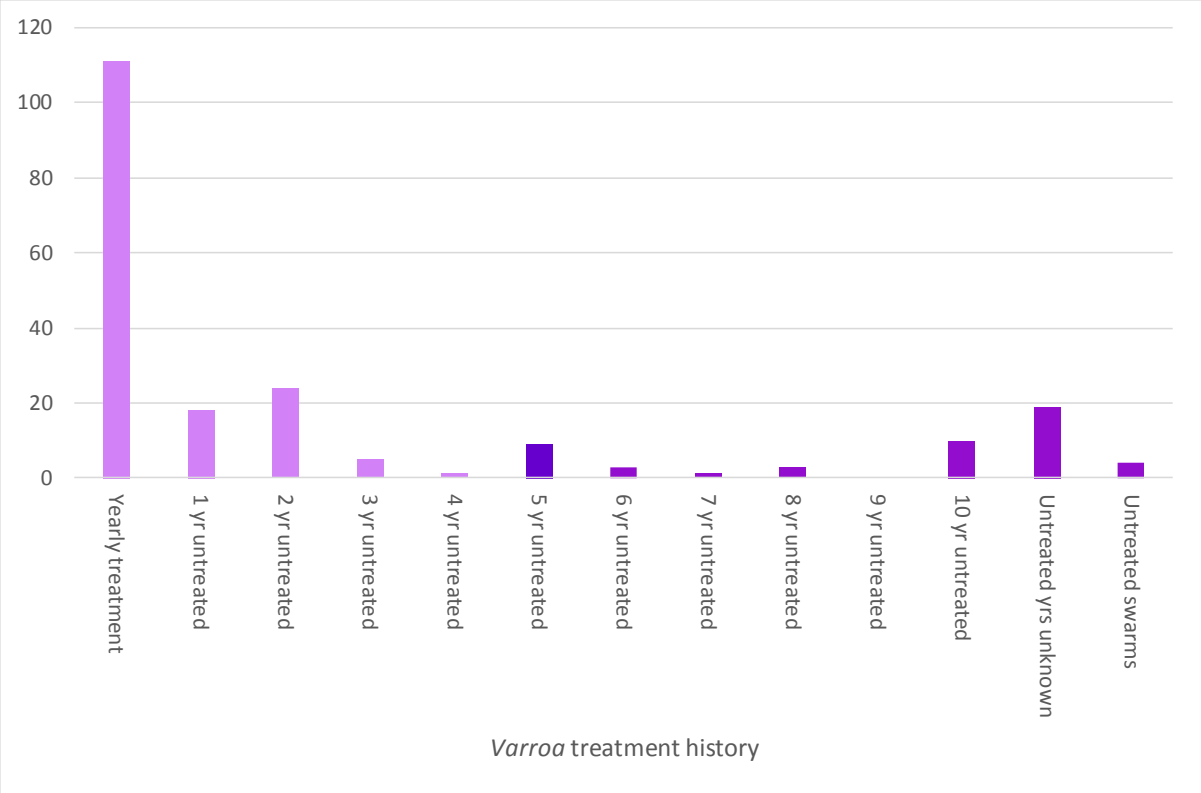

Figure 1. Number of samples provided from *Varroa* treated (pink) and untreated colonies (purple).

Samples were collected and stored in 100% ethanol, this was decanted prior transportation to the UK where upon arrival, molecular grade ethanol was added to the samples and stored at 4^{o}C. In total, 234 samples were received for analysis.

Sample protocol

Due to the large number of samples and time-consuming process of grinding up pooled whole bee samples for RNA extraction, an investigation into DWV load distribution for type-A and B between the bee head and body was conducted. This was done with the aim of speeding up sample processing but is also based on sound reasoning since this method has

been employed previously in studies for DWV (Yue & Genersch, 2005; Genersch et al., 2010, Zioni et al., 2011), acute bee paralysis virus (Siede et al., 2008) and Kukugo virus (Fujiyuki et al., 2004).

DWV type-A and B distribution in the heads and bodies of adult worker bees

Ten adult bees from the same colony, were removed from the ethanol, which was left to evaporate. Bees were inspected for any visual deformities and when seen these bees were discarded. The heads of honey bees were removed from the body and pooled separately into two groups, one containing heads only and the other group containing the remaining bee body parts (Thorax, abdomen and legs). The bee heads and body parts were then exposed to liquid nitrogen for approximately 30 secs. The bee heads were crushed into a homogenous powder within a 1.5 ml Eppendorf, whilst the bee bodies were crushed using a mortar and pestle. After each sample was crushed the bench and utensils were cleaned with bleach, Virkon and 70% ethanol to avoid cross contamination. In addition, an open test tube (blank) was left open during the crushing process. The blank was then subject to the RNA extraction process and analysed along with the samples using RT-qPCR, this step ensured a lack of airborne contamination.

RNA was extracted from 30mg of honey bee tissue from a pool of either ten bee heads or ten bee bodies using an RNeasy Mini prep kit (Qiagen) and quantified using a spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific), as per chapter one. RT-qPCR was conducted upon 50 ng/µl total RNA obtained from a pool of ten bee heads, using the

DWV-A, B, C specific primers and variant specific standard dilution series detailed in Chapter one. The data from this experiment shows that bee heads contain very similar DWV loads as bodies. On this basis and reasons given above all the subsequent USA samples had the RNA extracted from pools of 10 bee heads, processed in the same way as above.

RT-qPCR

RT-qPCR was performed on the 1 µl total RNA (50 ng/µl) extracted from a pool of 10 bee heads using a SensiFAST™ SYBR® No-Rox One Step kit (Bioline). Each reaction contained 10 µL SensiFAST™ SYBR® No-ROX One-Step mix (2×), 0.75 µL DWV forward primer (10uM), 0.75 µL reverse primer (A, B or C; Table 1), 0.2 µL reverse transcriptase, 0.4 µL RiboSafe RNase Inhibitor and 7.5 µL RNAse free H2O. The Reverse transcriptase step occurred at 45 °C for 10 min and denaturation at 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C 15 sec, annealing at 58.5 °C for 15 sec, and extension at 72 °C for 15 sec. A melt curve analysis was performed between 72 °C and 90 °C, at 0.1 °C increments, each with a 5 sec hold period. A no template control (NTC) was also included where 1 µl RNAse free water was used instead of total RNA. Both the amplification plot and melt curve were used to confirm there was no contamination of the controls.

Samples were analysed in triplicate, any samples which had a 3 Ct value deviation between triplicates were removed from the analysis, as 3 Ct values are the equivalent to a ten-fold change in viral load. Therefore, 209 samples were analysed, the RT-qPCR failed for 35 samples (more than 3 Ct value deviation between triplicates) and these results were

removed. In total, the analysis was conducted upon three samples for 61 apiaries, two samples from nine apiaries and one sample from eight apiaries.

Analysis of the results

To calculate the copy number of DWV master variant capsid genes RT-qPCR data for each DWV master variant was imported from the Rotor gene q series software® into Microsoft excel.

Copy numbers were calculated as follows as per Kevill et al., 2017:

$$\text{Copy number RNA} = (\text{Concentration RNA (ng/µL)} \times 6.022 \times 10^{23}) / (\text{Fragment length base pairs} \times 109 \times 325)$$

The genome equivalents per bee (DWV load), per variant were calculated as follows as per Kevill et al., 2017:

$$\text{Genome equivalents} = (\text{average copy number}) \times (\text{RNA dilution factor}) \times (\text{elution volume of RNA}) \times (\text{proportion of bee material})$$

The genome equivalent data for each variant was then summed and proportional DWV amount per variant was calculated as a percentage for each colony. The average DWV load per apiary was calculated from apiaries where more than one sample was received (n = 70). Apiary averages were converted into percentages to show the proportional DWV loads. Average DWV load per state was calculated by the sum of all DWV data per state and then the average calculated. Where only one apiary was sampled, the apiary average was used. In total, samples were provided from 34 states, 19 of these states provided samples from

more than one apiary. This data was inputted into excel, along with the state loss data obtained from the bee informed partnership.

Participating beekeepers were also contacted and asked to provide details of colony mortality for the overwintering period of 2016-2017. Briefly the questionnaire asked if the colonies which had been sampled survived or died and also asked for confirmation as to if a colony was untreated or treated for *Varroa*. Out of the 78 apiaries contacted, 45 returned an overwinter survey questionnaire.

Statistical analysis was performed using non-parametric tests upon the type-A and B data, as data were not normally distributed. Type-C positive samples were rare; therefore, the number of positive samples was too low to include in any statistical analysis e.g. n = <5. Spearman's Rho was calculated to test for correlations between DWV load per state and state losses. A Kruskal- Wallis test was used to analyse DWV variant viral load when comparing hobbyist and commercial beekeepers, the colonies that died or survived the overwintering period, and the *Varroa* susceptible and tolerant colonies, as this data is independent. When a significant result occurred post-hoc analysis was conducted using the Dunn's multiple pairwise comparisons (Dunn, 1961) which allowed for the identification of groups which differed significantly. The significance threshold was set at .05 for all three tests and when multiple comparisons were made the p values were adjusted using a Bonferroni correction (significance threshold / number of comparisons). The overall aim of the statistical analysis was to identify differences in DWV load and variant between the different groups.

To investigate inter-apiary diversity of viral strains, a 'city block' method was used as per Martin et al., (2012b). This test was carried out upon the proportional (%) DWV data for type-A, B, and C. An example of how this method is conducted is provided in Figure 2, here the differences between DWV type-A, B, and C percentage per colony were calculated and then summed. The sum of the absolute difference was then divided by three (number of sample groups) and further divided by 2 (degree of freedom (n-1)). This method produces scores between 0 (not diverse) - 100 (extremely diverse) and was used as it was considered a more transparent way to look at the data rather than using a complex analysis such as a three-way MANOVA. An example of how the city block scores were created is displayed in Figure 2.

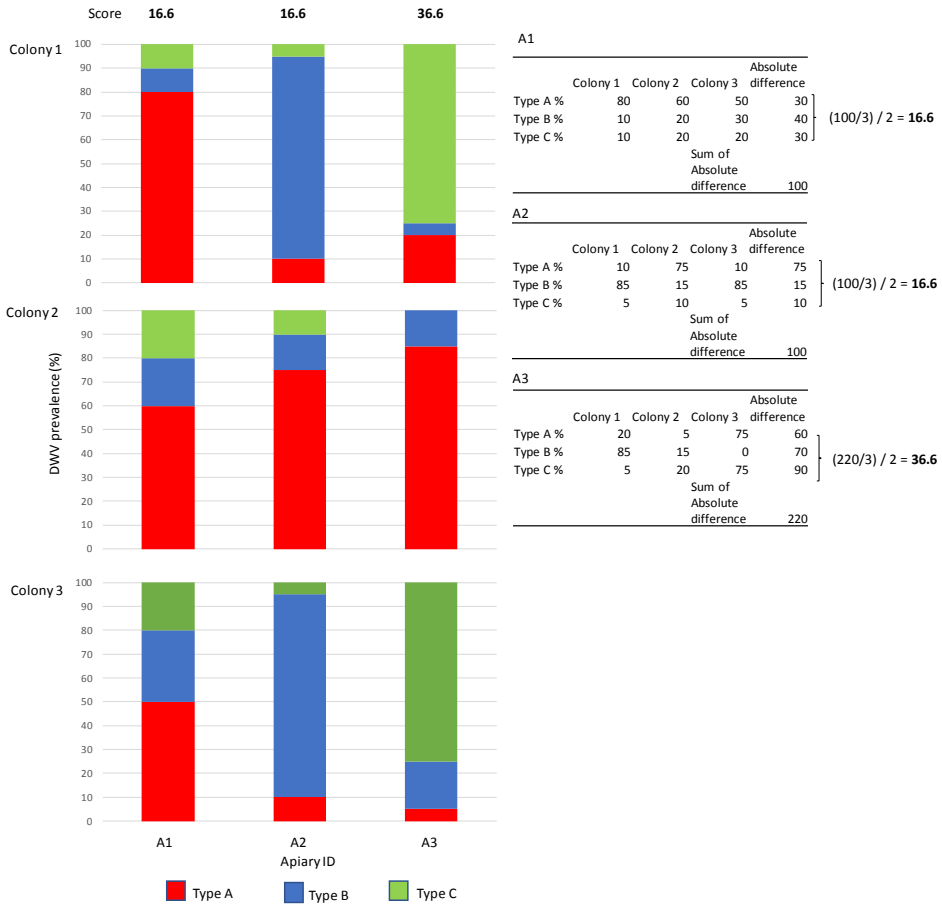

Figure 2. Example of how the 'city block' analysis was performed. Three colonies (colony 1, 2, and 3) per apiary (A1, A2, and A3) were analysed by calculating the sum of the absolute differences between each colony and dividing by the number of colonies and then further divided by the degrees of freedom (n-1). The 'city block' diversity scores are displayed in bold, 0 = no diversity, 50 = equal diversity and 100 = extremely diverse.

Results

DWV load in honey bee bodies verses heads

A comparison of DWV loads detected in individual adult worker bee heads and corresponding bodies reveals that DWV viral loads are similar between the bee head and bee body (Figure 3). A less than one-fold deviation between the head and corresponding body was witnessed for 83% of the type-A data and 80% of the type-B data, therefore confirming that the analysis of heads was sufficient for analysing DWV variant viral load.

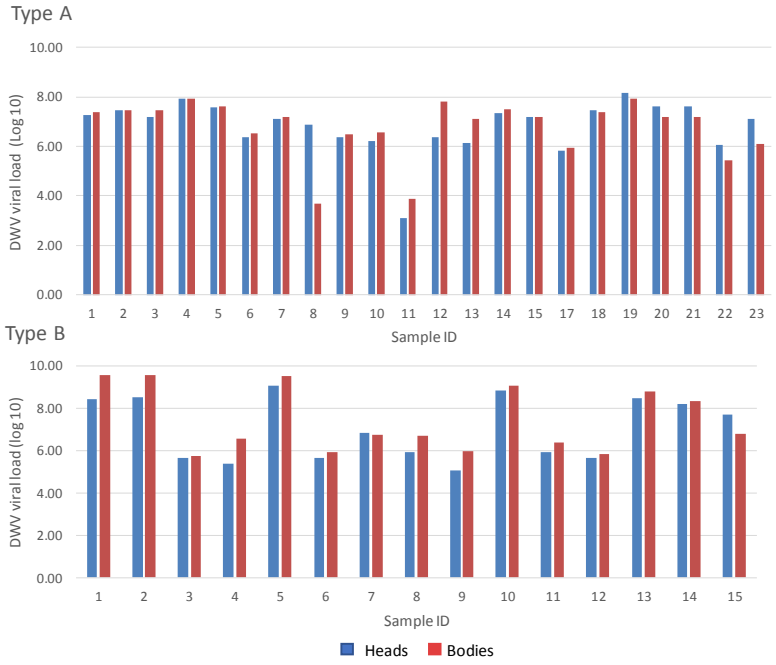

Figure 3. A comparison of DWV viral loads (type-A and B) found in an individual adult bee head and corresponding body.

Apiary level Analysis

DWV was prevalent in the USA 2016 pre-wintering bee population (Figure 4), type A dominated 63% of apiaries, whilst only 23% and 1% were dominated by type B and C, respectively. The remaining 13% of apiaries were positive below the limit of quantification. Analysis of the 209 colonies tested within the 78 apiaries prior to the overwintering period of 2016 reveals that at the apiary level DWV is widespread and DWV type-A has greater prevalence than type-B or C (Figure 4). When comparing the state average DWV load for all variants (type-A, B, and C) to the state average overwinter colony loss reported by the Bee informed partnership for the winter of 2016-17 the trends were non-significant (Spearman's Rho - Type-A - $r_s = 0.052$, $p = 0.76$; Type-B - $r_s = 0.066$, $p = 0.74$; Type-C - $r_s = 0.20$, $p = 0.45$; DWV total load - $r_s = 0.20$, $p = 0.25$) (Figure 5).

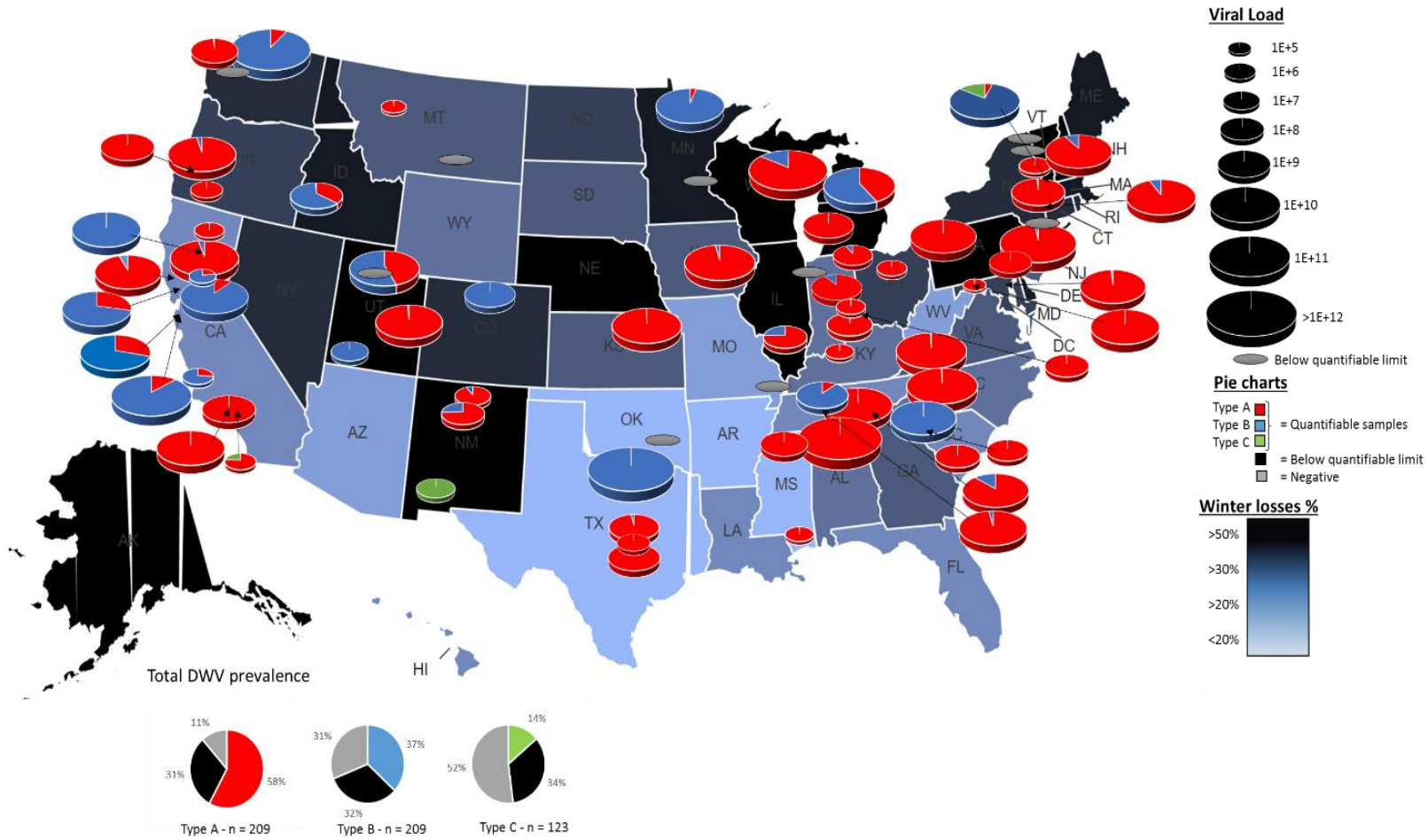

Figure 4. A map of the USA showing the average winter colony losses in 2016-2017 (Bee Informed Partnership, 2017), overlaid with the average proportional DWV load per variant, per apiary. Viral load is indicated by size of pie chart and DWV variant by colour. The map was created using https://createaclickablemap.com/.

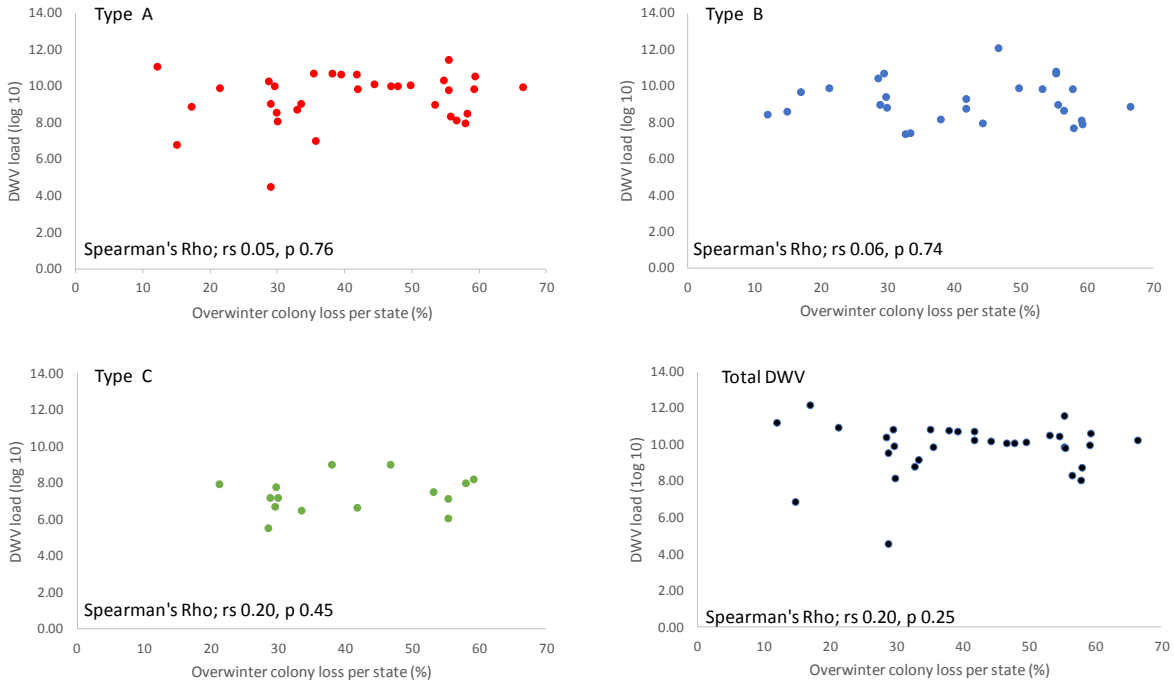

Figure 5. Comparison between the state average DWV load per variant and the average winter losses per state (Bee informed partnership, 2017).

DWV variant prevalence and load in hobbyist and commercial colonies

A high proportion of samples were co-infected with type-A and B. A comparison of DWV variants A and B reveals that hobbyist (n = 67) and commercial (n =11) colonies have a similar prevalence of type-A positive samples at 89% and 84%, respectively. Overall the percentage of type-A quantifiable samples (>1E+03) were higher in the hobbyist group, then the commercial at 61% and 42%, respectively (Figure 6). Conversely, quantifiable DWV type-B prevalence was greater in commercial colonies than hobbyist at 45% and 36%, respectively. Whilst prevalence varied, DWV type-A and type-B loads between hobbyist and commercial colonies (Figure 6) were found to have no significant difference between the

two groups for both DWV variants (Kruskal-Wallis - H = 2.53, df = 3, p = 0.46). Therefore, DWV prevalence and load is not affected by beekeeping operation.

