THE UNIVERSITY OF SYDNEY

# Disentangling the Relationship Between *Deformed wing virus*, the Honey Bee Host (*Apis mellifera*) and the Viral Vector, the Ectoparasitic Mite *Varroa destructor*

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*A thesis submitted to fulfil requirements for the degree of Doctor of Philosophy*

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# STATEMENT OF ORIGINALITY

*I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged. The work in this thesis has not been previously submitted for a degree at The University of Sydney or any other institution.*

**Amanda M. Norton**

# PREFACE

This thesis contains three independent manuscripts presented as research chapters, which have been published or are being prepared for submission to an academic journal. As each manuscript investigates the same study organisms, but from the perspective of the virus, host or vector, there is some overlap in the background material of each chapter. The manuscripts have been formatted to the specifications of each different journal, excluding the reference list and the use of Australian English throughout.

# AUTHORSHIP ATTRIBUTION STATEMENT

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AMN, EJR, and MB conceived the study. AMN conducted the experimental work. AMN and GB carried out molecular laboratory work. AMN analysed the data. AMN, EJR, and MB contributed to data interpretation. AMN wrote the original drafts, and EJR and MB contributed to manuscript revisions. All authors contributed critically to the drafts and gave final approval for publication

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AMN, EJR and MB conceived the study. AMN conducted the experimental work. AMN and GB carried out molecular laboratory work. GB conducted the small RNA library preparation. AMN analysed the qPCR and statistical data. AMN and EJR analysed the siRNA data. AA conducted the miRNA analysis. OTW analysed the piRNA data. AA and EJR determined the total composition of known small RNAs. AMN wrote the original drafts, and EJR and MB

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*I certify that I am the corresponding author of all research chapters.*

**Amanda M. Norton** | ***25th February 2021***

*As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.*

**Madeleine Beekman** | ***25th February 2021***

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*'Bats can hear shapes. Plants can eat light. Bees can dance maps.*

*We can hold all these ideas at once and feel both heavy and weightless with the absurd beauty of it all.'*

**Jarod K Anderson**

Field Guide to the Haunted Forest

# ABSTRACT

The ectoparasitic mite *Varroa destructor* is indisputably the most significant driver of global colony losses of the Western honeybee, *Apis mellifera*. Colony deaths are frequently attributed to *Deformed wing virus* (DWV), which is vectored by the mite. In this thesis I attempt to disentangle the tripartite relationship between DWV, *A. mellifera* and *V. destructor*, by investigating whether the two major DWV genotypes, A and B, differ from the point of view of the virus, the honey bee and the mite. First, I assessed the viral accumulation dynamics of multiple DWV genotypes during single or co-infection in Australian pupae (naïve to both DWV and *Varroa*). I found that DWV-B accumulated to higher levels than DWV-A when singly and co-injected, suggesting that DWV-B is able to outcompete DWV-A. Yet despite higher viral loads, DWV-B was associated with the lowest level of mortality. Therefore, I next investigated if the bees' immune system reacted differently to the two DWV genotypes. I examined the expression of 19 immune genes and analysed the small RNA response of pupae exposed to DWV-A and DWV-B. Overall, I found little evidence to indicate that *A. mellifera* responds differently to either genotype. Finally, to uncover what role vector transmission by *V. destructor* plays in DWV genotype prevalence at the colony level, I experimentally increased and decreased the number of mites within *A. mellifera* colonies and analysed viral loads over a period of ten months. I found that DWV-A was strongly affected by mite numbers, whereas DWV-B persisted in the presence and absence of *V. destructor*. Overall, my thesis furthers our understanding of the intricate relationship between DWV, *A. mellifera* and *V. destructor*, and provides insight into some of the factors that may be contributing to the increasing prevalence of DWV-B.

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# General Introduction

## The importance of pollinators and the challenges they face

Mutualism between angiosperms and their animal pollinators is fundamentally one of the most important evolutionary relationships in terrestrial ecosystems. Animals are estimated to pollinate 94% and 78% of flowering plant species in tropical and temperate environments, respectively (Ollerton et al., 2011). Animal pollinators are rewarded with sources of food (chiefly nectar and pollen) while transferring pollen grains between conspecifics (Figure 1.1). Animal pollination is performed by numerous invertebrate and vertebrate species, and provides a crucial ecosystem service across wild and managed environments. Pollination maintains genetic diversity in plant populations, and increases fruit set and seed production, which in turn provide sources of food for animals. Needless to say that pollination is also essential for many agricultural crops.

**Figure 1.1** A classic example of co-evolution between an angiosperm and an animal pollinator: photograph of the Madagascar orchid (*Angraecum sesquipedale*) and its co-adapted pollinator Morgan's sphinx moth (*Xanthopan morganii praedicta*), famously predicted to exist by Charles Darwin in 1862 (Arditti et al., 2012). Image source: Minden Pictures.

Increased human consumption and the globalisation of food trade has altered agricultural demands. Approximately 35% of crops grown for the human food supply rely on animal pollination, and over the last ~ 60 years there has been a > 300% increase in the production of pollinator-dependent crops (Klein et al., 2007; Aizen and Harder, 2009). While the bulk of the human diet is made up of a few staple crops that do not require animal pollination, pollinator-dependent crops provide rich sources of vitamins, antioxidants and minerals (Marshman et al., 2019). Pollinating insects, particularly bees, are heavily relied upon to improve yield in 39 of the 57 major crops worldwide (Klein et al., 2007). In many regions, a vast proportion of commercial crop species are introduced and very few native plant species are consumed (Shelef et al., 2017), meaning that many crops do not grow in the appropriate environmental conditions. In the US and Australia for example, almost all agricultural crops are introduced species (Cunningham et al., 2002; Pimentel et al., 2005). While growing such crops is important for meeting food supply and economic demands, they may require the use of introduced pollinators where native pollinators are absent or in decline.

There is mounting evidence of a substantial loss of insect pollinator species (Ollerton et al., 2014; Powney et al., 2019; Zattara and Aizen, 2021). Alarmingly, wild bee species are estimated to have declined by 25% since the 1990s (Zattara and Aizen, 2021). Pollinator losses are occurring across each continent. For example, Australia has three native bee species currently listed as critically endangered (Department of the Environment, 2021). Canada and the US have seven and eight endangered native bee species, respectively (Marshman et al., 2019; US Fish and Wildlife Service, 2021). In 2017, 9% of bee species in Europe were characterised as threatened with ~ 0.5% considered critically endangered, however this is likely to be a conservative estimate as data is deficient for 56% of species (Nieto et al., 2017). The true global scale at which wild pollinator species are declining is unclear, but some of the best evidence of a pollinator crisis has been obtained from bumble bees. In Europe and North America, many bumble bee species have experienced significant range contractions and regional declines in abundance, with at least four species extinctions within the last century (Goulson, 2003a; Winter et al., 2006; Grixti et al., 2009; Cameron et al., 2011; Graves et al., 2020; Simanonok et al., 2021). As with other wild bees, bumble bee declines are thought to be driven by the combined effects of habitat loss, reduced floral diversity, agricultural intensification, climate change, pesticides and disease (Goulson et al., 2008; Cameron et al., 2011; Goulson et al., 2015; Soroye et al., 2020).

With declines in wild pollinators and increasing agricultural demands, the world has become more and more reliant on managed pollinators. The Western honey bee, *Apis mellifera*, is native to Europe, Africa and the Middle East (Ruttner, 1988). *A. mellifera* was introduced to North America (1600s), Australasia and parts of Asia (1800s) for pollination services and the production of apicultural products (honey, wax and propolis) (Hopkins, 1901; Moritz et al., 2005; Arundel, 2011; Chantawannakul et al., 2016). *A. mellifera* is widely considered to be one of the most important pollinator species of commercial crops. Domesticated since at least 2600 BC for their honey and wax (Graystock et al., 2016), *A. mellifera* colonies are easy to manipulate and manage, and can be transported over long distances between multiple foraging sites. Honey bees are also generalist foragers, meaning that they will visit a wide variety of flowers to collect pollen and nectar. However, honey bees are not necessarily efficient pollinators of all plant species (Greenleaf and Kremen, 2006).

Sonication or 'buzz pollination' is the process of vibrating anthers in order to obtain pollen granules from small pores (Buchmann and Hurley, 1978). The requirement of sonication has evolved in 65 plant families, including important agricultural food crops such as those in the Solanaceae (e.g. tomatoes, eggplants, peppers, potatoes), Ericaceae (e.g. blueberries and cranberries) and Actinidiaceae (e.g. kiwifruit) families (De Luca and Vallejo-Marin, 2013). Over 50 genera of bees have the ability to sonicate but the trait is notably absent in *Apis* sp. (De Luca and Vallejo-Marin, 2013). The demand for buzz pollinated crops and agricultural production within greenhouses (particularly tomatoes) led to the domestication of bumble bees (Velthuis and van Doorn, 2006). *Bombus terrestris* colonies (Figure 1.2) are now mass produced and exported to 57 countries, 16 of which are outside the native range of Eurasia (Chandler et al., 2019). In addition, *B. impatiens* and *B. occidentalis* from North America, and *B. lucorum* and *B. ignitus* from Asia are now commercially reared for pollination purposes (Velthuis and van Doorn, 2006).


**Figure 1.2** An example of a commercial *B. terrestris* colony utilised for tomato pollination within a greenhouse. Image source: Koppert Biological Systems.

While the production of commercial bumble bee colonies is a relatively new industry, introducing bumble bee species outside of their native range is not a new phenomenon. Four bumble bee species (*B. terrestris, B. hortorum*, *B. ruderatus* and *B. subterraneus*) were introduced to New Zealand in 1885 and 1906, and *B. ruderatus* to Chile in the early 1980s to improve clover (*Trifolium* sp.) pollination (Donovan, 1980; Arretz and Macfarlane, 1986).

Humans have introduced exotic taxa into non-native regions for thousands of years. Throughout history, there are abundant examples of devastating consequences upon local ecosystems as a result of introducing species in to non-native regions (Suarez and Tsutsui, 2008). While bees are largely considered to be beneficial insects, as with any other organism, introduction into a new range can alter selective pressures and the evolution of both the introduced species and native taxa. Firstly, introduced bees have the potential to become

invasive and displace native species. For instance, bumble bees often escape from greenhouses (Morandin et al., 2001; Whittington et al., 2004). This has led to *B. terrestris* becoming established in Japan and potentially contributing to the decline of native *B. hypocrita sapporoensis* by outcompeting the latter for nest sites (Inoue et al., 2008). Secondly, introduced bee species can alter plant diversity and abundance. Following introduction to New Zealand and Chile, bumble bees have spread to Tasmania, Australia (*B. terrestris*), and Argentina (*B. ruderatus* and *B. terrestris*) (Abrahamovich et al., 2001; Montalva et al., 2011), where they have since become established. Introduced bumble bees and honey bees may be contributing to the spread of invasive weeds in Argentina, Australia and New Zealand (Goulson, 2003b; Morales and Aizen, 2006; Aizen et al., 2019). Furthermore, introduced bees may disrupt native plant and pollinator mutualism by depleting or robbing nectar, thereby rendering nectar unavailable to beneficial pollinators (Paton, 2000; Aizen et al., 2019).

Introducing exotic taxa in non-native ranges also increases the risk of alterations to parasite and pathogen dynamics (Vilcinskas et al., 2013). Commercial bumble bee colonies have been found to harbour elevated parasite loads (*Crithidia bombi*, *Nosema bombi*, and *Locustacarus buchneri*) compared to wild colonies. Bumble bee escapes from greenhouses has likely resulted in the spill-over of *C. bombi* and *N. bombi* from commercial to wild bumble bee populations (Colla et al., 2006). Furthermore, introduction of *N. bombi* and other parasites to wild colonies may have caused the sudden and widespread declines of seven bumble bee species in the US (Cameron et al., 2011; Cameron et al., 2016). Arguably, one of the most recent significant shifts in host-parasite and pathogen dynamics as a result of introducing species into non-native ranges has occurred between *A. mellifera* and the ectoparasitic mite *Varroa destructor*.

## Co-evolution between *Varroa* sp. and *Apis cerana*

*Varroa* mites were first described in 1904 (*V. jacobsoni*) when they were detected on *Apis cerana* in Java, Indonesia (Oudemans, 1904). The mites regained attention in the 1970/80s when they were detected outside of Asia having spread to parts of Europe, North Africa, North and South America at that time (De Jong et al., 1982; Martin, 1994). Originally, it was thought that *V. jacobsoni* had spread outside of Asia until Anderson and Trueman (2000) recognised that the mite that had increased in global distribution and prevalence was actually

a different species, which was aptly named *V. destructor*. Currently we recognise four *Varroa* species, all native to Asia: *V. destructor*, *V. jacobsoni*, *V. rindereri*, and *V. underwoodi. V. destructor* is the most well characterised and thus far the most damaging species, with the largest global distribution. *Varroa* are honey bee parasites. *V. underwoodi* parasitises *A. cerana, A. nigrocincta* and *A. nuluensis* (Chantawannakul et al., 2016; Wang et al., 2019). *V. rindereri* has been found to parasitise *A. koschevnikovi* and has been detected in colony debris of *A. dorsata* (de Guzman and Delfinado-Baker, 1996; Koeniger et al., 2002). *A. cerana* is the ancestral host of *V. jacobsoni* and *V. destructor,* however both mite species have developed the ability to switch-hosts and now parasitise *A. mellifera* (Techer et al., 2019; Roberts et al., 2020) as a direct result of the anthropogenic introduction of *A. mellifera* colonies into the native range of *A. cerana* in Asia.

*Varroa* mites have two life stages: the reproductive and dispersal (previously referred to as the phoretic) phases. *Varroa* reproduction takes place within honey bee brood cells. Mated female mites (foundress) enter late stage larval cells prior to capping and hide in the larval food (Figure 1.3). After the cell is capped by nurse bees and the larva has developed into a pre-pupa, the foundress creates a feeding-site and begins feeding upon the bee's fat body (Donzé and Guerin, 1994; Ramsey et al., 2019). After approximately 60-70 h after entering the cell, the foundress begins laying her eggs. The first egg laid is a haploid male produced via arrhenotokous parthenogenesis (Häußermann et al., 2019). The foundress subsequently lays ~ 4 diploid eggs approximately 30 h apart, which develop into females (Martin, 1994). Offspring develop into mature adults within 6-7 or 8-9 days for males and females, respectively, and feed on the developing pupae from the site created by the foundress (De Jong et al., 1982). The male mates with his freshly moulted adult sisters at the faecal accumulation site (Donzé and Guerin, 1994; Ziegelmann et al., 2013). The foundress and her mated daughter(s) leave the brood cell along with the newly emerged adult bee. At this point the mites begin the dispersal phase (Traynor et al., 2020), where they feed upon adult bees. The dispersal phase is crucial for successful reproduction. During the dispersal phase, it takes at least 5 days before the young female mites are able to lay eggs, as transferred spermatazoa mature within the female genitals (capacitation) (Häußermann et al., 2016). Each foundress typically has 2-3 reproductive cycles under field conditions (Martin and Kemp, 1997).

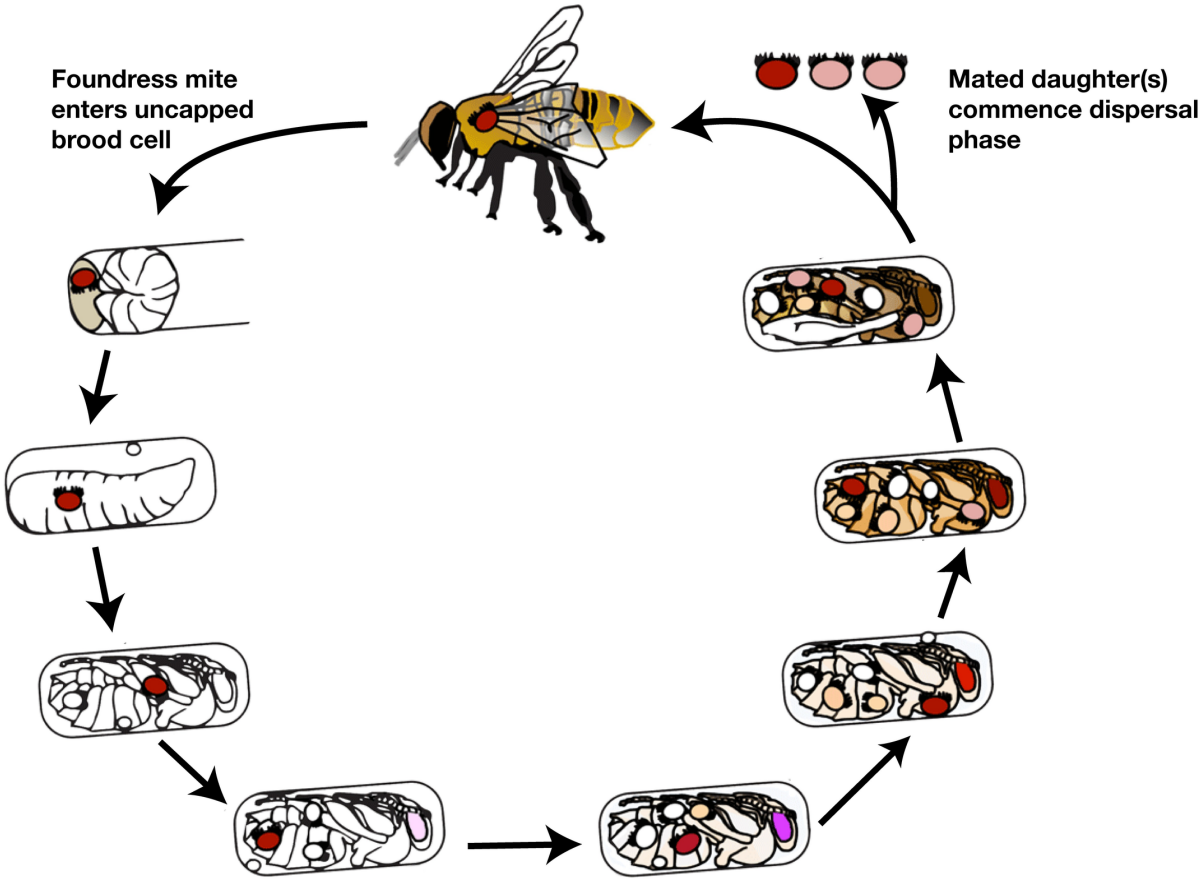


**Figure 1.3.** The *Varroa* life cycle is closely tied to the developmental cycle of honey bees. Image adapted from Harris et al. (2019).

*V. destructor* and *A. cerana* exist in a stable host-parasite relationship and the mite has little negative effect upon the Asian honey bee. Host-parasite co-evolution has resulted in numerous mechanisms that essentially keep *V. destructor* numbers low in *A. cerana* colonies. Firstly, while *V. destructor* enters both worker and brood cells, reproduction in *A. cerana* colonies is largely restricted to drone brood (Boot et al., 1997; Wang et al., 2020). Limiting reproduction to drone cells appears to be an adaptation strategy of the mites in *A. cerana* colonies rather than a behavioural trait of the bees (Boot et al., 1999). Avoiding reproducing in *A. cerana* worker cells likely increases mite fitness as fewer daughter mites would reach maturity during the 11-day developmental time of workers (Boot et al., 1995). Restricting mite reproduction to drone cells also benefits *A. cerana* by limiting the reproductive period to a shorter time window, as drone brood is not reared year round. *A. cerana* workers have a number of co-evolved defensive traits that further reduce mite numbers within the colony. They perform elevated grooming behaviour, removing mites in the dispersal phase from

themselves and other adult bees (Peng et al., 1987; Büchler et al., 1992). *A. cerana* workers are also adept at detecting mites within brood cells, and will remove the infested brood and the mites from the cell (*Varroa*-sensitive hygiene) (Peng et al., 1987; Rath and Drescher, 1990). In addition, *A. cerana* drone pupae experience elevated mortality when parasitised by two or more mites; workers subsequently entomb dead drone brood, further limiting the successful emergence of the mites in *A. cerana* colonies (Rath, 1999).

## Novel *Varroa* host-shifts to *A. mellifera*

Introducing *A. mellifera* colonies into the native range of *A. cerana* for beekeeping purposes allowed *V. destructor* and *V. jacobsoni* to switch hosts. It appears that *V. destructor* has shifted to *A. mellifera* in at least three independent events in Korea, Japan and the Philippines (Solignac et al., 2005; Beaurepaire et al., 2015; Techer et al., 2020), however very little is known about the adaptive processes that facilitated host-switching. In the early 1990s, *V. jacobsoni* was found to infest *A. mellifera* colonies in Papua New Guinea (PNG), but only 0.4% of the female mites found in drone brood had attempted to reproduce (Anderson, 1994). By 2008, two distinct *V. jacobsoni* lineages were found to have successfully switched hosts to *A. mellifera* in PNG, where mites were reproducing in drone and worker brood (Roberts et al., 2015). Roberts et al. (2015) hypothesised that host-shifts may arise from a lack of competition from an existing mite parasite (niche availability) and the availability of drone brood in *A. mellifera* colonies when *A. mellifera* and *A. cerena* are brought into sympatry.

In both *V. destructor* and *V. jacobsoni,* host-switching to *A. mellifera* has led to an important change in mite behaviour: the ability to reproduce in both drone and worker cells. The large number of worker brood cells and longer brood rearing period relative to drone brood results in substantially higher mite infestations than in *A. cerana* colonies (Fries et al., 1991; Martin and Kemp, 1997). Modelling suggests that ten *V. destructor* foundress mites can reproduce to numbers exceeding 10,000 in one to four years in untreated *A. mellifera* colonies, depending on the length of the brood rearing period in different climates (Fries et al., 1994; Calis et al., 1999). In its native range *A. mellifera* was not parasitised by any *Varroa* species (Eickwort, 1994). Therefore, *A. mellifera* lacks the co-evolved defence traits displayed by *A. cerana*, excluding a small number of *A. mellifera* populations that have developed *Varroa*-tolerant and resistant characteristics over-time when left untreated (Mondet et al., 2020).

*A. mellifera* colonies infested with *V. destructor* were transported through Europe and the Americas (Oldroyd, 1999), leading to further range expansion of the mite (Figure 1.4). *V. destructor* now has a near global distribution, with Australia being the only major beekeeping country to remain *Varroa* free (Roberts et al., 2017). The global spread of *V. destructor* had a devastating impact on *A. mellifera* colonies worldwide (Schroeder and Martin, 2012). For the vast majority of colonies, untreated *V. destructor* infestations typically result in colony death within as little as 6-24 months (Le Conte et al., 2010). Mite parasitism increases metabolic costs, reduces bee body mass and longevity, and impacts immune function (Bowen-Walker and Gunn, 2001; Nazzi et al., 2012; Annoscia et al., 2019; Aldea and Bozinovic, 2020). Yet, the most harmful impact of *V. destructor* appears to be its ability to vector viruses.

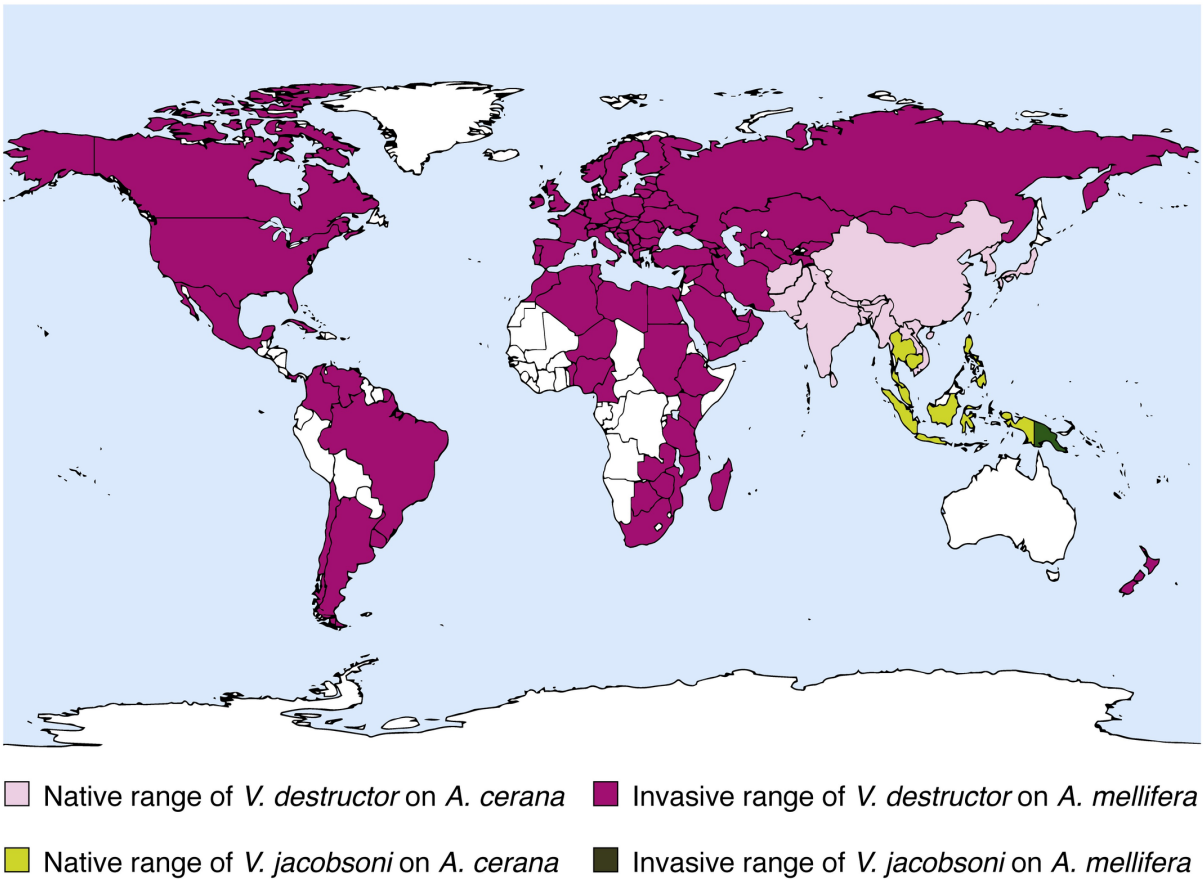


**Figure 1.4.** Range expansion of *V. destructor* and *V. jacobsoni* after host shifts to *A. mellifera* and transport of mite infested *A. mellifera* colonies. Geographical distribution illustrated by country and adapted from Techer (2020), Wilfert et al. (2016) and Koetz (2013).

*V. destructor* altered the dynamics of viral infections in *A. mellifera* by introducing a new route of transmission: vector transmission. *V. destructor* is capable of vectoring a number of different RNA viruses, notably *Deformed wing virus* (DWV) and three paralysis viruses belong to the 'AKI species complex' [*Acute bee paralysis virus* (ABPV), *Kashmir bee virus* (KBV) and *Israeli acute paralysis virus* (IAPV)] (Bailey and Ball, 1991; Boecking and Genersch, 2008; de Miranda et al., 2010; de Miranda and Genersch, 2010). The latter three paralysis viruses are highly virulent, meaning that vector transmission typically results in rapid viral replication and honey bee death, and consequently low levels of viral persistence (Bailey and Ball, 1991; de Miranda et al., 2010). DWV is comparatively less virulent, and viral loads are sustained at high levels within the colony in the presence of the mite. This characteristic has likely contributed to the continued success of DWV once *V. destructor* is established within a population (Mondet et al., 2014). The worldwide spread of *V. destructor* transformed DWV from a relatively rare and harmless virus to the most prevalent viral pathogen in *A. mellifera* colonies worldwide (Martin and Brettell, 2019). DWV is now detected in virtually all *A. mellifera* populations infested with the mite. Australian *A. mellifera* colonies are in a rare position, being free of both DWV and *V. destructor* (Roberts et al., 2017).

The close association between DWV and *V. destructor* has been associated with the death of millions of honey bee colonies worldwide (Martin et al., 2012; Schroeder and Martin, 2012). The tripartite relationship between *A. mellifera, V. destructor* and DWV has had detrimental effects on honey bee health with significant implications for apiarists, agriculture and eco-systems globally. Nevertheless, this relationship affords us an interesting opportunity to study co-evolution between host, vector and viral pathogen, which is the crux of my thesis.

## Thesis Outline

There are two major genotypes of DWV (A and B) commonly found in bees and mites. Past studies have found that DWV-A and DWV-B affect *A. mellifera* differently, although virulence (damage to the host attributed to the virus) differs between honey bee life stages and populations (McMahon et al., 2016; Gisder et al., 2018; Dubois et al., 2019; Tehel et al., 2019). DWV-A was associated with the initial spread of *V. destructor*, whereas DWV-B has recently increased in global prevalence (Kevill et al., 2017; Ryabov et al., 2017; Kevill et al., 2019; Manley et al., 2019b). This thesis explores the factors that may explain the increasing

prevalence of DWV-B at the expense of DWV-A. The main objective of my thesis is to disentangle the tripartite relationship, by investigating whether DWV-A and DWV-B differ from the point of view of the virus (Chapter 2), the honey bee (Chapter 3), and the mite vector (Chapter 4).

In Chapter 2, I investigate the accumulation and competition of multiple DWV genotypes in Australian *A. mellifera*, naïve to both DWV and *V. destructor*. I singly injected pupae with equal doses of DWV-A, DWV-B, and a recombinant strain isolated from a *V. destructor* tolerant bee population. I also co-injected pupae with DWV-A and DWV-B to assess the level of competition between the two major genotypes during co-infection. I monitored pupal survival throughout pupation and measured viral accumulation by RT-qPCR at multiple time-points, up to 192 h post-injection.

In Chapter 3, I examine whether differences in DWV accumulation and virulence, observed in Chapter 2 and previous studies, can be explained by the two major DWV genotypes eliciting a differential response in the *A. mellifera* immune system. I examined the expression of 19 immune genes by RT-qPCR and comprehensively analysed the small RNA response in the pupae experimentally injected in Chapter 2. My study is the first to investigate the honey bee immune response in relation to the two major DWV genotypes and within the same *V. destructor* and DWV-naïve population, removing confounding effects of mite feeding and pre-exposure to the virus.

In Chapter 4, I explore whether vector transmission by *V. destructor* is a driver of increased DWV-B prevalence globally. I experimentally increased or decreased the number of *V. destructor* mites in honey bee colonies, and tracked DWV-A and DWV-B loads over a period of ten months by RT-qPCR. My study is the first to experimentally manipulate mite numbers within full sized *A. mellifera* colonies in order to determine what role vector transmission plays on DWV genotype prevalence.

Finally, in Chapter 5, I provide a general discussion of my findings and make suggestions for future research directions.

# Accumulation and competition amongst *Deformed wing virus* genotypes in naïve Australian honey bees provides insight into the increasing global prevalence of genotype B


## ABSTRACT

Honey bee colony deaths are often attributed to the ectoparasitic mite *Varroa destructor* and *Deformed wing virus* (DWV), vectored by the mite. In the presence of *V. destructor* both main genotypes (DWV-A and DWV-B) have been correlated with colony loss. Studies show that DWV-B is the most prevalent genotype in the United Kingdom and Europe. More recently DWV-B has increased in prevalence in the United States. The increasing prevalence of DWV-B at the expense of DWV-A suggests that competition exists between the genotypes. Competition may be due to disparities in virulence between genotypes, differences in fitness, such as rate of replication, or a combination of factors. In this study we investigated if DWV genotypes differ in their rate of accumulation in Australian honey bees naïve to both *V. destructor* and DWV, and if viral load was associated with mortality in honey bee pupae. We singly and co-infected pupae with DWV-A, DWV-B, and a recombinant strain isolated from a *V. destructor* tolerant bee population. We monitored viral accumulation throughout pupation, up to 192 hours post-injection. We found significant differences in accumulation, where DWV-A accumulated to significantly lower loads than DWV-B and the DWV-recombinant. We also found evidence of competition, where DWV-B loads were significantly reduced in the presence of DWV-A, but still accumulated to the highest loads overall. In contrast to previous studies, we found significant differences in virulence between pupae injected with DWV-A and DWV-B. The average mortality associated with DWV-B (0.4% ± 0.33 SE) and DWV-recombinant (2.2% ± 0.83 SE) injection were significantly less than observed for DWV-A (11% ± 1.2 SE). Our results suggest that a higher proportion of DWV-B infected pupae will emerge into adults, compared to DWV-A. Overall, our data suggest that low mortality in pupae and the ability of DWV-B to accumulate to higher loads relative to DWV-A even during co-infection may favour vector transmission by *V. destructor,* and may thus be contributing factors to the increasing prevalence of DWV-B globally.

## INTRODUCTION

*Varroa destructor* is arguably one of the biggest threats to Western honey bee (*Apis mellifera*) populations worldwide. Over the past 60 years, *V. destructor* has spread globally from its origin in Asia where the mite originally parasitised the Asian honey bee *Apis cerana* (Solignac et al., 2005). *V. destructor* parasitism is particularly destructive to *A. mellifera* (hereafter simply honey bees), and is associated with significant colony losses. Australia is currently the only major beekeeping country to remain free from *V. destructor* (Oldroyd, 1999; Roberts et al., 2017).

Honey bee colony losses associated with *V. destructor* have often been attributed to viruses vectored by mites during feeding. One virus in particular, *Deformed wing virus* (DWV), is frequently associated with *V. destructor* (Highfield et al., 2009; Martin et al., 2012; Mondet et al., 2014; Martin and Brettell, 2019). DWV is a single-stranded positive sense RNA virus belonging to the *Iflaviridae* family. Prior to the spread of *V. destructor*, DWV was rarely detected whereas now the virus is found in virtually all honey bee populations worldwide, excluding Australia (Roberts et al., 2017). In some *Varroa*-free honey bee populations, DWV has been shown to have low prevalence and accumulate to very low levels (Martin et al., 2012; Ryabov et al., 2014; Shutler et al., 2014; McMahon et al., 2016). In contrast, numerous studies have found a positive correlation between *V. destructor* infestation levels and increased DWV loads (Martin et al., 2012; Nazzi et al., 2012; Mondet et al., 2014; Wu et al., 2017). Within a *V. destructor* infested colony, vector transmission of DWV is associated with approximately 20% pupal mortality (Martin, 2001; Martin et al., 2013). Such relatively low mortality allows the majority of DWV infected brood to emerge as adults. And because *V. destructor* reproduces within honey bee brood cells (Martin, 1995), low brood mortality results in a continuing increase in the number of mites and transmission of DWV.

Three DWV genotypes have been described: DWV-A, DWV-B and DWV-C (Mordecai et al., 2016b); only DWV-A (formally DWV) and DWV-B [formally *Varroa destructor virus* 1 or VDV-1 (Ongus et al., 2004)] are currently recognised by the International Committee on Taxonomy of Viruses. For clarity, we have employed the type A and B nomenclature widely adopted in recent publications (Martin et al., 2012; McMahon et al., 2016; Mordecai et al., 2016a; Mordecai et al., 2016b; Brettell and Martin, 2017; Kevill et al., 2017; Gisder et al.,

2018; Brettell et al., 2019; Dubois et al., 2019; Kevill et al., 2019; Remnant et al., 2019; Tehel et al., 2019).

An immediate effect of *V. destructor* appears to be a reduction in the genetic diversity of DWV in honey bees both in the field (Martin et al., 2012) and in experiments using injection of DWV to mimic vector transmission (Ryabov et al., 2014). Over time, the distribution of DWV genotypes changes so that one DWV genotype prevails within honey bee populations. DWV-B has become the most common variant in the United Kingdom (UK) and Europe (McMahon et al., 2016; Kevill et al., 2019; Manley et al., 2019b). In North-America DWV-A remains the most common genotype (Ryabov et al., 2017; Kevill et al., 2019). However, Ryabov et al. (2017) found that DWV-B prevalence in the US increased from 3% in 2010 to 65% in 2016. Similarly, Kevill et al. (2019) found that DWV-B was prevalent in 56% of tested colonies in 2016, and the dominant genotype in 23% of those colonies. Kevill et al. (2019) predicted that DWV-B prevalence will continue to increase and supersede DWV-A with time, as observed in England and Wales. Such change in relative prevalence suggests that the different DWV genotypes compete within their host. The increased prevalence of DWV-B may potentially be explained by differences in replication rate within the host, difference in virulence and associated host mortality, or a combination of both.

Understanding the exact relationship between DWV genotype and host virulence is far from straightforward. Not all studies distinguish between DWV genotypes. In those studies that do both DWV-A (Highfield et al., 2009; Martin et al., 2012; Mondet et al., 2014; Kevill et al., 2017; Barroso-Arévalo et al., 2019b; Kevill et al., 2019) and DWV-B (Natsopoulou et al., 2017) have been associated with colony deaths in the presence of *V. destructor*. At the same time, high viral loads of DWV-B have been associated with low levels of colony mortality in the UK and Spain (Mordecai et al., 2016a; Barroso-Arévalo et al., 2019b; Kevill et al., 2019). High DWV-B loads in surviving colonies that were untreated for *V. destructor* led Mordecai et al. (2016a) to hypothesise that DWV-B may outcompete DWV-A via 'superinfection exclusion'. If DWV-B is a superior competitor, it could prevent DWV-A from replicating to high levels. If, then, DWV-B causes less damage to the host, the exclusion of the more harmful DWV-A genotype could result in the association between DWV-B and low honey bee mortality.

Despite evidence of colonies surviving with high DWV-B loads, experimental evidence thus far indicates that DWV-B is more harmful to adult honey bees than DWV-A. After DWV-B was injected into adult bees, the virus was detected in brain tissue which was associated with impairment of cognitive function (Gisder et al., 2018). The inocula were then serially passaged in pupae before a second round of adult injections. After one round of serial passage viral particles were not detected in the bees' brain and the bees did not suffer from cognitive impairment. Gisder et al. (2018) associated the decreased tissue tropism and virulence of the passaged inoculum with a concurrent sequence shift from DWV-B to DWV-A. In a different study, injection of DWV-B into newly emerged adults resulted in significantly altered foraging behavior and higher mortality compared to controls (Benaets et al., 2017). However, the same experiment was not conducted on DWV-A (Benaets et al., 2017). When DWV-A and DWV-B were compared in a separate study, injection of DWV-B into newly emerged adults resulted in a significant reduction in survival compared to DWV-A (McMahon et al., 2016).

The most likely life stage to be infected with DWV is the pupal stage. Models have suggested that vector transmission of DWV to pupae results in reduced longevity in emerging adult honey bees and can lead to colony death in temperate climates, due to significantly reduced overwinter workforce (Martin, 2001; Sumpter and Martin, 2004). Thus, some studies have assessed whether DWV genotypes affect pupae differently. Gisder et al. (2018) found that injection of DWV-B into pupae resulted in significantly higher mortality compared to pupae injected with the passaged inoculum. Lamp et al. (2016) did not test DWV-B, but showed that both DWV-A directly isolated from infected bees and a constructed molecular clone both caused pupal death. However, Tehel et al. (2019) found no difference in survival between pupae injected with DWV-A or DWV-B when pupae were injected with the same source inocula as McMahon et al. (2016). Dubois et al. (2019) also found no difference in mortality between pupae infected with DWV-A and DWV-B obtained from heads of naturally infected bees. Similarly, a study using *V. destructor* and DWV naïve Australian honey bee pupae found no significant pupal mortality when white-eyed pupae were injected with DWV-A isolated from adult bees with overt disease symptoms, including deformed wings (Remnant et al., 2019).

Clearly while the global association between *V. destructor* and DWV seems irrefutable, determining whether virulence differences exist between DWV genotypes remains a challenge. The aforementioned experimental studies differ in many attributes, such as source inocula [with the exception of McMahon et al. (2016) and Tehel et al. (2019)], bee populations, life stage infected, experiment duration, and potential presence of other pathogens. In addition, covert infections with DWV may affect results as injection with buffered salt solutions can activate DWV replication (Dubois et al., 2019; Posada-Florez et al., 2019; Tehel et al., 2019). Similarly, previous infestations with *V. destructor* may have changed the viral landscape within honey bee populations by selecting for particular DWV genotypes that are better adapted to vector-based transmission. Australian honey bees are naïve to both *V. destructor* and DWV and are therefore an ideal model to determine the dynamics between different DWV genotypes. We infected white-eyed pupae to reflect the life stage at which *V. destructor* first vectors DWV to honey bees (Bailey and Ball, 1991). We infected pupae by injecting either a single DWV genotype or two genotypes (co-infection). Co-infection allowed us to determine the extent to which different DWV genotypes compete within the same host. We further determined if there is a relationship between viral load and host damage (mortality).

## MATERIALS AND METHODS

### 1. DWV source material and strain confirmation

Inocula were prepared from individual asymptomatic adult bees collected from Blenheim, New Zealand (DWV-A) and Amsterdam Water Dunes, the Netherlands (DWV-B and DWV-recombinant). The New Zealand bees were collected from *V. destructor* treated colonies and the Netherlands bees were collected from colonies that were part of a selection program for *V. destructor* tolerance (Panziera et al., 2017). The bees were imported on dry ice and stored at -80°C (Import permit and Quarantine details below). As we intended to use the source material as inocula, we firstly extracted viral material from individual bees from each population [protocol adapted from Remnant et al. (2019)]. We homogenised the thorax and abdomen [as eye pigments have been shown to inhibit PCR reactions (Boncristiani et al., 2011)] of individual adults in 2 mL 0.5M potassium phosphate buffer (PPB) pH 8. Within a fume hood, we added 5% v/v diethyl ether and 10% v/v chloroform and shook vigorously for 30 seconds,

before centrifuging at max speed (> 20000 × *g*) for 2 min. We then collected the supernatant and passed it through a 0.22 μm nitrocellulose filter to remove bacterial or particulate contaminants. We portioned the filtrate into aliquots, which were later used for RNA extraction or inoculation of pupae after strain identification.

For inocula identification, we obtained RNA from 100 μL of the partitioned filtrate using the RNeasy mini kit (Qiagen). For our sequencing negative control, we extracted RNA from a single juvenile velvet worm (*Euperipatoides rowelli*) using the Direct-zol RNA MiniPrep Plus (Zymo Research). To avoid any potential cross contamination we prepared the velvet worm sample in the Evolutionary and Integrative Zoology Laboratory, University of Sydney. All RNA samples were treated with DNase (Ambion® TURBO DNA-free kit) according to manufacturer's instructions. We shipped treated RNA (80-150 ng/μL) on dry ice to the Australian Genome Research Facility (AGRF) laboratory (Melbourne, Australia) for preparation of whole transcriptome, 150 bp paired-end libraries with ribosome depletion and MiSeq (Illumina) sequencing.

Sequencing reads were checked for quality using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and trimmed to remove residual adaptor sequences and low-quality sequences using Trimmomatic (Bolger et al., 2014). Trimmed reads were assembled *de novo* into contigs using the metagenomic assembler Megahit (Li et al., 2015). Resulting contigs were compared to a custom reference library containing previously identified honey bee virus genome sequences using BLASTn, including but not limited to *Acute bee paralysis virus* (ABPV), *Apis rhabdovirus* (ARV) (Remnant et al., 2017), *Black queen cell virus* (BQCV), *Chronic bee paralysis virus* (CBPV), *Israeli acute paralysis virus* (IAPV), *Kashmir bee virus* (KBV), *Lake Sinai virus* (LSV) and *Sacbrood virus* (SBV). The DWV-A and DWV-rec inocula were negative for all other honey bee viruses, including BQCV. The DWV-B inoculum contained low amounts of LSV and ARV-1 and ARV-2, however this did not impact our study as we found that these viruses were not transmissible to pupae via injection of our DWV-B inoculum (see Results). In addition, we examined a general viral reference database containing a comprehensive library of viral protein sequences downloaded from GenBank by using BLASTx to identify any potential novel viral sequences. Identified DWV contigs from each source inoculum were aligned to the DWV-A and DWV-B reference genomes in Geneious (Version 10.2.4, (Kearse et al., 2012); accession numbers

AJ489744 and AY251269), to produce alternate DWV strain sequence assemblies for each source inoculum. The DWV-A and DWV-B inocula sequences only contained the DWV genotype of interest. The DWV-rec inoculum contained one predominant genotype (Figure 2.1), where the average coverage per base was approximately 2800-fold, as estimated by Megahit. Additionally within the DWV-rec inoculum, we detected low frequencies of DWV-B, and an additional recombinant with an extended DWV-A fragment to position 2140, with low coverage per base values of 26 and 408, respectively (partial contig sequences available as Text A.1 and A.2). The DWV-A, DWV-B and DWV-rec inocula sequences used in this study were deposited to GenBank [accession numbers MN538208- MN538210]. We also compared our inocula sequences to strains previously injected by Gisder et al. (2018), Remnant et al. (2019) and Tehel et al. (2019). We performed pairwise comparisons and nucleotide alignments in Geneious using Muscle, and generated a maximum likelihood phylogenetic tree using PhyML (Figure A.1; Table A.1). We found that our DWV-A and DWV-B inoculum were the most similar to the references genomes, and more closely related to the inocula used by Remnant et al. (2019) and Tehel et al. (2019), compared to the isolates injected by Gisder et al. (2018).

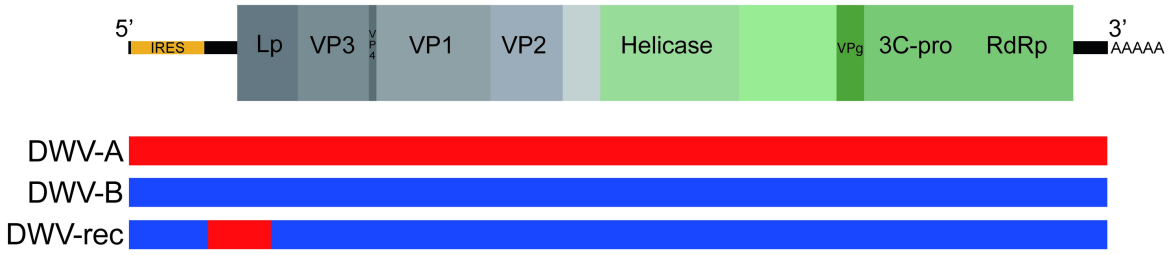


**Figure 2.1.** Schematic of the DWV genome structure. Location of coat proteins as per de Miranda and Genersch (2010). DWV-A and DWV-B sequence shown in red and blue, respectively. The Netherlands recombinant (DWV-rec) predominantly corresponds to DWV-B, with a DWV-A region between nucleotide positions 829 and 1487. The first recombination breakpoint occurs after the predicted internal ribosome entry site (IRES), which is predicted to fall within the first 810 nucleotides (Ongus et al., 2006).

## 2. Inocula standardisation

### 2a. Quantification of inocula viral load

First strand cDNA was synthesised from DNase treated RNA (0.2-0.4 µg) (Ambion® TURBO DNA-free kit) using SuperScript III Reverse Transcriptase (Invitrogen) with random hexamer primers, in 10 µL reaction volumes. The resulting cDNA was diluted in 30 µL UltraPure nuclease-free $dH_2O$ (Invitrogen). To determine the viral load of each inoculum we used quantitative PCR (qPCR) to compare quantitation cycle (Cq) values against the DWV-A and DWV-B standard curves (described below), and multiplied by dilution factor of 1/6400. We diluted inocula in 0.5M PPB pH 8.0 to standardise DWV concentration to $1 \times 10^7$ genome equivalents (GE). Next, we added 10% green food coloring (Queen, Australia) to visually aid injection.

### 2b. Preparation of DWV-A and DWV-B qPCR standards

We used absolute quantification with DWV-A and DWV-B plasmid standards to accurately determine viral loads in inocula and injected pupae (Figure A.2). DWV-A and DWV-B RdRp plasmid standards were prepared from the source material cDNA after strain confirmation (above), using the method adapted from Kevill et al. (2017). We analysed the cDNA by PCR with the Kapa2G Robust PCR Kit (Kapa Biosystems), as per manufacturer's instructions, using DWV strain specific RdRp primers (Table A.2). PCR cycling conditions for all reactions were 94°C (3 min), followed by 35 cycles of 94°C, 58°C and 72°C (30 sec), and 72°C (5 min). We analysed PCR products by gel electrophoresis on 1% agarose gel with SB buffer and SYBR Safe DNA stain (Life Technologies). We cleaned the PCR fragments with GF-1 PCR Clean-up Kit (Vivantis). Plasmid vectors containing DWV-A or DWV-B fragments were prepared with TOPO Cloning reaction and transfected into Transform One Shot TOP10 competent *Escherichia coli* cells (Invitrogen). LB plates with 50 µg/mL kanamycin were prepared as per manufacturer's instruction, plated with 100 µL cells and incubated at 37°C overnight. We performed colony PCR with DWV strain specific primers to ensure that transformation had occurred, and visualised PCR products on 1% agarose gel (as above). Colonies positive for DWV-A or DWV-B clones were added to 2 mL LB broth with 2 µL kanamycin and incubated at 37°C overnight. We then isolated plasmid DNA with Wizard SV Plus Minipreps DNA Purification System (Promega). As circular plasmids are known to supercoil and produce unreliable absolute qPCR results (Hou et al., 2010), we linearised our plasmids with *PmeI* restriction digest (New England Biolabs). We confirmed linearisation on

1% agarose gel, cleaned plasmids (as above), and quantified DNA concentration with Qubit Broad Range Assay. We calculated plasmid copy numbers as per Staroscik (2004), and diluted DWV-A and DWV-B linear plasmids to 5 ng/μL, which was equivalent to $1 \times 10^9$ genome copies of DWV.

### 2c. qPCR of DWV plasmid standards

We prepared ten-fold serial dilutions from the $1 \times 10^9$ plasmid stock to generate DWV-A and DWV-B standard curves from $10^8$ to $10^2$, prior to qPCR analysis with a Bio-Rad CFX384 Touch real-time PCR detection system. We performed all 5 μL qPCR reactions in triplicate with SsoAdvanced Universal SYBR Green supermix (Biorad), forward and reverse primers (final concentration 500 nM each), and 1 μL cDNA, in both DWV-A and DWV-B master mixes. We used the following cycling conditions: 95°C (10 min), followed by 35 cycles of 95°C (30 sec), 58°C (30 sec) and 72°C (30 sec). Melt curve analysis immediately followed between 55°C and 95°C, at 0.5°C increments. We plotted average Cq values against the $\log_{10}$ of the plasmid copy number to give a standard curve for DWV-A and DWV-B. PCR primer efficiency ($E = 10^{[-1/slope]}$) was 1.91 for DWV-A (slope = -3.5557, Y-intercept = 35.165, $R^2$ = 0.9998) and 1.92 for DWV-B (slope = - 3.5343, Y-intercept = 35.125, $R^2$ = 0.9998).

## 3. Pupal injection assay and sample preparation

### 3a. Experimental injection of pupae

We collected approximately 650 white-eyed pupae per colony, from capped brood cells of three unrelated *A. mellifera* colonies kept at the University of Sydney's apiary. These colonies are naïve to both *V. destructor* and DWV, neither of which are established in Australia (Roberts et al., 2017). Pupae that showed signs of melanisation or damage from uncapping were excluded from the assay prior to injection. Mated *V. destructor* females enter a honey bee brood cell just prior to the cell being capped and the bee undergoing pupation (Donzé and Guerin, 1994). The mother mite and her offspring feed on the fat bodies of the developing bee (Ramsey et al., 2019), during which the mother mite can transmit viruses acquired from her previous meal (Bowen-Walker et al., 1999). To mimic the natural vector-mediated infection route as closely as possible, we injected 475 white-eyed pupae per colony; each colony consisted of five treatment groups of 95 pupae. We adapted the injection protocol used by McMahon et al. (2016), to reflect the same viral load and similar DWV treatments. In our study, we injected pupae with either 2 μL of: (1) 0.5 M PPB pH 8 ('buffer control'), (2-4), 1

$\times 10^7$ GE of DWV-A, DWV-B, or the recombinant strain (DWV-rec), or (5) an equal mixture ('co-injection') containing $5 \times 10^6$ GE of DWV-A and DWV-B. Like McMahon et al. (2016), we co-injected pupae with the mixture to assess strain competition during co-infection.

We injected white-eyed pupae with a 32G needle attached to a 10 mL Hamilton syringe inserted between the 3rd and 4th tergites at the side of the abdomen [the typical *V. destructor* feeding site (Donzé and Guerin, 1994)], underneath but parallel to the cuticle to avoid puncturing the gut. After injection, we immediately placed pupae into Petri dishes lined with sterile filter paper (10 pupae/Petri dish). We placed the Petri dishes onto shallow racks within clip-locked plastic tubs (Sistema) and incubated at 34.5°C for 8 days (192 hours) in the dark. To keep the humidity high, we added 30 mL sterile $H_2O$ to the plastic tubs housing the Petri dishes.

