RNA viruses in Australian bees

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This thesis is dedicated to my father Wing Kock,

Who had the most beautiful soul and hearth….The greatest man I ever knew. He gave me the best he could and encourage me to dream big, set goals and work to achieve them. He is responsible for the person I am.

Although he is not here to celebrate with me, I know he is always by my side looking after me with his unique smile ☺

I MISS HIM everyday..!!

I LOVE HIM 

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Abstract

Bees play an important role as pollinators of angiosperms in most terrestrial ecosystems and they are exposed to numerous threats. In many regions in the world, bee abundance and species richness are in decline due to the combined effects of habitat loss, pesticide use, and parasites and disease. Worldwide, diseases caused by RNA viruses are among the greatest threats to the health of the European honey bee (*Apis mellifera*) predominantly when the parasitic Varroa mite (*Varroa destructor*) functions as a vector and incubator of these viruses. While research on RNA viruses in bees has been intensifying around the world, in Australia, information about RNA viruses is limited to managed hives of *A. mellifera*, but no information is available for unmanaged, wild colonies of *A. mellifera*, introduced bumble bees (*Bombus terrestris*) or solitary bees.

While knowledge of the distribution of RNA viruses is important in the context of managing and understanding bee declines, it is also important to have baseline data of prevalence and distributions of RNA viruses prior to an incursion of the Varroa mite. The mite is known to influence the infectivity and virulence of different viruses, but so far, baseline data that allow proper monitoring of this process have been scant. Hence, a survey of the RNA viruses carried by Australian bees is timely and necessary.

For many decades, *A. mellifera* has been perceived as the original and only host of a range of RNA viruses. However, recently "honey bee" RNA viruses have been detected in different species of non-*Apis* bees. This raises questions regarding the original hosts and the direction of transmission of these RNA viruses. Our study confirms the association of some RNA viruses with native bees and show that the probability of South Australian native bees carrying *Black queen cell virus* (BQCV) and S*acbrood virus* (SBV) is higher in non-arid areas with abundant managed and feral *A. mellifera*. Furthermore, the results indicate that BQCV and SBV were introduced into Australia with *A. mellifera*.

Since the introduction of *B. terrestris* onto the Australian island of Tasmania in 1992 from New Zealand, no research has been undertaken to determine whether these bees had brought new viruses to the island. Australia is free of a number of RNA viruses including the epidemic *Deformed wing virus* (DWV), which is present in New Zealand. Using RT-PCR, we found that *Kashmir bee virus* (KBV) and SBV are present and shared between Tasmanian *B. terrestris* and *A. mellifera*, while BQCV was detected only in *A. mellifera*. Because we did not find DWV in either *A. mellifera* or *B. terrestris*, we conclude that introduction of the latter species did not coincide with introduction of this virus. While this is the first report of KBV in Tasmania, we believe it may have been previously detected but misclassified.

Recent studies have reported RNA interference (RNAi) as an immune response of *A. mellifera* to different RNA viruses. The RNAi pathway is activated by presence of double-stranded RNA and degrades the viral genome in 21-22 nucleotides-long small interfering RNAs (siRNAs). siRNAs matching different RNA viruses have been reported in *A. mellifera*, but generation of a complete viral genome using assembly of siRNAs has not been achieved. Our results show that *A. mellifera* larvae activate the RNA interference (RNAi) immune response in the presence of SBV. We generate three complete SBV genomes from three individual larvae from different hives in a single apiary, and demonstrated the presence of different SBV quasispecies within the country.

In summary, this study provides new insights into the epidemiology and ecology of bee RNA viruses. This information is important for understanding the impact of RNA viruses in bee health and for elaboration of mitigation or control strategies.

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Abbreviations

(-)ssRNA	negative- and single-strand RNA
(+)ssRNA	positive- and single-strand RNA
%	Percentage
°C	Degree Celsius
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BQCV	Black queen cell virus
CBPV	Chronic bee paralysis virus
CCD	Colony Collapse Disorder
cDNA	complementary DNA
CP	Conservation Park
CSBV	Chines sacbrood virus
CsCl	Caesium chloride
CWV	Cloudy wing virus
dNTPs	Deoxynucleotide solution mix
dsDNA	double-strand DNA
dsRNA	double-strand RNA
DWV	Deformed wing virus
IAPV	Israel acute paralysis virus
ICTV	International Committee on Taxonomy of Viruses

ICTV	International Committee on Taxonomy of Viruses (ICTV)
kb	Kilobase
KBV	Kashmir bee virus
KI	Kangaroo Island
KSBV	Korean sacbrood virus
LD50	Lethal dose 50% or median lethal dose
m^2	Square meters
$MgCl_2$	Magnesium chloride
Ml	Millilitre
mM	Millimolar
NCBI	National Center for Biotechnology Information
NH_4	Ammonium
NP	National Park
nt	Nucleotides
NZ	New Zealand
ORF	Open Reading Frame
RdRp	RNA-dependent RNA-polymerase region
RNAi	RNA interference
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
s	Seconds
SA	South Australia
SBPV	Slow bee paralysis virus

SBV	Sacbrood virus
siRNA	Small interfering RNA
SNP	Single Nucleotide Polymorphism
ssDNA	single-strand DNA
ssRNA	single-strand RNA
TAS	Tasmania
TSBV	Thai sacbrood virus
UK	United Kingdom
USA	United States of America
UV	Ultra-violet
M	Molar
μM	Micromola

Chapter 1

Introduction and Review of Literature

Chapter 1

1. General Introduction

Bees are the most important pollinators of managed and natural ecosystems. They are responsible for the high yields and good quality of many important crop products (fruits, vegetables and seed crops). Although European honey bees (*Apis mellifera*) are the most recognised pollinators worldwide, bumble bees (*Bombus* spp.) and solitary bees can substitute or complement the pollination provided by honey bees (Delaplane & Mayer 2000; Kleijn et al. 2015; Kremen 2008; Velthuis & van Doorn 2006; Winfree et al. 2007). In this chapter, we separate the bees in three groups, namely "honey bees", "bumble bees", and "solitary bees". We define solitary bees to include all other species of bees excluding honey bees and bumble bees, regardless of their social behaviour.

Decline in abundance and richness of bees has been reported in many areas around the world and it has been linked to individual and/or combined biological, environmental and anthropogenic factors (e.g. Goulson & Hughes 2015; Neumann & Carreck 2010; Potts, Biesmeijer, et al. 2010; Vanbergen et al. 2013; Williams & Osborne 2009). Among the causes of bee decline, diseases caused by RNA viruses are one of the major threats to the health and fitness of honey bees. While the impacts of RNA viruses in honey bees are relatively well understood, and there has been a recent increase in the number of studies focusing on the role of these viruses in bumble bees (e.g. Fürst et al. 2014; McMahon et al. 2015), very little is known about the presence and infectivity of these viruses in solitary bees. In order to understand and manage the risks associated with RNA viruses in bees, a better understanding of the epidemiology, pathogenesis, and genome organisation is crucial (Chen, Y. P. & Siede 2007).

This study focuses on RNA viruses in introduced honey bees, bumble bees and native bees from Australia. In particular, it assesses the incidence of RNA viruses in South Australian native bees, followed by identification of the possible factors influencing this. The incidence and

prevalence of RNA viruses in introduced *B. terrestris* and *A. mellifera* collected from the Australian island of Tasmania is also determined in order to investigate whether new RNA viruses were introduced together with *B. terrestris* in 1992. Antiviral immune response to SBV infection is analysed in larval *A. mellifera,* followed by generation and variability analysis of SBV complete genomes.

This introduction contains a brief review of the literature of the main aspects related to research on RNA viruses and bees. While the emphasis of this chapter is on RNA viruses in bees, a short description of the bees and their value as pollinators and the causes of declines in bee abundance and diversity is included as these aspects relate to the scientific basis and rationale for this study. Furthermore, molecular characterization of the RNA viruses and their infection strategies are briefly described. In addition, the current knowledge about host species, biological properties, transmission and worldwide prevalence of RNA viruses are described for honey bees, bumble bees and solitary bees.

2. Bees

Bees are specialised insects that, together with wasps and ants, make up the order Hymenoptera (Michener 2006). Unlike other hymenopterans, adult and immature bees are specialised plant feeders that only use pollen and nectar as food (Delaplane & Mayer 2000; Michener 2006; Pitts-Singer & James 2008). Based on their behaviour, bees can be classified as: (a) solitary when females work alone to construct their nests and to provide food to their offspring; (b) communal when two or more females share the same nest but each one is responsible for constructing her own cells and providing for her offspring; (c) social when females live together as a colony and they have a reproductive division of labour which, in its most extreme form, consist of a reproductive queen and sterile workers (Michener 2006). When eusocial queens and workers are morphologically different, and the queen relies on workers for survival, while the workers depend on the queen for reproduction, they are classified as highly eusocial (Michener 2006).

Bee species can be categorised as mono-, oligo- or polylectic, according to their foraging preferences. Polylectic bees forage on a large range of flowering plants while mono- and oligolectic bees forage respectively, on only one or few related species of plants (Michener 2006; Pitts-Singer & James 2008). Both polylectic and oligolectic bees are beneficial for pollination of natural vegetation and agricultural crops, and essential for pollination of a range of plant species (Delaplane & Mayer 2000).

Worldwide, more than 17 500 species of bees are recognized and classified into seven families (Michener 2006). In Australia, more than 1 600 species of native bees are listed by the Australian Faunal Directory but between 300 and 400 species are still undescribed (Batley & Hogendoorn 2009). Five families are present in Australia, namely Colletidae, Halictidae, Megachilidae, Apidae and Stenotritidae, while Andrenidae and Mellitidae are absent (Batley & Hogendoorn 2009; Michener 2006). More than 75% of bees present in Australia belong to the families Colletidae, Halictidae and Stenotritidae, with Collectidae being the most diverse (Batley & Hogendoorn 2009). The length of the bees ranges between 1.5 mm to 40 mm and their colouration varies from black to metallic blue, green, red, yellow or bronze (O'Toole & Raw 1991). The majority of bees present in Australia are dependent on the Myrtaceae for pollen and nectar, and the most attractive genera are *Angophora*, *Baeckea*, *Callistemon*, *Eucalyptus*, *Eugenia*, *Leptospermum*, *Melaleuca* and *Tristania* (Naumann 1991).

The family Colletidae has the highest abundance and diversity in Australia (Michener, 2006). Nearly all colletid bees are solitary but some species are communal. Stenotritid bees are endemic to Australia and before 1980 they were classified as a subfamily of Colletidae (Naumann, 1991). They are solitary and nest in the ground (Naumann, 1991; Michener, 2006). Halictid bees, also known as sweat bees or furrow bees, are the most common bees of temperate areas worldwide (Michener, 2006). Australian halictid bees are solitary or communal and nest in the ground or sometimes in rotting wood (Michener, 2006). The Megachilidae contains the most easily recognisable species of bees due to their often large head and the positioning of pollen collection

hairs on the ventral part of the abdomen. All members of this family are solitary and a few species parasitise other megachilid nests. This family is divided into two subfamilies, namely Megachilinae and Fideliinae, but in Australia only Megachilinae bees are present (Naumann, 1991). The Apidae is one of the most diverse families of bees and is distributed worldwide (Michener, 2006). These bees vary from high eusocial to solitary bees (Naumann, 1991). Apid bees can construct their own nest in the ground or wood or they can inhabit pre-existing hollows (Michener, 2006). They are also capable of forming exposed (hanging) nests. All subfamilies are present in Australia (Naumann, 1991), and some apid species were introduced. That is, *A. mellifera* was introduced into mainland Australia from the United Kingdom in the 1820s (Paton 1996), into Tasmania in 1931 (Hopkins 1886), and is now abundant wherever floral resources and water are available. In 1992, *B. terrestris* was also introduced into Tasmania where it has become widespread and common (Hingston et al. 2002; Semmens, Turner & Buttermore 1993). In addition, the Asian honey bee, *A. cerana* was detected at Cairns in 2007 and is now considered established in northern Queensland (Koetz 2013).

2.1. Pollination value of bees

The majority of flowering crops and native plants rely on animals for pollination (Ollerton, Winfree & Tarrant 2011; Winfree 2010). The estimated number of angiosperms that rely on animal pollination is around 308,006 species, which covers 87.5% of global flowering plants (Ollerton, Winfree & Tarrant 2011).

Pollination by bees enhances quantity, quality and market value of crops (Bommarco, Marini & Vaissiere 2012; Garratt et al. 2014). For example, bee pollination improved quantity of production, size, shape and shelf life of apples (Garratt et al. 2014), seed weight and oil content of oilseed rape (Bommarco, Marini & Vaissiere 2012), and shape and shelf life of strawberries (Klatt et al. 2014).

Based on the global production volumes for human consumption, 35% of crop output depends on biotic pollination for fruit and seed set (Klein et al. 2007). Worldwide, in 2005, the total

economic value of pollination was approximately $US215 billions. This represented 9.5% of the value of the agricultural production worldwide (Gallai et al. 2009). In Europe, between 1991 and 2009, the annual economic value of crops pollinated by insects was around €16.25 billion, which was approximately 12% of total crop production (Leonhardt et al. 2013). In USA, the economic value of crops pollinated by insects decreased from $US14.29 billion in 1996 to $US10.69 billion in 2001, after which it increased attaining $US15.12 billion by 2009 (Calderone 2012).

Honey bees are the best known and the most exploited crop pollinator worldwide (Delaplane & Mayer 2000). For this reason, crop pollination is often perceived as dependent on honey bees, and their pollination value is overestimated (Breeze et al. 2011; Smith & Saunders 2016; Winfree et al. 2007). For instance, the economic value of honey bee pollination of USA crops in USA was estimated to be around $US 14.6 billion in 2000 (Morse & Calderone 2000), which is slightly higher than the total economic value of pollinated crops in 2001 ($US 10.69 billion; Calderone 2012) and therefore ignores the substantial contributions made by wild bees and other pollinators (e.g. Garibaldi et al. 2013; Kleijn et al. 2015; Rader et al. 2012).

Over the last century, bumble bees have displayed a distinct value as pollinators of greenhouse crops around the world since they reduce production costs, and improve yield and fruit quality. The bumble bee rearing and marketing industry is worth several billion dollars (Guerra-Sanz 2008; Velthuis & van Doorn 2006). The main crop pollinated by bumble bees in greenhouses is tomato (95% of bumble bee sales), but they are also used to pollinate other crops including cucumber, strawberry, and blackberry (Velthuis & van Doorn 2006). However, the total economic value of pollination by bumble bees, including wild and managed hives, is unknown (Goulson et al. 2011).

For many decades, the pollination value of solitary bees to crops and wild plants remained underestimated (Kremen 2008), but recent studies have demonstrated their importance as pollinators of crops. Both in Canada and the USA, it has been demonstrated that solitary bees could provide all pollination services for watermelon without supplying additional honey bees

(Kremen, Williams & Thorp 2002; Winfree et al. 2007). In addition, solitary bees provided consistent pollination over four consecutive years in 43 commercial fields of the brassica "Pak Choi" in New Zealand (Rader et al. 2012). Furthermore, a study based on data from 90 studies from five continents showed that contribution of solitary bees to production of 20 pollination-dependent crops in more than 1000 fields was over $US 3.000 per hectare, which was similar to contribution of managed honey bees of around $US 2.900 (Kleijn et al. 2015). In addition, Garibaldi and colleagues (2013) found that enhancement of pollination by solitary bees doubled the fruit set of 27 crops compared to similar enhancement of pollination by honey bees. However, these studies also informed the importance of presence of natural habitat to support the pollination of solitary bees. Indeed, management practises that guarantee conservation and restoration of natural or semi-natural areas near or within croplands is recommended (Gallai et al. 2009; Kremen, Williams & Thorp 2002; Rader et al. 2012; Winfree et al. 2007).

3. Reduction of bee abundance and/or richness

3.1. Honey bees

The decrease in bee abundance and/or species richness have received public and scientific attention due to the putative syndrome "Colony Collapse Disorder" (CCD; Carreck 2016), which was associated with the decline of managed honey bee in USA in 2006 (vanEngelsdorp & Meixner 2010). Reductions of more than 30% of managed honey bee colonies during winter were reported between 2006/2007 and 2014/2015 (Seitz et al. 2016; Spleen et al. 2013; Steinhauer et al. 2014; vanEngelsdorp et al. 2012; vanEngelsdorp et al. 2011; vanEngelsdorp et al. 2008; vanEngelsdorp et al. 2010; vanEngelsdorp et al. 2007). Although CCD symptoms have been characterised and described in many studies, the causes of this syndrome are not fully resolved (Ratnieks & Carreck 2010). The general consensus is that the decline of honey bee colonies is caused by the interaction of different factors including pests and pathogens, pesticide use and poor nutrition (Neumann & Carreck 2010; vanEngelsdorp et al. 2009).

A decline in honey bee colonies was also reported in Europe, the Middle East and Japan with approximately 1.8-53%, 10-85% and 25% of total losses, respectively (Neumann & Carreck 2010). During the winters of 2008/2009 and 2009/2010, declines in managed honey bee colonies were reported in Canada (16-25%), China (4%), Europe (7–30%), Israel (11.2%) and Turkey (17.4%; Zee et al. 2012). A more recent one-year survey (between autumn 2012 and summer 2013) reported winter reductions of managed honey bees varying from five to 30% in 17 European countries (Chauzat et al. 2016). In Africa, losses of around 30% and 46% were reported in South African managed honey bees between 2009/2010 and 2010/2011, respectively (Pirk et al. 2014). However, no large-scale losses were reported in other African countries (Pirk et al. 2016). Large reductions of honey bee colony density have not been reported in South America or Australia (Neumann & Carreck 2010).

The number of managed honey bee hives decreased by 59% in North America between 1947 and 2005, and 25% in central European countries between 1985 and 2005 (Potts, Roberts, et al. 2010), but this trend has not been reported worldwide. Aizen and Harder (2009) reported increment of global population of managed honey bee hives of around 45% from 1961 to 2007, because of increased number of hives in countries such as Argentina, Spain and China. Some increases were also observed in managed honey bee colonies between 1965 and 2005 in Mediterranean European countries (Potts, Roberts, et al. 2010). Likewise, Moritz and Erler (2016) reported reductions in the number of honey bee colonies in Western Europe and the USA, but increment in other regions of the world.

3.2. Bumble bees

Over recent decades, abundance and richness of bumble bee species have declined in Europe, North America, and Asia (Williams & Osborne 2009). In Europe, several bumble bee species have become extinct in certain regions, and four no longer exist throughout the continent (Goulson, Lye & Darvill 2008). In North America, a comparative study of historical and current distribution of bumble bees reported decline in abundance by up to 96% and contraction of

geographical ranges by 23-87% in four out of eight species (Cameron et al. 2011). For instance, since the late 1990s onward, some abundant and widespread bumble bee species have declined and reduced their range to a small fraction of their previous scope. In northern California, a bumble bee species is presumed extinct since it has not been seen since 2006. A survey of bumble bee fauna from Illinois over a century reported decline of bumble bee species richness during 1940-1960 and complete extinction of four species during early 2000s (Grixti et al. 2009). In Asia, declines in bumble bee species richness and abundance have been reported in China and Japan (Williams & Osborne 2009). For other areas in the world, data are limited or unavailable.

3.3. Solitary bees

Abundance and species richness of solitary bees are also in decline in some areas of the world. Species richness has declined both in the UK and The Netherlands since 1980 (Biesmeijer et al. 2006). Similarly, Carvalheiro and colleagues (2013) reported loss of species in the UK, The Netherlands and Belgium between 1930 and 1990 afterward this trend reduced substantially. Between 2008 and 2013, 23% of wild bees, including solitary bees, declined across USA (Koh et al. 2016). In Illinois, plant-pollinator interactions degraded over the last 30 years and 50% of bee species were locally eradicated (Burkle, Marlin & Knight 2013). Overall, the analysis of changes in abundance and diversity of solitary bees is hampered by a lack of good long-term data (Brown, MJF & Paxton 2009) and this is particularly the case for Australia (Batley & Hogendoorn 2009).

4. Causes of bee decline

Bee decline has been linked to the interaction of different biological, environmental and anthropogenic stressors. Biological stressors include pests and pathogens, and exotic plants and bees (Figure 1). Poor weather conditions and climate changes are the main environmental stressors associated with bee decline. Anthropogenic factors incorporate the pressures caused by human activity, namely habitat loss, application of pesticides, and trade (Figure 1). Normally, two or more factors will occur simultaneously and interact in their impact on bee diversity and

abundance. Some stressors cause more pressure on pollinators when combined than alone, for example RNA viruses (Potts, Biesmeijer, et al. 2010; vanEngelsdorp & Meixner 2010). In the following, we give a brief overview of the factors influencing bee declines, after which we focus on the role of RNA viruses on their own and in interaction with other stressors.

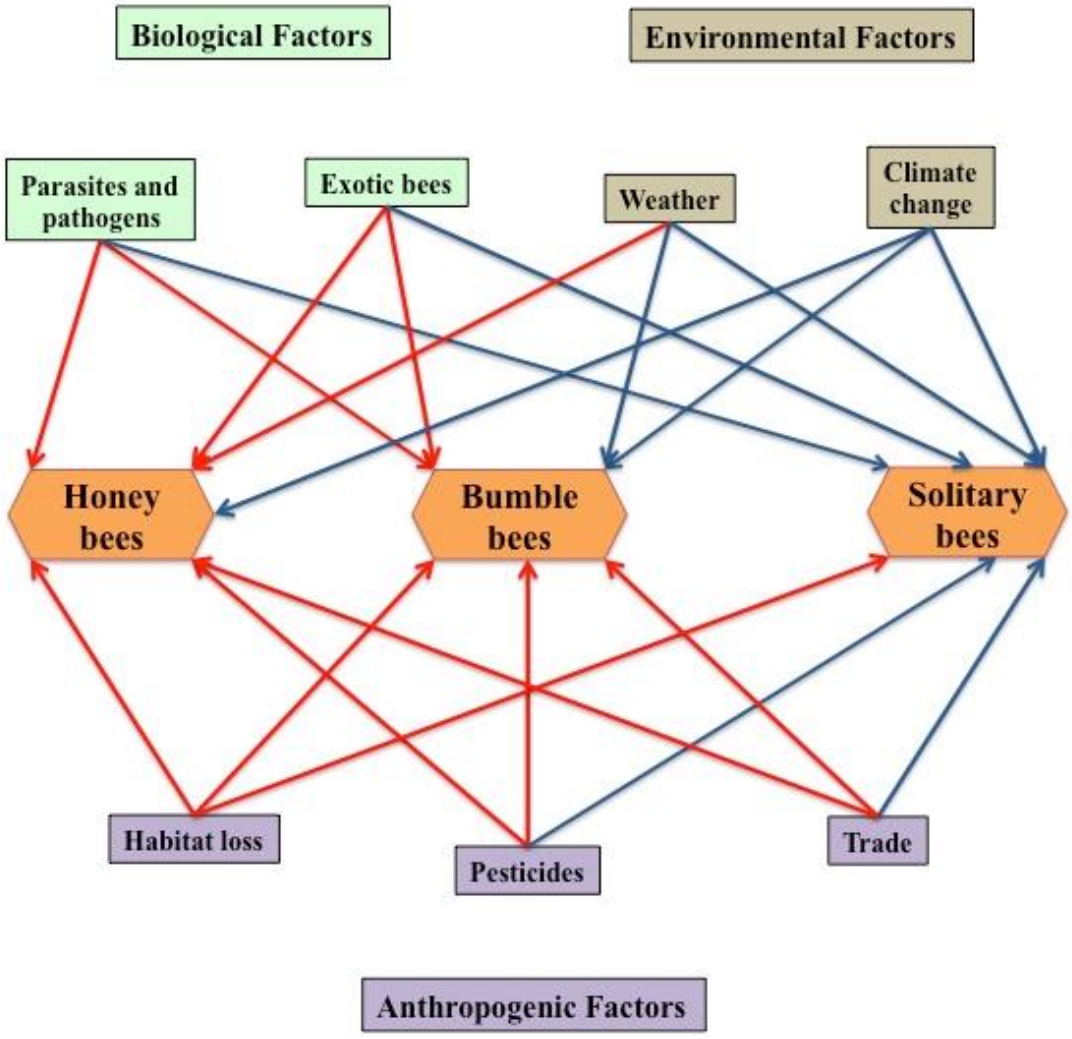


Figure 1: Illustration of the main biological, environmental and anthropogenic factors associated with reduction of abundance and/or richness of honey bees, bumble bees and solitary bees worldwide. Red and blue arrows represent demonstrated and postulated factors, respectively.

4.1. Pests and pathogens

4.1.1. Honey bees

Bees are susceptible to a broad range of pests and pathogens (including mites, bacteria, fungi, viruses and protozoans). Among these, the Varroa mite is the most destructive and the greatest threat to apiculture (Boecking & Genersch 2008; Rosenkranz, Aumeier & Ziegelmann 2010). This obligate parasite of the genus *Apis* feeds on haemolymph of brood and adult honey bees, but developing larvae and pupae are the most susceptible stages since the reproduction of mites occurs in their cells. The loss of haemolymph causes weight reduction and malformation of the bees. This leads to decreased flight performance of drones, and to reduced life span and return rates to the colony of foraging workers (Boecking & Genersch 2008; Rosenkranz, Aumeier & Ziegelmann 2010). Honey bee colonies can collapse within two to three years if not treated. Varroa mites are present all around the world with exception of Australia and Newfoundland (Canada; Rosenkranz, Aumeier & Ziegelmann 2010; Wilfert et al. 2016).

4.1.2. Bumble bees

The invasive pests and pathogens of bumble bees include parasitic flies, nematodes, mites, bacteria, fungi and protozoans (Schmid-Hempel 2005). Bees from the genus *Bombus* are alternate hosts of RNA viruses (e.g. Levitt et al. 2013; McMahon et al. 2015; Singh et al. 2010), and symptomatic bumble bees infected with DWV have been found (Genersch et al. 2006). Although RNA viruses constitute a potential threat to bumble bees, no information is available on their influence on reduction of abundance and richness of bumble bees (Williams & Osborne 2009).

4.1.3. Solitary bees

Known pests and pathogens of solitary bees include parasitic flies, mites, bacteria and fungi. However, compared to the information about honey bees and bumble bees, very little is known about pests and pathogens of native bees (see Figure 1). Although RNA viruses have been

detected in some species of solitary bees (Ravoet et al. 2014; Singh et al. 2010), it is still unknown whether these viruses constitute an issue for the health of these bees. Indeed, pests and pathogens have not been associated with reduction of solitary bees abundance and richness (Brown, MJF & Paxton 2009).

