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

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Abstract – A number of bee RNA viruses, including *Deformed wing virus* (DWV), are so far unreported from Australia. These viruses can be introduced together with imported live honey bees (*Apis mellifera*) and their products, with other bee species, and 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 prevalence of RNA viruses in *B. terrestris* and *A. mellifera* collected in south-eastern Tasmania. We did not find DWV in either *A. mellifera* and *B. terrestris*. However, both bee species shared *Kashmir bee virus* (KBV) and *Sacbrood virus* (SBV), but *Black queen cell virus* (BQCV) was detected only in *A. mellifera*. This reinforces the importance of ongoing 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 attention due to their putative association with Colony Collapse Disorder (CCD), and as one of the causes of reduced health and size of populations of European honey bee (*Apis mellifera* L.; vanEngelsdorp et al. 2009; 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, recent studies demonstrate their presence in many species of pollinators including bees of the genus *Bombus* (Singh et al. 2010; Levitt et al. 2013) with suggested ongoing exchange between species (McMahon et al. 2015; Radzeviciute et al. 2017).

Observations and experiments have suggested that bumble bees can acquire virus from wild or managed *A. mellifera* (McMahon et al. 2015), and from managed *Bombus* spp. (Graystock et al. 2014). Such inter-species horizontal transmission is strongly linked to co-foraging (i.e. foraging on the same flowers; Singh et al. 2010; Mazzei et al. 2014). For instance, under controlled conditions, *Israel acute paralysis virus* (IAPV) was transmitted

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between *A. mellifera* and *B. impatiens* Cresson through co-foraging (Singh et al. 2010).

Horizontal transmission can also occur via other invertebrate vectors. For instance, the Varroa mite (*Varroa destructor* Anderson & Trueman) facilitates intra-species transmission between bees of the genus *Apis* from which they suck haemolymph. However, *V. destructor* is not directly responsible for inter-species transmission of viruses between *Apis* and non-*Apis* bees, and among non-*Apis* bees (Manley et al. 2015). Nonetheless, the presence of *V. destructor* in honey bee populations 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). Varroosis of *A. mellifera* leads to an increase in prevalence and titres of different RNA viruses including DWV (Mondet et al. 2014). Indeed, interaction between the *V. destructor* 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 et al. 2012; Mondet et al. 2014). Contrary to claims by Wilfert et al. (2016; see discussion), it is clear that both DWV and the Varroa mite are absent from Australia (Rosenkranz et al. 2010; Roberts et al. 2015).

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 (Manley et al. 2015; Wilfert et al. 2016). 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 probably acquired from *A. mellifera* pollen used for their rearing (Goulson and Hughes 2015; Graystock et al. 2016).

The bumble bee *B. terrestris* L. was first discovered in the Australian island state of Tasmania in 1992 (Semmens et al. 1993), and 9 years later it had become established in most regions of the island including remote areas (Hingston et al. 2002). The bees were accidentally or intentionally introduced from New Zealand (NZ) without the

approval of the Australian government (Semmens et al. 1993). The recent detection of DWV in *A. mellifera* from NZ (Mondet et al. 2014) raised fears that new RNA viruses, mainly DWV could have been introduced into Tasmania with *B. terrestris*. Before introduction of *B. terrestris* in 1992, only *Sacbrood virus* (SBV) and *Chronic bee paralysis virus* (CBPV) were reported in Tasmanian honey bees (Hornitzky et al. 1990). In *A. mellifera* on mainland Australia, *Black queen cell virus* (BQCV), *Kashmir bee virus* (KBV), *Cloudy wing virus* (CWV), CBPV, and SBV were detected and reported in different studies (e.g. Hornitzky 1987; Anderson and Gibbs 1988).

Here, we investigate (i) which RNA viruses are present in Tasmanian *B. terrestris*, (ii) which viruses are shared between Tasmanian *B. terrestris* and *A. mellifera*, and (iii) the possible geographic origin of RNA viruses found in *B. terrestris*.

