Prevalence and impact of honey bee RNA viruses on Africanized honey bees and native stingless bee species of the Yucatan Peninsula, Mexico

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To Jakelin, Georgina and Victor, mi familia.

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Abstract

There is a perceived worldwide decline in pollinators, which are important providers of the ecosystem service of pollination. Currently, there is evidence of a high prevalence of RNA viruses and their negative effects on managed honey bees (*Apis mellifera*) in temperate climates; the same viruses are also found in temperate wild bee species. One plausible cause of pathogen sharing is pathogen spillover, nowadays considered a major threat to biodiversity. The phenomenon likely is of global scope, although it is poorly documented, and its consequences for recipient species are largely unknown. The tropical Yucatan Peninsula of Mexico is considered an important region for honey production from Africanized honey bees (AHBs) and home to 17 different native species of eusocial stingless bees, including the iconic *Melipona beecheii*, a species of cultural, economic, and ecological importance for this region. It is plausible that viral spillover occurs in the Yucatan Peninsula, with a negative impact on native bee populations. To address this question, I developed three investigations to evaluate viral spillover in the Neotropics from AHBs, a managed and invasive species, into native stingless bees, a biodiverse taxon of pollinators, as well as to evaluate the potential negative effects of RNA viruses in stingless bee populations.

In **Chapter II**, using original viral prevalence data from two different time points, I explored the changes in the prevalence of DWV (genotypes A and B) in Africanized honey bees (AHBs), comparing drones (2010) and workers (2019). In **Chapter III**, building upon the information from **Chapter II**, which provides evidence of RNA viral presence in AHBs since 2010, I assessed the prevalence, viral sharing, and viral load of RNA viruses associated with AHBs and different stingless bee species from 12 locations in the Yucatan Peninsula. Finally, in **Chapter IV**, I investigated the potential negative impact of RNA viruses associated with honey bees on *Melipona beecheii* through controlled infections under laboratory conditions

In summary, firstly, I confirmed the presence of RNA viruses in AHBs since 2010, without marked changes in the prevalence of DWV genotypes A and B over the subsequent 9 years. This contrasts with what has been observed in temperate regions, where genotype A is being replaced by genotype B. Secondly, I identified so-called honey bee viruses in different stingless bee species across the Yucatan Peninsula, lending support to the concept of viral spillover from AHBs to native stingless bees. Thirdly, through experimental viral

infections of adult workers on the iconic stingless bee species *M. beecheii*, I illustrated the negative impact of these viruses on *M. beecheii* survival. Further investigation into the potential adverse effects of RNA viruses on other bee species under laboratory and semi-natural conditions is imperative. This research will provide valuable insights into the consequences of pathogen spillover and strengthen conservation efforts for pollinators in tropical regions.

Keywords: RNA viruses, Spillover, AHBs, Stingless bee, *Melipona beecheii*, Yucatan Peninsula.

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Chapter I: General introduction

Ecological, economic and cultural importance of bees in the Neotropics

Importance of Africanized honey bees

Pollinators are necessary for the reproduction of 87% of flowering plants, and therefore, for the maintenance of natural ecosystems as well as for crop production (Potts et al., 2010). Among the different groups of animals that provide these services, insects from various orders, such as Hymenoptera, which comprises managed and wild bees, represent the most important group of pollinators in natural landscapes, providing pollination services to wild plant species and agricultural areas. These pollinators benefit crops and various agronomic products (Potts et al., 2010, 2016; Klein et al., 2017; Garibaldi et al., 2013). Among the different managed species, the western honey bee (*Apis mellifera*) is considered the most important commercial species of the Anthophila, due to its role in pollination services and honey production. Given this significance, *A. mellifera* currently has a quasi-global distribution, being present in all major world regions except Antarctica (Beaurepaire et al., 2020).

In the American continent, the western honey bee arrived together with the first European settlers, who introduced different European honey bee subspecies such as *A. m. mellifera* and *A. m. iberiensis*. Partially because of the introduction of other subspecies, honey bees in the Americas were completely of European origin up to 1956 (Guzmán Novoa et al., 2020; Whitfield et al., 2006). After 1956, an Africanization process took place in the Americas following the accidental liberation in Brazil of pure African honey bee subspecies swarms: *A. m. scutellata* and *A. m. adansonii* (Guzmán Novoa et al., 2020). These swarms began to mix with local European subspecies, giving rise to Africanized honey bees (AHBs). Subsequently, Africanization extended throughout the entire continent, with a greater dominance in tropical areas (Guzmán Novoa et al., 2020). Nowadays, with the new honey bee hybrid present throughout tropical regions of America, including the Yucatan Peninsula in Mexico, beekeeping activities, such as honey production, have undergone changes with new adaptations and management adjustments focused to AHBs.

The Yucatan Peninsula, comprising three states (Yucatan, Campeche, and Quintana Roo), is considered an important apicultural region in Mexico and one of the top honey producing areas in the world, thanks to the production of honey and other products from AHBs (Quezada-Euán et al., 1996). Estimates from recent years indicate an annual honey production of 63,000 tonnes from the region and honey exports of 29,000 tonnes per year between 2016 and 2020 (Sader, 2023). Despite the lower honey production of AHBs when compared to European subspecies, Africanization has brought benefits to beekeepers by eradicating some diseases (Guzman-Novoa et al., 2020) and increasing pollination services for some crops. It has also revived ancient activities involving local stingless bees in different regions of the Neotropics (Guzmán Novoa et al., 2020; Quezada-Euán, 2018). Currently, due to the economic importance of AHBs in the Yucatecan Peninsula, there is a high density of managed AHBs colonies as well as feral swarms distributed throughout the

entire region (Moritz et al., 2013). These, along with solitary and social native bee species, share the same floral resources.

Importance of Stingless Bees

Despite the importance of *A. mellifera* for tropical America, this region holds 5000 valid names of social and solitary bee species (Freitas et al., 2009). This region is home to over 390 stingless bee species (Hymenoptera: Apidae: Meliponini) (Michener, 2007; Quezada-Euán, 2018), a high diversity compared with other tropical and subtropical areas of the world. Stingless bees are, like honey bees, eusocial and perennial, storing a small amount of honey to keep the colony alive during inclement weather or periods of floral dearth (Figure 1.). They are considered generalist pollinators in the tropics (Grüter, 2020); however, there is evidence of the floral preference of this group compared with honey bees. For example, in regions such as Manaus (Brazil) 60% (23 of 38) of commercial crop plants benefit from pollination by different stingless bee species, as evidenced by stored pollen inside their colonies (Absy et al., 2018; Gruter, 2020). Therefore, stingless bee species are likely important for the reproduction of many plant species. Recently, new research has pointed to the potential pollination services of stingless bees for commercial purposes (Osterman et al., 2021), with 15 different stingless bee species showing potential for management for pollination in different crops such as tomatoes, aubergines and habanero peppers among others. Although their management is not an established practice in the Neotropics (Osterman et al., 2021), is a promising activity that reinforces the importance of stingless bees for the pollination of native and crop plants.

In other areas of the Neotropics, where western honey bees are not native, stingless bees have a remarkable importance for different cultures as a source of carbohydrates (honey) and other products (cerumen, batumen and pollen) (Quezada-Euán 2018). The worldwide distribution of stingless bees is restricted to tropical areas (Michener. 2007; Quezada-Euán, 2018), where many native cultures have been using them since ancient times. Notably, the Maya culture in Mesoamerica embraced these bees, incorporating meliponiculture (the keeping of stingless bees) into their society for economic and religious purposes. Mayan meliponiculture is particularly associated with the iconic *Melipona beecheii* (Quezada-Euán, 2018; Grüter, 2020; (Figure 2), which is nowadays still being managed traditionally in regions such as the Yucatan Peninsula of SE Mexico.

Today, meliponiculture is a growing activity that is contributing to increase knowledge about the management of different bee species as well as promoting their conservation. However, this rising popularity is also bringing about new potential issues for stingless bee populations, primarily due to inexperienced management practices that lead to colony losses (Quezada-Euán, 2018) as well as the indiscriminate trade of colonies in countries such as Mexico and Brazil (Carvalho, 2022; Quezada-Euán, 2018). These factors, together with the main drivers of pollinator decline such as habitat loss, indiscriminate use of pesticides as well as the presence of exotic species and their pathogens (Potts et al. 2016), all considered important threats to bees, could lead to the decline of this important pollinator group in

different regions of the world, including in the Neotropics (Dicks et al., 2021; Freitas et al., 2009; Potts et al., 2010).

Figure 1. Nest of *Frieseomelitta nigra*, a stingless bee species present in the Yucatan Peninsula, Mexico, in a "rational" hive box for stingless bees, showing the distribution of food pots (to the sides) and brood pots (middle). Picture: Fernando Fleites.

Bee threats habitat loses, pesticides and pathogens (RNA viruses)

Habitat loss

Current evidence suggests the decline of different wild bees in various regions worldwide, including tropical areas (Zattara & Aizen 2021). This decline is believed to result from the interaction of various factors; however, three are considered the primary drivers of bee decline: changes in land use (habitat loss), agricultural intensification (the use of pesticides), and the presence of pathogen Changes in land use, such as deforestation, fragmentation, degradation, or the complete transformation of semi-natural landscapes into farming systems, can result in reduced food supplies for pollinators. This, in turn, leads to a modification of plant species that provide essential nutritional elements, increasing the likelihood of malnutrition at both the individual and colony levels in various bee species, a phenomenon likely experienced across the world.

In the Neotropics, changes in land use, particularly deforestation, have significant and detrimental effects on bee populations. Deforestation greatly impacts the availability of flower and nesting resources (Freitas et al., 2009), especially in countries such as Colombia, Brazil, and Mexico, which are among the most affected by deforestation. Native species like stingless bees could be the most severely affected due to habitat loss, which results in the loss of trees for nesting and underground nesting sites, and the loss of flowering plants critical for their food supply (Grüter, 2020). Unfortunately, in Mexico, which harbours a remarkable diversity of bee fauna representing 10% of the world's bee species (Ayala,

2006), there has been no assessment of the impact of habitat loss its native apifauna (Quezada-Euán, 2018). However, regional studies within Mexico indicate that the diversity and abundance of bees has declined in modified landscapes (Meneses-Calvillo et al. 2010; Quezada-Euán, 2018).

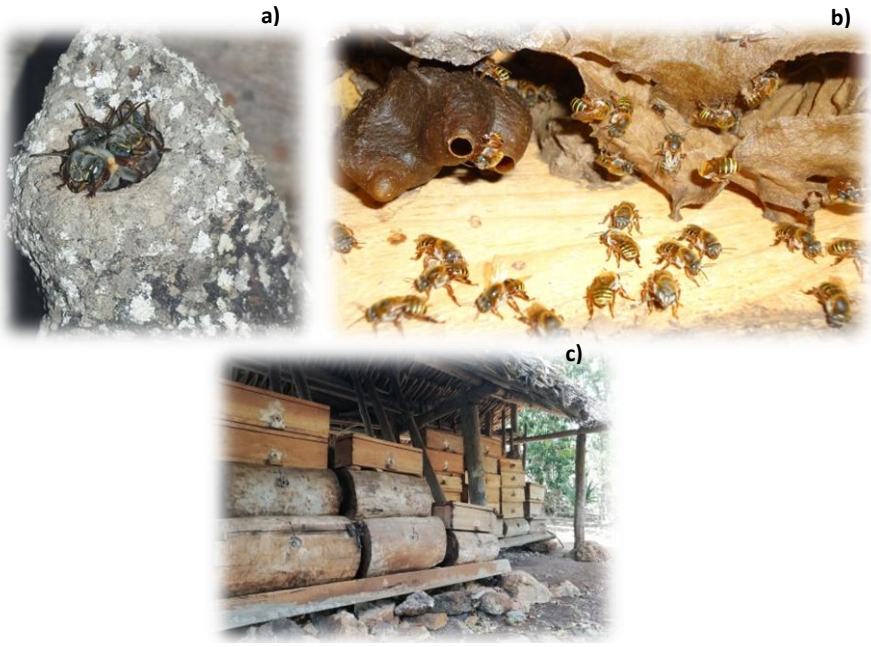

Figure 2. The stingless bee species *Melipona beecheii*: **a)** Colony entrance, **b)** Adults inside a colony **c)** *M. beecheii* colonies aggregated in a traditional meliponary and. Picture: Fernando Fleites.

Pesticides

The expansion and intensification of agriculture are closely linked to habitat loss, leading to a reduction in plant and animal diversity (Dicks et al., 2021). Furthermore, agricultural intensification can result in the indiscriminate use of pesticides, which is associated with the decline of pollinators, including bees (Kremen et al., 2002; IPBES, 2016). Pesticides, such as insecticides, acaricides, fungicides, molluscicides, and herbicides, can have negative effects on bee species due to a combination of toxicity and high exposure (Phalan et al., 2013; Costantini, 2015). Among the various pesticides, neonicotinoid insecticides in particular have a broad range of lethal and sublethal effects on various pollinators, including different bee species (Potts et al., 2016). The popularity of neonicotinoids arises from their potential to be less harmful to humans and vertebrates; however, they may be more toxic to insects such as bees (Casida and Durkin, 2013; Quezada-Euán, 2018).

It is considered that pesticides are important drivers of pollinator decline in multiple regions of the world; nevertheless, the impact of pesticides in Latin America and Asia-Pacific is considered to be particularly high (Dicks et al., 2021). Some pesticides such as DDT and other organochlorine compounds have been banned from Latin American countries such as Mexico. Nevertheless, other pesticides, including neonicotinoids, are still being used in several Latin American countries (Quezada-Euán, 2018). Despite concerns over pesticides and their effects on pollinators such as bees, information is scare regarding non-*A. mellifera*

species such as different solitary and social bee species, like stingless bees (Quezada-Euán, 2018). Therefore, more information is needed about the effect of pesticides in native bees from the tropics as well as the combination of those with other factors such as pathogen presence.

Exotic species and their pathogens

The presence of exotic species and their pathogens are considering another factor that could contribute to the loss of biodiversity by infecting native species; there is evidence of the disaster that exotic species and their pathogens could produce in other organisms (Goulson, 2003; Cameron et al., 2011). For example, the introduction of crayfish species from North America into Europe has had a considerable negative impact in two European freshwater crayfish species (*Astacus astacus* and *Austropotamobius pallipes*) because the presence of an exotic fungal pathogen *Aphanomyces astaci* carried by American crayfish, which reduced populations of native species that presented little resistance to the exotic fungus (Butler & Stein 1985; Goulson, 2003). Exotic species, whether managed or not, can serve as reservoirs of pathogens that remain and spread with the original host until they are transmitted to new hosts. This phenomenon has been well-documented in wild bumblebees infected with protozoans from managed bumblebees in North America (Colla et al., 2006) in native bumble bees from Europe (Murray et al., 2013) and in South America, also with native bumble bee species (Schmid-Hempel et al., 2014).

Similarly, the introduction of the western honey bee, an exotic and managed species in regions like the New World, has also brought with it parasites and pathogens such as fungus, bacteria, microsporidian, beetles, mites and viruses which represent a potential risk of disease spread to native bee populations (Goulson 2003). Among the principal pests and pathogens affecting western honey bee populations worldwide, the exotic mite *Varroa destructor* and associated RNA viruses represent arguably the greatest problem for apiculture; the same viruses are shared with wild bees, representing a risk to them, too (McMahon et al., 2015; Radzevičiūtė et al., 2017; Tehel et al., 2016). With the increase in honey bee populations in different world regions for commercial pollination, is very likely that associated pathogens and diseases, such as RNA viruses, could spread to wild bee species through spillover events, potentially negatively impacting their populations (Alaux et al., 2019).

RNA viruses associated with honey bees in wild bee species (spillover)

Over the past three decades, 24 different viruses have been associated with the Western honey bee (De Miranda et al., 2013; McMenamin et al., 2016), including deformed wing virus (DWV), chronic bee paralysis virus (CBPV), black queen cell virus (BQCV), sacbrood virus (SBV), slow bee paralysis virus (SBPV) and acute bee paralysis virus (ABPV). These viruses are known to cause diseases with recognizable symptoms and have adverse effects on honey bee colonies (De Miranda et al., 2013). All six are widespread in honey bee populations, though DWV is arguably the greatest challenge for *A. mellifera* (McMahon et al., 2016).

While the honey bee is likely the reservoir host for many of these viruses, especially DWV, in temperate regions of the world these RNA viruses have also been detected in other wild bee species such as bumble bees (*Bombus* spp.) and solitary bees, as well as insects from other orders (Fürst et al., 2014; McMahon et al., 2015; Nanetti et al., 2021; Radzevičiūtė et al., 2017; Tehel et al., 2016), even though *V. destructor*, the major vector of some of these viruses between honey bees, is restricted to *A. mellifera* and has never been found to parasitise bumble bees or other wild bee species. A possible explanation for the presence of these viruses in wild bee species is that honey bees from managed or feral colonies deposit virus on flowers during feeding or when defecating and that wild bee species subsequently take up the virus when foraging on the same flowers: pathogen spillover (Alger et al., 2019; Burnham et al., 2021; Singh et al., 2010; Tehel et al., 2022).

Pathogen spillover poses a significant threat to both wild and domestic animals, as well as human well-being (Daszak, 2000). This process occurs when pathogens are transmitted from infected hosts to naïve sympatric "non-reservoir" host populations, when spillover events occur among managed and wild animals, there is a high risk of species decline, which in turn threatens global biodiversity and disrupts ecosystem functions and their associated services (Alger et al., 2019; Colla et al., 2006; Daszak, 2000). A prime example is the recent Covid-19 pandemic in which the SARS-CoV-2 virus jumped from a wild host into the human population in late 2019 (Li et al., 2021). Insect pollinators are not exempt from the threat of pathogen spillover. Spillover represents a potential risk to individuals and populations of wild bee species. Honey bees infected by *V. destructor* have high titres of virus, particularly DWV (Martin et al., 2012; Mondet et al., 2014), which likely facilitates spillover into other insect species (Dalmon et al., 2021; Manley et al., 2019).

The presence of RNA viruses in *A. mellifera* and the potential for viral spillover are not limited to specific geographic regions, such as Europe, the USA and Canada, or particular subspecies of honey bees inhabiting temperate climates. For instance, both BQCV and DWV genotypes A and B (DWV-A and DWV-B, respectively) have been identified in European honey bees (EHBs) from North and South America (Paxton et al., 2022) and BQCV, DWV, SBV and ABPV have all been detected in Africanized honey bees (AHBs) from Central and South Africa (Luis et al., 2020; Reyes-Quintana et al., 2019; Tibatá et al., 2021). Furthermore, these viruses have been identified in diverse wild bee species of Latin America (LA); examples include DWV in *Xylocopa augusti* larvae (Lucia et al., 2014) and DWV-A, BQCV, and SBV in wild bumble bees of Argentina (Bravi et al., 2019; Reynaldi et al., 2013). Also RNA viruses have been founded in stingless bee species from the Neotropics in which BQCV, ABPV, Israeli acute paralysis virus (IAPV), DWV-A and DWV-B have all been detected e.g. in Argentina (Alvarez et al., 2018), Brazil (de Souza et al., 2019; Ueira-Vieira et al., 2015), and Mexico (Guzman-Novoa et al., 2016; Morfin et al., 2020; Tapia-González et al., 2019).

While the relatively low prevalence of RNA viruses in wild bee species supports the concept of virus spillover from honey bees, where viral prevalence can be notably high (Alger et al., 2019; Burnham et al., 2021; Fürst et al., 2014; McMahon et al., 2015; Singh et al., 2010), the subsequent effects of viral spillover on wild bees remain poorly

characterized. Earlier studies have suggested that DWV inoculation may lead to reduced survival in European bumble bees (Fürst et al., 2014; Graystock et al., 2015). However, subsequent experiments undertaken under benign laboratory conditions have suggested limited impact on *Bombus* survival (Tehel et al., 2020) as well as in semi-natural conditions (Streicher et al., 2022). There is currently a pressing need to comprehensively understand the extent to which pathogen spillover contributes to the decline of insect pollinators. Moreover, there is a necessity for studies that demonstrate the potential negative impact of viral spillover on populations of wild solitary and social bee species inhabiting tropical regions of the world, where honey bees and wild bees also share floral resources.

Problem, statement and justification

The Yucatan Peninsula is considered an important apicultural region in Mexico because of its high production of honey and other products from Africanized honey bees (Quezada-Euán et al., 1996). Additionally, this tropical region has 17 different species of eusocial stingless bees (Ayala, 1999), which contribute to the pollination of flowering wild and crop plants. These bee species include the culturally, biologically and economically important *Melipona beecheii*, a stingless bee species managed by ancient peninsular Maya people up to the present day (Quezada-Euán, 2018). Populations of stingless bees in the tropics have nowadays been reported to be decreasing; causes of decline include loss of habitat, indiscriminate use of pesticides, exotic invasive species, and their pathogens (Freitas et al., 2009). Interactions between co-occurring stingless bees and honey bees at flowers or at hives could lead to the spread of different pathogens, including RNA viruses, among stingless bees, as has been observed between managed and wild bee species in Great Britain and elsewhere in Europe (Fürst et al., 2014; McMahon et al., 2015; Manley et al., 2019).

Despite the growing number of investigations that reinforce the potential negative effects of RNA viruses associated with honey bees in wild bees through spillover events, these studies are all restricted to temperate areas. In tropical areas such as the Neotropics, where similar interactions among honey bees and wild bees occur, there is limited information on viral presence or prevalence in wild bees. The virulence of these viruses for stingless bee species is unknown. For this reason, identification of RNA viruses in Africanized honey bees and stingless bees, and the evaluation of their possible negative effects on stingless bee survival, is the focus of this thesis, as a priority in order to develop actions to conserve the health of AHBs as well as to conserve stingless bees, an economic and culturally important taxonomic group, as well as to maintain the ecosystem service of pollination in tropical regions.

Objectives and hypotheses

The overall aim of my thesis was to evaluate the presence, prevalence, and change in prevalence over time of six common honey bee associated viruses, namely DWV (genotypes A and B), BQCV, SBV, SBPV and ABPV in AHBs and stingless bee species of the Yucatan Peninsula as well as to evaluate the possible negative effects of the most common viruses presented in this tropical region on the longevity of the economic, ecological and culturally important stingless bee species *M. beecheii* under laboratory conditions.

Objectives

1. Document changes in prevalence of DWV (genotypes A and B) in Africanized honey bees as well as to evaluate viral sequence changes overtime.
2. Identify the presence of the six most common RNA viruses of honey bees in Africanized honey bees as well as in the most common stingless bee species in the Yucatan Peninsula, Mexico.
3. Test the virulence of the most common RNA viruses present in the Yucatan Peninsula on *M. beecheii*, a species with a remarkable cultural, economic and ecological importance, through controlled inoculation experiments in the laboratory.

Hypotheses

1. Currently, DWV prevalence data from temperate regions support the notion that DWV-A is being replaced by DWV-B. The presence of the same viral genotypes in Africanized honey bees from the tropics leads me to hypothesize a similar viral replacement pattern in honey bees in the Yucatan Peninsula of Mexico.
2. Due to (1) the presence of viral vectors such as the varroa mite *Varroa destructor*, (2) the negative impacts of DWV and BQCV on honey bees, and (3) the natural route of transmission in the field through shared use of flowers, there is a high probability of detecting the most common RNA virus of the honey bee in stingless bee species in the Yucatan Peninsula, Mexico.
3. The virulence of different RNA viruses is similarly high in stingless bee species as it is in honey bees, and these viruses reduce the longevity of infected stingless bees, in comparison with non-infected bees, under controlled laboratory conditions.

