Pathogen spill-over:

directionality and impact on biodiversity and the ecosystem service of pollination

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To all forgotten woman in science

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Émilie du Châtelet

Ester Lederberg

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Lise Meitner

Margarete von Wrangell

Hedy Lamarr

Jane Colden

Jane Cooke Wright

... and many more

Abstract

Over recent years, changes in the abundance of insects have been repeatedly reported in literature. Particularly the decline of insects like wild bees has gained wide attention due to their important contribution to the ecosystem service of pollination. Several studies undertaken in North America and Europe have revealed a significant reduction in the distribution and the relative abundance of many bumble bee species over the last decades. Causes of this decline, as well as for other insects, are thought to be habitat loss and degradation, fragmentation, intensification of land use including pesticide applications, and climate change as well as pathogens. Especially pathogens that spill over from domesticated animals into the wildlife are often considered major causes of biodiversity decline. Due to their biology, RNA viruses are one of the most important groups involved in these cross-species transmission events. Such viruses associated with honey bees that spill over and infect other wild bee species are suggested as one out of many possible reasons for wild bee decline. Deformed wing virus (DWV) in particular seems to be highly virulent for honey bees and is the major factor for high overwinter mortality. Already many studies have shown viruses like DWV or black queen cell virus (BQCV) at high prevalence in a variety of screened bumble bee species as well as in other wild bee species (reviewed in Chapter I). However, despite their presence in wild bees, the transmission of DWV and other viruses between honey bees and bumble bees as well as the effect of these viruses on the different potential hosts is not fully understood. To evaluate the possible harm by these viruses on wild bees, I performed highly controlled experiments with well characterised viral inocula, which I describe in the second and third chapters of this thesis.

After reviewing the literature in Chapter I, I tested for viral transmission between host bee species in Chapter II. I experimentally infected bees and checked for DWV genotype A (DWV-A) transmission within and between *Apis mellifera* and the widespread bumble bee *Bombus terrestris* when both were housed individually or together in the laboratory. I included all possible combinations of donor/recipient host species to reveal transmission routes. In the first experiment of this chapter, virus donor and virus recipient bees were kept together in one cage, while in the second experiment I kept virus donors and recipients in separate cages but transferred daily the feeding tube from the infected to the noninfected cage. Transmission could only happen in this case via the food or as virions (virus particles) that had been deposited on the feeding tubes. When I checked viral loads after 7 days and additionally after 14 days, I was able to record the same pattern in the first and in the second experiment. Both experiments showed spill-over from honey bees to bumble bees. When bumble bees, on the other hand, were infected, I did not record viral transmission to either bumble bees or honey bees.

In Chapter III, I tested for viral virulence after spill-over from honey bees to bumble bees. Infections with DWV-A, DWV genotype B (DWV-B) and BQCV were established

experimentally in commercially reared bumble bee workers (*B. terrestris*) with inocula containing one of the viruses. Workers were either fed once individually with 1×10^9 genome equivalents or were injected with 1µl of 1×10^7 genome equivalents of the respective inoculum. After infection, bees were kept in small groups in incubators at 30°C and were checked every day for mortality. While I show that the three viral isolates from honey bees readily replicate within *B. terrestris*, effects on the mortality of the bumble bee workers were negligible, even under starvation conditions.

I could show in my experiments that viruses can be transmitted from honey bees to bumble bees and, moreover, that they are able to replicate in bumble bees. RNA viruses are known for their propensity to infect new host species because of their high mutation rates that lie in the lack of a proof-reading function in their polymerase proteins and resulting error-prone replication cycles. This is underlined by my results, which support the assumption that honey bees are the original (reservoir) host of these viruses. Nevertheless, possible adaptation by a virus over time to a new host species, and resulting deleterious effects, need to be considered. As long as it is not clear whether these viruses have a negative effect on bumble bees or other wild bees, these important species deserve to be protected from the transmission of viruses from honey bees. Wild bees are an important component of our ecosystems and are essential for the maintenance of natural processes through provision of the ecosystem service of pollination. My results highlight the potential threat of viral spill-over from honey bees to novel wild bee species, though they also underscore the importance of additional studies on this and other wild bee species under field-realistic conditions to evaluate whether pathogen spill-over has a negative impact on wild bee individuals and population fitness. The ongoing decline of wild bees is likely to threaten the stability of the ecosystem service of pollination and, as a result, the stability of the entire ecosystem as well as the maintenance of food production.

Keywords: *Apis mellifera*, *Bombus terrestris*, deformed wing virus, black queen cell virus, spill-over, spill-back, RNA virus, multi-host pathogen, virulence

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General introduction

Parasitism

A significant proportion of the world's species are parasites and it is even assumed that most of the species on earth are parasites (Windsor, 1998). Measured by how often this lifestyle evolved and taking into account how many parasitic species are presently in existence, parasitism is one of the most successful life strategies by living organisms. The diversity of parasites is enormous, with variation across a wide range of body sizes due to the use of this lifestyle by a wide range of organisms like prokaryotes, viruses, fungi, protozoa, nematodes, annelids, insects, crustaceans and mites (Schmid-Hempel, 2011). Parasitism is defined as a relationship in which one of the participants, the parasite, either harms its host and reduces its fitness or in some sense lives at the expense of the host (Roberts & Janovy, 2009). Parasites can be infectious agents that cause diseases in the host and show different degrees of pathogenicity. But a parasite is not simply a pathogen or not, as was often postulated in history (Méthot & Alizon, 2014). The interaction between the host and its parasite is a complex system in which not solely the parasite causes a disease but also the ecological circumstances and host responses may module the interaction. The boundaries between commensalism, parasitism and mutualism are fluid, and these interactions may best be viewed as a continuum rather than as fixed categories in nature (Méthot & Alizon, 2014; Van Baalen & Jansen, 2001).

Some of the most pernicious and pathogenic parasites are arguably RNA viruses. They lead to some of the most harmful diseases ever encountered by humans. Aside from mortality, the economic costs associated with viral infections of humans or livestock are enormous. One prominent example is the outbreak of the severe acute respiratory syndrome corona virus (SARS-CoV; 2002–2003). Although only 8437 people were known to have been infected during the pandemic outbreak, with 813 deaths (WHO), the global economic loss has been estimated to be around US$50 billion. In the same way, the epidemic of foot-and-mouth disease in the UK in 2001 (due to foot-and-mouth disease virus, FMDV) was a serious blow to British agriculture, resulting in the death or culling of over 4 million domestic animals (cattle and sheep) and a total estimated cost of around £3.1 billion (Holmes, 2009; Thompson *et al.*, 2002). But these economic losses are slight when compared to the SARS-CoV 2 pandemic that started in March 2020 and that had within the first year 106 million confirmed cases with 2.3 million deaths worldwide. The overall global economic loss is not yet clear but vague calculations assume that the long-term economic cost of the SARS-CoV 2 pandemic may amount to $7trillion (Organisation for Economic Co-operation and Development; state: 16.09.2020).

RNA viruses are often highlighted as the most common class of pathogens found as new causes of emerging infectious diseases (EIDs) in humans, with 2 to 3 novel viruses being discovered each year (Rosenberg, 2015). Most emerging RNA viruses are zoonoses like the already mentioned corona viruses and jumped from mammals or birds to humans (Wolfe *et al.*, 2007). The transmission of pathogens from the reservoir population to a new host is called "pathogen spill-over" or "the spill-over effect" (Daszak *et al.*, 2000; Power & Mitchell, 2004). Such zoonoses can also potentially "spill back" and infect again local wildlife. In that case, domesticated animals represent reservoir hosts and a source of pathogens that spill over into wild species, e.g. *Mycoplasma ovipneumoniae* that spills over from domestic sheep and goats into bighorn sheep (*Ovis canadensis*) (Cassirer *et al.*, 2018).

The reason why RNA viruses are arguably the most important group involved in zoonotic disease transmission from wildlife lies in the biology of these pathogens (Parrish *et al.*, 2008; Woolhouse *et al.*, 2013). RNA viruses have higher probabilities to infect new host species because of their high mutation rates that lie in the lack of a proof-reading function in their polymerase proteins and resulting error-prone replication cycles (Holmes, 2009). Whereas dsDNA viruses tend to result in persistent infections and experience long-term virus-host co-divergence, in which the evolutionary history of the virus tracks that of its host species over many millions of years, RNA viruses tend to establish only short-term acute infections in their hosts and evolve by a mechanism of cross-species transmission (Holmes, 2009). They are often horizontally transmitted by aerosols, body fluids, faecal material, or vectors, which makes it easy for them to come into contact with a new host. Due to their extremely high rate of evolution, an immense number of deleterious but also beneficial mutations are generated in a short time, increasing adaptability to a new environment, the new host system (Moya *et al.*, 2004).

It is assumed that there are host phylogenetic constraints in the probability that viruses will be able to jump between closely related hosts and successfully establish productive infections because closely related hosts will be likely to present a comparable environment to the parasite (Holmes, 2009). This has been shown for several host-virus systems like for example for rabies virus in bats (Streicker *et al.*, 2010) or for the Drosophila sigma viruses in Diptera species (Longdon *et al.*, 2011). However, of course geographical overlap is always a requirement. Since the industrial revolution, global trade has immensely increased and changed the geographical distribution of many species. Global translocation of plants, animals and the products derived from them into new regions, e.g. for commercial or conservation reasons, increases the probability of a pathogen jumping from one host species to another (Cunningham, 1996; Daszak *et al.*, 2001). Moreover, increased human population and urbanization push wildlife into crowded habitat patches which together with climate change alter transmission conditions (Guth *et al.*, 2020; Tompkins *et al.*, 2015).

One prominent host-parasite system is between honey bees, the ectoparasitic mite *Varroa destructor* (Traynor *et al.*, 2020) (henceforth 'varroa') and its associated viruses. This mite

was originally linked to the Asian honey bee *Apis ceranae* (Oudemans, 1904) but jumped around the 1950s from its native host onto the European honey bee, *Apis mellifera* and managed to successfully increase in prevalence nearly globally within 50 years due to trading practices and honey imports (Rosenkranz *et al.*, 2010). From Asia, varroa spread to Europe, America and more recently also to New Zealand and Hawaii (Martin *et al.*, 2012; Oldroyd, 1999; Ruttner & Ritter, 1980; Traynor *et al.*, 2020). The only major beekeeping country that remained free from *V. destructor* currently is Australia (Roberts *et al.*, 2017). While varroa inflicts only limited damage to *A. ceranae* colonies due to several host defense mechanisms which have established during a long period of coevolution in a stable host-parasite relationship (Rath, 1999), *A. mellifera* is a naive new host with inadequate innate defences. Varroa is the greatest threat to the health of the European honey bee and it is linked to their worldwide decline. It has been accused of being responsible for the death of millions of colonies (Le Conte *et al.*, 2010; Rosenkranz *et al.*, 2010). Yet the severe impact on colony health is most likely caused indirectly by the several viruses that the mite vectors. The mite's ability to act as a reservoir for certain RNA viruses, where viruses are able to replicate prior to its vectoring, has established a new extremely efficient transmission route, leading to a rapid global spread and re-emergence of several bee viruses (Genersch & Aubert, 2010; Martin, 2001; Shen *et al.*, 2005; Sumpter & Martin, 2004; Tentcheva *et al.*, 2004).

Bee viruses

Around 20 viruses have already been associated with *A. mellifera*, of which many are positive single-stranded (+ss) RNA viruses. (Chen & Siede, 2007; Ellis & Munn, 2005; Grozinger & Flenniken, 2019; McMahon *et al.*, 2018; McMenamin & Flenniken, 2018). These known viruses mostly revealed themselves through physical, behavioural or developmental symptoms induced in their hosts. Due to ongoing research and development in detection methods like next-generation and third-generation sequencing, the number of viruses with known occurrence in honey bees is nowadays much higher and will likely increase in the coming years (Beaurepaire *et al.*, 2020; Bigot *et al.*, 2017; Daughenbaugh *et al.*, 2021; Levin *et al.*, 2019). A majority of these viruses will most likely be asymptomatic or at least without severe effects on honey bees. It will therefore be important to test if these viruses really infect the bees they were isolated from and whether these viruses are virulent.

One of the most common viruses in *A. mellifera* is the deformed wing virus (DWV), a positive single-stranded RNA virus from the Iflaviridae family within the Picornavirales order which is tightly linked to varroa infestation (de Miranda & Genersch, 2010; Martin *et al.*, 2012; Škubnik *et al.*, 2017). The co-occurrence of DWV and the mite causes severe symptoms in *A. mellifera.* DWV affects several tissues of a bee's body like the midgut and the brain (Fievet *et al.*, 2006; Shah *et al.*, 2009; Yue & Genersch, 2005). Typical symptoms are shrunken, deformed wings, discolouration and a shortened abdomen (de Miranda &

Genersch, 2010; Koziy *et al.*, 2019; Tehel *et al.*, 2019). Furthermore DWV increases mortality in adults (McMahon *et al.*, 2016) while it slightly elevates pupal death (Tehel *et al.*, 2019). Also histological changes in the mandibular and hypopharyngeal glands in bees with DWV are discussed (Koziy *et al.*, 2019). It has also been found that DWV accelerate the temporal polyethism schedule, although the host's behavioural repertoire is not reduced (Benaets *et al.*, 2017; Natsopoulou *et al.*, 2016).

Several studies indicate that DWV is one of the most prevalent viral pathogen detected in honey bees, with a worldwide distribution and a minimum average of 55% of colonies/apiaries infected across 32 countries (Martin & Brettell, 2019). Prior to *V. destructor*, DWV was associated with mostly asymptomatic infections (Bailey & Ball, 1991; Genersch & Aubert, 2010; Möckel *et al.*, 2011) with occasional outbreaks of acute disease (Forsgren *et al.*, 2012). The mite not only led to a global distribution of DWV in honey bees (Wilfert *et al.*, 2016), it also seems that the presence of varroa over time is reducing DWV diversity by selecting for particular variants, possibly leading to the predominance of a single DWV strain accompanied by an increase in viral titre (Martin *et al.*, 2012).

DWV nowadays comprises at least three distinct genotypes (Kevill *et al.*, 2017; Mordecai *et al.*, 2016; Ongus *et al.*, 2004), two of which (DWV-A and DWV-B) are widespread and differentially virulent in adult honey bees (McMahon *et al.*, 2016) while they do not exhibit clear differences in honey bee pupae (Dubois *et al.*, 2020; Norton *et al.*, 2020; Tehel *et al.*, 2019). DWV-B, which shares 84% sequence identity with DWV-A, was initially called *Varroa destructor* virus 1 (VDV-1) since it was extracted from the mite *V. destructor.* Recent studies have shown that DWV-B has become the most common variant in Europe (Kevill *et al.*, 2019; Manley *et al.*, 2019). Also in South Africa DWV-B is the dominant variant (de Souza *et al.*, 2021). In North-America DWV-A remains the most common genotype; but over the past 20 years, DWV-B prevalence has increased from 3% in 2010 to 65% in 2016 in the United States (Kevill *et al.*, 2019; Ryabov *et al.*, 2017) and also on the Hawaiian Islands the DWV-A variant is getting potentially replaced by genotype B (Grindrod *et al.*, 2021) while 10 years ago Hawaii honey bees were dominated by genotype A (Martin *et al.*, 2012). The reasons for the continual global increased prevalence of DWV-B over DWV-A has not been completely solved yet. Factors could be differences in replication rates or in the virulence of the two genotypes. DWV-B replicates to higher viral loads when injected into either pupae (Dubois *et al.*, 2020; Norton *et al.*, 2020; Tehel *et al.*, 2019) or adults (McMahon *et al.*, 2016) than DWV-A. Also in colonies infected with both genotypes, DWV-B loads were higher than DWV-A loads (Kevill *et al.*, 2019; Ryabov *et al.*, 2017). Pupal mortality could be an important factor limiting the reproduction of the virus. *Varroa destructor* feeds on host pupae within sealed brood cells and therefore DWV is just able to reproduce when the infected brood is emerging as adults. Pupal-induced mortality would lead to the failure of mite reproduction and limit viral transmission. There are contradictory studies about pupal mortality in DWV infected pupa, with studies showing no difference between DWV-A and DWV-B (Dubois *et al.*, 2020; Tehel *et al.*, 2019) and a study showing higher pupal

mortality in genotype A than in B (Norton *et al.*, 2020). Lower mortality of DWV-B in pupae may favour the increasing prevalence of DWV-B globally.

Another common virus is black queen cell virus (BQCV) (Spurny *et al.*, 2017). Even if highly prevalent and globally distributed (Ellis & Munn, 2005; Mondet *et al.*, 2014; Tentcheva *et al.*, 2004), it is one of the least understood honey bee viruses. Like DWV it is a single-stranded RNA virus but belongs to the family Dicistroviridae. It was first isolated from queen prepupae and pupae, found dead in their cells (Bailey & Woods, 1977). It can be frequently found in *A. mellifera* workers (McMahon *et al.*, 2015) but it persists mostly asymptomatically albeit chronically in bee colonies.

BQCV in a high dose (10^9 genome equivalents) fed to worker larvae causes significant mortality at 6 days post-infection (Doublet *et al.*, 2015) while fed to adults no difference in mortality was detectable compared to control bees (Doublet *et al.*, 2015; Retschnig *et al.*, 2014). Other studies also investigated the effects of injected BQCV directly into the hemolymph. It resulted in an increased mortality, increased viral titre as well as significant changes in the expression of key components of the RNAi pathway (Al Naggar & Paxton, 2020; Remnant *et al.*, 2019; Tehel *et al.*, 2020). If BQCV would be transmitted by the mite and would become vector-mediated, the effects on the health of honey bee colonies would drastically change. BQCV is also associated with co-infection by *Nosema* spp., a microsporidian found in the gut of honey bees (Bailey *et al.*, 1981; Bailey *et al.*, 1983). There is evidence that a co-infection of BQCV with *Nosema ceranea*, results in a synergistically increased mortality in adult honey bees (Doublet *et al.*, 2015).

Honey bee associated viruses in wild bees

Both, DWV and BQCV, even if mainly associated with honey bees, are frequently found in non-*Apis* bees (reviewed in Chapter I of this thesis (Tehel *et al.*, 2016)). Several studies show the presence of both viruses frequently in a lot of different *Bombus* species and solitary bee species (Jones *et al.*, 2021; Martin & Brettell, 2019; McMahon *et al.*, 2015; Melathopoulos *et al.*, 2017; Murray *et al.*, 2019; Radzevičiūtė *et al.*, 2017). The presence of honey bee apiaries seems to be highly correlated with an increase in viral prevalence in wild bees (Alger *et al.*, 2019a; Pritchard *et al.*, 2021), while the varroa infestation status of an apiary did not show an effect on the wild bee viral load (Brettell *et al.*, 2020). Even in other insects like wasps, ants and cockroaches, DWV is present (Bailes *et al.*, 2018; Brettell *et al.*, 2019, 2020; Payne *et al.*, 2020; Sébastien *et al.*, 2015). The at least 64 species in which the virus has been detected span eight orders of arthropods, from the class of insects as well as of arachnids, resulting in a very wide host range (Martin & Brettell, 2019). Usually studies reporting the presence of DWV in other insects use PCR primers developed originally using honey bee isolates. The number of individuals of species carrying other DWV variants adapted to other host species, which cannot be detected with these primers, is unknown. More studies using next-generation sequencing could reveal a till now unrecognised genetic virus diversity.

But one has to be careful when extrapolating from the presence of the virus to conclude viral replication and pathology.

