Emerging viral diseases of pollinating insects

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature: ……..

Abstract

The risks posed by rapidly evolving RNA viruses to human and animal health are well recognized. Epidemics in managed and wildlife populations can lead to considerable economic and biodiversity losses. Yet, we lack understanding of the ecological and evolutionary factors that promote disease emergence. Host-switching viruses may be a particular threat to species important for human welfare, such as pollinating bees. Both honeybees and wild bumblebees have faced sharp declines in the last decades, with high winter mortality seen in honeybees. Infectious and emerging diseases are considered one of the key drivers of declines, acting in synergy with habitat loss and pesticide use. Here I focus on multihost viruses that pose a risk to wild bumblebees. I first identify the risk factors driving viral spillover and emergence from managed honeybees to wild bumblebees, by synthesising current data and literature. Biological factors (i.e. the nature of RNA viruses and ecology of social bees) play a clear role in increasing the risk of disease emergence, but anthropogenic factors (trade and transportation of commercial honeybees and bumblebees) creates the greatest risk of viral spillover to wild bees. Basic knowledge of the pathogenic effect of many common pollinator viruses on hosts other than *A. mellifera* is currently lacking, yet vital for understanding the wider impacts of infection at a population level. Here, I provide evidence that a common bumblebee virus, Slow bee paralysis virus (SBPV), reduces the longevity of *Bombus terrestris* under conditions of nutrition stress. The invasion of *Varroa destructor* as an ectoparasitic viral vector in European honeybees has dramatically altered viral dynamics in honeybees. I test how this specialist honeybee vector affects multi-host pathogens that can infect and be transmitted by both honeybees and wild bumblebees. I sampled across three host species (*A. mellifera, B. terrestris and B. pascuorum*) from *Varroa*-free and *Varroa*-present locations. Using a combination of molecular and phylogenetic techniques I find that this specialist honeybee vector increases the prevalence of four multi-host viruses (deformed wing virus (type A and B), SBPV and black queen cell virus) in sympatric wild bumblebees. Furthermore, wild bumblebees are currently experiencing a DWV epidemic driven by the presence of virus-vectoring *Varroa* in *A. mellifera.* Overall this thesis demonstrates that wild bumblebees are at high risk of viral disease emergence. My research adds to the ever-expanding body of evidence indicating that stronger disease controls on commercial bee operations are crucial to protect our wild bumblebees.

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Statement of Contributions to this thesis

Chapter 1: The general introduction was written in its entirety by Robyn Manley (RM).

Chapter 2: RM researched, collated and analysed data, and wrote the review. Lena Wilfert (LW) provided advice on the content, structure and figures. LW and Mike Boots (MB) contributed to the writing through editing drafts before submission to the Journal of Applied Ecology.

Chapter 3: RM designed and carried out all the experimental and molecular work with training and advice from LW. Jess Lewis and Katherine Roberts assisted with the experiment set up. Devi Newcombe, Caroline Moussy, Corrina Lowry and John Hunt assisted with aspects of the sample processing. RM collected and analysed the data and wrote the article. LW and MB advised on and edited drafts before submission to Oecologia.

Chapters 4 and 5: In addition to NERC who funded this PhD, RM successfully applied for extra grants from The C.B. Dennis Trust and The Genetics Society that helped cover the costs of field and lab work for chapters 4 and 5. RM organised and carried out all field collections, with occasional help from field assistants (Martin Jones, Emma Davey and Daisy Gates). RM carried out all sample processing, molecular work, data collection and analysis, and wrote both chapters. LW provided training in molecular techniques and phylogenetic analysis, as well as advice on statistics and writing. Exeter Sequencing Service carried out sample library prep and sequencing on the Pacbio system. Dr Ben Temperton provided support and training for the Pacbio sequence analysis, and carried out the sliding windows analyses to identify DWV recombinants and novel strains presented in chapter 4. Daisy Gates, Megan Griffiths, Ciara McGinley and Sheera Abdulla carried out the DNA extractions, PCRs and ran gels to collect data on the prevalence of *N. ceranae*, presented in chapter 4.Toby Doyle and Lewis Campbell created the plasmids used in the qPCR assays in chapter 4 and 5. Lena Wilfert carried out the analysis to estimate the evolutionary rate of DWV-B, which we did not use, but is mentioned in the methods of chapter 4.

Chapter 6: The general discussion was written in its entirety by RM.

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Chapter 1 : General Introduction

Pathogens are an ever-present threat to their hosts' fitness and survival; infections can cause reductions in host longevity and fecundity, changes in behavioural and population dynamics, and even extinction (Anderson and May 1981; Boots and Sasaki 2002; Ebert and Herre 1996). Equally, a pathogen's fitness and survival depends entirely on its transmission to new hosts. Constant adaptation by host and pathogen to maintain their fitness can lead to long-term co-evolving interactions (Anderson and May 1982; Lambrechts et al. 2006). Evidence suggests that a trade-off between pathogen virulence (defined here as a reduction in host fitness by the pathogen (Anderson and May 1982)) and transmission leads to an optimum level of virulence in single host-pathogen systems (Bull 1994; Dwyer et al. 1990; Lipsitch and Moxon 1997). However, the majority of pathogens can infect and be transmitted by multiple hosts (Cleaveland et al. 2001; Pedersen et al. 2005), which complicates the dynamics of host-pathogen interactions. In a multi-host-pathogen system hosts may differ in their ecology, susceptibility and transmission potential of a pathogen, leading to unpredictable consequences for the evolution of virulence and pathogen epidemiology (Woolhouse et al. 2001). Although the complex population biology of multi host pathogens is not well understood, it is clear they create high potential for disease emergence and frustrate efforts for disease control. Indeed, novel and re-emerging diseases are continually arising (Jones et al. 2008) and present a significant threat to the health of human, animal and plant communities alike, with far-reaching impacts on our food security, biodiversity and the global economy (Cardinale et al. 2012; Jones et al. 2008; Morens et al. 2004).

A disease is said to be emerging when a pathogen spreads rapidly following a jump to a novel host (e.g. SARs, HIV, Myxoma virus), or when an established disease causes a new epidemic within the same hosts (Woolhouse et al. 2001). Re-emergence could occur because of pathogen evolution (e.g. tuberculosis, a disease previously believed to be in decline, has re-emerged as a current threat due to evolved multidrug-resistant variants of the bacteria *Mycobacterium tuberculosis* (Shah et al. 2007)), and/or changes in host-pathogen ecology such as the arrival of a novel vector or new transmission route (e.g. while HIV-1 is mainly transmitted via heterosexual sex, the practice of infected drug-users sharing needles substantially contributes to the rapid spread of HIV-1 outside of sub-Saharan Africa (Simon et al. 2006)). While arguably some of the worst epidemics have occurred due to host jumps

from animal reservoirs (both wild and domestic) to humans (Jones et al. 2008; Woolhouse et al. 2005), susceptible wildlife communities are also at risk of pathogen spillover from intensively managed domestic populations (Daszak et al. 2000). For example, the extinction of wild dogs in the Serengeti in 1991 coincided with an epidemic of canine distemper in sympatric domestic dogs (Macdonald 1992) and the recently eradicated Rinderpest virus of Eurasian cattle devastated African ruminants populations in the late 1800s (Sinclair and Norton-Griffiths 1995).

Not all host jumps result in epidemics, as the ecological, evolutionary and anthropogenic factors that determine whether a pathogen can establish in a new host are complex. One well documented risk factor is the nature of the pathogen: RNA viruses have been identified as high risk pathogens because of their high mutation rate (10^3 - 10^5 substitutions per site per year (Holmes 2008)) and poor error-correction ability, which facilitates adaption to new hosts (Taylor et al. 2001; Woolhouse and Gowtage-Sequeria 2005). Furthermore, pathogens that are transmitted indirectly (i.e. novel hosts become infected by contact with infectious stages of the pathogen contaminating the environment) are more likely to cause an epidemic compared to directly transmitted (i.e. by vertical, sexual transmission, or contact though close proximity) pathogens (Woolhouse et al. 2001). Vector-borne diseases are more likely to be zoonotic (Woolhouse et al. 2001), and high risk, with 75% of emerging and re-emerging diseases known to be zoonotic (Taylor et al. 2001). Broadly, changes in host-pathogen ecology can increase the opportunities for pathogens to access susceptible or novel hosts (Woolhouse et al. 2005). Such ecological changes are often the result of human interference (Daszak et al. 2000) i.e. urbanisation and agricultural intensification (e.g. a shift in land-use in Midwestern USA is suggested to have increased the incidence of Lyme disease), the globalisation of trade and travel (e.g. the spread of West Nile virus to North America by importation of infected vectors (Kilpatrick 2011)) or technology use (e.g. methods of food production caused the epidemic of bovine spongiform encephalopathy (BSE) in cattle that lead to the incidence of variant Creutzfeldt–Jakob disease (vCJD) in humans (Anderson et al. 1996). Changes in host diversity where key hosts for transmission are lost or gained can impact disease transmission and emergence. For example, in the USA the white-footed mouse is the most competent vector of *Borrelia burgdorferi*, the causative agent of Lyme disease; increased diversity of mammalian hosts increases the number of less competent hosts for the tick vector to feed on, thus high diversity reduces disease incidence (LoGiudice et al. 2003)). This has strong implications on the anthropogenic loss of diversity and increased risk

of disease emergence. Further, climate change has been tentatively associated with the range-shift of ticks and mosquitoes, important vectors of diseases such as Lyme disease and malaria (Rogers and Randolph 2006). Ecological changes can also lead to increased disease transmission by the introduction of a novel vector or transmission route (e.g. the invasive *Varroa* mite increases the transmission of Deformed wing virus (DWV) in western honey bees (Martin et al. 2012), or by increasing host susceptibility to pathogens (e.g. pesticide exposure in honey bees resulted in increased levels of the microporidian parasite *Nosema apis* and *N. ceranae* (Pettis et al. 2012)) and increased levels of DWV (Di Prisco et al. 2013).

The risk factors behind disease emergence are highly complex and still not well understood. The existence of multiple RNA viruses transmitted within and between the closely interacting pollinator community made up of social and solitary insects provides an accessible and fascinating population to study the impact of host behaviours and ecology on interspecific transmission and disease emergence. My focus throughout this thesis is on the epidemiology of RNA viruses in multi-host pollinator communities.

We rely on managed honeybees and bumblebees for the commercial pollination of crops; for example, honeybees are used to pollinate the intensive agriculture of Californian almond blossom, and commercial bumblebees are imported to the UK to pollinate sweet peppers, tomatoes and other soft fruits (Velthuis and van Doorn 2006). In addition, wild pollinators play an often underestimated role in the pollination of crops and wild plants (Garibaldi et al. 2013). As well as their pollination services, they form part of global biodiversity and should be conserved in their own right. However, regional yet dramatic declines have been seen in bee populations in recent years (Potts et al. 2010; Willams and Osborne 2009) with multiple interacting pressures believed to be responsible, including habitat loss and fragmentation, pesticide use, climate change and emerging pathogens (Goulson et al. 2015; Vanbergen et al. 2013).

The apiculture industry has experienced two major setbacks in recent years, both linked to emerging diseases. Firstly, the spread of the virus-vectoring *Varroa* mite, that jumped from its host *A. ceranae* (the Asian honeybee) to *A. mellifera* in the middle of the last century, and rapidly spread worldwide, providing a new route of virus transmission for an otherwise asymptomatic virus, DWV (Genersch 2010; Sumpter and Martin 2004). By feeding on developing honeybee pupae the mites can transmit viruses intra-specifically by injection

directly into the hemolymph, rather than faecal-oral transmission that would naturally occur within a colony (Chen et al. 2006b). This has caused dramatic increases in DWV prevalence and viral load (Martin et al. 2012; Mondet et al. 2014) and a dramatic decrease in viral strain diversity to a single master variant (Martin et al. 2012). Secondly, 'colony collapse disorder' hit the United States, in which the loss of a high proportion of hives made headlines (Oldroyd 2007); the cause of these losses remains unclear, although picorna-like RNA viruses, specifically Israeli acute paralysis virus, were implicated (Anderson and East 2008; Cox-Foster 2007).

In bumblebees there have been documented declines in the UK and Europe, mostly of rare and specialised species, and mainly associated with the loss of suitable habitat for nesting and foraging, climate change, pesticides and pathogens (Goulson et al. 2015; Willams and Osborne 2009). A recent study found that declining species were not randomly distributed across the *Bombus* phylogeny; certain subgenus' of *Bombus* (*Thoracobombus*) were more susceptible to declines, and species with a small geographic range were particularly vulnerable (Arbetman et al. 2017). Interestingly, Arbetman et al. (2017) also found that species that are not associated with three common pathogens (*Crithidia bombi*, *Nosema* spp. or *Locustacarus buchneri*) were more prone to decline, tentatively suggesting a reduced tolerance to infection in declining species. In North America, declines in bumblebee abundance are suggested to be due to the introduction of novel pathogens from non-native commercial bumblebees (Cameron et al. 2011; Colla and Packer 2008). Commercial bumblebees have been shown to harbour high levels of parasites (Graystock et al. 2013b) and spillover to wild bumblebees has been suggested to occur: gut parasites (*C. bombi* and *Nosema bombi*), which can be spread via flower sharing (Durrer and Schmid-Hempel 1994; Graystock et al. 2015) were found at higher prevalence surrounding commercial bumblebee-pollinated greenhouses (Colla et al. 2006; Murray et al. 2013). While there is no direct evidence of declines in wild pollinators directly linked to viral pathogen spillover, reduced population size makes species particularly vulnerable to epidemics (Dobson 2004).

Emerging diseases thus have the potential to be a major threat to pollinating insects. Honeybees are host to numerous RNA viruses, many of which are members of the Picornaviruses; 14 of which have been sequenced and made publically available (table 1:1). Many of these are now known to infect a broad host range of pollinator hosts (Manley et al. 2015). The same viral variants are found circulating between host species, suggesting the free

occurrence of interspecific transmission (Fürst et al. 2014; Singh et al. 2010). Managed honeybees have been implicated as the reservoir host for DWV (Fürst et al. 2014; Wilfert et al. 2016), with DWV identified as an emerging pathogen in bumblebees (Fürst et al. 2014). Wilfert et al. (2016) identified the movement of *A. mellifera* around the world as the source of the globally emerging DWV epidemic, likely driven by the concurrent spread of the *Varroa* mite. The negative effects of *Varroa* on apiculture have been clear. However, the capability of *Varroa* to alter the epidemiology of a multihost pathogen is likely to impact on wild pollinators.

Table 1-1: Some RNA viruses that infect honeybees and have been sequenced to date, with Genbank numbers.

Full virus name	Genbank Reference
Black queen cell virus	NC-0003784
Slow bee paralysis virus (Harpenden strain)	GU93876
Slow bee paralysis virus (Rothamsted strain)	EU035616
Deformed wing virus A	NC-004830
Deformed wing virus B	NC-006494
Deformed wing virus C	ENA: CEND01000001*
Sacbrood virus	AF092924
Acute bee paralysis virus	NC-002548
Kashmir bee virus	NC-004807
Israeli acute paralysis virus	KY243933
Chronic bee paralysis virus	NC-010711
Lake Sinai virus 1	HQ871931
Lake Sinai virus 2	HQ888865
Aphid lethal paralysis virus	KJ817182

* This sequence is available at http://www.ebi.ac.uk/ena/data/view/CEND01000001

Within multi-host systems, host species vary in their contribution to disease transmission because of differences in their abundance, ecology and behaviours, as well as in their susceptibility to pathogens and subsequent transmission potential (Haydon et al. 2002; Streicker et al. 2013). Consideration of the epidemiology of pathogens across multiple host species is important to identify the key hosts and mechanisms driving disease emergence, and for disease control. Controlling hosts that dominate transmission can be an important way to control disease (for example, vaccination of abundant and highly infectious domestic dogs to control rabies in rural Africa, (Karre et al. 2009)). However, the key host is not always that obvious; for example, a key host driving the West Nile virus epidemic in New York was

identified as the relatively rare but highly competent host, the American robin, due to preferential feeding behaviour of a mosquito vector (Kilpatrick et al. 2006). Yet, the conditions that determine if a pathogen can establish in a new host are not well understood (Jones et al. 2008; Parrish et al. 2008; Woolhouse et al. 2005). The multi-pathogen, multi-host pollinator community provides a useful system to better understand ecological, evolutionary and anthropogenic drivers of disease emergence. In **chapter two**, published in the Journal of Applied Ecology in 2015, I identify the risk of spillover and emergence of viral disease from managed honeybees to wild pollinators by reviewing the extensive and disparate literature and analysing all available data on RNA viruses in pollinators. I examine and identify risk factors at three stages necessary for a disease to emerge: first, the opportunity for a viral pathogen to access a novel host, followed by the ability to establish infection, and transmission between individuals of the novel host population. The greatest risk of pathogen spillover comes from commercial honeybees and bumblebee operations, combined with the inherent ecology of pollinators with their overlapping ranges and shared niches, which promotes interspecific transmission.

While there is growing data on the presence and prevalence of multiple RNA viruses in bumblebee hosts, there is a scarcity of data on the pathogenicity of these viruses, which is essential to recognise the wider implications of viral infection to individuals and populations. In **chapter three,** published in Oecologia in 2017**,** I measure mortality rates and sub-lethal effects of one RNA virus, Slow bee paralysis virus (SBPV), on one of its natural bumblebee hosts, *B. terrestris*. Based on prevalence and phylogenetic data (McMahon et al. 2015 and chapter 5 of this thesis), SBPV appears to be a bumblebee virus. However, previous infection studies have only been carried out in honeybees. SBPV is believed to exist asymptomatically in *A. mellifera* except in association with the *Varroa* mite (Carreck et al. 2010; Santillán-Galicia et al. 2014) or by injection in the laboratory, where it was found to cause paralysis in the anterior legs ten days after injection (Bailey and Woods 1974). I isolated virus from wild bumblebees to emulate a natural infection in the laboratory and dosed the worker bees orally. I found condition-dependent virulence, with longevity only reduced when the bumblebees were nutritionally stressed.

The *Varroa* mite has had a dramatic effect on DWV transmission in honeybees, increasing prevalence and titre, and reducing diversity to a single strain during the invasion of remote honeybee populations in Hawaii (Martin et al. 2012). While, *Varroa* only parasitizes

honeybees, DWV is a multi-host virus known to replicate in bumblebees and to dramatically reduce longevity (Fürst et al. 2014); thus I hypothesised that *Varroa*-DWV infection of honeybees would indirectly affect wild sympatric bumblebees. In **chapter four** I test this hypothesis with an extensive field study - collecting three species of bee (*A. mellifera*, *B. terrestris* and *B. pascuorum*) across 12 locations - and comparing DWV prevalence, viral load and sequence diversity between *Varroa*-present and *Varroa*-free locations. I present novel evidence that *Varroa* presence drives DWV prevalence and titre in bumblebee hosts; as well as in honeybees as expected. DWV is a complex of three known variants (DWV-A, -B and –C): Importantly, I differentiate between DWV variants: while DWV-A is responsible for the current global epidemic (Wilfert et al. 2016), I find DWV-B to be the dominant variant, particularly common in bumblebee hosts compared to DWV-A, which in my samples is extremely rare. Using phylogenetic analysis I identify DWV-B as an emerging viral variant that has expanded exponentially within the last decade. This is of concern given a recent infection experiment, which showed that DWV-B was more virulent than DWV-A in honeybees (McMahon et al. 2016). I found extremely little genetic variation, with no population structuring across host species, or in relation to *Varroa* presence. The invasion of *Varroa* has been shown to decrease genetic variation of DWV to a single strain over a short time scale, suggested to be due to rapid selection on the virus by the vector (Martin et al. 2012). I did not find the expected higher diversity of DWV strains in *Varroa*-free locations, but suggest this is because *Varroa* has been present in my population for over 20 years, and *Varroa*-free sites would have experienced spillover of the same strains via transportation of infected honeybees. While the same strains are present across all sites, the direct presence of *Varroa* is necessary to increase prevalence and titre, suggesting that *Varroa* drives infection in pollinators by amplifying the virus in honeybees dramatically increasing their cross-species transmission potential.

While DWV is in the limelight because of dramatic overwintering mortality in honeybee populations, a complex community of numerous multi-host viral pathogens exists in pollinator species. The viruses vary in their life histories but little is known about their epidemiology in wild bumblebees. Using the same field collections as above, in **chapter 5,** I use next generation sequencing (single molecule sequencing) to identify the viral composition within two host species (*A. mellifera* and *B. terrestris*) across four locations, without PCR bias towards known and common viruses. I find that viral composition and diversity is clearly structured by location, not by host species or by *Varroa*-presence. Further,

I focus on two very different viruses: black queen cell virus (BQCV), which is prevalent in honeybees but not associated with transmission by *Varroa* (note, there have been no infection studies of BQCV on bumblebees to my knowledge); and SBPV, which is prevalent in bumblebees, associated with *Varroa* transmission (Carreck et al. 2010; Santillán-Galicia et al. 2014) and known to cause pathogenic effects in *B. terrestris* (chapter three of this thesis). I test the effect of host species, location and *Varroa* presence on the prevalence and titre of BQCV and SBPV, and find unexpectedly, that *Varroa* drives the prevalence of both viruses. Interestingly, titre is not influenced by *Varroa*, suggesting an absent or passive association with transmission by *Varroa*. I use phylogenetic analysis and population genetics to determine population structure and test for epidemic growth. In contrast to DWV, both viruses are highly structured by location and show no signs of exponential growth, suggesting stable populations. Here again we find no structure by host species, with the same strains circulating in sympatric bee populations.

In chapter 6, I discuss the general findings and applied implications of this work. Overall I find that wild bumblebees are at high risk of pathogenic emerging viral diseases from managed honeybees, specifically in association with the *Varroa* mite. There are clear implications for better control of *Varroa*, and restrictions on bee keeping to protect wild bumblebees. I highlight fundamental research questions that are essential for mitigation of disease control, and also interesting evolutionary and ecological questions that can be answered using this system.

Chapter 2 : Emerging viral disease risk to pollinating insects: ecological, evolutionary and anthropogenic factors

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REVIEW

Emerging viral disease risk to pollinating insects: ecological, evolutionary and anthropogenic factors

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Summary

1. The potential for infectious pathogens to spillover and emerge from managed populations to wildlife communities is poorly understood, but ecological, evolutionary and anthropogenic factors are all likely to influence the initial exposure and subsequent infection, spread and impact of disease. Fast-evolving RNA viruses, known to cause severe colony losses in managed honeybee populations, deserve particular attention for their propensity to jump between host species and thus threaten ecologically and economically important wild pollinator communities.
2. We review the literature on pollinator viruses to identify biological and anthropogenic drivers of disease emergence, highlight gaps in the literature, and discuss potential management strategies.
3. We provide evidence that many wild pollinator species are exposed to viruses from commercial species, resulting in multiple spillover events. However, it is not clear whether species become infected as a result of spillover or whether transmission is occurring within these wild populations. Ecological traits of pollinating insects, such as overlapping ranges, niches and behaviours, clearly promote cross-species transmission of RNA viruses. Moreover, we conclude that the social behaviour and phylogenetic relatedness of social pollinators further facilitate within- and between-host transmission, leaving these species particularly vulnerable to emerging diseases.
4. We argue that the commercial use of pollinators is a key driver of disease emergence in these beneficial insects and that this must be addressed by management and policy.
5. *Synthesis and applications*. There are important knowledge gaps, ranging from disease distribution and prevalence, to pathogen life history and virulence, to the impacts of disease emergence, which need to be addressed as research priorities. It is clear that avoiding anthropogenic pathogen spillover is crucial to preventing and managing disease emergence in pollinators, with far-reaching effects on our food security, ecosystem services and biodiversity. We argue that it is crucial to prevent the introduction of diseased pollinators into natural environments, which can be achieved through improved monitoring and management practices.

Key-words: pollinators, emerging disease, anthropogenic, biological risk factors, RNA viruses, transmission, infection, multihost pathogens, pollination, niche overlap

Introduction

Emerging infectious diseases can have devastating impacts on both managed and wild species (e.g. Strauss, White & Boots 2012) and indirectly threaten human welfare by depleting ecosystem services (Daszak, Cunningham & Hyatt 2000). Pathogen spillover from intensively managed populations poses a particular risk to susceptible wildlife communities that lack evolved resistance to novel pathogens (Daszak, Cunningham & Hyatt 2000; Colla *et al*. 2006). Pollinating insects are increasingly experiencing such viral disease spillover from managed honeybee (*Apis mellifera* and *A. cerana*) populations, and this has led to a burgeoning but disparate literature on disease occurrence in pollinators. Here, we review this literature to gain a better understanding of the various drivers of disease emergence, to highlight key knowledge gaps and to make management recommendations.

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Insect pollinators are important for agriculture, food security and ecosystem function (Vanbergen *et al.* 2013), being responsible for the pollination of most flowering crops and wild plants (Klein *et al.* 2007). Indeed, Gallai *et al.* (2009) estimated the global value of insect pollinators at €153 billion per annum. Commercial pollination services are provided predominantly by honeybees *A. mellifera* and some bumblebee species, mainly *Bombus terrestris* (Europe and world-wide), *B. impatiens* (North America) and *B. ignitus* (East Asia) (Velthuis & van Doorn 2006). In addition, wild pollinators play an important and often underestimated role in pollination of crops as well as native plants (Garibaldi *et al.* 2013). Yet, extinctions, reduced abundance and range contractions of wild and managed pollinator populations have been recorded in the Northern Hemisphere (reviewed by Vanbergen *et al.* 2013). Multiple interacting pressures, including habitat loss and fragmentation, agriculture intensification, climate change and emerging pathogens, are believed to be responsible for these recent declines (e.g. Vanbergen *et al.* 2013).

Pathogens have emerged as a significant threat to the apicultural industry in recent years, with dramatic declines seen in populations of *A. mellifera*. While viral infections have been invoked as a potential cause of colony collapse syndrome (Cox-Foster 2007; but see van Engelsdorp *et al.* 2009), the main culprit in pathogen-related honeybee colony losses is infestation by the invasive mite *Varroa destructor*. This ectoparasite facilitates the spread of viral diseases and may increase their virulence (Martin 2001; Genersch 2010; Martin *et al.* 2012); see Table S1 in Supporting Information). In particular, one of these viruses (deformed wing virus, DWV) has recently been identified as an emerging disease in pollinators, with its prevalence in honeybees linked to its prevalence in wild bumblebees (Fürst *et al.* 2014). Although virological research has focused on honeybees, recent data suggest that many of the 24 viruses isolated from honeybees so far (de Miranda *et al.* 2013) have a broad host range, infecting some bumblebee, solitary bee, wasp, ant and hoverfly species (Fig. 1).

Here, we review the potential for disease emergence within the pollinator community, based on data from the best-studied honeybee RNA viruses [seven members of the Picornavirales; acute bee paralysis virus (ABPV), black queen cell virus (BQCV), DWV, Israeli acute paralysis virus (IAPV), Kashmir bee virus (KBV), sacbrood virus (SBV), slow bee paralysis virus (SBPV) and the unassigned chronic bee paralysis virus (CBPV)] (Table S2). We identify the biological and anthropogenic drivers that may promote successful disease emergence within the pollinator community, from (1) the initial exposure of the pathogen to novel hosts, to (2) successful infection in a novel host and, finally, (3) transmission within a novel host species (Fig. 2).

Exposure of novel hosts to viruses

The first step in disease emergence is the exposure of a potential novel host to the pathogen. Both biological and anthropogenic drivers can influence the frequency and extent of contact between a reservoir and novel host population, thus increasing the risk of transmission and disease emergence.

BIOLOGICAL DRIVERS: TRANSMISSION AND DISTRIBUTION

Prevalence and geographic range of viruses

High prevalence and large geographic range increase a pathogen's potential to encounter novel host species. Honeybees are now kept in most inhabited areas of the world, and many pathogens have accompanied their host in this global spread (Ellis & Munn 2005). Of the eight commonly studied viruses, most are reported globally (Table S1). Comparisons between studies are difficult because viral prevalence can vary between castes and through seasons (Chen & Siede 2007) and sampling effort and methods differ. Despite this, it is clear from available data that some viral pathogens (particularly DWV and BQCV) generally have high prevalence, infecting the majority of honeybee hives where they are present (Table S1). This high prevalence in honeybees is mirrored by the high DWV presence in other pollinator species surveyed in the USA and UK (Fig. 3). The near-ubiquitous presence of honeybees and the generally high prevalence of both asymptomatic and pathogenic virus infections across apiaries provides ample opportunity for cross-species transmission.

Mode of transmission

A pathogen's transmission mode can determine the likelihood of disease emergence. Indirect transmission routes (such as food-borne, faecal-borne or vector-borne), where hosts do not need to come into direct contact with each other, may increase opportunities for cross-species exposure and transmission (Woolhouse, Haydon & Antia 2005). In contrast, direct transmission (such as sexual and vertical transmission) characteristically occurs within, rather than between, host species. Viral infections within *A. mellifera* have been well studied and evidence suggests transmission can occur both directly and indirectly (Table S3).

Indirect transmission: flower sharing and vectors. Viruses have been detected in a variety of food resources (e.g. pollen, honey, royal jelly) (Shen *et al.* 2005; Chen, Evans & Feldlaufer 2006; Singh *et al.* 2010) as well as in the gut and faeces (Hung 2000; Chen *et al.* 2006; Ribière *et al.* 2007), providing evidence for faecal–oral transmission within *A. mellifera* colonies (Table S3). Most insect pollinators are generalist flower visitors (Waser *et al.* 1996), and flower sharing provides a route for cross-species transmission by faecal–oral transmission, as has been

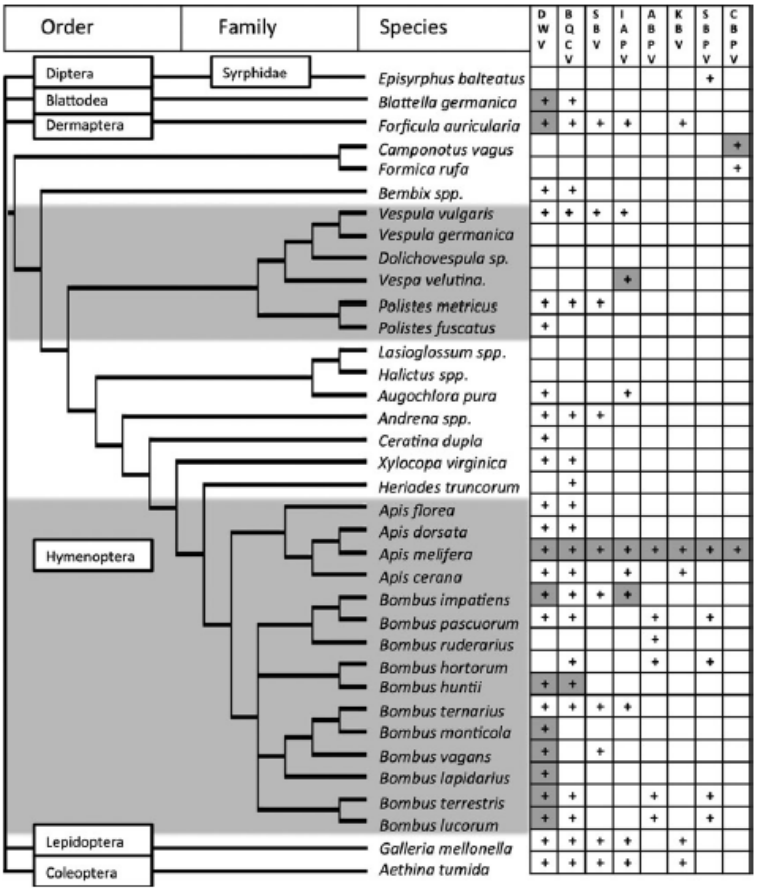

Fig. 1. Phylogeny of pollinator species, and other insects associated with honeybee colonies, focussing on the Hymenoptera. Shaded species are social insects. '+' indicates that the species has been identified as positive for virus, '✱' indicates virus replication has been demons trated. Virus abbreviations: DWV, deformed wing virus; BQCV, black queen cell virus; SBV, sacbrood virus; IAPV, Israeli acute paralysis virus; ABPV, acute bee paralysis virus; KBV, Kashmir bee virus; SBPV, slow bee paralysis virus; CBPV, chronic bee paralysis virus. Note that some data are based on small sample sizes, see Table S4.

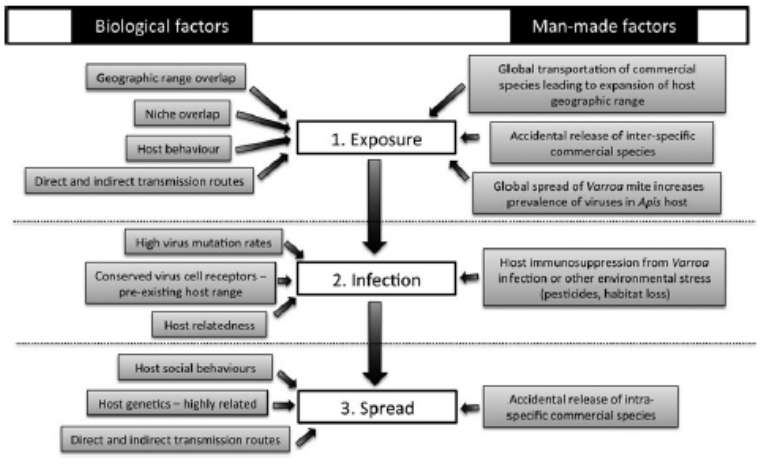

Fig. 2. Identifying the main factors increasing the risk of RNA virus emergence in social pollinators.

experimentally shown for the gut parasite *Crithidia bombi* in bumblebees (Durrer & Schmid-Hempel 1994). IAPV was demonstrated to pass from infected bumblebees to uninfected honeybees and vice versa in a controlled greenhouse experiment, with shared flowers as the only source of contact (Singh *et al.* 2010).

For successful transmission via flower sharing, infected faeces must first be deposited on the flower and then remain viable until acquired by a new host. Floral morphology will influence the likelihood of infected faeces being deposited on flowers, while floral traits such as antimicrobial volatiles, compounds in pollen and nectar, and

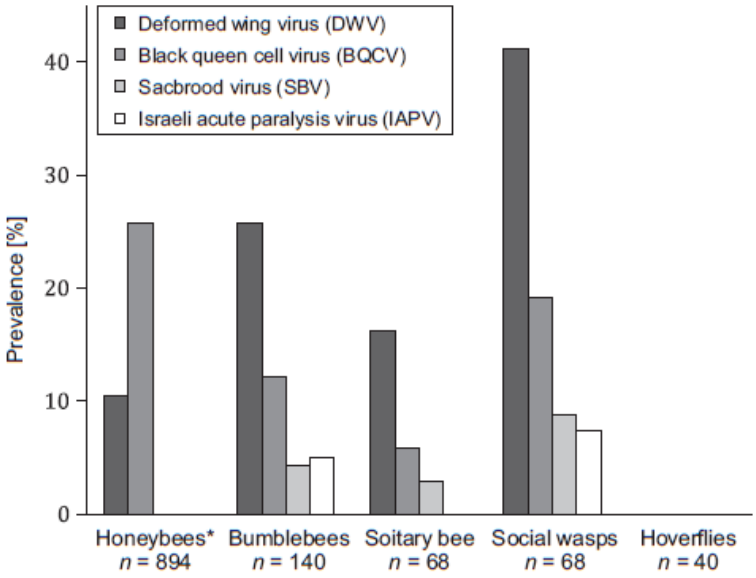

Fig. 3. Cumulative percentage prevalence of DWV, BQCV, SBV and IAPV across pollinator species groups. *Note that 'honeybees' exclude *A. mellifera* – (data from Singh *et al.* 2010; Evison *et al.* 2012; Li *et al.* 2012; Zhang *et al.* 2012; Levitt *et al.* 2013), *n* = total number of individuals sampled within each species group. See Table S4 for a list of species and raw data.

exposure of flower surfaces to ultraviolet radiation, could influence virus viability and survival time (McArt *et al.* 2014). Additionally, pollinator behaviour will influence virus transmission. Social pollinators can learn to recognize flower resources from conspecifics and heterospecifics and are attracted to flowers by the presence of other pollinators (Dawson & Chittka 2012). Conversely, virus presence may alter floral traits causing pollinators to avoid contaminated flowers (McArt *et al.* 2014) as was the case for flowers experimentally inoculated with *C. bombi* (Fouks & Lattorff 2011).

Vector-borne transmission is a frequent source of zoonoses. In *A. mellifera*, the vector *V. destructor* has played an important role for viral disease emergence (e.g. Martin *et al.* 2012), but is not directly relevant for cross-species transmission beyond honeybees, as it is specific to *Apis*. Bumblebees are associated with several phoretic and tracheal mite species. Little is known about their biology or impact on bumblebee populations. However, Schwarz & Huck (1997) found that four species of phoretic mite could actively transfer between flowers and foraging bumblebees, raising the possibility that these mites could spread pathogens. Whether tracheal or phoretic mites of non-*Apis* pollinators contribute to inter- and intraspecific viral transmission are currently unknown and warrants future research. Similarly, it is conceivable that conopid flies, parasitoid diptera that lay their eggs predominantly in adult aculeate hymenoptera, could contribute to disease transmission. Some of these species are known to locally parasitise multiple bumblebee species (Schmid-Hempel & Schmid-Hempel 1996), but their potential role in disease transmission has so far remained unexplored.

Direct transmission: social parasitism and predation. Social pollinators suffer from a range of social parasites and predatory behaviours that promote direct inter- and intraspecific pathogen transmission. For example, both wasps and bumblebees are known to rob honeybee nests; Genersch *et al.* (2006) discovered DWV in a wild *B. pascuorum* colony that was observed robbing honey from nearby DWV-positive honeybee colonies. Further, in a recent survey, only those wasp (*Vespula vulgaris*) and bumblebee species (*B. terrestris* and *B. pascuorum*) known to rob honeybee colonies were positive for DWV (Evison *et al.* 2012). However, sample sizes were too low to confirm virus absence in the nonrobbing species.

Pollinator colonies are also a valuable resource for social and larval parasitism, which has the potential to lead to disease transmission between the parasite and its host and vice versa. In bumblebees, cuckoo bees from the *Psithyrus* subgenus are obligate parasites, where the female cuckoo bee enters a bumblebee nest, kills the queen and lays eggs that are reared by the social bumblebee workers. Additionally, the larvae of several hoverfly species scavenge in social insect nests, for example *Volucella zonaria* (social wasps, Sommaggio 1999) and *V. pellucens* (social bees and wasps, Coe 1953).

Social bumblebees may also engage in some level of social parasitism that could lead to intraspecific transmission: dubbed 'egg dumping', there is microsatellite-based evidence that conspecific queens may lay eggs in foreign nests (O'Connor, Park & Goulson 2013). Additionally, direct transmission can occur where adult workers 'drift', that is when they enter an unrelated nest of the same species. This is a common phenomenon in *A. mellifera* (e.g. Chapman, Beekman & Oldroyd 2010) and has also been experimentally documented in artificial bumblebee colonies (Birmingham *et al.* 2004; Lopez-Vaamonde *et al.* 2004). While this behaviour is rare in natural bumblebee colonies (O'Connor, Park & Goulson 2013), direct trans-

mission via drifting could be highly relevant where artificial bumblebee colonies are used in close proximity to each other for pollination services.

ANTHROPOGENIC DRIVERS: SPILLOVER BETWEEN MANAGED AND WILD POLLINATORS

Poor husbandry and management

The husbandry techniques used in commercial pollination have potential to increase pathogen exposure. In bumblebees, for example, the cause of the symptomatic DWV infection in *B. terrestris* reported in Genersch *et al.* (2006) was assumed to be the once common practice of housing honeybee workers with bumblebee queens in commercial breeding facilities to encourage the queens to nest. Besides the increased potential for transmission by rearing large numbers of individuals in close proximity, virus-contaminated pollen (e.g. Singh *et al.* 2010) is a risk to commercial pollinators. Pollen is an essential protein and vitamin source that cannot readily be substituted. In captivity, both bumblebees and honeybees are often fed with pollen collected through traps attached to honeybee colonies, and a number of studies have suggested that feeding untreated virus-contaminated pollen can result in infected individuals and colonies (Singh *et al.* 2010; Graystock *et al.* 2013).

Unsurprisingly then, studies have found that several pathogens are more prevalent in commercial than wild bumblebee populations (Colla *et al.* 2006; Goka, Okabe & Yoneda 2006). Despite existing regulations and the commitment of commercial breeders to produce pathogen-free colonies (Meeus *et al.* 2011), a recent molecular study detected five pathogens (DWV, *Nosema bombi*, *N. ceranae*, *C. bombi* and *Apicystis bombi*) across 77% of 48 commercially produced bumblebee colonies (Graystock *et al.* 2013). This agrees with Murray *et al.* (2013), who found that 73·5% of 68 commercial *B. terrestris* colonies were infected either with *Crithidia spp.*, *N. bombi*, or both.

Accidental release of infected commercial bumblebees from agricultural systems poses a real risk of transmission to wild pollinators. First, local commercial bumblebee populations can be large: Colla *et al.* (2006) estimated that up to 23 000 bumblebees may pollinate a greenhouse. Secondly, bumblebees regularly escape and forage on non-commercial flower resources. Murray *et al.* (2013) found that pollen collected by commercial *B. terrestris* contained between 31 and 97% noncrop pollen, depending on the agricultural system (i.e. greenhouse, polytunnel and open field), in accordance with a previous study finding 73% noncrop pollen collected by bumblebees released in greenhouses (Whittington *et al.* 2004).

Global transportation of commercial species

The globalization of the pollinator industry provides unprecedented opportunities for pathogens to cross geographic and host boundaries. For example, the commercial production of *B. occidentalis* in North America collapsed in the last decade, although direct evidence is lacking (Brown 2011), this has been attributed to infection with the microsporidian *N. bombi,* introduced through commercial European *B. terrestris* colonies in the 1990s. Similarly, *C. bombi* has been found in native bumblebee populations at greenhouse sites where commercial imported colonies were used, but not at control sites (Colla *et al.* 2006). This corresponds with modelled predictions of primary pathogen spillover (Otterstatter & Thomson 2008). Such patterns are not limited to North America: in South America, *C. bombi* and *A. bombi* may have been introduced by the invasive *B. terrestris*, originally imported for greenhouse pollination and now the dominant species across much of Chile and Argentina (Plischuk & Lange 2009; Schmid-Hempel *et al.* 2014). European haplotypes of the bumblebee tracheal mite *Locustacarus buchneri* were found in commercial *B. ignitus* originally reared in European commercial operations (Goka *et al.* 2001), and later in native Japanese bumble bees, while Japanese haplotypes were found in commercial bees (a 'spill back' from wild populations to managed ones) (Goka, Okabe & Yoneda 2006).

Global spread of Varroa mite increases prevalence of viruses in Apis host

The most poignant case of disease emergence caused by beekeeping practices is the spread of *Varroa* together with the viruses it promotes. *Varroa* has spread globally since the 1950s after it jumped from its original host *A. cerana* (the Asian honeybee) to *A. mellifera* as a result of the commercial transportation of honeybees (Oldroyd 1999). Much of its pathogenicity is caused by spreading viral diseases and increasing the virulence of otherwise often asymptomatic viral infections, such as DWV (e.g. Martin *et al.* 2012). This increased prevalence may in turn increase transmission to wild pollinators.

Establishing an infection in the new host

To establish an acute infection, an emerging pathogen has to replicate within its novel host. Pathogen type (Woolhouse, Haydon & Antia 2005) and host relatedness (Davies & Pedersen 2008; Longdon *et al.* 2011) are generally the primary factors determining the range of host species a pathogen can infect. The currently available data suggest common RNA viruses, pathogenic to honeybees, are present in many hosts (Fig. 1). However, most studies have only screened for viral genomes in pollinator field samples using RT-PCR. Importantly, testing positive for virus presence does not necessarily imply that the pathogen is replicating in its host, but may simply reflect that an individual has ingested viral particles, for example through contaminated pollen. It should be noted, however, that an individual passively carrying a pathogen may still be infectious to others.

Genersch *et al.* (2006) inferred virus replication through identification of DWV symptoms in about 10% of queens in a commercial *B. terrestris* colony. Symptomatic bees were confirmed to be DWV-positive by RT-PCR. However, virus symptoms tend to be generic and are rarely diagnostic. For example, *N. bombi* may cause DWV-like symptoms in *B. terrestris* (Otti & Schmid-Hempel 2008), while viruses may often persist as asymptomatic infections (Chen & Siede 2007). Other symptomatic viral infections have not been reported in non-*Apis* pollinators, which may partly be due to biased collection methods: typically in these surveys, foraging pollinators are tested for viral infection (e.g. Singh *et al.* 2010; Evison *et al.* 2012; Levitt *et al.* 2013), so these individuals are capable of flying and are suffering no obvious ill effects. However, sublethal effects have been demonstrated in *B. terrestris* under laboratory conditions, infection with IAPV and KBV reduced worker reproduction (Meeus *et al.* 2014) and DWV reduced mean longevity by 6 days (Fürst *et al.* 2014). In positive-sense RNA viruses, virus replication can be detected through the specific amplification of the negative-strand replication intermediate. Several studies have used this diagnostic method across a limited range of host species for DWV, BQCV, IAPV and CBPV, generally finding that RNA viruses replicate in several species, particularly ones closely associated with *A. mellifera* through parasitism or flower sharing (Fig. 1, Table S4).

BIOLOGICAL DRIVERS: HOST AND PARASITE GENETICS

The nature of the pathogen

Pathogens vary greatly in host range breadth according to their type. For example, in contrast to the broad host range of 'honeybee' RNA viruses, trypanosome gut parasites tend to be more host specific, *that is C. bombi* infecting only bumblebee species, and *C. mellificæ* infecting only honeybees (Schmid-Hempel 1998). RNA viruses have the highest propensity for host shifting (Woolhouse, Haydon & Antia 2005); their high mutation rate, poor mutation-correction abilities and short replication time allow them to adapt rapidly to new host environments. The accumulation of various mutated viruses, called viral quasi-species (Domingo & Holland 1997), further increases the probability of successful adaptation to a new host. Viruses use cell receptors to enter host cells and these cell receptors are often conserved across host species, making them susceptible to infection (Woolhouse, Haydon & Antia 2005). For example, the broad host range of the foot-and-mouth disease virus (FMDV, a picorna-like virus related to common pollinator viruses) may be due to the use of conserved receptors (Baranowski, Ruiz-Jarabo & Domingo 2001). In addition, RNA viruses can often adapt to use novel receptors through few point mutations on the viral capsid, for example, a single amino acid substitution enabled FMDV to infect a new host, the guinea pig (Núñez *et al.* 2001). Identifying conserved receptors and virus mutations may allow for better predictions of the potential host range of viral diseases.

Relatedness of hosts

Pathogens are more likely to infect closely related hosts due to their shared evolutionary history (Engelstädter & Hurst 2006). This assumption has been experimentally documented primarily using Drosophila pathogens (Perlman & Jaenike 2003; Engelstädter & Hurst 2006; Longdon *et al.* 2011). Longdon *et al.* (2011) found that host relatedness was the main factor determining a virus' ability to persist and replicate in a host in the Drosophila–sigma virus system. This suggests that viruses are generally less well adapted to novel cellular environments or immune defence systems of distantly related hosts, even though they may jump phylogenetic divides to cause emerging diseases.

The host range of 'honeybee' RNA viruses reported so far includes closely related and phylogenetically diverse species (Fig. 1). While there are no studies systematically comparing infection spread, DWV seems to have a particularly broad host range, with replication detected in several bumblebee species and wasps as well as *V. destructor* and arthropods associated with honeybee hives, whereas other viral infections seem to have a more phylogenetically limited distribution (Fig. 1, Table S4). For DWV, Levitt *et al.* (2013) (USA) and Fürst *et al.* (2014) (UK) found that viral isolates were circulating amongst a range of species. While data are not conclusive at the moment, this suggests that disease emergence in pollinator communities is facilitated by consisting of many closely related species, but is not limited to, for example the social Hymenoptera.

ANTHROPOGENIC FACTORS: IMMUNOSUPPRESSION THROUGH ENVIRONMENTAL STRESS

It may be costly for an insect to mount an immune response against pathogens (Moret & Schmid-Hempel 2000). Immunosuppression through environmental stressors could increase the risk of infection and lower the threshold for disease emergence. Such stressors can include malnutrition caused by a lack of pollen sources and pollen diversity in areas under intense agricultural use, the use of chemical plant protection agents and the presence of *Varroa*, which can affect the honeybee's immune system (Yang & Cox-Foster 2005) leading to increased susceptibility to viruses (Vanbergen *et al.* 2013). Pollinators, and especially honeybees, can be exposed to a high level of diverse chemicals (Mullin *et al.* 2010). Neonicotinoid pesticides, for example, three of which are currently under a 2-year moratorium restricting their use in the EU, can increase susceptibility to DWV infections (Di Prisco *et al.* 2013) by affecting the immune system. Beyond the relatively well-understood threat of pesticides, the full breadth of chemicals to which pollinators may be exposed needs to be considered.

For example, it has become clear that an intact gut microbiome is essential for pathogen resistance (Koch & Schmid-Hempel 2011), which could be disrupted by chemicals with an antibiotic function.

Transmission within a new host species

Once the pathogen has established an infection in a novel host individual, its ability to transmit within the novel host population (either in isolation or as a multihost pathogen) will determine whether this remains an isolated spillover event or results in an emerging disease. In other words, it depends on each new infected host individual infecting, on average, more than one individual in its population (i.e. the basic reproductive number R_0 is >1) (Woolhouse, Haydon & Antia 2005). The data currently available are not sufficient to test whether infections in pollinator species represent transient spillovers or if they are part of a sustained transmission cycle. Neither are they sufficient to determine directionality of cross-species transmission. However, the evolutionary ecology of pollinators as well as management practices may increase the risk of spillovers leading to disease emergence (Fürst *et al*. 2014).

BIOLOGICAL DRIVERS: SOCIALITY

Pollinator species span a gradient of sociality, ranging from solitary species (such as solitary bees or hoverflies), through primitively eusocial species that live in annual colonies of a few hundred individuals (bumblebees and social wasps) to the eusocial honeybees. Although social living brings with it fitness benefits such as cooperative brood care, efficient foraging, mass defence and social immunity (reviewed by Cremer, Armitage & Schmid-Hempel 2007), it also provides an ideal environment for intraspecific pathogen transmission.