Chapter II: Viral prevalence in Africanized honey bees of the Yucatan Peninsula in SE Mexico suggests that DWV-A blocks DWV-B through superinfection exclusion

Abstract

An RNA virus population often comprises multiple variants that may co-circulate in a host population, with potentially complex dynamics that can impact the health of their hosts, be they wildlife, domestic species or humans. Deformed wing virus (DWV) is widely considered the most harmful RNA virus of honey bees because it is associated with colony losses in temperate regions; like other RNA viruses, it nowadays exists as two major variants, genotypes A (DWV-A) and B (DWV-B), which provides an amenable window into the dynamics of multi-variant viruses. The prevalence of DWV-B has recently increased in honey bee populations of Europe, where DWV-B now seems to be replacing DWV-A. DWV-B seems to have arrived more recently in the New World, where its prevalence has also increased in the last decade in the USA, though DWV-A continues as the dominant genotype. The Yucatan Peninsula of Mexico is home to a high density of managed and feral Africanized honey bees (AHBs), which are also known to be infected by DWV-A and DWV-B, yet there is a lack of information on the epidemiology of DWV variants in AHBs, which show remarkable tolerance to other honey bee pests and pathogens. Here, I present two temporally separated viral prevalence datasets that inform on the dynamics of DWV genotypes in AHBs, and develop an epidemiological model to account for genotype dynamics. I demonstrate the presence of both DWV genotypes in Yucatecan AHBs in 2010, with surprising little change in prevalence through to 2019. Phylogenetic analysis of viral sequences suggests that DWV-A has been presented from 2010 until 2019 without significant genetic changes. Finally, our epidemiological model suggests that "inverted pattern" of DWV genotypes seen in the Yucatan Peninsula, in which DWV-A has maintained its dominance despite the long-standing presence of DWV-B, may be due to a process of super infection exclusion, possibly due to recombination meltdown when the two genotypes co-infect the same host cells. As well as informing on the epidemiology of a major honey bee virus in the Neotropics, our results provide broader insight into the evolutionary dynamics of viruses that comprise two or more variants.

Keywords: AHBs, DWV, viral prevalence, Yucatan Peninsula, epidemiological modelling

I Introduction

The western honey bee (*Apis mellifera*) is considered one of the most important managed animals worldwide because it provides commercial pollination services as well as honey and other hive products (Osterman et al., 2021). Consequently, *A. mellifera* has been traded widely, resulting in a quasi-global distribution (Beaurepaire et al., 2020). Its parasites and pathogens have similarly achieved near-worldwide distribution, as in the case of the ectoparasitic mite *Varroa destructor* (Traynor et al. 2020) and numerous viruses (Beaurepaire et al., 2020), for some of which *V. destructor* acts as a vector (Yañez et al. 2020). Among the viruses closely associated with honey bees and vectored by *V. destructor*, deformed wing virus (DWV) in particular has risen to prominence (Grozinger & Flenniken

2020; Martin & Brettell, 2019) because it has been closely linked to colony decline and loss in temperate regions (Dainat et al. 2012; Francis et al. 2013; Natsopoulou et al. 2017).

DWV, a positive sense single-stranded RNA virus in the family *Iflaviridae*, is nowadays found as two common and widespread variants, the original genotype A (DWV-A; Wilfert et al. 2016) and the more recent genotype B (DWV-B; Paxton et al. 2022). Since the first description of DWV-B (synonym *Varroa destructor virus – 1*) isolated from *V. destructor* and *A. mellifera* in the Netherlands in 2001 (Ongus et al., 2004), it has subsequently spread around the globe (Paxton et al. 2022), presumably due to its high rate of transmission, which we here attribute to its higher rate of replication (e.g. McMahon et al. 2016) as well as its more efficient vectoring by *V. destructor* in comparison to DWV-A; whilst both genotypes of DWV are transmitted between honey bees primarily by *V. destructor*, DWV-B can additionally replicate within *V. destructor* whereas DWV-A cannot (Gisder & Genersch 2021).

RNA viruses in particular show high rates of mutation that can lead to their emergence and rapid evolutionary dynamics within a host population (Holmes 2009). Recently, during the SARS-Cov-2 pandemic, with its outbreak in Wuhan in 2019, variants of the virus were replaced in a short lapse of time within human populations, for example from alpha to delta and then to omega (Li et al., 2021). Replacement events, whereby one viral variant replaces another, might also be common to other viruses such as DWV, though the mechanisms of replacement probably differ among viruses.DWV seems to exhibit an evolutionary dynamic in which one variant replaces another. DWV genotype C seems to have recently disappeared from the UK (Kevill et al., 2019), possibly having been displaced by DWV-A and, more recently, DWV-B. Moreover, DWV-D exhumed from Egyptian honey bees collected in the 1960s is seemingly absent from extant host populations (de Miranda et al., 2022). Currently, DWV-B seems to be displacing DWV-A in many honey bee populations across the world (Paxton et al. 2022). Despite the higher virulence of DWV-B over DWV-A in adult honey bees (McMahon et al. 2016), the higher rate of transmission of DWV-B over DWV-A may explain why DWV-B has increased markedly in prevalence in many northern temperate regions during the last decade (USA: Ryabov et al. 2017; UK: Gindrod et al. 2021; Germany and Italy: Paxton et al. 2022).

That DWV-B is not only increasing in prevalence within honey bee populations but also replacing DWV-A is more of an enigma. Epidemiological modelling suggests that inter-genotype interference likely plays a role in explaining genotype replacement within a host population infected by both DWV-A and DWV-B because, when there is no interaction between genotypes, the prevalence of both genotypes rise; evidence suggests that when both genotypes are present in a host population, DWV-B replaces DWV-A (Paxton et al. 2022). Superinfection exclusion (SIE) has been coined for one form of inter-genotype interference, whereby a pre-existing virus variant may block the establishment of another variant. Indeed, it has already been suggested that DWV exhibits SIE; DWV-B has been hypothesised to block the establishment of DWV-A (Mordecai et al., 2016). One potential mechanism of SIE is recombinational meltdown, whereby high rates of recombination may lead the elimination of one genotype and improve the establishment of another (Moore et

al., 2011; Ryabov et al., 2014). SIE through recombinational meltdown could explain the current replacement of DWV-A by DWV-B in some regions of the world.

In the Americas, the prevalence of RNA viruses, including DWV and its genotypes A and B, have been linked with the expansion of ectoparasitic *V. destructor* in host honey bee populations (Hasegawa et al., 2023). Both DWV-A and DWV-B have been detected in many countries of the Continent (Paxton et al., 2022). In the USA, honey bee populations have witnessed a dramatic increase in the geographic range and prevalence of DWV-B in the last decade, including on Hawaiian islands (2010-2020) and mainland USA (2010-2016), though DWV-A remains the dominant variant.Current DWV prevalence data from tropical American countries is limited to a single time-point: Brazil (de Souza et al., 2019), Argentina (Brasesco et al., 2020), Colombia (Tibatá et al., 2021) and Chile (Riveros et al., 2020) and the Yucatan Peninsula of Mexico (Fleites-Ayil et al., 2023). They have confirmed the presence of genotypes A and B in honey bees, with DWV- A being more prevalent than B, presumably because DWV-B has recently entered these countries' honey bee populations as part of its global expansion (Paxton et al. 2022). But they do not allow examination of the dynamics of viral genotypes, which may differ from that in temperate regions of the world, where DWV-B seemingly replaces DWV-A.

Here, I fill this knowledge gap by examining the dynamics of DWV genotypes in one tropical American region, the Yucatan Peninsula, for which I present original data on DWV-genotype prevalence in drone honey bees collected in 2010 as well as with data from worker honey bees from the same region (Fleites-Ayil et al., 2023), with the surprising result that DWV-A has not (yet) been replaced by DWV-B. Through epidemiological modelling I then explore possible mechanisms to explain the dynamics of both genotypes A and B under three different scenarios: independent spreading, mutual inhibition and recombination meltdown. Our data and modelling give insight into why DWV-B generally, but not always, replaces DWV-A in host populations, and may inform on variant replacement in other host-virus systems exhibiting high viral prevalence.

2. Material and methods

2.1 Sample collection

The Yucatan Peninsula in tropical southeast Mexico is considered one of the most important apicultural regions in Mexico and the world because of its high honey production from managed Africanized honey bees (Güemes Ricalde et al., 2003). AHBs have dominated these regions since their arrival in the late 1980s (Clarke et al., 2001; Quezada-Euán et al., 1996). Nowadays, the Yucatan Peninsula, has a very high density of both managed and feral AHBs colonies (Moritz et al., 2013). Following Africanization of the honey bee population, *V. destructor* arrived in the Yucatan Peninsula, where it is now widespread (Medina & Martin, 1999). Recently, I (Fleites-Ayil et al. 2023) have shown that AHBs workers sampled from the field in 2019 were infected with both DWV-A and DWV-B, wherein DWV-A was more prevalent that DWV-B.

Sampling of honey bees in the Yucatan Peninsula of Mexico was undertaken at two time points (2010 and 2019) at different locations (SM Table SM 1). In 2010, at each of four locations (SM. Fig 1), 100 drones were captured from drone congregation areas (DCAs) using an aerial trap baited with synthetic queen mandibular pheromone (E-9-oxo-2-decenoic acid; 12.5 µg/ml in 70% ethanol). The trap was raised to ca. 15 m above the ground using weather balloons filled with helium (Williams, 1987) and checked every 30 min to collect trapped drones. Sampling took place between 14:00 and 18:00 hrs. Each drone was separated into head, thorax and abdomen and stored individually in RNA-Later® (QIAGEN, Hilden, Germany), then transported to the laboratory, where samples were stored at -80°C until RNA was extracted (Human et al., 2013).

In January to April 2019 during the flowering season, worker honey bees were sampled from 1000 m^2 flower patches (dimensions 10 m x100 m or 20 m x 50m) at 12 locations (SM. Fig 2). To do so, I walked a continuous transect within a flower patch, collecting maximally 10 honey bees per 10 minutes until I had collected ca. 30 honey bees (Fleites-Ayil et al., 2023; Fürst et al., 2014). Flower patches were embedded in a rural matrix composed of gardens, parks, and wildflower meadows. Bees were transferred immediately to individual 1.5 ml vials filled with RNA-Later® (QIAGEN, Hilden, Germany) and maintained in the field on dry ice (ca. -80°C) to avoid RNA degradation (Human et al., 2013) before storage at -80°C.

2.2 RNA extraction and viral detection

I extracted RNA from bees individually, using abdomens of drones or whole bodies of workers. Samples were crushed in 500 µl RLT-buffer containing 1 % β–mercaptoethanol using a plastic pestle, from which total RNA was extracted using an RNeasy Mini kit (QIAGEN, Hilden, Germany) in a QIAcube extraction robot (QIAGEN) (De Miranda et al., 2013; Radzevičiūtė et al., 2017; Tehel et al., 2019) and eluted into 30 µl RNAse-free water. cDNA was synthesised from 800 ng of the RNA using Oligo-dT oligonucleotides (Thermo Scientific) and reverse transcriptase (M-MLV and Revertase, Promega, Mannheim,

Germany) following the manufacturer's instructions and then diluted 1:10 before use in qPCRs.

I used RT-PCR to detect DWV-A and DWV-B using primers given in Table SM 2. I performed duplicate qPCR reactions per sample in a Bio-Rad C1000 thermal cycler (Bio-Rad, Munich, Germany) using SYBRgreen Sensimix with the following program: 5 minutes at 95ºC, followed by 40 cycles of 10 seconds at 95ºC, 30 seconds at the primer's t_m and 30 seconds at 72ºC. Two positive (extract of an infected bee) and two negative (template-free) control wells were included per 96 well plate; they were consistently positive (Cq<35) or negative Cq>35) respectively. I set the PCR cycle quantification (Cq) threshold at <35 to consider a sample as positive for a viral target and, if technical duplicates differed by more than one cycle, I re-ran qPCRs and averaged the nearest two values. I also ran a melt curve profile for each qPCR product in which PCR products were denatured for one minute at 95°C, cooled to 55°C for one minute, and then a melting profile was generated from 55°C to 95°C at an increment of 0.5°C per second to ensure a single product of the correct dissociation ('melt') temperature had been generated. Data from the honey bees collected in 2019 are already presented in Fleites-Ayil et al. (2023).

2.3 Phylogenetic analysis of DWV-A sequences

Six DWV-A positive samples, three from 2010 and three from 2019, were selected for sequencing. To do so, I PCR amplified a 451 bp partial sequence of the RdRp gene using primers F15 (5′-TCC ATC AGG TTC TCC AAT AAC GGA-3′) and B23 (5′-CCA CCC AAA TGC TAA CTC TAA GCG-3′) (Yue & Genersch, 2005) with the PCR conditions of 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 54.3°C for 1 min and at 72°C for 30 sec and a final extension step at 72°C for 5 min. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and cloned directly using the pGEM T Easy Vector System II (Promega, Mannheim, Germany) following the manufacturer's instructions. Three randomly selected clones per sample were Sanger sequenced in forward and reverse orientation (GATC Biotech, Constance, Germany), assembled into CONTIGs and aligned manually using Geneious v7.0.6 (Kearse et al., 2012) to a reference genome of DWV-A (NC_004830).

Merging identical sequences resulted in 11 unique sequences, each with a length of 403 nucleotides, corresponding to the nucleotide positions 9280-9680 on our NCBI reference genome. To determine if the DWV-A variant was shared among these time points at our study area, I constructed a phylogenetic tree using RAxML 1.0.3 (Kozlov et al., 2019) for maximum likelihood tree reconstruction and a Median-Joining haplotype network in PopART v1.7 (Leigh & Bryant, 2015). In addition to our samples from Mexico, I also include the RdRp fragment from 12 complete genomes of DWV-A from across the world as well as a DWV-B (NC_006494) as an outgroup to investigate relationships among sequences.

2.4 Epidemiological model describing the dynamics of DWV genotypes

Here I extend my deterministic epidemiological model first presented in Paxton et al. (2022) to describe the dynamics of DWV-A and DWV-B, with a focus on the case in which DWV-B enters a host population with a pre-existing high prevalence of DWV-A, as found in the Yucatan Peninsula (see Results). The current model focuses on the frequencies of different viral types in a population of honey bees instead of a compartmental model. In this way, some of the model equations are analytically solvable. The model runs in continuous time within an infinitely large honey bee population, likely approximating the very large Africanized honey bee population in the tropical Yucatan Peninsula of Mexico (Moritz et al., 2013).

Firstly I define a bee individual as being in one of the four states:
• H (healthy, more accurately defined as uninfected by either DWV genotype),
• A (infected only with DWV-A),
• B (infected only with DWV-B), or
• M (mixed), when co-infected (by both DWV-A and DWV-B).

Symbols A and B (and corresponding lower case letters a and b) are used to indicate the viral genotypes A and B (and their corresponding frequencies) in the population of honey bees respectively. In addition, symbols $\hat{a}$ and $\hat{b}$ are used to indicate the frequency of DWV-A and DWV-B at equilibrium. I initiated simulations by setting the frequency of DWV-A at 0.3 (a = 0.3) and of DWV-B at 0.01 (b = 0.01) to reflect a population of honey bees in which DWV-A initially predominates and is first invaded by DWV-B, the most plausible real-life scenario in the Yucatan Peninsula.

A viral genotype X (with the frequency of x in the population of honey bees) is described by two parameters: transmission rate (μ_X), which is defined as a fraction of X-uninfected individuals which will be infected in the next time step, conditional on the availability of infected individuals in the current time step; and their fatality rate (ν_X), defined as the fraction of X-infected individuals which will die in the next time step. I set $\mu_B > \mu_A$, as suggested by the increasing prevalence of DWV-B during the last decade in Europe and USA. DWV-B's higher rate of replication (McMahon et al. 2016) and more efficient transmission by Varroa mite vectors (Gisder & Genersch, 2021), its major route of transmission, could underpin the higher rate of transmission of DWV-B over DWV-A.

The model incorporates the idea that both viral genotypes can potentially limit the spread of each other. There are then two ways in which the frequency of genotype A (a) can increase:

•When an uninfected individual is infected with genotype A; this event occurs with the rate of $a(1-a)(1-b)\mu_A$.
•When a B-infected individual is infected with genotype A; this event occurs with the rate of $(1-a).a.b.m_A.\mu_A$, where the transmission rate of genotype A is inhibited by genotype B which is already present in the host. The coefficient m_A captures the extent of this inhibition; it varies from 0 (complete inhibition) to 1 (no inhibition).

The frequency of genotype A decreases in only one way when an A-infected individual or a co-infected individual dies; this event occurs at the rate of $a.v_A$, which depends only on the fatality rate of genotype A.
Symmetrically the same events happen to genotype B. The change in the frequency of genotypes A and B are then given by Model 1:

$$Model1: \begin{cases} da/dt = a(1-a)(1-b)\mu_A + (1-a).a.b.m_A\mu_A - a.v_A \\ db/dt = b(1-b)(1-a)\mu_B + (1-b).b.a.m_B.\mu_B - b.v_B \end{cases}$$

I explore the dynamics of this model under three different conditions (see Results). As these equations are in general non-linear, I used simulations with the package "deSolve" (Soetaert et al., 2010) in R v. 4.1.1 (R Core Team) to describe the dynamics of the frequency of DWV genotypes in a population of honey bee individuals across plausible parameter values. Figures were generated in R v. 4.1.1 (R Core Team).

2.5 Statistical analysis

Statistical analyses and plots of prevalence were in R v. 4.1.3 (R Core Team). I calculated the viral prevalence per virus and per time point (drones and workers) with 95% of confidence intervals using the R package epiR v. 2.0.63 (Stevenson et al., 2023).

3. Results

3.1 Viral prevalence

I found two viral targets (DWV-A and DWV-B) in drones and workers from both sampling time points in the Yucatan Peninsula, Mexico. In 2010, DWV-A was the most prevalent at 96% (95% CI: 90-98% n = 102) and DWV-B at 0.98% (95% CI: 0.02-5% n = 102) in drones sampled at DCAs (Figure SM 1). The only DWV-B infected drone was co-infected with DWV-A and Cq values suggested high DWV-A titres and low DWV-B titre. In workers sampled from flowers in 2019, DWV-A had a prevalence of 12% (95% CI: 7-19% n = 114) and DWV-B a prevalence of 2% (95% CI: 0.2-6% n = 114) (Figure SM 2).The two workers positives to DWV-B did not presented co-infection with DWV-A. Thus, in contrast to data from elsewhere in the world (Paxton et al. 2022), DWV-B has not risen rapidly in prevalence and DWV-A has not been replaced over time. DWV-B has remained at low frequency and DWV-A has maintained at high prevalence (Fig. 1).

3.2 Phylogenetic relations of DWV-A

DWV RdRp partial sequences from drone and worker AHBs from the Yucatan Peninsula of Mexico are closely related to each other, with a similarity > 97% (Fig. 2). Their similarity with 12 DWV-A sequences from the NCBI database reflects geographic proximity; Yucatecan isolates are more similar to other American sequences and less similar to European and Asian isolates (Fig. 2). Haplotype network analysis of DWV-A sequences confirms the close relationship of Yucatecan sequences with DWV-A from the USA and one from Europe (France) in a cluster. Additional clusters in the network are composed of

sequences from Europe and Asia in a pattern reflecting isolation by geographic distance (Fig. SM 3).

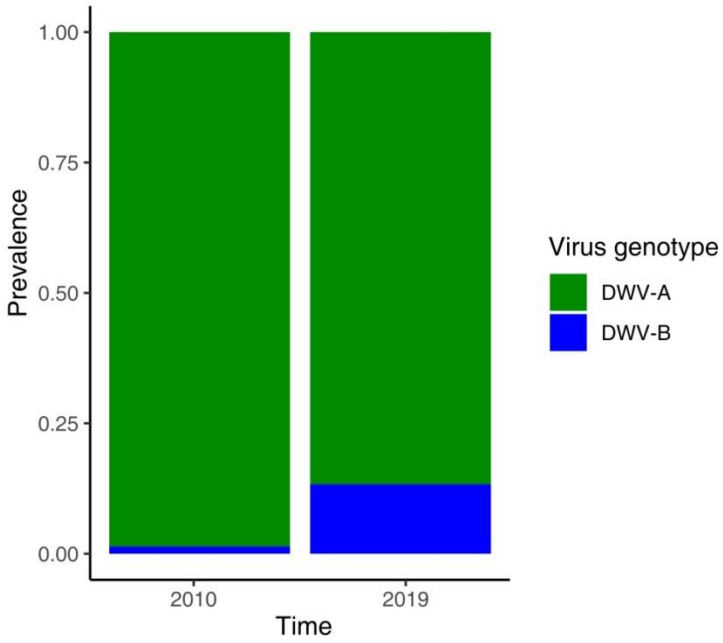

Figure 1. Temporal change in the prevalence of DWV-A and DWV-B in Africanized honey bees in the Yucatan Peninsula Mexico. The change in the viral prevalence among drones from 2010 (n = 249) and workers from 2019 (n = 114) was significant for DWV-A. On the other hand, despite the increase of DWV-B, differences between sampling years were not significant.

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3.3 Epidemiological model describing the blocking of DWV-B's spread by DWV-A

Independent spreading

Assuming DWV-A and DWV-B do not interact and therefore that they spread through the population independently, Model 1 reduces to:

$$Model2: \begin{cases} da/dt = a(1-a)\mu_A - a\nu_A \\ db/dt = b(1-b)\mu_B - b\nu_B \end{cases}$$

For this model, there are four points at which viral frequencies are fixed, among which the last is stable:

$$\begin{bmatrix} \alpha \\ \beta \end{bmatrix} = \begin{bmatrix} 0 \\ 0 \end{bmatrix}, \begin{bmatrix} 0 \\ 1 - \frac{\nu_B}{\mu_B} \end{bmatrix}, \begin{bmatrix} 1 - \frac{\nu_A}{\mu_A} \\ 0 \end{bmatrix}, \begin{bmatrix} 1 - \frac{\nu_A}{\mu_A} \\ 1 - \frac{\nu_B}{\mu_B} \end{bmatrix}$$

and where α and β are the frequencies of DWV-A and DWV-B at equilibrium. The trajectory (the change in frequency with time) of each viral genotype is then given by:

$$Equation 1: x(t) = \frac{\Delta_X}{\left[\frac{\Delta_X}{x_0} - \mu_X\right] e^{-t\Delta_X} + \mu_X}$$

where $\Delta_X = \mu_X - \nu_X$ and x_0 is the initial frequency of viral genotype X.

Figure 3 shows the change in frequency of both genotypes of DWV with time when the transmission rate of DWV-B is higher than DWV-A (μ_B = 0.12 vs μ_A = 0.1) and no interaction between them is assumed, and the fatality rate (ν_X) of both viral types is assumed the same. In the absence of any interaction between viral types, the frequency of both viruses increases until they reach equilibrium. The equilibrium values for the two genotypes are independent of each other and are determined by the ratio of fatality rate to transmission rate (ν_X / μ_X).

Mutual inhibition

Model 2 assumes no interaction between the two viral genotypes DWV-A and DWV-B. However, the pattern observed in the frequency of these two genotypes in UK, Germany and Italy (Grindrod et al., 2021; Paxton et al., 2022) suggests some form of negative interaction between them because an increase in the frequency of genotype B coincides with a decrease in the frequency of genotype A. Under the scenario of a population of host and two pathogen variants, the order of infection of a host individual may determine the probability that it becomes co-infected; a pathogen variant which is already established in a host may inhibit infection by other variant in a form of SIE (Mordecai et al., 2016). In order to capture this interaction, I set the mutual interaction terms to be nonzero.

For simplicity, I set equal fatality rates for both viral types. I am interested in the conditions under which $\alpha > \beta$, which occurs when:

$$Equation 2: m_B < \frac{1}{2\mu_B(\mu_A - \nu)} \left[\mu_A(\mu_A + \mu_B - 2\nu)m_A + (\mu_A - \mu_B)\left(2\nu + \sqrt{\mu_A}\sqrt{\mu_A . m_A^2 + 4\nu - 4\nu m_A}\right) \right]$$

When m_B drops below this threshold, genotype A has a strong enough effect to prevent genotype B establishment in host already infected by DWV-A. Figure 4 shows the corresponding parameter landscape described in Equation 2, where for some parameter values of interaction terms m_A and m_B, DWV-B (with higher transmission rate) can replace DWV-A ("Normal pattern" regime; DWV-B replaces DWV-A) and for some values it cannot ("Inverted pattern" regime, DWV-A blocks DWV-B). Panels b and c in Figure 5 show the dynamics of the model under two conditions, one from the "Normal pattern" scenario and another from the "Inverted pattern". Here I set the initial frequency of DWV-A to 0.8, which more realistically reflects our observational data from 2010.