4.2. Exotic bees

The introduction of exotic bees can cause several ecological impacts on native bees and competition for resources and nesting is one of the most reported worldwide (Goulson 2003; Stout & Morales 2009). Competition can occur when floral resources are limited, niche overlap occurs, and when exotic bees are present in high densities (Stout & Morales 2009). For example, high densities of honey bees have been correlated with low abundance and shifts in forage plants for bumble bees (Forup & Memmott 2005; Walther-Hellwig et al. 2006). In addition, the presence of honey bees was suggested to coincide with small body size of worker bumble bees (Goulson & Sparrow 2009). However, many studies assessing the impacts of competition with introduced species on the decline of bees have been inconclusive, as they have been beset by statistical challenges such as insufficient replication, study length or lack of controls (Glatz 2015; Potts, Biesmeijer, et al. 2010). Indeed, an Australian study showed that competition by *A. mellifera* reduced fecundity of a solitary bee although the effect was only significant when tested across two seasons (Paini & Roberts 2005). In addition to direct competitive effects, exotic bees can introduce and transmit new pathogens and pests to native bees and this is well supported by evidence.

4.3. Weather

Extreme weather conditions affect the productivity of honey bee colonies by increasing metabolic demands of foragers and reducing foraging activities (vanEngelsdorp & Meixner 2010). For instance, severe weather conditions has been reported as one of the five main causes of honey bee decline during consecutive winters from 2006 to 2014 in the USA (Spleen et al.

2013; Steinhauer et al. 2014; vanEngelsdorp et al. 2012; vanEngelsdorp et al. 2008; vanEngelsdorp et al. 2010). Severe winter conditions also caused losses of up to 30% in some European countries such as the UK and Ireland (Carreck 2016; van der Zee et al. 2014). No information is available on impact of severe weather conditions on decline of bumble bees and solitary bees.

4.4. Climate change

Climate change is suspected to influence abundance and richness of bees, but its putative impacts are still not well understood (Brown, MJF & Paxton 2009). A possible impact of climate change is range shifting, which can cause spatial divergence between plants and pollinators. This is can theoretically lead to decline or even extinction of specialist bees, but this has not as yet been observed (Vanbergen et al. 2013). Another possible impact is asynchrony between flowering plants and pollinators' foraging stage (Vanbergen et al. 2013).

Increment of temperature is one of the effects of current climate change. Therefore, warmer temperature may well affect bumble bees from temperate regions that are adapted to relatively cool conditions (Carreck 2016)) and may locally increase extremes so that solitary bees that nest above ground may not be able to survive. It is also predicted that climate change is accompanied by augmentation of extreme weather conditions such as floods, droughts, bushfires, and storms. For instance, floods are possible threats to ground nesting bees. While droughts and bushfires are known to impact wild honey bee populations (Oldroyd et al. 1997) and have been suggested to be implicated in the demise of the green carpenter bee from Victoria (Glatz 2015), very little is known about the impact of these threats on solitary bee populations.

4.5. Habitat loss

Habitat loss has been reported as the most important cause of bee decline worldwide. Many anthropogenic activities contribute to habitat loss such as agricultural intensification, urbanization, grazing, deforestation, and fire (De la Rua et al. 2009; Winfree et al. 2009). It is

well recognised that intensification of agriculture is negatively associated with pollination services provided by natural ecosystems (Allen-Wardell et al. 1998; Kleijn & van Langevelde 2006) quite possibly as a result of the reduction of floral resources for bees (Potts, Biesmeijer, et al. 2010; Vanbergen et al. 2013). For example, Ricketts and colleagues (2008) found that abundance and richness of native bees is negatively correlated with distance to natural vegetation. Although mass flowering crops can provide abundant food for pollinators, these mostly flower for a short period of time and provide one-sided nutrition, which can be exacerbated with the use of pesticides (Goulson et al. 2015; vanEngelsdorp & Meixner 2010). However, it is unknown how this poor diet affects the fitness of bees (Potts, Biesmeijer, et al. 2010; Vanbergen et al. 2013). Agriculture intensification also reduces nesting resources for wild honey bees, bumble bees, stingless bees and solitary bees (Vanbergen et al. 2013; Williams & Osborne 2009). Obviously, lack of nesting resources has no effect on managed honey bees (Ricketts et al. 2008; Winfree et al. 2009).

Urbanisation has also been postulated to contribute to a reduction of bee abundance and richness, but its impacts have not been clearly demonstrated (Carreck 2016). Urban gardens can provide floral and nesting resources for bees, and urban areas can provide nest substrate for bees that use cavities to build their nests. On the other hand, infrastructures and roads contribute to loss or degradation of habitats.

4.6. Pesticides

Agricultural intensification is generally also associated with the use of agrochemicals such as insecticides and fungicides, to control weeds, fungi and insects in order to increase yields and are another potential cause of pollinator decline (Goulson et al. 2015). Bees can be exposed to a variety of chemicals during brood and adult stages (vanEngelsdorp & Meixner 2010).

Among pesticides, neonicotinoids have been suggested as cause of bee decline and CCD. However, there are clearly other factors involved, as CCD is not reported from Australia while

neonicotinoids are used widely. Nevertheless, mortality of honey bees by poisoning can be a direct effect of these pesticides (vanEngelsdorp & Meixner 2010). In addition, neonicotinoids can reduce colony growth and queen production in bumble bees (Whitehorn et al. 2012) and cause sub-lethal effects such as reduction in the ability to learn, forage and return to the colony in both honey bees and bumble bees. However, negative impacts of foraging on canola that had been seed-treated have been shown experimentally for bumble bees and solitary mason bees, but not for honey bees (Carreck 2016; Rundlof et al. 2015).

Varroacides such as coumaphos or fluvalinate have been used to kill Varroa mites in honey bee colonies. These chemicals have been found in 100% of all honey bee wax tested in the USA and France, and the impacts of long-term exposure on honey bee health are unknown (vanEngelsdorp & Meixner 2010).

There is very little known about the effects of pesticides on solitary bees (Carreck 2016). A meta-analysis comparing LD50 values for different chemical classes reported high variability between bee species and that the impact may vary according to their life cycle, foraging and nesting behaviour. More research is required to elucidate the impacts of pesticides in solitary bees (Arena & Sgolastra 2014) and their interactions with other stressors.

4.7. Trade

Global trade in bees and their products has increased over the last several decades. Because of their value as pollinators, honey bees and bumble bees have been introduced into many areas outside of their natural habitat (Goulson & Hughes 2015). The main impact of trade of bees is to increase movement of bees and the potential for introduction of new pests, pathogens, different strains of pathogens, or haplotypes of parasites. The best known example is the Varroa mite, which assisted by humans has become the most important and widespread parasite of *A. mellifera* (Goulson, Dave & Hughes 2015). Parasites were detected in commercial bumble bees

produced between 2011 and 2012 in Europe and imported as being free of parasites and in pollen supplied with the colonies (Graystock et al. 2013).

5. RNA viruses

Worldwide, about 24 viruses have been identified as pathogens of honey bees (de Miranda et al. 2013). The majority of RNA viruses were identified and characterised between 1960s and 1980s using infectivity tests and serological techniques (de Miranda et al. 2013; Ribière, Ball & Aubert 2007). However, in the past 15 years new viruses have been identified using molecular sequencing (Sanger Sequencing and Next Generation Sequencing). Some of the new viruses are very similar to previously identified viruses (de Miranda et al. 2013). For example, *Acute bee paralysis virus* (ABPV), *Kashmir bee virus* (KBV) and *Israel acute paralysis virus* (IAPV) are likely to be a complex of related viruses (de Miranda, Cordoni & Budge 2010). Combining the closely related viruses into complexes reduces the number of viruses to between 16 and 18 (de Miranda 2007).

With exceptions of *Apis mellifera* filamentous virus and *Apis* iridescent virus, which are double stranded DNA, the majority of viruses infecting bees are single-strand RNA (ssRNA) viruses (Figure 2; de Miranda et al. 2013). Here, I will focus on nine RNA viruses, namely ABPV, *Black queen cell virus* (BQCV), Chronic bee paralysis virus (CBPV), Cloudy wing virus (CWV), DWV, IAPV, KBV, *Sacbrood virus* (SBV), and Slow bee paralysis virus (SBPV) since they were the targeted viruses in this study. These viruses are classified according the International Committee on Taxonomy of Viruses (ICTV). Following King and co-authors (2012) recognized taxa are written in italics whereas unassigned viruses are not.

Initially, RNA viruses have been described as specific to *A. mellifera* since they were first isolated from honey bees (Ball & Bailey 1991). However, over the last decade, RNA viruses have been detected in different species of bees and other pollinators (Table 1; Genersch et al. 2006; Levitt et al. 2013; Li et al. 2011; Peng et al. 2011; Ravoet et al. 2014; Singh et al. 2010).

Detailed biological properties and distribution of these RNA viruses in honey bees, bumble bees and solitary bees are given in Tables 1 and 2.

RNA viruses normally occur in honey bees as covert (inapparent) infections characterised by presence of low virus titres, absence of symptoms, persistence over many generations, and no other discernible impacts on bee fitness (Hails, Ball & Genersch 2007). Viruses that are present as a covert infection remain fully competent and can produce an overt infection if circumstances are conducive (Boecking & Genersch 2008; Brown, MJF & Fries 2007). The symptoms of RNA viruses in honey bees under controlled conditions are well known (Table 2), but these symptoms are hardly observed in the field (Chen, Y. P. & Siede 2007; Ribière, Ball & Aubert 2007). Although symptoms of DWV were observed in bumble bees (Fürst et al. 2014; Genersch et al. 2006), no information is available on overt infections caused by RNA viruses in solitary bees.

Individual honey bees (adult workers, brood and queens) can harbour two or more viruses simultaneously (Chen, Y. P., Pettis & Feldlaufer 2005; Ribière, Ball & Aubert 2007; Tentcheva et al. 2004). Similarly, two or more RNA viruses have been detected in individual bumble bees and solitary bees (Levitt et al. 2013; Ravoet et al. 2014; Singh et al. 2010). However, it is unknown whether these viruses interact or if they affect one anothers' epidemiology. It is also not known whether interactions between two or more viruses would lead to genetic recombination between interacting viral genomes (Ribière, Ball & Aubert 2007).

Table 1: Summary of worldwide distribution, host species and seasonal incidence of RNA viruses in honey bees, bumble bees and solitary bees. Also listed are the affected developmental stages (E: egg, L: larvae, P: pupae and A: adult) of bees. '+' represent present or previously reported, while '?' symbolise unknown or indeterminate.

Virus species	Host species			Distribution			Seasonal incidence			Affected host stage											
										Honey bees				Bumble bees				Solitary bees			
	Honey bees	Bumble bees	Solitary bees	Honey bees	Bumble bees	Solitary bees	Honey bees	Bumble bees	Solitary bees	E	L	P	A	E	L	P	A	E	L	P	A
***Acute bee paralysis virus* (ABPV)**	*Apis mellifera*	*Bombus agrorum* *B. atratus* *B. hortorum* *B. impatiens* *B. lapidarius* *B. lucorum* *B. pascuorum* *B. terrestris*	*Melipona scutellaris*	North and South America; Europe; Asia; Middle East, Africa and Asia	Belgium	?	Summer Autumn	?	?	+	+	+	+	?	?	?	+	?	?	?	?
***Black queen cell virus* (BQCV)**	*A. cerana* *A. mellifera*	*B. atratus* *B. huntii* *B. impatiens* *B. lapidarius* *B. lucorum* *B. pascuorum* *B. ternarius,* *B. terrestris,* *B. vagans,* *Bombus sp.*	*Andrena vaga, Andrena sp., Heriades truncorum, Megachile rotundata, Nomia melanderi, Osmia cornuta, Xylocopa virginica*	North and Central America; Europe; Africa; Middle East; Asia and Oceania (including Australia)	USA	Belgium USA	Summer	?	?	+	+	+	+	?	?	?	+	?	?	?	+
Chronic bee paralysis virus (CBPV)	*A. cerana* *A. mellifera*	*B. impatiens*	?	All continents with exception of Central and South America	?	?	Spring Summer	?	?	?	+	+	+	?	?	?	?	?	?	?	?
Cloudy wing virus (CWV)	*A. mellifera*	?	?	North and South America; Europe; Asia; Middle East, Africa and Oceania (including Australia)	?	?	No seasonality	?	?	?	?	?	+	?	?	?	?	?	?	?	?

Table 1: Continuation

Virus species	Host species			Distribution			Seasonal incidence			Affected host stage											
	Honey bees	Bumble bees	Solitary bees	Honey bees	Bumble bees	Solitary bees	Honey bees	Bumble bees	Solitary bees	Honey bees				Bumble bees				Solitary bees			
										E	L	P	A	E	L	P	A	E	L	P	A
Deformed wing virus (DWV)	*A. cerana, A. florea A. mellifera*	*B. atratus B. lapidarius B. huntii B. impatiens B. lucorum B. monticola B. pascuorum B. ternarius B. terrestris B. vagans Bombus sp.*	*Andrena* sp., *Augochlora pura, Ceratina dupla, M. rotundata, N. melanderi, O. bicornis, O. cornuta, X. virginica*	North and South America; Europe; Asia; Africa; Middle East and Asia	Belgium, Island of Man, UK, USA	Belgium, USA	Autumn Summer	?	?	+	+	+	+	?	?	?	+	?	?	?	+
***Israel acute paralysis virus* (IAPV)**	*A. cerana A. florea A. mellifera A. pura*	*B. impatiens, B. ternarius, B. vagans*	*A. pura,*	North, Central and South America; Middle East; Asia; Europe; Australia	USA	USA	Summer Autumn	?	?	+	+	+	+	?	?	?	+	?	?	?	+
***Kashmir bee virus* (KBV)**	*A. cerana A. mellifera*	*B. terrestris Bombus* sp.	?	North and Central America; Europe and Oceania (including Australia)	Belgium, USA	?	Autumn Summer	?	?	+	+	+	+	?	?	?	+	?	?	?	?
***Sacbrood virus* (SBV)**	*A. cerana A. mellifera*	*B. atratus B. impatiens B. lapidaries B. lucorum B. ternarius B. pascuorum B. vagans Bombus sp.*	*A. vaga, N. melanderi, X. virginica,*	All continents	USA	Belgium, USA	Spring Summer	?	?	?	+	+	+	?	?	?	+	?	?	?	+
Slow bee paralysis virus (SBPV)	*A. mellifera*	*B. pascuorum*	?	?	Belgium	?	No seasonality	?	?	?	+	+	+	?	?	?	+	?	?	?	?

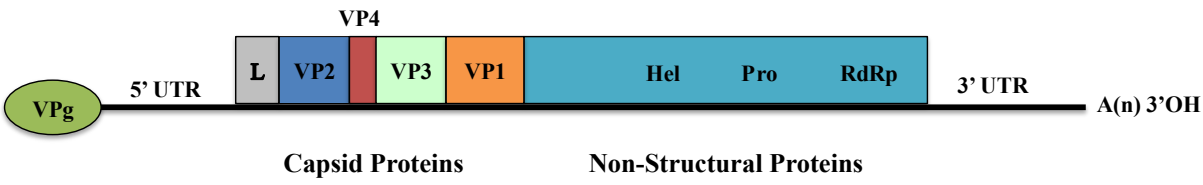
Generated from (Ai, Yan & Han 2012; Bailey & Gibbs 1964; Chen, Y. P. & Siede 2007; Choi, Jung & Lee 2015; de Miranda et al. 2013; Ellis & Munn 2005; Forsgren et al. 2015; Fürst et al. 2014; Levitt et al. 2013; Mazzei et al. 2014; McMahon et al. 2015; Meeus et al. 2014; Niu et al. 2016; Parmentier et al. 2016; Ravoet et al. 2014; Ribière, Ball & Aubert 2007; Singh et al. 2010; Ueira-Vieira et al. 2015)

5.1. Virion properties

All reported bee RNA viruses have a positive-sense ssRNA genome, icosahedral virion symmetry (except anisometric CBPV) and lack a lipid-containing envelope, but their biological properties are different (Chen, Y. P. & Siede 2007; de Miranda et al. 2013; Ribière, Ball & Aubert 2007).

Most RNA viruses of bees belong to the *Dicistroviridae* and *Iflaviridae* with an exception of the few that remain unclassified. The *Dicistroviridae* are subdivided into two genera *Cripavirus* (containing BQCV) and *Aparavirus* (containing ABPV, KBV and IAPV), while *Iflaviridae* contains only one genus *Iflavirus* (containing DWV, SBV and SBPV). In addition, CBPV and CWV have not been placed into any genus or family (Chen, Y. P., Becnel & Valles 2012; King et al. 2012). The genome of the *Dicistroviridae* is monopartite and dicistronic with two non-overlapping open reading frames (ORFs) that are separated and flanked by untranslated regions. In addition, the ORFs adjacent to the 5' and 3' ends encode the non-structural and structural protein precursors, respectively (Figure 2B; King et al. 2012). While the other hand, the *Iflavirus* genome is also monopartite, it encompasses one large polyprotein ORF. Moreover, the 5' end of the polyprotein gene encodes the structural proteins and the 3' end the non-structural proteins (Figure 2A; King et al. 2012). The genome size of the RNA viruses does not exceed 10 kb (de Miranda et al. 2013).

A



B

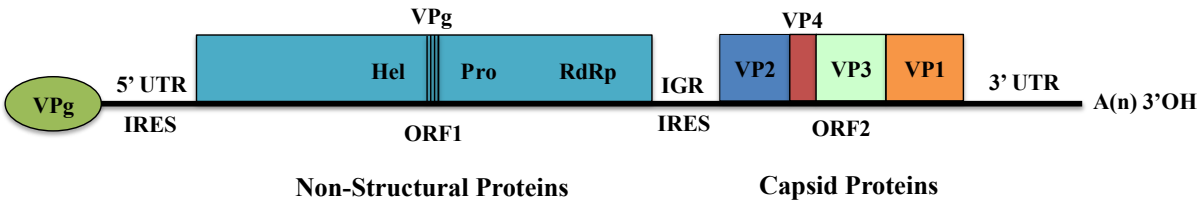


Figure 2: Genome structure of *Iflaviridae* (A) and *Dicistroviridae* (B) RNA viruses that encode a single and two non-overlapping open reading frames (ORFs), respectively. *Dicistroviridae* ORFs are separated by an intergenic region (IGR), and contains distinct internal ribosome entry sites (IRES) that are located at the 5' end untranslated region (UTR) and IGR. The 5' end of the genomes carries a covalently linked protein (VPg) and the 3' end is polyadenylated, and both ends comprise an UTR. Both genomes contain the non-structural proteins: RNA helicase (Hel), cysteine protease (Pro) and RNA-dependent RNA polymerase (RdRp), and three major structural proteins (VP1, VP2, VP3). VP4 is the minor structural component of the virion and is presumed to be an N-terminal extension of VP3 that is cleaved from the precursor. A short leader protein (L) precedes the capsid proteins of *Iflaviridae*.

Table 2: Description of the symptoms, fitness cost at individual level and symptomatic developmental stages (E: egg, L: larvae, P: pupae and A: adult) of honey bees, bumble bees and solitary bee. '+' represent present or previously reported, while '?' symbolise unknown or indeterminate.

Virus	Symptoms			Fitness cost at individual level			Symptoms visible											
	Honey bees	Bumble bees	Solitary bees	Honey bees	Bumble bees	Solitary bees	Honey bees				Bumble bees				Solitary bees			
							E	L	P	A	E	L	P	A	E	L	P	A
***Acute bee paralysis virus* (ABPV)**	Abnormal trembling of wings and bodies of adult workers, paralysis and flightless (acute paralysis)	**?**	**?**	Death of adult workers within 1 or 2 days	**?**	**?**	-	-	?	+	?	?	?	?	?	?	?	?
***Black queen cell virus* (BQCV)**	Queen larvae (early stages of infection): pale yellow appearance and hard saclike skin. Larvae fail to pupate. Queen larvae (late stages of infection) and pupae: decomposed individuals inside dark cells. Cell walls almost black in patches.	**?**	**?**	Death of queen larvae and pupae	**?**	**?**	-	-	+	-	?	?	?	?	?	?	?	?
Chronic bee paralysis virus (CBPV)	Two types of syndromes: Type 1 - abnormal trembling of the body and wings, paralysis, dislocated wings, flightless, and bloated abdomens. Type 2 - hairless, shiny, dark or almost black appearance, greasy appearance in bright light. Workers appearance cause nibbling attacked by the older bees from the colony, and rejection at the entrance of the hives.	**?**	**?**	Death of adult workers within few days	**?**	**?**	-	-	-	+	?	?	?	?	?	?	?	?
Cloudy wing virus (CWV)	Transparency of wings of adult workers	**?**	**?**	Death of adult workers within few days	**?**	**?**	-	-	-	+	?	?	?	?	?	?	?	?

Table 2: Continuation

Virus	Symptoms			Fitness cost at individual level			Symptoms visible											
	Honey bees	Bumble bees	Solitary bees	Honey bees	Bumble bees	Solitary bees	Honey bees				Bumble bees				Solitary bees			
							E	L	P	A	E	L	P	A	E	L	P	A
Deformed wing virus (DWV)	Newly emerging adults have small and deformed wings. Adults have shrunken and crumpled wings, as well as bloated, shortened and discoloured abdomens.	Adult bees have crumpled and vestigial wings	?	Early death of adult workers	Non-viable offspring and reduction of longevity	?	-	-	+	+	?	?	?	+	?	?	?	?
***Israel acute paralysis virus* (IAPV)**	Adults have a dark brown to black abdomen and thorax, and a hairless thorax. The bees are flightless and barely move, and undergo a period of spasms.	No clear symptoms	?	Death of adult workers	Death of adult workers	?	-	-	?	+	?	?	?	?	?	?	?	?
***Kashmir bee virus* (KBV)**	No clear symptoms	?	?	Death of brood and adult workers within few days.	?	?	-	-	+	+	?	?	?	?	?	?	?	?
***Sacbrood virus* (SBV)**	Workers larvae fail to pupate, become pale yellow and the skin turn leathery. When removed from the cells, the sac looks water-filled.	?	?	Death of workers larvae	?	?	?	+	-	?	?	?	?	?	?	?	?	?
Slow bee paralysis virus (SBPV)	The front two pairs of legs of adult workers become paralysed	?	?	Death of adult workers within 1 or 2 days	?	?	?	-	-	+	?	?	?	?	?	?	?	?

Generated from (Chen, Y & Siede 2007; de Miranda, Joachim R. et al. 2013; Genersch et al. 2006; Ribière, Ball & Aubert 2007; Rosalind & Zengzhi 2012)

5.2. Transmission of viruses

Virus transmission can occur from one individual to another from the same generation via direct or indirect transfer of virus particles (Brown, MJF & Fries 2007; Chen, Y. P. & Siede 2007; Manley, Boots & Wilfert 2015).

Direct transfer of RNA viruses occurs between infected and healthy individuals through venereal and vertical transmission, which allows exchanging of virus particles within individuals and generations of the same species (Figure 3). Indirect transfer requires some kind of vector such as food (pollen and/or nectar) or a biological vector, which facilitates intra and inter-species transmission (Brown, MJF & Fries 2007; Chen, Y. P. & Siede 2007; Manley, Boots & Wilfert 2015). Transmission of RNA viruses in honey bees is well understood and documented (Figure 3). Several studies have analysed transmission of RNA viruses in bumble bees, and no information is available for solitary bees (Figure 3).

Food-borne transmission occurs when healthy bees consume contaminated food, and it is the most common pathway of transmission (Chen, Y. P. & Siede 2007). Detection of SBV and KBV in brood food, honey, pollen, and royal jelly from honey bee colonies also infected by the same viruses suggested food as a pathway of virus transmission (Shen, Cui, et al. 2005). Another study conducted by Chen and colleagues (2006) detected six viruses namely ABPV, BQCV, CBPV, DWV, KBV and SBV in pollen collected from honey bee colonies, and BQCV and DWV in honey.

Singh and colleagues (2010) detected viruses in pollen pellets from foraging worker honey bees, but several of these bees were uninfected, indicating that pollen is a vector that can be carried by hosts or non-hosts of the virus. Infectivity of virus-contaminated food was demonstrated when healthy worker honey bees were fed with contaminated pollen and honey causing infection of the entire colony including the queen and eggs (Singh et al. 2010). DWV was detected in pollen collected directly from flowers visited by honey bees demonstrating food contamination outside the hives (Mazzei et al. 2014). Contaminated food has been reported as

the main cause of infection of commercialised bumble bees that had been fed with honey bee pollen during rearing (Goulson & Hughes 2015; Graystock et al. 2016; Graystock et al. 2013). Graystock and co-authors (2016) tested pollen used in bumble bee rearing and detected diverse parasites including RNA viruses.

Faecal-oral transmission happens when infected faeces is ingested by healthy individuals (Chen, Y. P. & Siede 2007). This route of transmission was hypothesised when KBV was detected in faeces of worker and queen honey bees (Hung 2000). BQCV and DWV were also detected in faeces of queen honey bees (Chen, Y. P. et al. 2006). Detection of high titres of BQCV and DWV in the gut of queen honey bees suggested faecal-oral or foodborne transmission, and the gut as the primary site of infection (Chen, Y. P. et al. 2006).

Venereal transmission occurs between sexes during mating (Chen, Y. P. & Siede 2007). DWV has been detected in adult drones (Chen, Y. P., Higgins & Feldlaufer 2005; Fievet et al. 2006), and ABPV and DWV in drone semen (Yue et al. 2006; Yue et al. 2007), suggesting potential for venereal transmission. This pathway of transmission was demonstrated when DWV was detected in spermatheca and ovaries of queen honey bees artificially inseminated with DWV-infected semen (de Miranda & Fries 2008). In bumble bees, detection of systemic infection of BQCV and DWV in males suggest that venereal transmission through semen is possible (Li et al. 2011; Peng et al. 2011), but this pathway still needs to be demonstrated. No information is available for solitary bees (Figure 3).

Vectors acquire virus particles from one host and deliver them to another, facilitating horizontal transmission (Chen, Y. P. & Siede 2007). The parasitic Varroa mite is a vector of many RNA viruses, namely ABPV, BQCV, DWV, IAPV, KBV, SBV, and SBPV. However, its association with CBPV and CWV is unknown (Boecking & Genersch 2008; de Miranda 2007). The mite functions both as a vector and supports viral replication within its body, delivering virions directly into the haemolymph of hosts. Because of that, over time, the virulence of the virus increases in association with the mite (Boecking & Genersch 2008; Rosenkranz, Aumeier

& Ziegelmann 2010; Shen, Yang, et al. 2005; Tentcheva et al. 2006). The synergistic effect between the Varroa mite and RNA viruses was demonstrated in studies performed in two countries with the most recent introduction of this parasite, Hawaii and New Zealand. After introduction of Varroa mite in New Zealand, prevalence of BQCV, CBPV, DWV, KBV, and SBV increased over ten years (Mondet et al. 2014). Similarly, in Hawaii, prevalence and viral load of DWV increased while strain diversity reduced within three years of Varroa mite infestation (Martin et al. 2012). Although Varroa mite has been identified as a vector of many RNA viruses in honey bees, it is still unknown if it is a vector of all bee RNA viruses, particularly the viruses most recently identified (de Miranda et al. 2013). No information is available on vectors of RNA viruses in bumble bees and solitary bees (Figure 3).