2. MATERIALS AND METHODS

2.1. Sample collection

A total of 200 foraging bees (100 *B. terrestris* and 100 *A. mellifera*) were collected between January and February of 2015 across southeast Tasmania (Figure 1). This region was chosen because *B. terrestris* is commonly found throughout, and because it includes the capital city of Hobart where *B. terrestris* was first found (Hingston et al. 2002). Of the 100 *B. terrestris*, 29 were queens and 71 were workers. For *A. mellifera*, only workers were collected. The bees were collected from flowers at 15 sites on the main island of Tasmania, and at five sites on Bruny Island. The sites were separated by at least 2 km. Workers of *B. terrestris* normally do not forage further than 1 km away from their nests (Osborne et al. 1999), and their maximum flight distance is 2.5 km (Hagen et al. 2011). Therefore, we expected that *B. terrestris* collected at different sites, originated from different colonies. On the other hand, *A. mellifera* can forage close to but also at distances of more than 10 km from their hive (Beekman and Ratnieks 2000). We consider *B. terrestris* flight distance instead of *A. mellifera* because it was our main object of study.

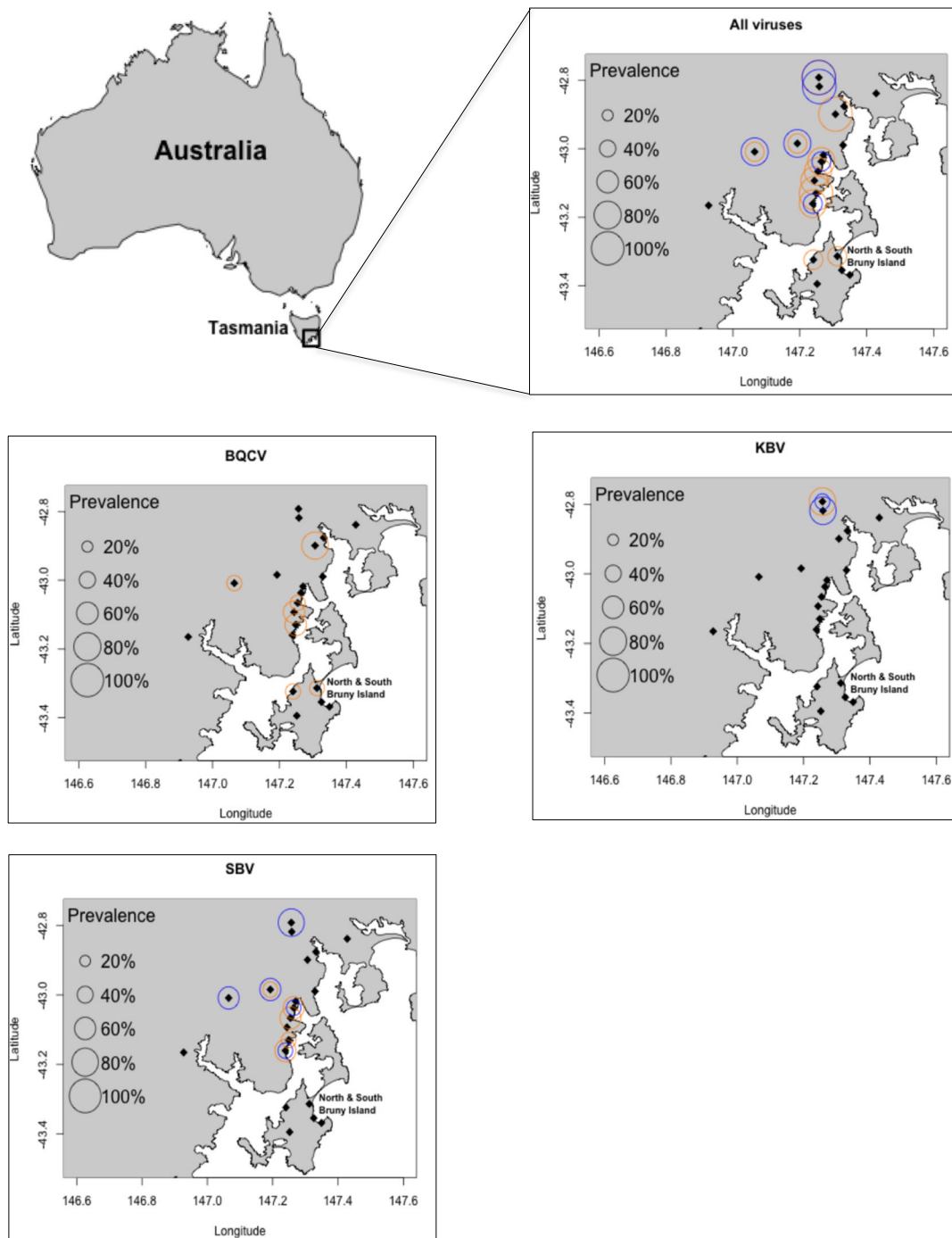


Figure 1. Prevalence (%) and geographical distribution of combined viruses as single response, BQCV, KBV, and SBV. Black diamonds represent collection sites. Blue and orange circles represent virus detection in *Bombus terrestris* and *Apis mellifera*, respectively. Sizes of circles correspond to prevalence (%) of the viruses. Prevalence of all viruses include a purple circle, which results from combination of blue and orange circles of the same size.

At each site, five specimens per species were captured individually with a net while foraging on flowers of the same plant or on adjacent plants of the same species. Specimens were stored on ice while in the field. After, they were individually 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 a 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 a 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 eight RNA viruses, namely *Acute bee paralysis virus* (ABPV), BQCV, CBPV, DWV, IAPV, KBV, SBV, and *Slow bee paralysis virus* (SBPV). To achieve this, 0.5–5 μg of total RNA was reverse transcribed using random hexamers (Bioline) and a Bioscript reverse transcriptase kit (Bioline) according to the manufacturer's guidelines. Two microliter 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 5 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, internal, and positive 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 et al. 2000). This was used as house-keeping gene to control for the quality of the RNA extracted from bees and the correct application of each batch of RT-PCRs. All primers used in this study are listed in [Online Resource -ESM1](#). Positive samples were confirmed by sequencing of PCR products.