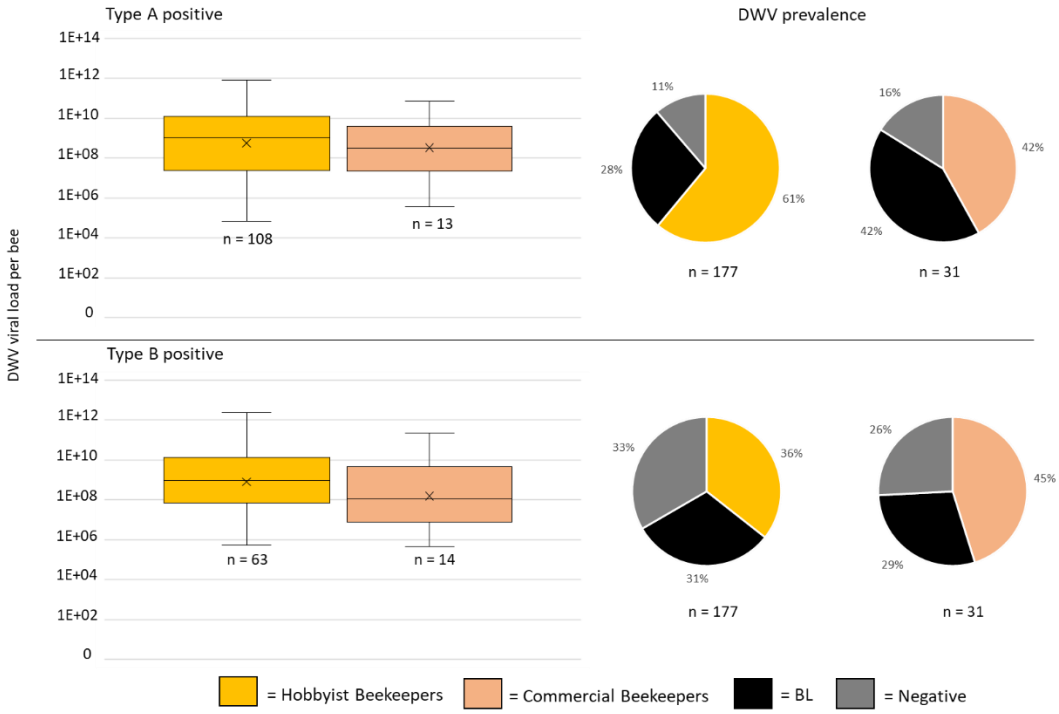

Figure 6. A comparison of DWV type-A and B viral loads and prevalence in commercial (pink) and hobbyist colonies (yellow). DWV prevalence and loads were similar between the two groups. Quantifiable DWV positives for hobbyist (yellow) and commercial (pink), positives below the quantifiable limit (BL) (black) and negative results (grey) are shown in the pie charts.

Colony level analysis

Across the whole data set, 95% of the 209 colonies, tested positive for DWV of these, 31% tested positive below the quantifiable limit, and 5% were negative of any DWV variant, showing that DWV is common and widespread throughout the USA (Figure 7). Prevalence of DWV variants at a colony level reveals a pattern of type-A > type-B > type-C (Figure 7). Type-A dominated infection represents 73% of DWV positive colonies, type-B was dominant in 24%, and type-C in 3%, showing type-A to be the most prevalent and dominant DWV variant in the USA (Figure 7).

Inter apiary DWV diversity analysis of colonies using the 'city block' method reveals that DWV infection in USA apiaries between colonies is highly conserved, as 75% (n = 61) of the 'city block' scores were <50, showing little diversity. Inter apiary diversity (city block score = > 50) did occur but in only 25% (n = 61) (Figure 8), here colonies had multiple DWV infections.

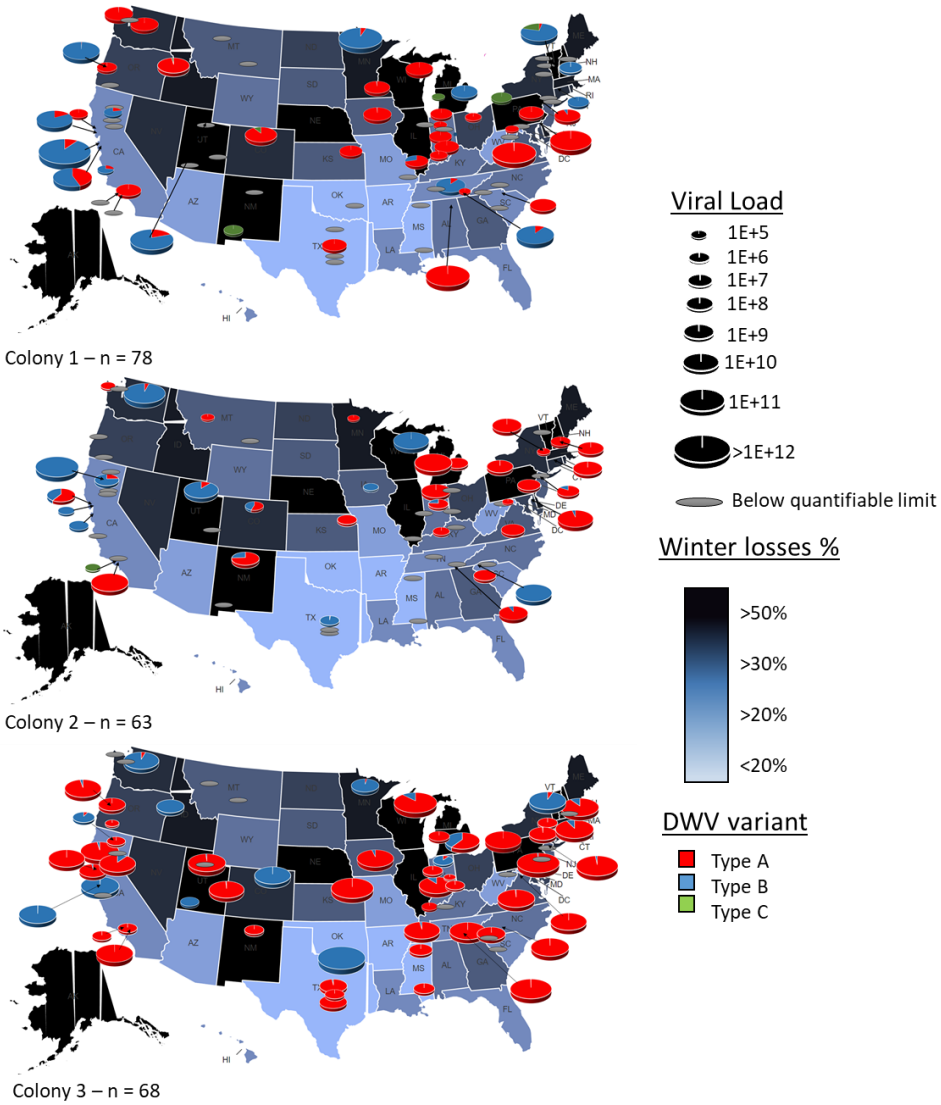

Figure 7. Map of winter losses and showing the proportional amount of DWV load per variant, per colony. Viral load is indicated by size of pie chart and variant by colour. The colour of each state represents the average percentage of winter colony loss, darker shade indicates high losses whilst paler shades represent low losses. Overwinter loss data was obtained from the Bee informed partnerships 2016-2017 colony loss survey. Preliminary loss data was plotted on to a map of the USA using https://createaclickablemap.com/.

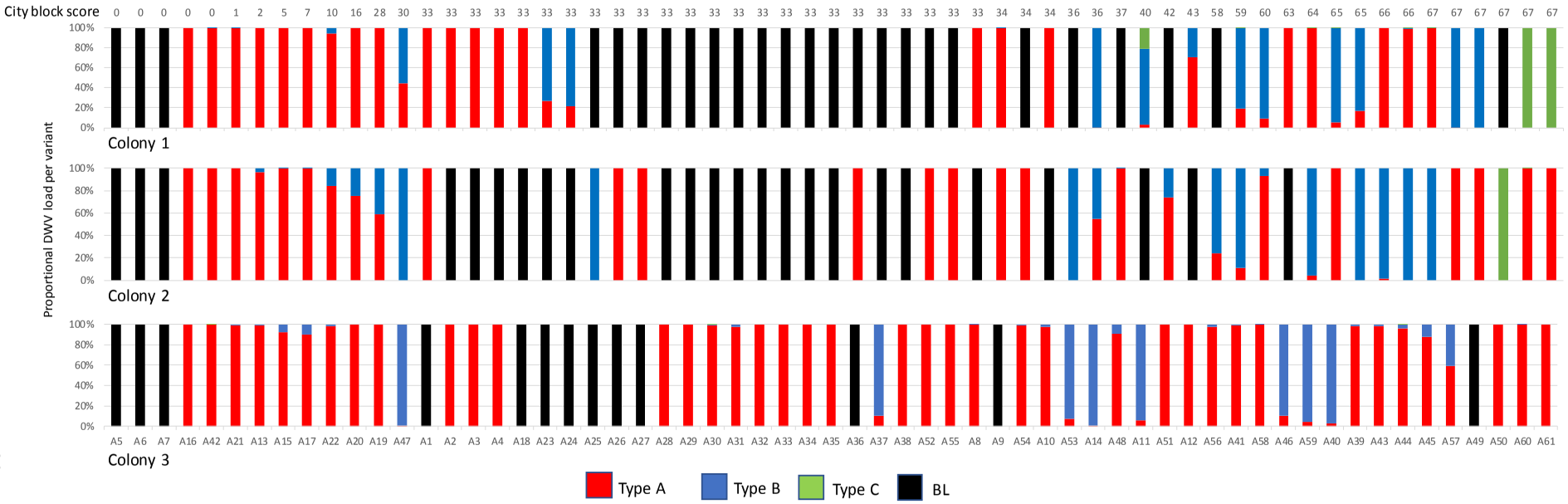

Figure 8. Inter apiary DWV diversity analysis, using the 'city block' method. Here the results of three colonies (colony 1, 2 and 3) from the same apiary have been stacked upon one another (reading downwards) and have been compared to test inter- apiary diversity. Black spaces in the data set represent colonies which were DWV positive below the quantifiable limit of detection (BL) and were not included in the analysis. The result of the 'city block' analysis is shown as the score, 0 = no diversity, 50 = equal diversity and 100 = extremely diverse. Results have been arranged from 0 – 100, reading left from right.

Colony Mortality

The returned overwinter loss surveys provided mortality data for 45 colonies. Colonies were separated into those which died (n = 27) and survived (n = 18). The results show a higher prevalence of DWV type-A and B quantifiable colonies (89% and 56%, respectively) for those that died during the winter of 2016-2017 (Figure 9). Whereas, DWV type-A and B prevalence were reduced (49% and 38%, respectively) in the colonies which survived (Figure 9). Statistical analysis revealed at least one significant difference between the groups (dead or alive) (Kruskal-Wallis – H = 15.2, df = 3, p = 0.0016), additional post-hoc analysis using a Dunn's test revealed that all groups had significantly different viral loads for both DWV type-A and B (Table 1). The box plots (Figure 9) show that type-A viral load in dead colonies (1E+08 – 1E+11) exceeded those of surviving colonies (1E+06 – 1E+10), furthermore type-A loads (1E+08 - 1E+11) in dead colonies exceeded those of type-B (1E+06 – 1E+10). Whereas, the surviving colonies had greater type B (1E+06 – 1E+12) loads than type-A and B for those which died (Figure 9). The difference in viral load accounts for the significant difference witnessed in the post-hoc analysis (Table 1).

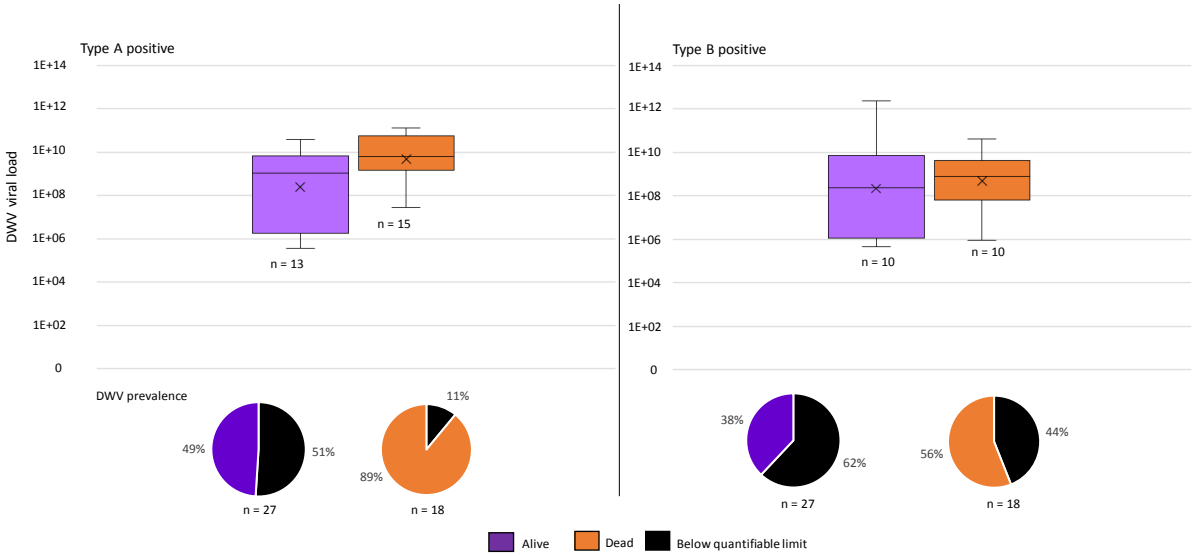

Figure 9. Box plots of DWV load for type-A and B positive colonies which survived the over winter period (alive – purple) and those which did not (dead - orange). DWV type-C was not found in the colonies which survived but was detected in two of the dead colonies. Pie charts show the percent of DWV quantifiable colonies and those which tested positive below the limit of quantification (BL).

Table 1. The p values as per Dunn's pairwise comparison of DWV Type A and B positive samples from colonies that survived or died during the overwintering period.

	Alive type-A	Dead type-A	Alive type-B	Dead type-B
Alive type-A		<0.001	<0.001	<0.001
Dead type-A			<0.001	<0.001
Alive type-B				<0.001
Dead type-B				

Varroa susceptible and tolerant colonies

DWV prevalence was similar between *Varroa* susceptible (n = 182) and tolerant colonies (n = 26), for the three DWV variants (A, B, C) (Figure 10). The percentage of type-A quantifiable *Varroa* susceptible and tolerant colonies was 54% and 59%, respectively. Type-A results which were positive below the quantifiable limit (BL) were also similar at 31% in susceptible colonies and 30% for the tolerant group. Type-B was quantifiable in 35% of susceptible colonies and 37% in the tolerant bees. Again, the percentage of samples below the quantifiable limit was similar for the susceptible and tolerant colonies at 27% and 32%, respectively (Figure 10). Susceptible colonies had a greater percentage of type-C quantifiable colonies (10%), however type-C prevalence was similar between the two groups as the percentage of colonies below the quantifiable limit was larger (>14%) in the tolerant group (Figure 10). In addition, no significant differences in viral load for type-A and B between *Varroa* susceptible (n = 182 from 70 apiaries) and tolerant colonies (n = 26 from 11 apiaries) occurred (Kruskal-Wallis - $H = 3.15$, $df = 3$, $p = 0.36$) (Figure 10). Therefore, these results indicate that tolerant bees may exhibit a trait which maintains DWV loads at <1E+10, similar to colonies which under-go *Varroa* control (susceptible) (Figure 10).

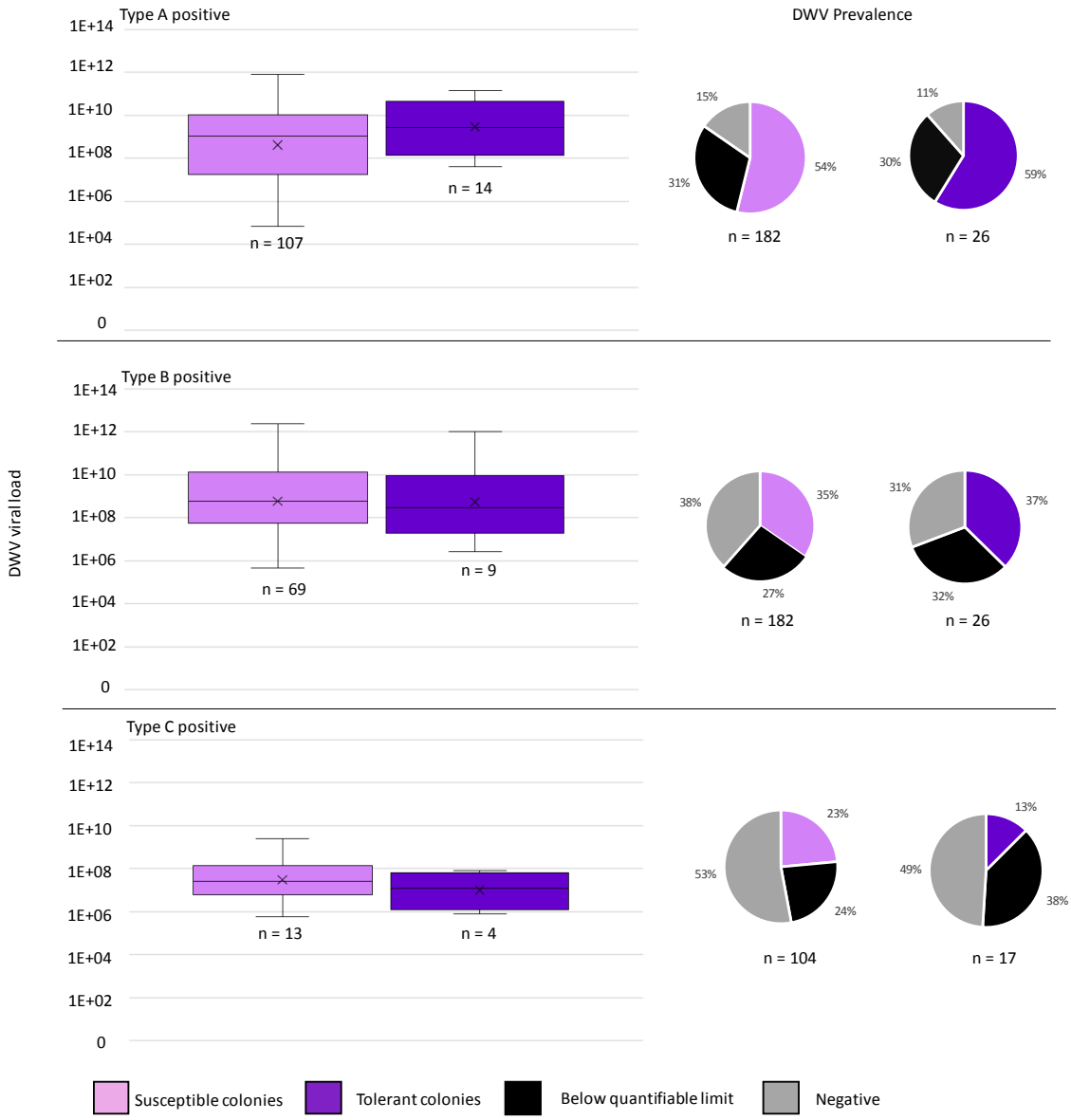

Figure 10. DWV load (box plots) and prevalence (pie charts) in *Varroa* susceptible (lilac) and tolerant (purple) colonies.

Discussion

In this study 95% of all 209 colonies sampled tested positive for DWV; of these 73% were predominately compromised of type-A, whilst type-B dominated 24% of the colonies and type-C was rare at 3% prevalence (Figures 4 & 7), dominance was defined as colonies with a greater than 50% proportional DWV load of one master variant. Non-significant correlations between the overwinter colony state loss data (Bee informed partnership) and DWV load per variant were witnessed (Figure 5) (Spearmans Rho - type-A - rs = 0.052, p = 0.76; type-B - rs = 0.066, p = 0.74, type-C - rs = 0.20, p = 0.45 and total load - rs = 0.20, p = 0.25), inferring that the correlations are very weak. However, further analysis of the results obtained from the overwinter mortality questionnaire provides evidence in support of type-A being a factor in overwinter colony losses when comparing colonies which survived the overwintering period to those that died (Figure 9). Here, type-A prevalence and viral loads were elevated in colonies which died, a result which was also reported in UK colonies sampled in 2007 (Highfield et al., 2009). In addition, the UK data presented in Chapter 2 shows that DWV type-B is dominant in England and Wales where overwinter losses for the same period (2016-2017) were recorded at 13.2%, which are considered low post-*Varroa* losses.

The DWV proportional load data was plotted onto data collected as part of the bee informed partnerships annual survey for 2016-2017. The bee informed partnership provides two data sets; one for total winter loss i.e. the total number of USA colonies reported lost that year and one for average winter loss per state. Total losses are considered to be representative of commercial losses (Randy Oliver personal communication). The average

loss rates are calculated per apiary, then the data is summed for each apiary and an average calculated per beekeeping operation per state, therefore these data are more representative of beekeeper losses. Study samples were largely provided by hobbyist beekeepers (n = 67). As such, the bee informed partnership's average winter loss rates are more representative of this studies data. No discernible difference between DWV prevalence or load when compared to hobbyist and commercial beekeepers was evidenced within these results (Figure 6). Therefore, there was no need to differentiate between commercial or hobbyist beekeepers during the data analysis.

The findings in the study reveal that when multiple infections are present between type-A and B (32% of the data, n = 66), type-A is found to be dominant (61%), however it is predicted that over time, type-B will outcompete type-A and slowly become the dominant DWV variant in the USA. This has been demonstrated in studies where DWV type-B was experimentally injected into pupae (Ryabov et al., 2014; Amanda Norton, personal communication) and adult bees (McMahon et al., 2017) and was shown to out-competed type-A. Type-B dominance is also seen in honey bee colonies screened in England and Wales, where DWV type-B dominated apiaries and colonies (Chapter 2). In 2010, few (2.6%) colonies tested positive for DWV type-B in the USA whereas DWV type-B is now widespread (64.5% of colonies positive) across the states (Ryabov et al., 2017; this study). Only 37% of colonies screened in this study showed quantifiable levels of type-B, however a total of 69% of colonies tested positive, a number in-line with that of Ryabov et al., (2017). It appears that DWV type-B needs time to establish itself in the viral landscape. Due to its ability to outcompete other variants of the virus, I predict that it will only be a matter of time before

DWV saturates the USA, as has happened in the UK. If type-B superinfection exclusion allows colonies to be resistant to more lethal DWV variants (Mordecai et al., 2016), then it is predicted that the number of overwinter colony losses recorded in the states will be reduced to a level currently witnessed in the UK.

Furthermore, the occurrence of type-B was higher in Californian colonies, where overwinter losses were lower compared to the rest of the USA (Bee Informed Partnerships preliminary data, 2017), which also fits the assumption that DWV type-B is avirulent under the host threshold (i.e. type-B loads can exceed those of type-A and bees survive). It is also worth noting that 6 of the 13 Californian apiaries were commercial operations in which some had selectively bred bees from survivor populations, in a bid to increase resistance (Randy Oliver, personal correspondence). Here, it appears that DWV type-B has been inadvertently selected for, a situation which also occurred in a seemingly resistant population of UK honey bees (Mordecai et al., 2016). California was not solely comprised of type-B but also type-A; of all surveyed colonies which died (n = 18) only two were from a Californian apiary, these colonies were dominated by type-A. The presence of type-A in high loads, in pre-winter bees which went on to die, further supports that type-A is a causative factor behind high losses witnessed in the USA. A similar situation occurred in Hawaii after the arrival of *Varroa*, here colonies were subject to pressures from *Varroa* but also DWV infection predominately comprised of type-A, this resulted in the subsequent loss of *Varroa* untreated colonies (n = 274 out of 419) and the loss of feral colonies from Oahu Island (Martin et al., 2012).

An interesting result occurred when comparing the *Varroa* susceptible and tolerant bees, as no notable difference was seen in DWV load and prevalence between variants regardless of treatment. The susceptible bees were subject to yearly *Varroa* treatments. Treating for *Varroa* is considered an important factor in managing honey bee health and when mite numbers are reduced, DWV infection is also reduced (Martin et al., 2010; Locke et al., 2017). The tolerant bees were untreated for *Varroa* for greater than 4 years, the maximum number of years untreated was 17, these colonies are considered true survivors as colonies typically die between 4-5 years (Korpela et al., 1992). This result indicates that tolerant bees have developed a trait which keeps DWV infection in-line with those of treated bees. European honey bees which exhibit *Varroa* resistance have also been reported elsewhere in the world, (Rinderer et al., 2001; Le Conte et al., 2007; Seeley, 2007; Locke & Fries, 2011), with a wide range of tolerance factors being present; Grooming (Rinderer et al., 2001), Hygienic behaviour (Rinderer et al., 2001; Mikheyev et al., 2015), small colony size (Locke & Fries, 2011; Fries & Bommarco, 2007), frequent swarming (Loftus et al., 2017) and more recently the recapping of honey bee brood (Oddie et al., 2018), to name a few. Therefore, tolerance to *Varroa* may be attributed to any trait which hinders *Varroa* reproduction, presumably reducing *Varroa*-DWV viral transmission, however more research needs to be conducted upon *Varroa* reproduction in colonies exposed to long term untreated *Varroa* infestations and the relationship of DWV variants.