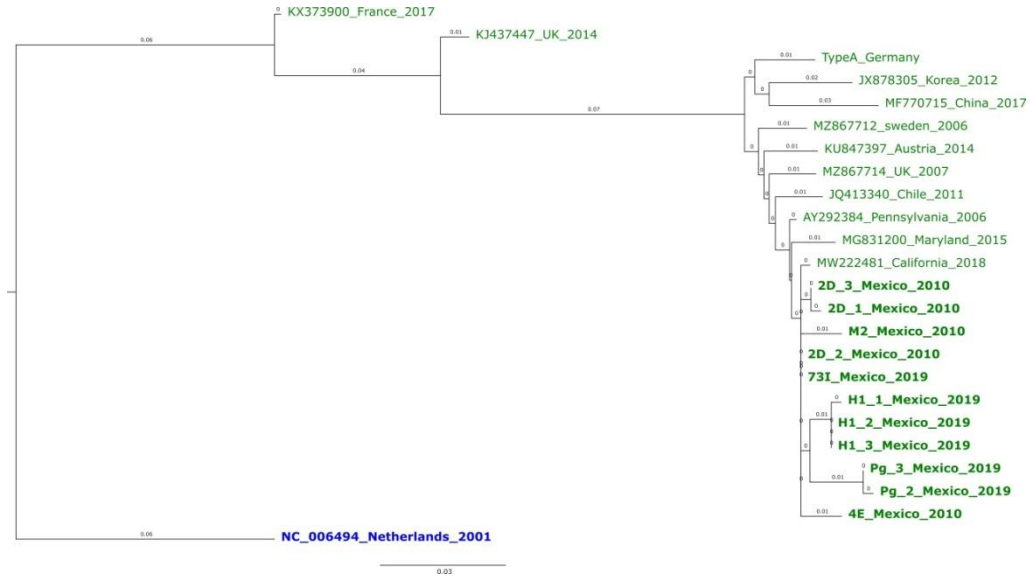

Figure 2. Phylogenetic tree showing the relationship between 11 DWV RdRp partial sequence amplified from samples from four different geographical locations in Yucatan, Mexico (Bold green), and 12 selected complete genomes of DWV-A across the world retrieved from GenBank. The sequence of DWV-B from the Netherlands (Bold Blue), collected in 2001, was used as an outgroup to root the tree. Phylogenetic analysis was performed using maximum likelihood with RAxML. Figtree was used for tree drawing. Numbers give branch lengths.

Recombination meltdown

I now propose a scenario in which, a newly immigrant virus (DWV-B) enters an already established population of DWV-A and, whenever these two viral types mix, they undergo a recombination process and produce a recombinant variant with high fatality rate. As a result, DWV-B will be quickly removed from the population, leaving DWV-A at high frequency despite the fact that DWV-B has a higher transmission rate compared to DWV-A. Model 3 describes these dynamics of a high recombination rate when two genotypes of DWV coexist. In this model, R (with transmission rate of μ_R, fatality rate of ν_R, and the frequency of r) indicates a recombinant. I assume for simplicity that there is only one type of DWV recombinant.

$$Model3: \begin{cases} da/dt = (1-a).a.\mu_A - a.b.(1-r).\alpha - a.\nu_A \\ db/dt = (1-b).b.\mu_B - a.b.(1-r).\alpha - b.\nu_B \\ dr/dt = (1-r).r.\mu_R + a.b.(1-r).\alpha - r.\nu_R \end{cases}$$

where α indicates the recombination rate of viral genotypes in a coinfected host. In this model, different genotypes of DWV do not influence each other's transmission rates. When α=0 and r=0, this model reduces to model 1. Since the frequency of each genotype depends on the frequency of other genotypes, this model cannot be solved analytically; however, I can inspect the equilibrium profile numerically. Figure 5 shows the equilibrium values of three types of the virus (DWV-A, DWV-B, and recombinant) under different values of recombination rate, recombinant fatality rate, and initial frequency of genotype A. When either the recombination rate, the recombinant fatality rate, or the initial frequency of genotype A are sufficiently high, DWV-B cannot enter and spread to high frequency in a host population already infected by DWV-A as a consequence of recombinational meltdown.

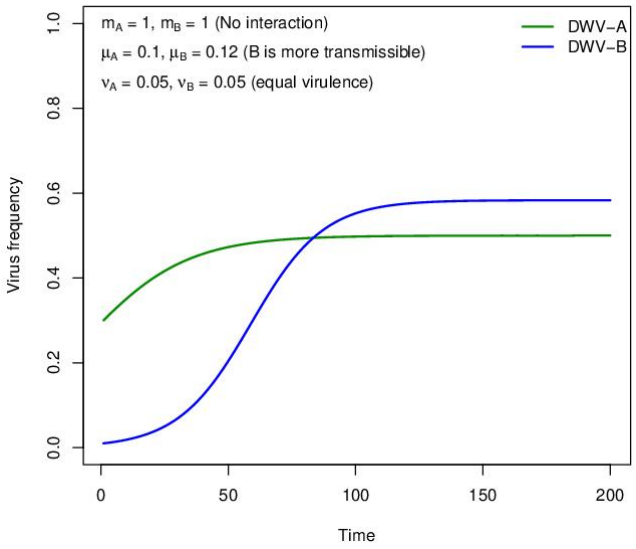

Figure 3. Simulation of the frequency of two viral genotypes when the transmission rate of genotype B is higher than type A, inspired by epidemiological data from the literature. The frequency of genotype B increases over time even if its virulence is higher compared with type A. The equilibrium frequency of type B can even cross that of type A if its virulence does not exceed a threshold, which is 0.06 in this case.

4 Discussion

Here I confirm the presence of DWV genotypes A and B in Africanized honey bees from the Yucatan Peninsula since 2010. However, DWV-B has not risen markedly in prevalence in a decade whereas DWV-A appears to have maintained its high relative prevalence. Our epidemiological modelling makes clear that this "inverted pattern" of apparent dominance of DWV-A over DWV-B in tropical Yucatan may arise through the negative interaction (mutual inhibition) between the two viral genotypes, possibly through a form of superinfection exclusion.

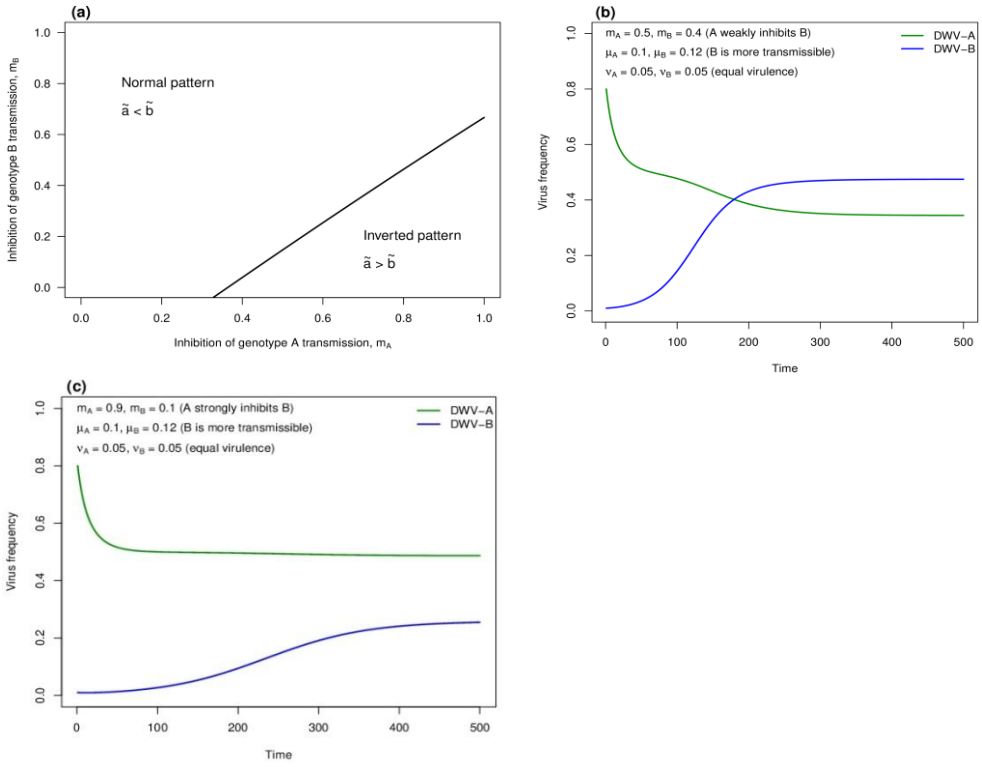

Figure 4. (a) The parameter landscape from the basic model (Model 1) where two different patterns (normal pattern vs inverted pattern) occur for different values of interaction between two viral types: mA and mB. The genotype A can prevent the establishment of type B in a small part of the landscape as the transmission rate of genotype B is higher than type A. (b) When genotype A weakly inhibits genotype B, the latter dominates at equilibrium. (c) When the inhibition of genotype B transmission imposed by genotype A exceeds some threshold, given in Equation 2, genotype B cannot dominate the pre-existing type A.

4.1 Viral prevalence in Africanized honey bees

Our data demonstrate that DWV-A and DWV-B have been present in AHBs from the Yucatan Peninsula of Mexico since 2010, at that DWV-A is at very high prevalence. Other studies support the view that DWV is widespread in Mexico (Correa-Benítez et al., 2023; Guzman-Novoa et al., 2016).

Considering the major role that Varroa plays as a vector of DWV (Martin et al., 2012; Mondet et al., 2014), the expansion of Varroa to encompass South America (Paraguay in 1971) and North America (USA in 1987) (Rosenkranz et al., 2010; Traynor et al., 2020) as well as the Yucatan Peninsula, where the first Varroa records are dated to 1994 (Medina & Martin, 1999), were likely accompanied by RNA viruses, including DWV (Hasegawa et al., 2023). Varroa and associated viruses likely entered the Yucatan Peninsula from North America (Traynor et al. 2020), possibly with imported honey bees. This view is supported by our phylogeny of DWV-A isolates. The first ever record of DWV-B is in honey bees and Varroa mites collected in 2001 in the Netherlands (Ongus et al., 2004). DWV-B's first detection on the American continent is from 2010, when it was found in only two of 71 US colonies (Ryabov et al., 2017), suggesting that DWV-B is a relatively recent (2010) arrival to continental America. It is highly likely, therefore, that DWV-A was first brought to the

Yucatan Peninsula, possibly in or before 1994 with Varroa mites (Medina & Martin, 1999), and has subsequently risen to very high prevalence in the region's honey bees.

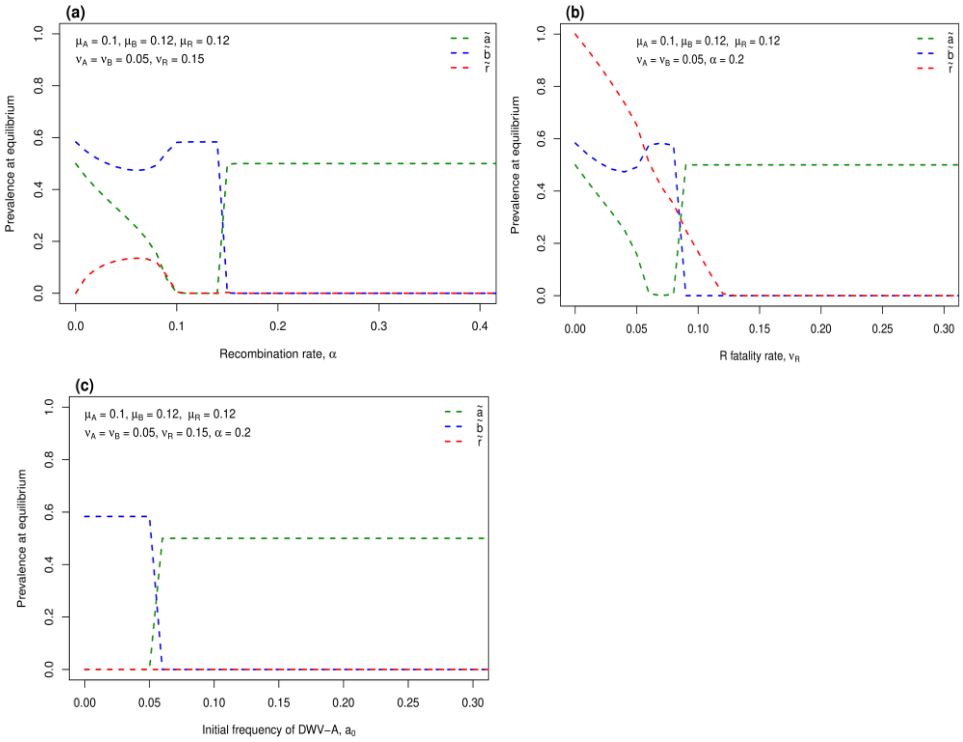

Figure 5. Equilibrium profile of model 3. In a pre-established DWV-A population with high recombination rate and high recombinant fatality rate, the genotype B is suppressed and the inverted pattern is observed. Releasing any of these three conditions concludes the dominance of genotype B.

Our viral prevalence data show little change across two time points (2010-2019). Of bees infected by DWV, all drones were infected with DWV-A in 2010 and in 2019 less number of workers were infected with DWV-A; on the other hand, DWV-B prevalence among dates did not differ. Differences in the absolute prevalence of DWV in honey bees between the two dates likely arises because drone pupae are preferred over worker pupae as hosts of Varroa mites (Rosenkranz et al., 2010) and therefore often have a high prevalence of viruses transmitted by Varroa mites, including DWV (Forfert et al., 2016), compared to workers.

Data from North America as well as from the Hawaiian islands reveal a rise in the prevalence of DWV-B (Ryabov et al., 2017; Grindrod & Martin, 2021), as seen in European countries (Kevill et al., 2021), where DWV-A is also seemingly being replaced by DWV-B (Paxton et al., 2022). Though DWV-A remained the dominant genotype in USA in 2016 (Ryabov et al., 2017), European data as well as epidemiological modelling predict an increase in DWV-B's prevalence in North America in the next two decades and a decline or loss of DWV-A (Paxton et al., 2022).

South and Central America paint a different picture, where DWV-A seems to be dominant over DWV-B and DWV-C (Brasesco et al., 2020; Fleites-Ayil et al., 2023; Riveros et al.,

2020). Even after ten years of presence in tropical SE Mexico, DWV-B remains at very low prevalence and DWV-A continues to be dominant.

4.2 Phylogenetic analysis

Our two phylogenetic analyses corroborate the presence of the same DWV-A variant in honey bees from the Yucatan Peninsula at both of our sampling time points, 2010 and 2019. Our sequence data also reveal close genetic identity among samples from 2010 and 2019, suggesting long-term stability of DWV-A in Yucatecan honey bees. The broad host range and low apparent virulence of DWV-A in arthropod communities suggest that DWV is a generalist virus of many host species (Martin & Brettell, 2019), which could explain why DWV-A remains dominant and without marked genetic change in a decade.

Additionally, our haplotype network supports the idea that DWV-A entered the Yucatan Peninsula from North America; our DNA sequences showed a closer relation with sequences from North America than with European and Asian sequences, as demonstrated more forcefully by Hasegawa et al. (2023) using whole genome sequences.

4.3 Inverted pattern of DWV genotypes predicted by model

Extending the epidemiological model describing the co-occurrence of DWV-A and DWV-B (Paxton et al., 2022), I show that DWV-A can prevent the establishment of DWV-B, likely through a form of superinfection exclusion. Our basic model describes the change in frequencies of two DWV genotypes (A and B) in a population of honey bees assuming a higher transmission rate of DWV-B and a higher initial frequency of DWV-A, with possible mutual inhibition between two variants of the virus. In the absence of inhibition (our Model 2), the frequency of each viral genotype changes with time independently until they reach equilibrium. Hence, both of these viruses can reach and remain at high prevalence in a population of honey bees. However, the pattern observed in the prevalence of DWV in Europe (Kevill et al., 2021; Paxton et al., 2022) and USA (Grindrod et al., 2021; Ryabov et al., 2017) suggests some form of interaction between these two variants because the prevalence of DWV-A decreases as the prevalence of DWV-B increases.

Based on this observation, I introduce mutual interaction (inhibition) between viral types to explain patters of variant prevalence. Since the transmission rate of DWV-B is higher than that of DWV-A, weak inhibition of DWV-B by DWV-A cannot theoretically block the spread of DWV-B. However, strong inhibition by DWV-A can suppress the spread of DWV-B despite the latter's higher rate of transmission (our Equation 2). I now explore how superinfection exclusion might account for the dominance of DWV-A in the Yucatan Peninsula through a variety of forms of strong inhibition (SIE).

Mordecai et al. (2015), through observations of an isolated UK honey bee population dominated by an apparently avirulent genotype B and absence of a virulent genotype A, described two possible mechanisms of SIE: (i) the resources of the host are already consumed by the first pathogen and (ii) host immunity is already triggered by the first pathogen, which makes it hard for the second pathogen to establish. These possibilities

might explain priority effects, which have been observed during co-infection by two or more viral variants (Jokinen et al., 2023), including in DWV infecting honey bees (Gusachenko et al., 2021). Our modelling of DWV genotype dynamics in which DWV-A is already established in a honey bee population (our Equation 2) demonstrates that genotype B may not be able to establish in the host population if DWV-A is at high enough initial frequency, possibly through these two mechanisms of SIE.

Another possible mechanism of SIE explaining the observed "inverted pattern" of DWV viral variants in the Yucatan Peninsula is recombination meltdown, which is a special case of negative interaction or mutual inhibition. Recombination events between two major genotypes of DWV have been reported before (Moore et al., 2011; Ryabov et al., 2014), suggesting a significant rate of recombination in DWV. Since DWV is a RNA virus, the correct spatial structure of the genome molecule is necessary for viral replication (Holmes 2009). As such, viral recombinants are likely not viable. The consequence of this process will then be recombination meltdown in which two viral RNA molecules (one of each variant) recombine when co-infecting the same host cell and perish. This is equivalent to error catastrophe in the quasispecies theory of viruses (Lauring & Andino, 2010).

A small number of surviving recombinants with a higher fatality rate represent a potentially higher risk for honey bees (e.g. Ryabov et al., 2014). Under this scenario, I hypothesize that a recombination process could eventually leads to suppression of DWV-B in the Yucatan Peninsula despite DWV-B's higher fitness if it enters as a rare variant into a host population already harbouring DWV-A at a high prevalence. I parsimoniously note that mutual inhibition through recombinational meltdown (or another mechanism of SIE) might also help explain why DWV-B has so rapidly replaced DWV-A in many temperate regions of the world where DWV-A prevalence was initially formerly lower than in the Yucatan Peninsula and DWV-B has entered and risen to high prevalence.

It is possible that the negative interaction through superinfection exclusion could be influenced by other factors that, when combined, could benefit the establishment or dominance of one viral variant over another, as in the case of DWV. For example, honey bees from tropical regions such as SE Mexican may show less susceptibility to viral infections, with consequences for viral epidemiology. The genetic origin of the honey bees could play an important role in host susceptibility to viral infections. Is well documented that AHBs show resistance or tolerance to numerous pathogens, including *V. destructor* (Guzmán-Novoa et al., 1999; Martin & Medina, 2004), and including viral infections (Hamiduzzaman et al., 2015), compared with European *A. mellifera*. Also, viral differences have been recorded among other honey bee species co-occurring with European *A. mellifera*; in North Thailand, European *A. mellifera* has higher viral prevalence compared with other sympatric and native honey bee species (Chantaphanwattana et al., 2023). Possible differences in viral tolerance among honey bee species and hybrids (AHBs) could explain why DWV-A continues its dominance over DWV-B in the Yucatan Peninsula. That honey bee genetic background may impact viral epidemiology needs closer investigation.

Finally, the prediction that DWV-B expansion is in process is an interesting idea that should be tested in the next decades. The evidence of the negative impact of DWV-B in European honey bee populations as well as its presence in different host species (de Souza et al., 2019; Martin & Brettell, 2019; Tehel et al., 2016) suggests that DWV-B may be in an expansion process in the Americas and other regions. We should consider it as an emerging threat for honey bee populations as well as for other bee species and pollinator diversity, including in the Neotropics (Fleites-Ayil et al., 2023).

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Chapter III: RNA viral prevalence in bee species from the Yucatan Peninsula of Mexico

Abstract

Pathogen spillover is a major threat to biodiversity. Insect pollinators, important providers of the ecosystem service of pollination that are in global decline, are no exception to this threat, with mounting evidence of pathogen spillover from managed into wild bee species in temperate regions. The phenomenon is likely global in scope, though poorly documented, and its consequences for recipient species are largely unknown. To address these knowledge gaps, I investigated viral spillover in the neotropics from the honey bee (*Apis mellifera*), where it is a managed and invasive species, into native stingless bees, a biodiverse taxon of pollinators. Samples of AHBs and different stingless bee species were captured in flower patches from 12 different locations in the Yucatan peninsula Mexico, then those samples were analyzed to find viral prevalence of six different RNA viruses associated to honey bees. Our results showed the prevalence of three different viruses (BQCV, DWV-A and DWV-B), as well as through our haplotype network analysis I found evidence of viral sharing among stingless bee species and AHBs. I conclude that viral spillover from managed to wild insect pollinators is likely a global phenomenon and poses a serious threat worldwide to native insect species.

Keywords: Spillover, stingless bees, RNA viruses, viral prevalence, Yucatan Peninsula

1 Introduction

The western honey bee (*Apis mellifera*) is the world's most important managed insect pollinator (Osterman et al., 2021), dominating as a flower-visitor in diverse agricultural and semi-natural terrestrial biomes, yet it is often infected with RNA viruses that are capable of causing disease with recognizable symptoms and adverse effects on honey bees (De Miranda et al., 2013; Kevill et al., 2019; McMahon et al., 2016). In temperate regions, these RNA viruses have also been found in wild bee species, particularly bumble bees (*Bombus* spp.) and solitary bees (Tehel et al., 2016; Alger et al. 2019; Nanetti et al., 2021). A possible explanation for their presence in wild bee species is that honey bees from managed or feral colonies deposit virus on flowers during feeding or when defecating and that wild bees subsequently take up the virus when foraging on the same flowers, leading to pathogen spillover (Alger et al., 2019; Burnham et al., 2021; Singh et al., 2010; Tehel et al., 2022), a potential risk to individuals and populations of wild bee species.

Spillover represents a potential risk to individuals and populations of wild bee species. Honey bees infected by *V. destructor* have high titres of virus, particularly of DWV (Martin et al., 2012; Mondet et al., 2014), which likely drives spill over into other insect species (Manley et al., 2019). The presence of RNA viruses in *A. mellifera* (and the potentially for spillover) is not confined to Europe, the USA, and Canada or temperate subspecies of honey bees. BQCB and DWV (genotype A and B) have been found in European honey bees (EHBs) from North and South America (Anguiano-Baez et al., 2016; Brasesco et al., 2020;

Guzman-Novoa et al., 2013; Riveros et al., 2020; Ryabov et al., 2017) and BQCV, DWV, SBV, and ABPV have all been detected in Africanized honey bees (AHBs) from Central and South America (Luis et al., 2020; Reyes-Quintana et al., 2019; Tibatá et al., 2021). Moreover, in the last decade, these bee viruses associated with honey bees have been identified in diverse wild bee species of Latin America (LA); examples include DWV in *Xylocopa augusti* larvae (Lucia et al., 2014) and DWV-A, BQCV, and SBV in wild bumble bees of Argentina (Bravi et al., 2019; Reynaldi et al., 2013).

Other bee group from the tropics susceptible to pathogens through spillover events are the stingless bees, the stingless bees (Hymenoptera: Apidae: Meliponini) represent the most diverse group whiting the corbiculate bees (>500 spp) (Michener 2007; Quezada-Euán., 2018). This bee group is considered important for their contributions to the biodiversity richness, to pollination services of native and commercial plats, as well as the close relationship with ancient cultures in different tropical regions (Grutter Quezada). Currently there is evidence of the prevalence of RNA iruses associated with honey bees in different stingless bee species from the Americas. Viruses as BQCV, ABPV, IAPV, and DWV genotypes-A and B have been detected in Argentina (Alvarez et al., 2018), Brazil (de Souza et al., 2019; Ueira-Vieira et al., 2015), and Mexico (Guzman-Novoa et al., 2016; Tapia-González et al., 2019, Morfin et al., 2020). Nevertheless, still poor information about other regions from Latin America where this corbiculate bee group brings benefits to the ecosystems as well as the society.