After injection, we randomly selected four pupae per treatment and colony at regular time-points (1, 4, 8, 12, 24 hours) and every subsequent 24 hours for 192 hours (just prior to eclosion). Sampled pupae were immediately frozen at -80°C. We continued to incubate the pupae not collected for RNA extraction until 192 hours [when remaining pupae were terminated due to Quarantine permit conditions (see below)]. We visually monitored the survival of pupae throughout the experiment, using an adapted version of the method described by Remnant et al. (2019). We used the continual pigment changes in pupal eye and body colour (Jay, 1962) as indicators of healthy development. A pupa was classed as dead when eye or body pigments has ceased changing color for 48 hours.

### 3b. RNA extraction and cDNA synthesis

We extracted RNA from each frozen pupa separately in 1 mL of TRI Reagent (Sigma) with a TissueLyser, according to the manufacturer's protocol. We suspended RNA pellets in 200 µL ultra-pure water (Invitrogen) and quantified the concentration with Qubit Broad Range Assay (Life Technologies). Samples were standardised to 200 µg/mL RNA to account for body mass differences between individual pupae. First strand cDNA was synthesised from 0.8 µg DNase treated RNA in 10 µL reaction volumes, as described above. The resulting cDNA was diluted in 30 µL UltraPure nuclease-free $dH_2O$ (Invitrogen).

## 4. qPCR analysis

### 4a. Viral analysis of pupae

For DWV analysis, cDNA from all individual pupae were analysed in both DWV-A and DWV-B master mixes alongside DWV-A and DWV-B plasmid standards, and positive and negative controls. cDNA from the source inocula was used as positive DWV controls and water served as a negative (no template) control. In addition, we screened all samples for BQCV, SBV and amplified the endogenous control gene, *Actin* (Table A.2). Whole transcriptome sequencing results indicated that the DWV-B source material was positive for LSV, and ARV-1 and ARV-2. We screened DWV-B injected pupae for ARV-1 and ARV-2 by qPCR, and LSV by endpoint PCR with primers that amplify multiple LSV strains (Table A.2). The qPCR and 1% agarose gel results showed that these viruses were not transmitted to pupae via injection of DWV-B inoculum.

## 5. Data analyses

### 5a. Relative viral loads

Average Cq values from triplicate qPCR analyses were confirmed to have a standard deviation of $\leq 0.3$ and we considered Cq values $\geq 35$ to be DWV free. A small number of samples (3.2% of 720 pupae) randomly distributed across each treatment and colony had abnormal amplification of DWV or *Actin*. These cases included two pupae which had very high average Cq values of 31.2 and 28.3 for *Actin*, three pupae with abnormally low DWV loads for their time-point, and 17 pupae where DWV-A or DWV-B was detected in pupae injected with the opposite genotype. We excluded these pupae from further analyses and attributed these anomalies to possible pupal death, error during injection, and contamination during downstream processing, respectively. Though we suspected it unlikely, we wanted to ensure that the three pupae with low DWV loads for their time point (one pupa injected with DWV-rec from Colony 2 at 192 hours post-injection, and two co-injected pupae from Colony 3 at 144 hours post-injection) were not true reflections of natural variation between individual pupae. Thus, we repeated the statistical analyses with these three individuals included (see Results for further details).

We measured the accumulation of viral loads in pupae from 8 to 192 hours post-injection, relative to housekeeping gene *Actin* to account for any precision error during preparation and

handling of samples. Primer efficiencies were calculated using the slope of the standard curve constructed with a ten-fold dilution series of cDNA, from $10^0$ to $10^{-6}$ (Table A.2). We determined the relative loads of DWV strains or BQCV with the Pfaffl expression ratio (Pfaffl, 2001), which mathematically corrects for differences in primer efficiencies. The calculation compares the primer efficiency (E) and Cq difference (Δ) of the target virus (DWV strain or BQCV) to those of reference gene *Actin*, in individual pupae versus buffer controls. We assigned buffer injected pupae a Cq of 40 for their viral value, as they were negative for DWV and BQCV.

### 5b. Absolute DWV viral loads

Absolute viral loads in DWV injected pupae were interpolated from mean Cq values against the associated standard curve and multiplied by dilution factor (9/4000). This gave the absolute viral load as DWV genome equivalents in cDNA synthesised from 0.8 μg RNA. Mean absolute viral loads per treatment and colony (8 to 192 hours post-injection) have been provided in the supplemental materials (Figure A.2) so that our results can be compared to other studies.

## 6. Statistical analyses

### 6a. Accumulation and competition

To determine if there were significant differences in mean viral loads (relative to *Actin*) between genotypes over time we used a two-way ANOVA followed by Tukey (HSD) post-hoc analysis. As viral loads rapidly increased over many orders of magnitude within the first 48 hours (exponential phase of replication) and were visibly different between genotypes, we chose to analyze the most linear and consistent phase of the data, from 48 to 192 hours post injection. A visual assessment of the homogeneity of variance (Residual vs. Fitted plot) and normality (Normal QQ plot) assumptions showed that a fourth root transformation of the response variable (mean DWV load) substantially improved the model. We used backward elimination based on Akaike's Information Criterion (AIC) values to fit the most parsimonious model. All statistical analyses were performed with RStudio software (R version 3.5.0).

### 6b. Survival

We analysed the survival of pupae throughout the incubation period when exposed to the five different injection treatments. At each specific time-point pupae were assigned a survival value of 0 if alive or censored (removed for viral analysis), or 1 if dead. As the data did not meet the Cox proportional hazards assumption, we analysed the mean proportion of pupal survival with a generalised linear mixed effects model (glmer) with binomial distribution and logit link function ("lme4" package) (Bates et al., 2018). Again, the most parsimonious model was determined with backward elimination based on AIC values. We then analysed the final model as a type II ANOVA ("car" package) (Fox et al., 2018), followed by Tukey pairwise comparison analysis using lsmeans function ("lsmeans" package) (Lenth, 2018).

## 7. Quarantine permit

Frozen adult honey bees (workers) containing DWV were imported from New Zealand and the Netherlands under our Department of Agriculture and Water Resources import permit 0000917783. The permit allows us to infect local honey bee pupae with DWV within our strictly controlled Quarantine approved laboratory at the University of Sydney; however, all pupae must be terminated prior to eclosion. Thus, the remaining pupae that were not collected at earlier time points for viral analysis were terminated at 192 hours post-injection, prior to eclosion.

## RESULTS

### Viral accumulation post-injection

We measured the accumulation of viral loads relative to housekeeping gene *Actin* and as absolute genome copy equivalents by standard curve (Figure A.2) in individual pupae using qPCR with cDNA synthesised from 0.8 μg RNA. Our method does not measure any level of viral degradation by the honey bee immune response and therefore reflects the net virus levels, assuming that a combination of viral replication and degradation occurs. DWV was not detected in any of our buffer injected pupae ($n$ = 144). The average Cq values for DWV in pupae at 1 and 4 hours post-injection were >30, inconsistent between samples, and in some individuals DWV was not detected at all. Thus, these time points were excluded from further analyses. DWV was detected in all DWV-injected pupae from 8 hours post-injection onwards. Viral loads of all genotypes (DWV-A, DWV-B and DWV-rec) rapidly increased within the first 48 hours post-injection, either when injected alone (Figure 2.2; Figure A.3) or co-injected (DWV-A and DWV-B) (Figure 2.3; Figure A.4), and plateaued between 72-96 hours post-injection. We found that accumulation patterns were more dynamic within the first 48 hours, with high variation between genotypes and colonies. Despite this variation, DWV-B loads were generally lower than DWV-A and DWV-rec, particularly in colonies 1 and 2. However, DWV-B loads in all colonies exceeded DWV-A from 72 hours post-injection. This remained true when DWV-A and DWV-B were co-injected excluding DWV-B loads in colony 1 compared to DWV-A loads in colonies 2 and 3 (Figure 2.3).

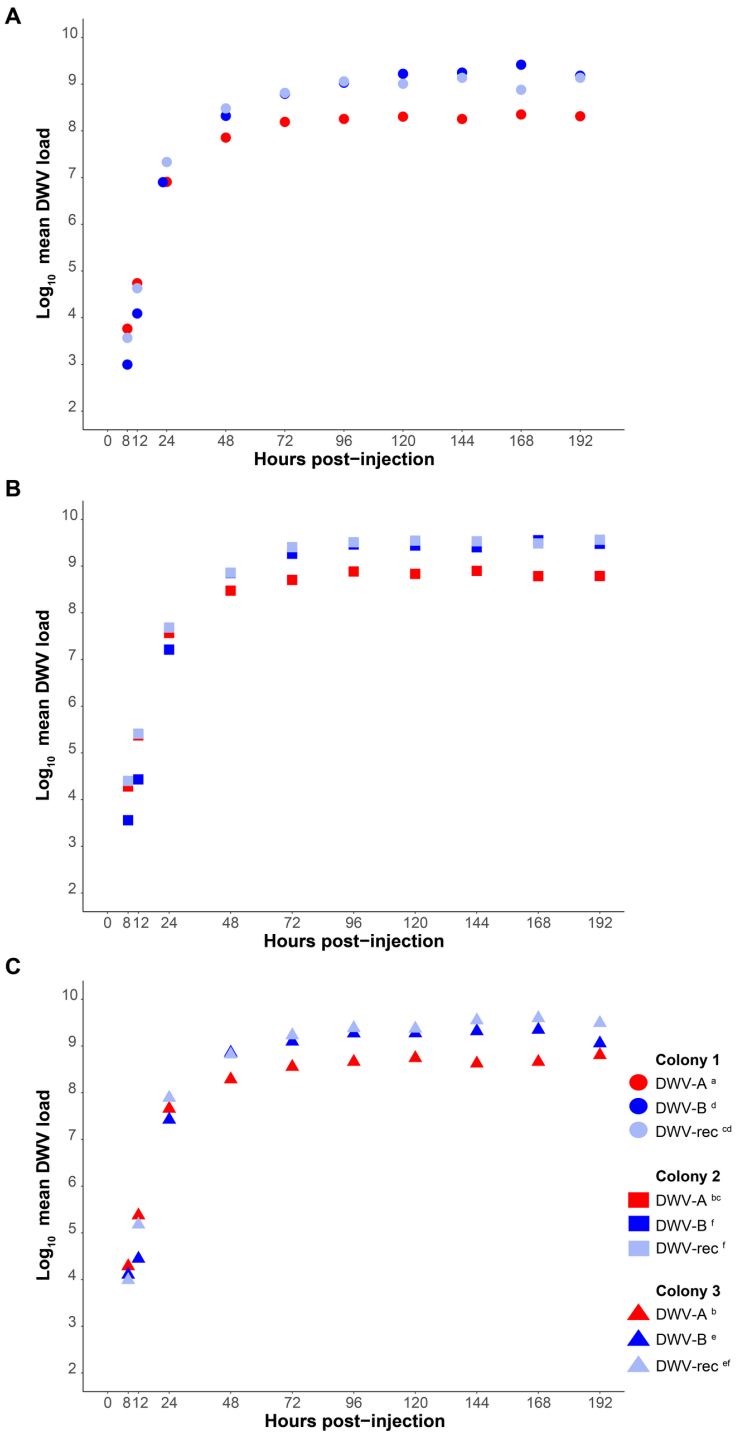


**Figure 2.2.** Mean DWV viral loads of individually infected pupae from 8 to 192 hours post-injection ($n$ = 120 per treatment), relative to housekeeping gene *Actin* in cDNA synthesised from 0.8 µg RNA. White-eyed pupae from three naïve colonies (Figures 2A, 2B, 2C) were singly injected with $1 \times 10^7$ genome equivalents of DWV-A, DWV-B or recombinant strain ('DWV-rec'). Viral loads rapidly increased over several orders of magnitude within the first 48 hours of infection (exponential replication phase). Statistical analyses were performed in the linear phase of the data, from 48 to 192 hours post-injection. Significant ($p < 0.05$) differences between genotypes and colonies indicated with lettering. See supplementary material Tables A.3 and A.4 for details of the statistical analyses.

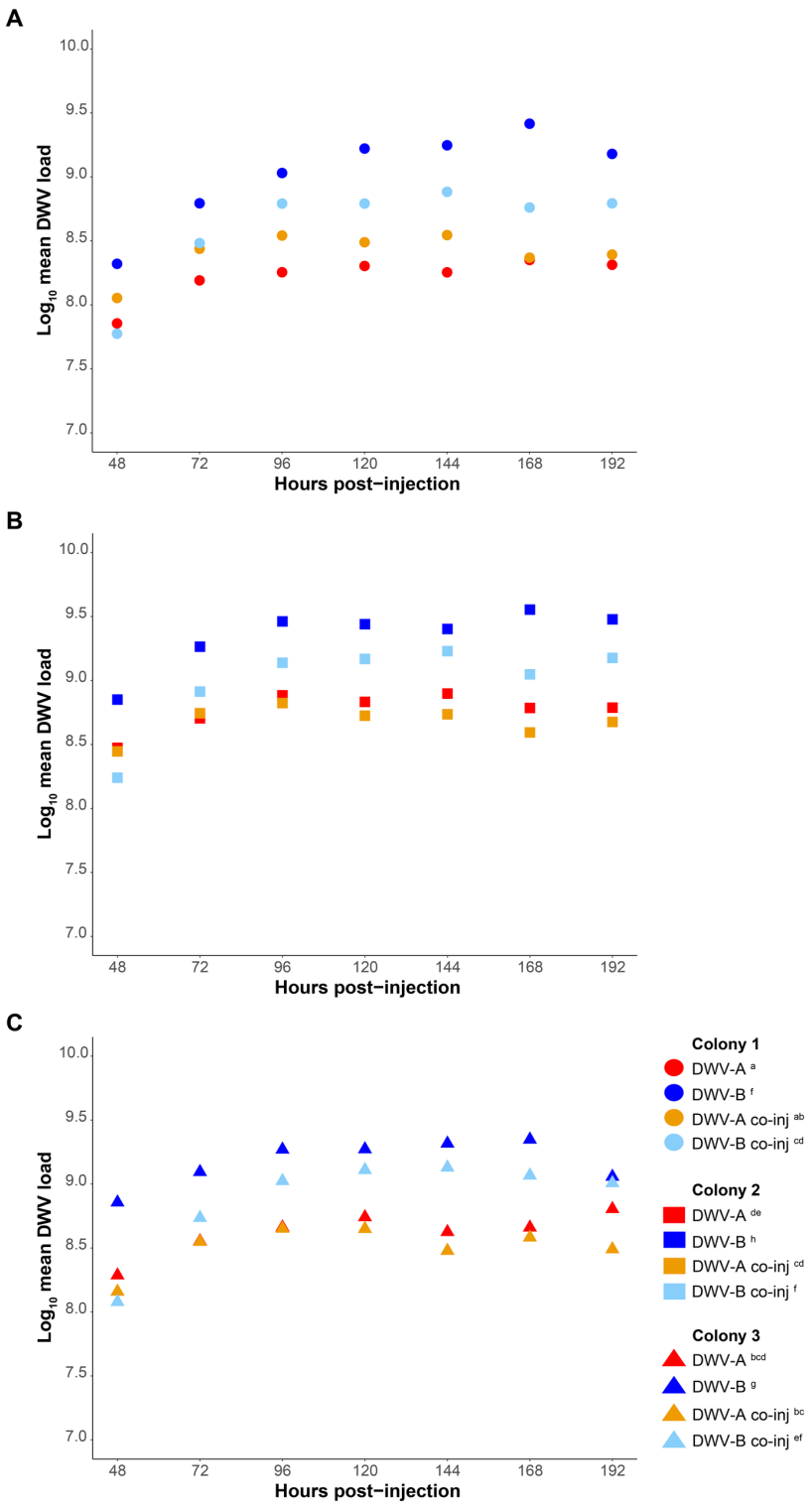


**Figure 2.3.** Comparison of mean DWV viral loads of individual pupae singly infected with DWV-A or DWV-B versus pupae co-injected with $5 \times 10^6$ genome equivalents of DWV-A and DWV-B, from 48 to 192 hours post-injection. Viral loads are relative to housekeeping gene *Actin* in cDNA synthesised from 0.8 µg RNA. Accumulation loads are displayed by colony (Figures 2.3A, 2.3B, 2.3C). Significant ($p < 0.05$) differences between genotypes and colonies indicated with lettering. Viral loads of singly injected pupae also shown in Figure 2.2.

To determine if there was a significant difference in the net accumulation of viral loads from 48 to 192 hours post-injection, we compared the mean viral loads of all genotypes relative to *Actin* (injected singly or co-injected) with an ANOVA followed by Tukey (HSD) post-hoc analysis. We found significant differences in mean viral loads amongst all genotypes ($F$ = 248.642; $df$ = 4, 381; $p < 0.0001$), and found that the strains differed in the rate at which they accumulated, as indicated by a significant strain × time interaction ($F$ = 15.325; $df$ = 4, 381; $p < 0.0001$) (Table A.3). DWV-B accumulated to significantly higher loads compared to DWV-A in all colonies (Tukey (HSD) posthoc $p < 0.0001$; Figure 2.2; Table A.4), and viral loads were 5 to 10-fold higher than DWV-A. The accumulation of DWV-B and DWV-rec were not significantly different within the same colonies ($p < 0.05$). However, mean viral loads were significantly affected by colony ($F$ = 147.876; $df$ = 2, 381; $p < 0.0001$), although the overall pattern of increase over time remained the same. We found that colony 1 pupae injected with DWV-A or DWV-rec had significantly lower loads compared to colonies 2 and 3 ($p < 0.0001$), and that DWV-rec loads in colony 1 were not significantly different to DWV-A loads in colony 3 ($p$ = 0.1358). Similarly, DWV-B loads were significantly different between all colonies ($p < 0.05$). We also found a significant interaction between strain and colony ($F$ = 4.837; $df$ = 8, 381; $p < 0.0001$). Upon reanalysis, the inclusion of the three pupae with low loads for their time point did not alter the significance of any of the predictor variables (Table A.5). However, we did find slight differences in the pairwise comparisons of colonies; DWV-B loads between colonies 1 and 3, and 2 and 3 were no longer significantly different ($p > 0.05$) (Table A.6).

We classified competition between DWV genotypes as a significant reduction in mean viral loads of DWV-A or DWV-B when co-injected compared to singly from 48-192 hours post-injection, relative to *Actin*. Co-injected DWV-B loads were significantly lower than when DWV-B was injected alone in all colonies ($p < 0.0001$; Figure 2.3; Table A.4). In contrast, DWV-A loads did not differ when injected singly or when co-injected ($p > 0.05$). As when injected singly, we found that DWV-A loads in co-injected pupae were significantly lower in colony 1 compared to colony 2, and DWV-B loads were significantly lower in colony 1 compared to colonies 2 and 3 ($p < 0.05$). We found slight differences in these results with the inclusion of the three additional pupae, whereby DWV-A loads in co-injected pupae were significantly higher ($p < 0.05$) in colony 2 compared to both colony 1 and 3 (Table A.6).

**Survival**

We monitored the survival of pupae throughout the incubation period when exposed to the five different injection treatments. At each specific time-point pupae were assigned a survival value of 0 if alive or censored (removed for viral analysis), or 1 if dead. While the vast majority of pupae survived the injections, survival was significantly affected by treatment ($\chi^2$ = 44.472; *df* = 4; *p* = < 0.0001) (Table A.7). The survival of pupae singly or co-injected with DWV-A did not significantly differ (*p* = 0.1095; Figure 2.4A; Table A.8). However, only pupae singly injected with DWV-A had mortality that significantly differed from the buffer control (*p* = < 0.0001), and only after 120 hours post-injection (Figure 2.4B). The survival of pupae injected with DWV-B or DWV-rec did not significantly differ from that of the buffer controls (*p* > 0.05). 'Colony' had no effect on survival (using Akaike's information criterion during backward elimination; Table A.9).

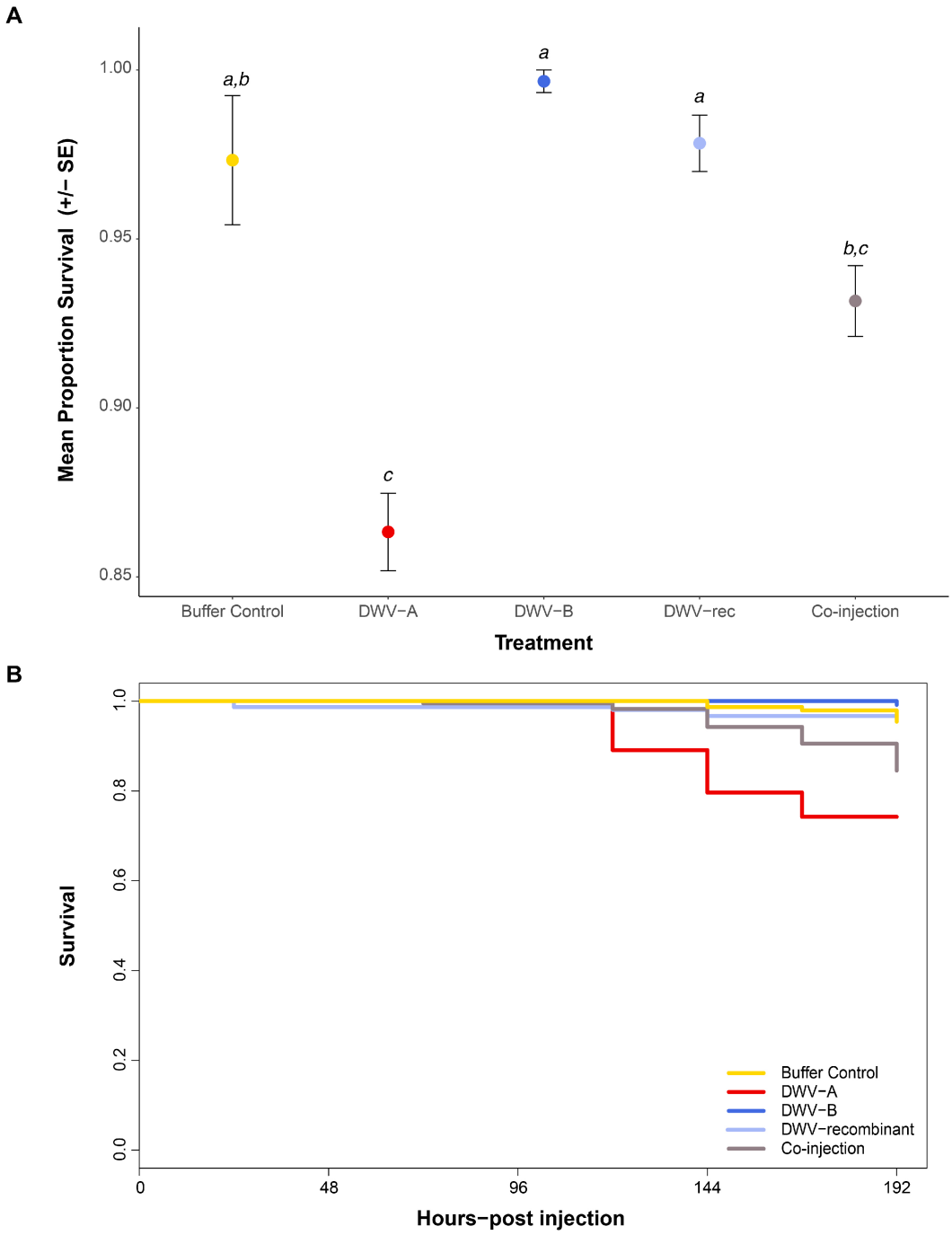


**Figure 2.4.** Survival of individual naïve pupae singly injected with DWV-A, DWV-B, DWV recombinant ('DWV-rec'), buffer control, or co-injected with DWV-A and DWV-B ($n$ = 285 per treatment). Pupal survival was monitored throughout the incubation period, up to 192 hours post-injection. Pupae collected at regular time points for viral analysis were recorded as censored, thus the final number of remaining pupae at 192 hours post-injection was $n \leq 141$ per treatment (depending on mortality). (A) Mean proportion and standard error of pupal survival at 192 hours post-injection. Letters show significant differences between treatments ($p < 0.05$) based on pairwise comparisons of the final model. See the supplementary material, Tables A.7 to A.9 for details of statistical analyses. (B) Survival curve of pupae by treatment up to 192 hours post-injection. Data did not meet the Cox proportional hazards assumption, thus Figure 2.4B only used to illustrate the pattern of mortality over time.

**Presence of other viruses in injected pupae**

Because our DWV-B inoculum contained LSV, and ARV-1 and ARV-2 we screened the DWV-B injected pupae for LSV by endpoint PCR with primers designed to amplify multiple variant strains, and ARV-1 and ARV-2 by qPCR (Table A.2). LSV, ARV-1 and ARV-2 were not detected in any of the DWV-B injected pupae, suggesting that these viruses were not transmissible via injection of the DWV-B inoculum. The DWV-A and DWV-rec inocula were negative for all other known honey bee viruses. However, we additionally chose to screen all injected pupae for BQCV and SBV via qPCR as Remnant et al. (2019) previously found covert infections of both viruses in our honey bee population. We did not detect SBV in any pupae across all colonies. In contrast, we detected BQCV in some of the DWV-A and co-injected pupae, yet BQCV was not detected in any of the DWV-B, DWV-rec or buffer injected pupae across the three colonies. The relative BQCV loads varied highly between individuals and colonies (Figure 2.5). BQCV had higher prevalence in pupae singly injected with DWV-A, where the virus accumulated to loads $\geq 1 \times 10^7$ in 4.9% of pupae, predominantly between 48 to 96 hours post-injection (Figure 2.5). Less than 1% of co-injected pupae had BQCV loads of $10^7$ or more. Unlike the DWV strains, BQCV loads did not continuously increase over time. Peak BQCV loads coincided with the commencement of mortality observed with DWV-A injections, at 120 hours post-injection (Figure 2.4B).

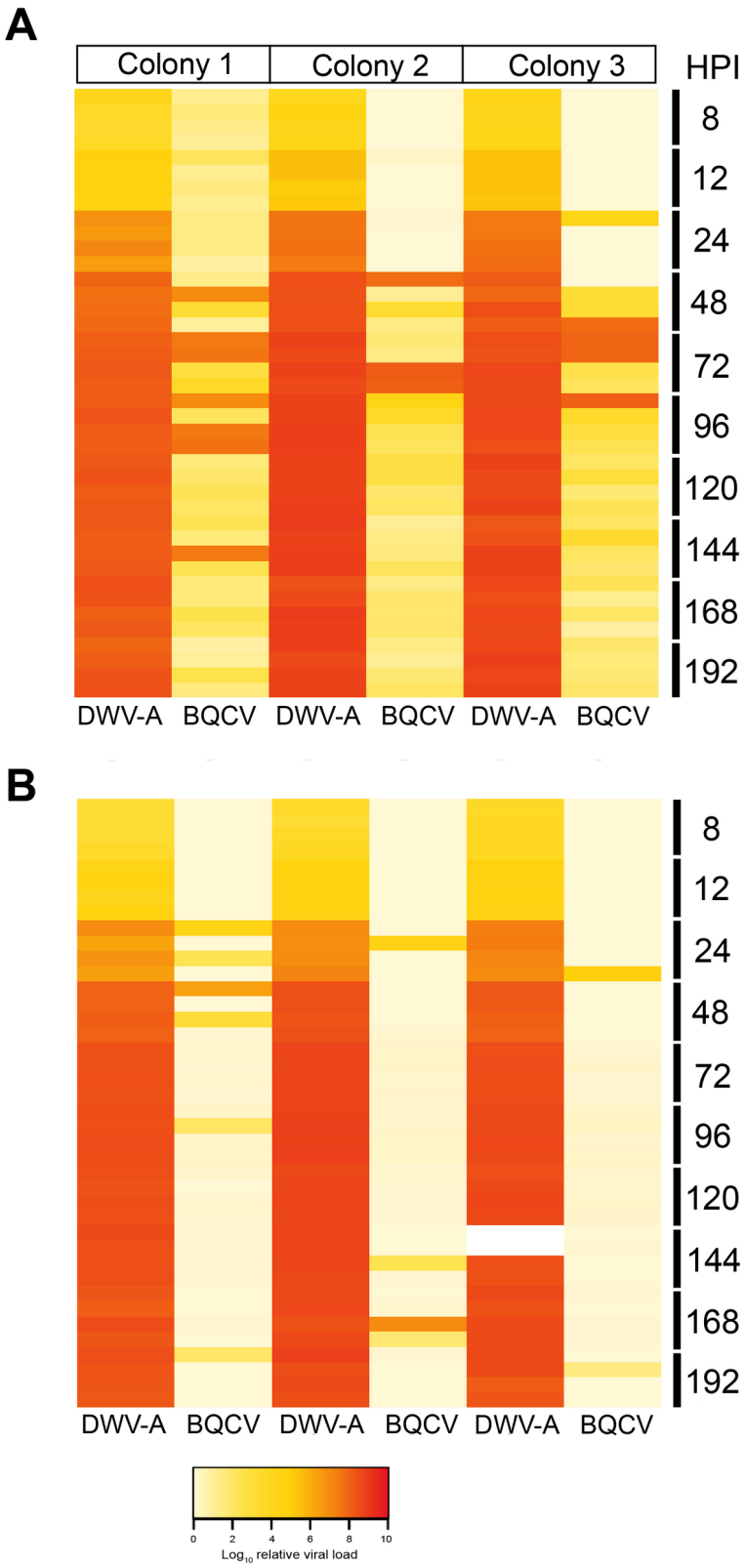


**Figure 2.5.** Heat map showing the $\log_{10}$ relative viral loads of DWV-A in comparison to BQCV per pupa and colony, in single DWV-A (A) and co-injected pupae (B), from 8 to 192 hours post-injection. The two white cells in colony 3 (co-injected DWV-A at 144 hours post-injection) had abnormally low DWV loads for their time-point, thus were excluded from analyses.

## DISCUSSION

Our aim was to assess the ability of three genotypes of DWV (DWV-A, DWV-B and DWV-rec) to accumulate in honey bee pupae naïve to DWV and *V. destructor*, both in isolation and during co-infection. Our experimental protocol resulted in a rapid infection with all DWV genotypes accumulating to viral loads exceeding $1 \times 10^7$ (relative to housekeeping gene *Actin* or $1 \times 10^9$ genome copy equivalents by standard curve) within 48 hours. Viral loads typically plateaued at 96 hours post-infection, in agreement with previous analysis by ELISA (Martin et al., 2013). We found significant differences in the relative mean viral loads of the two master variants, which differed by an order of magnitude in colony 1, and approximately 5-fold in colonies 2 and 3. While DWV-A loads initially accumulated faster, DWV-B ultimately reached significantly higher levels from 72 hours post-injection. Our results are consistent with the experimental findings of Tehel et al. (2019), Dubois et al. (2019), and McMahon et al. (2016) who also found that DWV-B accumulates to higher loads than DWV-A when injected into pupae or adults, despite all four studies using different honey bee populations. While Tehel et al. (2019) and McMahon et al. (2016) used the same source of inocula, the inocula used by Dubois et al. (2019) and our study were different. Given the consistency in results despite the differences amongst the four studies, we can safely conclude that DWV-B reaches higher viral loads than DWV-A after injection. Similarly to our study, in English and Welsh colonies (Kevill et al., 2019) and in the USA (Ryabov et al., 2017; Kevill et al., 2019), mean DWV-B loads were approximately 7-fold higher than DWV-A when colonies contain both genotypes. Yet there are exceptions. In some co-infected colonies in the USA that died over winter, Kevill et al. (2019) found significantly higher DWV-A loads relative to DWV-B.

Only DWV-B appears to be affected by competition when co-injected. We found that DWV-B loads were significantly reduced in pupae co-injected with DWV-A across all colonies. Interestingly by 96 hours post-infection, DWV-B still accumulated to higher loads than DWV-A during co-infection, excluding colony 1. DWV-A loads, when co-injected, were not significantly different to single DWV-A injections, despite containing half the starting dose. Thus, competition appears to be independent of the initial dose. Our results are in accordance with Tehel et al. (2019) even though Tehel et al. (2019) injected a much lower dose ($10^2 – 10^4$ genome equivalents) and quantified virus levels in far fewer samples ($n$ = 4 to 11). It thus seems there is a maximum level that DWV-A can accumulate to. This is in agreement with Ryabov et al. (2019), who found that five divergent DWV-A clones all accumulated to the

same level. While we observed evidence of competition between DWV-A and DWV-B, we did not see strong competitive exclusion between the genotypes, suggesting that the reduction in DWV-B loads was not due to a lack of some critical resource required for viral replication. In contrast, *Israeli acute paralysis virus* (IAPV) and closely related *Kashmir bee virus* (KBV) appear to compete directly for cellular resources. In the presence of KBV, accumulation of IAPV was reduced by four orders of magnitude (Carrillo-Tripp et al., 2016). Lastly, we found significant differences in both single and co-injected DWV loads between colonies, indicating that colony-level factors, such as immune response (Niu et al., 2014; Brutscher et al., 2015), might additionally affect DWV accumulation.

Our recombinant strain (DWV-rec) accumulated to equally high loads as DWV-B within the same colonies. Previous studies have shown that some recombinant strains can replicate to higher loads than the master variants (Moore et al., 2011; Zioni et al., 2011; Ryabov et al., 2014). The genome structure of DWV-rec predominantly corresponds to DWV-B, with two recombination breakpoints at positions 829 and 1487 (when aligned to DWV-B AY251269), resulting in a DWV-A region from the 5' untranslated region (UTR) up to approximately the first half of the Leader protein (Lp). This structure differs from previously characterised recombinants, which have a breakpoint within the helicase region and subsequent non-structural proteins corresponding to DWV-A (Moore et al., 2011; Zioni et al., 2011; Ryabov et al., 2014; Dalmon et al., 2017). Interestingly, the 5' end of our DWV-rec strain is similar to the RecVT-Fr1 strain isolated from a *V. destructor* tolerant colony in France (Dalmon et al., 2017), although our breakpoints occur earlier.

The difference in accumulation amongst the genotypes may potentially be due to the way viruses interact with cellular translational machinery. Many RNA viruses, including *Dicistroviridae*, *Flaviviridae* and *Picornaviridae*, use internal ribosome entry site (IRES) secondary structures to initiate translation of their open read frame(s) (Martínez-Salas, 2008). The predicted IRES of DWV-A and DWV-B fall approximately within the first 810 nucleotides (Ongus et al., 2006), prior to our first breakpoint at position 829 for DWV-rec. Thus, the IRES of DWV-rec corresponds to DWV-B. DWV-A and DWV-B share approximately 84% nucleotide and 95% amino acid homology (Ongus et al., 2004). While their 5' UTR sequences differ, the overall IRES structures were predicted to be the same (Ongus et al., 2006). Nevertheless, small sequence differences may result in different

translational efficiencies. For example, a single nucleotide mutation (C472U) in the IRES reduces poliovirus type 3 replication and virulence in mouse neural tissue (La Monica et al., 1987), but does not affect organ tropism (Kauder and Racaniello, 2004). At this stage, it is unclear exactly what part of the genome is most important for DWV replication. However, it is possible that the increased accumulation of DWV-B and DWV-rec compared to DWV-A might be associated with sequence differences within the IRES.

We found differences in mortality between pupae injected with different genotypes. In agreement with Tehel et al. (2019) and Dubois et al. (2019), we found no relationship between viral accumulation and mortality in pupae. In our study, only pupae singly injected with DWV-A showed mortality statistically different from the buffer control, but mortality was low (11% ± 1.2 SE). Interestingly, the 0.4% mortality we observed in DWV-B injected pupae up to 192 hours (8 days) post-injection was less than the 18%, 55% and 0-75% mortality observed by Tehel et al. (2019), Gisder et al. (2018), and Dubois et al. (2019), respectively, after 7 or 10 days post-injection.

Gisder et al. (2018) postulate that their high pupal mortality is further evidence that DWV-B is more virulent than DWV-A, yet the independent DWV-B isolates injected in our study and by Tehel et al. (2019) were found to be more similar (99.3% and 98.9% pairwise identity, respectively) to the DWV-B reference genome [AY251269; isolated from *V. destructor* by Ongus et al. (2004)] than the three isolates injected by Gisder et al. (2018) (91.4% to 96.9%). Furthermore, the DWV-$_{P0}$ I isolate injected by Gisder et al. (2018) shows recombination with DWV-A, however Gisder et al. (2018) did not indicate whether mortality differed between their three DWV-$_{P0}$ isolates. Dubois et al. (2019) associated their high mortality with SBV, initially present at very low levels in their inocula. While Tehel et al. (2019) and Dubois et al. (2019) found no difference in mortality between DWV-A and DWV-B injected pupae, this may be affected by background DWV infection in their pupae. Both studies detected accumulation of both DWV genotypes upon injection of a single genotype, accumulation of both genotypes in buffer injected pupae and had higher control mortality (11-25%) than observed in our study (2.7% ± 1.9 SE).

While we did find significant mortality when pupae were injected with DWV-A, we caution that we cannot exclude that the mortality was attributed to BQCV and not DWV-A, particularly as BQCV is known to kill brood (Chen and Siede, 2007). Unfortunately, we were unable to screen our 48-hour dead pupae for viruses due to extreme RNA degradation, thus cannot determine if dead pupae were infected with BQCV. Nevertheless, we only detected significant mortality in pupae injected with DWV-A, and only detected BQCV in pupae injected with the DWV-A genotype. As BQCV was not detected in any of our inocula by whole transcriptome sequencing, it seems unlikely that we injected BQCV together with DWV. While we cannot completely exclude the possibility that BQCV was present in our DWV-A inoculum at levels too low to be detected, we think this unlikely because we did detect low amounts of LSV and ARV in the DWV-B inoculum. It could be that DWV-A has an immunosuppressive effect that then allows other viruses, such as BQCV, to replicate to high viral loads as suggested by Barroso-Arévalo et al. (2019a). In a 21 month study of honey bee colonies in Spain, Barroso-Arévalo et al. (2019a) found that BQCV, in addition to DWV and *V. destructor,* was highly prevalent and negatively correlated with colony vigor. As our study was conducted in the absence of *V. destructor*, our results may point to a synergistic interaction between DWV-A and BQCV, such that injection with DWV-A activates an endogenous BQCV infection, potentially by disrupting immune response of pupae more than other DWV genotypes. D'Alvise et al. (2019) also suggested a potential synergistic interaction between DWV and BQCV. Their regression analysis showed that DWV was the most significant predictor of BQCV accumulation in German honey bees, despite contrasting seasonal dynamics and BQCV being significantly correlated to virtually all of the tested viral pathogens and intestinal parasites (D'Alvise et al., 2019). In agreement with Barroso-Arévalo et al. (2019a), D'Alvise et al. (2019) postulated this interaction may be associated with a reduction in host immune defense by DWV. As DWV-A and DWV-B were combined for analysis (D'Alvise et al., 2019), it is unclear if their results would differ between DWV genotypes. We found no evidence of a relationship between DWV-B and BQCV, or between our DWV-rec strain and BQCV. Previous modeling has shown that 20% pupal mortality associated with *Varroa* transmission of DWV to pupae can lead to colony mortality, due to a reduction in workforce longevity (Martin, 2001). While the mortality observed for DWV-A in our study was less than this, any increased effect of DWV-A on the mortality of pupae, with or without an interaction with BQCV, can explain the shift from DWV-A to DWV-B observed globally. Because the reproductive success of *V. destructor* depends on the pupa

surviving to adulthood, DWV-A associated pupal mortality will negatively affect the transmission of DWV-A in favor of the transmission of DWV-B.

Our data provide some explanation for the continued global increase in prevalence of DWV-B over DWV-A. Low mortality in pupae and the ability of DWV-B to accumulate to higher loads relative to DWV-A, even during co-infection, are likely to be contributing factors to the increasing prevalence of DWV-B. Further, our observed interaction between DWV-A and BQCV highlights the complex relationships between viruses. Previous studies have suggested, implicitly and explicitly, that studying a single virus in isolation does not provide the whole picture (Mondet et al., 2014; Carrillo-Tripp et al., 2016; Dubois et al., 2019; Remnant et al., 2019), particularly as honey bees are frequently infected with multiple pathogens (Bailey et al., 1981; Chen et al., 2004; Berényi et al., 2006; Berthoud et al., 2010; Nguyen et al., 2011; Cornman et al., 2012; Locke et al., 2014; Mondet et al., 2014; Amiri et al., 2015; Natsopoulou et al., 2017; D'Alvise et al., 2019). While a direct relationship between DWV-A and BQCV requires experimental validation, our results suggest that future studies should continue to incorporate a broader ecological approach by experimentally investigating how multiple pathogens interact with their honey bee hosts.

## ACKNOWLEDGEMENTS

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## CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**FUNDING**

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# *Deformed wing virus* genotypes A and B do not elicit immunologically different responses in naïve honey bee hosts


## ABSTRACT

*Deformed wing virus* (DWV), in association with *Varroa destructor*, is currently the leading factor associated with global honey bee deaths. With the exception of Australia, the virus and mite have a near global distribution, making it difficult to separate the effect of one from the other. Over time, the prevalence of the two main DWV genotypes (DWV-A and DWV-B) has changed, leading to the suggestion that the two strains elicit a different immune response by the host, the western honey bee *Apis mellifera*. Here we use a honey bee population naïve to both the mite and the virus to investigate if honey bees show a different immunological response to DWV genotypes. We examined the expression of 19 immune genes by RT-qPCR and comprehensively analysed the small RNA response in honey bees after experimental injection with DWV-A and DWV-B. We found no evidence to indicate that DWV-A and DWV-B elicit a different immune response in honey bees. We found that RNA interference genes are up-regulated during DWV infection and that the small interfering RNA (siRNA) response is proportional to viral loads, yet does not inhibit the virus from accumulating to high loads. We also found that the siRNA response towards DWV was weaker than the response to another honey bee pathogen, *Black queen cell virus*. This suggests that DWV is comparatively better at evading antiviral host defences. There was no evidence for the production of virus-derived PIWI-RNAs in response to DWV infection. In contrast to previous studies, and in the absence of *V. destructor*, we found no evidence that DWV has an immunosuppressive effect in honey bees. Overall, our results advance our understanding of the immunological effect DWV elicits in honey bees.

## AUTHOR SUMMARY

Global honey bee colony deaths are strongly associated with the spread of the parasitic mite *Varroa destructor* and RNA viruses vectored by it. *Deformed wing virus* (DWV) has become the most prevalent honey bee virus and is now almost ubiquitous with *V. destructor*. Some previous studies suggest that DWV has an immunosuppressive effect. Differences in host immune response could explain why some studies find that the two main genotypes of DWV, A and B, differ in their virulence. The almost ubiquitous association between DWV and *V. destructor* makes it challenging to disentangle the effect of DWV upon its host in the absence of the mite. Using a honey bee population naïve to both the mite and the virus, we investigated the immune response of honey bees experimentally infected with DWV-A and DWV-B. Our data provide little evidence to indicate that honey bees respond differently to the DWV genotypes. Further, our results contradict previous findings that DWV has an immunosuppressive effect. This suggests that the effect of feeding by *V. destructor* on the bees' immune system may be larger than previous findings have indicated.

## INTRODUCTION

Obligate pathogens need to evade or suppress host immune defences in order to hijack the host's cellular machinery. The success of the pathogen largely depends on how effectively it can enter and replicate within a host cell, and conversely, how successfully the host can inhibit, degrade or tolerate the infectious agent (Hedrick, 2017). Avoidance is often the first defence strategy against an infectious agent (Curtis, 2014; Townsend et al., 2020). Disease avoidance through the use of quarantine was first practiced in 14$^{th}$ century, whereby ships carrying sick individuals were prohibited from docking at port in Venice, Italy, for 40 days to stop the transmission of the bubonic plague (Kilwein, 1995). Avoiding transmission can be challenging when living in dense populations with frequent social interactions, due to the increased probability of encountering an infected individual. Rapid human population growth in cities, overcrowded slums, poor sanitation and contaminated water supplies, create ideal conditions for the transmission of human diseases such as tuberculosis, typhoid and cholera (Duffy, 1971).

Social insects such as ants, termites, and some bee and wasp species, which live in often densely populated colonies, are particularly vulnerable to infectious disease. Pathogen

avoidance is the first defence against disease transmission. Social insects have numerous behavioural adaptations that collectively provide 'social immunity' (Simone-Finstrom, 2017; Cremer et al., 2018), such as corpse removal in bees (Rosengaus et al., 1999) and ants (Wilson et al., 1958), pathogen alarm signalling in termites (Rosengaus et al., 1999), use of plant resins as antimicrobial agents in bees (Simone et al., 2009), and specific worker castes to deal with waste in ants (Hart and Ratnieks, 2001). These behavioural responses are so effective that they are thought to have contributed to the reduction in the number of immune genes that social insects have compared to their solitary counterparts (Harpur and Zayed, 2013). Nonetheless, social insects still rely upon an innate immune response at the individual level, as even the most hygienic colonies cannot entirely avoid pathogens.

Social insects have a number of immune response pathways involved in dealing with pathogens. Small interfering RNAs, operating within the RNA interference (RNAi) pathway, are the major antiviral defence mechanism. Double stranded RNA produced during the replication of DNA and positive sense RNA viruses is recognised by the protein dicer, which subsequently cleaves the RNA into 21-23 nt fragments termed virus-derived small interfering RNAs (vsiRNAs). The vsiRNAs bind to an argonaute protein and are loaded into an RNA-induced silencing complex (RISC), which catalyses the degradation of targeted viral RNA (Hammond et al., 2001). The Toll, Imd and JAK/STAT pathways are microbial sensing pathways that detect pathogens via cell surface receptors and initiate a downstream transcriptional response (Lemaitre and Hoffmann, 2007). The Toll pathway is generally associated with fungi and Gram-positive bacteria, and the Imd pathway with Gram-negative bacteria. *Dorsal* (Toll), and *relish* (Imd) are both NF-κB family transcription factors, involved in the production of antimicrobial peptides (AMPs) in fat bodies, a tissue analogous to the mammalian liver (Lemaitre and Hoffmann, 2007). The Toll and Imd pathways may also be involved in antiviral defence (Brutscher et al., 2015).

Parasites and pathogens are a particularly serious problem for the Western honey bee *Apis mellifera* due to its commercial use. As a worldwide pollinator of commercial crops, colony densities are unnaturally high in many areas. Almond orchards in Australia and the US are a good example. Each year approximately 200,000 and 2,000,000 honey bee colonies are moved to almond-growing areas in Australia and the US, respectively, for the purpose of pollination (Le Feuvre, 2017; Lee et al., 2018). In addition, beekeepers' practices lead to

increased colony size, as bigger colonies are better pollinators, and produce more wax and honey (Farrar, 1937). This facilitates the transmission of parasites and pathogens within and between colonies (Brosi et al., 2017; Alger et al., 2018). *Deformed wing virus* (DWV) is a good example of a pathogen that has benefitted from facilitated transmission within and between honey bee colonies. The viral dynamics of DWV were considerably altered when an ectoparasitic mite from the Asian honey bee (*Apis cerana*) shifted hosts to *A. mellifera* (Martin, 2001). This mite, *Varroa destructor,* turned out to be a competent vector of RNA viruses such as DWV (Allen and Ball, 1996). As a result, in the last 40 years DWV has spread across the globe, leaving no beekeeping country unaffected with the exception of Australia (Roberts et al., 2017; Martin and Brettell, 2019). The combination of *V. destructor* and DWV is considered to be the predominant driver of global honey bee colony deaths (Martin, 2001; Dainat et al., 2012a; Schroeder and Martin, 2012).

Two main genotypes of DWV are known: DWV-A and DWV-B. Their genome sequences differ by approximately 15% (Ongus et al., 2004). They also appear to differ in their effect on honey bees, but many studies find contradictory results. Some studies report increased mortality in colonies that harbour high DWV-A loads (Highfield et al., 2009; Martin et al., 2012; Mondet et al., 2014; Kevill et al., 2017; Barroso-Arévalo et al., 2019b; Kevill et al., 2019), while others report reduced survival and cognitive function in bees that were experimentally injected with DWV-B (McMahon et al., 2016; Benaets et al., 2017; Gisder et al., 2018). Our own earlier work found increased mortality in pupae injected with DWV-A (Norton et al., 2020), while others found no difference in survival in pupae injected with either genotype (Dubois et al., 2019; Tehel et al., 2019).

Many factors can potentially contribute to differences in observed mortality between studies. One explanation is that the bees used between studies differ in their immune response to DWV genotypes. Some honey bee populations may be more susceptible to one genotype of DWV, whereas others may be more susceptible to the other genotype. Such differences could be a result of pre-exposure to a particular strain, resulting in immune priming- improved survival if a sublethal dose of pathogen has been previously encountered (Hernández López et al., 2014). Secondly, it is possible that honey bees elicit a specific and different immune response to different genotypes of the same pathogen. Specific genotype-host immune interactions are known from parasites in bumblebees (Barribeau and Schmid-Hempel, 2013)

and mosquitos (Molina-Cruz et al., 2012), bacteria in crustaceans (Auld et al., 2012) and humans (Sela et al., 2018), and viruses in fish (Moreno et al., 2020).

Here we ask if honey bees mount a different response when experimentally injected with DWV-A compared to DWV-B. To test for differing responses, we used a honey bee population naïve to DWV and *V. destructor*. Our study thus minimises confounding factors such as exposure or adaptation to a particular DWV genotype, or direct damage inflicted by mite feeding (Yang and Cox-Foster, 2005; Kuster et al., 2014; Annoscia et al., 2019; Ramsey et al., 2019). We analysed the expression of 19 genes that represent several immune response pathways and have previously been associated with DWV (Table 3.1). We additionally sequenced the small RNA profiles of honey bees infected with DWV-A and DWV-B to better understand the role of RNAi during DWV infection.

## RESULTS AND DISCUSSION

**Table 3.1.** Immune gene expression in DWV infected honey bees in this study compared to previous studies. Treatment indicates if bees were parasitised by *V. destructor* or collected from mite-infested colonies (DWV+), or experimentally injected (DWV*i*) in past studies. Asterisks indicate variability in expression across time points, and in our study this is in comparison to the buffer injected or unmanipulated controls.

| Pathway | Gene | Past studies | | | This study | References |
|---|---|---|---|---|---|---|
| | | Treatment | life stage | expression | expression | |
| RNAi | *dicer* | DWV+ | adults | up-regulated | up-regulated | (Zhao et al., 2019)* |
| | | DWV*i* | adults | up-regulated | | (Al Naggar and Paxton, 2021) |
| | | DWV+ | pupae | equal to controls | | (Ryabov et al., 2014) |
| | *argonaute-2* | DWV+ | adults | up-regulated | up-regulated* | (Zhao et al., 2019)* |
| | | DWV*i* | adults | up-regulated | | (Al Naggar and Paxton, 2021) |
| | | DWV+ | pupae | equal to controls | | (Ryabov et al., 2014) |
| | | | | | | |
| Toll | *dorsal-1a* | DWV+ | adults | down-regulated | equal to both controls | (Nazzi et al., 2012; Barroso-Arévalo et al., 2019c)* |
| | | DWV+ | pupae | down-regulated | | (Annoscia et al., 2019) |
| | *cactus* | DWV+ | adults | up-regulated | mostly equal to controls* | (Zanni et al., 2017) |
| | *spaetzle* | DWV+ | pupae | down-regulated | equal to buffer inj. | (Khongphinitbunjong et al., 2015) |
| | | DWV+ | pupae | | | (Ryabov et al., 2014) |
| | *PGRP-S2* | DWV+ | adults | up-regulated | mostly equal to controls* | (Nazzi et al., 2012) |
| | | DWV+ | adults | down-regulated | | (Zanni et al., 2017) |
| | | | | | | |
| Imd | *relish* | DWV+ | adults | up-regulated | up-regulated* | (Barroso-Arévalo et al., 2019c)* |
| | | DWV+ | larvae + pupae | up-regulated | | (Kuster et al., 2014)* |
| | | DWV+ | adults | up-regulated | | (Barroso-Arévalo et al., 2019c)* |
| | | DWV+ | pupae | down-regulated | | (Khongphinitbunjong et al., 2015) |
| | | | | | | |
| AMPs | *defensin-1* | DWV*i* | pupae | up-regulated | mostly equal to buffer inj.* | (Ryabov et al., 2016) |
| | | DWV+ | pupae | | | (Khongphinitbunjong et al., 2015) |
| | | DWV+ | adults + pupae | | | (Aronstein et al., 2012) |
| | | DWV+ | adults | | | (Zhao et al., 2019)* |
| | | DWV+ | adults | down-regulated | | (Barroso-Arévalo et al., 2019c)* |
| | *defensin-2* | DWV+ | larvae + pupae | up-regulated | mostly equal to buffer inj.* | (Kuster et al., 2014)* |

| | | | | | | |
|---|---|---|---|---|---|---|
| | *abaecin* | DWV+ | adults | up-regulated | mostly equal to buffer inj.* | (Zanni et al., 2017; Zhao et al., 2019)* |
| | | DWV+ | adults + pupae | no significant change | | (Aronstein et al., 2012) |
| | *hymenoptaecin* | DWV *i* | pupae | up-regulated | equal to buffer inj. up to 48 HPI* | (Ryabov et al., 2016) |
| | | DWV+ | adults | | | (Zanni et al., 2017; Zhao et al., 2019)* |
| | | DWV+ | larvae + pupae | | | (Kuster et al., 2014)* |
| | | DWV+ | adults + pupae | no significant change | | (Aronstein et al., 2012) |
| | | DWV+ | adults | down-regulated | | (Yang and Cox-Foster, 2005) |
| | *apidaecin* | DWV+ | adults | up-regulated | mostly equal to buffer inj.* | (Zanni et al., 2017; Zhao et al., 2019) |
| | | DWV+ | larvae | down-regulated | | (Di Prisco et al., 2016) |
| | | | | | | |
| **JAK/ STAT** | *domeless* | DWV+ | pupae | down-regulated | up-regulated in DWV-B* | (Khongphinitbunjong et al., 2015) |
| | *hopscotch* | DWV+ | pupae | down-regulated | up-regulated from 72 HPI * | (Ryabov et al., 2014) |
| | | DWV+ | adults | up-regulated | | (Zhao et al., 2019)* |
| | | | | | | |
| **Other** | *lysozyme* | DWV+ | adults | down-regulated | mostly equal to controls* | (Yang and Cox-Foster, 2005) |
| | *malvolio* | DWV+ | adults | up-regulated | up-regulated | (Zanni et al., 2017) |
| | *vago* | DWV+ | pupae | up-regulated | mostly equal to controls* | (Ryabov et al., 2014) |
| | *PPOact* | DWV+ | pupae | down-regulated | mostly equal to controls* | (Khongphinitbunjong et al., 2015) |

Our main objective was to determine if honey bees naïve to both *V. destructor* and DWV differ in their immune response when exposed to DWV-A and DWV-B. Injecting honey bees with buffer is known to activate covert viral infections (Anderson and Gibbs, 1988; Dubois et al., 2019) and is likely to trigger an immune response to wounding (Kuster et al., 2014). We therefore felt it was important to include two control groups. By comparing the immune response of DWV or buffer injected pupae (BC) to unmanipulated pupae (UC), we were able to separate the effect of DWV genotypes from the effect of experimental injection.