Host genetics

Social Hymenoptera live in large, crowded colonies of closely related haplo–diploid individuals. In bumblebees, it has been demonstrated experimentally that parasite transmission is higher between genetically homogeneous individuals (Shykoff & Schmid-Hempel 1991) and that genetically diverse colonies have decreased parasite loads and higher reproductive success (Baer & Schmid-Hempel 1999). While multiple mating may increase the risk of venereal disease, it reduces disease burden in *A. mellifera* (Seeley & Tarpy 2006), which is naturally promiscuous, unlike most bumblebee species (Schmid-Hempel & Crozier 1999).

Direct and indirect transmission routes via host social behaviours

Social behaviours such as trophallaxis (exchanging food among colony members), brood care, grooming and hygienic removal of diseased individuals, can increase the potential for disease transmission by faecal-oral or direct contact routes (Cremer, Armitage & Schmid-Hempel 2007). Disease transmission may also increase with individual and colony life span, ranging from a few months in bumblebees to years in honeybees. For example, infection intensities of the multihost pathogen *N. bombi* reach a higher level in *B. terrestris* than in *B. lucorum*, the latter of which has a shorter life cycle and smaller colonies (Rutrecht & Brown 2009). Sexual transmission, as demonstrated for DWV in honeybees (de Miranda & Fries 2008), may play a particular role in rapidly spreading pathogens at a landscape scale. In bumblebees, the queens and males show dispersal ranges of several kilometres (Lepais *et al*. 2010), while workers' foraging trips are typically less than 300 m.

In solitary pollinators, pathogens face different challenges for transmission. In hoverflies, for example, brood care is absent and generations occupy separate niche space, with larvae of different species being carnivorous, phytophagous or scavengers. They inhabit various environments from tree holes to foul water (Branquart & Hemptinne 2000), where they may in turn acquire a different pathogen range. Adults meet only to mate and while feeding on flowers, severely limiting the opportunities for disease transmission as compared to social insects.

ANTHROPOGENIC FACTORS: THE USE OF MANAGED POLLINATORS

High densities within breeding facilities and in commercial pollination operations increase the contact rate between infected and uninfected conspecifics, thereby lowering the threshold for disease emergence. A particular issue in managed populations is the potential for transmission between genetically diverse hosts which could lead to the evolution of general transmission strategies and higher virulence, as has been demonstrated in fish farms (Pulkkinen *et al*. 2010).

CONCLUSIONS

There is potential for cross-species transmission of RNA viruses in pollinators world-wide. Exposure does not appear to be a limiting step for virus emergence in pollinators, with current data suggesting that virus spillover events across a broad range of closely and distantly related host species have occurred multiple times in different parts of the world (Fig. 1, Table S4). However, it is still unclear to what degree viruses can then replicate in novel hosts (Fig. 1). Whether these viruses then are able to spread within the new host population has so far not been addressed. Based on identified risk factors (Fig. 2), we propose that the risk of establishing viral transmission within social pollinator populations is higher than for solitary species as their life history and relatedness should lower the threshold for disease emergence.

To assess the threat and impact of disease emergence in pollinator populations, we have to fill a number of key knowledge gaps (Table 1). Fundamentally, we need to understand the true spread of viral diseases in pollinators, how they are transmitted and maintained and what harm they cause. While there is clearly a need for much additional research, it is equally clear that the risk of disease emergence increases with every opportunity for pathogen host switching. Given the key role of pollinators in agriculture and the natural environment, it is evident that we need to minimize the risk of spillover events to mitigate disease emergence.

There are many inherent biological factors that increase the risk of disease emergence in pollinator species (Fig. 2), but anthropogenic drivers may be equally important in this system. Crucially, it is these drivers that can be changed by policy and management. Currently, intensively bred alien and/or native species are repeatedly introduced in large numbers, with potentially high pathogen loads, transported globally and released into an environment where they can often freely interact with native populations of related species. Correlative and circumstantial evidence strongly suggests that pathogen spillover from commercial species has occurred to the detriment of native populations (Colla *et al.* 2006; Goka, Okabe & Yoneda 2006; Plischuk & Lange 2009; Cameron *et al.* 2011; Szabo *et al.* 2012), even though there is no direct evidence to date that spillovers have caused epidemics or declines in wild populations (Meeus *et al.* 2011).

To address this risk, first, viruses need to be managed and better monitored in apiculture. If *A. mellifera* acts as a reservoir host for spillover into the pollinator community, then disease control and monitoring in managed populations is essential not only for honeybee health, but for the sake of the wider pollinator community (Fürst *et al.* 2014). It is necessary to routinely screen for pathogens, including viruses, prior to movement across countries (i.e. migratory honeybees in the USA) as well as imports and exports. The *Varroa* mite appears to be the main cause of virus spread throughout *A. mellifera* populations, which by increasing viral prevalence, virulence and geographic range may indirectly affect virus spillover into non-*Apis* hosts. Thus, controlling the *Varroa* mite and keeping them out of currently *Varroa*-free areas is essential.

Secondly, the commercial use of bumblebees needs to be more tightly managed. Despite tightened regulations and mandatory screening in some countries, two recent studies worryingly report that over 70% of 'pathogen-free' commercially produced bumblebees were carrying pathogens (Graystock *et al.* 2013; Murray *et al.* 2013). Additionally, Graystock *et al.* (2013) found that the pollen supplied to feed these colonies was also carrying pathogens, including DWV. Irradiating pollen prior to use (Singh *et al.* 2010) and avoiding using honeybee workers to encourage egg laying in captive queens are easy and necessary precautions. While it may not be practical or economically feasible to keep breeding facilities entirely pathogen-free, routine checks to ensure breeding facilities are not introducing known or novel pathogens and/or strains into wild populations are necessary. The next best policy for elimination is to prevent the escape of commercially bred individuals into the wild by implementing biosecurity measures (Goka 2010). In addition, to prevent introducing invasive pathogens, native pollinator species should be used for commercial pollination and bred locally whenever possible.

Environmental stressors, such as pesticides and habitat degradation, are anthropogenic factors that may potentially increase disease emergence through immunosuppression. Thus, minimizing the exposure to chemicals such as pesticides or acaricides through integrated pest management (Smith & Smith 1949), which aims to balance the need for pest control with minimal pesticide use, is crucial. It is also critical to prevent malnourishment of individuals and colonies, which can be achieved by providing varied floral resources throughout the pollinator season through large-scale land management. This is an issue the public can be directly involved in: already, gardens provide prime floral resources for pollinators in temperate

Table 1. Gaps in our knowledge of viral diseases of pollinating insects and future research

Knowledge gaps	Further research
Prevalence and infection outside the *Apis*-genus	Field studies across a broad taxonomic and geographic range verifying both viral presence and infection status
Viral life cycle -Transmission routes -Transmission through hibernation	Experimental infection studies both in *Apis* and novel hosts. Genetic studies to confirm results in nature
Virulence -Pathogenicity -Effect of host-switching on virulence	Field and experimental studies to identify lethal and sublethal pathogenic effects across species
Disease emergence -Which viruses have successfully emerged as novel diseases? -What are the epidemiological dynamics in multihost systems? -Is it the cause of pollinator declines?	Field and experimental studies to determine whether transmission is maintained within species and whether there are source/sink dynamics in natural multihost systems

regions such as the UK (e.g. Goulson *et al.* 2002). By providing such resources and nesting opportunities, the public cannot only bolster pollinator populations themselves but potentially help prevent disease emergence.

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Data accessibility

This review contains no new data. See Supporting Information for references and data used in this review.

References

Baer, B. & Schmid-Hempel, P. (1999) Experimental variation in polyandry affects parasite loads and fitness in a bumblebee. *Nature*, **397**, 151–153.

Baranowski, E., Ruiz-Jarabo, C. & Domingo, E. (2001) Evolution of cell recognition by viruses. *Science*, **292**, 1102–1105.

Birmingham, A., Hoover, S.E., Winston, M.L. & Ydenberg, R.C. (2004) Drifting bumble bee (Hymenoptera: Apidae) workers in commercial greenhouses may be social parasites. *Canadian Journal of Zoology*, **82**, 1843–1853.

Branquart, E. & Hemptinne, J. (2000) Selectivity in the exploitation of floral resources by hoverflies (Diptera: Syrphinae). *Ecography*, **23**, 732–742.

Brown, M.J.F. (2011) Conservation: the trouble with bumblebees. *Nature*, **469**, 169–170.

Cameron, S.A., Lozier, J.D., Strange, J.P., Koch, J.B., Cordes, N., Solter, L.F. & Griswold, T.L. (2011) Patterns of widespread decline in North American bumble bees. *Proceedings of the National Academy of Sciences of the United States of America*, **108**, 662–667.

Chapman, N.C., Beekman, M. & Oldroyd, B.P. (2010) Worker reproductive parasitism and drift in the western honeybee *Apis mellifera*. *Behavioral Ecology and Sociobiology*, **64**, 419–427.

Chen, Y.P., Evans, J. & Feldlaufer, M. (2006) Horizontal and vertical transmission of viruses in the honey bee, *Apis mellifera*. *Journal of Invertebrate Pathology*, **92**, 152–159.

Chen, Y. & Siede, R. (2007) Honey bee virus. *Advances in Virus Research*, **70**, 33–80.

Chen, Y., Pettis, J., Collins, A. & Feldlaufer, M. (2006) Prevalance and transmission of honeybee viruses. *Applied and Environmental Microbiology*, **72**, 606–611.

Coe, R.L. (1953) *Handbooks for the Identification of Bristish Insects: Diptera, Syrphidae*, 1953 edn. Royal Entomological Society of London, London.

Colla, S.R., Otterstatter, M.C., Gegear, R.J. & Thomson, J.D. (2006) Plight of the bumble bee: Pathogen spillover from commercial to wild populations. *Biological Conservation*, **129**, 461–467.

Cox-Foster, D.L. (2007) A metagenomic survey of microbes in honey bee colony collapse disorder. *Science*, **318**, 283–287.

Cremer, S., Armitage, S.A.O. & Schmid-Hempel, P. (2007) Social immunity. *Current Biology*, **17**, R693–R702.

Daszak, P., Cunningham, A.A. & Hyatt, A.D. (2000) Emerging infectious diseases of wildlife – threats to biodiversity and human health. *Science*, **287**, 443–449.

Davies, T.J. & Pedersen, A. (2008) Phylogeny and geography predict pathogen community similarity in wild primates and humans. *Proceedings of the Royal Society of London Series B*, **275**, 1695–1701.

Dawson, E.H. & Chittka, L. (2012) Conspecific and heterospecific information use in bumblebees. *PLoS ONE*, **7**, e31444.

Di Prisco, G., Cavaliere, V., Annoscia, D., Varricchio, P., Caprio, E., Nazzi, F., Gargiulo, G. & Pennacchio, F. (2013) Neonicotinoid clothianidin adversely affects insect immunity and promotes replication of a viral pathogen in honey bees. *Proceedings of the National Academy of Sciences of the United States of America*, **110**, 18466–18471.

Domingo, E. & Holland, J.J. (1997) RNA virus mutations and fitness for survival. *Annual Review of Microbiology*, **51**, 151–178.

Durrer, S. & Schmid-Hempel, P. (1994) Shared use of flowers leads to horizontal pathogen transmission. *Proceedings of the Royal Society of London Series B*, **258**, 299–302.

Ellis, J.D. & Munn, P.A. (2005) The worldwide health status of honey bees. *Bee World*, **86**, 88–101.

Engelstädter, J. & Hurst, G.D.D. (2006) The dynamics of parasite incidence across host species. *Evolutionary Ecology*, **20**, 603–616.

vanEngelsdorp, D., Evans, D.J., Saegerman, C., Mullin, C., Haubruge, E., Nguyen, B.K. *et al.* (2009) Colony collapse disorder: a descriptive study. *PLoS ONE*, **4**, e6481.

Evison, S.E.F., Roberts, K.E., Laurenson, L., Pietravalle, S., Hui, J., Biesmeijer, J.C., Smith, J.E., Budge, G. & Hughes, W.O.H. (2012) Pervasiveness of parasites in pollinators. *PLoS ONE*, **7**, e30641.

Fouks, B. & Lattorff, H.M.G. (2011) Recognition and avoidance of contaminated flowers by foraging bumblebees (*Bombus terrestris*). *PLoS ONE*, **6**, e26328.

Fürst, M.A., McMahon, D.P., Osborne, J.L., Paxton, R.J. & Brown, M.J.F. (2014) Disease associations between honeybees and bumblebees as a threat to wild pollinators. *Nature*, **506**, 364–366.

Gallai, N., Salles, J., Settele, J. & Vaissière, B.E. (2009) Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. *Ecological Economics*, **68**, 810–821.

Garibaldi, L.A., Steffan-Dewenter, I., Winfree, R., Aizen, M.A., Bommarco, R., Cunningham, S.A. *et al.* (2013) Wild pollinators enhance fruit set of crops regardless of honey bee abundance. *Science*, **339**, 1608–1611.

Genersch, E. (2010) Honey bee pathology: current threats to honey bees and beekeeping. *Applied Microbiology and Biotechnology*, **87**, 87–97.

Genersch, E., Yue, C., Fries, I. & De Miranda, J.R. (2006) Detection of Deformed Wing Virus, a honey bee viral pathogen, in bumble bees (*Bombus terrestris* and *Bombus pascuorum*) with wing deformities. *Journal of Invertebrate Pathology*, **91**, 61–63.

Goka, K. (2010) Biosecurity measures to prevent the incursion of invasive alien species into Japan and to mitigate their impact. *Revue Scientifique et Technique – Office International Des Epizooties*, **29**, 299–310.

Goka, K., Okabe, K. & Yoneda, M. (2006) Worldwide migration of parasitic mites as a result of bumblebee commercialization. *Population Ecology*, **48**, 285–291.

Goka, K., Okabe, K., Yoneda, M. & Niwa, S. (2001) Bumblebee commercialization will cause worldwide migration of parasitic mites. *Molecular Ecology*, **10**, 2095–2099.

Goulson, D., Hughes, W.O.H., Derwent, L.C. & Stout, J.C. (2002) Colony growth of the bumblebee, *Bombus terrestris*, in improved and conventional agricultural and suburban habitats. *Oecologia*, **130**, 267–273.

Graystock, P., Yates, K., Evison, S.E.F., Darvill, B., Goulson, D. & Hughes, W.O.H. (2013) The Trojan hives: pollinator pathogens, imported and distributed in bumblebee colonies. *Journal of Applied Ecology*, **50**, 1207–1215.

Hung, A.C.F. (2000) PCR detection of Kashmir bee virus in honey bee excreta. *Journal of Apicultural Research*, **39**, 103–106.

Klein, A.M., Vaissière, B.E., Cane, J.H., Steffan-Dewenter, I., Cunningham, S.A., Kremen, C. & Tscharntke, T. (2007) Importance of pollinators in changing landscapes for world crops. *Proceedings of the Royal Society of London Series B*, **274**, 303–313.

Koch, H. & Schmid-Hempel, P. (2011) Socially transmitted gut microbiota protect bumble bees against an intestinal parasite. *Proceedings of the National Academy of Sciences of the United States of America*, **108**, 19288–19292.

Lepais, O., Darvill, B., O'Connor, S., Osborne, J.L., Sanderson, R.A., Cussans, J., Goffe, L. & Goulson, D. (2010) Estimation of bumblebee queen dispersal distances using sibship reconstruction method. *Molecular Ecology*, **19**, 819–831.

Levitt, A.L., Singh, R., Cox-Foster, D.L., Rajotte, E., Hoover, K., Ostiguy, N. & Holmes, E.C. (2013) Cross-species transmission of honey bee viruses in associated arthropods. *Virus Research*, **176**, 232–240.

Li, J., Qin, H., Wu, J., Sadd, B.M., Wang, X., Evans, J., Peng, W. & Chen, Y. (2012) The prevalence of parasites and pathogens in Asian honeybees *Apis cerana* in China. *PLoS ONE*, **7**, e47955.

Longdon, B., Hadfield, J.D., Webster, C.L., Obbard, D.J. & Jiggins, F.M. (2011) Host phylogeny determines viral persistence and replication in novel hosts. *PloS Pathogens*, **7**, e1002260.

Lopez-Vaamonde, C., Koning, W., Brown, R.M., Jordan, W.C. & Bourke, A.F.G. (2004) Social parasitism by male-producing reproductive workers in a eusocial insect. *Nature*, **430**, 557–560.

Martin, S.J. (2001) The role of *Varroa* and viral pathogens in the collapse of honeybee colonies: a modelling approach. *Journal of Applied Ecology*, **38**, 1082–1093.
Martin, S.J., Highfield, A.C., Brettell, L., Villalobos, E.M., Budge, G.E., Powell, M., Nikaido, S. & Schroeder, D.C. (2012) Global honey bee viral landscape altered by a parasitic mite. *Science*, **336**, 1304–1306.
McArt, A.H., Koch, H., Irwin, R.E. & Adler, L.S. (2014) Arranging the bouquet of disease: floral traits and the transmission of plant and animal pathogens. *Ecology Letters*, **17**, 624–636.
Meeus, I., Brown, M.J.F., De Graaf, D.C. & Smagghe, G. (2011) Effects of invasive parasites on bumble bee declines. *Conservation Biology*, **25**, 662–671.
Meeus, I., de Miranda, J.R., de Graaf, D.C., Wäckers, F. & Smagghe, G. (2014) Effect of oral infection with Kashmir bee virus and Israeli acute paralysis virus on bumblebee (*Bombus terrestris*) reproductive success. *Journal of Invertebrate Pathology*, **121**, 64–69.
de Miranda, J.R. & Fries, I. (2008) Venereal and vertical transmission of Deformed Wing Virus in honeybees (*Apis mellifera* L.). *Journal of Invertebrate Pathology*, **98**, 184–189.
de Miranda, J.R., Bailey, B., Ball, B.V., Blanchard, P., Budge, G.E., Chejanovsky, N. *et al.* (2013) Standard methods for virus research in *Apis mellifera*. *Journal of Apicultural Research*, **52**, doi:10.3896/IBRA.1.52.4.22.
Moret, Y. & Schmid-Hempel, P. (2000) Survival for immunity: the price of immune system activation for bumblebee workers. *Science*, **290**, 1166–1167.
Mullin, C.A., Frazier, M., Frazier, J.L., Ashcraft, S., Simonds, R., van Engelsdorp, D. & Pettis, J.S. (2010) High levels of miticides and agrochemicals in North American apiaries: implications for honey bee health. *PLoS ONE*, **5**, e0009754.
Murray, T.E., Coffey, F.C., Kehoe, E. & Horgan, F.G. (2013) Pathogen prevalence in commercially reared bumble bees and evidence of spillover in conspecific populations. *Biological Conservation*, **159**, 269–276.
Núñez, J.I., Baranowski, E., Molina, N., Ruiz-Jarobo, C.M., Sánchez, C., Domingo, E. & Sobrino, F. (2001) A single amino acid substitution in nonstructural protein 3A can mediate adaptation of foot-and-mouth disease virus to the guinea pig. *Journal of Virology*, **75**, 3977–3983.
O'Connor, S., Park, K.J. & Goulson, D. (2013) Worker drift and egg dumping by queens in wild *Bombus terrestris* colonies. *Behavioral Ecology and Sociobiology*, **67**, 621–627.
Oldroyd, B.P. (1999) Coevolution while you wait: *Varroa jacobsoni*, a new parasite of western honeybees. *Trends in Ecology and Evolution*, **14**, 312–315.
Otterstatter, M.C. & Thomson, J.D. (2008) Does pathogen spillover from commercially reared bumble bees threaten wild pollinators? *PLoS ONE*, **3**, e2771.
Otti, O. & Schmid-Hempel, P. (2008) A field experiment on the effect of *Nosema bombi* in colonies of the bumblebee *Bombus terrestris*. *Ecological Entomology*, **33**, 577–582.
Perlman, S.J. & Jaenike, J. (2003) Infection success in novel hosts: an experimental and phylogenetic study of *Drosophila*-parasitic nematodes. *Evolution*, **57**, 544–557.
Plischuk, S. & Lange, C.E. (2009) Invasive *Bombus terrestris* (Hymenoptera: Apidae) parasitized by a flagellate (Euglenozoa: Kinetoplastea) and a neogregarine (Apicomplexa: Neogregarinorida). *Journal of Invertebrate Pathology*, **102**, 261–263.
Pulkkinen, K., Suomalainen, L.R., Read, A.R., Ebert, D., Rintamaki, P. & Vaitonen, E.T. (2010) Intensive fish farming and the evolution of pathogen virulence: the case of columnaris disease in Finland. *Proceedings of the Royal Society of London Series B*, **277**, 593–600.
Ribière, M., Lallemand, P., Iscache, A.L., Schurr, F., Celle, O., Blanchard, P., Olivier, V. & Faucon, J.P. (2007) Spread of infectious Chronic bee paralysis virus by honeybee (*Apis mellifera* L.) feces. *Applied and Environmental Microbiology*, **73**, 7711–7716.
Rutrecht, S.T. & Brown, M. (2009) Differential virulence in a multiple-host parasite of bumblebees: resolving the paradox of parasite survival? *Oikos*, **118**, 941–949.
Schmid-Hempel, P. (1998) *Parasites in Social Insects*. Princeton University Press, Princeton, NJ..
Schmid-Hempel, P. & Crozier, R.H. (1999) Polyandry versus polygyny versus parasites. *Philosophical transactions of the Royal Society of London Series B*, **354**, 507–515.
Schmid-Hempel, R. & Schmid-Hempel, P. (1996) Host choice and fitness correlates for conopid flies parasitising bumblebees. *Oecologia*, **107**, 71–78.
Schmid-Hempel, R., Eckhardt, M., Goulson, D., Heinzmann, D., Lange, C., Plischuk, S. *et al.* (2014) The invasion of southern South America by imported bumblebees and associated parasites. *Journal of Animal Ecology*, **83**, 823–847.
Schwarz, H.H. & Huck, K. (1997) Phoretic mites use flowers to transfer between foraging bumblebees. *Insectes Sociaux*, **44**, 303–310.
Seeley, T. & Tarpy, D. (2006) Queen promiscuity lowers disease within honeybee colonies. *Proceedings of the Royal Society B: Biological Sciences*, **274**, 67–72.
Shen, M., Cui, L., Ostiguy, N. & Cox-Foster, D.L. (2005) Intricate transmission routes and interactions between picorna-like viruses (Kashmir bee virus and Sacbrood virus) with the honeybee host and the parasitic varroa mite. *Journal of General Virology*, **86**, 2281–2289.
Shykoff, J.A. & Schmid-Hempel, P. (1991) Parasites and the advantage of genetic variability within social insect colonies. *Proceedings of the Royal Society of London Series B*, **243**, 55–58.
Singh, R., Levitt, A.L., Rajotte, E.G., Holmes, E.C., Ostiguy, N., van Engelsdorp, S. *et al.* (2010) RNA viruses in hymenopteran pollinators: evidence of inter-taxa virus transmission via pollen and potential impact on non-*Apis* hymenopteran species. *PLoS ONE*, **5**, e14357.
Smith, R.F. & Smith, G.L. (1949) Supervised control of insects utilizes parasites and predators and makes chemical control more efficient. *Californian Agriculture*, **3**, 3–12.
Sommaggio, D. (1999) Syrphidae: can they be used as environmental bioindicators? *Agriculture, Ecosystems and Environment*, **74**, 343–356.
Strauss, A., White, A. & Boots, M. (2012) Invading with biological weapons: the importance of disease-mediated invasions. *Functional Ecology*, **26**, 1249–1261.
Szabo, N.D., Colla, S.R., Wagner, D.L., Gall, L.F. & Kerr, J.T. (2012) Do pathogen spillover, pesticide use, or habitat loss explain recent North American bumblebee declines? *Conservation Letters*, **5**, 232–239.
Vanbergen, A.J., Baude, M., Biesmeijer, J.C., Britton, N.F., Brown, M.J.F., Brown, M. *et al.* (2013) Threats to an ecosystem service: pressures on pollinators. *Frontiers in Ecology and the Environment*, **11**, 251–259.
Velthuis, H.H.W. & van Doorn, A. (2006) A century of advances in bumblebee domestication and the economic and environmental aspects of its commercialization for pollination. *Apidologie*, **37**, 421–451.
Waser, N.M., Chittka, L., Price, M.V., Williams, N.M. & Ollerton, J. (1996) Generalization in pollination systems, and why it matters. *Ecology*, **77**, 1043–1060.
Whittington, R., Winston, M.L., Tucker, C. & Parachnowitsch, A.L. (2004) Plant-species identity of pollen collected by bumblebees placed in greenhouses for tomato pollination. *Canadian Journal of Plant Science*, **84**, 599–602.
Woolhouse, M.E.J., Haydon, D.T. & Antia, R. (2005) Emerging pathogens: the epidemiology and evolution of species jumps. *Trends in Ecology and Evolution*, **20**, 238–244.
Yang, X. & Cox-Foster, D.L. (2005) Impact of an ectoparasite on the immunity and pathology of an invertebrate: evidence for host immunosuppression and viral amplification. *Proceedings of the National Academy of Sciences of the United States of America*, **102**, 7470–7475.
Zhang, X., He, S.Y., Evans, J.D., Pettis, J.S., Yin, G.F. & Chen, Y.P. (2012) New evidence that Deformed wing virus and Black queen cell virus are multi-host pathogens. *Journal of Invertebrate Pathology*, **109**, 156–159.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1. The global presence and prevalence of eight common viruses of *Apis mellifera*.

Table S2. A list of sequenced viruses isolated from *Apis mellifera* to date.

Table S3. Transmission routes of eight common viruses of *Apis mellifera*.

Table S4. Data on host range for eight common viruses of *Apis mellifera*.

Supplementary tables

Table S1. The global presence and estimated prevalence of the eight most common viruses of *Apis mellifera*, plus conditions that trigger pathogenicity and where they've been implicated in recent serious colony losses. Virus abbreviations: DWV - Deformed wing virus, SBV - Sacbrood virus, BQCV - Black queen cell virus, KBV - Kashmir bee virus, ABPV - acute bee paralysis virus, IAPV - Israeli acute paralysis virus, SBPV - Slow bee paralysis virus, CBPV - Chronic bee paralysis virus. Prevalence was estimated using data collected from the following surveys: (Berényi et al. 2006; Carreck et al. 2010; Chen et al. 2006a; Gauthier et al. 2007; Genersch et al. 2010; Nielson et al. 2008; Runckel et al. 2011; Singh et al. 2010; Tentcheva et al. 2004; Ward et al. 2007; Welch et al. 2009) (High prevalence +++, medium prevalence ++ and low prevalence +).

*An expanded survey could not confirm this result (vanEngelsdorp et al. 2009)

Virus	Global presence	% prevalence estimate	Pathogenicity association	Colony losses
DWV	Every continent except Oceania	>80% (+++)	*Varroa* (Bowen-Walker et al. 1999)	Germany (Genersch et al. 2010); UK (Carreck et al. 2010)
SBV	Every continent	10-90% (++)	Seasonal, brood density	
BQCV	Every continent	>80% (+++)	*Nosema* (Bailey 1982)	
KBV	N. America, Oceania and Europe	<20% (++)	*Varroa* (Chen et al. 2004)	
ABPV	Every continent except Oceania	High, variable (++)	*Varroa* (Genersch et al. 2010)	Germany (Genersch et al. 2010)
IAPV	N. America, Oceania and Europe	<25% in Europe, high elsewhere (++)	*Varroa* (Di Prisco et al. 2011)	USA (Cox-Foster 2007)*
SBPV	Europe and Oceania	<2% (+)	*Varroa* (Carreck et al. 2010)	UK (Carreck et al. 2010)
CBPV	Every continent except S. America	<10% (+)	Colony Density (Ribière et al. 2010)	

Table S2. A list of the viruses isolated from *Apis mellifera* that have been sequenced to date (all positive single-stranded RNA genotype), their families, and references.
Note: several viruses are so closely related that it is yet to be determined if they are actually members of same species e.g. DWV/VDV-1/KV; ABPV/KBV/IAPV; and LSV-1/LSV-2.

Virus	**Abbr.**	**Family**	**References**
Deformed Wing Virus	DWV	Iflaviridae	de Miranda and Genersch (2010)
Kakugo Virus	KV	Iflaviridae	Fujiyuki et al. (2004)
Varroa Destructor Virus-1	VDV-1	Iflaviridae	Ongus et al. (2004)
Sacbrood Virus	SBV	Iflaviridae	Bailey and Fernando (1972), Ghosh et al. (1999)
Slow Bee Paralysis Virus	SBPV	Iflaviridae	Bailey and Woods (1974), de Miranda et al. (2010b)
Acute Bee Paralysis Virus	ABPV	Dicistroviridae	Bailey et al. (1963), de Miranda et al. (2010a)
Israeli Acute Paralysis Virus	IAPV	Dicistroviridae	Maori et al. (2007)
Kashmir Bee Virus	KBV	Dicistroviridae	Bailey and Woods (1977), de Miranda et al. (2004)
Aphid Lethal Paralysis virus	ALPV	Dicistroviridae	Runckel et al. (2011)
Big Sioux River virus	BSRV	Dicistroviridae	Runckel et al. (2011)
Black Queen Cell Virus	BQCV	Dicistroviridae	Bailey and Woods (1977), Leat et al. (2000)
Chronic Bee Paralysis Virus	CBPV	Unassigned, related to Nodaviridae and Tombusviridae	Bailey et al. (1963), Olivier et al. (2008), Ribière et al. (2010)
Lake Sinai Virus 1 & 2	LSV	Unassigned, related to Nodaviridae and Tombusviridae	Runckel et al. (2011)
Tobacco Ring Spot Virus	TRSV	Comoviridae	Li et al. (2014)

Table S3. Routes of transmission for the eight most common RNA viruses of *Apis mellifera* with references. Virus abbreviations: DWV - Deformed wing virus, BQCV - Black queen cell virus, ABPV - Acute bee paralysis virus, SBPV - Slow bee paralysis virus, KBV - Kashmir bee virus, CBPV - Chronic bee paralysis virus, SBV - Sacbrood virus, IAPV - Israeli acute paralysis virus. Key: ✓evidence suggestive, ☑ solid evidence (note: evidence was considered 'solid' if a transmission route was directly tested and proven to occur without the possibility of alternative transmission routes occurring simultaneously; evidence was considered 'suggestive' if transmission was shown indirectly (i.e. virus presence in pollen does not prove faecal-oral transmission occurs), was not the main focus of the study, or alternative routes of transmission were not ruled out).

	Indirect transmission			**Direct transmission**		
Viruses	**Food**	**Faecal**	**Vector (*V.destructor*)**	**Contact**	**Sexual**	**Transovarial**
DWV	✓ 1,15	✓ 10	☑ **3**,11,12,14		✓ 7,16,19	✓ 10,17,19
BQCV	✓ 1,15	✓ 10				✓ 10
ABPV	✓ 1		✓ 11,18	✓ 5		
SBPV			☑ 14, **20**			
KBV	✓ 1,2	✓ 4	☑ **2,**9,11			✓ 10,2
CBPV	✓ 1	☑ **6**		✓ 8		✓ 10
SBV	✓ 1,2,15		✓ 11			✓ 10,2
IAPV			☑ **13**			

1. Chen et al. (2006b)
2. Shen et al. (2005)
3. Bowen-Walker et al. (1999)
4. Hung (2000)
5. Bailey et al. (1963)
6. Ribière et al. (2007)
7. Yañez et al. (2012a)
8. Bailey et al. (1983)
9. Chen et al. (2004)
10. Chen et al. (2006a)
11. Tentcheva et al. (2004)
12. Yue and Genersch (2005)
13. Di Prisco et al. (2011)
14. Carreck et al. (2010)
15. Singh et al. (2010)
16. Yue et al. (2006)
17. Yue et al. (2007)
18. Genersch et al. (2010)
19. de Miranda and Fries (2008)
20. Santillán-Galicia et al. (2014)

Table S4. Virus host range for the eight most common RNA viruses of *Apis mellifera* (virus abbreviations: DWV - Deformed wing virus, BQCV - Black queen cell virus, ABPV - Acute bee paralysis virus, SBPV - Slow bee paralysis virus, SBV - Sacbrood virus, KBV – Kashmir bee virus, IAPV - Israeli acute paralysis virus, CBPV - Chronic bee paralysis virus).

Virus	**Host**	**Location**	**Sample n**	**~ % n infected**	**Replication**	**Symptomatic**	**Ref.**
DWV	*Bombus terrestris*	Europe commercial	no data	10%	Not tested	Y*	Genersch et al. (2006)
		UK, mainly southwest	57	30%	Not tested	N	Evison et al. (2012)
		Scotland, UK	Unpublished data	Unpublished data	Not tested	N	McMahon pers. comm.
		UK	170	9	Y	N	Fürst et al. (2014)
	Bombus pascuorum	Germany	1 wild nest	No data	Not tested	N	Genersch et al. (2006)
		UK, mainly southwest	36	14%	Not tested	N	Evison et al. (2012)
		UK	60	4	N	N	Fürst et al. (2014)
	Bombus lucorum	Scotland, UK	Unpublished data	Unpublished data	Not tested	N	McMahon pers. comm.
		UK	60	18	Y	N	Fürst et al. (2014)
	Bombus lapidarius	UK	175	16	Y	N	Fürst et al. (2014)
	Bombus huntii	Utah, USA	30	no data	Y (gut only)	N	Li et al. (2011)
	Bombus impatiens	Pennsylvania, USA	5	100%	Not tested	N	Singh et al. (2010)
		Pennsylvania, USA	13	62%	Y	N	Levitt et al. (2013)
	Bombus vagans	Pennsylvania, USA	2	50%	Y	N	Levitt et al. (2013)
	Bombus tenarius	Pennsylvania, USA	2	100%	Not tested	N	Singh et al. (2010)
	Bombus horturum	UK	20	0	n/a	n/a	Fürst et al. (2014)
	Bombus monticola	UK	10	11	Y	N	Fürst et al. (2014)
	Osmia bicornis	Belgium	3 pooled samples (10 per sample)	n/a	Not tested	N	Ravoet et al. (2014)
	Vespula vulgaris	UK, mainly southwest	45	29%	Not tested	N	Evison et al. (2012)
		Pennsylvania, USA	12	92%	Not tested	N	Singh et al. (2010)
	Vespula spp.	Pennsylvania, USA	7	57%	Y	N	Levitt et al. (2013)
	Apis cerana	China (19 provinces)	570	33, 51 and 92% in 3 provinces	Not tested	n/a	Li et al. (2011)
	Apis florae	Xishuang province, China	134	15.6%	Not tested	n/a	Zhang et al. (2012)
	Apis dorsata	Xishuang province, China	190	11.6%	Not tested	n/a	Zhang et al. (2012)
	Blatella germanica	Pennsylvania, USA	8	100%	Y	N	Levitt et al. (2013)
	Aethina tumida	Pennsylvania, USA	21	72%	Not tested	N	Levitt et al. (2013)
	Forficula auricularia	Pennsylvania, USA	10	100%	Y	N	Levitt et al. (2013)
	Galleria mellonella	Pennsylvania, USA	21	71%	N	N	Levitt et al. (2013)

	Araneae (order)	Pennsylvania, USA	10	80%	Not tested	N	Levitt et al. (2013)
	Varroa destructor	UK apiaries (Devon)	462 (3 hives)	No data	Not tested	n/a	Bowen-Walker et al. (1999)
		France apiaries (nationwide)	22,000 (22 apiaries)	100%	Not tested	n/a	Tentcheva et al. (2004)
		France (360 apiaries)	36,000 (36 apiaries)	No data	Not tested	n/a	Gauthier et al. (2007)
		Germany	40 per hive (5 hives)	45-100% per hive	Y (only mites from symptomatic bees)	n/a	Yue and Genersch (2005)
BQCV	*B. huntii*	Utah, USA	30	No data	Y (gut only)	N	Peng et al. (2011)
	B. terrestris	Scotland, UK	Unpublished data	Unpublished data	Not tested	N	McMahon pers. comm.
	B. pascuorum	Scotland, UK	Unpublished data	Unpublished data	Not tested	N	McMahon pers. comm.
	B. lucorum	Scotland, UK	Unpublished data	Unpublished data	Not tested	N	McMahon pers. comm.
	Bombus hortorum	Scotland, UK	Unpublished data	Unpublished data	Not tested	N	McMahon pers. comm.
	B impatiens	Pennsylvania, USA	5	60%	Not tested	N	Singh et al. (2010)
		Pennsylvania, USA	13	61%	Not tested	N	Levitt et al. (2013)
	B. tenarius	Pennsylvania, USA	2	50%	Not tested	N	Singh et al. (2010)
	B. vagans	Pennsylvania, USA	1	100%	Not tested	N	Singh et al. (2010)
		Pennsylvania, USA	2	100%	Not tested	N	Levitt et al. (2013)
	Osmia cornuta	Belgium	3 pooled samples (10 per sample)	n/a	Not tested	N	Ravoet et al. (2014)
	Andrena vaga	Belgium	1 pooled sample (10 in pool)	n/a	Not tested	N	Ravoet et al. (2014)
	Heriades truncorum	Belgium	3 pooled samples (10 per sample)	n/a	Not tested	N	Ravoet et al. (2014)
	A. cerana	China (19 provinces)	570	12% – 98% across 6 provinces	Not tested	N	Li et al. (2012)
	A. florae	Xishuang province, China	134	52%	Not tested	N	Zhang et al. (2012)
	A. dorsata	Xishuang province, China	190	21.6%	Not tested	N	Zhang et al. (2012)
	V. vulgaris	UK, mainly southwest	45	2.2%	Not tested	N	Evison et al. (2012)
		Pennsylvania, USA	12	66.7%	Not tested	N	Singh et al. (2010)
	Vespula sp.	Pennsylvania, USA	7	29%	Not tested	N	Levitt et al. (2013)
	Blatella germanica	Pennsylvania, USA	8	50%	Not tested	N	Levitt et al. (2013)
	Aethina tumida	Pennsylvania, USA	21	5%	Not tested	N	Levitt et al. (2013)
	Forficula auricularia	Pennsylvania, USA	10	60%	Not tested	N	Levitt et al. (2013)
	Galleria mellonella	Pennsylvania, USA	21	33%	Not tested	N	Levitt et al. (2013)
	Araneae (order)	Pennsylvania, USA	10	10%	Not tested	N	Levitt et al. (2013)

ABPV	*B. lucorum*	n/a experimental infections	No data	No data	Not tested	Y	Bailey and Gibbs (1964)
		Scotland, UK	Unpublished data	Unpublished data	Unpublished data	N	Wilfert unpublished
	B. ruderarius	n/a experimental infections	No data	No data	Not tested	Y	Bailey and Gibbs (1964)
	B. terrestris	n/a experimental infections	No data	No data	Not tested	Y	Bailey and Gibbs (1964)
		Scotland, UK	Unpublished data	Unpublished data	Unpublished data	N	Wilfert unpublished
	B. hortorum	n/a experimental infections	No data	No data	Not tested	Y	Bailey and Gibbs (1964)
		Scotland, UK	Unpublished data	Unpublished data	Unpublished data	N	Wilfert unpublished
	B pascuorum	n/a experimental infections	No data	No data	Not tested	N	Bailey and Gibbs (1964)**
		Scotland, UK	Unpublished data	Unpublished data	Unpublished data	N	Wilfert unpublished
	V. destructor	France (nationwide)	22,000 (22 apiaries)	36%	Not tested	n/a	Tentcheva et al. (2004)
		France (360 colonies)	36,000	low	Not tested	n/a	Gauthier et al. (2007)
SBPV	*B. terrestris*	Scotland, UK	Unpublished data	Unpublished data	Unpublished data	N	Wilfert unpublished
	B pascuorum	Scotland, UK	Unpublished data	Unpublished data	Unpublished data	N	Wilfert unpublished
	B. lucorum	Scotland, UK	Unpublished data	Unpublished data	Unpublished data	N	Wilfert unpublished
	B. horturum.	Scotland, UK	Unpublished data	Unpublished data	Unpublished data	N	Wilfert unpublished
	Eristalis pertinax	Scotland, UK	Unpublished data	Unpublished data	Unpublished data	N	Wilfert unpublished
	Episyrphus balteatus	Scotland, UK	Unpublished data	Unpublished data	Unpublished data	N	Wilfert unpublished
SBV	*B. ternarius*	Pennsylvania, USA	2	50%	Not tested	N	Singh et al. (2010)
	B vagans	Pennsylvania, USA	1	100%/	Not tested	N	Singh et al. (2010)
		Pennsylvania, USA	2	50%	Not tested	N	Levitt et al. (2013)
	B. impatiens	Pennsylvania, USA	13	23%	Not tested	N	Levitt et al. (2013)
	V. vulgaris	Pennsylvania, USA	7	57%	Not tested	N	Singh et al. (2010)
		UK, mainly southwest	45	0.45%	Not tested	N	Evison et al. (2012)
	Vespula spp.	Pennsylvania, USA	7	0.07%	Not tested	N	Levitt et al. (2013)
	Blatella germanica	Pennsylvania, USA	8	75%	Not tested		Levitt et al. (2013)
	Aethina tumida	Pennsylvania, USA	21	11%	Not tested	N	Levitt et al. (2013)
	Forficula auricularia	Pennsylvania, USA	10	50%	Not tested	N	Levitt et al. (2013)
	Galleria mellonella	Pennsylvania, USA	21	29%	Not tested	N	Levitt et al. (2013)
	Araneae (order)	Pennsylvania, USA	10	10%	Not tested	N	Levitt et al. (2013)
	V. destructor	Penn State, USA	111 (one hive)	no data	Not tested	n/a	Shen et al. (2005)
		France (nationwide)	22,000 (22 apiaries)	45%	Not tested	n/a	Tentcheva et al. (2004)
KBV	*A. cerana*	India, Kashmir	No data	No data	Not tested	Y	Bailey and Woods (1977)

	Aethina tumida	Pennsylvania, USA	21	44%	Not tested	N	Levitt et al. (2013)
	Forficula auricularia	Pennsylvania, USA	10	20%	Not tested	N	Levitt et al. (2013)
	Galleria mellonella	Pennsylvania, USA	21	29%	Not tested	N	Levitt et al. (2013)
	V. destructor	France (nationwide)	22,000 (22 apiaries)	5%	Not tested	n/a	Tentcheva et al. (2004)
	V. destructor	Penn State, USA	111 (one hive)	No data	Not tested	n/a	Shen et al. (2005)
IAPV	*B. impatiens*	Pennsylvania, USA	13	31%	Y	N	Levitt et al. (2013)
	B. ternarius	Pennsylvania, USA	2	100%	Not tested	N	Singh et al. (2010)
	B. vagans	Pennsylvania, USA	1	100%	Not tested	N	Singh et al. (2010)
	V. vulgaris	Pennsylvania, USA	7	~71%	Not tested	N	Singh et al. (2010)
	Vespa velutina	Hangzhou, China	10	100%	Y (head, thorax & abdomen)	N	Yañez et al. (2012b)
	A. cerana	Hangzhou, China	180 (6 hives)	1 hive only	N***	N	Yañez et al. (2012b)
	Aethina tumida	Pennsylvania, USA	21	78%	Not tested	N	Levitt et al. (2013)
	Forficula auricularia	Pennsylvania, USA	10	40%	Not tested	N	Levitt et al. (2013)
	Galleria mellonella	Pennsylvania, USA	21	14%	Not tested	N	Levitt et al. (2013)
	Araneae (order)	Pennsylvania, USA	10	10%	Not tested	N	Levitt et al. (2013)
	V. destructor	n/a (experimental)	40	n/a	Y	n/a	Di Prisco et al. (2011)
CBPV	*Camponotus vagus*	France	22 (from 2 apiaries)	No data	Y	N	Celle et al. (2008)
	Formica rufa	France	20 (from 2 apiaries)	No data	N	N	Celle et al. (2008)
	V. destructor	France	19 (from 1 apiary)	No data	Y	n/a	Celle et al. (2008)

* Bumblebees had deformed wings and were found to be positive for DWV by PCR. However, it is not proven that the symptoms are linked to the virus.

** The authors use the now out-of-date name *Bombus agrorum* to refer to B. pascuorum

***The authors do not believe this result to be true as replication occurred in *A. mellifera* and the two species are so similar that it seems unlikely replication does not occur in *A. cerana* as well (Yañez et al. 2012b)

Chapter 3 : Condition-dependent virulence of slow bee paralysis virus in Bombus terrestris: are the impacts of honeybee viruses in wild pollinators underestimated?

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HIGHLIGHTED STUDENT RESEARCH

Condition-dependent virulence of slow bee paralysis virus in *Bombus terrestris*: are the impacts of honeybee viruses in wild pollinators underestimated?

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Abstract Slow bee paralysis virus (SBPV)—previously considered an obligate honeybee disease—is now known to be prevalent in bumblebee species. SBPV is highly virulent in honeybees in association with *Varroa* mites, but has been considered relatively benign otherwise. However, condition-dependent pathogens can appear asymptomatic under good, resource abundant conditions, and negative impacts on host fitness may only become apparent when under stressful or resource-limited conditions. We tested whether SBPV expresses condition-dependent virulence in its bumblebee host, *Bombus terrestris*, by orally inoculating bees with SBPV and recording longevity under satiated and starvation conditions. SBPV infection resulted in significant virulence under starvation conditions, with infected bees 1.6 times more likely to die at any given time point (a median of 2.3 h earlier than uninfected bees), whereas there was no effect under satiated conditions. This demonstrates clear condition-dependent virulence for SBPV in *B. terrestris*. Infections that appear asymptomatic in non-stressful laboratory assays may nevertheless have significant impacts under natural conditions in the wild. For multi-host pathogens such as SBPV, the use of sentinel host species in laboratory assays may further lead to the underestimation of pathogen impacts on other species in nature. In this case the impact of 'honeybee viruses' on wild pollinators may be underestimated, with detrimental effects on conservation and food security. Our results highlight the importance of multiple assays and multiple host species when testing for virulence, in order for laboratory studies to accurately inform conservation policy and mitigate disease impacts in wild pollinators.

Keywords Pollinators · Virulence · Virus · Bees · Longevity

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We show for the slow bee paralysis virus that virulence is condition dependent in a bumble bee host. This virus attacks also honey bees and therefore our results have consequences to evaluate the importance of pathogen pressure on wild bees. We hope that our result stimulates further studies on the impact of multi-host pathogens for the conservation of native pollinators.

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Introduction

Emerging infectious diseases are one of several key factors linked to pollinator decline (Manley et al. 2015; Vanbergen et al. 2013). Several fast-evolving Picorna-like RNA viruses, known to be pathogenic to honeybees (Carreck et al. 2010; Cox-Foster 2007; Genersch et al. 2010) have a broad host range, threatening ecologically and economically important wild pollinators (Manley et al. 2015; McMahon et al. 2015). Recent large-scale field studies have found deformed wing virus (DWV), black

queen cell virus (BQCV), acute bee paralysis (ABPV) and slow bee paralysis virus (SBPV) to be prevalent in bumblebee species (Fürst et al. 2014; McMahon et al. 2015). High disease prevalence in wild, unmanaged pollinators—who play an important role as an ecosystem service (e.g. Garibaldi et al. 2013)—has raised concerns over the impact of diseases and the risk of spillover from managed honeybees (Fürst et al. 2014; Manley et al. 2015; Vanbergen et al. 2013). However, with the exception of DWV (Fürst et al. 2014), Israeli acute paralysis virus (IAPV) and Kashmir bee virus (KBV) (Meeus et al. 2014), the actual impact of viral diseases on wild pollinators is unknown.

Here, we used a controlled infection study to examine the impact of SBPV in a natural host, the bumblebee *Bombus terrestris*. SBPV is a picornavirus (de Miranda et al. 2010) which was first discovered in 1974 in an English honeybee (*Apis mellifera*) population. However, this virus is common in a range of bumblebee species and at its highest prevalence in *B. hortorum* in the UK (McMahon et al. 2015). In *A. mellifera*, SBPV only causes high mortality in association with the recently emerged ectoparasitic mite *Varroa destructor,* while otherwise appearing asymptomatic (Carreck et al. 2010; Santillán-Galicia et al. 2014). SBPV causes paralysis in the two anterior pairs of legs 10 days after injection into the haemolymph (Bailey and Woods 1974). *Varroa*, which exclusively infests honeybees, feeds on hemolymph and can thus serve as a vector directly transmitting viral infections into the hemolymph. Field studies that have found virus-positive pollinator species have collected foraging individuals (Evison et al. 2012; Levitt et al. 2013; Singh et al. 2010). Recent UK-wide data finds on average 5% of apparently healthy foraging bumblebees harbouring SBPV, with more than a third of SBPV-positive individuals carrying high viral titres (>10^9 virus particles per individual) (McMahon et al. 2015). Based on these field-surveys, it is clear that SBPV is found in nature but seems to exist asymptomatically.

Virulence in its broad sense is a measure of pathogen impact on host fitness. While some pathogens are highly virulent, imposing fitness costs on hosts that lead to mortality and reduced fecundity, others often seem to have no obvious impact. However, in seemingly benign pathogens, costs may be hidden if the host is able to compensate for the impacts through increased resource use, but revealed under starvation conditions when resources are too low to maintain defence costs (Moret and Schmid-Hempel 2000). Increased virulence under environmental stress is a common phenomenon across a range of pathogens and their hosts (Boots and Begon 1994; Jaenike et al. 1995; Jokela et al. 2005; Koella and Offenberg 1999; Restif and Kaltz 2006; Steinhaus 1958) and has been documented in honeybees and bumblebees (Brown et al. 2000; Goulson et al. 2015; Moret and Schmid-Hempel 2000). It is therefore important to examine the impact of pathogens across a range of host resource conditions.