DWV or BQCV in wild bee species or other insects could reflect just a contamination of the exterior of a wild bee by the virus as well as viruses in the gut from the consumption of contaminated food resources. To test if the virus is able to replicate in the host from which it was obtained, the detection of the replicative intermediate form of the virus genome, the negative strand, is a good method to provide additional evidence about the infectivity of a +ss RNA virus like DWV or BQCV to the host. DWV replication has been shown in numerous bumble bee species like *Bombus huntii* (Li *et al.*, 2011), *B. impatiens* (Levitt *et al.*, 2013; Li *et al.*, 2011), *B. lapidarius* (Fürst *et al.*, 2014), *B. lucorum* (Fürst *et al.*, 2014), *B. monticola* (Fürst *et al.*, 2014), and *B. vagans* (Levitt *et al.*, 2013) as well as for other Apidae like the stingless bees *Melipona colimana* and *Trigona fluviventris* (Morfin *et al.*, 2020; Tapia-González *et al.*, 2019) and in solitary bees like *Osmia cornuta* (Mazzei *et al.*, 2014), *Andrena haemorrhoa* (Radzevičiūtė *et al.*, 2017) and *Colletes* spp. (Tapia-González *et al.*, 2019). For BQCV the negative strand has been found in several bumble bees like *B. soroeensis*, *B. laesus*, *B. vestalis* and *B. huntii* (Peng *et al.*, 2011; Radzevičiūtė *et al.*, 2017) as well as in other Apidae like *Anthophora plumipes* or stingless bees as well as in members of the Megachilidae e.g. *Osmia bicornis* (Morfin *et al.*, 2020; Radzevičiūtė *et al.*, 2017; Tapia-González *et al.*, 2019). But caution is advised, as active replication should not be equated with symptomatic infection and does not necessarily represent a threat to the host individual or the host population.

Symptomatic infection has only been described in *B. terrestris* and *B. pascuorum* (Genersch *et al.*, 2006) and just two studies have to date evaluated experimentally the virulence of DWV for *B. terrestris* (Fürst *et al.*, 2014; Graystock *et al.*, 2015b). Fürst *et al.* (2014) found that a mixed DWV-A/DWV-B inoculum fed to *B. terrestris* workers kept under lab conditions led to a significant increase in mortality over 20 days while Graystock *et al.* (2015b) injected DWV derived from *B. terrestris* fat bodies into conspecific, caged workers and revealed a 50% increase in mortality. From other RNA viruses that are common in honey bees and are also frequently detected in wild bees, few studies already reveal effects on *B. terrestris* fitness. Slow bee paralysis virus (SBPV) infection showed no effect on hosts under satiated conditions but under starvation conditions resulted in significant virulence, with infected bees 1.6 times more likely to die at any given time point (Manley *et al.*, 2017). Also Kashmir bee virus (KBV) and Israeli acute paralysis virus (IAPV) infections resulted in significantly decreased offspring production as well as, in the case of KBV, in a slower colony start-up, both viruses reducing the fitness of *B. terrestris* (Meeus *et al.*, 2014). More controlled experiments under laboratory conditions that prevent or control for conflating factors, are urgently required if one wants to understand the impact of these viruses on wild bees. Particularly necessary are experiments that shed light on the effects on fitness and reproductive success, as well as experiments taking other stressors like climate change, pesticides, malnutrition or habitat destruction into account. Seemingly benign pathogens

can reveal severe effects when resources are too low to maintain defence and the host is not able to compensate these with increased resource intake or when other stressors add additional costs (Moret & Schmid-Hempel, 2000).

Even if it is known that wild bees harbouring a lot of honey bee associated viruses, the process of virus transmission is not fully understood. Fürst *et al.* (2014) could for the first time show active local transmission of DWV between honey bees and different species of bumble bees. DWV-A and DWV-B were regionally distinct but identical in honey bees and the different collected bumble bee species at the same site. This finding speaks strongly for DWV jumping between honey bees and bumble bees, leaving open questions about directionality or transmission routes. Other studies also showed in the meantime that the same viral strains of DWV are shared across bee taxa (Radzevičiūtė *et al.*, 2017) or even insect taxa (Brettell *et al.*, 2020).

Transmission of viruses

Pathogen transmission is a complex field and can happen through a lot of different routes. These routes are split in two major types: vertical and horizontal transmission. Vertical transmission is the transmission of infectious agents to the next generation. In honey bees this is primarily from queens to eggs, where the virus can be either inside the egg or on the surface of it. Also transmission from the male via sperm could be a possibility. While this transmission route just appears within species, horizontal transmission (transmission of pathogens among individuals of the same generation) can also occur between species and is therefore of relevance for the transmission between honey bees and wild bees.

One highly likely route for viral transmission between bee species is the shared use of floral resources (Burnham *et al.*, 2021; Dalmon *et al.*, 2021; Durrer & Schmid-Hempel, 1994; McArt *et al.*, 2014; Singh *et al.*, 2010). Flowers are usually visited by a wide range of pollinators and other species and are therefore predestined to be hotspots of pathogen transmission, but very little empirical work has addressed this (Koch *et al.*, 2017). Different characteristics like floral traits such as nectar availability or inflorescence architecture may thereby affect the transmission rate (Durrer & Schmid-Hempel, 1994). Transmission via flowers has been already shown for bee pathogens like e.g. the trypanosomatid gut parasite, *Crithidia bombi* (crithidia). *Bombus lucorum* workers foraging on contaminated flowers had a rate of infection of 39% (Durrer & Schmid-Hempel, 1994). Furthermore shared flower use by *A. mellifera* and *B. terrestris* allowed interspecific transmission of three bee pathogens (Graystock *et al.*, 2015a). New studies about viral transmission via flowers are becoming more numerous but are still just scratching the surface of how this potential pathogen route is contributing to the disease dynamics of this system (Alger *et al.*, 2019b; Burnham *et al.*, 2021).

It is assumed that infected bees while visiting flowers probably contaminate the flower surface during foraging and other pollinators thereafter visiting the flowers pick up the deposited pathogens. Most likely, pollinators contaminate the pollen and the nectar by pathogenic particles shed via their faeces. In honey bee faeces, viruses like DWV, BQCV and others have already been identified (Chen *et al*., 2006b, 2014; Hung, 2000; Ribière *et al*., 2007) and it has also been shown that honey bees deposit viruses on flowers (Alger *et al*., 2019b). A study with *B. impatients* showed that bumble bees also defecate on floral surfaces during foraging (Bodden *et al*., 2019), when they also seem to deposit viruses (Burnham *et al*., 2021). For another pathogen, *C. bombi*, it is known that an infection of the host even increases the defecation rates on flowers (Figueroa *et al*., 2019). Flower shape also seems to have a huge impact on defecation (Adler *et al*., 2018; Bodden *et al*., 2019; Figueroa *et al*., 2019; Graystock *et al*., 2015a). This could be due, among other things, to the fact that floral morphology is known to influence the amount of time spent on a flower by a visitor (Zung *et al*., 2015). The longer the handling time and extraction time of a visitor on a flower, the greater the likelihood that the visitor will defecate. But the location of the faeces also seem to play a role in the resulting infection intensity (Figueroa *et al*., 2019). On the one side, it seems important that floral morphologies result in an overlap of spots where pollinator faeces are deposited and acquired compared to morphologies for which deposition and acquisition may be disjointed (Figueroa *et al*., 2019). On the other side, the location of the deposition on a flower has a huge impact on the contact of the virus to potentially harmful phytochemicals and UV radiation. Until pathogens are picked up by a new host, they are exposed without external defence to environmental conditions. *C. bombi* viability decreases when treated with pulsed UV radiation (Naughton *et al*., 2017). And also *C. bombi* on sun-exposed flowers had a shorter survival time than on shaded plants, probably due to UV radiation but also due to temperature, and/or increased desiccation (Figueroa *et al*., 2019). When *Nosema apis* spores were exposed to UV light, their viability was reduced by 51.6% within 5 min and 99% after 45-min exposure (Zheng *et al*., 2014). Air-dried *N. apis* spores also lost viability after 3 days at 40°C. While heating *N. apis* spores for at least 15 min at 60°C completely kills the parasite (Cantwell & Shimanuki, 1970), treatment at 60°C maintained a good viability of the spores of *N. ceranae* (Fenoy *et al*., 2009). This better adaption of *N. ceranae* than *N. apis* to high temperatures has been shown in several studies (Fenoy *et al*., 2009; Martín-Hernández *et al*., 2009). Despite the close relationship between the two species, their resistance to external environmental factors is very different. So even if for viruses in general several studies have shown an inactivating function of solar UV (McLeod *et al*., 1977), there is still a lack of data that show how stable viruses like DWV or BQCV are outside a host when confronted with abiotic factors like UV radiation, humidity or temperature. Nevertheless, it would be reasonable to assume that the survival and infectiousness of pathogens can vary greatly across floral parts. Pathogens on the outside of the corolla and on the flower bracts, which are generally more exposed to UV light, would therefore have lower chances of survival (Figueroa *et al*., 2019). But also biotic

factors can have a huge impact. Depending on where on a flower pathogens are deposited, phytochemicals in pollen or nectar can interact with the pathogen.

Nectar often contains secondary plant metabolites that provide a broad spectrum of antimicrobial protection for the flower. These compounds can have either an effect on the pathogen directly or can impact immune processes in the bees when consumed together with the pathogen. To date, several studies on crithidia and bumble bees give evidence that nectar secondary compounds reduce disease load in bees. These studies showed that consumtion of floral nectar containing secondary metabolites can reduce crithidia infections in already infected bumble bees (Biller *et al.*, 2015; Manson *et al.*, 2010; Richardson *et al.*, 2015). Secondary metabolites strongly reduced crithidia infection, with significant effects of alkaloids, terpenoids and iridoid glycosides ranging from 61 to 81% when mixed in sucrose solutions in ecologically relevant concentrations (Richardson *et al.*, 2015). Studies have also investigated if direct exposure of crithidia to nectar secondary compounds affects the ability to infect hosts. Crithidia pre-infection exposure to the alkaloids nicotine (Baracchi *et al.*, 2015) and gelsemine (Manson *et al.*, 2010) as well as catalpol, thymol and anabasine (Michaud *et al.*, 2019) did not reduce infectivity, even if these compounds show to have a beneficial effect post-infection. But there are also compounds that impact cell viability of crithidia: both citric acid and aucubin reduced crithidia cell counts compared with controls (Michaud *et al.*, 2019), suggesting that some floral nectars may reduce the viability of pathogens when directly exposed to them. The interactions between plant compounds and bee parasites have only been studied in a few cases till now and especially in regards to viruses there is still a huge data gap. Furthermore the pathogens will not be exposed to single plant compounds but to a whole range; they may interact additively or synergistically in reducing pathogen survival.

Besides transmission via flowers, other inter- and intra-species horizontal transmission routes of bee pathogens are also imaginable. In social bees, individuals can have very intense contact with each other and viruses can be easily transmitted within a colony of the same species. Social behaviour such as trophallaxis, the mouth-to-mouth sharing of food between colony members, is known to lead to the transmission of viruses between honey bee workers (Amiri *et al.*, 2019; Chen *et al.*, 2014). Also in the hypopharyngeal glands and in the larval food of honey bees, viruses have been detected, indicating a potential virus transmission route via brood care (Chen *et al.*, 2014; Fievet *et al.*, 2006; Shen *et al.*, 2005; Yue & Genersch, 2005). Food prosessing like packing pollen and handling nectar can also lead to contamination of the food, or to intake of viruses from contaminated food sources (Ravoet *et al.*, 2015; Singh *et al.*, 2010). Transmission via contaminated hive prodcuts like wax or honey has also been shown (Schittny *et al.*, 2020). And even simple body contact between infected individuals and others seem to be enough to spread a virus because e.g. chronic bee paralysis virus (CBPV) when topically applied to freshly broken cuticular hairs of honey bees can be readily transmitted (Bailey *et al.*, 1983).

Furthermore, in the social honey bee, virus transmission via other organisms, so called vectors, are a well-known phenomenon. Next to the intensively studied and already mentioned mite *V. destructor*, the most damaging parasite of honey bees (Rosenkranz *et al.*, 2010), the role of other endo- and ectoparasitic mites as vectors for viruses is still unclear. One example is the tracheal mite, *Acarapis woodi* (Liu, 1991). Neither *V. destructor* nor similar virus-transmitting mites are known to parasitise non-*Apis* bees; nonetheless these wild bees are host to a variety of other parasites, ranging from ecto-parasites to parasitoids that could potentially vector viruses. While DWV was successfully detected in several species of parasitoid phorid flies (Menail *et al.*, 2016), further research is still needed to establish the true potential of these species as intraspecific and potentially inter-specific vectors of bee viruses

In solitary bees, intra as well as inter-species transmission could for example happen on nest aggregations where bees of the same species are appearing in huge numbers or when old nests are reused that are maybe contaminated with viruses from the previous owner (Krunic & Stanisavljevic, 2006). The reuse of nesting sites is very common in solitary bees, but there are also rare reports of bumble bee species reusing old nests (Taylor & Cameron, 2003). Already used nests can e.g. contain pollen from the previous year harbouring contagious virus particles. Of course, these nests are exposed to environmental conditions like varying temperature and humidity, which may lead to viral degradation. More work on the stability of viruses under these conditions is needed to assess the risks posed by reused nests.

The nests can also be the place of transmission while the nest owner is still present. Several social and solitary bees are the target of brood parasites. A parasitic species may invade the nest and, while using the host's resources to its own reproductive benefit, it may get exposed to potentially infectious viruses present in the host bees, the brood or on the nest structures. Interspecies transmission can also happen when other insects like wasps or bumble bees rob honey bee hives and come into contact with viruses via honey (Genersch *et al.*, 2006). Several viruses have been detected in the food sources of hives (Chen *et al.*, 2006a; Shen *et al.*, 2005; Singh *et al.*, 2010) and it has already been shown that transmission of DWV-A in honey bees via hive products such as honey is feasible (Schittny *et al.*, 2020). But till now there is no direct evidence for virus transmission to other potential hosts through honey.

Ecological and economical importance of bees

If viruses spill over from honey bees into our wild bee fauna and show similar effects in the new hosts, these pathogens could be a huge potential threat to wild bees and also other pollinators, and therefore also for the health of humans and nature. The ecosystem service of pollination is fundamental e.g. for natural ecological processes due to the dependency of up to 87.5% of all wild plant species on pollination (Ollerton *et al.*, 2011). But also for crop

production bees are indispensable. More than 75% of our globally important crops (Klein *et al*., 2007) are pollination depended, which leads to a recent economic evaluations of this ecosystem service at between 1 to 2% of global GDP (Lippert *et al*., 2021). Especially these animal-pollinated plants are important suppliers of micronutrients such as vitamins A and C (Eilers *et al*., 2011), which are essential for a balanced human diet (Smith *et al*., 2015).

Apis mellifera, the Western honey bee, is the most intensively managed pollinator and is often used by farmers to increase their crop yield (Breeze *et al*., 2019). It has been shown that wild bees enhanced fruit set regardless of the density of honey bee colonies (Garibaldi *et al*., 2013). Also, non-bee pollinators contribute significantly to the pollination of crops and even seem to provide a unique benefit to flowers that is not provided by bees (Rader *et al*., 2016). But it is very difficult to differentiate the pollination contribution by different pollinators to the crop yield where complex parameters like e.g. visitation frequency or per visit efficiency have to be taken into account (Ne'eman *et al*., 2010). It has been shown that wild bees have a higher pollination efficiency per visit than honey bees for many pollinator dependent crops (Eeraerts *et al*., 2020; Greenleaf & Kremen, 2006; Vicens & Bosch, 2000a). Some crops are also depended on certain specialised pollinators (Rader *et al*., 2020) or require specific pollinator traits, such as tomato flowers that have to be buzz-pollinated (Buchmann, 1983), a capacity honey bees do not possess. Furthermore, compared to other pollinators, it is known that honey bees can be attracted to nearby mass-flowering crops like oil seed rape (Bänsch *et al*., 2021; Osterman *et al*., 2021) and also that their activity drops during inclement weather (Vicens & Bosch, 2000b), both of which can lead to an increased importance of non *Apis*-pollinators.

In the last two decades, however, bees have been of particular concern, with numerous reports of declines in wild and managed pollinators in temperate regions of the world (Bartomeus *et al*., 2010; Biesmeijer *et al*., 2006; Nieto *et al*., 2014; Potts *et al*., 2010; Powney *et al*., 2019; Zattara & Aizen, 2020). Causes of bee decline, as well as for other insects, are thought to revolve around the fragmentation, degradation and loss of habitat, intensification of land use (including pesticides), and climate change as well as, potentially, pathogens (Brown & Paxton, 2009; Cameron *et al*., 2011; Goulson *et al*., 2008, 2015; Vanbergen *et al*., 2013). The decline of insect pollinators threatens the stability of the ecosystem service of pollination and as a result the stability of the entire ecosystem as well as the maintenance of our food production (Potts *et al*., 2016).

Aims of this thesis

The overall aim of my thesis is to understand the general impact of honey bee associated viruses on wild bees like *Bombus terrestris*. Studying these viruses in the new system is fundamental to minimize its potential threat. Each of the following three chapters contributes in its own way to the understanding of this topic (Fig. 1).

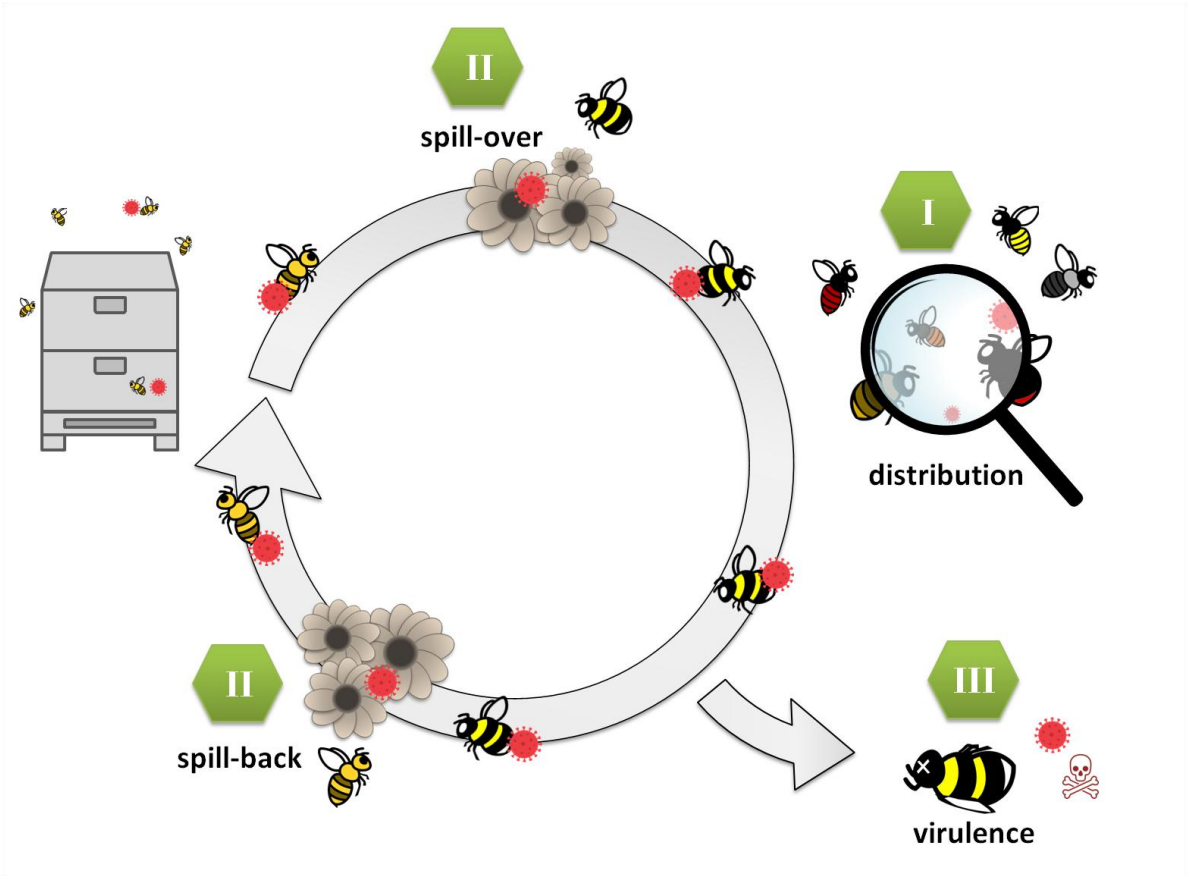

Fig. 1 Graphical representation of the three chapters presented in this thesis. **Chapter I** (*Impact of managed honey bee viruses on wild bees*) reviews the current literature and focuses on the distribution of honey bee-associated viruses in wild bees. **Chapter II** (*Experimental cross-species transmission of a major viral pathogen in bees is predominantly from honey bees to bumble bees*) is focused on spill-over and spill-back of DWV-A in the hosts *Apis mellifera* and *Bombus terrestris*. **Chapter III** (*Experimental infection of bumble bees with honey bee-associated viruses: no direct fitness costs but potential future threats to novel wild bee hosts*) explores potential effects of DWV-A, DWV-B and BQCV on the lifespan of *B. terrestris* workers.