**Up regulation of immune genes in DWV infected pupae**

Of the 19 immune genes examined, the majority [13 genes, excluding *domeless* and the antimicrobial peptides (AMPs)] were expressed at higher levels in pupae from 48-72 HPI onwards (Figure 3.1). Some genes (*dicer*, *argonaute-2*, *relish*, *malvolio*, *hopscotch*) were

strongly up-regulated from 48 HPI in DWV injected pupae. The point at which genes become up-regulated coincides with the accumulation of DWV. In our previous study using the same bee population, loads of both DWV genotypes exceeded $1 \times 10^7$ (relative to *Actin*) at 48 HPI, and exceeded $1 \times 10^8$ and $1 \times 10^9$ in DWV-A and DWV-B pupae by 96 HPI, respectively (Norton et al., 2020).

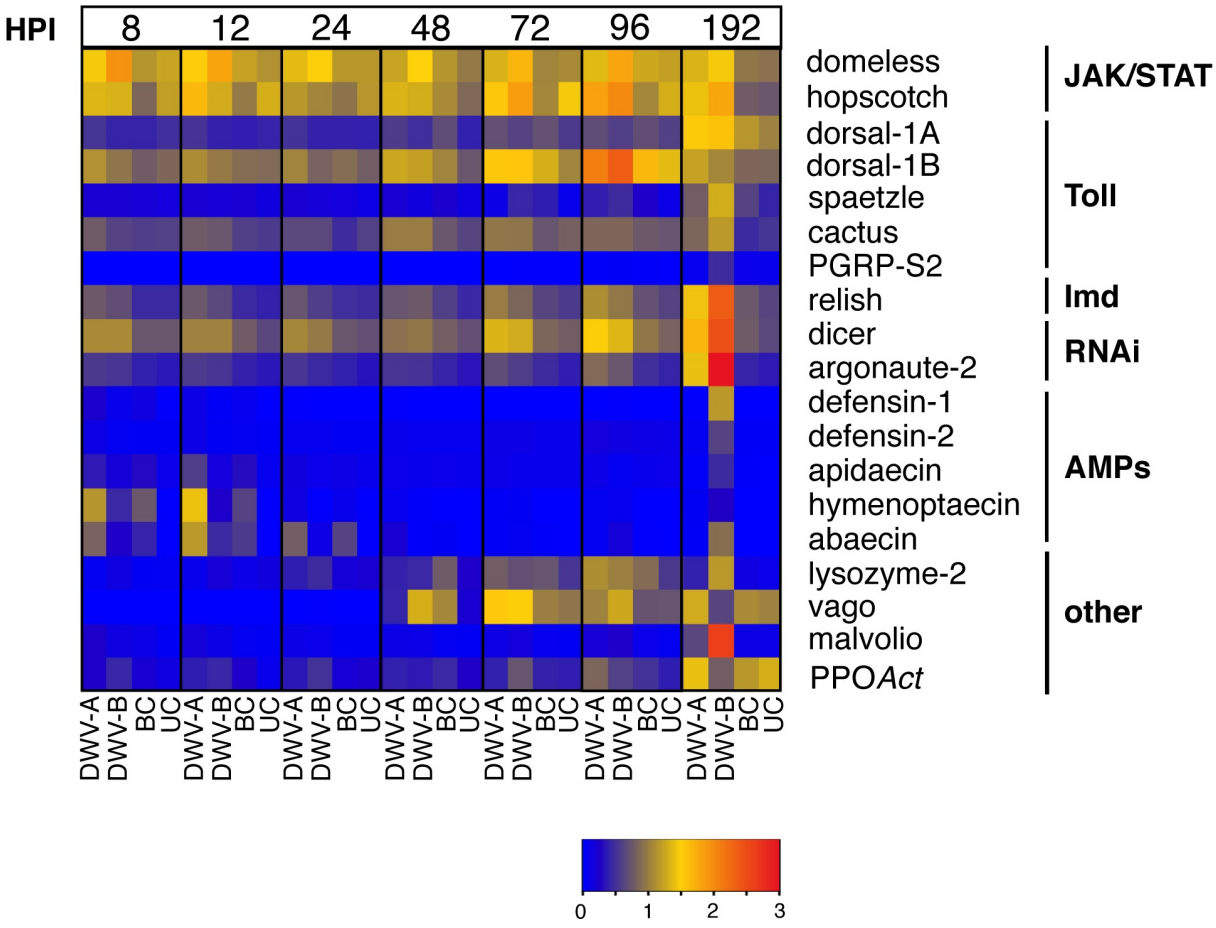


**Figure 3.1.** Heat map showing the median relative expression of 19 immune genes in unmanipulated pupae (UC), or pupae injected with buffer (BC), DWV-A or DWV-B at 8 to 192 hours-post injection. Each row corresponds to a gene, and each column to the median expression of DWV-A, DWV-B, BC or UC pupae at each time point. The immune pathways corresponding to each gene are indicated on the far right. Blue illustrates low gene expression and red high expression relative to two housekeeping genes (*Actin* and *RpS5*).

RNAi is the major antiviral mechanism in insects (Gammon and Mello, 2015). It was therefore expected that honey bees would mount an RNAi response when injected with DWV. The expression of both *dicer* and *argonaute-2* increased in parallel with DWV viral loads in pupae over time. *Dicer* was the only gene that was up-regulated in DWV injected pupae compared to both control groups at all time points (Figure 3.2). Median expression of *dicer* was approximately 0.5-fold higher in DWV injected pupae compared to the UC group from 8-48 HPI, and increased 0.6-2.8 fold between 72-192 HPI (Table S1-S2). The expression of *argonaute-2* ranged from 0.4 to 7.1-fold higher in DWV injected pupae compared to the UC group between 8-192 HPI, but did not differ to the BC group at 48 HPI and 24-72 HPI in DWV-A and DWV-B pupae, respectively (Figure 3.2; Table S3). In a previous study, neither *dicer* or *argonaute-2* were found to be differentially expressed in purple-eyed pupae parasitised by *V. destructor* (approximately equivalent to our 24 HPI), despite detecting a siRNA response (Ryabov et al., 2014). However, our results show that the RNAi response is time-dependent with increased expression associated with accumulating viral loads over time.

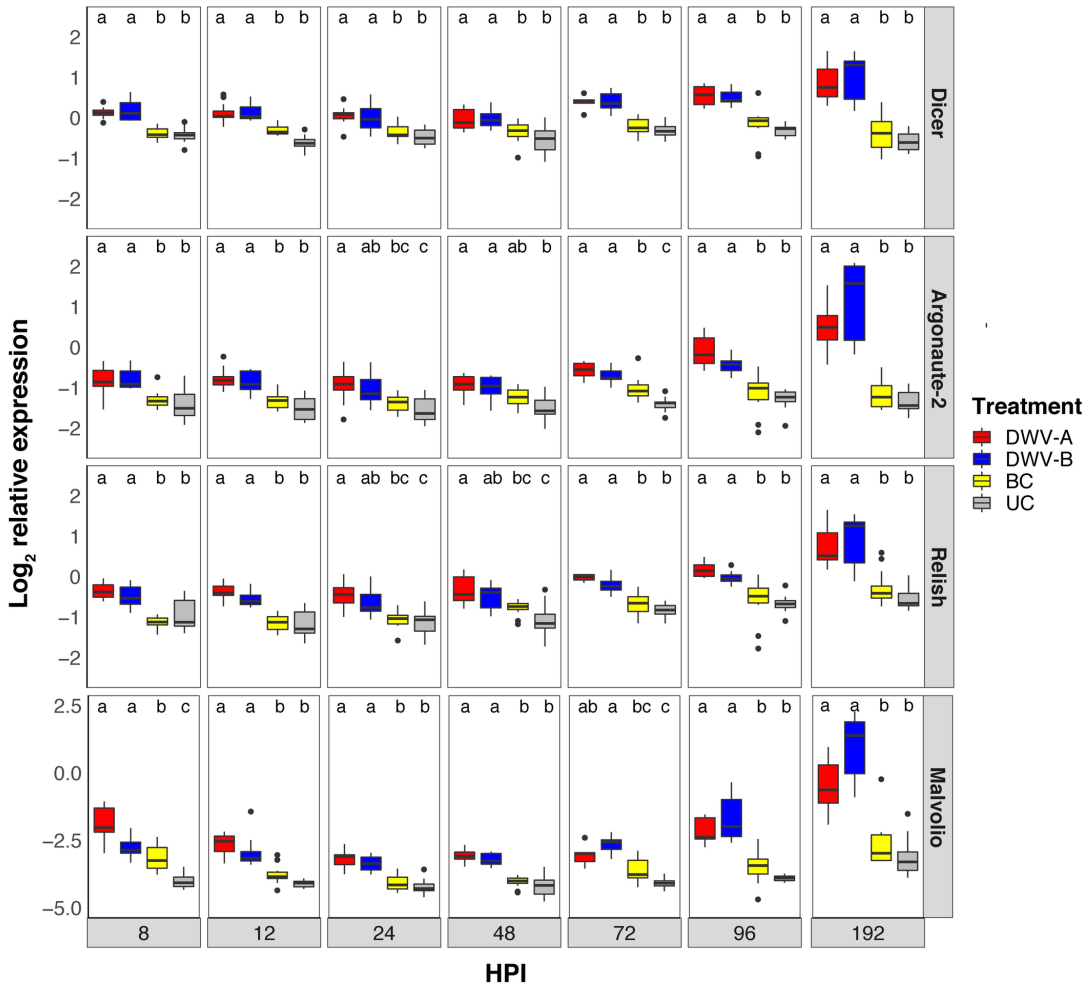


**Figure 3.2.** $Log_2$ relative expression of *dicer*, *argonaute-2*, *relish* and *malvolio* in honey bee pupae. Treatment groups that do not share a common letter differ at $p < 0.05$ at each hour post-injection (HPI).

*Relish* is a transcription factor in the Imd pathway, which upon cleavage, leads to the production of antimicrobial peptides (AMPs) *abaecin* and *hymenoptaecin* (Schlüns and Crozier, 2007). Similarly to *dicer* and *argonaute-2,* we found increased expression of *relish* in DWV injected pupae, which differed from both controls at all time points, except DWV-B and BC pupae between 24-48 HPI (Figure 3.2; Table S4). The highest expression of *relish* occurred at 192 HPI, which was 1.3-2.8-fold higher than in UC pupae. A previous study found an association between high DWV loads and mite infestation with increased expression of *relish* in honey bee colonies, suggesting that *relish* is an immunological marker of DWV-*Varroa* parasitism (Barroso-Arévalo et al. (2019c). Our results indicate that DWV alone (in the absence of *V. destructor*) triggers up-regulation of *relish.*

The metal ion ($Mn^{2+}$, $Fe^{2+}$ and $Cu^{2+}$) transporter *malvolio* was up-regulated in DWV injected pupae compared to both control groups at all time points, excluding DWV-B compared to BC pupae at 8 HPI ($Z = 1.21$, $p = 0.226$) and DWV-A relative to BC pupae at 72 HPI ($Z = 1.67$, $p = 0.114$) (Figure 3.2; Table S5). Expression was 1.1-5.3 and 0.8-24.7-fold higher in DWV-A and DWV-B pupae, respectively, compared to the UC group. We found the highest expression at 192 HPI, yet due to the variability between samples, differences between DWV-A and DWV-B pupae were not statistically significant ($Z = -1.36$, $p = 0.207$). *Malvolio* is involved in the sucrose responsiveness of insects, and thus affects foraging behaviour (Søvik et al., 2017). Foraging honey bees, who consume a carbohydrate rich diet, express higher levels of *malvolio* compared to nurse bees who consume higher amounts of protein. Feeding manganese to newly emerged honey bees results in increased expression of *malvolio* and premature foraging (Ben-Shahar et al., 2004), suggesting a cause and effect relationship. *Malvolio* is also up-regulated in mite-parasitised honey bees infected with DWV (Alaux et al., 2011; Zanni et al., 2017). Interestingly, experimental injection of DWV also induces precocious foraging in honey bees (Natsopoulou et al., 2016; Benaets et al., 2017). Taken together, increased expression of *malvolio* could potentially explain premature foraging in DWV infected honey bees.

The transmembrane receptor *domeless* and the kinase *hopscotch* are both part of the JAK/STAT pathway, associated with responses to immune challenge and septic injury in mosquitoes and *Drosophila* (Lematire and Hoffman 2007). We found increased expression of *domeless* in DWV-B injected pupae where expression was approximately 0.5-fold higher compared to both controls, excluding 24 HPI (Figure B.1; Table S6). However, the highest expression was observed at 8-12 HPI when DWV-B viral loads were $< 1 \times 10^6$ (Norton et al., 2020). This suggests that increased expression in DWV-B pupae was independent of viral replication. In contrast, *domeless* expression in DWV-A pupae was equal to both control groups, excluding 12 and 192 HPI. In *Drosophila*, *hopscotch* is activated by *domeless* and subsequent phosphorylation creates STAT92E binding sites, which induces the transcription of genes involved in antiviral activity (Merkling and van Rij, 2013; Öhlund et al., 2019). Despite limited differences in *domeless* expression in our DWV injected bees compared to the controls, we detected 0.4 to 1.4-fold higher expression of *hopscotch* in DWV injected bees compared to both control groups at 96-192 HPI (Figure B.1; Table S7). In *Drosophila*, up-regulation of *hopscotch* induces the transcription of *vir-1*, which counters the replication of

*Drosophila C virus* (Dostert et al., 2005). It is unclear whether the JAK/STAT pathway plays a role in antiviral defence against DWV in honey bees. Previous studies have found that *domeless* and *hopscotch* are up-regulated in DWV infected bees parasitised by *V. destructor* (Tesovnik et al., 2019; Zhao et al., 2019), while others found the opposite (Ryabov et al., 2014; Khongphinitbunjong et al., 2015).

**Toll pathway response to DWV**

It has previously been suggested that the Toll pathway, particularly expression of NF-κB factor *dorsal-1A,* is a key element in regulating the honey bee immune response against DWV (Nazzi et al., 2012; Nazzi and Pennacchio, 2018). *Dorsal-1* has two splice variants (*1A and 1B*), which are primarily expressed in the fat body tissue of honey bees, and *dorsal-1A* regulates the expression of *defensin-1* (AMP) (Lourenco et al., 2018). *Cactus* is a NF-κB inhibitor, which must be degraded to allow nuclear translocation of *dorsal* (Zuo et al., 2016). Increased *cactus* expression in DWV-infected adult bees parasitised by *V. destructor* has been associated with the down-regulation of *dorsal* (Zanni et al., 2017). Thus, we expected to find increased expression of *cactus* coupled with the down-regulation of *dorsal-1A* in our DWV injected pupae. However, our data showed that the expression of *dorsal-1A* (Figure 3.3; Table S8) and *dorsal-1B* (Figure B.2; Table S9) in DWV injected pupae was very similar to both control groups with only minor differences between treatment groups over time. The expression of *cactus* was also generally very similar between DWV injected pupae and both controls (Figure 3.3). We did, however, find elevated expression of *cactus* in DWV injected pupae at 48 and 192 HPI (Table S10), where median expression was 0.6-1.2 fold higher than in the UC group. However, this increase was not associated with any notable changes in *dorsal-1A* expression. As RNAi knockdown of *dorsal-1A* resulted in increased DWV loads, Nazzi et al. (2012) suggested that the transcription factor plays in important role in antiviral defence. *Dorsal-1A* was also down-regulated in DWV infected bees but mite parasitism did not have an effect on expression (Nazzi et al., 2012), even though *V. destructor* feeds on the fat bodies of honey bees (Ramsey et al., 2019). Annoscia et al. (2019) later found that experimental removal of honey bee haemolymph reduces *dorsal-1A* expression, although this was also associated with increased accumulation of DWV. Our results suggest that either *dorsal-1A* expression is only down-regulated in honey bees that have been pre-exposed to DWV, or that DWV alone (in the absence of *V. destructor* feeding) does not down-regulate *dorsal-1A*. Further, *spaetzle* was not down-regulated in our DWV injected bees, in contrast to

previous findings in DWV infected and mite infested pupae (Ryabov et al., 2014; Khongphinitbunjong et al., 2015). Instead, we found that the expression of *spaetzle* was equally elevated in DWV and BC injected pupae (Figure 3.3; Table S11), suggesting that the up-regulation of *spaetzle* was likely attributed to wounding during injection rather than DWV infection.

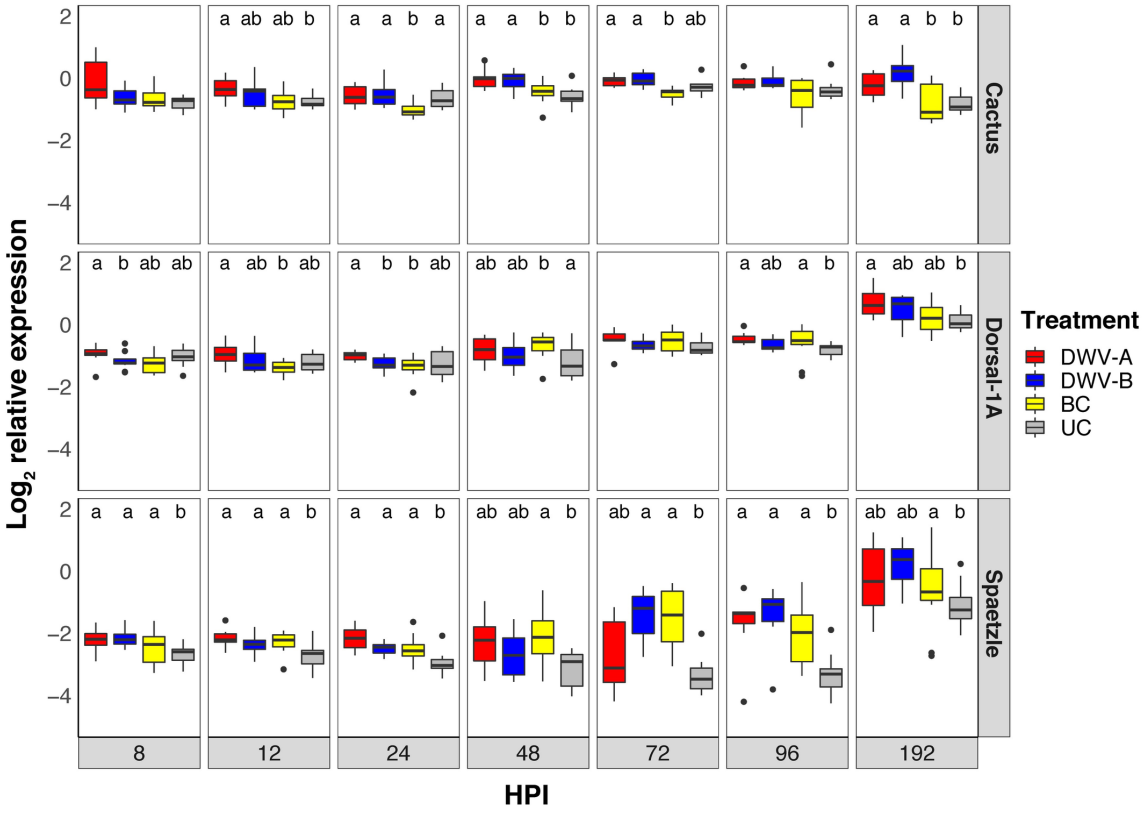


**Figure 3.3.** $Log_2$ relative expression of *cactus*, *dorsal-1A* and *spaetzle* in honey bee pupae. Treatment groups that do not share a common letter differ at $p < 0.05$ at each hour post-injection (HPI). No letters within a given time point indicates that no significant differences were detected between treatment groups.

### Up-regulation of AMPs at early time points due to wounding

The majority of previous studies have found an increase in expression of antimicrobial peptides (AMPs) in DWV infected bees (Table 3.1), both when experimentally injected (Ryabov et al., 2016) and mite parasitised (Kuster et al., 2014; Khongphinitbunjong et al., 2015; Zanni et al., 2017; Zhao et al., 2019). We also observed increased AMP expression, but only at specific time points. Within the first ~ 48 hours of infection, expression of AMPs was

often equal in the BC and DWV injected pupae (Tables S12-S16). This was most evident in the expression of *abaecin, hymenoptaecin*, and *defensin-1* (Figure 3.4), where up-regulation from 8 to ~48 HPI was likely due to wounding. However, we did find an approximate 1-fold increase in expression of *abaecin* in DWV-A injected pupae at 8 ($Z = 2.23$, $p = 0.031$) and 12 HPI ($Z = 2.07$, $p = 0.046$) (Figure 3.4) and *defensin-2* at 8 HPI ($Z = 2.71$, $p = 0.013$) relative to the BC group (Figure B.3). Expression of *abaecin, hymenoptaecin*, and *defensin-1* in the BC pupae was more similar to the UC group in the later time points. *Hymenoptaecin* was up-regulated in both DWV-A and DWV-B injected bees from 72-192 HPI, which might be explained by the increased expression of *relish* at the same time points. We found a similar increase for *abaecin* at 96 HPI, but only DWV-B injected pupae differed from the BC group at 192 HPI. The down-regulation of *defensin-1*, which is under the control of *dorsal-1A* (Lourenco et al., 2018), was associated with increased DWV loads and mite infestation in a previous study (Barroso-Arévalo et al., 2019c). In contrast, we found no evidence to indicate that *defensin-1* is down-regulated as a result of DWV infection. Conversely, we often found that AMP expression was higher in DWV-B injected pupae at 192 HPI compared to the BC group, including *defensin-1*, whereas expression in DWV-A pupae often did not differ from the BC group at 192 HPI (Figure 3.4; Figure B.3).

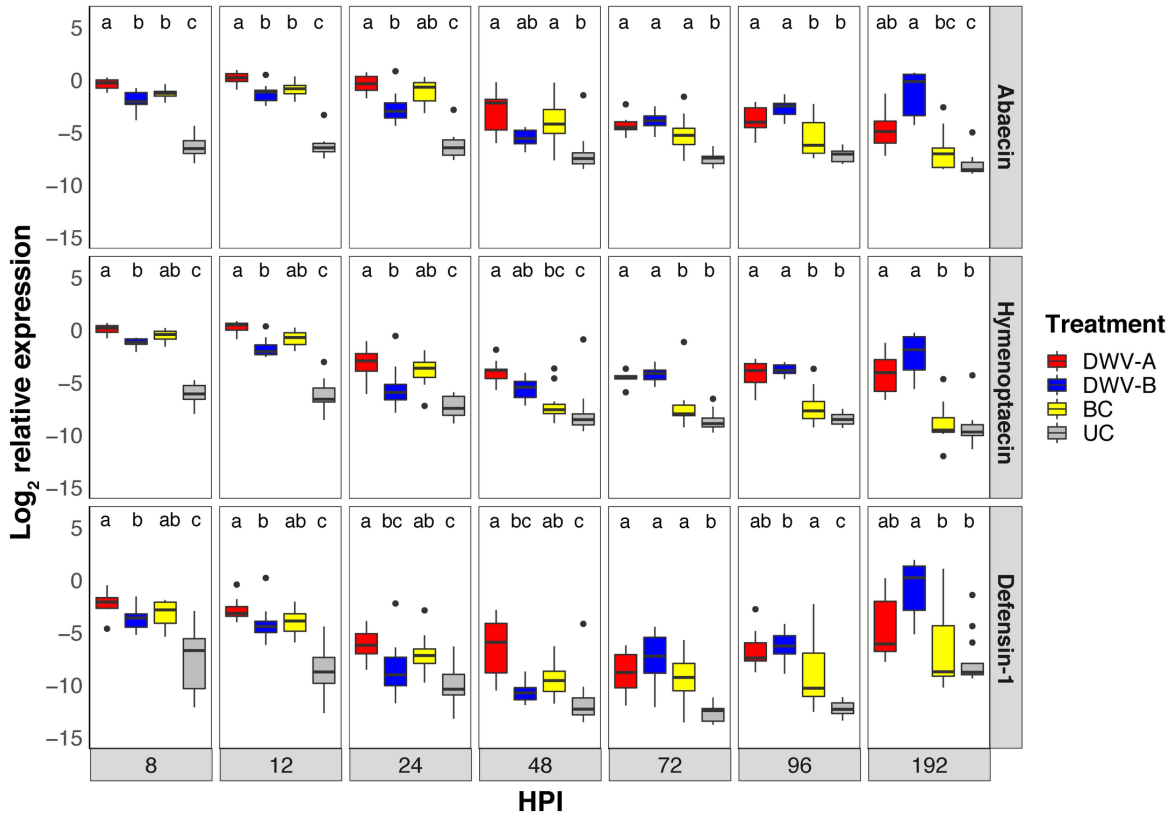


**Figure 3.4** $Log_2$ relative expression of *abaecin, hymenoptaecin* and *defensin-1* in honey bee pupae. Treatment groups that do not share a common letter differ at $p < 0.05$ at each hour post-injection (HPI).

**Differential expression in response to DWV-A and DWV-B**

We find very little evidence to indicate that honey bees respond differently to DWV-A and DWV-B. If differences in immune response were biologically significant, we would have expected a consistent difference in gene expression over time, or within a given immune pathway. However, this was not the case. We only found differences at isolated time points, where the expression in pupae exposed to one genotype was also different to both controls. When we did see a difference between two genotypes, this was often associated with higher expression in DWV-A injected pupae at early time points [*malvolio:* (8 HPI, 0.8-fold; $Z = 2.10$, $p = 0.043$); *defensin-2:* (8 HPI, 1-fold; $Z = 2.76$, $p = 0.017$); and *abaecin:* (8 HPI, 2.4-fold $Z = 3.24$, $p = 0.002$; and 12 HPI, 1.6-fold; $Z = 2.98$, $p = 0.006$)]. DWV-A accumulates to higher loads than DWV-B within the first 12 hours post-injection [Figure 3.5B, Norton et al. (2020)], so it is possible that these differences are associated with faster replication of DWV-A during the initial stage of infection. In contrast, we found that gene expression was often

elevated in DWV-B injected pupae at later time-points (192 HPI), although this was only statistically different from DWV-A for *PGRP-S2* (Toll) (5-fold; $Z$ = -2.36, $p$ = 0.036) (Figure B.2; Table S17), and *defensin-2* (9-fold; $Z$ = -2.40, $p$ = 0.033) (Figure B.3), other than increased expression of *domeless* at 8 HPI (0.3-fold; $Z$ = -2.67, $p$ = 0.015) and *PPOact* at 72 HPI (0.9-fold; $Z$ = -2.71, $p$ = 0.013) (Figure B.4; Table S18-S20). This is consistent with elevated viral loads of DWV-B at later time points [(Figure 3.5B; Norton et al. (2020)].

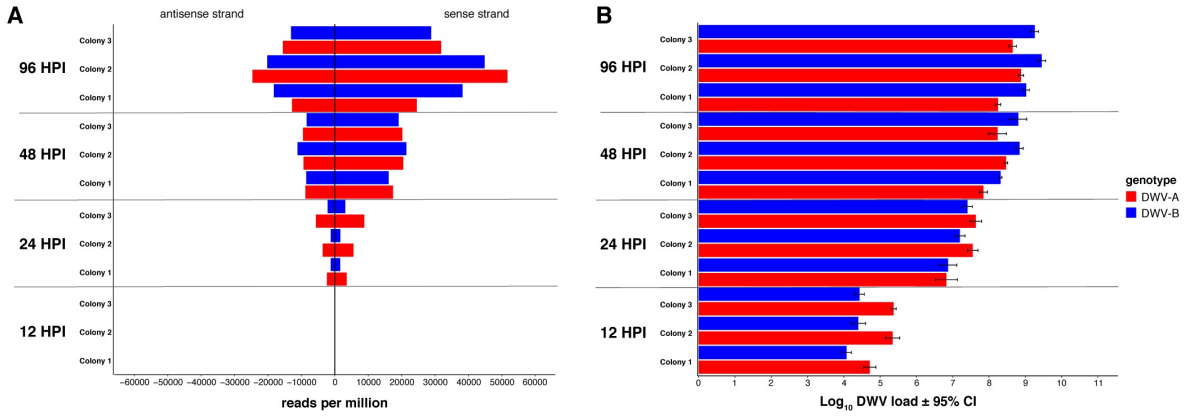


**Figure 3.5** Small RNA analysis in honey bee pupae. (A) The total sense and antisense vsiRNA (21-23 nt) reads mapping to DWV-A (red) and DWV-B (blue), in four pooled honey bee pupae per time point and colony. Reads at 12 HPI are not visible at this scale. (B) The mean DWV loads ± 95% CI of four pupae per treatment and colony at 12, 24, 48 and 96 hours post-injection. Viral loads are relative to housekeeping gene *Actin* and were previously published in Norton et al. (2020), but are presented here to illustrate the relationship between viral accumulation and siRNA response to DWV-A and DWV-B.

## Small RNA analysis

Our expression analysis showed that RNAi mechanism genes *dicer* and *argonaute-2* are clearly up-regulated in DWV injected pupae. We previously found that DWV-B accumulated to 5 to 10-fold higher loads than DWV-A (Norton et al., 2020) from 48-192 HPI, suggesting that DWV-B may be better able to evade RNAi degradation compared to DWV-A. Hence, we investigated the small RNA profiles of DWV injected pupae to determine if honey bees elicit a different siRNA response to either genotype. We analysed four pooled DWV-A and DWV-B injected pupae per colony at 12, 24, 48 and 96 HPI. The number of reads mapping to DWV increased over time, ranging from < 1.6% of the total reads for each sample at 12 and 24 HPI, to 3-4% at 48 HPI and 4.4-8.5% at 96 HPI. We examined the read size profiles of each sample and saw that a clear antiviral small RNA response was elicited in pupae injected with DWV-A and DWV-B. A signature *dicer* response was observed in pupae collected at 24, 48,

and 96 HPI, whereby vsiRNA reads (mapping to DWV-A and DWV-B genomes) were predominantly 22 nt in length, followed by 21 and 23 nt. Reads showed a 1.3-2.3-fold bias towards the sense orientation (Figure B.5A), consistent with previous observations of DWV infected honey bees (Wang et al., 2013; Chejanovsky et al., 2014; Ryabov et al., 2014). At 12 HPI there were higher counts of 15-16 nt fragments followed by less abundant 21-23 nt fragments. We found that the vsiRNAs (21-23 nt) in both DWV-A and DWV-B injected pupae increased over time, with counts ranging from 8.4-182/million reads at 12 HPI to 37400-76300/million reads at 96 HPI (Figure 3.5A). In agreement with Ryabov et al. (2014), we found that vsiRNA response in pupae strongly correlated with viral load of DWV-A ($r_s$ = 0.98, $p < 0.0001$) and DWV-B ($r_s$ = 0.99, $p < 0.0001$) (Figure 3.5B). However, we found no difference in the vsiRNA response between pupae injected with DWV-A or DWV-B ($\chi^2$= 0.27, df = 1, $p$ = 0.6033) (Figure B.5B), or between the three colonies ($\chi^2$= 0.455, df = 2, $p$ = 0.7965). This suggests that previously observed differences in viral accumulation (Norton et al., 2020), are likely explained by replicative characteristics of the DWV genotypes themselves rather than degradation by the host. We also found that the vsiRNAs were randomly distributed across both DWV genomes. Yet, we consistently detected a large peak starting at nucleotide position 8180 in DWV-A injected pupae (Figure B.5C) that was absent in DWV-B injected bees (Figure B.5D). Interestingly, a peak at a similar position was observed in Israeli colonies (Chejanovsky et al., 2014). The strong peak could due to active targeting by RNAi or could be indicative of a viral miRNA (Hussain et al., 2011; Bruscella et al., 2017), however further analysis is required to establish this.

The PIWI-interacting RNA (piRNA) response (RNAi) is known to play a role in germline protection against transposable elements in *Drosophila* (Czech and Hannon, 2016). piRNAs typically range from 26-31 nt in length and are produced independently of *dicer*, from single-stranded RNA (Hirakata and Siomi, 2016). The piRNA response also plays a role in antiviral defence against *Chikungunya* and *Dengue virus* in *Aedes aegypti* and *Ae. albopictus* mosquitos (Goic et al., 2016; Miesen et al., 2016). We analysed the larger sized sRNA reads (26-31 nt) that would be indicative of viral piRNAs, but did not detect any obvious peaks. We did, however, detect a low abundance of 26-31 nt fragments in all samples, which increased over time. Counts ranged from ~ 0-122/million reads at 12-24 HPI and ~22-200000/million reads at 48-96 HPI, with a strong bias towards the sense orientation. To confirm that viral piRNAs were not produced at low levels, the 26-31 nt reads were analysed for the 'ping-

pong' (1U/10A) signature associated with piRNA amplification (Czech and Hannon, 2016). Our results showed slightly (< 40%) elevated frequencies of uridine at the 5' end, however we found no evidence of adenine bias at position 10 (Figure B.6). We also observed elevated frequencies of uridine at the 3' end. Overall, the data suggested that the 26-31 nt reads were not piRNAs, but products of random degradation.

We also mapped the small RNA reads to the honey bee (*Apis mellifera*) genome and determined the composition of small RNA subtypes within each sample (Figure 3.6). There was some variability between the samples. The samples were predominantly made up of rRNA (15-58%), followed by miRNA (13-38%), tRNA (2-13%) and mRNA (0.8-2%). We then analysed the miRNA profiles. We found a small number of differentially expressed miRNAs (FDR-corrected $P < 0.01$) between pupae at 24, 48 and 96 compared to the 12 HPI pupae. The number of differentially expressed miRNAs increased over time in both DWV-A (0 to 28) and DWV-B (3 to 38) injected pupae. However, as all of our libraries were generated from DWV-A or DWV-B injected samples, we were unable to distinguish whether this was associated with viral infection or physiological changes at different time points during pupation. We subsequently compared the miRNAs expressed between DWV-A and DWV-B injected pupae at each specific time point, and found no differentially expressed miRNAs at 12-48 HPI, but found three at 96 HPI. Mir-3720 (FDR corrected $p < 0.0001$) and mir-6052 ($p < 0.0001$) were expressed 6.7 and 11.5-fold higher in the DWV-A injected pupae, respectively, whereas mir-279d was expressed 1.6-fold higher in DWV-B injected pupae ($p = 0.004$). However, we cannot exclude the possibility that differential miRNA expression was associated with *Black queen cell virus* [BQCV (see below)]. All three miRNAs have previously been identified in honey bees (Chen et al., 2010; Liu et al., 2012; Qin et al., 2014; Macedo et al., 2016), however their function is currently unknown.

We additionally performed a *de novo* assembly of the unmapped reads and used the resulting contigs to perform a BLAST search. We detected BQCV in five of our DWV-A injected samples (Figure 3.6A), as expected based on our previous detection of BQCV in DWV-A injected pupae (Norton et al., 2020). We detected very low reads for BQCV at 12 (< 0.001%) and 24 HPI (< 0.3%), consistent with our previous qPCR results where BQCV was undetected or $\leq 1 \times 10^2$ (relative to *Actin*) at these time-points. The number of small RNA reads aligning to BQCV ranged from 14-23% at 48 HPI and 0-25% at 96 HPI, as BQCV was

not detected in colony 2 at 96 HPI. BQCV was not detected in DWV-B injected pupae (Figure 3.6B), in agreement with our previous qPCR results (Norton et al., 2020).

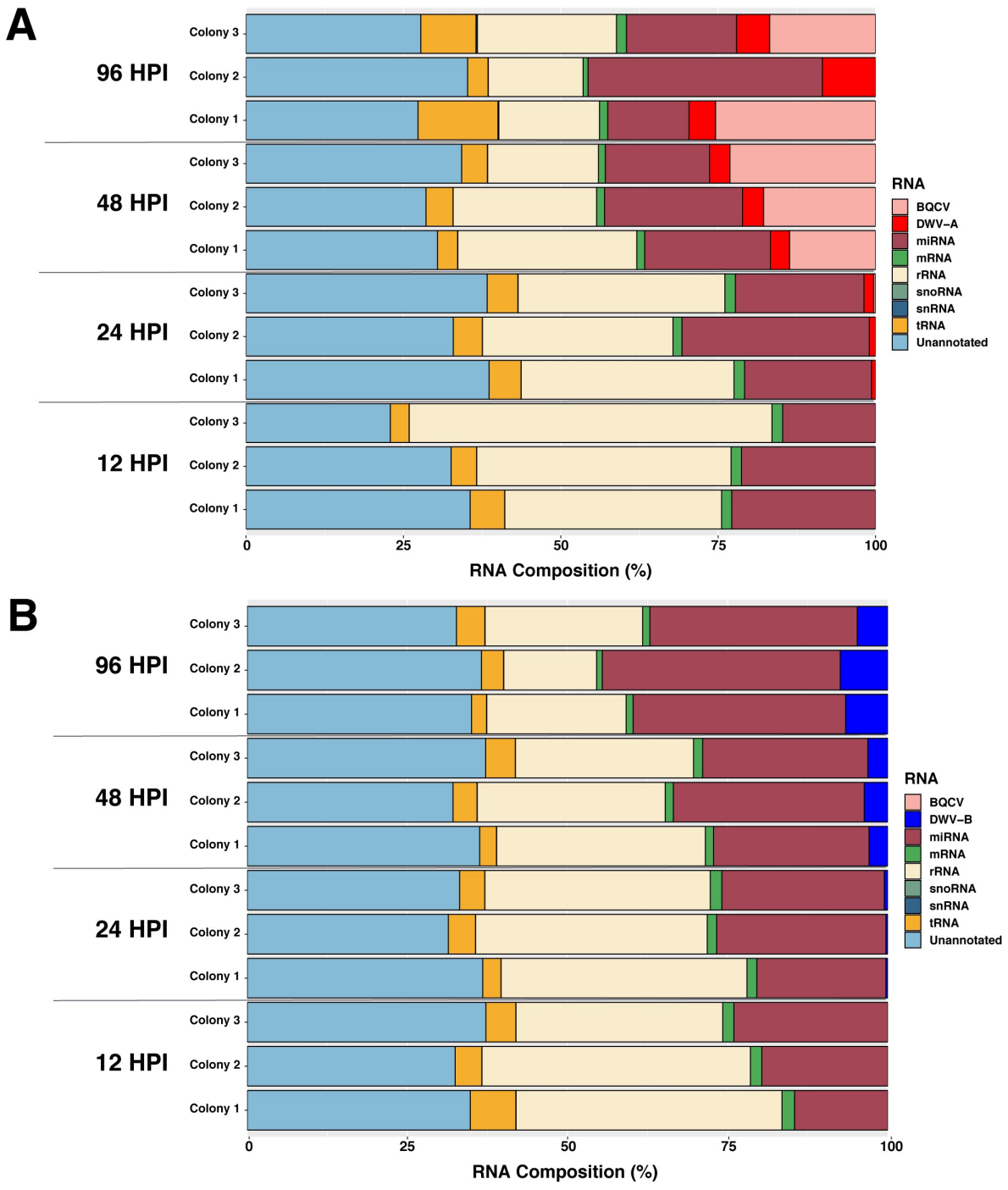


**Figure 3.6**. Percentage composition of RNA in (A) DWV-A and (B) DWV-B injected pupae. DWV-A and DWV-B at 12 HPI, and snoRNA and snRNA at all time points constituted < 1% of total RNAs.

We analysed the small RNA profiles of the reads aligning to BQCV and found that samples containing both viruses exhibited a stronger siRNA response to BQCV compared to DWV. Again, the reads were predominantly 22 nt in length, with a 1.5 to 2-fold bias to the sense orientation (Figure B.7A). However, this time the vsiRNAs (21-23 nt) ranged from 1200000-3300000/million reads (Figure B.8A), which exceeded the siRNA response to DWV by approximately two orders of magnitude. This is in contrast to the viral loads observed in the same samples, where high BQCV loads were ~5 to 10-fold lower than DWV-A loads in individuals containing both viruses [Figure B.8B; Norton et al. (2020)]. BQCV loads were highly variable between pupae (Figure B.8B), due to the majority of pupae per pooled group harbouring low BQCV loads $< 3 \times 10^4$, and there was no correlation between BQCV load and small RNA response ($r_s = 0.2$; $P = 0.7471$). We found that vsiRNAs were randomly distributed across the BQCV genome (Figure B.7B). BQCV has been found to exist at covert levels in our population of honey bees (Remnant et al., 2019) and is common pathogen of honey bees worldwide (McMahon et al., 2015; Roberts et al., 2017; Murray et al., 2018). Yet, observing such a large siRNA response to BQCV was surprising, considering so few pupae harboured high BQCV loads. Our results suggest that our population of bees may be better able to target BQCV due to pre-exposure to the virus, or that DWV is comparatively better at evading siRNA degradation. Some RNA viruses are known to evade RNAi by encoding suppressors (VSR). These VSRs can inhibit RNAi through a number of different mechanisms, such as binding long dsRNA or double stranded siRNAs thereby inhibiting *dicer* cleavage or RISC loading (Sullivan and Ganem, 2005; Van Rij et al., 2006; Nayak et al., 2010), or may interfere with RNAi by directly interacting with *dicer*, *argonaute* or other siRNA processes (Singh et al., 2009; Nayak et al., 2010; Qi et al., 2012). It is unknown whether DWV uses VSRs to evade RNAi, however a putative VSR has been identified in *Israeli acute paralysis virus* (Chen et al., 2014), a dicistrovirus honey bee pathogen related to BQCV.

## CONCLUSION

Previous studies have found that the two main DWV genotypes, A and B, affect honey bees differently (McMahon et al., 2016; Gisder et al., 2018; Dubois et al., 2019; Tehel et al., 2019; Norton et al., 2020), although results differ between life stages and honey bee populations, leaving our understanding of DWV genotype virulence unclear. Over time, the prevalence of the two main DWV genotypes has changed, where DWV-B has increased at the expense of DWV-A (Ryabov et al., 2017; Kevill et al., 2019). The elevated prevalence of DWV-B

suggests that the two genotypes may interact differentially with the honey bee immune system (Mordecai et al., 2016a). Here we investigated the effect the two genotypes have in isolation, and in the same *V. destructor* and DWV-naïve population. Overall, we found no evidence to indicate that the honey bee's immune system responds to either genotype differently. We only detected isolated differences in gene expression, and these may be attributed to differences in viral loads. This suggests that global changes in DWV-B prevalence are not explained by an immunologically different response in honey bee hosts. Our work highlights that the siRNA pathway is up-regulated in response to DWV infection, but that the host degradation response does not inhibit the virus from accumulating to high loads. We found that differences in viral accumulation between the two DWV genotypes are not associated with differential RNAi expression. Further, our results showed that the siRNA response towards BQCV far exceeded the response to DWV, implying that DWV is comparatively better at evading antiviral host defences. Future studies are required to investigate what mechanisms DWV may utilise to evade RNAi defence by honey bee hosts.

*Deformed wing virus*, in association with the parasitic mite *V. destructor*, has been associated with the death of millions of honey bee colonies worldwide (Martin, 2001; Dainat et al., 2012a; Schroeder and Martin, 2012). *V. destructor* was thought to have an immunosuppressive effect on honey bees (Yang and Cox-Foster, 2005). Recent studies conclude that mite feeding upon fat bodies directly weakens bees and disrupts their immune response (Kuster et al., 2014; Annoscia et al., 2019; Ramsey et al., 2019), particularly as fat bodies are a major site of immune and metabolic function (Arrese and Soulages, 2010). Indeed, Annoscia et al. (2019) showed that experimental removal of haemolymph (likely containing fat body tissue) resulted in the down-regulation of *dorsal-1A* and parallel increase of DWV loads naturally present within honey bees. The authors concluded that mite feeding disrupts the balance between honey bee immune response leading to increased viral replication. The fact that DWV is now found in virtually all honey bee populations worldwide (Martin and Brettell, 2019), makes it challenging to distinguish whether the down regulation of *dorsal-1A* was attributable to viral replication as previously suggested (Nazzi et al., 2012) or a result of haemolymph removal. Our results show that actively replicating DWV, in the absence of *V. destructor*, does not down-regulate *dorsal-1A*, supporting previous conclusions that mite feeding on bee tissue disrupts immune function (Kuster et al., 2014; Annoscia et al., 2019; Ramsey et al., 2019).

## MATERIALS AND METHODS

### Sample preparation

We collected white-eyed pupae from three unrelated *A. mellifera* colonies in Sydney, Australia, which are naïve to both *V. destructor* and DWV. Pupae not showing signs of damage or melanisation from uncapping were assigned to four treatment groups where pupae were unmanipulated ('unmanipulated control'; UC), or injected under aseptic conditions with either: 2 µL 0.5 M potassium phosphate buffer pH 8 ('buffer control'; BC), or $1 \times 10^7$ genome equivalents of DWV-A or DWV-B. All pupae were placed into Petri dishes lined with sterile filter paper (10 pupae/Petri dish). We then placed all Petri dishes per treatment in individual plastic tubs and incubated at 34.5°C for 8 days (192 hours). During incubation, four random pupae per colony and treatment group were collected at specific time points post-injection (1, 4, 8, 12, and 24 h) and every subsequent 24 h for 192 h. Selected pupae were frozen at −80°C prior to RNA extraction. Explicit details of inocula preparation, experimental injection of pupae, RNA extraction and RNA standardisation to 200 µg/mL are given in Norton et al. (2020).

We synthesised cDNA from 800 ng RNA with SuperScript IV VILO Master Mix with ezDNase enzyme (Invitrogen) in 10 µL reaction volumes. The cDNA was then diluted in 150 µL UltraPure nuclease-free $dH_2O$ prior to qPCR analysis (below). DWV levels in the pupae were analysed in a separate study, where we found that DWV-A and DWV-B accumulated exponentially between 8 and 48 hour post-injection (HPI), and reached their maximum viral loads between 72-96 HPI (Norton et al., 2020). We therefore decided to analyse the immune response of pupae at seven time points: during rapid viral accumulation (8, 12, 24 and 48 HPI), when viral loads reach their maximum load (72 and 96 HPI), and just prior to eclosion (192 HPI). Where possible, we analysed the gene expression of four pupae per colony and time point. However, our previous qPCR analysis showed that 2.1% of pupae injected with either DWV-A or DWV-B were contaminated with the other genotype during downstream processing (Norton et al., 2020). We also found that two UC pupae (0.7%) were contaminated with DWV-A and DWV-B. Pupae were screened for additional viruses by qPCR [*Black queen cell virus* (BQCV), *Sacbrood virus* (SBV), *Apis rhabdovirus* 1 and 2 (ARV-1/ARV-2)] and endpoint PCR (*Lake Sinai virus*) as described (Norton et al., 2020). These viruses were not detected in our samples, excluding 4.9% of DWV-A injected pupae, that were found to carry high loads ($\geq 1 \times 10^7$ relative to *Actin*) of BQCV, despite BQCV not being detected in

the sequenced inocula (Norton et al., 2020). Generally only one pupa out of four replicate pupae (per time point) had high BQCV loads, excluding 72 HPI, where this occurred in two out of four pupae in each of the three colonies, and in colony 1 at 96 HPI where three out of four pupae had high BQCV loads. To reduce any confounding effects, we removed pupae contaminated with BQCV or the alternate DWV strain from the qPCR analyses and our treatment groups consisted of: 82 (UC), 84 (BC), 76 (DWV-A) and 78 (DWV-B) pupae. As we conducted the small RNA analysis of pooled, rather than individual, pupae (below), we included all four pupae per time point/colony to prevent skewing the data with different sample sizes.

**qPCR analysis**

We analysed 19 immune related genes and three endogenous reference genes (*Actin*, *RpL8* and *RpS5*) by qPCR (Table S21). All 5 µL qPCR reactions were performed in duplicate in 384-well plates (Bio-Rad) using a Pipetmax 268 (Gilson) with SsoAdvanced Universal SYBR Green supermix (Bio-Rad), forward and reverse primers (final concentration 500 nM each), and 1 µL cDNA. qPCR analysis was conducted on a Bio-Rad CFX384 Touch real-time PCR detection system with cycling conditions 95°C (10 min), followed by 40 cycles of 95°C (15 s), 55-63.2°C depending on primer pair (15 s), and 72°C (10-30 s), and then followed by melt curve analysis between 55 and 95°C, at 0.5°C increments. Each run contained duplicate positive and negative (no template) controls. We validated the specificity of each primer pair with melt curve analysis and gel electrophoresis on 1% agarose gel with SB buffer and SYBR Safe DNA stain (Life Technologies). We calculated primer efficiencies from standard curves prepared from a six-step 10-fold dilution series of cDNA. Primer efficiencies ranged from 95-110%. We determined the stability of the three reference genes with BestKeeper (Pfaffl et al., 2004), and found that *Actin* and *RpS5* were the most stable. Estimates of the expression level of each immune related gene were calculated as $E^{CqMin\_Cqi}$ and normalised against the geometric mean of the two reference genes (Vandesompele et al., 2002), as described by Brito et al. (2010).

**Gene expression statistical analysis**

For each given gene, we stratified the expression data by time-point and compared the mean rank of gene expression between the four treatment groups with a Kruskal-Wallis test, followed by Dunn's posthoc test with Benjamini-Hochberg adjustment ['FSA' (Ogle et al., 2018)]. We used non-parametric analysis as parametric assumptions of homogeneity of variance and/or normality were violated for 11 genes, and could not be improved with transformations. All statistical analyses were performed with RStudio software (R version 3.5.0). We generated an expression heatmap with 'gplots' (Warnes et al., 2016) to visualise the gene expression patterns across all time points and all genes.

**Small RNA library preparation and sequencing**

To more closely examine active viral degradation by the immune system via the RNA interference pathway, we sequenced small RNAs in DWV-A and DWV-B injected pupae at 12, 24, 48 and 96 HPI. We chose these specific time points as we were interested in investigating the small RNA response in relation to increasing viral loads. The RNAi mechanism includes three small RNA pathways that may all have a role in antiviral defence: the small interfering (siRNA), PIWI-interacting (piRNA), and microRNA (miRNA) pathways. While the siRNA pathway is the major antiviral response in insects (Gammon and Mello, 2015), virus derived piRNAs are associated with antiviral defence in mosquitos (Goic et al., 2016; Miesen et al., 2016), and host-derived miRNAs can play an antiviral response and be differentially expressed during viral infections (Slonchak et al., 2014; Yan et al., 2014; Monsanto-Hearne and Johnson, 2018). It is unknown if honey bees produce either piRNAs or miRNAs in response to viral infection.