2.4. Sequence and phylogenetic analyses

PCR products were purified using the Ultra-Clean PCR Clean-Up Kit (MOBIO Laboratories, Inc.) and sent for bidirectional Sanger sequencing. Sequences were then matched against those in the National Center for Biotechnology Information (NCBI) and used for phylogenetic analysis. Detailed methodology of phylogenetic analyses can be found in [Online Resource -ESM2](#).

2.5. Statistical analysis

All statistical analyses were executed in R version 3.2.1 (R Core Team, 2016). The prevalence of all RNA viruses and 95% confidence intervals were calculated for both bee species using the function `epi.prev` (R library 'epiR' package version 0.9-62). 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 prevalence of SBV and BQCV for *A. mellifera* and SBV for *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 patterns. Spatial autocorrelation of KBV was not determined because of the low prevalence ($n < 5$) in both *B. terrestris* and *A. mellifera*. 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 adjacent sites (spatial clustering; Fürst et al. 2014).

In order to investigate the impact of the 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 BQCV in *B. terrestris*, and low prevalence of KBV ($n < 5$) in both bee species. The best model was selected based on the Akaike Information Criterion (AIC).

3. RESULTS

The 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 10–27%) and 0% (95% CI 0–12%), 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 I). 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 I; Figure 1) and the prevalence did not differ between bee species (KBV: $P = 1$; SBV: $P = 0.21$). BQCV was detected only in *A. mellifera* (Table I; Figure 1) and its higher prevalence was statistically significant ($P = 0.0003$). It was also the most prevalent virus in *A. mellifera* (Table I). None of the *B. terrestris* or *A. mellifera* individuals testing positive to RNA virus showed obvious symptoms of virus infection. ABPV, CBPV, DWV, IAPV, and SBPV were not detected in our samples.

No evidence of spatial clustering was found for BQCV ($P = 0.84$) and SBV ($P = 0.17$) in *A. mellifera*. Similarly, for *B. terrestris*, no spatial clustering was detected for SBV ($P = 0.99$) and latitude and longitude did not influence its prevalence (Table II). Mapping the prevalence of all RNA viruses as single response, in order to avoid underestimation of disease prevalence, suggested that prevalence of RNA viruses in *B. terrestris* and *A. mellifera* was associated (Figure 1). The prevalence of SBV in *B. terrestris* was positively

associated with its occurrence in *A. mellifera* (Table II) and vice versa (Online Resource -ESM3).