The Yucatan Peninsula is considered the most important beekeeping region in Mexico and the world due to its high density of managed and feral colonies of *A. mellifera*, predominantly AHBs (Güemes Ricalde et al., 2003; Guzman-Novoa et al., 2020; Quezada-Euán et al., 1996; Moritz et al. 2013). Additionally, 17 species of eusocial stingless bee are present in this tropical region (Ayala, 1999; González-Acereto, 2012; Quezada-Euán, 2018), which are known to contribute to the pollination of its wild and crop plants (Grüter, 2020; Quezada-Euán, 2018). Among these stingless bee species, *Melipona beecheii* is emblematic through the millennial tradition of management (meliponiculture) by the ancient Maya people, a practice that continues up to the present day (González-Acereto, 2012; Quezada-Euán et al., 2018). Despite the importance of stingless bees for the tropics, and *M. beecheii* in particular for the Yucatan Peninsula, their populations are threatened by a variety of factors that also impact bee species in temperate regions (Vanbergen et al. 2013) such as habitat loss, agricultural intensification, pesticides, the presence of exotic invasive species, and pathogens (Freitas et al., 2009; Galetto et al., 2022; Toledo Hernández et al., 2022).

Here, I evaluated the prevalence, and viral sharing of RNA viruses associated with honey bees in honey bees and different stingless bee species from The Yucatan Peninsula, Mexico. I hypothesized that due to the presence of bee virus vectors such as the mite *Varroa destructor*, the evidence of negative impacts of DWV and BQCV on honey bees, and the presence of possible transmission routes in the field through shared use of flowers, there is a high probability of detecting the most common RNA virus of the honey bee on different stingless bee species from the Yucatan Peninsula, Mexico. Evaluating the prevalence of

RNA viruses associated with honey bees on different stingless bee species is, therefore, a priority to identify risks to this culturally, ecologically, and economically important bee group, as well as to maintain the ecosystem service of pollination in tropical regions.

2 Materials and methods

2.1 Field sampling of bees

Sampling was carried out from January to April 2019 during the flowering season at 12 locations in the three states of Campeche, Quintana Roo and Yucatan that comprise the Yucatan Peninsula of Mexico (Figure 1). Honey bees are not native to the New World, i.e. *A. mellifera* is an exotic species, though the Yucatan Peninsula is one of the world's most important beekeeping regions due to its high density of managed and feral colonies of *A. mellifera*, referred to as Africanized honey bees (Guzmán-Novoa et al., 2020; Quezada-Euán et al., 1996). The Yucatan Peninsula also supports 17 eusocial stingless bee species (Ayala, 1999; Quezada-Euán, 2018), which are known to contribute significantly to the pollination of its wild and crop plants (Caro et al., 2017; Grüter, 2020; Quezada-Euán, 2018). One of them, *Melipona beecheii*, is emblematic through the millennial tradition of management (meliponiculture) by the ancient Maya people, a practice that continues to this day, yet is also declining in abundance (Quezada-Euán et al., 2018). Its ability to be managed lends it to experimentation.

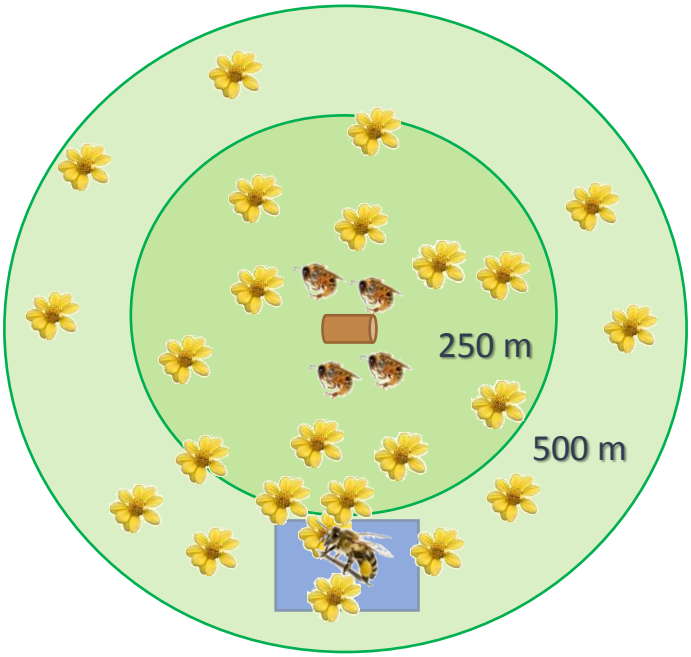

Figure 1. Sampling scheme (meliponary and flower patches) at each location in **Table SM 1**. *Melipona beecheii* was collected from within the meliponary, whilst honey bees were collected at flowers in transects at up to 500 m distance from the meliponary.

At each location, >15 km apart from one another to ensure independence, given the typical foraging ranges of 2-5 km for honey bees and, based on its similar body size, *M. beecheii* (Greenleaf et al., 2007), I sampled from one meliponario (stingless bee apiary containing 10-30 colonies of *M. beecheii*), collecting a total of 30 *M. beecheii* foraging worker bees

returning to their hives on a single day. Additionally, on the same day, I collected honey bee workers as well as different stingless bee species (which I term the Meliponinos group) along continuous transects at flower patches within 500 meters of the meliponario (Figure 1). At each flower patch, I performed a transect in an area of 1000 m^2 (10 x 100 m or 20 x 50m), where I collected maximally 10 bees per species per 10 m stretch before moving to the next 10 m stretch of flowers until I had collected ca. 30 bees per specie (Fürst et al., 2014). As meliponarios were in the backyards of villagers, flower patches were embedded in a rural matrix composed of gardens, parks, and wildflower meadows. Upon collection, bees were immediately transferred to individual 1.5 ml vials filled with RNA-Later® (QIAGEN, Hilden, Germany) and kept in the field on dry ice (-80°C) to avoid RNA degradation (Human et al., 2013).

2.2 *M. beecheii* RPL6 (Ribosomal protein L6) primers

To identify an internal reference marker for our stingless bee species, I selected candidate genes (Argk, alphaTub, GAPDH and RPL6) based on information from potential reference genes in stingless bees and honey bees (Freitas et al., 2019; Walker & Allen, 2010). The sequences of the candidate genes were retrieved from the *M. quadrifasciata* genome (Assembly ASM127656v1) and were also compared and identified in a *M. beecheii* transcriptome dataset (E. Stolle, unpublished). Intron-spanning primers were designed from *M. quadrifasciata* gene sequences using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) that were conserved in *M. beecheii*. In primer design I applied specific parameters of an optimal annealing temperature (t_m = 60°C) and amplicon length ranging from 151 to 275 bp.

Using conventional PCR, I checked the amplification of cDNA from *M. beecheii* adults with the different candidate gene primers. PCR products were verified by electrophoresis on 1 % agarose gels. From the different reference genes tested, all *M. beecheii* samples amplified unambiguously with the RPL6 primers. Finally, the QIAxcel capillary electrophoresis system was used to confirm the optimal t_m prior to perform qPCR assays, which proved to be 55°C. PCR products were denatured for 15 seconds at 95°C, cooled to 55°C for 30 seconds, followed by 39 cycles of 30 s at the t_m (55°C), 30 s at 72°C, and 1 min at 95°C. Finally, a melt curve profile was generated by cooling the sample to 50°C then raising the temperature to 95°C at 0.5°C per second increments to check that the correct PCR product had been amplified (clear trace rising quickly to peak at the primer pair's expected dissociation ('melt') temperature.

2.3 RNA extraction, virus detection, and absolute quantification

RNA extraction, viral detection and absolute viral quantification by qPCR were performed on individual bees using standard methods developed for honey bees (De Miranda et al., 2013) and following Tehel et al. (2019). I randomly selected 10 *M. beecheii* bees (total n = 120) ca. 10 honey bees (total n = 114) and ca.10 stingless bee per species (total n = 205), the number of stingless bee per species was variable; all the samples were processed per location for viral screening.

To extract RNA from bees, an individual was crushed in 500 µl RLT-buffer containing 1 % β–mercaptoethanol using a plastic pestle, from which total RNA was extracted using an RNeasy Mini kit (QIAGEN, Hilden, Germany) in a QIAcube extraction robot (QIAGEN) (De Miranda et al., 2013b; Radzevičiūtė et al., 2017; Tehel et al., 2019) and eluted into 30 µl RNAse-free water. cDNA was synthesised from 800 ng of the RNA using Oligo-dT oligonucleotides (Thermo Scientific) and reverse transcriptase (M-MLV and Revertase, Promega, Mannheim, Germany) following the manufacturer's instructions and then diluted 1:10 before use in qPCRs.

I used qPCR to detect ABPV, BQCV, DWV-A, DWV-B, SBPV and SBV using primers given in Table SM 2. I performed duplicate qPCR reactions per sample in a Bio-Rad C1000 thermal cycler (Bio-Rad, Munich, Germany) using SYBRgreen Sensimix with the following program: 5 minutes at 95 ºC, followed by 40 cycles of 10 seconds at 95 ºC, 30 seconds at the primer's t_m and 30 seconds at 72 ºC. I used a t_m of 57°C for all primers except those amplifying ABPV, for which I used a t_m of 53°C. Two positive (extract of an infected bee) and two negative (template-free) control wells were included per 96 well plate; they were consistently positive (< 30) or negative (>40) respectively. I set the PCR cycle quantification (Cq) threshold at < 35 to consider a sample as positive for a viral target and, if technical duplicates differed by more than one cycle, I re-ran qPCRs (5% of samples) and averaged the nearest two values.

I ran a melt curve profile for each qPCR product to ensure a single product of the correct dissociation ('melt') temperature had been generated. For absolute viral quantification, duplicate qPCR reactions were performed for each sample and run on the same 96-well PCR plate as duplicate standard curves generated from a 10-fold dilution series of PCR products of DWV-A, DWV-B, and BQCV-positive samples, with efficiencies of 106.82% (DWV-A), 104.54% (DWV-B), and 94.16% (BQCV), and correlation coefficients (R^2) from 0.961 to 0.999. For our qPCR conditions, a Cq of 35 represented ca. 10^3 viral genome equivalents (GEs) per bee.

To control for RNA degradation or extraction failure, I also performed duplicate qPCR amplifications of honey bee β–actin (primers in Table SM 2) for all honey bees and ribosomal protein L6 (RPL6, primers in Table SM 3) for all *M. beecheii* and different stingless bee species as host internal reference marker gene. Samples with an average reference gene Cq > 35 were excluded. In total, I rejected one *A. mellifera* sample, six *M. beecheii* samples and three stingless bee species either because of RNA degradation or extraction failure.

2.4 Sequencing of BQCV to test for host specificity

I found Africanized honey bees and *M. beecheii* positive to BQCV from different locations. To determine if sequences were identical, which would suggest sharing of the same virus, qPCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and cloned directly using the pGEM T Easy Vector System II (Promega, Mannheim, Germany) following the manufacturer's instructions. Plasmid DNA was

isolated using a Spin Miniprep Kit (Qiagen, Hilden, Germany). Three clones per sample were sequenced in forward and reverse orientation (GATC Biotech, Constance, Germany), and aligned manually using Geneious v6.0.6 (Kearse et al., 2012) to a reference genome of BQCV (NC_003784) to describe viral sharing among host bee species and between localities.

2.5 Statistical analyses

All analyses were performed in R v. 4.1.3 (R Core Team). Model assumptions were checked using the R package "DHARMa" (Hartig 2020) and were found to conform to expectations (residuals were normally distributed and homogeneity of variances was observed).

To test BQCV viral prevalence varied across host groups, I used generalized linear mixed models (GLMMs) with binomial error structure for BQCV and generalized mixed models for DWV-A. Because DWV-B presented few positive individuals, I did not run any model; bee group identity (Africanized honey bees, *M. beecheii* or Meliponinos group of additional stingless bee species) was used as a fixed factor and sampling location was included as a random factor. To investigate whether pathogen prevalence in *M. beecheii* was related to pathogen prevalence in Africanized honey bees at the same locality, I also used GLMMs with binomial error structure in which honey bee pathogen prevalence was used as a fixed effect and sampling location was included as a random factor. The analyses were implemented using the function glmer within the R package lme4 (Bates et al., 2015).

3 Results

3.1. RNA viruses in honey bees and stingless bees in the Yucatan Peninsula

Three of the six screened RNA viral targets were detected in honey bees: BQCV, DWV-A and DWV-B. In *A. mellifera* the most prevalent was BQCV at 80% (91 of 114 bees) and was detected at all 12 locations (Figure 2), followed by DWV-A at 13% (14 of 114 bees) detected at nine locations (Figure SM 1), then DWV-B at 2% (2 of 114 bees) at two locations (Figure SM 2). For the stingless bee species, in *M. beecheii*, I detected BQCV (prevalence 15%, 19 of 120 bees) at 7 locations (Figure 1) thought DWV-A at only one location (prevalence 1%, 1 of 120 bees; Figure SM1); DWV-B was not detected in *M. beecheii* (Figure SM 2). I identify that *M. beecheii* have a far lower average BQCV prevalence than honey bees (BQCV: GLMM, z = -3.08; Tukey's HSD, p = 0.001; DWV-A: χ^2_1 = 12.76 p = 0.001; Figure 3). I note one exceptional location (location 9, Bacalar) at which the prevalence of BQCV in *M. beecheii* was higher (60%) than in *A. mellifera* (10%, Figure 1). For the Meliponinos group, I detected DWV-A in two bees from *Nannotrigona perilampoides* at two different locations (prevalence 1%, 2 of 205 bees; Figure SM 2); I also identify that DWV-A prevalence was lower than in honey bees and DWV-B was detected in one bee, *Trigona nigra*, in one location (prevalence 0.5%, 1 of 205 bees; Figure SM 2). I did not find positive samples of BQCV in the Meliponinos group.

Across locations, the prevalence of BQCV in *M. beecheii* was unrelated to that in honey bees (GLMM, χ^2_1 = 2.55 p = 0.11; Figure SM 3).

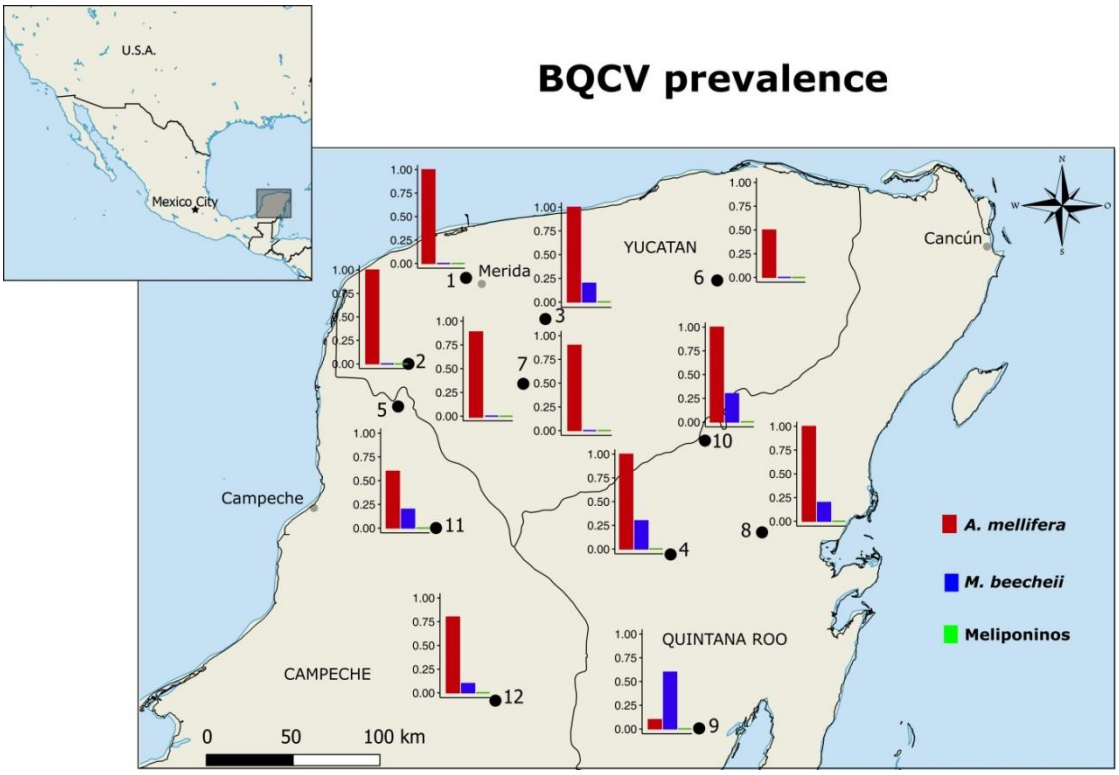

Figure 2. BQCV prevalence by location in the Yucatan Peninsula. Location codes: 1. UADY, 2. Maxcanú, 3. Hocabá, 4. Polyuc, 5. Calkiní, 6. Espita, 7. Mama, 8. Felipe Carrillo Puerto, 9. Bacalar, 10. Tihosuco, 11. Hopelchén and 12. Calakmul.

BQCV viral titres were significantly higher in qPCR-positive honey bees (n = 91; median 10^5, range 10^3-10^7 GEs per bee) compared to qPCR-positive *M. beecheii* (n = 19; median 10^4, range 10^2-10^5 GEs per bee) (GLMM, $\chi^2_1 = 349$ p = 0.001; Figure SM 4). DWV-A viral titres in qPCR-positive honey bees were quite variable (10^2-10^8 GEs, n = 14). The only *M. beecheii* sample qPCR-positive for DWV-A had a titre of 10^5 GEs; moreover, from the stingless bee group one *N. perilampoides* from Maxcanu had titres of 10^8 GEs and from Mama 10^5 GEs. Finally, the viral titres of the two honey bees as well as the only species from the Meliponinos group infected with DWV-B were low, and at the threshold of detection (~10^3 GEs).

3.2 BQCV sequences analysis

Haplotype network analysis of BQCV revealed two main clusters (Figure 4), one shared by *A. mellifera* and *M. beecheii* at the same locality (Figure SM 5) and one restricted to *M. beecheii*. The latter haplotype cluster included *M. beecheii* isolates from locality Bacalar (locality 9), at which the prevalence of BQCV in *M. beecheii* was also high (Figure 1). Coded by geographic origin, the haplotype network revealed that BQCV variants from both clusters were widely distributed across two or all three states of the Yucatan Peninsula (Figure SM 6).

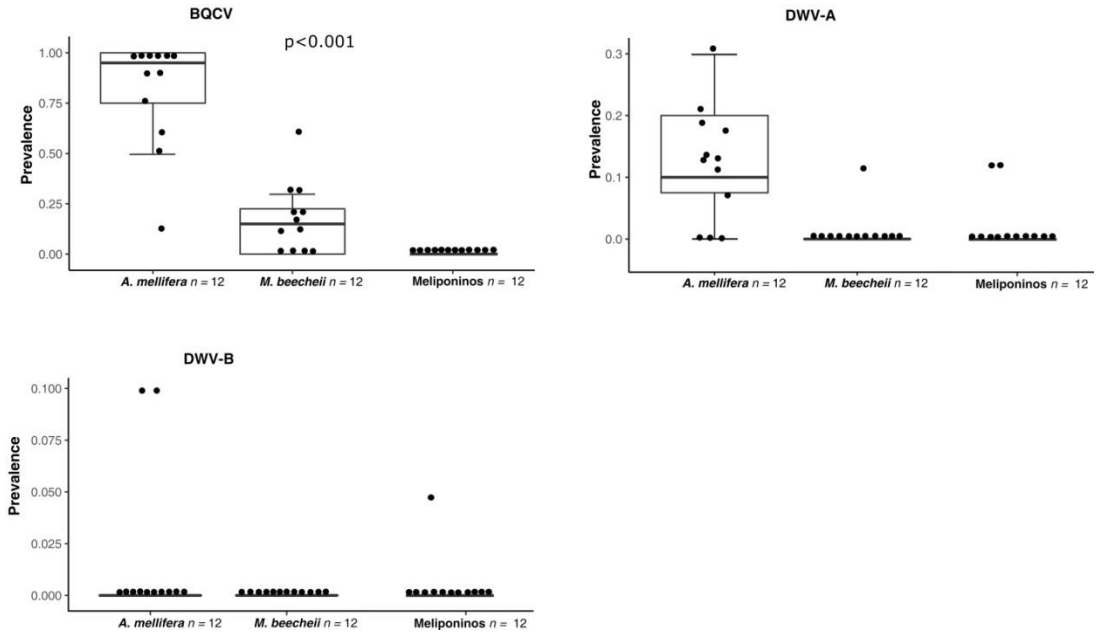

Figure 3. Viral prevalence (BQCV, DWV-A and DWV-B) in Africanized honey bees, *M. beecheii* and Meliponinos from the Yucatan Peninsula.

4 Discussion

The results of my investigation confirm the presence and prevalence of BQCV, DWV-A, and DWV-B in bees from Yucatan. These three viral targets have been reported in honey bees and stingless bees from elsewhere in Latin America, where BQCV was also found to be more prevalent than DWV-A (Alvarez et al., 2018; Guimarães-Cestaro et al., 2020; Guzman-Novoa et al., 2016; Maggi et al., 2016; Morfin et al., 2020; Ramos-cuellar et al., 2022; Tapia-González et al., 2019). On the other hand, I found the presence of DWV-B in an African honey bee (AHN) and in one stingless bee species, though I did not find this genotype in *M. beecheii* samples from meliponaries.

I hypothesize that DWV-B could be present at low prevalence or in other wild bee species, including other stingless bee species, as is the case in temperate regions (Fürst et al., 2014; Jessica L. Kevill et al., 2019; Riveros et al., 2020). Interestingly, of the six viral targets, I did not find SBV, SBPV or ABPV in honey bee or *M. beecheii* samples. As these viruses have already been reported in honey bees (Antúnez et al., 2015; Maggi et al., 2016; Tibatá et al., 2021) and stingless bees (Alvarez et al., 2018; Guimarães-Cestaro et al., 2020; Ueira-Vieira et al., 2015) from other regions in Latin America, the likelihood is that, if they are present in the Yucatan Peninsula, then they are at a very low prevalence.

My data support the notion of spillover of virus from honey bees to stingless bees because the prevalence and viral titre of BQCV and DWV-A and DWV-B in stingless bees were generally lower than those in honey bees in the Yucatan Peninsula. This pattern is also found in *Melipona colimana* from the west of Mexico, where BQCV and DWV-A were found at lower viral loads compared with honey bees (Morfin et al., 2020). Though the higher BQCV prevalence in *A. mellifera* at 12 Yucatecan sites suggests that virus may spill

over from honey bees to stingless bees, I did not find a statistically significant relationship between BQCV prevalence in honey bees and in *M. beecheii* across Yucatan, contrary to temperate regions with wild bees (bumble bees) and honey bees (Fürst et al., 2014); indeed, at one Yucatecan location (Bacalar), BQCV prevalence was higher in *M. beecheii* compared to honey bees. These data suggest more complex pattern of viral sharing, with spillover from honey bees to *M. beecheii* and onward transmission within *M. beecheii*.