We pooled 0.8 µg RNA from each pupa, to give a single DWV-A or DWV-B sample per colony and time point. We then used 0.6 µg of the pooled RNA to generate small RNA libraries using NEBNext® Multiplex Small RNA Library Prep Set for Illumina. We followed the manufacturer's protocol with the following modifications: libraries were generated from half-reactions, we used the Qiagen MinElute PCR Purification Kit for PCR purification and adjusted pH with 10 µl 3M sodium acetate pH 5.5. Acrylamide gel separation of the libraries was conducted on a 6% Novex TBE PAGE gel with 5 µL Novex Hi-Density TBE Sample Buffer (5X). Gels were stained with SYBR Gold in TBE buffer for 20 min. The excised gel slices were passed through gel breaker tubes. Gel elution was performed overnight at 4°C. We

used 2 µl of glycogen instead of linear acrylamide for precipitation and elution was performed with 10 µl TE elution buffer from the kit. The libraries were sequenced at the Australian Genome Research Facility (AGRF) laboratory (Melbourne, Australia) with HiSeq 2500 100 bp single end sequencing.

**siRNA analysis**

We checked the quality of the sequencing data with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and trimmed adapters with Trimmomatic (Bolger et al., 2014). We used consensus DWV-A (accession MN538208), DWV-B (MN538209) reference genomes and a BQCV reference genome (MW390818) assembled *de novo* (see below) to produce index libraries and used Bowtie2 (Langmead and Salzberg, 2012) to align the small RNA reads to either the DWV-A, DWV-B or BQCV genome. The mapped reads were exported as BAM files using SAMtools (Li et al., 2009), and analysed with the R package viRome (version 0.10).

**piRNA analysis**

We filtered the mapped reads to remove reads less than 26 and greater than 31 nucleotides in length, corresponding to the expected lengths of piRNAs (Aravin et al., 2006). SAMtools was used to remove duplicate reads for analysis of unique piRNA species and for BAM to fastQ file conversion (Li et al., 2009). The nucleotide frequencies for unique and total reads of each sample were plotted with R package SeqTools (Barson and Griffiths, 2016).

**miRNA analysis**

FastQ files were imported into CLC Genomics and passed through quality control and trimming tools. Differential expression analysis was conducted by performing the extract and count tool, followed by annotate and merge tool with miRBase (release 21), allowing for a maximum of 1 mismatch. Differential expression analysis was performed using the Empirical Analysis of DGE tool with false discovery rate (FDR) correction.

### Total RNA composition

Total composition of known small RNAs was calculated in CLC Genomics by performing annotate and merge on known small RNAs extracted from amel_OGSv3.2. In order to determine the total reads mapping to *A. mellifera*, DWV and BQCV genomes, trimmed reads were mapped first to the *A. mellifera* genome (amel_OGSv3.2) using the map reads to reference tool, unmapped reads were subsequently mapped to DWV and BQCV.

A final check of the unmapped reads was performed by generating a *de novo* assembly for each sample using Megahit (Li et al., 2015). As well as generating contigs representing DWV and BQCV genomes, multiple contigs were assembled that showed near 100% identity to *A. mellifera* ribosomal RNA in BLASTn searches. Unmapped reads from each sample were mapped back to the assembled rRNA contigs using Bowtie2 (Langmead and Salzberg, 2012), to assign any remaining ribosomal reads from the unmapped reads to their correct category.

## DATA AVAILABILITY

*De novo* assembled BQCV genome sequence deposited to GenBank (accession number MW390818). Supporting data files (including Tables S1-S21) and raw small RNA reads are available at https://doi.org/10.6084/m9.figshare.13473696


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**Chapter 4**

# Adaptation to vector-based transmission in a honey bee virus


## ABSTRACT

1. Global pollinator declines as a result of emerging infectious diseases are of major concern. Managed honey bees (*Apis mellifera*) are susceptible to numerous parasites and pathogens, many of which appear to be transmissible to sympatric non-*Apis* taxa. The ectoparasitic mite *Varroa destructor* is considered to be the most significant threat to honey bees (*Apis mellifera*) due to its role in vectoring RNA viruses, particularly *Deformed wing virus* (DWV). Vector transmission of DWV has resulted in the accumulation of high viral loads in honey bees and is often associated with colony death. DWV has two main genotypes, A and B. DWV-A was more prevalent during the initial phase of *V. destructor* establishment. In recent years, the global prevalence of DWV-B has increased, suggesting that DWV-B is better adapted to vector transmission than DWV-A.
2. We aimed to determine the role vector transmission plays in DWV genotype prevalence at a colony level.
3. We experimentally increased or decreased the number of *V. destructor* mites in honey bee colonies, and tracked DWV-A and DWV-B loads over a period of ten months.
4. Our results show that the two DWV genotypes differ in their response to mite numbers. DWV-A accumulation in honey bees was positively correlated with mite numbers yet DWV-A was largely undetected in the absence of the mite. In contrast, colonies had high loads of DWV-B even when mite numbers were low. DWV-B loads persisted in miticide treated colonies, indicating that this genotype has a competitive advantage over DWV-A irrespective of mite numbers.
5. Our findings suggest that the global increase in DWV-B prevalence is not driven by selective pressure by the vector. Rather, DWV-B is able to persist in colonies at higher viral loads relative to DWV-A in the presence and absence of *V. destructor*. The interplay between *V. destructor* and DWV genotypes within honey bee colonies may have broad consequences upon viral diversity in sympatric taxa as a result of spill-over.

## INTRODUCTION

Novel pathogens continue to emerge in animal and plant populations, placing significant medical, economic and environmental burdens worldwide. Emerging infectious diseases often occur when a pathogen is introduced to a naïve region by trade or travel, acquires the ability to infect a novel species, or enlists a vector (Woolhouse, 2002). The introduction of a vector can have a significant impact on disease emergence by both directly transmitting novel pathogens to new hosts and by altering the transmission modes of existing pathogens within a given population. Indeed, the emergence of numerous medically important arboviruses (viruses vectored by arthropods to vertebrate hosts) are attributed to a change of host due to the encroachment of humans into previously uninhabited locations (Tabachnick, 1998). For example, chikungunya, dengue, Zika and yellow fever viruses normally occur in non-human primates and are vectored by forest-dwelling mosquito species (Diallo et al., 1999; Vasconcelos et al., 2004; Musso and Gubler, 2016; Gutiérrez-Bugallo et al., 2019). Outbreaks in human populations are attributed to the viruses being vectored by anthrophilic urban mosquito species (primarily *Aedes aegypti*) when they feed on viremic humans infected during contact with forested areas or during travel (Couto-Lima et al., 2017; Gutiérrez-Bugallo et al., 2019).

When multiple pathogens share the same host and ecological niche, the introduction of a competent vector may also facilitate co-transmission, whereby a vector simultaneously transmits multiple pathogenic species or strains to the same host. Co-transmission of pathogens is known to occur in various vectors, including the bacteria *Borrelia burgdorferi* and *Ehrlichia phagocytophila* by *Ixodes scapularis* ticks (Levin and Fish, 2000), multiple strains of the malaria parasite (*Plasmodium chabaudi*) by mosquito *Anopheles stephensi* (Taylor et al., 1997), dengue-1 and chikungunya viruses by *Aedes albopictus* (Vazeille et al., 2010), and double or triple co-transmission of Zika, chikungunya and dengue-2 viruses by *Ae. aegypti* (Göertz et al., 2017; Rückert et al., 2017). Co-transmission by a vector and co-infection of a host may lead to complementary or competitive interactions between pathogens, provide opportunity for genetic exchange, increase host or vector specificity, and modify selective pressures acting upon evolutionary dynamics and epidemiological outcomes (Singer, 2017). When one pathogen infects a host that is already infected with a different pathogen, competitive exclusion can result, whereby the primary infection inhibits a secondary

infection, typically with different strains of the same virus (DaPalma et al., 2010; Mascia and Gallitelli, 2016).

The aforementioned dengue, chikungunya, Zika and yellow fever are all RNA viruses. RNA viruses characteristically have high mutation and replication rates, undergo frequent recombination and exist in diverse populations. These characteristics contribute to the probability of producing mutations that increase relative fitness and allow RNA viruses to quickly adapt to new hosts (Holmes, 2009), as the RNA virus SARS-CoV-2, the causative agent of COVID-19, has shown (Lu et al., 2020). The characteristic traits of RNA viruses most likely also facilitate their adaptation to vector-based transmission, as vector-based transmission requires the virus to be able to survive and potentially reproduce in both the vector and host species.

The last decade or so has seen a focus on RNA viruses of honey bees and their potential vector, the ectoparasitic mite *Varroa destructor*. Honey bees are naturally infected with a range of RNA viruses (Remnant et al., 2017; McMenamin and Flenniken, 2018; Roberts et al., 2018) so the arrival of a potential vector provides a unique opportunity to study the dynamics between pathogen, host and vector. The virus most commonly associated with *V. destructor* is *Deformed wing virus* (DWV).

*V. destructor* historically parasitised the Asian honey bee *Apis cerana* and shifted hosts to *Apis mellifera* in the early-mid 20$^{th}$ century (Techer et al., 2019). The anthropogenic movement of honey bees has resulted in a near global spread of *V. destructor* over the last 70 years. Prior to the arrival of *V. destructor*, DWV was a rare honey bee pathogen that was seldom detected in dead colonies (Bowen-Walker et al., 1999; Martin and Brettell, 2019). The virus is predominantly thought to have co-existed with honey bees, at very low or below detection levels, without causing any obvious harm (Martin and Brettell, 2019). In the absence of the mite, the virus is primarily horizontally transmitted during bee-to-bee contact and orally acquired in food (Chen et al., 2006a), whereby the virus accumulates to very low levels in its honey bee host. This equilibrium between host and virus changed dramatically with the arrival of *V. destructor*. Whilst feeding on the fat bodies of pupae and adult bees (Ramsey et al., 2019), *V. destructor* directly transmits viral particles into the bees' circulatory system. The change from predominantly horizontal transmission to vector-based transmission

of DWV has fundamentally changed the dynamics of the pathogen. An immediate effect of the change in mode of transmission has been a reduction in DWV genetic diversity within honey bee hosts, such that only a single strain remained (Martin et al., 2012; Ryabov et al., 2014), suggesting that certain strains are better adapted to the change in transmission route. At the same time, the presence of the vector has led to the accumulation of higher DWV loads in honey bees (Bowen-Walker et al., 1999; Tentcheva et al., 2006; Martin et al., 2012; Mondet et al., 2014; Locke et al., 2017). High viral loads in the presence of the mite have been associated with colony death (Highfield et al., 2009; Dainat et al., 2012b; Francis et al., 2013; Kevill et al., 2019).

Currently three DWV genotypes are associated with honey bees (DWV-A, -B and -C), with DWV-A and DWV-B being the most common in both honey bees and mites. DWV-A and DWV-B are estimated to have diverged from each other ~ 180 years ago (Mordecai et al., 2016b). DWV-A is thought to have been the predominant genotype in Western Europe and North America during the initial period of *V. destructor* establishment (de Souza et al., 2020). DWV-B was first detected in the Netherlands in 2001 (formally *Varroa destructor virus-1*) (Ongus et al., 2004). Since then, DWV-B has become the dominant genotype in the UK and has increased in prevalence in the USA (Ryabov et al., 2017; Brettell et al., 2019; Kevill et al., 2019; Manley et al., 2019b). The shift in DWV-B prevalence at the expense of DWV-A may be due to competition between the genotypes. Previous studies have found that honey bee colonies and mites can be co-infected with both genotypes, and that when both genotypes are present, DWV-B typically accumulates to higher loads (Ongus, 2006; Ryabov et al., 2017; Kevill et al., 2019). Similarly, DWV-B accumulates to higher loads than DWV-A when individual honey bees are simultaneously co-infected with both genotypes via experimental injection (McMahon et al., 2016; Norton et al., 2020). Furthermore, high DWV-B loads and the relative absence of DWV-A in *Varroa*-tolerant colonies led Mordecai et al. (2016a) to hypothesise that DWV-B may inhibit the accumulation of DWV-A via superinfection exclusion.

In addition to competition, the observed global shift from DWV-A to DWV-B could also, in part, be driven by DWV-B being better adapted to vector transmission by *V. destructor*. It could also be that some genotypes replicate in the mite, thereby increasing their prevalence. If we want to understand the relationship between *V. destructor* and DWV, we need to

experimentally manipulate the number of mites in a colony and determine the effect of mite numbers on the prevalence of both genotypes. Here we determine the role vector transmission plays in DWV prevalence at a colony level, by experimentally increasing or decreasing the number of *V. destructor* mites in honey bee colonies and tracking DWV-A and DWV-B loads in both treatment groups over a period of ten months.

## MATERIALS AND METHODS

### Experimental overview

We experimentally increased or decreased the number of mites within a given colony to determine what effect a change in mite numbers has on DWV genotypes A and B at the colony level over time. To increase the number of *V. destructor* in a colony, we added frames containing mites captured within capped brood frames to 'Mite Added' (M+) colonies (see below). To reduce the number of mites in a colony, we treated the colony with a miticide ('Mite Reduced'; M-). We monitored mite levels throughout the study to determine if our method successfully increased or reduced mite numbers by recording mite fall (Branco et al., 2006; Martin et al., 2010). To determine viral loads, we took monthly samples of adult honey bees (from July 2018 until April 2019) from both M+ and M- colonies, and mites from M+ colonies. We then analysed DWV-A and DWV-B viral loads in pooled honey bee or mite samples by qPCR to determine the effect on DWV genotypes over time. We also checked for the presence of other known honey bee viruses in our bee and mite samples.

### Honey bee colonies

In June 2018 we moved twenty-four colonies to the Grebbedijk apiary in Wageningen, the Netherlands. All colonies used in the experiment remained in the same apiary until April 2019 when the experiment ended, or until they died. No other hives were kept at the apiary during the experiment.

### Experimental set up

We aimed to rapidly increase the number of mites in six of our experimental colonies without introducing any bees from foreign colonies. Initial mite fall analysis indicated that mite numbers were very low in all colonies in early July 2018. In order to obtain a large number of

mites we adapted a protocol from Panziera et al. (2017), which involved moving capped brood frames containing mites between three colonies (per replicate group). This essentially pooled the mites from three colonies into one colony. We therefore placed eighteen of our twenty-four colonies into six groups of three. Within each group of three, one colony was designated as the 'Mite Added' (M+) colony. The second colony was designated as the 'Mite Shower' (MS) in which mites from the three colonies were concentrated, before they were transferred to the M+ (see below). The third colony was the 'Companion Colony' (CC), which donated frames of capped brood containing mites to the MS. The six M+ colonies were the experimental colonies in which we increased the number of mites and tracked the changes in viral loads over time.

As *V. destructor* reproduce inside capped brood cells, we moved capped brood frames from the M+ and CC colonies into the bottom box of the MS weekly for two or five weeks, respectively (Figure 4.1A). The mites present in the brood cells then emerged into the MS colonies along with newly eclosed adult bees, thereby increasing mite numbers in the MS colonies. This process also increased the honey bee population size of the MS colonies and reduced the CC population. In order to control the size of the MS and CC colonies, we transferred uncapped larval frames from the MS colonies to the CC colonies, thereby transferring brood without removing mites from the MS.

On the third week, we began capturing mites in order to transfer them to the M+ colonies. Late stage larvae are extremely attractive to *V. destructor* (Le Conte et al., 1989); thus we captured mites by exploiting the reproductive behaviour of *V. destructor*. We achieved this by transferring one-two marked frames of uncapped late stage larvae from the M+ colonies to the MS colonies (Figure 4.1B). After seven days, we returned the capped M+ brood frames to their M+ colony, thereby introducing the pooled mites into the M+ colonies (Figure 4.1C), along with the returning brood that belonged to that colony. We repeated the latter process three times.

The six remaining colonies were designated as 'Mite Reduced' (M-) colonies. Our intention was to have close to zero mites within these colonies throughout the duration of the experiment. We attached PolyVar yellow® (Bayer) strips to the outside of the hive entrances on July 12$^{th}$ 2018 (Figure 4.1D). The miticide strips contain 275 mg flumethrin and have

multiple holes that bees pass though thereby making contact with the active compound (Blacquière et al., 2017). The strips remained in place until the colony died or the experiment ended in April 2019.

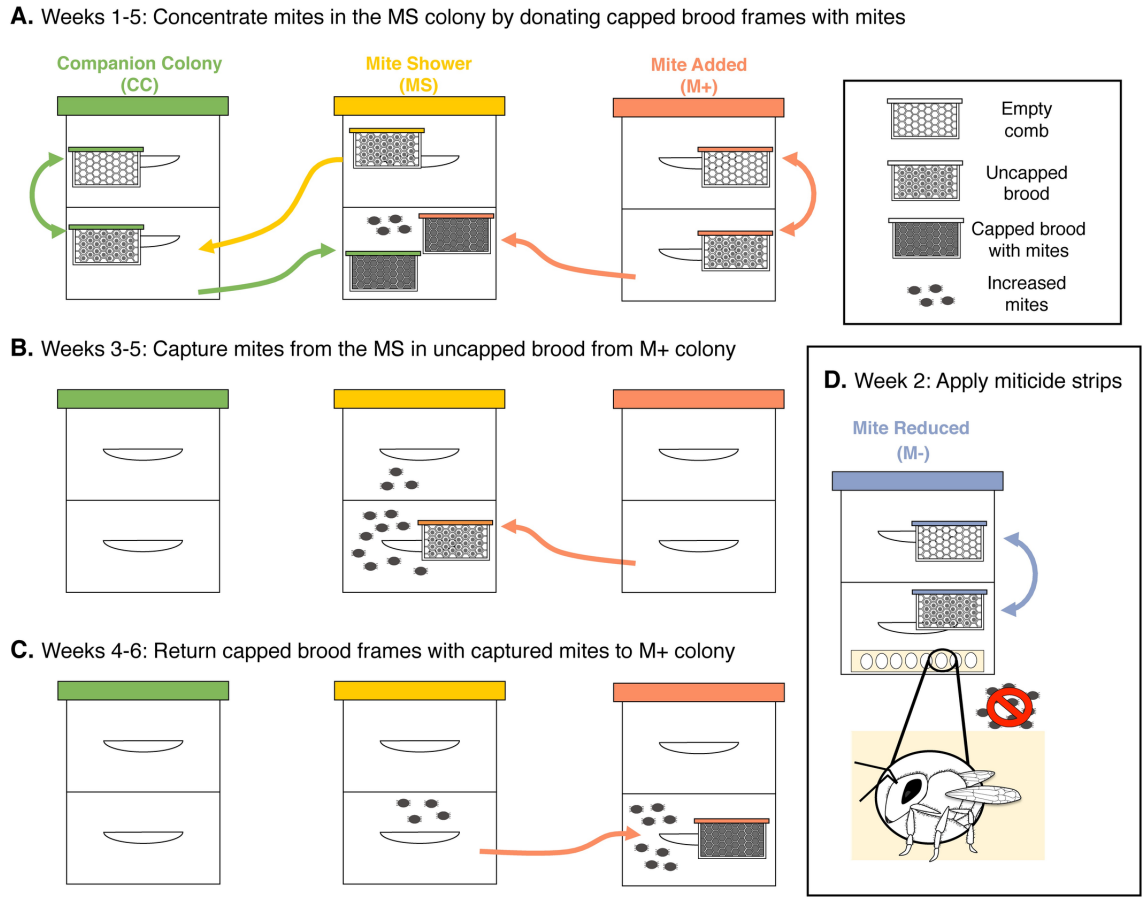


**Figure 4.1**. Diagram of the method used to rear a large number of *V. destructor* prior to introduction to the Mite Added (M+) colonies, or decrease mites in Mite Reduced (M-) colonies. The arrows show the direction of frame movement every seven days. **A)** Capped brood frames with mites were transferred to the Mite Shower (MS) colonies from M+ or Companion Colonies (CC) for two or five weeks, respectively. To increase the number of mites in M+ colonies without introducing foreign bees we **(B)** transferred frames of uncapped late stage larvae from the M+ colonies to the MS colonies. Mites then entered the brood cells before the cells were capped. After seven days **(C)**, the capped frames were returned to the M+ colonies, where the newly eclosed bees emerged with mites. **B** and **C** were repeated three times. **(D)** M- hives were fitted with PolyVar yellow® strips containing 275 mg flumethrin, which remained in place until the colony died or the experiment ended in April 2019.

At the start of the experiment, all colonies were housed in two boxes containing ten frames each, and separated by a queen excluder which confined the queen to the top box of each colony, as per Panziera et al. (2017). To ensure brood was of a similar age, the positions of the brood frames within each colony were changed every seven days. Excluding the MS colonies, combs containing brood > 4 days old were transferred from the top to the bottom box, which prevented the queens laying eggs amongst older brood. In MS colonies, open brood were transferred to the bottom box of CC colonies. Frames with empty comb (emerged brood cells) were transferred to the top box so that the queens could continue laying eggs. This intra-colony frame movement was conducted in all colonies for the first 6 weeks of the experiment, including the M- colonies.

**Mite levels within colonies**

To determine if mite numbers had been successfully increased in M+ colonies and decreased in M- colonies we recorded the number of mites that had fallen off bees and were removed from brood. To do this, we placed a wooden frame fitted with an aluminium mesh floor that allows mites to fall through and a sliding 'mite board' underneath each hive (Branco et al., 2006). We then counted the number of mites that had fallen onto the board within a 48-hour period prior to each sampling point

**SAMPLE COLLECTION**

**Adult bees**

Every month, from July 2018 until April 2019, we collected a random sample of ~ 50 adult worker bees from a centre frame from the bottom box of each M+ and M- colony. The bees were stored in an icebox in the field until we returned to the laboratory, where they were stored at -80°C. We then randomly chose ten bees from each month's sample for pooled viral analysis (see below). Seven out of twelve colonies died between February and April 2019, resulting in a total of 105 pooled bee samples.

**Mites**

We also collected mites for viral analyses. We could not use the fallen mites, as it is very difficult to extract high quality RNA from dead hosts. We therefore collected live mites. We either used the 'sugar shake' (Macedo et al., 2002), or 'washing' (Dietemann et al., 2013) method, depending on the season. When the humidity levels are high, sugar shakes are not

very effective. We used the sugar shake method from July through to December. From January to April we used the mite wash method. Sugar shakes or mite washes were performed each month on the M+ colonies. While we also performed sugar shakes and mite washes on the M- colonies in July and each month from December to April, no mites were found.

For sugar shakes we collected approximately 300 workers into a glass jar furnished with a mesh lid. We then added approximately 10 g of powdered sugar to the jar before it was gently rolled to evenly coat the bees in sugar. After one minute, we inverted the jar and shook the jar to dislodge the mites. Dislodged mites were collected into a plastic container, counted, cleaned and collected with a moist paintbrush. We kept the mites in an icebox until laboratory storage at -80°C. We returned the bees to their colony.

For mite washes we collected ~ 30 bees in plastic containers and transported them to the laboratory where we euthanised them at -20°C. To dislodge the mites from the bees, we added water to each sample jar, secured the nylon mesh lid and shook the bees for 30 s. We collected the mites by inverting the jar over a nylon mesh sieve (1 mm hole). After counting the number of mites we collected them using a paintbrush and stored the mites at -80°C for viral analysis.

## VIRAL ANALYSIS

### RNA extraction

To determine viral levels for each colony, each month (from July 2018 to April 2019, or until colony death), we crushed ten bees per colony in an extraction bag with a hand homogenising tool (Bioreba) with 5 mL cold 0.5M potassium phosphate buffer (pH 8). The pooled bee homogenate (3 mL) was portioned into aliquots for subsequent analysis and stored at -80°C. We extracted RNA from 250 µL pooled bee homogenate with 750 µL TRI Reagent (Sigma) according to the manufacturer's protocol with the following modification: in addition to 500 µL isopropanol, we added 500 µL RNase free $dH_2O$ and 1 µL glycogen to the transferred aqueous phase before samples were vortexed and chilled at -80°C overnight to enhance precipitation. Subsequent RNA pellets were suspended in 150 µL UltraPure nuclease-free $dH_2O$ (Invitrogen). RNA concentration was determined with Qubit Broad Range Assay (Life Technologies) before being standardised to 65 ng/µL.

We chose to analyse pooled mites per colony from three time points. As infestation levels in M+ colonies were too low in July to obtain samples from each colony, we selected August and October 2018, and January 2019 as our three time points. We extracted RNA from 6-10 mites per time point ($n$ = 18) with 500 µL TRI Reagent crushed with an RNase*Zap* treated pestle as per the manufacturer's protocol; as above, we added 250 µL isopropanol and 1 µL glycogen to the transferred aqueous phase before being chilled at -20°C overnight. RNA pellets were suspended in 10 µL UltraPure nuclease-free $dH_2O$, before being standardised to 65 ng/µL as above.

**cDNA synthesis**

First strand cDNA of all samples was synthesised from 260 ng of ezDNase (Invitrogen) treated RNA using SuperScript III Reverse Transcriptase (Invitrogen) with random hexamer primers in 10 µL reaction volumes. The resulting cDNA was diluted in 40 µL UltraPure nuclease-free $dH_2O$.

**qPCR Analysis**

All pooled bee or mite samples were screened for endogenous control gene *Actin* in addition to multiple viruses, including *Acute bee paralysis virus* (ABPV), *Apis rhabdovirus* (ARV-1 and ARV-2), *Black queen cell virus* (BQCV), *Bee macula like virus* (BeeMLV), *Chronic bee paralysis virus* (CBPV), *Deformed wing virus* (DWV; genotypes A, B and C), *Israeli acute paralysis virus* (IAPV), *Kashmir bee virus* (KBV), *Lake Sinai virus* (LSV), *Sacbrood virus* (SBV), and *Slow bee paralysis virus* (SBPV) (primers given in Table C.1). The 384-well plates (Biorad) were prepared with a Pipetmax 268 (Gilson), and cDNA of bees previously determined to be positive for a given virus served as positive controls and $dH_2O$ served as a negative (no template) control. We performed all 5 µL qPCR reactions in duplicate with SsoAdvanced Universal SYBR Green supermix (Bio-Rad), forward and reverse primers (final concentration 500 nM each), and 1 µL cDNA. Analysis was conducted on a Bio-Rad CFX384 Touch real-time PCR detection system with the following cycling conditions: 95°C (10 min), followed by 40 cycles of 95°C (15 s), 58-61.7°C depending on primer pair (15 s), and 72°C (30 s), followed by melt curve analysis between 55 and 95°C, at 0.5°C increments. Primer specificity was validated using melt curve analysis and gel electrophoresis. Samples were considered positive for a virus where the average Cq was < 35 and if replicate melt curves were consistent with the positive control. The standard deviation from duplicate qPCR

analyses in virus positive samples was confirmed to be ≤ 0.3 and no positive samples required re-analysis.

As virtually all bee and mite samples contained at least one strain of DWV, we used absolute quantification to determine DWV-A and DWV-B viral loads in bees and mites. We interpolated mean Cq values against a plasmid standard curve as per Norton et al. (2020), and multiplied by the associated dilution factor for each sample. This gave the DWV viral load for 3 mL of pooled bee homogenate or 10 μL of mite RNA, which was subsequently divided by 10 to give an average DWV load per bee, or divided by 6-10 for the average viral load per mite. To determine which DWV genotypes were present within bee or mite samples, we used the DWV-A, -B and -C primers designed by Kevill et al. (2017), which amplify a fragment of the highly conserved RdRp region (Table C.1). We independently validated the sensitivity and specificity of these primers in our previous study where we found no evidence of cross priming between genotypes (Norton et al., 2020). As viruses apart from DWV were detected less frequently in bee samples, we determined viral loads relative to housekeeping gene *Actin*, using the Pfaffl expression ratio (Pfaffl, 2001), to account for differences in primer efficiencies. Samples negative for the target virus were used as comparative controls, by assigning an arbitrary Cq value of 40 for the target virus. Primer efficiencies were determined by a standard curve, using a 10-fold dilution series (from $10^{0}$-$10^{-6}$) of cDNA positive for the target virus (Table C.1). As with absolute values, the relative viral load values were divided by 10 to give an average relative load per bee. For the mite analysis, we were unable to calculate relative viral loads as there were insufficient samples to assign as comparative controls. We therefore provide the average Cq values for viruses detected in mites, apart from DWV.

**DWV loads**

There is currently no defined aetiology between DWV viral loads and pathogenesis. Colony mortality is typically associated with loads > $10^{10}$, however mortality has been observed with DWV viral loads as low as $10^{6}$ genome equivalents (GE) (Kevill et al., 2019). We considered mean DWV loads of > $10^{9}$ GE per bee to be high, based on mortality observed in experimentally injected adults (McMahon et al., 2016) and pupae (Tehel et al., 2019; Norton et al., 2020). We viewed viral loads between $10^{6}$ and $10^{9}$ GE/bee as moderate, and < $10^{6}$ GE/bee as low in accordance with Barroso-Arévalo et al. (2019a).

**Statistical analyses**

We used the number of mites fallen within 48 h to determine whether our experimental methods successfully increased and reduced the number of mites in our M+ and M- colonies, respectively. We compared mite fall in M+ and M- colonies using a one-way repeated measured analysis of variance with pairwise Bonferroni adjusted post hoc tests, with RStudio software (R version 3.5.0) using 'ez' (Lawrence, 2016). We evaluated sphericity with Mauchly's test, thus p values are reported with the Greenhouse-Geisser correction. We restricted this statistical analysis to data collected from July 2018 to January 2019 because some of our colonies died between February and April 2019, and the model cannot handle missing data.

Prior to statistical analyses, we added a constant value (+1) to all viral data points to accommodate viral loads of zero during log transformation. We used a generalised additive model (GAM) with negative binomial error family and log link with 'mgcv' (Wood, 2018), to determine if there were differences in the mean viral loads of DWV-A and DWV-B between M+ and M- colonies between July 2018 to April 2019 (or until colony death). Secondly, we used a linear mixed effects model (LMM) to determine if DWV-A and DWV-B loads differed in mites collected from M+ colonies in August, October and January. Colony ID was used as a random factor in the GAM and LMM models. Homogeneity of variance and normality in the LMM were visually assessed, which illustrated that a $\log_{10}$ transformation of the response variable (mean DWV load per mite) substantially improved the model. The LMM was performed with 'lme4' (Bates et al., 2018), and random effects were assessed with ranova function in 'lmerTest' (Kuznetsova et al., 2017). To determine pairwise differences, we analysed the GAM and LMM models as a type III ANOVA [with anova.gam or 'car' (Fox et al., 2018), respectively], followed by Tukey post hoc analysis with estimated marginal means using 'emmeans' (Lenth, 2019). In addition, we used a repeated measures correlation to determine the relationship between mite numbers and viral loads. We compared the ($\log_{10}$) mean DWV-A or DWV-B load per bee to mite fall in M+ and M- colonies using 'rmcorr' (Bakdash and Marusich, 2017). Again, this analysis was restricted to July 2018 to January 2019 due to the missing data.

To assess the relationship between the abundance (viral loads) of all RNA viral species detected in honey bee samples between treatment groups and seasons, we performed non-

metric multidimensional scaling (NMDS). We first $\log_{10}$ transformed the viral loads of nine pathogens detected in our colonies (DWV-A, DWV-B, ABPV, ARV-1, ARV-2, BeeMLV, BQCV, LSV and SBV) as viral loads differed by many orders of magnitude between species. We created a viral community dissimilarity matrix using the Bray-Curtis index and plotted the NMDS by treatment (M+ vs M-). We performed a permutational multivariate analysis of variance (PERMANOVA) on the dissimilarity matrix to determine which factors (treatment, mite fall and season) best explained the structure of the viral community, followed by pairwise analysis with Bonferroni correction for multiple comparisons. NMDS and PERMANOVA (adonis) were performed with 'vegan' (Oksanen et al., 2013), and pairwise multilevel analysis was performed with 'pairwiseAdonis' (Martinez Arbizu, 2017). All figures were generated with 'ggplot2' (Wickham et al., 2016).

## QUARANTINE PERMIT

Frozen adult honey bees (workers) were imported into Australia for analysis on dry ice under Department of Agriculture and Water Resources import permit 0000917783.

# RESULTS

### Mite numbers in M+ and M- colonies

Our experimental method successfully increased the number of mites within M+ colonies ($F_{6,30}$ =11.46; $p$ = 0.003). At the start of the experiment (July 2018), the average mite fall in M+ colonies was 5.5 ± 1.5 SE mites. Mite fall increased by approximately 10-fold by August (mean 58.2 ± 12.8 SE) and peaked in September and October (Figure 4.2; Figure C.1A), when we observed an approximately 35-fold increase in average mite fall, which was higher than July ($p < 0.001$) and August ($p < 0.05$). Average mite fall declined in December ($p < 0.01$) and January ($p < 0.001$), compared to levels observed in September and October. Mite fall remained low in M- colonies from July 2018 to January 2019 (mean 2.0 ± 0.7 SE) (Figure C.1B). We found that mite fall did not differ across the seven months in our M- colonies ($F_{6,30}$ = 1.10; $p$ = 0.358), despite a very slight increase in mite fall in October (mean 5.2 ± 1.6 SE).

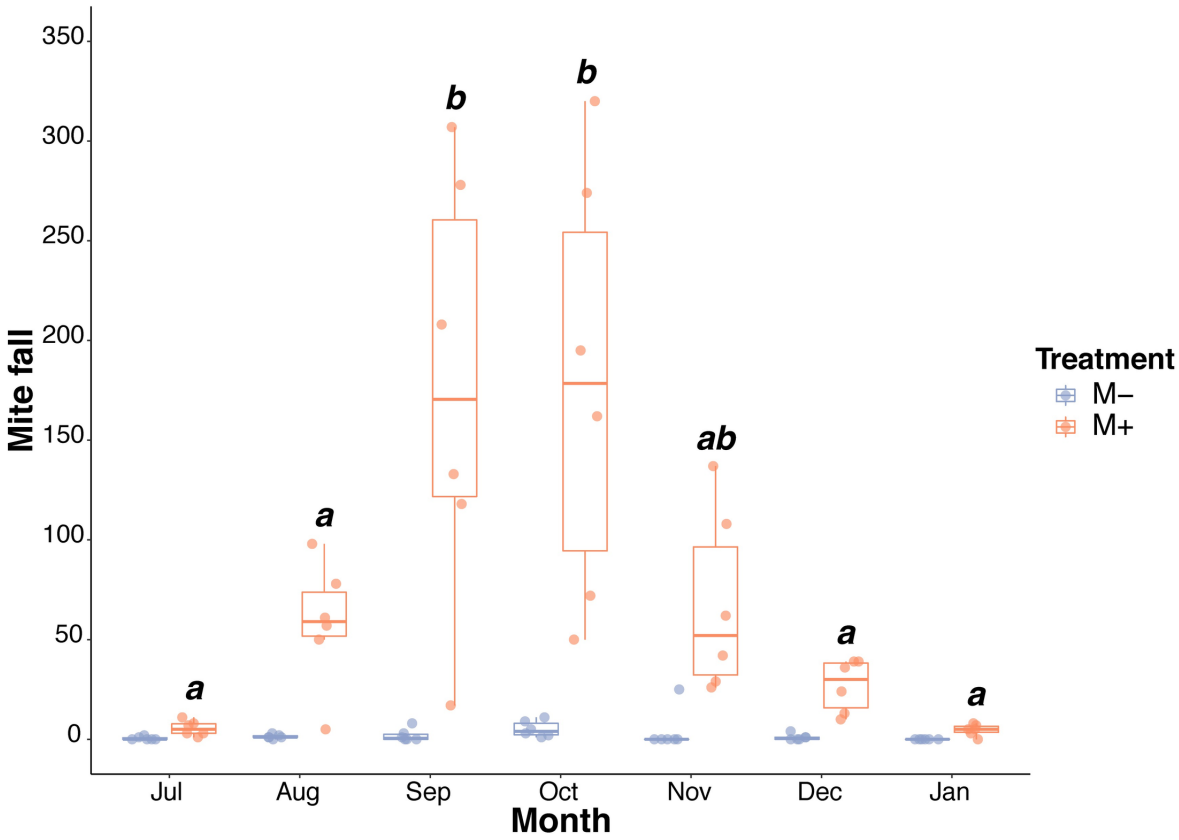


**Figure 4.2**. Boxplot comparison of mite fall within 48 h in Mite Added (M+) and Mite Reduced (M-) colonies. Each point represents the number of fallen mites for each colony between July 2018 and January 2019. Mite numbers were successfully increased in M+ colonies, and peaked in September and October. In contrast, mite numbers remained low in M- colonies, and mite fall did not differ across the seven months. Months that do not share a common letter differ at $p < 0.05$. No letters above the M- boxplots illustrates that no differences in mite fall were detected between each month.

**Colony-level DWV loads**

DWV loads differed between M+ and M- colonies ($\chi^2 = 71.41$; df = 1; $p < 0.0001$) and between DWV-A and DWV-B genotypes ($\chi^2 = 273.74$ ; df = 1; $p < 0.0001$) (Figure 4.3). We also found a significant interaction between treatment and genotype ($\chi^2 = 15.61$; df = 1; $p < 0.0001$), indicating that DWV-A and DWV-B differ in their response to high or low mite numbers. At the start of the experiment in July, DWV-A was only detected in two out of the twelve colonies (one M+ and one M- colony) and only at low levels (mean GE $\leq 7 \times 10^4$) (Figure C.2A and B). As mite numbers increased in M+ colonies, so did the frequency of DWV-A detection. In August DWV-A was detected in 50% of M+ colonies. By September all M+ colonies carried DWV-A. DWV-A remained present in all M+ colonies throughout the

remainder of the study, with the exception of one colony in October. This colony also had the lowest mite fall throughout the study.

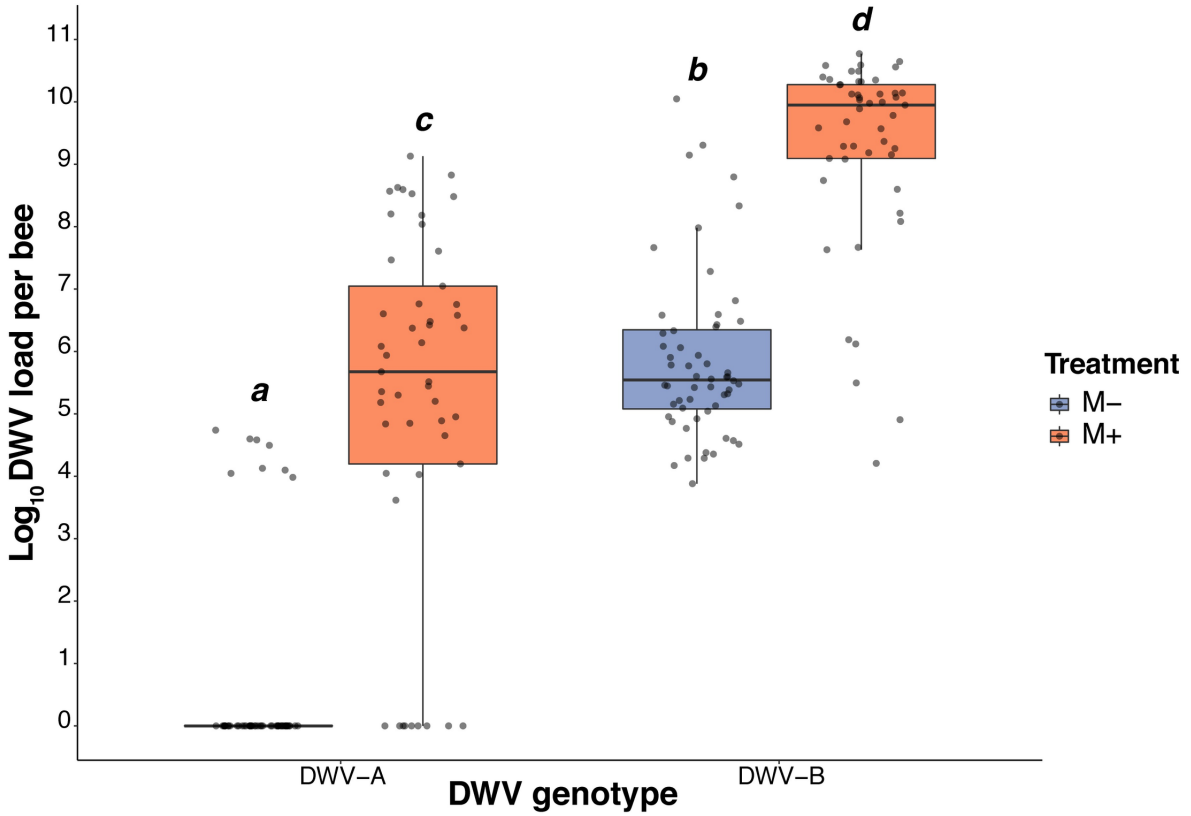


**Figure 4.3.** Boxplot comparison of $\log_{10}$ DWV-A and DWV-B loads per bee in Mite Added (M+) or Mite Reduced (M-) colonies. Each point represents the mean DWV load calculated from ten pooled honey bees, sampled monthly between July 2018 and April 2019 ($n$ = 105). The different letters above each boxplot indicates that DWV loads differed ($p < 0.05$) between treatment groups (M+ vs. M-) and between DWV-A and DWV-B genotypes.

DWV-B was consistently detected each month in all colonies of both treatment groups although loads varied amongst colonies at the start of the experiment. Four M+ colonies had moderate to high DWV-B loads in July ($1.6 \times 10^8$ - $2.2 \times 10^{10}$ GE), while the remaining two M+ colonies had low to moderate DWV-B loads ($1.6 \times 10^4$ – $1.3 \times 10^6$ GE) (Figure C.2C). DWV-B increased with increasing mite numbers, however the level of DWV-B accumulation differed between colonies depending on initial loads observed in July. Overall, the average

DWV-B load in M+ colonies was 1.2 x $10^{10}$ GE, which was higher than the average DWV-A load of 9 x$10^{7}$ GE (Tukey; $p < 0.0001$).

Reduced mite numbers had different effects on DWV-A and DWV-B. Both genotypes had lower loads in M- colonies compared to M+ colonies (Tukey; $p < 0.0001$). DWV-A was only detected in 12.5% of M- samples across the entire study period (until April 2019). We only detected DWV-A in one M- colony in July (Figure C.2B). This colony had the highest average DWV-A load (5.5 x $10^{4}$ GE) observed in all M- colonies. We did not detect DWV-A in any M- colonies in August, October, and between December to February. In contrast, we consistently detected DWV-B in all M- colonies across each month until April 2019, or colony death (Figure C.2D). In July, DWV-B loads in M- colonies ranged from 2.0 x $10^{4}$ to 1.9 x $10^{7}$ GE. There was an initial 10-1000 fold decrease in DWV-B loads in four colonies by August. However average DWV-B loads increased by 1-4 orders of magnitude by September. Overall, average DWV-B loads in M- colonies remained moderate over time (~ 1 x$10^{5}$-$10^{6}$ GE) and mean DWV-B loads in M- colonies were slightly lower DWV-A loads in M+ colonies [(Tukey; $p = 0.0399$) Figure 4.3].

When we compared the relationship between mite numbers and DWV-A or DWV-B viral loads within M+ and M- colonies, we found that only DWV-A was affected by increasing mites ($R_{rm\,(71)} = 0.28$, 95% CI [0.05, 0.49], $p = 0.017$) (Figure C.3A). In contrast, we found no correlation between mite numbers and DWV-B loads ($R_{rm\,(71)} = 0.10$, 95% CI [-0.14, 0.32], $p = 0.42$) (Figure C.3B).

**DWV loads in mites**

Average DWV loads in mites collected from M+ colonies in August, October and January differed between the three months ($\chi^2$ =41.8, df = 2, $p < 0.0001$). We did not detect DWV-A in any mites collected in August (Figure C.4A). DWV-A was detected in 83% and 100% of mites collected in October and January, respectively, and loads were highest in October. In contrast, we detected DWV-B in 67% of mites in August, and in 100% of mites in October and January (Figure C.4B). As with DWV-A, DWV-B loads were highest in October. We found that DWV-B loads were less variable and higher in mites compared to DWV-A ($\chi^2$ = 48.1, df = 1, $p < 0.0001$) (Figure 4.4). We did not detect DWV-C in any honey bee or mite samples throughout the study.

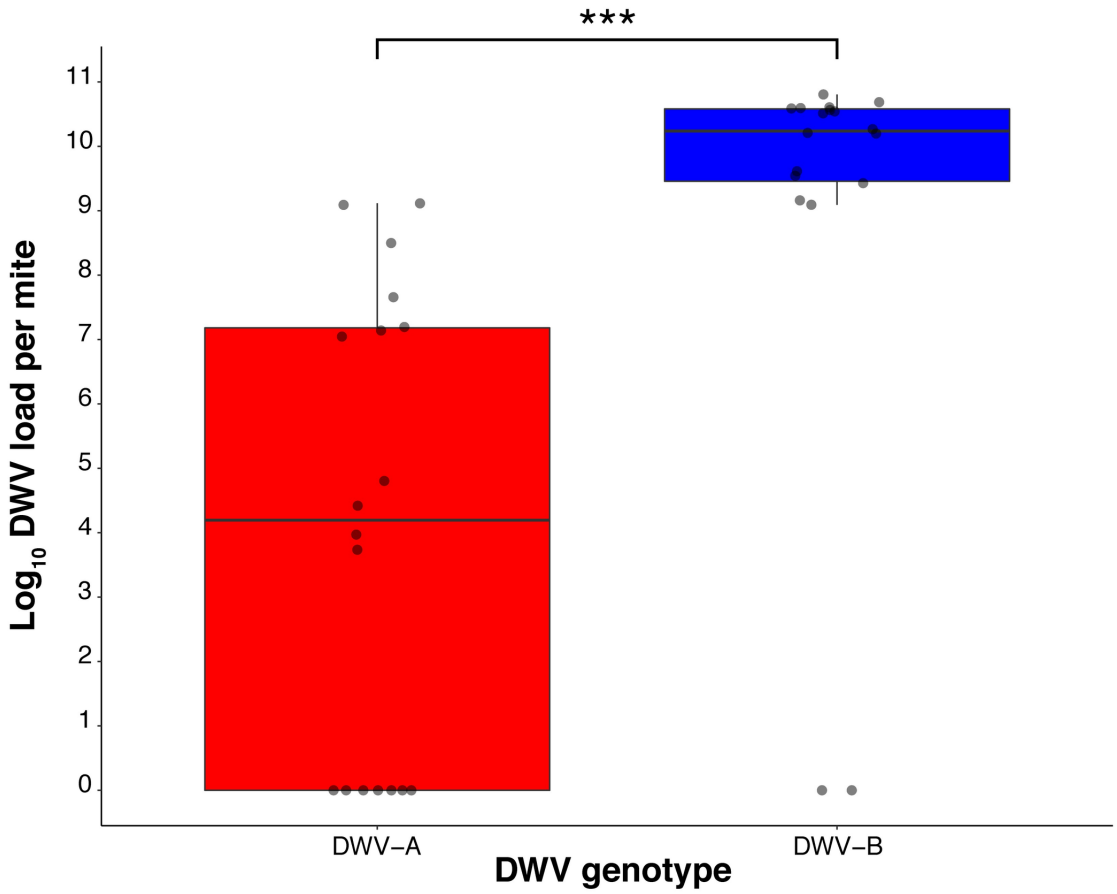


**Figure 4.4.** Boxplot comparison of $\log_{10}$ DWV-A and DWV-B loads per mite. Each point represents the mean DWV load calculated from six-ten pooled *V. destructor* collected from M+ colonies in August, October and January ($n$ = 18). DWV-B loads were higher than DWV-A (*** $p < 0.0001$).

**Other viruses in honey bee colonies and mites**

In addition to DWV-A and DWV-B, we detected BQCV, LSV and SBV in honey bees, and ABPV, ARV-1 and ARV-2, and BeeMLV in both bees and mites (Table 4.1). The presence and viral load (relative to *Actin*) of each virus differed widely, but all viral loads in bees (excluding DWV) were $\leq 2.2 \times 10^5$ (Figure C.5). We frequently detected multiple viruses within pooled bee ($n$ =105) and pooled mite samples ($n$ = 18) at any given time. Our two-dimensional NMDS (Figure 4.5) and PERMANOVA showed that viral communities in honey bee samples differed between M+ or M- treatments ($F_{1,95} = 100.5$, $R_2 = 0.44$, $p = 0.001$). Mite fall was not a predictor of the community structure ($F_{1,95} = 2.0$, $R_2 = 0.009$, $p = 0.114$), however we did find an interaction between mite fall × treatment ($F_{1,95} = 2.9$, $R_2 = 0.013$, $p$ =

0.030). The NMDS showed that DWV-A was strongly clustered in the M+ group, whereas DWV-B clustered between M+ and M- treatments. BeeMLV clustered closely to DWV-A, which was expected as BeeMLV was more frequently detected in bees of M+ colonies and often at higher viral loads compared to M- group. ARV-1 and ARV-2 were infrequently detected (< 10%) in honey bees and viral loads did not appear to be influenced by increasing mite numbers over time, however, both species were predominantly found in M+ colonies. We did not observe any evidence to suggest a relationship between increased mite levels and the abundance of LSV, BQCV and SBV. LSV and BQCV were detected in 48% and 60% of colonies, respectively; prevalence and viral loads of both viruses were very similar between M+ and M- colonies. SBV was infrequently detected (0.7%) throughout the study, yet was found more commonly in M- colonies. In contrast, ABPV (16%) was predominantly detected in M+ colonies.

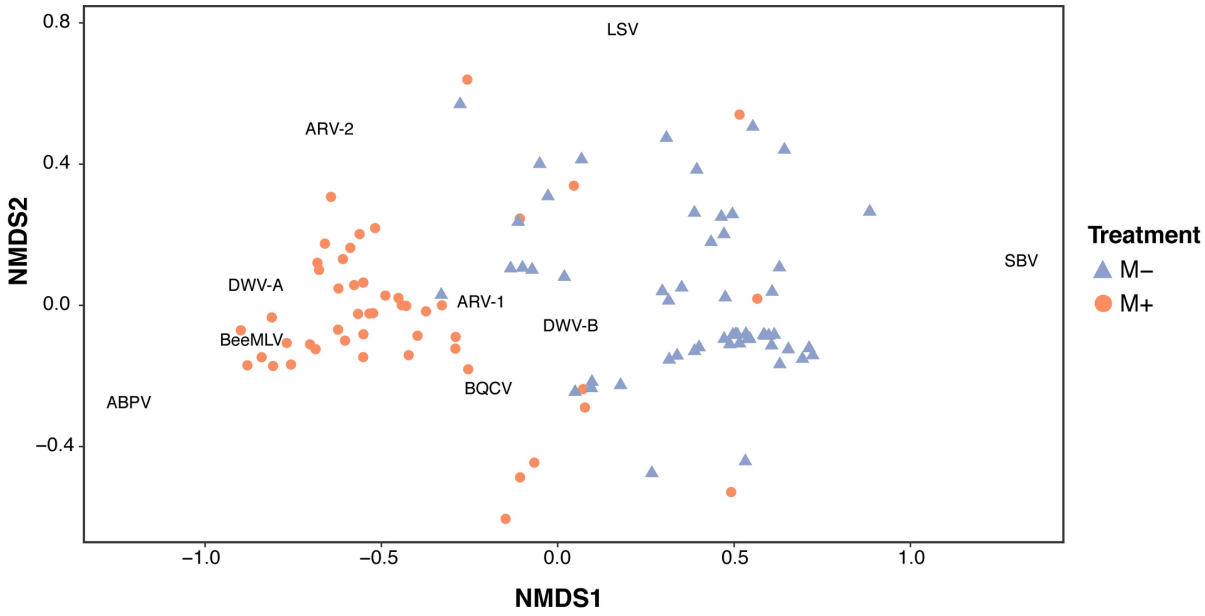


**Figure 4.5**. Non-metric multidimensional scaling (NMDS) plot (k = 2) of viral species abundance in pooled honey bee samples in M+ and M- colonies. Differences between species were analysed with a Bray-Curtis dissimilarity matrix (stress = 0.12). Our corresponding PERMANOVA indicated that treatment explained the largest amount of variance in the pathogen composition.