It has been known for some years that honey bee viruses are widespread in wild bees, as revealed through several correlative studies. **Chapter I** of my thesis summarises the current research of this topic, exploring former hypotheses, pointing out weaknesses in the current evidence and highlighting research areas that may help test them. This chapter is thereby mainly focussing on DWV.

In **Chapter II** I focused on the transmission of viruses between honey bees and bumble bees. In a fully crossed experimental set up, I aimed to investigate the potential for viral spill-over from honey bees to wild bee species in the field when robbing resources from heterospecific nests or when visiting the same flowers. To do so I experimentally infected bees and checked for DWV-A transmission within and between *A. mellifera* and *B. terrestris* when both were housed individually or together in the laboratory. In the first experiment,

donor (injected with virus) and recipient (untreated) bees were mixed in the same cage, while in the second experiment donor and recipient bees were maintained in separate cages and only had contact via the same food resource.

In honey bees, the mentioned RNA viruses cause enormous damage and impose increased mortality on host honey bees. However, not much is known about the possible consequences of a virus infection for a wild bee. Therefore I explore in **Chapter III** of my thesis the effects of three different RNA viruses on the lifespan of bumble bee workers using experimental infections in the laboratory. I have therefore infected workers of the species *B. terrestris*, the Dark earth bumble bee, with the viruses DWV-A, DWV-B or BQCV in order to detect possible effects of the viruses on the lifespan of the bee. Workers of social insects, such as bumble bees, do not have their own offspring. They increase their own genetic fitness by helping to raise their siblings, either by gathering food or performing brood care. Thus, any reduction in their lifespan is a reduction in the fitness of the colony. Worker mortality is thus a highly appropriate metric with which to measure the effect of a pathogen on social insect colonies (Schmid-Hempel, 2011).

A greater knowledge of the dynamics of virus transmission can help to reveal how transmission takes place in the field. This would be an important basis for the protection of both honey bees and wild bees. Furthermore, a sound knowledge of the actual effects of the viruses on wild bee species could help to assess which wild bees might be more or less at risk and thus in need of special protection.

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Chapter I

Impact of managed honey bee viruses on wild bees

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Abstract

Several viruses found in the Western honey bee (*Apis mellifera*) have recently been detected in other bee species, raising the possibility of spill-over from managed to wild bee species. Alternatively, these viruses may be shared generalists across flower visiting insects. Here we explore the former hypothesis, pointing out weaknesses in the current evidence, particularly in relation to deformed wing virus (DWV), and highlighting research areas that may help test it. Data so far suggest that DWV spills over from managed to wild bee species and has the potential to cause population decline. That DWV and other viruses of *A. mellifera* are found in other bee species needs to be considered for the sustainable management of bee populations.

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Chapter II

Experimental cross-species transmission of a major viral pathogen in bees is predominantly from honey bees to bumble bees

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Abstract

Cross-species transmission of a pathogen from a reservoir to a recipient host species, spill-over, can have major impacts on biodiversity, domestic species and human health. Deformed wing virus (DWV) is a panzootic RNA virus in honey bees that is causal in their elevated colony losses, and several correlative field studies have suggested spill-over of DWV from managed honey bees to wild bee species such as bumble bees. Yet unequivocal demonstration of DWV spill-over is lacking whilst spill-back, the transmission of DWV from a recipient back to the reservoir host, is rarely considered. Here we show in fully crossed laboratory experiments that transmission of DWV (genotype A) from honey bees to bumble bees occurs readily, yet we neither detected viral transmission from bumble bees to honey bees nor onward transmission from experimentally infected to uninoculated bumble bees. Our results support the potential for viral spill-over from honey bees to other bee species in the field when robbing resources from heterospecific nests or when visiting the same flowers. They also underscore the importance of studies on the virulence of DWV in wild bee species so as to evaluate viral impact on individual and population fitness as well as viral adaption to new host species.

Key words: *Apis mellifera*, *Bombus terrestris*, DWV-A, RNA virus, spill-over, spill-back

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1. Introduction

Pathogen spill-over, the cross-species transmission of a pathogen from a reservoir to a recipient host species, may lead to disease emergence in the recipient host, impacting host community structure and acting as an important cause of biodiversity decline and risk to domestic animal and human health (Daszak *et al*., 2000; Lloyd-Smith *et al*., 2009; Power & Mitchell, 2004). Domesticated animals may represent reservoir hosts and a source of pathogens that spill over into wild species, e.g. *Mycoplasma ovipneumoniae* that spills over from domestic sheep and goats into bighorn sheep (*Ovis canadensis*) (Cassirer *et al*., 2018). The domestic Western honey bee (*Apis mellifera*), the world's most numerous commercial pollinator (Osterman *et al*., 2021), may also act as a reservoir host from which pathogens spill over and pose a harm to wild bee species, a worldwide threatened (Zattara & Aizen, 2020) yet economically and ecologically important taxon for their pollination services (Garibaldi *et al*., 2013).

Apis mellifera is the presumed reservoir host of deformed wing virus (DWV), a (+)ssRNA virus that has become a global emerging infectious disease of honey bees ((Wilfert *et al*., 2016); reviewed in (Grozinger & Flenniken, 2019; Martin & Brettell, 2019)) as a consequence of vector-based transmission by the exotic ectoparasitic mite *Varroa destructor* (Martin *et al*., 2012). Mounting correlation evidence supports the view that DWV spills over from domesticated honey bees into sympatric wild bee species, particularly bumble bees (*Bombus* spp.) (Alger *et al*., 2019a; Fürst *et al*., 2014; Manley *et al*., 2019; McMahon *et al*., 2015). Given DWV's high virulence in honey bees (McMahon *et al*., 2016), the ubiquity of both *A. mellifera* (Hung *et al*., 2018) and DWV (Wilfert *et al*., 2016) across terrestrial biomes, and the ongoing decline of wild bee species (Zattara & Aizen, 2020) that may be attributed to pathogen spill-over (Dicks *et al*., 2021), it is important to study DWV's potential for cross-species transmission together with the involved pathways if we are to understand and control its spread and impact.

To explain the first record of DWV in bumble bees, Genersch *et al*. (2006) hypothesised cross-species transmission during colony robbing, wherein a bumble bee robs DWV-contaminated honey or hive debris from a collapsing, infected honey bee hive. Supporting this route of horizontal transmission, DWV has been found in a range of other insect species associated with heavily DWV-infected honey bee hives on Hawaii islands (Brettell *et al*., 2019a, 2020). Yet *Bombus* spp. and other wild bee species are rarely seen at honey bee hives, arguing against the general importance of this transmission route (Yañez *et al*., 2020). For sympatric communities of bee species, shared flowers more likely act as important transmission hubs for a range of bee pathogens (McArt *et al*., 2014; Proesmans *et al*., 2021), including viruses such as DWV (Yañez *et al*., 2020). In support of this route of transmission, mounting correlational evidence relates DWV prevalence in honey bees to that in bumble bees collected on flowers at the same field sites (Alger, *et al*., 2019a; Fürst *et al*., 2014; Manley *et al*., 2019; McMahon *et al*., 2015).

Yet evidence that flowers act as transmission hubs for the virus is not unequivocal. In support of this hypothesis, Fürst *et al.* (2014) found viral sequences of co-occurring honey bees and bumble bees to be identical, suggesting on-going transmission in the field, presumably at flowers. Contrary to this, in a first experiment with the North American *Bombus impatiens* visiting DWV-infected flowers in flight cages, bumble bees failed to acquire DWV (Alger *et al.*, 2019b). Moreover, in laboratory assays with genetically labelled DWV, Gusachenko *et al.* (2020) were able to demonstrate that DWV actively replicates when injected into the Western Palearctic *Bombus terrestris* (see also Tehel *et al.*, 2020) but failed to demonstrate viral acquisition and replication by feeding, questioning the spill-over of DWV from honey bees to bumble bees through shared resource use at flowers in the field. In a more recent series of flight cage experiments with *B. impatiens* and DWV, Burnham *et al.* (2021) have now demonstrated the potential for viral transmission from honey bees to bumble bees as well as transmission from *Bombus* back to *Apis*; DWV-infected honey bees deposited DWV onto red clover (*Trifolium pratense*), *B. impatiens* foraging on DWV-infected flowers became infected with DWV, and DWV-infected *B. impatiens* themselves deposited DWV onto artificial flowers in a laboratory setting. It is unclear whether differences among studies in the potential for transmission of DWV (from *Apis* to *Bombus*) reflect the choice of *Bombus* species or experimental paradigm. Furthermore, there is a need to characterise the onward transmission of DWV from *Bombus* to conspecifics and heterospecifics, including spill-back to *Apis*, to understand the epidemiology of DWV and the impact of spill-over on host populations (Wasik *et al.*, 2019).

To characterise the potential for, and directionality of, horizontal transmission of DWV between honey bees and bumble bees, we undertook fully crossed laboratory experiments in which we inoculated either the reservoir host *A. mellifera* or the common and widespread *B. terrestris* with DWV derived from honey bees and tested for transmission to uninfected individuals. We provide unequivocal support for transmission of DWV from *Apis mellifera* to *Bombus terrestris* through physical contact and at a shared food resource but detected neither onward transmission of DWV from *B. terrestris* to *B. terrestris* nor transmission back to *A. mellifera*.

2. Material and methods

2.1. Experimental set up

We established an experimental paradigm to test for transmission of DWV within and between *A. mellifera* and *B. terrestris* by housing virus-inoculated 'donor' bees and uninoculated 'recipient' bees in metal cages (10cm x 10cm x 6cm), either mixed in one cage or in single species cages (Fig. 1). Additional details on housing bees are given in the Supplementary Methods. For viral quantification, bees where removed from the experimental cages after respectively 7 or 14 days, freeze killed and stored at -80°C till further analysis.

2.1.1 Experiment 1, mimicking *intracolony transmission*

In the first experiment, donor and recipient bees were mixed in the same cage, and cages held either a single bee species, mimicking intraspecific transmission, or both honey bees and bumble bees, mimicking interspecific transmission. Either honey bees or bumble bees were used as virus donors and as virus recipients in a fully crossed design (Fig. 1). Though this experiment permitted multiple plausible routes of transmission (faecal-oral, via trophallaxis, shared food or grooming), it maximised bee-to-bee transmission, thereby mimicking the scenario in which bees interact with conspecifics within a hive or when a heterospecific robs the honey stores of the other species.

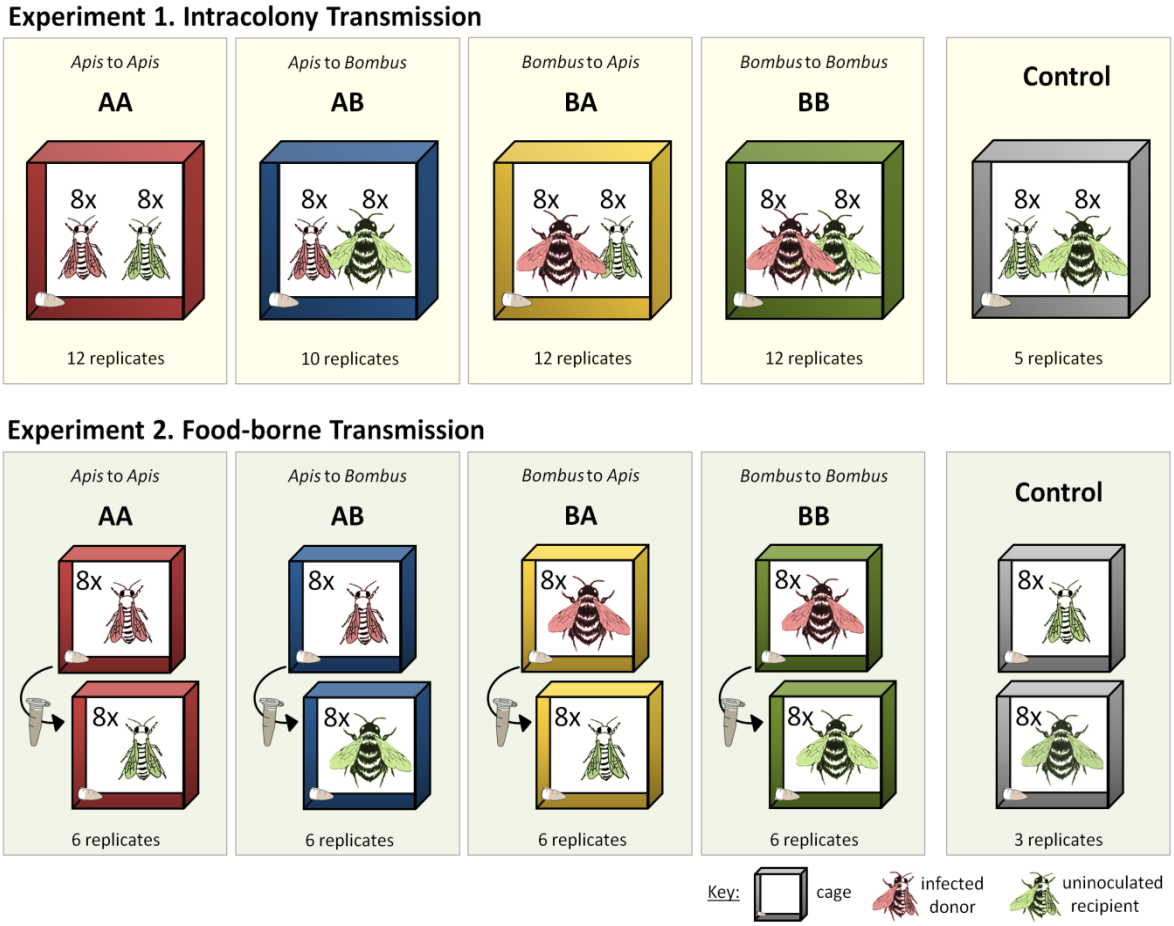

Fig. 1 Schematic of experimental set-up. Viral transmission within and between honey bees and bumble bees was investigated in two experiments in which half of the bees were experimentally inoculated by injection with 10^7 viral genome equivalents of DWV-A (red, virus donors) while the other half were uninoculated and therefore initially considered uninfected with DWV-A (green, virus recipients). In Experiment 1, mimicking *intracolony transmission*, donor (red) and recipient (green) bees were held together in one cage, permitting multiple horizontal transmission routes. In Experiment 2, mimicking *food-borne transmission*, donor (red) and recipient (green) bees were held in different cages and every 24h the feeding tube was transferred from a donor to its paired recipient cage to allow horizontal transmission only via shared food. Both the number of independent replicates (cages per treatment) and bees per cage (8x = eight bees) are given.

The donor-recipient combination *Apis* to *Apis* (treatment AA) acted as a positive test of our experimental paradigm as several of the plausible transmission routes within this treatment are well established for DWV among honey bees (Yañez *et al*., 2020). We excluded the most important *Apis-Apis* vector-based transmission route via the ectoparasitic mite *V. destructor* because it is restricted to *Apis* spp. and is not known to parasitise *Bombus* (Yañez *et al*., 2020). We did not detect *V. destructor* in any of our cages.

Under the plausible assumption, that *A. mellifera* is the reservoir host of DWV, the combination *Apis* to *Bombus* (treatment AB) mimicked virus spill-over. The combination *Bombus* to *Apis* (treatment BA) mimicked potential spill-back, though we infected our donor *B. terrestris* experimentally and therefore our protocol did not strictly fulfil the definition of spill-back, which is the transmission of a pathogen from reservoir to recipient host species and its subsequent transmission back to the reservoir host. Finally, the treatment *Bombus* to *Bombus* (treatment BB) tested for potential onward viral transmission e.g. within a *Bombus* colony (Fig. 1) of a honey bee derived viral inoculum.

Viral donor bees were generated by briefly placing individual workers on ice and then injecting them laterally between the 2nd and 3rd tergite with 10^7 viral genome equivalents of DWV genotype A using a Hamilton syringe (hypodermic needle outer diameter: 0.235mm), sufficient to guarantee infection of all individuals (Tehel *et al*., 2020). Donor bees were held for 24 hours in a cage (16 bumble bees per cage or 24 honey bees per cage) to ensure they survived physical handling (injection). Surviving bees were anaesthetised with CO_2 for 3 minutes to facilitate handling and then 8 of them were transferred to a new cage simultaneously with 8 anaesthetised but un-injected recipient bees, representing day 1 of the experiment. Donor and recipient bees were labelled by clipping 3mm off their right or left forewing respectively.

Each treatment was replicated 10-12 times (Supplementary Table S1). As a negative control, five cages each with eight untreated honey bees and eight untreated bumble bees were established and maintained as described above to check for background infection as a consequence of the experimental paradigm; none was infected (Supplementary Table S1).

Bees (donor and recipient) were analysed at day 7 post introduction by real time quantitative PCR (qPCR; see Supplementary methods) to test for transmission and to quantify DWV titre. To do so, bees were removed from the experimental cages, freeze killed and immediately thereafter stored individually at -80°C till further analysis. Aggressive behaviour between honey bees and bumble bees in heterospecific treatments was frequently observed, potentially leading to a lowered force of infection from donors to recipients. We did not, however, record a difference in viral titre in recipient bees in cages with a high versus low force of infection, measured as the number of donor bees alive at day 5 (Supplementary Fig. S1). Survival in single-species cages was high through to day 5, averaging > 70% across cages (Supplementary Table S1).

2.1.2. Experiment 2, mimicking *food-borne transmission*

In the second experiment, mimicking food-borne transmission via a common food source, donor and recipient bees were established as described for Experiment 1 but maintained in separate cages throughout. Cage establishment represented day 1 of the experiment. To mimic transmission (faecal-oral and oral-oral) at e.g. flowers, a donor cage's feeding tube was transferred every 24 hours to its paired recipient cage whilst donor cages received a new feeding tube. Both honey bees and bumble bees were used as virus donors and as virus recipients in a fully crossed experimental design comprising 6 pairs of cages per treatment (Fig. 1; Supplementary Table S2), with the treatment *Apis* to *Apis* (AA) acting as a positive control (Yañez *et al*., 2020), *Apis* to *Bombus* (treatment AB) mimicking spill-over, *Bombus* to *Apis* (treatment BA) the potential for spill-back, and *Bombus* to *Bombus* (treatment BB) onward transmission to other *Bombus* individuals.