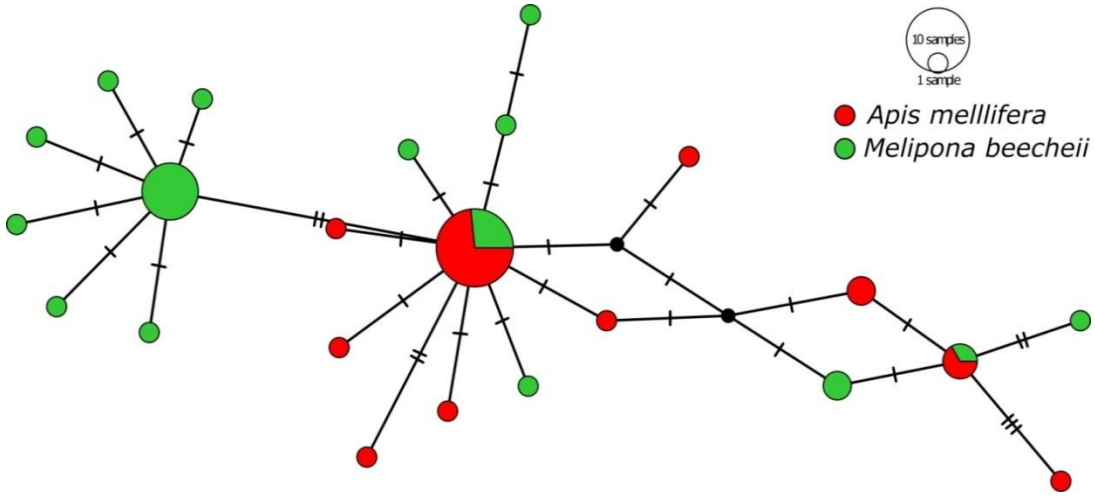

Figure 4. Median-Joining haplotype network of BQCV sequences from *A. mellifera* (n = 11 bees) and *M. beecheii* (n = 13 bees) at seven locations. The size of the circle representing a haplotype is proportional to the haplotype's frequency. Hatch marks indicate mutational steps and black dots represent inferred haplotypes.

The distribution of viral haplotypes in honey bees and *M. beecheii* across Yucatan suggests a more complex pattern of viral spillover and onward transmission of BQCV within the *M. beecheii* population. On the one hand, I found that one widespread BQCV haplotype was shared among *A. mellifera* and *M. beecheii*, supporting interspecific transmission. Similar patterns of virus sharing between honey bees and wild bee species have been detected for DWV-A and DWV-B in Europe and Asia (Manley et al., 2019; Radzevičiūtė et al., 2017) and between honey bees and stingless bees for DWV in Brazil (de Souza et al., 2019) and for DWV and BQCV in Mexico (Morfin et al., 2020). On the other hand, I also found another widespread BQCV haplotype that was apparently restricted to *M. beecheii*, suggesting onward transmission of a novel BQCV variant in *M. beecheii*.

Interspecific interactions at flower patches (Dalmon et al., 2021), wherein honey bees and wild bees sequentially or simultaneously visit the same flower to collect resources such as pollen and nectar, is considered one of the most plausible scenarios for viral sharing among bee species (Alger et al., 2019; Graystock et al., 2015; McArt et al., 2014).Stingless bee species and honey bees are very likely to visit the same flowers in Yucatan because of the high density of managed honey bees for honey production (Magaña Magaña et al., 2016) as well as the high density of Africanized honey bee feral swarms (J J G Quezada-Euán, 2007). On the other hand, as colonies of *M. beecheii* and other stingless bee species are often traditionally managed, I do not discard other potential inter- or intraspecific routes of transmission though human management. The use of honey bee products in meliponicultural activities (Matthijs et al., 2020; Schittny et al., 2020; Yañez et al., 2020)

and the indiscriminate transport of stingless bee species for commercial purposes (Carvalho, 2022) could lead to viral spread within and among stingless bees and other wild bee species. Future research should focus on exploring these potential intraspecific and interspecific routes of viral transmission.

The results of my investigation are indications that suggest a spillover scenario from AHBs to stingless bee species in the Yucatan Peninsula, Mexico. The relation between viral presence and prevalence, as well as the evidence of viral sequences sharing among honey bees and wild bees, have been recorded in the last decades in different world regions (Fürst et al., 2014; Manley et al., 2019; McMahon et al., 2015; Nanetti et al., 2021; Radzevičiūtė et al., 2017). Nevertheless, despite that we nowadays have more evidence about the possible routes of infections among honey bees and wild bees that could explain pathogen spillover (Alger et al., 2019; Burnham et al., 2021; Durrer & Schmid-Hempel, 1994; Tehel et al., 2022), it is still not clear if pathogens such as RNA viruses associated with honey bees could pose a threat to wild bees populations.

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Chapter IV: Impact of honey bee RNA viruses on the stingless bee *Melipona beecheii* in the Yucatan Peninsula, Mexico

Abstract

The western honey bee has a global distribution, and so do the pathogens associated with this commercially important pollinator. Among the different pathogens associated with honey bees, RNA viruses are considered a main driver of colony losses reported in temperate regions. The same viruses are present in different insect orders, including managed and wild bee species where honey bees are present. Nowadays, there is growing evidence of viral spillover events between solitary and social bees, which contribute to an understanding of the distribution of those pathogens within and between communities of flower-visitors; nevertheless, the impact of those RNA viruses is poor documented and limited to commercial non-*Apis* bees, such as the bumble bee *Bombus terrestris* from temperate regions. In the Neotropics, despite the presence of Africanized honey bees, there is a rich diversity of solitary and social bees, including the stingless bees group that comprise >350 spp. for this region. Among this different stingless bee species, *Melipona beecheii* represent one of the most important stingless bees of the Neotropics because of its cultural, ecological and economic importance for the Mexican South-East. Here, to elucidate the potential negative impact of RNA viruses associated with honey bees on *M. beecheii*, pupae and adult worker bees of this species were exposed to BQCV, DWV-A, and DWV-B by injection and by feeding in laboratory experiments. I then tested for potential negative impacts of these RNA viruses on *M. beecheii*. Evidence for viral replication of the different viruses in *M. beecheii* pupae through injections confirms that this species is a competent host for these viruses. Moreover, host *M. beecheii* survival was reduced when inoculated with virus by feeding. These findings confirm for first time the negative impact of RNA viruses associated with honey bees on *M. beecheii*. Future research, such as viral exposition in semi-natural conditions as well as the replication of similar experiments with other stingless bee species, is necessary to evaluate the impact of RNA viruses on this important tropical pollinator group.

Keywords: *Melipona beecheii*, *Apis mellifera*, Spillover, RNA viruses, negative impact

1 Introduction

Among the principal pests and pathogens that affect western honey bee populations around the world, the exotic mite *Varroa destructor* and associated RNA viruses represent arguably the most significant problem for apiculture (Rosenkranz et al., 2010; Traynor et al., 2020). Many viruses are associated with *A. mellifera*, including deformed wing virus (DWV), chronic bee paralysis vi*rus* (CBPV), black queen cell virus (BQCV), sacbrood virus (SBV), slow bee paralysis virus (SBPV), and acute bee paralysis virus (ABPV) (Beaurepaire et al., 2020). All six viruses and their variants are widespread in honey bee populations and capable of causing disease with recognizable symptoms and adverse effects (De Miranda et al., 2013; Jack & Ellis, 2021; Kevill et al., 2019; McMahon et al., 2016).

In temperate regions, these RNA viruses have been found not only in *A. mellifera* but also in other wild bee species such as bumble bees (*Bombus* spp.) and solitary bees as well as in insects from other orders, even though *V. destructor* is restricted to *A. mellifera* (Fürst et al., 2014; McMahon et al., 2015; Radzevičiūtė et al., 2017; reviewed in Tehel et al., 2016; Nanetti et al., 2021). A possible explanation for the presence of honey bee viruses in wild bee species is that honey bees from managed or feral colonies deposit virus on flowers during feeding or when defecating and that wild bee species subsequently take up the virus when foraging on the same flowers: pathogen spillover (Alger et al., 2019; Burnham et al., 2021; Singh et al., 2010). Nowadays, there is evidence of viral prevalence associated to honey bees in different bee groups from tropical regions, including Mexico (Martin and Tehel), making pathogen spillover from *A. mellifera* may therefore be not only a temperate region problem but also potentially one of global extent.

Invasive species may be a source of particularly pernicious pathogens that mediate displacement of native species ahead of competition for resources or habitat, particularly when the invasive is an exotic species, as are Africanized honey bees in the neotropics, because native species are naive to the invasive's pathogens. Examples include the decline of the South American native bumble bee *Bombus dahlbomii* Guérin-Méneville, attributed to the introduction and spread through Chile and Argentina of the Eurasian bumble bee *B. terrestris* and its protozoan pathogens (Schmid-Hempel et al., 2014). While there is growing evidence for pathogen spillover from honey bees to other insects, the subsequent effects of viral spillover on native, non-*Apis* insect pollinators are less well characterised. Earlier studies in Europe using native honey bees and *Bombus* spp. demonstrated that inoculation with DWV leads to reduced survival of *B. terrestris* (Fürst et al., 2014; Graystock et al., 2016), though subsequent experiments in the laboratory and the field have suggested limited impact of honey bee viruses on commercially sourced *B. terrestris* (Tehel et al., 2020, Streicher et al. 2022). We lack studies on the impact of viral spillover on populations of wild non-*Apis* bee species from tropical regions, where honey bees and wild bees also share floral resources and spillover is likely to be prevalent and where, for the neotropics, *A. mellifera* is an exotic invasive species.

In tropical Yucatan Peninsula (Mexico), Africanized honey bees and 17 different stingless bee species are found sympatrically (Ayala, 1999; Quezada-Euán, 2018), which together contribute pollination services to different ecosystems of this region (Caro et al., 2017; Grüter, 2020; Quezada-Euán, 2018). Among the different stingless bee species, *Melipona beecheii* has outstanding for this region because of its ecological, cultural and economic importance (Quezada-Euán, 2018). Despite its importance, there is little knowledge about the potential threats to its populations, such as the presence in it of RNA viruses associated with honey bees.

Here, to elucidate the potential negative impact of RNA viruses associated with honey bees on *M. beecheii*, pupae and adult worker bees of *M. beecheii* were exposed to BQCV, DWV-A, and DWV-B by injection and by feeding. I then tested for potential negative impacts of these RNA viruses on *M. beecheii*. I hypothesised that the virulence of different RNA viruses on *M. beecheii* was similar to their virulence in honey bees and bumble bees,

reducing the longevity of exposed stingless bees in comparison with non-infected bees under controlled laboratory conditions.

2 Material and methods

2.1 Source of bees

AHN and *M. beecheii* worker bees (pupae and adults) were taken from an experimental apiary as well as a traditional meliponario at the Department of Apiculture of the Campus of Biological Sciences and Animal Husbandry, the Autonomous University of Yucatán in Xmatkuil Mexico. To evaluate the viability of viral inocula, I collected white-eyed pupae from combs from three different AHN colonies, as well as white-eyed pupae from combs from five different colonies of *M. beecheii*; combs from both species were placed in an incubation chamber (36ºC with 70% RH and 32ºC with 70% RH for AHBs and *M. beecheii*, respectively) to obtain newly hatched worker bees for the feeding-inoculation experiment.

2.2 Viral propagation

Previous to my experiments addressing viral impact on *M. beecheii*, I propagated DWV-A, DWV-B, and BQCV by injecting one µl of one of the three viral inocula of Tehel *et al.* (2020) into white-eyed honey bee pupae. Three to five days thereafter, pupae were collected in groups of three and crushed in 0.5 M of cold potassium phosphate buffer (PPB pH 8.0). RNA extraction, cDNA synthesis, and screening by qPCR were performed for the newly generated virus inocula (DWV-A, DWV-B, and BQCV) as well as for other common RNA viruses (ABPV, SBV, and SBPV) as described above (RNA extraction and virus detection) to ascertain that inocula were free of contaminating viruses. Viral inocula were aliquoted and stored at -80 °C until use.

Inocula were injected into *M. beecheii* pupae using a Hamilton syringe (hypodermic needle outer diameter: 0.235 mm) in UADY (Yucatan) laboratories to check on the competence of *M. beecheii* in supporting viral replication. To avoid viral cross-contamination, needles and syringes were specific to each virus or the control. In addition, injections were performed by one person that injected 15 honey bee pupae per viral treatment on the same day. Treated pupae were placed in a 96-well microtiter plate and maintained in an incubator at 35°C and 50% RH. After 24 hr, dead pupae (ca. 10%) were discarded as they likely succumbed to handling during injection. Five days after inoculation, pupae were harvested and stored individually in vials at -80°C for subsequent evaluation of their viral titres, as described above for absolute viral quantification, as a measure of the competence of *M. beecheii* to support viral replication.

2.3 Testing the viability of viral inocula

To test the viability of viral inocula, I transferred white-eyed honey bee pupae from three colonies at an experimental apiary at the Autonomous University of Yucatán to CT chambers (36ºC, 70% RH). I then diluted the inocula in 0.5 M cold PPB (pH 8.0) to a final concentration of 10^5 viral genome equivalents (GEs) per µl, of which I injected 1 µl into

each *A. mellifera* pupa using a Hamilton syringe (hypodermic needle outer diameter: 0.235 mm). As a control treatment, I injected 1 µl of 0.5 M cold PPB (pH 8.0) into white-eyed *A. mellifera* pupae. Five days post inoculation, injected honey bee pupae had high viral loads (>10^9 GEs) of the respective virus whereas control pupae were devoid of these viruses, indicating that viral inocula were indeed infective (Figure SM 1).

2.4 Viral impact on *M. beecheii*

To test the virulence of the three prevalent viruses in Yucatecan honey bees (BQCV, DWV-A and DWV-B; see results) in *M. beecheii*, I performed a viral exposure experiment using inocula of each virus generated and tested for purity as described in Tehel *et al.* (2019) (see Supplementary Methods). To test the viability of viral inocula, I transferred white-eyed pupae from three honey bee colonies at an experimental apiary at the Autonomous University of Yucatán to CT chambers (36ºC, 70 % RH). I then diluted the inocula in 0.5 M cold PPB (pH 8.0) to a final concentration of 10^5 viral genome equivalents (GEs) per µl, of which 1 µl was injected into each pupa using a Hamilton syringe (hypodermic needle outer diameter: 0.235 mm). As a control treatment, I injected 1 µl of 0.5 M cold PPB (pH 8.0) into white-eyed *A. mellifera* pupae. Further methodological details are given in Supplementary Methods. Five days post inoculation, honey bee pupae injected with 10^5 GEs of either BQCV, DWV-A, or DWV-B had high viral loads (>10^9 GEs) of the respective virus whereas control pupae were devoid of these viruses, indicating that viral inocula were indeed infective (Figure 1).

2.5 Testing the competence of *M. beecheii* to support viral replication

To determine whether *M. beecheii* is a competent host for BQCV, DWV-A and DWV-B, I removed white-eyed pupae from five colonies of *M. beecheii* housed in a traditional meliponario at the Autonomous University of Yucatán and transferred them to a CT chamber (32ºC, 70 % RH). I then injected 1 µl of 10^5 viral GEs into the abdomens of white-eyed *M. beecheii* pupae (15 pupae per virus) and included a control group (n = 15 pupae) injected with 1 µl of 0.5 M cold PPB (pH 8.0). After five days, the pupae were collected individually in vials and stored at -80 °C for subsequent measurement of viral titre. Injections were performed as described for honey bee pupae (see Supplementary Methods).

2.6 Experimental viral inoculation by feeding *M. beecheii* adult worker bees

To determine the impact of viral inocula on *M. beecheii* worker bees of known age, simulating viral spillover in the field at flowers, brood combs from five *M. beecheii* colonies were stored under the same environmental conditions as the white-eyed pupae in the laboratory (32ºC, 70% RH) until worker emergence. Newly emerged *M. beecheii* worker bees (within 24 hours of emergence) were starved in 1.5 ml vials for one hour. Bees were then individually fed with 10µl of a viral inoculum consisting of 1 µl of 10^8 viral genomes equivalents (GEs) of either DWV-A or DWV-B, or 10^6 viral GEs of BQCV mixed with 9µl of sucrose solution 50% (w/v). Control bees were fed with 10µl of sucrose solution 50% (w/v). Immediately after inoculation, bees were transferred in groups of 10 bees per treatment to plastic cages (15 cages per treatment) with a removable base, multiple

ventilation holes, and *ad libitum* access to two vials containing a 50% w/v sucrose solution (Evans et al., 2009). Though inoculations were undertaken across 7 days, an equal number of bees was inoculated for all four treatments on any one day and by the same person. The survival of adult *M. beecheii* was recorded for 24 days, by which time all bees had died.

To identify changes in viral load of inoculated bees over time, I removed one bee per cage at three time points (two, four, and six days) post-inoculation (d.p.i) to quantify viral titres. Bees were stored individually at -80ºC to avoid RNA degradation. Eight bees per treatment and time point were then processed for RNA extraction, cDNA synthesis, virus screening and absolute viral quantification by qPCR, as described above. None of the bees from our control treatment showed a viral signal by qPCR.

Ethical approval is not required for experiments on insects in Germany or Mexico. The insects used in Germany (honey bees) and in Mexico (honey bees, *Melipona beecheii*) are not under conservation protection because they are managed.

2.7 Statistical analysis

All analyses were performed in R v. 4.1.3 (R Core Team). Model assumptions were checked using the R package "DHARMa" (Hartig 2020) and were found to conform to expectations (residuals were normally distributed and homogeneity of variances was observed).

Survival analysis of adult *M. beecheii* after oral virus exposure was performed with a Cox proportional hazards model using the R package *coxme* (Therneau, 2022). Experimental treatment (control, BQCV, DWV-A or DWV-B) was used as a fixed factor and cage as a random factor. To test for differences between experimental treatments, Tukey post-hoc tests were implemented with the R package *multcomp* (Hothorn et al., 2008), adjusting the family-wise error rate.

3 Results

3.1 Experimental inoculation of RNA viruses in *M. beecheii* pupae

Injection of 10^5 GEs of BQCV, DWV-A or DWV-B into *M. beecheii* pupae revealed that this stingless bee species is a competent host for all three viral targets. Three to five days after viral injection, *M. beecheii* pupae contained 6 x 10^7 GEs BQCV, $10^{7,}$ DWV-A, and 6 x 10^6 DWV-B (Figure 1). Control *M. beecheii* pupae that were injected with buffer were devoid of BQCV, DWV-A, DWV-B or other viruses (ABPV, SBV, and SBPV).

3.2 Experimental inoculation of RNA viruses in *M. beecheii* adults

The survival of *M. beecheii* adults was significantly reduced when experimentally fed with BQCV, DWV-A or DWV-B compared to control bees (*coxme*, $\chi^2 = 43.47$, df = 3 $p < 0.001$; Control vs. BQCV: Hazard Ration (HR) 2.13, Control vs. DWV-A: HR 2.28, Control vs. DWV-B: HR 2.40; Figure 2 and Table SM 1). The median survival of control bees was 7

days (95% CI: 6-8) versus 6 days for all viral treatments (BQCV, 6±6-8; DWV-A, 6±5-7; DWV-B, 6±5-7).

3.3 Viral load of *M. beecheii* infected by feeding decrease over the time

Melipona beecheii inoculated with virus by feeding had detectable titres of the inoculum's respective virus at two, four and six days post inoculation, though viral titres decreased slightly over time. I registered a decrease of the viral load over the time in each of the viral treatments (Figure 3). Bees infected with 10^6 viral (GE) of BQCV showed differences between the second fourth day post infection (GLMM, z= 1970; Tukey's HSD, p=0.001) and between the second and sixth day post infection (GLMM, z= 3362; Tukey's HSD, p=0.001). Nevertheless I did not find differences between the fourth and sixth day post-infection (GLMM, z= -1034; Tukey's HSD, p=0.44).

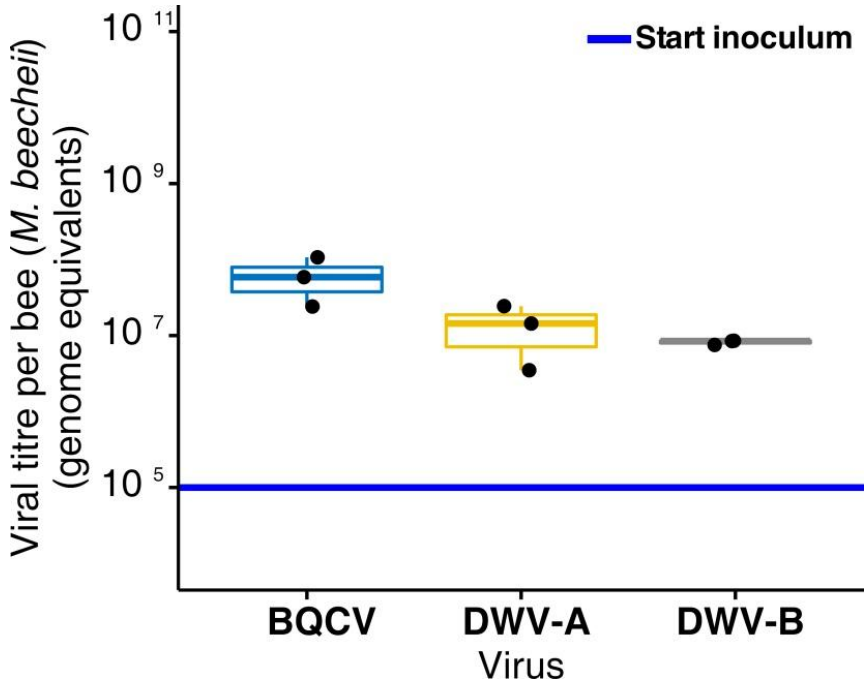

Figure 1. RNA viral titres in *M. beecheii* pupae five days after injection with 10^5 genome equivalents of viral inoculum (shown as a blue bar). Control pupae (n=3) were devoid of BQCV, DWV-A and DWV-B (data not shown).

The viral loads from DWV genotype A and B infected with 10^8 GE similarly decreased over the time. DWV-A viral loads where different between the second and fourth day (GLMM, z= 1827.2; Tukey's HSD, p=0.038), second and sixth day (GLMM, z= 1425.7; Tukey's HSD, p=0.001), as well as among the fourth and sixth day post-infection (GLMM, z= -967.3; Tukey's HSD, p=0.015). On the other hand, DWV-B viral loads from the second and fourth day were not different (GLMM, z= 1667.2; Tukey's HSD, p=0.065). Whereas the second and sixth day where slightly different (GLMM, z= 477.3; Tukey's HSD, p=0.038), I did not find differences in titre between the fourth and sixth day post-infection (GLMM, z= 939.5; Tukey's HSD, p=0.51). Bees from the control treatment did not show signs infections and gave no signal of viral presence by qPCR.

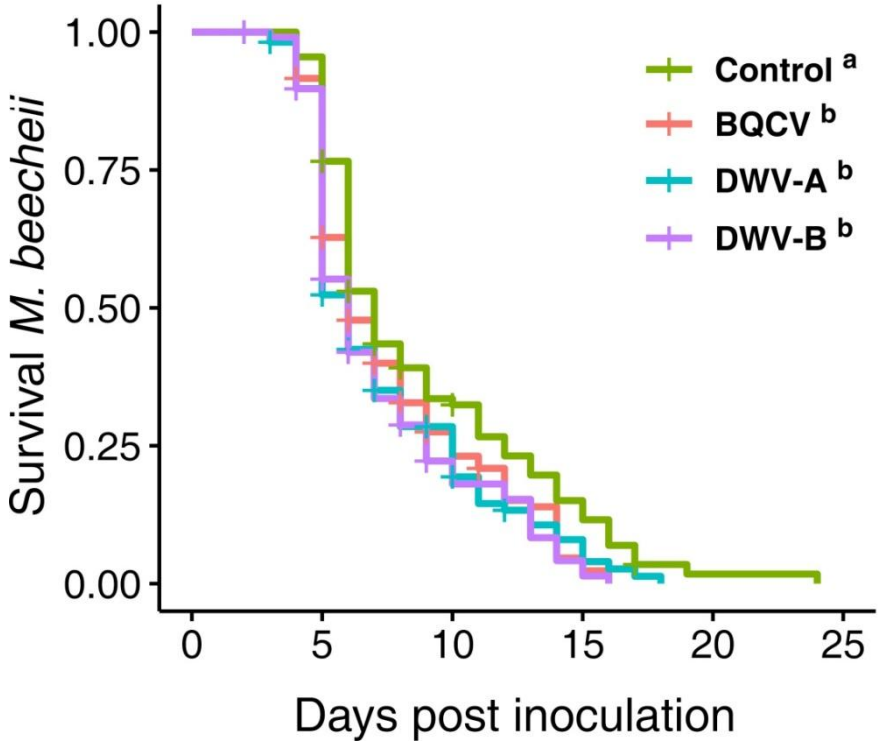

Figure 2. Kaplan-Meier curves showing reduced survival of *M. beecheii* adult worker bees exposed by feeding with BQCV, DWV-A and DWV-B; Cox proportional hazard model, $\chi^2 = 43.47$, df = 3 $p < 0.001$; different lower case letters following a treatment show significance of differences in survival ($p < 0.05$).