**Table 4.1.** Summary of additional viruses detected in honey bee colonies (M+ and M-), or mite samples collected from M+ colonies. Proportions calculated from pooled honey bee samples repeatedly collected from each colony between July 2018 to April 2019 (*n* = 105), or pooled mites collected in August and October 2018, and January 2019 from M+ colonies (*n* = 18).

| Virus | Proportion in pooled bee samples | Proportion in pooled mite samples | Viral loads in bees associated with increased mite numbers (Y/N) | Seasonal detection in bees | Seasonal detection in mites |
|---|---|---|---|---|---|
| ABPV | 0.16 | 0.94 | Y | Infrequently detected in summer and spring | N |
| ARV-1 | 0.08 | 1 | N; predominantly detected in M+ samples | Not detected in winter | N |
| ARV-2 | 0.07 | 1 | N; predominantly detected in M+ samples | Predominantly detected in autumn | N |
| BeeMLV | 0.48 | 0.72 | Y; predominantly M+ samples and relative loads increase with increased mite levels | Infrequently detected in summer | Y; higher loads in autumn and winter |
| BQCV | 0.60 | 0 | N | N | NA |
| LSV | 0.48 | 0 | N | N | NA |
| SBV | 0.07 | 0 | N | Predominantly detected in summer | NA |

Our NMDS indicated an effect of season on the clustering of viral species found in our colonies (Figure C.6). PERMANOVA indicated that season alone ($F_{3,95} = 6.59$, $R_2 = 0.085$, $p = 0.001$) and an interaction between treatment × season ($F_{3,95} = 3.44$, $R_2 = 0.045$, $p = 0.004$) explained a small amount of the variability in the viral community structure. It is important to note though, that there were very few data points for DWV-A, BeeMLV and ABPV in samples from summer. These species were largely undetected until mite numbers had reached high levels (in M+ colonies) from September onwards. After correction for multiple comparisons, the pairwise analysis showed that there was only a small difference between summer and autumn ($F_1 = 6.21$, $R_2 = 0.097$, $p = 0.036$).

We found that ABPV, ARV-1, ARV-2, and BeeMLV were more frequently detected (72-100%) in pooled mite samples compared to honey bees. In addition, our results suggest that mites harbour high loads of BeeMLV (Figure C.7).

**Mortality**

Our experimental protocol was not designed to establish a relationship between mite infestation, genotype presence and viral loads as predictors of colony death. However, we observed five M+ colonies (83%) and two M- colonies (33%) die between February and April 2019. We have intentionally excluded analysing what factors may have contributed to colony death in our study due to (i) our small sample size of six colonies per treatment, and (ii) the lack of unmanipulated control colonies to determine the natural rate of colony mortality within the environmental conditions of the experiment.

## DISCUSSION

We aimed to investigate what effect increasing or decreasing mite numbers have on DWV genotypes A and B over a period of ten months. Our results suggest the two genotypes differ in their response to the mite. We found that accumulation of DWV-A in honey bees was more strongly influenced by mite numbers than DWV-B. DWV-A was only detected in one M+ colony at the start of our experiment, yet we observed a clear increase in DWV-A prevalence and accumulation with increasing mite numbers over time. This indicates that DWV-A was circulating in our honey bee population, but at low/below detection levels until mite numbers reached sufficiently high levels. While we found a significant correlation between mite

numbers and DWV-A loads, the correlation coefficient was weak (0.28). This was likely due to DWV-A not being detected in some colonies in August, despite increasing mites, and conversely, DWV-A loads remaining at moderate levels within M+ colonies in winter despite mite numbers decreasing. DWV-A has been associated with initial establishment of *V. destructor* in the UK and North America (Highfield et al., 2009; Martin et al., 2012; Wilfert et al., 2016; Kevill et al., 2017), leading Martin et al. (2012) to suggest that certain DWV-A strains have increased fitness or have a competitive advantage when transmitted by *V. destructor*. Our data suggests that DWV-A is more dependent on vector transmission by *V. destructor* compared to DWV-B. Our results are strikingly similar to Manley et al. (2019b) despite very different studies. DWV-A was undetected in honey bees and bumble bees on *Varroa*-free islands in England and France, whereas DWV-B was detected in both the presence and absence of the mite but at lower loads in mite free areas (Manley et al., 2019b).

Overall, we observed that DWV-B accumulates to higher loads than DWV-A in co-infected colonies, both in the presence and absence of *V. destructor*. This is consistent with past studies of co-infected colonies parasitised by the mite (Ryabov et al., 2017; Kevill et al., 2019), and individual bees experimentally co-injected with both genotypes (McMahon et al., 2016; Norton et al., 2020). Co-injection of equal amounts of DWV-A and DWV-B reduces DWV-B accumulation in individual pupae (Tehel et al., 2019; Norton et al., 2020), yet we found that DWV-B still accumulates to higher loads than DWV-A during co-infection (Norton et al., 2020). The results of this study and our previous work indicate that DWV-B outcompetes DWV-A during co-infection by accumulating to higher viral loads, but does so without strongly excluding DWV-A replication. Mordecai et al. (2016a) hypothesised that a primary DWV-B infection may inhibit DWV-A through superinfection exclusion, but our study finds no evidence to support this at a colony level. We note that our analysis characterises DWV-A and DWV-B based on amplification of a small region of the RdRp, but does not distinguish whether other regions of the genome have undergone recombination. It is common for DWV genotypes to recombine in co-infected colonies (Moore et al., 2011; Zioni et al., 2011; Ryabov et al., 2014; Dalmon et al., 2017; Brettell et al., 2020). This may have influenced which strains predominate in our M+ colonies, such that our DWV-A or DWV-B genotypes could in fact be a recombinant containing genomic portions of both A and B, as suggested by Daughenbaugh et al. (2021).

There are two modes by which vectors can transmit pathogens: biological transmission, whereby the pathogen replicates within the vector, or mechanical transmission where the pathogen is transmitted in a non-propagative manner (Kuno and Chang, 2005). There is some conjecture in the literature on whether DWV can replicate in the mite (Martin and Brettell, 2019), and whether this differs between DWV-A and DWV-B (Yañez et al., 2020). Some studies have detected the negative (replicative) strand of both DWV-A and DWV-B in whole homogenised mites (Ongus et al., 2004; Yue and Genersch, 2005; Gisder et al., 2009; Bradford, 2019) and DWV-B mite tissue (Campbell et al., 2016). Using reverse genetics, Gusachenko et al. (2020) found that a constructed DWV strain could replicate to some extent within mites. Furthermore, Gisder and Genersch (2020) detected DWV-B in the intestinal epithelium and salivary glands of *V. destructor* using fluorescence-*in situ*-hybridisation, but found no evidence of DWV-A infecting mite tissue; however, it is unclear if DWV-A was present within the population assayed.

In our colonies, DWV-A was only found at moderate-high viral loads after mite numbers increased. If this relationship is best explained by *V. destructor* predominantly acting as a biological vector, then we would expect to find high DWV-A levels within mites coinciding with low/below detection levels in honey bees. However, we found the opposite. We did not detect DWV-A in mites until October, after DWV-A was already established within M+ colonies. This indicates that at a colony level, *V. destructor* predominantly transmits DWV-A via mechanical transmission, whereby DWV-A must first accumulate within honey bees. This directly supports the experimental findings of Posada-Florez et al. (2019) who showed that *V. destructor* transmission of DWV-A is non-propagative and decreases if mites do not consecutively feed on infected pupae.

If DWV-A does not predominantly replicate in *V. destructor*, what would cause DWV-A to emerge in our M+ colonies? A previous study showed that experimental injection of $10^2$ GE is sufficient for DWV to accumulate to loads > $10^9$ GE per bee (Tehel et al., 2019). Möckel et al. (2011) suggested that mites must harbour viral loads > $10^8$ GE in order to transmit ~ $10^2$ GE to a pupa during feeding. Our data show that at a colony level, DWV-A can emerge to moderate levels within bees without mites harbouring detectable levels of virus. This supports the findings of Tehel et al. (2019), however, we acknowledge that we did not explicitly pair bees to their parasitising mites. It is also possible that DWV-A was introduced via mites

originating from the MS or CC colonies. While we cannot exclude this possibility, we think it unlikely; brood are capped for ~ 12 days (Human et al., 2013), and we sampled the colonies in the middle of each month. This means that the majority of the mites we introduced into the M+ colonies would have emerged from brood cells at the August sampling point and were likely to be in the dispersal phase (Traynor et al., 2020), where they could be captured with the sugar shakes. If DWV-A was introduced from MS or CC mites, then we should have detected DWV-A in mites in August, and yet, DWV-A was not detected in mites until October when brood rearing had ceased.

The persistence of DWV-B in the absence of the vector illustrates the importance of alternative transmission modes. DWV is transmissible in pollen and larval food (Yue and Genersch, 2005; Singh et al., 2010), and acquired through the cannibalisation of infected pupae (Posada-Florez et al., 2020). Transovum vertical transmission from queen to offspring can occur, but is not the most efficient transmission pathway (Chen et al., 2006b; Amiri et al., 2018). Thus, in agreement with Locke et al. (2017), we believe the most parsimonious explanation for the persistence of DWV-B in our M- colonies is food-borne transmission. Past studies have shown that DWV can persist within miticide treated colonies (Martin et al., 2010; Locke et al., 2012; Locke et al., 2017), yet curiously these studies most likely detected DWV-A. It is not clear what would cause DWV-B to exploit alternative transmission routes in our study, and not DWV-A. It is widely accepted that DWV-A was the prevailing genotype during the initial stage of *V. destructor* establishment (Martin et al., 2012; Wilfert et al., 2016; Kevill et al., 2017; Kevill et al., 2019; de Souza et al., 2020). DWV-B was first characterised in Wageningen (Ongus et al., 2004; Ongus, 2006), where we conducted our study. It is possible that DWV-B has historically been the dominant genotype in this area. Unfortunately, to the best of our knowledge, no further studies investigated whether DWV-B was present in honey bees until ~ 2010. Nevertheless, the increase in DWV-B prevalence across the UK and US indicates that competitive displacement of DWV-A is occurring across landscapes (Ryabov et al., 2017; Kevill et al., 2019). Our results suggest that displacement of DWV-A is not driven by selective pressure by the vector.

Vector transmission by *V. destructor* has inarguably altered the dynamics of DWV infections in honey bees. Understanding what factors may drive shifts in DWV genotype prevalence has broad ecological implications far beyond the health of *A. mellifera*. While DWV is widely

referred to as a 'honey bee virus', the virus has been detected in numerous orders of arthropods (Levitt et al., 2013). DWV is frequently detected in insects that live in sympatry with honey bees, such as hoverflies (Bailes et al., 2018), wasps (Lester et al., 2015; Forzan et al., 2017; Santamaria et al., 2018; Brettell et al., 2019; Loope et al., 2019), ants (Gruber et al., 2017; Brettell et al., 2019), and non-*Apis* bees (Genersch et al., 2006; Li et al., 2011; Tehel et al., 2016; Radzevičiūtė et al., 2017; Santamaria et al., 2018; de Souza et al., 2019).

Pathogen spill-over between managed and wild pollinators is an area of major ecological concern (Fürst et al., 2014), particularly with mounting evidence of a substantial loss of global pollinator species within the last three decades (Zattara and Aizen, 2021). This has global implications for biodiversity and food security (Klein et al., 2007; Marshman et al., 2019). There is correlational evidence to suggest that DWV primarily spills-over from *A. mellifera* to wild pollinator species (McMahon et al., 2015; Tehel et al., 2016). Furthermore, previous studies have suggested that *V. destructor* parasitism of honey bees may drive concurrent shifts in DWV diversity in other sympatric species (Brettell et al., 2019; Loope et al., 2019; Manley et al., 2019b). The introduction of *V. destructor* in Hawaii resulted in a simultaneous reduction in DWV-A strain diversity in honey bees and the predatory wasp *Vespula pensylvanica* (Loope et al., 2019).

Pathogen spill-overs have the potential to result in emerging infectious diseases (Power and Mitchell, 2004). RNA viruses, with their extremely high mutation and substitution rates, appear to be well-adapted to cross species barriers. Given that DWV is able to infect multiple hosts, it seems prudent we understand the epidemiology and evolutionary history of this economically important virus.

## DATA ACCESSIBILITY

All of our data (including qPCR, viral loads, mite numbers and colony data) and R scripts used to conduct statistical analyses are available online at https://doi.org/10.6084/m9.figshare.12838187

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# General Discussion

Many viruses that infect *A*. *mellifera* appear to have co-evolved with their host. Viruses such as DWV and ABPV normally cause limited damage when transmitted orally or vertically, and historically were only problematic for colonies in rare instances (Bailey and Ball, 1991). These viruses were typically not associated with symptoms of disease, and are thought to have persisted at low viral loads and in viral populations that are genetically highly diverse (Ribière et al., 2008; Martin et al., 2012; Martin and Brettell, 2019). The global spread of *V*. *destructor* altered viral dynamics in *A*. *mellifera* by introducing a new route of transmission: vector transmission. By feeding on the fat bodies of both pupae and adult bees, the mites transmit viral particles directly into the bee's circulatory system, thereby bypassing physical barriers (e.g., gut barrier) and co-evolved immune defences. Evolutionary theory predicts that a change in the mode of transmission will lead to a change in virulence (the damage incurred by the host owing to infection), although the direction of change is not necessarily easy to predict (Ewald, 1983). As the vector is now responsible for the pathogen's spread instead of the host, the arrival of a vector could select for more virulent pathogen strains, strains that have a higher rate of replication. It is then implicitly or explicitly assumed that a high rate of replication equals high levels of damage to the host. The introduction of *V*. *destructor* to Hawaii appears to support the theory that arrival of a vector selects for more virulent strains, as the accumulation of a single DWV-A variant increased with increased exposure to the mite (Martin et al., 2012). Numerous studies have found a positive correlation between DWV loads in honey bees and mite infestation levels (Genersch et al., 2010; Dainat et al., 2012b; Francis et al., 2013; Mondet et al., 2014; Barroso-Arévalo et al., 2019a). Colonies that die often have high viral loads of DWV (Highfield et al., 2009; Genersch et al., 2010; Dainat et al., 2012b; Francis et al., 2013; Kevill et al., 2019). A conclusion is then quickly drawn: *V*. *destructor*, by vectoring DWV has selected for more virulent strains of the virus, resulting in death of bees and colonies (Martin et al., 2012). But the relationship between the virus, honey bee host and mite vector is far from straightforward. Furthermore, the global prevalence of the two major DWV genotypes has shifted over time, with DWV-B increasing in prevalence at the expense of DWV-A (Ryabov et al., 2017; Kevill et al., 2019; Manley et al., 2019b). This shift in prevalence suggests that the two genotypes differ in their dynamics, or in their

relationship with the honey bee host and vector, or both. Finally, a recent study has suggested that there is no simple relationship between DWV titres in bees and mortality (Remnant et al., 2019).

The overarching aim of this thesis was to disentangle the tripartite relationship between DWV, *A. mellifera* and *V. destructor*, while exploring the factors that may explain the increase in DWV-B prevalence and displacement of DWV-A. To better understand this relationship, I have looked at each level (virus, host and vector) in isolation within each chapter.

In Chapter 2, I began by examining the characteristics of the genotypes in the absence of the mite. I investigated the accumulation and competition of DWV genotypes throughout pupation, to determine if DWV-A and DWV-B differ when singly and co-injected. I found that DWV-B accumulated to higher viral loads than DWV-A in both single and simultaneous co-infection, in agreement with McMahon et al. (2016). I also found that DWV-A appears to be unaffected by competition during co-infection, and while DWV-B loads are reduced, DWV-B still accumulates to higher loads overall. Interestingly, despite accumulating to substantially higher viral loads, DWV-B was associated with the lowest level of pupal mortality, whereas pupae singly injected with DWV-A had the highest level of mortality. This suggests that mortality, at least in pupae, cannot simply be attributed to high DWV loads. My results suggest that increased viral accumulation and reduced pupal mortality could both contribute to the increasing prevalence of DWV-B. A higher proportion of individual bees infected with DWV-B will survive to adulthood, compared to bees infected with DWV-A.

By definition a pathogen depends on its host. Conversely, hosts employ physical and innate defence strategies to protect themselves against invading pathogens. It seems possible that the bee's immune system could respond differently to different genotypes of a particular pathogen, as has been observed in other taxa (Auld et al., 2012; Molina-Cruz et al., 2012; Barribeau and Schmid-Hempel, 2013; Sela et al., 2018; Moreno et al., 2020). In Chapter 3, I examined the immune response of the *A. mellifera* pupae injected with DWV-A and DWV-B from Chapter 2. Overall, I found little evidence to indicate that *A. mellifera* pupae respond to DWV-A and DWV-B differently. Only minor differences in immune gene expression were found between pupae injected with DWV-A or DWV-B. This suggests that the increasing prevalence of DWV-B is not attributable to an immunologically different response in honey

bee hosts. Because RNAi is the main antiviral defence mechanism in insects (Gammon and Mello, 2015), it was not surprising to find that RNAi genes *dicer* and *argonaute-2* were up-regulated in DWV injected pupae. By performing small RNA sequencing analysis, I found that DWV-naïve *A*. *mellifera* actively mount a small interfering RNA (siRNA) response that is proportional to viral loads, in agreement with Ryabov et al. (2014). Despite the pupae's response, DWV-A and DWV-B were both able to escape complete degradation by RNAi and accumulate to high loads. As I found no increased degradation of DWV-A compared to DWV-B, a stronger immune response against DWV-A cannot explain the difference in accumulation between DWV-A and DWV-B that I documented in Chapter 2.

RNAi genes are under strong selective pressure and are in a continual 'arms race' with rapidly evolving RNA viruses. For example, RNAi pathway genes are the fastest evolving immune genes in *Drosophila* (Obbard et al., 2006). While I assayed DWV-naïve pupae, the viral accumulation loads I observed were consistent with previous studies (McMahon et al., 2016; Tehel et al., 2019), indicating that my RNAi results were not biased by using a naïve host. Nevertheless, RNA viruses are known for their ability to encode RNAi suppressors (VSR) to counter host-defence (Betting and Van Rij, 2020). It is possible that the wild-type DWV strains obtained from *A*. *mellifera* in New Zealand (DWV-A) and the Netherlands (DWV-B) have undergone selection to evade RNAi detection by *A*. *mellifera*. To the best of my knowledge, no studies have investigated whether DWV encodes VSRs, however this warrants further investigation.

Past studies have suggested that DWV has an immunosuppressive effect on *A*. *mellifera,* particularly upon expression of the NF-κB family transcription factor *dorsal-1A*. Reduced expression of *dorsal-1A* was observed in mite parasitised bees (Nazzi et al., 2012), and in bees exposed to experimental haemolymph removal (Annoscia et al., 2019). Yet, both studies associated expression with increasing DWV loads rather than (simulated) mite feeding. The maximum DWV-A and DWV-B loads observed in Chapter 2 appear to be lower than those reported by Nazzi et al. (2012) ($\sim 10^{10}$ to $10^{15}$ GE) and Annoscia et al. (2019) ($\sim 10^{10}$ to $10^{12}$ GE). Nevertheless, I found very little change in *dorsal-1A* expression over time, even as DWV loads increased over numerous orders of magnitude throughout pupation, with no substantial difference in expression to the virus free controls. Overall, in the absence of *V. destructor*, I find no evidence that that DWV down regulates immune expression.

The third party that needs to be taken into consideration is of course the vector itself. Could it be that DWV-A and B differ in their dependence on vector transmission? If DWV-B is better adapted to vector transmission by the mite, then this might contribute to its increased prevalence. Recent studies have convincingly shown that DWV-B can replicate within the mite (Campbell et al., 2016; Gisder and Genersch, 2020), however, thus far, there has been little evidence to indicate this is also the case for DWV-A (Posada-Florez et al., 2019). It is not unlikely that DWV-B and *V. destructor* have become co-adapted, as pathogens and hosts (including biological vectors) exert selective pressure upon each other (Gulbudak et al., 2017). Phylogenetic analysis of DWV-B isolates in the UK and France shows that DWV-B has rapidly expanded after a bottleneck event, potentially driven by adaptation to *V. destructor* (Manley et al., 2019b). The increased survival of DWV-B infected pupae shown in Chapter 2 would certainly be of benefit to *V. destructor.* When pupae are killed by the virus, the foundress mite and her mated daughters will fail to reach the dispersal phase (Martin, 2001). To investigate the role of vector transmission on DWV genotype prevalence at the colony level, in Chapter 4, I experimentally increased and decreased the number of mites within *A. mellifera* colonies. I found that the two DWV genotypes indeed differ in their response to mite numbers. In agreement with Chapter 2, DWV-B accumulated to higher loads than DWV-A in the presence and absence of *V. destructor.* Perhaps surprisingly, I found that DWV-A is more dependent on mite vectoring than DWV-B. DWV-A only appeared in my colonies when mite numbers were increased. Overall, my results suggest that the increase in DWV-B prevalence is not driven by selective pressure by the vector, rather by DWV-B being able to persist in colonies at moderate levels even when colonies are free of *V. destructor.*

## The curious case of *Black queen cell virus*

One unexpected element to emerge from my research was the accumulation of BQCV in pupae injected with DWV-A in Chapter 2. I found no evidence of BQCV in the sequenced viral inocula, suggesting that an endogenous BQCV infection in our population of *A. mellifera* was activated in pupae infected with DWV-A. Interestingly, this result was also echoed by Manley et al. (2019a) who suggested that the presence of DWV-A increases the likelihood of being infected with BQCV (and SBPV) in honey bees and bumble bees. I note that in Chapter 4, I did not observe increased prevalence of BQCV in M+ colonies, which had elevated loads of DWV-A. This could potentially be an artefact of my experimental design, as I only analysed pooled adult workers and BQCV is known to cause high mortality in pupae

(Chen and Siede, 2007; Remnant et al., 2019). Thus, pupae harbouring high loads of DWV-A and BQCV would likely not have survived to emergence. It is unclear if the increased mortality I observed in DWV-A injected pupae in Chapter 2 was singly attributable to DWV-A, BQCV or both. Nevertheless, my results suggest that increased pupal mortality as a result of DWV-A alone or activated endogenous viruses, such as BQCV, may contribute to the increased dominance of DWV-B over time.

## Future Research Directions

Mordecai et al. (2016a) hypothesised that DWV-B may inhibit DWV-A accumulation at a colony level via superinfection exclusion. My results from Chapter 4 suggest that this is unlikely, as I found that DWV-A can emerge from a covert infection and accumulate to moderate loads with increasing mite numbers. Nevertheless, the results from Chapter 2 and Chapter 4 do indicate that there is a level of competition between DWV-A and DWV-B, albeit without strong competitive exclusion during co-infection. It is possible that the competition dynamics I observed in pooled samples (Chapter 4) may not fully reflect what occurred in individual bees. Moreover, I have not explicitly tested superinfection exclusion within this thesis. Superinfection exclusion refers to a distinct process where a secondary infection transmitted to a host is inhibited by a pre-existing primary infection, typically between closely related pathogens (Mascia and Gallitelli, 2016; Syller and Grupa, 2016). I believe it would be worthwhile experimentally validating whether or not a primary infection with DWV-B can inhibit a subsequent infection with DWV-A and vice versa.

When I commenced this PhD, infecting honey bees with wild-type strains of DWV or other viruses, as I did in Chapter 2, was the only method employed in the available literature. While this is still broadly practiced, recent studies have begun using engineered strains, such as the green fluorescent protein (eGFP) clones designed by Gusachenko et al. (2020) and Ryabov et al. (2020). Using infectious engineered clones is advantageous when investigating viral accumulation dynamics and tissue tropism, and any confounding effects from endogenous infections are more readily distinguishable. As, outside of Australia, DWV is one of the most common pathogens to infect honey bees globally (Martin and Brettell, 2019), I would recommend that future studies use engineered clones when experimentally infecting honey bees.

The rapid increase in DWV-B prevalence in the US (Ryabov et al., 2017) highlights the need to conduct nationwide screening to better understand how viral genotypes shift over time. Unfortunately, historical data for DWV-B are severely lacking (particularly prior to 2010) and only within the last 5-10 years did studies start to distinguish between multiple DWV genotypes within the same study. The best opportunities to track temporal changes between DWV-A and DWV-B might be in New Zealand and Sweden. DWV-A is the dominant genotype in New Zealand (Sébastien et al., 2015; Gruber et al., 2017; Dobelmann et al., 2020). DWV-B was detected in honey bee workers from Otago via RNA sequencing (Mondet et al., 2015), and in workers recently collected from the North Island by RT-PCR (personal communication Thomas Gillard, 2021). Similarly, DWV-A has been the predominant genotype in Sweden (Locke et al., 2012; Locke et al., 2014), although low levels of DWV-B were recently detected in samples from Gotland (Thaduri et al., 2018). It will be interesting to see if DWV-B becomes the dominant genotype in these populations, as it now is in England and Wales (Kevill et al., 2017; Kevill et al., 2019).

Understanding what factors may have caused BQCV to accumulate in my DWV-A infected pupae warrants further investigation. Injecting buffer into honey bees can activate covert infections (Anderson and Gibbs, 1988), although I did not observe this in any of my buffer injected pupae. Past studies have assessed synergistic interactions between honey bee viruses and parasites, such as *Nosema apis* and BQCV (Bailey et al., 1983), *N. ceranae* and BQCV (Doublet et al., 2015), or *N. ceranae* and *Chronic bee paralysis virus* (Toplak et al., 2013). To the best of my knowledge, no studies have investigated potential synergistic interactions between viral pathogens of honey bees. Yet, synergistic interactions are known to occur between plant viruses (Syller, 2012; Mascia and Gallitelli, 2016; Syller and Grupa, 2016). For example, co-infection with *Potato virus X* (PVX) and *Potato virus Y* (PVY) in tobacco results in elevated accumulation of PVX and increased disease severity in plant tissue, compared to single infections (Goodman and Ross, 1974; Vance, 1991). It is unclear what mechanisms could explain the activation of BQCV in the presence of DWV-A, particularly as I found that pupae exhibited an elevated siRNA response towards BQCV, suggesting that BQCV was not exploiting DWV-A to evade host degradation. I suggest that future studies experimentally explore the dynamics between BQCV and DWV-A. Assuming BQCV is present within the population, one could investigate whether a synergistic relationship exists between DWV-A and BQCV by using anti-BQCV antisera as described by Anderson and Gibbs (1988). By comparing pupae injected with both DWV-A and antisera to those that have only been

injected with DWV-A one could determine if DWV-A indeed activates BQCV.

## Conclusion

Overall, the work I have presented within this thesis has expanded our understanding of the intricate relationship between DWV, *A. mellifera*, and *V. destructor*. While initially the change in viral dynamics with the arrival of *V. destructor* seemed to fit the predictions of evolutionary epidemiology, my work has shown that the relationship between DWV-A and DWV-B with respect to *A. mellifera* and *V. destructor* is more complicated. My research indicates that the success of DWV-B appears to be driven by an ability to accumulate to higher loads than DWV-A under multiple conditions, whilst causing less mortality in pupae thereby favouring the reproductive cycle of *V. destructor*. My findings also indicate that the dynamics of DWV-A are comparatively more complex. Will DWV-B eventually displace DWV-A in all infected populations? Is DWV-A more damaging to pupae, or is that damage associated with synergistic interactions with other, more virulent viruses? Only time will tell.

# REFERENCES


Abrahamovich, A., Telleria, M., and Díaz, N. (2001). *Bombus* species and their associated flora in Argentina. *Bee World* 82(2)**,** 76-87. doi: 10.1080/0005772X.2001.11099505.

Aizen, M.A., and Harder, L.D. (2009). The global stock of domesticated honey bees is growing slower than agricultural demand for pollination. *Current Biology* 19(11)**,** 915-918. doi: 10.1016/j.cub.2009.03.071.

Aizen, M.A., Smith-Ramírez, C., Morales, C.L., Vieli, L., Sáez, A., Barahona-Segovia, R.M., et al. (2019). Coordinated species importation policies are needed to reduce serious invasions globally: The case of alien bumblebees in South America. *Journal of Applied Ecology* 56(1)**,** 100-106. doi: 10.1111/1365-2664.13121.

Al Naggar, Y., and Paxton, R.J. (2021). The novel insecticides flupyradifurone and sulfoxaflor do not act synergistically with viral pathogens in reducing honey bee (*Apis mellifera*) survival but sulfoxaflor modulates host immunocompetence. *Microbial Biotechnology* 14(1)**,** 227-240. doi: 10.1111/1751-7915.13673.

Alaux, C., Dantec, C., Parrinello, H., and Le Conte, Y. (2011). Nutrigenomics in honey bees: digital gene expression analysis of pollen's nutritive effects on healthy and *Varroa*-parasitized bees. *BMC Genomics* 12(496)**,** 1-13. doi: 10.1186/1471-2164-12-496.

Aldea, P., and Bozinovic, F. (2020). The energetic and survival costs of *Varroa* parasitism in honeybees. *Apidologie* 51**,** 997–1005. doi: 10.1007/s13592-020-00777-y.

Alger, S.A., Alexander Burnham, P., Lamas, Z.S., Brody, A.K., and Richardson, L.L. (2018). Home sick: Impacts of migratory beekeeping on honey bee (*Apis mellifera*) pests, pathogens, and colony size. *PeerJ* 2018(11)**,** 1-22. doi: 10.7717/peerj.5812.

Allen, M., and Ball, B. (1996). The incidence and world distribution of honey bee viruses. *Bee World* 77(3)**,** 141-162. doi: 10.1080/0005772X.1996.11099306.

Amiri, E., Kryger, P., Meixner, M.D., Strand, M.K., Tarpy, D.R., and Rueppell, O. (2018). Quantitative patterns of vertical transmission of deformed wing virus in honey bees. *PLoS ONE* 13(3)**,** e0195283. doi: 10.1371/journal.pone.0195283.

Amiri, E., Meixner, M., Nielsen, S.L., and Kryger, P. (2015). Four categories of viral infection describe the health status of honey bee colonies. *PLoS ONE* 10(10)**,** e0140272. doi: 10.1371/journal.pone.0140272.

Anderson, D. (1994). Non-reproduction of *Varroa jacobsoni* in *Apis mellifera* colonies in Papua New Guinea and Indonesia. *Apidologie* 25(4)**,** 412-421.

Anderson, D., and Gibbs, A. (1988). Inapparent virus infections and their interactions in pupae of the honey bee (*Apis mellifera* Linnaeus) in Australia. *Journal of General Virology* 69(7)**,** 1617-1625.

Anderson, D., and Trueman, J. (2000). *Varroa jacobsoni* (Acari: Varroidae) is more than one species. *Experimental & Applied Acarology* 24(3)**,** 165-189. doi: 10.1023/A:1006456720416.

Annoscia, D., Brown, S.P., Di Prisco, G., De Paoli, E., Del Fabbro, S., Frizzera, D., et al. (2019). Haemolymph removal by *Varroa* mite destabilizes the dynamical interaction between immune effectors and virus in bees, as predicted by Volterra's model. *Proceedings of the Royal Society B* 286(1901)**,** 20190331. doi: 10.1098/rspb.2019.0331.

Aravin, A., Gaidatzis, D., Pfeffer, S., Lagos-Quintana, M., Landgraf, P., Iovino, N., et al. (2006). A novel class of small RNAs bind to MILI protein in mouse testes. *Nature* 442(7099)**,** 203-207. doi: 10.1038/nature04916.

Arditti, J., Elliott, J., Kitching, I.J., and Wasserthal, L.T. (2012). 'Good Heavens what insect can suck it'– Charles Darwin, *Angraecum sesquipedale* and *Xanthopan morganii praedicta*. *Botanical Journal of the Linnean Society* 169(3)**,** 403-432. doi: 10.1111/j.1095-8339.2012.01250.x.

Aronstein, K.A., Saldivar, E., Vega, R., Westmiller, S., and Douglas, A.E. (2012). How *Varroa* parasitism affects the immunological and nutritional status of the honey bee, *Apis mellifera*. *Insects* 3(3)**,** 601-615. doi: 10.3390/insects3030601.

Arrese, E.L., and Soulages, J.L. (2010). Insect fat body: energy, metabolism, and regulation. *Annual Review of Entomology* 55**,** 207-225. doi: 10.1146/annurev-ento-112408-085356.

Arretz, P., and Macfarlane, R. (1986). The introduction of *Bombus ruderatus* to Chile for red clover pollination. *Bee World* 67(1)**,** 15-22. doi: 10.1080/0005772X.1986.11098855.

Arundel, J. (2011). *Understanding the spread of honey bee pests and diseases: An agent-based modelling approach*, Rural Industries Research and Development Corporation. Canberra.

Auld, S.K., Edel, K.H., and Little, T.J. (2012). The cellular immune response of *Daphnia magna* under host–parasite genetic variation and variation in initial dose. *Evolution* 66(10)**,** 3287-3293. doi: 10.1111/j.1558-5646.2012.01671.x.

Bailes, E.J., Deutsch, K.R., Bagi, J., Rondissone, L., Brown, M.J.F., and Lewis, O.T. (2018). First detection of bee viruses in hoverfly (syrphid) pollinators. *Biology Letters* 14(2). doi: 10.1098/rsbl.2018.0001.

Bailey, L., and Ball, B.V. (1991). *Honey bee pathology.* San Diego, USA: Academic Press.

Bailey, L., Ball, B.V., and Perry, J. (1981). The prevalence of viruses of honey bees in Britain. *Annals of Applied Biology* 97(1)**,** 109-118.

Bailey, L., Ball, B.V., and Perry, J. (1983). Association of viruses with two protozoal pathogens of the honey bee. *Annals of Applied Biology* 103(1)**,** 13-20.

Bakdash, J.Z., and Marusich, L.R. (2017). Repeated measures correlation. *Frontiers in Psychology* 8**,** 456-456. doi: 10.3389/fpsyg.2017.00456.

Barribeau, S.M., and Schmid-Hempel, P. (2013). Qualitatively different immune response of the bumblebee host, *Bombus terrestris*, to infection by different genotypes of the trypanosome gut parasite, *Crithidia bombi*. *Infection, Genetics and Evolution* 20**,** 249-256. doi: 10.1016/j.meegid.2013.09.014.

Barroso-Arévalo, S., Fernández-Carrión, E., Goyache, J., Molero, F., Puerta, F., and Sánchez-Vizcaíno, J.M. (2019a). High load of deformed wing virus and *Varroa destructor* infestation are related to weakness of honey bee colonies in Southern Spain. *Frontiers in Microbiology* 10(JUN). doi: 10.3389/fmicb.2019.01331.

Barroso-Arévalo, S., Vicente-Rubiano, M., Molero, F., Puerta, F., and Sánchez-Vizcaíno, J.M. (2019b). Nucleotide sequence variations may be associated with virulence of deformed wing virus. *Apidologie* 50**,** 1-15. doi: 10.1007/s13592-019-00660-5.

Barroso-Arévalo, S., Vicente-Rubiano, M., Puerta, F., Molero, F., and Sánchez-Vizcaíno, J.M. (2019c). Immune related genes as markers for monitoring health status of honey bee colonies. *BMC Veterinary Research* 15(1)**,** 2-15. doi: 10.1186/s12917-019-1823-y.

Barson, G., and Griffiths, E. (2016). SeqTools: visual tools for manual analysis of sequence alignments. *BMC Research Notes* 9(39)**,** 1-10. doi: 10.1186/s13104-016-1847-3.

Bates, D., Maechler, M., Bolker, B., and Walker, S. (2018). Lme4: Linear mixed-effects models using Eigen and S4. R package version 1.1-21.

Beaurepaire, A.L., Truong, T.A., Fajardo, A.C., Dinh, T.Q., Cervancia, C., and Moritz, R.F. (2015). Host specificity in the honeybee parasitic mite, *Varroa* spp. in *Apis mellifera* and *Apis cerana*. *PLoS ONE* 10(8)**,** e0135103. doi: 10.1371/journal.pone.0135103.

Ben-Shahar, Y., Dudek, N.L., and Robinson, G.E. (2004). Phenotypic deconstruction reveals involvement of manganese transporter malvolio in honey bee division of labor. *Journal of Experimental Biology* 207(19)**,** 3281-3288. doi: 10.1242/jeb.01151.

Benaets, K., Van Geystelen, A., Cardoen, D., De Smet, L., de Graaf, D.C., Schoofs, L., et al. (2017). Covert deformed wing virus infections have long-term deleterious effects on honey bee foraging and survival. *Proceedings of the Royal Society B: Biological Sciences* 284(1848)**,** 20162149. doi: 10.1098/rspb.2016.2149.

Berényi, O., Bakonyi, T., Derakhshifar, I., Köglberger, H., and Nowotny, N. (2006). Occurrence of six honeybee viruses in diseased Austrian apiaries. *Applied and Environmental Microbiology* 72(4)**,** 2414-2420. doi: 10.1128/AEM.72.4.2414–2420.2006.

Berthoud, H., Imdorf, A., Haueter, M., Radloff, S., and Neumann, P. (2010). Virus infections and winter losses of honey bee colonies (*Apis mellifera*). *Journal of Apicultural Research* 49(1)**,** 60-65. doi: 10.3896/IBRA.1.49.1.08.

Betting, V., and Van Rij, R.P. (2020). Countering Counter-Defense to Antiviral RNAi. *Trends in Microbiology* 28(8)**,** 600-602. doi: 10.1016/j.tim.2020.05.018.

Blacquière, T., Altreuther, G., and Krieger, K.J. (2017). Evaluation of the efficacy and safety of flumethrin 275 mg bee-hive strips (PolyVar Yellow®) against *Varroa destructor* in

naturally infested honey bee colonies in a controlled study. *Parasitology Research* 116(1)**,** 109-122. doi: 10.1007/s00436-017-5497-8.

Boecking, O., and Genersch, E. (2008). Varroosis – the ongoing crisis in bee keeping. *Journal of Consumer Protection and Food Safety* 3(2)**,** 221-228. doi: 10.1007/s00003-008-0331-y.

Bolger, A.M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30(15)**,** 2114-2120. doi: 10.1093/bioinformatics/btu170.

Boncristiani, H., Li, J., Evans, J.D., Pettis, J., and Chen, Y. (2011). Scientific note on PCR inhibitors in the compound eyes of honey bees, *Apis mellifera*. *Apidologie* 42(4)**,** 457-460. doi: 10.1007/s13592-011-0009-9.

Boot, W.J., Calis, J.N., Beetsma, J., Hai, D.M., Lan, N.K., Van Toan, T., et al. (1999). Natural selection of *Varroa jacobsoni* explains the different reproductive strategies in colonies of *Apis cerana* and *Apis mellifera*. *Experimental & Applied Acarology* 23(2)**,** 133-144.

Boot, W.J., Tan, N.Q., Dien, P.C., Van Huan, L., Van Dung, N., and Beetsma, J. (1997). Reproductive success of *Varroa jacobsoni* in brood of its original host, *Apis cerana*, in comparison to that of its new host, *A. mellifera* (Hymenoptera: Apidae). *Bulletin of Entomological Research* 87(2)**,** 119-126.

Boot, W.J., van Baalen, M., and Sabelis, M.W. (1995). Why do *Varroa* mites invade worker brood cells of the honey bee despite lower reproductive success? *Behavioral Ecology and Sociobiology* 36(4)**,** 283-289.

Bowen-Walker, P., Martin, S., and 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(1)**,** 101-106. doi: 10.1006/jipa.1998.4807.

Bowen-Walker, P.L., and Gunn, A. (2001). The effect of the ectoparasitic mite, *Varroa destructor* on adult worker honeybee (*Apis mellifera*) emergence weights, water, protein, carbohydrate, and lipid levels. *Entomologia Experimentalis et Applicata* 101(3)**,** 207-217.

Bradford, E.L. (2019). *Transmission of deformed wing virus (DWV) between Varroa destructor and the European honeybee (Apis mellifera): in vitro and in vivo studies.* PhD thesis, University of Aberdeen.

Branco, M.R., Kidd, N.A., and Pickard, R.S. (2006). A comparative evaluation of sampling methods for *Varroa destructor* (Acari: Varroidae) population estimation. *Apidologie* 37(4)**,** 452-461. doi: 10.1051/apido:2006010.

Brettell, L., and Martin, S. (2017). Oldest *Varroa* tolerant honey bee population provides insight into the origins of the global decline of honey bees. *Scientific Reports* 7**,** 45953. doi: 10.1038/srep45953.

Brettell, L.E., Schroeder, D.C., and Martin, S.J. (2019). RNAseq analysis reveals virus diversity within hawaiian apiary insect communities. *Viruses* 11(5)**,** 397. doi: 10.3390/v11050397.

Brettell, L.E., Schroeder, D.C., and Martin, S.J. (2020). RNAseq of deformed wing virus and other honey bee-associated viruses in eight insect taxa with or without *Varroa* infestation. *Viruses* 12(11)**,** 1229. doi: 10.3390/v12111229.

Brito, R., McHale, M., and Oldroyd, B. (2010). Expression of genes related to reproduction and pollen foraging in honey bees (*Apis mellifera*) narcotized with carbon dioxide. *Insect Molecular Biology* 19(4)**,** 451-461. doi: 10.1111/j.1365-2583.2010.01003.x.

Brosi, B.J., Delaplane, K.S., Boots, M., and de Roode, J.C. (2017). Ecological and evolutionary approaches to managing honeybee disease. *Nature Ecology & Evolution* 1(9)**,** 1250. doi: 10.1038/s41559-017-0246-z.

Bruscella, P., Bottini, S., Baudesson, C., Pawlotsky, J.-M., Feray, C., and Trabucchi, M. (2017). Viruses and miRNAs: more friends than foes. *Frontiers in Microbiology* 8(824)**,** 1-11. doi: 10.3389/fmicb.2017.00824.

Brutscher, L.M., Daughenbaugh, K.F., and Flenniken, M.L. (2015). Antiviral defense mechanisms in honey bees. *Current Opinion in Insect Science* 10**,** 71-82. doi: 10.1016/j.cois.2015.04.016.

Büchler, R., Drescher, W., and Tornier, I. (1992). Grooming behaviour of *Apis cerana*, *Apis mellifera* and *Apis dorsata* and its effect on the parasitic mites *Varroa jacobsoni* and *Tropilaelaps clareae*. *Experimental & Applied Acarology* 16(4)**,** 313-319.

Buchmann, S.L., and Hurley, J.P. (1978). A biophysical model for buzz pollination in angiosperms. *Journal of Theoretical Biology* 72(4)**,** 639-657.

Calis, J.N., Fries, I., and Ryrie, S.C. (1999). Population modelling of *Varroa jacobsoni* Oud. *Apidologie* 30(2-3)**,** 111-124.

Cameron, S.A., Lim, H.C., Lozier, J.D., Duennes, M.A., and Thorp, R. (2016). Test of the invasive pathogen hypothesis of bumble bee decline in North America. *Proceedings of the National Academy of Sciences* 113(16)**,** 4386-4391. doi: 10.1073/pnas.1525266113.

Cameron, S.A., Lozier, J.D., Strange, J.P., Koch, J.B., Cordes, N., Solter, L.F., et al. (2011). Patterns of widespread decline in North American bumble bees. *Proceedings of the National Academy of Sciences* 108(2)**,** 662-667. doi: 10.1073/pnas.1014743108.

Campbell, E.M., Budge, G.E., Watkins, M., and Bowman, A.S. (2016). Transcriptome analysis of the synganglion from the honey bee mite, *Varroa destructor* and RNAi knockdown of neural peptide targets. *Insect Biochemistry and Molecular Biology* 70**,** 116-126. doi: 10.1016/j.ibmb.2015.12.007.

Carrillo-Tripp, J., Dolezal, A.G., Goblirsch, M.J., Miller, W.A., Toth, A.L., and Bonning, B.C. (2016). *In vivo* and *in vitro* infection dynamics of honey bee viruses. *Scientific Reports* 6**,** 22265. doi: 10.1038/srep22265.

Chandler, D., Cooper, E., and Prince, G. (2019). Are there risks to wild European bumble bees from using commercial stocks of domesticated *Bombus terrestris* for crop pollination? *Journal of Apicultural Research* 58(5)**,** 665-681. doi: 10.1080/00218839.2019.1637238.

Chantawannakul, P., de Guzman, L.I., Li, J., and Williams, G.R. (2016). Parasites, pathogens, and pests of honeybees in Asia. *Apidologie* 47(3)**,** 301-324. doi: 10.1007/s13592-015-0407-5.

Chejanovsky, N., Ophir, R., Schwager, M.S., Slabezki, Y., Grossman, S., and Cox-Foster, D. (2014). Characterization of viral siRNA populations in honey bee colony collapse disorder. *Virology* 454**,** 176-183. doi: 10.1016/j.virol.2014.02.012.

Chen, X., Yu, X., Cai, Y., Zheng, H., Yu, D., Liu, G., et al. (2010). Next-generation small RNA sequencing for microRNAs profiling in the honey bee *Apis mellifera*. *Insect Molecular Biology* 19(6)**,** 799-805. doi: 10.1111/j.1365-2583.2010.01039.x.

Chen, Y., Evans, J., and Feldlaufer, M. (2006a). Horizontal and vertical transmission of viruses in the honey bee, *Apis mellifera*. *Journal of Invertebrate Pathology* 92(3)**,** 152-159. doi: 10.1016/j.jip.2006.03.010.

Chen, Y., Zhao, Y., Hammond, J., Hsu, H., Evans, J., and Feldlaufer, M. (2004). Multiple virus infections in the honey bee and genome divergence of honey bee viruses. *Journal of Invertebrate Pathology* 87(2)**,** 84-93. doi: 10.1016/j.jip.2004.07.005.

Chen, Y.P., Pettis, J.S., Collins, A., and Feldlaufer, M.F. (2006b). Prevalence and transmission of honeybee viruses. *Applied and Environmental Microbiology* 72(1)**,** 606-611. doi: 10.1128/AEM.72.1.606-611.2006.

Chen, Y.P., Pettis, J.S., Corona, M., Chen, W.P., Li, C.J., Spivak, M., et al. (2014). Israeli acute paralysis virus: epidemiology, pathogenesis and implications for honey bee health. *PLoS Pathogens* 10(7)**,** e1004261. doi: 10.1371/journal.ppat.1004261.

Chen, Y.P., and Siede, R. (2007). 'Honey bee viruses' in *Advances in virus research,* eds. K. Maramorosch, S.A. Shabalina & F.A. Murphy. San Diego: Elsevier Academic Press, 33-80.

Colla, S.R., Otterstatter, M.C., Gegear, R.J., and Thomson, J.D. (2006). Plight of the bumble bee: pathogen spillover from commercial to wild populations. *Biological Conservation* 129(4)**,** 461-467. doi: 10.1016/j.biocon.2005.11.013.

Cornman, R.S., Tarpy, D.R., Chen, Y., Jeffreys, L., Lopez, D., Pettis, J.S., et al. (2012). Pathogen webs in collapsing honey bee colonies. *PLoS ONE* 7(8)**,** e43562. doi: 10.1371/ journal.pone.0043562.

Couto-Lima, D., Madec, Y., Bersot, M.I., Campos, S.S., de Albuquerque Motta, M., Dos Santos, F.B., et al. (2017). Potential risk of re-emergence of urban transmission of yellow fever virus in Brazil facilitated by competent *Aedes* populations. *Scientific Reports* 7(1)**,** 1-12. doi: 10.1038/s41598-017-05186-3.

Cremer, S., Pull, C.D., and Fuerst, M.A. (2018). Social immunity: emergence and evolution of colony-level disease protection. *Annual Review of Entomology* 63**,** 105-123. doi: 10.1146/annurev-ento-020117-043110.

Cunningham, S.A., FitzGibbon, F., and Heard, T.A. (2002). The future of pollinators for Australian agriculture. *Australian Journal of Agricultural Research* 53(8)**,** 893-900. doi: 10.1071/AR01186.

Curtis, V.A. (2014). Infection-avoidance behaviour in humans and other animals. *Trends in Immunology* 35(10)**,** 457-464. doi: 10.1016/j.it.2014.08.006.

Czech, B., and Hannon, G.J. (2016). One loop to rule them all: the ping-pong cycle and piRNA-guided silencing. *Trends in Biochemical Sciences* 41(4)**,** 324-337. doi: 10.1016/j.tibs.2015.12.008.

D'Alvise, P., Seeburger, V., Gihring, K., Kieboom, M., and Hasselmann, M. (2019). Seasonal dynamics and co-occurrence patterns of honey bee pathogens revealed by high-throughput RT-qPCR analysis. *Ecology and Evolution* 00**,** 1-12. doi: 10.1002/ece3.5544.

Dainat, B., Evans, J.D., Chen, Y.P., Gauthier, L., and Neumann, P. (2012a). Dead or alive: deformed wing virus and *Varroa destructor* reduce the life span of winter honeybees. *Applied and Environmental Microbiology* 78(4)**,** 981-987. doi: 10.1128/AEM.06537-11.

Dainat, B., Evans, J.D., Chen, Y.P., Gauthier, L., and Neumann, P. (2012b). Predictive markers of honey bee colony collapse. *PLoS ONE* 7(2)**,** e32151. doi: 10.1371/journal.pone.0032151.

Dalmon, A., Desbiez, C., Coulon, M., Thomasson, M., Le Conte, Y., Alaux, C., et al. (2017). Evidence for positive selection and recombination hotspots in deformed wing virus (DWV). *Scientific Reports* 7**,** 41045. doi: 10.1038/srep41045.

DaPalma, T., Doonan, B.P., Trager, N.M., and Kasman, L.M. (2010). A systematic approach to virus–virus interactions. *Virus Research* 149(1)**,** 1-9. doi: 10.1016/j.virusres.2010.01.002.

Daughenbaugh, K.F., Kahnonitch, I., Carey, C.C., McMenamin, A.J., Wiegand, T., Erez, T., et al. (2021). Metatranscriptome analysis of sympatric bee species identifies bee virus variants and a new virus, Andrena-associated bee virus-1. *Viruses* 13(2)**,** 291. doi: 10.3390/v13020291.

de Guzman, L., and Delfinado-Baker, M. (1996). A new species of *Varroa* (Acari: Varroidae) associated with *Apis koschevnikovi* (Apidae: Hymenoptera) in Borneo. *International Journal of Acarology* 22(1)**,** 23-27. doi: 10.1080/01647959608684077.

De Jong, D., Morse, R.A., and Eickwort, G.C. (1982). Mite pests of honey bees. *Annual Review of Entomology* 27(1)**,** 229-252.

De Luca, P.A., and Vallejo-Marin, M. (2013). What's the 'buzz' about? The ecology and evolutionary significance of buzz-pollination. *Current Opinion in Plant Biology* 16(4)**,** 429-435. doi: 10.1016/j.pbi.2013.05.002.

de Miranda, J.R., Cordoni, G., and Budge, G. (2010). 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, J.R., and Genersch, E. (2010). Deformed wing virus. *Journal of Invertebrate Pathology* 103**,** S48-S61. doi: 10.1016/j.jip.2009.06.012.

de Souza, F.S., Allsopp, M.H., and Martin, S.J. (2020). Deformed wing virus prevalence and load in honeybees in South Africa. *Archives of Virology* 166**,** 237–241 doi: 10.1007/s00705-020-04863-5.

de Souza, F.S., Kevill, J.L., Correia-Oliveira, M.E., de Carvalho, C.A.L., and Martin, S.J. (2019). Occurrence of deformed wing virus variants in the stingless bee *Melipona subnitida* and honey bee *Apis mellifera* populations in Brazil. *The Journal of General Virology* 100(2)**,** 289-294. doi: 10.1099/jgv.0.001206.

Department of the Environment (2021). *Species Profile and Threats Database* [Online]. Canberra. Available: https://www.environment.gov.au/cgi-bin/sprat/public/publicthreatenedlist.pl?wanted=fauna - other_animals_endangered [Accessed 3rd Feb 2021].

Di Prisco, G., Annoscia, D., Margiotta, M., Ferrara, R., Varricchio, P., Zanni, V., et al. (2016). A mutualistic symbiosis between a parasitic mite and a pathogenic virus undermines honey bee immunity and health. *Proceedings of the National Academy of Sciences* 113(12)**,** 3203-3208. doi: 10.1073/pnas.1523515113.

Diallo, M., Thonnon, J., Traore-Lamizana, M., and Fontenille, D. (1999). Vectors of Chikungunya virus in Senegal: current data and transmission cycles. *The American Journal of Tropical Medicine and Hygiene* 60(2)**,** 281-286.

Dietemann, V., Nazzi, F., Martin, S.J., Anderson, D.L., Locke, B., Delaplane, K.S., et al. (2013). Standard methods for *Varroa* research. *Journal of Apicultural Research* 52(1)**,** 1-54. doi: 10.3896/IBRA.1.52.1.09.