Vertical transmission occurs when virus particles are passed from a mother to her offspring (Chen, Y. P. & Siede 2007). Detection of BQCV, DWV, CBPV, KBV, and SBV in queen honey bees and their ovaries, and in infected offspring of the same hive (eggs, larvae and resultant adult workers) implicated vertical transmission as a route of virus infection (Chen, Y. P. et al. 2006). Another study detected ABPV, DWV, KBV, and IAPV in ovaries of queen honey bees (Francis, Nielsen & Kryger 2013). Vertical transmission was demonstrated when DWV was detected in both unfertilized and fertilized eggs of healthy virgin queen honey bees inseminated with DWV-positive semen (de Miranda & Fries 2008; Yue et al. 2007). No information is available on vertical transmission of RNA viruses in bumble bees and solitary bees (Figure 3).

Although many studies have reported honey bees as the primary host of RNA viruses, the origin and directionality of movement of virus particles between bee species is still unclear. Singh and colleagues (2010) showed that IAPV does not have specific directionality between honey bees and bumble bees. In addition, in the UK, prevalence of DWV in honey bees was a good predictor of its prevalence in bumble bees, but the direction of the transmission was not investigated (McMahon et al. 2015).

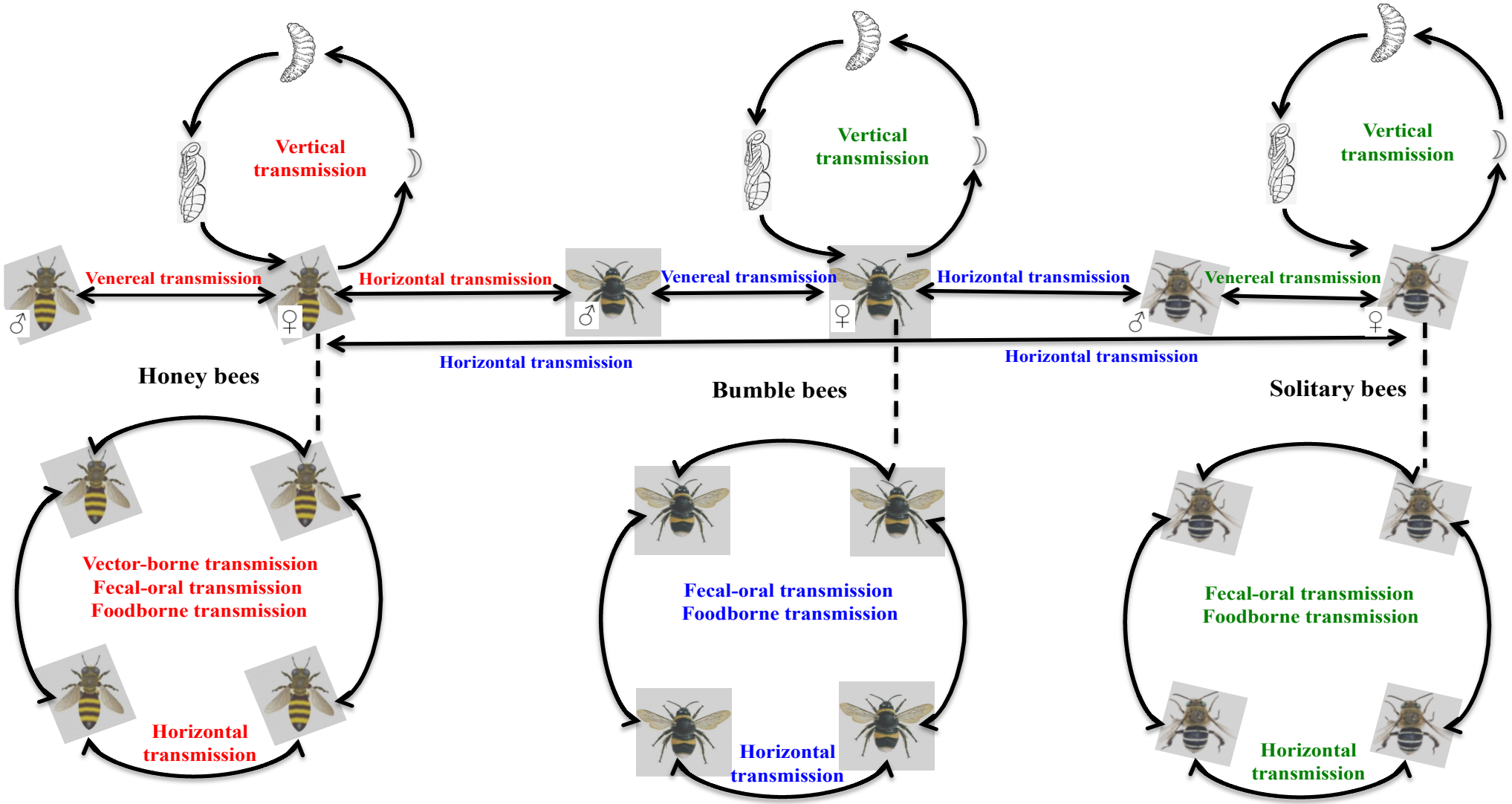

Vertical transmission
Vertical transmission
Vertical transmission
Venereal transmission
Horizontal transmission
Venereal transmission
Horizontal transmission
Venereal transmission
Horizontal transmission
Horizontal transmission
Honey bees
Bumble bees
Solitary bees
Vector-borne transmission
Fecal-oral transmission
Foodborne transmission
Horizontal transmission
Fecal-oral transmission
Foodborne transmission
Horizontal transmission
Fecal-oral transmission
Foodborne transmission
Horizontal transmission

Figure 3: Schematic representation of the transmission pathways of RNA viruses in honey bees, bumble bees and solitary bees. Inter-species transmission can occur vertically and horizontally via foodborne, faecal-oral and vector-borne transmission. Intra-specific transmission occurs horizontally potentially between all susceptible bees. No information is available for transmission pathways written in green. Blue and red writing represents transmission pathways previously speculated and demonstrated, respectively. It should be noted that the degree of characterisation of the respective pathways is proportional to the amount of research that has been performed, which has been focussed on honey bees, to a lesser extent on *Bombus* spp., and minimally on solitary bees.

6. Antiviral response of bees

Bees have three lines of defence that act in cascade to avoid and fight pathogen infection. The outer body wall and the cuticle of the exoskeleton are examples of the primary and nonspecific line of defence that provides protection against pathogen invasions. If these barriers are overcome by the pathogens, the humoral and cellular immune responses may be activated (Chen, Y. P. & Siede 2007). The host recognises the pathogen associated molecular patterns such as viral dsRNA, bacterial peptidoglycan, and fungal β-glucans (humoral response) and activates the cellular immune responses in order to eliminate or kill the pathogen (Chen, Y. P. & Siede 2007; Evans & Spivak 2010; Schmid-Hempel 2005).

Different cellular immune response pathways have been associated with honey bee antiviral responses, namely RNA interference (RNAi), Janus kinase/Signal Transducer and Activator of Transcription (JAK/STAT), Toll, Immune deficiency, endocytosis, and Mitogen-Activated protein Kinases, but they are largely uncharacterised (Brutscher & Flenniken 2015; Evans et al. 2006). RNAi is an antiviral response of insects, plants, fungi, nematodes and vertebrates (Ding 2010).

The RNAi mechanism is a post-transcriptional gene silencing mechanism that involves three distinct pathways, namely short-interfering RNA (siRNA), microRNA (miRNA), and piwi-

interacting RNA (piRNA). These pathways have distinct biological functions and interactions with distinct proteins (Brutscher & Flenniken 2015; Ding 2010; Hammond, Caudy & Hannon 2001). RNAi has been successfully used to regulate and alter gene expression of different organisms, and to reverse genetics of plants, flies and nematode *Caenorhabditis elegans* (Brutscher & Flenniken 2015; Hammond, Caudy & Hannon 2001). In addition, RNAi technology has been used as a tool to investigate the function and characterise genes of insects (Belles 2010; Brown, S et al. 1999; Lucas & Raikhel 2013). RNAi is also responsible for antiviral defence in plants and invertebrates via siRNA pathway (Brutscher & Flenniken 2015).

The siRNA pathway is the most important antiviral response in honey bees (Brutscher, Daughenbaugh & Flenniken 2015; Brutscher & Flenniken 2015; Niu et al. 2014). This pathway is activated by dsRNA (e.g. viral genome or replicative intermediates of ssRNA) and a ribonuclease III enzyme named *Dicer-like* degrades the viral genome into 21-22 nucleotide (nt) long short-interfering RNAs (siRNAs; Brutscher & Flenniken 2015; Niu et al. 2014).

Under controlled conditions, siRNA pathway was demonstrated when feeding honey bees with IAPV-dsRNA prior to IAPV-infection reduced bee mortality and virus titres (Maori et al. 2009). Likewise, second instar larvae of *A. cerana* had reduced levels of Chinese sacbrood virus (CSBV) when pre-treated with virus-specific dsRNA prior to infection (Liu et al. 2010). Another study showed increased longevity and reduction of DWV titres after feeding adult honey bees with DWV-dsRNA prior to infection under controlled conditions (Desai et al. 2012). Hunter and colleagues (2010) validated this defence mechanism in honey bee hives kept under normal beekeeping conditions. The numbers of bees per hive and honey yield increased in honey bee colonies treated for six weeks with IAPV-dsRNA. Administration of non-specific dsRNA also trigged antiviral response and reduced virus infection in honey bees (Flenniken & Andino 2013). On the other hand, the antiviral response was not activated in honey bees fed with ABPV-dsRNA before infection, which may be associated RNAi silencing by ABPV (Azzami et al. 2012). The successful use of RNAi in silencing some RNA viruses via ingestion and feeding has

led to increased interest in using it as either a preventive technique or as a treatment (Hunter et al. 2010).

In bumble bees, injecting IAPV and SBPV before infection activated the siRNA pathway and produced 22nt virus-derived siRNAs (Niu et al. 2016). Similarly, oral administration of IAPV-dsRNA and non-specific dsRNA reduced virus titres of IAPV infection in bumble bees (Piot et al. 2015). As for many aspects of RNA virus epidemiology in bees, no information is available about the immune response of solitary bees.

The siRNA pathway has been shown to silence different types of viral genomes such as (+)ssRNA, (-)ssRNA, dsRNA, ssDNA, and dsDNA (Ding 2010). In mosquitos, fruit flies, plants and nematodes, deep sequencing of total siRNAs and assembly of contigs showed continuous coverage of genomic (+)ssRNA and dsRNA viruses. This allowed assembly of entire or partial viral genomes of known viruses and discovery of novel viruses (Barrero et al. 2017; Kreuze et al. 2009; Wu et al. 2010). Therefore, deep sequencing of siRNAs can be used as a tool for diagnosis, discovery and sequencing of viruses (Barrero et al. 2017; Kreuze et al. 2009).

Despite the demonstration of the siRNA mechanism as antiviral defence in honey bees and bumble bees (e.g. Desai et al. 2012; Maori et al. 2009; Niu et al. 2016), deep sequencing of vsiRNAs has not yet yielded full genomes of infecting viruses (Cheijanovsky 2014). For instance, Chejanovsky and colleagues (2014) deep sequenced honey bee samples originating from colonies with and without CCD symptoms and detected abundant siRNAs matching IAPV, DWV and KBV only in CCD-affected bees. However, they were able to generate only partial genomes of these bee viruses.

7. Aim and significance of the project

Worldwide, the knowledge about parasites and pathogens infecting honey bees has grown substantially over the last three decades (Figure 1), through a welth of studies of the epidemiology and ecology of RNA viruses. However, less is known about RNA viruses in bumble bees and information about solitary bees is scant (Figures 1 & 3, and Tables 1 & 2).

In Australia, all studies on RNA viruses have been performed in the genus *Apis*. The majority of these studies have been performed on European honey bees in 1990s, while two recent studies have investigated the incidence of RNA viruses in *A. cerana* and *A. mellifera* in Cairns region (Roberts & Anderson 2013) and performed a survey of viruses in apiaries in Australia (Roberts, Anderson & Durr 2015). Although RNA viruses have recently been reported in bumble bees and solitary bees in Europe and the USA, no work has been done in Australia to assess RNA viruses in native (solitary) bees or in bumble bees that were introduced to Tasmania. Australia is one of the last countries without the parasitic Varroa mite, so baseline data on RNA viruses are required to better understand and manage the impacts in case of its introduction into the country.

The main objective of my project was to study the distribution and possible exchange of RNA viruses between bee species in Australia. In order to address this objective, three studies were performed. These studies addressed the following questions: (1) Is the prevalence of RNA viruses in South Australian native bees associated with managed honey bees (*A. mellifera*) and can we find evidence for directionality in inter-species transmission (Chapter 2)?; (2) Did Bumble bees (*B. terrestris*) carry new RNA viruses onto the Australian island of Tasmania (Chapter 3)?; (3) Is small interfering RNA a new technique for detection, sequencing and complete genome assembly of bee RNA viruses (Chapter 4)?. The results of these studies provide new insights into origin, host range, prevalence, and genome variability of RNA viruses in Australian bees.

8. Scope and structure of thesis

The main body of this thesis by publication includes an introduction and review of literature, three manuscripts ready for submission, and an overarching discussion. The introductory chapter includes a review of the literature on the topic of RNA viruses and the manuscripts may contain some repetition since they are intended to be published and read independently. The references of the introduction and review of literature (Chapter 1), and the general discussion (Chapter 5) are listed at the end of the thesis.

Chapter 2

Association between RNA viruses of Australian native bees and managed honey bees (*Apis mellifera*)

To submit to Plos One

Chapter 2

Association between RNA viruses of Australian native bees and managed honey bees (*Apis mellifera*)

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Short title: RNA viruses in non-*Apis* bees

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Abstract

Over the last decade, RNA viruses have been associated with population declines of *Apis mellifera* in many areas around the world. RNA viruses were initially characterised as pathogens of just *A. mellifera* until recent detection in different species of non-*Apis* bees. This raised questions about the origin and direction of transmission of RNA viruses. Therefore, in this study we determine whether Australian native bees carry similar RNA viruses to American and

European *A. mellifera* and non-*Apis* bees, and deduce whether the RNA viruses carried by Australian native bees were introduced together with *A. mellifera* or were already present in the native bees. To investigate this, we performed a survey in six geographical regions of South Australia with different median annual rainfall. This allowed collection of native bees both co-foraging with *A. mellifera* and foraging in absence of *A. mellifera*. When present, workers of *A. mellifera* were also collected. We used reverse transcription polymerase chain reaction (RT-PCR) to test the bees for eight viruses. Our results confirm the association of some RNA viruses with native bees and show that the probabilities of (a) native bees carrying *Black queen cell virus* (BQCV) and S*acbrood virus* (SBV) and (b) worker *A. mellifera* carrying viruses are higher in areas with beekeeping activities. The results indicate that BQCV and SBV were introduced into Australia with *A. mellifera*.

Introduction

To date a total of 22 RNA viruses have been reported to infect European honey bees (*Apis mellifera*) worldwide [1]. Because RNA viruses were first isolated from *A. mellifera*, they were described as specific to this bee species and called 'honey bee viruses' [2, 3]. However, over the last decade, honey bee viruses have been detected in non-*Apis* species of bees including bumble bees (*Bombus* spp.) and solitary bees, showing that these viruses are not specific to *A. mellifera* [e.g. 4-8]. This suggests that known honey bee viruses may have spilled over into non-*Apis* bees from *A. mellifera* or *vice versa*. Hence, the origin, host range and direction(s) of interspecies transmission of known honey bee viruses are uncertain. With this in mind, we will use the term 'RNA viruses' to refer to known honey bee viruses.

Insights into the origin and transmission of RNA viruses have been gathered during different studies that analysed the movement of virus particles between *A. mellifera* and non-*Apis* bees. Intra- and inter-species transmission of RNA virus particles has been associated with bees' foraging activities [7, 9] as RNA virus particles were detected in *A. mellifera* honey, pollen, and

royal jelly [10, 11]. In addition, particles of *Deformed wing virus* (DWV) were detected in pollen suspension collected directly from flowers visited by *A. mellifera*. These particles caused infection after direct injection into haemolymph of *Osmia cornuta* and *A. mellifera* [12]. Ingestion of contaminated food was suggested as mode of virus particles acquisition in the natural environment after detection of high DWV titres and replicative forms in the gut of *B. huntii* workers [4]. Contaminated food has been reported as the main cause of infection of commercialised *Bombus* spp. since they are fed with pollen of *A. mellifera* during rearing [9, 13, 14]. The direction of transmission of *Israeli acute paralysis virus* (IAPV) between *B. impatiens* and *A. mellifera* was investigated under controlled conditions but the results were not conclusive [7]. Similarly, the prevalence of DWV in *A. mellifera* in natural conditions was a good predictor of its prevalence in *Bombus* spp., but the direction of transmission also remained unclear [15, 16].

The presence of *A. mellifera* has been associated with prevalence of RNA virus particles in arthropods and pollinator communities. RNA virus particles were detected in arthropods including different species of spiders, cockroaches, beetles, earwig, bugs, butterflies, flies, wasps, ants and bees collected inside, under or around *A. mellifera* hives, and on flowering plants near apiaries [6-8, 17]. Several other studies have reported presence of RNA virus particles in *Bombus* bees collected from the fields, greenhouses, and breeding programs [18-20].

Decline of *A. mellifera* populations has been reported in many areas of the world and have been linked to biological, anthropogenic and environmental factors, and putative Colony Colapse Disorder (CCD) [13, 21, 22]. Among these factors, diseases caused by RNA viruses feature as very important contributors to these declines particularly in association with the parasitic Varroa mite (*Varroa destructor*). The Varroa mite increases the incidence and severity of RNA viruses since it supports viral replication within its body and delivers virions directly into haemolymph of *A. mellifera* [13, 23, 24]. The Varroa mite is widely distributed around the world with exception of Australia and Newfoundland (Canada) [23, 25].

In addition to the effects of the Varroa mite on the prevalence and virulence of RNA viruses, the practise of bee keeping itself has been associated with the spread of diseases among both honey bees and native bees, and this may have three non-exclusive causes. Firstly, managed hives often have reduced genetic variation and hence they can be susceptible to certain diseases [26, 27]. Secondly, when traveling with hives, beekeepers may inadvertently assist in the spread of diseases between different regions [26, 28, 29] as virus-infected hives in an apiary can influence the viral load of native bees in the proximity [17, 28, 30]. Thirdly, while a viral infection may weaken feral hives and make them more susceptible to other diseases, thus creating a sink for viruses and other diseases, beekeepers often control colony strength and disease development in managed hives and has the potential to cause RNA viruses to remain present in the hives at low levels. In light of the increasing evidence of inter-species transmission of bee viruses, surprisingly little attention has been paid to the potential role of pathogens in the reductions in density and diversity of solitary bees reported in some areas of the world [31-34]. Generally, the latter declines have been attributed to loss of foraging habitat and use of pesticides, factors which undoubtedly have an impact, but which might exacerbate the effects of introduced pathogens [13, 35, 36].

Worldwide, by far the majority of studies of RNA viruses have focused on *A. mellifera* because of its obvious benefits to humans, and very few on solitary bees [13]. In Australia, research into RNA viruses has been performed only on bees of the genus *Apis*, with a focus on the introduced *A. cerana* and managed hives of *A. mellifera* [e.g. 37-40]. Detailed assessment of the prevalence and infectivity are not available for viruses present in wild *A. mellifera* and native bees. Comparing the distribution of viruses among managed *A. mellifera* and unmanaged bees (both wild *A. mellifera* and native bees) in Australia serves two main purposes. Firstly, differences in the prevalence of viruses in native bees in the absence and presence of beekeeping and/or of co-foraging wild *A. mellifera* could provide indirect clues for the directionality and infectivity of the viruses. Secondly, it will provide baseline data for an assessment of the impact

of the interaction between RNA viruses and the Varroa mite on native bees should the mite become established in Australia.

This study investigates, for the first time, the presence and transfer of RNA viruses in Australian native bees. We collected native bees in different regions in South Australia (SA), which either had no *A. mellifera* due to high levels of aridity, wild *A. mellifera* only due to lack of suitability for beekeeping, or both wild *A. mellifera* and beekeeping activity. Whenever co-foraging *A. mellifera* were present, these were collected as well. We tested all bees for the presence of RNA viruses in order to determine (i) whether the native bees carry RNA virus particles, (ii) which RNA viruses are shared between *A. mellifera* and native bees, (iii) other hosts of RNA viruses, (iv) factors associated with presence of RNA viruses in both *A. mellifera* and native bees, and (v) whether the data would allow us to gain insights on origin and transmission of RNA viruses between *A. mellifera* and native bees.

Materials and methods

Sample collection

We collected more than 1,800 bees including native bees and *A. mellifera* between February 2012 and March 2015 from six geographical regions of SA namely, Adelaide, Kangaroo Island (KI), Eyre Peninsula, Flinders Ranges, Witchelina Station, and South East (SE). These regions are separated by at least 400 km, or by approximately 15 km of sea (KI) to minimise the probability of natural, unassisted transmission of RNA viruses between bees from sample regions. At each region we collected bees from at least one area, and within one area we collected bees from different sites and plants. Since this is the first study on RNA viruses in native bees and we also intended to identify other hosts of these viruses, we performed a convenience sampling (also known as availability sampling). That is, we collected native bees present or available at the time of the sampling [41]. In order to identify the possible origin and direction of transmission of virus particles, we also collected *A. mellifera*, when the latter occurred. We collected as many bees possible within 60 minutes, per site. We used a direct

searching and collected bees foraging on flowers or in flight near flowers using sweep nets. Blue vein traps were only used at Flinders Ranges. The collections were performed on flowering plants in vegetation along roads or tracks.

The South Australian climate varies between the regions. Our northern regions (e.g. Flinders Ranges and Witchelina) are mainly deserts and experience very hot and dry summers, and cool and dry winters (around 100 mm of annual average rainfall). However, the southeast coast and Mount Lofty Ranges experience very hot and dry summers, but cool mild wet winters (up to 1000 mm of annual average rainfall) [42]. Because of this variability, we were able to collect bees from areas with different annual average rainfall. Since accessibility of water is an important factor for occurrence of *A. mellifera* [43], this species was not found at the arid areas of Witchelina and Port Augusta.

Managed and feral *A. mellifera* colonies are patchily distributed around SA with exception of arid areas [43]. Managed colonies are usually shifted depending on the local availability of water and key floral resources, weather conditions, and diseases. Feral colonies are less abundant and their occurrence is dependent on suitable food resources, water, and suitable hollows in the area, which can vary over time. Hence, presence of both managed and feral hives can vary over time and between seasons, no exact information is available the distribution of managed colonies, or on abundance and distribution of feral colonies [43]. Therefore, we distinguish between beekeeping and non-beekeeping areas based on the latest records of beekeepers' movement around SA. The movement of beekeepers around SA, which is mainly associated with availability of flowering *Eucalyptus* spp. and flowering crops. Both resources are available mainly around Adelaide, KI and SE regions, so these are generally utilised as beekeeping areas while Northern regions do not have significant commercial beekeeping activity. *Apis mellifera* caught in Northern regions were therefore classified as wild (non-managed).

Individuals were immersed in RNALater and stored in -20°C until transported to The Waite Research Institute, in Adelaide, Australia. Before long-term storage, bees were grouped into families, genus or species and identified based on Michener (2006) key classification of bees

(Table 3) [44]. Only bees identified to species or genus were analysed in this study. Bees were stored at -80°C until molecular analysis. Vouchers of species of bees collected in this study will be donated to the tissue collection of the SA Museum.

Detection of RNA viruses using reverse transcription PCR

We tested for the presence of eight RNA viruses namely *Acute bee paralysis virus* (ABPV), *Black queen cell virus* (BQCV), Chronic bee paralysis virus (CBPV), DWV, IAPV, *Kashmir bee virus* (KBV), *Sacbrood virus* (SBV), and Slow bee paralysis virus (SBPV). Prior to extraction, bees were washed in 1% sodium dodecyl sulphate to guarantee elimination of pollen from the scopa of the bee and other possible contaminants. Total RNA was extracted from a pool of abdomens of bees from the same species and site using GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) following the protocol of the manufacturer. Any DNA contamination was removed from the RNA preparations using the On-Column DNase I Digestion (Sigma-Aldrich). The concentration and purity of the total RNA were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). RNA supernatants were stored at -80^{o}C until used.

Reverse transcription (RT) reactions were performed to synthesise the complementary DNA (cDNA) using random hexamer primers (Bioline) and Bioscript Reverse Transcriptase kit (Bioline) according to the manufacturer's guidelines. Two µl of RT reaction contents were added to a final 25 µl polymerase chain reaction (PCR) volume: 2.5 µl 10x NH_4 buffer, 0.7 µl of 10 mM dNTPs, 0.75 µl of 50 mM $MgCl_2$, 1 µl forward and reverse primer (20 µM), and 0.15 µl BIOTAQ DNA Polymerase (Bioline). PCR reactions were performed using the following conditions: initial denaturation for 5 min at 94^{o}C, 35 cycles of denaturation for 30 s at 94^{o}C, 30 s annealing at 55^{o}C, 90 s extension at 72^{o}C, and final extension for 5 min at 72^{o}C. PCR products were visualised under UV light on 1% agarose gels stained with GelGreen Nucleic Acid Gel Stain (Biotium). Negative (no template) and internal controls were included in each group of RT-PCR reactions. The internal control used in this study amplifies the D2 region of the gene

encoding the 28S ribosomal subunit [45]. The internal control was used as indicator of the quality of RNA extracted and the success of the RT-PCR reactions. Positive detections were confirmed by sequencing the PCR products. All primers used in this study are listed in Table 1. These primers were chosen because they were successfully used in previous studies to detect RNA viruses in *A. mellifera* [7, 46-48]. In addition, the IAPV, DWV, KBV, BQCV, and SBV primers used here were previously used to detect virus particles in different species of pollinators and other arthropods [6, 7].

Table 1: Primers pairs used in this study and their literature sources.

Virus	Amplicon size (bp)	Gene	Primer sequence (5'-3')	Source
IAPV	840	Capsid	GGTCCAAACCTCGAAATCAA TTGGTCCGGATGTTAATGGT	Singh, Levitt *et al*. [7]
DWV	424	Capsid	GGCGTGGTTCATTAGAATATAGG AAGCAGATCCCCACCTAAAAA	Singh, Levitt *et al*. [7]
KBV	625	Capsid	TGTTTGTGGCAATCCAGCTA TACGTCTTCTGCCCATTTCC	Singh, Levitt *et al*. [7]
BQCV	700	Capsid/ 3'UTR	TGGTCAGCTCCCACTACCTTAAAC GCAACAAGAAGAAACGTAAACCAC	Singh, Levitt *et al*. [7]
SBV	693	Capsid	GCACGTTTAATTGGGGATCA CAGGTTGTCCCTTACCTCCA	Singh, Levitt *et al*. [7]
ABPV	900	Capsid	TTATGTGTCCAGAGACTGTATCCA GCTCCTATTGCTCGGTTTTTCGGT	Benjeddou, Leat *et al*. [46]
CBPV	570	RdRP	TCAGACACCGAATCTGATTATTG ACTACTAGAAACTCGTCGCTTCG	Blanchard, Olivier *et al*. [47]
SBPV	868	RdRP	GATTTGCGGAATCGTAATATTGTTTG ACCAGTTAGTACACTCCTGGTAACTTCG	de Miranda, Dainat *et al*. [48]
Internal control	560	Ribosomal DNA	CGTGTTGCTTGATAGTGCAGC TTGGTCCGTGTTTCAAGACGG	Campbell, Heraty *et al*. [45]

IAPV, *Israeli acute paralysis virus*; DWV, *Deformed wing virus*; KBV, *Kashmir bee virus*; BQCV, *Black queen cell virus*; SBV, *Sacbrood virus*; ABPV, *Acute bee paralysis virus*; CBPV, Chronic bee paralysis virus; CWV, Cloudy wing virus; SPV, Slow paralysis virus.