In addition to the studies mentioned above, it would be sensible not to ignore the fact that Africanised bees are present in some of the southern states of the USA, these are; South California, Nevada, Arizona, South Utah, New Mexico, Texas, Arkansas, Louisiana, Florida

and Oklahoma. Africanised bees exhibit a range of natural tolerance mechanisms to *Varroa*, which are not exhibited in the closely related European honey bees. Africanised bees build slightly smaller worker brood cells (4.84mm) than other races of bee (>5mm) (Piccirillo & De Jong, 2003), the worker brood of Africanised bees is two times less attractive to reproductive *Varroa* females than the worker brood of European honey bees (Guzman-Novoa et al., 1996) and Africanised bees are also more sensitive to *Varroa* volatiles which triggers the removal of mites and brood (Aumeier & Rosenkranz, 2001), this explains why the reproductive rates of *Varroa* are poor in Africanised colonies (Martin & Medina, 2004). Only four apiaries analysed in this study had Africanised bees, these were located in New Mexico, Texas, Oklahoma, and Tennessee (H. Kevill, 2016, master's dissertation). The apiaries in Oklahoma and New Mexico were from colonies which were considered true survivors, whilst the apiaries in Texas and Tennessee were treated for *Varroa*. In addition, the beekeepers which provided these samples were not aware that they had Africanised bees in their apiary. DWV infection in these bees was below the quantifiable limit of detection in 7 out of 11 colonies screened. The remaining four colonies had DWV infections comprised mostly of type-A (n = 3) with viral loads <1E+09. Therefore, it is speculated that Africanisation results in increased resistance to *Varroa* and therefore DWV, however studies which concentrate upon Africanised bees are required to evidence this further due to the low number of Africanised colonies in this study.

Conclusion

DWV type-A is the most dominant and prevalent variant in colonies screened, whilst type-B was commonly detected in colonies it was only found to dominate 24%, and type-C was

found to be rare. All of the colonies sampled in this study were from pre-winter bees, therefore colonies generally entered the overwinter period with high loads of DWV type-A, particularly on the east coast of the USA where overwinter colony losses were typically higher than the west coast. Colonies which are considered tolerant to *Varroa* had similar prevalence, load and variants of DWV as those which are susceptible, indicating that these bees have developed a mechanism which allows them to survive infections that may otherwise be fatal. Further field and lab studies need to be conducted to assess the function behind *Varroa* and DWV resistance. The situation in the USA appears to be hopeful considering that the less virulent variant of DWV (type-B) is currently becoming established in honey bee populations. If type-B infections are maintained it is predicted that a reduction in the number of overwinter colony losses will be witnessed in the upcoming years, however the situation will need to be closely monitored.

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Chapter 4: The role of DWV avirulence and superinfection exclusion in wild colonies of honey bees located in the Arnot forest and Ithaca, New York

Abstract

Overwinter colony losses of European honey bees are regularly reported in the northern hemisphere. These losses are now suspected to be caused by the *Varroa* mites ability to vector a number of viruses, with Deformed wing virus (DWV) often detected in colonies which die. However, these losses are less severe in some populations of bee which have developed a natural tolerance to *Varroa* over time. One such population are located outside of Ithaca, upstate New York, in the Arnot forest and are the focus of this study. Researchers suggest that *Varroa* resistance occurs in the Arnot honey bee colonies due to a number of life history traits and avirulent *Varroa* mites. However, no viral work has ever been conducted. Over a three-year period (2015-2017), honey bee colonies were collected from the Arnot forest and Ithaca. These were screened for the three DWV master variants to establish if these are avirulent. The results show that avirulence is not a factor in survival as DWV type-A infection steadily increased between sample years (2015, 2016, 2017) whilst type-B and C steadily decreased. Furthermore, the type-B variant was often detected below the quantifiable limit, ruling out superinfection exclusion as a mechanism for resistance. Whilst DWV avirulence and superinfection exclusion have been disregarded as resistant factors, type-A DWV loads rarely exceeded 1E+10 and were maintained between seasons (summer and autumn). This result suggests that Arnot forest bees are surviving due to their ability to halt a seasonal increase in DWV load.

Introduction

Populations of European honey bees (*Apis mellifera*) have been exploited by humans since 7000 BC when early Neolithic farmers in Europe, the near east, and North Africa started to harvest bee products (Roffet-Salque et al., 2015). The popularity of apiculture is still present in modern day society and European honey bees have been exported outside of their native range to Eastern and Central Asia, America, and Australia (Moritz et al., 2005).
Both the America's and Australia/New Zealand did not have a native *Apis* species before the introduction of European honey bees.

The movement of European honey bees, into areas with endemic honey bee populations led to a jump in host species of the ectoparasite *Varroa destructor,* which originally infected Asian honey bee (*Apis cerana*) colonies as *Varroa jacobsoni*. The host jump from *A. cerana* to *A. mellifera* was first reported in Japan and in 1957 (Sakai & Okada, 1973), followed by reports in the former Soviet Union, Korea (Crane, 1978), South America (Goncalves et al.,1987), North America and then Europe, with Australia being the only exception (Rosenkranz et al., 2010). Co-evolution between *Varroa* and its original host *A. cerana* has allowed for the adaptation of behavioural and physiological traits which prevent exponential growth of *Varroa* mites within a colony. Asian honey bees typically maintain mite numbers of less than 800 mites per colony (Rath, 1999), whereas *Varroa* populations can increase 2000-fold within one year in a European colony (Martin, 1998), as a host-parasite equilibrium has not yet evolved (Oldroyd, 1999).

European honey bees have little tolerance to *Varroa* and heavily infested colonies are severely affected. Once *Varroa* is prevalent within a colony, viral infection increases; this has been well evidenced for *Varroa* and Deformed wing virus (DWV) (Genersch & Aubert, 2010; Martin et al., 2012; 2013). DWV is now the most commonly detected virus of honey bees due to *Varroa*-mediated transmission (Sumpter & Martin 2004; De Miranda & Genersch 2010; Rosenkranz et al. 2010). It is often during the winter months when DWV loads reach their maximum and colonies succumb to disease (Berthoud et al., 2010; Dainat, et al., 2012; Highfield et al., 2009; Martin et al., 2010; 2013; Nazzi et al., 2012; Francis et al., 2013). Therefore, beekeepers of European honey bee colonies use a number of mite control methods to reduce mite numbers and in turn the adverse effects of a *Varroa* infestation.

To date, beekeepers have reduced the number of *Varroa* mites in apiaries by using several control mechanisms from chemical treatments to colony manipulations (Rosenkranz et al., 2010). These control methods come with consequences from disrupting normal colony growth, chemical residue in bee products, to queen death (Le Conte, Ellis, & Ritter, 2010). Therefore, beekeepers are eager to see *Varroa* abolished from colonies, with breeding programs concentrating on resistance being one of the only feasible solutions to date (Neumann & Carreck, 2010; Carreck, 2011). However, human intervention is limiting natural selection for *Varroa* resistance in managed populations of honey bees (Neumann & Blacquiere, 2016). Therefore, bees which have evolved a natural tolerance to *Varroa* are of interest to researchers and beekeepers alike.

Fortunately, European honey bees which have developed a natural tolerance to *Varroa* have been reported from around the world; These populations are located in France (Le Conte et al., 2007), Brazil (DeJong & Soares, 1997), Sweden (Fries et al., 2003; Fries & Bommarco, 2007), UK (Mordecai et al., 2016) and the USA (Seeley, 2007). Factors which contribute to resistance are; low-stress factors, good environmental conditions (Le Conte et al., 2007), grooming, hygienic behaviour (Rinderer et al., 2001), small colony size (Locke & Fires, 2011, Loftus et al., 2016), frequent swarming episodes (Seeley, 2017; Loftus et al., 2016), avirulence of *Varroa* mites (Seeley, 2007) and associated disease (Mordecai et al., 2016; Brettell & Martin, 2017).

The most researched population of *Varroa* resistant honey bees are those found in the Arnot forest located just outside of Ithaca in New York. The Arnot forest is Cornell University's 4200-acre research forest, and the bee population here has been under investigation since 1978 (Seeley, 2007). In 1978 Tom Seeley conducted a census of the honey bee population, which was repeated in 2002, post-*Varroa's* arrival. The census found that honey bee populations are at similar levels to those pre-*Varroa*. Since then, several studies have been conducted to understand the mechanism which enables the Arnot forest bees to co-exist with *Varroa*.

Past studies have found that Arnot colonies have similar *Varroa* reproductive rates as Carniolan bees (Seeley, 2007), colonies will die once *Varroa* mite drop exceeds 100 mites (Seeley, 2017), and that *Varroa* mite populations increase along with available brood space

(Loftus et al., 2016), however wild populations of bees exist without any human intervention. Genetic analysis of Arnot queen mating frequencies found that they are the same as those in managed bees. Therefore resistance cannot be attributed to increased intra-colony genetic diversity (Seeley et al., 2015). Further genetic studies found that the Arnot bees endured a mitochondrial genetic bottleneck shortly after the arrival of *Varroa* (Mikheyev et al., 2015). The selection of 232 genes were shown in this population. However, none of the genes under selection were associated with an immune response against viral infection, suggesting that resistance to viral disease is not a factor in this population's survival (Mikheyev et al., 2015; Locke, 2016). The genes under selection in the Arnot bees are implied with hygienic behaviour and development. Changes to bee development can hamper the *Varroa* mite's reproductive success and is a factor behind tolerance to *Varroa* in Africanised bees (Martin & Medina, 2004). To explain if these genes are expressed due to trade-offs made in response to *Varroa*, Seeley conducted a six year study upon Arnot forest bees from 2010-2016 (Seeley, 2017) and compared the results of life history traits to similar data obtained pre-*Varroa*. He found that the Arnot bees life history traits remained the same as they were pre- *Varroa*, these are; slow development of brood, an extended adult stage, and few but costly offspring. He also discovered that queen turnover is vital in established colonies, as colonies which did not requeen (e.g. by swarming) in the summer months had increased mite populations. Once mite drop exceeded 100+ mites per colony, they began to succumb to the negative effects of *Varroa*. This result inferred that bees are resistant to *Varroa* due to regular swarming episodes mediated by small nest size (Loftus et al., 2016), but also led Seeley to question to the role of avirulent mites and viruses in these populations.

The long-term survival of this population of bees suggests that mechanisms for resistance to *Varroa* do exist and may be partly explained by DWV viral populations. This was found to be the case for one of the oldest *Varroa* resistant honey bee populations located in Brazil (Brettell & Martin, 2017), which were found to have avirulent forms of DWV present in low viral loads (<1E+05). It was also the case for a population of honey bees located in Swindon, UK. The Swindon colonies were infected with DWV type-B (Mordecai et al., 2016), which was evidenced to out-compete and suppress the replication of DWV type-A in honey bee colonies. Therefore, Seeley (2017) may be correct when suggesting that mites are avirulent or have avirulent forms of viruses. However, no viral studies have so far been conducted upon the Arnot forest bees.

As the Arnot forest bees have apparent *Varroa* infestations, and there is a proven association between DWV and *Varroa*, it is likely that DWV is present within the colonies. Therefore, this research aims to assess the prevalence and viral load of the three known DWV master variants (type-A, B, and C) in colonies from the Arnot forest and surrounding area of Ithaca. The role of superinfection exclusion via DWV type-B infection and seasonal DWV variant prevalence and viral loads are under investigation. A comparison of DWV infections in Arnot and Ithaca bees, *Varroa* susceptible bees from surrounding states (New York, Connecticut, Pennsylvania, and Vermont), and also *Varroa* tolerant bees from the USA (See Chapter three) will be also made to see if the Arnot forest bees differ from those in surrounding areas. Thus, allowing DWV via superinfection exclusion or avirulence to be considered as a factor in the long-term survival of these colonies.

Methods

Sample history and collection

Honey bee samples from the Arnot forest and Ithaca were provided by David Peck, Cornell University, New York, for the years 2015 (n = 20), 2016 (n = 49), and 2017 (n = 16). Approximately 30 adult honey bees were received from 85 colonies sampled. All samples were collected from the front of the hive entrance and contained older foragers and young bees taking orientation flights. Honey bee samples were stored and collected in 100% ethanol; this was decanted prior to transportation to the UK, where upon arrival molecular grade ethanol was added to the samples which were then stored at 4^{o}C. Only the colony ID and date of sample collection were provided with the samples; colony histories were unknown until analysis was complete, allowing for a blind study.

The data from all sample years (2015, 2016, 2017) dependant of colony history were categorised into the following groups; yard summer (n = 18), yard autumn (n = 28), wild summer (n = 16), wild autumn (n = 11), control summer (n = 4), and control autumn (n = 8). Yard bees are colonies founded by pristine Arnot forest swarms caught within the forest interior. The pristine Arnot swarms were kept in experimental bee yards (Figure 1) on the Arnot forest perimeter, at a distance of 21-73 meters apart from each other. Yard bees were only managed to prevent swarming; this involved adding extra supers (honey storage space) to create more room, and when colonies became too large they were split into two. The yard bee samples for 2015 are primarily (6 samples out of 9) from pristine Arnot forest swarms which had just been experimentally placed and as such will closely represent the viral population of Arnot forest bees. Wild bee samples are from long-term surviving feral

colonies of unknown origin. These colonies were located in the Ithaca area and occupy buildings, trees and experimentally placed bait hives (Empty hive containing empty brood comb in which swarms will move into). Control colonies are from commercial stock purchased from Vermont and California, these colonies were kept in separate bee yards in the Ithaca area and are considered to exhibit no resistance to *Varroa* or associated viruses.

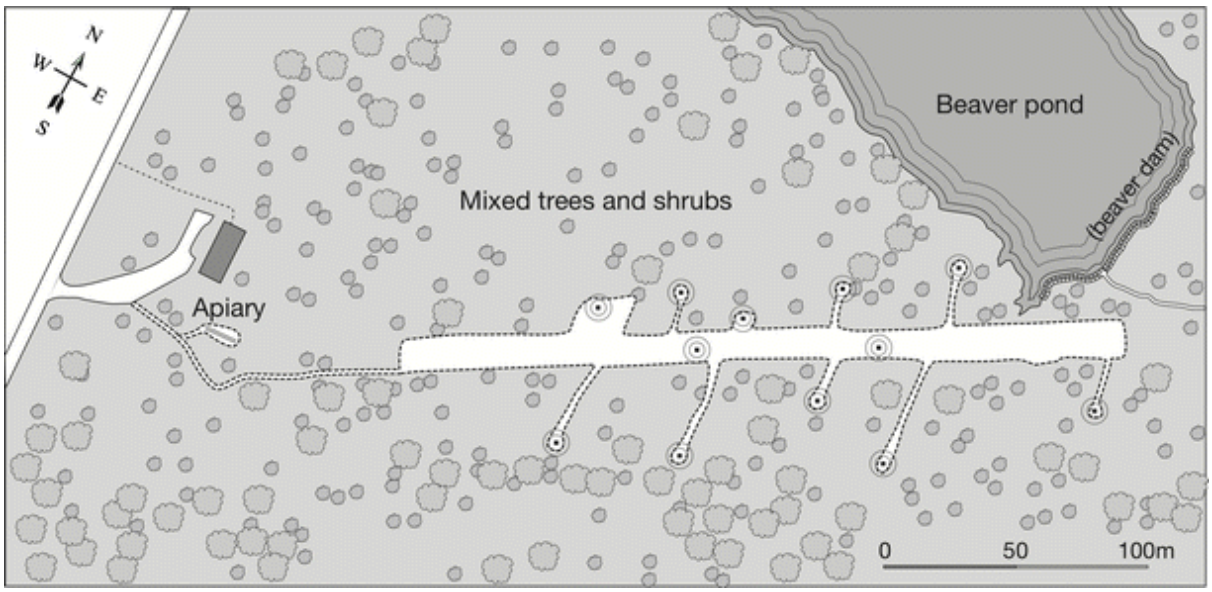

Figure 1. Example of the experimental bee yard in which the yard bees were kept. The experimental bee yard is shown by the large white area surrounded by mixed trees and shrubs. Colonies are indicated by black dots surrounded by circles (Seeley & Smith, 2015).

Additional samples collected in 2016 were also included in the analysis. These samples were grouped as 2016-SS and 2016-U. The 2016-SS sample group were collected from *Varroa* susceptible colonies located in New York (n = 8) (outside of the Arnot forest and Ithaca) and the surrounding states of Connecticut (n = 3), Pennsylvania (n = 3), and Vermont (n =6). Therefore, allowing a comparison of DWV viral diversity, prevalence, and load between

colonies that are *Varroa* susceptable and tolerant colonies. The 2016-U samples are from colonies which have survived untreated for *Varroa* > 5 years and are considered *Varroa* tolerant (see Chapter three), therefore allowing for a comparison with other resistant populations of bees. These colonies were located in Alabama (n = 1), California (n = 5), Iowa (= 1), New Hampshire (n = 1), New Mexico (n = 2), South Carolina (n = 2), and Virginia (n = 2).

Sample processing

Total RNA was extracted from a pool of ten adult worker bees per colony for 2015, 2016, and 2017 Arnot and Ithaca samples. Bees were removed from the ethanol, which was left to evaporate. Once the ethanol had evaporated bees were inspected for any visible deformities and when seen these bees were discarded. The ten bees selected for analysis were then exposed to liquid nitrogen and crushed into a homogenous powder using a mortar and pestle. The homogenate of the ten whole bees was then transferred into a 1.5 ml Eppendorf tube.

RNA was extracted from 30mg of pooled honey bee homogenate, using an RNeasy Miniprep kit (Qiagen) and quantified on a spectrometer (Nanodrop 2000, Thermo Fisher Scientific), as per Chapter one. RT-qPCR was conducted upon 50 ng/µl total RNA, using the DWV A, B, C primers and variant specific standard dilution series detailed in Chapter one. The 2016-SS and 2016-U had already had the RNA extracted as per Chapter two, i.e. using pools of 10 bee heads per sample.

RT-qPCR

RT-PCR was performed on the 1 µl total RNA (50 ng/µl) extracted from a pool of ten whole bees, using a SensiFAST™ SYBR® No-Rox One Step kit (Bioline). Each reaction contained 10 µL SensiFAST™ SYBR® No-ROX One-Step mix (2×), 0.75 µL DWV forward primer, 0.75 µL reverse primer (A, B or C; Table 1), 0.2 µL reverse transcriptase, 0.4 µL RiboSafe RNase Inhibitor, 7.5 µL RNase free H2O. The reverse transcriptase step occurred at 45 °C for 10 min and denaturation at 95 °C for 10 min, followed by 35 cycles of denaturaturation at 95 °C for 15 sec, annealing at 58.5 °C for 15 sec and extension at 72 °C for 15 sec. A melt curve analysis was performed between 72 °C and 90 °C, at 0.1 °C increments, each with a 5 sec hold period. RT-qPCR also contained a no template control (NTC) where 1 µl RNase free water was used instead of total RNA. Both the amplification plot and melt curve were used to assess if contamination occurred in the controls.

Samples were analysed in triplicate, against a ten-fold dilution series, specific to each DWV variant (A, B, or C). Any samples which had a 3 Ct (critical threshold) value deviation between triplicates had the RT-qPCR step repeated, as 3 Ct values are the equivalent of a ten-fold change in viral load. This ensured that a precise DWV copy number was obtained for each sample.

Data analysis

RT-qPCR data for each DWV master variant was imported from the Rotor-gene q series software® into Microsoft Excel. Sample data which fell inside the range of the standard curve at 7 Ct's – 30 Ct's was deemed a quantifiable positive, whilst samples which fell beyond the standard curve at 30 Ct values were deemed below the quantifiable limit (BL), samples with no amplification curve on the amplification plot and melt curve were considered DWV negative. This data was further categorised into sample year (2015, 2016, 2017) and the percentage of positive, BL (positive below the quantifiable limit) and negative samples per DWV variant, for each year, were calculated allowing for an annual comparison of DWV prevalence. Colonies were categorised per their life history regardless of year to allow for a comparison of DWV prevalence between groups (yard, wild, and control) with different life histories.

Viral copy numbers were determined using the following equation as per Kevill et al (2017):

$$\text{Copy number RNA} = (\text{Concentration RNA (ng/µL)} \times 6.022 \times 10^{23}) /$$

$$(\text{Fragment length base pairs} \times 109 \times 325)$$

The DWV genome equivalents per bee (DWV viral load), per variant (DWV type-A, B or C) were calculated for positive samples using the following equation as per Kevill et al., (2017):

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

The DWV load for each variant were then transformed using the $\log^{10}$ function in excel. Positive samples were divided by year and also the groups; yard, wild, and control, per season (summer and autumn). The $\log^{10}$ data for positive samples were then used to create the box plots shown in the results section. Statistical analysis was performed using non-parametric tests as the data were not normally distributed. A Kruskal- Wallis test was used to compare DWV type-A load per year, season and group (yard, wild, control, 2016-SS, and 2016-U) as this data is independent of each other. When a significant result occurred post-hoc analysis was conducted using the Dunn's multiple pairwise comparisons (Dunn, 1961) which allowed for the identification of groups which differed significantly. The significance threshold was set at $p = <0.05$ for all three tests and when multiple comparisons were made the p values were adjusted using a Bonferroni correction (significance threshold / number of comparisons). The overall aim of the statistical analysis was to identify differences in DWV load and variant between the different groups. Type-B and C positive samples were rare between years and groups; therefore, the number of positive samples were too low to include in the statistical analysis, e.g., $n = < 5$.

Results

DWV master variant prevalence per year

DWV infection in honey bees from the Arnot forest and surrounding area of Ithaca in 2015, 2016, and 2017 were primarily comprised of DWV type-A (Figure 2). A steady increase in the percentage of type-A positive and quantifiable samples was witnessed for all sample years (2015 - 45%; 2016 - 70%; 2017 - 75%) (Figure 2). This corresponds with a yearly reduction in DWV type-B positive colonies, which fell from 20% in 2015 to 0% in 2017 (Figure 2). DWV type-C was rare and only detected in two colonies in 2016 (Figure 2). Therefore, the remaining analysis mainly concentrated upon DWV type-A.

A Kruskal Wallis test of the type-A viral load between sample years, colonies from surrounding states (2016-SS) and USA *Varroa*-tolerant colonies (2016-U) were conducted (Figure 2, Table 1). This result provided strong evidence of a difference in median rank between at least one pair of groups (Kruskal-Wallis test - n 53, H = 12.99, df = 4, p = 0.015). Therefore, Dunn's pairwise comparisons were made for all five groups (Table 1). A significant difference in DWV type-A load was witnessed between the 2015 and 2016 Arnot forest and Ithaca colonies (Dunn's post-hoc test – p = 0.029). This result is attributed to an increase in mean viral load from 1E+07 in 2015 to 1E+09 in 2016 (Figure 3). A significant difference also occurred between the 2015 Arnot and Ithaca colonies, and 2016-U colonies (Dunn's post-hoc test; p = 0.008). Again, this result is due to a difference in viral load between Arnot and Ithaca 2015 (1E+07) and 2016-U (1E+10) colonies (Figure 3, Table 1). No difference in type-A viral load was recorded between 2016, 2017, 2016-SS, and 2016- U

colonies (Figure 3, Table 1). This result indicates that for sample years 2016 and 2017 the Arnot forest and Ithaca bees had DWV type-A loads similar to surrounding states and also USA tolerant bees.