Condition-dependent virulence has the potential to impact on individual bees, colonies and populations of wild pollinators, potentially contributing to population declines under poor environmental conditions. The availability of multiple reservoir species may also affect a pathogen's virulence and mask the fitness effects of pathogen infection in wild hosts (Leggett et al. 2013). Moreover, in a multi-host-pathogen system there is potential to underestimate the impact of pathogens, as sentinel species used in lab studies may not be representative if they are assayed under conditions of resource superabundance. For example, *A. mellifera*, the sentinel species in many pesticide studies, are less susceptible to dietary imidacloprid, a systemic neonicotinoid, than *B. terrestris*; therefore raising concern about the true impact of this pesticide on wild pollinator populations (Cresswell et al. 2012). In a survey of honeybees and five bumblebee species in the UK, the mean prevalence of SBPV in *B. terrestris* was comparable to honeybees and other bumblebee species at ~6%, while *B. hortorum* had a significantly higher prevalence (McMahon et al. 2015), thus differences in host susceptibility and potentially host tolerances may exist. An understanding of the condition dependence of virulence, the impact on non-*apis* hosts, and the likely environmental changes that populations are experiencing is therefore critical to an assessment of the impact of these pathogens on wild pollinator communities.

Materials and methods

We used three *B. terrestris* colonies (Biobest Belgium N.V.) in the experiments and kept them at 28 °C with ad libitum irradiated pollen (Biobest, gamma radiation) and sugar water. We used Invertbee feed sugar (*BelgoSuc*), which has a sugar content of 71.4%, and was diluted 1:1 with water. We confirmed the absence of four common bee viruses (deformed wing virus (DWV), black queen cell virus (BQCV), SBPV and acute bee paralysis virus (ABPV) (McMahon et al. 2015) by RT-PCR (see methods below and supplementary tables S1 and S2 for details) in ten bees from each colony (>20% of the young colony). Graystock et al. (2013) found that in colonies where DWV was present, >10% of bees within the colony were infected with the virus. Thus, screening >20% of the colony is sufficient to determine virus-free status. Phase-contrast microscopy of faeces and gut tissue samples from the same ten bees per colony confirmed the absence of the gut parasites *Nosema* spp, *Crithidia* spp and *Apicystis bombi*. Bees were handled

throughout the experiment using forceps that were flamed between individuals.

To imitate a naturally occurring infection, we prepared virus inoculum from five naturally SBPV-infected wild-caught bees from Scotland (2× *B. hortorum* and 3× *B. pascuorum*), known to be uninfected by DWV, BQCV and ABPV by previous PCR. We homogenised their abdomen in 400 µl of insect ringer solution per bee and combined them into one inoculum solution. We prepared the control inoculum in the same way from uninfected colony bees. SBPV and control inocula were confirmed by RT-PCR to be SBPV positive and negative, respectively.

Experimental set-up

Infection time course

To determine the pattern of SBPV infection in the bee guts over time, we collected 50 bees from each of the three colonies of random age and size. We confined all bees individually in tubes and starved them for 2 h before dosing them with 10 µl of sugar water solution, containing 5 µl of SBPV extract. The 10 µl droplet was pipetted onto the upper side of the tube and all bees were observed to drink the droplet until no liquid was visible. We then maintained bees individually with ad libitum pollen and sugar water. We killed three bees per colony every 2 days for 14 days, then once a week until day 28. Guts were dissected from each bee for qPCR analysis (see below). Full guts were used in this assay to track viral loads in the gut specifically, as faecal-oral transmission is thought to be a primary transmission route for insect viruses. Dose was determined from analysis of day zero bees (killed 2 h post dosing) to be directly comparable to the other time points.

Longevity under satiated conditions

We collected 142 newly emerged worker bees over 4 days from the same three colonies (46, 59 and 37, respectively, from colonies A, B and C). It was essential to use same-age bees when comparing time to death between treatment groups under satiated conditions because of the bees's long lifespan (up to 3 months). Gut fauna was reconstituted by feeding all bees a 10 µl preparation of faeces mixed with sugar water, which we collected from worker bees in the corresponding colony (Koch and Schmid-Hempel 2011). At 6 days old, we dosed and maintained the bees as detailed above. The experiment continued for 95 days, by which point 94% of bees had died; we killed the eight surviving bees. We collected dead bees daily and stored them at −80 °C .

Longevity under starvation conditions

It is paramount to use bees from the same colonies in each assay, as colony-specific gut microbiota play a role in immunity (Koch and Schmid-Hempel 2012). Thus, to reach a sufficient sample size we collected 150 worker bees (age range 0–3 week-old) from the same three colonies (50 per colony); bees were randomly allocated to either the treatment or the control group, each individually fed with 10 µl of sugar water solution, containing either 5 µl of SBPV extract or control extract, respectively. To control for age and allow for the acquisition of the normal gut flora, all workers in the colonies were marked on a set day and experimental bees were then randomly chosen and allocated from unmarked bees that had emerged over a 3-week period to randomise age differences across treatments. We maintained workers individually with ad libitum pollen and sugar water. On day 10 post-infection, we starved all surviving individuals ($N = 144$) of pollen and sugar water and recorded time to death every 15 min until 56 h, at which point we killed the six surviving individuals. Death was confirmed by lack of movement when we turned the tubes at each 15 min time point.

RNA extractions and RT-PCR

To allow for multiple assays, we cut all bees from the two longevity assays in half laterally. Guts were extracted from infection time course bees. One half bee, or gut extract, was used to determine infection status through RT-PCR. We extracted RNA individually from each sample using Trizol© (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Briefly, samples were homogenised with glass beads in 1.3 ml Trizol© in a tissue-lyser (note; 500 µl of Trizol was used for the guts). RNA was separated using bromo-chloropropane and precipitated in isopropanol. The RNA was washed with 75% ethanol and re-suspended in 400 µl of diethylpyrocarbonate (DEPC)-treated water. We converted 2 µl of RNA into first-strand cDNA using GoScript™ Reverse Transcriptase, according to the manufacturer's instructions (Promega) using random hexamer primers and RNasin® to avoid RNA degradation. SBPV positive RNA and water negatives were run as controls.

SBPV specific forward (5′-GAGATGGATMGRCCTGAAGG-3′) and reverse primers (5′-CATGAGCCCAKGARTGTGAA-3′) were used to amplify a 915 bp cDNA fragment by PCR. We designed primers based on the published Rothamsted strain coding region [EU035616 (de Miranda et al. 2010)]. We carried out PCR in 20 µl reactions using GoTaq® DNA Polymerase, with 35 amplification cycles, an annealing temperature of 55 °C for 30 and 20 s of extension at 72 °C (table S1). Every run included

a known positive SBPV sample and a water negative as controls. 5 µl of PCR product were run on 1.5% TAE agarose gel and RedSafe™ nucleic acid staining solution. In addition, we carried out PCR with arginine kinase (AK), a stable reference gene for *B. terrestris* (Horňáková et al. 2010), to check the quality of RNA extractions (primers: 5′-TGACAAGCATCCACCAAAAG-3′, 5′-TCGTCGATCAGTTTCTGCTG-3′), amplifying a 263 bp fragment using the PCR programme as above.

Real-time quantitative RT-PCR

RNA extractions from all bee guts in the infection time course were measured on a Nanodrop 1000 spectrometer. 260/280 ratios ranged from 1.81 to 2.03 with 64% of samples falling between 1.9 and 2.03. Two samples fell below 1.8 and were excluded from qPCR analysis. All remaining samples were run on a Qubit® 3.0 Fluorometer using the Qubit® RNA quantification assay: RNA concentrations ranged from 1.8 to 207 ng/µl. This range was accounted for by diluting all sample RNA to 5 ng/µl (using RNase/DNase free water). Samples that were already at 5 ng/µl or below ($N = 10$) were used directly.

One-step absolute quantification of SBPV was carried out on the Step One ABI Applied Biosystems qPCR machine. Taqman® primer and probe assays were designed and optimised by Primerdesign© for SBPV and an endogenous control bee gene, Actin beta (ACTB) (SBPV; Accession Number EU035616, context sequence length -138 and the anchor nucleotide—4189: and ACTB; Accession Number FN391379, context sequence length—211 bases and the anchor nucleotide—641).

Each sample was run in duplicate for both SBPV and ACTB assays on each plate, along with a no-template control, and a five point standard curve (1:10 dilution series) also run in duplicate. Reactions were carried out with Precision one-step mastermix and 2.5 µl of RNA in 12.5 µl reactions, according to the manufacturer's instructions. Reverse transcription occurred at 42 °C for 10 min, enzyme activation took place at 95 °C for 8 min, followed by 40 cycles of amplification at 95 °C for 10 s and data collection at 60 °C for 1 min. The standard curve was created using a SBPV positive control provided by Primerdesign with a known quantity of 2×10^5 viral particles per µl, thus SBPV could be quantified between 5×10^1 and 5×10^5 viral copies (figure S1). SBPV assay efficiency ranged from 90.3 to 92.4% across plates. Quantitation cycle (Cq) values and absolute viral quantities were calculated using StepOne software. ACTB Cq values were in the range of 17.4–24.8 Cq and stayed stable relative to SBPV Cq values (figure S2), confirming extraction of a valid biological template.

Body size and fat content

For both longevity assays, we measured the length of the radial wing cell, using a Leica camera microscope and ImageJ, as a proxy for body size (Brown et al. 2000). For the starvation assay, we used the second half of each individual to measure fat content—as a proxy for condition—by adapting methods from Ellers (1996): the half abdomen were dried at 70 °C for 3 days and then weighed with a precision balance, before being placed in 2 ml of dichloromethane:methanol (2:1 mix) for 2 days, dried at 70 °C for another 3 days and weighed again. The difference between the two weights is taken as a proxy for the amount of fat. The relative fat content is the ratio of amount of fat (mg) and radial wing cell length (mm).

Statistical analysis

We carried out all analyses in R v3.2.3. We used the *lme4* package (Bates et al. 2015) to run a GLMM (generalised linear mixed model) modelling log viral load as dependent on time post-infection and host colony, with qPCR plate ($N = 7$) as a random effect. *Lmertest* was used to determine significance. Model simplification, using term removal and Anova for model comparison, was used to determine the minimum adequate model of best fit. After model simplification, goodness of fit was determined using residual plots. One-sided Kolmogorov–Smirnov tests were used to determine significant differences in viral load between time points.

For survival analysis we used the *Survival* package (Therneau 2008). Possible confounding correlations between body size and relative fat content were tested and found to be insignificant. Kaplan–Meier survival curves were produced using the *survfit* function; the *survdiff* function was used to test the difference between curves with a log-rank test. We used the *coxph* function to determine the effects of infection status, body size and relative fat content on the survival of bees, with the frailty function used to fit colony as a random term in the survival models. We checked for correlations between fixed factors using the *cor.test* function. Model simplification, using term removal and Anova for model comparison, was used to determine the minimum adequate model of best fit. We checked models for the assumption of proportionality of hazards using the *cox.zph* function.

Results

SBPV infection course

To confirm that SBPV replicates in bumblebees, we tracked SBPV in inoculated bees for 28 days (Fig. 1). Day post-inoculation is a significant factor determining

log viral load (GLMM: $t = -3.8_{59.4}$, $p < 0.001$). Bees killed on day zero, 2 h post-inoculation, had a mean viral load of 1.5×10^4 copies per ng of RNA (ranging from 8×10^3 to 2.5×10^4 copies, $N = 9$), corresponding to a mean of 3×10^7 copies per bee gut. There was a reduction in mean viral load between day zero and day two to less than 50 copies per ng of RNA (ranging from viral absence to 103 copies, $N = 8$). Compared to day two, viral loads were significantly higher on day four [mean $= 3.4 \times 10^5$ viral copies ($N = 9$), $D = 0.9$, $p < 0.01$], day six [mean $= 10 \times 10^5$ viral copies ($N = 9$), $D = 0.9$, $p < 0.01$], day eight [mean $= 1.6 \times 10^3$ viral copies ($N = 9$), $D = 0.7$, $p = 0.01$], day ten [mean $= 1.4 \times 10^2$ viral copies ($N = 7$), $D = 0.7$, $p = 0.02$] and day fourteen [mean $= 3.8 \times 10^2$ viral copies ($N = 4$), $D = 1$, $p = 0.006$] (Fig. 1), implying that the virus results in a replicating infection. Two bees out of 64, both from colony A, replicated the virus beyond the initial viral load, reaching viral loads of 3×10^6 and 9.7×10^6 per ng of RNA, on day four and six, respectively).

Survival assays

There is a significant difference in survival between SBPV-positive and SBPV-negative bees when stressed by starvation (Fig. 2; $N = 119$, log-rank test: $\chi^2 = 4.5_1$, $p = 0.03$) with a median difference in survival of 2.3 h, but no difference under satiated conditions (Fig. 3; $N = 121$, log-rank test: $\chi^2 = 2.3_1$, $p = 0.1$). Longevity did not vary according to host colony in our experiment (log-rank test for starvation: $\chi^2 = 4.2_2$, $p = 0.1$, and satiated conditions: $\chi^2 = 2.2_2$, $p = 0.3$).

SBPV infection status

SBPV inoculated bees are able to clear infection: with 34 of 77 starvation bees clearing infection before being killed at day 10 post-inoculation, and 14 of 73 satiated

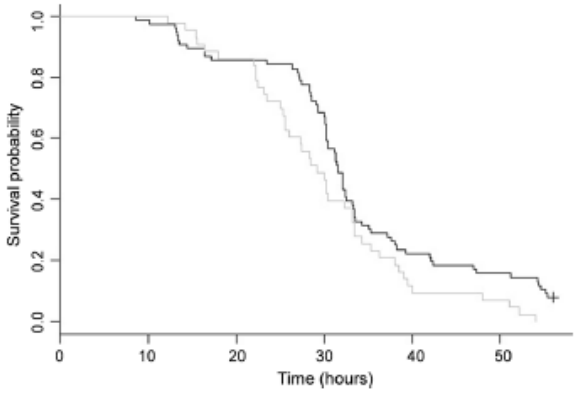

Fig. 2 Kaplan–Meier survival curves for bees infected with slow bee paralysis virus (SBPV) (*grey*) and disease-free bees (*black*) during the starvation assay (contaminated controls excluded)

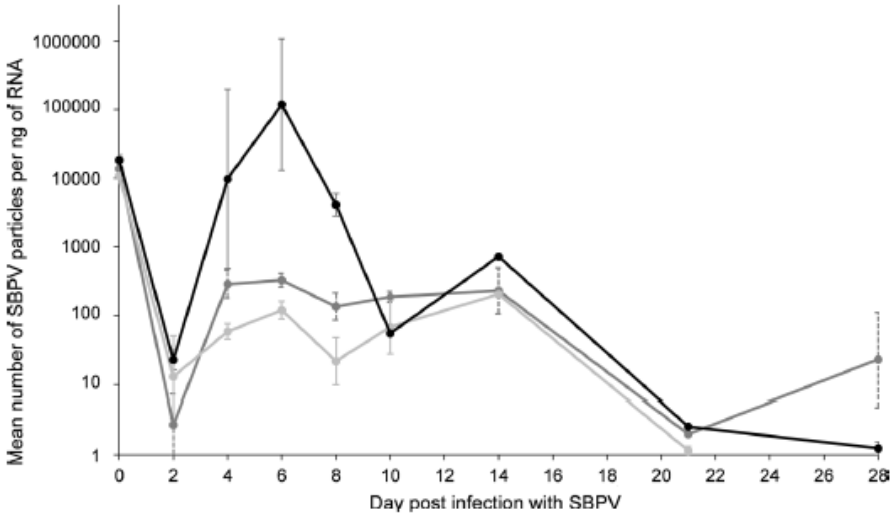

Fig. 1 Mean slow bee paralysis virus (SBPV) load (viral copies per ng of RNA) per colony over a 28-day time course of SBPV infection in the guts of *B. terrestris*. Three guts per colony were analysed per time point on day 0, 2, 4, 6, 8, 10, 14, 21, and 28 (note; only 1 or 2 samples were analysed per colony at days 14 and 21, and no bees remained on day 28 to kill for colony C). Standard error bars are included (note; not for days 14 and 21 when there were less than two data points. Key: colony A (*black*), colony B (*dark grey*) and colony C (*light grey*). *Asterisk* above a time point indicates a significant increase in viral load compared to day two, across all colonies (one-sided Kolmogorov–Smirnov tests). Note log scale used on Y axis

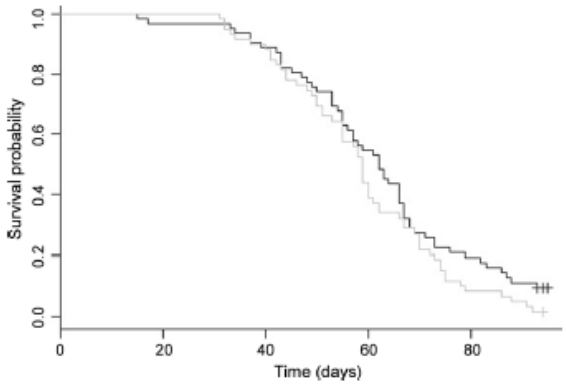

Fig. 3 Kaplan–Meier survival curves for bees infected with slow bee paralysis virus (SBPV) (*grey*) and disease-free bees (*black*) during the satiated assay (contaminated controls excluded)

bees clearing infection before they died naturally at various ages. Interestingly, there is a significant difference in virus clearance rates between the two assays (test of proportions; $\chi^2 = 9.6_1$, $p < 0.01$). SBPV appears to be highly contagious by a transmission route other than direct inoculation. Even though the experimental set-up prevented any direct contact between control bees and SBPV, 26 of 68 starvation control bees and 21 of 69 satiated control bees became contaminated with SBPV during the experiment. Note, there is no significant difference in number of contaminated controls between assays (test of proportions; $\chi^2 = 0.4_1$, $p = 0.5$). Thus, for the analysis we compared infected versus uninfected bees, rather than inoculated versus control bees (table S3). Based on qualitative electrophoresis gel data, it is notable that inoculated bees have a range of viral loads, while contaminated control bees generally have a low viral load (figure S3).

It is important to note that the route of infection—whether inoculation or contamination—does not affect the overall results. Bees positive for SBPV had reduced longevity under starvation conditions when all bees are included in the analysis [median difference in survival = 1.34 h; $N = 145$, HR = 1.4 (1.0–2.0), $\chi^2 = 3.8_1$, $p = 0.05$], if all control bees are removed from analysis [median difference in survival = 3 h; Cox regression: $N = 77$, HR = 1.87 (1.1–3.16), $\chi^2 = 5.42_1$, $p = 0.02$], or if only infected controls are removed (median difference in survival = 2.3 h (Table 1) (table S3). Although interesting, we do not know enough about the route of infection or the dose of SBPV received by contaminated controls to draw any conclusions, and have thus excluded them from the main survival analysis for both starvation and satiated conditions.

Starvation conditions

Of the 119 workers in the starvation assay (excluding the infected controls), 113 subsequently died during the experiment. A maximal Cox proportional hazards regression model included the effects of infection status, body size, relative fat content and host colony, as well as an interaction between infection status and fat content, on the survival of bees. Infection status was significant (hazard ratio (HR) = 1.6, 95% CIs [1.0, 2.3], $\chi^2 = 4.5_1$, $p = 0.03$). Based on the hazard ratio, SBPV-infected bees are 1.6 times more likely to die at a given time point compared to uninfected bees. The data also suggests that the risk of death decreases with increasing relative fat content (HR = 0.4, 95% CIs [0.2, 1.0], $\chi^2 = 3.3_1$, $p = 0.07$). Host colony was fitted as a random effect, and although not significant, it was retained as an important variable within the model. There was no interaction between fat content and body size with infection status. All variables satisfied the assumption of proportional hazards (table S4a). The overall model was significant (likelihood ratio test: $13.9_{3,94}$, $p = 0.007$) (Table 1).

As a result of the ability of inoculated bees to clear infection, the data can be divided into three groups: (1) SBPV-positive bees that were inoculated and maintained infection ($N = 43$), (2) SBPV-negative bees that were inoculated but cleared infection ($N = 34$), and (3) SBPV-negative control bees ($N = 42$). Note, we assume 100% of inoculated bees initially became infected based on qPCR data from our infection time course (Fig. 1). Interestingly, bees that cleared infection did not differ in survival from control bees; but both these SBPV-negative groups differ in survival from SBPV-positive inoculated bees (Table 2 and figure S4: note, these also includes pairwise comparison with contaminated controls, indicating that survival of SBPV-positive contaminated control bees does not significantly differ from survival of SBPV-positive inoculated bees).

Satiated conditions

Of the 121 workers in the satiated assay (excluding the infected controls), 114 subsequently died during the experiment. Infection had no significant effect on survival but was retained in the model as an important variable. Batch had no effect on survival and was removed from the model by model simplification. Only body size had a positive significant effect on survival (HR = 0.4 CIs [0.2, 0.8], $\chi^2 = 6.49_1$, $p = 0.01$) (Table 1). Host colony was not significant but was retained as an important variable within the model. The overall model was significant (likelihood ratio test: $10.3_{3,94}$, $p < 0.03$). All variables satisfied the assumption of proportional hazards (table S4b). Under satiated

Table 1 Cox regression models comparing survival of slow bee paralysis virus (SBPV)-infected bees to SBPV-free bees under starvation conditions and favourable conditions. Colony was fitted as a random effect

Variable	Regression coefficient (b)	SE (b)	*p* value	HR (e^b)	95% CIs for HR	
Starvation conditions ($N = 119$, events = 113)					Lower	Upper
Infection status (0 = not infected, 1 = infected)	0.4	0.2	0.03**	1.6	1.0	2.4
Colony A				0.8	0.2	2.6
Colony B	n/a	n/a	0.12	0.9	0.3	3.0
Colony C				1.3	0.4	3.0
Fat ratio	−0.8	0.5	0.07*	0.4	0.2	1.0
Body size (wing)	0.3	0.4	0.4	1.4	0.6	3.4
Overall model	Likelihood ratio = $13.9_{3,94}$			$p = 0.007$**		
Favourable conditions ($N = 121$, events = 114)						
Body size (wing)	−1.0	0.4	0.01**	0.4	0.2	0.8
Infection status (0 = not infected, 1 = infected)	0.2	0.2	0.2	1.3	0.9	1.9
Batch number (1,2,3,4)	0.1	0.1	0.2	1.2	1.0	1.4
Colony A	n/a	n/a	0.3	1.2	0.4	3.8
Colony B				1.0	0.3	3.2
Colony C				0.8	0.2	2.7
Overall model	Likelihood ratio = $10.3_{3,94}$			$p = 0.03$**		

HR hazard ratio, *CIs* 95% confidence intervals for HR. Variables underlined are included in the final model, variables that are not underlined were included in maximal models but removed by model simplification

** Indicates significance at 95% and *at 90%. Overall model fit refers to the minimum adequate model

Table 2 Kaplan–Meier log-rank test results between the survival probabilities of four groups of bees under starvation conditions: inoculated (+) are SBPV inoculated bees that maintained infection, inoculated (−) are inoculated bees that cleared infection, controls (+) are control bees that became infected indirectly, and controls (−) are control bees that remained clean

	Inoculated (+)	Inoculated (−)	Controls (+)
Inoculated (+) ($n = 43$)			
Inoculated (−) ($n = 34$)	$\chi^2 = 4_1, p = 0.045$ **		
Controls (+) ($n = 26$)	$\chi^2 = 0.4_1, p = 0.506$	$\chi^2 = 1.3_1, p = 0.2$	
Controls (−) ($n = 42$)	$\chi^2 = 3.7_1, p = 0.077$ *	$\chi^2 = 0.1_1, p = 0.7$	$\chi^2 = 0.7_1, p = 0.415$

The number of bees in each group is recorded in parentheses

** Indicates significant difference in survival between two groups at 95% and * at 90%

conditions there was no difference in survival between the four groups mentioned above [(1) SBPV-positive bees that were inoculated and maintained infection ($N = 59$), (2) SBPV-negative bees that were inoculated but cleared infection ($N = 14$), (3) SBPV-negative control bees ($N = 48$)] and (4) SBPV-positive contaminated control bees ($N = 21$) ($\chi^2 = 2.4_3$, $p = 0.5$).

Colony effects

There are colony differences in susceptibility to SBPV, with colony A showing significantly higher titres compared to the other two colonies (Fig. 1, GLMM; $t = 5.3_{58}$, $p < 0.001$). In addition, colony A was significantly less able to clear infection in the starvation assay ($\chi^2 = 12.1$, $p = 0.002$): post-starvation (i.e. day 10 post-infection) 44% ($N = 77$) of inoculated bees were clear of infection—in colony A only 19% (5 of 26) of inoculated bees had cleared infection by day 10, while 60% (15 of 25) and 58% (15 of 26) of individuals had cleared the infection in colony B and C, respectively. In the longevity assay, few bees had cleared infection at the time of death [12.5% ($n = 24$), 27% ($n = 30$) and 15.8% ($n = 19$) in colony A, B and C, respectively, with no significant differences between colonies ($\chi^2 = 1.9$, $p = 0.38$).

Discussion

Slow bee paralysis virus is widespread and prevalent across bumblebee species in the UK and occurs in apparently healthy foraging individuals (McMahon et al. 2015). Our results demonstrate that SBPV has the potential to exert a hidden cost on its host *B. terrestris*. Under satiated conditions, when *B. terrestris* have unlimited access to food, SBPV infection has no effect on longevity; while, under starvation conditions, the longevity of infected bees is significantly reduced—they are 1.6 times more likely to die at each time point. Under lab conditions, infected bees survived on average 2.3 h less under starvation. In the wild, periods of starvation are likely to occur frequently for bumblebees due to adverse weather conditions such as high winds, rain or cold weather. Unlike honeybees, bumblebees do not have significant honey stores in their nests. A decreased survival of a median of 2.3 h would thus be ecological significant for bumblebees in the wild (Brown et al. 2000); more importantly, this effect would most likely be exacerbated under real life conditions where individuals need to expend additional energy for, e.g. thermoregulation and flight, therefore the impact of viral infection may be underestimated here.

The experimental starvation conditions used in this experiment were designed to imitate natural situations where poor weather can prevent bees foraging for extended periods. Both pollen and nectar are critical energy resources for bumblebees, and colonies suffering from an energy shortfall are less effective at protecting the colony from predators, social parasites (Cartar and Dill 1991) and pathogens (Alaux et al. 2010; Moret and Schmid-Hempel 2000). As our results show, the virulence of a viral pathogen, which may appear asymptomatic under benign conditions, is also unmasked under such energy-limited conditions. Given such synergistic effects of environmental stress and disease there is clearly the need for more consideration of spillover effects of viral disease between managed bees and wild pollinators.

Condition-dependent virulence has been demonstrated in bumblebees for the gut parasite *Crithidia bombi*, which also decreases survival under starvation (Brown et al. 2000). *C. bombi* additionally exerts fitness costs during stressful times in the bumblebee life cycle, such as queen hibernation and colony foundation (Brown et al. 2003). For bee viruses, condition-dependent virulence of DWV has been demonstrated under pesticide stress (Di Prisco et al. 2013) as well as nutritional stress (Degrandi-Hoffman et al. 2010). In some sense, co-infection of viruses with the ectoparasitic honeybee mite *V. destructor* is probably the best documented example of condition-dependent virulence [SBPV (Carreck et al. 2010), DWV (Martin et al. 2012), ABPV (Genersch et al. 2010) and IAPV (Di Prisco et al. 2011)]. However, the mechanisms of increased virulence in this case are more complicated. Although *V. destructor* weakens the bees and may cause immunosuppression (Nazzi et al. 2012; Yang and Cox-Foster 2005) similar to other environmental stresses, the mite itself increases viral virulence in its capacity as a virus vector (Martin 2001).

It is probable that, like *C. bombi*, other condition-dependent pathogens of bumblebees such as SBPV will affect colony fitness beyond mere survival to starvation. Clearly these individual and colony effects can lead to population level responses and pathogens are well documented to exert large effects on their host's ecology (e.g. Anderson and May 1981; Hatcher et al. 2006). Theoretical models and eventually species specific simulation models would be useful in determining the likely population level effects of the individual impact of condition-dependent virulence under variable resource conditions.

The precise mechanism by which SBPV reduces host longevity under starvation conditions is unclear. It is possible that infection induces a costly response by the host immune system; in this scenario, the host is unable to maintain a defence against the virus when resources are withheld, resulting in increased virus virulence and reduced lifespan (Moret and Schmid-Hempel 2000). It is also possible that infection reduces the resources in the gut that are available to the bee, or inhibits uptake of resources, as may be the case for trypanosomal gut parasites (Gorbunov 1987, 1996; Jensen et al. 1990). Such effects may be exacerbated by any damage caused by viral replication within its host, with the pathogen's virulence likely to be determined by a combination of these factors.

Bees with higher body fat have reduced risk of death under starvation conditions, while body size itself has no effect. The fat body is key for immunity and longevity; it is the main site of energy and protein storage, synthesis of immunoproteins, and vitellogenin synthesis involved in longevity (Amdam and Omholt 2002). In *B. terrestris* workers, fat body increases with age (Moret and Schmid-Hempel 2009). As the bees in this assay were of mixed age (within 3 weeks of each other), although randomised across treatments, it is possible the relationship between fat body and survival could be linked to age. Body size had no effect on longevity over short-term starvation but had a significant positive effect on longevity under satiated conditions. Body size is determined by conditions during larval development. Maintaining brood at 30 °C requires high energy consumption (Heinrich 1974). When resources are low, workers cease incubating and the brood temperature drop, which slows the development and potentially causes developmental defects (Barrow and Pickard 1985). Low body size and body fat are both symptoms of a lack of resources

and reduce individual longevity, which could have consequences at a colony and population level.

Many measured immune defences decrease with age in *B. terrestris* workers, i.e. antibacterial activity, encapsulation and melanisation, haemocyte concentration and phenoloxidase activity, with declines seen within a biologically relevant age range (Doums et al. 2002; Moret and Schmid-Hempel 2009). It is interesting that the ability of bees in the starvation assay to clear viral infection is significantly higher than for bees in the satiated assay. Because of the mixed age of starvation bees it is important to consider how immunosenescence could influence viral clearance rates. The starvation bees were older (on average) at the time point of inoculation because of the age range 0–3 weeks (1–21 days old), while satiated bees were all inoculated at 6 days old. Thus, a higher viral clearance rate in older bees is contrary to the immunosenescence reported for the aspects of humoral and cellular immune defences in *B. terrestris,* mentioned above. However, not all immune measures decrease with age, e.g. fat body (Moret and Schmid-Hempel 2009). It is conceivable that anti-viral defences against oral-faecal infections might be stronger in older bees. In addition, in contrast to individual age, some immune measures increase with colony age. Moret and Schmid-Hempel (2009) found that *B. terrestris* workers born when the colony is young have lower concentration of haemocytes and lower PO activity than those born later in the colony life cycle. As starvation bees emerged at a later point in each colony's life cycle, compared to satiated bees, increased immunity with colony age could also explain why starvation bees cleared infection at significantly higher rates. It is clear that experiments dedicated to studying immunosenescence of anti-viral defences in social insects are needed to understand the impact of viral infections on wild populations.

The dose of SBPV used in this study came from a natural infection and is high enough to cause an initial infection in the majority of inoculated bees, with condition-dependent effects on longevity. The infection persisted up to 95 days in our bees kept in satiated conditions, which would mean a life-long infection for worker bees in the wild. However, our data show a significant variation between colonies in viral replication levels (across several orders of magnitude at day four and six post-infection) and their ability to clear infection, suggesting a genetic basis for defence that is likely to be reflected in wild bumblebees.

Over 20% of the control bees became infected with SBPV indirectly. While contamination via plastic-ware cannot be categorically ruled out, this raises the possibility that SBPV may be an airborne pathogen. In addition, Graystock et al. (2016) recently showed that commercial irradiated pollen can still contain pathogens, and although they did not test for SBPV this is a possibility. It is noteworthy that there was no difference in survival of SBPV-infected bees, whether they were orally inoculated with a 5 µl dose or indirectly infected; it appears that SBPV is highly transmittable at low doses.

In summary, we have demonstrated that a common honeybee and bumblebee pathogen, that may appear asymptomatic in field collections and under optimal lab conditions, exerts a fitness cost on bumblebees under adverse conditions. Our results show the importance of examining subtle fitness effects when assessing a pathogen's effect on its host. Additionally, we found that larger bumblebees—indicating energy-rich conditions during larval growth—had a higher longevity and individuals that survived starvation conditions for longer had larger fat reserves. Providing good forage opportunities for pollinators may thus directly contribute to their longevity and resistance to stressful conditions. This highlights the importance of providing forage opportunities for pollinators throughout the season, as laid out, for example, in the UK's National Pollinator Strategy (DEFRA 2014). A wider uptake of conservation measures in land management under schemes such as the Countryside Stewardship in the UK could directly impact longevity and disease tolerance in pollinators.

Conditions in the lab are extremely favourable, e.g. individual lifespan under laboratory conditions exceeds the natural lifespan of worker bees in the field (Schmid-Hempel and Heeb 1991), such that laboratory studies can underestimate the impact of a pathogen. The use of sentinel species in studies of a multi-host-virus system may misrepresent the true impact of a pathogen on wild pollinator populations, as hosts can vary in their susceptibility to viruses (McMahon et al. 2015). In addition, estimates of virus prevalence are believed to be underestimated (McMahon et al. 2015). Thus, the impact of SBPV on natural populations may be greater than predicted. Indeed, it is possible that SBPV does affect longevity under satiated nutritional conditions in the wild, as workers would face additional ecological and environmental stressors such as inclement weather, energetically costly foraging and exposure to pesticides and pollutants. More broadly, this demonstrates that impact assessments of emerging multi-host pathogens, such as West Nile Virus in the USA (Kilpatrick 2011) need to take into account the pathogen's ecology rather than narrowly focusing on the most tractable laboratory model system.

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Author contribution statement RM and LW conceived and designed the experiments. RM performed the experiments. RM analysed the data with statistical advice from MB. RM and LW wrote the manuscript; MB provided editorial advice.

Compliance with ethical standards

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

Data accessibility If this paper is accepted for submission I intend to archive the original data using Dryad.

References

Alaux C, Ducloz F, Crauser D, Le Conte Y (2010) Diet effects on honeybee immunocompetence. Biol Lett 6:562–565

Amdam GV, Omholt SW (2002) The regulatory anatomy of honeybee lifespan. J Theor Biol 216:209–228

Anderson RM, May RM (1981) The population dynamics of microparasites and their invertebrate hosts. Philos Trans R Soc Lond B 291:451–524

Bailey L, Woods RD (1974) Three previously undescribed viruses from the honey bee. J Gen Virol 25:175–186

Barrow DA, Pickard RS (1985) Larval temperature in brood clumps of *Bombus pascuorum* (Scop.). J Apic Res 24:69–75

Bates D, Maechler M, Bolker B, Walker S (2015) Fitting linear mixed-effects models using lme4. J Stat Softw 67:1–48. doi:10.18637/jss.v067.i01

Boots M, Begon M (1994) Resource limitation and the lethal and sublethal effects of a viral pathogen in the Indian meal moth, *Plodia interpunctella*. Ecol Entomol 19:319–326

Brown MJF, Loosli R, Schmid-Hempel P (2000) Condition-dependent expression of virulence in a trypanosome infecting bumblebees. Oikos 91:421–427

Brown MJF, Schmid-Hempel R, Schmid-Hempel P (2003) Strong context-dependent virulence in a host-parasite system: reconciling genetic evidence with theory. J Anim Ecol 72:994–1002

Carreck NL, Ball BV, Martin SJ (2010) Honey bee colony collapse and changes in viral prevalence associated with *Varroa destructor*. J Apic Res 49:93–94

Cartar RV, Dill LM (1991) Costs of energy shortfall for bumblebee colonies: predation, social parasitism, and brood development. Can Entomol 123:283–293

Cox-Foster DL (2007) A metagenomic survey of microbes in honey bee colony collapse disorder. Science 318:283–287

Cresswell JE et al (2012) Differential sensitivity of honey bees and bumble bees to a dietary insecticide (imidacloprid). Zoology 115:365–371

de Miranda JR et al (2010) Genetic characterization of Slow Bee Paralysis Virus of the honeybee (*Apis mellifera* L.). J Gen Virol 91:2524–2530

DEFRA (2014) National pollinator strategy: for bees and other pollinators in England, https://www.gov.uk/government/publications/national-pollinator-strategy-for-bees-and-other-pollinators-in-england. Accessed 04 May 2015

Degrandi-Hoffman G, Chen Y, Huang MH (2010) The effect of diet on protein concentration, hypopharyngeal gland development and virus load in worker honey bees (*Apis mellifera IL.*). J Insect Physiol 56:1184–1191

Di Prisco G, Pennacchio F, Caprio E, Boncristiani HF Jr, Evans J, Chen Y (2011) *Varroa destructor* is an effective vector of Israeli acute paralysis virus is the honeybee, *Apis mellifera*. J Gen Virol 92:151–155

Di Prisco G et al (2013) Neonicotinoid clothianidin adversely affects insect immunity and promotes replication of a viral pathogen in honey bees. PNAS 110:18466–18471. doi:10.1073/pnas.1314923110

Doums C, Moret Y, Benelli E, Schmid-Hempel P (2002) Senescence of immune defence in *Bombus* workers. Ecol Entomol 27:138–144

Ellers J (1996) Fat and eggs: an alternative method to measure the trade-off between survival and reproduction in insect parasitoids. Neth J Zool 46:227–235

Evison SEF et al (2012) Pervasiveness of parasites in pollinators. PLoS One 7:e30641. doi:10.1371/journal.pone.0030641

Fürst MA, McMahon DP, Osborne JL, Paxton RJ, Brown MJF (2014) Disease associations between honeybees and bumblebees as a threat to wild pollinators. Nature 506:364–366. doi:10.1038/nature12977

Garibaldi LA et al (2013) Wild pollinators enhance fruit set of crops regardless of honey bee abundance. Science 339:1608–1611. doi:10.1126/science.1230200

Genersch E et al (2010) The German bee monitoring project: a long term study to understand periodically high winter losses of honey bee colonies. Apidologie 41:332–352. doi:10.1051/apido/2010014

Gorbunov PS (1987) Endoparasitic flagellates of the genus *Crithidia* (Trypanosomatidae, Zoomastigophorea) from alimentary canal of bumblebees. Zoologicheskii Zhurnal 66:1775–1780

Gorbunov PS (1996) Peculiartities of life cycle in flagellate *Crithidia bombi* (Protozoa, Trypanosomatidae). Zoologicheskii Zhurnal 75:803–810

Goulson D, Nicholls E, Botías C, Rotheray EL (2015) Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. Science 347:1255957

Graystock P, Yates K, Evison SEF, Darvill B, Goulson D, Hughes WOH (2013) The Trojan hives: pollinator pathogens, imported and distributed in bumblebee colonies. J Appl Ecol 50:1207–1215

Graystock P et al (2016) Hygienic food to reduce pathogen risk to bumblebees. J Invertebr Pathol 136:68–73. doi:10.1016/j.jip.2016.03.007

Hatcher MJ, Dick JTD, Dunn AM (2006) How parasites affect interactions between competitors and predators. Ecol Lett 9:1253–1271

Heinrich B (1974) Thermoregulation in bumblebees. 1. Brood incubation by *Bombus vosnesenkii* queens. J Comp Physiol 88:129–140

Horňáková D, Matoušková P, Kindl J, Valterová I, Pichová I (2010) Selection of reference genes for real-time polymerase chain reaction analysis in tissues from *Bombus terrestris* and *Bombus lucorum* of different ages. Anal Biochem 397:118–120

Jaenike J, Benway H, Stevens G (1995) Parasite-induced mortality in Mycophagous *Drosophila*. Ecology 76:383–391

Jensen C, Schaub GA, Molyneux DH (1990) The effect of *Blastocrithdia triatomae* (Trypanosomatidae) on the midgut of the reduviid bug *Triatoma infestans*. Parasitology 100:1–10

Jokela J, Taskinen J, Mutikainen P, Kopp K (2005) Virulence of parasites in hosts under environmental stress: experiments with anoxia and starvation. Oikos 108:156–164

Kilpatrick AM (2011) Globalization, land use, and the invasion of West Nile virus. Science 334:323–327

Koch H, Schmid-Hempel P (2011) Socially transmitted gut microbiota protect bumble bees against an intestinal parasite. PNAS 108:19288–19292. doi:10.1073/pnas.1110474108

Koch H, Schmid-Hempel P (2012) Gut microbiota instead of host genotype drive the specificity in the interaction of a natural host-parasite system. Ecol Lett 15:1095–1103

Koella JC, Offenberg J (1999) Food availability and parasite infection influence the correlated responses of life history traits to selection for age at pupation in the mosquito *Aedes aegypti*. J Evol Biol 12:760–769

Leggett HC, Buckling A, Long GH, Boots M (2013) Generalism and the evolution of parasite virulence. Trends Ecol Evol 28:592–596

Levitt AL et al (2013) Cross-species transmission of honey bee viruses in associated arthropods. Virus Res 176:232–240

Manley R, Boots M, Wilfert L (2015) Emerging viral disease risk to pollinating insects: ecological, evolutionary and anthropogenic factors. J Appl Ecol 52:331–340

Martin SJ (2001) The role of *Varroa* and viral pathogens in the collapse of honeybee colonies: a modelling approach. J Appl Ecol 38:1082–1093

Martin SJ et al (2012) Global honey bee viral landscape altered by a parasitic mite. Science 336:1304–1306. doi:10.1126/science.1220941

McMahon DP, Fürst MA, Caspar J, Theodorou P, Brown MJF, Paxton RJ (2015) A sting in the spit: widespread cross-infection of multiple RNA viruses across wild and managed bees. J Anim Ecol 84:615–624

Meeus I, de Miranda JR, de Graaf DC, Wäckers F, Smagghe G (2014) Effect of oral infection with Kashmir bee virus and Israeli acute paralysis virus on bumblebee (*Bombus terrestris*) reproductive success. J Invertebr Pathol 121:64–69

Moret Y, Schmid-Hempel P (2000) Survival for immunity: the price of immune system activation for bumblebee workers. Science 290:1166–1167

Moret Y, Schmid-Hempel P (2009) Immune responses of bumblebee workers as a function of individual and colony age: senescence versus plastic adjustment of the immune function. Oikos 118:371–378

Nazzi F et al (2012) Synergistic parasite-pathogen interactions mediated by host immunity can drive the collapse of honeybee colonies. PLoS Pathog 8:e1002735. doi:10.1371/journal.ppat.1002735

Restif O, Kaltz O (2006) Condition-dependent virulence in a horizontally and vertically transmitted bacterial parasite. Oikos 114:148–158

Santillán-Galicia MT, Ball BV, Clark SJ, Alderson PG (2014) Slow bee paralysis virus and its transmission in honey bee pupae by *Varroa destructor*. J Apic Res 53:146–154

Schmid-Hempel P, Heeb D (1991) Worker mortality and colony development in bumblebees, *Bombus lucorum* (L.) (Hymenoptera, Apidae). Mitt der Schweize entomologischen Ges 64:93–108

Singh R et al (2010) RNA viruses in hymenopteran pollinators: evidence of inter-taxa virus transmission via pollen and potential impact on non-*Apis* hymenopteran species. PLoS One 5:e14357. doi:10.1371/journal.pone.0014357

Steinhaus M (1958) Stress as a factor in insect disease. In: Proceedings of the 10th international conference of entomology 4, pp 725–730

Therneau T (2008) Survival: survival analysis, including penalised likelihood, http://cran.r-project.org/web/packages/survival. Accessed 08 Aug 2015

Vanbergen AJ et al (2013) Threats to an ecosystem service: pressures on pollinators. Front Ecol Environ 11:251–259. doi:10.1890/120126

Yang X, Cox-Foster DL (2005) Impact of an ectoparasite on the immunity and pathology of an invertebrate: evidence for host immunosuppression and viral amplification. PNAS 102:7470–7475

Supplementary Material

Supplementary tables

Table S1. The molecular and thermal conditions used for each pair of primers.

Primers	Assay mix								Thermal cycling			Amplicon size (bp)
	5x buffer (ul)	$MgCl_2$ (mM)	dNTPS (mM)	Primer F(uM)	Primer R (uM)	Taq (ul)	Template (ul)	Final volume (ul)	Denaturing (Temp|Min)	Replication (Temp|sec)	Elongation (Temp|min)	
SBPV	4	2.5	0.2	0.4	0.4	0.15	2	20	94|5	X 35 94|15 55|30 72|20	72|7	915
ABPV	4	2.5	0.2	0.4	0.4	0.15	2	20	94|5	X 35 94|15 55|30 72|70	72|7	1034
DWV	4	2.5	0.2	0.4	0.4	0.15	2	20	94|5	Touchdown PCR X10 94|15 62|30 72|60 X30 94|15 [Fst-62|30 Lst-52|30] 72|60	72|7	644
BQCV	4	2.5	0.2	0.4	0.4	0.15	2	20	95|2	x35 95|15 60|30 72|60	72|7	986
AK (Arginine Kinase)	3	2.5	0.2	1	1	0.15	3	15	94|5	x35 94|15 55|30 72|20	72|7	263

Table S2. Primer Sequences

Primer name	Sequence 5'-3'
SBPV 774F	GAGATGGATMGRCCTGAAGG
SBPV 1689R	CATGAGCCCAKGARTGTGAA
ABPV 5088F	CyATGGACACACCCTATGTG
ABPV 6122R	CGCCATTTTGGTACTTCTCC
DWV 8577F *	AACTGGCGAYCATACTCAGC
DWV 7933R	WCCAGGCACMCCACATACAG
BQCV 4119F	TCCyCCAGTTCAACCATCTA
BQCV 5376R	AACGTTGCCTAGrTTCGTCA
ACTB F	TGACAAGCATCCACCAAAAG
ACTB R	TCGTCGATCAGTTTCTGCTG

*DWV primers not-specific to any strain of DWV, taken from Wilfert et al. (2016)

Table S3. The number of bees in each experimental group, followed by the groups that were used in the survival analysis. Note: we tested infected vs uninfected bees, rather than inoculated vs control bees because of the number of bees that cleared or gained infection within each group. Note 2: 68 of the original 73 control bees in the starvation assay were used in the experiment because five bees died prior to the starvation assay (day 10). Importantly, the main results remain unchanged whether contaminated/cleared individuals are removed or not. The result in bold at the end of the table is the main result we report in the paper.

	Starvation		Survival analysis (Cox proportional hazards regression for infection variable reported)	Satiated		Survival analysis
	Inoculated	**Controls**		**Inoculated**	**Controls**	
Total bees	77	73		73	69	
Used in experiment	77	68	n/a	73	69	n/a
Bees Cleared infection	34	n/a	n/a	14	n/a	n/a
Bees gained infection	n/a	26	n/a	n/a	21	n/a
Total bees without positive controls	77	42	n/a	73	48	n/a
	Infected	**Uninfected**		**Infected**	**Uninfected**	
Total bees	69	76	Sig median difference in survival = 1.34hr; N = 145, HR = 1.4 (1.0 – 2.0), $\chi^2 = 3.8_1$, p = 0.05	80	62	Not sig
Total bees without controls	43	34	Sig median difference in survival = 3hr; Cox regression: N = 77, HR = 1.87 (1.1-3.16), $\chi^2 = 5.42_1$, p = 0.02)	59	14	Not sig
Total without positive controls	**43**	**76**	**Sig median difference in survival = 2.3hr: Cox regression: N = 119, HR = 1.6,** (1.0 –	**59**	**62**	**Not sig**

			2.3), $\chi^2 = 4.5_1$, p = 0.03			

Table S4. The output from testing the assumption of proportional hazards for the Cox proportional hazards model (using coxph function) under starvation (a) conditions and satiated (b) conditions. Rho is the Pearson product moment correlation between the scaled Schoenfeld residuals and log(time) for each variable. The final row contains the global test for all the interactions tested at once. A p value <0.05 shows a violation of the proportionality assumption.

a.

Variable	**rho**	**Chi sq**	**p**
Infected	-0.026	0.079	0.778
Fat ratio	-0.009	0.006	0.940
Colony A	0.221	0.359	0.549
Colony B	0.117	0.087	0.768
Colony C	-0.310	0.352	0.553
Global	n/a	10.421	0.064

b.

Variable	**rho**	**chisq**	**p**
Infected	0.072	0.611	0.435
Wing	-0.067	0.569	0.450
Colony A	-0.065	0.020	0.888
Colony B	0.110	0.068	0.794
Colony C	-0.043	0.017	0.896
Global	n/a	2.156	0.827

Supplementary figures

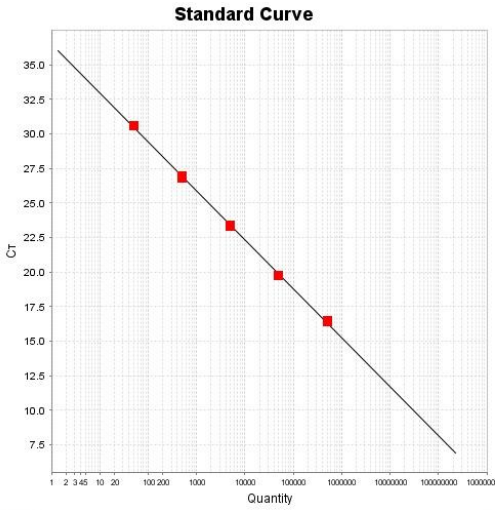

Figure S1. Standard curve for SBPV; Slope = -3.5, Y-inter: 36.5, R2 = 1, eff% = 91.8. Diagram from StepOne Software v 2.3

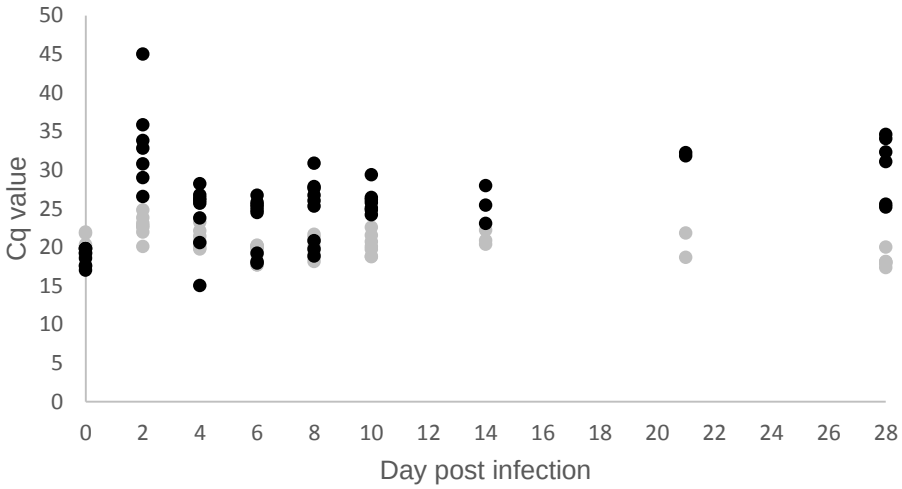

Figure S2. Cq values of individual bees in the gut time course for both Slow bee paralysis virus (SBPV) (black) and Actin Beta (ACTB) (grey) confirming extraction of a valid biological template and demonstrating that ACTB Cq stayed stable relative to SBPV Cq.