UTR, untranslated region; RdRP, RNA-dependent RNA polymerase; bp, base pairs

Statistical analysis

We analysed each area of collection as separate populations, and the probability of detecting viruses on a bee was estimated for *A. mellifera* and native bees. Inference about differences between populations was determined using Fisher's exact test. The population from Adelaide (Waite campus) was used as reference since it represented the highest proportions of occurrence of RNA viruses in *A. mellifera* and native bees. Also, the distribution of *A. mellifera* to native bees was almost equal in this area. Separately, the distribution of viruses on native bees was compared to the highest estimated probability for native bees in non-beekeeping areas, which was seen at Port Augusta in 2015.

Results

The proportion of *A. mellifera* carrying RNA viruses was significantly higher in beekeeping areas of Adelaide, KI and Nangwarry, while no RNA viruses were detected in wild *A. mellifera* from the drier regions: Flinders Ranges, Gawler Ranges and Lake Gilles (Table 2, Fig 1). In addition, there were no significant differences in the probability of occurrence of RNA viruses in *A. mellifera* populations between Adelaide and KI (Table 2).

The probability that a random native bee carried an RNA virus particle was higher in Adelaide and KI, and was significantly lower in the more Northern arid areas (Table 2). While some RNA virus particles were detected in native bees from Flinders Ranges and Port Augusta (2015), their frequency did not differ significantly from the areas where no viruses were found. RNA virus particles were not detected in native bees from Witchelina, Lake Gilles, Gawler Ranges and Nangwarry (Fig 1, Table 2). No detection were found in native bees from Nangwarry although this is a beekeeping area and *A. mellifera* tested positive to viruses (Table 2, Fig 1). Native bees collected from Port Augusta in 2015 tested positive to RNA virus particles while bees collected in 2014 did not (Table 2, Fig 1).

Table 2: Analysis of proportion of RNA viruses in both wild *Apis mellifera* and native bees, and comparison between South Australian sites. Each area of collection was analysed as individual population. The proportion of detection was determined based on probability of at least one native bee or *A. mellifera* carrying a RNA virus particle. Confidence intervals for proportion of detection are shown in brackets. Fisher's exact test was used to compare presence of RNA viruses between populations of native bees and *A. mellifera* (odds). The collection from Adelaide and Port Augusta (2015) were used as reference to infer the odds in the population interest.

Year		**2012**	**2013**	**2014**				**2015**		
Region		**Adelaide**	**Kangaroo Island**	**South East**	**Eyre Peninsula**	**Flinders Ranges**	**Witchelina**	**Eyre Peninsula**		
Area		**Adelaide**	**Kangaroo Island**	**Nangwarry**	**Port Augusta**	**Flinders Ranges**	**Witchelina**	**Port Augusta**	**Lake Gilles**	**Gawler Ranges**
Significant beekeeping activities		Yes	Yes	Yes	No	No	No	No	No	No
Apis mellifera	**Collected**	61	9	26	0	69	0	0	4	13
	Positive detection	44	8	12	0	0	0	0	0	0
Native bees	**Collected**	107	101	93	21	140	186	450	121	264
	Positive detection	32	14	0	0	2	0	9	0	0
Proportion of *A. mellifera* to native bees		0.76	0.15	0.41	0.00	0.49	0.00	0.00	0.03	0.05
Proportion of detection in *A. mellifera*		0.42 (0.32-0.52)	0.47 (0.23-0.72)	0.32 (0.18 - 0.49)	NA	0	NA	NA	0	0
Proportion of detection in native bees		0.23 (0.16-0.31)	0.12 (0.07-0.20)	0	0	0.01 (0.001 - 0.05)	0	0.02 (0.01 - 0.03)	0	0
Odds_*A. mellifera*		Reference	0.69	0.56						
Odds_Native bees			0.03	0.00*	0.01*	0.00*	0.00*	0.00*	0.00*	0.00*
Odds_Native bees		0.00*	0.00*	0.37	0.99	0.99	0.07	Reference	0.22	0.03

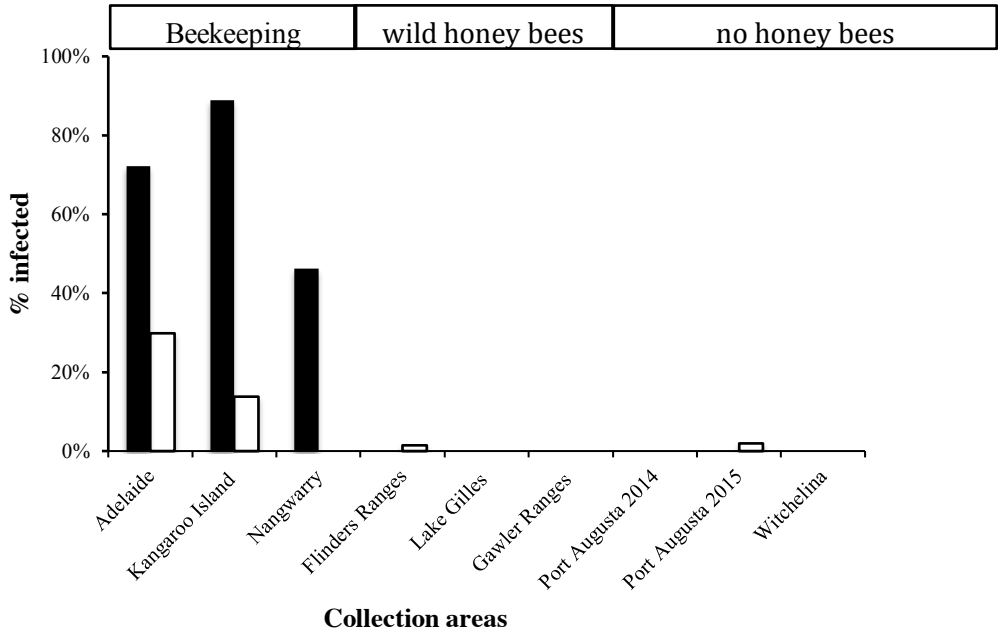

Fig 1. The percentage of wild *Apis mellifera* (black) and native bees (white) carrying RNA viruses in different regions, in relation to the presence of beekeeping, an absence of beekeeping, or total absence of *A. mellifera*.

RNA virus particles were detected in various species of South Australian native bees (Table 3). Out of 80 native bee species tested for the presence of RNA virus particles, only 16 species tested positive. There was no correlation between the numbers of specimens tested with proportion of positive detection. Bees from the families Apidae, Colletidae and Halictidae carried RNA virus particles while megachilid bees did not. Between all families of bees, members of the Apidae had the highest percentage of individuals positive to RNA viruses. Within this family, bees from the genus *Apis* that is represented by *A. mellifera* had the highest occurrence of RNA viruses (Table 3). There was no difference between the probability of halictid and colletid bees of carrying virus particles (Table 3). Virus particles were detected in bees with a wide range of nesting behaviours namely, solitary, communal, parasitic, primitive eusocial and highly eusocial.

BQCV and SBV were the most common viruses while IAPV was only detected in Adelaide. These viruses were detected in both wild *A. mellifera* and native bees indicating that these bees share BQCV and SBV. IAPV was detected only in native bees from Adelaide but not in *A. mellifera*. The presence of more than one virus particles was detected in individuals of both *A. mellifera* and native bees collected in Adelaide. Disease symptoms were not observed in either wild *A. mellifera* or native bees. Male native bees were also collected and tested positive to RNA viruses.

Table 3: Summary of abundance and richness of bee species collected and tested in this study, and the percentage of individuals carrying RNA virus particles.

Family	Tribe	Genus	Nr species	Nr specimens collected per species	Nr of species testing positive	Nr specimens tested	% Individuals testing positive* [Min	Max]	Average per family [Min	Max]
Apidae	Allodapini	*Exoneura*	2	15	1	52	2%	19%	8%	21%
		Exoneurella	3	104	0	86	0%	0%		
	Anthophorini	*Amegilla*	3	89	1	75	5%	13%		
	Apini	*Apis*	1	350	1	198	14%	53%		
	Melectini	*Thyreus*	1	5	1	5	20%	20%		
Colletidae	Colletini	*Leioproctus*	5	53	3	46	7%	15%	2%	3%
	Euryglossini	*Euhesma*	2	11	0	8	0%	0%		
		Euryglossa	5	37	2	37	5%	11%		
		Euryglossina	1	12	0	12	0%	0%		
		Pachyprosopis	3	350	0	125	0%	0%		
		Xanthesma	2	35	0	21	0%	0%		
	Hylaeini	*Hylaeus*	8	180	1	175	1%	2%		
		Hyleoides	1	2	0	2	0%	0%		
Halictidae	Halictini	*Homalictus*	4	81	0	67	0%	0%	3%	5%
		Lasioglossum	26	295	5	272	2%	4%		
	Nomiini	*Lipotriches*	3	145	2	105	6%	10%		
Megachilidae	Megachilini	*Megachile*	11	91	0	89	0%	0%	0%	0%
		Total	81	1 855	17	1 375				

Nr, total number; Min, minimum; Max, maximum

*Minimum and maximum percentage of individuals testing positive to RNA viruses were calculated assuming infection of one and all specimens in pool of bees tested

Discussion

We detected, for the first time in Australia, RNA virus particles in different species of native bees showing that, similarly to the European and American non-*Apis* bees, Australian native bees can carry RNA virus particles [6-8]. This underscores that the host range of "honey bee" viruses is wide and may include many species of bees. The relative large proportion of native bees carrying RNA viruses from Adelaide and KI, but not in the more arid regions where beekeeping does not occur, suggests that beekeeping may be associated with spreading of virus particles between co-foraging species. Because *A. mellifera* had a higher proportion of individuals testing positive to RNA viruses than any other species, it seems likely that *A. mellifera* is the original host of RNA viruses in Australia. However, the potential for beekeeping practises, such as moving hives and placing them in close proximity to each other, which increases viral transfer and infection, makes this unclear. There was no detection of viruses in native bees from Witchelina, where *A. mellifera* was not found, also supporting an assumption of *A. mellifera* as the original host of RNA viruses in Australia. In the USA and Belgium, non-*Apis* bees reported as positive to RNA viruses were collected near *A. mellifera* apiaries suggesting viral spillover from managed *A. mellifera* to wild non-*Apis* bees [7, 8]. Transmission of RNA viruses between managed and wild *A. mellifera* has been reported in different studies [e.g. 8, 10]. Foraging has been linked with spillover of RNA viruses intra- and inter-species of bees [7, 26, 49, 50].

RNA viruses were not detected in wild *A. mellifera* from Flinders, Lake Gilles and Gawler Ranges. Similarly, native bees from Nangwarry (where beekeeping occurs), Witchelina, Lake Gilles and Gawler Ranges also tested negative to virus particles. Although these data support a correlation between beekeeping and viruses in wild bees, another contributing factor could be associated with the potential occurrence of different RNA virus strains or variants that are not detectable with the primers used in this study. Generation of RNA virus variants or strains is linked to their high mutation rates caused by their large population size, short generation time

and high replication rates [51]. The role of recombination in generation of RNA viruses variability is still unidentified [51]. On the other hand, it is also possible that native bees harbour other viruses that do not occur in *A. mellifera* or viruses never reported before. It would be interesting to further test South Australian native bees for presence of RNA viruses but using deep sequencing that would allow detection of novel viruses and variants of known viruses.

So far, no more than ten species of non-*Apis* have been previously tested for presence of RNA viruses [e.g. 6-8]. As our study included 80 species and found virus presence in 16 of these, our study makes an important contribution to the knowledge of non-*Apis* bees as hosts of RNA viruses. While the drawback of testing large number of species of native bees is the smaller sample size per species, our study suggests that the sample size does not impact the probability of detection of viruses. For instance, virus particles were detected in pools of 10 as well as in single bees.

Our finding that the abundance and distribution of *A. mellifera* seems to be linked to the presence of RNA virus particles in native bees concurs with earlier findings. Graystock and colleagues demonstrated that 60-80 workers *B. terrestris*, and three frames consisting of adult and brood *A. mellifera* could disperse parasites onto flowers followed by successful acquisition by non-hosts within three hours under controlled conditions. Although this work did not concentrate on RNA viruses, it provides insights on factors associated with dispersal of parasites within bees and the speed with which this can occur [50]. Hence, a high density of bees may be able to spread more parasites in shorter period of time. It is possible that the relatively low virus detection in *A. mellifera* from Nangwarry combined with a relatively low density of *A. mellifera* could explain the lack of viruses observed in native bees in the area.

The prevalence of RNA viruses in bees did not seem to be influenced by the nesting behaviour and flower preferences of species. The bees that carried RNA virus particles seemed to have a wide range of nesting behaviour. For instance, bees of the genus *Exoneura* (primitive eusocial), *Amegilla* (communal), *Thyreus* (parasitic), and *Euryglossa* (solitary) tested positive to RNA viruses. In addition, both widely polylectic bees (*Lasioglossum* spp.) and species that are

only ever observed foraging on native plants (e.g. Euryglossine bees) carried RNA viruses. Further research in correlation between species/family of bees, flower preference and nesting behaviour with prevalence of RNA viruses may provide more insights in transmission of these viruses.

The presence of wild *A. mellifera* in non-beekeeping areas, namely Lake Gilles, Gawler and Flinders Ranges can be a result of beekeeping activities in these areas or in nearby regions in the past. For instance, current beekeepers know of previous beekeeping activities that have occurred at Lake Gilles, where twenty years ago wild *A. mellifera* were locally common (K. Hogendoorn pers. obs.). Additionally, wild *A. mellifera* have been present across much of Australia for well over 100 years [52, 53]. However, lack of water, due to hot summers and droughts are likely to have locally reduced some wild *A. mellifera* populations. This suggests that, without an influx of managed hives, RNA virus particles may only persist at very low levels where there are lower densities of *A. mellifera* that are affected by aridity. This might be the reason behind the low incidence of RNA viruses in native bees from Flinders Ranges. This information is important for Australia since most of it is arid country and it may become drier as consequence of climate change [54, 55].

Similarly to Flinders Ranges, low levels of RNA viruses were also detected in native bees from Port Augusta. In Port Augusta, we collected only native bees at the Australian Arid Lands Botanic Gardens, and although it is quite dry (median annual rainfall 250 - 500mm) [42] surrounding areas have ecological conditions allowing survival of *A. mellifera*. Therefore, exchange of virus particles may have occurred between *A. mellifera* and native bees in that area.

We detected BQCV and SBV in both *A. mellifera* and native bees. The same viruses were detected in a 2015 survey of Australian *A. mellifera* apiaries [38]. Detection of more than one RNA virus occurred in our samples, similar to previous reports of multiple-infection in *A. mellifera* [e.g. 56, 57] and *Bombus* spp. [e.g. 7, 17]. Symptoms of BQCV and SBV are visible in the larval stage of *A. mellifera* [26, 58], so were not expected in our samples of adults. We found male native bees were also carrying RNA viruses suggesting venereal transmission during

mating, vertical transmission from the mother, or spillover via contaminated nectar since they generally only feed on nectar [44]. Venereal transmission [59-61] and vertical transmission [61-63] are well described and demonstrated in *A. mellifera*, but no information is available in native bees. No information is available on presence of RNA viruses in nectar while pollen has been demonstrated as viral reservoir [7, 10].

In summary, our results strongly suggest that the original host of the RNA viruses present in Australian bees is *A. mellifera*, and that prevalence of these viruses in *A. mellifera* and in native bees is associated with beekeeping. The detection of the same RNA viruses in co-foraging *A. mellifera* and native bees in some areas raises further questions about epidemiology of RNA viruses and their potential to impact native pollinators. This has important consequences for the management of the risks associated with RNA viruses in bees, particularly in light of the potential for the Varroa mite to establish in Australia, the reliance of many native plants on pollination by native bee species, and the contribution made to crop pollination by wild and managed *Apis*, and native bees.

Our results may be distinct to those from Varroa-infested countries since Australia still has high local densities of wild *A. mellifera* colonies in many areas [64] and there have not been reports of managed colony decline as in the USA and elsewhere. Although spillover of RNA viruses is shown in this study, it is as yet unclear whether these viruses have negative impacts on native bees health. Hence, further research is necessary to determine infectivity of RNA viruses in Australian native bees and to better understand the pathways of inter- and intra-species transmission involving native bee species. Recently, it was shown that anthropogenic movement of honey bee hives had contributed to the global spread of DWV [25]. Similarly, our data suggest that anthropogenic factors, i.e. movement and maintenance of hives may be contributing to spread of other RNA viruses. This is because movement of hives increases the probability of having high densities of bees, of foraging with bees from different apiaries and with different populations of wild bees, while beekeeping could help to maintain the viral presence in the managed hives. The consequences of beekeeping and commercial travel with hives needs to be

investigated as it can provide new management strategies for Australian beekeepers in order to reduce the impact of a Varroa-mite incursion and the background levels of transmission of RNA viruses and other bee diseases, between bee species.

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Chapter 3

Co-occurrence of RNA viruses in Tasmanian introduced bumble bees (*Bombus terrestris*) and honey bees (*Apis mellifera*)

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Chapter 3

Co-occurrence of RNA viruses in Tasmanian introduced bumble bees (*Bombus terrestris*) and honey bees (*Apis mellifera*)

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Short title: RNA viruses in Tasmanian bumble bees (*Bombus terrestris*)

Abstract

A number of bee RNA viruses, including *Deformed wing virus* (DWV), are so far absent from Australia. These viruses can be introduced together with imported live honey bees (*Apis mellifera*), their products such as pollen, semen and royal jelly, with other bee species, and with bee parasites. Given that bee viruses have a profound impact on bee health, it is surprising that, since the introduction of bumble bees (*Bombus terrestris*) onto Tasmania in 1992 from New Zealand, no work has been done to investigate which RNA viruses are associated with these bees. Consequently, we investigate the current presence and the prevalence of RNA viruses in *B. terrestris* and *A. mellifera* collected in south-eastern Tasmania and compare them to available data. Both species shared some RNA viruses, namely *Kashmir bee virus* (KBV) and S*acbrood*

virus (SBV), but *Black queen cell virus* (BQCV) was detected only in *A. mellifera*. Here we report the presence of KBV for the first time in Tasmania. However, we believe it may have been previously detected but misclassified. Furthermore, while DWV is present in New Zealand we did not find this virus in either *B. terrestris* or *A. mellifera*. This reinforces the importance of a strong regulation of the anthropogenic movement of live bees and their products.

***Apis mellifera* / *Bombus terrestris* / *RNA viruses* / Tasmania / Australia**

1. Introduction

Over the last decade, RNA viruses of bees have received increasing research attention due to their putative association with Colony Collapse Disorder (CCD), and with reduced health and population size of European honey bee (*Apis mellifera* L.; Genersch 2010; Neumann and Carreck 2010). RNA viruses were initially called "honey bee viruses" and described as being specific to *A. mellifera* (Ball and Bailey 1991). However, recently studies have detected RNA viruses in many species of pollinators including bees of the genus *Bombus* or bumble bees (e.g. Singh, Levitt et al. 2010; Levitt, Singh et al. 2013; Graystock, Goulson et al. 2014; McMahon, Fürst et al. 2015; Parmentier, Smagghe et al. 2016). Experiments have shown that bumble bees can acquire virus particles from wild or managed *A. mellifera* (Li, Peng et al. 2011; Peng, Li et al. 2011), and from managed *Bombus* spp. (Colla, Otterstatter et al. 2006; Graystock, Goulson et al. 2014).

Intra- and inter-species horizontal transmission can occur indirectly via co-foraging on the same flowers (Manley, Boots et al. 2015). Foraging is strongly linked to inter-species transmission of RNA viruses (Singh, Levitt et al. 2010; Mazzei, Carrozza et al. 2014). For instance, under controlled conditions, *Israel acute paralysis virus* (IAPV) was transmitted between *A. mellifera* and *B. impatiens* Cresson through foraging on the same flowers (Singh, Levitt et al. 2010). The potential of pollen to harbour RNA virus particles was demonstrated

when injection of a suspension of pollen, visited only by *A. mellifera* infected with DWV, directly into haemolymph of *A. mellifera* and *Osmia cornuta* Latreille, caused the associated virus infection in the treated bees (Mazzei, Carrozza et al. 2014).

Horizontal transmission can also occur via other invertebrate vectors. For instance, Varroa mite (*Varroa destructor* Anderson & Trueman) facilitates intra-species horizontal transmission between bees of the genus *Apis* as they are specific to this genus. However, they are not directly responsible for inter-species transmission of viruses between *Apis* and non-*Apis* bees, and among non-*Apis* bees (Manley, Boots et al. 2015). Nonetheless, the presence of Varroa mite increases the likelihood that bumble bees and other native pollinators encounter pathogens on flowers, because the mite increases the incidence and severity of RNA viruses in hives (Brown and Fries 2007; vanEngelsdorp and Meixner 2010). Infestation of Varroa mite in *A. mellifera* led to an increase in prevalence and titres of different RNA viruses including DWV (Mondet, de Miranda et al. 2014). Indeed, the interaction between the Varroa mite and DWV has been hypothesised as the greatest threat to the health of *A. mellifera* and the main contributor of *A. mellifera* colony collapse (Martin, Highfield et al. 2012; Mondet, de Miranda et al. 2014). In addition, both DWV and the Varroa mite are widely distributed around the world with the exception of Australia (Rosenkranz, Aumeier et al. 2010; Roberts, Anderson et al. 2015) although there is conflicting evidence about status of DWV in Australia (Wilfert, Long et al. 2016). Spreading of Varroa mite together with the RNA viruses it vectors (DWV in particular) is proposed as the most serious consequence of commercial transportation of *A. mellifera* worldwide (Manley, Boots et al. 2015; Wilfert, Long et al. 2016).

The spread of these and other diseases and their vectors is mainly caused by global transportation of *A. mellifera* and *Bombus* spp., for commercial use as pollinators, is one of the main causes of spreading disease agents between geographical areas and species (Manley, Boots et al. 2015). Goulson and Hughes (2015) emphasise that commercial trade of *Bombus* spp. is redistributing pathogens and parasites of bees into new areas around the world. This risk is

increased by the fact that commercially reared *Bombus* spp. can carry RNA viruses acquired from *A. mellifera* pollen used for their rearing (Goulson and Hughes 2015; Graystock, Jones et al. 2016).

So far, it seems that DWV is still absent from Australia while a recent report (Wilfert, Long et al. 2016) indicated DWV sequences generated from a few *A. mellifera* imported from Australia (Singh, Levitt et al. 2010). However, Roberts and colleagues (2015) did not detect DWV despite the fact that they surveyed managed hives much more extensively and around the whole Australia and used more sensitive techniques. Hence, it is hard to reconcile that DWV was detected in several *A. mellifera* imported from Australia and not in large number of managed *A. mellifera* tested recently. Intriguingly, Roberts and colleagues (2015) reported a small number of short nucleic acid sequences sharing 77-92% similarity with DWV, these were considered as different viruses but closely related to DWV.

New Zealand (NZ) was one of the last countries reporting the incursion of the Varroa mite. This parasite was first observed in managed hives of *A. mellifera* in 2000. However, when and from where the mite was introduced is unknown (Zhang 2000) and it is speculated that the mite was present for several years prior to its detection. It is unclear whether DWV was introduced to NZ together with Varroa mite as it was not detected in *A. mellifera* collected from infested colonies between 2001 and 2003 (Todd, De Miranda et al. 2007). Detection of DWV in a colony from an area where Varroa mite was not yet present (Mondet, de Miranda et al. 2014) suggested that DWV could have been circulating in both NZ *A. mellifera* and/or *B. terrestris* since their multiple introductions between 1880's and early 1900's (Macfarlane and Gurr 1995; Mondet, de Miranda et al. 2014; Goulson and Hughes 2015).

The large earth bumble bee *B. terrestris* L. was first discovered in the Australian island state of Tasmania in 1992 (Semmens, Turner et al. 1993), and nine years later it had become established in most regions of the island including remote areas (Hingston, Marsden-Smedley et al. 2002). The bees were accidentally or intentionally introduced from NZ without the approval

of the Australian government (Semmens, Turner et al. 1993). The recent detection of DWV in *A. mellifera* from NZ (Mondet, de Miranda et al. 2014) raised fears that new RNA viruses, mainly DWV could have been introduced into Tasmania with *B. terrestris* in 1992.

This study is the first to provide insights into RNA viruses associated with *B. terrestris* from Tasmania. We collected *B. terrestris* and *A. mellifera* across southeast Tasmania and analysed them using reverse transcription polymerase chain reaction (RT-PCR) for presence of RNA viruses in order to (i) determine which RNA viruses are present in Tasmanian *B. terrestris*, (ii) determine which viruses are shared between Tasmanian *B. terrestris* and *A. mellifera*, and (iii) infer possible geographic origin of RNA viruses found in *B. terrestris*.

2. Materials and methods

2.1. Sample collection

A total of 200 bees (100 *B. terrestris* and 100 *A. mellifera*) were collected between January and February of 2015 across southeast Tasmania (Figure 1). Of the 100 *B. terrestris*, 29 were queens and 71 were workers. For *A. mellifera*, only workers were collected. The bees were collected at 15 sites on the main island of Tasmania, and at five sites on Bruny Island. At each site, five specimens per species were captured allowing a theoretical detection threshold of 20%. The sites were separated by at least two km and specimens were collected with a net while foraging on flowers of the same plant or on adjacent plants of the same species. Workers of *B. terrestris* normally do not forage further than one km away from their nests (Osborne, Clark et al. 1999), and their maximum flight distance is 2.5 km (Hagen, Wikelski et al. 2011). Therefore, we expected that *B. terrestris* collected at different sites belong to different colonies. On the other hand, the flight distance of *A. mellifera* depends on quality and availability of foraging plants in surrounding areas. They can forage close to the hives but they can also fly more than 10 km away from the hives in some circumstances (Beekman and Ratnieks 2000). We consider *B. terrestris* flight distance instead of *A. mellifera* because it was our main object of study. Specimens were transferred to plastic bags and stored on ice while in the field. After, they were

placed at -20°C for approximately two hours, before individuals were immersed in aliquots of RNALater and stored at -80°C until shipment to Waite Research Institute, Adelaide, South Australia, for processing.