Dobelmann, J., Felden, A., and Lester, P.J. (2020). Genetic strain diversity of multi-host RNA viruses that infect a wide range of pollinators and associates is shaped by geographic origins. *Viruses* 12(3)**,** 358. doi: 10.3390/v12030358.

Donovan, B. (1980). Interactions between native and introduced bees in New Zealand. *New Zealand Journal of Ecology* 3**,** 104-116.

Donzé, G., and Guerin, P.M. (1994). Behavioral attributes and parental care of *Varroa* mites parasitizing honey bee brood. *Behavioral Ecology and Sociobiology* 34(5)**,** 305-319. doi: 10.1007/BF00197001.

Dostert, C., Jouanguy, E., Irving, P., Troxler, L., Galiana-Arnoux, D., Hetru, C., et al. (2005). The Jak-STAT signaling pathway is required but not sufficient for the antiviral response of *Drosophila*. *Nature Immunology* 6(9)**,** 946-953. doi: 10.1038/ni1237.

Doublet, V., Labarussias, M., Miranda, J.R., Moritz, R.F., and Paxton, R.J. (2015). Bees under stress: sublethal doses of a neonicotinoid pesticide and pathogens interact to

elevate honey bee mortality across the life cycle. *Environmental Microbiology* 17(4)**,** 969-983. doi: 10.1111/1462-2920.12426.

Dubois, E., Dardouri, M., Schurr, F., Cougoule, N., Sircoulomb, F., and Thiéry, R. (2019). Outcomes of honeybee pupae inoculated with deformed wing virus genotypes A and B. *Apidologie* 51**,** 18–34. doi: 10.1007/s13592-019-00701-z.

Duffy, J. (1971). Social impact of disease in the late nineteenth century. *Bulletin of the New York Academy of Medicine* 47(7)**,** 797-810.

Eickwort, G.C. (1994). 'Evolution and life-history patterns of mites associated with bees' in *Mites: Ecological and Evolutionary Analyses of Life-History Patterns,* ed. M.A. Houck. Dordrecht: Springer, 218-251.

Ewald, P.W. (1983). Host-parasite relations, vectors, and the evolution of disease severity. *Annual Review of Ecology and Systematics* 14**,** 465-485.

Farrar, C. (1937). The influence of colony populations on honey production. *Journal of Agricultural Research* 54(12)**,** 945-954.

Forzan, M., Sagona, S., Mazzei, M., and Felicioli, A. (2017). Detection of deformed wing virus in *Vespa crabro*. *Bulletin of Insectology* 70(2)**,** 261-265.

Fox, J., Weisberg, S., and Price, B. (2018). Car: Companion to applied regression. R package version 3.0-0.

Francis, R.M., Nielsen, S.L., and Kryger, P. (2013). *Varroa*-virus interaction in collapsing honey bee colonies. *PLoS ONE* 8(3)**,** e57540. doi: 10.1371/journal.pone.0057540.

Fries, I., Aarhus, A., Hansen, H., and Korpela, S. (1991). Development of early infestations by the mite *Varroa jacobsoni* in honey-bee (*Apis mellifera*) colonies in cold climates. *Experimental & Applied Acarology* 11(2-3)**,** 205-214.

Fries, I., Camazine, S., and Sneyd, J. (1994). Population dynamics of *Varroa jacobsoni*: a model and a review. *Bee world* 75(1)**,** 5-28. doi: 10.1080/0005772X.1994.11099190.

Fürst, M.A., McMahon, D.P., Osborne, J.L., Paxton, R.J., and Brown, M.J.F. (2014). Disease associations between honeybees and bumblebees as a threat to wild pollinators. *Nature* 506(7488)**,** 364-366. doi: 10.1038/nature12977.

Gammon, D.B., and Mello, C.C. (2015). RNA interference-mediated antiviral defense in insects. *Current Opinion in Insect Science* 8**,** 111-120. doi: 10.1016/j.cois.2015.01.006.

Genersch, E., Von Der Ohe, W., Kaatz, H., Schroeder, A., Otten, C., Büchler, R., et al. (2010). The German bee monitoring project: a long term study to understand periodically high winter losses of honey bee colonies. *Apidologie* 41(3)**,** 332-352. doi: 10.1051/apido/2010014.

Genersch, E., Yue, C., Fries, I., de Miranda, J.R., and Sveriges, l. (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(1)**,** 61-63. doi: 10.1016/j.jip.2005.10.002.

Gisder, S., Aumeier, P., and Genersch, E. (2009). Deformed wing virus: replication and viral load in mites (*Varroa destructor*). *Journal of General Virology* 90(2)**,** 463-467. doi: 10.1099/vir.0.005579-0.

Gisder, S., and Genersch, E. (2020). Direct evidence for infection of *Varroa destructor* mites with the bee-pathogenic deformed wing virus variant B-but not variant A-via fluorescence-in situ-hybridization analysis. *Journal of Virology* JVI.01786-20. doi: 10.1128/JVI.01786-20.

Gisder, S., Möckel, N., Eisenhardt, D., and Genersch, E. (2018). *In vivo* evolution of viral virulence: switching of deformed wing virus between hosts results in virulence changes and sequence shifts. *Environmental Microbiology* 20(12)**,** 4612–4628. doi: 10.1111/1462-2920.14481.

Göertz, G.P., Vogels, C.B.F., Geertsema, C., Koenraadt, C.J.M., and Pijlman, G.P. (2017). Mosquito co-infection with Zika and chikungunya virus allows simultaneous transmission without affecting vector competence of *Aedes aegypti*. *PLoS Neglected Tropical Diseases* 11(6)**,** e0005654. doi: 10.1371/journal.pntd.0005654.

Goic, B., Stapleford, K.A., Frangeul, L., Doucet, A.J., Gausson, V., Blanc, H., et al. (2016). Virus-derived DNA drives mosquito vector tolerance to arboviral infection. *Nature Communications* 7(1)**,** 12410. doi: 10.1038/ncomms12410.

Goodman, R.M., and Ross, A.F. (1974). Enhancement of potato virus X synthesis in doubly infected tobacco occurs in doubly infected cells. *Virology* 58(1)**,** 16-24.

Goulson, D. (2003a). *Bumblebees: behaviour, ecology and conservation.* New York, USA: Oxford University Press.

Goulson, D. (2003b). Effects of introduced bees on native ecosystems. *Annual Review of Ecology, Evolution, and Systematics* 34**,** 1-26. doi: 10.1146/annurev.ecolsys.34.011802.132355.

Goulson, D., Lye, G.C., and Darvill, B. (2008). Decline and conservation of bumble bees. *Annual Review of Entomology* 53**,** 191-208. doi: 10.1146/annurev.ento.53.103106.093454.

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

Graves, T.A., Janousek, W.M., Gaulke, S.M., Nicholas, A.C., Keinath, D.A., Bell, C.M., et al. (2020). Western bumble bee: declines in the continental United States and range-wide information gaps. *Ecosphere* 11(6)**,** e03141. doi: 10.1002/ecs2.3141.

Graystock, P., Blane, E.J., McFrederick, Q.S., Goulson, D., and Hughes, W.O. (2016). Do managed bees drive parasite spread and emergence in wild bees? *International Journal for Parasitology: Parasites and Wildlife* 5(1)**,** 64-75. doi: 10.1016/j.ijppaw.2015.10.001.

Greenleaf, S.S., and Kremen, C. (2006). Wild bees enhance honey bees' pollination of hybrid sunflower. *Proceedings of the National Academy of Sciences* 103(37)**,** 13890-13895. doi: 10.1073 pnas.0600929103.

Grixti, J.C., Wong, L.T., Cameron, S.A., and Favret, C. (2009). Decline of bumble bees (*Bombus*) in the North American Midwest. *Biological Conservation* 142(1)**,** 75-84. doi: 10.1016/j.biocon.2008.09.027.

Gruber, M.A.M., Cooling, M., Baty, J.W., Buckley, K., Friedlander, A., Quinn, O., et al. (2017). Single-stranded RNA viruses infecting the invasive Argentine ant, *Linepithema humile*. *Scientific Reports* 7(1). doi: 10.1038/s41598-017-03508-z.

Gulbudak, H., Cannataro, V.L., Tuncer, N., and Martcheva, M. (2017). Vector-borne pathogen and host evolution in a structured immuno-epidemiological system. *Bulletin of Mathematical Biology* 79(2)**,** 325-355. doi: 10.1007/s11538-016-0239-0.

Gusachenko, O.N., Woodford, L., Balbirnie-Cumming, K., Campbell, E.M., Christie, C.R., Bowman, A.S., et al. (2020). Green bees: reverse genetic analysis of deformed wing virus transmission, replication, and tropism. *Viruses* 12(5)**,** 532. doi: 10.3390/v12050532.

Gutiérrez-Bugallo, G., Piedra, L.A., Rodriguez, M., Bisset, J.A., Lourenço-de-Oliveira, R., Weaver, S.C., et al. (2019). Vector-borne transmission and evolution of Zika virus. *Nature Ecology & Evolution* 3(4)**,** 561-569. doi: 10.1038/s41559-019-0836-z.

Hammond, S.M., Boettcher, S., Caudy, A.A., Kobayashi, R., and Hannon, G.J. (2001). Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science* 293(5532)**,** 1146-1150.

Harpur, B.A., and Zayed, A. (2013). Accelerated evolution of innate immunity proteins in social insects: adaptive evolution or relaxed constraint? *Molecular Biology and Evolution* 30(7)**,** 1665-1674. doi: 10.1093/molbev/mst061.

Harris, J., Sheridan, A.B., and MacGown, **J.A.** (2019). *Managing Varroa Mites in Honey Bee Colonies* [Online]. Available: http://extension.msstate.edu/sites/default/files/publications/publications/p2826_web.pdf [Accessed 18 Feb 2021].

Hart, A.G., and Ratnieks, F.L. (2001). Task partitioning, division of labour and nest compartmentalisation collectively isolate hazardous waste in the leafcutting ant *Atta cephalotes*. *Behavioral Ecology and Sociobiology* 49(5)**,** 387-392. doi: 10.1007/s002650000312.

Häußermann, C.K., Giacobino, A., Munz, R., Ziegelmann, B., Palacio, M.A., and Rosenkranz, P. (2019). Reproductive parameters of female *Varroa destructor* and the impact of mating in worker brood of *Apis mellifera*. *Apidologie* 51**,** 342–355. doi: 10.1007/s13592-019-00713-9.

Häußermann, C.K., Ziegelmann, B., and Rosenkranz, P. (2016). Spermatozoa capacitation in female *Varroa destructor* and its influence on the timing and success of female reproduction. *Experimental and Applied Acarology* 69(4)**,** 371-387. doi: 10.1007/s10493-016-0051-4.

Hedrick, S.M. (2017). Understanding immunity through the lens of disease ecology. *Trends in Immunology* 38(12)**,** 888-903. doi: 10.1016/j.it.2017.08.001.

Hernández López, J., Schuehly, W., Crailsheim, K., and Riessberger-Gallé, U. (2014). Trans-generational immune priming in honeybees. *Proceedings of the Royal Society B: Biological Sciences* 281(1785)**,** 20140454. doi: 10.1098/rspb.2014.0454.

Highfield, A.C., El Nagar, A., Mackinder, L.C., Laure, M.-L.N., Hall, M.J., Martin, S.J., et al. (2009). Deformed wing virus implicated in overwintering honeybee colony losses. *Applied and Environmental Microbiology* 75(22)**,** 7212-7220. doi: 10.1128/AEM.02227-09.

Hirakata, S., and Siomi, M.C. (2016). piRNA biogenesis in the germline: from transcription of piRNA genomic sources to piRNA maturation. *Biochimica et Biophysica Acta* 1859(1)**,** 82-92. doi: 10.1016/j.bbagrm.2015.09.002.

Holmes, E.C. (2009). The evolutionary genetics of emerging viruses. *Annual Review of Ecology, Evolution, and Systematics* 40**,** 353-372. doi: 10.1146/annurev.ecolsys.110308.120248.

Hopkins, I. (1901). *The Illustrated Australasian Bee Manual and Complete Guide to Modern Bee Culture in the Southern Hemisphere.* Wellington, New Zealand: Gordon and Gotch.

Hou, Y., Zhang, H., Miranda, L., and Lin, S. (2010). Serious overestimation in quantitative PCR by circular (supercoiled) plasmid standard: microalgal *pcna* as the model gene. *PLoS ONE* 5(3)**,** e9545. doi: 10.1371/journal.pone.0009545.

Human, H., Brodschneider, R., Dietemann, V., Dively, G., Ellis, J.D., Forsgren, E., et al. (2013). Miscellaneous standard methods for *Apis mellifera* research. *Journal of Apicultural Research* 52(4). doi: 10.3896/IBRA.1.52.4.10.

Hussain, M., Torres, S., Schnettler, E., Funk, A., Grundhoff, A., Pijlman, G.P., et al. (2011). West Nile virus encodes a microRNA-like small RNA in the 3' untranslated region which up-regulates GATA4 mRNA and facilitates virus replication in mosquito cells. *Nucleic Acids Research* 40(5)**,** 2210-2223. doi: 10.1093/nar/gkr848.

Inoue, M.N., Yokoyama, J., and Washitani, I. (2008). Displacement of Japanese native bumblebees by the recently introduced *Bombus terrestris* (L.)(Hymenoptera: Apidae). *Journal of Insect Conservation* 12(2)**,** 135-146. doi: 10.1007/s10841-007-9071-z.

Jay, S.C. (1962). Colour changes in honeybee pupae. *Bee World* 43(4)**,** 119-122.

Kauder, S.E., and Racaniello, V.R. (2004). Poliovirus tropism and attenuation are determined after internal ribosome entry. *The Journal of Clinical Investigation* 113(12)**,** 1743-1753. doi: 10.1172/JCI200421323.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., et al. (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28(12)**,** 1647-1649. doi: 10.1093/bioinformatics/bts199.

Kevill, J.L., de Souza, F.S., Sharples, C., Oliver, R., Schroeder, D.C., and Martin, S.J. (2019). DWV-A lethal to honey bees (*Apis mellifera*): a colony level survey of DWV variants (A, B, and C) in England, Wales, and 32 states across the US. *Viruses* 11(5)**,** 1-12. doi: 10.3390/v11050426.

Kevill, L.J., Highfield, A., Mordecai, J.G., Martin, J.S., and Schroeder, C.D. (2017). ABC assay: method development and application to quantify the role of three DWV master variants in overwinter colony losses of European honey bees. *Viruses* 9(11)**,** 2-14. doi: 10.3390/v9110314.

Khongphinitbunjong, K., de Guzman, L.I., Tarver, M.R., Rinderer, T.E., Chen, Y., and Chantawannakul, P. (2015). Differential viral levels and immune gene expression in three stocks of *Apis mellifera* induced by different numbers of *Varroa destructor*. *Journal of Insect Physiology* 72**,** 28-34. doi: 10.1016/j.jinsphys.2014.11.005.

Kilwein, J.H. (1995). Some historical comments on quarantine: part one. *Journal of Clinical Pharmacy and Therapeutics* 20(4)**,** 185-187. doi: 10.1111/j.1365-2710.1995.tb00647.x.

Klein, A.M., Vaissiere, B.E., Cane, J.H., Steffan-Dewenter, I., Cunningham, S.A., Kremen, C., et al. (2007). Importance of pollinators in changing landscapes for world crops. *Proceedings of the Royal Society B: Biological Sciences* 274(1608)**,** 303-313. doi: 10.1098/rspb.2006.3721.

Koeniger, G., Koeniger, N., Anderson, D.L., Lekprayoon, C., and Tingek, S. (2002). Mites from debris and sealed brood cells of *Apis dorsata* colonies in Sabah (Borneo) Malaysia, including a new haplotype of *Varroa jacobsoni*. *Apidologie* 33(1)**,** 15-24. doi: 10.1051/apido: 2001005.

Koetz, A.H. (2013). Ecology, behaviour and control of *Apis cerana* with a focus on relevance to the Australian incursion. *Insects* 4(4)**,** 558-592. doi: 10.3390/insects4040558.

Kuno, G., and Chang, G.J.J. (2005). Biological transmission of arboviruses: reexamination of and new insights into components, mechanisms, and unique traits as well as their evolutionary trends. *Clinical Microbiology Reviews* 18(4)**,** 608-637. doi: 10.1128/CMR.18.4.608-637.2005.

Kuster, R.D., Boncristiani, H.F., and Rueppell, O. (2014). Immunogene and viral transcript dynamics during parasitic *Varroa destructor* mite infection of developing honey bee (*Apis mellifera*) pupae. *Journal of Experimental Biology* 217(10)**,** 1710-1718. doi: 10.1242/jeb.097766.

Kuznetsova, A., Brockhoff, P.B., and Christensen, R.H. (2017). LmerTest package: Tests in linear mixed effects models. *Journal of Statistical Software* 82(13)**,** 1-26. doi: 10.18637/jss.v082.i13.

La Monica, N., Almond, J., and Racaniello, V. (1987). A mouse model for poliovirus neurovirulence identifies mutations that attenuate the virus for humans. *Journal of Virology* 61(9)**,** 2917-2920.

Lamp, B., Url, A., Seitz, K., Eichhorn, J., Riedel, C., Sinn, L.J., et al. (2016). Construction and rescue of a molecular clone of deformed wing virus (DWV). *PloS ONE* 11(11)**,** e0164639. doi: 10.1371/journal.pone.0164639.

Langmead, B., and Salzberg, S.L. (2012). Fast gapped-read alignment with Bowtie 2. *Nature Methods* 9(4)**,** 357-360. doi: 10.1038/nmeth.1923.

Lawrence, M.A. (2016). Ez: Easy analysis and visualization of factorial experiments. R package version 4.4-0.

Le Conte, Y., Arnold, G., Trouiller, J., Masson, C., Chappe, B., and Ourisson, G. (1989). Attraction of the parasitic mite *Varroa* to the drone larvae of honey bees by simple aliphatic esters. *Science* 245(4918)**,** 638-639.

Le Conte, Y., Ellis, M., and Ritter, W. (2010). *Varroa* mites and honey bee health: can *Varroa* explain part of the colony losses? *Apidologie* 41(3)**,** 353-363. doi: 10.1051/apido/2010017.

Le Feuvre, D. (2017). Honeybee pollination services for the Australian almond industry. *Australasian Agribusiness Perspectives* 20(11)**,** 195-205.

Lee, H., Sumner, D.A., and Champetier, A. (2018). Pollination markets and the coupled futures of almonds and honey bees: simulating impacts of shifts in demands and costs. *American Journal of Agricultural Economics* 101(1)**,** 230-249. doi: 10.1093/ajae/aay063.

Lemaitre, B., and Hoffmann, J. (2007). The host defense of *Drosophila melanogaster*. *Annual Review of Immunology* 25**,** 697-743. doi: 10.1146/annurev.immunol.25.022106.141615.

Lenth, R. (2019). Emmeans: Estimated marginal means, aka least-squares means. R package version 1.4.6.

Lenth, R.V. (2018). Lsmeans: Least-Squares Means. R package version 2.27-62.

Lester, P.J., Bosch, P.J., Gruber, M.A., Kapp, E.A., Peng, L., Brenton-Rule, E.C., et al. (2015). No evidence of enemy release in pathogen and microbial communities of common wasps (*Vespula vulgaris*) in their native and introduced range. *PLoS ONE* 10(3)**,** e0121358. doi: 10.1371/journal. pone.0121358.

Levin, M.L., and Fish, D. (2000). Acquisition of coinfection and simultaneous transmission of *Borrelia burgdorferi* and *Ehrlichia phagocytophila* by *Ixodes scapularis* ticks. *Infection and Immunity* 68(4)**,** 2183-2186.

Levitt, A.L., Singh, R., Cox-Foster, D.L., Rajotte, E., Hoover, K., Ostiguy, N., et al. (2013). Cross-species transmission of honey bee viruses in associated arthropods. *Virus Research* 176(1-2)**,** 232-240. doi: 10.1016/j.virusres.2013.06.013.

Li, D., Liu, C.-M., Luo, R., Sadakane, K., and Lam, T.-W. (2015). MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. *Bioinformatics* 31(10)**,** 1674-1676. doi: 10.1093/bioinformatics/btv033.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., et al. (2009). The sequence alignment/map format and SAMtools. *Bioinformatics* 25(16)**,** 2078-2079. doi: 10.1093/bioinformatics/btp352.

Li, J., Peng, W., Wu, J., Strange, J.P., Boncristiani, H., and Chen, Y. (2011). Cross-species infection of deformed wing virus poses a new threat to pollinator conservation. *Journal of Economic Entomology* 104(3)**,** 732-739. doi: 10.1603/EC10355.

Liu, F., Peng, W., Li, Z., Li, W., Li, L., Pan, J., et al. (2012). Next-generation small RNA sequencing for microRNAs profiling in *Apis mellifera*: comparison between nurses and foragers. *Insect Molecular Biology* 21(3)**,** 297-303. doi: 10.1111/j.1365-2583.2012.01135.x.

Locke, B., Forsgren, E., and De Miranda, J.R. (2014). Increased tolerance and resistance to virus infections: a possible factor in the survival of *Varroa destructor*-resistant honey bees (*Apis mellifera*). *PLoS ONE* 9(6)**,** e99998. doi: 10.1371/journal.pone.0099998.

Locke, B., Forsgren, E., Fries, I., and de Miranda, J.R. (2012). Acaricide treatment affects viral dynamics in *Varroa destructor*-infested honey bee colonies via both host physiology and mite control. *Applied and Environmental Microbiology* 78(1)**,** 227-235. doi: 10.1128/AEM.06094-11.

Locke, B., Semberg, E., Forsgren, E., and de Miranda, J.R. (2017). Persistence of subclinical deformed wing virus infections in honeybees following *Varroa* mite removal and a bee population turnover. *PLoS ONE* 12(7)**,** e0180910. doi: 10.1371/journal.pone.0180910.

Loope, K.J., Baty, J.W., Lester, P.J., and Wilson Rankin, E.E. (2019). Pathogen shifts in a honeybee predator following the arrival of the *Varroa* mite. *Proceedings of the Royal Society B* 286(1894)**,** 20182499. doi: 10.1098/rspb.2018.2499.

Lourenco, A., Florecki, M., Simões, Z., and Evans, J. (2018). Silencing of *Apis mellifera dorsal* genes reveals their role in expression of the antimicrobial peptide *defensin-1*. *Insect Molecular Biology* 27(5)**,** 577-589. doi: 10.1111/imb.12498.

Lu, R., Zhao, X., Li, J., Niu, P., Yang, B., Wu, H., et al. (2020). Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *The Lancet* 395(10224)**,** 565-574. doi: 10.1016/S0140-6736(20)30251-8.

Macedo, L.M.F., Nunes, F., Freitas, F.C.d.P., Pires, C.V., Tanaka, E.D., Martins, J., et al. (2016). MicroRNA signatures characterizing caste-independent ovarian activity in queen and worker honeybees (*Apis mellifera* L.). *Insect Molecular Biology* 25(3)**,** 216-226. doi: 10.1111/imb.12214.

Macedo, P.A., Wu, J., and Ellis, M.D. (2002). Using inert dusts to detect and assess *Varroa* infestations in honey bee colonies. *Journal of Apicultural Research* 41(1-2)**,** 3-7. doi: 10.1080/00218839.2002.11101062.

Manley, R., Temperton, B., Boots, M., and Wilfert, L. (2019a). Contrasting impacts of a novel specialist vector on multihost viral pathogen epidemiology in wild and managed bees. *Molecular Ecology* 29**,** 380– 393. doi: 10.1111/mec.15333.

Manley, R., Temperton, B., Doyle, T., Gates, D., Hedges, S., Boots, M., et al. (2019b). Knock-on community impacts of a novel vector: spillover of emerging DWV-B from *Varroa*-infested honeybees to wild bumblebees. *Ecology Letters* 22(8)**,** 1306-1315. doi: 10.1111/ele.13323.

Marshman, J., Blay-Palmer, A., and Landman, K. (2019). Anthropocene crisis: climate change, pollinators, and food security. *Environments* 6(22)**,** 1-16. doi: 10.3390/environments6020022.

Martin, S. (1995). Reproduction of *Varroa jacobsoni* in cells of *Apis mellifera* containing one or more mother mites and the distribution of these cells. *Journal of Apicultural Research* 34(4)**,** 187-196.

Martin, S., and Kemp, D. (1997). Average number of reproductive cycles performed by *Varroa jacobsoni* in honey bee (*Apis mellifera*) colonies. *Journal of Apicultural Research* 36(3-4)**,** 113-123. doi: 10.1080/00218839.1997.11100937.

Martin, S.J. (1994). Ontogenesis of the mite *Varroa jacobsoni* Oud. in worker brood of the honey bee *Apis mellifera* L. under natural conditions. *Experimental & Applied Acarology* 18(2)**,** 87-100. doi: 10.1007/BF00055033.

Martin, S.J. (2001). The role of *Varroa* and viral pathogens in the collapse of honey bee colonies: a modelling approach. *Journal of Applied Ecology* 38(5)**,** 1082-1093. doi: 10.1046/j.1365-2664.2001.00662.x.

Martin, S.J., Ball, B.V., and Carreck, N.L. (2010). Prevalence and persistence of deformed wing virus (DWV) in untreated or acaricide-treated *Varroa destructor* infested honey bee (*Apis mellifera*) colonies. *Journal of Apicultural Research* 49(1)**,** 72-79. doi: 10.3896/IBRA.1.49.1.10.

Martin, S.J., Ball, B.V., and Carreck, N.L. (2013). The role of deformed wing virus in the initial collapse of *Varroa* infested honey bee colonies in the UK. *Journal of Apicultural Research* 52(5)**,** 251-258. doi: 10.3896/IBRA.1.52.5.12.

Martin, S.J., and Brettell, L.E. (2019). Deformed wing virus in honeybees and other insects. *Annual Review of Virology* 6**,** 227-253. doi: 10.1146/annurev-virology-092818-015700.

Martin, S.J., Highfield, A.C., Brettell, L., Villalobos, E.M., Budge, G.E., Powell, M., et al. (2012). Global honey bee viral landscape altered by a parasitic mite. *Science* 336(6086)**,** 1304-1306. doi: 10.1126/science.1220941.

Martinez Arbizu, P. (2017). PairwiseAdonis: Pairwise multilevel comparison using adonis. R package version 0.0.1.

Martínez-Salas, E. (2008). The impact of RNA structure on picornavirus IRES activity. *Trends in Microbiology* 16(5)**,** 230-237. doi: 10.1016/j.tim.2008.01.013.

Mascia, T., and Gallitelli, D. (2016). Synergies and antagonisms in virus interactions. *Plant Science* 252**,** 176-192. doi: 10.1016/j.plantsci.2016.07.015.

McMahon, D.P., Fürst, M.A., Caspar, J., Theodorou, P., Brown, M.J., and Paxton, R.J. (2015). A sting in the spit: widespread cross-infection of multiple RNA viruses across wild and managed bees. *Journal of Animal Ecology* 84(3)**,** 615-624. doi: 10.1111/1365-2656.12345.

McMahon, D.P., Natsopoulou, M.E., Doublet, V., Fürst, M., Weging, S., Brown, M.J., et al. (2016). Elevated virulence of an emerging viral genotype as a driver of honey bee loss. *Proceedings of the Royal Society B: Biological Sciences* 283(1833)**,** 20160811. doi: 10.1098/rspb.2016.0811.

McMenamin, A.J., and Flenniken, M.L. (2018). Recently identified bee viruses and their impact on bee pollinators. *Current Opinion in Insect Science*. doi: 10.1016/j.cois.2018.02.009.

Merkling, S.H., and van Rij, R.P. (2013). Beyond RNAi: antiviral defense strategies in *Drosophila* and mosquito. *Journal of Insect Physiology* 59(2)**,** 159-170. doi: 10.1016/j.jinsphys.2012.07.004.

Miesen, P., Ivens, A., Buck, A.H., and van Rij, R.P. (2016). Small RNA profiling in dengue virus 2-infected *Aedes* mosquito cells reveals viral piRNAs and novel host miRNAs. *PLOS Neglected Tropical Diseases* 10(2)**,** e0004452. doi: 10.1371/journal.pntd.0004452.

Möckel, N., Gisder, S., and Genersch, E. (2011). Horizontal transmission of deformed wing virus: pathological consequences in adult bees (*Apis mellifera*) depend on the transmission route. *Journal of General Virology* 92(2)**,** 370-377. doi: 10.1099/vir.0.025940-0.

Molina-Cruz, A., DeJong, R.J., Ortega, C., Haile, A., Abban, E., Rodrigues, J., et al. (2012). Some strains of *Plasmodium falciparum*, a human malaria parasite, evade the complement-like system of *Anopheles gambiae* mosquitoes. *Proceedings of the National Academy of Sciences* 109(28)**,** E1957-E1962. doi: 10.1073/pnas.1121183109.

Mondet, F., Alaux, C., Severac, D., Rohmer, M., Mercer, A.R., and Le Conte, Y. (2015). Antennae hold a key to *Varroa*-sensitive hygiene behaviour in honey bees. *Scientific Reports* 5**,** 10454. doi: 10.1038/srep10454.

Mondet, F., Beaurepaire, A., McAfee, A., Locke, B., Alaux, C., Blanchard, S., et al. (2020). Honey bee survival mechanisms against the parasite *Varroa destructor*: a systematic review of phenotypic and genomic research efforts. *International Journal for Parasitology* 50(6)**,** 433-447. doi: 10.1016/j.ijpara.2020.03.005.

Mondet, F., de Miranda, J.R., Kretzschmar, A., Le Conte, Y., and Mercer, A.R. (2014). On the front line: quantitative virus dynamics in honey bee (*Apis mellifera* L.) colonies along a new expansion front of the parasite *Varroa destructor*. *PLoS Pathogens* 10(8)**,** e1004323. doi: 10.1371/journal.ppat.1004323.

Monsanto-Hearne, V., and Johnson, K.N. (2018). miRNAs in insects infected by animal and plant viruses. *Viruses* 10(354)**,** 1-19. doi: 10.3390/v10070354.

Montalva, J., Dudley, L., Arroyo, M.K., Retamales, H., and Abrahamovich, A.H. (2011). Geographic distribution and associated flora of native and introduced bumble bees (*Bombus* spp.) in Chile. *Journal of Apicultural Research* 50(1)**,** 11-21. doi: 10.3896/IBRA.1.50.1.02.

Moore, J., Jironkin, A., Chandler, D., Burroughs, N., Evans, D.J., and Ryabov, E.V. (2011). Recombinants between *Deformed wing virus* and *Varroa destructor virus*-1 may prevail in *Varroa destructor*-infested honey bee colonies. *Journal of General Virology* 92(1)**,** 156-161. doi: 10.1099/vir.0.025965-0.

Morales, C.L., and Aizen, M.A. (2006). Invasive mutualisms and the structure of plant–pollinator interactions in the temperate forests of north-west Patagonia, Argentina. *Journal of Ecology* 94(1)**,** 171-180. doi: 10.1111/j.1365-2745.2005.01069.x.

Morandin, L., Laverty, T., Kevan, P., Khosla, S., and Shipp, L. (2001). Bumble bee (Hymenoptera: Apidae) activity and loss in commercial tomato greenhouses. *The Canadian Entomologist* 133(6)**,** 883-893.

Mordecai, G.J., Brettell, L.E., Martin, S.J., Dixon, D., Jones, I.M., and Schroeder, D.C. (2016a). Superinfection exclusion and the long-term survival of honey bees in *Varroa*-infested colonies. *The ISME Journal* 10(5)**,** 1182-1191. doi: 10.1038/ismej.2015.186.

Mordecai, G.J., Wilfert, L., Martin, S.J., Jones, I.M., and Schroeder, D.C. (2016b). Diversity in a honey bee pathogen: first report of a third master variant of the deformed wing virus quasispecies. *The ISME Journal* 10(5)**,** 1264-1273. doi: 10.1038/ismej.2015.178.

Moreno, P., Gemez-Mata, J., Garcia-Rosado, E., Bejar, J., Labella, A.M., Souto, S., et al. (2020). Differential immunogene expression profile of European sea bass (*Dicentrarchus labrax*, L.) in response to highly and low virulent NNV. *Fish and Shellfish Immunology* 106**,** 56-70. doi: 10.1016/j.fsi.2020.06.052.

Moritz, R.F.A., Härtel, S., and Neumann, P. (2005). Global invasions of the western honey bee (*Apis mellifera*) and the consequences for biodiversity. *Ecoscience* 12(3)**,** 289-301. doi: 10.2980/i1195-6860-12-3-289.1.

Murray, E.A., Burand, J., Trikoz, N., Schnabel, J., Grab, H., and Danforth, B.N. (2018). Viral transmission in honey bees and native bees, supported by a global black queen cell virus phylogeny. *Environmental Microbiology* 21**,** 972–983. doi: 10.1111/1462-2920.14501.

Musso, D., and Gubler, D.J. (2016). Zika virus. *Clinical Microbiology Reviews* 29(3)**,** 487-524. doi: 10.1128/CMR.00072-15.

Natsopoulou, M.E., McMahon, D.P., Doublet, V., Frey, E., Rosenkranz, P., and Paxton, R.J. (2017). The virulent, emerging genotype B of *Deformed wing virus* is closely linked to overwinter honeybee worker loss. *Scientific Reports* 7(1)**,** 5242. doi: 10.1038/s41598-017-05596-3.

Natsopoulou, M.E., McMahon, D.P., and Paxton, R.J. (2016). Parasites modulate within-colony activity and accelerate the temporal polyethism schedule of a social insect, the honey bee. *Behavioral Ecology and Sociobiology* 70(7)**,** 1019-1031. doi: 10.1007/s00265-015-2019-5.

Nayak, A., Berry, B., Tassetto, M., Kunitomi, M., Acevedo, A., Deng, C., et al. (2010). Cricket paralysis virus antagonizes argonaute 2 to modulate antiviral defense in *Drosophila*. *Nature Structural & Molecular Biology* 17(5)**,** 547-554. doi: 10.1038/nsmb.1810.

Nazzi, F., Brown, S.P., Annoscia, D., Del Piccolo, F., Di Prisco, G., Varricchio, P., et al. (2012). Synergistic parasite-pathogen interactions mediated by host immunity can drive the collapse of honey bee colonies. *PLoS Pathogens* 8(6)**,** e1002735. doi: 10.1371/journal.ppat.1002735.

Nazzi, F., and Pennacchio, F. (2018). Honey bee antiviral immune barriers as affected by multiple stress factors: A novel paradigm to interpret colony health decline and collapse. *Viruses* 10(4). doi: 10.3390/v10040159.

Nguyen, B.K., Ribière, M., vanEngelsdorp, D., Snoeck, C., Saegerman, C., Kalkstein, A.L., et al. (2011). Effects of honey bee virus prevalence, *Varroa destructor* load and queen condition on honey bee colony survival over the winter in Belgium. *Journal of Apicultural Research* 50(3)**,** 195-202. doi: 10.3896/IBRA.1.50.3.03.

Nieto, A., Roberts, S.P., Kemp, J., Rasmont, P., Kuhlmann, M., Criado, M.G., et al. (2017). *European red list of bees*, International Union for Conservation of Nature. Belgium.

Niu, J., Meeus, I., Cappelle, K., Piot, N., and Smagghe, G. (2014). The immune response of the small interfering RNA pathway in the defense against bee viruses. *Current Opinion in Insect Science* 6**,** 22-27. doi: 10.1016/j.cois.2014.09.014.

Norton, A.M., Remnant, E.J., Buchmann, G., and Beekman, M. (2020). Accumulation and competition amongst deformed wing virus genotypes in naïve Australian honeybees provides insight into the increasing global prevalence of genotype B. *Frontiers in Microbiology* 11(620). doi: 10.3389/fmicb.2020.00620.

Obbard, D.J., Jiggins, F.M., Halligan, D.L., and Little, T.J. (2006). Natural selection drives extremely rapid evolution in antiviral RNAi genes. *Current Biology* 16(6)**,** 580-585. doi: 10.1016/j.cub.2006.01.065.

Ogle, D., Wheeler, P., and Dinno, A. (2018). FSA: Fisheries stock analysis. R package version 0.8.22.

Öhlund, P., Lundén, H., and Blomström, A.-L. (2019). Insect-specific virus evolution and potential effects on vector competence. *Virus Genes* 55(2)**,** 127-137. doi: 10.1007/s11262-018-01629-9.

Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'hara, R., et al. (2013). Vegan: Community ecology package. R version 2.1-1.

Oldroyd, B.P. (1999). Coevolution while you wait: *Varroa jacobsoni*, a new parasite of western honey bees. *Trends in Ecology & Evolution* 14(8)**,** 312-315. doi: 10.1016/S0169-5347(99)01613-4.

Ollerton, J., Erenler, H., Edwards, M., and Crockett, R. (2014). Extinctions of aculeate pollinators in Britain and the role of large-scale agricultural changes. *Science* 346(6215)**,** 1360-1362. doi: 10.1126/science.1257259.

Ollerton, J., Winfree, R., and Tarrant, S. (2011). How many flowering plants are pollinated by animals? *Oikos* 120(3)**,** 321-326. doi: 10.1111/j.1600-0706.2010.18644.x.

Ongus, J.R. (2006). *Varroa destructor virus 1: A new picorna-like virus in Varroa mites as well as honey bees.* PhD thesis, Wageningen University.

Ongus, J.R., Peters, D., Bonmatin, J.M., Bengsch, E., Vlak, J.M., and van Oers, M.M. (2004). Complete sequence of a picorna-like virus of the genus *Iflavirus* replicating in the mite *Varroa destructor*. *Journal of General Virology* 85(12)**,** 3747-3755. doi: 10.1099/vir.0.80470-0.

Ongus, J.R., Roode, E.C., Pleij, C.W.A., Vlak, J.M., and van Oers, M.M. (2006). The 5′ non-translated region of *Varroa destructor* virus 1 (genus *Iflavirus*): Structure prediction and IRES activity in *Lymantria dispar* cells. *Journal of General Virology* 87(11)**,** 3397-3407. doi: 10.1099/vir.0.82122-0.

Oudemans, A.C. (1904). On a new genus and species of parasitic acari. *Notes from the Leyden Museum* 24(4)**,** 216-222.

Panziera, D., van Langevelde, F., and Blacquière, T. (2017). *Varroa* sensitive hygiene contributes to naturally selected *Varroa* resistance in honey bees. *Journal of Apicultural Research* 56(5)**,** 635-642. doi: 10.1080/00218839.2017.1351860.

Paton, D.C. (2000). Disruption of bird-plant pollination systems in southern Australia. *Conservation Biology* 14(5)**,** 1232-1234.

Peng, Y.-S., Fang, Y., Xu, S., and Ge, L. (1987). The resistance mechanism of the Asian honey bee, *Apis cerana* Fabr., to an ectoparasitic mite, *Varroa jacobsoni* Oudemans. *Journal of Invertebrate Pathology* 49(1)**,** 54-60.

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Research* 29(9)**,** e45-e45. doi: 10.1093/nar/29.9.e45.

Pfaffl, M.W., Tichopad, A., Prgomet, C., and Neuvians, T.P. (2004). Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper–Excel-based tool using pair-wise correlations. *Biotechnology Letters* 26(6)**,** 509-515.

Pimentel, D., Zuniga, R., and Morrison, D. (2005). Update on the environmental and economic costs associated with alien-invasive species in the United States. *Ecological Economics* 52(3)**,** 273-288. doi: 10.1016/j.ecolecon.2004.10.002.

Posada-Florez, F., Childers, A.K., Heerman, M.C., Egekwu, N.I., Cook, S.C., Chen, Y., et al. (2019). Deformed wing virus type A, a major honey bee pathogen, is vectored by the mite *Varroa destructor* in a non-propagative manner. *Scientific Reports* 9(1)**,** 12445. doi: 10.1038/s41598-019-47447-3.

Posada-Florez, F., Lamas, Z.S., Hawthorne, D.J., Chen, Y., Evans, J.D., and Ryabov, E.V. (2020). Pupal cannibalism by worker honey bees contributes to the spread of deformed wing virus. *bioRxiv*. doi: 10.21203/rs.3.rs-117474/v1.

Power, A.G., and Mitchell, C.E. (2004). Pathogen spillover in disease epidemics. *The American Naturalist* 164(S5)**,** S79-S89.

Powney, G.D., Carvell, C., Edwards, M., Morris, R.K., Roy, H.E., Woodcock, B.A., et al. (2019). Widespread losses of pollinating insects in Britain. *Nature Communications* 10(1)**,** 1-6. doi: 10.1038/s41467-019-08974-9.

Qi, N., Zhang, L., Qiu, Y., Wang, Z., Si, J., Liu, Y., et al. (2012). Targeting of dicer-2 and RNA by a viral RNA silencing suppressor in *Drosophila* cells. *Journal of Virology* 86(10)**,** 5763-5773. doi: 10.1128/JVI.07229-11.

Qin, Q.H., Wang, Z.L., Tian, L.Q., Gan, H.Y., Zhang, S.W., and Zeng, Z.J. (2014). The integrative analysis of microRNA and mRNA expression in *Apis mellifera* following maze-based visual pattern learning. *Insect Science* 21(5)**,** 619-636. doi: 10.1111/1744-7917.12065.

Radzevičiūtė, R., Theodorou, P., Husemann, M., Japoshvili, G., Kirkitadze, G., Zhusupbaeva, A., 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. doi: 10.1016/j.jip.2017.04.002.

Ramsey, S.D., Ochoa, R., Bauchan, G., Gulbronson, C., Mowery, J.D., Cohen, A., et al. (2019). *Varroa destructor* feeds primarily on honey bee fat body tissue and not hemolymph. *Proceedings of the National Academy of Sciences* 116(5)**,** 1792-1801. doi: 10.1073/pnas.1818371116.

Rath, W. (1999). Co-adaptation of *Apis cerana* Fabr. and *Varroa jacobsoni* Oud. *Apidologie* 30(2-3)**,** 97-110.

Rath, W., and Drescher, W. (1990). Response of *Apis cerana* Fabr towards brood infested with *Varroa jacobson*i Oud and infestation rate of colonies in Thailand. *Apidologie* 21(4)**,** 311-321.

Remnant, E.J., Mather, N., Gillard, T., Yagound, B., and Beekman, M. (2019). Direct transmission by injection affects competition amongst RNA viruses in honeybees. *Proceedings of the Royal Society B: Biological Sciences* 286(1895)**,** 20182452. doi: 10.1098/rspb.2018.2452.

Remnant, E.J., Shi, M., Buchmann, G., Blacquière, T., Holmes, E.C., Beekman, M., et al. (2017). A diverse range of novel RNA viruses in geographically distinct honey bee populations. *Journal of Virology*. doi: 10.1128/JVI.00158-17.

Ribière, M., Ball, B., and Aubert, M. (2008). 'Natural history and geographical distribution of honey bee viruses' in *Virology and the honey bee,* eds. M. Aubert, B. Ball, I. Fries, R. Moritz, N. Milani & I. Bernardinelli. Luxembourg: EEC Publications, 15-84.

Roberts, J., Anderson, D., and Tay, W. (2015). Multiple host shifts by the emerging honeybee parasite, *Varroa jacobsoni*. *Molecular Ecology* 24(10)**,** 2379-2391. doi: 10.1111/mec.13185.

Roberts, J.M., Anderson, D.L., and Durr, P.A. (2017). Absence of deformed wing virus and *Varroa destructor* in Australia provides unique perspectives on honeybee viral

landscapes and colony losses. *Scientific Reports* 7**,** 1-11. doi: 10.1038/s41598-017-07290-w.

Roberts, J.M., Anderson, D.L., and Durr, P.A. (2018). Metagenomic analysis of *Varroa*-free Australian honey bees (*Apis mellifera*) shows a diverse Picornavirales virome. *Journal of General Virology* 99(6)**,** 818-826. doi: 10.1099/jgv.0.001073.

Roberts, J.M., Simbiken, N., Dale, C., Armstrong, J., and Anderson, D.L. (2020). Tolerance of honey bees to *Varroa* mite in the absence of deformed wing virus. *Viruses* 12(5)**,** 575. doi: 10.3390/v12050575.

Rosengaus, R., Jordan, C., Lefebvre, M., and Traniello, J. (1999). Pathogen alarm behavior in a termite: a new form of communication in social insects. *Naturwissenschaften* 86(11)**,** 544-548.

Rückert, C., Weger-Lucarelli, J., Garcia-Luna, S.M., Young, M.C., Byas, A.D., Murrieta, R.A., et al. (2017). Impact of simultaneous exposure to arboviruses on infection and transmission by *Aedes aegypti* mosquitoes. *Nature Communications* 8**,** 15412. doi: 10.1038/ncomms15412.

Ruttner, F. (1988). *Biogeography and taxonomy of honeybees.* Berlin, Germany: Springer.

Ryabov, E.V., Childers, A.K., Chen, Y., Madella, S., Nessa, A., vanEngelsdorp, D., et al. (2017). Recent spread of *Varroa destructor virus*-1, a honey bee pathogen, in the United States. *Scientific Reports* 7(1)**,** 17447. doi: 10.1038/s41598-017-17802-3.

Ryabov, E.V., Childers, A.K., Lopez, D., Grubbs, K., Posada-Florez, F., Weaver, D., et al. (2019). Dynamic evolution in the key honey bee pathogen deformed wing virus: Novel insights into virulence and competition using reverse genetics. *PLoS Biology* 17(10)**,** e3000502. doi: 10.1371/journal.pbio.3000502.

Ryabov, E.V., Christmon, K., Heerman, M.C., Posada-Florez, F., Harrison, R.L., Chen, Y., et al. (2020). Development of a honey bee RNA virus vector based on the genome of a deformed wing virus. *Viruses* 12(4)**,** 374. doi: 10.3390/v12040374.

Ryabov, E.V., Fannon, J.M., Moore, J.D., Wood, G.R., and Evans, D.J. (2016). The Iflaviruses sacbrood virus and deformed wing virus evoke different transcriptional responses in the honey bee which may facilitate their horizontal or vertical transmission. *PeerJ* 4**,** e1591. doi: 10.7717/peerj.1591.

Ryabov, E.V., Wood, G.R., Fannon, J.M., Moore, J.D., Bull, J.C., Chandler, D., et al. (2014). A virulent strain of deformed wing virus (DWV) of honey bees (*Apis mellifera*) prevails after *Varroa destructor*-mediated, or *in vitro*, transmission. *PLoS Pathogens* 10(6)**,** e1004230. doi: 10.1371/journal.ppat.1004230.

Santamaria, J., Villalobos, E.M., Brettell, L.E., Nikaido, S., Graham, J.R., and Martin, S. (2018). Evidence of *Varroa*-mediated deformed wing virus spillover in Hawaii. *Journal of Invertebrate Pathology* 151**,** 126-130. doi: 10.1016/j.jip.2017.11.008.

Schlüns, H., and Crozier, R. (2007). Relish regulates expression of antimicrobial peptide genes in the honeybee, *Apis mellifera*, shown by RNA interference. *Insect Molecular Biology* 16(6)**,** 753-759. doi: 10.1111/j.1365-2583.2007.00768.x.

Schroeder, D.C., and Martin, S.J. (2012). Deformed wing virus: the main suspect in unexplained honey bee deaths worldwide. *Virulence* 3(7)**,** 589-591. doi: 10.1126/science.1220941.

Sébastien, A., Lester, P.J., Hall, R.J., Wang, J., Moore, N.E., and Gruber, M.A. (2015). Invasive ants carry novel viruses in their new range and form reservoirs for a honeybee pathogen. *Biology Letters* 11(9)**,** 20150610. doi: 10.1098/rsbl.2015.0610.

Sela, U., Euler, C.W., Correa da Rosa, J., and Fischetti, V.A. (2018). Strains of bacterial species induce a greatly varied acute adaptive immune response: the contribution of the accessory genome. *PLoS Pathogens* 14(1)**,** e1006726. doi: 10.1371/journal.ppat.1006726.

Shelef, O., Weisberg, P.J., and Provenza, F.D. (2017). The value of native plants and local production in an era of global agriculture. *Frontiers in Plant Science* 8**,** 2069. doi: 10.3389/fpls.2017.02069.

Shutler, D., Head, K., Burgher-MacLellan, K.L., Colwell, M.J., Levitt, A.L., Ostiguy, N., et al. (2014). Honey bee *Apis mellifera* parasites in the absence of *Nosema ceranae* fungi and *Varroa destructor* mites. *PloS one* 9(6)**,** e98599.

Simanonok, M.P., Otto, C.R.V., Cornman, R.S., Iwanowicz, D.D., Strange, J.P., and Smith, T.A. (2021). A century of pollen foraging by the endangered rusty patched bumble bee (*Bombus affinis*): inferences from molecular sequencing of museum specimens. *Biodiversity and Conservation* 30(1)**,** 123-137. doi: 10.1007/s10531-020-02081-8.

Simone, M., Evans, J.D., and Spivak, M. (2009). Resin collection and social immunity in honey bees. *Evolution* 63(11)**,** 3016-3022. doi: 10.1111/j.1558-5646.2009.00772.x.

Simone-Finstrom, M. (2017). Social immunity and the superorganism: behavioral defenses protecting honey bee colonies from pathogens and parasites. *Bee World* 94(1)**,** 21-29. doi: 10.1080/0005772X.2017.1307800.

Singer, M. (2017). The spread of Zika and the potential for global arbovirus syndemics. *Global Public Health* 12(1)**,** 1-18. doi: 10.1080/17441692.2016.1225112.

Singh, G., Popli, S., Hari, Y., Malhotra, P., Mukherjee, S., and Bhatnagar, R.K. (2009). Suppression of RNA silencing by Flock house virus B2 protein is mediated through its interaction with the PAZ domain of Dicer. *The FASEB Journal* 23(6)**,** 1845-1857. doi: 10.1096/fj.08-125120.

Singh, R., Levitt, A.L., Rajotte, E.G., Holmes, E.C., Ostiguy, N., Lipkin, W.I., 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(12)**,** e14357. doi: 10.1371/journal.pone.0014357.

Slonchak, A., Hussain, M., Torres, S., Asgari, S., and Khromykh, A.A. (2014). Expression of mosquito microRNA Aae-miR-2940-5p is downregulated in response to West Nile virus infection to restrict viral replication. *Journal of Virology* 88(15)**,** 8457-8467. doi: 10.1128/JVI.00317-14.

Solignac, M., Cornuet, J.M., Vautrin, D., Le Conte, Y., Anderson, D., Evans, J., et al. (2005). The invasive Korea and Japan types of *Varroa destructor*, ectoparasitic mites of the Western honey bee (*Apis mellifera*), are two partly isolated clones. *Proceedings of the Royal Society of London B: Biological Sciences* 272(1561)**,** 411-419. doi: 10.1098/rspb.2004.2853.

Soroye, P., Newbold, T., and Kerr, J. (2020). Climate change contributes to widespread declines among bumble bees across continents. *Science* 367(6478)**,** 685-688. doi: 10.1126/science.aax8591.

Søvik, E., LaMora, A., Seehra, G., Barron, A.B., Duncan, J.G., and Ben-Shahar, Y. (2017). *Drosophila* divalent metal ion transporter malvolio is required in dopaminergic neurons for feeding decisions. *Genes, Brain and Behavior* 16(5)**,** 506-514. doi: 10.1111/gbb.12375.

Staroscik, A. (2004). *Calculator for determining the number of copies of a template* [Online]. Available: https://cels.uri.edu/gsc/cndna.html [Accessed 2017].

Suarez, A.V., and Tsutsui, N.D. (2008). The evolutionary consequences of biological invasions. *Molecular Ecology* 17(1)**,** 351-360. doi: 10.1111/j.1365-294X.2007.03456.x.

Sullivan, C.S., and Ganem, D. (2005). A virus-encoded inhibitor that blocks RNA interference in mammalian cells. *Journal of Virology* 79(12)**,** 7371-7379. doi: 10.1128/JVI.79.12.7371–7379.2005.

Sumpter, D.J., and Martin, S.J. (2004). The dynamics of virus epidemics in *Varroa*-infested honey bee colonies. *Journal of Animal Ecology* 73(1)**,** 51-63. doi: 10.1111/j.1365-2656.2004.00776.x.

Syller, J. (2012). Facilitative and antagonistic interactions between plant viruses in mixed infections. *Molecular Plant Pathology* 13(2)**,** 204-216. doi: 10.1111/J.1364-3703.2011.00734.X.

Syller, J., and Grupa, A. (2016). Antagonistic within-host interactions between plant viruses: molecular basis and impact on viral and host fitness. *Molecular Plant Pathology* 17(5)**,** 769-782. doi: 10.1111/mpp.12322.