4 Discussion

Through controlled inoculations by injection and feeding, I confirmed that *M. beecheii* pupae are competent hosts for BQCV, DWV-A, and DWV-B, as all three viruses successfully replicated to high viral titres. Similar results have been recorded in commercial bumble bees (Fürst et al., 2014; Gusachenko et al., 2020; Tehel et al., 2020). Gusachenko et al. (2020) found evidence of replication in *Bombus terrestris* pupae inoculated by injection with DWV (10^8) after five days post-inoculation (p.i.). Also, Tehel et al. (2020), working with BQCV, DWV-A, and DWV-B in *B. terrestris* adults, found an increase in the titre of two different treatments (satiated and starved) after 10 days p.i., confirming viral replication in this host species. Despite lower viral inoculation doses (10^5) in my study being less, I recorded an increase in viral titre after five days p.i., confirming viral replication in *M. beecheii* and confirming that this stingless bee species is a competent host for RNA viruses associated with honey bees.

Moreover, I observed a negative impact of oral viral exposure on the survival of adult *M. beecheii* worker bees. Information on the impact of RNA viruses on wild bees is limited and contradictory. Early studies that evaluated the effect of DWV on the survival of *B. terrestris*, found negative effects on bee survival exposing bees to either injection or feeding (Fürst et al., 2014; Graystock et al., 2015). However, later studies, despite finding evidence of BQCV, DWV-A, and DWV-B viral replication in *B. terrestris*, were not able to find negative effects on the survival of exposed *B. terrestris* (by feeding or injection) at different

development stages (Gusachenko et al., 2020) or when satiated and starved (Tehel et al., 2020).

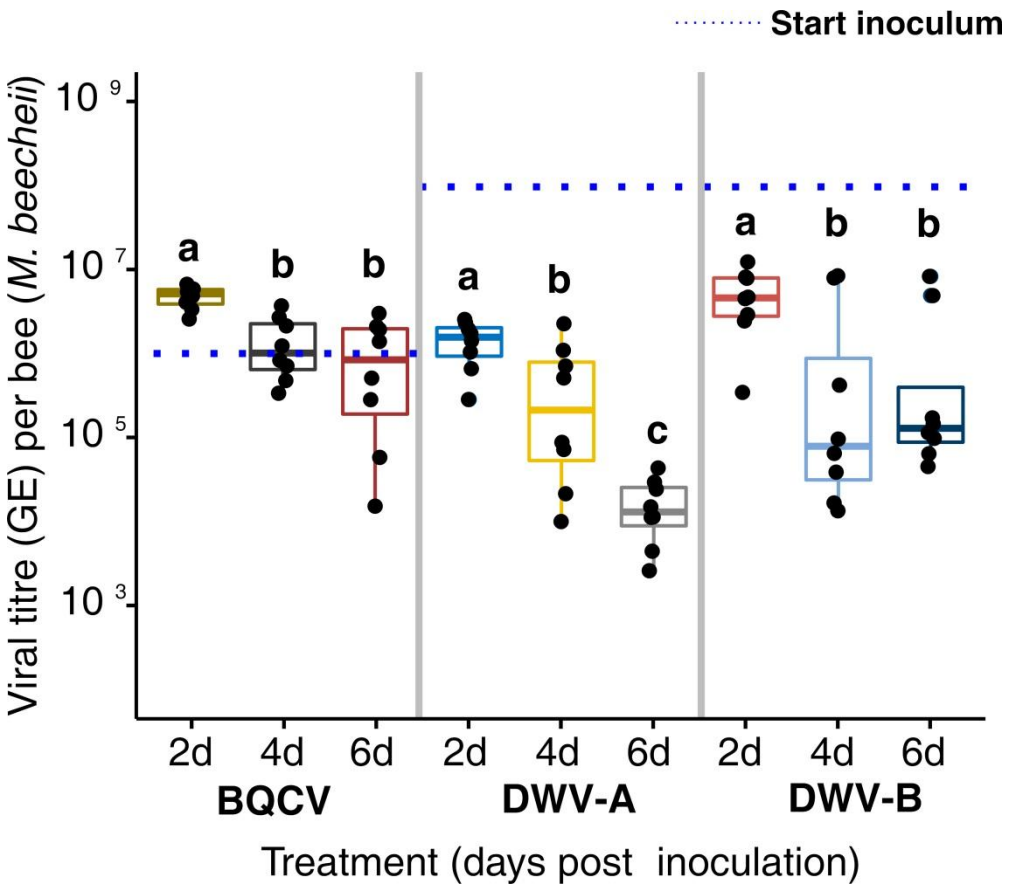

Figure 3. Change in viral load (genome equivalents: GEs) over time (two, four and six days post inoculation) of individual *M. beecheii* bees that had been individually fed virus inoculum. The blue dotted line represents the amount of virus initially fed to each and every bee, while the black dots represent the individually analysed bees (n=8 per treatment). Those fed with 10^6 GEs of BQCV and those fed 10^8 GEs of DWV-B or DWV-A showed a significant decrease in viral titre between day two and day four p.i. (post-hoc Tukey HSD; BQCV: p=0.001, DWV-A: p=0.038 and DWV-B: p=0.045) and day two and day six p.i. (BQCV: p=0.001, DWV-A: p=0.001 and DWV-B: p=0.038,) while a significant decrease between day four and day six p.i. was observed for DWV-A (p=0.015) but not for BQCV or for DWV-B (BQCV: p=0.44, DWV-B: p=0.51). Within a viral treatment, significant differences ($p < 0.05$) in titre across days are shown by different lower case letters. Variation in viral titre among bees at the same time point may be due to regurgitation or trophallactic exchange of the inoculum once bees were housed in cages, or to inter-individual differences in innate immune response. Control adults fed sucrose solution (n=72) were devoid of BQCV, DWV-A and DWV-B (data not shown).

Apart from these studies, all involving bumble bees from temperate regions, there is only one report of potential pathogen spillover together with confirmation of a negative fitness impact among honey bees and stingless bees involving the Australian stingless bee species *Tetragonula hockingsi*, namely when *T. hockingsi* adults are exposed to the pathogen *Nosema ceranae* (Purkiss & Lach, 2019). Although that study evaluated the impact of a different pathogens on the survival of *T. hockingsi*, the results of that study together with the results of my own study highlight the potential negative effects of pathogen spillover for stingless bees.

Interestingly, and despite the negative fitness impact of viral exposure on *M. beecheii* survival, my results also revealed a decrease of viral titre in *M. beecheii* after exposure by feeding. Similar reports from bumble bees exposed to DWV recorded a decrease in viral titre to 10^5 seven days after a feeding inoculation of 10^7. Further, Tehel et al. (2020) reported changes in the viral load of adult *B. terrestris* exposed to 10^9 viral titre of BQCV, DWV-A, and DWV-B by feeding at 18-25 days p.i. They found that, of seven bumble bees inoculated with DWV-B, three increased their viral load; instead, DWV-A and BQCV showed viral loads below the initial infection dose. Though I recorded a decrease in viral titer over time in *M. beecheii*, I do not discard the potential for possible increases in titre, as is reported in bumble bees over a longer observation time period (Tehel et al., 2020).

Information on the impact of RNA viruses on wild bees is limited and contradictory. Early studies that evaluated the effect of DWV on the survival of *B. terrestris* in the laboratory found negative effects on bee survival when exposing bees either by injection or feeding (Fürst et al., 2014; Graystock et al., 2013). However, later laboratory studies were not able to find negative effects on the survival of exposed *B. terrestris* (by feeding or injection) of different development stages (Gusachenko et al., 2020) or of adults, except when stressed by food deprivation (Tehel et al., 2020), suggesting that only under an additional stress does a honey bee virus impact a wild bee species. Indeed, bumble bee workers held in a natural environment, where they are forced to forage for their colony, exhibited reduced longevity when experimentally inoculated with DWV-A (Streicher et al. 2022). Condition-dependent mortality nevertheless supports the view that honey bee viruses can harm wild bee populations.

The evidence of a negative impact of RNA viruses associated with honey bees in *M. beecheii* highlights the potential risk that these pathogens pose for stingless bees and wild bee populations. Further research using controlled and field-realistic conditions on pathogen impact in stingless bees and other wild bee species is needed. Generating this information will contribute to informing on actions for their conservation and for the responsible management of tropical American stingless bees, a valuable and native resource that contributes to the health of tropical ecosystems as well as the economic and cultural development of society that depends of this pollinator group.

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Chapter V: General discussion

At present, insect pollinators are facing declines due to a multitude of factors, including climate change, GMOs, pesticides, poor pollinator management, and poor land management practices as well as the presence of exotic species and their associated pathogens (Dicks et al., 2021). In this thesis, I have highlighted the risks associated with viral spillover from honey bees to wild bees, specifically stingless bees, within the Mexican tropics. The original data, collected and analyzed by me, is presented in three distinct chapters. These data have revealed the presence of RNA viruses within a diverse community that includes native stingless bee species and managed Africanized honey bees. The general problem that I addressed in my thesis is the lack of knowledge about the potentially harmful effects of RNA viruses on native bee species. The information that I generated and described in this research has the intention to produce more scientific evidence pointing out the risks of exotic species, their pathogens, and the associated presence of these pathogens in native species so as to inform conservation actions for this pollinator group.

In this thesis, I demonstrate that honey bee-associated RNA viruses are found in AHBs drones, and thereby in AHBs populations (both managed and feral colonies) from the Yucatan Peninsula since 2010, which suggests a long-standing viral prevalence in this region (Chapter II). Later, with data collected in 2019 from worker AHBs and different stingless bee species, I confirmed the presence of the same viruses in their populations (Chapter III). The viral prevalence in AHBs population over time indicates an establishment of these pathogens without significant changes in their prevalence, in contrast to what is currently reported in honey bee populations from temperate regions (Chapter II).

It is likely that virus present in stingless bees is a consequence of spillover from honey bees; these are not only the first records of honey bee-associated RNA viruses in honey bees from this tropical region in Mexico but also the very first records in different stingless bee species, including the iconic species *M. beecheii* from the Yucatan Peninsula. Moreover, in an experiment simulating a realistic mode of viral transmission in the field, I found a negative impact of honey bee viruses on *M. beecheii* worker survival.

RNA viral prevalence in AHBs in the Yucatan Peninsula

In **Chapter II**, I revealed RNA viral presence in AHBs in this neotropical region since 2010. The first record of viruses in honey bees from Mexico was given by Guzmán-Novoa et al. (2012), who quantified the prevalence of four different viruses, including DWV, whose high prevalence is remarkable given that this virus is associated with and can cause colony losses in temperate regions (Paxton et al., 2022). It was possible for me to quantify the prevalence of the two main DWV genotypes (A and B); demonstrating that both genotypes had been present from 2010 and possibly much more long in this region. Chronologically, DWV prevalence data from Mexico coincides with the first reports in the USA, which were found in samples collected in 2010 from the mainland in the USA (Ryabov et al., 2017) and from the Hawaiian islands in 2012 (Mordecai et al., 2016). A possible explanation for their high prevalence could be the intense commercialization of

honey bees and other bee products in both US regions, making possible the transportation of honey bees and *V. destructor*, a honey bee ectoparasite considered the a main viral vector in honey bees (Martin et al., 2012; Mondet et al., 2014).

Interestingly, after the arrival of varroa and presumably RNA viruses, including DWV (Hasegawa et al., 2023), between the 1970s and 1980s in South and North America, respectively (Rosenkranz et al., 2010; Traynor et al., 2020), massive colony losses linked with viral prevalence have not been recorded in Latin America (Vandame & Palacio, 2010), though they have in the USA, where massive colony losses were reported for first time in winter 2006/2007 (Cox-Foster et al., 2007). Nevertheless, a possible reason for the lack of colony losses records in Latin American countries may be the absence of monitoring programs, making it difficult to know if, in some regions, there was a similar tendency of colony losses, as seen in the USA and Europe, as well as if those losses were related to viral prevalence (Requier et al., 2018). In Europe, from the different RNA viruses associated with honey bees, DWV-B is considered the principal cause of colony losses (Paxton et al., 2022); recently the change in prevalence of DWV genotypes to a preponderance of DWV B in Europe (Paxton et al 2022).

In the USA, besides a dramatic increase in DWV-B prevalence in the last decades (Ryabov et al., 2017), genotype A still maintains its dominance. My data, as is described in **Chapter II**, show that genotype B has been present since 2010 in the Yucatan Peninsula. Nevertheless, the prevalence of DWV-B is not increasing, as is reported in the USA. My data is similar to findings from Riveros et al (2020), who found that genotype B is present in Chilean colonies, but at a low prevalence. Unfortunately, the information from Chilean colonies, including other records from Argentina (Brasesco et al., 2020), Brazil (de Souza et al., 2019) or Colombia (Tibatá et al., 2021), do not have another time point from the past to undertake similar analyses as I presented in my thesis. Should it become important to perform similar viral prevalence studies in the future in these countries (including Mexico) to evaluate DWV dynamics, for example because of new evidence of viral replacement of DWV-A by DWV-B in the USA and elsewhere (Paxton el at., 2022), then this could have a negative impact on managed honey bee colonies for LA, impacting negatively the industry that depends on this pollinator.

The use of epidemiological modelling is an interesting approach that could help to understand viral dynamics in honey bee populations. As I mention in **Chapter II,** extending the model describing the co-occurrence of DWV-A and DWV-B by Paxton et al. (2022), I described together with my colleagues an epidemiological model that shows how DWV-A can prevent the establishment of DWV-B, possibly a form of superinfection exclusion. Through this 'inverted' patter (in which DWV-A is not replaced by DWV-B), I predict that DWV-A will continue being the dominant genotype over genotype B in AHBs in the Yucatan Peninsula. My predictions could be supported by different factors related with AHBs from Latin America, including AHN genetic background, climatic conditions or genetic differences among viral variants present in AHBs. Regarding this last point, our evidence from the DWV-A phylogenetic analysis at both time points showed no genetic change in the sequences but rather a consistent geographic pattern, where sequences from

the Yucatan Peninsula are similar to sequences from the USA and distant to sequences from Asia. It is feasible that, given the geographic distances as well as the differences in host species among America and Asia, it is not a surprise to find a geographic pattern to the genetic clustering of sequences. Unfortunately, it was not possible to undertake a similar analysis with DWV-B sequences because so few individuals were infected. For the future, it will be necessary to conduct similar analyses with genotype B to confirm if the genotype found in areas with low prevalence is the same as in locations where genotype B is rapidly increasing. Monitoring the dynamics of virus populations is crucial for future research because there is a possibility that DWV-B is currently undergoing an expansion process in the rest of the Americas and other regions. In this regard, it should be considered an emerging threat to honey bee populations, as well as to other bee species and overall pollinator diversity, especially in the Neotropical region (Fleites-Ayil et al., 2023).

Spillover in the sun: viral sharing among bees from the Yucatan peninsula

An important achieve from my thesis in **Chapter III** was to quantify viral prevalence of BQCV, DWV-A and DWV-B in stingless bee species from the Yucatan Peninsula. My findings support the notion of viral spillover events from AHBs to stingless bees. Also, I found that viral sequences were shared among AHBs and *M. beecheii*. Taken together, this information is similar to records from other world regions, where viral spillover is suggest when RNA viruses associated with honey bees are presented in other bee species (Fürst et al., 2014; Manley et al., 2019; Radzevičiūtė et al., 2017). Currently, the presence of the same viral sequence in different bee species is used as evidence of a spillover event; I found for first time the presence of BQCV and DWV-A and DWV-B in stingless bee species from my study region, and use sequences to suggest spillover. The presence of a 'honey bee virus' in stingless bees could be due to spillover at flower patches, where honey bees and wild bees shared resources (Alger et al., 2019; Graystock et al., 2016; McArt et al., 2014). However, this idea of flowers acting as transmission hubs should be tested in future research in different insect species (interspecific interactions).

Currently, there is no evidence of viral transmission by ectoparasites of stingless bees, in stark contrast to the case of *V. destructor* in honey bees. Therefore, spillover at flower patches could be the principal route of viral transmission in stingless bees. However, other routes could be involved in viral transmission. Interestingly *M. beecheii* had a higher viral prevalence compared with other stingless bee species evaluated in this thesis. *Melipona beecheii* is a traditionally managed species in the Yucatan Peninsula (Quezada-Euán, 2018) whose populations are currently mostly present in traditional Meliponarios (Grüter, 2020;Quezada-Euán, 2018). In fact, all my *M. beecheii* samples were from traditional meliponarios. Therefore, I cannot discard other potential inter or intra-specific routes of transmission through human management. The use of honey bee products in meliponicultural activities (Matthijs et al., 2020; Schittny et al., 2020; Yañez et al., 2020) and the indiscriminate transport of stingless bee species for commercial purposes (Carvalho, 2022; Quezada-Euán et al., 2022) could lead to viral spread within and among stingless bees and other wild bee species. However, these routes of transmission should be tested in

future investigation of other stingless be species that are closely related with human activities, as is *M. beecheii* in the Yucatan Peninsula.

Troubles in the tropics: evidence of negative impact of RNA viruses on *M. beecheii*

Finally, in **Chapter IV** I undertook a series of controlled inoculations of virus into *M. beecheii* in the laboratory by injection and feeding of the viruses BQCV, DWV-A and DWV-B, all of which I had detected in AHBs and stingless bee species in the study region described in **Chapter III**. Here, for first time I demonstrated the virulence of these pathogens in the stingless bee species *M. beecheii*; these findings reveal the potential negative effects of RNA viral spillover in this species. This hypothesis of viral spillover could be extended to other stingless bee and wild bee species in the tropics because I found a wide range of other stingless bee species to harbour the same viruses (see Chapter III and Tehel et al., 2016). However, it is rare to see studies that evaluate the harmful effects of pathogens in stingless bees; the only investigation of stingless bees exploring this topic was performed in *Tetragonula hockingsi*, a stingless bee species from Australia, to evaluate *Nosema ceranae* virulence in controlled conditions; the authors found harmful effects of this pathogen for the survival of this species (Purkiss & Lach, 2019).

Bombus terrestris is arguably the best studied host/recipient species with regard to viral pathogen spillover from honey bees. Negative effects of spillover on *B. terrestris* survival have been detected in the laboratory when exposing bumble bees to virus either by injection or feeding (Fürst et al., 2014; Graystock et al., 2013). However, later laboratory studies were not able to find negative effects of viral inoculation on the survival of exposed *B. terrestris* (by feeding or injection) at different development stages (Gusachenko et al., 2020) or of adults, except when stressed by food deprivation (Tehel et al., 2020). A possible explanation is related with the differences in the origin of colonies. In experiments with *B. terrestris*, commercial colonies were used, which themselves may have inadvertently been selected for viral tolerance or resistance during 'domestication' to commercial rearing facilities. In the case of managed stingless bee species, colony reproduction is performed using traditional techniques that allow colonies to develop in semi-natural conditions i.e. they have not been selected to survive in a state of high exposure to honey bee pathogens. My experiments should be replicated in other managed stingless bee species, with the objectives of determining if different stingless bee species are competent hosts for RNA viruses and, if so, whether viral replication impacts their survival.

Further investigation of RNA viral impact on stingless bees under semi-natural conditions is necessary to evaluate if their foraging activity is affected by viral infection. It is well known that there is a negative impact on bumble bee worker bees when infected by injection with DWV-A, reducing host life-span and causing a negative impact on foraging activity, with negative consequences for colony development (Cartar and Dill, 1991; Plowright and Pendrel, 1977; Sutcliffe and Plowright, 1990). Currently few studies have been able to address this question in bumble bees. One exception is Streicher et al. (2022), who found that *B. terrestris* workers infected with DWV-A by injection exhibited reduced longevity

when held in the field but no change in longevity when experimentally inoculated by feeding.

The viral loads in the feeding experiment that I generated in **Chapter IV** showed a decrease of in titre of all three different viruses over time. Currently, I can only speculate on the underlying reasons for these decreases. Similar to honey bees and bumble bees, physical and chemical barriers in the gut of stingless bees are likely to protect against different pathogens, (Evans & Spivak, 2010; Yañez et al., 2020). Moreover, gut microbiome associations in stingless bees could play a significant role in defending against different pathogens, including RNA viruses, as has been recorded in honey bees and bumble bees (Dosch et al., 2021; Engel et al., 2016; Kwong and Moran, 2016). Recent studies have shown the benefits of microbial association in insect development (Paludo et al., 2019) as well as the potential benefits of microorganisms with antimicrobial properties to defend their hosts against bee pathogens (de Paula et al., 2021). These and other factors could make *M. beecheii* less susceptible to viral infections and other pathogens, a field of research that deserves greater attention.

Summary

In this thesis I have emphasized the importance of evaluating the risk posed to native species of pathogens that are present in exotic species through spillover events. The presence of exotic species and their pathogens are considering one of the drivers of pollinator decline (Dicks et al., 2021). Currently the western honey bee has a worldwide distribution because different economic sectors (beekeepers, orchardists) depend of this pollinator (Beaurepaire et al 2020), which in turn leads to the widespread distribution of their pathogens. In this sense, is not a novelty to find pathogens of honey bees, including RNA viruses, in native bee populations where honey bees are considering an alien species, as is the case in the Neotropics. In **Chapter II**, I addressed the importance of evaluating the prevalence of DWV (genotypes A and B) in AHBs over time because to the major negative impact of this virus on temperate region honey bees. Interestingly, my data show an inverted pattern of viral dynamics of DWV, where DWV-A continues to remain dominant over DWV-B in AHBs population from the Yucatan Peninsula after nine years of its presence on the peninsula. In temperate areas such as Europe, this pattern is not seen; DWV-A is being replaced by DWV-B (Paxton et al., 2022).

In **Chapter III**, through my viral prevalence data in stingless bees and worker AHBs, I showed evidence of viral sharing among honey bees and different stingless bee species, supporting the notion of viral spillover from AHBs to stingless bee species on the Yucatan Peninsula. Moreover, in **Chapter IV,** I described for first time the harmful effects of three RNA viruses (BQCV, DWV-A and DWV-B) on *M. beecheii;* in this section of my thesis, I demonstrated by controlled infections: first, that pupae are competent hosts for viral replication by injection inoculation (biologically unrealistic) and, secondly, that adults fed inoculum (biologically realistic) reduced *M. beecheii* survival. But I also found that, after viral inoculum ingestion, viral load decreased over time.

My research adds support to a growing number of studies on viral sharing and pathogen spillover among honey bees and wild bees. The evidence of a negative impact of RNA viruses associated with honey bees on *M. beecheii* demonstrates the potential risk of these pathogens for stingless bees and other wild bee populations of Latin America and elsewhere. My results also underscore the importance of further research using controlled and field-realistic conditions to reveal the impact of pathogens on stingless bees and other wild bee species. Generating this information will contribute to the conservation and sustainable management of stingless bees, valuable components of terrestrial biodiversity that contribute to the health of tropical ecosystems as well as the economic and cultural development of societies that depend on this important pollinator group.

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I would like to thank to Dr. Theodorou, and Dr. Stolle, for their valuable contributions and support during my PhD. A special acknowledgment goes to Dr. Simon Tragust, my second mentor at MLU, for the insightful talks, advice, and support throughout my doctoral journey. I hold great respect for your honesty and passion for science. Many thanks to the excellent technicians from the Zoologie group: Anja Manigk, Claudia Castillo, and Henriette Kühnert, for their consistent help and support. I want to thank to Dra. Soro for her continuous kindness and support, which I truly appreciate. A big thanks to all the members of Robert's group, including visitors, Bachelor, Master, PhD students and Postdocs over these five years. Thank you for making a nice environment in Rob's group.