2.2. Viral RNA extraction

Prior to extraction, bees were washed with 1% sodium dodecyl sulphate to remove pollen from their bodies and other possible contaminants. Total RNA was extracted using GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) following the manufacturer's protocol. Any DNA contamination was removed from the RNA preparations using On-Column DNase I Digestion (Sigma-Aldrich). The purity of the total RNA was measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific), and the concentration was measured using Qubit® 3.0 Fluorometer (Thermo Fisher Scientific). RNA samples were stored at -80°C until used.

2.3. Virus detection

We aimed to determine the presence of nine RNA viruses, namely *Acute bee paralysis virus* (ABPV), BQCV, *Chronic bee paralysis virus* (CBPV), Cloudy wing virus (CWV), DWV, IAPV, *Kashmir bee virus* (KBV), SBV and Slow bee paralysis virus (SBPV). To achieve this, RNA was extracted from all bee specimens and reverse transcribed using random hexamer (Bioline) and Bioscript Reverse Transcriptase kit (Bioline) according to the manufacturer's guidelines. Two µl of RT reaction contents were added to a final 25 µl PCR solution, with amplification occurring under the following conditions: initial denaturation for 5 min at 94°C, 35 cycles of denaturation for 30 s at 94°C, 30 s annealing at 55°C, 90 s extension at 72°C, and a final extension for five min at 72°C. PCR products were visualised under UV light on 1% agarose gels stained with GelGreen Nucleic Acid Gel Stain (Biotium). No-template and internal controls were included in each group of RT-PCR reactions. The internal control used in this study amplifies the D2 region of 28S ribosomal DNA (Campbell, Heraty et al. 2000). This was used as housekeeping gene to control for the quality of the RNA extracted from bees and the correct application each batch of

RT-PCRs. All primers used in this study are listed in Table 1. Positive detections were confirmed by sequencing of PCR products.

2.4. Sequence and phylogenetic analyses

In order to increase the yield of the DNA templates sent for sequencing, we performed several identical PCRs using the conditions above described. The PCR products from a single bee were subsequently pooled and purified using the UltraClean PCR Clean-Up Kit (MOBIO Laboratories, Inc.) and sent for bidirectional Sanger sequencing (AGRF, Adelaide) using the relevant virus primers (Table 1). Forward and reverse sequences for each of the virus PCR products were aligned using BioEdit (Hall 1999) and ambiguities corrected using sequence chromatograms. Sequences were then matched against sequences in National Center for Biotechnology Information (NCBI) GenBank using Blastn. Only sequences matching to the database (more than 90%), and with the best quality were used in our phylogenetic analysis.

Phylogenetic relationships were inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model (Tamura 1992). The percentage of trees in which the associated taxa clustered together is shown next to the branches. The heuristic trees were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. Evolutionary analyses were conducted in MEGA6 (Tamura, Stecher et al. 2013).

If available, corresponding partial viral sequences from both *B. terrestris* and *A. mellifera* from different countries were included in our analyses. BQCV sequences from Australia and New Zealand were not available for *B. terrestris* or *A. mellifera* in NCBI. Similarly, SBV sequences from New Zealand were also not available in NCBI for both bee species. The only Australian SBV sequences available in NCBI encode the RNA-dependent RNA-polymerase (RdRp) gene while the sequences generated in this study encode the capsid protein. The KBV

sequences from New Zealand are from a non-structural gene and from a different region of the polyprotein to that used here. Therefore, our phylogenetic analyses suffer from absence of directly comparable sequences from Australia and New Zealand. IAPV sequences from *A. mellifera* and *B. terrestris* were included in the KBV phylogenetic analyses because of the uncertainty of the origin of this virus (Chen and Siede 2007), and it is believed that some isolates previously reported as KBV might be IAPV (Palacios, Hui et al. 2008).

2.5. Statistical analyses

All statistical analyses were executed in R version 3.2.1 (R Core Team, 2016). The true prevalence of all RNA viruses and 95% confidence intervals were calculated for both species on the basis of an imperfect test using the function epi.prev (R library 'epiR' package version 0.9-62) and Blaker's method with 95% and 99% set for sensitivity and specificity, respectively (Reiczigel, Foldi et al. 2010). True prevalence of RNA viruses was mapped according to the collection sites using the 'Mapplots' package version 1.5. Fisher's exact test was used to infer any relationship between the proportions of infected *A. mellifera* and *B. terrestris*.

Spatial autocorrelation of the true prevalence of each virus for both *A. mellifera* and *B. terrestris* from all locations was computed using Moran's I test (R library 'ape' package version 3.3) to assess disease hotspots and spatial distribution. Moran's I is an indicator of spatial clustering and in this case it was used to assess whether presence of RNA viruses in one location was influenced by the presence of the same viruses in near sites (spatial clustering; Fürst, McMahon et al. 2014).

In order to investigate the impact of prevalence of SBV in *A. mellifera* on SBV in *B. terrestris*, we ran a generalised linear model (GLM) with binomial error and logit link (glm function from R library 'stats' package version 3.2.1). The other viruses detected, BQCV and KBV, were not modelled because of absence of positive detections of BQCV in *B. terrestris*, and low positive detections of KBV ($n<5$) for both bee species. The best model was selected based on a smaller Akaike Information Criterion (AIC) value.

3. Results

The true prevalence of RNA viruses considering all viruses as single response (i.e. assessing individuals carrying at least one RNA virus) associated with workers and queens of *B. terrestris* was 17% (95% CI: 9-28%) and 0% (95% CI: 0-10%), respectively, but this difference was not statistically significant ($P = 0.41$). For this reason, we treated both workers and queens of *B. terrestris* as the same for the following analyses. The overall prevalence of RNA viruses associated with *A. mellifera* was higher than in *B. terrestris* (Table 2). The majority of viruses occurred as a single infection in individual bees, but detection of co-infections occurred for both *A. mellifera* and *B. terrestris*. KBV and SBV were detected in both *B. terrestris* and *A. mellifera* (Table 2) and the prevalence did not differ between them (KBV: $P = 1$; SBV: $P = 0.21$). BQCV was detected only in *A. mellifera* (Table 2) and its higher prevalence was statistically significant ($P = 0.0003$). It was also the most prevalent virus in *A. mellifera* (Table 2). No *B. terrestris* or *A. mellifera* testing positive to RNA virus particles showed obvious symptoms of virus infection. ABPV, CBPV, CWV, DWV, IAPV and SBPV were not detected in our samples (Table 2).

No evidence of spatial clustering was found for BQCV ($P = 0.82$), SBV ($P = 0.17$), and KBV ($P = 0.81$) in *A. mellifera*. Similarly, for *B. terrestris* no spatial clustering was detected for SBV ($P = 0.99$). However, a disease hotspot (spatial clustering) was found for KBV ($P < 0.001$) in *B. terrestris* by mapping the prevalence of individual RNA viruses (Figure 1). A combined map of all viruses as single response was presented to avoid undervaluation of disease prevalence, as these viruses can co-occur in the same area and as co-infections in individuals. This illustrated that presence of RNA viruses in *B. terrestris* and *A. mellifera* were associated (Figure 1).

Our data suggest that presence of SBV in *B. terrestris* was positively associated with occurrence of this virus in *A. mellifera* (Table 3) and *vice versa* (S1). Latitude and longitude did not influence the incidence of SBV in *B. terrestris* (Table 3). KBV and SBV sequences from *B. terrestris* and *A. mellifera* from Tasmania clustered together, distinct from *A. mellifera* sequences from other countries (S3 and S4). The BQCV sequence from *A. mellifera* from

Tasmania clustered with sequence from UK (S2), in agreement with historical reports of Europe as source of *A. mellifera* imported to Australia. The KBV sequences from Tasmania and other countries clustered together, and separately from IAPV sequences (S4).

4. Discussion and conclusions

We found that *B. terrestris* and *A. mellifera* in Tasmania have two RNA viruses in common: KBV and SBV, while BQCV was detected only in *A. mellifera*. It is likely that BQCV is less amenable to inter-species transmission through co-foraging than SBV because laboratory experiments have shown that ingestion of BQCV particles does not cause infection in *A. mellifera*, while ingestion of SBV particles causes infection in larvae and adult bees (Chen and Siede 2007; Ribière, Ball et al. 2007). Overall prevalence of BQCV in *B. terrestris* was lower than in *A. mellifera*. This may be entirely due to BQCV being the most prevalent virus in *A. mellifera* while not being detected in *B. terrestris*. A recent survey of RNA viruses in Australian *A. mellifera* also detected BQCV and SBV in Tasmania (Roberts, Anderson et al. 2015). Our data also confirms previous studies that have shown susceptibility of *B. terrestris* to SBV infection (Singh, Levitt et al. 2010; Levitt, Singh et al. 2013).

Our findings suggest that indirect transmission of KBV and SBV by foraging might have occurred between both *B. terrestris* and *A. mellifera* in Tasmania. Prevalence of SBV in *A. mellifera* was a good predictor of prevalence of the same virus in *B. terrestris*, and *vice versa* (Table 4, S1). Also, both species appeared to carry the same strains of these two viruses in Tasmania (S3 and S4) which where distinct from viral sequences obtained for both bee species in other regions. Likewise, for DWV, MacMahon and colleagues (2015) reported that prevalence in *A. mellifera* is an important indicator of prevalence in *B. terrestris*. There is a high chance of viral transfer between these species through co-foraging because they are both polylectic and they forage on a wide variety of Australian native and introduced plants in Tasmania (Goulson, Stout et al. 2002; Hingston 2005), often using the same plant species as food sources (Hingston

and McQuillan 1998). At the time of collection, these species had been foraging together in Tasmania for 23 years (Semmens, Turner et al. 1993).

Latitude and longitude were not good predictors of prevalence of SBV in either bee species (Table 4). This may be an artefact of the sampling points, which are all located in south-eastern Tasmania and not across the island. SBV was not detected on Bruny Island in the 50 individuals we tested. This is intriguing since the bee population of this island is not isolated from the mainland Tasmania. That is, managed *A. mellifera* are transported between Bruny Island and the mainland Tasmania, and the distance between the island and the mainland is short and would not stop bees from crossing the water. It would be useful to further assess presence of SBV and other viruses around Tasmania including Bruny Island using a more comprehensive sample size that encompasses a large proportion of resident colonies. This would elucidate the contribution of latitude and longitude in prediction of disease prevalence and strengthen or refute the putative absence of RNA viruses that were not detected in this study.

It seems likely that BQCV, KBV and SBV were introduced into Tasmania with *A. mellifera* and/or possibly with *B. terrestris*. However, although several studies have previously reported BQCV in *Bombus* spp. (Singh, Levitt et al. 2010; Peng, Li et al. 2011; Levitt, Singh et al. 2013), we did not detect it in Tasmanian *B. terrestris*, which strongly suggests that the *B. terrestris* introduced into Tasmania were free of BQCV. We cannot say whether *B. terrestris* introduced into Tasmania were carrying SBV and KBV or whether they became infected after arrival. SBV was the only virus reported in the island before introduction of *B. terrestris* (Hornitzky, McDonald et al. 1990). However, SBV and KBV were both present in *A. mellifera* from NZ before introduction of *B. terrestris* into Tasmania (Anderson 1985) leaving open the possibility of virus introduction onto Tasmania via NZ-derived *B. terrestris*. KBV was detected in Australian mainland *A. mellifera* after their introduction into Tasmania (Rhodes and Teakle 1978; Dall 1985; Hornitzky 1987; Anderson and Gibbs 1988; Anderson 1991). However, we are cautious in postulating the origin or the original host of all viruses we detected. Indeed, we

cannot discard the possibility that other native bees or insects could have been the primary host of the viruses. RNA viruses were previously associated with solitary bees and other Hymenoptera such as wasps and ants, as well as non-hymenopteran insects such as butterflies, beetles and flies (Singh, Levitt et al. 2010; Levitt, Singh et al. 2013; Ravoet, De Smet et al. 2014).

Interestingly, for the first time in Tasmania, KBV was detected in very low prevalence in both *A. mellifera* and *B. terrestris* (Table 2). The other recent survey of RNA viruses did not detect KBV in Tasmania, although they detected IAPV in Tasmania and the Australian mainland (Roberts, Anderson et al. 2015). The phylogenetic tree generated from KBV and IAPV sequences from Tasmania and other countries showed that they are distinct since they clustered separately (S3). The position of IAPV in the Dicistroviridae phylogenetic tree implies that it could have been incorrectly classified as a KBV strain in earlier studies in particular those from Australia (Palacios, Hui et al. 2008; de Miranda, Cordoni et al. 2010). Analysis of IAPV isolates from the United States, Canada, Australia and Israel using RNA-dependent RNA polymerase sequences showed high similarities to previously reported KBV sequences from France, Australia and Russia (Palacios, Hui et al. 2008). Although IAPV and KBV are classified as different virus species by the International Committee on Taxonomy of Viruses (King, Adams et al. 2012), early literature and public sequences databases still have data of both viruses misclassified. This emphasises problems with online sequence databases and molecular virus diagnostics (de Miranda, Cordoni et al. 2010), particularly with regard to design of specific diagnostic primers. Bees positive to KBV were spatially clustered in our study. However, to decisively consider its presence restricted to a certain, further investigation is necessary with samples collected extensively around Tasmania, particularly as our sampling covers a relative small area of the island (in the southeast) and the KBV sites are at the northern margin of survey area.

We detected RNA viruses in asymptomatic *A. mellifera* and *B. terrestris*. KBV does not cause symptomatic disease, and SBV and BQCV symptoms are visible in worker larvae, and in queen larvae and worker brood, respectively (Chen and Siede 2007; Hails, Ball et al. 2007). As our samples consisted of adult bees, it is not surprising that we did not observe BQCV and SBV symptoms. However, it is likely that most of these commonly occurring RNA viruses are present as covert infections (Boecking and Genersch 2008), characterised by absence of disease symptoms, but with persistence over many generations through vertical transmission and low impact on bee fitness (Hails, Ball et al. 2007; Boecking and Genersch 2008).

Detection of two virus species in a single individual *A. mellifera* and *B. terrestris* from different locations was found in our samples. Similarly, more than one virus was detected per individual *Bombus* spp. in the USA and Belgium (Singh, Levitt et al. 2010; Parmentier, Smagghe et al. 2016). Likewise, co-infection has been reported in individual *A. mellifera* (e.g. Tentcheva, Gauthier et al. 2004; Chen, Pettis et al. 2005; Singh, Levitt et al. 2010).

Importantly, we did not detect bees carrying ABPV, CBPV, CWV, DWV, IAPV and SBPV. ABPV and SBPV have not been reported in Australia before; therefore, our data although limited supports Australia remaining free of these viruses. Our results suggest absence of DWV in Australia contrary to its putative occurrence recently reported. The presence and variability of DWV-like sequences in Australian bees needs further research in order to unambiguously define DWV status in Australia and at present we consider occurrence of IAPV but not DWV. Bee RNA virus populations do not occur as a single and homogenous genome but rather by a pool of related variants that are genetically distinct (Carter and Genersch 2007). Therefore, we cannot exclude the possibility of occurrence of different RNA virus variants that were unable to be amplified using the set of primers we employed. Further research is required with a larger sample size of *B. terrestris* and using deep sequencing to allow detection of all virus variants and novel viruses.

The low overall prevalence of RNA viruses is in line with expectations as in absence of the Varroa mite, the prevalence of RNA viruses it vectors is generally low (Martin 2001; Rosenkranz, Aumeier et al. 2010; Mondet, de Miranda et al. 2014). After incursion of the Varroa mite, the prevalence of some RNA viruses increased in New Zealand and Hawaii (Martin, Highfield et al. 2012; Mondet, de Miranda et al. 2014). For example, in New Zealand, prevalence of SBV, DWV, CBPV and KBV in *A. mellifera* increased significantly after Varroa mite arrival (Mondet, de Miranda et al. 2014). This study therefore contributes baseline data regarding virus prevalence before an incursion of the Varroa mite, should it occur in Tasmania.

Our study highlights the importance of stringent policy measures to restrict anthropogenic movement of bees and associated products in order to prevent introduction of *V. destructor* and DWV in Australia, as we consider this virus to be absent. Hence, importation of live bees including *A. mellifera* and *Bombus* spp., and honey bee products such as semen, pollen and royal jelly should be restricted and extremely carefully monitored to avoid introduction of this epidemic virus into Australia. Manley and co-authors (2015) highlight the importance of keeping the few current Varroa-free areas free of *V. destructor*. Goulson and Hughes (2015) suggest prioritisation of use of native bees for pollination, reduction or ceasing importation of non-native bees, and monitoring prevalence of RNA viruses in native bees to allow early detection of disease-associated decline of populations. This may be of eminent importance for Australia as its diverse native bee population is highly endemic and has not coevolved with DWV. Aside from the disease implications for native bee species, they also contribute significant (though poorly quantified) pollination benefits to crops (Kleijn, Winfree et al. 2015), and are crucial for pollination of many Australian native plant species (Paton 1993). Our data also highlight the value of islands for inferring virus introductions and potential transmission between hosts, due to their isolation from frequent outcrossing or co-foraging with other populations.

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Table 1: Primer pairs used in this study and their literature sources

Virus	Amplicon (bp)	Gene	Primer sequence (5'-3')	Source
IAPV	840	Capsid	GGTCCAAACCTCGAAATCAA TTGGTCCGGATGTTAATGGT	Singh *et al*. (2010)
DWV	424	Capsid	GGCGTGGTTCATTAGAATATAGG AAGCAGATCCCCACCTAAAAA	Singh *et al*. (2010)
KBV	625	Capsid	TGTTTGTGGCAATCCAGCTA TACGTCTTCTGCCCATTTCC	Singh *et al*. (2010)
BQCV	700	Capsid/ 3'UTR	TGGTCAGCTCCCACTACCTTAAAC GCAACAAGAAGAAACGTAAACCAC	Singh *et al*. (2010)
SBV	693	Capsid	GCACGTTTAATTGGGGATCA CAGGTTGTCCCTTACCTCCA	Singh *et al*. (2010)
ABPV	900	Capsid	TTATGTGTCCAGAGACTGTATCCA GCTCCTATTGCTCGGTTTTTCGGT	Benjeddou *et al*. (2001)
CBPV	570	RdRP	TCAGACACCGAATCTGATTATTG ACTACTAGAAACTCGTCGCTTCG	Blanchard*et al*. (2008)
CWV*	361	RdRP	GATGAACGTCGACCTATTGAAAAAG TGTGGGTTGGCTATGAGTCATCATG	This work
SBPV	868	RdRP	GATTTGCGGAATCGTAATATTGTTTG ACCAGTTAGTACACTCCTGGTAACTTCG	De Miranda *et al*. (2010)
Internal control	560	Ribosomal DNA	CGTGTTGCTTGATAGTGCAGC TTGGTCCGTGTTTCAAGACGG	Campbell *et al*. (2000)

IAPV, *Israeli acute paralysis virus*; DWV, *Deformed wing virus*; KBV, *Kashmir bee virus*; BQCV, *Black queen cell virus*; SBV, *Sacbrood virus*; ABPV, *Acute bee paralysis virus*; CBPV, Chronic bee paralysis virus; CWV, Cloudy wing virus; SBPV, Slow bee paralysis virus.
UTR, untranslated region; RdRP, RNA-dependent RNA polymerase
*also amplifies KBV

Table 2: True prevalence (%) of RNA viruses in *Bombus terrestris* (n=100) and *Apis mellifera* (n=100) with 95% confidence interval in square brackets. ABPV, CBPV, CWV, DWV, IAPV, and SBPV were not detected in either species.

Species of bee	BQCV	KBV	SBV	Overall	*P*-value
A. mellifera	11[5-19]	2[0-8]	9[4-16]	21[14-30]	0.04*
B. terrestris	-	3[0-9]	9[4-16]	12[6-20]	

Table 3: Generalised Linear Models parameter values for the best model explaining prevalence of SBV in *B. terrestris* using lowest AIC value for model selection.

Response	Virus	Parameters	Estimate	SE	*z*-value	*P*-value
B. terrestris virus prevalence	SBV	Intercept	-278.99	674.63	-0.41	0.68
		Apis SBV	2.52	1.01	2.5	0.01 *
		Latitude	-1.66	2.24	-0.74	0.46
		Longitude	1.39	4.49	0.31	0.76

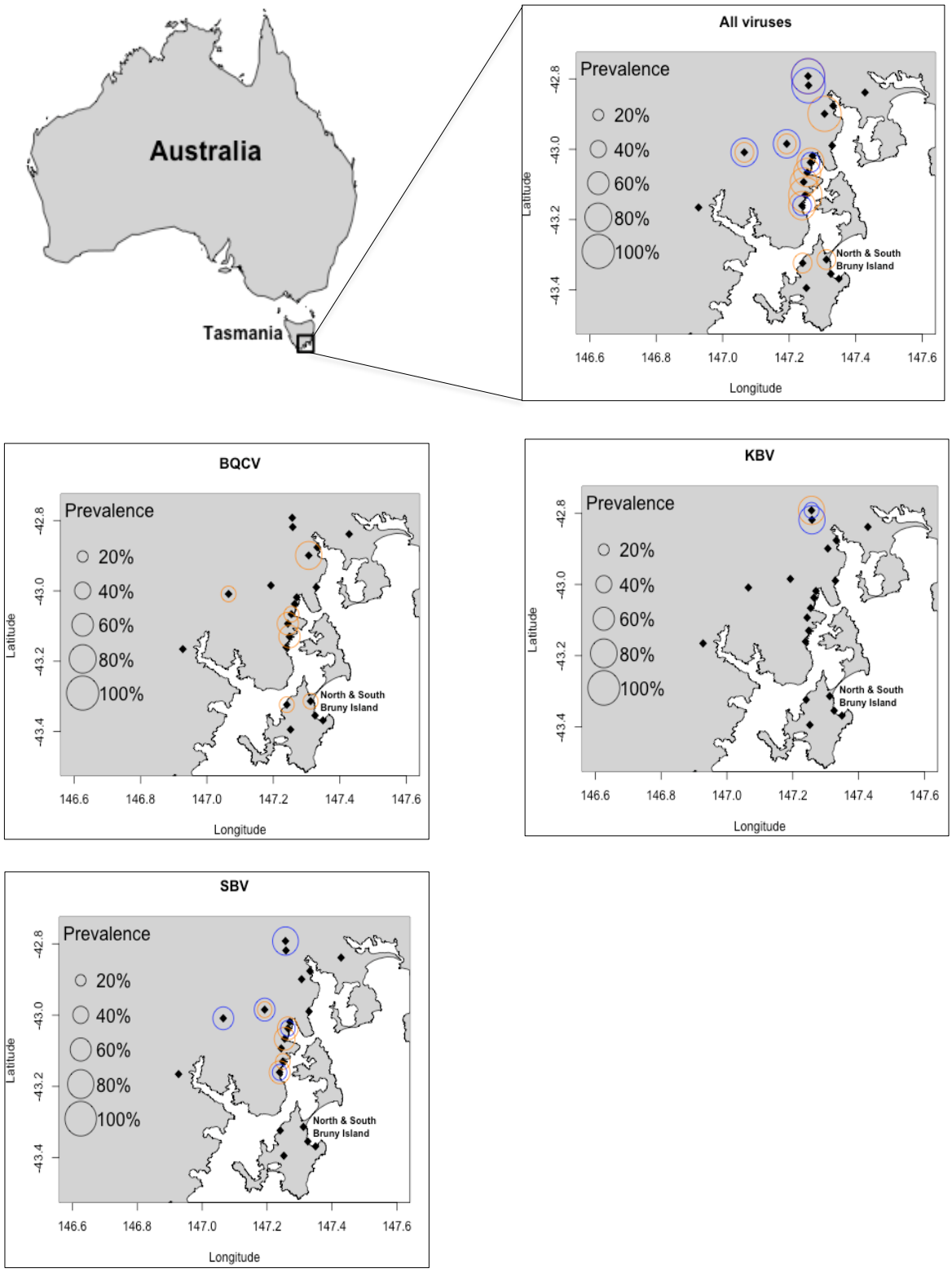
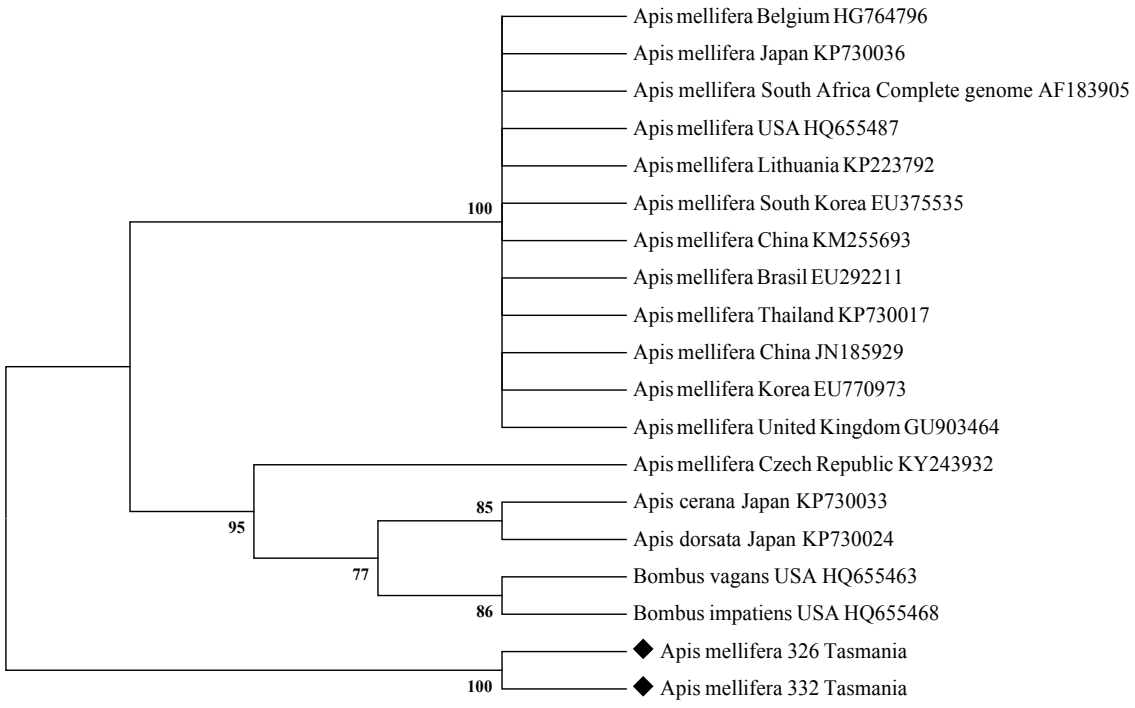


Figure 1: Prevalence (%) and geographical distribution of combined viruses as single response, BQCV, KBV and SBV. Black diamonds represent collection sites. Blue and orange circles represent virus detection in *Bombus terrestris* and *Apis mellifera*, respectively. Purple circle (top panel only) represents the same prevalence for both bee species. Sizes of circles correspond to prevalence (%) of the viruses.