The KBV and SBV sequences from *B. terrestris* and *A. mellifera* from Tasmania clustered together, distinct from *A. mellifera* sequences from other countries (Online Resources -ESM4 and 5). The BQCV sequence from *A. mellifera* from Tasmania clustered with a sequence from UK (Online Resource -ESM6), in agreement with historical reports of Europe as source of *A. mellifera* imported to Australia (Hopkins 1886; Glatz 2015). The KBV sequences from Tasmania and other countries clustered together, and separately from IAPV sequences (Online Resource -ESM4).

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*. Although our results suggest BQCV to be less amenable to inter-species transmission through co-foraging than SBV, it is known that BQCV can infect different *Bombus* spp. including *B. terrestris* (Peng et al. 2011; Radzeviciute et al. 2017) possibly through ingestion of virus contamination, a common pathway of intra- and inter-species transmission of RNA viruses. BQCV and SBV were also detected during a recent survey of RNA viruses in Australian *A. mellifera* in Tasmania (Roberts et al. 2015). Our data confirm previous studies that have shown susceptibility of *B. terrestris* to SBV infection (Singh et al. 2010; Levitt et al. 2013).

Our findings suggest that indirect transmission of KBV and SBV by co-foraging might have occurred between *B. terrestris* and *A. mellifera* in Tasmania. This is supported by the result that prevalence of SBV in *A. mellifera* was a good predictor of prevalence of the same virus in *B. terrestris*, and vice versa as well as by the result that both species appeared to carry the same strains of these two viruses in Tasmania, which were distinct from viral sequences obtained for both species in other regions. Similar local sharing of viruses between *A. mellifera* and *B. terrestris* has been found for DWV (Fürst et al. 2014; McMahon et al. 2015; Radzeviciute et al. 2017).

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

Species of bee	BQCV	KBV	SBV	Overall	<i>P</i> value
<i>A. mellifera</i>	11[6–19]	3[1–8]	9[5–16]	21[14–30]	0.04*
<i>B. terrestris</i>	–	4[2–9]	9[5–16]	12[7–20]	

Viral transfer between these species is likely to be brought about through co-foraging because they are both polylectic and forage on a wide variety of native and introduced plants in Tasmania (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 et al. 1993).

It seems likely that BQCV, KBV, and SBV were introduced into Tasmania with *A. mellifera*, as they are also found on the mainland of Australia. Moreover, on the basis of our data, we cannot exclude introduction with *B. terrestris*. For BQCV, introduction with *A. mellifera* is likely, as we did not detect it in *B. terrestris*, despite the fact that it has been reported in *Bombus* spp. elsewhere (Singh et al. 2010; Levitt et al. 2013). Whether *B. terrestris* introduced into Tasmania were carrying SBV and KBV, or whether they became infected after arrival or both remains unclear. Because SBV was reported in the island before the introduction of *B. terrestris* (Hornitzky et al. 1990), it likely was introduced with *A. mellifera*. However, because SBV and KBV were both present in *A. mellifera* from NZ before introduction of *B. terrestris* into Tasmania (Anderson 1985), the possibility of virus introduction onto Tasmania via NZ-derived

B. terrestris remains open. KBV was detected in Australian mainland *A. mellifera* after their introduction into Tasmania (Anderson and Gibbs 1988; Anderson 1991) and the data are therefore inconclusive. Therefore, we are cautious in postulating the origin or the original host of all viruses we detected, and we cannot discard the possibility that other native bees or insects could have been the primary host of the viruses. The RNA viruses found by us have also been detected in solitary bees and other Hymenoptera such as wasps and ants, as well as non-hymenopteran insects such as butterflies, beetles, and flies (Singh et al. 2010; Levitt et al. 2013).

Interestingly, for the first time in Tasmania, KBV was detected at very low prevalence in both *A. mellifera* and *B. terrestris*. The other recent survey of RNA viruses did not detect KBV in Tasmania, although it detected IAPV in Tasmania and the Australian mainland (Roberts et al. 2015).