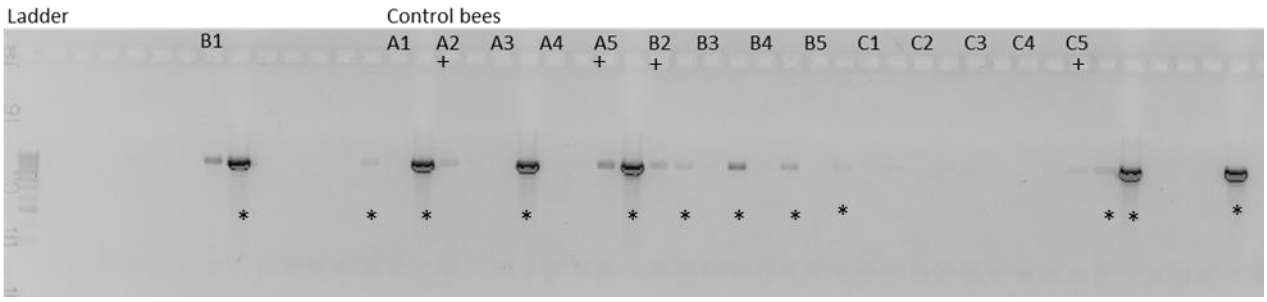

Figure S3. Electrophoresis gel picture of an SBPV PCR product (915 bp fragment) on a number of inoculated and control bees from the starvation assay. Unlabelled wells are inoculated bees; the bands with an asterisk are inoculated bees positive for SBPV. A1-A5, B1-B5 and C1-C5 wells are control bees from colonies A, B and C, respectively; + identifies controls positive for SBPV.

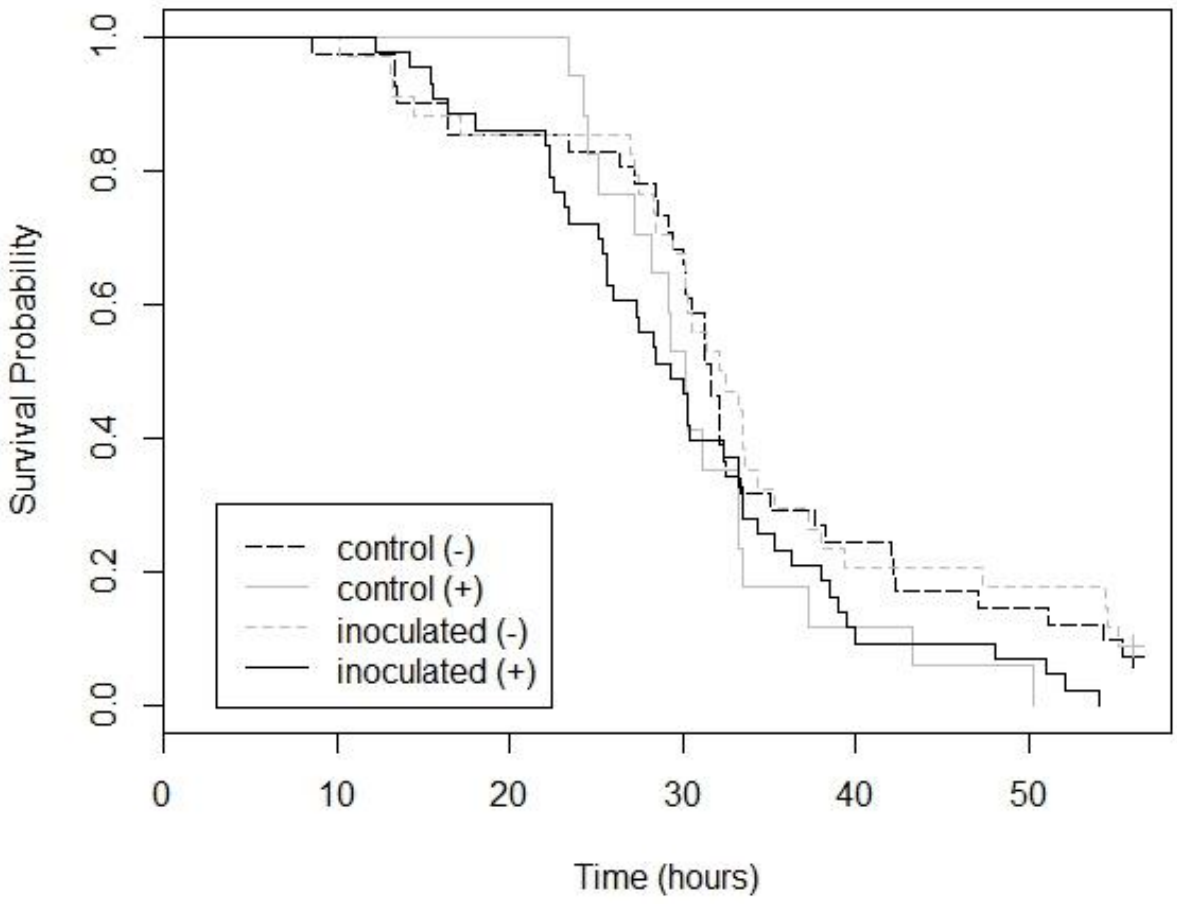

Figure S4. A comparison of the survival probability curves between four groups of bees in the starvation assay: Inoculated (+) are bees that were still infected at day 10 post inoculation (p.i.), Inoculated (-) are bees that were inoculated but cleared infection by day 10 p.i., Controls (-) are clean control bees and control (+) are infected controls.

Chapter 4 : Community-level impact of a specialist vector: the honeybee ectoparasite *Varroa* destructor drives DWV prevalence and viral load in sympatric wild bumblebees

Abstract

Emerging infectious diseases (EIDs) in managed and wild bees have the potential to compromise pollination services, and consequently our food security. Honeybees carry a range of harmful pathogens, many of which are shared across pollinator species. The spillover of shared pathogens from managed honeybees may contribute to declines seen in wild pollinators. The Deformed wing virus-complex (DWV type A and B) and the microsporidian *Nosema ceranae* are two emerging pathogens known to infect and cause harm to both honeybees and bumblebees. *Varroa destructor* is an ectoparasitic mite, specific to honeybees, that vectors and changes the dynamics of DWV: increasing prevalence, titre and virulence. In contrast, transmission of *N. ceranae* has no links with *Varroa*. To test the effect of *Varroa* on these pathogens on pollinators, we collected honeybees (*A. mellifera*) and bumblebees (*B. terrestris* and *B. pascuorum*) from matched *Varroa*-free and *Varroa*-present sites; using phylogenetic methods, we tested for spillover of DWV from honeybees to bumblebees. We found that *Varroa* presence increases the prevalence and titre of DWV not only in honeybees, but also in sympatric bumblebees. Honeybees are likely the source population for infections in bumblebees, as the same strains infect sympatric populations, and prevalence of DWV is more than three times higher in honeybees. In contrast, the prevalence and titre of *N. ceranae*, which is not known or expected to be vectored by ectoparasites, is unaffected by *Varroa*-presence. In a multi-host-pathogen system, we show that changes to host-parasite ecology in a single host – in this case the invasion of a novel virus vector – can have community level-effects on transmission dynamics. For wild and managed bees, these results highlight the need for further controls on *Varroa*, and potentially on beekeeping, for wild pollinator health.

Introduction

Emerging infectious diseases (EIDs) are an ever-present threat to human, animal and plant populations alike. Disease emergence can be driven by ecological, evolutionary and anthropogenic factors (Woolhouse et al. 2005). One factor is the acquisition of novel transmission routes by existing pathogens, leading to disease spread, and increasing the

potential of spillover to naive populations or species. An important example is that of *Varroa destructor*, an ectoparasitic mite that is capable of vectoring numerous bee viruses. This ectoparasite jumped hosts from the Asian honeybee (*Apis cerana*) to the European honeybee (*Apis mellifera*) in the middle of the last century. The *Varroa* mite has since spread rapidly across the globe (Oldroyd 1999) and provided a new route of virus transmission in *A. mellifera*, leading to a dramatic increase, in particular of Deformed wing virus (DWV) prevalence, viral load (Martin et al. 2012) and virulence (Genersch et al. 2010; Ryabov et al. 2014). Wilfert et al. (2016) show trade and movement of *A. mellifera* around the world is the source of the globally emerging DWV epidemic, likely driven by the concurrent spread of the *Varroa* mite. This worldwide epidemic is no doubt a grave threat to apiculture and the pollination services it provides. However, while *Varroa* is a specialist parasite of honeybees, it indirectly poses a risk to wild pollinators by altering the disease dynamics of the multihost pathogens it transmits.

Pollination services are essential for our food security and the biodiversity of flowering plants. *A. mellifera* are heavily relied upon for commercial pollination, while wild pollinators also play an important role in pollination of crops and wild flowers (Albrecht et al. 2012; Breeze et al. 2011; Gallai et al. 2009). Two emerging multihost pollinator pathogens, DWV and the gut parasite *N. ceranae*, are suggested to be a driving force of dramatic regional declines seen in recent years in the apiculture industry (Fürst et al. 2014; Graystock et al. 2013a; Potts et al. 2010; Wilfert et al. 2016). *N. ceranae* is a microsporidian parasite that recently jumped hosts from *A. cerana* to *A. mellifera*, spreading worldwide, and has since been implicated in substantial colony losses (Higes et al. 2006; Klee et al. 2007). *N. ceranae* is transmitted horizontally via faecal-oral transmission (Fries 2010), which has no known link to *Varroa*. Conversely, the damage caused at individual and colony level by the association between DWV and *Varroa* is well documented. The *Varroa* mite feeds off developing pupae and by doing so transmits virus directly into the bee haemolymph, bypassing the natural infection barriers of the honeybee such as the exoskeleton and physiological inhibitors in the gut (Evans and Spivak 2010). Recent invasions of *Varroa* in Hawaii and New Zealand led to an increase in DWV prevalence and viral load (Martin et al. 2012; Mondet et al. 2014), with a severe loss of viral diversity (Martin et al. 2012). There is also evidence that *Varroa* increases mortality of colonies over winter (Dainat et al. 2012; Genersch et al. 2010; Highfield et al. 2009).

DWV is a single-stranded RNA virus comprising of at least three distinct clades: DWV-A is the strain currently responsible for the reported global epidemic (Martin et al. 2012; Wilfert et al. 2016) (which also contains Kakugo virus (Fujiyuki et al. 2004) and the Southeast-Asian variant found in Thailand and Pakistan (Wilfert et al. 2016)); sequence data for DWV-B (also known as Varroa destructor virus 1 (Ongus et al. 2004)) has so far only been deposited from Europe and the Middle East (Zioni et al. 2011), with unconfirmed individuals in Hawaii (Martin et al 2012), mainland USA (Kielmanowicz et al. 2015) and South Africa (Strauss et al. 2013); DWV-C is a newly discovered strain from a UK apiary (Mordecai et al. 2015).

In contrast to honeybees, little is known of the impact of DWV-complex on wild bumblebees. The *Varroa* mite does not directly parasitise bumblebees. However, DWV has been identified as an emerging disease in wild pollinators with *A. mellifera* implicated as the reservoir host (Fürst et al. 2014). Recent field studies have shown that the DWV-complex is prevalent in wild bumblebee populations, with 11% of *Bombus* carrying DWV compared to 36% of *A. mellifera* (Fürst et al. 2014); further analysis of these field samples demonstrated that DWV-B was more prevalent than DWV-A, although this difference was not significant (McMahon et al. 2016). Experimental infections have shown DWV (mixed variants) reduces longevity of *B. terrestris* workers by a mean of six days (Fürst et al. 2014); a significant decrease for a worker that could negatively affect colony dynamics. Further infection experiments have shown that DWV-B is potentially more virulent to *A. mellifera* than DWV-A at both an individual and colony level (McMahon et al. 2016). *N. ceranae* has also been shown to be infective for *B. terrestris*, although the impact on survival was low (Fürst et al. 2014); while another study showed a dramatic 48% reduction in survival and sub-lethal behaviour effects (Graystock et al. 2013a). Spillover of pathogens into bumblebee populations is believed to occur through interspecific transmission via shared foraging sites (reviewed by Manley et al. (2015)). Many populations of bumblebee and solitary bee have experienced declines and range contractions, some of which can be attributed to disease (Biesmeijer et al. 2006; Cameron et al. 2011; Goulson et al. 2015). Widespread pesticide use and loss of nesting and foraging habitat also play an important role and put wild bee populations under pressure. This is important, as small or declining populations are particularly vulnerable to repeated spillover events from a sympatric reservoir host, with the potential to drive them to extinction (Dobson 2004).

Although *Varroa* has spread globally, a few *Varroa*-free refugia still exist on islands around the UK and France. We would not expect absence of DWV in *Varroa*-free refugia because firstly, DWV is known to exist in populations without *Varroa* (Martin et al. 2012), and secondly pathogens can spread ahead of their vectors and primary hosts if there is a competent novel host, as is the case with other wildlife diseases such as squirrel pox (Tomkins, White and Boots, 2003). Thus, anthropogenic movement of *A. mellifera* can spread DWV beyond the range of *Varroa* itself. We test whether the specialist honeybee vector *Varroa* leads to spillover of DWV from honeybees to wild bumblebees. We use a structured field survey across 12 sites, comprising of four *Varroa*-free and eight *Varroa*-present sites, to test the indirect impact of *Varroa* infestation of honeybees on DWV intensity in sympatric *B. terrestris* and *B. pascuorum* populations. Since all *Varroa*-free sites are islands, we also sampled three *Varroa*-present islands, to enable testing of the potentially confounding island effect. We compare and contrast prevalence and titre of DWV and *N. ceranae*, two pathogens with very different associations with *Varroa*, to isolate the impact of *Varroa*.

Materials and methods

Bee samples

We collected ~30 *A. mellifera*, ~30 *B. pascuorum* and ~60 *B. terrestris*/*lucorum* individuals from 12 sites across the British Isles and Brittany (France), between June-August 2015 (table S1 for sample details). The sites comprised four *Varroa*-free islands (Isle of Man (Douglas), Scilly Isles (St Mary's), Ushant and Alderney (St Anne); three *Varroa*-present islands (Guernsey (St Peter Port), Jersey (St Helier) and Belle Ile (Le Palais) and five *Varroa*-present mainland sites (Liverpool, Penryn, Cherbourg, Le Conquet and Quiberon) (figure S1 for map of sites). In total we collected 355 *A. mellifera*, 640 *B. terrestris*, 37 *B. lucorum* (see methods below for molecular differentiation of the *B. terrestris*/*lucorum* species complex) and 280 *B. pascuorum.* Bees were collected from a 1x1km area whilst foraging on flowers. We used individual collecting tubes and kept samples on ice, before sacrificing and storing them at -190° in a dry shipper on the day of collection. For Belle Ile and Jersey, bees were sacrificed and stored at in the dry shipper within 48 hours of collection. All samples were then stored at -80° on return to the laboratory.

RNA isolation and RT-PCR

To be able to measure the *N. ceranae* titre microscopically while obtaining whole-body RNA, we removed the gut from each individual and macerated them individually in 200µl of insect ringer solution. For individual RNA extractions, we used half the head and thorax of individuals (bisected laterally) and 80µl of the gut solution described above, using Trizol© (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction. Briefly, samples were homogenised with glass beads in 1.3ml Trizol© in a tissue-lyser. RNA was separated using bromo-chloropropane and precipitated in isopropanol. The RNA was washed with 75% ethanol and re-suspended in 400µl diethylpyrocarbonate (DEPC)-treated water. We converted 2µl of RNA into first-strand cDNA using GoScript™ Reverse Transcriptase, according to the manufacturer's instructions (Promega), using random hexamer primers and RNasin® to prevent RNA degradation. Honeybee RNA was eluted in 100µl of RNase-free water and bumblebees in 400µl to allow for variation in RNA pellet size between the species.

DNA extraction

DNA was extracted from the gut solution described above using a modified Chelex® method: 100µl of 10% Chelex® 100 resin (Biorad) solution was added to 35µl of gut solution, with 2µl of Proteinase K (20µg/µl) before incubation at 56°C for one hour, and vortexed twice during this time. Samples were incubated at 95°C for 15 minutes, centrifuged and the supernatant was stored at -20°C until use.

Prevalence

We differentiated between the *B. terrestris/lucorum* species complex via a DNA length polymorphism in the mitochondrial IGS region, using the primer pair BBMI_IGSF1 and BBM1_IGSR1 (*pers comm* Regula Schmid-Hempel, table 4:1). To determine pathogen prevalence of DWV-A, DWV-B and *N. ceranae*, cDNA (for viral detection) and DNA (for *N. ceranae* detection) were diluted 1:10 prior to PCR. We used previously published primer pairs to identify positive samples by PCR and carried out PCR in 20µl reactions using GoTaq® DNA Polymerase, with PCR programs specific to each primer pair (table 4:1). Every run included a known positive sample and a water negative as controls. 5µl of PCR product were run on 1.5% TAE agarose gel with ethidium bromide nucleic acid staining solution.

Table 4-1: Primers and protocols used to differentiate the *B. terrestris/lucorum* complex, and detect the prevalence of pathogens deformed wing virus (type A and B) and *Nosema ceranae*

Target	Primer name	Sequence	Amplification program	Amplicon (bp)	Reference
B. terrestris/ B. lucorum complex	BBM1IGSF BBM1IGSR	GGAGCAATAATTTCAATAAATAG AARTTCAAAGCACTAATCTGC	15 s at 95C 15 s at 55C 45 s at 72C X38 cycles	180 210	Regular Schmid-Hempel (*pers comm*)
DWV-A DWV-B	DWV F1a VDV F1a DWV-VDV 7aR	GGAAACATCTGGAATTAGCGACAA GAAAACATTTGGAATTAGCAACGAC AATCCGTGAATATAGTGTGAGG	10 s at 95C, 30 s at 60C, 30 s at 72C X35 cycles	360 360	McMahon et al. (2015)
N. ceranae (Multiplex PCR differentiates between three species of *Nosema*)	Mnapis_F MnBombi-F Mnceranae_F Mnuniv_R	GCATGTCTTTGACGTACTATG TTTATTTTATGTRYACMGCAG CGTTAAAGTGTAGATAAGATGTT GACTTAGGAGTAGCCGTCTCTC	30 s at 95C 30 s at 56C 60 s at 72C X35 cycles	224 171 143	Fries et al. (2013)

Quantitation of viral load by reverse transcription-PCR (qRT-PCR)

All DWV-A positive samples were analysed by qRT-PCR (N = 94). For DWV-B, the sample size was much larger, thus we randomly chose ten positive samples per site per species (or total number if there were less than ten positives available) for qRT-PCR analysis (N = 184). We measured nucleic acid quality (Nanodrop™ 2000 spectrophotometer) and concentration (Qbit™ Fluorometer) for each individual. No samples were excluded for poor quality as all Nanodrop 280/260 ratios were greater than 1.8. Based on Qubit concentrations, each RNA sample was diluted to 100ng/µl in RNase free water. cDNA was synthesised from 400ng of RNA template using GoScript™ Reverse Transcriptase, as reported above. cDNA was then diluted 1:10 prior to use in qPCR. For absolute quantification, duplicate reactions were performed for each sample on a Strategene machine (Mx3005P) using GoTaq® qPCR Master mix for dye-based detection (Promega) on the following program: 2 mins at 95 °C, followed by 40 cycles of 10s at 95 °C, and data collection for 60s at 57°C. We used virus specific primers for the detection DWV-A and DWV–B, and the internal reference marker Rp49 was amplified in duplicate for all *A. mellifera* samples (table 4:2). Two no-template negative samples containing RNase-free water were run on each plate. No virus detection threshold was set because only samples already identified as positive by PCR were used in these reactions. Quantification was calculated using duplicate eight-point standard curves of plasmid DNA in a 1:10 serial dilution on each plate. DWV-A and DWV-B plasmids were generated using Promega pGEM®-T Easy Vector, according to the manufacturer's

instructions, to clone a 360bp fragment and 89bp fragment of the *rdrp*-gene in DWV-A and –-B, respectively, from purified PCR products (primer details in table 4:2). Successful transformants were selected via blue/white screening, and plasmids were extracted using GeneJET Plasmid Miniprep Kit (ThermoFisher). M13 primers (designed to sequence inserts inside pGEM®-T Easy Vector: forward 5´- GTTTTCCCAGTCACGAC -3´, reverse 5´- CAGGAAACAGCTATGAC -3´) were used to determine the correct product had been cloned. We linearlised the plasmids using the restriction enzyme Apa 1 (New England Biolabs), according to the manufacturer's instructions, and diluted them 1:1000 with RNase-free water. Mean efficiency across plates for DWV-A was 90.6% (four plates with range 89.2 - 92.1%) and DWV-B was 92.2% (seven plates ranging from 90.8 - 92.2%) with $R^2 > 0.98$ across assays (for standard curves see figure S2).

Table 4-2: Primers and protocols used for qPCR amplification and to create plasmids for standard curves.

Primer	Sequence	Amplification program	Reference
DWV_F2*	TGTCTTCATTAAAGCCACCTGGAA	10 s at 95C,	McMahon et al. (2015)
DWV_R2a*	TTTCCTCATTAACTGTGTCGTTGAT		
DWV_F1a **	GGAAACATCTGGAATTAGCGACAAA	30 s at 60C,	
DWV_7Ra**	AATCCGTGAATATAGTGTGAGG		
VDV_F2	TATCTTCATTAAAACCGCCAGGCT	X40 cycles	
VDV_R2a	CTTCCTCATTAACTGAGTTGTTGTC		
Rp49_qF	AAGTTCATTCGTCACCAGAG	Melting curve profile: 55 – 95C (0.5C per second increments)	de Miranda and Fries (2008)
Rp49_qB	CTTCCAGTTCCTTGACATTATG		

Note * primers were used for DWV-A qPCR assays, while ** primers were used to create the DWV-A plasmid.

Nosema spore counts

For all samples positive for *N. ceranae* by PCR (above), we diluted the gut homogenate 1:10 and counted spores on a hemocytometer (Immune Systems FastRead 102) under 40x magnification (Motic BA300 phase contrast light microscope). Each sample was first scanned for four minutes for the presence of spores; if a single spore was detected in this time then spores were counted across eight small squares of the counting chamber.

Sanger sequencing

DWV-A and DWV-B have positive, single-stranded monocistronic RNA genomes comprising 10,140 (Lanzi et al. 2006) and 10,112 (Ongus et al. 2004) nucleotides, respectively, which have a nucleotide similarity of 84% (Mordecai et al 2015). Both viruses

are consigned to the Iflavirus family and contain a single open-reading-frame (Lanzi et al. 2006; Ongus et al. 2004). Following Wilfert et al. (2016), all individuals identified as positive for DWV-A by PCR (as detailed above) were assayed by PCR for four genomic regions; L-protein, *vp3*, *helicase* and RNA-dependent RNA polymerase (*Rdrp*) (table S2a). We designed four additional primer pairs to sequence the same regions of all samples positive for DWV-B (table S2b). 15µl of PCR product were purified using Exonuclease 1 and Antarctic phosphatase (NEB) incubated at 37°C for 60 minutes and denatured at 80°C for 20 mins. Big Dye Terminator v3.1 (ThermoFisher Scientific) was used for florescence-based direct sequencing of amplicons, following manufacturer's instructions. The Big Dye PCR products were purified by filtering through Sephadex® G-50 (Sigma-Aldrich) and directly sequenced in the reverse direction (except for *helicase* DWV-B samples, which were sequenced in the forward direction) on an ABI 3730 Genetic Analyser using the appropriate PCR primer (table S2). Sequencing direction was chosen to maximise the overlap between DWV-A and -B sequences.

Single-molecule real-time (SMRT) sequencing (Pacbio)

The concentration of individual RNA samples were measured using a Qbit™ Fluorometer, and 1000ng of RNA from each of 30 *A. mellifera* and 30 *B. terrestris* from two *Varroa*-free sites (Ushant and the Isle of Man) and their paired *Varroa*-present mainland sites (Le Conquet and Liverpool) were pooled to create eight populations for SMRT sequencing. Note: *B.terrestris* was rare on Ushant, thus only 13 individuals were collected and used in this pool. The individual RNA samples were quality checked with the Nanodrop™ 2000 spectrophotometer (all samples had 280/260 ratio >1.8), and the pooled RNA run on Agilent 2200 Tapestation (pools all had high RIN values (>9), except for Liverpool honeybees with RIN=7.6). Full-length cDNA libraries (using the BluePippin System) were prepared using the Clontech SMARTer PCR cDNA Synthesis Kit. Following this, the PacBio Template Prep Kit was used to generate SMRTbell™ libraries, which were then sequenced on the PacBio System (library prep and sequencing was carried out by the Exeter Sequencing Service). Non-chimeric reads from each pool were mapped first against their respective host species genomes using BWA (Li and Durbin 2009) (v. 0.7.12) with the following parameters: "bwa mem -x pacbio" to remove host-derived sequences. Remaining reads were mapped against all sequenced bee RNA viruses and 23 novel bumblebee viruses (Pascall et al, *pers comm*) (table S3). Reads mapping to the genomes of DWV-A, B and C were then extracted for further analysis. To identify recombinants, reads mapping to known DWV variants from each pool

were fragmented into 100bp windows with a 50bp overlap (windows of 200-2000nt were also tested, but resulted in reduced sensitivity in the detection of synthetic *in silico* recombinants). Windows were then mapped back to known DWV genomes using bbmap to identify the best mapping for each window. Putative recombinants were identified when three consecutive windows (150bp) aligned to a different variant compared to the rest of the windows from the parent read. Further, the presence of unknown DWV variants was investigated by aligning windows against the genomes of DWV-A, DWV-B and DWV-C separately using BLASTN (Camacho et al. 2008) and recording the percentage identity of the best alignment (with a minimum cut-off of 70%) against each of the genomes. Percentage identity of the parent read to each of the DWV genomes was then calculated as the mean percentage identity of its windows.

Statistics

All statistical analyses were carried out in RStudio (v0.99.896). Note, *B. lucorum* samples were excluded from prevalence and viral load analyses because of low sample size. True prevalence with 95% confidence intervals were calculated to account for assay efficiency and sensitivity, which was conservatively set at 95% (Reiczigel et al. 2010) using R library epiR v.0.9-82 and the function epi.prev. Within the package, confidence intervals are calculated based on methods in Blaker (2000).

To examine if disease prevalence was affected by *Varroa*-presence, we used generalised linear mixed models (GLMMs) (with DWV-complex, DWV-A, DWV-B and *N. ceranae* prevalence tested in four separate models) with binomial error distribution and logit link function, using the lme4 package (v1.1-12) (Bates and Sarkar 2006). Full models included three-way interactions between the fixed effects *Varroa*-presence, species (a factor with three levels: *A. mellifera, B. terrestris and B. pascuorum*) and island/mainland location, with latitude and sunshine hour duration as additional fixed effects; field site and individual were included as random effects (individual was added to account for over-dispersion in the model (Harrison 2014)). Sunshine hours provided a proxy for favourable disease transmission conditions (Fürst et al. 2014) and were calculated as the mean sunshine hours from monthly data between March and July 2015 collected from MET office data (*pers comms*) and Meteo France (http://www.meteofrance.com/climat/france). The minimum adequate model (MAM) was found through removal of non-significant terms and comparison of models using anova – if the simplified model was not significantly different at $p > 0.05$ the term was removed from

the model. To test the full effect of our test predictors we compared the MAM with the null model (which only included random effects) using anova. Residual plots were examined to assess model fit.

Varroa-free refugia only exist on islands, thus it was necessary to also sample on *Varroa*-present islands, as well as paired *Varroa*-present mainland sites, to test a possible island effect on disease prevalence. Further, we ran models on reduced datasets 1) comparing island sites with and without *Varroa*, and 2) comparing *Varroa* present islands and mainland sites. If the island/mainland effect is not a confounding factor, in 1) we expect to maintain a significant effect of *Varroa*, and in 2) we expect island to remain a non-significant effect.

To investigate if viral load was affected by *Varroa*-presence, we ran GLMMs (DWV-A and DWV-B were tested in separate models) with Gamma error distribution and reciprocal link function. Viral load data varied across orders of magnitude from 10^3 to 10^{10}, thus these data were log transformed. Full models, model simplification and testing model fit were carried out as described above.

Alignments

Sequences were individually manually inspected in Geneious® (v.6.8); only high-quality non-heterozygous sequences (less than three ambiguous base pairs) of a minimum length were included in further sequence analysis. Not all fragments from all samples were amplified successfully (of the 94 DWV-A positive samples $N_{lp} = 11$, $N_{vp3} = 8$, $N_{helicase} = 19$, $N_{rdrp} = 8$: and of 294 DWV-B positives $N_{lp} = 117$, $N_{vp3} = 200$, $N_{helicase} = 146$, $N_{rdrp} = 147$). Thus distinct datasets were used for each fragment, optimising information by maximising the alignment length whilst keeping as many samples as possible (table 4:3). Alignments were created using Geneious® (v.6.8) by mapping the sequences to DWV-A and DWV-B reference sequences (NC_004830 and NC-006494), assigning each sequence to a viral strain. We created an alignment for each fragment for DWV-A and –B sequences combined, and for DWV-B alone (table 4:3). Note there were too few DWV-A sequences to focus on this variant alone. In addition, to maximise the genetic information, we concatenated the DWV-B sequences from all samples that amplified across all four fragments (N = 67), as well as across three fragments excluding the *lp*-gene (N = 103) to increase the number of samples from bumblebee hosts (table 4:3) (using Fasta Alignment Joiner (http://users-birc.au.dk/biopv/php/fabox/alignment_joiner.php). To confirm that there was no

recombination within fragments at a p value of 0.05, we used the GENECONV (Padidam et al. 1999), MaxChi (Maynard Smith 1992), BootScan (Martin et al. 2005) and SiScan (Gibbs et al. 2000) algorithms in the Rdp4 package (v4.56) (Martin et al. 2015). Only the *lp*-gene fragment contained recombinants (N = 13), and these were removed for phylogenetic analysis. We found two points of recombination within the *lp* region: one at ~1550 and one ~1660, with both DWV-A and DWV-B seen at the 5' and 3' end of the sequenced fragment.

Table 4-3: Alignments details: number of sequences and alignment length, genetic diversity (π), number of segregating sites and parsimony informative sites, and the number of sequences from each host species (*A. mellifera, B. terrestris* and *B. pascuorum*).

Note, recombinant *lp* samples were excluded from alignments.

Fragment (viral spp.)	N. seqs	Length (bp)	π	N. seg. sites	N. parsimony inf. sites	N. *A.mel*	N *B.ter*	N. *B.pas*
lp (A&B)	128	132	0.058	75	73	108	17	3
lp (B)	117	329	0.0083	42	21	98	16	3
vp3 (A&B)	208	209	0.01	40	23	139	51	18
vp3 (B)	200	209	0.00015	21	2	135	48	17
helicase (A&B)	165	214	0.057	60	52	128	32	5
helicase (B)	146	214	0.00018	12	5	110	31	5
rdrp (A&B)	155	176	0.018	38	30	130	22	3
rdrp (B)	147	176	0.0023	17	4	122	22	3
Concat-4 (B)	67	906	0.0042	60	27	62	5	0
Concat-3 (B)	102	617	0.0022	38	8	87	14	1

Phylogenetic reconstruction: model selection, evolutionary rates and migration routes

For individual and concatenated DWV-B fragment alignments (table 4:3), we used Jmodeltest (v.2.1) (Darriba et al. 2012; Guindon and Gascuel 2003) to compare evolutionary substitution models based on the Bayesian Information Criterion (Alizon and Fraser 2013) (table S4). In Beast 1.8, we partitioned substitution rates between the 1^{st}, 2^{nd} and 3^{rd} codon positions. For the concatenated alignments we fit discrete trait models with asymmetric substitution models for host species and geographic location (note, there was insufficient genetic information in the individual fragment alignments to include traits). The evolutionary rate prior was set at 1.35×10^{-3} changes per site per year (95% HPD 5.41×10^{-4} - 2.63×10^{-3}), with a lognormal distribution and standard deviation of 0.4 (Mordecai et al. 2015) to estimate time since divergence from a common ancestor. This rate was chosen as there was little temporal information in the currently available DWV-B sequences; only the *lp*-fragment

showed a weak temporal signal (correlation coefficient with best fitting root 0.27, $R_2 = 0.07$) based on a MrBayes-generated phylogenetic tree analysed in TempEst (Rambaut et al. 2016). There was no signal for the *vp3*- or *rdrp*-fragments (correlation coefficients -0.12 and -0.19, $R_2 = 0.012$ and 0.0035 respectively). For the *lp*-fragment, we estimated the evolutionary clock using a lognormal relaxed clock and an exponential growth prior, based on our standard model selection procedure. We used the SRD06 model of sequence evolution (Shapiro et al. 2006). Based on this procedure, the clock rate for DWV-B was estimated as 1.99×10^{-3} (95% HPD 4.58×10^{-4} to 4.07×10^{3}); this overlaps with the 95% HPD of the mean DWV-A clock mean so we therefore chose to use the better supported prior previously estimated from DWV-A (Mordecai et al. 2015). We ran and compared nine models per fragment with different demography and molecular clock rates, and used path sampling maximum likelihood estimator, implemented in Beast 1.8, to determine the best model (table S4). All models were run long enough to obtain effective sample sizes >200 for all parameters, with a 10% burn in of MCMC generations, sampling the chain at equal distances to obtain a total of 10,000 trees per analysis. By analysing the output in Tracer (v1.6) we checked models for convergence, determined if a strict clock could be excluded or not by examining the relaxed lognormal clock's coefficient of variation statistic (if it shoulders the zero boundary a strict clock cannot be excluded), and for models with an exponential growth prior, we determined if there was significant exponential growth (Drummond and Bouckaert 2015). We produced Maximum Clade Credibility (MCC) trees (TreeAnnotator (v1.8.4)) to infer host ancestral state probabilities. Phylogenetic trees were also produced for each alignment using MrBayes 3.2.6. For the concatenated data sets, well supported rates for migration routes between host species and sites were identified using a Bayes factors analysis (SPREAD v1.0.6 (Bielejec et al. 2011)), with a Bayes Factor of 3 as a cut-off.

Population genetics measures

For DWV-B concatenated fragment (including *lp*-gene), a median joining phylogenetic networks was produced using PopArt (v.1.7) (http://popart.otago.ac.nz). Using DNASPv5.10.1 (Librardo and Rozas 2009) we calculated Kst, a measure of population differentiation based on the proportion of between-population nucleotide differences (Hudson et al. 1992) and the nearest neighbour statistic SNN, which calculates the proportion at which the genetic nearest neighbours are found within same population (Hudson 2000).

Results

Prevalence

We analysed the prevalence of DWV-A, DWV-B and *N. ceranae* in 355 *A. mellifera*, 640 *Bombus terrestris* and 280 *B. pascuorum* across 12 locations, illustrated in figure 4:1. We find that *Varroa* presence is a significant predictor of prevalence of DWV-complex for all bee species, predicting a ~10 fold increase in *A. mellifera* and ~15 fold increase in both *B. terrestris* and *B. pascuorum* (table S5) (GLMM: estimate ± s.e. of the fixed factor '*Varroa* presence' in the model = 3.06 ±1.02, p = 0.003, table 4:4). There was no evidence that honeybees and bumblebee species respond differently to *Varroa* presence, as the interaction between species and *Varroa* did not contribute to the model fit (anova: $\chi^2 = 4.25_2$, p = 0.119). Importantly, given that all *Varroa*-free sites were islands, there was no evidence that island/mainland location influenced DWV prevalence (anova: $\chi^2 = 4.25_2$, p = 0.12). [To further confirm that *Varroa* presence, rather than an island effect, explained DWV prevalence, we ran the model on a dataset excluding *Varroa*-free sites and with island as an explanatory model: there was no significant difference in DWV prevalence on *Varroa*-present islands compared to *Varroa*-present mainland (estimate ± s.e. of the fixed factor 'island/mainland' in the model = 0.51 ±0.89, p=0.57). In addition, we ran a model on a dataset including only island sites; here *Varroa* presence remained a significant explanatory factor of DWV prevalence, with higher prevalence on *Varroa*-present islands compared to *Varroa*-free islands (estimate ± s.e. = 3.06 ±1.03, p < 0.001)]. Our full model for DWV prevalence fitted the data significantly better than the null model with only random factors included (anova: $\chi^2 = 25.49_3$, p < 0.001). Examining the DWV variants individually, *Varroa* presence influences DWV-B prevalence (estimate ± s.e. = 3.16 ±1.11, p = 0.004, table 4:4); DWV-A was notably absent from samples collected from all *Varroa*-free sites (save for one *B. terrestris* from the Isle of Man): in a GLMM without *Varroa* as a predictor, sunshine hours was a positive significant predictor of DWV-A (estimate ± s.e. = 8.71 ± 2.82, p = 0.022) (table 4:4). Coinfection rates of DWV-A and -B are also influenced by *Varroa* presence ($\chi^2 = 48.61_2$, p < 0.001), linked to low DWV-A presence in *Varroa*-present sites only.

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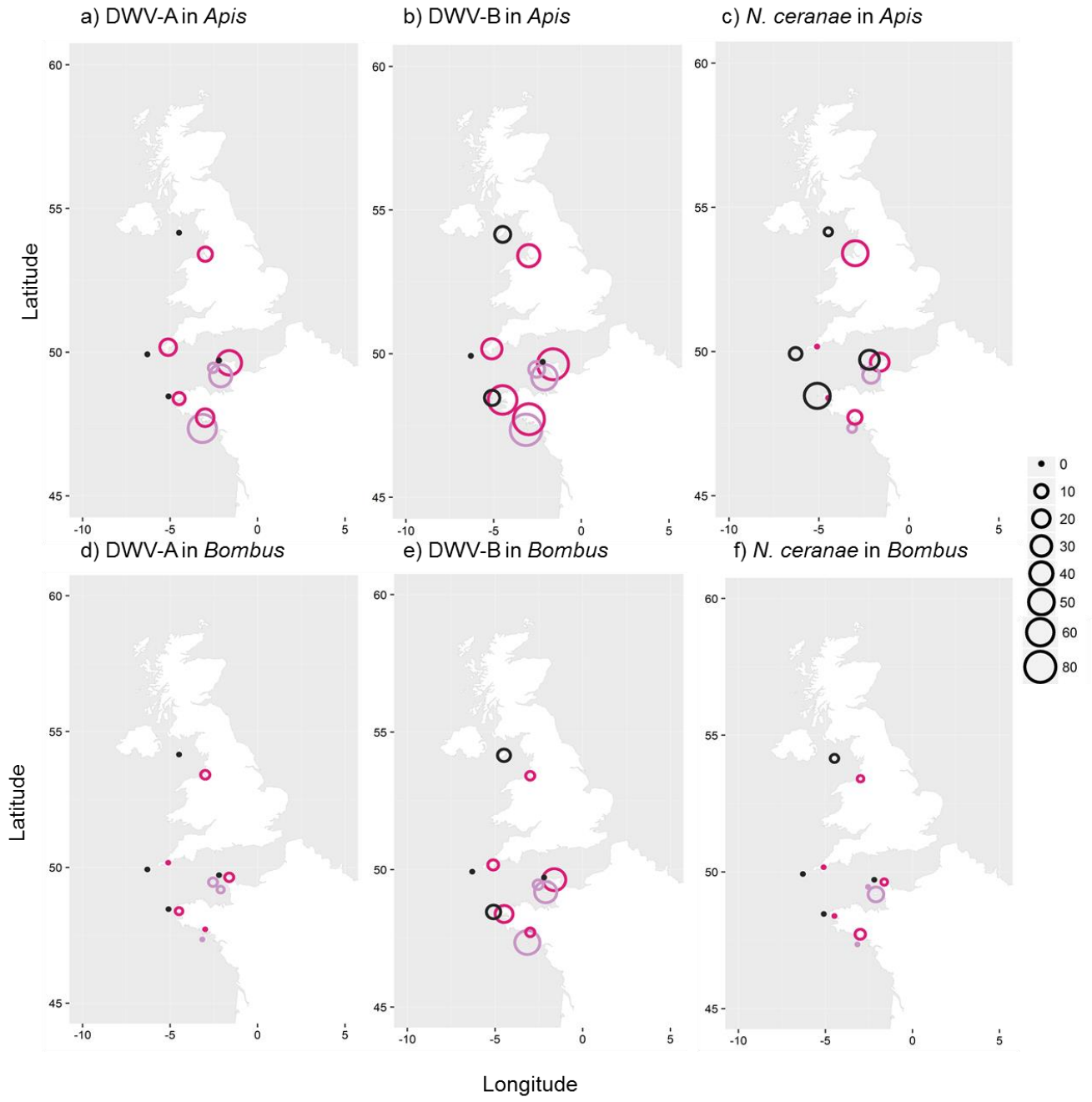


Figure 4:1: Prevalence data mapped by pollinator genus (*Apis* a-c, *Bombus* d-f), location and *Varroa* presence/absence. *Varroa*-free sites are black, *Varroa*-present islands are light purple, and *Varroa*-present mainland sites are fuchsia pink.

Table 4-4: Best model explaining pathogen prevalence using GLMMs with binomial error structure and logit function

Pathogen (response)	Parameters (predictors)	Estimate	SE	z-value	P-value
DWV-complex prevalence	Intercept	-2.46	0.88	-2.77	0.0056
	B. pascuorum	-2.32	0.39	-6.04	<0.001
	B.terrestris	-2.38	0.35	-6.88	<0.001
	Varroa presence	3.06	1.02	2.97	0.0030
DWV-A	Intercept	-1.10	0.39	-2.81	0.0050
	B. pascuorum	-4.64	1.10	-4.24	<0.001
	B.terrestris	-4.36	0.7	-6.23	<0.001
	Log sunshine hours duration	8.73	2.82	2.29	0.022
DWV-B	Intercept	-2.49	0.87	-2.86	0.0042
	B. pascuorum	-2.22	0.38	-5.19	<0.001
	B.terrestris	-2.38	0.35	-6.89	<0.001
	Varroa presence	2.96	1.01	2.95	0.0032
N. ceranae	Intercept	-2.35	0.64	-3.70	<0.001
	B. pascuorum	-3.31	0.99	-3.33	<0.001
	B.terrestris	-2.97	0.81	-3.68	<0.001
	Varroa presence	-0.33	1.17	-0.28	0.78

There were differences in DWV prevalence between honeybee and bumblebee hosts in association with *Varroa* presence, which are shown in figure 4:2 and illustrated in figure 4:1. In tests of proportions, both DWV-A and DWV-B were significantly less prevalent in *A. mellifera* on *Varroa*-free islands compared to *Varroa*-present sites (including island and mainland sites) ($\chi^2 = 56.98_2$, $p < 0.0001$ and $\chi^2=93.17_2$, $p < 0.0001$, respectively) (figure 4:2a&b). Prevalence of DWV-A was equally low in *B. terrestris* and *B. pascuorum* across all sites ($\chi^2 = 2.77_2$, $p = 0.25$; $\chi^2 = 1.58$, $p = 0.45$) (figure 4:2a), but prevalence of DWV-B was significantly lower in *Varroa*-free sites for both *B. terrestris* and *B. pascuorum* ($\chi^2 = 60.84$, $p < 0.001$ and $\chi^2 = 12.04_2$, $p = 0.002$, respectively) (figure 4:2b).

In contrast, the prevalence of *N. ceranae* – for which there is no evidence that *Varroa* effects transmission - was unaffected by the presence of *Varroa* (GLMM estimate ± s.e. = -0.33 ±1.17, $p = 0.78$, table 4:4) as shown in figure 4:2c and illustrated in figure 4:1. There was also no evidence that island/mainland location influenced *N. ceranae* prevalence (anova: $\chi^2 = 1.04_1$, $p = 0.31$). *N. ceranae* prevalence was low generally; but there was a significant difference between species, with prevalence in *A. mellifera* more than 6 times higher than *B. terrestris* and *B. pascuorum* (table S5, illustrated in figure 4:1).

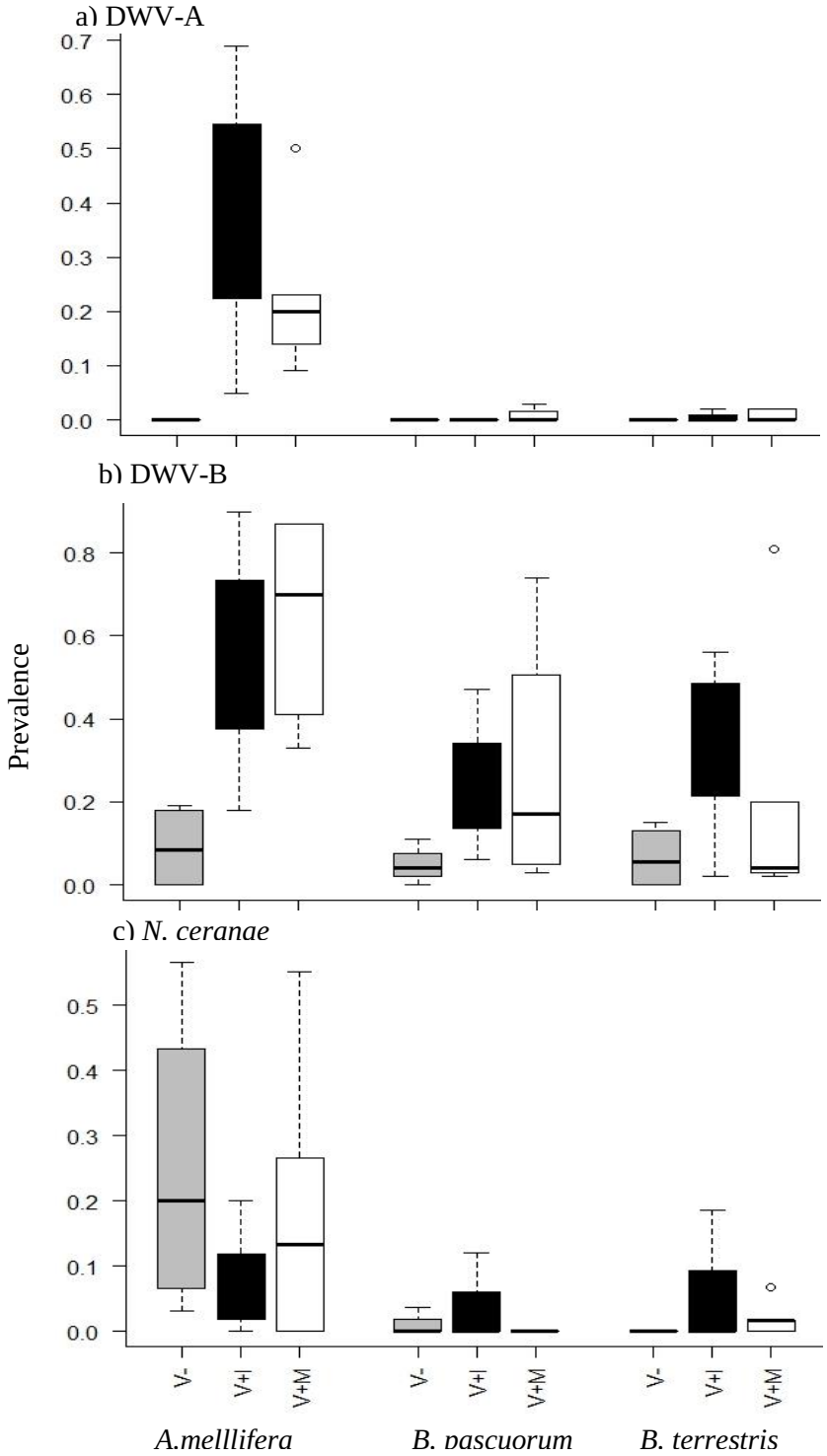


Figure 4:2: Prevalence (with 95% confidence intervals) of Deformed wing virus (DWV) type A (a) and type B (b), and *Nosema ceranae* (c) by host species and *Varroa*-free (V-, grey), *Varroa*-present islands (V+I, black) and *Varroa*-present mainland sites (V+M, white).

Viral load

We quantified DWV-A and DWV-B from positive bees and detected a wide range of viral loads, from $10^3 – 10^{10}$, for both *A. mellifera* and *Bombus* (figure 4:3a and b). There was a significant difference in DWV-B viral load between *Varroa*-free islands and *Varroa*-present sites (both mainland and islands) (two-sided Kolmogorov-Smirnov, $D = 0.697$, $p < 0.001$ and $D = 0.70$, $p < 0.001$, respectively) with low level infections ($10^2 – 10^4$ virus particles) found in *Varroa*-free sites compared to high level infections in *Varroa*-present sites (10^3 -10^{10}) (figure 4:3b): there was no difference in DWV-B load between mainland and island *Varroa*-present sites ($D = 0.11$, $p = 0.85$). It is clear from GLMMs that *Varroa* presence is a significant driver of DWV-B viral load (estimate ± s.e. of the fixed factor '*Varroa* presence' in the model = -0.089 ± 0.026, $p < 0.001$). For *A. mellifera* the mean DWV-B viral load is 1.5×10^4 on *Varroa*-free sites, compared to 4.2×10^6 in *Varroa*-present sites, thus *Varroa* presence drives a predicted increase in mean viral load by two orders of magnitude. DWV-B viral load is also significantly lower in *B. terrestris* and *B. pascuorum* in *Varroa*-free sites (estimate ± s.e. of the fixed factors '*B. terrestris*' and '*B. pascuorum*' in the model = 0.04 ± 0.01, $p < 0.001$; 0.06 ± 0.002, $p < 0.01$, respectively) and *Varroa* presence significantly increases the predicted mean DWV-B viral load in these bumblebee species from $3.7 \times 10^3 – 1.7 \times 10^5$ and $2.4 \times 10^3 – 7.1 \times 10^4$, respectively. Sample size for DWV-A from *Varroa*-free sites was too low to estimate the effect of *Varroa* on DWV-A load; however, there was no difference in DWV-A load between *Varroa*-present mainland and islands (KS test: $D = 0.14$, $p = 0.87$). In a GLMM, DWV-A loads are significantly higher in *A. mellifera* compared to *B. terrestris* and *B. pascuorum* (estimate ± s.e. = 0.22 ± 0.023, $p < 0.001$). In both DWV-A and DWV-B models, island/mainland location did not contribute to the model fit and was removed from the models (anova: $\chi^2 = 0.13_1$, $p = 0.72$, $\chi^2 = 0.0948_1$, $p = 0.76$, respectively).