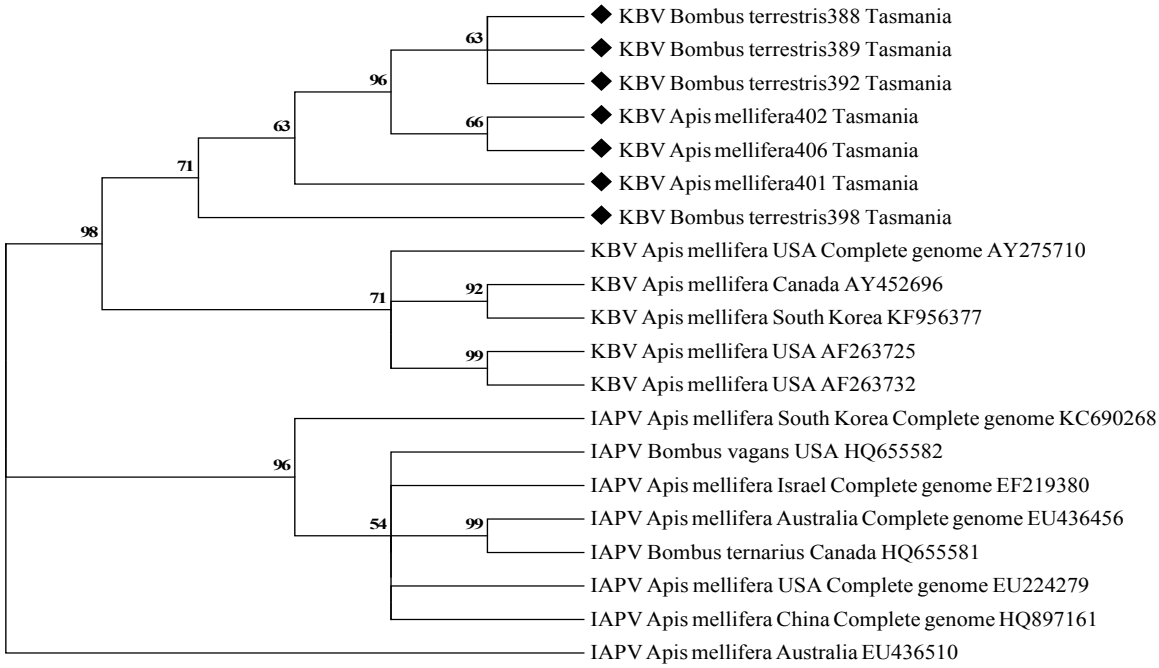
Supplementary material

Supplementary 1: Generalised Linear Model parameter values of the best model explaining prevalence of SBV in *A. mellifera* using lowest AIC value for model selection.

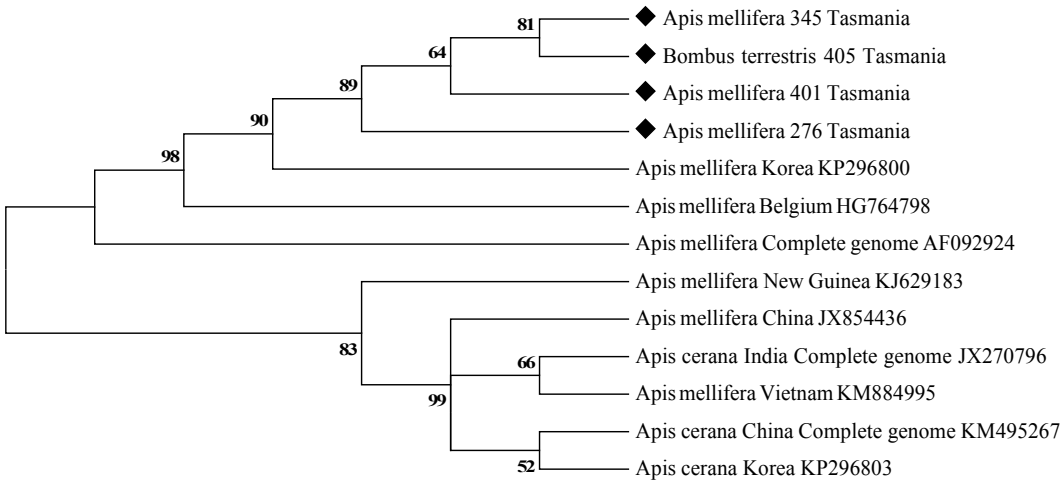
Response	**Virus**	**Parameters**	**Estimate**	**SE**	***z*-value**	***P*-value**
A. mellifera	SBV	Intercept	1.01e+06	1.31e+06	0.77	0.44
		Bombus SBV	4.5e+00	1.82e+00	2.47	0.01*
		Latitude	2.34e+04	3.05e+04	0.77	0.44
		Longitude	-6.86e+03	8.91e+03	-0.77	0.44
		Latitude:Longitude	-1.59e+02	2.07e+02	-0.77	0.44



Supplementary 2: Unrooted maximum likelihood phylogeny of BQCV from various *Apis* spp. and *Bombus terrestris*, using Tamura 3-parameter model. The analysis involved 19 nucleotide sequences and 582 base pairs of the capsid gene. The reliability of the tree was assessed by bootstrap replication (n = 1000 replicates), and node values >50% were considered as indicative of clustering. Viral sequences NCBI GenBank accession number. Diamonds indicate sequences generated in this study.



Supplementary 3: Unrooted maximum likelihood phylogeny of KBV from *Bombus terrestris* and *Apis mellifera* using Tamura 3-parameter model. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The analysis involved 20 nucleotide sequences and 383 base pairs of the capsid gene. The reliability of the tree was assessed by bootstrap replication (n = 1000 replicates), and node values >50% were considered as indicative of clustering. Viral sequences are identified by host species, country of isolation and NCBI GenBank accession number. Diamonds indicate sequences generated in this study.



Supplementary 4: Unrooted maximum likelihood phylogeny of SBV from *Apis mellifera* and *A. cerana* using Tamura 3-parameter model. The rate variation model allowed for some sites to be evolutionarily invariable. The analysis involved 13 nucleotide sequences and 560 base pairs of the capsid gene. The reliability of the tree was assessed by bootstrap replication (n = 1000 replicates), and node values >50% were considered as indicative of clustering. Viral sequences are identified by host species, country of isolation and NCBI GenBank accession number. Diamonds indicate sequences generated in this study.

Chapter 4

De novo assembly of complete bee RNA viral genomes by tapping into the innate insect antiviral response pathway

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Chapter 4

De novo assembly of complete bee RNA viral genomes by tapping into the innate insect antiviral response pathway

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Abstract

Recent studies have reported RNA interference (RNAi) as an antiviral immune response to infection of a number of RNA viruses in *Apis mellifera*. The RNAi pathway is activated by the presence of double-stranded RNA and degrades the viral genome into small interfering RNAs

(siRNAs) of 21-22 nucleotides in length. Although siRNAs that perfectly match several RNA viruses have been reported in *A. mellifera* previously, generation of complete viral genomes using assembly of siRNAs has not been performed. Therefore, in this study, we used deep sequencing to analyse siRNAs from symptomatic *A. mellifera* larvae. Our results show that *A. mellifera* larvae activate the RNA interference (RNAi) immune response in the presence of *Sacbrood virus* (SBV). We assembled three complete SBV genomes from three individual larvae from different hives in a single apiary, with 1-2% variability among them. The sequence divergence of SBV genomes suggests either multiple hive infection events at a local scale or occurrence of similar mutation rates in different hives, and possibly movement of an original SBV strain to nearby hives. Furthermore, we found 3-4% variability between SBV genomes generated in this study and earlier published Australian variants suggesting the presence of different SBV quasispecies within the country.

Importance

Over the last decade, honey bee population decline has been reported in North America, Europe and few regions of Asia. This has been associated with different factors including RNA viruses. This has led to an increase in bee virus research. Recent studies on the immune response of honey bees to RNA virus infection have shown that deep sequencing of siRNA can be used as tool for detection and sequencing of these viruses. For the first time, we demonstrate that this tool can be used to assemble complete bee RNA viral genomes, and to confirm infectivity in the host. Applications might include discovery, diagnosis and epidemiological study of RNA viruses, and analysis their genomic diversity.

Introduction

The European honey bee (*Apis mellifera*) is the best known and most used pollinator worldwide (1). Because of its value as honey producer and pollinator, *A. mellifera* has been deliberately

introduced into Australia and the Americas which has resulted in an almost global distribution with exception of Antarctica (2). About 22 RNA viruses have been reported infecting *A. mellifera* worldwide (3, 4) although not all are recognised and classified by the International Committee on Taxonomy of Viruses (5). These viruses affect the health and fitness of the bees and have been linked to widespread disease and loss of managed *A. mellifera* colonies in USA, Europe and Asia (e.g. 3, 6-11).

It has been recently reported that *A. mellifera* individuals can reduce the impact of infection of RNA viruses through their antiviral immune response. Among all the antiviral responses, RNA interference (RNAi) is the most important in plants and insects (12-14). RNAi is a post-transcriptional gene silencing mechanism that involves three distinct pathways, namely small interfering RNA (siRNA), microRNA (miRNA), and piwi-interacting RNA (piRNA). They have distinct biological functions and characteristics such as biogenesis, cleaved RNA length and modifications, and targets. siRNA is the major antiviral response of *A. mellifera* and its defence role is well characterised in comparison with the other pathways (12, 14-16).

siRNA is triggered by occurrence of double-stranded RNA (dsRNA), which can be either the viral genome itself or an intermediate dsRNA product generated during the virus replication. The host recognizes the dsRNA and uses ribonuclease III (*Dicer-like*) to cleave the viral genome into 21-22 nucleotide (nt) long pieces called virus-derived small interfering RNAs (vsiRNAs; 13, 17). siRNA antiviral response was first demonstrated when mortality level and *Israel acute paralysis virus* (IAPV) titres reduced in individual *A. mellifera* fed with IAPV-dsRNA prior to infection under controlled conditions (18). Later, this response was demonstrated in *A. mellifera* colonies under normal beekeeping conditions (19).

siRNA was also reported in naturally infected *A. mellifera* via deep sequencing of vsiRNAs. Adult *A. mellifera* from colonies affected by Colony Collapse Disorder (CCD) had abundant

vsiRNAs of 21-22 nt matching *Deformed wing virus* (DWV), IAPV, and *Kashmir bee virus* (KBV; 20). vsiRNAs matching *Acute bee paralysis virus* (ABPV), *Sacbrood virus* (SBV), and Varroa destructor virus-1 (VDV; unassigned) were observed in the same study but in low incidence.

Deep sequencing of siRNAs and subsequent assembly of viral genomes was previously demonstrated for plants, mosquitoes, fruit flies and nematodes. This process was used successfully to reassemble entire or partial genomes of known viruses and discovery of novel viruses (20-22). The use of deep sequencing of vsiRNAs for diagnosis and genome assembly of bee viruses is still limited although there is an increasing interest in *A. mellifera* antiviral defence which will likely see this technique used more widely for this purpose.

SBV was the first virus identified in *A. mellifera* and it has become distributed in all continents where *A. mellifera* is present (23). Three serotypes of this single-strand RNA (ssRNA) virus have been characterised worldwide, namely European, Asian and New Guinea serotypes (24-27). The New Guinea serotype was briefly mentioned in late 1970's (28), and it was recently characterised from an *A. mellifera* isolate. It was hypothesised that it evolved from the European serotype via mutations (24, 28). The European serotype has been detected in *A. mellifera* and is normally reported as SBV (3, 27). The Asian serotype has been detected in the Asian honey bee *A. cerana*, and it includes several variants, namely Thai, Chinese and Korean SBV (TSBV, CSBV, KSBV; 3, 25, 26). Although this serotype has been mainly reported in *A. cerana,* latest studies have reported it in *Apis* bees other than *A. mellifera* (24, 29). While the European and Asian serotypes cause the same disease symptoms, there are physiochemical, pathogenic and genome variation between them (3, 26). However, the variation between genomes of both serotypes has not exceeded 10% (e.g. 30-33).

The genomic variability of around 10% between the Asian and European serotypes and the phenotypic differences of symptoms caused in infected hosts, separate them as two distinctive viral

strains (34). Clouds of *mutant genomes* (*mutant spectra*) that form viral strains are called *quasispecies* (34-36). Viral quasispecies are a collection of non-identical but closely related viral genomes that can be generated by mutation or recombination, and are continuously subjected to genetic variation, competition and selection. Quasispecies surround a central master species that is the most frequently occurring and fitted variant (23, 37-39). Although the term quasispecies was introduced to describe variants of RNA virus of a phylogenetically related population present in one infected organism, it has been used to describe genome heterogeneity of RNA virus populations (35). The term quasispecies was used to describe genome diversity of DWV variants detected in *A. mellifera* from the same apiary (40) and hence, in this study, we will also use 'quasispecies' to reflect variants of RNA viruses in several species and individuals. In Australia, very little information is available on quasispecies of RNA viruses circulating in the country including SBV, which was one of the most common and consistently detected viruses in the most recent national survey (41). It is important to know the sequence variability of the viruses present in Australia to better evaluate the impact of distinct variants.

This study investigates the occurrence of the antiviral RNAi pathway in symptomatic SBV-infected *A. mellifera* larvae, and it is the first to generate complete bee RNA virus genomes using assembly of sequenced siRNAs. Three SBV-infected larvae from different hives within one South Australian apiary were analysed in order to (i) confirm SBV replication detected using strand-specific reverse-transcription polymerase chain reaction (RT-PCR), (ii) confirm the occurrence of antiviral response by deep sequencing of siRNAs, and (iii) use SBV as model to confirm our methodological approach of generating complete RNA virus genomes for comparison with those available in public databases.

Materials and methods

Sample collection

Three symptomatic larvae of *A. mellifera* were collected by the South Australian state apiary inspector from different hives in the same apiary located near Scott Creek Conservation Park, Mount Lofty Ranges, near Adelaide, South Australia. Samples were immersed in RNALater and stored in -20°C until transported to Waite Research Institute in Adelaide, where the larvae were stored at -80°C until molecular analysis.

Molecular analyses and library preparation

Prior to RNA extraction, larvae were washed in 1% sodium dodecyl sulphate to guarantee elimination of any brood food and any other possible contaminants. Total RNA and enriched small RNA were extracted from individual larvae simultaneously using mirVana miRNA Isolation kit (Life Technologies) following the manufacturer's instructions. Concentration and purity of the total RNA was measured using Qubit®3.0 Fluorometer and a NanoDrop ND-1000 spectrophotometer (both Thermo Fisher Scientific), respectively. Solubilised RNA was stored at -80ºC until used.

Total RNA was used to test for (+)SBV (positive-strand) and (-)SBV (negative-strand) using the strand-specific RT-PCR method based on the work of Boncristiani et al. (42) but with several modifications (Figure 1). Two types of RT reactions were performed using Bioscript Reverse Transcriptase kit (Bioline). First, RT was conducted using biotinylated (fwd) and biotinylated (rev) to generate complementary DNA (cDNA) of (-)SBV and (+)SBV, respectively. Second, RT was performed using non-specific random hexamer primers (Bioline) to generate conventional (non-biotinylated) cDNA (Figure 1). Prior to PCR amplification, biotinylated-cDNA were magnetically separated from any non-target cDNA using Dynabeads® kilobaseBINDER™ kit (Invitrogen) according to the manufacturer's instructions (Figure 1). Two µl of both conventional- and biotinylated-cDNA were added to a final 25 µl PCR reaction solution: 2.5 µl 10x NH_4 buffer, 0.7 µl of 10 mM dNTPs, 0.75 µl of 50 mM $MgCl_2$, 1 µl forward and reverse conventional primers (20 µM), and 0.15 µl BIOTAQ DNA Polymerase (Bioline). PCR reactions were performed using the

following conditions: initial denaturation for 8 min at 94ºC followed by 35 cycles of denaturation for 30 s at 94ºC, 55 s annealing at 51ºC, 90 s extension at 72ºC, and a final extension for 10 min at 72ºC. Biotinylated and conventional sequence-specific primers used in this study were forward (5'-GCACGTTTAATTGGGGATCA-3') and reverse (5'-CAGGTTGTCCCTTACCTCCA-3'), which amplified fragments of 693 base pairs. RT without template (negative control) and internal control (ribosomal DNA) were included in each series of RT-PCR reactions. PCR products were analysed on 1% agarose gels stained with GelGreen Nucleic Acid Stain (Biotium) and visualised by UV light. EasyLader II (Bioline) was included on each gel for determination of the size of PCR products.

Enriched small RNA was sent to BGI Tech Solutions Co. in Hong Kong for library preparation and sequencing using Illumina HiSeq 4000. In summary, enriched molecules of 18-30 nt were selected and ligated to adapters on the 5'- and 3'- terminals of the small RNAs. The samples were subjected to RT for synthesis of cDNAs, which was followed by PCR amplification for production of the sequence libraries, which were subjected to Illumina high-throughput sequencing (Figure 1). The library was prepared using the NEBNext Multiplex Small RNA Library Prep Set for Illumina.

Bioinformatics data analysis

Our Illumina small RNA sequencing datasets were analysed using YABI (Centre for Comparative Genomics, Murdoch University, Western Australia; 43). This web-based bioinformatics toolkit was customised by Barrero and colleagues (2017) with specific tools for sequence analysis to diagnose plant viruses and viroids. This toolkit allows execution of different operations such as quality control, assembly, mapping, and similarity searches, using a single access point. We analysed our data based on Barrero and co-authors (2017) YABI workflow (Figure 1 – bioinformatics data analysis A), but additional mapping and *de novo* assembly steps were introduced for one sample (Figure 1 – bioinformatics data analysis B). The top hit reference genome used in workflow B was obtained via Basic Local Alignment Search Tool (BLAST) from the National

Center for Biotechnology Information (NCBI). We analysed reads with only 22 nt contrary to a previously reported study in *A. mellifera* that used 21-22 nt reads (44) since it reduced the number of assembled contigs (Supplementary 1) and gaps between contigs. We used ConDeTri (45) and SPAdes 3.5 (46) to extract trimmed readings from the raw data and to *de novo* assemble the contigs using sequential kmer lengths of 15, 17 and 19, respectively (22). Then, we used CAP3 (47) to assemble the overlapping contigs generated from SPAdes followed by screening of the nucleotide queries in NCBI. We used the Open Reading Frame Finder program from NCBI to predict the sequences open reading frames (ORFs).

Illumina sequencing is highly accurate and the majority of bases rate 30 or more quality scores (>Q30). That is, the probability of incorrect base call by the sequencer is 1 in 1,000 and the accuracy of the sequencing platform is 99.9%. After sequencing, BGI Tech Solutions Co. filtered the data and removed low quality reads (<Q20). After that, we trimmed the raw reads from the 3'-end and extracted reads of 22 nt long. This assured exclusion of the low quality base reads at the end of the sequences and the use of siRNA sequences that are specifically derived from the host antiviral response. The quality control and assembly strategies used in this study guarantee unparalleled accuracy of analysable data.

Phylogenetic and sequence analysis

SBV genomes generated in this study were aligned and compared. This analysis was performed using nucleotide and amino acid sequences of the single ORF. Then, complete nucleotide genomes were compared with National Center for Biotechnology Information (NCBI) top hit reference (AmSVB-Kor1), and representatives of European (Rothamstead) and Asian serotypes (CSBV-FZ). All alignments were performed in MUSCLE (48). We used Simplot version 3.5.1 (49) to visualise resultant similarities (Figure 3).

In order to analyse the relationship of our samples with other SBV strains from *A. mellifera* and *A. cerana* from various countries, we used the Maximum Likelihood method based on the Tamura 3-parameter model (50). Heuristic search of initial trees were obtained by applying the Neighbor-Joining method to a matrix of pairwise distance estimated using the Maximum Composite Likelihood (MCL) approach. A discrete Gamma distribution was used to model evolutionary rate differences among sites. All positions with less than 95% site coverage were eliminated. That is, less than 5% chance of alignment gaps, missing data, and ambiguous bases occurring at any position. The analyses include 40 nucleotide sequences of the highly conserved RNA-dependent RNA-polymerase region (RdRp; Figure 5) and a total of 448 base pairs in the final dataset. Analyses were conducted in MEGA6 (51).

Nucleotide sequence accession numbers

The GeneBank nucleotide sequences used in this study are under accession numbers AF092924 (AmSVB-Kor1), AF092924 (Rothamstead), and KM495267 (CSBV-FZ).

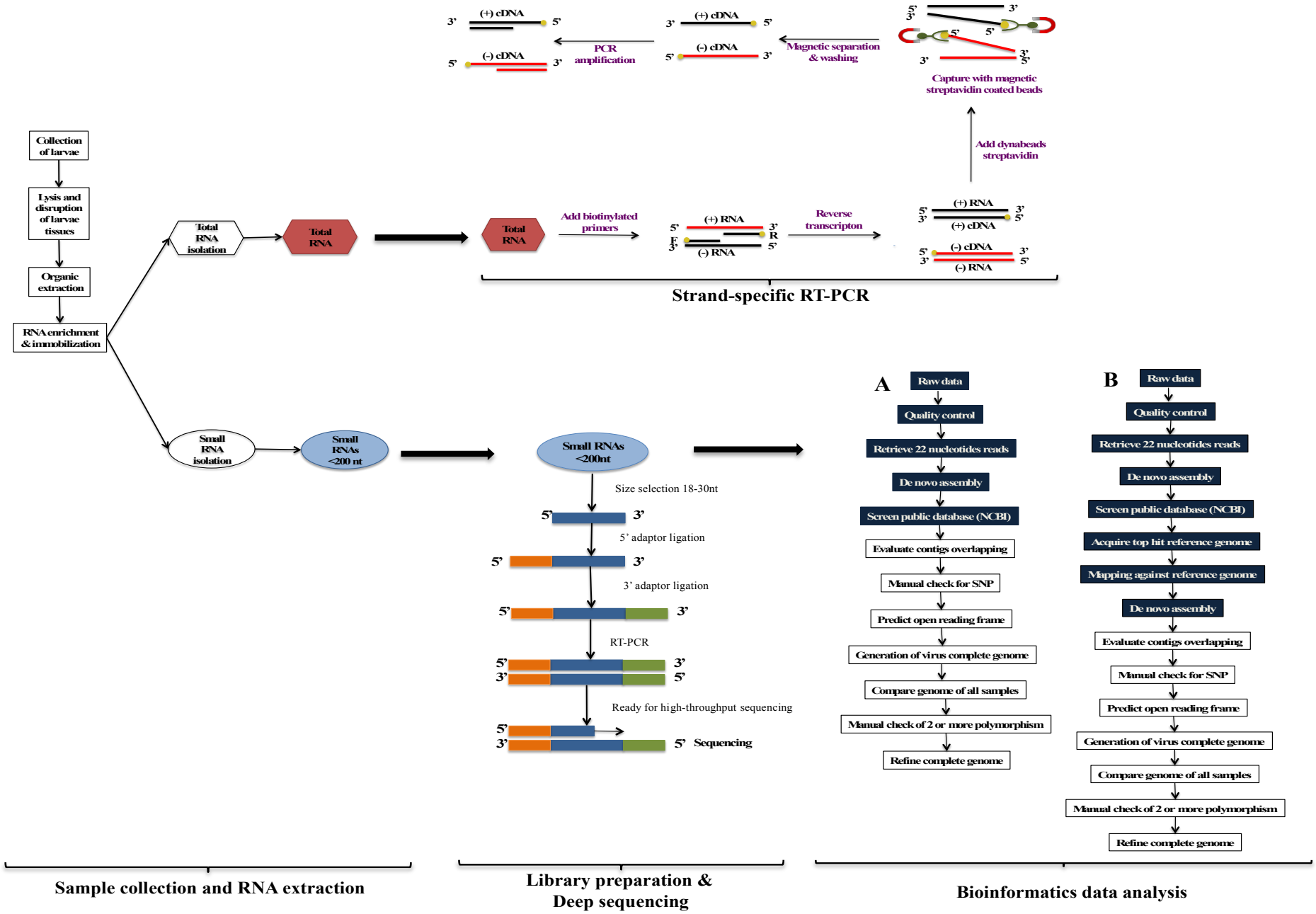
Collection of larvae
Lysis and disruption of larvae tissues
Organic extraction
RNA enrichment & immobilization
Total RNA isolation
Total RNA
Small RNA isolation
Small RNAs <200 nt
Add biotinylated primers
Reverse transcripton
Add dynabeads streptavidin
Capture with magnetic streptavidin coated beads
Magnetic separation & washing
PCR amplification
(+) cDNA
(-) cDNA
(+) RNA
(-) RNA
Strand-specific RT-PCR
Small RNAs <200nt
Size selection 18-30nt
5' adaptor ligation
3' adaptor ligation
RT-PCR
Ready for high-throughput sequencing
Sequencing
A
Raw data
Quality control
Retrieve 22 nucleotides reads
De novo assembly
Screen public database (NCBI)
Evaluate contigs overlapping
Manual check for SNP
Predict open reading frame
Generation of virus complete genome
Compare genome of all samples
Manual check of 2 or more polymorphism
Refine complete genome
B
Raw data
Quality control
Retrieve 22 nucleotides reads
De novo assembly
Screen public database (NCBI)
Acquire top hit reference genome
Mapping against reference genome
De novo assembly
Evaluate contigs overlapping
Manual check for SNP
Predict open reading frame
Generation of virus complete genome
Compare genome of all samples
Manual check of 2 or more polymorphism
Refine complete genome
Sample collection and RNA extraction
Library preparation & Deep sequencing
Bioinformatics data analysis

Figure 1: Overview of the methodology used in this study. This study started with collection of *Apis mellifera* larvae showing symptoms of SBV, followed by extraction of small and total RNA. Total RNA was used for strand-specific RT-PCR for viral diagnosis and detection of virus replication; the RT used biotinylated primers followed by magnetic separation with Dynabeads® kilobaseBINDER^{TM} kit and PCR amplification with conventional primers. Small RNA was sent to Hong Kong for Illumina sequencing at BGI Tech Solutions Co. Deep sequencing data were analysed following the workflows of the bioinformatics data analysis (A and B). Shaded steps represent the analyses performed in YABI (Centre for Comparative Genomics, Murdoch University, Western Australia). Open reading frames were predicted using Open Reading Frame Finder program from NCBI. SNP, Single Nucleotide Polymorphism; nt, nucleotides.

Results

All three larvae were symptomatic and tested positive for (+)SBV but intriguingly only one indicated presence of the replicative form (-)SBV (Figure 2). Detection of (+)SBV showed presence of viral particles in symptomatic *A. mellifera* larvae, but it does not confirm whether these viruses were infecting and replicating in these hosts.