The phylogenetic tree generated from KBV and IAPV sequences from Tasmania and other countries showed that they are distinct since these viruses clustered separately. Literature and public sequence databases continue to misclassify IAPV and KBV although they are classified as different virus species by the International Committee on Taxonomy of Viruses (King et al. 2012). Phylogenetic analysis of IAPV and KBV RNA-

Table II. 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	<i>z</i> value	<i>P</i> value
<i>B. terrestris</i> virus prevalence	SBV	Intercept	– 278.99	674.63	– 0.41	0.68
		<i>Apis</i> 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

dependent RNA polymerase sequences suggest that some IAPV isolates from Australia, France, and Russia may have been incorrectly classified as KBV in earlier studies (Palacios et al. 2008; de Miranda et al. 2010). This emphasises problems with online sequence databases and molecular virus diagnostics (de Miranda et al. 2010), particularly with regard to design of specific diagnostic primers.

Importantly, we did not detect bees carrying ABPV, CBPV, DWV, IAPV, and SBPV. ABPV and SBPV have not been reported in Australia before (Allen and Ball 1996; Roberts et al. 2015); therefore, our data, although limited, support that Australia remains free of these viruses. Similarly to our results, CBPV was not detected during the most recent survey of RNA viruses of hived Australian *A. mellifera* (Roberts et al. 2015). We cannot exclude the possibility of occurrence of different RNA virus variants that did not amplify with 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.

Contrary to a recent report (Singh et al. 2010; Wilfert et al. 2016), our results together with those of Roberts and Anderson (2013) and Roberts et al. (2015) suggest the absence of DWV in Australia. Roberts et al. (2015), who did not detect DWV, performed a very extensive survey of managed hives around Australia and they used very sensitive techniques for detection of viruses (deep sequencing). Hence, it is hard to reconcile the detection of DWV in a small sample of *A. mellifera* imported from Australia with the lack of detection in a large number of recently tested *A. mellifera*. Intriguingly, Roberts et al. (2015) reported a small number of short nucleic acid sequences with 77–92% similarity to DWV; these were considered to be different viruses but closely related to DWV. DWV occurs as two main variants, genotype A (conventional) and genotype B, which is closely related to *Varroa destructor virus* (VDV-1) and around 82% identical to conventional DWV (Ongus et al. 2004; Martin et al. 2012). Therefore, we cannot eliminate the risk of occurrence of DWV genotype B, although Roberts et al. (2015) reported short nucleic acid sequences

78% similar to VDV-1. Indeed, the presence and variability of DWV-like sequences in Australian bees need further research in order to unambiguously define DWV status in Australia. At present, we consider IAPV but not DWV to be present in Australia.

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. The combination of both *V. destructor* and DWV causes serious harm to the health of *A. mellifera*, whether introduced simultaneously or separately. In New Zealand, while DWV and *V. destructor* were introduced separately (Zhang 2000; Mondet et al. 2014), the impact of combination of both in *A. mellifera* colony losses was significant (Mondet et al. 2014). Therefore, it is important to consider separate as well as simultaneous introduction of DWV and *V. destructor*. Manley et al. (2015) highlight the importance of maintaining the few current Varroa-free areas free of *V. destructor*. 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 diligently monitored to avoid introduction of DWV into Australia. In addition, measures to avoid the independent introduction of the Varroa mite or of associated viruses should be explored. Such measures include prioritisation of the use of native bees for pollination, reduction, or cessation of the importation of non-native bees, and monitoring the prevalence of RNA viruses in native bees to allow early detection of disease-associated decline in populations (Goulson and Hughes 2015).

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AUTHOR CONTRIBUTIONS

EF: designed and performed experiment and data analyses, and wrote the paper. KH, KH, RG: experiment design, technical assistance, and edition of the paper. AH: sample collection and edition of the paper. All authors read and approved the final manuscript.

Virus à ARN présents dans des bourdons (*Bombus terrestris*) et des abeilles (*Apis mellifera*) introduits en Tasmanie

Apis mellifera / *Bombus terrestris* / introduction de colonies / virus/ Australia

Gemeinsames Auftreten von RNA-Viren in nach Tasmanien eingeführten Hummeln (*Bombus terrestris*) und Honigbienen (*Apis mellifera*)

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

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