Tabachnick, W.J. (1998). 'Arthropod-borne pathogens: issues for understanding emerging infectious diseases' in *Emerging Infections,* ed. R.M. Krause. New York: Academic Press, 411-429.

Taylor, L.H., Walliker, D., and Read, A.F. (1997). Mixed–genotype infections of malaria parasites: within–host dynamics and transmission success of competing clones. *Proceedings of the Royal Society B: Biological Sciences* 264(1383)**,** 927-935.

Techer, M.A. (2020). *World distribution of Varroa mites mtDNA lineages* [Online]. Available: https://mikheyevlab.github.io/varroa-mtDNA-world-distrib/ [Accessed 18 Feb 2021].

Techer, M.A., Rane, R.V., Grau, M.L., Roberts, J.M.K., Sullivan, S.T., Liachko, I., et al. (2019). Divergent evolutionary trajectories following speciation in two ectoparasitic

honey bee mites. *Communications Biology* 2(1)**,** 357. doi: 10.1038/s42003-019-0606-0.

Techer, M.A., Roberts, J.M.K., Cartwright, R.A., and Mikheyev, A.S. (2020). The first steps toward a global pandemic: Reconstructing the demographic history of parasite host switches in its native range. *bioRxiv***,** 2020.2007.2030.228320. doi: 10.1101/2020.07.30.228320.

Tehel, A., Brown, M.J., and Paxton, R.J. (2016). Impact of managed honey bee viruses on wild bees. *Current Opinion in Virology* 19**,** 16-22. doi: 10.1016/j.coviro.2016.06.006.

Tehel, A., Vu, Q., Bigot, D., Gogol-Döring, A., Koch, P., Jenkins, C., et al. (2019). The two prevalent genotypes of an emerging infectious disease, deformed wing virus, cause equally low pupal mortality and equally high wing deformities in host honey bees. *Viruses* 11(2)**,** 114. doi: 10.3390/v11020114.

Tentcheva, D., Gauthier, L., Bagny, L., Fievet, J., Dainat, B., Cousserans, F., et al. (2006). Comparative analysis of deformed wing virus (DWV) RNA in *Apis mellifera* and *Varroa destructor*. *Apidologie* 37(1)**,** 41-50. doi: 10.1051/apido:2005057.

Tesovnik, T., Zorc, M., Gregorc, A., Rinehart, T., Adamczyk, J., and Narat, M. (2019). Immune gene expression in developing honey bees (*Apis mellifera* L.) simultaneously exposed to imidacloprid and *Varroa destructor* in laboratory conditions. *Journal of Apicultural Research* 58(5)**,** 730-739. doi: 10.1080/00218839.2019.1634463.

Thaduri, S., Locke, B., Granberg, F., and de Miranda, J.R. (2018). Temporal changes in the viromes of Swedish *Varroa*-resistant and *Varroa*-susceptible honeybee populations. *PLoS ONE* 13(12)**,** e0206938. doi: 10.1371/journal.pone.0206938

Toplak, I., Jamnikar Ciglenečki, U., Aronstein, K., and Gregorc, A. (2013). Chronic bee paralysis virus and *Nosema ceranae* experimental co-infection of winter honey bee workers (*Apis mellifera* L.). *Viruses* 5(9)**,** 2282-2297. doi: 10.3390/v5092282.

Townsend, A.K., Hawley, D.M., Stephenson, J.F., and Williams, K.E. (2020). Emerging infectious disease and the challenges of social distancing in human and non-human animals. *Proceedings of the Royal Society B* 287(1932)**,** 20201039. doi: 10.1098/rspb.2020.1039.

Traynor, K.S., Mondet, F., de Miranda, J.R., Techer, M., Kowallik, V., Oddie, M.A., et al. (2020). *Varroa destructor*: a complex parasite, crippling honey bees worldwide. *Trends in Parasitology* 36(7)**,** 592-606. doi: 10.1016/j.pt.2020.04.004.

US Fish and Wildlife Service (2021). *Environmental conservation online system: listed animals* [Online]. USA. Available: https://ecos.fws.gov/ecp0/reports/ad-hoc-species-report?kingdom=V&kingdom=I&status=E&status=T&status=EmE&status=EmT&status=EXPE&status=EXPN&status=SAE&status=SAT&mapstatus=3&fcrithab=on&fstatus=on&fspecrule=on&finvpop=on&fgroup=on&header=Listed+Animals [Accessed 4 Feb 2021].

Van Rij, R.P., Saleh, M.-C., Berry, B., Foo, C., Houk, A., Antoniewski, C., et al. (2006). The RNA silencing endonuclease argonaute 2 mediates specific antiviral immunity in

*Drosophila melanogaster*. *Genes & Development* 20(21)**,** 2985-2995. doi: 10.1101/gad.1482006.

Vance, V.B. (1991). Replication of potato virus X RNA is altered in coinfections with potato virus Y. *Virology* 182(2)**,** 486-494. doi: 10.1016/0042-6822(91)90589-4.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., et al. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* 3(7)**,** research0034.0031. doi: 10.1186/gb-2002-3-7-research0034.

Vasconcelos, P.F., Bryant, J.E., Da Rosa, A.P.T., Tesh, R.B., Rodrigues, S.G., and Barrett, A.D. (2004). Genetic divergence and dispersal of yellow fever virus, Brazil. *Emerging Infectious Diseases* 10(9)**,** 1578.

Vazeille, M., Mousson, L., Martin, E., and Failloux, A.-B. (2010). Orally co-infected *Aedes albopictus* from La Reunion Island, Indian Ocean, can deliver both dengue and chikungunya infectious viral particles in their saliva. *PLoS Neglected Tropical Diseases* 4(6)**,** e706. doi: 10.1371/journal.pntd.0000706.

Velthuis, H.H., and van Doorn, A. (2006). A century of advances in bumblebee domestication and the economic and environmental aspects of its commercialization for pollination. *Apidologie* 37(4)**,** 421-451. doi: 10.1051/apido:2006019.

Vilcinskas, A., Stoecker, K., Schmidtberg, H., Röhrich, C.R., and Vogel, H. (2013). Invasive harlequin ladybird carries biological weapons against native competitors. *Science* 340(6134)**,** 862-863. doi: 10.1126/science.1234032.

Wang, H., Xie, J., Shreeve, T.G., Ma, J., Pallett, D.W., King, L.A., 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(9)**,** e74508. doi: 10.1371/journal.pone.0074508.

Wang, S., Lin, Z., Chen, G., Page, P., Hu, F., Niu, Q., et al. (2020). Reproduction of ectoparasitic mites in a coevolved system: *Varroa* spp.—Eastern honey bees, *Apis cerana*. *Ecology and Evolution* 10(24)**,** 14359-14371. doi: 10.1002/ece3.7038.

Wang, S., Lin, Z., Dietemann, V., Neumann, P., Wu, Y., Hu, F., et al. (2019). Ectoparasitic Mites *Varroa underwoodi* (Acarina: Varroidae) in Eastern Honeybees, but not in Western Honeybees. *Journal of Economic Entomology* 112(1)**,** 25-32. doi: 10.1093/jee/toy288.

Warnes, M.G.R., Bolker, B., Bonebakker, L., Gentleman, R., and Huber, W. (2016). Gplots: Various R programming tools for plotting data. R package version 3.1.0.

Whittington, R., Winston, M.L., Tucker, C., and Parachnowitsch, A.L. (2004). Plant-species identity of pollen collected by bumblebees placed in greenhouses for tomato pollination. *Canadian Journal of Plant Science* 84(2)**,** 599-602.

Wickham, H., Chang, W., Henry, L., Pedersen, T.L., Takahashi, K., Wilke, C., et al. (2016). Ggplot2: Create elegant data visualisations using the grammar of graphics. R package version 3.3.2.

Wilfert, L., Long, G., Leggett, H., Schmid-Hempel, P., Butlin, R., Martin, S., et al. (2016). Deformed wing virus is a recent global epidemic in honey bees driven by *Varroa* mites. *Science* 351(6273)**,** 594-597. doi: 10.1126/science.aac9976.

Wilson, E.O., Durlach, N.I., and Roth, L.M. (1958). Chemical releasers of necrophoric behavior in ants. *Psyche* 65(4)**,** 108-114.

Winter, K., Adams, L., Thorp, R., Inouye, D., Day, L., Ascher, J., et al. (2006). *Importation of non-native bumble bees into North America: potential consequences of using Bombus terrestris and other non-native bumble bees for greenhouse crop pollination in Canada, Mexico, and the United States*, North American Pollinator Protection Campaign. San Francisco.

Wood, S. (2018). Mgcv: Mixed GAM computation vehicle with GCV/AIC/REML smoothness estimation and GAMMs by REML/PQL. R package version 1.8-23.

Woolhouse, M.E. (2002). Population biology of emerging and re-emerging pathogens. *Trends in Microbiology* 10(10)**,** s3-s7. doi: 10.1016/S0966-842X(02)02428-9.

Wu, Y., Dong, X., and Kadowaki, T. (2017). Characterization of the copy number and variants of deformed wing virus (dwv) in the pairs of honey bee pupa and infesting *Varroa destructor* or *Tropilaelaps mercedesae*. *Frontiers in Microbiology* 8**,** 1558. doi: 10.3389/fmicb.2017.01558.

Yan, H., Zhou, Y., Liu, Y., Deng, Y., Puthiyakunnon, S., and Chen, X. (2014). MiR-252 of the Asian tiger mosquito *Aedes albopictus* regulates dengue virus replication by suppressing the expression of the dengue virus envelope protein. *Journal of Medical Virology* 86(8)**,** 1428-1436. doi: 10.1002/jmv.23815.

Yañez, O., Chávez-Galarza, J., Tellgren-Roth, C., Pinto, M.A., Neumann, P., and de Miranda, J.R. (2020). The honeybee (*Apis mellifera*) developmental state shapes the genetic composition of the deformed wing virus-A quasispecies during serial transmission. *Scientific Reports* 10(1)**,** 5956 doi: 10.1038/s41598-020-62673-w.

Yang, X., and 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* 102(21)**,** 7470-7475. doi: 10.1073 pnas.0501860102.

Yue, C., and Genersch, E. (2005). RT-PCR analysis of *Deformed wing virus* in honeybees (*Apis mellifera*) and mites (*Varroa destructor*). *Journal of General Virology* 86(12)**,** 3419-3424. doi: 10.1099/vir.0.81401-0.

Zanni, V., Galbraith, D.A., Annoscia, D., Grozinger, C.M., and Nazzi, F. (2017). Transcriptional signatures of parasitization and markers of colony decline in *Varroa*-infested honey bees (A*pis mellifera*). *Insect Biochemistry and Molecular Biology* 87**,** 1-13. doi: 10.1016/j.ibmb.2017.06.002.

Zattara, E.E., and Aizen, M.A. (2021). Worldwide occurrence records suggest a global decline in bee species richness. *One Earth* 4(1)**,** 114-123. doi: 10.1016/j.oneear.2020.12.005.

Zhao, Y., Heerman, M., Peng, W., Evans, J.D., Rose, R., DeGrandi-Hoffman, G., et al. (2019). The dynamics of deformed wing virus concentration and host defensive gene expression after *Varroa* mite parasitism in honey bees, *Apis mellifera*. *Insects* 10(1)**,** 1-19. doi: 10.3390/insects10010016.

Ziegelmann, B., Lindenmayer, A., Steidle, J., and Rosenkranz, P. (2013). The mating behavior of *Varroa destructor* is triggered by a female sex pheromone. *Apidologie* 44(3)**,** 314-323. doi: 10.1007/s13592-012-0182-5.

Zioni, N., Soroker, V., and 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(1)**,** 106-112. doi: 10.1016/j.virol.2011.05.009.

Zuo, H., Yuan, J., Chen, Y., Li, S., Su, Z., Wei, E., et al. (2016). A microRNA-mediated positive feedback regulatory loop of the NF-κB pathway in *Litopenaeus vannamei*. *The Journal of Immunology* 196(9)**,** 3842-3853. doi: 10.4049/jimmunol.1502358.

# Supplementary Material for Chapter 2

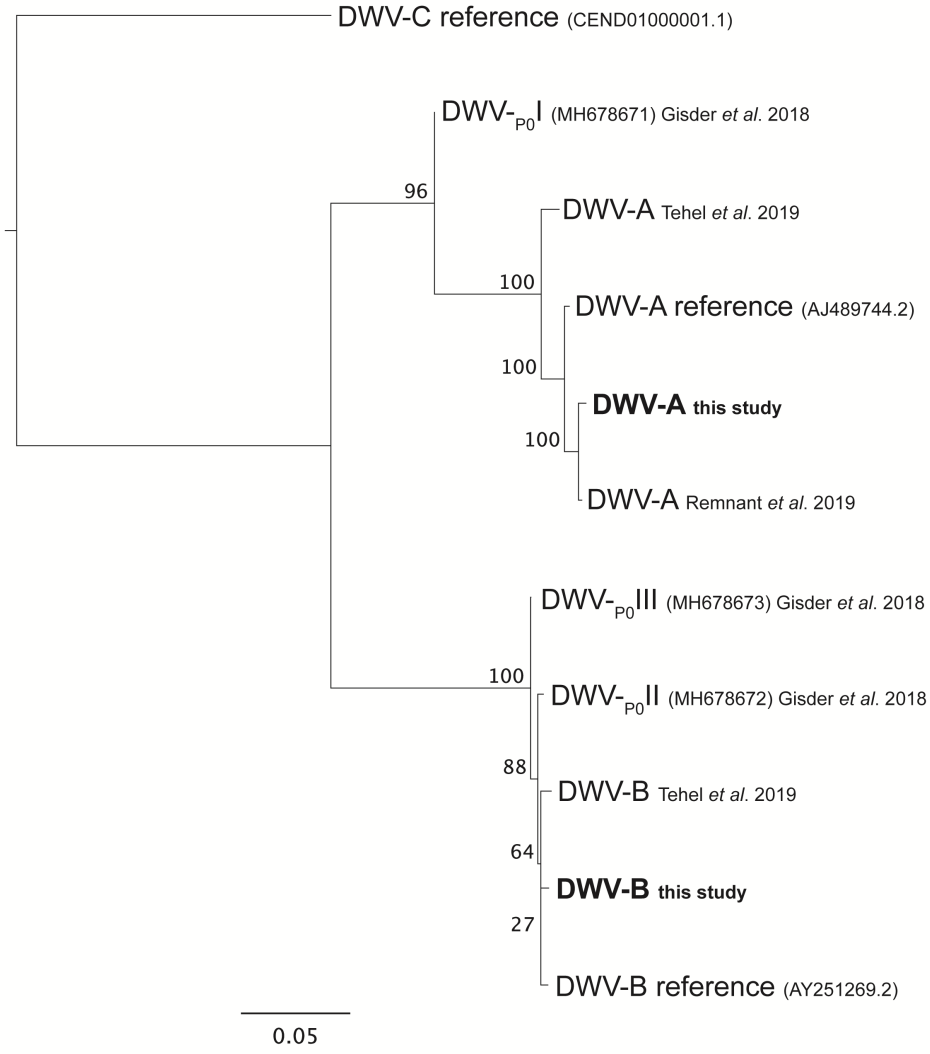


**Figure A.1.** Phylogenetic tree comparing whole genome sequences of the DWV-A (accession number MN538208) and DWV-B (MN538209) inocula obtained in this study to isolates experimentally injected by Remnant et al. (2019), Tehel et al. (2019) and Gisder et al. (2018) (MH678671-73). DWV-A [AJ489744.2; Lanzi et al. (2006)], DWV-B [AY251269.2; Ongus et al. (2004)] and DWV-C [CEND01000001; (Mordecai et al., 2016)] were used as reference sequences, with DWV-C as the outgroup. The DWV-A and DWV-B whole genome sequences from this study were obtained from transcriptome sequencing (see Materials and Methods) and are highlighted in bold. Sequences were aligned in Geneious using Muscle and trimmed where required. Maximum likelihood trees were produced using PhyML HKY85 model after 100 bootstrap replicates.

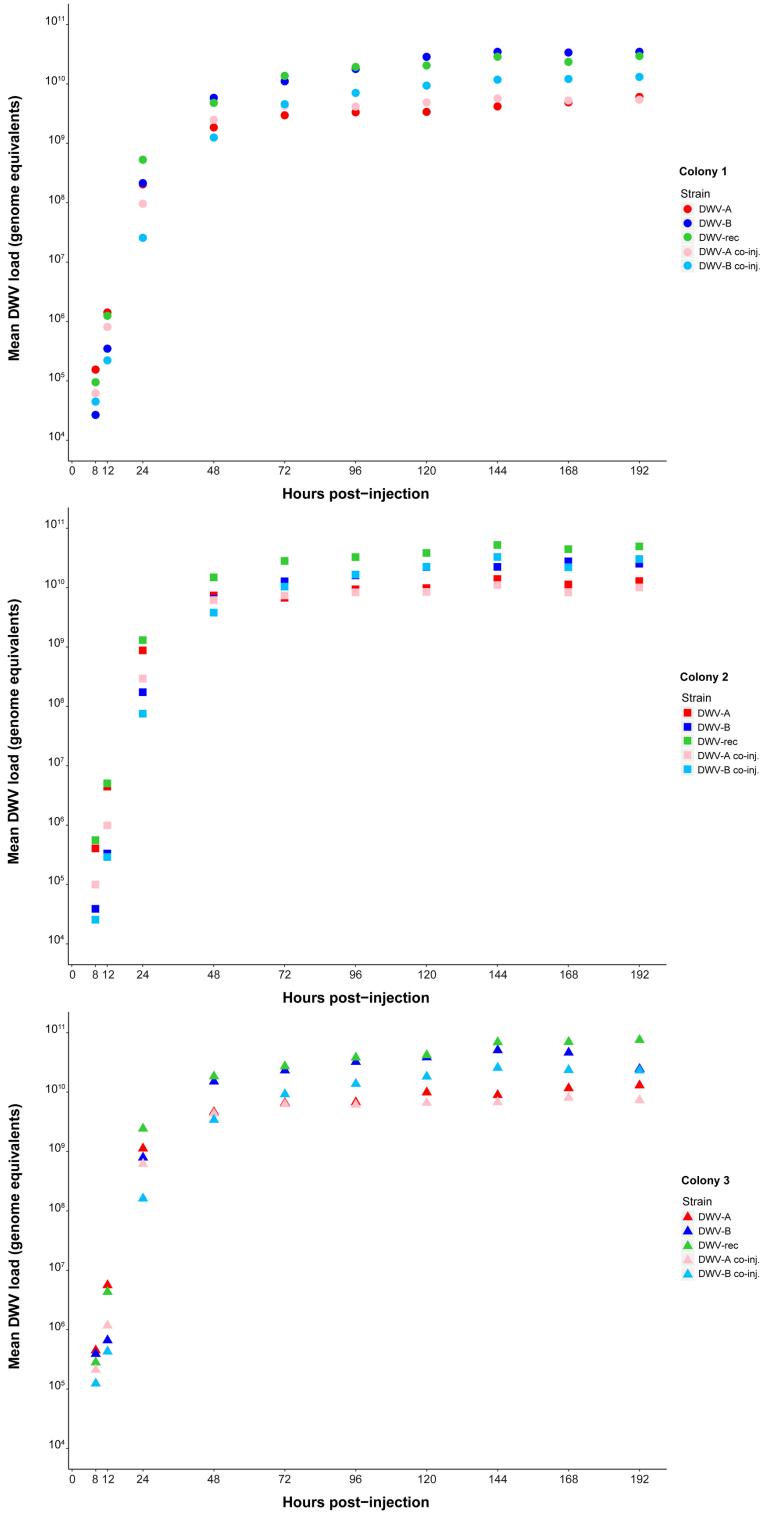


**Figure A.2.** Mean DWV viral loads (genome equivalents) of individually infected pupae from 8 to 192 hours post-injection ($n$ = 120 per treatment), as calculated by standard curve in cDNA synthesized from 0.8 µg RNA. White-eyed pupae from colonies one to three were singly injected with $1 \times 10^7$ genome equivalents of DWV-A, DWV-B or recombinant strain ('DWV-rec'), or co-injected with $5 \times 10^6$ genome equivalents of DWV-A and DWV-B. Each figure displays the mean viral load by colony to illustrate colony differences.

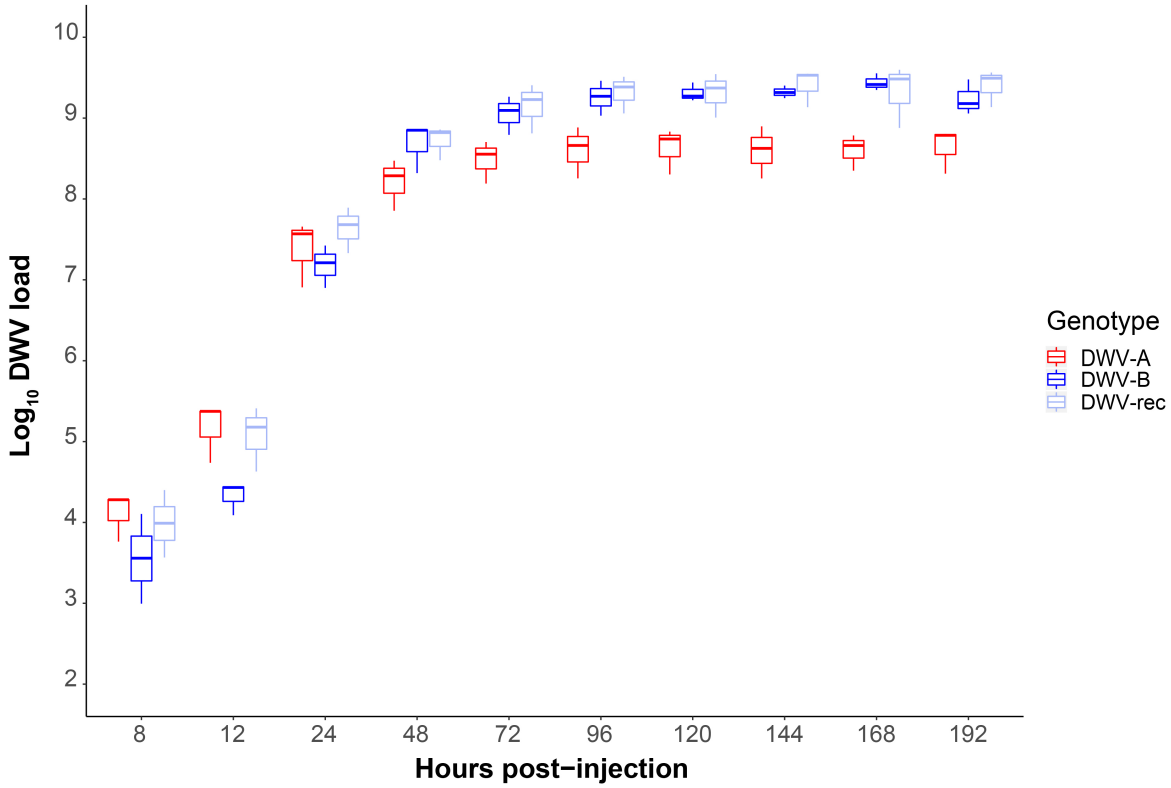


**Figure A.3.** $Log_{10}$ DWV viral loads of singly infected pupae from 8 to 192 hours post-injection, relative to housekeeping gene *Actin*. Colonies 1-3 have been combined (per treatment) to illustrate the distribution of viral accumulation per genotype.

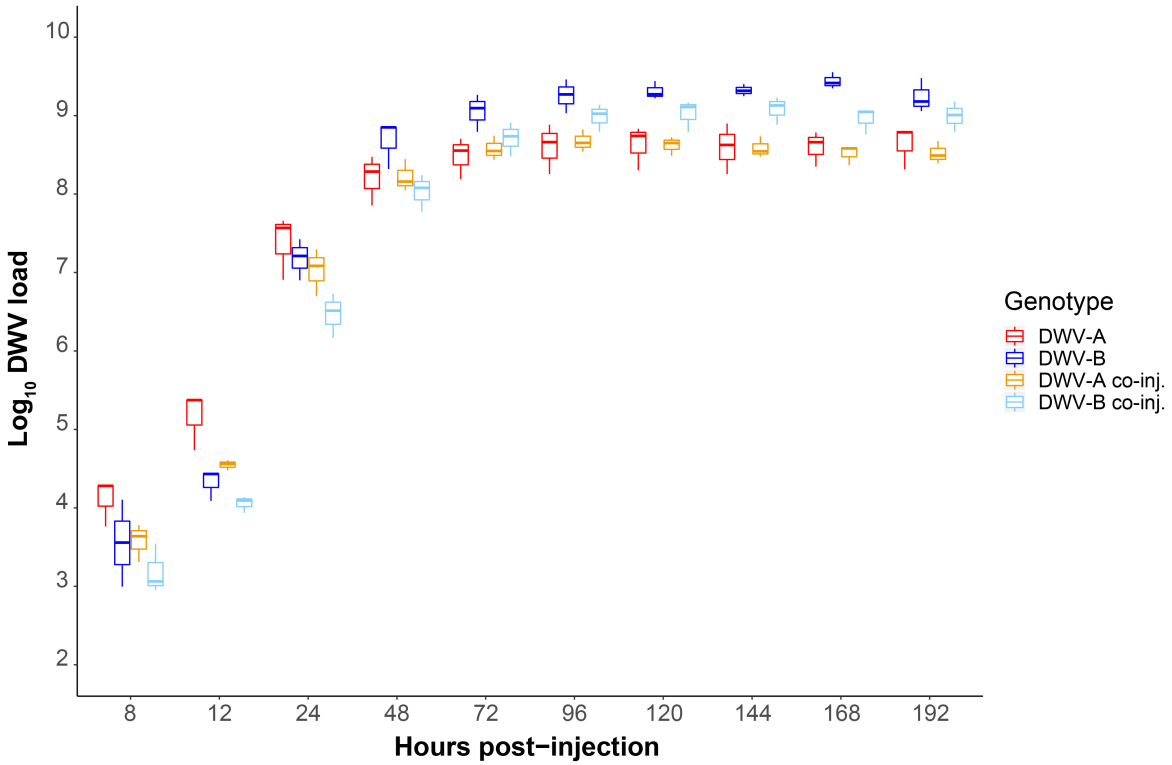


**Figure A.4.** Comparison of $\log_{10}$ DWV viral loads of pupae singly infected with DWV-A or DWV-B versus pupae co-injected with DWV-A and DWV-B, relative to housekeeping gene *Actin*. Colonies 1-3 have been combined (per treatment) to illustrate the distribution of viral accumulation per genotype.

**Table A.1.** Comparison of pairwise identity values, generated from pairwise Muscle alignments of whole genome sequences in Geneious. DWV-A (MN538208) and DWV-B (MN538209) inocula sequences obtained in this study were compared to DWV-A (AJ489744.2) and DWV-B (AY251269.2) reference sequences, in addition to the DWV-A isolate we previously injected in Remnant et al. (2019) and those experimentally injected by Tehel et al. (2019) and Gisder et al. (2018).

| | DWV-A (this study) MN538208 | DWV-A (AJ489744.2) (Lanzi et al., 2006) | DWV-A Remnant et al. (2019) | DWV-A Tehel et al. (2019) | DWV-$_{P0}$ I Gisder et al. (2018) | DWV-$_{P0}$ II Gisder et al. (2018) |
|---|---|---|---|---|---|---|
| DWV-A (AJ489744.2) | 98.50% | | | | | |
| DWV-A Remnant et al. (2019) | 99.50% | 98.72% | | | | |
| DWV-A Tehel et al. (2019) | 97.30% | 97.70% | 97.46% | | | |
| DWV-$_{P0}$ I Gisder et al. (2018) | 91.20% | 91.50% | 91.29% | 91.80% | | |
| DWV-$_{P0}$ II Gisder et al. (2018) | 84.70% | 85.00% | 84.87% | 84.90% | 90.70% | |
| DWV-$_{P0}$ III Gisder et al. (2018) | 85.80% | 85.90% | 85.85% | 86.00% | 91.60% | 94.50% |

| | DWV-B (this study) MN538209 | DWV-B (AY251269.2) (Ongus et al., 2004) | DWV-B Tehel et al. (2019) | DWV-$_{P0}$ I Gisder et al. (2018) | DWV-$_{P0}$ II Gisder et al. (2018) |
|---|---|---|---|---|---|
| DWV- B (AY251269.2) | 99.30% | | | | |
| DWV-B Tehel et al. (2019) | 99.20% | 98.90% | | | |
| DWV-$_{P0}$ I Gisder et al. (2018) | 91.40% | 91.40% | 91.40% | | |
| DWV-$_{P0}$ II Gisder et al. (2018) | 95.00% | 94.90% | 94.90% | 90.70% | |
| DWV-$_{P0}$ III Gisder et al. (2018) | 96.90% | 96.80% | 96.80% | 91.60% | 94.50% |

**Table A.2.** List of the primers used for viral detection by endpoint PCR and quantitative PCR. (ARV: *Apis rhabdovirus*, BQCV: *Black queen cell virus*, DWV: *Deformed wing virus*, LSV: *Lake Sinai virus*, SBV: *Sacbrood virus*).

| Virus | Primer name | Sequence | Product size (bp) | Primer efficiency | Reference |
|---|---|---|---|---|---|
| **LSV** | LSV_2108_F | TCATCCMAAGAGAACCA | 400 | - | This study |
| | LSV_uni_R | GTCAAAGGTGTCGTATCC | | | |
| **qPCR primers** | | | | | |
| **DWV** | DWV_F | TACTAGTGCTGGTTTTCCTTT | 155 | 1.91 | Kevill et al. (2017) |
| | DWV-A_R | CTCATTAACTGTGTCGTTGAT | | | |
| | DWV-B_R | CTCATTAACTGAGTTGTTGTC | 155 | 1.92 | |
| **BQCV** | BQCV_qPCR_F | AGGTTTACGCTCCAAGATCG | 112 | 1.73 | Remnant et al. (2019) |
| | BQCV_qPCR_R | TTTGTTCAGCAGGTAAATTGTTC | | | |
| **SBV** | SBV_ qPCR_F | CGAGGAGGGAAAAACTACGC | 115 | - | Remnant et al. (2019) |
| | SBV_ qPCR_R | GTGGCTTAACTGGATCATAGCC | | | |
| **ARV-1** | ARV-1_F | ATGAGGCTTGGAGACACAGC | 100 | - | This study |
| | ARV-1_R | GGAGCTTTCCTGAGGACACG | | | |
| **ARV-2** | ARV-2_F | CTAAACCCCACCTGTCTGCC | 150 | - | This study |
| | ARV-2_R | ATTGAGCACTGGAGCGTTGG | | | |
| ***Actin*** | Actin_F | TGCCAACACTGTCCTTTCTG | 155 | 1.70 | Scharlaken et al. (2008) |
| | Actin_R | AGAATTGACCCACCAATCCA | | | |

**Table A.3.** Two-way ANOVA comparing mean DWV loads relative to housekeeping gene *Actin* in pupae singly injected with DWV-A, DWV-B, DWV-rec, or co-injected with DWV-A and DWV-B, from 48 to 192 hours post injection ($n$ = 401). Comparison of DWV loads between genotype and colonies (1-3) over time. Data plotted in Figure 2.2 and Figure 2.3, where we have presented the mean relative viral loads as single injection by colony (DWV-A, DWV-B and DWV-rec), and single injection versus co-injection (DWV-A and DWV-B), respectively.

| **Summary** | ***df*** | **Sum sq** | **Mean sq** | **F value** | ***p*** |
|---|---|---|---|---|---|
| DWV genotype | 4 | 344421 | 86105 | 248.642 | <0.0001 |
| hour post injection (HPI) | 1 | 76077 | 76077 | 219.685 | <0.0001 |
| colony | 2 | 102420 | 51210 | 147.876 | <0.0001 |
| DWV genotype:HPI | 4 | 21228 | 5307 | 15.325 | <0.0001 |
| DWV genotype:colony | 8 | 13400 | 1675 | 4.837 | <0.0001 |
| residuals | 381 | 131941 | 346 | | |

**Table A.4.** Tukey HSD post-hoc analysis of two-way ANOVA (S3 Table), showing pairwise comparisons of mean relative viral loads by genotype and colony. Lettering denotes genotype followed by numbering 1-3, which indicates colony (A: DWV-A single injection, B: DWV-B single injection, R: DWV-rec single injection, CA: DWV-A co-injection, and CB: DWV-B co-injection). Significant comparisons ($p < 0.05$) denoted as letters on Figure 2.2 and Figure 2.3.

| Comparison | *df* | t.ratio | *p* | Comparison | *df* | t.ratio | *p* |
|---|---|---|---|---|---|---|---|
| A,1 - B,1 | 381 | -13.138 | <.0001 | R,1 - A,2 | 381 | 3.061 | 0.1358 |
| A,1 - CA,1 | 381 | -2.643 | 0.3451 | R,1 - B,2 | 381 | -9.501 | <.0001 |
| A,1 - CB,1 | 381 | -6.152 | <0.0001 | R,1 - CA,2 | 381 | 4.412 | 0.0013 |
| A,1 - R,1 | 381 | -11.214 | <.0001 | R,1 - CB,2 | 381 | -1.563 | 0.9654 |
| A,1 - A,2 | 381 | -8.205 | <0.0001 | R,1 - R,2 | 381 | -9.900 | <.0001 |
| A,1 - B,2 | 381 | -21.109 | <.0001 | R,1 - A,3 | 381 | 5.429 | <.0001 |
| A,1 - CA,2 | 381 | -6.931 | <.0001 | R,1 - B,3 | 381 | -5.388 | <.0001 |
| A,1 - CB,2 | 381 | -13.02 | <.0001 | R,1 - CA,3 | 381 | 6.912 | <.0001 |
| A,1 - R,2 | 381 | -22.646 | <.0001 | R,1 - CB,3 | 381 | 0.546 | 1.0000 |
| A,1 - A,3 | 381 | -5.679 | <.0001 | R,1 - R,3 | 381 | -9.075 | <.0001 |
| A,1 - CA,3 | 381 | -16.008 | <.0001 | R,2 - A,3 | 381 | 16.592 | <.0001 |
| A,1 - CA,3 | 381 | -4.175 | 0.0033 | R,2 - B,3 | 381 | 5.018 | <.0001 |
| A,1 - CB,3 | 381 | -10.871 | <.0001 | R,2 - CA,3 | 381 | 18.093 | <.0001 |
| A,1 - R,3 | 381 | -20.457 | <.0001 | R,2 - CB,3 | 381 | 11.874 | <.0001 |
| A,2 - B,2 | 381 | -12.71 | <.0001 | R,2 - R,3 | 381 | 1.955 | 0.0631 |
| A,2 - CA,2 | 381 | 1.337 | 0.9916 | CA,1 - CB,1 | 381 | -3.509 | 0.0371 |
| A,2 - CB,2 | 381 | -4.696 | <.0004 | CA,1 - R,1 | 381 | -8.62 | <.0001 |
| A,2 - R,2 | 381 | -14.311 | <.0001 | CA,1 - A,2 | 381 | -5.585 | <.0001 |
| A,2 - A,3 | 381 | 2.418 | 0.5027 | CA,1 - B,2 | 381 | -18.467 | <.0001 |
| A,2 - B,3 | 381 | -8.31 | <.0001 | CA,1 - CA,2 | 381 | -4.289 | 0.0021 |
| A,2 - CA,3 | 381 | 3.915 | 0.0091 | CA,1 - CB,2 | 381 | -10.377 | <.0001 |
| A,2 - CB,3 | 381 | -2.567 | 0.3956 | CA,1 - R,2 | 381 | -20.028 | <.0001 |
| A,2 - R,3 | 381 | -12.221 | <.0001 | CA,1 - A,3 | 381 | -3.089 | 0.1263 |
| A,3 - B,3 | 381 | -10.497 | <.0001 | CA,1 - B,3 | 381 | -13.572 | <.0001 |
| A,3 - CA,3 | 381 | 1.481 | 0.9783 | CA,1 - CA,3 | 381 | -1.582 | 0.9617 |
| A,3 - CB,3 | 381 | -4.983 | <.0001 | CA,1 - CB,3 | 381 | -8.229 | <.0001 |
| A,3 - R,3 | 381 | -14.502 | <.0001 | CA,1 - R,3 | 381 | -17.863 | <.0001 |
| B,1 - CA,1 | 381 | 10.545 | <.0001 | CA,2 - CB,2 | 381 | -6.089 | <.0001 |
| B,1 - CB,1 | 381 | 7.102 | <.0001 | CA,2 - R,2 | 381 | -15.779 | <.0001 |
| B,1 - R,1 | 381 | 1.891 | 0.8565 | CA,2 - A,3 | 381 | 1.118 | 0.9987 |
| B,1 - A,2 | 381 | 4.969 | <.0001 | CA,2 - B,3 | 381 | -9.62 | <.0001 |
| B,1 - B,2 | 381 | -7.576 | <.0001 | CA,2 - CA,3 | 381 | 2.626 | 0.3557 |
| B,1 - CA,2 | 381 | 6.337 | <.0001 | CA,2 - CB,3 | 381 | -3.94 | <.0083 |
| B,1 - CB,2 | 381 | 0.363 | 1.000 | CA,2 - R,3 | 381 | -13.655 | <.0001 |
| B,1 - R,2 | 381 | -9.206 | <.0001 | CA,3 - CB,3 | 381 | -6.493 | <.0001 |
| B,1 - A,3 | 381 | 7.319 | <.0001 | CA,3 - R,3 | 381 | -15.989 | <.0001 |
| B,1 - B,3 | 381 | -3.602 | <.0001 | CB,1 - R,1 | 381 | -5.177 | <.0001 |
| B,1 - CA,3 | 381 | 8.802 | <.0001 | CB,1 - A,2 | 381 | -2.109 | 0.7284 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| B,1 - CB,3 | 381 | 2.471 | 0.4639 | CB,1 - B,2 | 381 | -14.958 | <.0001 |
| B,1 - R,3 | 381 | -7.184 | <.0001 | CB,1 - CA,2 | 381 | -0.779 | 1.000 |
| B,2 - CA,2 | 381 | 14.178 | <.0001 | CB,1 - CB,2 | 381 | -6.869 | <.0001 |
| B,2 - CB,2 | 381 | 8.089 | <.0001 | CB,1 - R,2 | 381 | -16.551 | <.0001 |
| B,2 - R,2 | 381 | -1.730 | 0.9227 | CB,1 - A,3 | 381 | 0.353 | 1.000 |
| B,2 - A,3 | 381 | 15.026 | <.0001 | CB,1 - B,3 | 381 | -10.338 | <.0001 |
| B,2 - B,3 | 381 | 3.445 | 0.0454 | CB,1 - CA,3 | 381 | 1.862 | 0.8704 |
| B,2 - CA,3 | 381 | 16.54 | <.0001 | CB,1 - CB,3 | 381 | -4.720 | <.0003 |
| B,2 - CB,3 | 381 | 10.238 | <.0001 | CB,1 - R,3 | 381 | -14.42 | <.0001 |
| B,2 - R,3 | 381 | 0.259 | 1.000 | CB,2 - R,2 | 381 | -9.745 | <.0001 |
| B,3 - A,1 | 381 | -16.008 | <.0001 | CB,2 - A,3 | 381 | 7.091 | <.0001 |
| B,3 - CA,3 | 381 | 11.894 | <.0001 | CB,2 - B,3 | 381 | -4.008 | <.0064 |
| B,3 - CB,3 | 381 | 5.988 | <.0001 | CB,2 - CA,3 | 381 | 8.602 | <.0001 |
| B,3 - R,3 | 381 | -3.154 | 0.1062 | CB,2 - CB,3 | 381 | 2.149 | 0.7009 |
| | | | | CB,2 - R,3 | 381 | -7.680 | <.0001 |
| | | | | CB,3 - R,3 | 381 | -9.788 | <.0001 |

**Table A.5.** Two-way ANOVA repeated as above, with the inclusion of three pupae with low DWV loads for their time-point.

| **Summary** | ***df*** | **Sum sq** | **Mean sq** | **F value** | ***p*** |
|---|---|---|---|---|---|
| DWV strain | 4 | 351858 | 87965 | 167.09 | <0.0001 |
| hour post injection (HPI) | 1 | 65820 | 65820 | 125.03 | <0.0001 |
| colony | 2 | 94129 | 47064 | 89.40 | <0.0001 |
| DWV strain:HPI | 4 | 21683 | 5421 | 10.20 | <0.0001 |
| DWV strain:colony | 8 | 16258 | 2032 | 3.86 | <0.001 |
| residuals | 384 | 202685 | 526 | | |

**Table A.6.** Tukey HSD post-hoc analysis of repeated two-way ANOVA (Table A.5), showing the three pairwise comparisons that were significantly different in the repeated analysis.

| **Comparison** | ***df*** | **t.ratio** | ***p*** |
|---|---|---|---|
| B,1 - B,3 | 384 | -3.038 | 0.144 |
| B,2 - B,3 | 384 | 2.74 | 0.2848 |
| CA,2 - CA,3 | 384 | 3.592 | 0.0282 |

**Table A.7.** Analysis of the mean proportion of survival of pupae subjected to five treatments (buffer control, DWV-A, DWV-B, DWV-rec and co-injection) at 192 hours post injection. We fit a generalized linear mixed effects model (glmer) with binomial distribution and logit link function, comparing mean proportion of survival per treatment. Colony had no effect on survival (using Akaike's information criterion during backward elimination; Table A.9), thus was included in the model as a random factor. We then analysed the final model as a type II ANOVA. As treatment was a significant predictor of survival, we subsequently ran a Tukey pairwise comparison (Table A.8).

| **Response: Proportion Survival** | **Chi sq** | ***df*** | ***p*** |
|---|---|---|---|
| (Intercept) | 92.583 | 1 | < 0.0001 |
| treatment | 44.472 | 4 | < 0.0001 |

**Table A.8.** Tukey post-hoc analysis of ANOVA (Table A.7); pairwise comparisons of mean proportion of survival between treatments.

| **Comparison** | **z. ratio** | ***p*** |
|---|---|---|
| Buffer Control - DWV-A | -4.384 | < 0.0001 |
| Buffer Control - DWV-B | 1.835 | 0.3532 |
| Buffer Control - DWV-rec | 0.280 | 0.9987 |
| Buffer Control - Co-injection | -2.586 | 0.0728 |
| DWV-A - DWV-B | 3.747 | 0.0017 |
| DWV-A - DWV-rec | 4.467 | < 0.0001 |
| DWV-A - Co-injection | 2.422 | 0.1095 |
| DWV-B - DWV-rec | -1.671 | 0.4522 |
| DWV-B - Co-injection | -3.036 | 0.0203 |
| DWV-rec - Co-injection | -2.778 | 0.0435 |

**Table A.9.** Model testing of mean proportion of survival data per treatment (buffer control, DWV-A, DWV-B, DWV-rec and co-injection) and colony. Akaike's information criterion obtained during backward elimination showed that colony was not a significant predictor of survival. Colony was thus included as a random factor in survival analyses.

| | ***df*** | **Deviance** | **AIC** | **LRT** | ***p*** |
|---|---|---|---|---|---|
| | | 512.07 | 526.07 | | |
| treatment | 4 | 579.34 | 585.34 | 67.275 | < 0.0001 |
| colony | 2 | 514.71 | 524.71 | 2.641 | 0.267 |

**Text A.1.** Partial contig sequence of the DWV-B isolate detected in low frequency in the DWV-rec inoculum, where Megahit average coverage per base was 408.0453.

>DWV-Rec_Netherlands_k141_295_multi=408.0453

AATCATATATTACTTTCTTAATTTTAAATAATAACATGGGCGATCGATTACAAACGGGCCTAACAACTTTCTCGCGCACAAAT
TTTGATGCCACCCGTTTCACTTTCTTACAAATCTTTGTATATTTGGGTTTCTTAAATACGAACTCACCCGCGTCTTTTCCTACCT
CAATCTTAAGGGATTCTAAAGGGGAATATCTATTACTGATTGAAATGGGGACATGTTCTTGAACAGGGCGGCGGGTACATCTT
TCAGCTATAGAATATATCGTGTATAAATTATTCAATTGTTCCGTAAGGAACTCATTATCACGCGCATCTTCTTGCTCCCACGTT
GTATGATCATACACAGTGACGTCAAGAACGTTTCGAATCCGTTCCTGTTCCAACGCCAAACGCTTAATAACGCGTCGACGCCT
AGCTTCATCAATCTCCCAACTACGGGGAGCATGAGCTACAGAGGGAGCTTGGGCAACAGCAGCATAAGAAAGAGTTCCACA
ACTAAATGCCATTTTTATATATATTTACCTTCAAAGTCAAAATCAAATTGAAAATATAAAATAAAAAGAATTGAATCTAATAT
ATAAAGCAAAAATAGCAAAGAAATATATTTAGCAATAATGAAAATAGCAAAATATAATAGCAAATAAAAACAGCAAAATAA
AACAGCAATAATCGTAGCATTAAAATATTAAATAATGTCTTTCCATTCGTCATCGTCTGGGCTATGTGACATTAACGGGTGCC
CTGCGAACGTAACGTACCGTGGCATAAATTATACTGTATTAAAAGGATTCTTGCCAAAGGAAATCCCAATACAGTCACTTATG
TATAGAGACATAAATTACTTTCACACTTTCGCCTCATACA

**Text A.2.** Partial contig sequence of the secondary DWV-recombinant isolate detected at very low frequency in the DWV-rec inoculum, where Megahit average coverage per base was 26.3092.

>DWV-Rec_Netherlands_k141_7331_multi=26.3092

AAGAATGGATATACGTGTTTAAAAGGTATCACTAATTTTGCTTCATTACTCGCTGATGCGCTAATCAAAGCATGATCCATATG
CGAAAAACCATACACACTTCGCTTCGTCTGGATATTCAAATTTTCATGATCCGAATAATACCAAGTTGCCTGTAATTGACCAA
CTTGGAATTTATTTGAATTAATTTGAACTCTAACTTCCATATCGCCTCGCCAATATGCGTGCACCTTAAATGGGATAGTATTAG
GCACATCACATATAGCATCAGAATTAGCCTCTATACTAGATAACAAAGCACGAGGCAAAATTAAACGTGCTAACTCCTTATC
AAATGGATCATCCTTCGACCAAACAAATTCAGCAATCTGATACCATCGAGATGTAATTGTGGCATAATCATCTACTACATCAT
TACTAGTCCATCTACTCCATTTTACGCTGACCGGCGCTGGAATAGATGTACTAGGATCTCGTTGAGTCGTTAAAACAACATTA
CTATCCTTTTCTAATTCAACTTCACCCTCGCCATCAGGTCCTGGATTAGGGTTATCCATCTCTGGTTTTGCCTGCACCGGATTCG
ATAATTGTAACAGACTAGTGACACACTCTAACTCATAATCGCGCTGTTTTTGACGTCGCAACATCCTAATCTGTTTTCTTAATC
TATATAAATGCAAATCATAAATTATCTTCTTAAGCTTAAATAATAGCATAGGGGATCTAGAACATATAGGACGAACAACTTTT
TCACGAACGAAGCGAGTTGCAACGCGCTTCACTTTCTTGCAAACGCGCGTATATTTAGGTTTCTTAAATATACATTCGCCTGCT
TCTTGACCGACCTCGACCTTAAGGGATTCCAGTGGAGCAAACCTATTCGAAACTGATATAGGAGAGTACTCTTTGATAGGCCG
ACGCGTACAACGTTCAGCGATCGAATAAATAGTATATAAGTTGTTTAATTGTTCCATTAGGAACTCATTATCGCGCGCGTCCT
CCTGTTCCCACGTTGCCTGGTCATAGACGTCAGCGTCAAGCACGTTACGAATACGTTCTTGCTCCAACGCCAAACGTTTAATA
ACTCGGCGCCGCCTAGCTTCATCTACTTCCCATGTACGAGGTGCATGGGCGACAGATGGAGCTTGAGCGACGGCAGAGTAAG
AAAGAATTCCACAACTAAATGCCATTTTTATATATATTTACCTTGAAAATCAAAATCAAATTGAAAATATAAAATAAAAAGA
ATTGAATCTAATATATAAAGCAAAAATAGCAAAGAAATATATTTAGCAATAATGAAAATAGCAAAATATAATAGCAAATAA
AAACAGCAAAATAAAACAGCAATAA

# Supplementary Material for Chapter 3

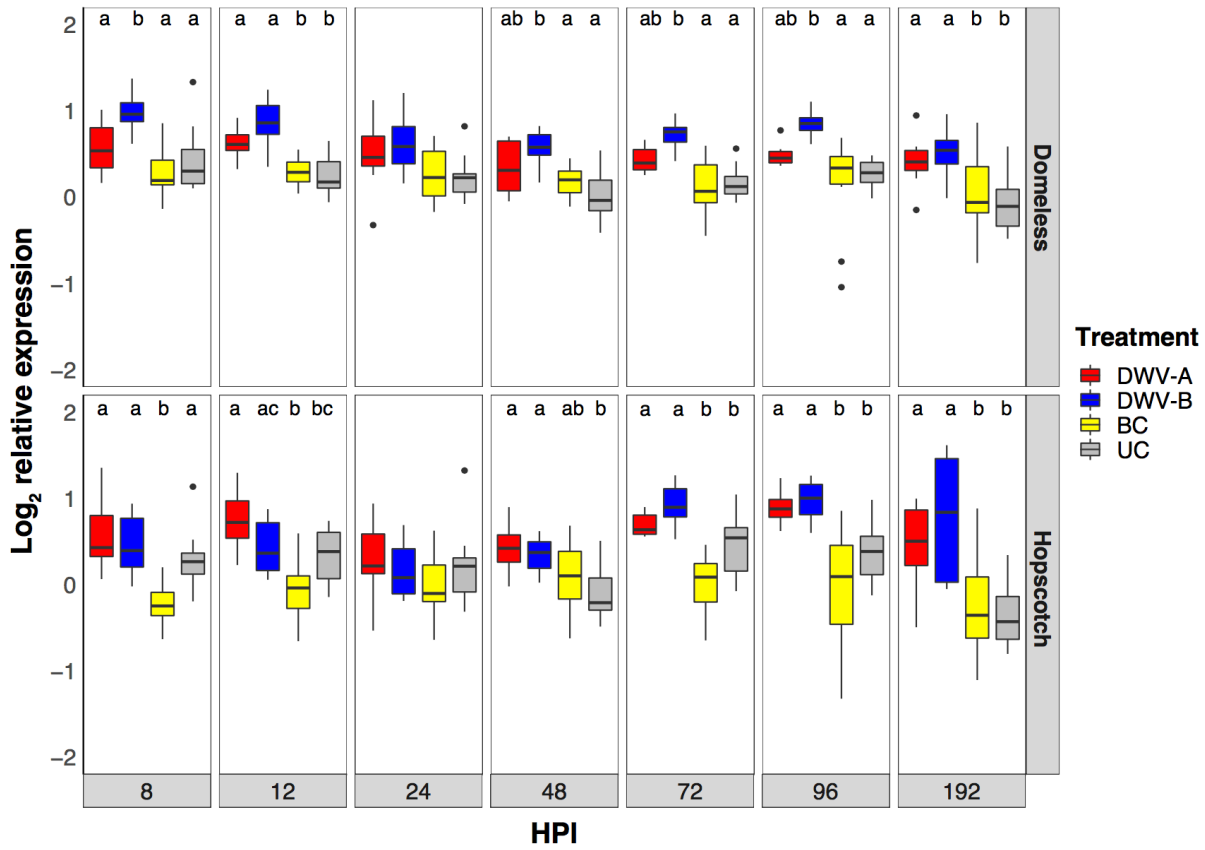


**Figure B.1.** $Log_2$ relative expression of JAK/STAT pathway genes *domeless* and *hopscotch* in honey bee pupae. Treatment groups that do not share a common letter differ at $p < 0.05$ at each hour post-injection (HPI). No letters above a given time point indicates that no significant differences were detected between treatment groups.