Donor and recipient bees were tested by qPCR for DWV titre at days 7 and 14 post introduction as a measure of transmission (sample sizes in Supplementary Table S2); removed bees were immediately frozen and stored individually at -80°C until analysis. To hold constant the force of infection throughout the experiment, bees that died or were removed for viral quantification from donor cages were replaced with additional, experimentally infected conspecifics of the donor. As in Experiment 1, we did not detect *V. destructor* in any of our cages.

Additionally, we established 3 cages per bee species with either 8 untreated honey bees or 8 untreated bumble bees and maintained them with their unique feeding tube. As for Experiment 1, these 'negative control' cages were checked for background infection as a consequence of the experimental paradigm.

2.2 Source of bees

Bombus terrestris is a dominant bee species in temperate European ecosystems that harbours several honey bee-associated viruses in the wild, including DWV (Fürst et al., 2014; Manley et al., 2019; McMahon et al., 2015). We used commercial *B. terrestris* colonies that were fed UV-radiated, freshly defrosted pollen pellets and honey bee colonies originating from our institute apiary in Halle (Germany) and originally purchased from local beekeepers. All colonies and pollen were tested by real-time quantitative PCR (qPCR) prior to use for the presence of six common honey bee viruses, including DWV (Supplementary Table S3). Bees from different colonies were evenly distributed between experiments and treatments (donor, recipient, control) to exclude potential genetic effects. Age of bees was not controlled as we deemed it unnecessary; our experimental end-point was viral titre and not survival or behaviour. Additional details on the source of bees as well as the number of colonies and individuals used in experiments is given in the Supplementary Methods.

2.3. Viral inoculum

Two widespread genotypes of DWV, namely A and B, can be found in co-occurring honey bees and bumble bees (Fürst *et al*., 2014). We chose to use DWV genotype A (DWV-A) in experiments because our previous study suggested that it (but not DWV genotype B) compromised bumble bee survival when stressed by starvation (Tehel *et al*., 2020). Our DWV inoculum was the same as that of Tehel *et al.* (2020). In short, we propagated DWV-A, originally derived from a honey bee, in honey bee pupae using methods described in Tehel *et al.* (2019). Absolute viral quantification was by qPCR. Ultradeep next-generation sequencing on an Illumina platform confirmed the identity of our DWV-A inoculum and the absence of other pathogens (see Tehel *et al*., 2019) and BioProject ID PRJNA515220).

2.4 Viral quantification

Absolute quantification of viral titre by qPCR was performed on individual bees throughout. It followed previously described methods (Tehel *et al*., 2019, 2020; see Supplementary Material for RNA extraction, cDNA synthesis and qPCR protocols and quality checks, which included technical duplication of qPCRs, inclusion of positive and negative controls on each qPCR plate, and a qPCR quantification cycle (Cq) threshold of $Cq < 35$ (approximately equivalent to 106 genome equivalents per bee)) to define a positive sample. Sample sizes of bees analysed for DWV are given in Supplementary Tables S1 and S2 (Experiments 1 and 2 respectively) and viral titres are presented in the text as mean genome equivalents per bee ± SEM. Samples used for laboratory analysis were randomly selected from all frozen bees when available for a given treatment.

2.5. Statistics

All analyses were performed in R v. 3.5.1 (R Core Team).

We used Fisher exact tests (package "stats") to compare proportions of infected versus non-infected bees. In Experiment 1, analyses were based on individual bees, assuming independence of individuals in a cage (results were qualitatively similar when analysing data at the level of the cage). In Experiment 2, transmission could only have occurred between cages via the shared source of food but, once one individual within a recipient cage became infected, transmission within a cage could have subsequently occurred via additional routes; for Experiment 2, cage was therefore used as the statistical unit of replication.

To assess differences in $\log_{10}$ transformed viral titres, we used two different models for the donor – recipient combinations in Experiment 1, one for viral titres with *Apis* as a donor (treatment AA and AB with four levels: donor in AA, recipient in AA, donor in AB, and recipient in AB) and one with *Bombus* as a donor (treatment BA and BB with only two levels: donor in BA and donor in BB, as the recipients never became infected). We used a

linear mixed model (LMM, package "lme4" (Bates *et al*., 2015), with experimental cage as a random factor for the donor – recipient combinations with *Apis* as a donor to account for the fact that we measured two bees from the same cage for donors in treatment AA and receivers in treatment AB in one out of five and two out of seven cages, respectively. A linear model (LM) was used to analyse donor – recipient combinations with *Bombus* as a donor because only one donor was analysed per cage.

In Experiment 2, as in Experiment 1, we used two different models to analyse viral titre data for the donor-recipient combinations, an LMM for viral titres with *Apis* as a donor (treatment AA and AB with two levels: donor in AA and donor in AB, as only very few recipients became infected) and an LM for viral titres with *Bombus* as a donor (treatment BA and BB with only two levels: donor in BA and donor in BB, as the recipients never became infected). An LMM with experimental cage as a random factor was again used for the donor – recipient combinations with *Apis* as a donor to account for the fact that we measured two bees from the same cage for donors in treatment AA in one out of five cages. An LM was instead used to analyse donor – recipient combinations with *Bombus* as a donor as we only analysed one donor per cage. For all analyses, models were compared to null (intercept only) models to assess whether levels of donor – recipient combinations in the respective models were significant predictors of viral titre.

Pairwise comparisons between factor levels of a significant predictor were performed using post-hoc tests, adjusting the family-wise error rate according to the method of Westfall (package "multcomp" (Bretz *et al*., 2011)). Model assumptions were checked with diagnostic tests and plots implemented in the package "DHARMa" (Hartig, 2021) for LMMs, or via diagnostic plots, the Shapiro-Wilks-Test, and the Bartlett-Test in base R for LMs.

3. Results

3.1 Experiment 1, mimicking intracolony transmission

In Experiment 1, we could clearly demonstrate viral transmission from infected honey bees to uninoculated recipient bumble bees. All 9 recipient bumble bees from the subset we analysed (treatment AB: 9 of 9 individuals) were infected with DWV-A after seven days of contact with infected donor honey bees (Fig. 2A) whereas none of the 5 analysed bumble bee was infected in a control cage (control: 5 of 5 *Bombus* individuals analysed; comparison of infection status of treatment vs. control, Fisher exact test $P < 0.001$; Supplementary Table S1). All donor bees were successfully infected (Fig. 2A). These data demonstrate that infected honey bees readily transmit virus to the bumble bee *B. terrestris* when in close contact.

In the treatment AA, all 9 analysed recipient honey bees (9 of 9 individuals) were infected by donor honey bees (Fig. 2A) whereas no analysed honey bee was infected in a control

cage (Control: 5 of 5 *Apis* individuals; comparison of infection status of treatment vs. control, Fisher exact test $P < 0.001$; Supplementary Table S1 and Supplementary Fig. S2A), confirming *Apis-Apis* transmission and that our experimental paradigm functioned as expected.

In the two treatments AB and AA in which honey bees were viral donors, viral titres in all donor honey bees and all recipient bees were consistently high (Fig. 2B), often orders of magnitude greater than the inoculum (10^7) injected into donors (mean ± s.e.; donor honey bees of both treatments: $1.19^{13} \pm 4.27^{12}$, n = 12; recipient *Bombus* in treatment AB: $3.44^8 \pm 5.86^8$; recipient *Apis* in treatment AA: $3.63^{11} \pm 2.16^{11}$, n = 9 each), evidence for transmission from donor to recipient and replication within recipients. Additional statistical results comparing viral titres across groups are found in the Supplementary Results.

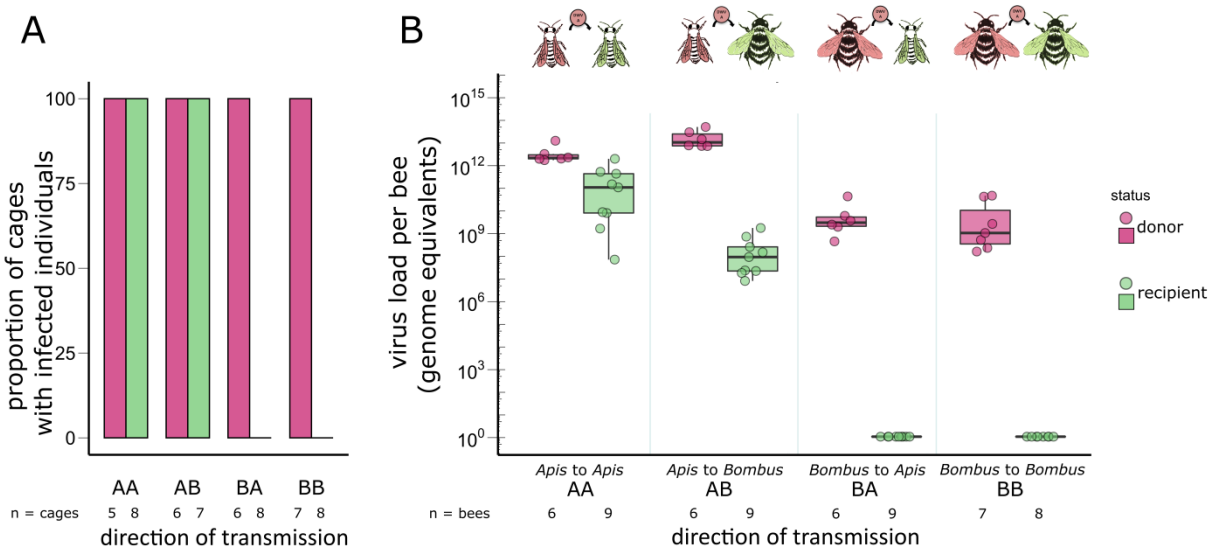

Fig. 2 Experiment 1, mimicking intracolony transmission. A. The proportion of cages in which experimentally infected donor bees (red, 1st column of a treatment) and uninoculated recipient bees (green, 2nd column of a treatment) were infected with DWV by day 7. **B.** Viral (DWV) titres of donor and recipient bees at day 7. Donors (red, injected with 10^7 viral genome equivalents of DWV-A) and recipients (green, DWV-uninoculated) shared one cage, permitting multiple horizontal transmission routes and mimicking elements of intracolony transmission. All donor bees had high viral titres in all cages by day 7. All recipient *Bombus* from the treatment *Apis* to *Bombus* showed high titres by day 7, indicative of infection, whereas none of the recipients from the transmission treatments in which *Bombus* was the donor (*Bombus* to *Apis* and *Bombus* to *Bombus*) was infected. Sample sizes given as: **A**, number of cages; **B**, number of analysed bees.

In stark contrast to our results in which honey bees were viral donors, we found no evidence of viral transmission from donor bumble bees, either to recipient honey bees or to recipient bumble bees in treatments BA and BB respectively. All 9 analysed recipient honey bees (treatment BA: 9 of 9 individuals) and all 8 analysed recipient bumble bees (treatment BB: 8 of 8 individuals) were devoid of DWV-A after seven days of contact with infected donor bumble bees (Fig. 2A; Supplementary Table S1). All donor bumble bees were

infected (treatment BA: 6 of 6 donor individuals in 6 of 6 cages, Fisher exact test of difference in infection status between donor and recipient, $P < 0.001$; treatment BB: 7 of 7 donor individuals in 7 of 7 cages, Fisher exact test of difference between donor and recipient, $P < 0.001$; Fig. 2A and Supplementary Table S1A). Bumble bees in control cages remained uninfected with DWV-A (Control: 5 of 5 *Bombus* individuals; Supplementary Table S1B and Supplementary Fig. S2A), confirming that our experimental paradigm to introduce DWV-A infected donors and DWV-A uninfected recipients into cages had functioned.

Viral titres in all donor bumble bees were consistently high (mean ± s.e.; donor bumble bees of both treatments: $1.19^{10} \pm 5.25^{9}$, n = 13), indicating successful viral infection of donor bumble bees (Fig. 2B). Additional statistical results comparing viral titres across groups are given in the Supplementary Results.

3.2 Experiment 2, mimicking food-borne transmission

In Experiment 2, we could again demonstrate viral transmission from donor honey bees to recipient bumble bees and to recipient honey bees, though with reduced efficiency compared to Experiment 1. At day 7, recipient *Bombus* and recipient *Apis* in one of six cages apiece were infected with DWV (treatments AB and AA respectively; Fig. 3A and Supplementary Table S2A). This is a significantly lower probability of transmission than in Experiment 1 (Fisher exact test $P = 0.002$ for both treatment AB and for treatment AA). By day 14, two of six and four of five recipient cages contained infected *Bombus* and *Apis* in treatments AB and AA respectively (Fig. 3A and Supplementary Table S2A), demonstrating that, with time, sharing of food resources leads to successful viral transmission from honey bees to bumble bees (and to conspecific honey bees). Summing across both days 7 and 14, recipient bees became infected in a total of 8 of 12 cages with *Apis* as donors (the cage-wise transmission from *Apis* donors to recipients (honey bees and bumble bees) was 0.67).

Recipient honey bee cages in the treatment AA were either all infected or all non-infected at either of the two time points of sampling (n = 6 cages over two time points; Fig. S3 and Supplementary Table S2A). In contrast, infected recipient bumble bee in the treatment AB were singletons in two of three cages housing infected recipients (Fig. S3 and Supplementary Table S2S). These data suggest that, if a *B. terrestris* becomes infected through consumption of virus-laden food, there is a low probability that the virus is transmitted onwards to other conspecific bumble bees.

Few recipient bumble bees in treatment AB were infected at day 7 (n = 3 of 18 bees) or at day 14 (n = 2 of 18 bees; Supplementary Table S2A). Yet the substantive DWV titres of infected recipient bumble bees (mean ± s.e.: $5.53^{7} \pm 3.49^{7}$, n = 5; Fig. 3B) support viral transmission to and subsequent replication within recipient bumble bees. Additional

statistical details comparing viral titres between groups are given in the Supplementary Results.

Viral titres in donor honey bees inoculated with 10^7 genome equivalents of DWV were consistently high at day 7 (*Apis* donors in treatment AA: $2.03^{13} \pm 2.76^{12}$, n = 12; Fig. 3B), demonstrating successful viral replication in donors. Few recipient honey bees in treatment AA were infected at day 7 (n = 3 of 18 bees) but many more were infected by day 14 (n = 13 of 17 bees; Supplementary Table S2A). Viral titres of infected recipient honey bees were also > 10^7 (Fig. 3B), supporting horizontal transmission to conspecific honey bees of virus in shared food and subsequent replication of the virus in recipients. Additional statistical details comparing viral titres between groups are given in the Supplementary Results.

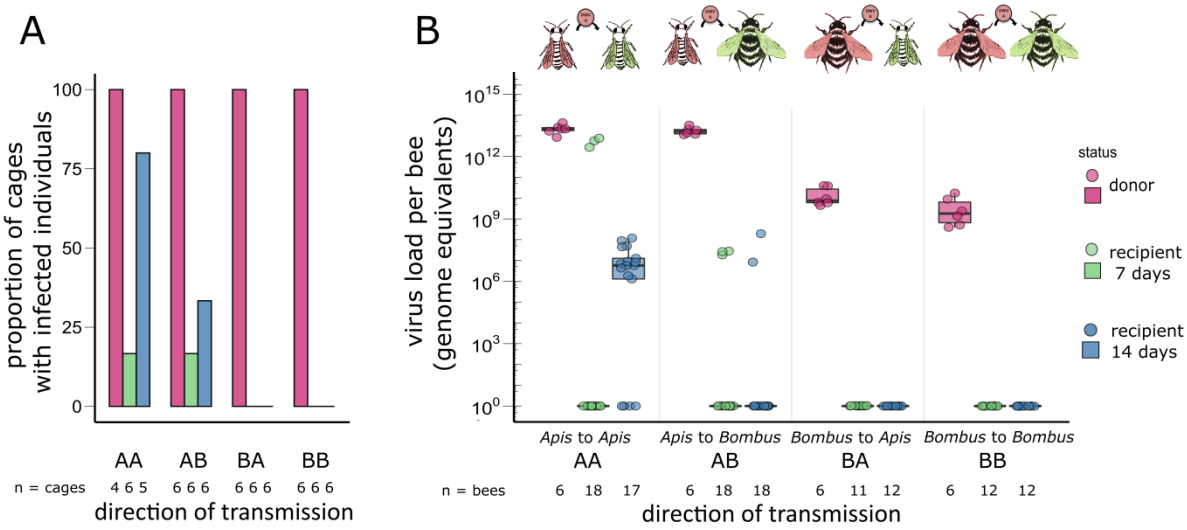

Fig. 3 Experiment 2, mimicking food-borne transmission. A. The proportion of cages in which experimentally infected donor bees (red, 1st column of a treatment) and uninoculated recipient bees (2nd and 3rd columns of a treatment) were infected with DWV by day 7 (2nd column: green) and day 14 (3rd column: blue). **B.** Viral (DWV) titres of experimentally infected donor bees at day 7 and of recipient bees at days 7 (green) and 14 (blue). Donors (red, injected with 10^7 viral genome equivalents of DWV-A) and recipients (green and blue, DWV-uninoculated) were held in separated cages but the donor feeding tube was moved from a donor to its paired recipient cage every day, mimicking the use of a shared food resource. All donor bees showed high viral titres at day 7. Recipient bees from treatments in which honey bees were the donors (*Apis* to *Apis* and *Apis* to *Bombus*) showed high titres at days 7 and 14, indicative of infection, whereas recipient bees from treatments in which bumble bees were the donors (*Bombus* to *Apis* and *Bombus* to *Bombus*) did not. Sample sizes given as: **A**, number of cages; **B**, number of analysed bees.

We found no evidence for viral transmission from donor bumble bees to recipient bees, either uninoculated bumble bees or uninoculated honey bees, suggesting that virus is not transmitted onward from one infected *Bombus* to another or transmitted back to *Apis* (Fig. 3A and Supplementary Table S2A). Cages with *Apis* as donors led to far higher transmission to recipient bees than cages with *Bombus* as donors (Fisher exact text P = 0.001,

odds ratio infinity, 95% confidence intervals 3.055 – infinity). To account for the low number of cages per treatment, we use the binomial theorem to state with 95% confidence that the cage-wise transmission from *Bombus* donors to recipients (honey bees and bumble bees) was less than 0.22.

Inoculated donor bumble bees had high viral titres ($1.13^{10} \pm 4.02^{9}$, n = 12; Fig. 3B), demonstrating their competence as hosts of DWV. Furthermore, bumble bees were observed to sit on feeding tubes, which were spotted with excretions, suggesting that feeding tubes offered a plausible route of food-borne transmission to recipient bees. Additional statistical details comparing viral titres between groups are given in the Supplementary Results.

Of the Control treatments in Experiment 2 (*Bombus* n = 6, *Apis* n = 6), one bumble bee had a background infection on day 14 of 3.79^{7} genome equivalents, probably because its source colony carried a low-titre infection (Supplementary Fig. S2B). We could not detect virus in any other controls.

4. Discussion

Our experiments demonstrate that infected honey bees readily transmit DWV-A to the bumble bee *B. terrestris*, both when in close contact and indirectly, when sharing a common food resource (sugar solution). But we could not detect viral transmission from *B. terrestris* to conspecifics or to honey bees, either through direct contact or indirectly via shared food. Our data support the view that DWV infected honey bees readily transmit virus to *B. terrestris* which causes an infection but DWV-infected bumble bees are far less likely to transmit virus back to honey bees or onward to other *B. terrestris*.