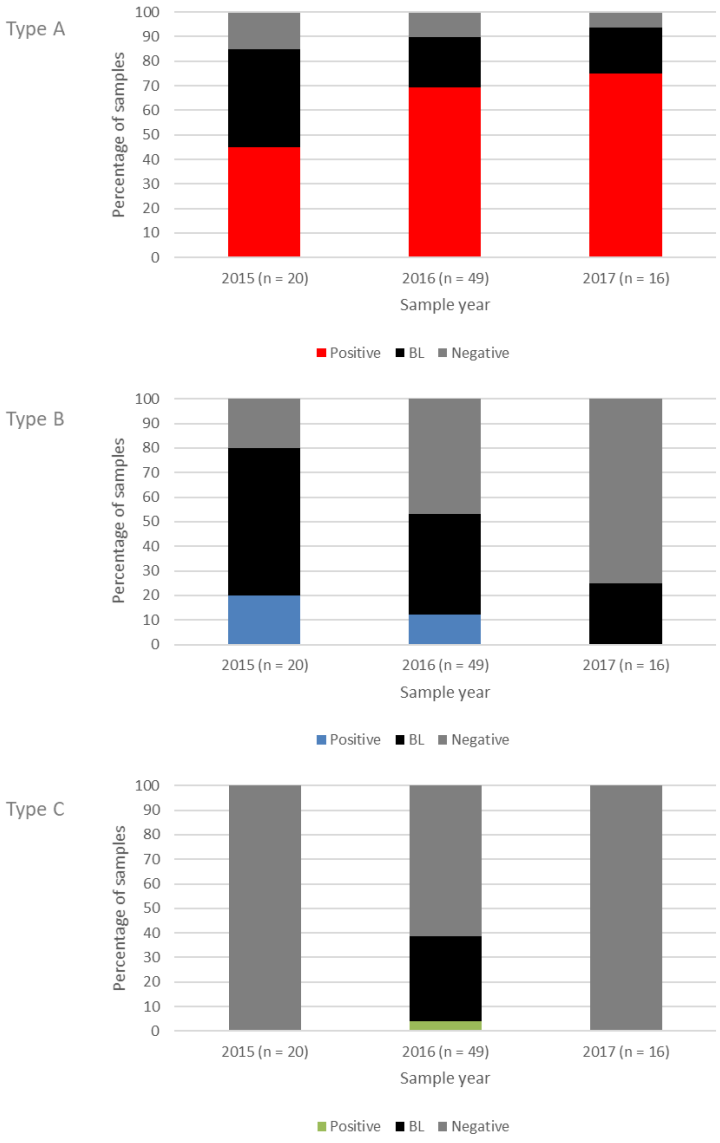

Figure 2. The proportion of Arnot and Ithaca honey bee samples which were DWV positive (type-A = red; type-B = blue; type-C = green), positive below the limit of quantification (BL) (black) or negative (grey) for the years 2015, 2016 and 2017.

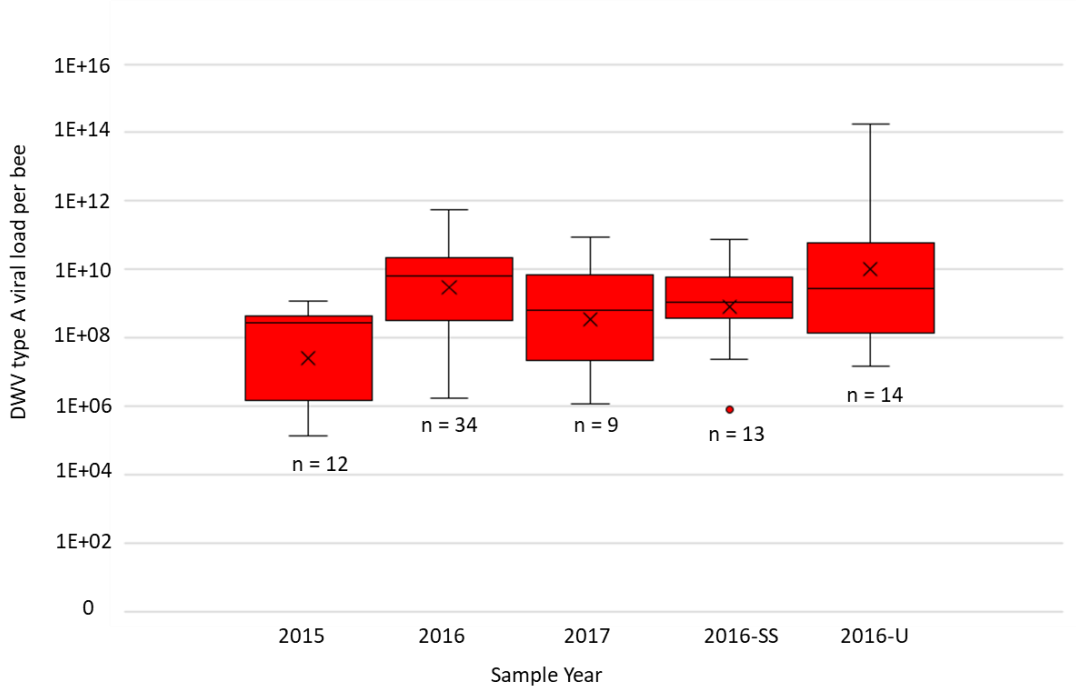

Figure 3. A comparison of DWV type-A viral load between each group (yard, wild, and control, 2016-SS, 2016-U), per year.

Table 1. The p values as per Dunn's pairwise comparison of positive samples from 2015, 2016, 2017, 2016 colonies from surrounding states (2016-SS) and USA *Varroa*-tolerant colonies (2016-U). Significant p values are highlighted in bold and underlined.

	2015	**2016**	**2017**	**2016-SS**	**2016-U**
2015		**<u>0.029</u>**	0.149	0.065	**<u>0.008</u>**
2016			0.086	0.204	0.807
2017				0.686	0.205
2016 -SS					0.382
2016-U					

DWV seasonal prevalence and load between groups

As expected an increase in DWV prevalence was detected between summer and autumn samples, with an increased autumn prevalence of DWV type-A for all groups (Figure 4). Whereas, a similar trend was not detected for the prevalence of type-B (Figure 4), due to the low number of positive DWV type-B samples (Summer – Yard n = 1, Wild n = 1, Control n = 1; Autumn - Yard n = 4, Wild n = 2, Control n = 1). Therefore, the remaining seasonal analysis of viral load was conducted upon DWV type-A.

Interestingly, whilst prevalence increased between seasons (Figure 4), type-A viral load remained at a similar level between seasons (Kruskal-Wallis – n = 48, H = 2.117, df = 3, p = 0.54). Furthermore, no significant differences in type-A viral load were found between the yard and wild bee's sampled in summer (Kruskal-Wallis test - n = 14, H = 0.102, df = 1, p = 0.255) or the autumn samples (Kruskal-Wallis test - n = 40, H = 1.505, df = 2, p = 0.471). This result suggests that the bees sampled as part of this study do exhibit a mechanism for resistance to DWV as viral loads were maintained between seasons (Figure 5).

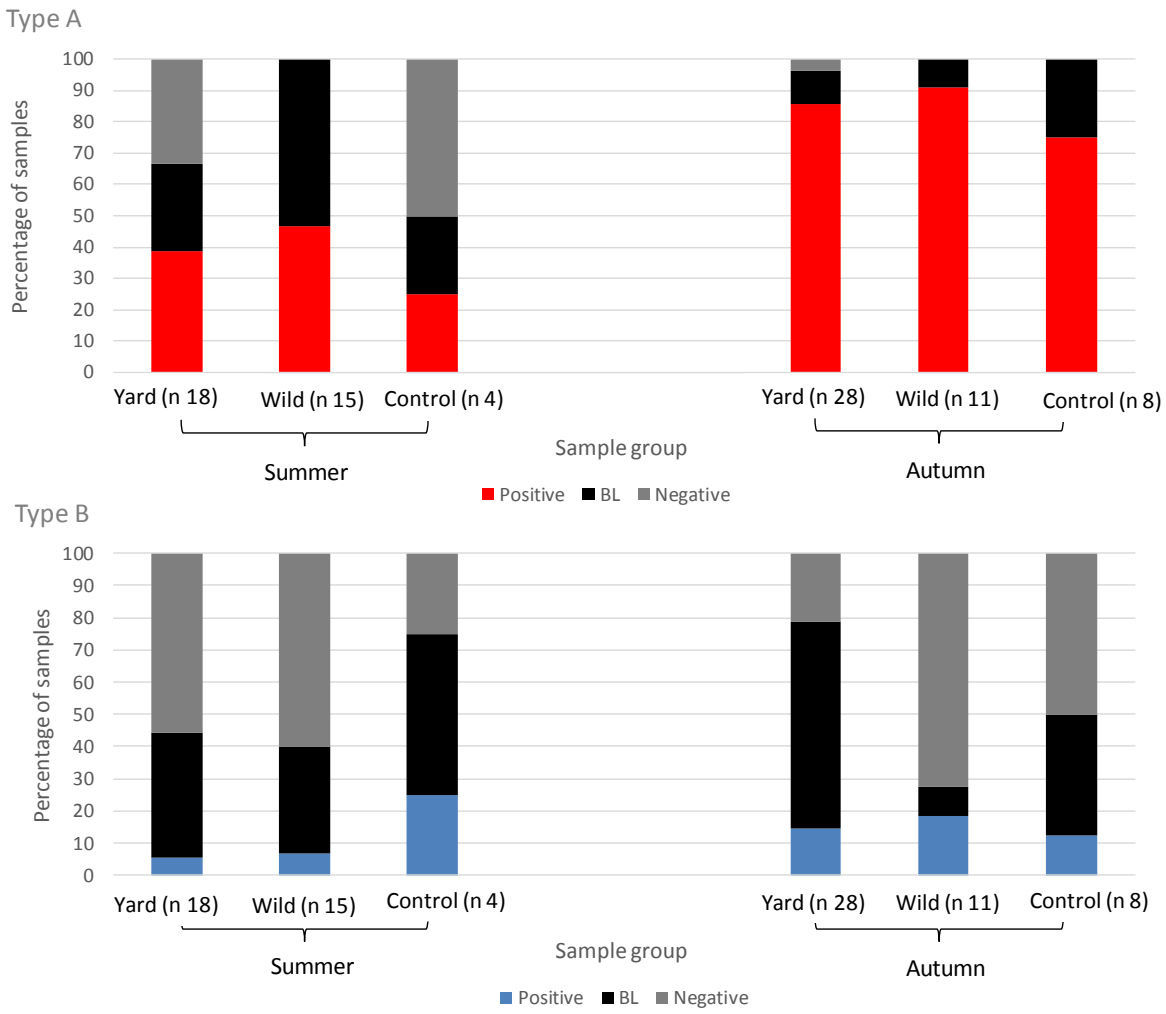

Figure 4. A comparison of DWV type-A (red) and B (blue) prevalence between seasons (summer & autumn) and group (yard, wild, control). This chart shows the percentage of quantifiable results (red or blue), results below the limit of quantification (BL) (black) and negative results (grey).

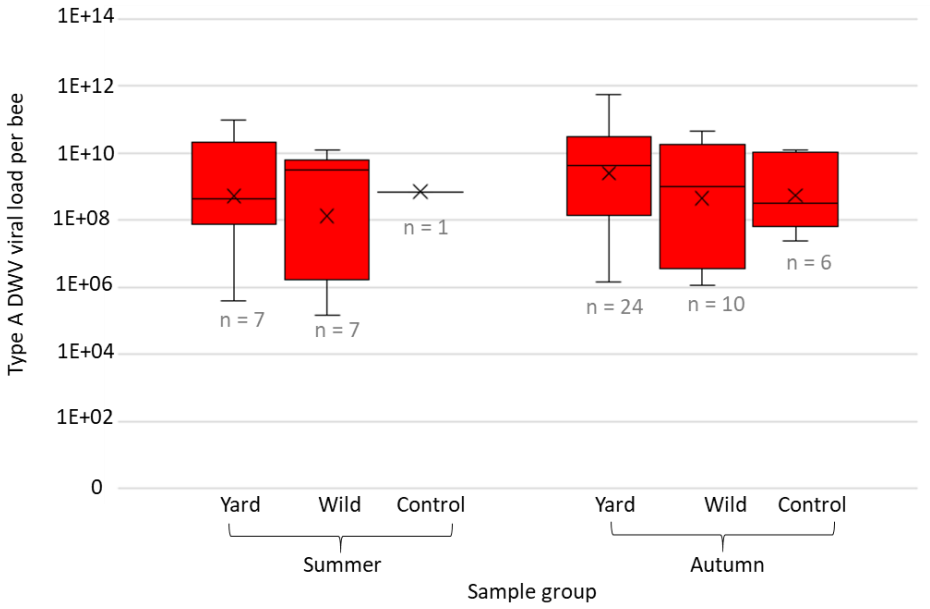

Figure 5. A comparison of DWV type-A viral load between each group (yard, wild, and control), per season (summer and autumn).

DWV autumn infection in Arnot and Ithaca bees compared to surrounding states and USA *Varroa* tolerant bees

Pooling all the data from across the three sample years, a comparison of DWV type-A samples collected in autumn from the three groups in the Arnot forest (yard, wild, and control) were compared to treated colonies from the surrounding areas and states (New York, Connecticut, Pennsylvania, and Vermont), as well as tolerant colonies found in the USA (Chapter three). This result revealed no significant difference between the viral load across groups (Kruskal-Wallis test; n = 67, H = 2.499, df = 4, p = 0.654,) (Figure 6) as median viral load were below 1E+10 (Figure 6) for each group.

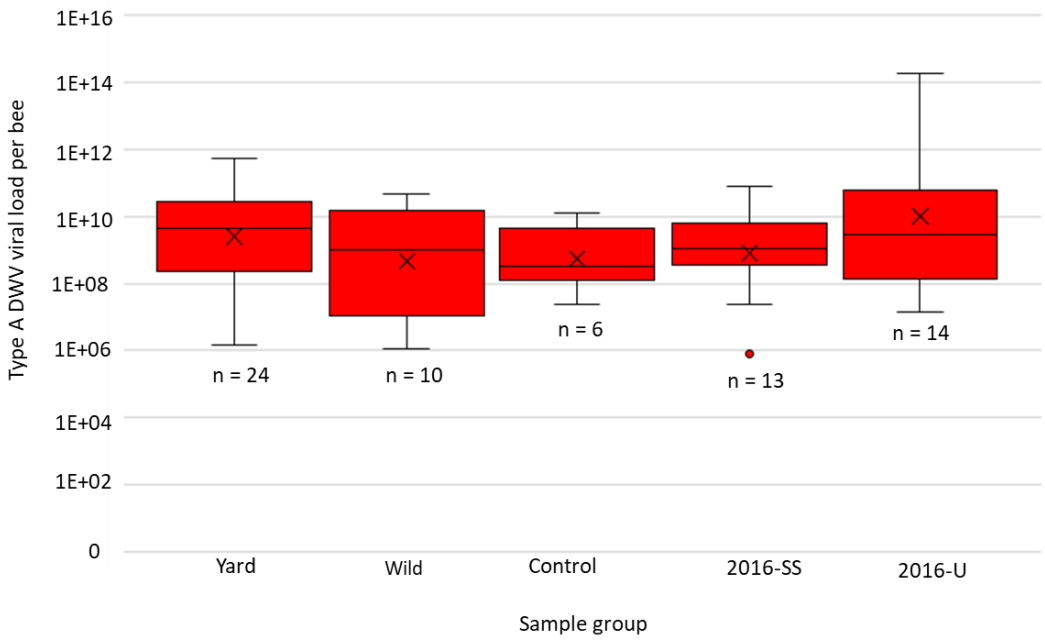

Figure 6. A comparison of Autumn DWV type-A viral load between each group (yard, wild, control, 2016-SS and 2016-U), for all sample years.

Discussion

This study reveals that DWV viral infections in Arnot forest and Ithaca bees are primarily comprised of DWV type-A. DWV type-B positive colonies were present for all years; however, loads were often too low to quantify. Therefore, superinfection exclusion via DWV type-B (Mordecai et al., 2016) is not a mechanism for *Varroa* resistance in the Arnot bees. The yearly DWV result (Figure 2 and 3) also shows a significant two-fold increase of DWV type-A in Arnot colonies from 2015 to 2016. This result is believed to be the effect of placing wild swarms into spacious honey bee hives, as increased brood rearing will lead to increased *Varroa* infestation (Loftus et al., 2016) and a subsequent increase in DWV load (Martin et al., 2010; 2013). This data provides evidence that avirulence of DWV is not a resistance factor in

these bees as DWV prevalence increased over the years. In addition, previous studies have shown that once mite drop exceeds 100+ mites then Arnot colonies will die (Seeley, 2017). Avirulent viruses do not cause any detectable damage to the host and have low viral replication rates (Casadevall & Pirofski, 2001), this is not the case for DWV infection in the Arnot colonies, as loads are maintained between 1E+07-1E+10 for the majority of the data (Figures 5 and 6).

When the analysis was broken down into seasons (summer and autumn) between groups (yard, wild and control), no change in viral load was witnessed. Type-A viral loads were maintained at sub-lethal levels (<1E+10) in the majority of the data for all Arnot forest and Ithaca sample groups. This result was particularly true for the free-living, wild bees. Providing evidence that the Arnot forest bees have the ability to maintain DWV viral load over the season. Typically, colonies which are susceptible to *Varroa* have elevated pre-winter DWV loads and are at risk of collapsing during the winter period (Berthoud et al., 2010; Dainat, et al., 2012; Highfield et al., 2009; Martin et al., 2010; 2013; Nazzi et al., 2012; Francis et al., 2013). When the DWV results of the Arnot colonies were compared to colonies which undergo a *Varroa* treatment each year (2016-SS) and colonies which are *Varroa* tolerant (2016-U), viral loads were similar. Therefore, the Arnot bee's entering the winter season do so with viral loads similar to the colonies sampled from elsewhere with different *Varroa* treatment histories.

The exact mechanism behind the seasonal maintenance of viral loads in these bees remains unclear. Small nest size, which causes frequent swarming events is almost certainly a factor

in the wild Arnot bees, as *Varroa* numbers are reduced during the swarming season (Loftus et al., 2016; Figure 7). Regular swarming and small nest size is also an essential factor found in the *Varroa* resistant bees studied in the 'live and let die' experiment on Gotland island, Sweden (Fries et al., 2003; Locke & Fries 2011). However as the bees in this study were placed into large hives, this does not explain the full story.

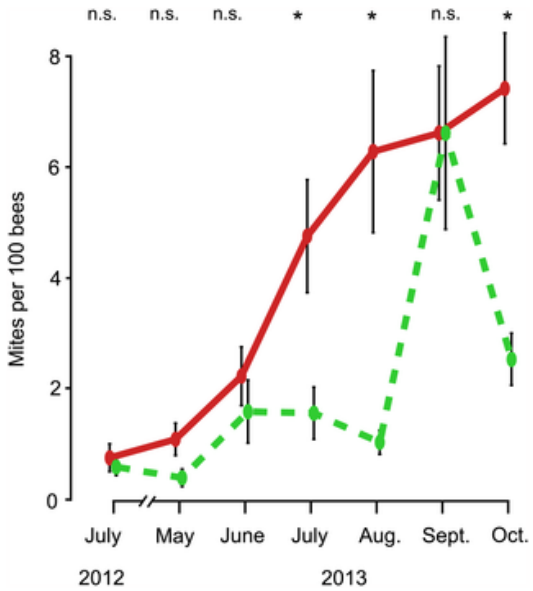

Figure 7. Dynamics of *Varroa* infestation rates on adult bees in colonies housed in small hives (dashed, green line) and colonies housed in large hives (solid, red line), from July 2012 to October 2013 (Loftus et al., 2016).

The data from Loftus et al., (2016), show that *Varroa* numbers are drastically reduced in small sized Arnot colonies between September and October (Figure 7), whilst brood production in September remains at a level similar to that seen in August. A reduction in *Varroa* numbers in pre-winter Arnot bees may explain why viral loads are maintained

between seasons, as the removal of *Varroa* mites has been shown to cause a gradual decrease in DWV viral loads (Locke et al., 2012), whilst DWV oral transmission from bee to bee is evidenced to maintain DWV loads in colonies of bees (Lock et al., 2017). Therefore, the reduction of *Varroa* mites in the pre-winter months suppresses *Varroa* mediated DWV transmission, allowing for sub-lethal DWV viral loads to be maintained horizontally via bee to bee, within the colony. However, once viral loads exceed host tolerance colonies will die and this was witnessed post-sampling in the winter of 2016-2017. This leads to the assumption that Arnot bees exhibit some tolerance to *Varroa* but not to DWV and colonies will die once viral loads become unsustainable.

A recent study by Oddie et al., (2018) found colonies of long-term (> 17 years) *Varroa* tolerant bees, maintain low *Varroa* populations via the recapping of sealed brood. The recapping of sealed brood exposes reproducing *Varroa* mites to shifts in temperature and humidity (Le Conte et al., 1990; Kraus & Velthuis, 1997) which impairs mite reproduction leading to a reduction in mite populations in colonies which display this behaviour (Oddie et al., 2018). Small nest size and frequent swarming appear to be an important factor in these colonies as this behaviour maintains low *Varroa* numbers, however, *Varroa* numbers increased in Arnot colonies post-swarming season (Loftus et al., 2016) (Figure 7). Therefore, it is suspected that an increase in numbers of *Varroa* induces a *Varroa* control response in the bees, which limits the *Varroa* mediated transmission of DWV. To understand the exact mechanism for resistant behaviour in the Arnot bees it is imperative that field research in the wild population is conducted as viruses do not play apart in this resistance story.

Conclusion

The long-term survival of Arnot forest bees depends upon their ability to maintain sub-lethal DWV loads in pre-wintering colonies. The results of the study show that *Varroa* mites do not have avirulent forms of DWV, as colonies exhibited an increase in type-A viral loads between 2015 and 2016. Colonies are not subject to superinfection exclusion via DWV type-B, as this variant was not prevalent in any of the colonies screened. A drastic reduction in mite numbers at the end of October has previously been evidenced (Loftus et al., 2016) (Figure 6), which may account for why DWV viral loads remained similar between seasons and did not significantly increase as is the case elsewhere (Berthoud et al., 2010; Dainat, et al., 2012; Highfield et al., 2009; Martin et al., 2010; 2013; Nazzi et al., 2012; Francis et al., 2013). The method in which viral loads are maintained in the Arnot forest bees remains unclear. Therefore further research into how colonies reduce mite numbers is required, and it is predicted that colonies may exhibit the recapping behaviour witnessed in other populations of *Varroa* resistant European honey bees (Oddie et al., 2018).

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Chapter 5: DWV recombinant prevalence in DWV positive colonies from England, Wales and the USA

Abstract

There is now a general consensus amongst the scientific community that *Varroa* mediated Deformed wing virus (DWV) infections are a major factor in the untimely demise of European honey bee (*Apis mellifera*) colonies around the globe. Three master variants of DWV have been formally identified (Type-A, B, and C), with current research efforts focusing on the impact of each DWV variant upon colony health. These DWV master variants readily recombine forming a mosaic of novel genomes, however no landscape scale comparative studies have been undertaken. Therefore, the aim of this study is to address the prevalence of DWV master variants and various recombinant forms of the virus in honey bee colonies from across England, Wales, and the USA, screening both *Varroa* tolerant and susceptible colonies. The data reveal that master variants of DWV type-A and B persist in a higher percentage of colonies than any of their recombinant forms. In addition, recombinants comprising the type-B structural genes (cp) and non-structural genes (RdRp) originating from all DWV master variants were detected in each data set, with type-B structural (cp) genes far exceeding the non-structural (RdRp) genes, suggesting that type-B has an evolutionary advantage over type-A and C. The data also confirmed the striking difference seen between DWV variants detected in the English, Welsh, and USA data sets (Chapters two and three) as master variant type-A was rare in England and Wales, whilst type-B was rare the USA.

Introduction

Deformed wing virus (DWV) is now considered a major factor in the global decline of overwintering honey bee colonies (Berthoud et al., 2010; Dainat, et al., 2012; Highfield et al., 2009; Martin et al., 2010, 2012; 2013; Cornman et al., 2012; Nazzi et al., 2012; Francis et al., 2013). DWV was first discovered in symptomatic honey bees from Japan in the early 1980's (Bailey & Ball, 1991) and was present in colonies prior to the arrival of *Varroa* (Martin et al., 2012). Pre-*Varroa* DWV viral loads were low and infections were diverse, often causing no visible disease symptoms (Martin et al., 2012). In association with *Varroa*, DWV loads are typically present in high amounts (Carreck et al., 2010; Martin et al., 2012) accompanied with a loss in viral diversity, allowing for the selection of one dominant master variant in the honey bee host (Martin et al, 2012; Ryabov et al, 2014; Chapters 2 and 3).