Many thanks to the wonderful people I have had the pleasure of meeting during my time in Germany. Special thanks to Nele, my German sister, thank you for the warm welcome to your country and for sharing our incredibly cool trip with Gina. To my friend Richard and his family/crew aus Dresden, you are very nice people who will always have a place in my heart. A big thank you to my Parcero Adrian Osorio; brother, your constant presence, insightful discussions on science and life, and our numerous adventures in "la gran ciudad" mean a lot to me. We survived it all! I extend my sincere gratitude to my family/friends in

Halle: Alice Seguret, Jonathan Kidner, my coma Claudia Castillo, Sara Hellström, and my sister from another mother, Andreia Texeira. It has been a pleasure to share these years with you in Halle and many other places with many nice and crazy adventures! Your presence made this significant step in my life much easier and incredibly fun. Thank you, amigos! Very special thanks to my dear Anna Friedel, thank you for all your support, for spend all this nice time together, for show me your country, your Bayreuth and your family; thanks too for being patient with my bad German but for above all for being the great person that you are, thanks a lot Anna.

Thanks to my wonderful friends in Mexico, who have always stayed in touch and eagerly awaited my return. Your warm welcomes and cheerful farewells during my various trips to Mexico mean a lot to me. A special thanks to my brother, Jorge Escalante, who has been by my side through both good and challenging times. Your constant presence is deeply appreciated. Tony and Mariel, your friendship and the super cool trips we have shared to Italy, Berlin and Halle hold a special place in my heart. Every year, the posadas with Rayo, Mariana, Leny, Diablo, Pop, and Raúl from mi Comarca & Juanas are moments I cherish. To Ursula Lopez, my sister from another mother, thank you for your unwavering friendship, support, and comforting words during those days when your kindness and yoga techniques transcended the distance.

Thank you to my dear family in Yucatán, to the Torres Barrera and Fleites Canto families, for taking care of my parents, my sister, and little Bosquito. This year was challenging for all of us, but, as always, we faced it together as a family. I am deeply grateful for your support and for consistently celebrating each of my milestones. Finally, I want to express my profound gratitude to my Mum, Dad, and Sister. There are no words or measures that can capture the extent of my appreciation for having you as my family. You have always believed in me and my ideas, and I am truly fortunate to be a part of you. Thank you for your patience, respect, and boundless love. Jake, my dear carnala, your care for our parents and Bosco, your constant presence, your visit in Germany, and affective and positive words mean a lot to me. You've been my unwavering support, and I'm endlessly grateful. To my Dad and Mom, your advice, discussions, opinions, and understanding have been invaluable to me. Your continuous support fuels my journey, and there's no greater joy than seeing you happy and proud of my achievements. Thank you, from the bottom of my heart, for everything.

¡Gracias totales!

Appendix Chapter II

Figure SM 1

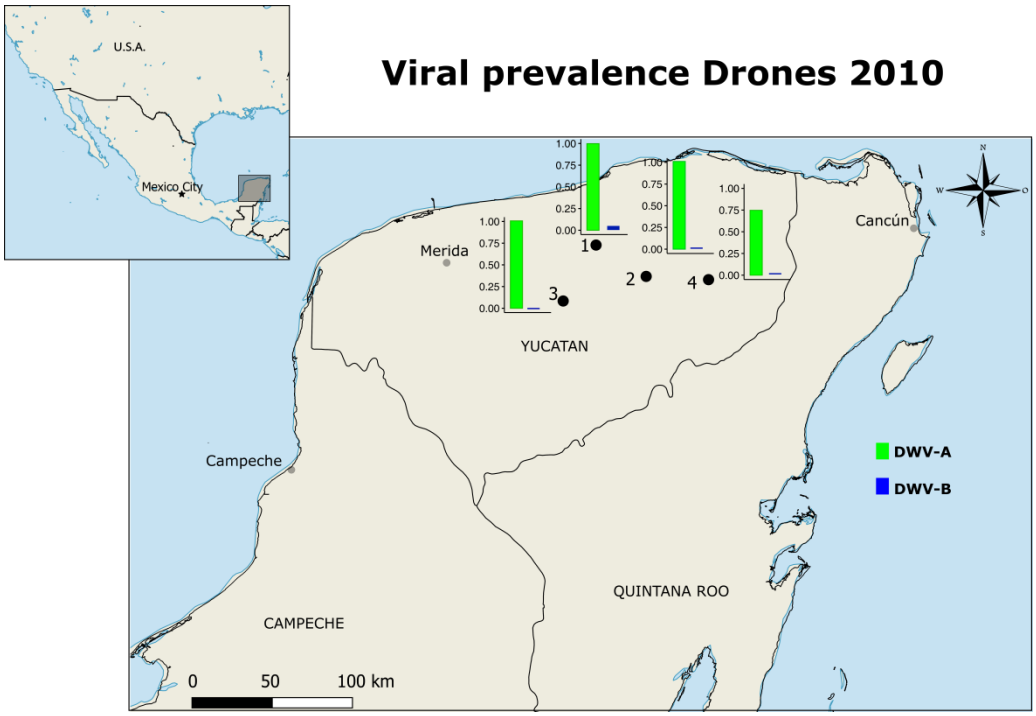

Figure SM 1. DWV-A and DWV-B prevalence in AHBs drones from DCA's, mapped by location in the Yucatan Peninsula of Mexico. Sample sites: 1. Dzoncahuich, 2. Cenotillo, 3. Izamal and 4. Espita. The DWV-A prevalence at each location from was of 100 % in Dzoncahuich (30 of 30 bees), Cenotillo (30 of 30 bees) and Izamal (25 of 25 bees); Espita (13 of 17 bees) presented a prevalence of 75 %. DWV-B was just present in Dzoncahuich 3 % (1 of 30 bees). DWV-A and DWV-B are represented by green and blue bars, respectively.

Figure SM 2

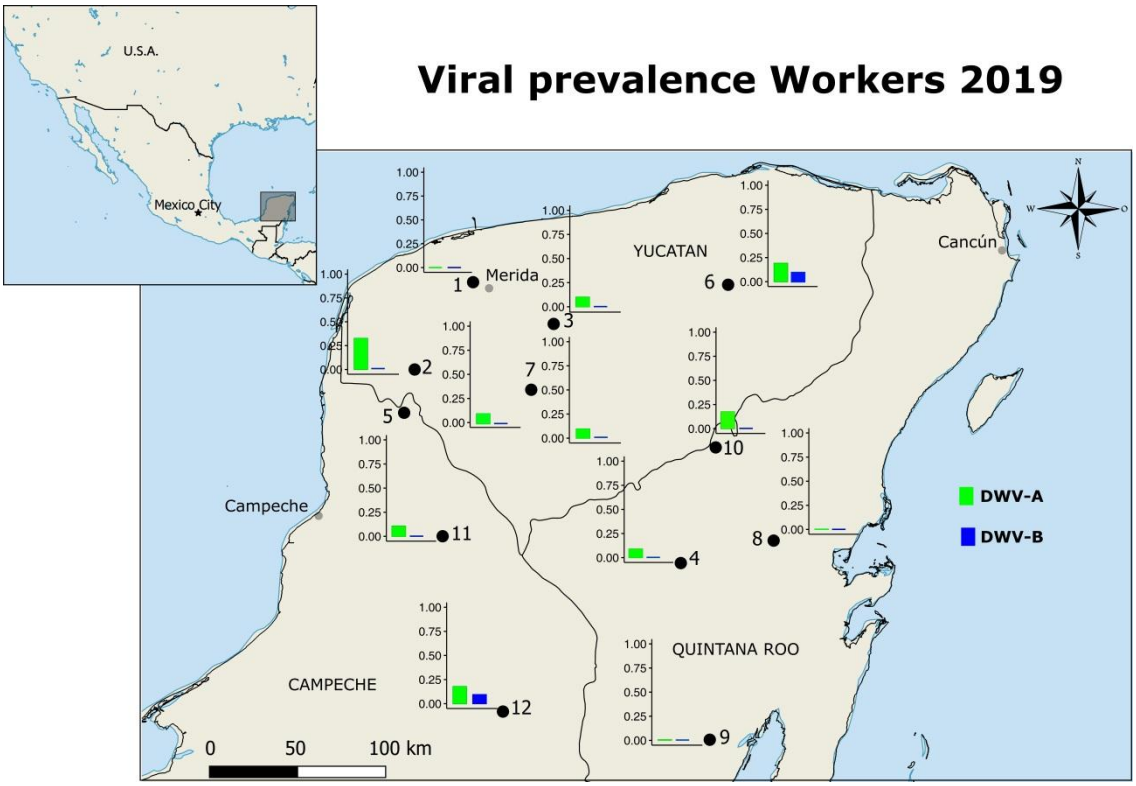

Figure SM 2. DWV-A and DWV-B prevalence in AHBs drones from DCA's, mapped by location in the Yucatan Peninsula of Mexico. Sample sites: 1. UADY, 2. Maxcanú, 3. Hocabá, 4. Polyuc, 5. Calkiní, 6. Espita, 7. Mama, 8. Felipe Carrillo Puerto, 9. Bacalar, 10. Tihosuco, 11. Hopelchén and 12. Calakmul. DWV-A and DWV-B are represented by green and blue bars, respectively.

Figure SM 3

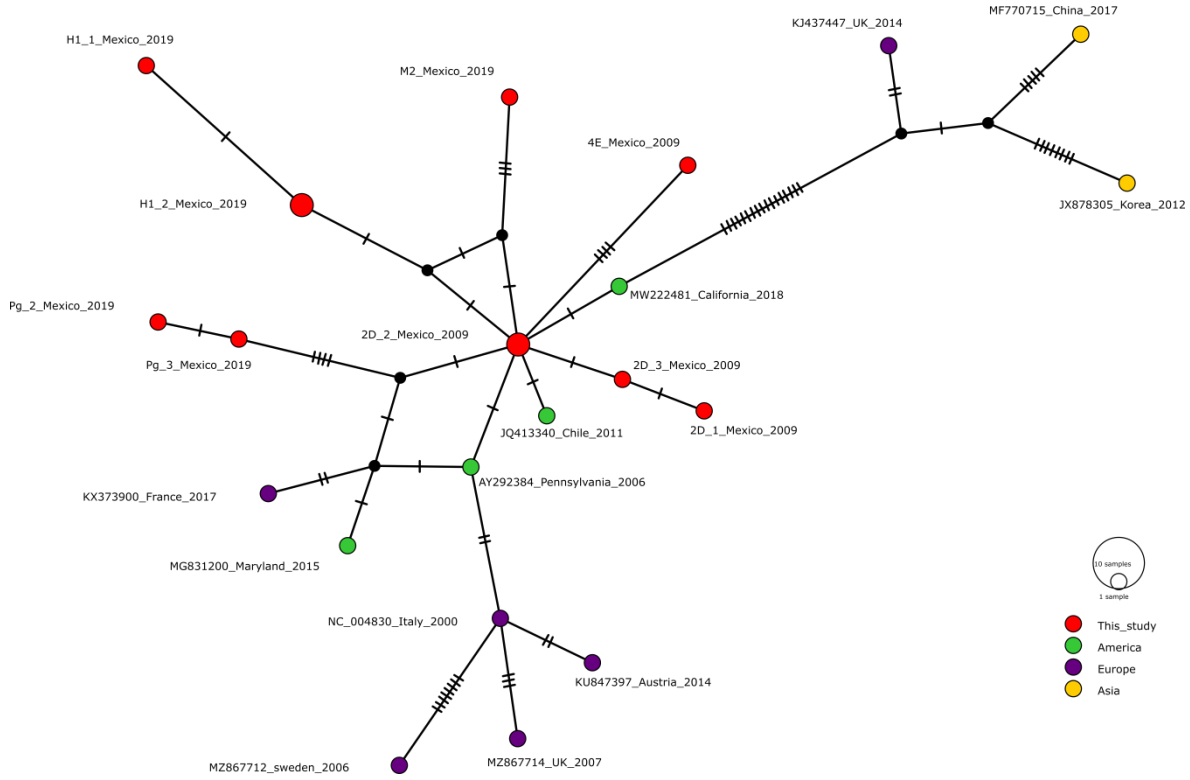

Figure SM 3. Haplotype network analysis of the 11 DWV-A RdRp sequences from this study as well as 12 DWV-A RdRp sequences from across the world (downloaded from NCBI). Identical sequences (from this study) are removed. The name of each sample is made of three parts in which the second and the third parts denote the location and the date of sample collection. Sequences are coloured based on the location from which the samples have been collected; red for Yucatan, Mexico (this study), green for elsewhere in N and S America, purple for Europe and yellow for East Asia (China and Korea).

Tables SM

Table SM 1. Sampling information, ID and locations at which drones or worker AHBs were sampled on the Yucatan Peninsula of Mexico.

ID	Site	State	Longitude (W)	Latitude (N)	N AHBs	Year
1	Dzoncahuich	Yucatan	-88.89368	21.05247	100	2010
2	Cenotillo	Yucatan	-88.60438	20.96621	100	2010
3	Izamal	Yucatan	-89.01802	20.93537	100	2010
4	Espita	Yucatan	-88.30781	21.01325	100	2010
1	UADY	Yucatan	-90.0000000	20.9698352	30	2019
2	Maxcanu	Yucatan	-89.9932666	20.5878314	21	2019
3	Hocaba	Yucatan	-89.24574466	20.81670164	30	2019
4	Polyuc	Quintana Roo	-88.56223253	19.61150749	30	2019
5	Calkini	Campeche	-90.04978002	20.37003207	20	2019
6	Espita	Yucatan	-88.30781489	21.01325066	25	2019
7	Mama	Yucatan	-89.36643358	20.48597127	22	2019
8	Carrillo Puerto	Quintana Roo	-88.06315205	19.72513655	5	2019
9	Bacalar	Quintana Roo	-88.40871002	18.7177042	30	2019
10	Tihosuco	Quintana Roo	-88.37450218	20.19562046	30	2019
11	Hopelchen	Campeche	-89.84275692	19.74731966	13	2019
12	Calakmul	Campeche	-89.51854627	18.85858972	30	2019

Table SM 2. qPCR primers used to amplify viruses and β–actin in honey bees.

Target	Name	Sequence	Reference
DWV-A	DWV-F8688 (F) DWV-B8794 (R)	GGTAAGCGATGGTTGTTTG CCGTGAATATAGTGTGAGG	Mondet et al., 2014
DWV-B	VDVq-R2a VDVq-F2	CTTCCTCATTAACTGAGTTGTTGTC TATCTTCATTAAAACCGCCAGGCT	McMahon et al. 2015
β–actin	Am-actin2-qF Am-actin2-qB	CGTGCCGATAGTATTCTTG CTTCGTCACCAACATAGG	Locke et al., 2012

Appendix Chapter III

Figure SM 1

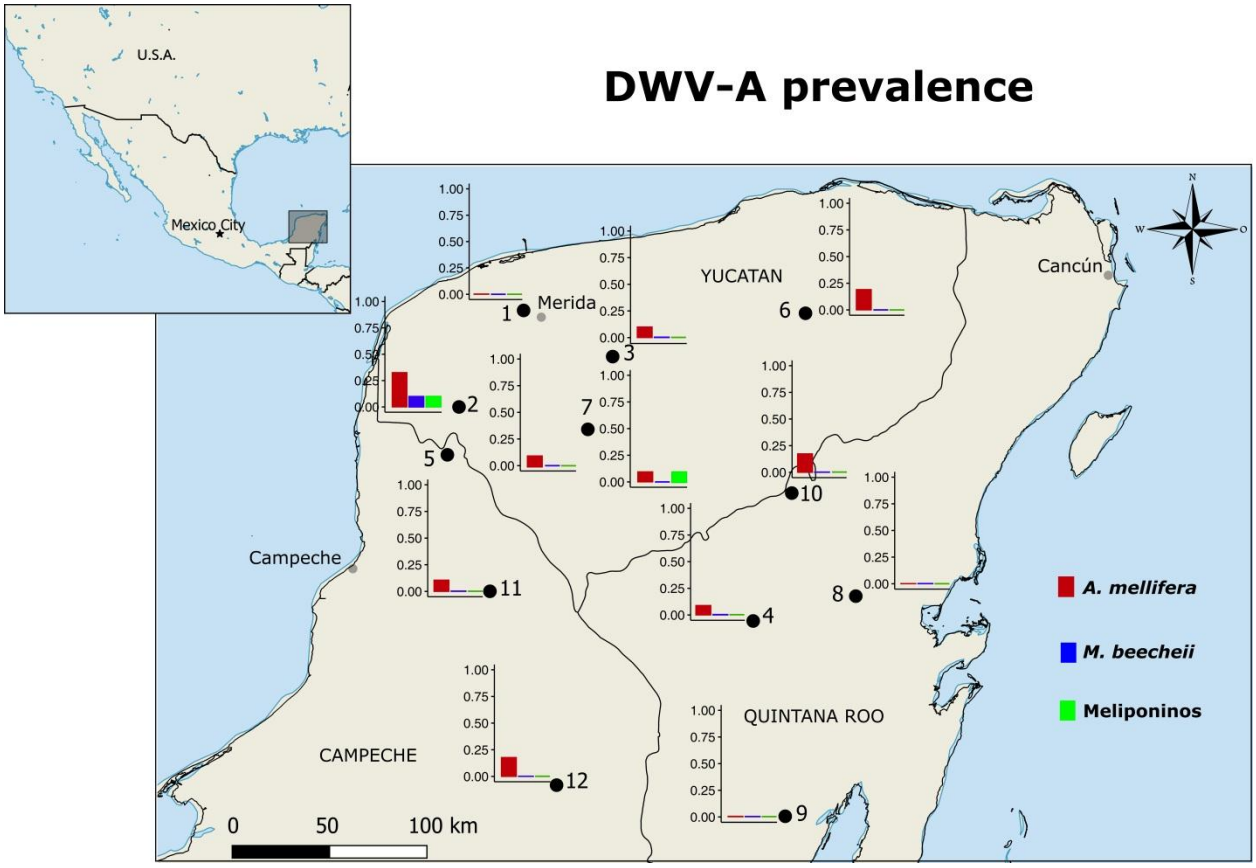

Figure SM 1. DWV-A prevalence mapped by location in the Yucatan Peninsula of Mexico. Sample sites: 1. UADY, 2. Maxcanú, 3. Hocabá, 4. Polyuc, 5. Calkiní, 6. Espita, 7. Mama, 8. Felipe Carrillo Puerto, 9. Bacalar, 10. Tihosuco, 11. Hopelchén and 12. Calakmul. AHBs and *M. beecheii* are represented as red and blue bars, respectively.

Figure SM 2

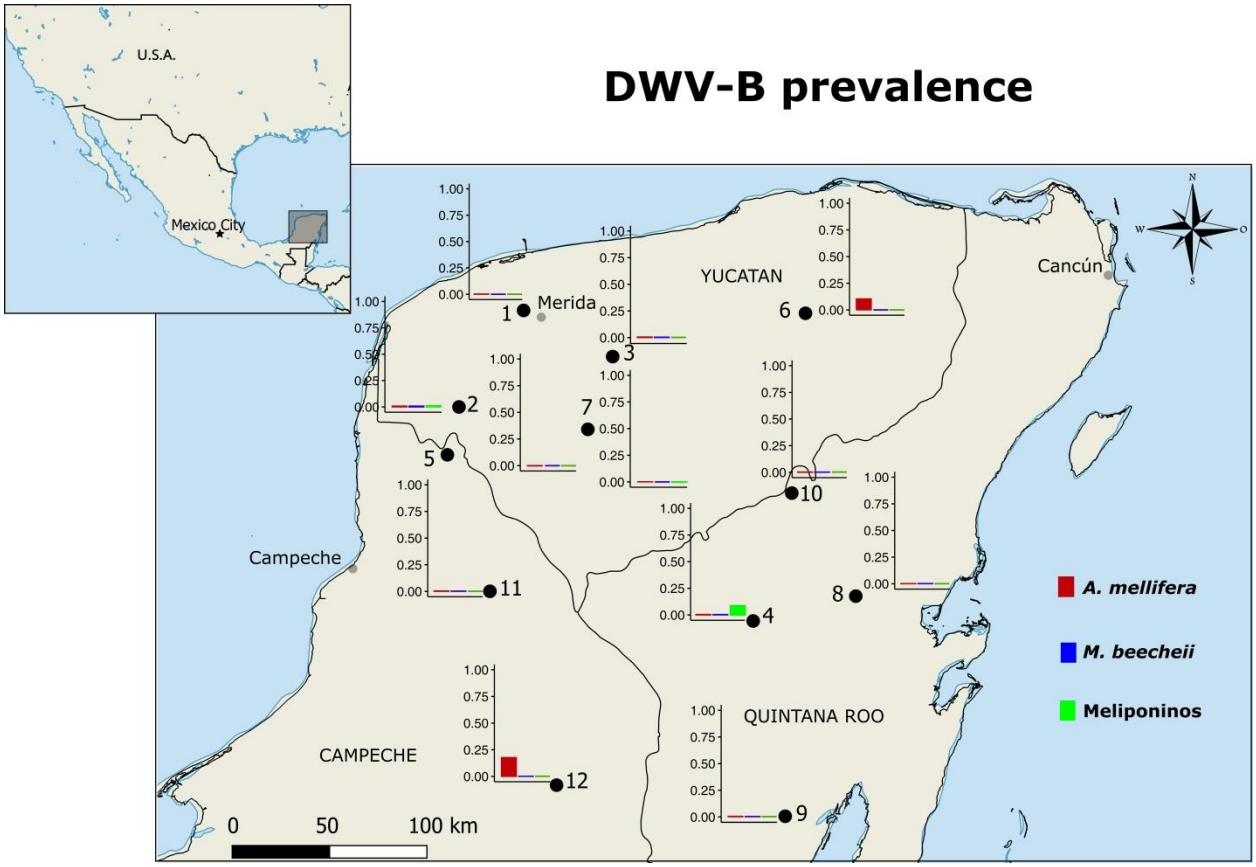

Figure SM 2. DWV-B prevalence mapped by location in the Yucatan Peninsula of Mexico. Sample sites: 1. UADY, 2. Maxcanú, 3. Hocabá, 4. Polyuc, 5. Calkiní, 6. Espita, 7. Mama, 8. Felipe Carrillo Puerto, 9. Bacalar, 10. Tihosuco, 11. Hopelchén and 12. Calakmul. AHBs and *M. beecheii* are represented as red and blue bars, respectively.

Figure SM 3

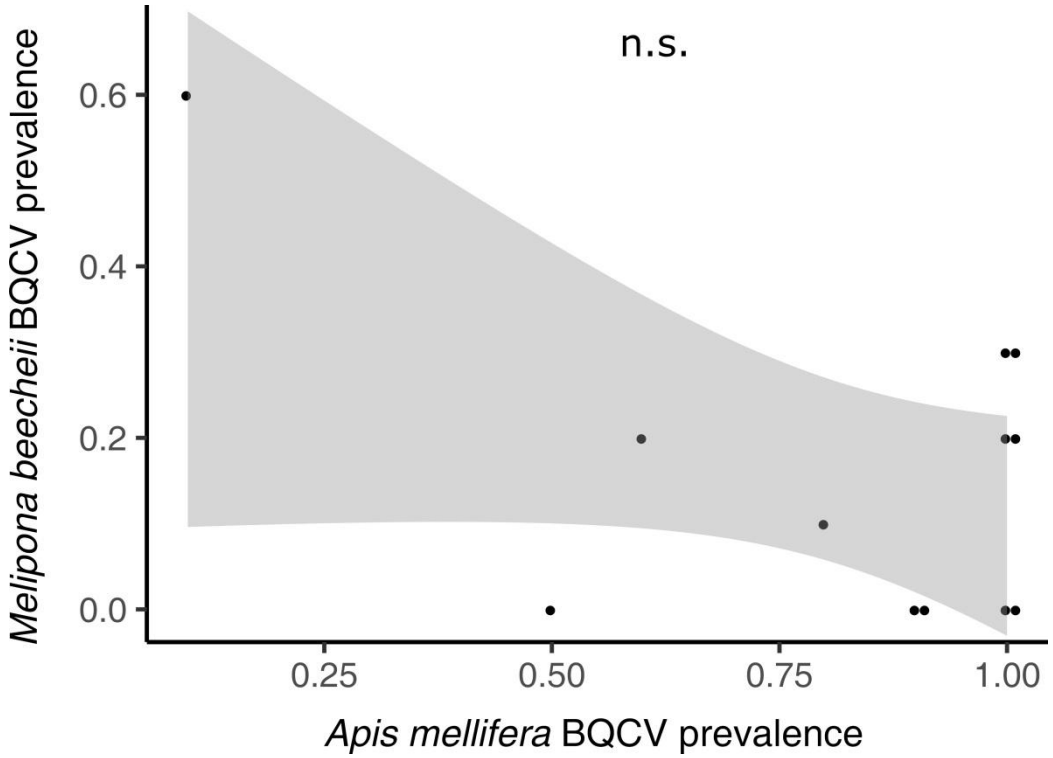

Figure SM 3. Relationship between BQCV prevalence in *M. beecheii* and *A. mellifera* across locations (GLMM, χ^2_1 = 2.55 p = 0.11). Black dots (jittered) represent the 12 sampling locations and shaded area indicates the 95 % confidence intervals.