Previous studies have been contradictory, arguing either that DWV is unlikely (for *B. terrestris*: Gusachenko *et al*., 2020) or is likely (for *B. impatiens*: Burnham *et al*., 2021) to be transmitted from honey bees to bumble bees. Differences amongst studies might be due to variation among recipient host species in their competence for viral replication or in the mode of transmission used in experiments. *Bombus terrestris* is a susceptible host for DWV when inoculated by injection or by feeding (Gusachenko *et al*., 2020; Tehel *et al*., 2020). We now show that *B. terrestris* also readily becomes infected when housed with, or when sharing a common source of food with, infected *A. mellifera* under our experimental conditions.

We found that viral spill-over from honey bees to bumble bees was more efficient when insects were in direct contact (our Experiment 1 mimicking intracolony transmission) than through a shared food resource (our Experiment 2 mimicking food-borne transmission). Both experiments permitted multiple modes of transmission: faecal-oral and oral-oral for both experiments, as well as via grooming and trophallaxis for Experiment 1 mimicking intracolony transmission. Though vector-based transmission could lead to more efficient

transmission within colonies in nature, we did not detect any mites in our cages that could lead to viral transmission and therefore assume that this route does not explain our results. Furthermore honey bees and bumble bees generally host their own mite species (Alford, 1980; Rosenkranz *et al*., 2010), additionally arguing against a role for viral vectors in explaining the efficiency of transmission when heterospecific hosts were in direct contact. We hypothesise that interspecific and intraspecific (honey bee to bumble bee, honey bee to honey bee) transmission was likely more efficient in Experiment 1 because it permitted a higher dose of infective virus to be transferred from donor to recipient. Dose is considered critical for cross-species transmission in other cases of viral spill-over e.g. MERS-CoV and Nipah virus (Lunn *et al*., 2019). In support of our hypothesis, the first detection of DWV in bumble bees was in *Bombus* bearing deformed wings and collected from a honey bee apiary, suggesting that spill-over was facilitated by intracolony transmission following bumble bee entry into infected honey bee colonies (Genersch *et al*., 2006).

That infected bumble bees in our experiment did not lead to the transmission of DWV back to honey bees or onward to uninoculated bumble bees suggests that infected *B. terrestris* are incapable of shedding infective DWV or of shedding sufficient virions to represent an infective dose for a recipient host. In a cage experiment, Burnham *et al.* (2021) have shown that *B. impatiens* inoculated with 3×10^6 DWV *per os* subsequently deposit detectable virus on artificial flowers, demonstrating the potential for infected bumble bees to transmit DWV. However, there is no information on the viability and the infectious potential of these shed viruses. Donor *B. terrestris* in our experiments had high viral titres ($> 10^9$) but had been inoculated by injection. Differences between studies may therefore reflect variation among *Bombus* species in response to viral infection or mode of inoculation. Furthermore, the origin of the inoculum might also determine its transmissibility. The source of our DWV-A was an infected honey bee and we amplified it in honey bee pupae to generate our experimental inoculum. DWV-A derived from bumble bees might be more transmissible from bumble bee hosts to recipient conspecifics and heterospecifics. Additional analyses of the infectivity of the viruses in oral and anal excretions of bumble bees infected orally or by injection with *Bombus*-derived *versus Apis*-derived inocula would help to resolve these questions.

Shared food resources such as bird feeders or waterholes are a common site of pathogen transmission (Nunn *et al*., 2014; Paull *et al*., 2012). For bees and other flower-visiting insects, flowers are considered important transmission hubs for their pathogens (McArt *et al*., 2014), and observational and experimental data support their role in the transmission of numerous eukaryote pathogens (Durrer & Schmid-Hempel, 1994; Figueroa *et al*., 2019, 2020; Graystock *et al*., 2020; Graystock *et al*., 2015a; Purkiss & Lach, 2019). Their role in viral transmission is less well documented; flower-based transmission may theoretically represent a barrier to transmission as many viruses are sensitive to UV light (Lytle & Sagripanti, 2005; McLeod *et al*., 1977), and flowers represent an alien and potentially hostile environment for viruses (McArt *et al*., 2014). DWV in particular is considered unstable

outside of its host (De Miranda *et al*., 2013). However, pollen collected from honey bee-visited flowers has been shown to contain infective DWV (Mazzei *et al*., 2014). Furthermore, DWV is excreted in the faeces of infected honey bees (Chen *et al*., 2006), and faeces are deposited on flowers by bees when foraging (for *Bombus*: Bodden *et al*., 2019). Our results in Experiment 2 mimicking food-borne transmission also support the view that DWV is readily transmitted by honey bees to conspecifics and heterospecifics at flowers, either oral-faecally (via faeces) or oral-orally (via cephalic secretions or regurgitation). Floral transmission may well account for the presence of DWV in a wide diversity of flower-visiting insects (Nanetti *et al*., 2021). We note, however, that our experimental paradigm may well have accentuated food-borne transmission beyond that which may occur naturally at flowers because donors had 24 hours to walk over, defecate upon and regurgitate onto a feeding tube whereas flowers are usually visited briefly by foraging bees. Whether *Bombus* spp. transmit DWV (or other viruses) at flowers in the field remains an open question, though an important one to answer to understand the epidemiology of DWV in pollinator communities.

Pathogen spill-over *sensu stricto* has been conceptually represented as a series of hierarchical steps, from the release of viable, transmissible environmental stages (virions in the case of viruses like DWV) from a reservoir host species through to successful acquisition by a recipient host species (Becker *et al*., 2019; Plowright *et al*., 2017). Successful replication in and subsequent transmission among recipient host individuals are additional bottleneck steps that, if overcome by a pathogen, may then lead to disease emergence (Wasik *et al*., 2019). We have here shown that DWV-A has the potential to spill over from *A. mellifera* to *B. terrestris*, though we have no support for its onward transmission among recipient bumble bees. That bumble bees, other wild bee species and many other flower-visiting insects often harbour DWV (Nanetti *et al*., 2021), sometimes to titres as high as in honey bees (Jones *et al*., 2021), demonstrates the potential for DWV to spill over into other host species and replicate in them. The correlation across field sites in the prevalence of DWV in honey bees and bumble bees (Alger *et al*., 2019a; Fürst *et al*., 2014; Manley *et al*., 2019) as well as the sequence identity of viral variants in *Apis* and *Bombus* from the same site (Daughenbaugh *et al*., 2021; Fürst *et al*., 2014; Manley *et al*., 2019; Radzevičiūtė *et al*., 2017) support the notion of pervasive, ongoing spill-over. Given the considerable evolutionary potential of RNA viruses (Holmes, 2009), there is a tangible risk of local adaptation of DWV to a bumble bee or other flower-visiting host, with negative knock-on effects on biodiversity and the ecosystem service of pollination.

There is mounting evidence for the impact of pathogens on pollinator species (Dicks *et al*., 2021); transcriptome analysis of the rare and declining *Bombus terricola* of North America points to pathogen (and pesticide) driven decline (Tsvetkov *et al*., 2021). In our experiments, we employed commercially sourced *B. terrestris*, a common and widespread species (Estoup *et al*., 1996; Nieto *et al*., 2014) which may have been inadvertently selected for tolerance to or limited transmission of DWV in breeding facilities. Given the ubiquity of

DWV in terrestrial biomes worldwide, its role in driving population loss of rare or declining species needs to be taken seriously.

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Author contributions
Conceptualization: all co-authors; formal analysis: A.T.; investigation: A.T., T.S.; statistics: A.T., S.T. writing - original draft: A.T., R.J.P. All authors reviewed and commented on the submitted manuscript.

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Competing interests
The authors declare no competing or financial interests.

Ethics statement
The insect research described in this paper does not require ethics approval.

Data accessibility
All data supporting the findings of this study are available from the Dryad Data Repository (doi:10.5061/dryad.w0vt4b8t7).

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Chapter III

Experimental infection of bumble bees with honey bee associated viruses: no direct fitness costs but potential future threats to novel wild bee hosts

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Abstract

Pathogen spill-over represents an important cause of biodiversity decline. For wild bee species such as bumble bees, many of which are in decline, correlational data point toward viral spill-over from managed honey bees as a potential cause. Yet impacts of these viruses on wild bees are rarely evaluated. Here, in a series of highly controlled laboratory infection assays with well characterised viral inocula, we show that three viral types isolated from honey bees (deformed wing virus genotype A, deformed wing virus genotype B and black queen cell virus) readily replicate within hosts of the bumble bee *Bombus terrestris*. Impacts of these honey bee-derived viruses – either injected or fed – on the mortality of *B. terrestris* workers were, however, negligible and likely dependent on host condition. Our results highlight the potential threat of viral spill-over from honey bees to novel wild bee species, though they also underscore the importance of additional studies on this and other wild bee species under field-realistic conditions to evaluate whether pathogen spill-over has a negative impact on wild bee individuals and population fitness.

Key words: *Apis mellifera*, *Bombus terrestris*, DWV, BQCV, multi-host pathogen, virulence

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1. Introduction

A wealth of evidence points to massive biodiversity loss in the Anthropocene, resulting in range declines, local extirpations and species extinctions (Maxwell *et al.*, 2016). Though generally considered mobile and numerous, mounting evidence demonstrates that many insect species and communities are also in decline (Hallmann *et al.*, 2017; Seibold *et al.*, 2019; Thomas *et al.*, 2004), with potential consequences for the functioning of terrestrial ecosystems (Wagner, 2020). Bees are a particular focus of concern because of their importance in pollination (Potts *et al.*, 2016b), with strong support for range decline and species loss in temperate regions of the world (Bartomeus *et al.*, 2010; Biesmeijer *et al.*, 2006; Nieto *et al.*, 2014). Causes of bee decline, as for the fate of other insects, are thought to revolve around the fragmentation, degradation and loss of habitat, intensification of land use (including pesticides), and climate change as well as, potentially, pathogens (Brown & Paxton, 2009; Goulson *et al.*, 2015; Vanbergen *et al.*, 2013).

Pathogen spill-over is an important cause of biodiversity decline as well as a risk to human health (Daszak *et al.*, 2000). Recent examples of pathogen spill-over causing population decline include European whitenose fungus killing North America bats (Leopardi *et al.*, 2015), Asiatic chitrid fungus decimating European populations of the amphibian *Salamandra salamandra* (Stegen *et al.*, 2017), and Ebola virus that spills over from wild mammal reservoir hosts into humans to cause life-threatening disease (Carroll *et al.*, 2015). Yet pathogen spill-over may have variable, and sometimes benign, consequences for novel hosts. For example, the exotic *Nosema ceranae* microsporidian of the Asiatic honey bee *Apis cerana* is nowadays an emerging infectious disease (EID) of *Apis mellifera* (Klee *et al.*, 2007) throughout much of the world. It spills over into wild bee species, where it has been reported to reduce the lifespan of the Australian stingless bee *Tetragonula hockingsi* (Purkiss & Lach, 2019), though causes little apparent harm to the Eurasian *Bombus terrestris* (Fürst *et al.*, 2014; Graystock *et al.*, 2013) and European mason bee *Osmia bicornis* (Müller *et al.*, 2019). In these cases, the pathogen might be merely vectored through novel bee hosts (Graystock *et al.*, 2015) rather than cause them harm.

There is mounting correlational evidence that the Western honey bee *A. mellifera*, the world's most important commercial pollinator, is a source of pathogens that spill over into wild bee species (Alger *et al.*, 2019a; Fürst *et al.*, 2014; Genersch *et al.*, 2006; Manley *et al.*, 2019; McMahon *et al.*, 2015; Radzevičiūtė *et al.*, 2017; Tehel *et al.*, 2016), in which those pathogens may cause population decline. While black queen cell virus (BQCV) is the most prevalent virus in honey bees (Alger *et al.*, 2019b; McMahon *et al.*, 2015; Murray *et al.*, 2019; Radzevičiūtė *et al.*, 2017), temperate regions of the world have seen elevated honey bee colony losses (Neumann & Carreck, 2010), likely caused by the exotic invasive ectoparasitic mite *Varroa destructor* and deformed wing virus (DWV), which the mite transmits (Dainat & Neumann, 2013; Highfield *et al.*, 2009; Natsopoulou *et al.*, 2017). DWV is an EID which has

become panzootic in honey bee populations to which *V. destructor* has been introduced i.e. worldwide excluding Australia (Wilfert *et al*., 2016).

Bumble bees (*Bombus* spp.) are widespread wild bee species in northern temperate regions (Goulson, 2010) yet many are decreasing in abundance or distribution, with parasites being a potential cause of their decline (Cameron & Sadd, 2020; Meeus *et al*., 2011). BQCV is the most prevalent virus in bumble bees (Alger *et al*. 2019b; McMahon *et al*., 2015; Murray *et al*., 2019; Radzevičiūtė *et al*., 2017), it exhibits broad tissue tropism in the American *Bombus huntii* (Li *et al*., 2011; Peng *et al*., 2011), and its prevalence in *Bombus* spp. covaries with that in *Apis*. Though *Bombus* spp. are not known to host *V. destructor*, spill-over of DWV from honey bees to bumble bees has been inferred from the tight relationship between DWV prevalence in populations of *A. mellifera* and *Bombus* spp. and higher prevalence in the former (Fürst *et al*., 2014), with pathogen transmission presumably occurring through shared use of flowers (Alger *et al*., 2019a; McArt *et al*., 2014). DWV is a highly virulent pathogen of *A. mellifera* comprising two main genotypes: the original DWV genotype A (DWV-A) and the more virulent DWV genotype B (DWV-B) (McMahon *et al*., 2016), both of which have been inferred to spill over from honey bees to bumble bees (Fürst *et al*., 2014). A leading hypothesis is that *Varroa destructor* parasitism of honey bees, by elevating DWV prevalence and intensity of infection (pathogen load) in honey bees, may help drive pathogen spill-over from honey bees to bumble bees (Manley *et al*., 2019). We note, though, that most data suggesting viral spill-over from honey bees to bumble bees are correlational; directionality has rarely been demonstrated and wild bees may also be a source of infection for honey bees (McMahon *et al*., 2015).

Two studies have to date evaluated the virulence of DWV to bumble bees. Firstly, Fürst *et al*. (2014) found that a mixed DWV-A/DWV-B inoculum fed to caged worker *B. terrestris* led to a significant increase in mortality over 20 days. It is not known whether observed mortality was due to DWV-A, DWV-B, enhanced virulence due to co-infection, or an A-B recombinant. Though DWV-A and DWV-B are widespread, have high prevalence in British and US honey bees, and often co-occur in the same host (McMahon *et al*., 2016; Ryabov *et al*., 2017), A-B recombinants were rarely detected in US honey bees (Ryabov *et al*., 2017), suggesting they may be infrequent. However, A-B recombinants have been shown to exhibit elevated virulence in honey bees (Ryabov *et al*., 2014) and may have comprised the inoculum of Fürst *et al*. (2014). In the second study, Graystock *et al*. (2015b) injected DWV derived from *B. terrestris* fat bodies into conspecific, caged workers and revealed a 50% increase in mortality. In this second study (Graystock *et al*., 2015b), DWV was isolated from *B. terrestris* hosts, to which it had potentially adapted, thus not reflecting a spill-over scenario from honey bees to bumble bees. In both studies (Fürst *et al*., 2014; Graystock *et al*., 2015b), viral titre in bumble bees following experimental inoculation was not quantified, making it unclear how well DWV replicated in *B. terrestris* and whether it *per se*, as opposed to a potentially pre-existing pathogen in experimental bees or inoculum, induced elevated mortality.

To clarify the potential impact of honey bee associated viruses on bumble bees, we experimentally inoculated *B. terrestris* workers with either BQCV, DWV-A or DWV-B derived from honey bees and thereafter quantified host mortality and viral titre. Inoculation of bumble bees was done by injection, so as to determine the capacity of the virus to replicate in a novel host, as well as by feeding, representing the more likely natural route of infection in the field (McArt *et al*., 2014). These experiments were carried out under *ad libitum* food conditions. However, fitness costs when responding to an immune challenge may be dependent on host nutritional state, and have been shown for bumble bees when diet restricted (Moret & Schmid-Hempel, 2000). We therefore complemented our investigation with an experiment under starvation conditions.

2. Material and methods

2.1 Source of bees

Commercial *B. terrestris* colonies (Koppert B.V., Berkel en Rodenrijs, Netherlands) were kept in an incubator at 30°C and 50% relative humidity with *ad libitum* 50% (w/v) sucrose solution. Every 2-3 days they were fed with fresh-frozen honey bees pollen pellets (Imkerei Schachtner, Schardenberg, Austria) that had been freshly defrosted. Pollen was UV-irradiated before use to destroy pathogens. Honey bees for experiments and for generating viral inocula were taken from our local apiary (University of Halle, Germany), originally purchased as the subspecies *Apis mellifera carnica*, as is typical for beekeeping in the region. To check that bumble bees (12 source colonies: labelled B1 to B12) and honey bees (2 source colonies, labelled 5.1 and G) as well as the fresh-frozen pollen pellets were devoid of viral pathogens, we tested them by real-time quantitative PCR (qPCR) for seven common honey bee viral targets and three Microsporidia (Supplemental Methods). Bumble bee and honey bee colonies were largely free of virus (Supplementary Table S1), pollen was devoid of virus, and Microsporidia were not detected.

2.2. Propagation of viral inocula

To propagate DWV-A and DWV-B for experimental inocula, we used the inocula from Tehel *et al.* (2019). Our BQCV inoculum was prepared by propagating the BQCV inoculum of Doublet *et al.* (2015). Viral propagation in honey bee pupae and absolute quantification of virus followed precisely methods in Tehel *et al.* (2019). We always generated the correct virus inoculum from the original inoculum, which was devoid of other viruses (Supplementary Fig. S1).

Inocula containing only DWV-A, only DWV-B or only BQCV at known concentrations were aliquoted and stored at –80°C for use in experiments, as was the control inoculum devoid of virus. For each virus, a single inoculum derived from one preparation was used for all experiments with *Bombus* and *Apis*. Ultradeep next-generation sequencing (NGS) on an Illumina platform confirmed the identity of our DWV-A and DWV-B inocula (see Tehel

et al., 2019 for consensus sequences and the pipeline used to assemble them from NGS data as well as BioProject ID PRJNA515220 for the original NGS source files).

2.3. Experimental inoculation

Honey bees – injected with inoculum, satiated. We initially ensured that viral inocula were viable by injecting them into honey bee workers.

Freshly eclosed workers were cooled to 4°C and then injected laterally between the 2nd and 3rd tergite with 10^7 viral genome equivalents (or, as control, virus-free inoculum), a quantity sufficient to ensure 100% infection of adults (McMahon *et al.*, 2016), using a Hamilton syringe (hypodermic needle outer diameter: 0.235mm). To avoid cross-contamination, syringes were cleaned after each use, and different syringes were used for each inoculum (DWV-A, DWV-B, BQCV) and for the control inoculum devoid of virus. The 249individually injected honey bees were randomly assigned to injection treatments, held in groups of 20-22 in autoclaved metal cages (10cm x 10cm x 6cm) independent of their source colony but with bees of the same treatment per individual cage in an incubator (30°C), fed *ad libitum* with 50% (weight/volume) sucrose solution, and monitored daily till death, as in McMahon *et al.* (2016). At 10 days post inoculation (d.p.i.), one bee per cage was removed to quantify viral titre.