DWV is a small (30nm), single-stranded, positive-sense RNA virus belonging to the family *Iflaviridae*, within the order of the Picornavirales. Owing to the fact that RNA viruses have high mutation rates and frequently large population sizes, DWV exists as a diverse population of variants commonly referred to as a quasispecies (Domingo & Holland, 1997). A quasispecies consists of a number of master variants, with each variant having their own swarm of mutant progeny (Palacios et al., 2008; Lauring & Andino, 2010). This constant process of mutation provides clouds of variants with an evolutionary advantage, allowing them to occupy several biological niches (Domingo et al., 2012). DWV is regularly detected in *Varroa* exposed populations of honey bees as three closely related master variants; type-A (Lanzi et al., 2006), type-B (which was originally designated as *Varroa destructor*-Virus 1) (Ongus et al., 2004), and type-C (Mordecai et al., 2016a). Each DWV variant is evidenced to

impact honey bee colonies in different ways. Type-A has been linked to colony losses (Martin et al., 2012; Schroeder et al., 2012; Francis et al., 2013) and has recently been found dominating USA honey bee colonies (Chapter two). Type-B was shown to be slightly more lethal than type-A when experimentally injected in DWV naive caged bees (McMahon et al., 2017). Type-B's ability to out-compete type-A was also reported by Mordecai et al., (2015) in a UK *Varroa* resistant population of honey bees and it was suggested that type-B infection offered protection to the colonies via phenomena known as superinfection exclusion (SIE). DWV type-C has been evidenced as rare in both English, Welsh, and USA honey bee colonies (Chapters two and three).

Multiple DWV master variants are capable of co-infecting a honey bee, it is under these circumstances that viral recombination occurs. Recombination is the process where a new genome is formed by the covalent linkage of genetic material from two or more differing parent genomes and is a major factor for genetic variation in all living organisms. Several types of recombination have been described (Domingo, 2016); there are those in which genetic exchange can occur from similar or identical nucleotide sequences, termed homologous recombination. Non-homologous recombination occurs when genes are swapped between closely related, yet distinct genomes, such as in almost all bacteria (Vos, 2009). The process of generating a recombinant is either replicative or non-replicative. Replicative recombination occurs as a result of template switching during RNA synthesis and non-replicative recombination occurs via the random ligation of genome fragments (Galli & Bukh, 2014; Domingo, 2016).

Viral recombination was first described in RNA viruses belonging to the *Picornavirales*; polioviruses (Cooper, 1974) and *Aphthae epizooticae* virus (Foot and mouth disease) (King et al., 1985). Since then, the role of recombination has been well evidenced in a number of RNA viruses capable of infecting a wide range of host species. Recombinant viruses have been well evidenced in mammalian viruses such as Hepatitis C (Kalinina et al, 2002; Lee et al, 2010; Hoshino et al, 2012) and HIV-1 (Thomson & Najera, 2005; Delviks-Frankenberry et al, 2011); plant viruses such as tomato bushy stunt virus (TBSV) (White & Morris, 1994) and cucumber necrosis virus, (CNV) (Shapka & Nagy, 2004); and finally in viruses infecting invertebrates such as Israeli acute paralysis virus (IAPV) (Maori et al, 2007; Palacios et al, 2008) and DWV (Moore et al., 2011; Zioni et al., 2011; Ryabov et al 2014, 2017; Mordecai et al 2015a & b; Dalmon et al 2017, McMahon et al., 2017). However, the evolutionary advantage of viral recombination is disputed, as recombinants are often considered to be less fit than the parental genomes (Desbiez et al, 2011; Garcia-Arenal et al, 2003), therefore persisting recombinant viruses must be fit enough to evade selective pressures to become established in a population. Viral recombinants have been evidenced to become dominant in viral populations where the parental viruses are the target of anti-viral therapies, this leads to the rise of drug resistant variants (Moutouh et al., 1996; Morel et al., 2010).

DWV Recombinants between the type-B structural and non-structural genes of type-A have been most commonly detected (Zioni et al., 2011; McMahon et al., 2016; Ryabov et al., 2014; 2017; Dalmon et al., 2017). In addition, recombinants between DWV type-A structural and non-structural genes of type-B and C have also been reported (Mordecai et al., 2015a & b) as well as recombinants at the beginning of the open reading frame (ORF) at the 5’ end

(Dalmon et al., 2017). However, the extent at which recombination occurs on a landscape scale and how they affect the honey bee host remains unclear. Therefore, the main aim of this study is to assess the prevalence and viral load of DWV recombinants against the parental genomes of DWV infections present in honey bee colonies sampled from across England, Wales, and the USA. Both *Varroa* tolerant and susceptible colonies were selected. This work was conducted using a new primer combination method that amplifies DWV master variants and recombinant genomes from the VP1 region at the 5' end and RdRp region at the 3'end. In addition, a new RT-qPCR method was also used to quantify the structural (cp) genes of each DWV recombinant, the data was then compared to the non-structural (RdRp) genes quantified in Chapters one and two. This is the first time in which landscape scale analysis has been conducted upon DWV recombinant forms.

Methods

Sample collection

Approximately 30 adult worker honey bee samples were collected from 36 English and Welsh apiaries, and 57 USA apiaries. In total 112 colonies from England and Wales, and 79 USA colonies were sampled, see Chapters two and three for more details. From these a sub-sample of 93 colonies from England and Wales and 73 colonies from the USA were tested for the presence of DWV master variants and recombinants. To avoid subconscious bias, samples were selected without any prior thought given to season of collection, location, or colony mortality. Samples were screened for DWV viral recombinants between the non-structural (cp) and structural (RdRp) genes for all three DWV master variants (A, B, and C). Only during the analysis stage was the true identity of the sample known (*Varroa* tolerant or susceptible) and if these colonies survived the overwintering period. The English and Welsh samples were collected in both spring (April, n = 71) and late summer (mid-August to mid-September, n = 41), whilst USA samples were all collected in November. All samples were collected in 2016.

Sample processing

Honey bee Samples were processed as per Chapters two and three. Briefly, honey bees were checked for visual deformities and when found these were removed as were any *Varroa* mites. A pool of 20 whole bees (English and Welsh) or 10 heads (USA) per colony were homogenised. Total RNA was extracted from 30mg of homogenised bee tissue, using an RNeasy Mini prep kit (Qiagen) as per the manufacturer's instructions. Total RNA was quantified on a spectrophotometer (Nanodrop 2000, ThermoFisher Scientific) and samples diluted to 50ng/µl prior to PCR and RT-qPCR.

DWV master variant and recombinant PCR protocol

Nine combinations of DWV strain specific primers (Table 1), flanking between the structural VP1 genes and non-structural RdRp genes were used to amplify fragments >5 kb of DWV master variants and recombinants (Figure 1). Recombination hotspots (where differing genomes of parent viruses readily combine) for DWV were identified by Dalmon et al. (2017) and occur between the structural genes at the 5' end of the open reading frame (LP, VP1, VP2, VP3, and VP4) and the non-structural genes (Helicase, 3c-pro and RdRp) at the 3' end, with common breakpoints located in the Helicase. Other studies also confirmed recombinants between structural and non-structural genes are common (Moore et al., 2011; Zioni et al., 2011; Mordecai et al., 2015 a & b; Ryabov et al., 2014; 2017; McMahon et al., 2017), hence the selection of primers which bind at the 5' and 3'. The nine primer combinations are detailed in Table 2. These primers were designed and verified by A. Highfield, in the Schroeder Lab (paper in prep).

Firstly, total RNA was synthesised into cDNA using an Invitrogen SuperScript IV first strand synthesis system (ThermoFisher Scientific), as per the manufacturer's instructions. PCR reactions were prepared using a Q5 high-fidelity DNA polymerase kit and 10mM dNTPs obtained from New England Biolabs. Each reaction contained, 5 µl 5 x Q5 buffer, 0.5 µl 10mM dNTPs, 0.25 µl Q5 high fidelity DNA polymerase, 1.25 µl forward DWV variant specific capsid primer, 1.25 µl reverse DWV specific RdRp primer, 14.75 µl nuclease free H_2O, and 2 µl cDNA template. The thermocycler was set to; initial activation 98^{o}C for 30 secs, followed by 35 cycles of denature at 98^{o}C for 10 secs, annealing at 54^{o}C for 20 secs, extension at 72^{o}C for 3 mins, followed by a final extension at 72^{o}C for 2 mins. Results were

then visualised via gel electrophoresis using a 1.4% agarose gel containing gel red against a 1kb hyper ladder (Figure 2). A total of 93 UK and 73 USA honey bee colonies were screened using the nine combinations of strain specific primers for the recombinant PCR (Table 1).

Table 1. Primers used in this study to generate a >5 kb PCR product of DWV master variant and recombinant genomes (see Figure 1).

Primer	Name	Sequence (5'–3')
Capsid C forward	C_Capsid_F2_AH	GCATATTTGCGTATAAAAGATG
RdRp C reverse	C_RdRp_R_AH	ATAAGTTGCGTGGTTGAC
Capsid A forward	A_Capsid_F2_AH	TTTGTGGAGCAAAGAATTGA
RdRp A reverse	A_RdRp_R_AH	CTCATTAACTGTGTCGTTGAT
Capsid B forward	B_Capsid_F2_AH	CAAGACAACTTGCTCAGCAT
RdRp B reverse	B_RdRp_R_AH	CTCATTAACTGAGTTGTTGTC

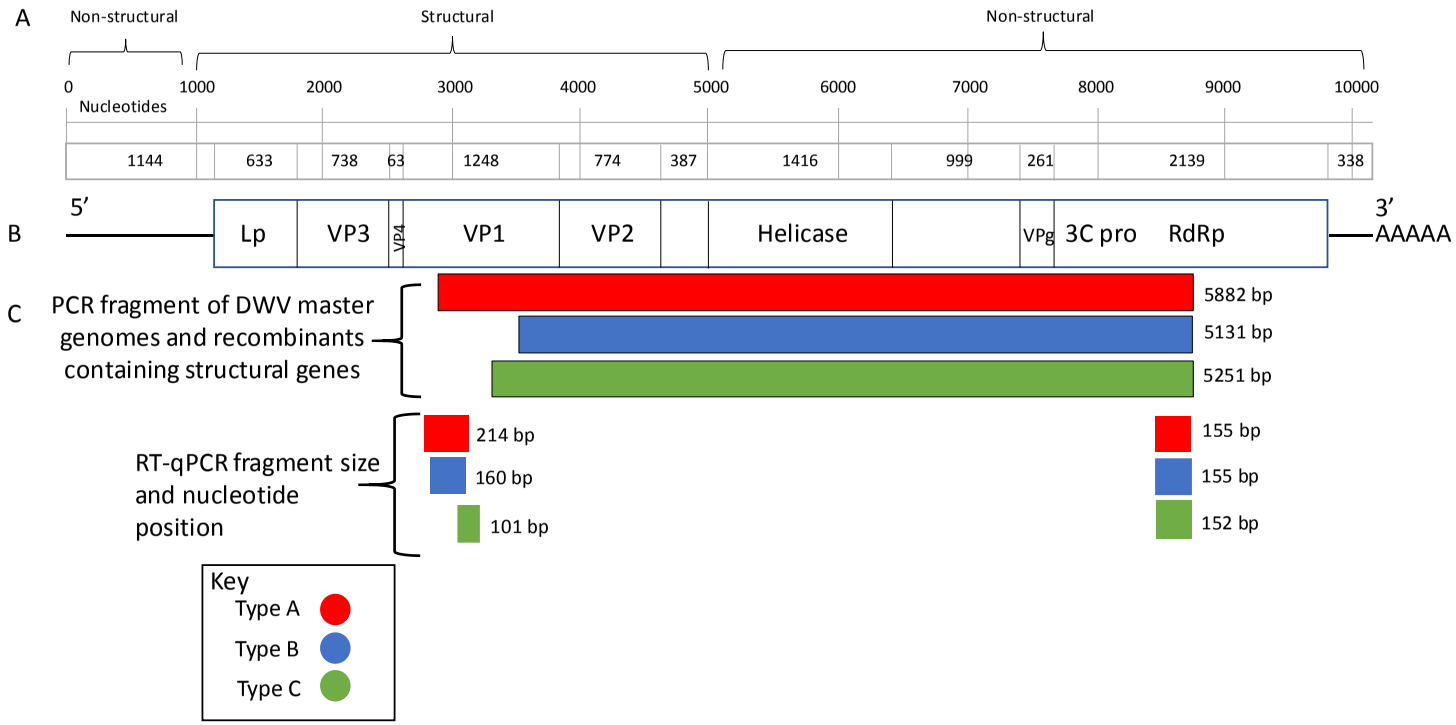


Figure 1. Shows the DWV genome against the position and size of DWV fragments generated in the PCR and RT-qPCR assays. (A) Non-structural and structural protein positions are shown above the nucleotide scale and number of nucleotides coding for each protein (Dalmon et al., 2017). (B) The DWV genome as described by De Miranda & Genersch (2010). (C) Position and size of DWV fragments generated in the PCR and RT-qPCR assays. Type-A, B and C variants describe PCR fragments of DWV master genomes and recombinants involving the specified variants structural genes.

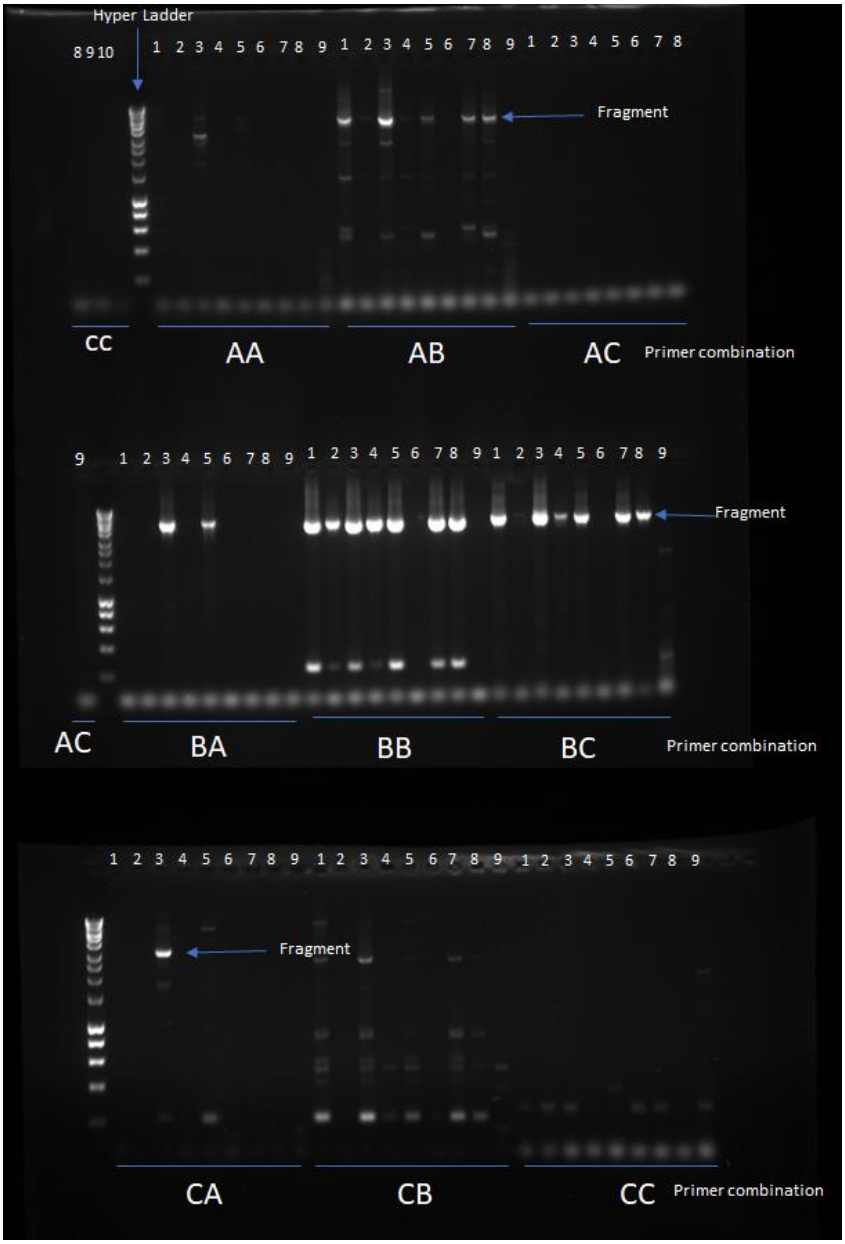


Figure 2. Example of a gel image used to identify the DWV fragments, which were amplified using the new recombinant PCR method. The position of the >5 kb fragment is indicated by the blue arrows, the 1 kb hyper ladder is shown to the left of the gel and primer combinations listed underneath the gel (AA, AB, AC, BA, BB, BA, BC, CA, CB, CC).

Table 2. Nine primer combinations used to identify DWV master variants and recombinant genomes.

Capsid	RdRp
A	A
A	B
A	C
B	A
B	B
B	C
C	A
C	B
C	C

Capsid standard curve

A capsid RT-qPCR protocol for the structural genes (cp) of each DWV master variant (A, B, and C) were also designed by A. Highfield (paper in prep). For the creation of a standard, capsid RT-PCR was performed using the primers detailed in Table 3. The results were visualised on a 2% agarose gel containing ethidium bromide. Positive bands were excised from the gel and purified before sanger sequencing to confirm target genes for each of the DWV master variants (VP1 region). Plasmid vectors containing the target genes were created (Biomatik, Table 4) and reconstituted as per the manufacturer's guidelines. Heat shock transformation was performed using One Shot® TOP10 chemically-competent *Escherichia coli* following the manufacturer's instructions (Life technologies). The transformants were selected on LB agar plates, containing kanamycin and left to incubate at 37 °C overnight. A single colony for each master variant was propagated in LB medium containing kanamycin and a Qiaprep® spin mini prep kit (Qiagen) was used per the manufacturer's instructions to extract plasmid DNA. Plasmid DNA was then synthesized in to

RNA using a mMessage machine T7 kit (ThermoFisher) as per the manufacturer's guidelines. This part of the work was conducted by A. Highfield, who supplied the capsid standards used in this study.

Table 3. Primers used in this study for DWV capsid RT-qPCR.

Primer	Name	Sequence (5'–3')	Size of product (bp)
Capsid A forward	A_capsidF_AH	GGTACTAATTTAGTTGAACCATT	214
Capsid A Reverse	A_capsidR_AH	TCAATTCTTTGCTCCACAAA	
Capsid B Forward	B_capsidF_AH	CTCGAGGAGAGTTGGCATTC	160
Capsid B Reverse	B_capsidR_AH	ATGCTGAGCAAGTTGTCTTG	
Capsid C Forward	C_capsidF_AH	GTTAGTATTTAGATGGGGCACG	101
Capsid C Reverse	C_CapsidR_AH	CATCTTTTATACGCAAATATGC	

Table 4. DWV master variant (A, B, C) structural (cp) insert used in the plasmids. Primer sequences are underlined and reverse primer sequences are highlighted in bold.

Target	Insert
DWV type A	GGTACTAATTTAGTTGAACCATTACATGCATTACGTTTGGATGCAGCCGGTACGA CACAACATCCTGTAGGTTGTGCTCCTGATGAAGATATGACTGTATCCTCCATTGC ATCTCGATATGGACTAATTAGACGGGTACAATGGAAGAAAGATCATGCTAAAGG ATCACTTTTGTTACAGTTAGATGCCGATCCA**TTTGTGGAGCAAAGAATTGA**A
DWV type B	CTCGAGGAGAGTTGGCATTCCTGCGTATCCGCGATGCTAAGCAAGCTGCTGTAG GAACGCAACCTTGGCGTACTATGGTCGTTTGGCCTTCAGGTCATGGATATAATAT TGGAATACCAACTTATAATGCTGAACGAG**CAAGACAACTTGCTCAGCAT**A
DWV type C	GTTAGTATTTAGATGGGGCACGTTAGCAGACCAGATAGCGCAATGGCCCTCTATT AATGTTCCACGCGGTGAATAT**GCATATTTGCGTATAAAAGATG**AA

To create the standard curve, a ten-fold dilution series was made for each DWV master variant, the standard curve ranged from 15 – 30 Ct (critical threshold) values. The following equation was used to determine the copy number as per Kevill et al., (2017):

$$\text{Copy number RNA} = (\text{Concentration RNA (ng/µL)} \times 6.022 \times 10^{23}) /$$

$$(\text{Fragment length base pairs} \times 109 \times 325)$$

RT-qPCR

RT-qPCR was performed on the 1 µl total RNA (50 ng/µl) extracted from the adult worker bees using a SensiFAST™ SYBR® No-Rox One Step kit (Bioline). Each 20 µL reaction contained 10 µL SensiFAST™ SYBR® No-ROX One-Step mix (2×), 1 µL DWV capsid forward primer, 1 µL capsid reverse primer (A, B or C; Table 3), 0.2 µL reverse transcriptase, 0.4 µL RiboSafe RNase Inhibitor, 5.4 µL RNAse free H_2O. The Reverse transcriptase step occurred at 45 °C for 10 min and denaturation at 95 °C for 10 min, followed by 35 cycles of denature 95 °C 15 s, annealing at 52 °C (type-A and C) or 62 °C (type-B) for 15 s and extension at 72 °C for 15 s. A melt curve analysis was performed between 72 °C and 95 °C, at 0.1 °C increments, each with a 5 s hold period. RT-qPCR's also contained a no template control (NTC) where 1 µl RNAse free water was used instead of total RNA. Both the amplification plot and melt curve were used to assess if contamination occurred in the controls. RT-qPCR was conducted in duplicate. A total of 112 UK samples and 79 USA samples were screened for DWV variants at the VP1 region of the genome. This data was then compared to the RT-qPCR data of the RdRp genes as per Chapters two and three.

Analysis of the data

DWV master variant and recombinant PCR

The percentage of honey bee colonies positive for DWV master variants (type-A, B, and C) and recombinant variants (Table 2) were established from the number of gel confirmed positive samples. The gel confirmed DWV recombinant positive (AB, BA, BC, CA, CB) samples were then selected for further analysis using RT-qPCR.

Capsid and RdRp RT-qPCR

RT-qPCR was performed on the capsid VP1 region (cp) for this study, which was analysed alongside the RdRp RT-qPCR data obtained in Chapters two and three.

Capsid RT-qPCR data for each DWV master variant was imported from the Rotor gene q series software® into Microsoft excel. Here the DWV genome equivalents per bee (DWV load), per variant were calculated as follows as per Kevill et al., (2017):

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

The corresponding genome equivalent data (viral load) for each variant and fragment (capsid or RdRp) were then transformed using the log ^{10} function allowing the data to become more manageable. Samples were removed from the analysis that returned a RT-qPCR negative result for both capsid (cp) and RdRp genes, and when more than 3 Ct (critical threshold) value deviation occurred, as a 3 Ct values is representative of a ten-fold change in viral load. Samples which were positive but below the quantifiable limit of detection were given an arbitrary value of 100 genome equivalents per bee (i.e. a log ^{10} value of 2). Samples which tested positive for just the capsid (cp) or RdRp were included in the analysis as these partial fragments either represent partially transcribed RNA or RNA which is involved in other DWV recombinant pairings.

Comparisons were made between the capsid (cp) and RdRp genes of the type-A and B RT-qPCR data. Recombinant viral loads were calculated by comparing the capsid (cp) and RdRp RT-qPCR data, this was performed upon the recombinant positive samples which had previously been confirmed via the recombinant PCR. Therefore, the total number of samples which had the RT-qPCR data analysed varies between the nine DWV viral recombinants. The mean and standard deviation (± 1 SD) of the structural (cp) and non-structural (RdRp) genes were calculated for RT-qPCR quantifiable samples. The total number of samples analysed are displayed in Figures 4, 5, and 6.

As the data were not normally distributed, Spearman's Rho were obtained for the capsid and RdRp genes from the RT-qPCR data. Spearman Rho closer to one suggest a strong correlation between capsid and RdRp genes forming either parent DWV sequences or recombinants. A Z score test of two population proportions was also conducted upon the colonies which died and those which survived in the USA. This test allows for significant differences between two groups of categorical data to be established and was used to test if the proportion of DWV master variants and recombinants differed between dead and surviving colonies. This test was not performed on the UK data as too few colonies died.