We found no difference in *N. ceranae* spore load between *Varroa*-present islands (mean = 78.18 spores (N = 23)) and *Varroa*-free islands (mean = 81.9 spores (N = 31)) (KS-test: $D = 0.36$, $p = 0.061$) or between *Varroa*-present mainland sites (mean = 180.18 spores (N=34) and *Varroa*-free islands ($D = 0.18$, $p = 0.69$) (figure 4:3c).

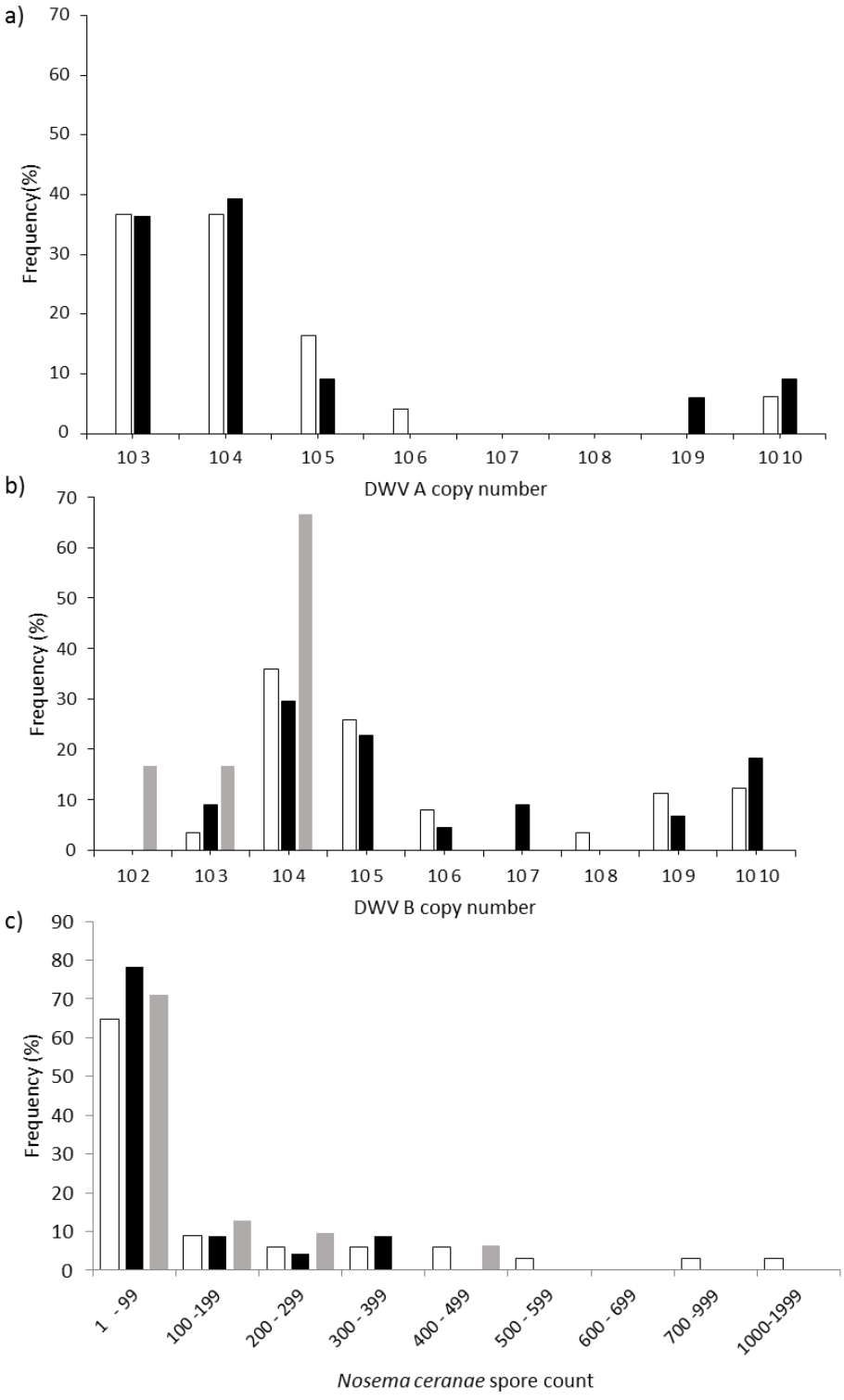


Figure 4:3: % frequency of pathogen load: DWV-A (a), DWV-B (b) and *Nosema ceranae* (c) on *Varroa*-free islands (grey), *Varroa*-present islands (black) and *Varroa*-present mainland sites (white).

Virus population genetics and phylodynamics

DWV–B was the most prevalent species, and all samples that tested positive for DWV–A were co-infected with DWV-B. While the prevalence primers (table 4:1) are species-specific (McMahon et al. 2016), the sequencing primers (table S2) are not, thus the majority of co-infected individuals were sequenced as DWV-B likely reflecting its dominance within co-infected individuals. Consequently, we focus on DWV-B for this analysis. We find that DWV-B populations for each fragment are not structured by host species (note, a Kst value nearing 1 indicates strong population differentiation: Kst_{lp} = 0.00017, Kst_{vp3} = 0.0021, $Kst_{helicase}$ = 0.0016, Kst_{rdrp} = 0.002; $p > 0.05$ for all fragments). However, when all four fragments are concatenated together there is an extremely modest indication of host differentiation (Kst = 0.016, p = 0.015) (note, this fragment only contains five bumblebees sequences, and this significance does not hold for the concatenated alignment without the *lp*-gene, which contains 15 bumblebee sequences (table 4:3) (Kst = 0.0015, p = 0.26)). There is evidence of moderate geographic population differentiation for all fragments (table S6a) supported by the concatenated fragment (Kst = 0.303, $p < 0.001$). Samples that are genetic nearest neighbours often come from the same population (Snn = 0.30; $p < 0.01$). We tested the effect of *Varroa* presence on the *vp3* fragment, the only alignment containing multiple sequences from *Varroa*-free sites, but there was no evidence for sequence differentiation due to *Varroa* presence (Kst = 0.0008, p = 0.58). These results hold for individual fragment alignments containing both DWV-A and DWV-B sequences combined (table S6b).

The low genetic diversity of the DWV-B phylogeny ($\pi = 0.0042$, with 60 polymorphic sites out of 906 sites of the concatenated fragment, examined over 67 sequences) suggests a recent bottleneck and subsequent exponential expansion, with the sequences forming a star-shaped network as expected following exponential expansion (figure 4.4). This result is supported by phylogenies for the concatenated fragment (figure 4:5) and each individual fragment (figure S3). The low levels of population structure enabled us to combine sequences from across populations to investigate the past demography of the virus. We found a large excess of rare variants compared with the neutral model, suggestive of an expanding population after a bottleneck (Tajima's D for DWV-B concatenated fragment = -2.4, p = 0.002). This is supported for each individual fragment across populations (Tajima's D for *lp* = -2.14, *vp3* = -2.5, *helicase* = -2.16, *rdrp* = -2.34; $p < 0.01$).

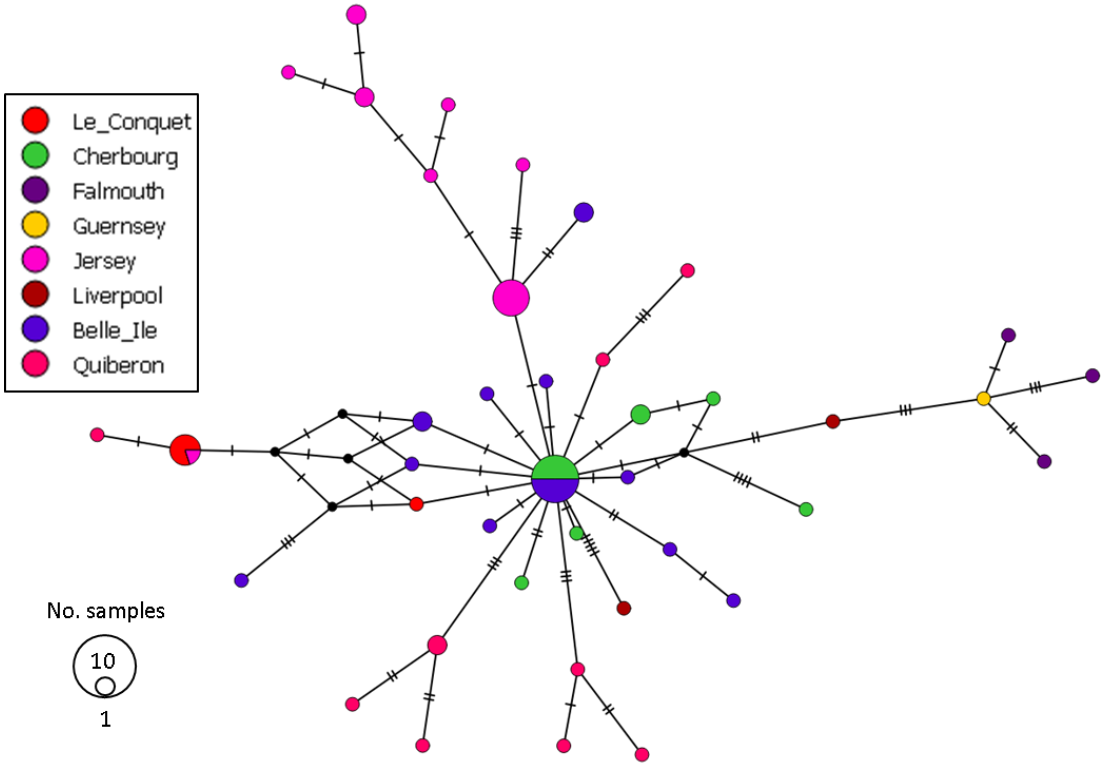


Figure 4:4: Median joining phylogenetic network of sequences from Deformed wing virus-B (N = 67). The colours represent sampling location, the size of the node represents the number of samples with the same sequence and the dashes on branches show the number of mutations between nodes.

To reconstruct past changes in the DWV-B populations we ran the concatenated fragments (both with and without the *lp*-gene fragment) in Beast 1.8 to estimate the routes of transmission by comparing geographic and host-specific patterns dated via the mean viral evolutionary rate (1.35×10^{-3} mutations per site per year, as discussed in the methods). We note that the MCC trees had low posterior support (the fragment with the *lp*-gene had high posterior support (>0.8) for one major clade, but <0.6 for another (figure S4) likely due to extremely low genetic variation across DWV-B sequences (note, the shorter fragment without the *lp*-gene had very poor posterior support at less than 0.5, thus this model was discarded). The most recent common ancestor for the concatenated fragment dates back to within this decade (mean root height for concatenated fragment (with *lp*-gene) = 4.46 (95% HPD 1.56 - 8.17), which corroborates findings for individual fragments (table S7). All DWV-B individual and concatenated fragments showed exponential growth, with doubling rates estimated to be less than a year (doubling rate for concatenated fragments (with *lp*-gene) =

0.7 years (95% HPD 0.38 – 2.28). These data, population genetics (figure 4:4) and the phylogeny (figure 4:5) suggest a recent bottleneck and subsequent exponential expansion of DWV-B. However, although the ancestral host was identified as *A. mellifera* in both concatenated phylogenetic reconstructions (state probability $P_{concatenated}$ = 99.9%), we found no support for transmission routes between host species (*A. mellifera* and *B. terrestris*) in either direction. There is strong support for DWV-B reciprocal transmission between geographic locations (table S8, figure S5). However, there is no obvious pattern - for example, locations that are closer together do not necessarily have the strongest support.

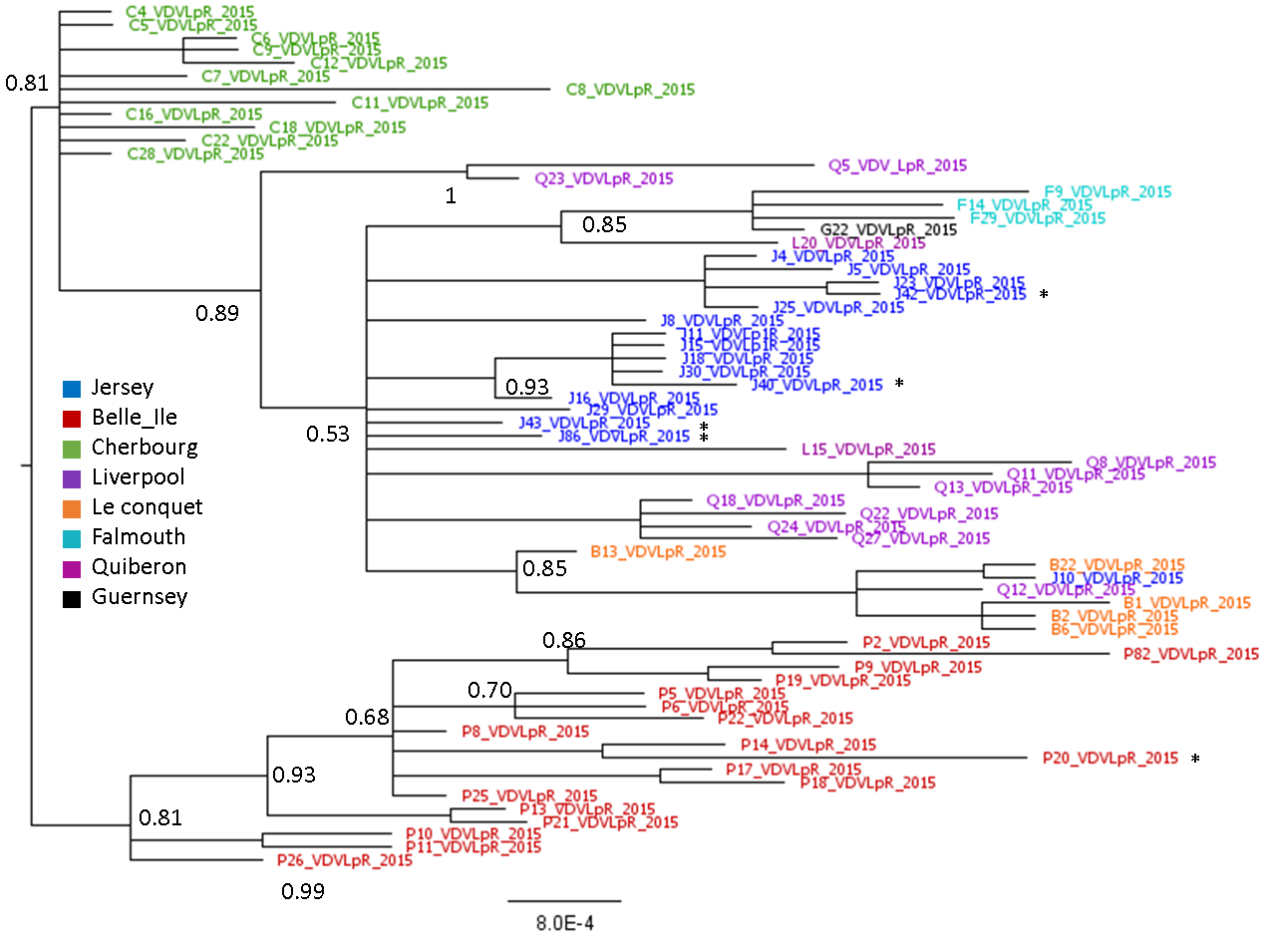


Figure 4:5: MrBayes tree showing phylogenetic relationships of DWV-B sequences isolated from *A. mellifera* and B. *terrestris* (sequences comprised of four concatenated fragments of DWV-B; regions of the *lp-*, *vp3-*, *helicase-* and *rdrp*-gene fragments, total length = 906bp). Host species are *A. mellifera* unless indicated by an asterisk to be *B. terrestris*. The tip ends are coloured by geographic location (see key). Posterior support is indicated for nodes up until the 4th node.

SMRT sequencing

Single molecule sequencing produced 31,297 non-chimeric reads greater than 1000bp long, with an average length of 4172 bp. 24,302 reads mapped to known viruses, with 20,578 reads mapping to DWV genomes. Reads mapping to DWV genomes reached a maximum of 6048bp from the 3' end, including the *helicase* and r*drp* genes but not the *vp3* and *lp* genes. DWV-B was shown to be the dominant species across both *Varroa*-present and *Varroa*-free sites, with 20,560 DWV-B reads, compared to 18 DWV-A reads across sites and zero DWV-C reads. Window analysis of DWV reads showed no evidence of either significant recombination or unknown variants. We found recombination between DWV-A and DWV-B to be rare in nature with only five potential recombinants (figure S6) within the r*drp*-gene region, found in *A. mellifera* from Liverpool, which was notably the pool with the largest population of viral sequences (N = 12,108, compared to a range of 146 viral reads (from the *B. terrestris* population in Brest) – 8703 viral reads (from the *A. mellifera* population in Brest). Similarity of reads to DWV-A, DWV-B and DWV-C showed no evidence of unknown variants with most reads placed in a large, single cluster containing reads with high similarity to both DWV-A and DWV-B (figure S7).

Discussion

We have shown that infestation of honeybees by the virus-vectoring *Varroa* mite is correlated with increased DWV prevalence and titre in sympatric wild bumblebee populations, who themselves are not parasitized by *Varroa* mites. In direct comparison, prevalence and intensity of *N. ceranae*, a microsporidian parasite not hypothesised to be vectored by *Varroa*, was unaffected by the presence of this honeybee ectoparasite. Together, our results show that *Varroa*-present honeybees are associated with a higher DWV disease prevalence and titre in wild bumblebees. This demonstrates how the introduction of a specialised vector can have community-level effects on pathogen transmission.

Data is scarce on DWV prevalence, titre and diversity preceding the arrival of *Varroa* into western honeybee populations, especially in relation to wild bees. However, low-level and apparently benign DWV infections likely circulated in *A. mellifera* (de Miranda and Genersch 2010; Genersch and Aubert 2010), suggested to be transmissible within its honeybee host via multiple routes both directly (faecal-oral and food contamination) and indirectly (sexual and transovarial) (reviewed in Manley et al. (2015)). Indeed, phylogenetic analyses suggest that the current DWV epidemic is a re-emergence of the virus driven by

anthropogenic movement of honeybees, coinciding with the invasion of a novel vector, the *Varroa* mite (Wilfert et al. 2016). The primary mechanism behind the dramatic effect of *Varroa* on DWV re-emergence is likely the increase in transmission events and titre through the direct injection of viruses into the honeybee haemolymph. It is also possible that the physical feeding activity of the mite itself (Kuster et al. 2014) or immunosuppression of the bee (Nazzi et al. 2012) could cause the increase in DWV. In addition, it has been suggested that *Varroa* drives selection on DWV leading to loss of variant diversity, and resulting in the dominance of a single master variant (Martin et al. 2012).

Vector-borne transmission places a strong selection pressure on pathogens regardless of whether a pathogen is actively or passively transmitted by the vector, because even with a mechanical mechanism of transmission, complex adaptations between vector and pathogen need to occur for transmission to take place (Gray and Banerjee 1999). Thus, pathogen evolution is constrained by selection because of the inevitable trade-offs between adaptations to the phylogenetically divergent vector and host species (Chare and Holmes 2004; Jenkins et al. 2002). For example, an amino acid substitution adaptive for DWV proliferation in the vector would likely be maladaptive to proliferation in the bee host; indeed, a study across the capsid proteins of 36 plant viruses observed increased purifying selection against amino acid change in those viruses transmitted by vectors (Chare and Holmes 2004). It is currently controversial as to whether DWV replicates in the *Varroa* mite (Erban et al. 2015; Ryabov et al. 2014), but still adaptations are likely to occur. Despite these selection pressures, DWV remains a multihost pathogen and we see community-wide effects of raised prevalence and titre amongst our wild bee populations. Given the variety of transmission pathways that will concurrently occur within a honey bee colony, it is plausible that selection on DWV by *Varroa*-transmission is diluted, thus not limiting interspecific transmission that likely occurs via flower sharing and other social behaviours (reviewed by Manley et al. (2015)).

In a comprehensive study of over 400 vector-borne plant viruses the majority of viruses showed high levels of vector-specificity coupled with a broad host range (Power and Flecker 2003). This suggests that the biological barriers to infecting a vector must be greater than those to overcome host defences and infect multiple hosts (Power 2000). This is logical given the disparate relatedness of vectors and their hosts; in this case the *Varroa* mite compared to pollinator hosts. Certainly, the immune repertoire across bee species, including social and solitary species, has been shown to be strikingly similar, which could reduce biological

barriers to infecting multiple pollinator hosts (Barribeau et al. 2015). However, evidence for co-evolution between anti-viral RNAi genes, which exhibit rapid adaptive evolution, and viruses in invertebrates, suggests selection pressures on adapting to multiple hosts likely exist (Obbard et al. 2009). We find the same viral variants circulating between hosts, yet differences in prevalence and viral load across hosts, indicating that there are few barriers to exposure and initial infection, but the host tolerances or susceptibilities exist. For DWV-A and –B we find that prevalence and titre is significantly higher in *A. mellifera* compared to both *B. terrestris* and *B. pascuorum*. Interestingly, while the two variants are equally common across honeybees, in our bumblebee hosts DWV-A is extremely rare while DWV-B is prevalent, suggesting the viral variants differ in their ability to cross host barriers.

We present compelling evidence, in line with other studies (Fürst et al. 2014; Wilfert et al. 2016), that *A. mellifera* is the ancestral and the reservoir host for DWV. Significantly higher prevalence of both DWV variants in *A. mellifera* compared to bumblebees found in this study, and others is consistent with this hypothesis. The prevalence of DWV in *A. mellifera* has been linked to prevalence in bumblebees, strongly suggesting spillover between managed honeybees and wild pollinator populations (Fürst et al. 2014). [Note, we did not find this effect, potentially because of the dominance of DWV-B in our samples that is highly prevalent in bumble bees compared to DWV-A, while in the 2011 survey by Fürst et al. (2014), DWV-B was prevalent, but not significantly different to DWV-A]. Replication of DWV in bumblebees has previously been demonstrated by detection of the negative strand, which is only present during replication for a positive-sense RNA virus (Fürst et al. 2014; Radzevičiùte et al. 2017). While we did not test for replication specifically, the high viral loads across bumblebees, specifically in DWV-B (figure S8), combined with the effect of *Varroa*-presence increasing DWV viral load in bumblebees, suggests we are detecting true DWV infections in bumblebee hosts. However, we cannot confirm if higher viral loads in bumblebee hosts are simply the result of spillover of higher viral loads also recorded in sympatric honeybees; or if the recently emerged DWV-B variant that dominates in our samples is better able to replicate to high level in bumblebee hosts. As the same strains are found in *Varroa*-free sites but at lower prevalence and titre, spillover is the likely explanation.

RNA viruses frequently recombine, which can result in phenotypic changes including increased virulence. It has been shown experimentally and in the field that recombination

occurs between DWV-A and -B (Moore et al. 2011; Ryabov et al. 2014; Wang et al. 2013; Zioni et al. 2011). Ryabov et al. (2014) suggest an association between *Varroa* transmission and a virulent recombinant DWV strain. In our populations, evidence from both SMRT and Sanger sequencing showed that while recombination occurs between DWV-A and -B, in both the *lp* gene (5' end) and *rdrp* gene (3'end), their occurrence was rare in nature. The recombination points we find differ from those determined by others (Moore et al. 2011; Ryabov et al. 2014). While the rare recombinants were found only in sites with *Varroa*, this may simply be explained by the higher prevalence and titre, especially of DWV-A, in *Varroa*-present sites; rather than *Varroa*-mediated selection as suggested by Ryabov et al. (2014). In addition, recombinants only occurred in honeybee hosts; again this likely reflects the higher prevalence of both virus variants in *A. mellifera*, rather than host specificity.

In contrast to Martin et al. (2012), we did not find greater viral diversity without *Varroa* and we found no evidence of *Varroa* causing a decrease in viral diversity. However, pathogens can spread ahead of their vector if a host can carry, replicate and transmit viruses. As our populations have lived through 20 years of *Varroa* infestation to date, we would expect to find a similar diversity of viral variants on *Varroa*-free islands due to spillover from imported infected bees over time. A surprising finding is that DWV-B is the dominant variant, rather than the globally distributed DWV-A variant implicated in the current worldwide DWV epidemic (Martin et al. 2012; Wilfert et al. 2016). We find little genetic variation across the DWV-B populations, with no population structure by host species and only modest population structure by location. Phylogenetic analyses suggest that DWV-B emerged within the last decade and expanded exponentially after this genetic bottleneck; this result is supported by a significant excess of rare mutations in these populations. Recent surveys across similar locations from 2009 (Wilfert et al. 2016), 2011 (McMahon et al. 2016) and the current 2015 data further support our data showing a recent exponential spread of DWV-B: in 2009 DWV-A dominated; in 2011 DWV-B was high but equal to that of DWV-A; and in 2015 we find DWV-B is dominant. Interspecific transmission clearly occurs because the same strains are found across all bee species. We find DWV-B to be far more prevalent than DWV-A in bumblebees across our samples. This is particularly concerning in light of McMahon et al. (2016) demonstrating in laboratory studies that DWV-B is a more virulent strain than DWV-A in *A. mellifera*, with unknown effects on *Bombus* species.

Emerging viral pathogens exert an additional pressure on already vulnerable wild bee populations. We demonstrate a clear impact of *Varroa* on disease prevalence in honeybees and also sympatric wild bumblebees, which highlights the importance of controlling the *Varroa* mite for the protection of all pollinators. Greater knowledge of the epidemiology and cross-species effects of DWV – specifically with regard to different genetic variants - will aid efforts for disease control. One controversial outcome of this research is that if honeybees are the main reservoir host and source of disease to wild bees, they need to be managed to protect wild pollinators, which could include limiting or regulating bee keeping in conservation areas.

Supplementary material

Supplementary tables

Table S1. The town and location of field site, and the total number of each bee species collected from each site. Note. *Bombus terrestris* and *B. lucorum* are cryptic and required molecular identification post-collection

Location	*Apis mellifera*	*B. terrestris*	*B. lucorum*	*B. pascuorum*
Guernsey (St Peter Port)	22	45	15	32
Jersey (St Helier)	30	59	1	33
Alderney (St Anne)	30	57	3	30
Cherbourg (marina area)	30	61	3	30
Ushant	30	13	2	29
Le Conquet, Brittany	33	59	2	19
Quiberon, Brittany	30	59	1	19
Belle Ile (Le Palais)	29	59	0	1
Penryn (University campus)	30	56	5	30
Isles of Scilly (St Mary's)	30	60	0	0
Liverpool	29	59	0	29
Isle of Man (Douglas)	32	53	5	29

Table S2. Primer sequences, protocols and amplicon product size for four genomic fragments across a) DWV–A and b) DWV-B genomes. Note for DWV-A *rdrp* primers, the genome position is given in brackets as it was not included in the name

a)

Primer name and genome position	Sequence	Amplification program 35 cycles	Amplicon (bp)	Reference
DWV–A primers				
Lp _F1153 Lp_ B1806	ATTAAAAATGGCCTTTAGTTG CTTTTCTAATTCAACTTCACC	30 s at 94C 30 s at 55C 30 s at 72C X35 cycles	653	Wilfert et al. (2016)
VP3_DWV F1 VP3_DWV B1	CCTGCTAATCAACAAGGACCTGG CAGAACCAATGTCTAACGCTAACCC	30 s at 94C 30 s at 55C 30 s at 72C X35 cycles	355	Genersch (2005)
Helicase_6285F Helicase_6693R	GAGCGTACACTATGGTCAGA GTTCACGACGCTTACTACAC	30 s at 94C 30 s at 56.6C 30 s at 72C X35 cycles	409	Berényi et al. (2007)
RdRp_F15 (9247) RdRp_B23 (9697)	TCCATCAGGTTCTCCAATAACGGA CCACCCAAATGCTAACTCTAAGCG	30 s at 94C 30 s at 49.2C 30 s at 72C X35 cycles	450	Yue and Genersch (2005)

b)

DWV–B primers				
Lp_1_1520F	AAGAAAGTGAAACGGGTGGC	30 s at 94C 30 s at 62.2C 30 s at 72C X35 cycles	437bp	This study
Lp_1_1998R	ATTAAGCGCGCCAATTCCTT			
VP3_1_3707	CAAGGACCCGGCAAAGTAAG	30 s at 94C 30 s at 63.3C 30 s at 72C X35 cycles	383bp	
VP3_1_4089	CCATCACGGCAGCGATTAAA			
Heli_1_6428	TATGCAGCAGGAATGAACGC	30 s at 94C 30 s at 59.6C 30 s at 72C X35 cycles	318bp	
Heli_1_6745	TGTAGAACGCTCGTGGACAT			
RdRp_2_9343	CGTGCTAGTTTGTTACGGTGA	30 s at 94C 30 s at 61.6C 30 s at 72C X35 cycles	435bp	
RdRp_2_9777	ACATCCATTTCTTCCCATGTGA			

Table S3. List of reference virus genomes used in BWA to align SMRT sequence reads. * DWV-C sequences can be found at http://www.ebi.ac.uk/ena/data/view/CEND01000001

Virus	Full virus name	Genbank Reference	
BQCV	Black queen cell virus	NC-0003784	
SBPV Harpenden	Slow bee paralysis virus	GU93876	
SBPV Roththamsted	Slow bee paralysis virus	EU035616	
DWV-A	Deformed wing virus A	NC-004830	
DWV-B	Deformed wing virus B	NC-006494	
DWV-C	Deformed wing virus C	ENA: CEN01000001*	
SBV	Sacbrood virus	AF092924	
ABPV	Acute bee paralysis virus	NC-002548	
KBV	Kashmir bee virus	NC-004807	
IAPV	Israeli acute paralysis virus	KY243933	
CBPV	Chronic bee paralysis virus	NC-010711 and NC010712	
LSv 1	Lake Sinai virus 1	HQ871931	
LsV 2	Lake Sinai virus 2	HQ888865	
Alpv	Aphid lethal paralysis virus	KJ817182	
AmFv	Apis mellifera filamentous virus	NC- 027925	
New bumblebee viruses	N/A	N/A	Pascall et al, *pers comm*
Acry1			
Bloom1			
Bloom2			
Bloom3			
Bou1			
Bou2			
Bou3			
Corn1			
Dia1			
Dicist_Full			
Dicist_Half			
Grange1			
I1			
I2			
Mut1			
N1			
N2			
Sac1			
Toti1			
Toti2			
Toti3			
Toti4			
Wuchang1			

Table S4. Substitution models for each DWV-B alignment based on jModelTest (Darriba et al. 2012; Guindon and Gascuel 2003). Results of path sampling maximum likelihood estimator analysis comparing demographic and molecular clock models run in Beast 1.8. Analyses of each model in Tracer showed that a strict molecular clock could not be excluded for any model, and all fragments had significant exponential growth, thus for these models * we override path sampling selection

Fragment and traits	Substitution model	Molecular clock	Population demography		
			Constant	Exponential	GMRF Skyride
DWV-B Lp (no traits)	HKY + G	Strict	-1335.06	**-1317.47**	-1323.90
		Exponential	-1332.10	-1313.63*	-1321.31
		Lognormal	-1332.00	-1319.72	-1327.38
DWV-B VP3 (no traits)	K80 + G	Strict	-1245.50	**-1226.50**	-1235.76
		Exponential	-1245.57	-1222.91	-1229.38
		Lognormal	-1241.78	-1220.23*	-1237.03
DWV-B Heli (no traits)	HKY + G	Strict	-749.74	**-743.06**	-750.49
		Exponential	-749.67	-745.22	-750.50
		Lognormal	-751.36	-744.81	-751.41
DWV-B RdRp (no traits)	K80 + G	Strict	-621.20	**-610.98**	-617.97
		Exponential	-621.35	-610.82	-618.76
		Lognormal	-621.00	-610.63*	-618.74
DWV_B concat 4 (host and site)	K80 + G	Strict	-2573.87	**-2556.42**	-2560.85
		Exponential	-2573.13	-2558.87	-2562.56
		Lognormal	-2573.58	-2558.83	-2560.00
DWV-B concat 3 (host and site)	HKY + G	Strict	-1941.46	**-1915.93**	-1923.10
		Exponential	-1940.47	-1918.40	-1926.44
		Lognormal	-1940.86	-1917.77	-1928.30

Table S5. Predicted proportions (%) of pathogen prevalence for three bee species when *Varroa* is present and absent (conversion of GLMM (table 4:1) estimates on the logit scale to proportions using the formula exp(x)/(1+exp(x)), where x equals the parameter estimate

Response	Species	Prevalence when *Varroa* absent	Prevalence when *Varroa* present
DWV-complex	*A. mellifera*	7.87	64.56
	B. pascuorum	0.83	15.19
	B. terrestris	0.78	14.43
DWV-B alone	*A. mellifera*	7.66	61.54
	B. pascuorum	0.89	14.80
	B. terrestris	0.76	12.89
N. ceranae	*A. mellifera*	8.71	6.42
	B. pascuorum	0.35	0.25
	B. terrestris	0.49	0.35

Table S6. Population genetics for DWV by individual fragment: Kst and Snn are calculated to determine if populations are structured by either host or geographic location; a) DWV-B only alignments and (b) DWV-A and B combined. Bold text indicates significance.

a)

Fragment	Trait	Kst	P	Snn	p
lp	host	-0.0017	>0.05	0.71	>0.05
lp	**location**	**0.21**	**<0.01**	**0.47**	**<0.01**
vp3	host	0.0021	>0.05	0.56	>0.05
vp3	**location**	**0.074**	**<0.01**	**0.19**	**<0.01**
vp3	*Varroa*	0.0001	>0.05	0.94	>0.05
helicase	host	0.0016	>0.05	0.64	>0.05
helicase	**location**	**0.068**	**<0.01**	**0.25**	**<0.01**
rdrp	host	0.0020	>0.05	0.72	>0.05
rdrp	**location**	**0.090**	**<0.01**	**0.025**	**<0.01**

b)

Fragment	Trait	Kst	P	Snn	p
lp	host	-0.0001	>0.05	0.075	>0.05
lp	**location**	**0.050**	**<0.01**	0.36	**<0.01**
vp3	host	-0.00054	>0.05	0.56	>0.05
vp3	**location**	**0.068**	**<0.01**	0.21	**<0.01**
vp3	*Varroa*	0.0008	>0.05	0.95	>0.05
helicase	host	0.00042	>0.05	0.59	>0.05
helicase	**location**	**0.054**	**<0.01**	0.29	**<0.01**
rdrp	host	0.0083	>0.05	0.79	>0.05
rdrp	**location**	**0.012**	**<0.01**	0.27	**<0.01**

Table S7. Mean root heights and exponential growth with 95% HPD (Highest posterior density) in brackets, estimated from Beast 1.8 models for concatenated and individual fragments. Note: Concat 3 is *vp3, helicase and rdrp* fragments concatenated, Concat 4 additionally includes *lp*-fragments).

Fragment	Partitions	Mean exponential growth rate (95% HPD)	Doubling rate	Mean root height (95% HPD)
Concat 3	no traits	1.46 (0.46 - 2.81)	0.47	4.40 (1.58 -7.98)
Concat 3	host and site	1.47 (0.44 - 2.71)	0.47	4.46 (1.56 -8.17)
Concat 4	no traits	0.99 (0.30 - 1.84)	0.70	5.69 (1.98 -10.37)
Concat 4	host and site	0.95 (0.31 -1.78)	0.73	5.83 (1.91 -10.64)
Lp-gene	no traits	1.07 (0.26 -2.11)	0.65	6.31 (2.11 -12.27)
Vp3-gene	no traits	0.92 (0.24 -1.87)	0.76	7.14 (2.05 -13.93)
Helicase-gene	no traits	1.32 (0.15 - 2.83)	0.52	4.12 (1.13 -8.21)
RdRp-gene	no traits	2.00 (0.31 -4.10)	0.35	3.45 (1.10 -6.61)

Table S8. Bayes factor support for DWV-B transmission between sites for concatenated fragments without *lp*-gene (a), and with *lp*-gene (b). Note, not all sites had samples where all four, or all three, fragments were amplified, thus not all locations could be included in this analysis (i.e. Isle of Man and Liverpool).

a)

Donor	Receiver Falmouth	Jersey	Cherbourg	Le Conquet	Belle Ile	Quiberon
Falmouth	NA	114	84	93	64	90
Jersey	ns	NA	255	40	ns	ns
Cherbourg	64	ns	NA	185	ns	ns
Le Conquet	99	58	52	NA	52	40
Belle Ile	60	78	31	23	NA	43
Quiberon	56	ns	ns	38	84	NA

b)

donor	receiver Liverpool	Falmouth	Jersey	Cherbourg	Le Conquet	Belle Ile	Quiberon
Liverpool	NA	228	230	ns	ns	ns	ns
Falmouth	ns	NA	88	86	ns	ns	ns
Jersey	ns	ns	NA	599	46	62	140
Cherbourg	ns	81	65	NA	81	ns	ns
Le Conquet	79	179	239	ns	NA	81	57
Belle Ile	ns	ns	ns	28714	45	NA	51
Quiberon	ns	62	259	ns	102	ns	NA

Supplementary figures

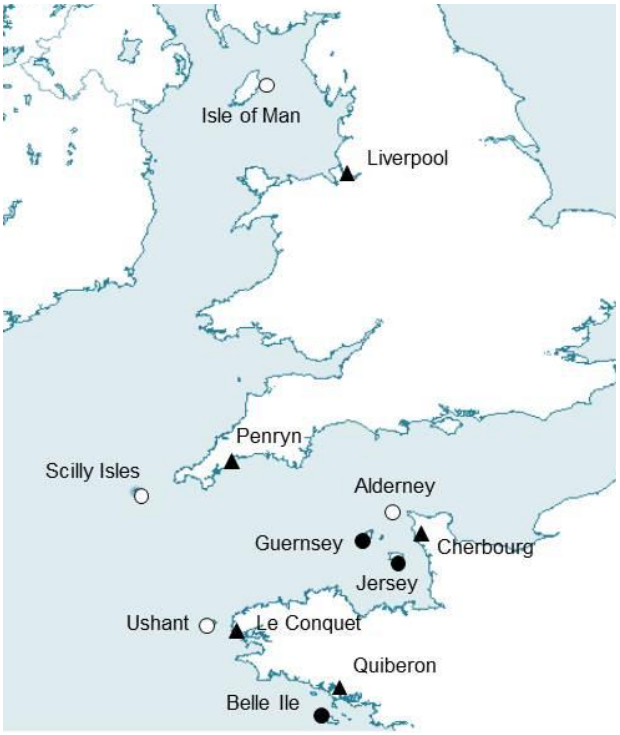


Figure S1. Location of field sites: white circles are *Varroa*-free islands, black circles are *Varroa*-present islands, and black triangles are *Varroa*-present mainland sites

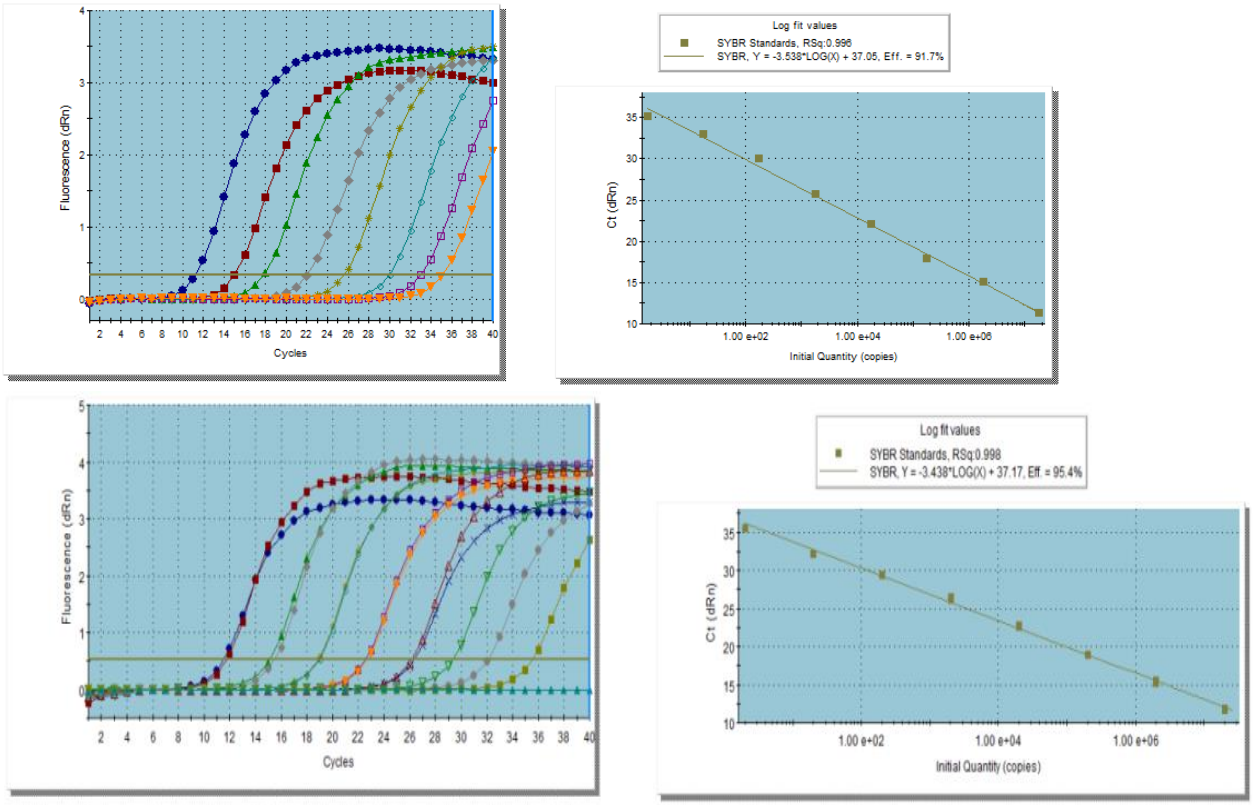


Figure S2. DWV-A (a) and DWV-B (b) 8 points standard curve and amplification plot

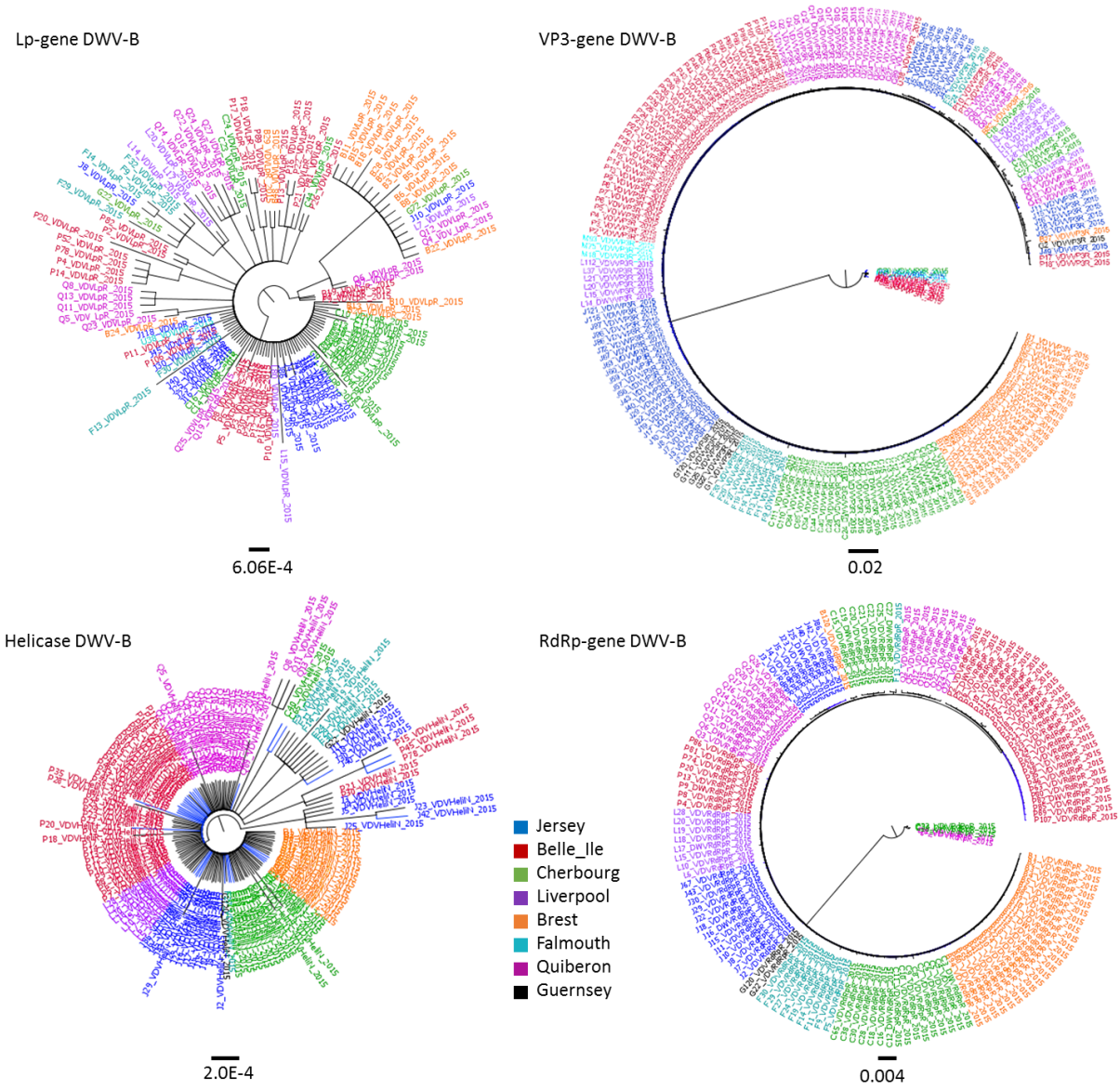


Figure S3. Individual fragment phylogenetic trees produced using MrBayes analysis with all major nodes showing high posterior support >0.8.

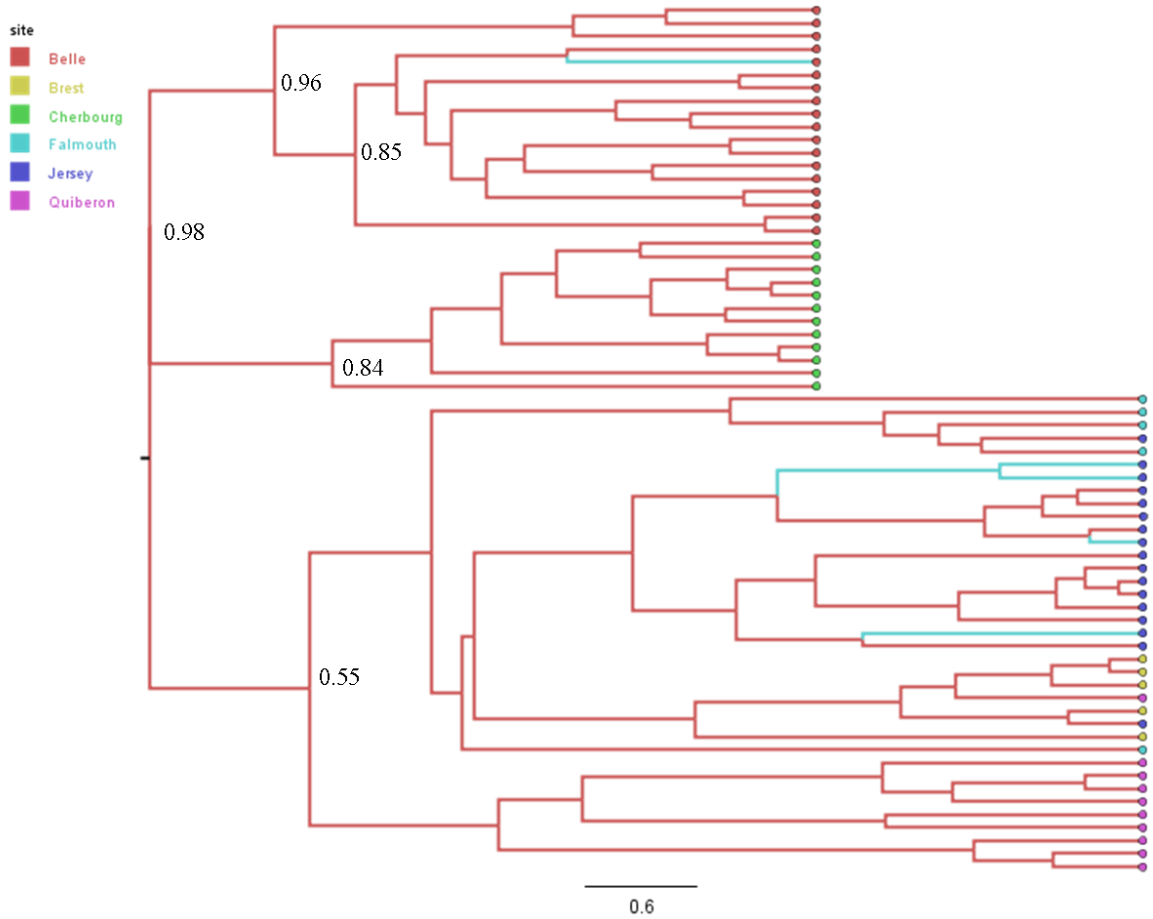


Figure S4. Maximum clade credibility (MCC) tree for Deformed wing virus (type B) (genomes comprised of four concatenated fragments of DWV-B; regions of the *lp*-, *vp3*-, *helicase*- and *rdrp*-gene fragments, total length = 906bp) showing host and location structure. Branches are coloured according to host species (*A. mellifera* in red, and *B. terrestris* in blue), and the branch tips are coloured by location (see key). Posterior support >0.5 is indicated for nodes up to the 3rd order. The scale is time, in years.