Bumble bees – general handling. Viral inocula were tested in freshly emerged *B. terrestris* workers as follows. Firstly, we marked all workers in our 12 *B. terrestris* colonies. Colonies were checked daily and unmarked, newly emerged workers were transferred to autoclaved metal cages (10cm x 10cm x 6cm), fed *ad libitum* with 50% (w/v) sucrose solution, and held in an incubator at 30°C. On the next day (i.e. 24-48h after eclosion), workers were inoculated with virus (or control solution), either by injection or orally by feeding, and then kept in groups of 5-10 of the same treatment per cage. In an experiment, the number of bees per cage was constant (±one bee) for every treatment within any one day of infection. This procedure was repeated across 25 days to allow for sufficient replication per experiment.

Bumble bees – fed inoculum, satiated. Inoculation of *B. terrestris* workers by feeding was designed to test the likely route of viral spill-over from honey bees at flowers in the field. Freshly emerged (24-48h after eclosion) bumble bee workers were individually fed with 10^9 viral genome equivalents or the equivalent control solution devoid of virus (Supplemental Methods), a quantity inducing an acute infection (Doublet *et al.*, 2015; Wang *et al.*, 2018). Then bees were transferred to a new, autoclaved metal cage in small groups (5 to 10 bees per cage, grouped according to treatment). In total 512 bees from five source colonies were evenly distributed between all four treatments (DWV-A, DWV-B, BQCV, Control; 128 bumble bee per treatment) and were randomly assigned to cages independent of source colony. They were monitored daily for mortality. One bee per cage was removed at 18-25 d.p.i. to quantify viral titre. In a preliminary trial following the identical protocol as

described above, we quantified viral titres at 10 and 20 d.p.i., but found no significant difference among them or with bees tested at 18-25 d.p.i. (Supplementary Fig. S2).

Bumble bees – injected with inoculum, satiated. Inoculation by injection was designed to test whether *B. terrestris* is a competent host for each virus. To inject workers, they were cooled on ice till immobile. Viral inoculation then followed that for honey bees; *B. terrestris* workers were then transferred to autoclaved metal cages in small groups (5 to 7 bees per cage).Bees were randomly assigned to cages independent of their 4 source colonies but grouped according to treatment per cage, resulting in n = 404 bees that were recorded daily for mortality (ca. 100 bees per treatment: DWV-A, DWV-B, BQCV, Control). One bee per cage was removed at 10 d.p.i. to quantify viral titre.

Bumble bees – injected with inoculum, starved. As *B. terrestris* workers did not exhibit elevated mortality over controls following viral inoculation under benign laboratory conditions with *ad libitum* food (see Results), we ran an additionally experiment in which we removed their food to determine whether viral inocula induced mortality under non-benign, starvation conditions. Bees from three colonies were collected over a 14 day period as they eclosed, held in autoclaved metal cages and individually injected as described above. To control statistically for effects of age, bees of approximately the same age were held in the same cage. All bees were injected on the same day. At 13 d.p.i., after the virus had time to replicate, bees were individually transferred to a plastic cup covered with netting, devoid of sucrose solution but with a small cotton wool ball soaked in water, held at 30°C and checked every hour for mortality (Supplementary Fig. S3).

At death, bee size was estimated because size might determine the ability to survive under starvation (Couvillon & Dornhaus, 2010; Supplementary Fig. S6). Viral titre was quantified in a subset of bees collected at 13 d.p.i.. In total, 326 *B. terrestris* where inoculated by injection in this experiment, of which 194 survived till 13 d.p.i. and therefore entered the starvation part of the experiment.

Viral titres. To quantify viral titres in adult worker bees arising from inoculation experiments, we crushed one whole honey bee or one bumble bee abdomen in 500µl of 0.5 M PPB (pH 8.0) using a plastic pestle, of which 100µl were used for RNA isolation. Absolute quantification of viral titre followed methods used for viral inocula described in Tehel *et al.* (2019) (Supplemental Methods), including all positive and negative controls.

2.4. Statistics

All analyses were performed in R v. 3.5.1 (R Core Team). We used generalized linear models (GLMs) with a quasi-Poisson error distribution to test for the effect of treatment or experiment on viral titre.

Survivorship of experimentally inoculated bees was analysed using Cox proportional hazards models with the R package *coxme* (Therneau & Grambsch, 2000; Therneau *et al*., 2003). 'Cage' was used as a random factor in all analyses and 'round of infection' as a random factor for *B. terrestris* experiments in which an experiment was initiated across multiple days. To assess the significance of predictors, statistical models including all predictors were compared to null (intercept only) or reduced models (for those with multiple predictors) using Likelihood Ratio (LR) tests. Pairwise comparisons between factor levels of a significant predictor were performed using pairwise post-hoc tests, adjusting the family-wise error rate according to the method of Bonferroni (package *multcomp*, (Bretz *et al*., 2011)). For the experiments with bumble bees under satiated conditions (inoculated by injection and by feeding), survival models retained 'cage' and date or 'round of infection' (for *B. terrestris* experiments in which an experiment was initiated across multiple days) as random factors and treatment as a fixed factor. For the *Bombus* experiment under starvation conditions, 'cage' was again retained as a random factor, and treatment together with bee age and bee size entered as fixed factors. Median survival was calculated using the Survfit function in *survival*. In all survival analyses, bees that died within one day (24h) post inoculation were eliminated from subsequent analyses as death was probably a consequence of physical damage by injection *per se* rather than the inoculum.

3. Results

3.1 Honey bees – injected with inoculum, satiated

All viral inocula, BQCV, DWV-A and DWV-B, resulted in rapid honey bee mortality (Supplementary Fig. S4a); which was significantly faster than control (Cox proportional hazard: BQCV, Exp. (β) = 562.259, $p < 0.001$; DWV-A: Exp. (β) = 2.489, $p = 0.006$; DWV-B: Exp. (β) = 4.461, $p < 0.001$; Supplementary Table S2). BQCV killed honey bees the fastest, followed by DWV-B and DWV-A (Supplementary Table S2). Injected virus grew to ca. 3 x 10^{13} viral genome equivalents at 10 d.p.i. (mean genome equivalents per bee ± SEM): BQCV, 2.39 x 10^{13} ± 8.96 x 10^{12}; DWV-A, 3.70 x 10^{13} ± 9.35 x 10^{12}; DWV-B, 3.85 x 10^{13} ± 4.81 x 10^{12}; Supplementary Fig. S4b). Honey bees suffered a slight background infection with DWV-B. However, all viral inocula were devoid of contaminating virus (Supplementary Figure S1), viable and highly virulent in their original host, *A. mellifera*.

3.2.1 Bumble bees – fed inoculum, satiated

Bombus terrestris workers inoculated orally and subsequently fed *ad libitum* did not differ in survival compared to controls (Cox proportional hazards: BQCV: Exp. (β) = 0.940, $p = 0.75$; DWV-A: Exp. (β) = 1.244, $p = 0.26$; DWV-B: Exp. (β) = 1.218, $p = 0.30$; see Fig. 1a and Supplementary Table S2). Though all viruses were detectable in bumble bee abdomens at 18-25 d.p.i. (Fig. 1a), viral titres were at or just below 10^9, the amount administered per bumble bee (mean genome equivalents per abdomen ± SEM: BQCV, 1.01 x 10^8 ± 6.70 x 10^7;

DWV-A, 1.51 x 10^8 ± 1.37 x 10^8; DWV-B, 4.42 x 10^{10} ± 3.73 x 10^{10}). Bumble bees were devoid of background infection. This experiment suggests that all three viruses can maintain themselves in *B. terrestris* following oral infection, but that they are not virulent when hosts are maintained in the laboratory under benign, satiated conditions.

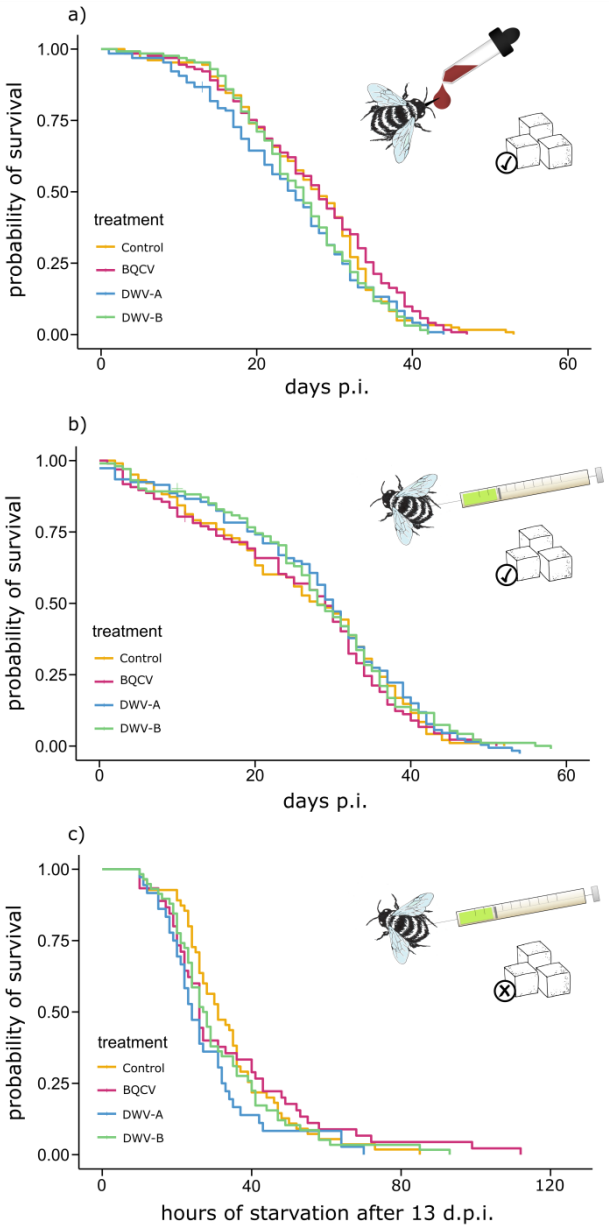

Fig. 1. Cox proportional hazards survival curves of bumble bees inoculated with virus. (a) Survival in days post infection (p.i.) of bumble bee workers when inoculated by feeding with 10^9 viral genome equivalents of BQCV, DWV-A or DWV-B then fed *ad libitum* (n = 128 bees per treatment); (b) Survival in days post infection (p.i.) of bumble bee workers when inoculated by injection with 10^7 viral genome equivalents of BQCV, DWV-A or DWV-B then fed *ad libitum* (Control, n = 102; BQCV, n = 97; DWV-A, n = 103; DWV-B, n = 102); c) Survival in hours of bumble bee workers when inoculated by injection with 10^7 viral genome equivalents of BQCV, DWV-A or DWV-B, fed *ad libitum* for 13 days then starved, defined as hour 0 (Control, n = 55; BQCV, n = 45; DWV-A, n = 36; DWV-B, n = 58). Symbols represent the method of infection and the availability of sucrose.

3.2.2 Bumble bees – injected with inoculum, satiated

In contrast to honey bees, bumble bees injected with viral inocula and fed *ad libitum* did not die any faster than controls (Cox proportional hazards BQCV: Exp. (β) = 0.623, p = 0.13; DWV-A: Exp. (β) = 1.240, p = 0.47; DWV-B: Exp. (β) = 0.923, p = 0.79; see Fig. 1b and Supplementary Table S2). Virus did, though, replicate very well in *B. terrestris* hosts (mean genome equivalents per abdomen ± SEM: BQCV, $5.51 \times 10^9 \pm 9.57 \times 10^8$; DWV-A, $7.10 \times 10^{10} \pm 2.21 \times 10^{10}$; DWV-B, $2.21 \times 10^{11} \pm 2.65 \times 10^{10}$; see Fig. 2). Bumble bees suffered a slight background infection with DWV-B (Supplementary Fig. S5). These results indicate that *B. terrestris* workers are competent hosts of BQCV, DWV-A and DWV-B, though these viruses seem not to impact host longevity under benign (satiated) laboratory conditions.

3.2.3 Bumble bees – injected with inoculum, starved

When inoculated by injection and then starved from 13 d.p.i., viral treatment had again no effect on *B. terrestris* mortality (Fig. 1c). When all treatments were analysed simultaneously through to the death of all bumble bees, statistically significant differences among control or treatments were not seen (Cox proportional hazards BQCV: Exp. (β) = 1.059, p = 0.87; DWV-A: Exp. (β) = 1.589, p = 0.10; DWV-B: Exp. (β) = 1.167, p = 0.57; Supplementary Table S2). However, DWV-A inoculated bees exhibited a subtly shorter lifespan (Fig. 1c), dying ca. 1.6 fold faster than controls, suggesting that DWV-A (but neither DWV-B nor BQCV) might subtly impact *B. terrestris* longevity (see Supplementary Fig. S7).

Though smaller worker bumble bees lived longer than larger workers (Cox proportional hazards: Exp. (β) = 1.665, p = 0.03), bee size did not differ between treatments (Supplementary Fig. S6) and bumble bee size did not differentially impact mortality across treatments (Supplementary Table S2).

Viral titres in inoculated bumble bees at 13 d.p.i where higher for all three viruses than the dose of virus administered: 10^7 viral genome equivalents (mean per abdomen± SEM: BQCV $1.67 \times 10^8 \pm 3.10 \times 10^7$; DWV-A, $6.70 \times 10^9 \pm 2.50 \times 10^9$; DWV-B, $6.58 \times 10^{10} \pm 3.47 \times 10^{10}$; Fig. 2). Bumble bees were not contaminated with other virus (Supplementary Fig. S5). This experiment confirms that all three viruses can replicate within *B. terrestris*, and that virus did not markedly shorten bumble bee worker lifespan under food deprivation.

3.3 Viral titres across experiments

All three viruses replicated to higher titres in *A. mellifera* than *B. terrestris*. Inoculation of honey bees by injection led to three orders of magnitude higher viral titre (ca. 3×10^{13} viral genome equivalents per bee at 10 d.p.i.) than the equivalent inoculation by injection of bumble bees (ca. 4×10^{10} viral genome equivalents per abdomen at 10 d.p.i.), for all three viruses (Fig. 2 and Supplementary Fig. S4b).

Bumble bee inoculation by injection led to higher viral titres than by oral inoculation (Fig. 2), despite variation in dose (dose injected: 10^7; dose fed: 10^9) and duration of infection across experiments (injected, duration of viral replication: 10 d.p.i. and 18-25 d.p.i.; fed, duration of viral replication: 13 d.p.i.). Notably, inoculation with DWV-B led to a significantly higher viral titre than with BQCV within each experiment with bumble bees (Fig. 2), whereas DWV-A titre lay below BQCV or between DWV-B and BQCV, though not significantly different from either (Fig. 2).

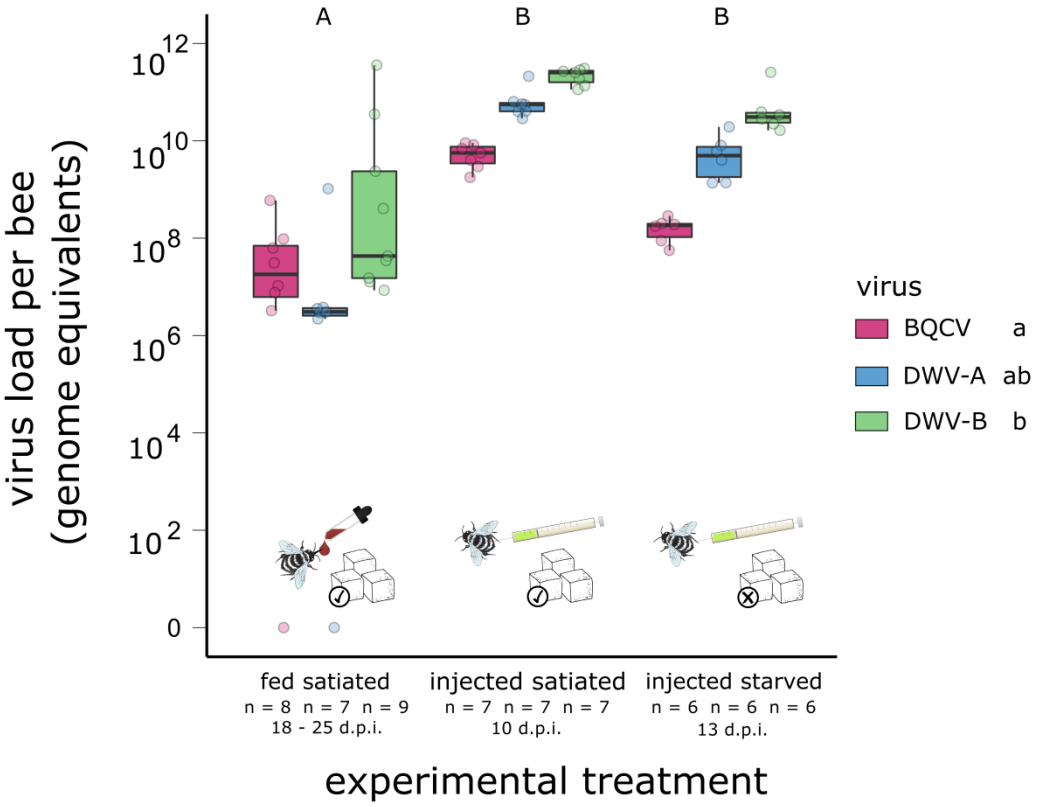

Fig. 2 Viral genome equivalents per bumble bee worker abdomen after infection by injection of 10^7 viral genome equivalents or feeding of 10^9 viral genome equivalents of BQCV, DWV-A or DWV-B. Across all three viruses, injection resulted in higher viral titres than feeding (horizontal bars: sat.inj – sat.fed z = 6.117, p ≤ 0.001; starv.inj – sat.fed z = 4.096, p ≤ 0.001; starv.inj – sat.inj z = -1.733, p = 0.083). Different uppercase letters indicate significant differences between experimental treatments overall. Across all three experiments, DWV-B titres were significantly higher than BQCV whilst DWV-A titre was intermediate and not significantly different from BQCV or DWV-B (vertical bar: DWV-A – DWV-B z = 1.005, p = 0.315; DWV-B – BQCV z = 2.826, p = 0.013 *; DWV-A – BQCV z = 1.776, p = 0.076); virus treatments followed by a different lower case letter, P < 0.05. Symbols represent the method of infection and the availability of sucrose.

4. Discussion

Here we show that *B. terrestris* is a competent host for BQCV, DWV-A and DWV-B, suggesting that spill-over from honey bees is a potential threat for this and likely other wild bee species. We did not, though, observe impacts of these viruses on bumble bee

mortality under laboratory conditions. Furthermore all three viruses replicated to higher titres in honey bees than in bumble bees which is surprising given that honey bees are generally smaller than bumble bees. The higher vial titre per honey bee suggests that these viruses are locally adapted to *A. mellifera*, which is likely their reservoir host.

BQCV, DWV-A and DWV-B have been frequently detected in bumble bees (*Bombus* spp.; (Alger *et al.*, 2019b; Fürst *et al.*, 2014; Li *et al.*, 2011; Manley *et al.*, 2019; McMahon *et al.*, 2015; Peng *et al.*, 2011; Radzevičiūtė *et al.*, 2017) and other wild bee species collected from the field (reviewed in Tehel *et al.*, 2016), as well as in other insect species associated with honey bees or the flowers they visit (e.g. Bailes *et al.*, 2018; Brettell *et al.*, 2019; Evison *et al.*, 2012; Loope *et al.*, 2019; Santamaria, 2018). Moreover, the negative strand of these (+)ssRNA viruses has also been detected in *Bombus* spp. and other wild bee species (Alger *et al.*, 2019a; Fürst *et al.*, 2014; Manley *et al.*, 2019; Radzevičiūtė *et al.*, 2017) as evidence that virus is actively replicating inside these non-*Apis* hosts. Here we have been able to show unequivocally that all three viruses can replicate to high titres in *B. terrestris*. Additional studies on other non-*Apis* bees, including non-commercial *B. terrestris*, as well as with other honey bee viruses are needed to understand the extent of their host tropism across wild bee species. It will also be important to determine how virulence evolves after a viral jump to a new wild bee species as this is central to disease emergence in the new host (Geoghegan & Holmes, 2018).