Results

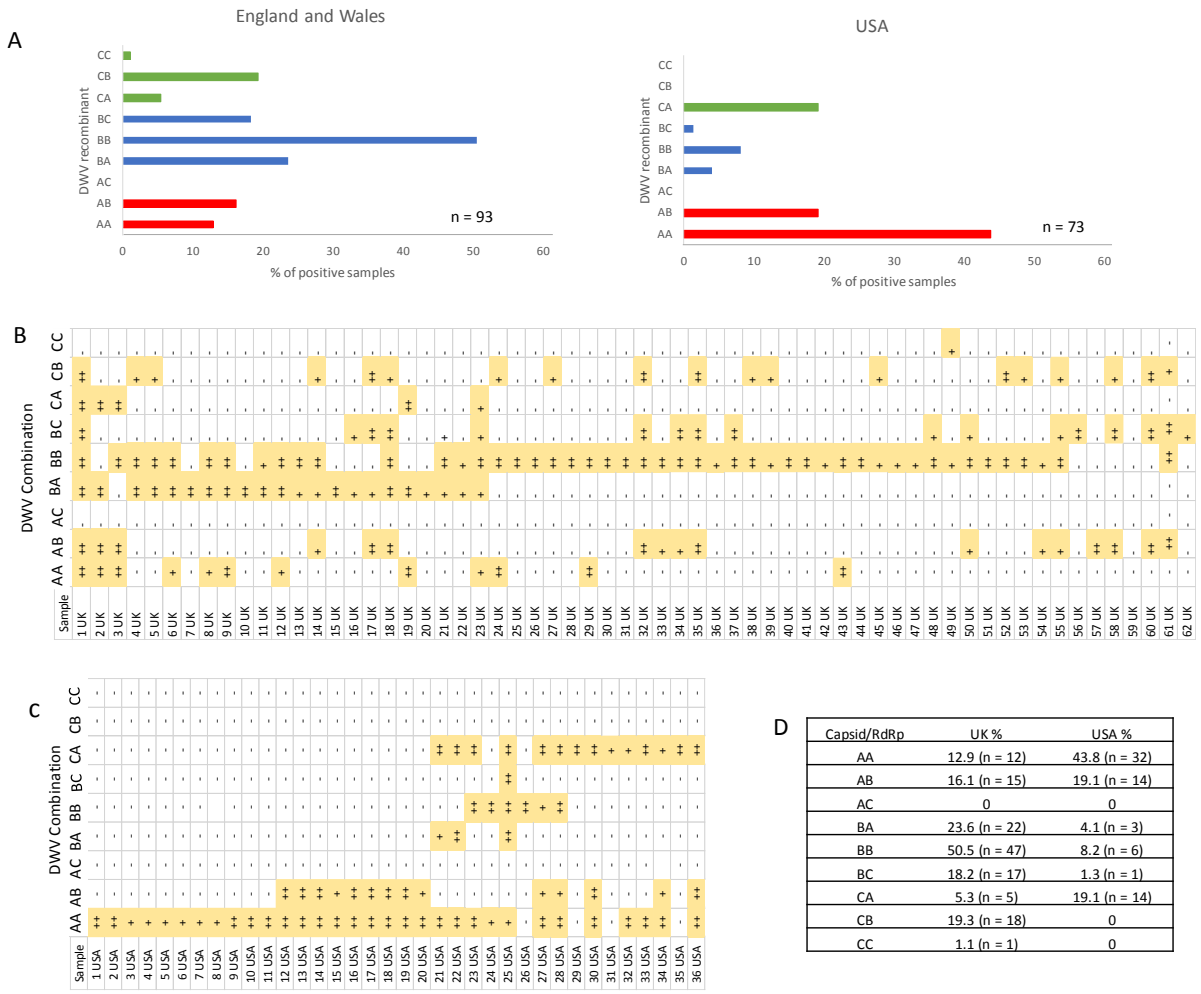
Identification of DWV recombinants in honey bees from England, Wales, and USA

The results of the PCR reveal that 68% (n = 112) of English and Welsh colonies and 49% (n = 73) of USA colonies were positive for at least one DWV master variant (Figure 3). Both DWV

type-A and B master variants were detected in the honey bee colonies, however a higher prevalence of type-A was witnessed in the USA than in England and Wales (44%, and 13%, respectively) (Figure 4). Conversely, type-B had a higher prevalence in English and Welsh colonies than those from the USA (51% and 8%, respectively). Master variant type-C was detected in 1.6% of the English and Welsh colonies screened and in none of the USA colonies (Figures 3 & 4).

The majority of positive samples contained a DWV recombinant for the English and Welsh (65% of positive samples) and USA data sets (58% of positive samples) (Figure 3). When only one positive result was returned it was often the dominating master variant rather than a recombinant; this occurred in 11 colonies out of 15 for type-A in the USA (Figure 3), and 13 out of 15 colonies for type-B in England and Wales (Figure 3).

The prevalence of recombinant DWV variants varied between England and Wales, and the USA samples and appear to be determined by which master variant is dominant (Figure 3). The English and Welsh honey bee colonies are mainly comprised of type-B and recombinants of type-B, whereas the USA is dominated by type-A and recombinants of type-A (Figure 3). Interestingly master variant type-C was rare; however, recombinants of type-C were detected.



D

Capsid/RdRp	UK %	USA %
AA	12.9 (n = 12)	43.8 (n = 32)
AB	16.1 (n = 15)	19.1 (n = 14)
AC	0	0
BA	23.6 (n = 22)	4.1 (n = 3)
BB	50.5 (n = 47)	8.2 (n = 6)
BC	18.2 (n = 17)	1.3 (n = 1)
CA	5.3 (n = 5)	19.1 (n = 14)
CB	19.3 (n = 18)	0
CC	1.1 (n = 1)	0

Figure 3. (A) Bar charts show the percentage of English/Welsh and USA honey bee samples which retuned a positive result using the DWV new PCR method. The tables show DWV master variants and recombinant positive samples confirmed using gel electrophoresis for English/Welsh (B) and USA (C) honey bee colonies. Negative samples are indicated as a -. DWV mater variants are shown as AA, BB, and CC, and recombinants between the capsid and RdRp region are displayed as AB, AC, BA, BC, CA, and CB. (D) shows the percentage and sample size of each recombinant in England/Wales (UK) and USA.

A comparison of DWV structural and non-structural genes

DWV RNA from the structural (cp) and non-structural (RdRp) genes were quantified against strain specific standard curves using RT-qPCR for the English and Welsh, and USA samples. Both the structural (cp) and non-structural (RdRp) RT-qPCR results were compared for type-A and type-B DWV master variants, as type-C was only found in one English sample. Samples which had gel confirmed DWV recombinants also had the structural (cp) and non-structural (RdRp) genes quantified. The results reveal a significant correlation in structural (cp) and non-structural (RdRp) genes for master variant type-A and the type-A (cp) / B (RdRp) recombinant in USA honey bee colonies (Spearman's Rho – $rs = 0.83$, $p = <0.001$ and $rs = 0.57$, $p = <0.03$, respectively) (Figure 4). Out of all the possible recombinant pairs involving the type-A (cp), only the USA type-A (cp) / B (RdRp) result returned a significant correlation.

A significant correlation was witnessed for type-B cp and RdRp genes in England and Wales, and the USA (Spearman's Rho – $rs = 0.76$, $p = <0.001$ and $rs = 0.66$, $p = <0.001$ respectively) (Figure 5). Type-B structural (cp) recombinants were more commonly detected in the English/Welsh data set when compared to the USA, however none of the positive samples from either data set had a significant correlation between the structural (cp) and non-structural (RdRp) genes quantified (Figure 5). Therefore, whilst samples were confirmed as positive, these recombinants are likely to be present in low viral loads (Figure 5). Type-C (cp) / type-A (RdRp) recombinants were detected in the English and Welsh, and USA colonies, whilst type-C (cp) / type-B (RdRp) were detected in England and Wales only. Type-C structural genes (cp) were unquantifiable in England and Wales and were not detected in the USA, therefore a comparison could not be made for the type-C data.

In addition, the mean DWV load for the type-A structural (cp) and non-structural (RdRp) genes were similar in the USA data set at 7.17 ± 2.84 and 7.62 ± 3.01 viral load per bee, respectively. Whereas in England and Wales the mean type-A structural (cp) and non-structural (RdRp) loads differed at 3.44 ± 2.50 and 7.97 ± 2.54 (Figure 6), further confirming that the majority of the USA is master variant type-A, and that DWV type-A loads in England and Wales are low. It is also expected that surplus type-A non-structural (RdRp) genes in both data sets would belong to the remaining recombinant pairs type-B (cp) / type-A (RdRp) and type-C (cp) / type-A (RdRp). A different situation occurs for type-B, as mean structural loads (cp) loads exceed those of the non-structural (RdRp) for both the English/Welsh (11.3 ± 3.20 and 9.85 ± 2.58 viral loads per bee, respectively) and USA data sets (10.98 ± 1.89 and 7.68 ± 2.87 viral loads per bee, respectively) (Figure 6). In addition, all three type-B structural (cp) recombinants were detected in both England and Wales, and the USA (Figure 3), suggesting that type-B has a competitive edge over type-A and C.

Overall these results confirm that DWV master variants, type-A (USA) and type-B (England/Wales) are better adapted for survival and replication in the honey bee host than any recombinant form (Figures 4 and 5) and that type-C is mostly likely to present as a recombinant rather than as a master variant.

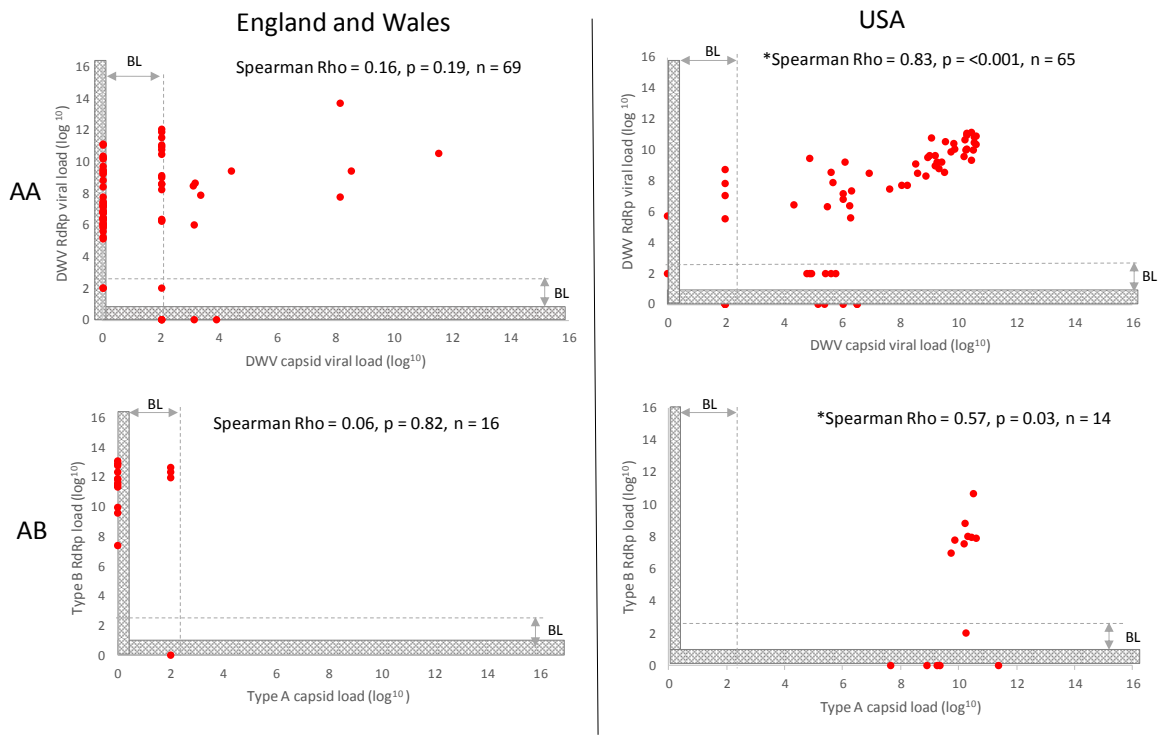


Figure 4. A comparison of the RT-qPCR data for the type-A structural (cp) and type-A and B non-structural (RdRp) genes quantified (log^{10}) in English/Welsh and USA colonies. Samples which are below the quantifiable limit fall below the dashed lines (BL). Samples which tested positive for only one gene (cp or RdRp) fall within the grey bars. A significant correlation * between the structural (cp) and non-structural (RdRp) genes of type-A was witnessed in the USA data sets (Spearman Rho – AA; rs = 0.83, p = < 0.001, AB; rs = 0.57, p = 0.03).

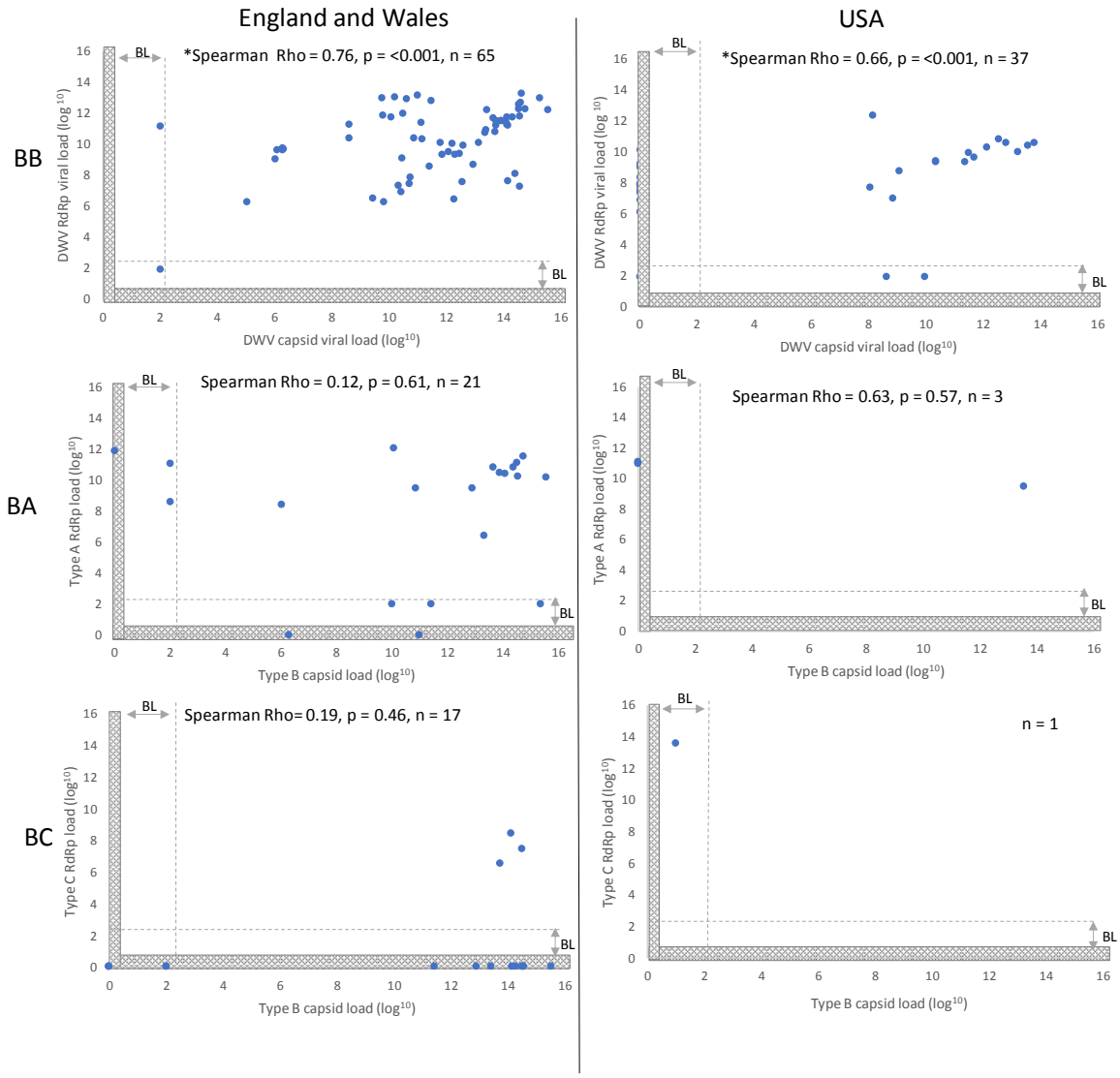


Figure 5. A comparison of the RT-qPCR data for the type-B structural (cp) and type-A, B, C non-structural (RdRp) genes quantified (log^{10}) in the English and Welsh, and USA colonies. Samples which are below the quantifiable limit fall below the dashed lines (BL). Samples which tested positive for only one gene (cp or RdRp) fall within the grey bars. A significant correlation * between type-B structural (cp) and non-structural (RdRp) genes was witnessed in the USA and English/Welsh data sets (Spearman Rho – UK; rs = 0.76, p = <0.001, USA; rs = 0.66, p = <0.001).

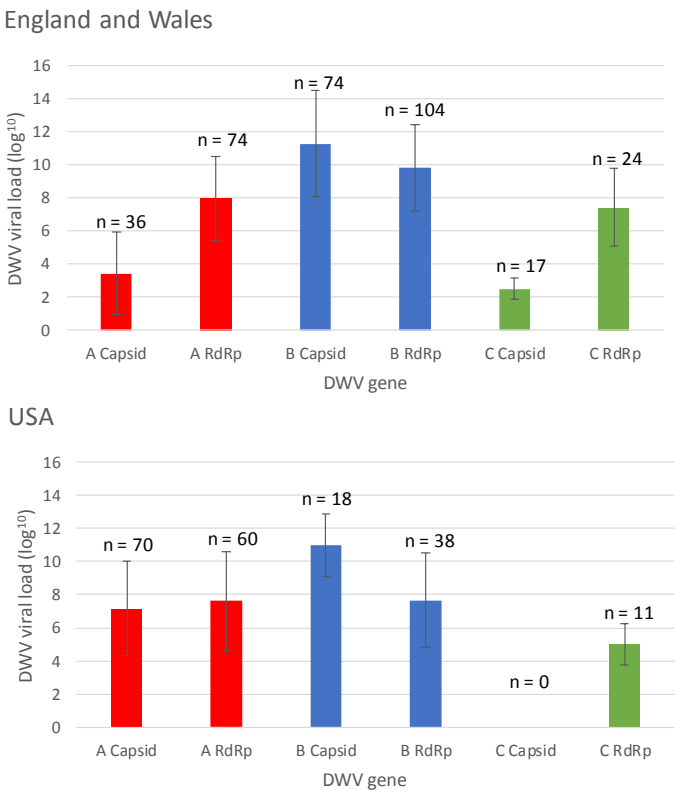


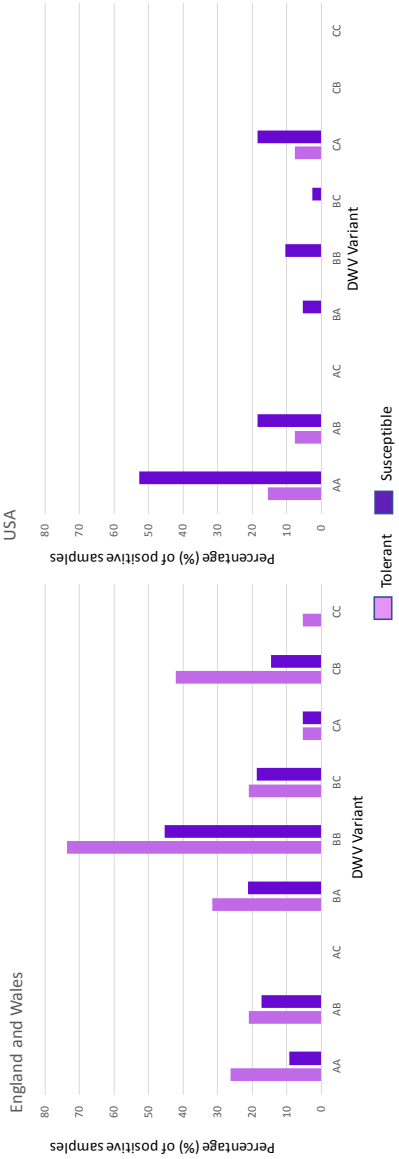
Figure 6. Bar charts comparing the mean (± 1 SD) DWV structural (cp) and non-structural (RdRp) genes (log^{10}) obtained via RT-qPCR, for DWV variants detected in England and Wales (UK) and USA honey bee colonies.

DWV recombinants in *Varroa* tolerant and susceptible honey bee colonies

Dominating master variants were prevalent (Type-B England/Wales; Type-A USA), DWV in *Varroa* tolerant and susceptible colonies follows the overall trend seen in Figure 3. The *Varroa* tolerant colonies in England and Wales have a higher prevalence of DWV; both master variants and recombinants (excluding the recombinant of type-A [cp] / type-C [RdRp], as no colonies tested positive) when compared to *Varroa* susceptible colonies. The reverse is seen in colonies from the USA, in which *Varroa* susceptible colonies had a higher

prevalence of DWV master variants and recombinant forms than tolerant colonies (Figure 7). The majority (>84%) of USA tolerant colonies where negative for any of the DWV master variants and recombinants.

A



B

Eng/Wel		AA	AB	AC	BA	BB	BC	CA	CB	CC	Total n of samples analysed
	Tolerant (n)	5	4	0	6	14	4	1	8	1	19
	Susceptible (n)	7	13	0	16	34	14	4	11	0	75
USA	Tolerant (n)	2	1	0	0	0	0	1	0	0	13
	Susceptible (n)	20	7	0	2	4	1	7	0	0	38

Figure 7. Bar charts showing the percentage of *Varroa* tolerant and susceptible colonies in Engaland, Wales, and USA (A) that tested positive for DWV master variants and recombinants. The total number of positive samples and the total number of samples analysed per group (tolerant or susceptible) is shown in the table (B).

A comparison of DWV variants in English/Welsh and USA colonies that died and survived

Samples in which mortality data exists were categorised in to those which died unexpectedly during the overwinter period and those that survived. The prevalence data show that USA colonies which died had a higher prevalence of DWV type-A and recombinant type-A (cp) / type-B (RdRp) (Figure 8). Furthermore, the Z test for two population proportions reveals that proportional amount of DWV type-A and the A (cp) and B (RdRp) recombinant were significantly higher in the colonies that died when compare to those that survived in the USA (Z test - $p = <0.001$) (Figure 8). Due to the low number of overwinter colony losses seen in 2016 (Chapter two) in England, only four colonies which died were tested in. Two of the dead colonies were negative of DWV and the remaining two colonies had both type-B and a recombinant of type-B (Cp) / type-A (RdRp) (Figure 8). The English and Welsh surviving colonies had a high type-B prevalence and a diverse range of recombinants (Figure 8).

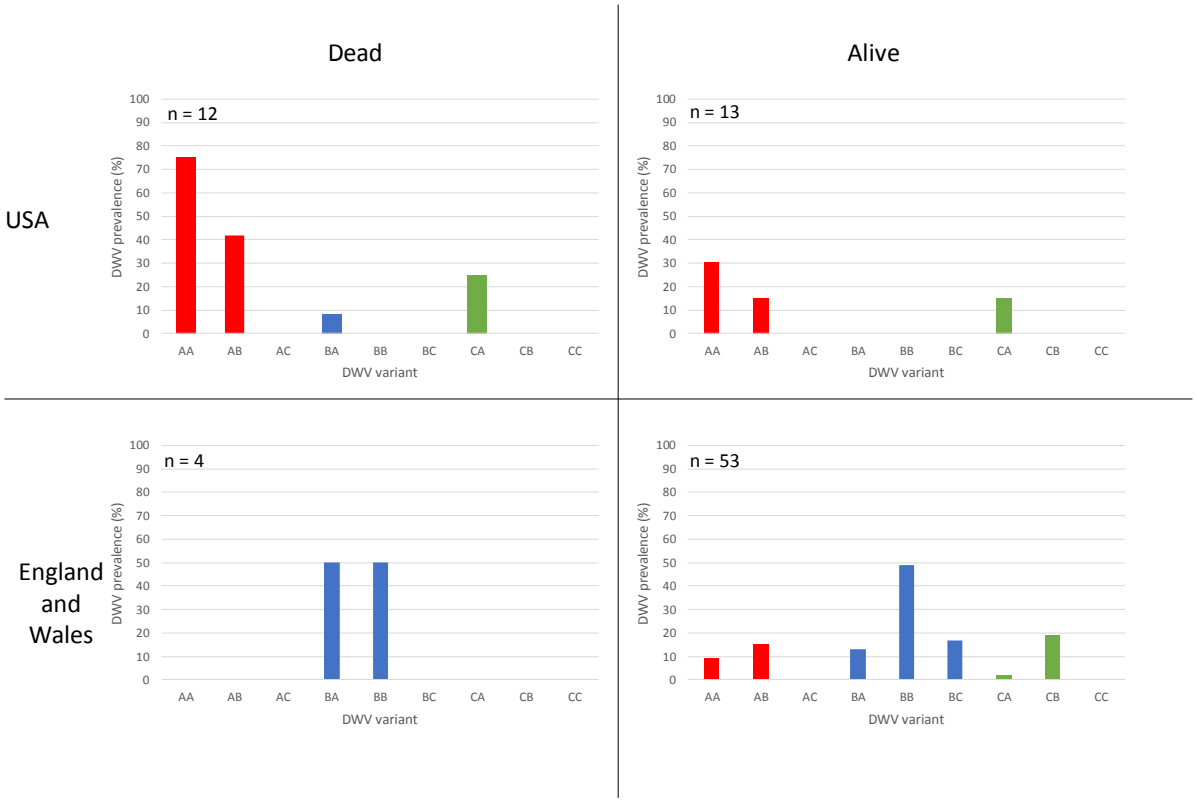


Figure 8. Shows the prevalence of DWV variants in England and Wales, and the USA colonies which survived or died the overwintering period of 2016/17.