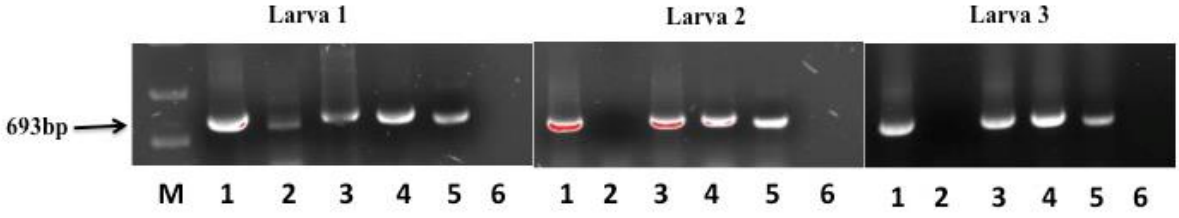

Figure 2: Detection of positive- and negative-strand SBV RNAs in three symptomatic *Apis mellifera* larvae. Biotinylated (fwd) and Biotinylated (rev) were used in initial reverse transcription (RT) reactions to generate cDNA of (-)SBV (Lane 2) and (+)SBV (Lane 1), respectively. Mix of biotinylated forward and reverse (Lane 3) and Random hexamer primers (Lane 4) were also used in RT. Conventional forward and reverse primers were used in PCR

reactions to amplify SBV fragments (693bp) from all larvae. Lane M represents molecular marker, and lanes 5 and 6 are internal and water (no RNA) controls, respectively.

To confirm infection in *A. mellifera* larvae and antiviral response to the same virus, we sequenced enriched small RNAs from the three larvae. Our results showed presence of siRNAs matching SBV in high incidence in all larvae including the ones that did not show (-)SBV in the RT-PCR reaction. Our sequencing outcomes reported millions of reads for each larvae (Figure 3). We observed a distinct maximum high peak of siRNAs at 22 nt followed by a much smaller peak at 21 nt for all larvae. Also, frequency of these siRNAs varied between 27 to 50% of total reads in these three samples (Figure 4).

Assembly of overlapping siRNA contigs allowed reconstruction of the complete genome of SBV occurring in each *A. mellifera* larva (denoted as "SA isolates"). The length of the genomes were 8821 nt (larva 1), 8831 nt (larva 2), and 8848 nt (larva 3), which contained a single large ORF of 8583 nt encoding 2860 amino acids (Figure 3, Supplementary 2). Comparison among these genomes indicated 1-2% variability between sequences although they were collected in the same apiary (Table 1).

Comparisons of SA isolates with other SBV genomes revealed highest similarities with the Korean *A. mellifera* variant (96.5%) followed by the UK *A. mellifera* (92.8%) and then Chinese *A. cerana* (89.6%), respectively (Table 2 & Figure 4). The divergence between SA isolates and other *A. mellifera* SBV genomes did not exceed 10%, which was the sequence difference found between CSBV and *A. mellifera* SBV.

Phylogenetic analysis of SBV RdRp region illustrated four distinct clusters, which included corresponded to the European, Asian, and New Guinea serotypes (Figure 5). The South African strain did not cluster with any other serotype suggesting that it could possibly be another distinct serotype. Within the Asian serotype clade, the same serotype was circulating in Asia independently of the host (*A. mellifera*, *A. cerana*, and *A. dorsata*; Figure 5). SA isolates clustered together but separately from Perth (western Australia) and Canberra (eastern Australia)

quasispecies, although all were the European serotype (Figure 5). In addition, nucleotide comparison of SA, Perth and Canberra quasispecies genomes revealed 3-4% variability. Australian SBV were more similar to Asian variants of the European serotype (from Japan, Korea and Nepal) than to European variants of the same serotype.

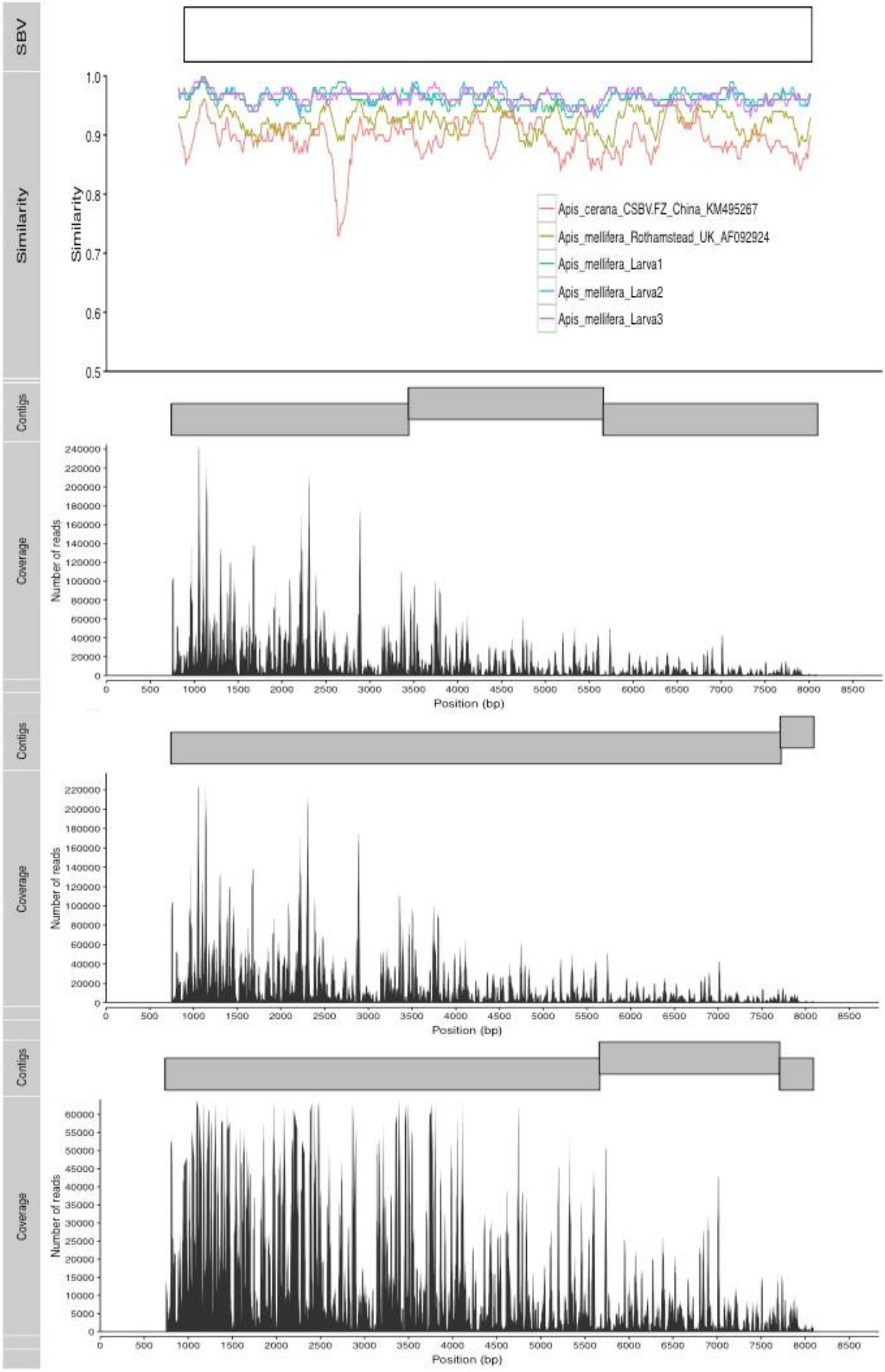
SBV
Similarity
Similarity
Apis_cerana_CSBV.FZ_China_KM495267
Apis_mellifera_Rothamstead_UK_AF092924
Apis_mellifera_Larva1
Apis_mellifera_Larva2
Apis_mellifera_Larva3
Contigs
Coverage
Number of reads
Position (bp)
Contigs
Coverage
Number of reads
Position (bp)
Contigs
Coverage
Number of reads
Position (bp)

Figure 3: Similarity plot of *Sacbrood virus* (SBV) complete genomes reconstructed in this work (*Apis mellifera* larvae 1 to 3) compared to each other and two other similar genomes available publically. The rectangles above bottom three plots represent the number of contigs and overlapping points. Small interfering RNA reads of larvae 1 to 3 (in increasing order) are illustrated in chromatograms. SBV virus polyprotein is shown at the very top.

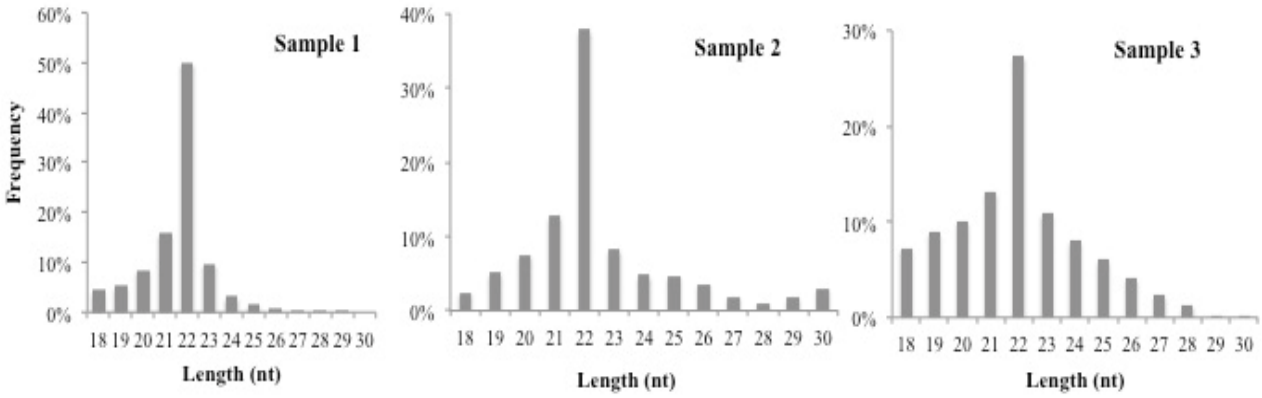

Figure 4: Length (in nucleotides) distribution of small RNAs from symptomatic SBV-infected *Apis mellifera* larvae that match *Sacbrood virus* (SBV) genome sequence. Only 22 nt RNAs were used for assembly of SBV genomes.

Table 1: Nucleotide and amino acid variability (% variability) of three *Apis mellifera* SBV genomes generated in this study. The three genomes were detected in different hives within the same apiary. Only the large open reading frame was used in this analysis

Genome variability	**Nucleotide**			**Amino Acid**		
Apis mellifera	Larva 1	Larva 2	Larva3	Larva 1	Larva 2	Larva3
Larva 1		2.2%	2.2%		0.5%	0.3%
Larva 2	2.2%		1.8%	0.5%		0.1%
Larva3	2.2%	1.8%		0.3%	0.1%	

Table 2: Nucleotide variability comparison (% variability) of *Apis mellifera* SBV complete genomes generated in this study and three other publically available genomes. The GeneBank® accession numbers for the strains Rothamstead, AmSBV-Kor1, and CSBV-FZ are AF092924, KP296800, and KM495267, respectively.

Complete SBV variant genomes			
SBV variant hosts	*Apis mellifera* AmSBV-Kor1	*Apis mellifera* Rothamstead	*Apis cerana* CSBV-FZ
Apis mellifera larva 1	3.5%	7.2%	10.5%
Apis mellifera larva 2	3.5%	7.3%	10.4%
Apis mellifera larva3	3.4%	7.2%	10.4%

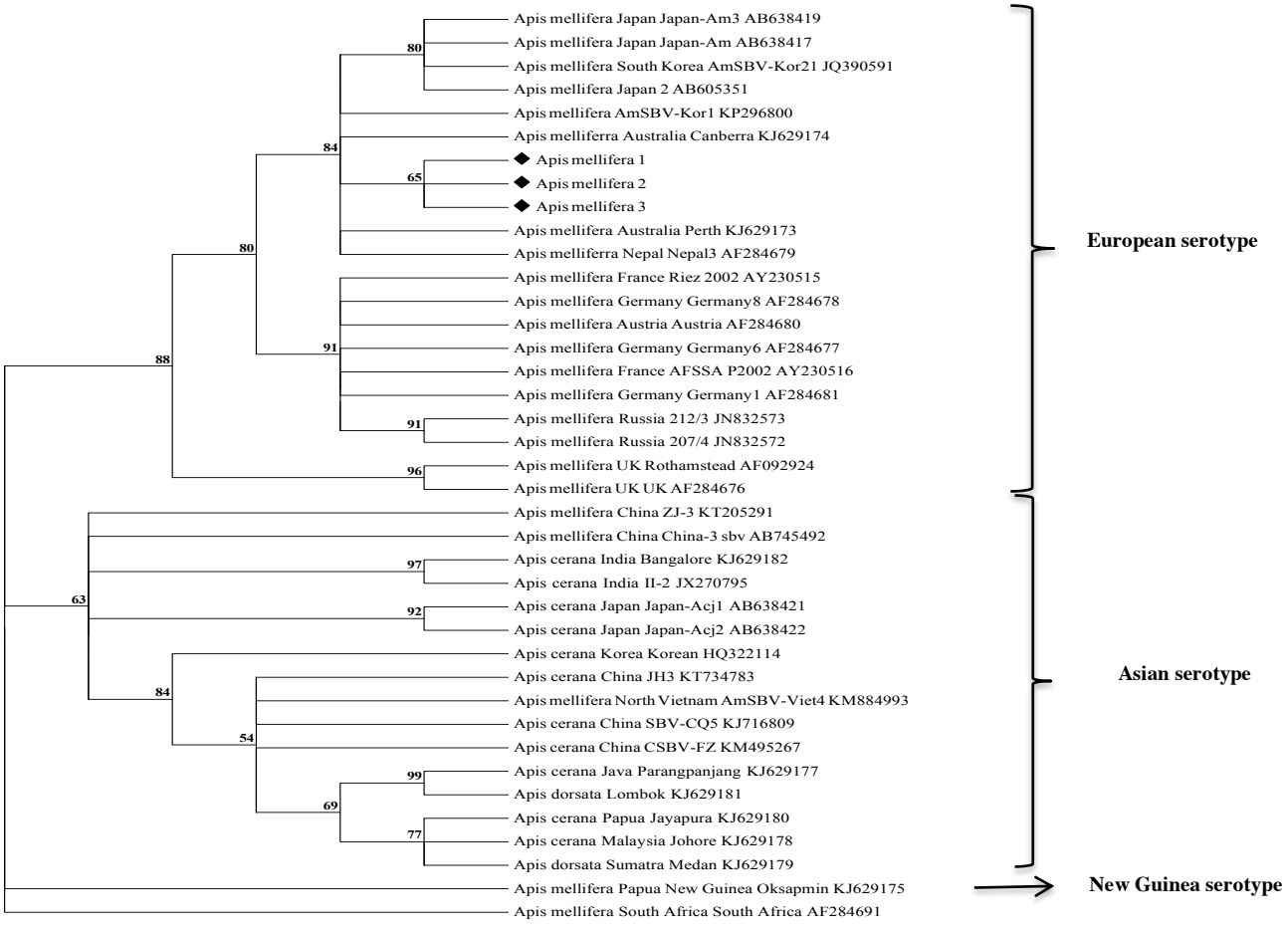

Figure 5: Unrooted maximum likelihood phylogeny of SBV variants using Tamura 3- parameter distance model of a 429 base pair region of the RNA-dependent RNA polymerase. Next to the branches is shown the percentage of trees in which associated taxa clustered together. The reliability of the phylogenetic trees was assessed by bootstrap replication (N = 1000 replicates), and node values >50% were considered as indication of clustering. Strains are identified by host species, country of origin, strain or isolate, and accession number. The diamonds indicate the larvae used in this study. Variants mostly fell into three clades that mirror known serotypes except for the South African (bottom) that did not fall into any of these clades.

Discussion

RNAi has been reported as an effective antiviral response of *A. mellifera* against infection of RNA viruses. Activation of this response requires occurrence of dsRNA, which is generated as an intermediate during ssRNA virus replication (13, 17). All three larvae were positive to (+)SBV and this was anticipated since they were symptomatic samples (52). Surprisingly, two larvae were negative to presence of (-)SBV when tested with RT-PCR, while later detection of siRNA in these larvae indicated generation of (-)SBV sometime during primary infection that triggered the RNAi response. Therefore, this result suggests that virus replication was not occurring or had ceased as result of the RNAi response. Alternatively, this result might indicate that the presence of (-)SBV was in very low titres, which fell below the limit of detection after the extraction process. Boncristiani and colleagues (42) detected 3000 times more (+)DWV than (-)DWV in infected adult honey bees. Although biotinylated strand-specific RT-PCR was used successfully used to detect (-)DWV in this study, it has not been previously used in any study to detect SBV. Consequently, sensitivity of this method for detection of (-)SBV is unknown.

Detection of siRNAs in three naturally infected *A. mellifera* larvae confirmed occurrence of RNAi antiviral response to SBV infection. This supports previous studies that reported occurrence of RNAi in response to virus infection in *A. mellifera* (e.g. 18, 44, 53). RNAi

response was also shown in *A. cerana* larvae (54) and adult bumble bees (*Bombus terrestris*; 55). Abundant siRNAs of 22 nt-long was previously detected in infected adult *A. mellifera* (44) and bumble bees (55). Similarly, our siRNA results also reported maximum reads at 22 nt, which matched perfectly to SBV and allowed generation of complete genomes.

For the first time, complete genomes were assembled for three *A. mellifera* SBV isolates using siRNAs. This result demonstrates the potential to generate the complete genome of other RNA viruses using siRNAs. Chejanovsky and colleagues (2014) assembled the majority of the genomes of IAPV, DWV and KBV, and partial genomes of ABPV and *Black queen cell virus* (BQCV) using siRNAs from *A. mellifera* colonies with CCD symptoms. Generation of a complete viral genome based on assembly of siRNAs contigs were previously reported in diseased and symptomless plants (21, 22).

Genome comparison of South Australian SBV isolates indicated the presence of at least three quasispecies in the same *A. mellifera* apiary (1-2% variability, Table 1). This variability resulted in several predicted amino acid changes in the resultant proteins (Supplementary 2), but it is not sufficient to suggest different genotypes (Figure 5). The divergence observed among larval *A. mellifera* may be linked to the relative high mutation rate of around 10^{-3} - 10^{-5} substitutions per nucleotide copied during replication of viruses (38) since it is unknown whether interaction of RNA viruses leads to their genetic recombination in *A. mellifera* (56). Also, the degree to which this might have occurred in Australia or the areas from which our data and comparisons derive also make the data hard to interpret with confidence. The genomic variability within the single apiary used in our study may be also associated with movement of Australian *A. mellifera* hives (57), which could expose hives to multiple quasispecies of the same virus.

The South Australian isolates were distinct although closely related to other broadly distributed Australian variants (3-4% variability, Figure 5) suggesting occurrence of six or more SBV quasispecies in Australia. Given the small number of hives that have been assessed nationwide, it is likely that many more quasispecies exist in Australia. Genomic variability of

less than 10% has been reported within SBV quasispecies of both Asian and European serotypes (30, 31, 58). Alignment of nucleotide sequences of *A. mellifera* SBV from different European countries showed less than 7% variability between them (33). Similarly, in China, analysis of seven *A. cerana* CSBV variants from three provinces revealed variability of less than 10% (59).

The Asian and European serotypes were initially reported as infecting only *A. cerana* and *A. mellifera*, respectively (31, 58, 60, 61) and it was speculated whether these serotypes were species specific (29). Our phylogenetic analysis indicates that *A. mellifera* and *A. dorsata* also carry the Asian serotype and confirms previous reports of these species harbouring strains that are closest to the Asian serotype (24, 30, 32). Other evidence that the Asian serotype is not species-specific is that it was used to infect *A. mellifera* under controlled conditions. Injection of TSBV into *A. mellifera* resulted in infection and death of pupae within days (25). Also, a CSBV variant was recently detected in *A. mellifera* colonies from China followed by detection of its replicative form under controlled experiments (29). Indeed, both European and Asian serotypes are circulating among different species with the *Apis* genus.

Analysis of the virus SBV RdRp region supports the distinctiveness of SBV serotypes (Figure 5). Interestingly, Australian quasispecies grouped with Asian *A. mellifera* variants (from Korea, Japan and Nepal) all of which fell into the European serotype clade, and were less similar to European variants. In addition, complete genome comparison of the SA SBV quasispecies with other genomes showed highest similarity with a Korean *A. mellifera* strain. These results propose introduction of the European serotype into Australia, Japan, Korea and Nepal via anthropogenic movement of *A. mellifera* (2). A recent study of DWV also showed that widespread anthropogenic movement of the virus had occurred and that Australian DWV-like sequences were of European origin (62). Roberts & Anderson (2014) also reported clustering of Australian, Japanese, Nepalese, and European SBV variants. In addition, similar homologies of *A. mellifera* variants from Korea, Australia and UK were illustrated in two other recent studies (30, 58).

Although the European and Asian serotypes have been well characterised, the New Guinean serotype requires full description of distribution, pathogenesis and epidemiology (24, 33).

In our study, siRNAs were detected in two symptomatic *A. mellifera* larvae that tested negative to presence of replicative form. If this is simply related to a titre of the replicative RNA strand that falls below the RT-PCR sensitivity threshold, this asserts that deep sequencing of siRNAs is the more sensitive method to investigate infection and replication of RNA virus in *A. mellifera*. Indeed, deep sequencing of siRNAs is a potential method for detection, sequencing and discovery of new RNA viruses in *A. mellifera* and other bees, which are subject to the RNAi immune response. This method is most suitable for detection and identification of novel viruses or viruses that are not well known or described genetically. On the other hand, this method cannot be used for detection of viruses that have counter-defence strategy capable of suppressing the RNAi. Several plant viruses have demonstrated their ability to supress their host RNA silencing machinery.

Further research is required to determine genome diversity of SBV and other RNA viruses in Australian *A. mellifera*. This information is fundamental for determining the impact of the possible introduction of the parasitic Varroa mite (*Varroa destructor*) in Australia and potentially for detection of possible novel invasions of *A. mellifera*. Moreover, future investigation on genetic variability of viruses in single infected bees or colonies can improve our understanding on the development of quasispecies in bee viruses. In addition, future research should focus on the antiviral RNAi response. For example, modulation of the RNAi response to multiple infections and interaction with stressors such as pesticides and poor nutrition would be beneficial, as these factors are linked to CCD (and bee health generally) and because RNAi is considered a control strategy against RNA viruses in order to reduce their impact on bee health.

Acknowledgement

We thank Michael Stedman from Primary Industries and Regions SA (PIRSA) for his kindness in providing the symptomatic SBV larvae analysed in this study. We also thank the Holsworth Wildlife Research Endowment - Equity Trustees for the financial support that allowed the molecular analysis of this study.

Supplementary material

Supplementary 1: Comparison of outcomes of using 21-22 versus only 22 nucleotide-long reads for RNA virus genome assembly. The virus summary tables were generated in YABI (Center for Comparative Genomics, Murdoch University, Western Australia). The number of contigs is higher for 21-22nt reads and therefore only 22nt reads were used for assembly.

22 nucleotide-long small interfering RNA reads						
Accession number	**Virus name**	**Nr of contig hits**	**Average % ID**	**Alignment length**	**Virus length**	**% Coverage by contigs**
AF092924	Sacbrood virus complete genome	3	93.03	8826	8832	99.93
HM237361	Sacbrood virus CSBV-LN/China/2009, complete genome	1	92.62	461	8863	5.2
KJ629183	Sacbrood virus isolate Oksapmin polyprotein gene, partial cds	2	91.32	8112	8359	97.05
AF469603	Sacbrood virus polyprotein gene, complete cds	1	90.36	2459	8740	28.14
JQ390592	Sacbrood virus strain AmSBV-Kor19, complete genome	1	91.54	461	8784	5.25
KP296800	Sacbrood virus strain AmSBV-Kor1, complete genome	3	96.63	8829	8837	99.91
JQ390591	Sacbrood virus strain AmSBV-Kor21, complete genome	3	96.96	8829	8855	99.71
KC007374	Sacbrood virus strain SBM2, complete genome	1	90.43	5918	8854	66.84

21-22 nucleotide-long small interfering RNA reads						
Accession number	**Virus name**	**Nr of contig hits**	**Average % ID**	**Alignment length**	**Virus length**	**% Coverage by contigs**
AF092924	Sacbrood virus complete genome	4	92.84	8826	8832	99.93
HM237361	Sacbrood virus CSBV-LN/China/2009, complete genome	1	92.62	461	8863	5.2
KJ629183	Sacbrood virus isolate Oksapmin polyprotein gene, partial cds	3	91.21	8112	8359	97.05
AF469603	Sacbrood virus polyprotein gene, complete cds	1	90.36	2459	8740	28.14
JQ390592	Sacbrood virus strain AmSBV-Kor19, complete genome	2	92.01	1777	8784	20.23
KP296800	Sacbrood virus strain AmSBV-Kor1, complete genome	4	96.48	8829	8837	99.91
JQ390591	Sacbrood virus strain AmSBV-Kor21, complete genome	4	96.92	8829	8855	99.71
KC007374	Sacbrood virus strain SBM2, complete genome	1	90.61	4618	8854	52.16