Under benign conditions of the laboratory, we found that BQCV, DWV-A and DWV-B were not virulent (i.e. did not reduce host fitness, *sensu* (Schmid-Hempel, 2011)). Gusachenko *et al.* (2019) have recently reported similar findings for DWV-A and DWV-B. These results are surprising because DWV has been associated with field-collected *Bombus* spp. exhibiting clinical symptoms (deformed wings; Genersch *et al.*, 2006), which is typical of honey bees when infected by DWV in the pupal stage (Tehel *et al.*, 2019). Also, when fed (Fürst *et al.*, 2014) or injected (Graystock *et al.*, 2015b) into *B. terrestris* workers, DWV has been previously shown to reduce *B. terrestris* lifespan. Differences between former studies and ours may reflect the genetic background of the host; Fürst *et al.* (2014) and Graystock *et al.* (2015) employed *B. terrestris* from a different commercial source to our study (though Gusachenko *et al.*, 2019 used the same source as Graystock *et al.*, 2013). Alternatively, it may reflect the source of virus; Fürst *et al.* (2014) used a mixed DWV-A/DWV-B inoculum and Graystock *et al.* (2015) used DWV isolated from *B. terrestris* whereas we used DWV-A and DWV-B isolated from *A. mellifera*. Recombination between DWV-A and DWV-B deserves greater attention as a source of virulent virus that may impact both honey bees and bumble bees (Ryabov *et al.*, 2014), as does the extent of local adaptation of DWV to a host species.

Another facet of virulence may be the size of the host in relation to viral titre. Honey bee workers are generally smaller than those of bumble bees and, in our experiments, we inoculated each host species with the same viral titre. A direct relationship between host

size and inoculum titre could therefore account for the higher mortality of honey bees *versus* bumble bees that we observed. However, viral titres were actually higher in honey bees than bumble bees, arguing against a relationship between host size and inoculum titre that is constant across host bee species. Furthermore, viral titre seems to asymptote after several days in each host species, high in honey bees (Tehel *et al.*, 2019; this study) and lower in bumble bees (Supplementary Fig. S2), suggesting that initial viral inoculum size is not related to ensuing viral titre in a host. The relationship between viral titre and host mortality nevertheless deserves greater attention, not only within but also across host species.

Not even under stressful, starvation conditions did we detect a marked effect of either BQCV, DWV-A or DWV-B in reducing *B. terrestris* longevity in the laboratory. Condition-dependent virulence of honey bee viruses in *Bombus* spp. hosts has been seen for Slow bee paralysis virus infecting *B. terrestris*, in which longevity was compromised only when hosts were starved (Manley *et al.*, 2017), and for other bumble bee pathogens such as *Crithidia bombi* (Brown *et al.*, 2000, 2003). We therefore urge caution in the interpretation of our result that viral virulence was non-existent in *B. terrestris*. Laboratory conditions may underestimate the impact of honey bee virus spilling over into wild bees in the field, where hosts may be exposed to far harsher environmental conditions and limited resources (e.g. Heinrich, 1979). Insecticides have been highlighted as playing a role in insect, including *Bombus* spp., decline (Potts *et al.*, 2016b; Vanbergen *et al.*, 2013), with sublethal impacts of novel classes of insecticide on colony fitness (Siviter *et al.*, 2019; Whitehorn *et al.*, 2012). Sublethal doses of insecticide can interact with pathogens to elevate host honey bee mortality (Di Prisco *et al.*, 2013; Doublet *et al.*, 2015; Nazzi *et al.*, 2012), and may represent another condition-dependent factor for bumble bees and other wild bee species that exacerbates the impact on them of viral spill-over from honey bees. Field-realistic experimental paradigms are now needed to reveal the role of viral spill-over for the individual, colony and population fitness of wild bee species as well as additional experiments examining other response variables than mere mortality e.g. offspring production, pupal development and foraging efficiency. Changes in sublethal parameters like these could decrease the success of a social bee colony enormously. Furthermore, our non-benign scenario (starvation) may have been too stressful to allow expression of condition-dependent virulence; use of more natural levels of stress, as may be typically experienced by bees in the field, are warranted to reveal condition-dependent virulence.

We found that viral titres were lower and the impact on host mortality was non-existent when BQCV, DWV-A or DWV-B was injected into *B. terrestris vs.* injected into *A. mellifera*. These results suggest that virus may be locally adapted to its host, and that *A. mellifera* may be the reservoir host for all three viruses. The immediate impact of viral spill-over from honey bees to bumble bees and other wild bee species might then indeed be low, as we found under our benign laboratory conditions. But transmission from bumble bee to bumble bee could lead to local adaption of a virus to a *Bombus* host, with unknown

consequences of pathogen spill-back from bumble bees and other wild bee species to honey bees if viral adaptation to the novel host (*Bombus*) trades off with a loss of virulence in the original host (*Apis*) (Barrett *et al.*, 2009; Ebert, 1998; Elena, 2017). The speed with which local adaptation to a novel host occurs, its relationship to virulence, and whether it results in a loss of viral fitness or virulence in the reservoir host, will help determine the impact of viral pathogen spill-over for the entire bee pollinator community (McMahon *et al.*, 2018).

It is unsurprising that we found inoculation by injection to lead to higher viral titres than by oral inoculation of bumble bees. Injection of a pathogen into the insect haemocoel gives the pathogen access to the entire host body tissue whereas oral infection initially gives it access to the gut alone. The former route of transmission, injection into the haemocoel, through *V. destructor* host feeding is thought to account for the huge increase in viral prevalence and intensity of infection of DWV in honey bees (Martin *et al.*, 2012; Mondet *et al.*, 2014). In support of this view, injection of another honey bee virus, Israeli acute paralysis virus, into *B. terrestris* led to systemic infection and rapid host death whereas oral infection led to infection of the host gut in a dose-dependent manner and with more limited impact on host health (Wang *et al.*, 2018).

That BQCV was extremely virulent in our honey bee assay is at first sight surprising because BQCV is widespread and highly prevalent in honey bee populations (Alger *et al.*, 2019b; McMahon *et al.*, 2015; Murray *et al.*, 2019; Radzevičiūtė *et al.*, 2017; Traynor *et al.*, 2016). Both Retschnig *et al.* (2014) and Doublet *et al.* (2015) found no effect of feeding BQCV on adult honey bee mortality, suggesting it is a benign pathogen, though lethal when fed to queen (Bailey & Woods, 1977)), drone (Siede & Büchler, 2003) and worker (Doublet *et al.*, 2015) pupae. The high virulence of BQCV in honey bees that we here and others (Remnant *et al.*, 2019) have observed is likely due to it having been injected into hosts. From epidemiological theory, pathogen prevalence is often inversely related to virulence in insect host populations (May & Anderson, 1979). To explain its high prevalence in honey bees, we suggest that BQCV is rather benign when infecting adult *A. mellifera* workers through its typical faecal-oral route of transmission.

The Western honey bee is the dominant flower visitor across most terrestrial ecosystems of the world (Hung *et al.*, 2018). Dominant species in a community often disproportionately influence pathogen transmission and dynamics (Keesing *et al.*, 2010) through their central role in contact networks (White *et al.*, 2017), exacerbated in the case of *A. mellifera* because it is likely the reservoir host of BQCV, DWV-A, DWV-B. Though we recorded little to no virulence of these viruses on *B. terrestris* under laboratory conditions, their impact on this and other bee species (and other flower visitors) under field-realistic conditions should be the focus of future studies to evaluate the role of viral spill-over in wild bee decline.

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Authors' contributions
A.T. carried out the experimental and molecular laboratory work, participated in data analysis, conceived the design of the study and drafted the manuscript; T.S. assisted with the experimental and molecular laboratory work, participated in the design of the study and data interpretation; S.T. contributed to the design of the study, to data interpretation and to statistical analyses; R.J.P. conceived of the study, assisted in data interpretation, coordinated the study and helped draft the manuscript. All authors gave final approval for publication.

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Competing interests
The authors have no competing interests.

Data accessibility
All data supporting the findings of this study are available from the Dryad Data Repository (doi:10.5061/dryad.fxpnvx0nt).

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General discussion

It has been shown in several correlative studies that BQCV and DWV are very common in bumble bees and other wild bees (reviewed in Chapter I). However, it is difficult to draw conclusions from observational data from the field about the true risks arising from these viruses or bring clarity to the directionality of viral spill-over. It is also fundamental to address experimentally the impact of virus on potential new hosts and to evaluate its transmission so as to be able to understand the consequences of virus spill-over for wild bees. The aim of my doctoral thesis was to investigate this topic in more detail by performing highly controlled lab experiments.

In Chapter II my co-authors and I were able to demonstrate that infected honey bees readily transmit DWV-A to the bumble bee *B. terrestris*, both when in close contact and indirectly, when sharing a common food resource (sugar solution). But we could not detect viral transmission from *B. terrestris* to conspecifics or to honey bees, either through direct contact or indirectly via shared food.

Previous studies have shown contradictory results, arguing either that DWV is unlikely (for *B. terrestris*: Gusachenko *et al*., 2020) or is likely (for *B. impatiens*: Burnham *et al*., 2021) to be transmitted from honey bees to bumble bees. Furthermore Burnham *et al.* (2021) have shown that *B. impatiens* inoculated with 3×10^6 DWV *per os* subsequently deposit detectable virus on artificial flowers, demonstrating the potential for infected bumble bees to transmit DWV, while I showed in Chapter II that infected bumble bees in my experiment did not lead to the transmission of DWV back to honey bees or onward to uninoculated bumble bees. Several differences between the two studies could have lead to the contradictory results. Besides using different bumble bee species and the resulting genetic differences between hosts (Burnham *et al. B. impatiens*; this study *B. terrestris*) also genetic differences between the inocula could be a reason for transmission being observed in the USA but not in Germany in my experiments. Furthermore the technique of infection, injection vs. feeding, could alter the results. While injection of a pathogen into the insect haemocoel gives the pathogen access to the entire host body tissue, oral infection initially gives it access to the gut alone, leading to higher viral titres in injected individuals compared to orally inoculated bees (Chapter III). But even with high viral titres found in injected individuals, the virus is likely not able to pass through the intestinal cells and to access the digestive tract. Viral transmission via flowers is assumed to happen due to faeces deposited on flowers. Maybe the virus is not able to reach the gut lumen when injected into a bumble bee and therefore cannot be deposited with the faeces. Viruses fed orally are automatically in the gut lumen and therefore are likely to be spread via the faeces.

It could also be that bumble bees in my experiments deposited virus but that the virus lost its viability. Burnham *et al.* (2021) showed that virus deposited by bumble bees was detectable on flowers but there is no information on the viability and the infectious potential of these shed viruses and if these viruses could infect another individual. On the other hand, I did not collect the faeces from my experimental bumble bees and screen them for the presence of DWV. It could therefore be that I would also detect viruses in the faeces but would not find ongoing transmission from them due to the lack of infectivity of the virus particles. The virus could have been inactivated in the bumble bee gut system by immune defence mechanisms or potentially interfering host microbiota. The role of the gut microbiota in viral defence has so far not been well investigated, but gut microbiota, food and other environmental factors are highly connected (Dosch *et al*., 2021). Alternatively, the virus may be selected in a way that it loses the possibility to infect another individual. Additional analyses of the infectivity of the viruses in oral and anal excretions of bumble bees infected orally or by injection with *Bombus*-derived *versus Apis*-derived inocula would help to resolve these questions.

For bees and other flower-visiting insects, flowers are considered important transmission hubs for their pathogens (McArt *et al*., 2014). But flowers represent an alien and potentially hostile environment for viruses (McArt *et al*., 2014). Furthermore many viruses are sensitive to UV light (Lytle & Sagripanti, 2005; McLeod *et al*., 1977). Therefore, the viruses shed onto flowers probably constantly decrease over time in infectivity, as found in other bee pathogens (*Crithidia bombi* Figueroa *et al*., 2019, *Nosema apis* Zheng *et al*., 2014), leading to a decreasing possibility of infection. Also some floral nectars are suggested to reduce the viability of pathogens. Citric acid and aucubin, two plant compounds, reduced crithidia cell counts compared with controls (Michaud *et al*., 2019). Therefore my study just shows whether transmission in general is possible. The highly controlled lab setting of the experiment allowed me to exclude these abiotic und biotic factors that act on viruses on flowers. Furthermore this setting forced all bees to use one feeding source for lack of alternatives. Fouks *et al.* (2011) were able to show that bumble bees have a strong preference for the non-contaminated flowers when *C. bombi*, a trypanosomatid Euglenozoa, is present (Fouks & Lattorff, 2011). This shows the ability of bumble bees to recognise the contamination of food sources with at least one pathogen. Therefore, there is a possibility for a reduced transmission of the virus in nature by bees avoiding resource collection from contaminated sources.

Assuming the hypothesis is correct that both abiotic factors like heat or uv radation, as well as biotic factors like nectar compounds, have an impact on the viability of bee pathogens, the duration of time between the visitations of donor and recipient pollinators to the same flower would be crucial for transmission to occur. High visitation frequency per flower by different host species would likely lead to higher transmission compared to low visitation frequency. Floral patches, like artificial flowering strips, especcially in semi-natural poor regions, are known for attracting bumble bees (Kleijn *et al*., 2015, 2018). Another study has

demonstrated an effect of sown wildflower fields on micro-parasite prevalence; the prevalence of parasites increased with increasing size of sown wildflower fields in resource poor landscapes (Piot *et al*., 2019). And also in a semi-field setup, it has been shown that high host density compared to low host density resulted in a higher viral prevalence and level of infection in bumble bees with SBPV (Bailes *et al*., 2020). On the other side, flowering strips are positively correlated with pollinator diversity (Buhk *et al*., 2018), leading to species-rich communities that are likely to have a lower viral prevalence due to dilution effects (Fearon & Tibbetts, 2021). More studies on flower visitation frequency at flowers and more studies on pathogen prevalence in flowering strips are needed to know whether the artificial creation of attractive flower patches is still beneficial (more resources) or counterproductive (more spill-over) for wild bees.

Based on the results in Chapter II, it seems crucial to examine the effects of honey bee associated viruses on *B. terrestris* (Chapter III). I did not, though, observe impacts of DWV-A, DWV-B or BQCV on adult bumble bee mortality under laboratory conditions. Furthermore, all three viruses replicated to higher titres in honey bees than in bumble bees, which is surprising, given that honey bees are generally smaller than bumble bees. The higher viral titre per honey bee suggests that these viruses are locally adapted to *A. mellifera*, which is probably their reservoir host.

But there are also experimental studies showing, contrary to my study, the reduced lifespan of DWV on worker bumble bees of the species *B. terrestris* compared to a control group (Fürst *et al*., 2014; Graystock *et al*., 2015b), as is seen in honey bees. I was not able to reproduce these results in my study, which did not detect an increase in mortality following inoculation by DWV or BQCV. There are a wide number of parameters that can be responsible for the differences between the studies. First, there could again be a genetic effect underlying differences between studies. Both mentioned studies were conducted in the UK where they worked with *Bombus terrestris audax* colonies from Biobest Group NV (Westerlo Belgium), whereas I used *B. terrestris* colonies from Koppert B.V (Berkel en Rodenrijs, Netherlands). Besides the genetic differences between these two subspecies *B. terrestris audax* and *B. terrestris*, there is probably also an additional genetic difference between bumble bees from Koppert and from Biobest. Colonies from commercial breeders are most likely partly inbred and therefore possibly exhibit a reduction in genetic variation, with unknown consequences for host tolerance to viral infection. In addition, over the years the bees from Koppert could have developed an unknown unintended tolerance against honey bee associated viruses. In the bumble bee rearing industry, some companies use honey bee workers during *Bombus* queen colony initiation (Velthuis & Van Doorn, 2006), which could have selected for such a tolerance. To address this topic further, experiments with wild caught bumble bees would be necessary. This would show the real impact of these viruses on the bumble bees in the wild. Of course, working with wild caught bees is more challenging due to difficulties in controlling for age, pre-infection with other viruses, life history or obtaining sufficient sample sizes.

Another important factor accounting for differences between studies could be the origin of the viral inoculum. In my study, the virus originated from honey bees and was propagated in honey bees. Although bumble bees are phylogenically closely related to honey bees and are within the family Apidae together in the subgroup of the corbiculate bees (Danforth *et al*., 2013), there are genetic differences between the species that could underpin differences in viral tolerance. Reconstructions of host shifts in nature have confirmed that pathogens are more likely to shift between closely related species (Faria *et al*., 2013; Longdon *et al*., 2011; Streicker *et al*., 2010). Close relatives of the natural host typically offer a comparable environment to which the pathogen is adapted, potentially making it more susceptible. With this in mind, one might predict that a honey bee-derived virus would readily infect bumble bees. Nonetheless often pathogens have to adapt to be successfully able to infect a new host. The virus has to be able to cope with the immune response of the host and it has to use specific cell surface receptors to enter the cell, to replicate and to persist (Holmes, 2009). Almost nothing is known about the mechanisms that enable DWV to enter host cells, so it is possible that DWV adapted to *A. mellifera* has a genetic predisposition to successfully enter cells of *B. terrestris* without a need for many or any mutations. An understanding of the mechanism of host cell entry is crucial to understand molecular compatibility within a novel host system (McMahon *et al*., 2018) and could thus be helpful for risk assessments of host shifts. Maybe *B. terrestris* provides a too restrictive host environment where the virus has to adapt further to improve its fitness. Therefore it could be really important to investigate the role of the host in which the virus was propagated. Virus derived from honey bees potentially needs more time to adapt to a new host compared to virus propagated in the new host. Indeed, comparing viral titres of the different viruses injected into honey bees and bumble bees revealed that viruses always replicated to lower titres in bumble bees (Chapter II, Chapter III) than in honey bees. Maybe the lower titres in bumble bees could be an indication that *B. terrestris* provides a more constrained host environment for the viruses than *A. mellifera* due to lack of adaptation to *Bombus*. One possibility to overcome this problem could be to propagate the virus over several generations in the new host that one wants to use for studies of virulence effects. Selecting the virus in this way potentially favours mutations that allow a pathogen to (a) enter a host cell with greater efficiency and (b) "fine tune" or optimise its fitness in a new host, for example by better utilising cellular machinery, enhancing immune avoidance, optimising virulence, and maximising transmission potential (Longdon *et al*., 2014). These adaptations could be then visible as an increase in virulence or virus load of the virus or as genetic change in its genome since certain mutations are often necessary for a successful establishment in a new host (Durrer & Schmid-Hempel, 1994; Longdon *et al*., 2014; Schmid-Hempel, 2011). Another option would be to start from the beginning with a field derived virus extracted from a bumble bee in the experiments I undertook. But it is difficult to check for the historical background of this infection. Since how long this virus is actually fluctuating already in bumble bees would be unknown. Keeping that in mind, the study from Graystock *et al.* (2015) is notable because it used an inoculum derived from the

fat bodies of 50 previously infected bumble bees. It is not known where the initial inoculum comes from that was used to infect the 50 bumble bees to propagate the virus. But maybe with that one step already some adaptations arose in the virus and the inoculum was more suitable to infect *Bombus* hosts whereas in my experiment the virus had no time to adapt to the host prior to my inoculating bumble bees.