Discussion

Six DWV recombinants along with the three master genomes were screened for using the new PCR primer combinations. The detection of at least one DWV recombinant occurred in the majority of DWV positive colonies screened (74% of English/Welsh colonies; 64% of USA colonies) (Figure 3). Diverse DWV populations were common in English/Welsh colonies, which had multiple master variants and recombinants. However, the six DWV recombinants were present in honey bee colonies at levels of < 25% prevalence (Figure 3), whilst viral loads were often detected <1E+03 for four of the recombinant pairs, for the majority of positive samples. Furthermore, the type-A and B master variants were shown to dominate

the colonies screened, confirming the results of Chapters two and three. It is possible that other DWV recombinants were present but not detected in the assay, such as those which have three recombinant breakpoints (Dalmon et al., 2017).

Recombinant forms of RNA viruses are considered less fit than the parental genomes (Desbiez et al., 2011; Garcia-Arenal et al., 2003), the results of this study show that the majority of DWV recombinants are no exception. Only in the USA data set were there a significant correlation between the structural (cp) and non-structural (RdRp) genes in samples which had gel confirmed positive recombinants of type-A (cp) / type-B (RdRp), furthermore these were detected in high loads, showing that this recombinant is virulent (Figure 4). The remaining recombinant pairs were not shown to be present in high loads. However, the prevalence data (Figure 3) shows that recombinants variants are present in honey bee colonies despite the detection of only one of the master variants, these results suggest that viral recombinants are capable of replicating in honey bees, but cannot compete with the fitter master genomes of type-A and type-B.

Type-B structural (cp) genes were shown to be present at levels ten-fold greater than the non-structural (RdRp) genes in both the English/Welsh and USA honey bee colonies, whilst the reverse was seen for type-A and C which had RdRp genes far exceeding the cp genes (Figure 6). These surplus genes could possibly be degraded RNA due to a host immune response such as RNA silencing, which occurs in a vast range of organisms as a result of viral infection (Ding & Voinnet, 2007). Alternately, they could reflect recombinant pairings of the type-B structural genes (cp) with non-structural (RdRp) genes of the type-A and C variants. It

has been evidenced that type-B can out-compete other DWV variants as a result of being able to replicate at a higher rate (McMahon et al., 2017) and its regular detection in high loads in Chapter two further confirms this. Therefore, it is suspected that when co-infection occurs in a host cell between type-B and the A or C master variants, the opportunity for replicative recombination via template switching is increased and occurs at random, giving rise to the diverse DWV population in English and Welsh colonies. This is an important aspect of viral evolution and eventually leads to the emergence of new viruses (Domingo & Perales, 2016), of which the impact upon a honey bee colony will be unknown.

No one overall mechanism for *Varroa* tolerance was found in the data. English/Welsh *Varroa* tolerant colonies had the highest prevalence of DWV recombinants and master variants whilst the USA tolerant colonies had few, with only the recombinants of type-A (cp) / type-B (RdRp) and type-C (cp) / type-B (RdRp) being detected in one colony each. The absence of more than one DWV master variant in the majority of USA *Varroa* tolerant colonies (11 out of 13 colonies) is attributed to this result. As the *Varroa* tolerant bees have previously been shown to have comparable DWV loads as susceptible colonies (Chapter two & three), it is suspected that the tolerant bees exhibit a behaviour which keeps *Varroa* numbers at levels similar to the treated (susceptible colonies), which then limits the opportunity for *Varroa* to act as a vector.

The prevalence of DWV recombinants and master variants in colonies which died unexpectedly overwinter were also investigated (Figure 8). The colonies which died in the USA were type-A dominated with an increased prevalence of the type-A (cp) / type-B (RdRp)

recombinant and viral loads 10-fold higher than those which survived. The opposite was seen in England and Wales where surviving colonies were type-B dominated, with diverse recombinant populations and viral loads 10-fold higher than dead colonies. Only four dead UK colonies were included in the data however in Chapter two it was concluded that the death of these colonies was not solely due to DWV infection due to a large number of colonies that survived with high type-B viral loads. The same conclusion is drawn here, however as the sample size is limited it is recognised that further sampling is required. UK colony losses for the winter of 2016-17 were lower (13% overwinter colony loss in 2016) (BBKA, 2017) than those seen in the USA (21% overwinter colony loss in 2016) (Bee informed partnership, 2017). Type-A prevalence and viral loads exceeding 1E+09 were evidenced in USA colonies that died overwinter (Chapter three) and the same result was also seen here, however the type-A (cp) / type-B (RdRp) recombinant was also prevalent in these colonies and provides new evidence that not only is this recombinant able to replicate in high loads (Figure 4), it was also prevalent in colonies which failed to successfully overwinter. In addition, other studies have previously raised concerns over increased virulence of recombinants of master variants type-A and B (Moore et al., 2011; Zioni et al., 2011; Ryabov et al., 2014). The result produced here suggests that the recombinant of type-A (cp) / type-B (RdRp) are virulent.

Master variant type-C was almost absent in both data sets, the type-A (cp) / type-C (RdRp) recombinant was totally absent in all the colonies screened. Whilst the type-C (cp) / type-A (RdRp) recombinant was found in higher prevalence in colonies that died over the winter period in the USA. The recombinant of type-A (cp) / type-C (RdRp) was previously found in

colonies which died unexpectedly during the winter period (Mordecai et al., 2016a). Our results suggest that this variant is implicated in colony losses, however more studies of this nature are required to confirm this result.

Conclusion

Overall, the results in this study imply that the majority of DWV recombinants are not as fit as their parent genotypes, as viral loads and prevalence were lower than those of master variants type-A and B. The results confirm pervious findings that England and Wales are mainly comprised of DWV master variant type-B, the USA is mainly type-A and type-C is rare (Chapters two and three). The type of recombinants present in a colony and the prevalence of these is driven by co-infection and also by the dominating master variant. No one overall mechanism aiding in *Varroa* resistance were found and the situation varies between the England/Wales and USA honey bee colonies.

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GENERAL DISCUSSION

The unacceptable percentage of honey bee colonies that die in the northern hemisphere during the overwinter months has led to a huge amount of concern amongst beekeepers and the scientific community (Aizen et al., 2009). The cause of overwinter colony losses has now been linked to the association between the *Varroa* mite and Deformed Wing virus (DWV) which the mite vectors with lethal consequences (Highfield et al., 2009; Martin et al., 2012; Schroeder & Martin, 2012; Nazzi et al., 2012; Francis et al., 2013). The recent discovery of three DWV master variants; Type-A (Lanzi et al., 2006), B (Ongus et al., 2004) and C (Mordecai et al., 2016a) has provided researchers with the opportunity to assess the impact of each variant on the honey bee host and also host tolerance, both of which have been a major focus of this PhD.

I have shown that DWV is a prevalent pathogen in English, Welsh and USA honey bee populations. In the English and Welsh data set every colony tested positive for DWV, whilst 95% of USA colonies tested positive. These results were not surprising since DWV is commonly detected in colonies with *Varroa* infestations (Carreck, Ball, & Martin, 2010; Martin et al., 2013; Martin et al., 2012; Mondet et al*.,* 2014). However, there was a striking difference between the DWV variants present in both the English, Welsh and USA colonies. The English and Welsh DWV population was dominated by type-B, type-A was detected in 99% of colonies but 84% of these had type-A proportional viral loads of <10%. The reverse was seen in the USA where 78% of colonies were dominated by type-A and 24% by type-B. DWV type-C was rare in both data sets (Chapters two and three).

Multiple DWV infections were often detected in honey bee colonies with either type-A or B achieving dominance (Chapter five). DWV co-infection allows for DWV viral recombinants to form, some of which have previously been identified between the structural and non-structural genes of type-A and B (Moore et al., 2011; Ryabov et al., 2014, Dalmon et al., 2017) and also type-A and C (Mordecai et al., 2016a). Therefore, I decided to screen colonies for the prevalence of DWV recombinants between the structural and non-structural genes. I found that DWV recombinants are commonly detected in colonies, however these are not as dominant as the master genomes. Viral recombinants are often considered less fit than the parent genomes (Desbiez et al, 2011; Garcia-Arenal et al, 2003), and for the majority of DWV recombinants this appears to be true. An increased virulence was previously reported for recombinants of type-A and B (Moore et al., 2011; Ryabov et al., 2014) and these recombinants were detected in high loads (1E+10) in the English, Welsh and USA data sets. The impact of these recombinants on colony health is still unknown.

The analysis of historic samples reveals that type-B prevalence has risen in the last decade in England. Samples collected in Devon in 2007 had DWV infections primarily comprised of type-A and C, whilst type-B was detected in low prevalence in my assay it was evidenced to be absent in works conducted by Baker & Schroeder (2008). In 2010 type-A dominated 33% of English colonies with viral loads exceeding 1E+08 in the majority (85%) of positive colonies, whilst type-B dominated 77% of colonies and viral loads were greater than 1E+10 in over half (54%) of the data. Only 7% of UK colonies were type-A dominated in 2016, the remaining 93% were dominated by type-B. Both Mordecai et al (2016b) and McMahon et al.

(2017) show that DWV type-B outcompetes type-A and their results explain why DWV type B prevalence increased overtime in the data presented in Chapter two.

A similar situation is currently occurring in the USA where Ryabov et al. (2017) reported only two type-B positive colonies in 2010, a number which increased to 121 colonies in 2016. The data presented in Chapter three confirms the result produced by Ryabov et al. (2017), but shows that DWV type-A is still the dominating master variant. The ability of type-B to dominate was shown in 24% of the colonies screened. Sequence data provided by Ryabov et al. (2017) found that type-B infections present in Florida colonies from 2016 were identical to European strains of the virus, furthermore Wilfert et al. (2016), show DWV fragments migrating from European populations of bees into the USA. Thus, allowing for the potential of European DWV type-B to exclude DWV type-A in USA colonies. The gradual increase in type-B dominated colonies suggests that type-B is a newly emerging or remerging variant in the USA (Ryabov et al., 2017). The gradual increase in the UK over the decade and the recent detection of type-B in the USA suggests that over time the USA may become dominated by type-B. I predict that this will occur in the next decade, however the situation will require monitoring.

Varroa tolerant and susceptible colonies were selected for analysis and careful consideration as to when a colony can be termed tolerant was taken. I deemed a *Varroa* tolerant colony as any colony which has been *Varroa* unmanaged for longer than 4 years. This is because susceptible colonies have been shown to die beyond this point (Korpela et al., 1982). The susceptible colonies were those managed for *Varroa* by receiving regular

Varroa treatments throughout the beekeeping year. These treatments involve a variety of methods, with the common goal of reducing mite numbers and in-turn DWV viral load (Martin et al., 2010; Locke et al., 2012). *Varroa* tolerant colonies were sampled from English, Welsh and USA apiaries, along with a long-term *Varroa* tolerant population of bees from the Arnot forest (Chapters two, three and four). The Arnot forest bees have survived with *Varroa* for over 20 years. All the data sets show that *Varroa* tolerant colonies have pre-winter DWV loads similar to the susceptible bees. In the English and Welsh populations colonies were dominated by type-B and in the USA type-A was dominant, therefore the variant present in a colony did not aid in the colonies long-term survival. It is more likely that it is the tolerant colonies ability to maintain pre-winter DWV viral loads which enables their survival. Early works by Martin et al. (2010), show an increase in DWV prevalence prior to the overwinter period in colonies that were not treated for *Varroa* which later went on to die. Highlighting the importance of *Varroa* control in managed colonies however, this no-longer appears to be the case as an increasing number of beekeepers have stopped managing for *Varroa* and colonies appear to survive despite this decision. I have shown that DWV loads did not increase prior to the overwintering period despite the lack of *Varroa* management in tolerant colonies. Recent work shows DWV infections are maintained in honey bee colonies despite the removal of *Varroa* (Locke et al., 2017) due to the feeding activities of bees, and could explain why no significant difference in viral load occurred between the *Varroa* susceptible and tolerant colonies sampled. However, this is not suspected to be the case for the late summer susceptible samples, as beekeepers were asked to provide these prior to applying pre-winter *Varroa* treatments.

Information about the role of *Varroa* tolerance and the DWV master variants present in a tolerant colony is almost non-existent, only two papers report on specific DWV master variants and their role in *Varroa* tolerance (Mordecai et al., 2016b; Brettell & Martin, 2017). Superinfection exclusion via type-B was proposed by Mordecai et al. (2016b) to be enabling the survival of a tolerant population of UK bees, whilst Brettell and Martin (2017) found avirulent DWV variants to be present in a Brazilian tolerant population of honey bees. This situation appears very rarely, and may have arisen due to the isolated nature of the population. Neither of these situations were evidenced when investigating DWV variants and viral load present in the tolerant colonies sampled in Chapters two, three and four. Another study involving tolerant bees located in Sweden found DWV loads in *Varroa* susceptible colonies and tolerant colonies were comparable between the two (Locke et al., 2014). The Swedish tolerant bees were later evidenced to exhibit a *Varroa* reducing trait. Oddie et al. (2018), discovered the parallel evolution of a *Varroa* reducing recapping trait in long-term *Varroa* surviving colonies from France, Sweden and Norway. Here, adult worker bees were shown to detect and recap *Varroa* infested brood which reduced mite reproductive success. The exact mechanism which keeps DWV loads at levels seen in *Varroa* tolerant bees similar to susceptible colonies in this Ph.D. remains unclear, however it is considered that tolerant populations mitigate *Varroa* infestations thereby hampering the DWV-*Varroa* vectoring cycle. It is possible that the *Varroa* tolerant colonies sampled may have also developed *Varroa* reducing traits such as the recapping trait, yet this needs to be evidenced further.

I also explored the role of DWV variants and how they influence colony survivorship (Chapters two and three). The UK mortality data revealed that only 4% of the colonies died, this result is also supported by the BBKA overwinter colony loss survey who reported a 13.2% loss, which was lower than the previous year (16%). Prior to my undertaking of this work DWV was suggested to offer a level of protection to tolerant colonies via superinfection exclusion (SIE), where less lethal variants of a virus out-compete more lethal variants and become established in a population (Mordecai et al., 2016b). The results in Chapter two show that this phenomenon is widespread across the England and Wales, and was prevalent prior to my research and also at the time of the Mordecai et al. (2016b) study. Furthermore, there are no reports in the literature of DWV type-B infection prevailing in colonies which failed to successfully overwinter; type-B has only been shown to kill worker bees when loads exceeded 1E+12 (McMahon et al., 2017). In addition, type-B was detected in mid-high loads (1E+09) in a honey bee population sampled in autumn which was sampled again in spring (Natsopoulou et al.,2017). Viral loads in spring were low (<1E+08) leading the authors to suggest that type-B is lethal to adult worker bees (Natsopoulou et al., 2017). I believe this result can be explained by the seasonal prevalence of DWV in colonies, as load and prevalence is generally lower in spring than it is in pre-wintering bees (Martin et al., 2010, Gleeny et al., 2017). The low number of losses witnessed in the Chapter two further provide evidence that below the host threshold of tolerance (which I suspect is > 1E+12) type-B offers a level of protection by inhibiting the replication of type-A, however this protective factor comes at a cost as long-term chronic infections have been evidenced to reduce the life span of bees (Dainat et al., 2012a; Benaets et al., 2018). All variants of the disease have negative consequences; however, the results indicate that these are reduced in type-B dominated colonies. Due to the competitive nature of RNA viruses of the same

species complex, it is possible that one of the DWV variants may evolve to out-compete type-B and this situation will need to be monitored. Additional monitoring should also focus on colony losses to further confirm that DWV type-B is less lethal to honey bee colonies than type-A or C.

The USA overwinter colony mortality data revealed 40% of apiaries experience unexpected overwinter colony losses, whilst the Bee informed partnership colony loss survey reported a 21% loss for the winter of 2016/17, however colony losses for the winter of 2017/18 increased to 31%. The losses reported in the UK could not be linked to DWV due to the high number of colonies surviving with similar DWV variants and viral loads, whereas in the USA an association between overwinter colony death and DWV type-A was witnessed. Type-A and the loss of over half the *Varroa* untreated colonies were also evidenced after the arrival of *Varroa* in Hawaii (Martin et al., 2012). Furthermore, Highfield et al., (2009) reported the loss of colonies that had high type-A loads in 2007. These colonies were rescreened using the ABC assay detailed in Chapter one, DWV type-A and C were found to be present in colonies that died. In addition, previous research shows overwinter losses of colonies with high type-A viral loads (Berthoud et al., 2010, Dainat et al, 2012a; 2012b; Francis et al., 2013). Therefore, the association between type-A, *Varroa* and colony death has already been evidenced and is a likely explanation as to why USA colony losses are periodically higher than those reported in the UK.

A link between type-B and *Varroa* has been established (Hawkins et al., appendix one) as *Varroa* infested larvae have significantly higher type-B loads than type-A and non-infested

larvae. Therefore, it is suspected that the type-B variant present in UK colonies is preferentially vectored by *Varroa* mites to honey bees. This also supports the theory that DWV type-B has evolved in the DWV-*Varroa* vectoring cycle (Mordecai et al., 2016b) and a trade-off between virulence (ability to replicate in a host) and pathogenicity (ability to cause disease symptoms) has been achieved, therefore it is suspected that DWV type-B is less virulent than other DWV variants at the colony level. *Varroa* mites require honey bee larvae to complete their reproductive cycle, during this time they will feed on developing larvae. It is the feeding activity of the mites that has been evidenced as an effective DWV transmission route (Ball, 1989; Bowen-Walker et al., 1999; Carreck, Ball & Martin, 2010). If a virulent virus is transmitted the larvae will die along with the mite, preventing the transmission of the virus. This is seen in colonies infected with Acute bee paralysis virus (ABPV) which can kill a host in as little as 3 days (Maori et al., 2007). Therefore, the viruses that the mite's vector must not kill the host. I suspect a trade-off has occurred where a less virulent has been selected for in the DWV-*Varroa* vectoring cycle and explains the low percentage of English and Welsh colonies lost in chapter two. Vector borne virulence trade-offs are hard to evidence in the field as the viral load of the vector has to be established and the virulence to the host monitored (Froissart et al., 2010). The spread of type-B across the USA provides the perfect opportunity to further evidence the *Varroa*-DWV virulence trade-off in the field, whilst lab assays can be used in conjunction to mimic what is happening in the wider environment.

All these findings were only made possible by establishing a new method (Chapter 1) which enabled the quantification of all three DWV variants. Since publication this method has now

been used in studies concentrating upon the *Varroa*- DWV vectoring cycle (Hawkins et al., paper in prep; Appendix one), viral spill-over from honey bees into colonies of stingless bees from Brazil (De Souza et al., 2018; Appendix two), and also in research involving DWV naïve bees found in Australia (Amanda Norton personal communication). Furthermore, the importance of using an RNA standard when quantifying RNA viruses was highlighted in this study to encourage it's use in the wider community.

Conclusion and future works

Overall, I set out to address the role, prevalence and viral load of each of the DWV variants and how these are implicated in *Varroa* tolerance and overwinter colony losses. I have provided evidence of the widespread prevalence and dominance of DWV master variants in honey bee colonies from England, Wales and the USA. This data shows type-A is prevalent in the USA and linked to overwinter colony losses. Type-B is prevalent in the UK and could not be linked to colony losses as colonies survived with type B infections. In addition, I have shown SIE via type-B is currently an English and Welsh phenomenon. I also provided evidence that honey bees will develop *Varroa* and disease tolerance if left untreated for greater than 4 years in the English, Welsh and USA populations however the exact mechanism for this tolerance remains unclear and is most likely linked to a *Varroa* reducing behaviours.

Further research is required to establish when DWV type-B infections become lethal at the colony level via both field and lab experiments. This work is important as DWV type-B has

not been shown to cause the death of overwintering colonies and currently appears to be less lethal than DWV type-A or C. If the protective factor of DWV type-B is continually evidenced than it could have the potential to hamper colony losses. I predict that type-B will gradually replace type-A in the USA and this will also coincide with a reduction in overwinter colony losses. This situation will require constant monitoring and will enable us to further understand type-B SIE. Further experiments are also required in the *Varroa* tolerant colonies to establish the exact mechanism which maintained seasonal DWV loads, a behavioural trait is suspected to be the cause.

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APPENDICES

Appendix 1: Effect of *Varroa* on the variation of transmission of DWV strains in honey bee pupae

Hawkins George, Kevill Jessica, Chambers Lucy, Martin Stephen J

Abstract

When transmitted by the ectoparasitic mite *Varroa* destructor, Deformed wing virus (DWV) is strongly associated with the death of colonies. Currently three master variants of DWV exist (A, B, and C), however little is known about the impact or dynamics of each variant. This study found that natural viral loads of type-B are increased by *Varroa* more consistently than type-A. This could lead type B becoming dominant variant in the population and helps support for the notions that type-B is the faster replicator and is becoming the dominant genetic variant of DWV in the USA and UK.

Introduction

The mite *Varroa* destructor is responsible for the collapse of millions of colonies via the transmission of Deformed wing virus (DWV) (Dainat et al., 2012; Martin et al., 2012). In its natural state, DWV is transmitted vertically via sexual reproduction (Yue et al., 2007) and horizontally via oral ingestion (Yue & Genersch, 2005). Infection via these pathways has been generally characterised by low prevalence, low viral loads (Ryabov et al., 2014), and high strain diversity (Martin et al., 2012). The nature of DWV in A. mellifera has however been drastically altered since exposure to *Varroa* (Martin et al., 2012), with elevated viral loads, reduction in strain diversity and high prevalence leading to reduced longevity and

eventually death of the host colonies (De Miranda & Genersch, 2010). The interrelations between DWV, the mites and colony health remain complex and dynamic. If the phenomena described above were absolute, the removal of *Varroa* would reduce viral prevalence as was originally demonstrated (Martin et al., 2010). However, now the viral landscape has been permanently altered by the spread of *Varroa*, an increasing number of studies are indicating the importance of viral transmission in the absence of *Varroa*, and have shown that high levels of DWV can persist even following treatment (Locke et al., 2017).

To date, three of these master variants of DWV have been described: type-A (Martin et al., 2012), type-B (formerly *Varroa* destructor virus-1, or VDV-1) (Ongus et al., 2004), and type-C (Mordecai et al., 2016b). Although the effects of natural and *Varroa* mite transmission on DWV have been extensively studied, no studies have compared whether *Varroa* transmission differentially affects any one of the three master variants. This preliminary study investigated whether natural and *Varroa* transmitted viral loads differed between the master variants present.