Supplementary 2: Alignment of amino acid sequence of three larvae *Apis mellifera*. The amino acid genomes were generated from the single open reading frames and using MUSCLE.

```
Larva 1 MDDISPLFYGDVRNTNRFLSSGGIRRNQSSEYSSRARIYKTKLEARNYGVERLSTILTSSKKTFDTVDSYTDLFNGWVSG
Larva 2 MDDISPLFYGDVRNTNRFLSSGGIRRNQSSEYSSRARIYKTKLEARNHGVERLSTILTSSKKTFDTVDSYTDLFNGWVSG
Larva 3 MDDISPLFYGDVRNTNRFLSSGGIRRNQSSEYSSRARIYKTKLEARNYGVERLSTILTSSKKTFDTVDSYTDLFNGWVSG

Larva 1 MFVDKNVHYTEMSSDESGRRIWNVRRAVSIKTAEGTVVWRKVITSYSCKVASELAAKSILVQFAGPIRTQSDEVPSKESI
Larva 2 MFVDKNVHYTEMSSDESGRRIWNVRRAVSIKTAEGTIVWRKVITSYSCKVASELAAKSILVHFAGPIRTQSDEVPSKESI
Larva 3 MFVDKNVHYTEMSSDESGRRIWNVRRAVSIKTAEGTIVWRKVITSYSCKVASELAAKSILVQFAGPIRTQSDEVPSKESI

Larva 1 QGDATQQSSKEENTIITRDQQQTVSEKIPSTVGDLVIASSEPTQQFRSLTNRWMPINSIRVTVNGKRNDLLAQYYIPEDF
Larva 2 QGDATQQSSKEENTIITRDQQQTVSEKIPSTVGDLVIASSEPTQQFRSLTNRWMPINSIRVTVNGKRNDLLAQYYIPEDF
Larva 3 QGDATQQSSKEENTIITRDQQQTVSEKIPSTVGDLVIASSEPTQQFRSLTNRWMPINSIRVTVNGKRNDLLAQYYIPEDF

Larva 1 LSTHAKCAPNTIPFETYVYGKYELEMKFVANGNKFQCGKVIISVKFDSYQADNINTGFQAALSRPHIMLDLSTNNEGVLK
Larva 2 LSTHAKCAPNTIPFETYVYGKYELEMKFVANGNKFQCGKVIISVKFDSYQADNINTGFQAALSRPHIMLDLSTNNEGVLK
Larva 3 LSTHAKCAPNTIPFETYVYGKYELEMKFVANGNKFQCGKVIISVKFDSYQADNINTGFQAALSRPHIMLDLSTNNEGVLK

Larva 1 VPFRYHRAFVRNQTHKTATAGVRPGKFASIYVQVLSPLQTGEGGANDMFIRPFYRYTRAEFAGMSYKVPLTQMDVIGTLI
Larva 2 VPFRYHRAFVRNQTHKTATAGVRPGKFASIYVQVLSPLQTGEGGANDMFIRPFYRYTRAEFAGMSYKVPLTQMDVIGTLI
Larva 3 VPFRYHRAFVRNQTHKTATAGVRPGKFASIYVQVLSPLQTGEGGANDMFIRPFYRYTRAEFAGMSYKVPLTQMDVIGTLI

Larva 1 SGGPTPALKDILVGVEKTLDQLGRSNNQDKPKDVSSITIIPKPRLGFPHGKGKSDAVAMRVNPVALTSFQEVSAYPDEPR
Larva 2 SGGPTPALKDILVGVEKTLDQLGRSNNQDKPKDVSSITIIPKPRLGFPHGKGKSDAVAMRVNPVALTSFQEVSAYPDEPR
Larva 3 SGGPTPALKDILVGVEKTLDQLGRSNNQDKPKDVSSITIIPKPRLGFPHGKGKSDAVAMRVNPVALTSFQEVSAYPDEPR

Larva 1 TTLDIARIWGLRSTFNWGSGDEHGKELFNTVLDPGLRFYDQDYEGQITPMEYVTGLYNFWSGPIELRFDFVSNAFHTGTV
Larva 2 TTLDIARIWGLRSTFNWGSGDEHGKELFNTVLDPGLRFYDQDYEGQITPMEYVTGLYNFWSGPIELRFDFVSNAFHTGTV
Larva 3 TTLDIARIWGLRSTFNWGSGDEHGKELFNTVLDPGLRFYDQDYEGQITPMEYVTGLYNFWSGPIELRFDFVSNAFHTGTV

Larva 1 IISAEYNRSSTNTDECQSHSTYTKTFHLGEQKSVHFTVPYIYDTVVRRNTASAYLPVTDYDKVDNVSRAQAMGIRAESKM
Larva 2 IISAEYNRSSTNTDECQSHSTYTKTFHLGEQKSVHFTVPYIYDTVVRRNTASAYLPVTDYDKVDNVSRAQAMGIRAESKM
Larva 3 IISAEYNRSSTNTDECQSHSTYTKTFHLGEQKSVHFTVPYIYDTVVRRNTASAYLPVTDYDKVDNVSRAQAMGIRAESKM

Larva 1 RVKVRVVNVLRPVASTTSTIEVLVYMRGGKNYALHGLKQSTYWPSNSVVPIDSFPPDGYDPVKPPNRSRRELASSDSDGG
Larva 2 RVKVRVVNVLRPVASTTSTIEVLVYMRGGKNYALHGLKQSTYWPSNSVVPIDSFPPDGYDPVKPPNRSRRELASSDSDGG
Larva 3 RVKVRVVNVLRPVASTTSTIEVLVYMRGGKNYALHGLKQSTYWPSNSVVPIDSFPPDGYDPVKPPNRSRRELASSDSDGG

Larva 1 KGEPVLAGSDNPHRFLPANVSNRWNEYSSAYLPRVQMDTGAKEDEDETANFSDGVTAMGFQSLDTQVSIKDILRRPVLLF
```

Larva 2 KGEPVLAGSDNPHRFLPANVSNRWNEYSSAYLPRVQMDTGAKEDEDETANFSDGVTAMGFQSLDTQVSIKDILRRPVLLF

Larva 3 KGEPVLAGSDNPHRFLPANVSNRWNEYSSAYLPRVQMDTGAKEDEDETANFSDGVTAMGFQSLDTQVSIKDILRRPVLLF

Larva 1 NHVELDPDYTGFFIPIMPPSRMMQYKSGDKETSFQRLIGRTPQAAIMNLFRFWRGSLRYTIIIHSTDGHPIYVTHVPHTG

Larva 2 NHVELDPDYTGFFIPIMPPSRMMQYKSGDKETSFQRLIGRTPQAAIMNLFRFWRGSLRYTIIIHSTDGHPIYVTHVPHTG

Larva 3 NHVELDPDYTGFFIPIMPPSRMMQYKSGDKETSFQRLIGRTPQAAIMNLFRFWRGSLRYTIIIHSTDGHPIYVTHVPHTG

Larva 1 NRVYGLMKVNNLHEYTKVPIFGCGLTTEMIIPSVNPSICVEVPFDTENNWAVTFDEDAQRNYSWRDKGDTVTGHLVVTPV

Larva 2 NRVYGLMKVNNLHEYTKVPIFGCGLTTEMIIPSVNPSICVEVPFDTENNWAVTFDEDAQRNYSWRDKGDTVTGHLVVTPV

Larva 3 NRVYGLMKVNNLHEYTKVPIFGCGLTTEMIIPSVNPSICVEVPFDTENNWAVTFDEDAQRNYSWRDKGDTVTGHLVVTPV

Larva 1 VSVYMSVWVEAGDDFEVSNFYGPPSVKTNDWNYAFSDEHVQVQMDDSTERVYDEGNQVYYYPPPPKPEGFSLNNVRTSVS

Larva 2 VSVYMSVWVEAGDDFEVSNFYGPPSVKTNDWNYAFSDEHVQVQMDDSTERVYDEGNQVYYYPPPPKPEGFSLNNVRTSVS

Larva 3 VSVYMSVWVEAGDDFEVSNFYGPPSVKTNDWNYAFSDEHVQVQMDDSTERVYDEGNQVYYYPPPPKPEGFSLNNVRTSVS

Larva 1 TLCNMIGKVVTPDRAVKTALCATPYFGSAYMTATTLDAIGSMQNTVTGAAHHLTASVDARLEQLSAKFGDSIDVITTAVK

Larva 2 TLCNMIGKVVTPDRAVKTALCATPYFGSAYMTATTLDAIGSMQNTVTGAAHHLTASVDARLEQLSAKFGDSIDVITTAVK

Larva 3 TLCNMIGKVVTPDRAVKTALCATPYFGSAYMTATTLDAIGSMQNTVTGAAHHLTASVDARLEQLSAKFGDSIDVITTAVK

Larva 1 EAIGKISSGMFNMVNYTGYCIDVILDILVAWIDRSWTAVGVGIIRFVTKVLGLGAISKVMHMATTFGQLIARVYEPPRPV

Larva 2 EAIGKISSGMFNMVNYTGYCIDVILDILVAWIDRSWTAVGVGIIRFVTKVLGLGAISKVMHMATTFGQLIARVYEPPRPV

Larva 3 EAIGKISSGMFNMVNYTGYCIDVILDILVAWIDRSWTAVGVGIIRFVTKVLGLGAISKVMHMATTFGQLIARVYEPPRPV

Larva 1 VQAPPPTEATLTGALAGILGTLMGVYISPLSGGSYFKNLMLRMTSSAGPSYLVGVLRFVEATFNTVKDMTLNALGYVSPE

Larva 2 VQAPPPTEATLTGALAGILGTLMGVYISPLSGGSYFKNLMLRMTSSAGPSYLVGVLRFVEATFNTVKDMTLNALGYVSPE

Larva 3 VQAPPPTEATLTGALAGILGTLMGVYISPLSGGSYFKNLMLRMTSSAGPSYLVGVLRFVEATFNTVKDMTLNALGYVSPE

Larva 1 NAALKMLSGTSTTIQNFITDAQLITTEANAALVGHPSFRAKYWNTVMQAYQIQKLLLTVPQSSASPILSRLCSDVIRNSN

Larva 2 NAALKMLSGTSTTIQNFITDAQLITTEANAALVGHPSFRAKYWNTVMQAYQIQKLLLTVPQSSASPILSRLCSDVIRNSN

Larva 3 NAALKMLSGTSTTIQNFITDAQLITTEANAALVGHPSFRAKYWNTVMQAYQIQKLLLTVPQSSASPILSRLCSDVIRNSN

Larva 1 EKFIDISSSPVRYEPFVICIEGPAGIGKSEIVETLATELLKGVNLKRPHSGATYFRMPGSRFWSGYRDQPVVVYDDWANL

Larva 2 EKFIDISSSPVRYEPFVICIEGPAGIGKSEIVETLATELLKGVNLKRPHSGATYFRMPGSRFWSGYRDQPVVVYDDWANL

Larva 3 EKFIDISSSPVRYEPFVICIEGPAGIGKSEIVETLATELLKGVNLKRPHSGATYFRMPGSRFWSGYRDQPVVVYDDWANL

Larva 1 TEPQALMQQISELYQLKSTSTFIPEMAHLEEKKIRGNPLIVILLCNHAFPDSAVTNMSLEPSAIYRRRDVLLYAERKPEY

Larva 2 TEPQALMQQISELYQLKSTSTFIPEMAHLEEKKIRGNPLIVILLCNHAFPDSAVTNMSLEPSAIYRRRDVLLYAERKPEY

Larva 3 TEPQALMQQISELYQLKSTSTFIPEMAHLEEKKIRGNPLIVILLCNHAFPDSAVTNMSLEPSAIYRRRDVLLYAERKPEY

Larva 1 EGVSLRDMSVNEQTTFAHLNFYKYKDSTNSASCTSKPVGYELTKDWLVAKFAKWHAQEQIKVQRRMENIRAGMYDAEVGS

Larva 2 EGVSLRDMSVNEQTTFAHLNFYKYKDSTNSASCTSKPVGYELTKDWLVAKFAKWHAQEQIKVQRRMENIRAGMYDAEVGS

Larva 3 EGVSLRDMSVNEQTTFAHLNFYKYKDSTNSASCTSKPVGYELTKDWLVAKFAKWHAQEQIKVQRRMENIRAGMYDAEVGS

Larva 1 LRLEDPFSLYYSVSSE**V**IENNEDVTTGFLPSEILAFECKRIANVIDAHQSSVREIVIPDEPKDPFVTTQGDLAGVFMGAA

Larva 2 LRLEDPFSLYYSVSSE**I**IENNEDVTTGFLPSEILAFECKRIANVIDAHQSSVREIVIPDEPKDPFVTTQGDLAGVFMGAA

Larva 3 LRLEDPFSLYYSVSSE**I**IENNEDVTTGFLPSEILAFECKRIANVIDAHQSSVREIVIPDEPKDPFVTTQGDLAGVFMGAA

Larva 1 LGRVV**M**EKVCSFSSELINYA**I**DWIISKHNVTHECCVCKETKGISWYCLDSAQLAPQATHYMCNSCMVASREANREVVQCP

Larva 2 LGRVV**L**EKVCSFSSELINYA**V**DWIISKHNVTHECCVCKETKGISWYCLDSAQLAPQATHYMCNSCMVASREANREVVQCP

Larva 3 LGRVV**L**EKVCSFSSELINYA**V**DWIISKHNVTHECCVCKETKGISWYCLDSAQLAPQATHYMCNSCMVASREANREVVQCP

Larva 1 MCRSPNFERWGTYQQMTGITIVGRALIMGLITVDKGVNVLRRMLGGTFGAMYAAIMRIAATLHPSMSERTADLLRMTGAL

Larva 2 MCRSPNFERWGTYQQMTGITIVGRALIMGLITVDKGVNVLRRMLGGTFGAMYAAIMRIAATLHPSMSERTADLLRMTGAL

Larva 3 MCRSPNFERWGTYQQMTGITIVGRALIMGLITVDKGVNVLRRMLGGTFGAMYAAIMRIAATLHPSMSERTADLLRMTGAL

Larva 1 VDMSEYTVRELQHVVTQIDDPFESDCE**E**DDV**D**TSKVHWRDIVTFDFEEDVARSLMRREREITNIPCLHILLGGALHHVSYR

Larva 2 VDMSEYTVRELQHVVTQIDDPFESDCE**D**DDV**G**TSKVHWRDIVTFDFEEDVARSLMRREREITNIPCLHILLGGALHHVSYR

Larva 3 VDMSEYTVRELQHVVTQIDDPFESDCE**D**DDV**G**TSKVHWRDIVTFDFEEDVARSLMRREREITNIPCLHILLGGALHHVSYR

Larva 1 DGGYNVPNGGTMVRVPELPCTSDCYFSDMEAFKSFAQRYKEEKKIEIQSHLLGFINNQHSHDYYRKRVPRIFQPHWMRAD

Larva 2 DGGYNVPNGGTMVRVPELPCTSDCYFSDMEAFKSFAQRYKEEKKIEIQSHLLGFINNQHSHDYYRKRVPRIFQPHWMRAD

Larva 3 DGGYNVPNGGTMVRVPELPCTSDCYFSDMEAFKSFAQRYKEEKKIEIQSHLLGFINNQHSHDYYRKRVPRIFQPHWMRAD

Larva 1 EDLALEISNITA**S**GWYQRVGDSFVNYRTLIVAAAGLVMAVGSIFGMYKFFSIGTDPARVEFVPSGDEITRNLKRTTRTLQ

Larva 2 EDLALEISNITA**N**GWYQRVGDSFVNYRTLIVAAAGLVMAVGSIFGMYKFFSIGTDPARVEFVPSGDEITRNLKRTTRTLQ

Larva 3 EDLALEISNITA**N**GWYQRVGDSFVNYRTLIVAAAGLVMAVGSIFGMYKFFSIGTDPARVEFVPSGDEITRNLKRTTRTLQ

Larva 1 RTRTERPHFQQVNEHPPLDSVVKKYVARNYITISLYKPAGRVKLTACGIYGTVALLPRHYVRAIKEAWEKSVKITITPAL

Larva 2 RTRTERPHFQQVNEHPPLDSVVKKYVARNYITISLYKPAGRVKLTACGIYGTVALLPRHYVRAIKEAWEKSVKITITPAL

Larva 3 RTRTERPHFQQVNEHPPLDSVVKKYVARNYITISLYKPAGRVKLTACGIYGTVALLPRHYVRAIKEAWEKSVKITITPAL

Larva 1 LEHEEHVYTYDAADFTISESTDLAIWVLSPSFGMFKDIRKFIATDEDLSKPITTEGSLLLAPTNRNPVLKEQSIEILGLQ

Larva 2 LEHEEHVYTYDAADFTISESTDLAIWVLSPSFGMFKDIRKFIATDEDLSKPITTEGSLLLAPTNRNPVLKEQSIEILGLQ

Larva 3 LEHEEHVYTYDAADFTISESTDLAIWVLSPSFGMFKDIRKFIATDEDLSKPITTEGSLLLAPTNRNPVLKEQSIEILGLQ

Larva 1 NEMQVSELNGTVFYANDVICYDYSQQGACGSLCFLSRSQRPIVGMHFAGRGEGSCGEGYGVILTKEAIGDILALKSQPVV

Larva 2 NEMQVSELNGTVFYANDVICYDYSQQGACGSLCFLSRSQRPIVGMHFAGRGEGSCGEGYGVILTKEAIGDILALKSQPVV

Larva 3 NEMQVSELNGTVFYANDVICYDYSQQGACGSLCFLSRSQRPIVGMHFAGRGEGSCGEGYGVILTKEAIGDILALKSQPVV

Larva 1 QLEDWEGPSLEEAKIILPETNVSYIGAVTKEQTPYLPKKTKIRPSLIQNVGDLHPVSEPCILDKTDSRYQHDDTPLVAGC

Larva 2 QLEDWEGPSLEEAKIILPETNVSYIGAVTKEQTPYLPKKTKIRPSLIQNVGDLHPVSEPCILDKTDSRYQHDDTPLVAGC

Larva 3 QLEDWEGPSLEEAKIILPETNVSYIGAVTKEQTPYLPKKTKIRPSLIQNVGDLHPVSEPCILDKTDSRYQHDDTPLVAGC

Larva 1 KKHGRLTVDFGTTRVES**V**KEALWDGWLSKMKPLVV**R**PKLLTPEEAASGFPDIQYYDPMILNTSAGFPYVATEKKRKEDYI

Larva 2 KKHGRLTVDFGTTRVES**A**KEALWDGWLSKMKPLVV**K**PKLLTPEEAASGFPDIQYYDPMILNTSAGFPYVATEKKRKEDYI

Larva 3 KKHGRLTVDFGTTRVES**A**KEALWDGWLSKMKPLVV**K**PKLLTPEEAASGFPDIQYYDPMILNTSAGFPYVATEKKRKEDYI

Larva 1 VFERNENEQPIGATIDPGVLEEMKRKSELRKRGVQPITPFIDTLKDERKLPEKVRKYGGTRVFCNPPIDYIVSMRQYYMH

Larva 2 VFERNENEQPIGATIDPGVLEEMKRKSELRKRGVQPITPFIDTLKDERKLPEKVRKYGGTRVFCNPPIDYIVSMRQYYMH

Larva 3 VFERNENEQPIGATIDPGVLEEMKRKSELRKRGVQPITPFIDTLKDERKLPEKVRKYGGTRVFCNPPIDYIVSMRQYYMH

Larva 1 FVAAFMEQRFKLMHAVGINVQSTEWTLLASKLLAKGNNICTIDYSNFGPGFNAQIAKAAMELMVRWTMEHVEGVNEIEAY

Larva 2 FVAAFMEQRFKLMHAVGINVQSTEWTLLASKLLAKGNNICTIDYSNFGPGFNAQIAKAAMELMVRWTMEHVEGVNEIEAY

Larva 3 FVAAFMEQRFKLMHAVGINVQSTEWTLLASKLLAKGNNICTIDYSNFGPGFNAQIAKAAMELMVRWTMEHVEGVNEIEAY

Larva 1 TLLHECLNSVHLVSNTLYQQKCGSPSGAPITVVINTLVNILYIFVAWETLVGSKERGQ**T**WESFKQNVELFCYGDDLIMSV

Larva 2 TLLHECLNSVHLVSNTLYQQKCGSPSGAPITVVINTLVNILYIFVAWETLVGSKERGQ**I**WESFKQNVELFCYGDDLIMSV

Larva 3 TLLHECLNSVHLVSNTLYQQKCGSPSGAPITVVINTLVNILYIFVAWETLVGSKERGQ**T**WESFKQNVELFCYGDDLIMSV

Larva 1 TDKYKDVFNALTISQFLAQYGIVATDANKGDEVEAYTTLL**N**STFLKHGFRPHEVYPHLWQSALAWSSINDTTQWIWECAD

Larva 2 TDKYKDVFNALTISQFLAQYGIVATDANKGDEVEAYTTLL**K**STFLKHGFRPHEVYPHLWQSALAWSSINDTTQWIWECAD

Larva 3 TDKYKDVFNALTISQFLAQYGIVATDANKGDEVEAYTTLL**N**STFLKHGFRPHEVYPHLWQSALAWSSINDTTQWIWECAD

Larva 1 LKLATRENCRAALYQAHGHGSTVYNRFKQQVNQALIKRKIQPIALSWKEIDDLFYPEISY

Larva 2 LKLATRENCRAALYQAHGHGSTVYNRFKQQVNQALIKRKIQPIALSWKEIDDLFYPEISY

Larva 3 LKLATRENCRAALYQAHGHGSTVYNRFKQQVNQALIKRKIQPIALSWKEIDDLFYPEISY

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Chapter 5

General Discussion

General Discussion

During the last half century, decline in abundance and richness of bees have been reported in some areas of the globe. Dramatic losses of managed honey bees (*Apis mellifera*) have been reported in the USA, many European and few Asian countries, respectively. However, managed honey bee populations increased in China, Argentina and Spain (Aizen & Harder 2009). Also, large reductions of managed African honey bees have not been reported yet (Pirk et al. 2016). Hence, evidence of reduced honey bee populations is irregular and incomplete. There is no evidence of reduced *A. mellifera* density on the Australian continent or in adjacent countries.

Reduced abundance and richness of solitary bees and bumble bees have been shown in several studies with some species becoming extinct. However, these studies are restricted to few European countries and the USA (Biesmeijer et al. 2006; Burkle, Marlin & Knight 2013; Cameron et al. 2011; Carvalheiro et al. 2013; Goulson, Lye & Darvill 2008). The decline of bee populations coincided with a global increased in the demand for pollination (more than 300%; Aizen & Harder 2009) and raised concerns about possible 'pollination crises'. Therefore, research on decline of bees and its causes has increased in recent years. So far, factors associated with decline of honey bees densities are pesticides, habitat loss, the parasitic Varroa mite (*Varroa destructor*), and RNA viruses (Carreck 2016; Goulson et al. 2015).

Worldwide, RNA viruses are well studied in honey bees, but very little is known in bumble bees and solitary bees. In Australia, no work has been done in Australian native bees, with exception of a single study performed in 1980's. In this study, 23 colonies of stingless bees were examined for RNA viruses, and no evidence was found on presence of seven viruses in these bee (Anderson & Gibbs 1982).

In this study, we aimed to address some questions about the prevalence and origin of RNA viruses in Australian native bees, and in the large earth bumblebee, *Bombus terrestris*, which was introduced to the Australian island state of Tasmania in the early 1990s, and is still restricted to that island. Firstly, we studied whether Australian native bees carry RNA viruses and deduced whether these viruses were introduced together with *A. mellifera* or were already present in the native bees. Our results showed that while Australian native bees carry some RNA viruses, BQCV and SBV were introduced into Australia with *A. mellifera* (Chapter 2).

Secondly, we investigated the presence and prevalence of RNA viruses in *B terrestris* and wild *A. mellifera* collected in Tasmania (Chapter 3). We found that the two species shared *Kashmir bee virus* (KBV) and S*acbrood virus* (SBV), but *Black queen cell virus* (BQCV) was only present in *A. mellifera*. There has been speculation that the Tasmanian *B. terrestris* were introduced from New Zealand where DWV is present and therefore, we tested the bees from Tasmania for DWV. However, we did not find this virus in either *A. mellifera* or *B. terrestris* on Tasmania.

Last, we analysed the RNA interference (RNAi) immune response in South Australian SBV-infected *A. mellifera* larvae by deep sequencing of small interfering RNAs (siRNAs; Chapter 4). We were able to successfully use this technique to reconstruct the entire SBV genome from several larvae and found that different strains were present in the same apiary.

Our results provide new insights into the epidemiology of bee RNA viruses. Association between occurrences of RNA viruses in native bees from South Australia (SA) with presence of managed *A. mellifera* point to the latter as the source of viral inoculum, both in Australian native bees and managed and unmanaged hives of *A. mellifera*. This suggests that minimising the contact between managed *A. mellifera* and both wild *A. mellifera* and native bees, could reduce the potential for spillover of RNA viruses and potentially mitigate the impact of these viruses on bee health in Australia. This can be achieved by restricting beekeeping or placement of managed *A. mellifera* hives to certain areas.

Goulson & Hughes (2015) also proposed mitigation strategies to reduce spillover of parasites and pathogens from honey bees to wild bees. In that context, this study provides important information for Australia that can be used to prepare for the potential incursion of the Varroa mite. Our data speculates that there might also be an impact on native bees to which the Australian flora is highly adapted. Monitoring the prevalence of parasites in wild bees is fundamental in order to identify and address issues such as endemic diseases at an early stages of occurrence (Goulson & Hughes 2015). Our research data could therefore be used as an initial estimate on prevalence of RNA viruses in SA native bees. Also, the techniques we used can be used for development of future studies including monitoring of RNA viruses in native bees.

We found that SA native bees and Tasmanian *B. terrestris* carry RNA viruses (Chapters 2 & 3). However, infection of the wild bees in our study was not demonstrated. That is, we could not test for replication of virus particles in SA native bees. Detection of the viral replicative form using strand-specific RT-PCR was performed in *A. mellifera* (Chapter 3), but we were not confident that the assay was optimised in terms of sensitivity and therefore were not confident that all negative results were accurate. In order to develop a more powerful way to assess the presence and infectivity of RNA viruses in native bees, we used symptomatic SBV-infected *A. mellifera* larvae as a model to extract and sequence siRNAs (Chapter 3). This was useful for confirming an immune response (and hence evidence of virus entry into larval cells) and obtaining an entire viral genome for analysis. Hence, high-throughput sequence of siRNAs would be a useful technique for further investigation on infectivity of any RNA virus that activates the RNAi pathway of bees; for our purposes this could include native bees and Tasmanian *B. terrestris*. Since this technique allows non-specific generation of complete genome of RNA viruses, it could also be used for detection and characterisation of new viruses in *A. mellifera* as its complete genome is available.

Adult Tasmanian *B. terrestris* and SA native bees did not show any disease symptoms. This might be interpreted to suggest that RNA viruses only occur in these bees as covert infections. However, this is not necessarily the case, as it is also possible that symptomatic adult bees are uncommon. In support of this, symptomatic worker *A. mellifera* are occasionally observed in the field and are nearly only detected in managed hives (Chapter 1 Table 2). In addition, the few researchers that occasionally open native bee nests to investigate brood numbers may not have been paying much attention to infected brood (K. Hogendoorn pers. com.). We derive further support of covert infection in native bees from our finding that RNA viruses did occur in areas where managed hives are absent.

Overseas DWV has been found to infect native bees in countries where the Varroa mite is present. While it is known that the mite has increased the infectivity of DWV for *A. mellifera*, we don't know whether the increased virulence and the consequential increased prevalence has also affected native bees. This needs to be urgently investigated overseas, to allow better predictions of the risks associated with the introduction of DWV, for example with imported bee semen.

Our data strongly suggest that the RNA viruses we detected did not originate from the non-*Apis* bees we assessed. Nevertheless, we cannot discount the occurrence of other RNA viruses in non-*Apis* bees that have not been identified or characterised. Given the potential for introduction of Varroa-mite into Australia, the potential for native bees to augment or partially replace pollination services provided by *A. mellifera*, and the requirement of native bees for efficient pollination of many native plant species, further research on possible occurrence of different viruses in non-*Apis* bees is warranted. Furthermore, other pollinators (e.g. wasps and flies) also carry RNA viruses (Levitt et al. 2013; Singh et al. 2010); therefore, it would be important to understand the role of these pollinators in transmission of these viruses, and if they carry their own RNA viruses distinct from *A. mellifera* and/or other bees.

Prevalence of RNA viruses in Tasmanian *A*. *mellifera* was higher than in *B*. *terrestris*, and SBV prevalence in both species were linked. However, we did not investigate presence and prevalence of RNA viruses in Tasmanian native bees. The co-occurrence of *A*. *mellifera*, *B*. *terrestris* and native bees in Tasmania provides a unique opportunity to further investigate the distribution and dynamics of RNA viruses in bees. This is because Tasmania provides a discrete landmass where the introductions of the exotic species *A*. *mellifera* (mid 1880s) and B. *terrestris* (early 1990s) are relatively recent but occurring far enough apart to draw conclusions about the origin of RNA viruses in Tasmania, as well as which host introduced them and if/how interspecies transmission has occurred.

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(Literature Review & General Discussion)

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