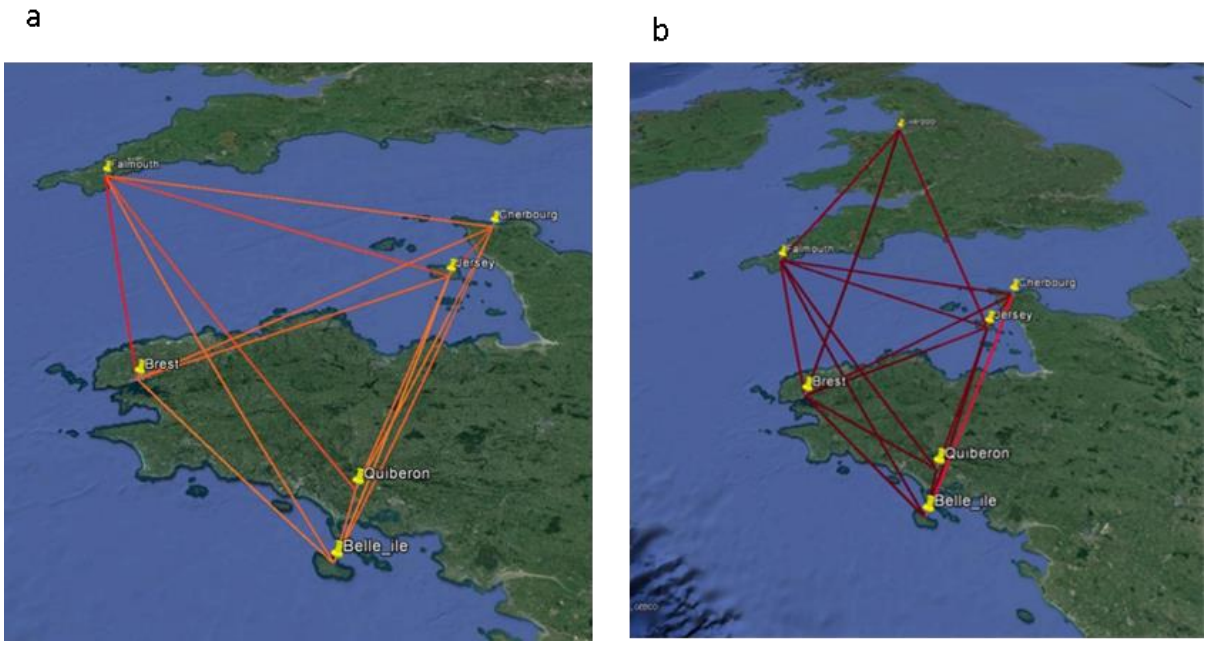


Figure S5. DWV-B transmission routes between sampling locations supported by Bayes factors >3 between field sites based on concatenated fragments - with (a) and without (b) *lp*-gene included. Note, the dataset that included the *lp*-gene didn't include any sequences from Liverpool or the Isle of Man.

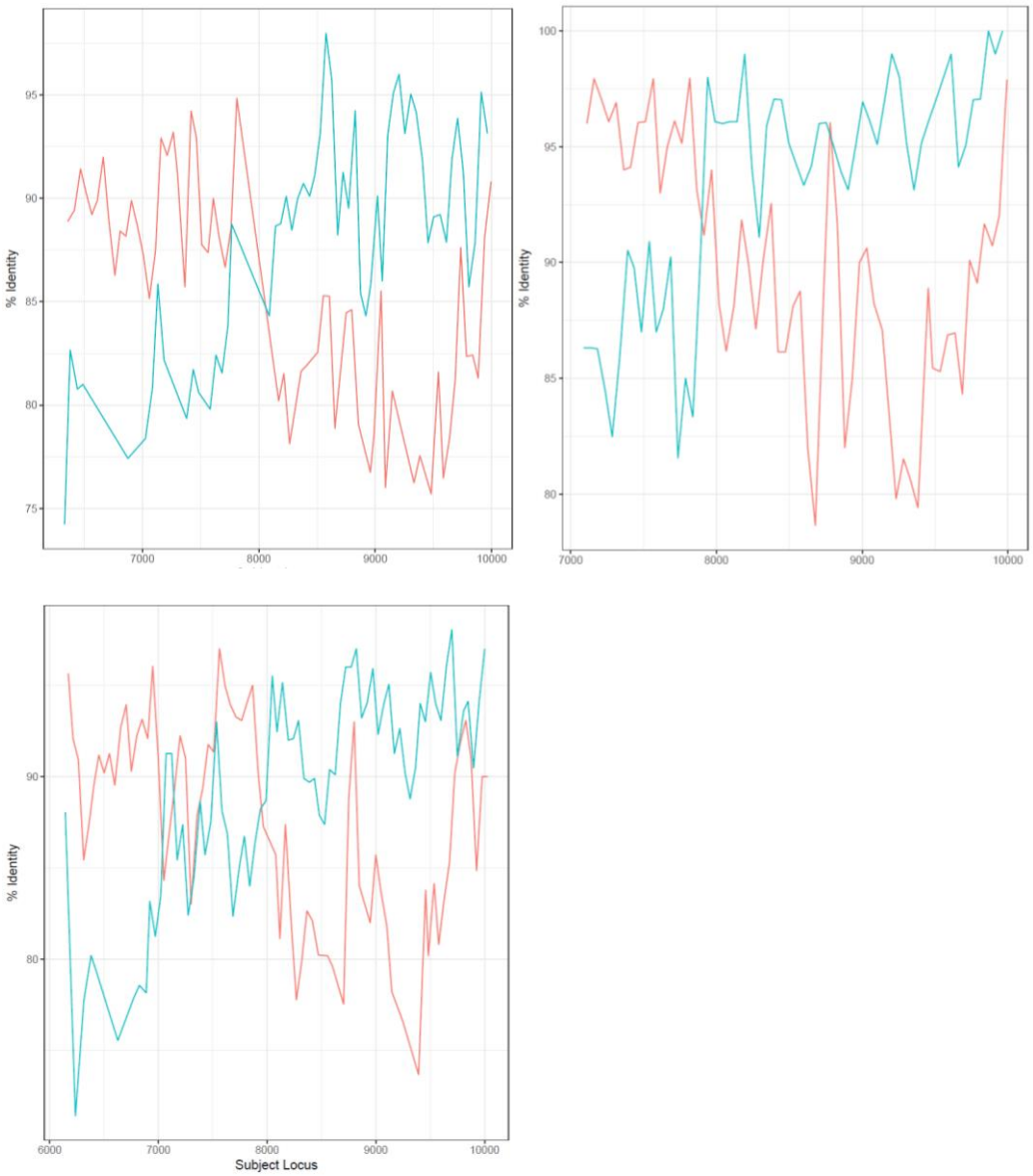


Figure S6. Three DWV reads from SMRT sequencing of *A. mellifera* pool collected from Liverpool, identified as recombinant within the *rdrp*-gene: DWV-A (red) and DWV-B (blue). Note, five reads were identified as recombinants by the analysis, but only the three displayed above are convincing.

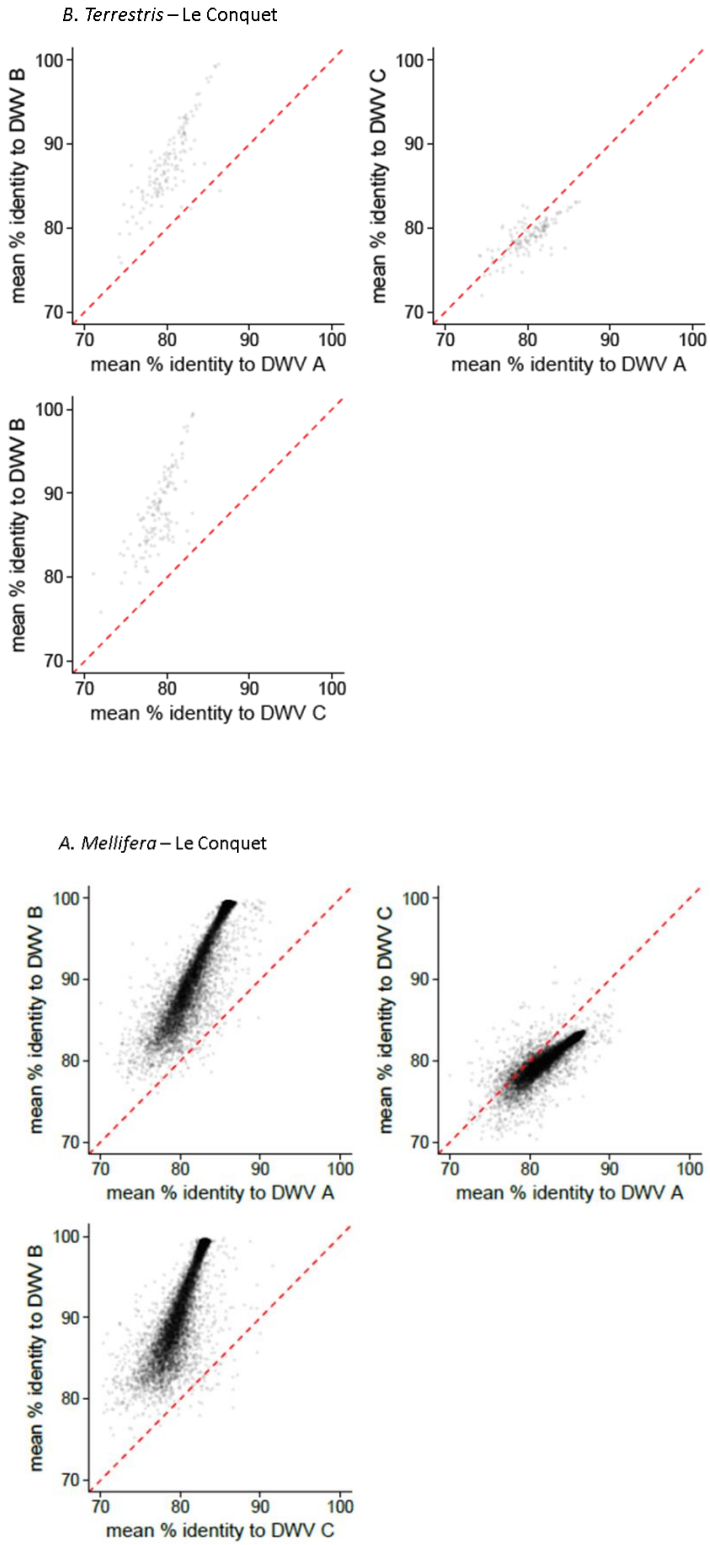


Figure S7. Similarity of SMRT sequencing reads to DWV-A, DWV-B and DWV-C showed no evidence of unknown variants with most reads placed in a large, single cluster containing reads with high similarity to both DWV-A and DWV-B. Example plots from two pools a) *B. terrestris* and b) *A. mellifera* collected from Le Conquet, France.

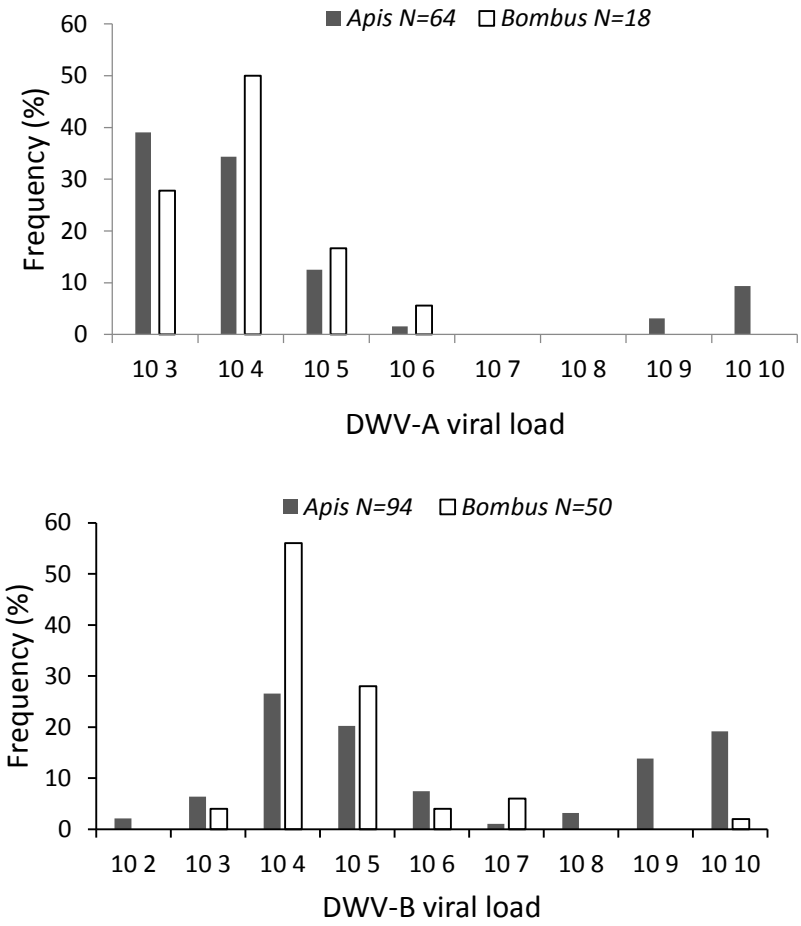


Figure S8. Viral titre by host for DWV-A and DWV-B: *A. mellifera* and *Bombus* species combined

Chapter 5 : The epidemiology of multihost RNA viruses in pollinator communities

Abstract

The majority of pathogens are generalists, infecting multiple hosts. Each host differs in their contribution to the persistence and transmission of a pathogen, often with a key host playing a disproportionate role. Pollinators are host to numerous multi-host RNA viruses, known to replicate and cause harm to both honeybees and bumblebees. The epidemiology of these viruses in their wild bumblebee hosts is largely unknown. The emergence and subsequent spread of the virus-vectoring *Varroa* mite, that is specific to honeybees, plays a particular role in the emergence of RNA viruses in honeybees and potentially indirectly in bumblebees. We carried out a large-scale field study, including *Varroa*-free locations, to test the effect of host species, location and *Varroa-presence* on RNA viral composition; we studied the genetics, prevalence and titre of two viral diseases, Slow bee paralysis virus (SBPV) and Black queen cell virus (BQCV), that have been shown to vary in their affinity to honeybees and bumblebees based on previous field studies. We find that viral communities and genetic variation within viral species are structured by location, not host species or *Varroa* presence. The same viral variants circulate between hosts in each location, indicating interspecific transmission is pervasive; yet, we find virus-specific host differences in prevalence and viral load. Significantly, the specialist honeybee mite, *Varroa destructor*, increases the prevalence of BQCV and SBPV in honeybees and, indirectly, in wild bumblebees. Importantly, in contrast to DWV, BQCV and SBPV viral loads are not increased by *Varroa* presence, indicating these viruses have a less active association with *Varroa*. Our findings provide further evidence that effective control of *Varroa* in managed honeybee colonies is necessary to mitigate further disease emergence, and alleviate the pressure on our vital wild bee populations.

Introduction

The majority of pathogens exist in complex communities infecting multiple host species (Pedersen et al. 2005; Woolhouse et al. 2001). Pathogen host-switching has been a major cause of epidemics in humans and vertebrates (Woolhouse et al. 2005). Within multi-host systems, host species are unlikely to contribute evenly to disease transmission as they vary in their abundance, ecology and behaviours, as well as in their susceptibility to pathogens and subsequent transmission potential (Haydon et al. 2002; Streicker et al. 2013). Thus, often a key species drives a disproportional amount of the disease persistence and transmission to

sympatric hosts, while other hosts are sub-optimal for pathogen transmission (Streicker et al. 2013). Further, the addition of a vector into these complex multi-host transmission cycles can complicate systems as the vector's behaviour and population dynamics must be taken into account (Dobson 2004). For example, a generalist vector can dilute transmission by parasitising multiple hosts with varying transmission potential e.g. the white-footed mouse is the most competent vector of *Borrelia burgdorferi* (the causative agent of Lyme disease), thus increased diversity of mammalian hosts that are hosts for the tick-vector but sub-optimal for the pathogen, dilute the impact of this key host on disease transmission (LoGiudice et al. 2003). Conversely, a specialist vector can increase the transmission potential of a key host e.g. the American robin is believed to be responsible for the West Nile virus epidemic in New York due to preferential feeding behaviour of a mosquito vector on this relatively rare but highly competent host (Kilpatrick et al. 2006). Thus, consideration of the epidemiology of pathogens across multiple host species, and vector behaviour, is essential to identify key hosts and mechanisms driving disease emergence, and ultimately for disease control.

Pollinators are host to a large number of RNA viruses known to be pathogenic to honeybees, and more recently discovered to be multi-host pathogens, prevalent in wild bee populations (Evison et al. 2012; Fürst et al. 2014; Levitt et al. 2013; McMahon et al. 2015; Singh et al. 2010). However, there are known differences in viral prevalence and load across honeybee and bumblebee hosts for many RNA viruses (McMahon et al. 2015). The mechanisms behind host heterogeneity for DWV are relatively well understood: *A. mellifera* has been strongly implicated as the ancestral and reservoir host for DWV (Fürst et al. 2014) in association with the virus-vectoring ectoparasitic mite, *Varroa destructor* (Wilfert et al. 2016, chapter 4 of this thesis). *Varroa* jumped from its native Asian host, *Apis ceranae*, to the European honeybee *A. mellifera*, in the middle of last century, and has since spread worldwide (Oldroyd 1999) causing high colony mortality by vectoring and increasing DWV (Dainat et al. 2012; Genersch 2010; Highfield et al. 2009). DWV potentially increases in the mite by replication (Gisder et al. 2009; Ryabov et al. 2014), or possibly through bioaccumulation of virus particles through blood feeding (Erban et al. 2015). While *Varroa* is a specialist vector to honeybees, it indirectly increases DWV prevalence and titre in sympatric honeybees by dramatically increasing the transmission potential of *A. mellifera*, i.e. *A. mellifera* becomes a 'superspreader' host (Lloyd-Smith et al. 2005) (chapter 4 of this thesis).

RNA viruses differ in their genetics and epidemiology, and in their association with the *Varroa* mite. Consequently, this will influence the risk of that virus emerging in wild bumblebees. Here we study the RNA viral composition and diversity across a honeybee and bumblebee host, with regard to geography and *Varroa* presence. Further, we focus on the epidemiology of two multi-host viruses SBPV and BQCV, and compare and contrast them with DWV. Based on their genomic organisation, SBPV has been assigned to the genus *Iflavirus* - the same genus as DWV (de Miranda et al. 2010b), while BQCV is a member of the *Dicistroviridae* family. *Varroa* has been shown experimentally to be capable of transmitting SBPV (Santillán-Galicia et al. 2014) and to be more prevalent in *Varroa*-present colonies (Carreck et al. 2010). However, in the wild, SBPV has been found at higher prevalence in certain bumblebee species (specifically in *B. horturum*), rather than *A. mellifera* (McMahon et al. 2015), suggesting that *A. mellifera* is not the reservoir host for this virus. Further, SBPV has been shown to be infective to *B. terrestris*, significantly reducing longevity under nutritional stress (Manley et al. 2015). This is important to realise the risk of viral prevalence to the health of wild bumblebees at a population level. BQCV is closely linked with honeybees, with significantly higher prevalence in *A. mellifera* compared to bumblebee species (McMahon et al. 2015), but there is currently no clear evidence associating this virus to *Varroa* (Locke et al. 2012; Ribière et al. 2008; Tentcheva et al. 2004), although one recent study found a weak correlation of BQCV titre with *Varroa* infestation rates (Mondet et al. 2014). To my knowledge, there have been no studies on the pathogenic effect of BQCV to non-*Apis* hosts.

We carried out a structured field survey across 12 sites, comprising of four *Varroa*-free and eight *Varroa*-present sites, to understand viral composition of pollinator populations, and to test the indirect impact of *Varroa* infestation of honeybees on prevalence and titre of multi-host viruses in sympatric *B. terrestris* and *B. pascuorum* populations. Since all *Varroa*-free sites are islands, we also sampled three *Varroa*-present islands, to enable testing of the potentially confounding island effect. We determine how host species, location and the presence/absence of the *Varroa* mite, influence viral composition and the cross-species epidemiology of viruses with different life histories.

Methods

Please note: The samples and many of the methods are used in both chapter 4 and 5. Details are repeated here for completeness, with additional methods added when necessary.

Bee samples

We collected ~30 *A. mellifera*, ~30 *B. pascuorum* and ~60 *B. terrestris*/*lucorum* from 12 sites across the British Isles and Brittany (France) in the summer of 2015 (for sample details see table S1 – chapter 4). The sites comprised four *Varroa*-free islands (Isle of Man (Douglas), Scilly Isles (St Mary's), Ushant and Alderney (St Anne); three *Varroa*-present islands (Guernsey (St Peter Port), Jersey (St Helier) and Belle Ile (Le Palais) and five *Varroa*-present mainland sites (Liverpool, Penryn, Cherbourg, Le Conquet and Quiberon) (figure S1 – chapter 4). In total we collected 355 honeybees (*Apis mellifera*), 640 *Bombus terrestris*, 37 *B. lucorum* (see methods below for molecular differentiation of the *B. terrestris*/*lucorum* species complex) and 280 *B. pascuorum*. Bees were collected from a 1x1km area whilst foraging on flowers. We used Individual collecting tubes and kept samples on ice, before sacrificing and storing them at -80 in a dry shipper on the day of collection. For Belle Ile and Jersey, bees were sacrificed and stored at -80 deg. within 48 hours of collection.

DNA and RNA isolation

To extract DNA as well as RNA from the samples, we removed the gut from each individual and macerated them individually in 200µl of insect ringer solution. DNA was extracted from 35µl of the gut homogenate using a modified Chelex® method: 100ul of 10% Chelex® 100 resin (Biorad) solution was added to the gut solution, with 2µl of Proteinase K (20µg/µl) before incubation at 56°C for one hour, and vortexed twice during this time. Samples were incubated at 95°C for 15 minutes, centrifuged and the supernatant was stored at -20°C until use. For individual RNA extractions, we used half the head and thorax of individuals (bisected laterally) and 80µl of the gut solution (described above), using Trizol© (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction. Briefly, samples were homogenised with glass beads in 1.3ml Trizol© in a tissue-lyser. RNA was separated using bromo-chloropropane and precipitated in isopropanol. The RNA was washed with 75% ethanol and re-suspended in 100µl (*A. mellifera*) or 400µl (*Bombus* species) of RNase-free water (Sigma), to allow for variation in RNA pellet size between the species.

Single molecule real-time (SMRT) sequencing to determine viral composition of populations

The concentration of individual RNA samples was measured using a Qbit™ Fluorometer. 1000ng of RNA from 30 *A. mellifera* and 30 *B. terrestris* individuals, from two *Varroa*-free sites (Ushant and the Isle of Man) and their paired *Varroa*-present mainland sites (Le

Conquet and Liverpool), were pooled to create eight populations for SMRT sequencing. Note: *B. terrestris* was rare on Ushant, thus only 13 individuals were collected and used in that pool. The individual RNA samples were quality checked with the Nanodrop™ 2000 spectrophotometer (all samples had 280/260 ratio >1.8), and the pooled RNA run on an Agilent 2200 Tapestation: pools all had high RIN values (>9), except for Liverpool honeybees with RIN=7.6. Full-length cDNA libraries (using the BluePippin System) were prepared using Clontech SMARTer PCR cDNA Synthesis Kit. Following this, the PacBio Template Prep Kit was used to generate SMRTbell™ libraries, which were then sequenced on the PacBio System (library prep and sequencing was carried out by the Exeter Sequencing Service). Non-chimeric reads from each pool were mapped first against their respective host species genomes using BWA (Li and Durbin 2009) (v. 0.7.12) with the following parameters: "bwa mem -x pacbio" to remove host-derived sequences. Remaining reads were mapped against all sequenced RNA viruses and 23 novel bumblebee viruses (Pascall et al. *pers comm*) (table S3 in chapter 4). We ran principle component analysis (PCA) in RStudio (v0.99.896) using the prcomp function to look at similarity in viral composition between pools. We calculated traditional measures of diversity for each pool: Simpson's Diversity Index (1-D) and Shannon's Diversity Index (H). Reads that did not map to either known viruses or hosts were assembled with canu (v.1.5) to look for novel viruses with the following parameters: 'genomeSize=100k useGrid=false -pacbio-raw contigFilter="2 1000 1.0 1.0 2". Assemblies were analysed using the gene annotator software Genemark (http://opal.biology.gatech.edu/GeneMark), with each putative gene examined individually using BLASTP+.

Prevalence by RT-PCR

We converted 2µl of RNA into first-strand cDNA using GoScript™ Reverse Transcriptase, according to the manufacturer's instructions (Promega), using random hexamer primers and RNasin® to prevent RNA degradation. We differentiated between the cryptic species *B. terrestris* and *B. lucorum* using the DNA extractions (diluted 10%) and PCR using primer pair BBMI_IGSF1 and BBM1_IGSR1 (*pers comm* Regula Schmid-Hempel, table S1). To determine viral prevalence of BQCV and SBPV, cDNA was diluted 1:10 prior to PCR; we used previously published primer pairs to identify positive samples and carried out PCR in 20µl reactions using GoTaq® DNA Polymerase, with PCR programs specific to each primer pair (table S1). Every run included a known positive sample and a water negative as controls. Five µl of PCR product were run on 1.5% TAE agarose gel with ethidium bromide nucleic

acid staining solution. We used Chi-square pairwise comparisons with Bonferroni corrected P-values and Yates' continuity correction to assess if co-infection of viruses occurred more often than expected based on individual prevalence rate. Note, we used prevalence data for DWV-A and DWV-B (chapter 4 of this thesis) for this analysis.

Quantitative reverse transcription PCR (qRT-PCR)

Ten positives per site per species (or total number if there were less than ten positives available) were randomly selected from samples that tested positive for BQCV and SBPV by PCR analysis (described above), and analysed by qRT-PCR. We measured nucleic acid quality (Nanodrop™ 2000 spectrophotometer) and concentration (Qbit™ Fluorometer) for each individual. No samples were excluded due to poor quality, as assessed with Nanodrop 280/260 ratios greater than 1.8. Based on Qubit concentrations, each RNA sample was diluted to 100ng/µl in RNase free water. cDNA was synthesised from 400ng of RNA template using GoScript™ Reverse Transcriptase, as reported above. cDNA was then diluted 1:10 prior to use in qRT-PCR. For absolute quantification, duplicate reactions were performed for each sample on a Strategene machine (Mx3005P) using GoTaq® qPCR Master mix for dye-based detection (Promega) on the following program: 2 mins at 95 °C, followed by 40 cycles of 10s at 95 °C, and data collection for 60s at 57°C. We used virus specific primers for the detection of BQCV and SBPV, and Rp49 was amplified in duplicate for all *A. mellifera* samples as an internal reference marker (table S2).Two no-template negative samples containing RNase-free water were run on each plate. Quantification was calculated using duplicate eight-point standard curves of plasmid DNA in a 1:10 serial dilution on each plate. BQCV and SBPV plasmids were generated using Promega pGEM®-T Easy Vector, according to the manufacturer's instructions, to clone a 257bp fragment of ORF 2 of the BQCV genome and 186bp fragment of the VP2-gene in SBPV from purified PCR products (primer details in table S2). Successful transformants were selected via blue/white screening, and plasmids were extracted using GeneJET Plasmid Miniprep Kit (ThermoFisher). M13 primers (designed to sequence inserts inside pGEM®-T Easy Vector: forward 5´-GTTTTCCCAGTCACGAC -3´, reverse 5´-CAGGAAACAGCTATGAC -3´) were used to determine the correct product had been cloned. We linearlised the plasmids using the restriction enzyme Apa 1 (New England Biolabs), according to the manufacturer's instructions, and diluted them 1:1000 with RNase-free water. Mean efficiency across plates for BQCV was 95.7% (four plates ranging from 93.5 - 96.7%) and SBPV was 95.2 (five

plates ranging from 91.6 – 101.8) with $R^2 > 0.98$ across assays (for standard curves see figure S1).

Statistics for prevalence and viral load

All statistical analyses were carried out in RStudio (v0.99.896). True prevalence with 95% confidence intervals were calculated to account for assay efficiency and sensitivity, which was conservatively set at 95% (Reiczigel et al. 2010) using R library epiR v0.9-82) and the function epi.prev. Within the package, confidence intervals are calculated based on methods in Blaker (2000). To examine if disease prevalence was affected by *Varroa*-presence we ran generalised linear mixed models (GLMMs) (with BQCV and SBPV tested in separate models) with binomial error distribution and logit link function, using the lme4 package (v1.1-12) (Bates & Sarkar, 2006). Full models included three-way interaction between the fixed effects; *Varroa*-presence/absence, species (a factor with three levels: *A. mellifera, B. terrestris and B. pascuorum*) and island/mainland location, with latitude and sunshine hours duration as additional fixed effects: field site and individual were included as random effects (individual was added to account for over-dispersion in the models (Harrison 2014)). Sunshine hours provided a proxy for favourable disease transmission conditions (Fürst et al. 2014) and were calculated as the mean sunshine hours from monthly data between March and July 2015 at each location (or as near to the sampling location as possible), collected from MET office data (*pers comm*) and Meteo France (http://www.meteofrance.com). The minimum adequate model (MAM) was determined through removal of non-significant terms and comparison of how much variation was explained by each model using the anova function: if the new model was not significantly different at $p<0.05$ the term was removed from the model. To test the full effect of our predictors we compared the MAM with the null model (which only included random effects) using ANOVA. Residual plots were examined to assess model fit.

Varroa-free refugia only exist on islands, thus it was necessary to also sample on *Varroa*-present islands, as well as paired *Varroa*-present mainland sites, to test a possible island effect on disease prevalence. Further, we ran further models on reduced datasets 1) comparing island sites with and without *Varroa*, and 2) comparing *Varroa* present islands and mainland sites. If the island effect is not confounding, in 1) we expect to maintain a significant effect of *Varroa*, and in 2) we expect island to remain a non-significant effect.

To investigate if viral load was affected by *Varroa*-presence we ran GLMMs: BQCV and SBPV were tested in separate models with Gamma error distribution and inverse link function. As viral load data varied across orders of magnitude from 10^3 to 10^{10} these data were log transformed before analysis. Full models, model simplification and testing model fit were carried out as described above.

Nucleotide sequencing

We sequenced the same samples that were selected for qPCR. We assayed these samples by PCR for one genomic region in BQCV and SBPV (primers detailed in table S1). We purified 15µl of PCR product using Exonuclease 1 and Antarctic phosphatase by incubation at 37°C for 60 minutes and denaturation at 80°C for 20 mins. We used Big Dye™ Terminator v3.1 (ThermoFisher) for florescence-based direct sequencing of amplicons, following the manufacturer's instructions. The Big Dye PCR products were purified by filtering through Sephadex® G-50 (Sigma-Aldrich) and directly sequenced in the reverse direction on an ABI 3730 Genetic Analyser using the appropriate PCR primer (table S1). Sequencing direction was chosen to optimise the number of sequences and the length of amplicon. Not all positive samples were successfully amplified for each chosen region. We inspected all sequences manually in Geneious® (v6.8) for quality and excluded any sequences based on the following quality criteria: heterozygosity, too short for the chosen region, and >3 unidentified base-pairs. Alignments were made in Geneious® using the 'map to reference' tool. To confirm that there was no recombination within fragments at a p-value of 0.05, we used the GENECONV (Padidam et al. 1999), MaxChi (Maynard Smith 1992), BootScan (Martin et al. 2005) and SiScan (Gibbs et al. 2000) algorithms in the Rdp4 package (v4.56) (Martin et al. 2015).

Population genetics

For BQCV and SBPV sequence alignments (table S3), median joining phylogenetic networks were produced using PopArt (v.1.7), (http://popart.otago.ac.nz). We used DNAsp (v5.10.1) (Librardo and Rozas 2009) to calculate population genetic measures: we looked at differences in populations by host species and geographic location with Kst (a measure of population differentiation based on the proportion of between-population nucleotide differences (Hudson et al, 1992), and the nearest neighbour statistic SNN (which calculates the proportion at which the genetic nearest neighbours are found within same population (Hudson, 2000). We calculated Tajima's D: a statistic that compares the average number of pairwise differences

with the number of segregating sites. A negative estimate indicates an excess of rare mutations, for example after a selective sweep there will be very little genetic variation, thus any mutations that occur will be rare within the population.

Reconstructing viral phylogenies

We ran Jmodel test to compare and select an appropriate evolutionary substitution model for each alignment based on Bayesian Information Criterion (Alizon and Fraser 2013) for use in phylogenetic reconstructions (table S4). We fit discrete trait models with asymmetric substitution models for host species and geographic location, which allows transitions to and from a host or location to occur at different rates (trait rate and indicators operators weight = 1), implemented in Beast v1.8 (Drummond and Bouckaert 2015). We ran and compared nine models concurrently for each alignment with different demography and molecular clock rates, and used path sampling maximum likelihood estimator, implemented in Beast 1.8, to determine the best model (table S4). Models were run without prior knowledge of the evolutionary rate. BQCV models were run for 10,000,000, and SBPV models for 20,000,000 MCMC steps with sampling every 1000 or 2000 generations, respectively. Posterior distribution, convergence and effective sample size was assessed using Tracer v1.6 (Drummond and Rambaut 2007): all models achieved high effective sample size (>200). In addition, we identified exponential population growth (if the 95% HPD of estimates for growth did not cross zero), and detected if the model follows or excludes a molecular strict clock (by examining the relaxed lognormal clock's coefficient of variation statistic – if it shoulders the zero boundary a strict clock cannot be excluded) (Drummond and Bouckaert 2015). We produced Maximum Clade Credibility (MCC) trees (TreeAnnotator (v1.8.4)) to infer host ancestral state probabilities. Phylogenetic trees were also produced for each alignment using MrBayes 3.2.6. Phylogenetic tree figures were created using Figtree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). Well-supported migration routes between host species and sites could be identified using Bayes factors (SPREAD v1.0.6.).

Results

Viral composition

We determined viral composition across host species, geographic location and *Varroa*-presence/absence using SMRT sequencing reads from eight populations (these were pools of *A. mellifera* and *B. terrestris* from four sites - two paired locations each comprising of a *Varroa*-free island and *Varroa*-present mainland site). From principle component analysis, it

is clear that geographic location, rather than host species or *Varroa* presence/absence, determines the viral composition of pollinator populations (figure 5:1). Both *A. mellifera* and *B. terrestris* populations from the geographically paired sites - Liverpool and the Isle of Man, carry a more diverse viral fauna (mean Simpson's Index of Diversity (1-D) = 2.33 (range: 1.91-2.62), and mean Shannon's diversity Index (H) = 1.17 (range: 0.92 – 1.36)) than populations from the geographically paired sites - Le Conquet and Ushant ((1-D) = 1.31 (range: 1.02-2.00), H = 0.31 (0.05-0.72)). While viral composition in *B. terrestris* populations mirror that of *A. mellifera* from the same location, the total number of virus reads from the *B. terrestris* populations are significantly lower (tests of proportions: Brest $\chi^2=16545_1$, $p < 0.001$; Liverpool - $\chi^2=676_1$ $p < 0.001$; Isle of Man - $\chi^2=2843_1$, $p < 0.001$; Ushant was the exception with significantly more reads in *B. terrestris* compared to *A. mellifera* ($\chi^2 = 84_1$, $p< 0.001$).

A striking finding was that DWV-B is the dominant virus across all eight populations, comprising 75% of all virus reads, while DWV-A is rare comprising only 0.07% of reads. Apis mellifera filamentous virus, a double-stranded DNA virus distantly related to Bracoviruses (Gauthier et al. 2015), is also common across all populations (6.8% of reads). SBPV (12.9% of reads), Sacbrood virus (4.4% of reads) and BQCV (0.5% of reads) are notably common in the Liverpool/Isle of Man populations but absent from the Le Conquet/Ushant populations (figure S2) (for map of sites see figure S1, chapter 4); reads from all other viruses (table S3, chapter 4) are rare (including four newly discovered bumblebee viruses (Pascall et al. *pers comm*) or absent across all populations. We found no evidence for further novel viruses within these pools from analysis of contigs assembled from reads that did not map to either host or viral genomes.

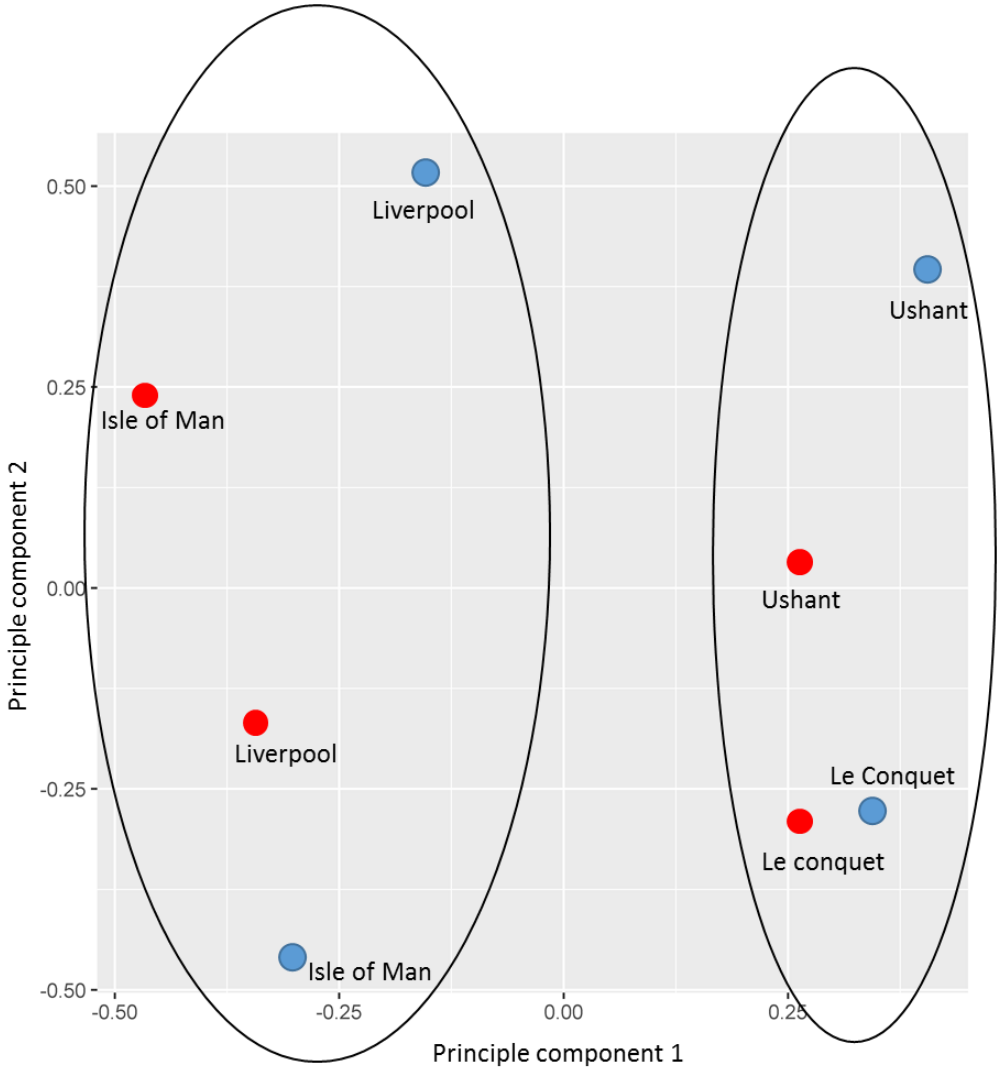


Figure 5:1: Principle component analysis on the viral composition of eight bee populations, based on single molecule real-time (SMRT) sequences mapped by BWA-mem to all previously sequenced 'honeybee' viruses and fragments from 23 newly discovered bumblebee viruses. *A. mellifera* populations are shown with red circles and *B. terrestris* populations shown with blue circles. The ovals indicate the clustering of populations from geographically close locations along principle component 1 (PC1). 38.8% of the variance is explained by PC1, and the cumulative % variance explained by PC1 and PC2 is 62.7%.

BQCV and SBPV prevalence and viral load across host species

We mapped the prevalence of BQCV and SBPV across all bees sampled from 12 locations by pollinator species (figure 5:2). In GLMMs, host species was a significant predictor of BQCV prevalence (table 5:1), but for SBPV host species was not significant and was removed from the model (anova: $\chi^2 = 2.62_2$, $p = 0.27$). BQCV is more prevalent in *A. mellifera* compared to *Bombus* species (tests of proportions: BQCV $\chi^2 = 229.75_1$, $p < 0.001$) (table 5:2). Prevalence

of SBPV, in contrast to the other viruses, was highest in *B. pascuorum* compared to both *B. terrestris* ($\chi^2 = 29.86_1$, $p < 0.001$) and *A. mellifera* ($\chi^2 = 4.89_1$, $p = 0.027$ – though note this difference is small) (table 5:2).

Table 5-1: GLMMs with binomial error structure and logit function: BQCV and SBPV prevalence as a response to *Varroa* presence and host species (minimum adequate models)

Pathogen prevalence	Parameters	Estimate	SE	z-value	P-value
BQCV	Intercept	-2.62	0.98	-2.65	0.008
	B. pascuorum	-4.73	0.62	-7.55	<0.001
	B.terrestris	-2.78	0.39	-6.99	<0.001
	Varroa presence	3.05	1.14	2.66	0.008
SBPV	Intercept	-7.74	2.22	-3.46	<0.001
	Varroa presence	4.98	2.44	2.05	0.0408

Table 5-2: True prevalence (with 95% confidence intervals) of Black queen cell virus (BQCV) and Slow bee paralysis virus (SBPV) detected using PCR (primer details table S1) *For populations where true prevalence = 0 (because it is based on 95% sensitivity and specificity of the PCR assay) we report actual prevalence based on our data (positives confirmed by Sanger sequencing)

	BQCV	SBPV
A.mellifera (N = 355)	46.4 (40.58-52.2)	16.97 (12.49 – 21.92)
B.terrestris (N = 640)	7.81 (5.14-10.88)	8.85 (6.09 – 12.01)
B.pascuorum (N = 280)	2.14* (0.9 – 4.5)	25.79 (20.13 – 32.08)

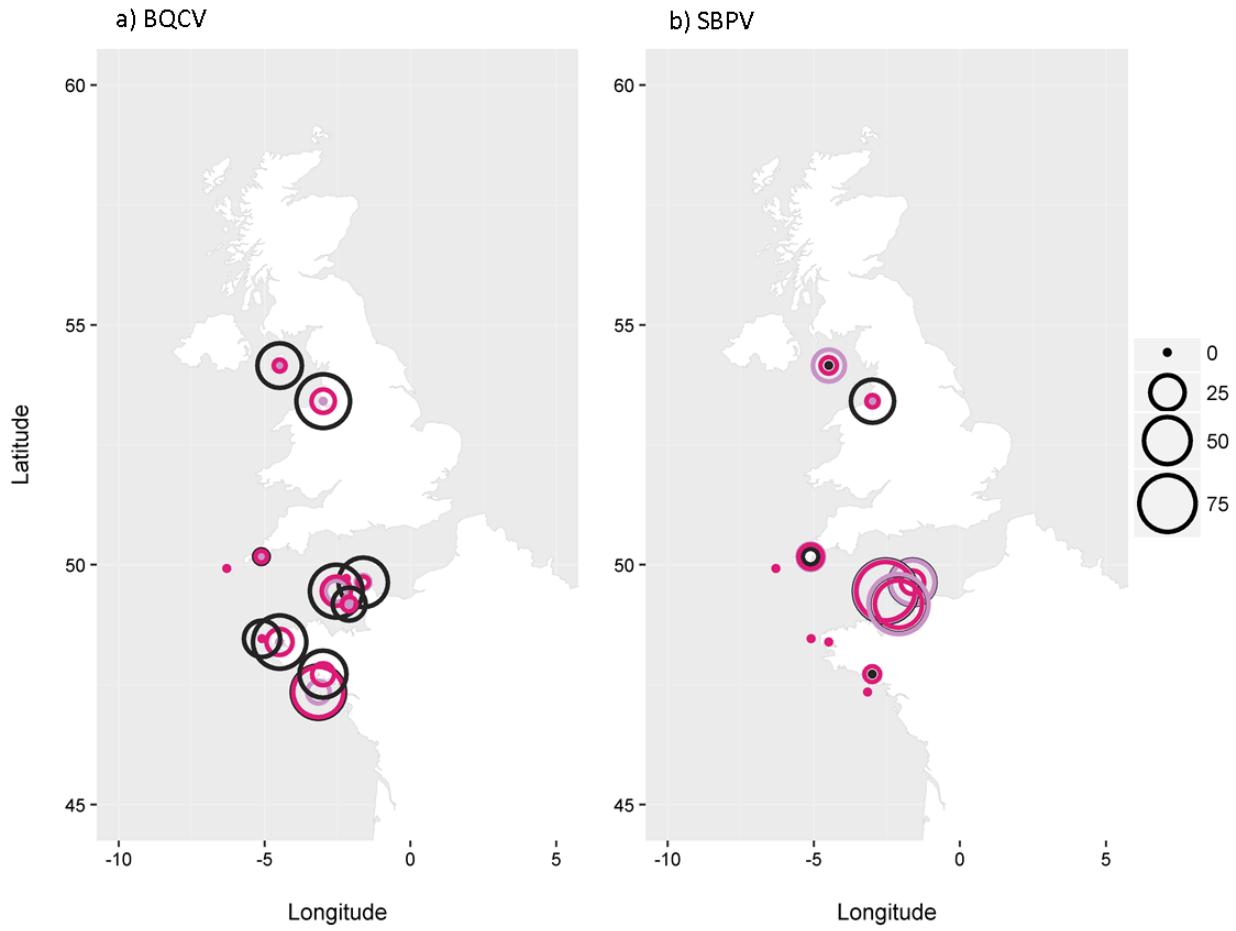


Figure 5:2: Individual prevalence of BQCV (a) and SBPV (b) mapped across 12 field sites. Scales represent % prevalence, colour represents host species (*A. mellifera* (black), *B. terrestris* (light purple) and *B. pascuorum* (fuchsia pink).

Viral loads ranging from $10^3 – 10^{10}$ were found for all viruses; host species is a significant factor predicting SBPV viral load (table 5:3, figure 5:3). *B. pascuorum* has significantly higher SBPV loads than *A. mellifera* (KS-tests: D = 0.71, p < 0.001); with SBPV loads in *B. pascuorum* predicted from the GLMM to be one order of magnitude higher than *A. mellifera* and *B. terrestris*. There were no significant differences in viral load between host species for BQCV (D = 0.18, p = 0.37).

Table 5-3: GLMMs on SBPV viral load with Gamma error structure

Pathogen viral load	Parameters	Estimate	SE	z-value	P-value
SBPV	Intercept	0.195	0.024	8.036	<0.001
	B. pascuorum	-0.046	0.013	-3.45	<0.001
	B. terrestris	-0.0016	0.0137	-1.176	0.239

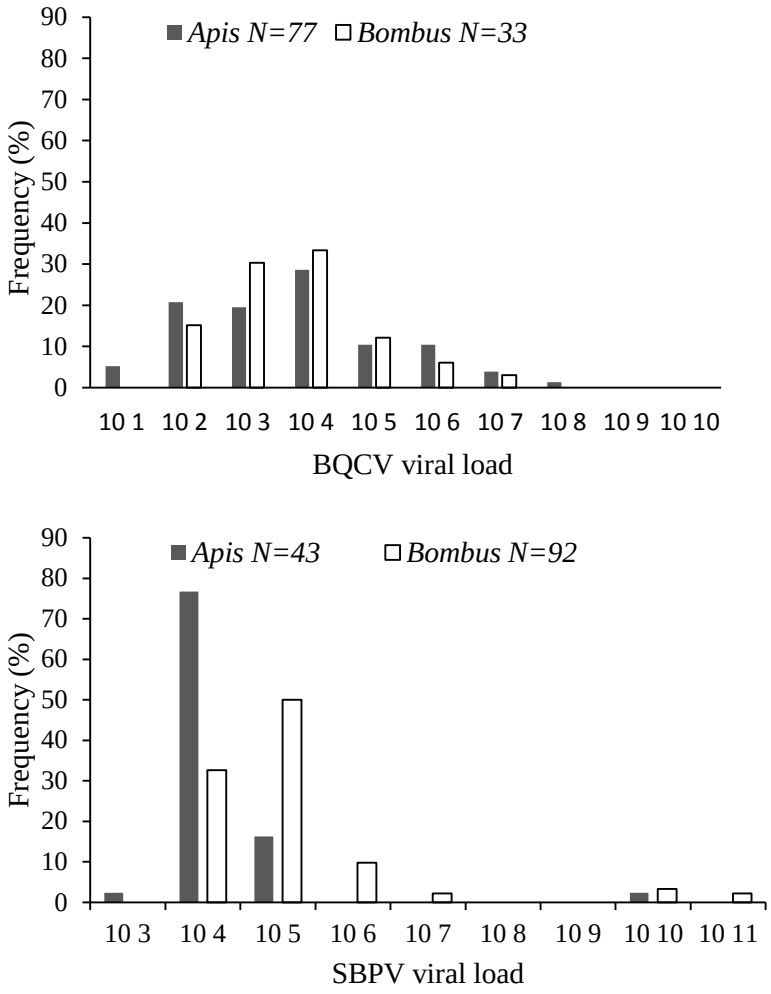


Figure 5:3: Percentage frequency of viral loads for BQCV and SBPV displayed by host genus: As samples were already identified as positive by PCR, a detection threshold was only set based on residual amplification of the no-template samples; this was only necessary for SBPV where a cut-off point of Cq = 31 was set.

Varroa drives prevalence but not viral load of BQCV and SBPV

The influence of *Varroa* on viral prevalence is highlighted by the apparent absence of viruses from two *Varroa*-free sites (Scilly Isles and Alderney). In GLMMs, *Varroa* presence was a significant predictor for virus prevalence for both viruses (table 5:1, figure 5:4). *Varroa* presence predicts an increase in BQCV prevalence by 10 times in *A. mellifera* and by 20 times in both bumblebee species; and an increase in SBPV prevalence across host species by over 100 fold (table S5). Interactions between *Varroa* presence and host species were included in each full model, but were removed by model selection indicating that *Varroa* presence increases prevalence in both honeybees and bumblebees equally. Sunshine hours

and latitude were removed from all models by model selection. Island-mainland location was also included as a fixed factor and excluded by model selection for both viruses (anova: BQCV $\chi^2 = 0.844_1$, p = 0.35; SBPV $\chi^2 = 3.3_1$, p = 0.070), suggesting that we are indeed seeing an effect of *Varroa* presence, rather than an island effect. We ran further tests and models that confirmed island location did not influence viral prevalence (see note S1). Our full models for viral prevalence fitted the data significantly better than the null model that contained random factors only (ANOVA: BQCV $\chi^2 = 41.37_3$, p < 0.001, SBPV $\chi^2 = 4.12_3$, p = 0.04).