Materials and Methods

Eight honey bee colonies were sampled in total from the North West region of England, UK; three in September 2016 from Manchester, and five in September 2017 from Merseyside. As the sampling took place prior to winter treatment *Varroa* infestation was present in all. One frame containing all brood stages was removed from each colony and freeze-killed at -20°C. Individual brood were removed and pooled (up to 10 per sample) according to their colony, developmental stage (egg, larvae, and pupae [pink-eyed or older]), and presence or

absence of *Varroa* on the pupae. Each pooled sample was frozen in liquid N2, crushed to a powder-like consistency using autoclaved pestle and mortars, and stored at -80°C. RNA extraction was preformed using a RNeasy mini kit and quantified via NanoDrop and diluted to obtain 50ng/ul. RT-qPCR was performed on a Rotor-Gene 6000 (Qiagen) using SensiFast SYBR No-Rox One-Step kit (Bioline) and primers developed by Kevill et al. (2017) (to detect types A, B and C). Each sample was run in triplicate to increase reliability. Any samples that generated readings below the standard detection threshold (Ct value >30), were not included in the analysis to rule out the possibility of quantifying false positives or contamination. If a difference greater than three was generated between any Ct values, the outlier was removed before calculating the mean. From these averages, the genome equivalents (G/E) were calculated to produce copy numbers that were the equivalent to an individual bee; the calculation used is as follows: (average copy number) x (RNA dilution factor) x (elusion volume of RNA) x (proportion of bee material) (Kevill et al., 2017).

As the G/E data did not fit normal distribution even following log10 transformation a series of nonparametric Mann-Whitney U tests were performed to ascertain significant differences.

Results & Discussion

In all 64 samples DWV types-A and B were detected and 28 tested positive for type-C. Within the 64 samples, one or more of the three master-variants were quantifiable in 35 samples (4 eggs, 8 larvae, 11 pupae and 12 mite-infested pupae). Type-C was only quantifiable in four sample, all low (median = 1.04E+08) amounting for <0.1% of all the copies. Types A and B were quantifiable in all brood stages, indicating an increasing viral

load as the brood developed (Fig. 1). This confirmed transmission via sexual reproduction (Yue et al., 2007; Amiri et al., 2016) for both A and B and that both variants (type-B in particular) can persist at high levels in the absence of *Varroa* (Highfield et al., 2009; Locke et al., 2017), implying that treatments that simply remove the mites are not sufficient in eradicating these variants from a population.

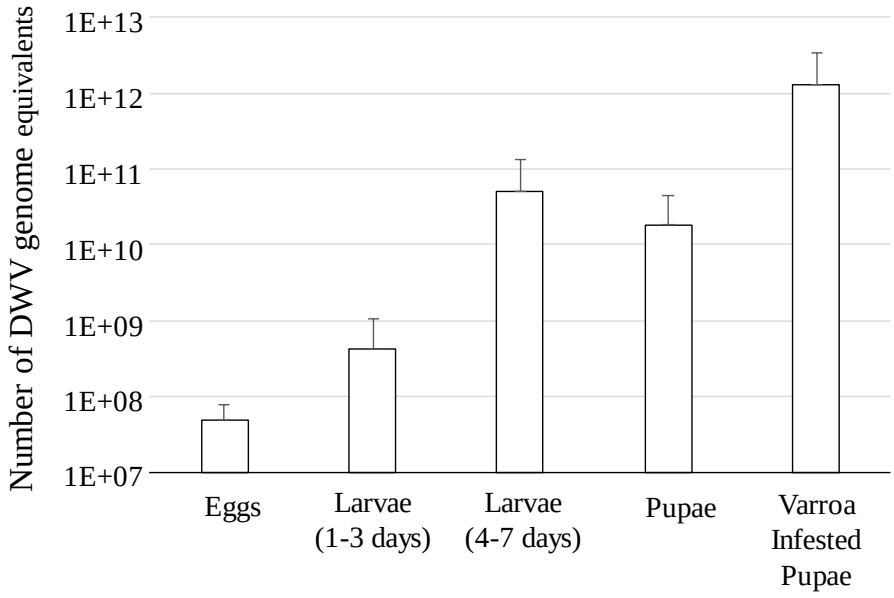

Fig. 1 The average DWV load measured in genome equivalents, in various developmental stages of worker brood of Apis mellifera, indicating that *Varroa* has a significant (w=610, p=0.01) effect on DWV viral load. The error bars indicate one S.D.

When the effect of *Varroa* on DWV transmission of the A and B variants were analysed the median average viral loads of the B-type were consistently higher when transmitted by *Varroa* (w=203, p=0.004) than when naturally transmitted (Fig. 2); a similar outcome was not generated for type-A (w=101, p=0.170) (Fig. 2).

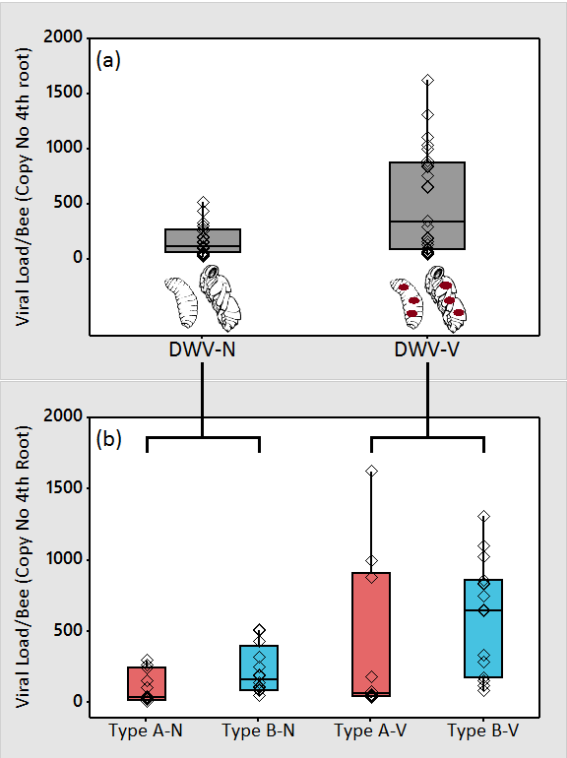

Fig. 2. DWV viral load in *Varroa* infested and non-infested larvae. (a) shows total DWV load. (b) Shows viral load for type-A (red) and type-B (blue)

Thus, the impact of *Varroa* mite transmission is more profound on Deformed wing virus (DWV) type-B in comparison to the classic type-A. This preferential selection during *Varroa*-mediated transmission provides evidence in support for type-B having a higher replication rate (McMahon et al., 2016), which can subsequently lead to the dominance over and exclusion of type-A from a population (Mordecai et al., 2016a). This may also help explain the recent discovery that type-B is widespread in Germany (Natsopoulou et al., 2017) and becoming the dominant variant of in USA (Ryabov et al., 2017) and UK (McMahon et al, 2016).

Given the significant increase in type-B viral loads in association with *Varroa* mite transmission, this master variant could now be outcompeting types-A and C. What this

means for the future of honey bees however is largely unknown. The danger that type-B poses has been demonstrated (McMahon et al., 2016; Benaets et al., 2017), yet colonies exist that paradoxically survive harbouring high loads of this variant (Mordecai et al 2016a).

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Appendix 2: Occurrence of Deformed wing virus variants in the stingless *Melipona subnitida* and honey *Apis mellifera* bee populations in North Eastern Brazil

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Key Words: spill-over, *Varroa*, viral variants

Abstract

Due the association with the parasitic honey bee *Varroa* mite, Deformed wing virus (DWV) has become one of the world's most widespread and studied insect viral pathogens. In Brazil, stingless bees (*Meliponini*) are a diverse and widespread group with many species being managed for honey production, often in close proximity to honey bees. The aim of this study was to investigate the prevalence and viral load of DWV in the stingless bee (*Melipona subnitida*) and honey bee (*Apis mellifera*) from NE Brazil. DWV prevalence was 100% in both species with the A and C-variants dominating in *M. subnitida* and the A-variant in *A. mellifera*. The average viral loads were 8.83E+07 and 7.19E+07 in *M. subnitida* and *A. mellifera* respectively. However, on the remote island of Fernando de Noronha DWV a previous study showed DWV very low (<1E+03) is the honey bees, but 1.6E+08 in the neighbouring nine *M.*

subnitida colonies, indicating that no spill-over of DWV from *M. subnitida* to *A. mellifera* has occurred during the past 34 years when these two species were introduced to the island and maintained in close proximity. Again, the ubiquitous present of the DWV-C variant in *M. subnitida* colonies, and rarity in *A. mellifera*, suggests limited viral exchange between these two species.

Introduction

The stingless bees (Apidae: *Meliponini*) are the most diverse group of eusocial bees, comprising of more than 400 species contained within 60 genera (Rasmussen and Cameron 2010). The majority of species occur in the Neo-tropics with colonies typically containing 200-700 adults and a perennial life-cycle (Wille 1983). Many species, particularly the large *Melipona* species have a long association with humans that harvest their highly prized honey (Jaffé et al. 2015), but they are also responsible for pollinating 40-90% of the native flora in some regions of Brazil (Nascimento et al. 2000). Relative to the honey bees (*Apis* spp), very little is known about the pests and pathogens of stingless bees despite their importance.

Brazil has a long history of managing honey bees (*Apis mellifera*) originally imported from Europe, but in 1957, 26 colonies of imported African *A. m. scutellata* escaped quarantine and spread throughout Brazil, hybridising with existing honeybees to form the Africanised honey bee (Winston 1992). However, when in 1971 the parasitic *Varroa* (*Varroa destructor)* mite arrived in Brazil, the Africanised honey bees were natural tolerance to the mite, whereas, the European honeybees suffered large scale losses. These losses are caused

by a viral pathogen called Deformed Wing Virus (DWV) that is transmitted by the *Varroa* mite (Rosenkranz et al., 2010).

Although *Varroa* can only survive on honey bees, Santamaria et al (2018) showed that the raised DWV levels in the honey bee population, initiated by the mite, has resulted in viral the spill-over into other species of bees and wasps. This may explain why DWV has been detected in a wide range of non-*Apis* insects (Singh et al. 2010, Evison et al., 2012; Levitt et al., 2013; Manley et al. 2015) and has even been detected in pollen (Mazzei et al 2014). The impact of DWV on these hosts remains unknown (Tehel et al. 2016), although there is growing concern (Li et al., 20011; Fürst et al. 2014,; Graystock et al. 2015, Manley et al. 2015).

In Brazil, the Africanised honey bee, *Varroa* mite and DWV have been present for decades so there have been ample opportunities for cross-species infections to occur, especially since both honey bees and stingless bees are often managed in close proximity, i.e. in nearby apiaries. Therefore, the aim of this study was to evaluate both the prevalence and viral load of the three described DWV master-variants (A, B and C) across a population of stingless bee (*Melipona subnitida*) and Africanised honey bees from North-Eastern Brazil. The stingless bee *M. subnitida* is swarm founding species, brood development takes around 40 days, and workers survive for a few months. This species is endemic to the dryland-shrub forest 'Caatinga biome' found in NE Brazil and is the typical stingless bee maintained by beekeepers throughout the region. These meliponiparies help towards the conservation of local biodiversity, as well as provide extra income to the beekeepers (Jaffé et al. 2015).

Materials and Methods

Samples

Pools of 30 *M. subnitida* workers were collected using a pooter directly at the entrance of 24 colonies from meliponiparies at ten mainland locations across NE Brazil. Samples from Fortaleza and Mossoro were collected in 2016 with all other samples collected in 2013. In addition, pools of ten *M. subnitida* workers from nine colonies located on the remote oceanic island of Fernando de Noronha were collected in 2013 using the same method. These samples are interesting since this population was originally established by 30 colonies brought to the island in 1983 from the mainland states of Ceara and Rio Grande do Norte (Kerr and Cabeda 1985). In 1984 Kerr also established a small population of European honey bees on Fernando de Noronha that were accidentally infested by the *Varroa* mite, although the typically high levels of DWV were not present in either the honey bees or *Varroa* (Brettell and Martin 2017).

During the same period pools of 30 healthy adult worker Africanised honey bees where collected from the brood area of 12 colonies from six states across NE Brazil. All samples were collected into absolute ethanol and stored at -20° C before transportation to the UK under license to be analysed.

Detection and quantification of DWV variants

Total RNA was extracted from a pool of 10 heads per colony for both stingless and honey bees. Heads were used to avoids possible viral contamination from the gut and DWV may have a greater impact if present in the head (Fujiyuki, et al 2004). The heads were ground in liquid Nitrogen into a fine homogeneous powder, a 30mg sub-sample had its RNA extracted using a Qiagen RNeasy mini kit, which was enhanced by using a QIAshredder kit for the *M.*

subnitida samples (Forsgren et al. 2017). Nanodrop (8000 series) quantification was used to standardise the amounts of total RNA to 50 ng/µl using RNase free water, before been stored at -80° C.

In order to quantify the viral load of each variant we used a recently developed method (Kevill et al., 2017). Briefly, cDNA was synthesised using one-step SensiFAST SYBR No ROX One-step kit (Bioline, London, UK), the reactions contained 1ul 50ng/µl RNA 10ul Senifast mix, 0.2ul Reverse transcriptase, 0.4ul RNase inhibitor, 0.75 pmol of each primer (DWV F and R-Type-A, B and C [Table 1]) and 7.5ul of H_20. Reactions were run on a Rotor-Gene Q Thermocycler (Qiagen) with an initial reverse transcription stage at 45° C for 10 min and a denaturation step of 95° C for 10 min, followed by 35 cycles of denaturation for 15 s at 95° C, annealing for 15 s at 58° C for primers A and B, and 61.5 °C for primer C, and extension for 15 s at 72° C. A final dissociation melt curve was performed between 72° C and 90° C, at 0.5° C increments, each with a 90 s hold. 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 (Ct) value was determined for each sample using the QIAGEN Rotor—Gene Q Series Analysis software and viral quantification was done by using serial dilutions of the standard DWV RNA, ranging from 1E+02 to 1E+07 copies of DWV per reaction. All samples were run in triplicate and the average taken. Those samples which had a standard deviation of ≥3 Ct were re-run. Furthermore, PCR products were run on a 2% agarose gel stained with 0.001% GelRed to confirm the correct sized band had been amplified. A control housekeeping gene β-actin (Highfield et al. 2009) was also run to ensure no degradation of the samples had occurred, due to large distances these samples were transported both within and between countries. Genome equivalents were calculated per sample using the following equation:

Genome equivalents = (average copy number) x (RNA dilution factor) x (elution of RNA) x (proportion of bee material)

Table 1. Primers used in this study were developed by Kevill et al. (2017).

Target	Primer Name	Sequence (5' - 3')	Size of product (bp)
DWV Forward	DWVnew-F1	TACTAGTGCTGGTTTTCCTTT	
DWV Type A	DWVA-R1	CTCATTAACTGTGTCGTTGAT	155
DWV Type B	DWVB-R1	CTCATTAACTGAGTTGTTGTC	155
DWV Type C	DWVC-R1	ATAAGTTGCGTGGTTGAC	152

Results

Prevalence of DWV

We detected DWV in every *M. subnitida* and *A. mellifera* colony. Negative controls indicated no contamination had occurred in any of the runs. Furthermore, the housekeeping gene indicated all samples contain intact RNA (Fig. 1). The average Ct values indicated more β-actin in the *A. mellifera* samples (19.7Ct ± 1.91 S.D.) relative to the *M. subnitida* samples (23.5Ct ± 0.70 S.D.).

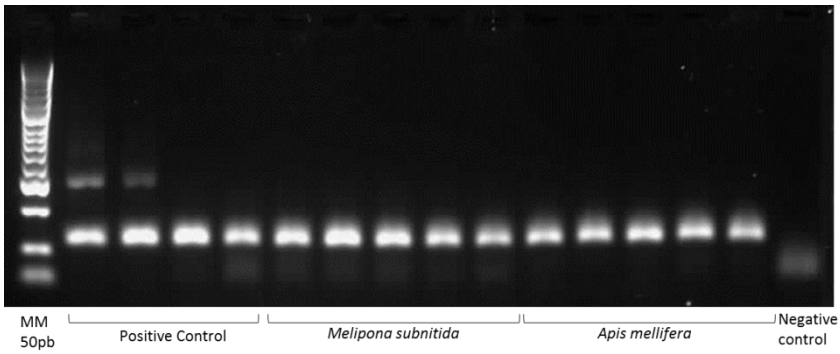

Figure 1. Typical gel showing the presence of β-actin in all samples of *Melipona subnitida*, *Apis mellifera* and positive controls, confirming that the samples contained intact RNA.

DWV viral loads

The DWV in the *M. subnitida* population, only the A and C master-variants were detected (Fig. 2). The DWV-A variant was dominate in 78% of the colonies (Fig. 2) and the C-variant dominating the remaining 22% of colonies. Whereas, in the honey bee colonies, 92% were dominated by the A-variant and only one colony (8%) was dominated by the C-variant. The DWV-B variant was quantifiable only in a single *A. mellifera* colony (Table 2) and detected on the gel but below the qPCR detection limit in three other *A. mellifera* colonies (Mossoro, Garanhus and Cruz das Almas). The viral loads detected in both species of bee averaged 8.8E+07 and 7.2E+07 in *M. subnitida* and *A. mellifera* respectively. On the remote Fernando de Noronha island, the *M. subnitida* colonies all colonies were dominated by the A-variant, and C-variant was widespread. However, the viral load was an order of magnitude higher on the island (1.6E+08) relative to the mainland (3.6E+07).

Table. 2. The mean viral load of each DWV master variant detected in the 21 *Melipona subnitida* and 12 *Apis mellifera* samples collected from across NE Brazil.

	Melipona subnitida Average viral load	*Apis mellifera* Average viral load
DWV-A	8.10E+07	6.96E+07
DWV-B	n.d.	2.35E+05
DWV-C	7.31E+06	2.06E+06
All	8.83E+07	7.19E+07

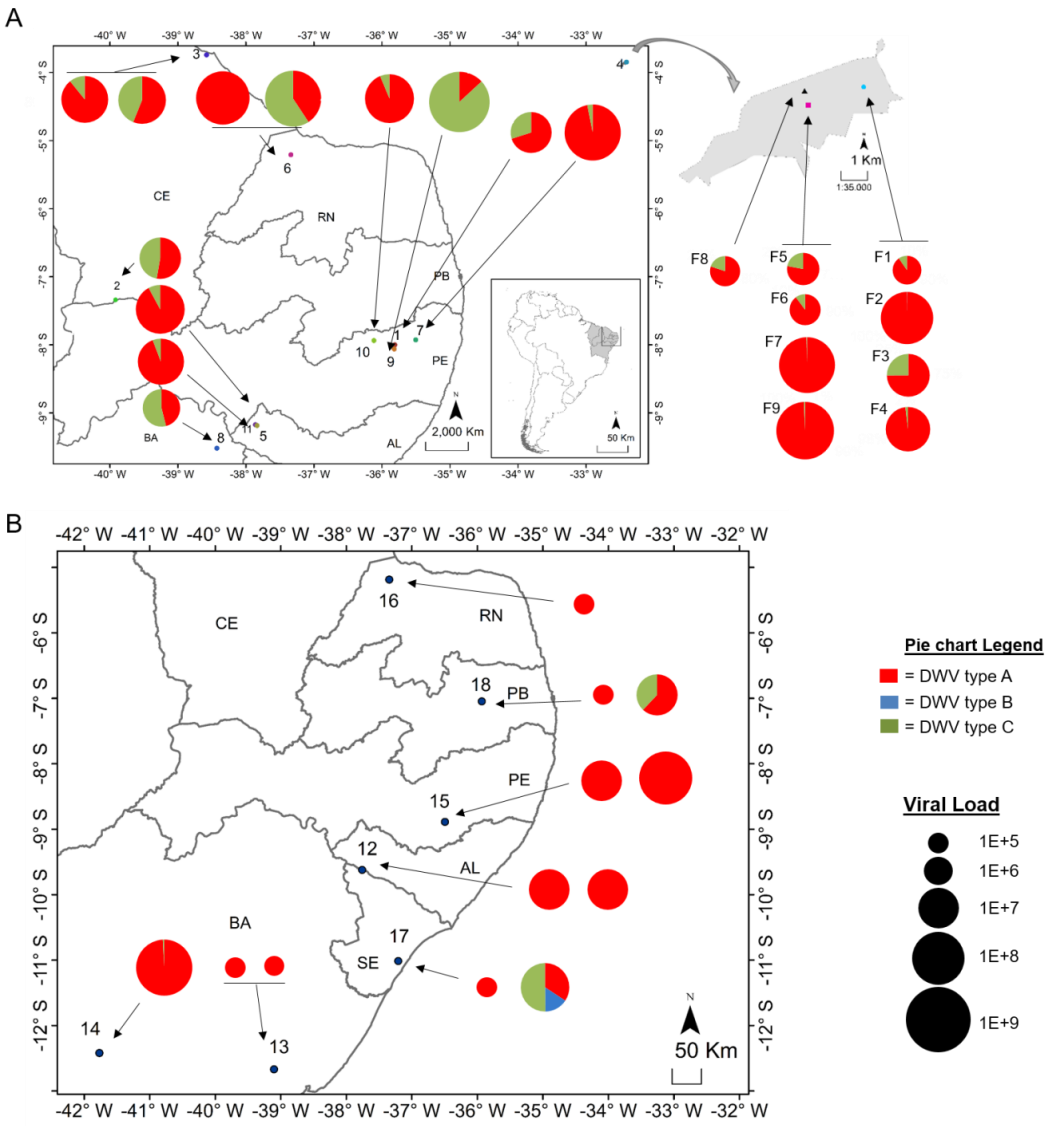

Figura 2. Proportions and viral loads DWV-A (red), B (blue) and C (green) variants detected in A) *Melipona subnitida* stingless bees and B) *Apis mellifera* from across NE Brazil. The sample locations are 1. Cumaru, 2. Exu, 3. Fortaleza, 4. Fernando de Noronha, 5. Mata Grande, 6. Mossoró, 7. Passira, 8. Paulo Afosnso, 9. Riacho das Almas, 10. Taquaritinga do Norte, 11. Água Branca, 12. Piranhas, 13. Cruz das Almas, 14. Seabra, 15. Garanhuns 16. Mossoró, 17. São Cristóvão, 18. Areial. The states are CE= Ceara, RN= Rio Grande Do Norte, PB= Paraiba, PE= Pernambuco, AL= Alagoas, SE= Sergipe and BA= Bahia.

Discussion

This study provides the first report of DWV in the *Meliponini* stingless bees, since DWV was not detected previously in the stingless bees *Melipona quadrifasciata quadrifasciata* and *M. torrida* (Alvarez et al. 2017), although they did detect the DWV-A variant in the Argentinian stingless bee (*Tetragonisca fiebrigi*). Furthermore, although *M. scutellaris* tested negative for six bee-associated viruses including DWV, but did test positive for the honey bee associated acute bee paralysis virus (Ueira-Vieira et al. 2005). The high prevalence of DWV in *A. mellifera* was expected since DWV is consistently the most prevalent viral pathogen of European and Africanised honey bees (Wilfert et al., 2016).

The dominance of the DWV-A variant found in this study reflects the situation found in honey bees in the USA in 2010 (Ryabov et al. 2017). Although the B-variant is replacing the A-variant in the USA (Ryabov et al., 2017) and appears common in Europe (McMahon, et al., 2016), it was only detected in any quantity in a single Africanised colony (Fig. 2). This is despite the likely long-term infection of both stingless and honey bees in Brazil. The rarely detected C-variant (Kevill et al., 2017, Ryabov et al. 2017) was presence in almost all the *M. subnitida* colonies.

Interestingly on the remote island of Fernando de Noronha where both *M. subnitida* and *A. mellifera* have been maintain in close proximity over the past 34 years, the DWV-A variant dominated all nine colonies with a mean viral load of 1.6E+08. Whereas in the European honey bees on this island have a low (~1E+03) viral load, and diverse range of DWV variants (Brettell & Martin 2017). This provides further evidence that DWV may be a general hymenopteran or insect virus rather than a honey bee pathogen that has spilled over into the pollinator community. Again, the ubiquitous presence of the DWV-C variant in *M. subnitida*

colonies, and rarity in *A. mellifera* colonies on the mainland again suggests limited viral exchange between these two species. The chance of spill-over may be reduced due to the low (8E+07) DWV viral loads present in both the stingless and honey bees of NE Brazil, relative to those found in asymptomatic (2.4E+09) and symptomatic (6.9E+11) European honey bees (Highfield et al. 2009). Whereas, when these high DWV loads are present in honey bees, DWV appears to spill-over into the neighbouring wasps and solitary bees (Santamaria et al., 2018). These low viral loads of DWV in Brazil may be attributed to hygienic habit of stingless bees (De Jesus et al. 2017), and *Varroa*-tolerance in Africanised bees, both which will reduce the viral load in a colony.

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