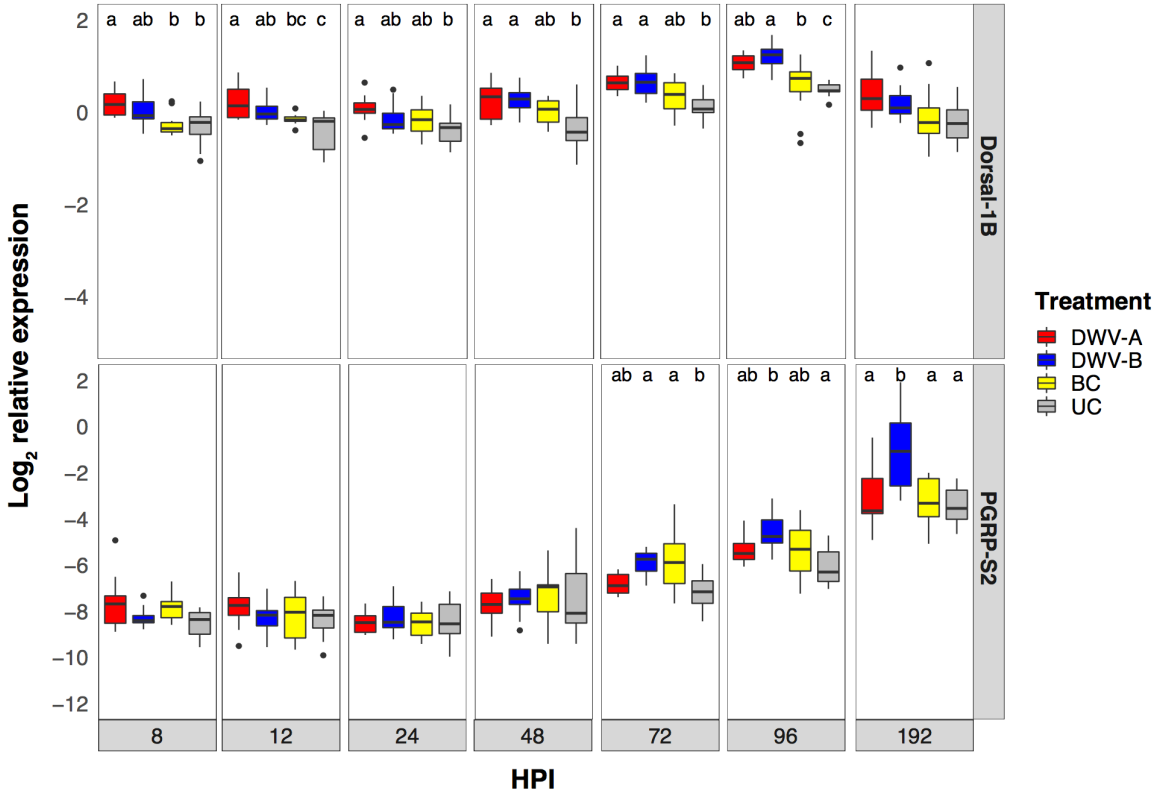


**Figure B.2**. $Log_2$ relative expression of Toll pathway genes *dorsal-1B* and *PGRP-2* in honey bee pupae. Treatment groups that do not share a common letter differ at $p < 0.05$ at each hour post-injection (HPI). No letters above a given time point indicates that no significant differences were detected between treatment groups.

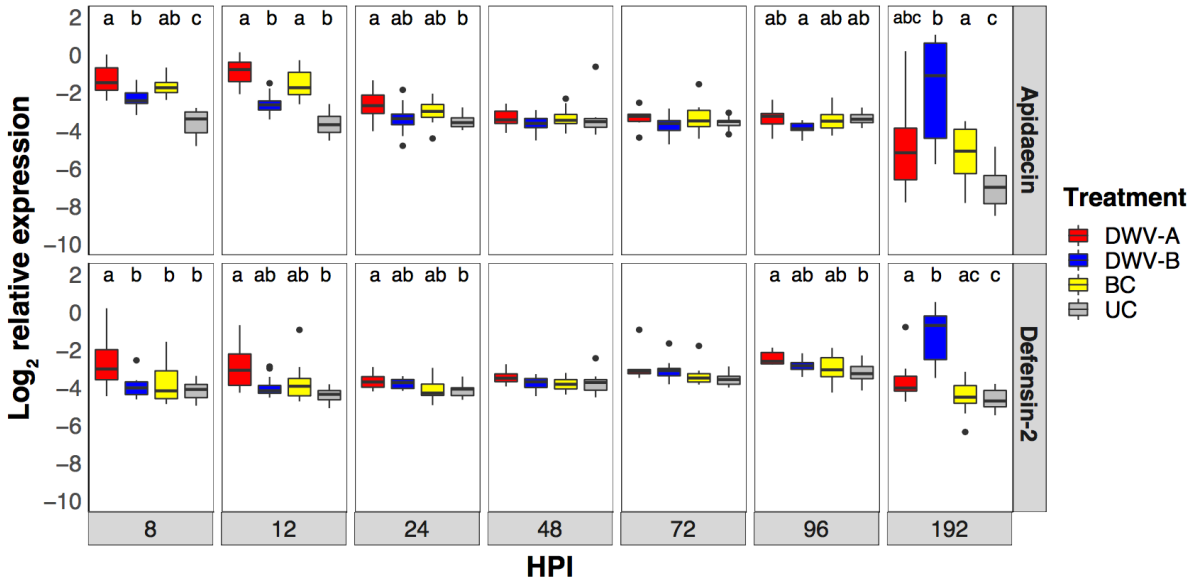


**Figure B.3.** $Log_2$ relative expression of AMP genes *apidaecin* and *defensin-2* in honey bee pupae. Treatment groups that do not share a common letter differ at $p < 0.05$ at each hour post-injection (HPI). No letters above a given time point indicates that no significant differences were detected between treatment groups.

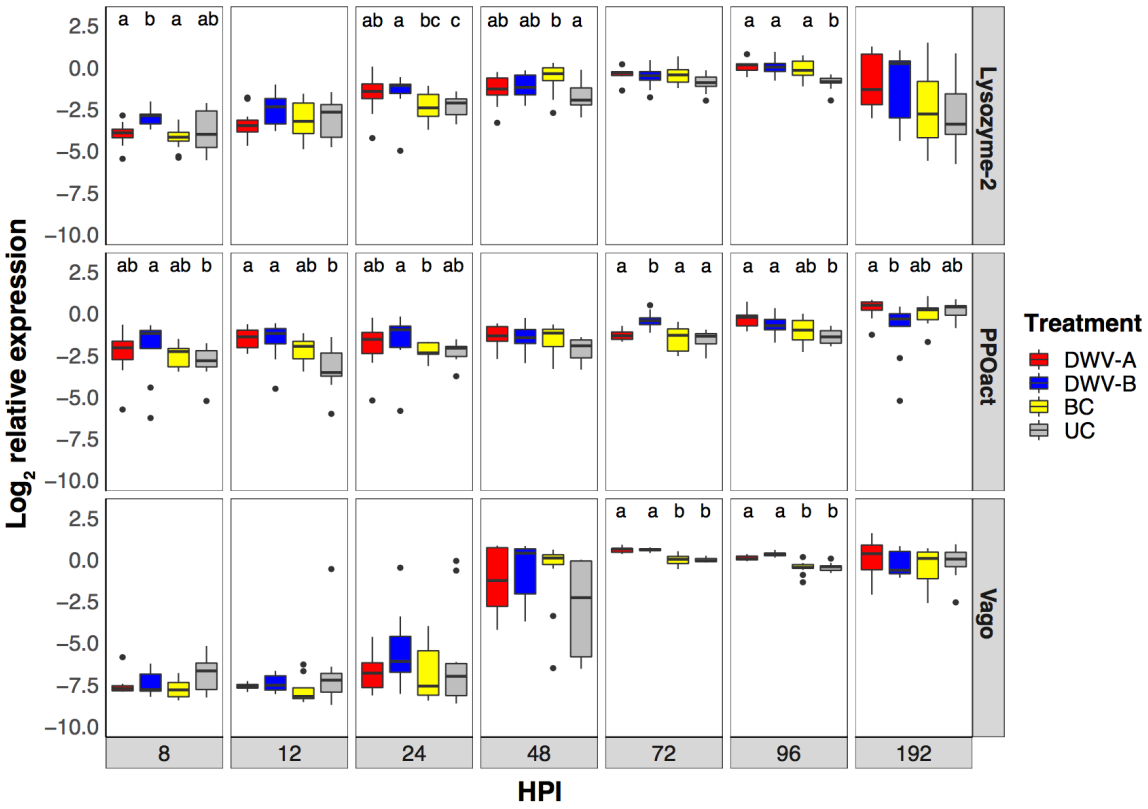


**Figure B.4.** $Log_2$ relative expression of genes *lysozyme-2, PPOact* and *vago* in honey bee pupae. Treatment groups that do not share a common letter differ at $p < 0.05$ at each hour post-injection (HPI). No letters above a given time point indicates that no significant differences were detected between treatment groups.

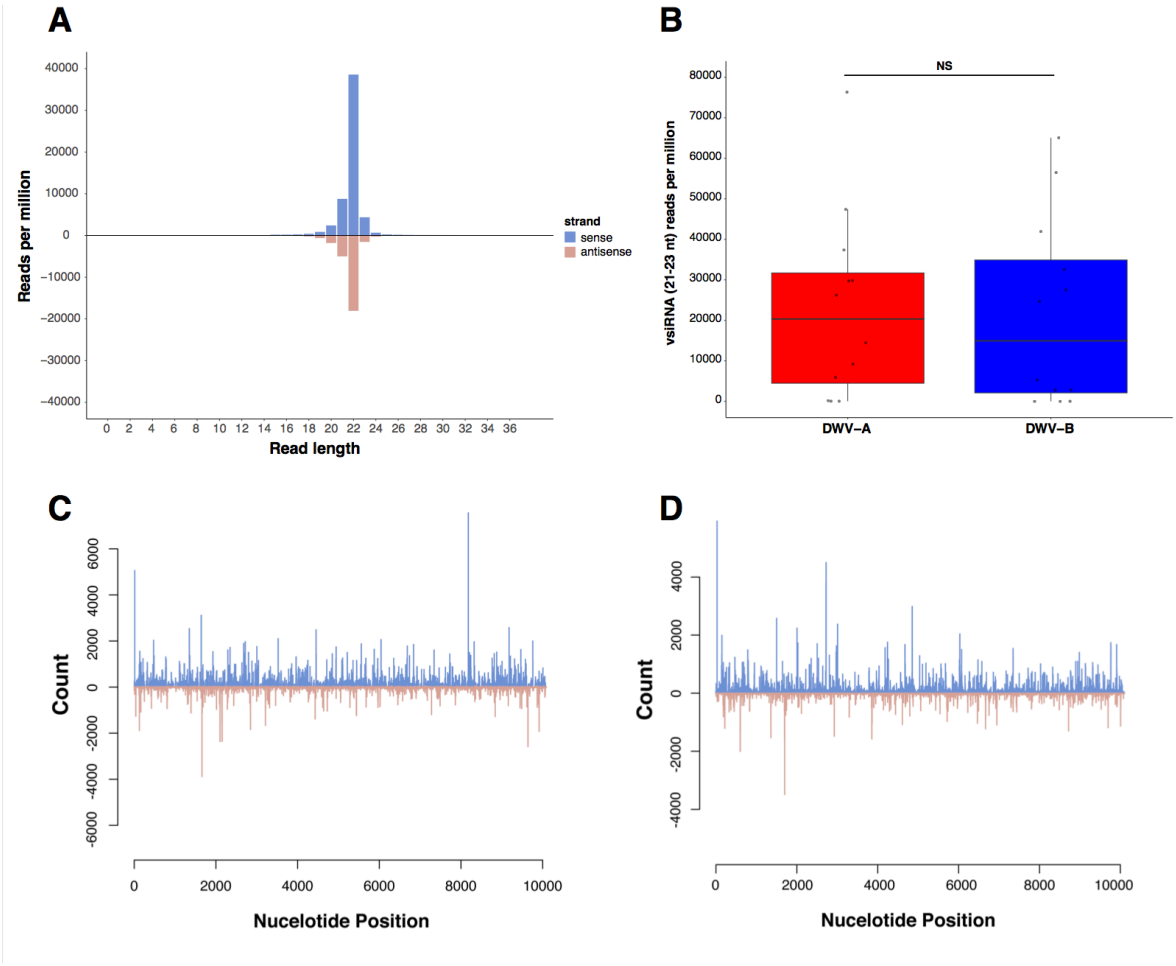


**Figure B.5.** (A) Size distribution small RNA read lengths mapping to DWV. (B) Number of normalised vsiRNA (21-23 nt) reads mapping to DWV in pupae injected with DWV-A or DWV-B did not differ between the two genotypes ($\chi^2$= 0.27, df = 1, $p$ = 0.6033). Distribution of vsiRNA (21-23 nt) reads mapping to the (C) DWV-A and (D) DWV-B genomes. Data from one representative sample shown in figures 5A, 5C and 5D.

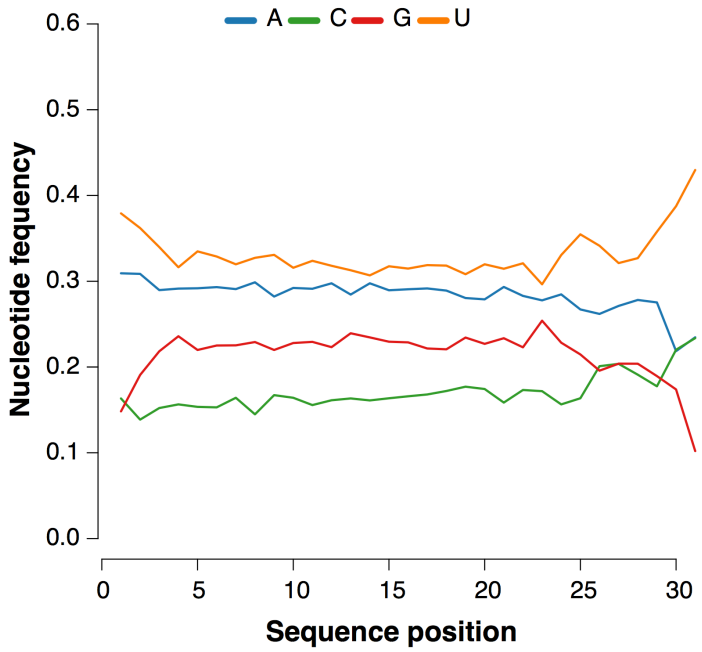


**Figure B.6.** Nucleotide frequency of small 26-31 nt length RNA reads. We observed elevated frequencies of uridine at position 1, but did not detected enrichment of adenine at position 10. Thus, no evidence of a 'ping-pong' signature associated with piRNA activity was detected.

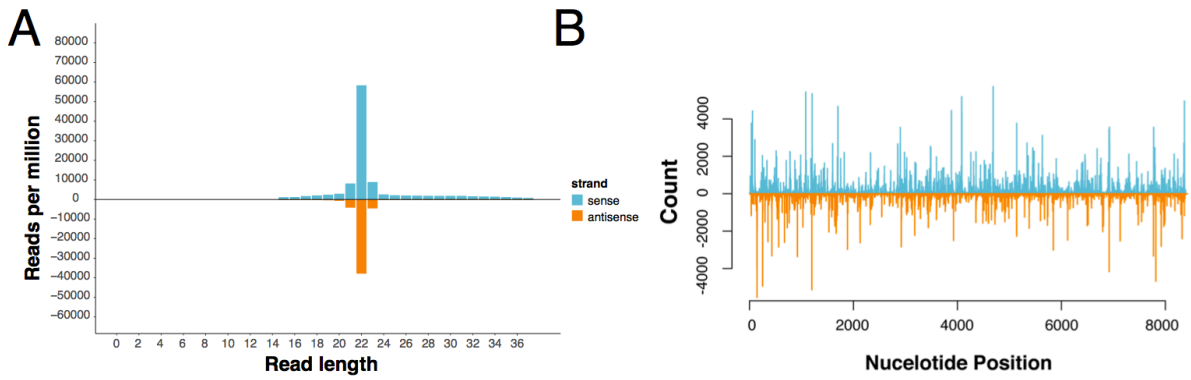


**Figure B.7.** A) Size distribution small RNA read lengths mapping to BQCV. Distribution of vsiRNA (21-23 nt) reads mapping to the BQCV genome. Data from one representative sample shown.

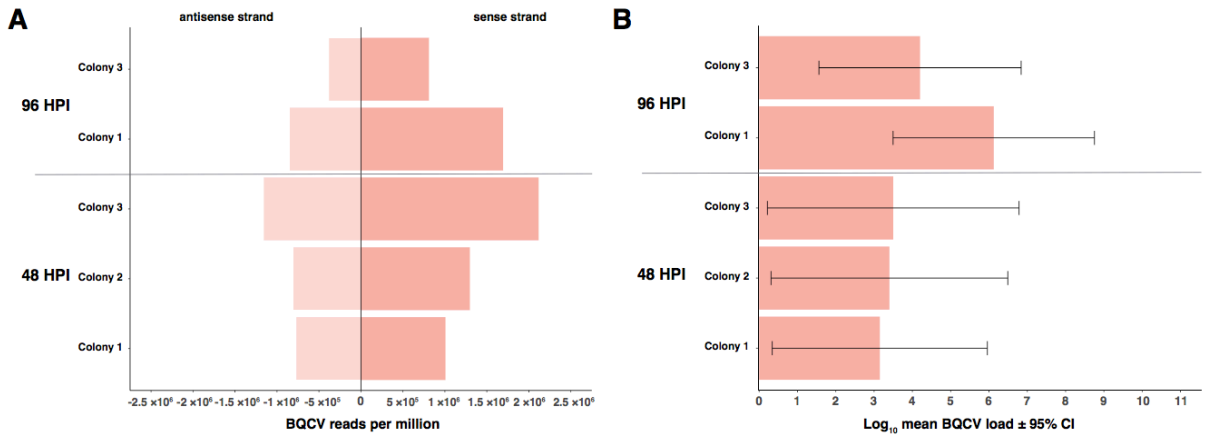


**Figure B.8.** (A) Normalised siRNA (21-23 nt) reads mapping to BQCV in four pooled DWV-A injected pupae at 48 and 96 HPI, per colony. No reads mapping to BQCV were detected in the pooled sample from colony 2 at 96 HPI. (B) The mean BQCV loads ± 95% CI of the same four pupae. The large confidence intervals indicate the high variability in BQCV loads between the four pupae. Viral loads are relative to housekeeping gene *Actin* and were previously published in Norton et al. (2020), but presented here to illustrate the relationship between viral accumulation and siRNA response to BQCV. We did not detect a correlation between BQCV load and small RNA response ($r_s = 0.2$; $P = 0.7471$).

# Supplementary Material for Chapter 4

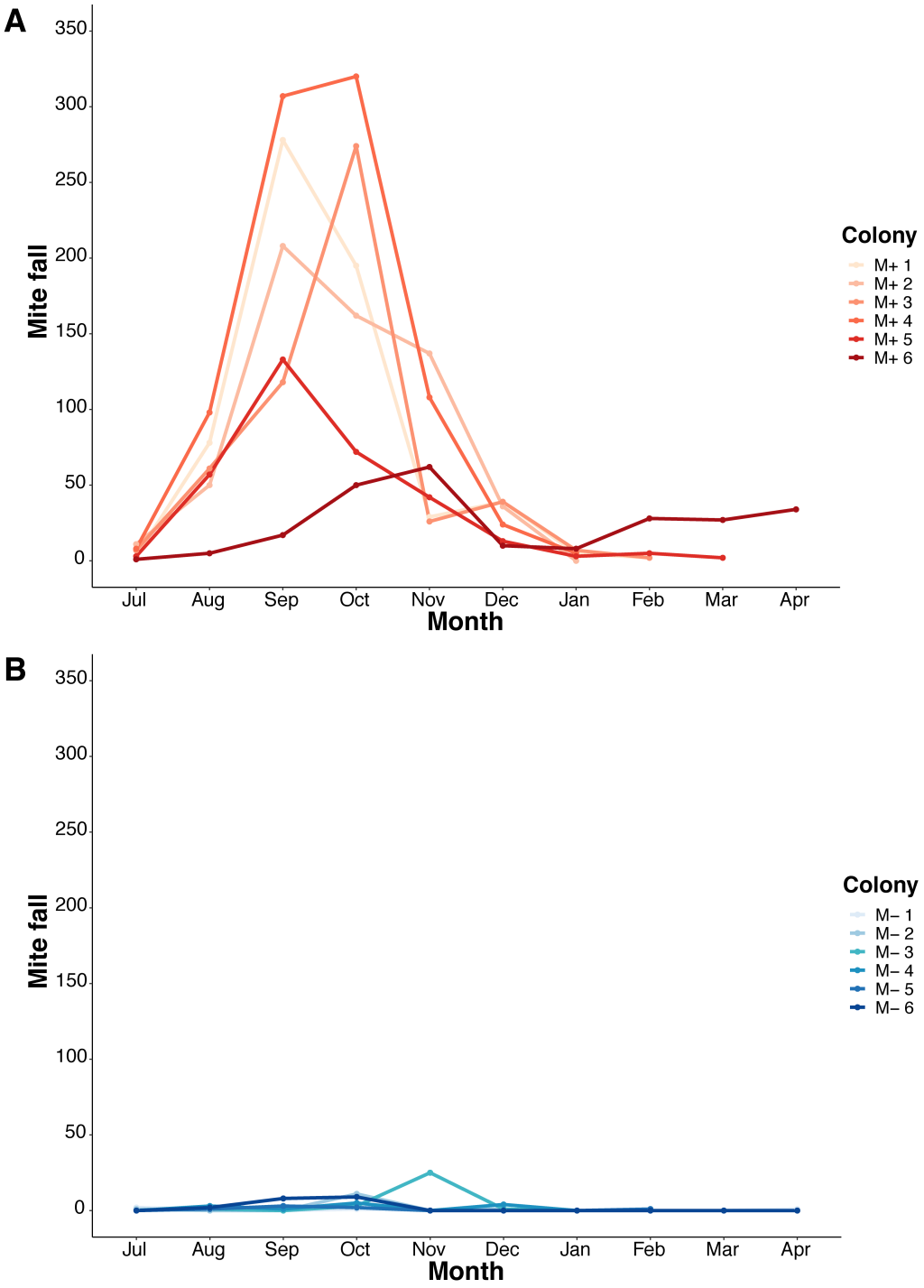


**Figure C.1.** Number of fallen mites within 48 h in **(A)** Mite Added (M+) and **(B)** Mite Reduced (M-) colonies between July 2018 and April 2019, or until colony death.

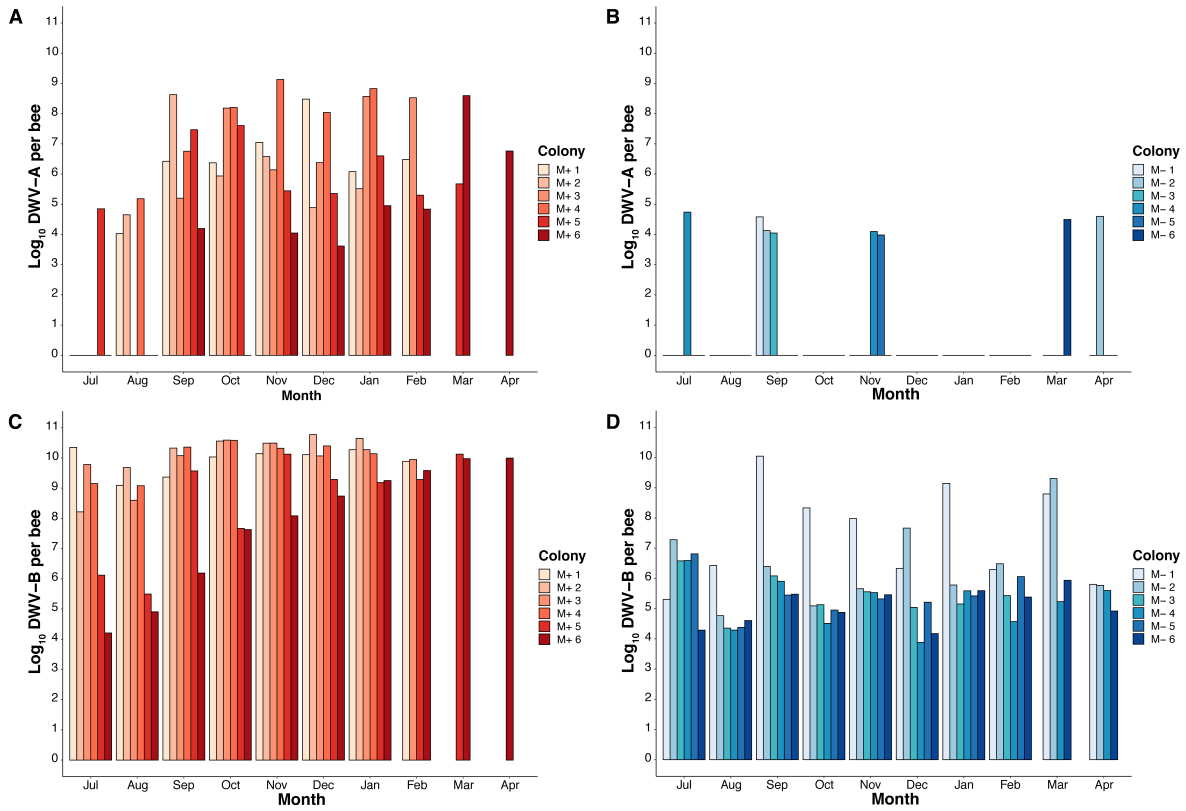


**Figure C.2.** $Log_{10}$ DWV-A (**A** and **B**) and DWV-B (**C** and **D**) loads per bee in individual Mite Added (M+) or Mite Reduced (M-) colonies. DWV loads calculated from ten pooled honey bees sampled monthly between July 2018 and April 2019, or up until colony death.

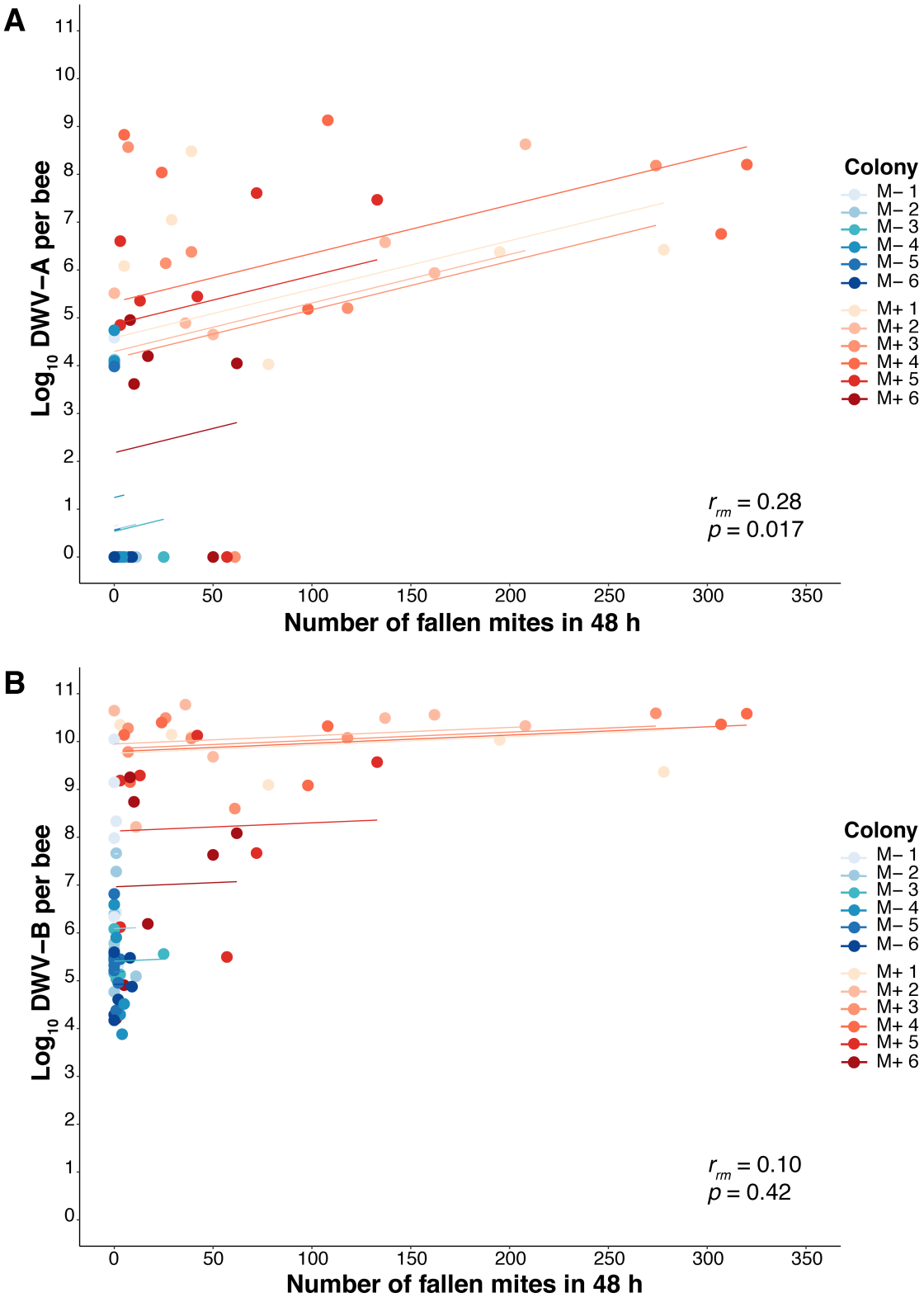


**Figure C.3.** Repeated measures correlation between (**A**) mean DWV-A or (**B**) DWV-B load per bee and mite fall in both M+ and M- colonies. The analysis determined the within-colony association between DWV load and mite fall measured monthly between July 2018 and January 2019.

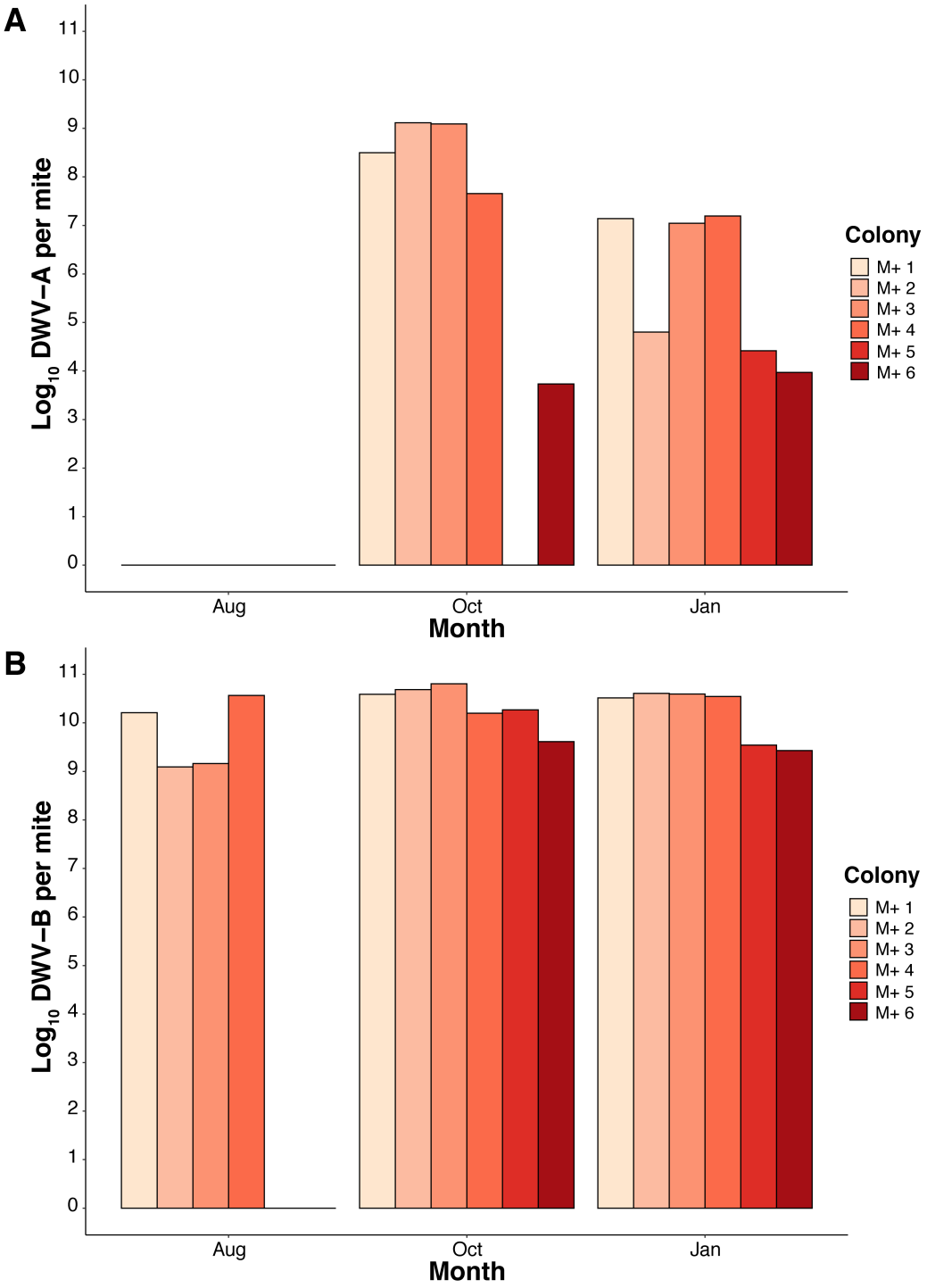


**Figure C.4.** $Log_{10}$ DWV-A (**A**) and DWV-B (**B**) loads per mite in individual Mite Added (M+) colonies. DWV loads calculated six-ten pooled *V. destructor* collected from M+ colonies in August, October and January. Missing data points indicate where either genotype was not detected in the pooled mite sample.

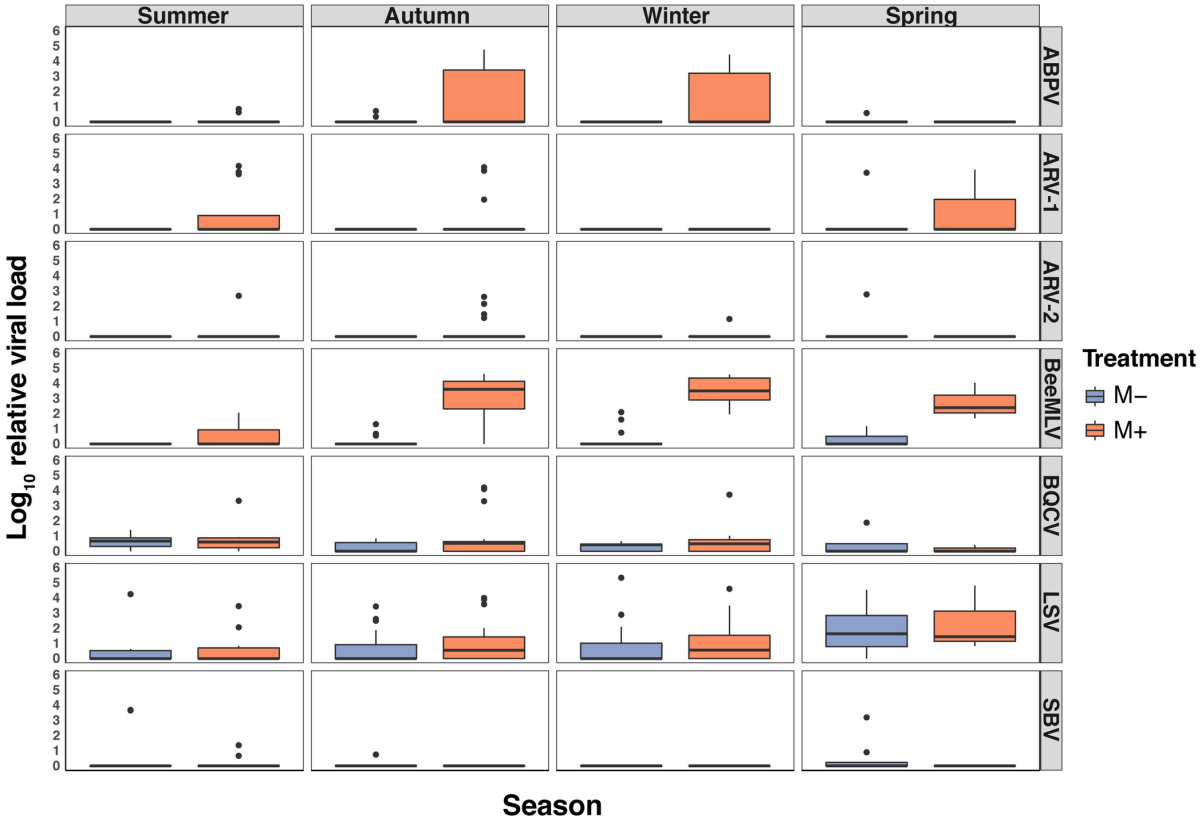


**Figure C.5.** Boxplot comparisons of the average viral load ($log_{10}$) per bee of the additional viruses detected in pooled honey bee samples from M- and M+ colonies across each season (*n* = 105). Viral loads are relative to endogenous control gene *Actin* and calculated using the Pfaffl expression ratio, which accounts for differences in qPCR primer efficiencies (Pfaffl, 2001).

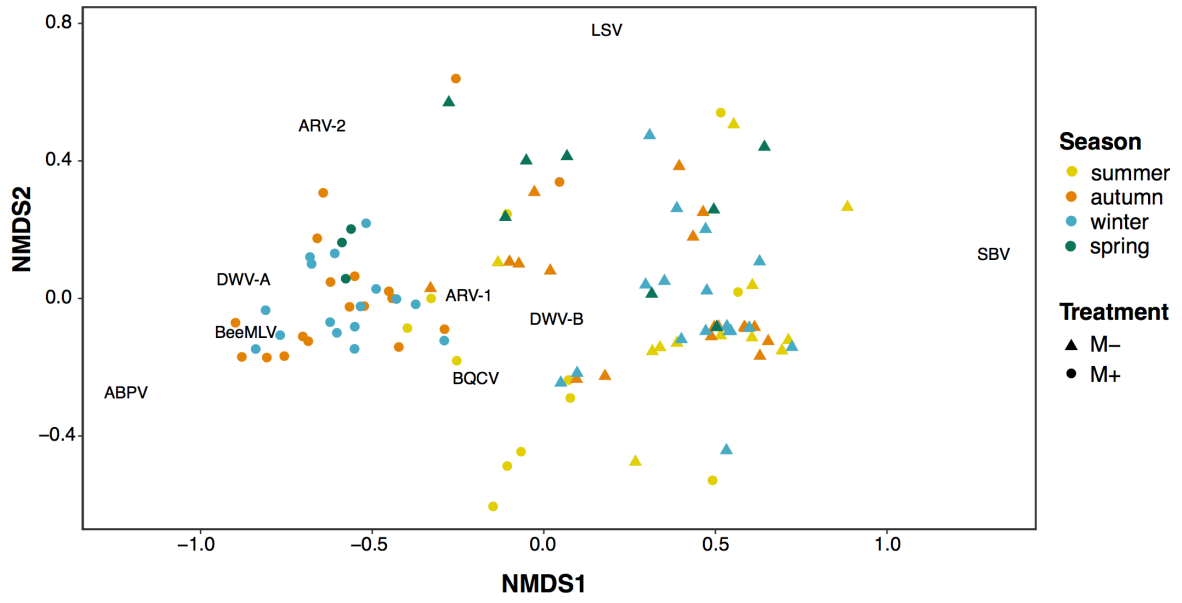


**Figure C.6.** Non-metric multidimensional scaling (NMDS) plot (k = 2) of viral species abundance in pooled honey bee samples in M+ and M- colonies. Each point is colour coded by season and shaped according to mite treatment. Our corresponding PERMANOVA indicated that treatment explained the largest amount of variance in the pathogen composition ($F_{1,95} = 100.5$, $R_2 = 0.44$, $p = 0.001$). The PERMANOVA indicated that season alone ($F_{3,95} = 6.59$, $R_2 = 0.085$, $p = 0.001$) and an interaction between treatment × season ($F_{3,95} = 3.44$, $R_2 = 0.045$, $p = 0.004$) explained a small amount of the variability in the viral community structure. After correction for multiple comparisons, only a small difference between summer and autumn ($F_1 = 6.21$, $R_2 = 0.097$, $p = 0.036$) was found.

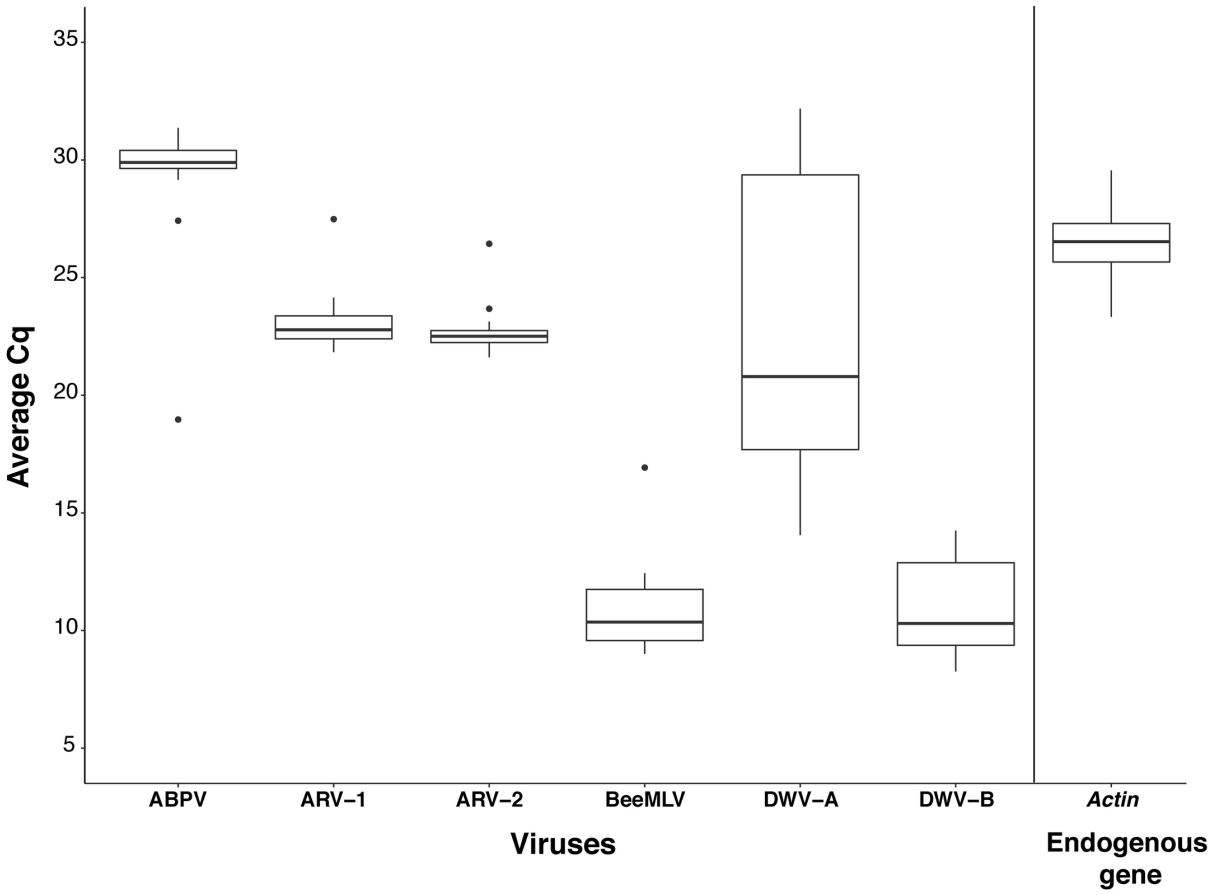


**Figure C.7.** Average Cq values of all viruses detected in pooled mite samples from M+ colonies, collected in August, October and January ($n$ = 18). Averages were obtained from duplicate qPCR reactions. Low Cq values are indicative of high amounts of the target sequence and *vice versa*. BQCV, LSV and SBV were not detected in mites. RNA was successfully extracted from all pooled mite samples, as illustrated by the amplification of the endogenous control gene *Actin*.

**Table C.1**. List of qPCR primers

| | Primer name | Sequence | Product size (bp) | Numerical position | Primer efficiency | Reference |
|---|---|---|---|---|---|---|
| **ABPV** | ABPV_F | ACCGACAAAGGGTATGATGC | 124 | 9104-9123 | 1.77 | (Glavinic et al., 2019) |
| | ABPV_R | CTTGAGTTTGCGGTGTTCCT | | 9208-9227 | | |
| **ARV-1** | ARV-1_F | ATGAGGCTTGGAGACACAGC | 100 | 13955-13974 | 1.86 | (Norton et al., 2020) |
| | ARV-1_R | GGAGCTTTCCTGAGGACACG | | 14035-14054 | | |
| **ARV-2** | ARV-2_F | CTAAACCCCACCTGTCTGCC | 150 | 11416-11435 | 1.90 | (Norton et al., 2020) |
| | ARV-2_R | ATTGAGCACTGGAGCGTTGG | | 11564-11565 | | |
| **BQCV** | BQCV_qPCR_F | AGGTTTACGCTCCAAGATCG | 112 | 5735-5754 | 1.73 | Remnant et al. (2019) |
| | BQCV_qPCR_R | TTTGTTCAGCAGGTAAATTGTTC | | 5824-5846 | | |
| **BMLV** | BeeMLV-F | ATCCCTTTTCAGTTCGCT | 142 | 5625-5642 | 1.76 | (de Miranda et al., 2015) |
| | BeeMLV-949R | CGAGCACGGCCTCAAGAG | | 5749-5766 | | |
| **CBPV** | qF1818 | CAACCTGCCTCAACACAG | 296 | 1801-1818 | - | (Locke et al., 2012) |
| | qB2077 | AATCTGGCAAGGTTGACTGG | | 2077-2096 | | |
| **DWV-A** | DWV_F | TACTAGTGCTGGTTTTCCTTT | 155 | 8626-8646 | 1.91 | Kevill et al. (2017) |
| | DWV-A_R | CTCATTAACTGTGTCGTTGAT | | 8781-8761 | | |
| **DWV-B** | DWV_F | TACTAGTGCTGGTTTTCCTTT | 155 | 8599-8619 | 1.92 | |
| | DWV-B_R | CTCATTAACTGAGTTGTTGTC | | 8754-8735 | | |
| **DWV-C** | DWV_F | TACTAGTGCTGGTTTTCCTTT | 152 | 8617-8636 | - | |
| | DWV-C_R | ATAAGTTGCGTGGTTGAC | | 8751-8768 | | |
| **IAPV** | IAPV_F_7762 | GCAGCTATTTTTGGCTGGTC | 114 | 7761-7780 | - | (Daughenbaugh et al., 2015) |
| | IAPV_R_7876 | CCAATGTACGCTCATATCG | | 7856-7875 | | |
| **KBV** | KBV_F | TGAACGTCGACCTATTGAAAAA | 127 | 5408-5492 | - | (vanEngelsdorp et al., 2009) |
| | KBV_R | TCGATTTTCCATCAAATGAGC | | 5514-5534 | | |
| **LSV** | LSV_F | TCATCCCAAGAGAACCAC | 114 | 2108-2125 | 1.73 | (D'Alvise et al., 2019) |
| | LSV_R | GCATGGAAGAGAGTAGGTA | | 2203-2221 | | |
| **SBV** | SBV_ qPCR_F | CGAGGAGGGAAAAACTACGC | 115 | 2202-2221 | - | Remnant et al. (2019) |
| | SBV_ qPCR_R | GTGGCTTAACTGGATCATAGCC | | 2295-2316 | | |
| **SBPV** | F3177 | GCGCTTTAGTTCAATTGCC | 226 | 3159-3177 | - | (Locke et al., 2012) |
| | B3363 | ATTATAGGACGTGAAAATATAC | | 3363-3384 | | |
| ***Actin*** | Actin_F | TGCCAACACTGTCCTTTCTG | 155 | 3307-3326 | 1.70 | Scharlaken et al. (2008) |
| | Actin_R | AGAATTGACCCACCAATCCA | | 3443-3462 | | |

# SUPPLEMENTARY REFERENCES


D'Alvise, P., Seeburger, V., Gihring, K., Kieboom, M., and Hasselmann, M. (2019). Seasonal dynamics and co-occurrence patterns of honey bee pathogens revealed by high-throughput RT-qPCR analysis. *Ecology and Evolution* 00**,** 1-12. doi: 10.1002/ece3.5544.

Daughenbaugh, K.F., Martin, M., Brutscher, L.M., Cavigli, I., Garcia, E., Lavin, M., et al. (2015). Honey bee infecting Lake Sinai viruses. *Viruses* 7(6)**,** 3285-3309. doi: 10.3390/v7062772.

de Miranda, J.R., Cornman, R.S., Evans, J.D., Semberg, E., Haddad, N., Neumann, P., et al. (2015). Genome characterization, prevalence and distribution of a macula-like virus from *Apis mellifera* and *Varroa destructor*. *Viruses* 7(7)**,** 3586-3602. doi: 10.3390/v7072789.

Gisder, S., Möckel, N., Eisenhardt, D., and Genersch, E. (2018). *In vivo* evolution of viral virulence: switching of deformed wing virus between hosts results in virulence changes and sequence shifts. *Environmental Microbiology* 20(12)**,** 4612–4628. doi: 10.1111/1462-2920.14481.

Glavinic, U., Tesovnik, T., Stevanovic, J., Zorc, M., Cizelj, I., Stanimirovic, Z., et al. (2019). Response of adult honey bees treated in larval stage with prochloraz to infection with *Nosema ceranae*. *PeerJ* 7**,** e6325. doi: 10.7717/peerj.6325.

Kevill, L.J., Highfield, A., Mordecai, J.G., Martin, J.S., and Schroeder, C.D. (2017). ABC assay: method development and application to quantify the role of three DWV master variants in overwinter colony losses of European honey bees. *Viruses* 9(11)**,** 2-14. doi: 10.3390/v9110314.

Lanzi, G., de Miranda, J.R., Boniotti, M.B., Cameron, C.E., Lavazza, A., Capucci, L., et al. (2006). Molecular and biological characterization of deformed wing virus of honeybees (*Apis mellifera* L.). *Journal of Virology* 80(10)**,** 4998-5009. doi: 10.1128/JVI.80.10.4998–5009.2006.

Locke, B., Forsgren, E., Fries, I., and de Miranda, J.R. (2012). Acaricide treatment affects viral dynamics in *Varroa destructor*-infested honey bee colonies via both host physiology and mite control. *Applied and Environmental Microbiology* 78(1)**,** 227-235. doi: 10.1128/AEM.06094-11.

Mordecai, G.J., Wilfert, L., Martin, S.J., Jones, I.M., and Schroeder, D.C. (2016). Diversity in a honey bee pathogen: first report of a third master variant of the deformed wing virus quasispecies. *The ISME Journal* 10(5)**,** 1264-1273. doi: 10.1038/ismej.2015.178.

Norton, A.M., Remnant, E.J., Buchmann, G., and Beekman, M. (2020). Accumulation and competition amongst deformed wing virus genotypes in naïve Australian honeybees provides insight into the increasing global prevalence of genotype B. *Frontiers in Microbiology* 11(620). doi: 10.3389/fmicb.2020.00620.

Ongus, J.R., Peters, D., Bonmatin, J.M., Bengsch, E., Vlak, J.M., and van Oers, M.M. (2004). Complete sequence of a picorna-like virus of the genus *Iflavirus* replicating in the mite *Varroa destructor*. *Journal of General Virology* 85(12)**,** 3747-3755. doi: 10.1099/vir.0.80470-0.

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Research* 29(9)**,** e45-e45. doi: 10.1093/nar/29.9.e45.

Remnant, E.J., Mather, N., Gillard, T., Yagound, B., and Beekman, M. (2019). Direct transmission by injection affects competition amongst RNA viruses in honeybees. *Proceedings of the Royal Society B: Biological Sciences* 286(1895)**,** 20182452. doi: 10.1098/rspb.2018.2452.

Scharlaken, B., De Graaf, D.C., Goossens, K., Brunain, M., Peelman, L.J., and Jacobs, F.J. (2008). Reference gene selection for insect expression studies using quantitative real-time PCR: The head of the honeybee, *Apis mellifera*, after a bacterial challenge. *Journal of Insect Science* 8(33)**,** 1-10. doi: 10.1673/031.008.3301.

Tehel, A., Vu, Q., Bigot, D., Gogol-Döring, A., Koch, P., Jenkins, C., et al. (2019). The two prevalent genotypes of an emerging infectious disease, deformed wing virus, cause equally low pupal mortality and equally high wing deformities in host honey bees. *Viruses* 11(2)**,** 114. doi: 10.3390/v11020114.

vanEngelsdorp, D., Evans, J.D., Saegerman, C., Mullin, C., Haubruge, E., Nguyen, B.K., et al. (2009). Colony collapse disorder: a descriptive study. *PLoS ONE* 4(8)**,** e6481. doi: 10.1371/journal.pone.0006481.