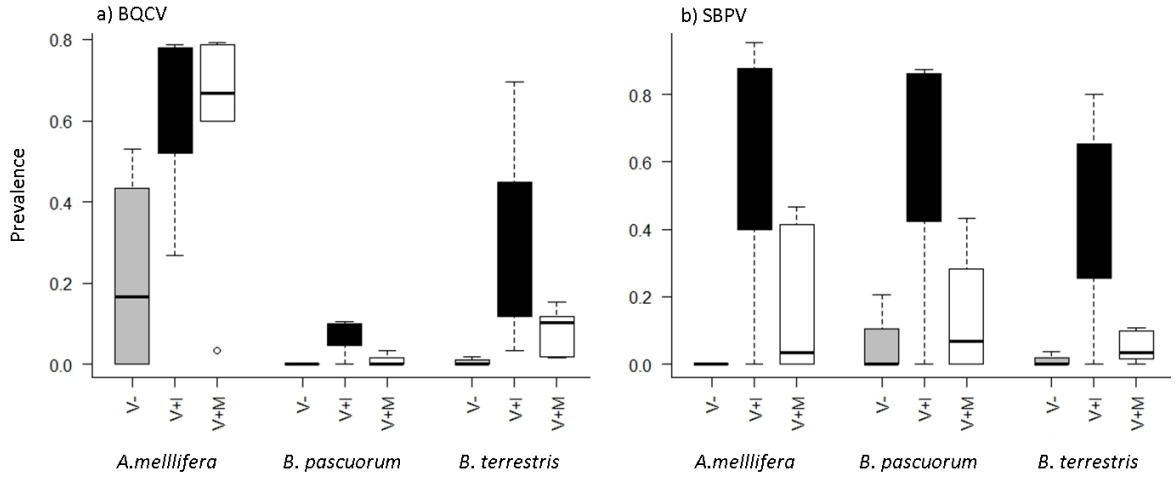


Figure 5:4: Prevalence of BQCV and SBPV across host species and *Varroa*-presence: *Varroa*-free sites (grey, V-), *Varroa*-present islands (black, V+I), *and Varroa*-present mainland (white, V+M)

*A*lthough *Varroa* influences prevalence of both BQCV and SBPV, it is not a significant predictor of viral load (figure 5:5). Latitude, sunshine hours duration and island/mainland location were included in the initial model, but had no influence on viral load and were removed from the models by model selection.

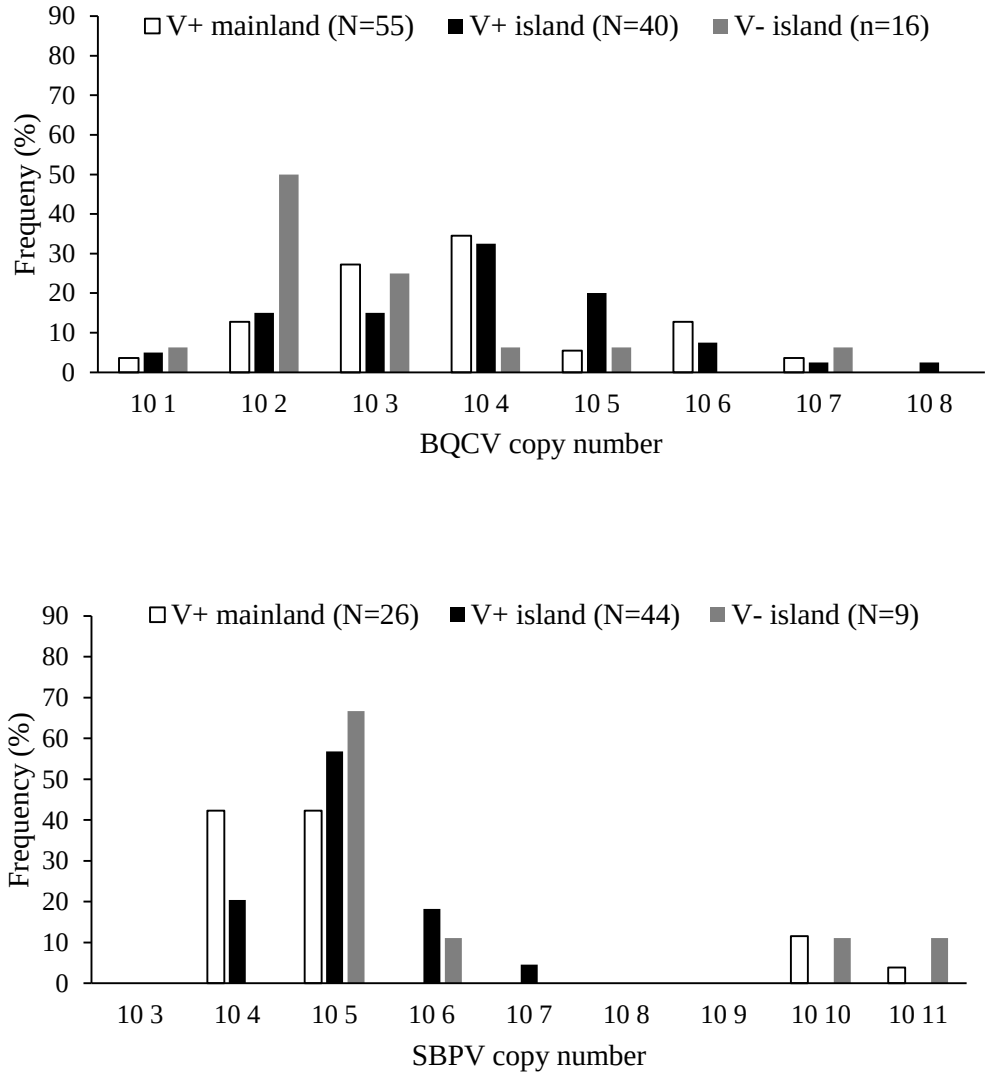


Figure 5:5: Percentage frequency of viral loads for BQCV and SBPV displayed by *Varroa* status: *Varroa*-present mainland sites = white (V+ mainland), *Varroa*-present island sites = black (V+ island) and *Varroa*-free sites = grey (V- island). As in figure 5:4, a cut-off point of Cq 31 was set for SBPV.

Coinfection

Co-infection occurred in all combinations between four viruses (BQCV, SBPV and DWV (type A and B)); co-infection was rare in bumblebees and highly common in *A. mellifera* but only in *Varroa*-present sites (figure S3). True prevalence of co-infection for two viruses was 8% (95% CI 6.11- 10.13); co-infection of three (N= 49 *A. mellifera* and 1 *B. terrestris*) and four viruses (N=11 *A. mellifera*) was rare, with true prevalence <1%. The proportion of co-infected individuals was higher than expected based on single infection rates ($\chi^2 = 150.33_3$, p

< 0.001). Further, pairwise comparisons of the four viruses, using Bonferroni corrected P-values (p = 0.008) and Yates' continuity correction, revealed that DWV-A presence was linked to DWV-B, BQCV and SBPV presence ($\chi^2 = 142.18_1$, $p < 0.001$, $\chi^2 = 102.3_1$, $p < 0.001$ and $\chi^2 = 106.96_1$, $p < 0.001$, respectively). Indeed, DWV-A only occurred in co-infection with DWV-B. In contrast, DWV-B, BQCV and SBPV presence were all independent of each other. We confirmed these results in an analysis excluding the DWV-A *Varroa* negative sites. Further, we carried out the same analysis on *Varroa*-free sites in the absence of DWV-A, SBPV presence was linked to DWV-B and BQCV presence ($\chi^2 = 7.69_1$, $p < 0.005$; $\chi^2 = 12.02_1$, $p < 0.001$, respectively). BQCV and DWV-B presence remained independent of each other.

Population genetics and phylogenies

SBPV and BQCV genomes are highly structured by geographic location, not host species. Kst values for genetic differences between locations are significant (note, Kst and Snn values close to 1 represent strong population differentiation (Hudson 2000; Hudson et al. 1992)); $Kst_{SBPV} = 0.78$, $p < 0.001$ and $Kst_{BQCV} = 0.79$, $p < 0.001$. In addition, samples that are genetic nearest neighbours largely come from the same populations: $Snn_{SBPV} = 0.97$, $p < 0.001$, $Snn_{BQCV} = 0.93$, $p < 0.001$. SBPV and BQCV show some weak host differentiation on the edge of significance ($Kst_{SBPV} = 0.034$, $p = 0.040$, $Kst_{BQCV} = 0.019$, $p = 0.068$).

We produced median joining phylogenetic networks for each virus (figure 5:6). Both BQCV and SBPV population are highly structured by location: BQCV ($\pi = 0.044$, with 64 polymorphic sites out of 432 examined over 69 sequences) and SBPV ($\pi = 0.074$, with 118 polymorphic sites out of 535 examined over 78 sequences) (figure 5:6a and 5:6b). As Tajima's D statistic is sensitive to population structure, we restricted our analyses of demography to populations within locations, choosing those with the largest sample size. For SBPV, we examined Jersey (N = 28) and Guernsey (N = 29), and for BQCV we examined Liverpool (N = 13) and Belle Ile (N = 11), and found no evidence for an excess of rare variants (Tajima's D = 1.58, 1.15, -1.054, -0.3219, respectively, $p > 0.05$). In addition, models of exponential growth for both viruses were rejected in a Beast analysis, as the 95% HPD of the growth rate overlapped zero. Phylogenetic trees using MrBayes are available in figures S4 and S5).

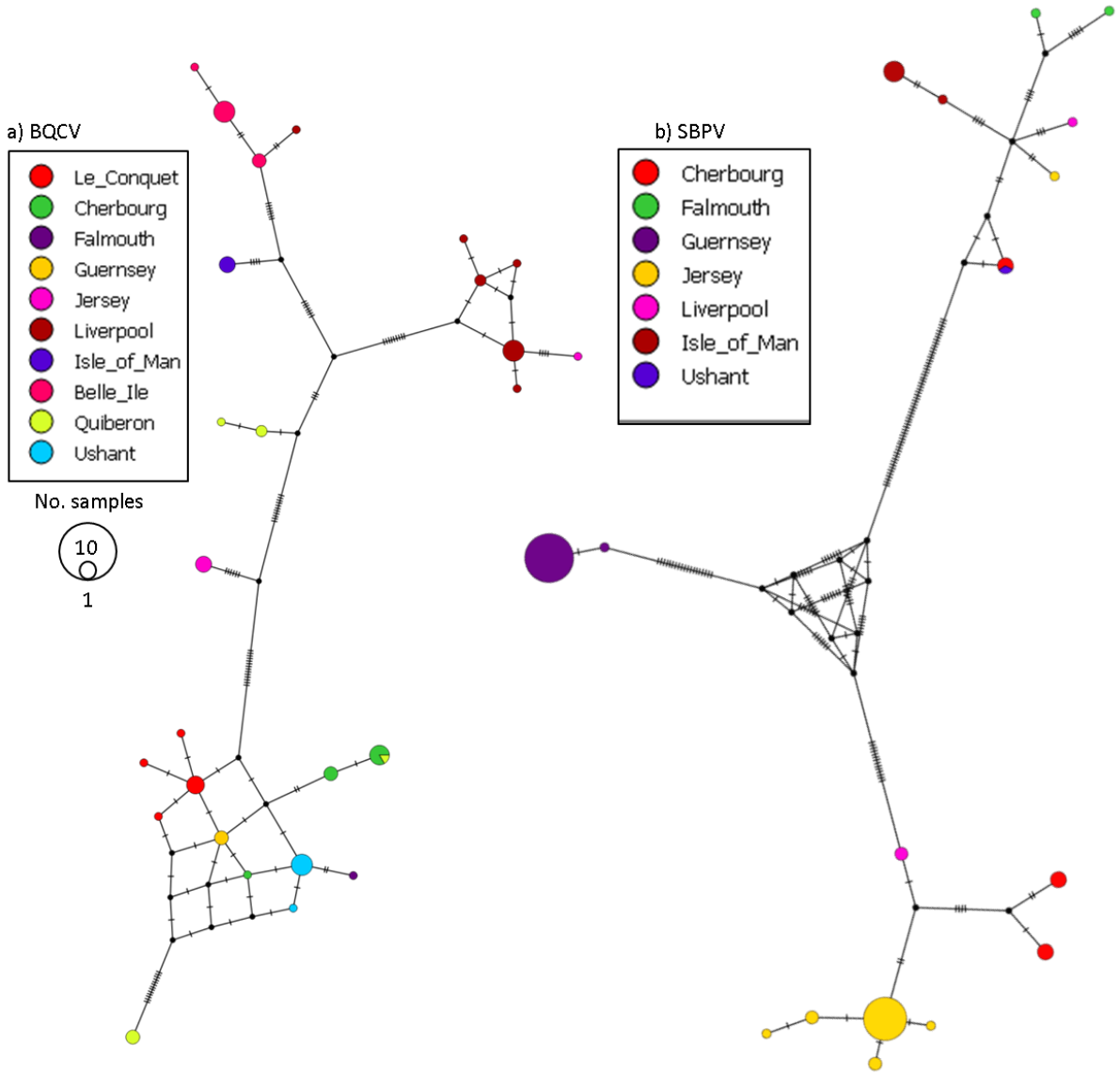


Figure 5:6: Median joining phylogenetic network of sequences from two viruses a) BQCV (N = 69) and b) SBPV (N = 78). The colours represent sampling location, the size of the node represents the number of samples with the same sequence and the dashes on branches show the number of mutations between nodes.

For BQCV and SBPV, genetic diversity was sufficiently high to estimate the routes of transmission by comparing geographic and host specific patterns in Beast. The BQCV ancestral host was identified as *A. mellifera* (state probability, P = 94%), while the SBPV

ancestral host was identified as *B. pascuorum* (state probability, P = 47%). For BQCV there is support for transmission between *A. mellifera* and *B. pascuorum* (BF = 7.91) and for transmission between *B. terrestris* and *B. pascuorum* (BF = 4.9) (table S6a). For SBPV, there is strong support for transmission between all species, but strongest support for transmission between *A. mellifera* and *B. pascuorum*, and vice-versa (BF = 30.14, BF = 28.78, respectively, table S7a). There is no clear pattern of transmission routes between locations; for example, locations nearest each other did not have the strongest support for routes of transmission (tables S6b and S7b, figure S6).

Discussion

RNA viruses, historically associated with honeybees, are now recognised to be multihost pathogens of a broad range of pollinator species (Evison et al. 2012; Fürst et al. 2014; Manley et al. 2015; McMahon et al. 2015). Our findings from the deep-sequencing of eight honeybee and bumblebee populations, and a large-scale field study focussing on viruses with different life histories confirm that RNA viruses are prevalent in wild bee populations across England and northern France and that communities are shaped by geography, not host species or the presence of *Varroa.*

We find the viral composition in *B. terrestris* mirrors that of *A. mellifera* hosts, with *B. terrestris* harbouring significantly lower level across all viruses. Further, we find the same viral variants of BQCV and SBPV circulating within sympatric *A. mellifera*, *B. terrestris* and *B. pascuorum* individuals within each location. These findings strongly suggest that these viruses, along with DWV (chapter 4 of this thesis) are continually transmitted inter-specifically across sympatric species. However, prevalence of viruses significantly varies across species and viral pathogen. *A. mellifera* has previously been implicated as the reservoir host for DWV-A and DWV–B (Fürst et al. 2014, chapter 4 of this thesis; McMahon et al. 2015), and results of this study suggest it is the reservoir host for BQCV, inferred from both prevalence data and phylogenetic analysis: BQCV is significantly more prevalent in *A. mellifera* compared to both *Bombus* species, with *A. mellifera* identified as the ancestral host. Conversely, we identified *B. pascuorum* as the ancestral host for SBPV and record the highest prevalence and titre of SBPV in *B. pascuorum*, compared to *A. mellifera* and *B. terrestris*. These differences indicate the existence of virus-specific host barriers to infection. The significantly lower prevalence of DWV and BQCV in bumblebee species compared to *A. mellifera* suggests that bumblebees are sub-optimal hosts for these viruses. The presence of

sub-optimal hosts in a multi-host system can have a dilution effect on disease transmission; or conversely, amplify transmission by increasing the abundance of both infected and susceptible hosts (Dobson 2004; Keesing et al. 2006). However, while our results suggest that *A. mellifera* is the key host for DWV and BQCV, and *B. pascuorum* is the key host for SBPV, we cannot identify the cause of these host differences, or the contribution of each host to transmission, from our data. It would be necessary to collect data on host relative abundance, transmission potentials and interspecific contact rates, all factors that will all influence the contribution of hosts to disease transmission. Further, the seasonal changes in host abundance and reproductive stage mean that pathogen prevalence and titre will vary temporally (Runckel et al. 2011); thus temporal data are necessary to acquire a true picture of the relative importance of host species to transmission.

We found that *Varroa* presence does not determine viral composition and suggest that the current viral populations are the result of long-term exposure to *Varroa.* The *Varroa* epidemic began in the middle of last century, reaching UK shores in 1992 and mainland France in the 1980s, leaving a few isolated islands *Varroa*-free. Thus, the populations in this study have been exposed to *Varroa* for over 20 years either directly (mainland sites), or indirectly (*Varroa*-free island sites) through interconnection by trade and transportation. Pathogens can spread ahead of their vector if a host can carry, replicate and transmit viruses (e.g. Squirrel pox, Tomkins et al. 2012). Therefore, finding similar viral composition on *Varroa*-free islands is likely due to transportation of infected bees and subsequent interspecific transmission. The apparent absence of viruses on two *Varroa*-free sites, Alderney and the Scilly Isles, suggests that these islands are less connected by trade and transportation.

While not impacting on viral composition, the direct presence of *Varroa* significantly increases prevalence across all viruses. Notably, we demonstrate that the presence of *Varroa* mites in honeybee populations' drive an increase in prevalence of BQCV and SBPV in honeybees, and indirectly, sympatric bumblebee hosts. The significant effect of *Varroa* on BQCV was unexpected because there is no evidence of *Varroa*-transmission or replication within *Varroa* for this virus, with only one study correlating BQCV viral load to *Varoa* infestation (Mondet et al. 2014). Further, a previous study found no effect of *Varroa* on SBPV prevalence (Martin et al. 2012), despite associations with *Varroa* transmission (Carreck et al. 2010; Santillán-Galicia et al. 2014).

Crucially, BQCV and SBPV viral load is not increased by *Varroa* presence, suggesting that *Varroa* does not increase the initial titre by replication or bioaccumulation; rather these results suggest that *Varroa* can passively transmit the virus between individuals. One possible explanation is that the natural titre is high enough to cause maximum effect without amplification by *Varroa*, but this is unlikely given the broad range of viral titres found across naturally infected individuals (10^3-10^{11}). A more likely explanation is that the indirect effects of *Varroa* infection such as immunosuppression (Nazzi et al. 2012) or simply the physiological damage from piercing the hemolymph (Bowen-Walker et al. 1999) could make *A. mellifera* more susceptible to other viruses. Moreover, the well documented pathogenic association of *Varroa* with DWV infection (Bowen-Walker et al. 1999; Evans and Schwarz 2011; Highfield et al. 2009; Martin et al. 2012) could lead to opportunistic co-infection of *A. mellifera* by other viruses. Indeed, we found that DWV-A co-infection with DWV-B, BQCV and SBPV occurred significantly more often than expected, suggesting that DWV-A infection increases the chances of infection with another virus by some mechanism. However, in models DWV-A prevalence was not determined to be a significant driver of the prevalence of other viruses.

In contrast to the low genetic variation and exponential expansion of DWV-B (chapter 4 of this thesis), BQCV and SBPV have higher genetic diversity, are highly structured by location, and show no exponential growth. Further, analysis restricted to populations from specific locations also showed no localised expansion for either virus. Although we were not able to date the common ancestor because evolutionary rates for these viruses have yet to be estimated, all evidence combined suggests that SBPV and BQCV are relatively stable infections compared to DWV-B, which we show in chapter 4 to be rapidly expanding. Interestingly, there is no clear pattern of transmission routes between locations, for example locations nearest each other did not have the strongest support for routes of transmission, which further suggests that these are long term stable virus populations with strong geographic structure, obscuring any pattern of geographic movement.

In combination, our results suggest that *Varroa* presence increases intra-specific BQCV and SBPV prevalence in *A. mellifera* by direct but passive (i.e. no direct replication or bioaccumulation of the viruses by the vector) transmission, and possibly opportunistic infection of individuals weakened by *Varroa* or *Varroa*-DWV infection; thereby, increasing

spillover to wild bumblebees resulting in increased prevalence across hosts, but not causing epidemic spread. This is in stark contrast to DWV-B, where *Varroa* dramatically increases DWV prevalence and viral loads in *A. mellifera*, leading to increased spillover of high viral loads to competent bumblebee hosts, resulting in emerging disease across hosts (chapter 4 of this thesis). It is clear that *Varroa* plays a complex role in facilitating disease emergence in wild bumblebees. Controlling the *Varroa* mite infection in managed honeybees is vital to prevent further impact of viral disease in wild bees, already under pressure from habitat loss and pesticide use.

Supplementary material

Supplementary tables

Table S1. Primers and protocols to differentiate between *Bombus terrestris/lucorum* species complex, and detect prevalence of Black queen cell virus (BQCV) and Slow bee paralysis virus (SBPV). Note *BQCV primers were used for nucleotide sequencing, while the same SBPV primers were used for both prevalence and sequencing.

Target	Primer name	Sequence	Amplification program	Amplicon (bp)	Reference
B. terrestris/ lucorum complex	BBM1_IGSF-1 BBM1_IGS_R	GGAGCAATAATTTCAATAAATAG AARTTCAAAGCACTAATCTGC	15 s at 95C 15 s at 55C 45 s at 72C x38 cycles	180 210	Regular Schmid-Hempel (*pers comm*)
BQCV	BQCV4119 BQCV5476	TCCyCCAGTTCAACCATCTA AACGTTGCCTAGrTTCGTCA	15 s at 95C, 30 s at 60C, 60 s at 72C x40 cycles	1257	Lena Wilfert (*pers comm*)
*BQCV	BQCVLF_6527 BQCVLR_7513	GCGKGCCAAAGAGAGTAAGG TTGyTGTTCAGTCCCCGAAT	Touchdown PCR 62C 15 s at 95C, 30 s at 62C, 60 s at 72C X10 cycles 15 s at 95C, 30 s at 602 → 52C, 60 s at 72C X30 cycles	986	Lena Wilfert (*pers comm*)
SBPV	SBPV_F3168 SBPV_B4193	ATGCGTGATGGTCATATTCC CGGTCGCTTGGTGAAAGTAT	Touchdown PCR 60C 15 s at 95C, 30 s at 60C, 60 s at 72C x10 cycles 15 s at 95C, 30 s at 60 → 50C, 60 s at 72C x30 cycles	955	Lena Wilfert (*pers comm*)

Table S2. Primers and protocols used for qPCR amplification and to create plasmids for standard curves used in qPCR assays.

Primer	Sequence	Amplification program	Amplicon (bp)	Reference
SBPV_F3177 SBPV_B3363	GCGCTTTAGTTCAATTGCC ATTATAGGACGTGAAAATATAC	10 s at 95C, 30 s at 60C, X40 cycles Melting curve profile: 55 – 95C (0.5C per second increments)	186	de Miranda et al. (2010b)
BQCV_F7893 BQCV_B8150	AGTGGCGGAGATGTATGC GGAGGTGAAGTGGCTATATC		257	Locke et al. (2012)
Rp49_qF Rp49_qB	AAGTTCATTCGTCACCAGAG CTTCCAGTTCCTTGACATTATG			de Miranda and Fries (2008)

Table S3. BQCV and SBPV alignment details: * note, 7 *B. lucourum* were also sequenced for SBPV but removed from other alignments because of small sample size

Virus	Genbank	Length	N total seqs	N *A.mellifera* sequences	N. *B.terrestris* sequences	N. *B pascuorum* sequences
BQCV	NC0003784	432	69	52	16	1
SBPV *	GU93876	535	78	20	19	32

Table S4. Substitution models for BQCV and SBPV alignments based on jModelTest (Darriba et al. 2012; Guindon and Gascuel 2003). Results of path sampling maximum likelihood estimator analysis comparing demographic and molecular clock models run in Beast 1.8. Analyses of each model in Tracer showed that a strict molecular clock could be excluded from both models; there was no significant exponential growth. Bold text highlights the chosen model for each alignment.

Fragment	Substitution model	Molecular clock	**Population demography**		
			Constant	Exponential	GMRFSkyride
BQCV	HKY + I + G	Strict	-1744.59	-1744.02	-1829.75
		Exponential	**-1741.92**	-1739.38	-1810.10
		Lognormal	-1744.27	-1740.74	-1801.56
SBPV	HKY + G	Strict	-1790.59	-1793.56	-1913.68
		Exponential	-1780.61	-1774.47	-1847.92
		Lognormal	**-1774.55**	-1775.58	-1787.82

Table S5. Predicted proportions (%) of pathogen prevalence for three bee species when *Varroa* is present and absent (conversion of GLMM (table 5:2) estimates on the logit scale to proportions using formula exp(x)/(1+exp(x)), where x equals the parameter estimate).

Response	Species	Prevalence when *Varroa* absent	Prevalence when *Varroa* present
BQCV	*A. mellifera*	6.78	60.61
	B. pascuorum	0.064	1.34
	B. terrestris	0.45	8.74
SBPV	All species	0.04	5.95

Table S6. Bayes factors a) support for BQCV transmission between hosts b) support for BQCV transmission between locations

a)

donor	receiver Am	Bt	Bp
Am	NA	ns	7.91
Bt	ns	NA	4.94
Bp	ns	ns	NA

b)

donor	receiver IOM	Liv	Fal	Jer	Guern	Cher	Brest	Ush	Bell	Qui
IOM	NA	250	72	ns	73	ns	1559	173	287	78
Liverpool	ns	NA	ns	ns	273	ns	ns	190	ns	333
Falmouth	283	83	NA	ns	160	348	2936	ns	301	81
Jersey	ns	ns	ns	NA	349	1150	39	72	ns	ns
Guernsey	152	71	84	69	NA	5804	ns	ns	116	120
Cherbourg	ns	ns	137	195	141	NA	ns	ns	130	ns
Brest	461	175	573	1013	ns	ns	NA	ns	75	77
Ushant	ns	591	ns	53	85	ns	544	NA	94	49
Belle Ile	129	ns	ns	101	49	ns	44	ns	NA	79
Quiberon	1544	598	69	ns	131	ns	172	87	143	NA

Table S7. Bayes factors SBPV a) support for SBPV transmission between hosts b) support for SBPV transmission between locations

a)

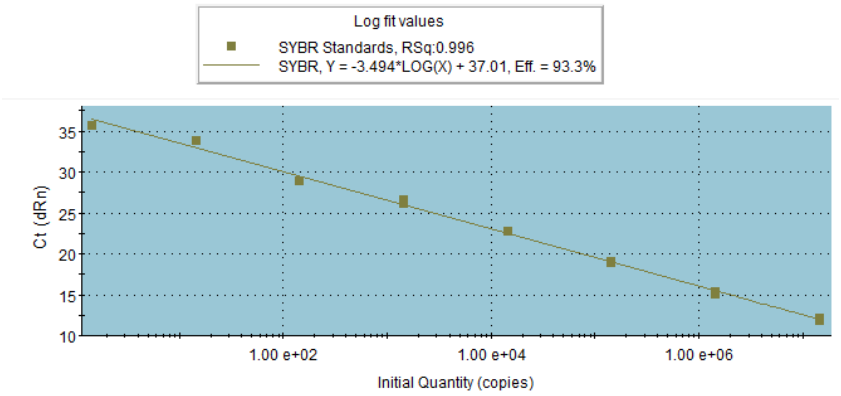
donor	receiver Am	Bt	Bl	Bp
Am	NA	9.89	ns	30.14
Bt	9.8456	NA	9.937	11.33
Bl	ns	6.95	NA	
Bp	28.78	10.705		NA

b)

donor	recipient Cherbourg	Jersey	Guernsey	IOM	Liverpool
Cherbourg	NA	61.15	39.69	ns	ns
Jersey	23.5	NA	19.72	21.54	42.17
Guernsey	27.25	no support	NA	23.54	31.24
IOM	ns	771.82	55.62	NA	ns
Liverpool	ns	ns	37.22	ns	NA

Supplementary figures

a)



b)

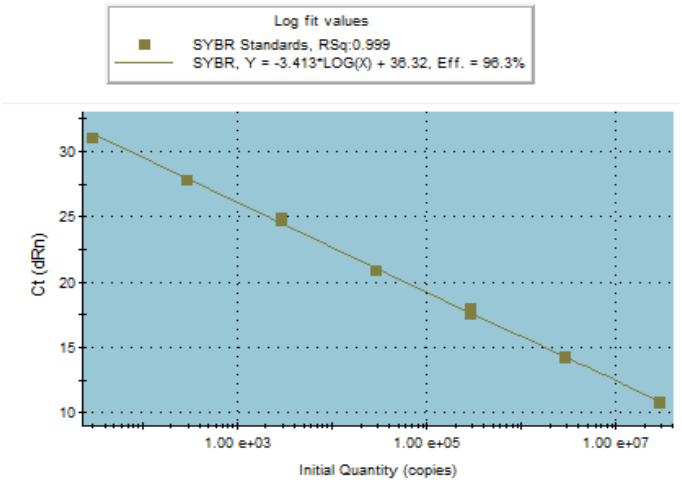


Figure S1. Standard curves used to quantify a) BQCV and B) SBPV; displaying R-squared value (RSq), curve equation and efficiency (Eff.)

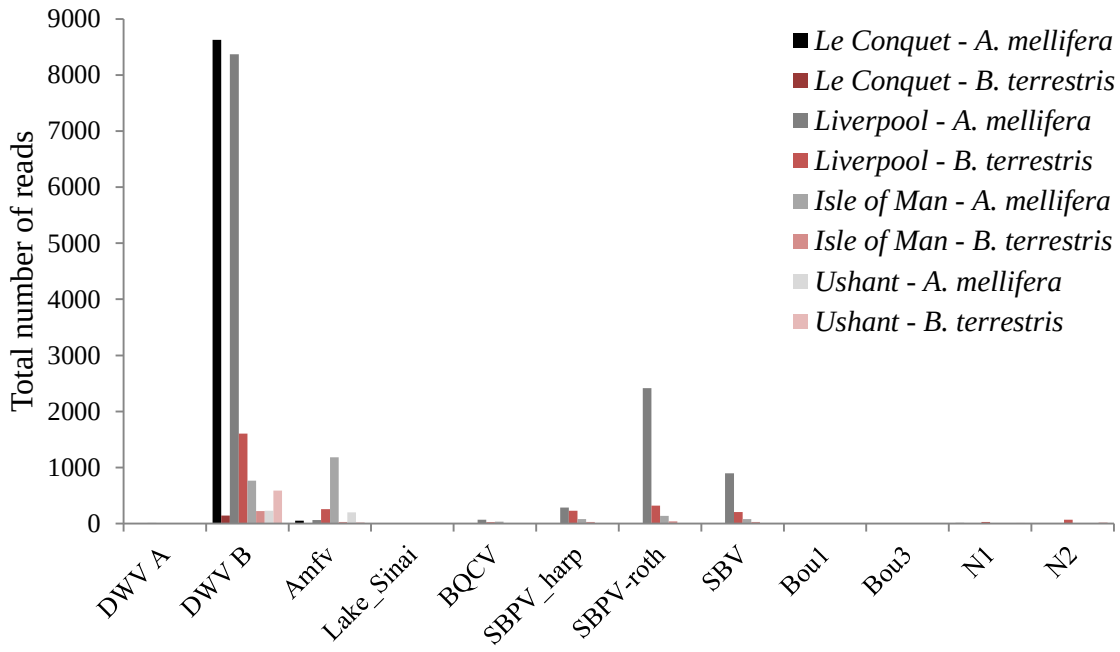


Figure S2. The number of SMRT sequencing reads that mapped against reference virus sequences (this included all sequenced 'honeybee' viruses and 23 novel bumblebee viruses. Only the viruses that had > 0 reads are displayed here. Note; Acute bee paralysis virus, Israeli acute paralysis virus, Kashmir bee virus, Aphid lethal paralysis virus and Chronic bee paralysis virus are all absent from these populations; and Bou1, Bou3, N1 and N2 are four of the new bumblebee viruses discovered by Pascal et al. (*pers comm*) present in these populations. *A. mellifera* populations are in grey scale, and *B. terrestris* populations in red scale.

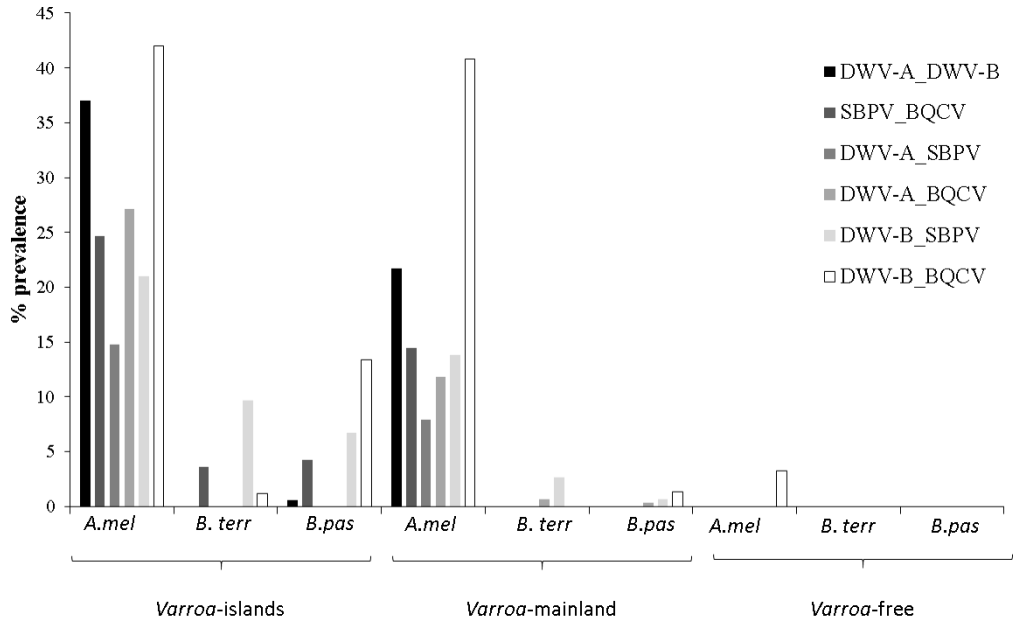


Figure S3. % prevalence of co-infections of four RNA viruses (DWV-A, DWV-B, SBPV and BQCV) by host species and *Varroa*-presence/absence (V+I = *Varroa*-present island, V+M = *Varroa*-present mainland, V- = *Varroa*-free islands.

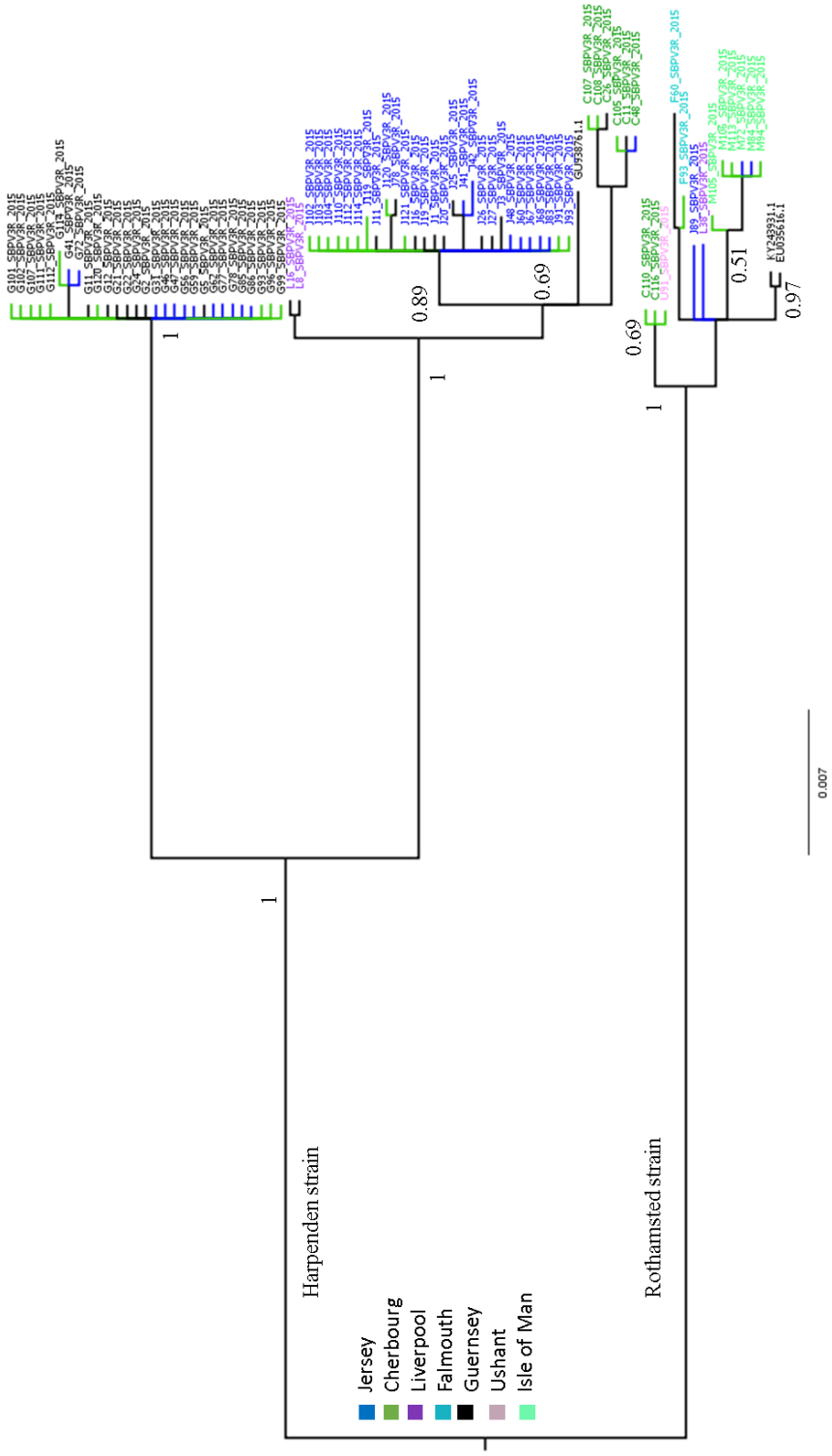


Figure S4. MrBayes phylogenetic tree for SBPV sequences showing posterior support (>0.5) for each node up until the 3rd order. The tip label colours represent locations (see key), branch colours represent species (*A. mellifera* black, *B. terrestris/lucorum* blue and *B. pascuorum* green). This three include three samples from Genbank, all isolated from *A. mellifera:* GU938761.1 (Harpenden strain), KY243931.1 and EU035616.1 (Rothamsted strain).

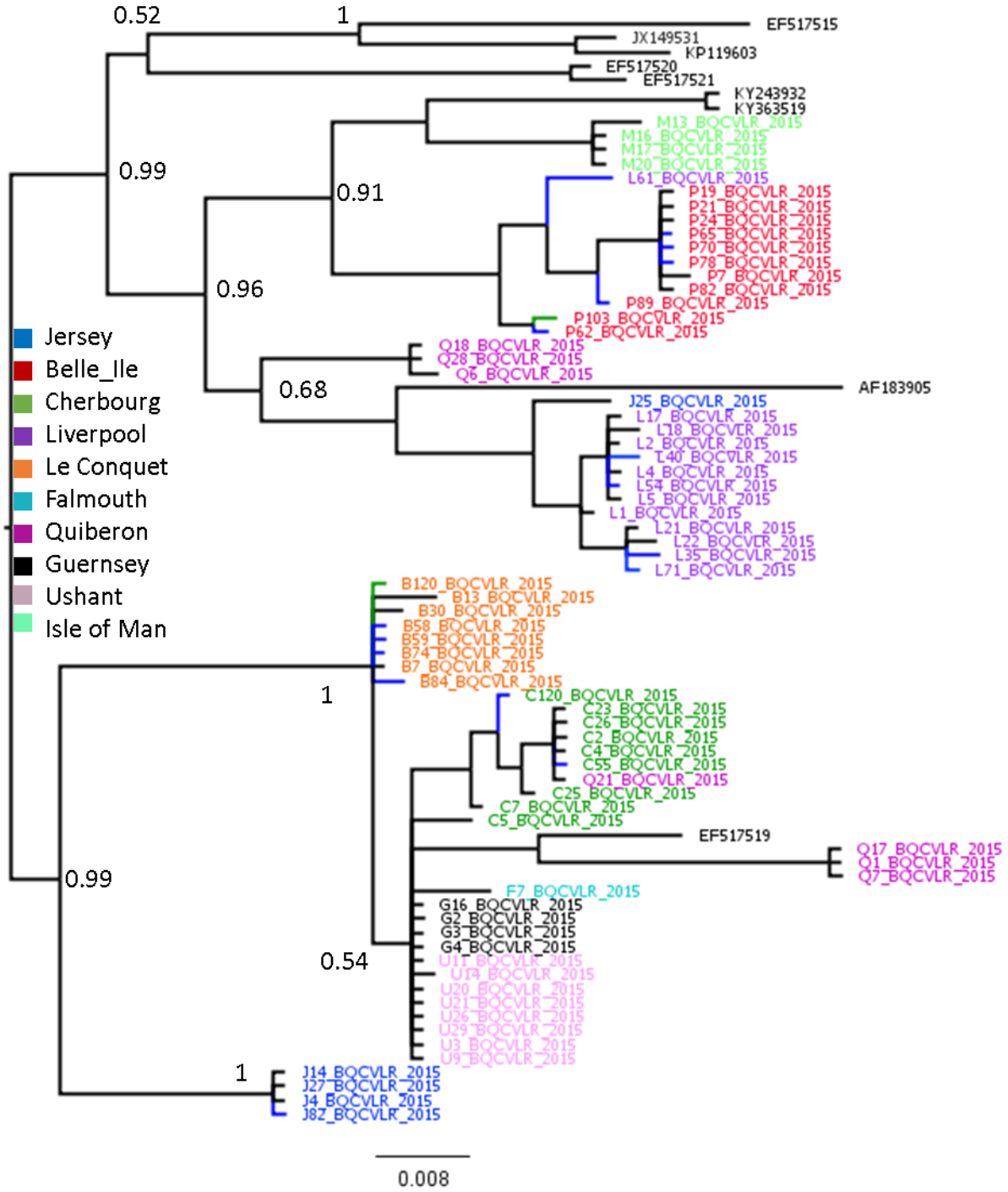


Figure S5. MrBayes phylogenetic tree for BQCV sequences showing posterior support (>0.5) for each node until the 3rd order. The tip label colours represent location, branch colours represent species (*A. mellifera* black, *B. terrestris* blue and *B. pascuorum* green). Samples include all sequenced isolates from the current study and eight Genbank sequences, all isolated from *A. mellifera*.

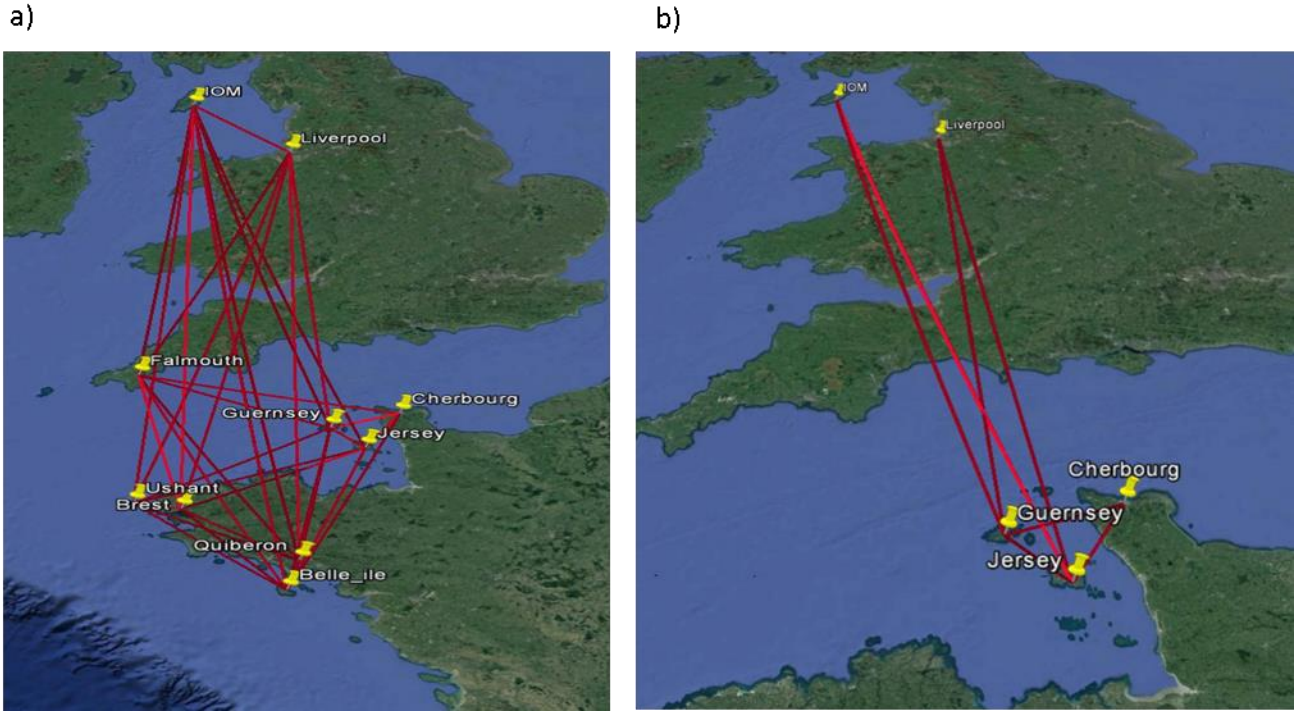


Figure S6. Map showing Bayes Factor support for BQCV (a) and SBPV (b) transmission routes between sampling locations, from Beast phylogenetic analysis

Note S1. Ruling out the island effect

To further confirm that *Varroa* presence, rather than an island effect, explained viral prevalence, we ran GLMMs on a dataset excluding *Varroa*-free sites and with island as an explanatory variable: there was no significant difference in BQCV or SBPV prevalence on *Varroa*-present islands compared to *Varroa*-present mainland (estimate ± s.e. of the fixed factor 'island/mainland' in the model = 1.03 ± 0.95, p = 0.27; 0.97±1.0, p = 0.26, respectively). In addition, we ran the same models on a dataset excluding mainland sites, here *Varroa* presence remained a significant explanatory factor of BQCV and SBPV prevalence, with higher prevalence on *Varroa*-present islands compared to *Varroa*-free islands (estimate ± s.e. of the fixed factor '*Varroa* presence in the model = 3.76 ± 1.49, p = 0.018; 7.19 ± 3.87, p = 0.063, respectively. It is notable that SBPV was found at extremely high prevalence on two *Varroa*-present islands, Guernsey and Jersey, but is absent on the Belle-Ile (the third *Varroa*-present island): however, prevalence is also high and absent on their paired mainland sites, Cherbourg and Quiberon respectively, thus this is likely a location difference rather than an island effect.

Chapter 6 : General Discussion

Emerging infectious diseases are a major threat to human health (Jones et al. 2008), ecosystem services and biodiversity, with dramatic declines in bats (Frick et al. 2010), amphibians (Lips et al. 2006) and pollinators (Cameron et al. 2011; Fürst et al. 2014) all linked to emerging pathogens. Understanding the ecological and evolutionary dynamics of viral disease emergence is fundamental to furthering our knowledge of disease epidemiology and host-pathogen co-evolution, and ultimately, for disease control. Throughout this thesis I focus on the epidemiology of multi-host RNA viruses in pollinating insects. This system enables the study of pathogen evolution and dynamics across multiple hosts. The inherent ecology and behaviours of social insects and the recent global spread of a specialist honeybee mite, *Varroa destructor*, that vectors and amplifies RNA viruses, have greatly increased the risk of viral emergence in this system.

The majority of earlier work in this field focussed on honeybees. *A. mellifera* are kept and managed at both a commercial and amateur level, thus signs of disease and their subsequent impacts are conspicuous. However, within the last decade many researchers have recognised the risks of interspecific disease transmission across the broader pollinator community. The work in this thesis contributes to this expanding field, presenting data on basic epidemiology, transmission dynamics and epidemics of RNA viruses in wild bumblebees.

Pinpointing the ecological, evolutionary and anthropogenic risks to viral disease in pollinators is fundamental to identifying gaps in our knowledge and to help optimise disease control. RNA viruses are known to be high risk pathogens because of their fast evolutionary rate and poor-error correction abilities that enables rapid adaptation to new hosts (Cleaveland et al. 2001; Taylor et al. 2001; Woolhouse and Gowtage-Sequeria 2005). The ecological traits of pollinating insects, particularly those of social bees, e.g. overlapping ranges, niches and behaviours, promote cross-species transmission of RNA viruses. While biological factors of both hosts and pathogens clearly impact on disease transmission, we find the intensive rearing of commercial honeybees and bumblebees, along with global trade and transportation, to be a key driver of disease emergence. The spread of the invasive *Varroa* mite, also caused by global trade, is the main cause of increased viral transmission in honeybees. The key knowledge gaps I identify are the scarcity of data on the pathogenicity of viruses to bumblebees; viral prevalence and infection outside of *A, mellifera*; and more broadly,

understanding the epidemiology of multiple RNA viruses in multi-host systems. I have addressed these knowledge gaps using controlled experiments, field collections, molecular and phylogenetic techniques.