Figure SM 4

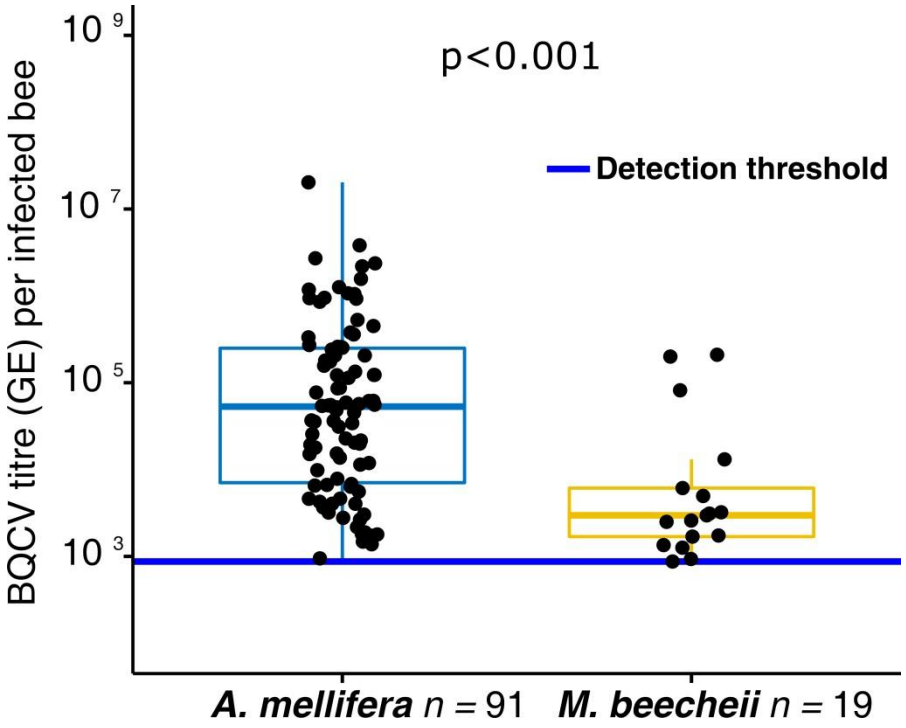

Figure SM 4. BQCV viral titres (GEs: genome equivalents) of qPCR-positive bees were lower in *M. beecheii* than in *A. mellifera* (GLMM, $\chi^2_1 = 349$ p = 0.001); blue line represents the qPCR detection threshold, equivalent to ca.10^3 GEs.

Figure SM 5

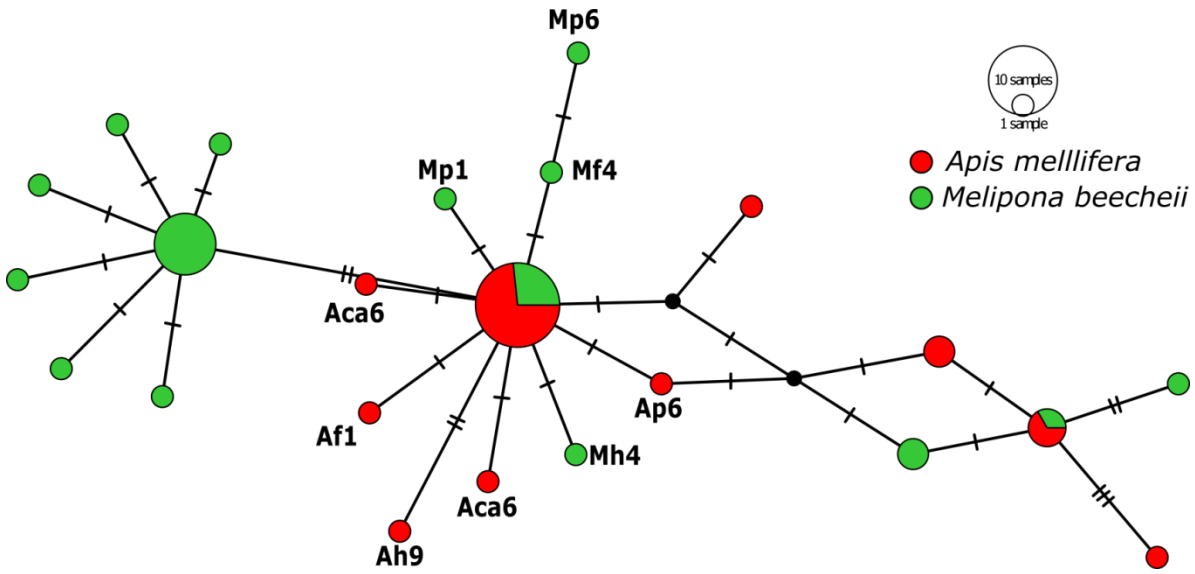

Figure SM 5. Median-Joining haplotype network of cloned partial sequences of BQCV coloured by species. Sanger sequenced viral fragments (294 bp, N sequences = 59 amplicons) of *A. mellifera* (n=11 bees comprising 27 amplicons) and *M. beecheii* (n=14 bees comprising 32 amplicons). Capital letters corresponding to species and lowercase letters to locations; viral haplotypes are shared at the same location as Carrillo Puerto (f), Polyuc (p) and Hocaba (h) and among species. The size of the circle representing a haplotype is proportional to the haplotype's frequency. Hatch marks indicate mutational steps and black dots represent hypothetical haplotypes that were not detected. Duplicate sequences from the same host individual were pruned.

Figure SM 6

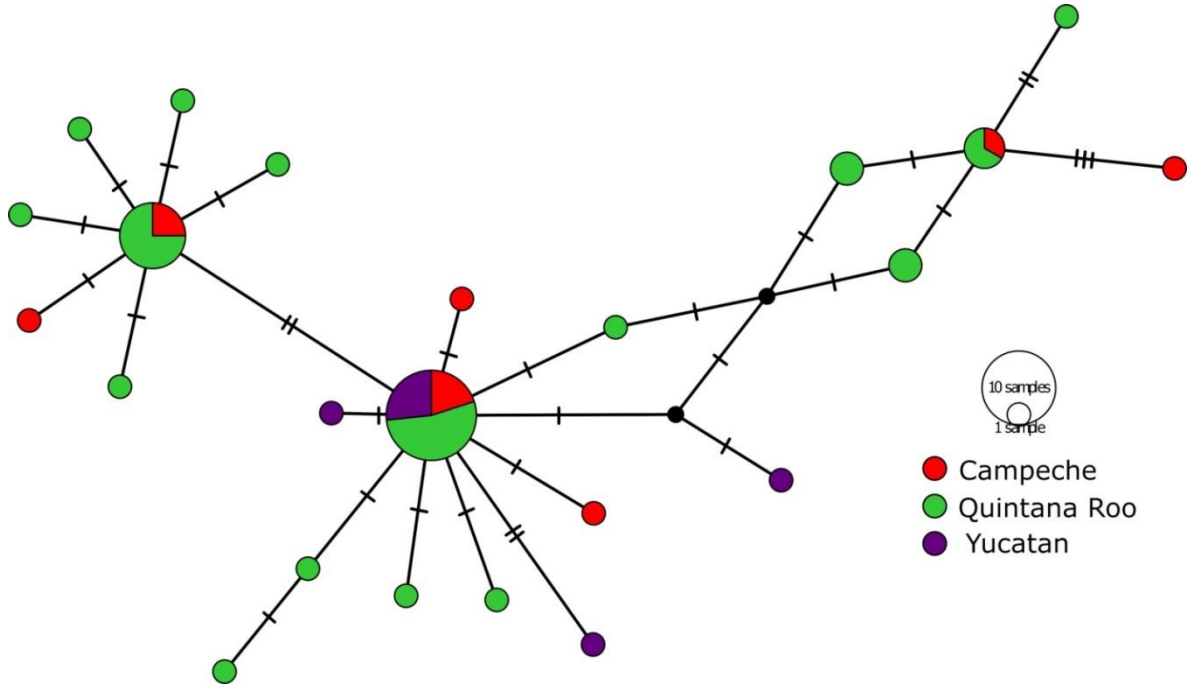

Figure SM 6. Median-Joining haplotype networks of cloned partial sequences of BQCV coloured by location rather than host species. Sanger sequenced viral fragments (294 bp, N sequences = 59 amplicons) of *A. mellifera* (n=11 bees comprising 27 amplicons) and *M. beecheii* (n=14 bees comprising 32 amplicons) from seven locations across the three different states of the Yucatan Peninsula. The size of the circle representing a haplotype is proportional to the haplotype's frequency. Hatch marks indicate mutational steps and black dots represent hypothetical haplotypes that were not detected. Duplicate sequences from the same host individual were pruned.

Tables SM

Table SM 1. Sampling information, ID and locations at which *M. beecheii* honey bees and Meliponinos species were sampled on the Yucatan Peninsula of Mexico.

ID	Site	State	Longitude (W)	Latitude (N)	N *A. mellifera*	N *M. beecheii*	Meliponinos
1	UADY	Yucatan	-90.0000000	20.9698352	30	30	44
2	Maxcanu	Yucatan	-89.9932666	20.5878314	21	30	35
3	Hocaba	Yucatan	-89.24574466	20.81670164	30	30	33
4	Polyuc	Quintana Roo	-88.56223253	19.61150749	30	30	16
5	Calkini	Campeche	-90.04978002	20.37003207	20	30	30
6	Espita	Yucatan	-88.30781489	21.01325066	25	30	19
7	Mama	Yucatan	-89.36643358	20.48597127	22	30	10
8	Carrillo Puerto	Quintana Roo	-88.06315205	19.72513655	5	30	2
9	Bacalar	Quintana Roo	-88.40871002	18.7177042	30	30	30
10	Tihosuco	Quintana Roo	-88.37450218	20.19562046	30	30	20
11	Hopelchen	Campeche	-89.84275692	19.74731966	13	30	2
12	Calakmul	Campeche	-89.51854627	18.85858972	30	30	33

Table SM 2. qPCR primers used to amplify viruses in all bees and β–actin in the honey bee.

Target	Name	Sequence	Reference
DWV-A	DWV-F8688 (F) DWV-B8794 (R)	GGTAAGCGATGGTTGTTTG CCGTGAATATAGTGTGAGG	Mondet et al., 2014
DWV-B	VDVq-R2a VDVq-F2	CTTCCTCATTAACTGAGTTGTTGTC TATCTTCATTAAAACCGCCAGGCT	McMahon et al. 2015
BQCV	BQCV-qF7893 (F) BQCV-qB8150 (R)	AGTGGCGGAGATGTATGC GGAGGTGAAGTGGCTATATC	Locke et al., 2012
ABPV	ABPV-F6548 (F) KIABPV-B6707 (R)	GATACCCCCATGGCTC CTGAATAATACTGTGCGTATC	Locke et al., 2012
SBV	SBV-qF3164 (F) SBV-qB3461 (R)	GCTCTAACCTCGCATCAAC TTGGAACTACGCATTCTCTG	Locke et al., 2012
SBPV	SBPV-F3133 SBPV-B3363	GCGCTTTAGTTCAATTGCC ATTATAGGACGTGAAAATATAC	Locke et al., 2012
β–actin	Am-actin2-qF Am-actin2-qB	CGTGCCGATAGTATTCTTG CTTCGTCACCAACATAGG	Locke et al., 2012

Table SM 3. Ribosomal protein L6 (RPL6) primers for *M. beecheii.*

Gene	Primer sequence	Annealing temperature	Amplicon size (bp)
RPL6 (Ribosomal protein L6)	F: AAAGCTGTCTACAAATTCATTGGC R: AAGCGTTGATCAAGAAAGGACC	55 °C	275

Appendix Chapter IV

Figure SM 1

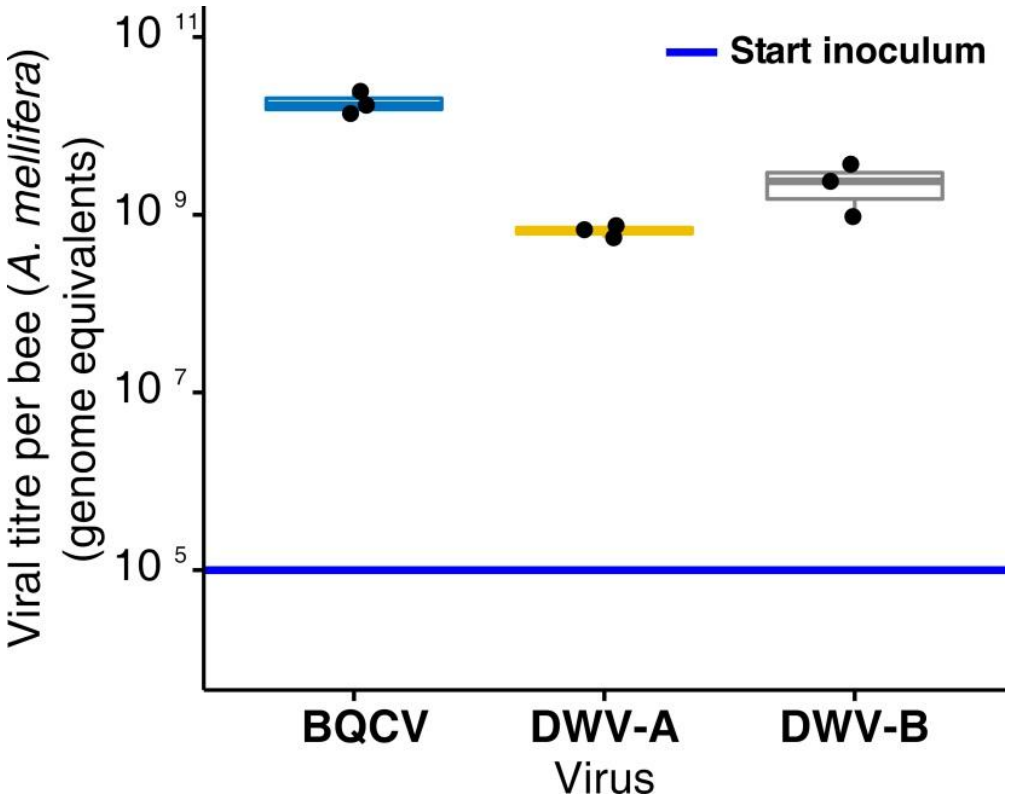

Figure SM 1. RNA virus titres in *A. mellifera* pupae five days after injection with 10^5 genome equivalents of viral inoculum (shown as a blue bar).

Table SM 1. Cox proportional hazards model of adult *M. beecheii* mortality from feeding exposure. In addition to model testing, I present the *ß* coefficient (standardised effect size) and the exp. *ß*, equivalent to the hazard ratio, the instantaneous risk of death for bees in each treatment compared with baseline treatment level (in this case, the control group). Higher *ß* indicates higher risk of death. Different lower case letters following *ß* show significant differences ($p < 0.05$, *a posteriori* Tukey test with Bonferroni correction for multiple comparisons).

M. beecheii	**Coefficients**						**model testing**		
	β	**s.e. (β)**	**exp. (β)**	**z**	**p**		**Chi^2**	**df**	**p**
Feeding inoculation						**treatment**	43.47	3	<0.001
Control	0 a		1						
BQCV	0.758 b	0.155	2.135	4.88	0.65				
DWV-A	0.823 b	0.156	2.279	5.27	0.70				
DWV-B	0.874 b	0.160	2.397	5.46	0.73				

CURRICULUM VITAE

MSc. Fernando Amin Fleites
Ayil

Education	
2018-Present	Martin Luther Universität Halle-Wittenberg. PhD Candidate. Scholarship-DAAD Research grants-Doctoral programs in Germany. "Prevalence and impact of honey bee RNA viruses on native stingless bee species of the Yucatan Peninsula, Mexico". Supervisor: Prof. Dr. Robert J. Paxton.
2013-2015	Universidad Autónoma de Yucatán Campus de Ciencias Biológicas y Agropecuarias. Master of Agricultural science "*Nosema ceranae* induces precocious foraging and reduces foraging activity and longevity in Africanized honey bees (*Apis mellifera*)" Supervisor: Professor Dr. Luis A. Medina Medina. Professor Dr. José Javier G. Quezada Euán.
2005-2010	Instituto Técologico de Conkal Bachelor of Biology "Potential distribution of *Selaginella convoluta* (Arn) Spring. Medicinal species of the Yucatan Peninsula." Supervisor: Professor Dr. Martha Mendez Gonzalez, Professor Dr. Rafael Durán García.

Internships and work experience

2022-Present	Martin Luther Universität Halle-Wittenberg. Research assistant. Professor Dr. Robert J. Paxton.
2017-2018	Universidad Autónoma de Yucatán Campus de Ciencias Biológicas y Agropecuarias Tropical Tropical Beekeeping department. Laboratory and field technician "Climate change and pollinators: effect of thermal increase on developmental stability and indicators of biological aptitude in tropicalbees in Mexico" Professor Dr. Jose Javier G. Quezada Euán.
2017-2018	Agricultural extension program SAGARPA-Minister of agriculture (Mexico). Professional service provider: "Agricultural sciences technical training work program: Beekeeping".
2015-2018	Universidad Autónoma de Yucatán Campus de Ciencias Biológicas y Agropecuarias Tropical Beekeeping department. Laboratory technician project: "Reversal of the fecundity/longevity trade-off in an orchid bee at the cusp of sociality" PhD student Alice Séguret, Professor Dr. Robert J. Paxton.
2015-2017	Universidad Autónoma de Yucatán Campus de Ciencias Biológicas y Agropecuarias Tropical Beekeeping department. Research assistant, Scholarship-CONACYT, Mexico "Climate change and pollinators: effect of thermal increase on developmental stability and indicators of biological aptitude in tropical bees in Mexico" Professor Dr.José Javier G. Quezada Euán.
2012-2013	Univeridad Autonoma de Yucatan Departamento de Apicultura Tropical. Laboratory and field technician "Determination and quantification of soy pollen grains and their relationship to bee behavior in areas of crops near apiaries in the Yucatan peninsula" Prof. Dr. Luis A. Medina Medina.
2010-2012	Centro de Investigacion Cientifica de Yucatan(CICY). Laboratory and field technician, "Conservación ex situ y Fortalecimiento del uso de las plantas medicinalesyucatecas" Prof. Dr. Martha Méndez.
Summer 2009	Academia Mexicana de ciencias (AMC) Scientific summer of investigation (Mexicanacademy of science) "Effects of fire on composition and plant structure during the secondary succession process in tropical forests". Prof. Dr. Ligia Esparza Olguin.

Publications list

Accepted Publication	*"Nosema ceranae* induces precocious foraging and reduces foraging activity and longevity in Africanized honey bees (*Apis mellifera*)". (2018). Fleites-Ayil Fernando Amin, Quezada-Euán José Javier G., Medina-Medina Luis A.
Accepted Publication	"Developmental stability, age at onset of foraging and longevity of Africanized honey bees (*Apis mellifera* L.) under heat stress (Hymenoptera: Apidae)"(2018). Rubén G. Medina,, Robert J. Paxton, Efraín De Luna, Fernando A. Fleites-Ayil, Luis A. Medina Medina, José Javier G. Quezada-Euán.
Accepted Publication	"Transcriptomic Signatures of Ageing Vary in Solitary and Social Forms of an Orchid Bee" (2021). Alice Séguret, Eckart Stolle, Fernando A. Fleites- Ayil, José Javier G. Quezada-Euán, Klaus Hartfelder, Karen Meusemann, Mark C. Harrison, Antonella Soro, Robert J. Paxton.
Accepted Publication (Included in this thesis)	PhD Thesis product: Trouble in the tropics: Pathogen spillover is a threat for native stingless bees (2023). Fernando A. Fleites-Ayil, Luis A. Medina- Medina, Jos´e Javier G. Quezada Euán, Eckart Stolle, Panagiotis Theodorou, Simon Tragust, Robert J. Paxton.
Unpublished data	PhD Thesis second product: "Viral prevalence in Africanized honey bees of the Yucatan Peninsula in SE Mexico, suggests that DWV-A blocks DWV-B through exclusion". Fernando A. Fleites- Ayil, Luis A. Medina-Medina, José Javier G. Quezada Euán, Shafiey Hassan, Robert J. Paxton.
Unpublished data	PhD Thesis third Product: "Prevalence and genetic diversity of honeybee virus on stingless bees in Mesoamerica". Fernando A. Fleites-Ayil, Luis A. Medina-Medina, José Javier G. Quezada Euán, Robert J. Paxton, Landaverde-González Patricia.
Skills	
Languages	Spanish (mother language). English (Good). German (Basic).
Computer Skills	Microsoft Office package, Statgraphics, Geneious Prime, Linux, Bash Scripting and R.

Declaration of own contribution to the original articles, product of this thesis

This thesis includes three data chapters (II-IV), which are organized in three scientific manuscripts. Chapter II is in preparation for submission, Chapters III and IV are together in a published scientific manuscript: "Trouble in the tropics: Pathogen spillover is a threat for native stingless bees". Some of the data from Chapter III will also be part of a third scientific manuscript also in preparation, as is described below (as the third ms listed below):

Fernando A. Fleites-Ayil, Luis A. Medina- Medina, José Javier G. Quezada Euán, Eckart Stolle, Panagiotis Theodorou, Simon Tragust, Robert J. Paxton. (2023). Trouble in the tropics: Pathogen spillover is a threat for native stingless bees. *Biological Conservation* 284 (2023) 110150. https://doi.org/10.1016/j.biocon.2023.110150.

Design of the project: 80%
Collection of field data: 100%
Laboratory work: 100%
Data analysis: 80%
Writing of the paper: 100%

Fernando A. Fleites- Ayil, Claudia A. Castillo Carrillo, Luis A. Medina-Medina, José Javier G. Quezada-Euán, Shafiey Hassan, Robert J. Paxton. Viral prevalence in Africanized honey bees of the Yucatan Peninsula in SE Mexico, suggests that DWV-A blocks DWV-B through exclusion. Manuscript in preparation.

Design of the project: 80%
Collection of field data: 50%
Laboratory work: 80%
Data analysis: 50%
Writing of the paper: 80%

Fernando A. Fleites-Ayil, Luis A. Medina-Medina, José Javier G. Quezada Euán, Robert J. Paxton, Landaverde González Patricia. Prevalence and genetic diversity of honeybee virus on stingless bees in Mesoamerica. Manuscript in preparation.

Design of the project: 50%
Collection of field data: 50%
Laboratory work: 50%
Data analysis: 30%
Writing of the paper: 50%

Fernando Amin Fleites Ayil, 29.11.2023 **Prof. Dr. Robert Paxton, 29.11.2023**

Eidesstattliche Erklärung

Münnerstadt, den 29.11.2023

Hiermit erkläre ich an Eides statt, dass diese Arbeit, in der gegenwärtigen bzw. in einer anderen Fassung, von mir bisher weder an der Naturwissenschaftlichen Fakultät I – Biowissenschaften der Martin-Luther-Universität Halle-Wittenberg noch an einer anderen wissenschaftlichen Einrichtung zum Zweck der Promotion eingereicht wurde.

Ich erkläre weiterhin, dass ich mich bisher noch nicht um den Doktorgrad beworben habe.

Ferner erkläre ich, dass ich diese Arbeit selbstständig und nur unter Zuhilfenahme der angegebenen Quellen und Hilfsmittel angefertigt habe. Die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen sind als solche kenntlich gemacht worden.

Fernando Amin Fleites Ayil