Multiple RNA viruses are prevalent across the pollinator community (this thesis, Evison et al. 2012; Levitt et al. 2013; McMahon et al. 2015; Singh et al. 2010), and I find that both *A. mellifera* and *Bombus* hosts share the same viruses and viral variants, indicating that host barriers are not a limiting factor to virus spillover and cross-species transmission is pervasive. Instead, viral composition, diversity and genetic variation are determined by geographic location. I have shown that DWV-B is the dominant virus across populations. Interestingly, DWV-A (the variant responsible for the current worldwide epidemic (Martin et al. 2012; Wilfert et al. 2016)) is rare in these populations. The timeline of prevalence data from the field surveys in 2009 (Wilfert et al. 2016) and 2011 (McMahon et al. 2016) and the current study collected in 2015, combined with phylogenetic reconstructions of the 2015 sequences, strongly suggest that DWV-B is a currently emerging viral variant and is exponentially expanding across the UK and Europe. In contrast, all evidence suggests that SBPV and BQCV populations are relatively stable infections, showing higher genetic variation and strong structuring by location. We were able to date the divergence of DWV-B from its common ancestor using the evolutionary rate previously estimated for DWV-A using sequence data across time (Mordecai et al. 2015). For SBPV and BQCV, because the viral populations are highly structured by location, it would be necessary to collect localised long term data from multiple locations to accurately estimate the rate of evolution; data that is currently not available.

I used next generation Pacbio single-molecule real-time (SMRT) sequencing combined with Sanger sequencing to generate genetic data for phylogenetic and evolutionary analysis. SMRT sequencing enables the detection of full RNA viral assemblage, without bias towards known viruses from PCR and Sanger sequencing. SMRT sequencing also has the potential to sequence full viral genomes, without the need to reassemble genomes; this is particularly useful for the study of rapidly evolving, genetically variable RNA virus populations (e.g. it has been used to characterise Hepatitis C virus populations, (Ho et al. 2016)) and may be a useful tool to study recombination. Recombination in DWV has been well documented (Dalmon et al. 2017; Moore et al. 2011), with potential impacts on virulence and epidemiology (Mordecai et al. 2015; Ryabov et al. 2014). Recombination is rare in my

foraging populations of honeybees, and absent from *B. terrestris* populations. I find points of recombination in the 5' *lp*-gene (from Sanger sequencing) and the 3' *rdrp*-gene (from SMRT sequencing – note, SMRT sequence reads did not cover the *lp*-gene region so we were unable to compare recombination rate or breakpoints), at different break points to previous studies (Dalmon et al. 2017; Moore et al. 2011; Ryabov et al. 2014). However, SMRT sequencing has its own inherent biases depending on the library prep. For example, we sequenced a maximum of ~6000bp (of viruses that are ~ 10,000bp long), with a bias from the 3' end due to the Clontech technology that uses the polyA tail to create a cDNA library. Further, the error rate is high and chimeric sequences can be produced during library prep, thus limiting the detection of single-base mutations and true recombination.

Significantly, the presence of *Varroa* had no effect on viral composition, diversity or genetic variation. I expected to see higher viral variant diversity on *Varroa*-free sites, based on a previous study that showed the arrival of *Varroa* in Hawaii reduced a formerly highly diverse DWV community to a single DWV-A variant (Martin et al. 2012). However, the pollinator populations from my *Varroa*-present sites have endured over 20 years of *Varroa* infestation; while *Varroa*-free sites would have felt the impact indirectly over the years through spillover via trade, travel, deliberate and accidental transportation, and possibly migration, of infected competent pollinator hosts across these highly connected locations. In contrast, Hawaii is extremely isolated and data were collected over the first few years of *Varroa* invasion; and by year three post-infestation, diversity had already been reduced to a single strain (Martin et al. 2012).

A novel and significant finding is that the presence of *Varroa* increases the prevalence of RNA viruses in honeybees, but also sympatric wild bumblebees. By piercing the hemolymph of developing honeybee pupae, *Varroa* not only cause physiological damage, but also provides a new route of infection for viruses that are currently circulating via established routes of transmission (Genersch 2010; Martin 2001; Sumpter and Martin 2004); i.e. social behaviours within colonies, such as trophallaxis, brood care, grooming and hygienic removal of infected bees, enable both direct and indirect transmission to occur (Cremer et al. 2007). The association between DWV and *Varroa* and subsequent negative impacts on *A. mellifera* are well documented (Genersch 2010; Highfield et al. 2009; Martin et al. 2012; Ryabov et al. 2014; Sumpter and Martin 2004) and I find the expected increase in prevalence and titre here. However, it is interesting that *Varroa* also increases the prevalence of BQCV – a virus with

no known association with *Varroa* transmission (except, Mondet et al. (2014) describe a weak correlation between BQCV titre and *Varroa* infestation rates), and SBPV – a virus that has been linked with *Varroa* (Carreck et al. 2010; Santillán-Galicia et al. 2014) but is also associated with higher prevalence in bumblebees (McMahon et al. 2015 and this thesis). *Varroa* has no impact on viral variants in this study, but significantly increases prevalence of all viruses irrespective of an active or passive association with transmission by *Varroa;* thus, I suggest that rather than selection on the virus, transmission by *Varroa* turns *A. mellifera* into a 'superspreader' species i.e. *Varroa* increases intra-specific transmission of viruses leading to increased spillover to wild bumblebees (figure 6:1).

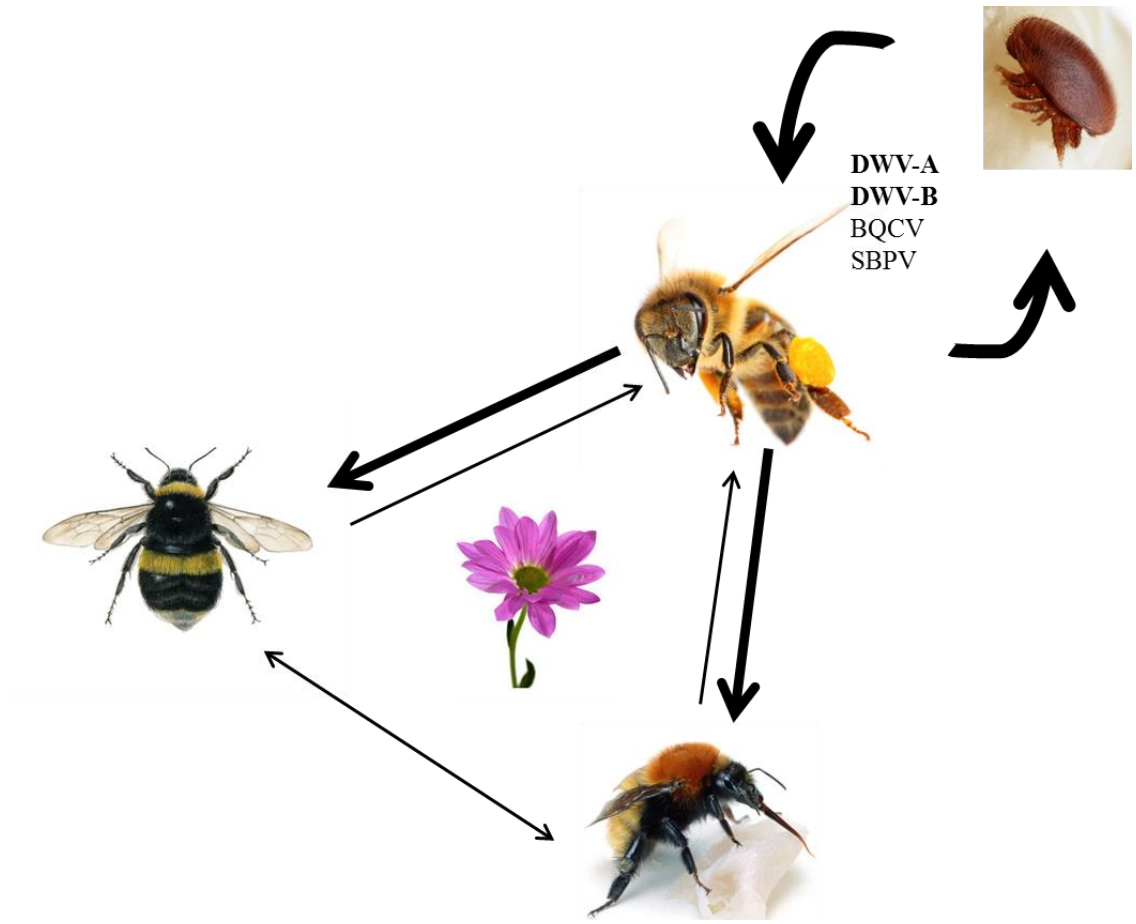


Figure 6:1: *A. mellifera* become 'superspreaders' of viral diseases in association with *Varroa* infestation. *Varroa* increases intra-specific viral transmission within colonies, increasing spillover to sympatric wild bumblebees

I also find *Varroa* increases titre in honeybees and sympatric bumblebees for DWV-B (note DWV-A could not be tested directly because it was absent on *Varroa*-free sites), but not

SBPV and BQCV; strongly implying that the viruses differ in their association with *Varroa*. *Varroa* increases DWV-B during transmission to *A. mellifera*, however, there is currently not enough evidence to confirm if amplification occurs by replication within the *Varroa* mite (Gisder et al. 2009; Ryabov et al. 2014), or by passive accumulation of viral particles during blood feeding (Erban et al. 2015), as can be the case for Norovirus in shellfish (Maalouf et al. 2011).

Within a multi-host-pathogen system, hosts differ in their contribution to transmission (Streicker et al. 2013), which has implications for the risk of disease emergence. I find differences in prevalence and titre across pollinator species – the pattern of which differs across viruses - suggesting that some hosts are more susceptible, or conversely, more tolerant, to infection than others. Deformed wing virus (type A and B) and BQCV are more prevalent with high titres in *A. mellifera*, while SBPV is more prevalent with high titres in *B. pascuorum*. These data support previous field studies (Fürst et al. 2014; McMahon et al. 2015; McMahon et al. 2016). However, caution must be taken when inferring direction of transmission or source/sink dynamics based on these data: viral prevalence and titre are known to vary temporally (Runckel et al. 2011) because of phenological traits such as the relative abundance at each reproductive stage in bumblebees to sympatric honeybees. The pollinator community is broad and even relatively rare species that are active for a short period of time that can harbour viruses (e.g. hoverflies and species of solitary bee) could be particularly effective at spreading disease; inherently, these are the less well studied species (e.g. the relatively rare but competent host American robin was identified as a 'superspreader' of West Nile virus (Kilpatrick et al. 2006)). Thus it is important to sample across all present host species, across locations and across time. Phylogenetic trait models in Beast combined with Bayes factor analysis can determine support for direction of transmission between hosts; however it is essential to sample evenly across the potentially extensive host populations, including rare species. My field survey was designed to sample equally across three host species to enable phylogenetic reconstruction of the host associations and the reconstruction of epidemics; and also to directly test the effect of *Varroa* across pollinator species. My data powerfully suggest that *A. mellifera* in combination with *Varroa* is the major cause of viral disease spread. However, we do lack the host abundance data to determine their relative contribution to disease transmission, and the temporal resolution required to test whether this picture remains stable throughout the year, or if some host species are of greater importance at certain times of year.

Critically, all these findings are only relevant if viruses cause harm to their non-*Apis* hosts. Controlled infection studies are sorely needed to answer basic questions on the impacts of viruses on non-*Apis* hosts. We know that DWV-A reduces longevity in bumblebees (Fürst et al. 2014); while DWV-B is more virulent in honeybees (McMahon et al. 2016), it is not yet known if this is the case for bumblebees. However, this is concerning given the dominance of this variant in our viral populations, specifically across bumblebee hosts. In addition, a study by Meeus et al. (2014) found that KBV and IAPV negatively impacted colony set up and fecundity in *B. terrestris*. Here, I provide the first evidence that SBPV is pathogenic to a bumblebee host (*B. terrestris*). In a controlled infection study, I find that *B. terrestris* infected with SBPV were 1.6 times more likely to die than uninfected bees at a given time point, but importantly this increased virulence was only seen under conditions of nutritional stress. This has clear implications for virulence in wild bees, as they face external stresses such as nutritional stress through bad weather or lack of forage, pesticides, predation and co-infection with other parasites. From my field study, SBPV prevalence was relatively low in *B. terrestris* compared to *B. pascuorum,* and McMahon et al. (2015) found highest prevalence in *B. horturum*, implying that the effect on longevity may be greater on other bumblebee species. However, there is a possibility that *B. pascuorum* and *B. horturum* are more tolerant to SBPV, while *B. terrestris* is more susceptible; in this case highly infected *B. terrestris* individuals might be excluded from field collections if they are unable to forage. This highlights the need for further studies on the pathogenicity of multiple viruses and their variants, across multiple host species and a range of viral loads.

The research within this thesis raises further questions, both for pollinator conservation and for understanding epidemiology of multi-host pathogens that can be addressed using this system:

What are the true infection levels across honeybees and bumblebees?

Social bees live in colonies, where intra-specific transmission occurs; thus analysing infection levels across randomly collected foragers with unknown ancestry could overestimate rates of interspecific transmission. Data on the relatedness of collected foragers is an important additional aspect of epidemiology that would greatly add to current prevalence data. For polyandrous honeybees matriline composition can be determined through direct sequencing of the mitochondrial CO1-CO11 region. It is possible to reconstruct colony composition for monandrous bumblebees using multiplexed microsatellite analysis (Lepais et al. 2010). As

well as confirming true infection levels, this will further our knowledge of the genetic diversity and population structure of bumblebee populations. This would benefit both pollinator conservation and increase our understanding of epidemiology across bumblebee hosts.

How does the transmission potential vary across pollinator hosts?

In a multi-host system, transmission potential will vary between hosts and determine if cross-species disease emergence is a risk (Haydon 2008). Prevalence and viral load vary across pollinator hosts, with the key host identified as *A. mellifera* for DWV and BQCV; it is less clear for SBPV, but evidence suggests a bumblebee (*B. horturum* or *B. pascuorum*) may be a key host for this virus. However, the factors behind this heterogeneity and the contribution of each host to transmission in this system are not currently known, but are important for effective disease control. Below I identify such factors and suggest means to test them:

- Host resistance: DWV and BQCV are far less prevalent in bumblebee hosts compared to *A. mellifera*. However, there is no evidence to confirm if species with low prevalence of a virus are more tolerant or more susceptible. Transmission potential of species can be estimate by measuring viral load. Yet, field studies have the potential for bias if highly infectious individuals avoid collection because they are unable to forage due to behavioural changes or increased mortality. Thus, experimental infection and transmission studies are necessary to identify transmission potential of each host species.
- Host abundance: A highly abundant host can often equate to greater transmission potential, but that assumption does not always hold true (e.g. the American robin is a rare yet highly competent spreader of West Nile Virus (Kilpatrick et al. 2006). Within the pollinator community there are species that are common throughout the year, but also those that are rare or active for a short time (e.g. solitary bees) that could be particularly effective at spreading disease. However, abundance of all present host species is essential data, in combination with transmission potential data, to determine hosts contribution to disease transmission.
- Host exposure: Contact between hosts will influence transmission rates. Observational field and semi-natural field experiments could identify foraging networks, generalist/specialist flower feeders, and lag time between pollinator visits. As well as measuring the effect of flower complexity, pollinator flower handling time and the

production of floral secondary components are all factors that are likely to influence transmission (McArt et al. 2014). As contact rates do not necessarily equate to transmission, this must be combined with the direct measures of transmission potential mentioned above.

- Behavioural changes: There is a possibility that viral infection can influence pollinator behaviour, and further, that these behaviours may vary depending on level of infection. For example, transmission potential of hosts could be altered by sub-lethal impacts of infection, such as reduced queen fecundity, foraging behaviours, longevity of workers and social worker behaviours. Infection experiments at an individual and colony level, in the laboratory and in semi-natural conditions, could measure these effects.
- Temporal change: Across host species there will be seasonal changes in abundance and in population composition e.g. the proportion of workers to sexuals in social bees, and thus, host transmission potential will change temporally. Sampling at different time points throughout the year, specifically when the different life stages are present (i.e. bumblebee queens in spring, workers throughout the colony lifecycle and males towards the end of colony life), is necessary to make sure the data represent more than just a snapshot in time.

In summary, I find that wild bumblebees are at high risk of disease transmission from managed honeybees, particularly in combination with *Varroa* mite infestation. Interspecific transmission across pollinators is pervasive, with the same viral variants crossing species. I show that DWV-B, a virulent variant, is amplified by *Varroa* and is currently emerging across the UK and Europe in both honeybees and wild bumblebees. Other RNA viruses, SBPV and BQCV, appear to be stable but their prevalence in honeybees and wild bumblebees is also increased by the presence of *Varroa*. Further, I provide the first evidence of SBPV pathogenicity to a bumblebee host, suggesting that increased spillover of this virus could impact wild bumblebees at a population level. Overall, a specialist vector can increase the prevalence of multi-host viruses, influencing disease emergence across the host community. This novel contribution to the field of multi-host pathogen dynamics further highlights the current risk of multi-viral pathogen spillover from managed honeybees to wild pollinators. Both commercial and wild pollinators contribute to pollination services, but managed honeybees, in combination with the invasive *Varroa* mite, represent a

disproportional threat to the system. It is clear from this thesis that further disease control measures are needed to protect pollination services and conserve wild pollinators.

References

Albrecht M, Schmid B, Hautier Y, Müller CB (2012) Diverse pollinator communities enhance plant reproductive success. Proceedings of the Royal Society of London Series B 279:4845-4852. doi: 10.1098/rspb.2012.1621

Alizon S, Fraser C (2013) Within-host and between-host evolutionary rates across the HIV-1 genome. Retrovirology 10:49. doi: 10.1186/1742-4690-10-49

Anderson DL, East IJ (2008) The latest buzz about colony collapse disorder. Science 319:724-725

Anderson RM et al. (1996) Transmission dynamics and epidemiology of BSE in British cattle. Nature 382:779-788

Anderson RM, May RM (1981) The population dynamics of microparasites and their invertebrate hosts. Philosophical Transactions of the Royal Society of London B 291:451-524

Anderson RM, May RM (1982) Coevolution of hosts and parasites. Parasitology 85:411-426

Arbetman MP, Gleiser G, Morales CL, Williams P, Aizen MA (2017) Global decline of bumblebees is phylogenetically structured and inversely related to species range size and pathogen incidence. Proceedings of the Royal Society B 284. doi: 10.1098/rspb.2017.0204

Bailey L (1982) Viruses of honeybees. Bee World 63:165-173

Bailey L, Ball B, Perry JN (1983) Honeybee paralysis: Its natural spread and its diminished incidence in England and Wales. Journal of Apicultural Research 22:191-195

Bailey L, Fernando EF (1972) Effects of Sacbrood Virus on adult honey bees. Annals of Applied Biology 72:27-&. doi: 10.1111/j.1744-7348.1972.tb01268.x

Bailey L, Gibbs AJ (1964) Acute infection of bees with paralysis virus. Journal of Insect Pathology 6:395-407

Bailey L, Gibbs AJ, Woods RD (1963) Two viruses from adult honey bees (*Apis mellifera* Linnaeus). Virology 21:390-395

Bailey L, Woods RD (1974) Three previously undescribed viruses from the honey bee. Journal of General Virology 25:175-186

Bailey L, Woods RD (1977) 2 more small RNA viruses from honey bees and further observations on Sacbrood and Acute Bee Paralysis Viruses. Journal of General Virology 37:175-182. doi: 10.1099/0022-1317-37-1-175

Barribeau SM et al. (2015) A depauperate immune repertoire precedes evolution of sociality in bees. Genome Biology 16. doi: 10.1186/s13059-015-0628-y

Bates D, Sarkar D (2006) lme4: Linear mixed models using S4 classes. R package version 0.99875-8. doi: http://CRAN.R-project.org/doc/Rnews/

Berényi O, Bakonyi T, Derakhshifar I, Koglberger H, Nowotny N (2006) Occurrence of six honeybee viruses in diseased Austrian apiaries. Applied and Environmental Microbiology 72:2414-2420

Berényi O et al. (2007) Phylogenetic analysis of deformed wing virus genotypes from diverse geographic origins indicates recent global distribution of the virus. Applied and Environmental Microbiology 73:3605-3611

Bielejec F, Rambaut A, Suchard MA, Lemey P (2011) SPREAD: Spatial phylogenetic reconstruction of evolutionary dynamics. Bioinformatics 27:2910-2912

Biesmeijer JC et al. (2006) Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. Science 313:351-354

Blaker H (2000) Confidence curves and improved exact confidence intervals for discrete distributions. Canadian Journal of Statistics 28:783-798

Boots M, Sasaki A (2002) Parsite-driven extinction in spatially explicit host-parsite systems. The American Naturalist 159:706-713

Bowen-Walker PL, Martin SJ, Gunn A (1999) The transmission of deformed wing virus between honeybees (*Apis mellifera* L.) by the ectoparasitic mite *Varroa jacobsoni* Oud. Journal of Invertebrate Pathology 73:101-106

Breeze TD, Bailey AP, Balcombe KG, Potts SG (2011) Pollination services in the UK: How important are honeybees? Agriculture, Ecosystems and Environment 142:137-143

Bull JC (1994) The evolution of virulence. Evolution 48:1423-1437
Camacho C et al. (2008) BLAST+: architecture and applications. BMC Bioinformatics 10:421
Cameron SA et al. (2011) Patterns of widespread decline in North American bumble bees. PNAS 108:662-667
Cardinale BJ et al. (2012) Biodiversity loss and its impact on humanity. Nature 486:59-67
Carreck NL, Ball BV, Martin SJ (2010) Honey bee colony collapse and changes in viral prevalence associated with *Varroa destructor*. Journal of Apicultural Research 49:93-94
Celle O et al. (2008) Detection of Chronic Bee Paralysis Virus (CBPV) genome and its replicative RNA form in various hosts and possible ways of spread. Virus. Res. 133:280-284
Chare ER, Holmes EC (2004) Selection pressures in the capsid genes of plant RNA viruses reflect mode of transmission. Journal of General Virology 85:3149-3157
Chen Y, Pettis J, Collins A, Feldlaufer M (2006a) Prevalance and transmission of honeybee viruses. Applied and Environmental Microbiology 72:606-611
Chen Y, Pettis JS, Evans JD, Kramer M, Feldlaufer M (2004) Transmission of Kashmir bee virus by the ectoparasitic mite *Varroa destructor*. Apidologie 35:441-448
Chen YP, Evans J, Feldlaufer M (2006b) Horizontal and vertical transmission of viruses in the honey bee, *Apis mellifera*. Journal of Invertebrate Pathology 92:152-159
Cleaveland S, Laurenson MK, Taylor LH (2001) Disease of humans and their domestic mammals: pathogen characteristics, host range and the risk of emergence. Philosophical transactions of the Royal Society of London Series B 356:991-999
Colla SR, Otterstatter MC, Gegear RJ, Thomson JD (2006) Plight of the bumble bee: Pathogen spillover from commercial to wild populations. Biological Conservation 129:461-467
Colla SR, Packer L (2008) Evidence for decline in eastern North American bumblebees (Hymenoptera: Apidae), with special focus on *Bombus affinis* Cresson. Biodiversity and Conservation 17:1379-1391
Cox-Foster DL (2007) A metagenomic survey of microbes in honey bee colony collapse disorder. Science 318:283-287
Cremer S, Armitage SAO, Schmid-Hempel P (2007) Social Immunity. Current Biology 17:R693-702
Dainat B, Evans DJ, Chen Y, Gauthier L, Neumann P (2012) Dead or alive: Deformed wing virus and *Varroa destructor* reduce the life span of winter honeybees. Applied and Environmental Microbiology 78:981-987
Dalmon A et al. (2017) Evidence for positive selection and recombination hotspots in Deformed wing virus (DWV). Scientific Reports 7:41045. doi: 10.1038/srep41045
Darriba D, Taboada GL, Doallo R, Posada D (2012) jModelTest 2: more models, new heuristics and parallel computing. . Nature Methods 9:772. doi: 10.1038/nmeth.2109
Daszak P, Cunningham AA, Hyatt AD (2000) Emerging infectious diseases of wildlife - Threats to biodiversity and human health. Science 287:443-449
de Miranda JR, Cordoni G, Budge G (2010a) The Acute bee paralysis virus-Kashmir bee virus-Israeli acute paralysis virus complex. Journal of Invertebrate Pathology 103:S30-S47. doi: 10.1016/j.jip.2009.06.014
de Miranda JR et al. (2010b) Genetic characterization of Slow Bee Paralysis Virus of the honeybee (*Apis mellifera* L.). Journal of General Virology 91:2524-2530
de Miranda JR et al. (2004) Complete nucleotide sequence of Kashmir bee virus and comparison with acute bee paralysis virus. Journal of General Virology 85:2263-2270
de Miranda JR, Fries I (2008) Venereal and vertical transmission of Deformed Wing Virus in honeybees (*Apis mellifera* L.). Journal of Invertebrate Pathology 98:184-189
de Miranda JR, Genersch E (2010) Deformed Wing Virus. Journal of Invertebrate Pathology 103:S48-S61
Di Prisco G et al. (2013) Neonicotinoid clothianidin adversely affects insect immunity and promotes replication of a viral pathogen in honey bees. PNAS 110:18466-18471. doi: 10.1073/pnas.1314923110

Di Prisco G, Pennacchio F, Caprio E, Boncristiani Jr HF, Evans J, Chen Y (2011) *Varroa destructor* is an effective vector of Israeli acute paralysis virus is the honeybee, *Apis mellifera*. Journal of General Virology 92:151-155

Dobson A (2004) Population dynamics of pathogens with multiple host species. The American Naturalist 164:S64-S78

Drummond AJ, Bouckaert RR (2015) Bayesian Evolutionary Analysis with BEAST. Cambridge University Press

Drummond AJ, Rambaut A (2007) BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evolutionary Biology 7:214. doi: 10.1186/1471-2148-7-214

Durrer S, Schmid-Hempel P (1994) Shared use of flowers leads to horizontal pathogen transmission. Proceedings of the Royal Society of London Series B 258:299-302

Dwyer G, Levin SA, Buttel L (1990) A simulation model of the population dynamics and evolution of myxomatosis. Ecological Monographs 60:423-447

Ebert D, Herre EA (1996) The evolution of parastic diseases. Parisitology Today 12:96-101

Erban T et al. (2015) In-depth proteomic analysis of Varroa destructor: Detection of DWV-complex, ABPV, VdMLV and honeybee proteins in the mite. Scientific Reports 5:13907. doi: 10.1038/srep13907 pmid:26358842

Evans DJ, Spivak M (2010) Socialized medicine: Individual and communal disease barriers in honey bees. Journal of Invertebrate Pathology 103:S62-S72

Evans JD, Schwarz RS (2011) Bees brought to their knees: microbes affecting honey bee health. Trends in Microbiology 19:614-620

Evison SEF et al. (2012) Pervasiveness of parasites in pollinators. PLoS ONE 7:e30641. doi: 10.1371/journal.pone.0030641

Frick WF et al. (2010) An emerging disease causes regional population collapse of a common North American bat species. Science 329:679-682

Fries I (2010) *Nosema ceranae* in European honeybees (*Apis mellifera*). Journal of Invertebrate Pathology 103:S73-79

Fries I et al. (2013) Standard methods for *Nosema* research. Journal of Apicultural Research 52:1-28

Fujiyuki T et al. (2004) Novel insect picorna-like virus identified in the brains of aggressive worker honeybees. Journal of Virology 78:1093-1100

Fürst MA, McMahon DP, Osborne JL, Paxton RJ, Brown MJF (2014) Disease associations between honeybees and bumblebees as a threat to wild pollinators. Nature 506:364-366. doi: 10.1038/nature12977

Gallai N, Salles J, Settele J, Vaissière BE (2009) Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. Ecological Economics 68:810-821

Garibaldi LA et al. (2013) Wild pollinators enhance fruit set of crops regardless of honey bee abundance. Science 339:1608-1611. doi: 10.1126/science.1230200

Gauthier L et al. (2015) The Apis mellifera filamentous virus genome. Viruses 7:3798-3815. doi: 10.3390/v7072798

Gauthier L et al. (2007) Viral load estimation in asymptomatic honey bee colonies using the quantitative RT-PCR technique. Apidologie 38:426-435

Genersch E (2005) Development of a rapid and sensitive RT-PCR method for the detection of deformed wing virus, a pathogen of the honeybee (*Apis mellifera*). The Veterinary Journal 169:121-123

Genersch E (2010) Honey bee pathology: Current threats to honey bees and beekeeping. Applied Microbiology and Biotechnology 87:87-97

Genersch E, Aubert M (2010) Emerging and re-emerging viruses of the honey bee (*Apis mellifera* L.). Veterinary Research 41. doi: 10.1051/vetres/2010027

Genersch E et al. (2010) The German bee monitoring project: a long term study to understand periodically high winter losses of honey bee colonies. Apidologie 41:332-352. doi: 10.1051/apido/2010014

Genersch E, Yue C, Fries I, De Miranda JR (2006) Detection of Deformed Wing Virus, a honey bee viral pathogen, in bumble bees (*Bombus terrestris* and *Bombus pascuorum*) with wing deformities. Journal of Invertebrate Pathology 91:61-63

Ghosh RC, Ball BV, Willcocks MM, Carter MJ (1999) The nucleotide sequence of Sacbrood Virus of the honey bee: an insect picorna-like virus. Journal of General Virology 80:1541-1549

Gibbs MJ, Armstrong JS, Gibbs AJ (2000) Sister-Scanning: a Monte Carlo procedure for assessing signals in recombinant sequences. Bioinformatics 16:573-582

Gisder S, Aumeier P, Genersch E (2009) Deformed wing virus: replication and viral load in mites (*Varroa destructor*). Journal of General Virology 90:463-467

Goulson D, Nicholls E, Botías C, Rotheray EL (2015) Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. Science 347. doi: 10.1126/science.1255957

Gray SM, Banerjee N (1999) Mechanisms of arthropod transmission of plant and animal viruses. Microbiology and Molecular Biology Reviews 63:128-148

Graystock P, Goulson D, Hughes WOH (2015) Parasites in bloom: flowers aid dispersal and transmission of pollinator parasites within and between bee species. Proceedings of the Royal Society B 282. doi: 10.1098/rspb.2015.1371

Graystock P, Yates K, Darvill B, Goulson D, Hughes WOH (2013a) Emerging dangers: deadly effects of an emergent parasite in a new pollinator host. Journal of Invertebrate Pathology 114:114-119

Graystock P, Yates K, Evison SEF, Darvill B, Goulson D, Hughes WOH (2013b) The Trojan hives: pollinator pathogens, imported and distributed in bumblebee colonies. Journal of Applied Ecology 50:1207-1215

Guindon S, Gascuel O (2003) A simple, fast and accurate method to estimate large phylogenies by maximum-likelihood. Systematic Biology 52:696-704

Harrison XA (2014) Using observation-level random effects to model overdispersion in count data in ecology and evolution. Peer J 2. doi: 10.7717/peerj.616

Haydon DT (2008) Cross-disciplinary demands of multihost pathogens. Journal of Animal Ecology 77:1079-1081

Haydon DT, Cleaveland S, Taylor LH, Laurenson MK (2002) Identifying reservoirs of infection: a conceptual and practical challenge. Biol Letters 8:1468-1473

Higes M, Martin R, Meana A (2006) *Nosema ceranae* in Europe: an emergent type C nosemosis. Apidologie 41:375-392

Highfield AC et al. (2009) Deformed wing virus implicated in overwintering honeybee colony losses. Applied and Environmental Microbiology 75:7212-7220

Ho CKY et al. (2016) Characterization of Hepatitis C virus (HCV) envelope diversification from acute to chronic infection within a sexually transmitted HCV cluster by using single-molecule, real-time sequencing. Journal of Virology 91:e02262-02216. doi: 10.1128/JVI.02262-16

Holmes EC (2008) Evolutionary history and phylogeography of human viruses. Annual Review of Microbiology 62:307-328

Hudson RR (2000) A new statistic for detecting genetic differentiation. Genetics 155:2011-2104

Hudson RR, Boos DD, Kaplan NL (1992) A statistical test for detecting geographic subdivision. Molecular Biology and Evolution 9:138-151

Hung ACF (2000) PCR detection of Kashmir bee virus in honey bee excreta. Journal of Apicultural Research 39:103-106

Jenkins GM, Rambaut A, Pybus OG, Holmes EC (2002) Rates of molecular evolution in RNA viruses: a quantitative phylogenetic analysis. Journal of Molecular Evolution 54:156-165

Jones KE et al. (2008) Global trends in emerging infectious diseases. Nature 451:990-994

Karre M et al. (2009) Rabies control in rural Africa: Evaluating strategies for effective domestic dog vaccination. Vaccine 27:152-160

Keesing F, Holt RD, Ostfeld RS (2006) Effects of species diversity on disease risk. Ecology Letters 9:485-498

Kielmanowicz MG et al. (2015) Prospective large-scale field study generates predictive model identifying major contributors to colony losses. PLoS Pathogens 11:e1004816. doi: 10.1371/journal.ppat.1004816

Kilpatrick AM (2011) Globalization, land use, and the invasion of west nile virus. Science 334:323-327

Kilpatrick AM, Daszak P, Jones MI, Marra PP, Kramer LD (2006) Host heterogeneity dominates West Nile virus transmission. Proceedings of the Royal Society B 273:2327-2333

Klee J, Mesana AM, Genersch E, Paxton R (2007) Widespread dispersal of the microsporidian *Nosema ceranae*, an emergent pathogen of the western honey bee, *Apis mellifera*. Journal of Invertebrate Pathology 96:1-10

Kuster RD, Boncristiani HF, Rueppell O (2014) Immunogene and vital transcript dynamics during parasitic *Varroa destructor* mite infection of developing honeybee (*Apis mellifera*) pupae. The Journal of Experimental Biology 217:1710-1718

Lambrechts L, Fellous S, Koella J, C, (2006) Coevolutionary interactions between host and parasites genotypes. Trends in Parasitology 22:12-16

Lanzi G et al. (2006) Molecular biological characterization of Deformed Wing Virus of honeybees (*Apis mellifera* L.). Journal of Virology 80:4998-5009

Leat N, Ball B, Govan V, Davison S (2000) Analysis of the complete genome sequence of Black Queen Cell Virus, a picorna-like virus of honey bees. Journal of General Virology 81:2111-2119

Lepais O et al. (2010) Estimation of bumblebee queen dispersal distances using sibship reconstruction method. Molecular Ecology 19:819-831. doi: 10.1111/j.1365-294X.2009.04500.x

Levitt AL et al. (2013) Cross-species transmission of honey bee viruses in associated arthropods. Virus. Res. 176:232-240

Li H, Durbin R (2009) Fast and accurate short read alignment with Burrows-Wheeler Transform. Bioinformatics 25:1754-1760

Li J, Peng W, Wu J, Strange JP, Boncristiani H, Chen Y (2011) Cross-species infection of deformed wing virus poses a new threat to pollinator conservation. Journal of Economic Entomology 104:732-739

Li J et al. (2012) The prevalence of parasites and pathogens in Asian honeybees *Apis cerana* in China. PloS ONE 7:e47955. doi: 10.1371/journal.pone.0047955

Li JL et al. (2014) Systemic spread and propagation of a plant-pathogenic virus in European honeybees, *Apis mellifera*. mBio 5:e00898-00813. doi: 10.1128/mBio.00898-13

Librardo P, Rozas J (2009) DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25:1451-1452

Lips KR et al. (2006) Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. PNAS 103:3165-3170. doi: 10.1073/pnas.0506889103

Lipsitch M, Moxon ER (1997) Virulence and transmissibility of pathogens: what is the relationship? Trends in Microbiology 5:31-37. doi: 10.1016/S0966-842X(97)81772-6

Lloyd-Smith JO, Schreiber SJ, Kopp PE, Getz WM (2005) Superspreading and the impact of individual variation on disease emergence. Nature 438:355-359

Locke B, Forsgren E, Fries I, De Miranda JR (2012) Acaricide tratment affects viral dynamics in *Varroa destructor*-infested honey bee colonies via both host physiology and mite control. Applied and Environmental Microbiology 78:227-235

LoGiudice K, Ostfeld RS, Schmidt KA, Keesing F (2003) The ecology of infectious disease: effects of host diversity and community composition on Lyme disease risk. PNAS 100:567-571

Maalouf H et al. (2011) Strain-dependent norovirus bioaccumulation in oysters. Applied and Environmental Microbiology 77:3189-3196

Macdonald DW (1992) Cause of wild dog deaths. Nature 360:633-634

Manley R, Boots M, Wilfert L (2015) Emerging viral disease risk to pollinating insects: ecological, evolutionary and anthropogenic factors. Journal of Applied Ecology 52:331-340

Maori E et al. (2007) Isolation and characterization of Israeli acute paralysis virus, a discistrovirus affecting honeybees in Israel: evidence for diversity due to intra- and inter-species recombination. Journal of General Virology 88:3428-3438

Martin DP, Murrell B, Golden M, Khoosal A, Muhire B (2015) RDP4: Detection and analysis of recombination patterns in virus genomes. Virus Evolution 1:vev003 doi: 10.1093/ve/vev003

Martin DP, Posada D, Crandall KA, Williamson C (2005) A modified bootscan algorithm for automated identification of recombinant sequences and recombination breakpoints. AIDS Research and Human Retroviruses 21:98-102

Martin SJ (2001) The role of *Varroa* and viral pathogens in the collapse of honeybee colonies: a modelling approach. Journal of Applied Ecology 38:1082-1093

Martin SJ et al. (2012) Global honey bee viral landscape altered by a parasitic mite. Science 336:1304-1306. doi: 10.1126/science.1220941

Maynard Smith J (1992) Analyzing the mosaic structure of genes. Journal of Molecular Evolution 34:126-129

McArt AH, Koch H, Irwin RE, Adler LS (2014) Arranging the bouquet of disease: floral traits and the transmission of plant and animal pathogens. Ecology Letters 17:624-636

McMahon DP, Fürst MA, Caspar J, Theodorou P, Brown MJF, Paxton RJ (2015) A sting in the spit: widespread cross-infection of multiple RNA viruses across wild and managed bees. Journal of Animal Ecology 84:615-624

McMahon DP et al. (2016) Elevated virulence of an emerging viral genotype as a driver of honeybee loss. Proceedings of the Royal Society B 283:20160811. doi: 10.1098/rspb.2016.0811

Meeus I, de Miranda JR, de Graaf DC, Wäckers F, Smagghe G (2014) Effect of oral infection with Kashmir bee virus and Israeli acute paralysis virus on bumblebee (*Bombus terrestris*) reproductive success. Journal of Invertebrate Pathology 121:64-69

Mondet F, De Miranda JR, Kretzschmar A, Le Conte Y, Mercer AR (2014) On the front line: Quantitative virus dynamics in honeybee (*Apis mellifera* L.) colonies along a new expansion front of the parasite *Varroa destructor*. PLoS Pathogens 10:e1004323. doi: 10.1371/journal.ppat.1004323

Moore JD, Jironkin A, Chandler D, Burroughs N, Evans DJ, Ryabov E (2011) Recombinants between Deformed wing virus and *Varroa destructor* virus-1 may prevail in *Varroa destructor*-infested honeybee colonies. Journal of General Virology 92:156-161

Mordecai GJ, Wilfert L, Martin SJM, Jones IM, Schroeder DC (2015) Diversity in a honey bee pathogen: first report of a third master variant of the Deformed wing virus quasispecies. International Society for Microbial Ecology 10:1264-1273. doi: 10.1038/ismej.2015.178

Morens DM, Folkers GK, Fauci AS (2004) The challenge of emerging and re-emerging infectious diseases. Nature 430:242-249

Murray TE, Coffey FC, Kehoe E, Horgan FG (2013) Pathogen prevalence in commercially reared bumble bees and evidence of spillover in conspecific populations. Biological Conservation 159:269-276

Nazzi F et al. (2012) Synergistic parasite-pathogen interactions mediated by host immunity can drive the collapse of honeybee colonies. PLoS Pathogens 8:e1002735. doi: 10.1371/journal.ppat.1002735

Nielson SL, Nicolaisen M, Kryger P (2008) Incidence of Acute bee paralysis virus, Black queen cell virus, Chronic bee paralysis virus, Deformed wing virus, Kashmir bee virus and sacbrood virus in honey bees (*Apis mellifera*) in Denmark. Apidologie 39:310-314

Obbard DJ, Gordon KHJ, Buck AH, Jiggins FM (2009) The evolution of RNAi as a defence against viruses and transposable elements. Philosophical transactions of the Royal Society of London Series B 364:99-115

Oldroyd BP (1999) Coevolution while you wait: *Varroa jacobsoni*, a new parasite of western honeybees. Trends in Ecology and Evolution 14:312-315

Oldroyd BP (2007) What's killing American honey bees? PloS Biology 5:1195-1199. doi: 10.1371/journal.pbio.0050168

Olivier V et al. (2008) Molecular characterisation and phylogenetic analysis of Chronic bee paralysis virus, a honey bee virus. Virus. Res. 132:59-68. doi: 10.1016/j.virusres.2007.10.014

Ongus JR, Peters D, Bonmatin J, Bengsch E, Vlak JM, van Oers MM (2004) Complete sequence of a picorna-like virus of the genus *Iflavirus* replicating in the mite *Varroa destructor*. Journal of General Virology 85:3747-3755

Padidam M, Sawyer S, Fauquet CM (1999) Possible emergence of new Geminiviruses by frequent recombination. Virology 265:218-225

Parrish CR et al. (2008) Cross-species virus transmission and the emergence of new epidemic diseases. Microbiology and Molecular Biology Reviews 72:457-470

Pedersen A, Altizer S, Poss M, Cunningham AA, Nunn (2005) Patterns of host specificity and transmission among parasites of wild primates. International Journal of Parisitology 35:647-657

Peng W, Li J, Boncristiani Jr HF, Strange JP, Hamilton M, Chen YP (2011) Host range expansion of honey bee Black Queen Cell Virus in the bumble bee, *Bombus huntii*. Apidologie 42:650-658

Pettis JS, vanEngelsdorp D, Johnson J, Dively G (2012) Pesticide exposure in honey bees results in increased levels of the gut pathogen Nosema. Naturwissenschaften 99:153-158

Potts SG, Biesmeijer JC, Kremen C, Neumann P, Schweiger O, Kunin WE (2010) Global pollinator declines: trends, impacts and drivers. Trends in Ecology and Evolution 25:345-353

Power AG (2000) Insect transmission of plant viruses: a constraint on virus variability. Current Opinion in Plant Biology 3:336-340

Power AG, Flecker AS (2003) Virus specificity in disease systems: are species redundant? In: Levin SA, Kareiva P (eds) The Importance of Species. Princeton University Press, Princeton, pp 330-346

Radzevičiùte R et al. (2017) Replication of honey bee-associated RNA viruses across multiple bee species in apple orchards of Georgia, Germany and Kyrgyzstan. Journal of Invertebrate Pathology 146:14-23

Rambaut A, Lam TT, Carvalho ML, Pybus OG (2016) Exploring the temporal structure of heterochronous sequences using TempEst (formerly Patho-O-Gen). Virus Evolution 2. doi: 10.1093/ve/vew007

Ravoet J, De Smet L, Meeus I, Smagghe G, Wenseleers T, de Graaf DC (2014) Widespread occurence of honey bee pathogens in solitary bees. Journal of Invertebrate Pathology 122:55-58

Reiczigel J, Foldi J, Ozsvari L (2010) Exact confidence limits for prevalence of a disease with imperfect diagnostic test. Epidemiology and Infection 138:1674-1678

Ribière M, Ball BV, Aubert M (2008) Natural history and geographical distribution of honey bee viruses. Virology and the honey bee. European Commission, pp 15-84

Ribière M et al. (2007) Spread of infectious Chronic bee paralysis virus by honeybee (*Apis mellifera* L.) feces. Applied and Environmental Microbiology 73:7711-7716

Ribière M, Olivier V, Blanchard P (2010) Chronic bee paralysis: a disease and a virus like no other? Journal of Invertebrate Pathology 103:S120-S131

Rogers D, Randolph S (2006) Climate change and vector-borne diseases. Advances in Parasitology 62:346-377

Runckel C et al. (2011) Temporal analysis of the honey bee microbiome reveals four novel viruses and seasonal prevalence of known viruses, *Nosema,* and *Crithidia*. PloS ONE 6:e20656. doi: 10.1371/journal.pone.0020656

Ryabov E et al. (2014) A virulent strain of Deformed wing virus (DWV) of honeybees (*Apis mellifera*) prevails after *Varroa destructor*-mediated, or *In Vitro*, transmission. PLoS Pathogens 10:e10004230. doi: 10.1371/journal.ppat.1004230

Santillán-Galicia MT, Ball BV, Clark SJ, Alderson PG (2014) Slow bee paralysis virus and its transmission in honey bee pupae by *Varroa destructor*. Journal of Apicultural Research 53:146-154

Shah NS et al. (2007) Worldwide emergence of extensively drug-resistant tuberculosis. Biol Letters 13:380-387

Shapiro B, Rambaut A, Drummond AJ (2006) Choosing appropriate substitition models for the phylogenetic analysis of protein coding sequences. Molecular Biology and Evolution 23:7-9

Shen M, Cui L, Ostiguy N, Cox-Foster DL (2005) Intricate transmission routes and interactions between picorna-like viruses (Kashmir bee virus and Sacbrood virus) with the honeybee host and the parasitic varroa mite. Journal of General Virology 86:2281-2289

Simon V, Ho DD, Karim QA (2006) HIV/AIDS epidemiology, pathogenesis, prevention, and treatment. The Lancet 368:489-504

Sinclair ARE, Norton-Griffiths M (1995) Serengeti: Dynamics of an Ecosystem. University of Chicago Press

Singh R et al. (2010) RNA viruses in hymenopteran pollinators: Evidence of inter-taxa virus transmission via pollen and potential impact on non-*Apis* hymenopteran species. PloS ONE 5:e14357. doi: 10.1371/journal.pone.0014357

Strauss U, Human H, Gauthier L, Crewe RM, Dietemann V, Pirk CWW (2013) Seasonal prevalence of pathogens and parasites in the savannah honeybee (*Apis mellifera scutellata*). Journal of Invertebrate Pathology 114:45-52

Streicker DG, Fenton A, Pedersen AB (2013) Differential sources of host species heterogeneity influence the transmission and control of multihost parasites. Ecology Letters 16:975-984

Sumpter DJT, Martin SJ (2004) The dynamics of virus epidemics in *Varroa*-infested honey bee colonies. Journal of Animal Ecology 73:51-63

Taylor LH, Latham SM, Woolhouse MEJ (2001) Risk factors for human disease emergence. Philosophical transactions of the Royal Society of London Series B 356:983-989

Tentcheva D et al. (2004) Prevalence and seasonal variations of six bee viruses in *Apis mellifera* L. and *Varroa destructor* mite populations in France. Applied and Environmental Microbiology 70:7185-7191

Tomkins DM, White AR, Boots M (2012) Ecological replacement of native red squirrels by invasive greys driven by disease. Ecology Letters 6:189-196

Vanbergen AJ et al. (2013) Threats to an ecosystem service: pressures on pollinators. Frontiers in Ecology and the Environment 11:251-259. doi: 10.1890/120126

vanEngelsdorp D et al. (2009) Colony collapse disorder: A descriptive study. PloS ONE 4:e6481. doi: 10.1371/journal/pone.0006481

Velthuis HHW, van Doorn A (2006) A century of advances in bumblebee domestication and the economic and environmental aspects of its commercialization for pollination. Apidologie 37:421-451

Wang H et al. (2013) Sequence recombination and conservation of Varroa destructor Virus-1 and Deformed wing virus in field collected honey bees (Apis mellifera). PLoS ONE 8:e74508

Ward L et al. (2007) First detection of Kashmir bee virus in the UK using real-time PCR. Apidologie 38:181-190

Welch A, Drummond F, Tewari S, Averill A, Burand JP (2009) Presence and prevalence of viruses in local and migratory honeybees (*Apis mellifera*) in Massachusetts. Applied and Environmental Microbiology 75:7862-7865

Wilfert L et al. (2016) Deformed wing virus is a recent global epidemic in honeybees driven by *Varroa* mites. Science 351:594-597

Willams PH, Osborne J (2009) Bumblebee vulnerability and conservation world-wide. Apidologie 40:367-387

Woolhouse MEJ, Gowtage-Sequeria S (2005) Host range and emerging and reemerging pathogens. Biol Letters 11:1842-1847

Woolhouse MEJ, Haydon DT, Antia R (2005) Emerging pathogens: the epidemiology and evolution of species jumps. Trends in Ecology and Evolution 20:238-244

Woolhouse MEJ, Taylor LH, Haydon DT (2001) Population biology of multihost pathogens. Science 292:1109-1112

Yañez O et al. (2012a) Deformed wing virus and drone mating flights in the honey bee (*Apis mellifera*): implications for sexual transmission of a major honey bee virus. Apidologie 43:17-30

Yañez O, Zheng H, Hu F, Neumann P, Dietemann V (2012b) A scientific note on Israeli acute paralysis virus infection of Eastern honeybee *Apis cerana* and vespine predator *Vespa velutina*. Apidologie 43:587-589

Yue C, Genersch E (2005) RT-PCR analysis of *Deformed wing virus* in honeybees (*Apis mellifera*) and mites (*Varroa destructor*). Journal of General Virology 86:3419-3424

Yue C, Schröder M, Bienefeld K, Genersch E (2006) Detection of viral sequences in semen of honeybees (*Apis mellifera*): evidence for vertical transmission of viruses through drones. Journal of Invertebrate Pathology 92:105-108

Yue C, Schröder M, Gisder S, Genersch E (2007) Vertical-transmission routes for Deformed wing virus of honeybees (*Apis mellifera*). Journal of General Virology 88:2329-2336

Zhang X, He SY, Evans JD, Pettis JS, Yin GF, Chen YP (2012) New evidence that Deformed wing virus and Black queen cell virus are multi-host pathogens. Journal of Invertebrate Pathology 109:156-159

Zioni N, Soroker V, Chejanovsky N (2011) Replication of Varroa destructor virus 1 (VDV-1) and a Varroa destructor virus 1–deformed wing virus recombinant (VDV-1–DWV) in the head of the honey bee. Virology 417:106-112