Next to the origin of the inoculum, the concentration of an inoculum can play a huge role in its virulence for a host. Several studies have shown that that a certain amount of viral particles is needed to start an oral infection (Doublet *et al*., 2015; Gusachenko *et al*., 2020; Piot *et al*., 2015). Also it has been shown that the duration of passage of orally ingested *Nosema* spores through the midgut was only 6h in bumble bees but was at least 24h in honey bees (Gisder *et al*., 2020). Rapid gastrointestinal passage in *Bombus* could also leads to the need of a high number of virus particles, or more than one infection event, to start an infection in a bumble bee. I therefore decided in my feeding assay to inoculate 10^9 virus particles per host. In the injection assays, I decided to use 10^7 to stick to established amounts of viruses that are used in honey bee research (McMahon *et al*., 2016). Of course it is possible that successful inoculation and subsequent virulence are dependent on body size differences between honey bees and bumble bees and that thus the same concentration of virus has a different effect on the two species. Whereas Fürst *et al.* (2014) used inocula of the same concentration as I did, even probably not pure DWV genotype A or DWV-B but a mix, Graystock *et al.* (2015) used an unknown concentration of DWV inocula, which was moreover not tested for contamination by other pathogens. It is known for some viruses that they show a high impact on the survival of honey bees and wild bees i.e. they are highly virulent; for example, Wang *et al.* (2017) showed that IAPV killed all bumble bees within five days when hosts were injected with just 500 virus particles. A small contamination with e.g. that virus could probably change the whole outcome of any viral inoculation experiment.

Because my experiments in Chapter III were carried out in an artificial setting, results have to be taken with caution. Under benign lab conditions, hosts can potentially be able to compensate for the pathogenic effects of a parasite. But if the conditions become more stressful, the host may lose this ability and so-called condition-dependent virulence is detectable. In nature, multiple stressors like climate, pathogens, malnutrition or pesticides hit the host and the individual may not be able to compensate all stressors. Especially pesticides are known for their synergistic effects with other stressors on bee survival. It seems there is an interaction between pesticides and honey bee pathogens like *Nosema* (Alaux *et al*., 2010a; Dussaubat *et al*., 2016; Vidau *et al*., 2011) or viruses (Coulon *et al*., 2018; Locke *et al*., 2012) that change their replication or virulence. It is known that there is an interaction between e.g. thiacloprid and BQCV when using *A. mellifera* as the host, with viral virulence increasing in the presence of the pesticide (Doublet *et al*., 2015). Additionally its known that clothianidin and imidacloprid induce proliferation of DWV in honey bees under lab conditions (Di Prisco *et al*., 2013). It is likely that similar results would be

detectable in wild bees. The starvation assay in my experiments in Chapter III represents a starvation period like in a natural situation, where foraging can be interrupted by cold weather and rain results in a costly energy shortfall in the colony if the workers fail to collect necessary amounts of pollen and nectar (Cartar & Dill, 1991). While I was not able to detect condition-dependent virulence of the tested viruses in *B. terrestris* workers, it has been shown that SBPV infection in workers of *B. terrestris* can result in significantly higher mortality under starvation conditions, with infected bees 1.6 times more likely to die compared to the control at any given time point whereas there is no effect of SBPV on bumble bees under satiated conditions (Manley *et al*., 2017). Also for *C. bombi* it is known that starvation has an impact on the host. Under favourable conditions the infection causes no mortality, while when *B. terrestris* workers were starved the infection increased the host mortality rate by 50% (Brown *et al*., 2000). Similar results were also found for other hosts-parasite relationships (Arnqvist & Mäki, 1990; Jokela *et al*., 1999; Vargas & Zeledón, 1985). Besides low quantity of food, also low quality or the absence of certain nutrients could be a huge stressor for bees.

To ensure comparable standardised results, I used for all my experiments a 50% sucrose solution instead of a diet based on carbohydrates as well as pollen, which would have been more natural and more nutritious. First trials with pollen resulted in bumble bee workers becoming pseudo-queens and starting to lay eggs. Under these conditions, a comparison between workers that remained as workers and those that had become pseudo-queens might have been compromised due to possible changes in survival (e.g. up-regulation of innate immunity) due to reproduction. But the presence or absence of pollen can have a huge effect on some parasites. It has been shown that a richer diet leads to better immunity (Alaux *et al*., 2010b; DeGrandi-Hoffman & Chen, 2015; Dolezal & Toth, 2018). The viral load of DWV decreased dramatically in honey bees fed pollen or protein supplement compared with controls (DeGrandi-Hoffman *et al*., 2010). Furthermore a good diet can help honey bees tolerate infections with IAPV (Dolezal *et al*., 2019) or *Nosema* (Di Pasquale *et al*., 2013). But on the other side it is for example known for the gut trypanosome *C. bombi* that size and temporal pattern in development of the parasite are different in pollen-fed and pollen-starved bees (Logan *et al*., 2005). In well-fed *B. terrestris* workers, more *C. bombi* was detectable. This parasite probably obtains significant nutritional supplies directly from its host and therefore a shortfall of nutrition in the host results in a reduced population of the parasite (Logan *et al*., 2005). Even if mechanisms of replication are fundamentally different between viruses and an Euglenozoa, DWV is often very present in the gut, too, and therefore it cannot be excluded that already the lack of pollen in the benign conditions on my experiments in Chapter III had a negative effect on the parasite, not just on the host.

Open questions for the future

Although numerous correlative publications concerning viruses in wild bees are now available, experimental work on this topic is still essential to understand the potential damage of viruses on wild pollinators. In my thesis I brought light onto some aspects of this problem, but still a lot of questions remain unanswered.

On the one side, even if hypotheses existing about transmission routes, experimental demonstration of these hypotheses are mostly lacking. Especially looking at Chapter II and the paper from Burnham *et al.* (2021), faeces are often assumed to be the route of cross-species transmission between bee species. But no studies have yet shown that bumble bee faeces contain viable virus particles that can again infect conspecifics or others; in honey bees, in contrast, it has been already shown that their faeces may contain viable virus (Chen *et al*., 2014; Hung, 2000; Ribière *et al*., 2007). For chronic bee paralysis virus (CBPV), it is known that infectious CBPV particles excreted in the faeces of infected bees can infect naive bees and provoke overt diseases. (Hung, 2000). Bailey and Gibbs (1964) even showed that dried faeces containing acute bee paralysis virus (ABPV) derived from infected bees still caused acute paralysis in bees when injected into them (Bailey & Gibbs, 1964). In the last two years, contradictory results appeared concerning the transmission of virus emanating from bumble bees. While Gusachenko *et al.* (2020) and my study in Chapter II do not show any transmission from bumble bees to either bumble bees or honey bees, Burnham *et al.* (2021) argue for transmission emanating from bumble bees. Additional analyses of the infectivity of the viruses in oral and anal excretions of bumble bees infected orally or by injection with viral inocula would help to resolve these questions.

All experiments in this thesis used bumble bees purchased by a commercial breeder. Colonies from commercial breeders are probably partly inbred and may therefore exhibit a reduction in genetic variation. It would be very interesting to repeat the experiments performed here with wild bumble bees to be able to assess the susceptibility to viruses of these wild specimens. Also looking at other bumble bee species or even other wild bees for their susceptibility to virus could be very interesting. Published studies (McMahon *et al*., 2015) and own unpublished results show that e.g. *B. pascuorum* harbours less DWV and BQCV than *B. terrestris* caught in the same locality. Maybe just slight differences in foraging behaviour and therefore less overlap in flower use with honey bees could explain the potential differential spill-over of the virus among bumble bee species (Alger *et al*., 2019a); see also Chapter II. If that would be the case, *B. lapidarius* that has a relatively short tongue and therefore considerable niche overlap with honey bees might be predicted to have higher virus titres and higher viral prevalence compared to other bumble bee species. Differences between species could also be aqccounted for by their differential susceptibility to these viruses. This could be investigated by controlled infection experiments in the laboratory with different bumble bee species, looking at transmission *per se* and mortality, as I have described in this thesis.

But not just bumble bees should be the focus of future research. These viruses have also been already detected in other wild bees (Radzevičiūtė *et al*., 2017). In some wild bees besides bumble bees, even the negative strand of DWV has been found (*Osmia cornuta* Mazzei *et al*., 2014; *Andrena haemorrhoa* Radzevičiūtė *et al*., 2017) or *Colletes spp*. (Tapia-González *et al*., 2019), or the negative strand of BQVB (Osmia *bicornis* Morfin *et al*., 2020; Radzevičiūtė *et al*., 2017; Tapia-González *et al*., 2019), a sign of viral replication in the host. Besides knowing more about the potential impacts these viruses have on these hosts, it would also be interesting to quantify the differences in host susceptibility, which might be hypothesised from the different prevalence of the viruses in the respective host species. Several hypotheses could be imaginable to explain the high viral prevalence found in bumble bees and the rather low prevalence in other bee species. Experimental studies looking at possible effects of host density due to sociality on the transmission behaviour of the virus (Schmid-Hempel, 1998) or higher susceptibility on the basis of close host ancestry, as seen in several other viruses (Longdon *et al*., 2011; Streicker *et al*., 2010), would be really interesting.

Concerning the effect of the viruses on bumble bees or other wild bees, also other parameters than worker mortality would be interesting to look at. Workers of social insects, such as bumble bees, do not reproduce themselves but increase their fitness by helping their mother to raise reproductively competent offspring. They so gain fitness by working for the colony in collecting food or in brood care. Thus, anything that reduces survival of workers also reduces their inclusive fitness, therefore worker survival is the ultimate fitness measure (Schmid-Hempel, 2011). But there are definitely other important traits of hosts that a virus may impact upon, too. KBV and IAPV are famous for their high virulence, inducing high mortality in honey bees. In experiments with bumble bees, no increase in worker mortality was detectable as a consequence of infection with KBV or IAPV (Meeus *et al*., 2014). But KBV infections resulted in significant slower colony development and offspring production, while only the latter can be reported for IAPV (Meeus *et al*., 2014). Also, foraging behaviour could have an effect on the colony performance and ultimately colony fitness. In honey bees Natsopoulo *et al.* (2016) found that DWV accelerated the temporal polyethism schedule, but did not reduce the behavioural repertoire of the host. Honey bee workers still performed all tasks but e.g. shortened significantly the time they performed brood care and started significantly earlier to forage (Benaets *et al*., 2017; Natsopoulou *et al*., 2016). Bumble bees, even less strict and probably not so age related but more size related in their temporal polyethism schedule, also exhibit a division of labour (Jandt *et al*., 2009; Spaethe & Weidenmüller, 2002). One could assume that due to the weak task specialization of bumble bees, workers tend to switch even more to foraging when infected with viruses so as to move away from the central area of the colony, where the brood and the queen are sited. Exploring other virulence parameters could open new insights into the impact of honey bee viruses on wild bees.

Furthermore my experiments were performed under relatively benign lab conditions. Field-realistic experiments are essential to fully understand the impact of virus on wild bees. As already mentioned, hosts under benign conditions may be able to compensate for the pathogenic effects of its parasites. But if conditions become more stressful, the host may lose this potential ability. Furthermore, studies in the field give the bees the opportunity to search for specific food resources. It has been shown that honey bees increase plant resin collection in response to a fungal infection for self-medical purposes (Simone-Finstrom & Spivak, 2012), a mechanism that has been already discussed for bumble bees too (Baracchi *et al*., 2015; Manson *et al*., 2010). In my experiments in Chapter II and Chapter III**,** bees had unlimited access to carbohydrates but not to protein. If not simply food limitation but also food quality is an important factor in determining host tolerance, experimental set-ups allowing bees to forage freely seem to be important to derive field-realistic results.

Last but not least, it would be important also to put more focus on the virus. In my studies, I focused mainly on the ecology of the bees and the responses of the bee without looking in more detail at the virus. Experiments investigating how DWV and BQCV evolve and potentially adapt when exposed to different host species could help a lot in assessing the risk of cross-species transmissions. Local adaptation is often studied using serial passage experiments due to the ability to monitor the genotypic and phenotypic evolution of the pathogen in real-time (Ebert, 1998). Comparing BQCV and DWV from honey bees and bumble bees could help to understand the differences that are found in experiments dealing with transmission (Chapter II) or virulence (Chapter III).

Bees are an important component of our terrestrial ecosystems and are essential for the healthy maintenance of natural processes through provision of the ecosystem service of pollination. In summary, I could show that it can be assumed that there is a potential threat posed by viruses for wild bees. I could show in my experiments that viruses can be transmitted from honey bees to bumble bees and, moreover, that they are able to replicate in bumble bees. RNA viruses are known for their higher probabilities to infect new host species because of their high mutation rates that lie in the lack of a proof-reading function in their polymerase proteins and resulting error-prone replication cycles (Holmes, 2009). This is underlined in the results shown in my thesis, under the assumption that honey bees are the original host of these viruses. However, in controlled experiments, no life-shortening effect was exhibited by the viruses in bumble bee workers comparable to those in honey bees. However, this does not preclude that these viruses may not otherwise cause lasting harm to bumble bees or other wild bees. Also, possible viral adaptation over time to a new host and resulting effects must be considered. As long as it is not clear whether these viruses have a negative effect on bumble bees or other wild bees, wild bees should be protected from the transmission of viruses from honey bees.

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Appendix

A. Supplementary Material

Chapter I:

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.coviro.2016.06.006.

Chapter II:

Contents:

Supplementary Methods: additional information to cage experimental and molecular biological (qPCR) methods used in this study

Cage experiments

Here we provide (1) additional rationale for our experimental design that excluded the DWV vector *Varroa destructor*, (2) additional details related to housing bees in cages and (3) information on the source of bees.

1) Rational for an experimental design that excluded *Varroa destructor*

We did not incorporate *Varroa destructor* into our experimental design. DWV is vectored among honey bees by the exotic invasive ectoparasitic mite *Varroa destructor*, leading to elevated viral titres in honey bees (Stephen *et al*., 2012), high virulence of DWV (McMahon *et al*., 2016; Norton *et al*., 2020; Tehel *et al*., 2019) and causing colony mortality. Though *V. destructor* mites represent the major route of *Apis* to *Apis* horizontal transmission in honey bee colonies (Yañez *et al*., 2020), we did not incorporate them into our experiments because they are restricted to host *Apis* spp. (Rosenkranz *et al*., 2010). They therefore play only an indirect role in the transmission of DWV to or from *Bombus* through elevation of the prevalence and titre of DWV in honey bees (Fürst *et al*., 2014; Manley *et al*., 2019), which we controlled by experimental infection of donor bees.

2) Cage maintenance

Cages were held in incubators at 30°C and 50% RH with *ad libitum* feeding of bees with sucrose (50% w/v) in a single feeding tube (1.5mL Eppendorf tube with two perforations to allow access to the sucrose solution) per cage, replaced daily.

3) Source of bees

As a source of bumble bees, we used commercial *Bombus terrestris* colonies (Koppert B.V., Berkel en Rodenrijs, Netherlands), which were held at 30°C and 50% relative humidity with *ad libitum* 50% (w/v) sucrose solution and UV-radiated, freshly defrosted pollen pellets (Imkerei Schachtner, Schardenberg, Austria). Honey bee colonies originated from our institute apiary, in which we use honey bees widely employed by beekeepers in Germany that are, or are derived from, the subspecies *Apis mellifera carnica*. . Colonies are treated regularly with Byvarol (flumethrin) or Apistan (tau-flumethrin) and oxalic acid (all have a strong miticide effect) according to manufacturers' specifications.

All colonies used for experiments (four bumble bee colonies and two honey bee colonies) as well as the pollen were tested by real-time quantitative PCR (qPCR) prior to use for the presence of six common honey bee viruses, including DWV-A as well as DWB-B (Supplementary Table S3). In total, we used ca. 800 bumble bees taken randomly from the four bumble bee colonies and ca. 800 adult honey bees taken randomly from brood frames of the two honey bee colonies.

Molecular biological (qPCR) methods

1) RNA extraction

To check that bumble bees (4 source colonies) and honey bees (2 source colonies) as well as the fresh-frozen pollen pellets were devoid of viral pathogens, we tested them by real-time quantitative PCR (qPCR) for six common honey bee viral targets: deformed wing virus genotype A (DWV-A), deformed wing virus genotype B (DWV-B), black queen cell virus (BQCV), chronic bee paralysis virus (CBPV), sacbrood virus (SBV), and slow bee paralysis virus (SBPV) (Supplementary Table S1). For viral screening, we collected 10-20 adult worker bees per colony or 2 x 0.3g of pollen pellets (i.e. two samples), crushed them in a plastic RNAse-free mesh bag (BioReba, Reinach, Switzerland) with ultrapure diethylpyrocarbonate (DEPC)-treated water (500µl per honey bee, 1000µl per bumble bee, 1000µl per 0.3g of pollen) after snap-freezing them on dry ice, and then recovered 100µL of homogenate from beyond the BioReba mesh for RNA extraction. This allowed us to quantify the viral titres of a pool of bees or pollen from each colony to check on whether there was a potential background viral infection in colonies used as sources of bees for experiments.

We quantified viral (DWV-A) titres in individual adult worker bees arising from inoculation experiments by crushing one whole honey bee in 500µl or one whole bumble bee in 1000µL of 1% beta-mercaptoethanol RTL buffer using a plastic pestle, of which 100µL were used for RNA isolation. RNA was extracted from the homogenate of an individual bee using an RNeasy mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions in a QiaCube robot (Qiagen). cDNA was synthesized from RNA extracts using oligo$(dT)_{18}$ primers (Thermo Scientific) and reverse transcriptase (M-MLV and Revertase, Promega, Mannheim, Germany) following the manufacturer's instructions. For cDNA synthesis, 800ng of RNA were used, after which the resultant cDNA was diluted 1:10 prior to use in qPCR. This allowed us to quantify the viral titres of individual bees arising from inoculation experiments.

2) Quantification of virus

We ran quality control checks on each 96-well qPCR reaction plate. To check that the correct template had been amplified, PCR products were denatured for one minute at 95°C, cooled to 55°C for one minute, and then a melting profile was generated from 55°C to 95°C (0.5°C per second increment) to ensure the expected product had been amplified, namely a single peak at the product's dissociation temperature (Tm). In addition, a virus-free (negative control) and a virus-infected (positive control) sample were included on each 96-well plate. A host reference (housekeeping) gene, *Apis mellifera*-β-actin, was also amplified for all bee samples as an internal reference marker using the primers given in Locke *et al.* (2012). All PCRs showed the expected signals for the negative (no signal) and positive ($Cq < 35$) controls and β-actin gave Cq values for all samples between 18 and 26, suggesting no contamination or error in pipetting, no RNA degradation, no error in RNA extraction, and no failure in cDNA synthesis. All qPCRs were run for 40 cycles. We set a threshold at $Cq = 35$. $Cq < 35$ was counted as positive, Cq values > 35 were counted as negative.

Absolute quantification of virus (DWV-A) titres in inocula and in individuals arising from experiments followed methods in Tehel *et al.* (2019) using a dilution series ($10^{-1} – 10^{-8}$) of an external DNA standard to generate calibration curves and the mean Cq of duplicate qPCRs performed for each sample. Cq values of qPCR duplicates did not differed by > 0.5 for all Cq < 35. 0.5% of qPCRs had to be rerun to match this criterion. Primer efficiencies were 96% for DWV-A with correlation coefficients (R^2) ≥ 0.9. Our methods, quality criteria and the thresholds conform to current best practice MIQE guidelines (Bustin *et al*., 2009). By comparison of Cq values of individual samples across the range of RNA concentrations (700ng/µl – 40ng/µl) to a standard curve, our Cq threshold of 35 approximates 10^6 genome equivalents of DWV-A per bee, a titre below which DWV likely has little or no pathological impact on a host bee (Gusachenko *et al*., 2020).

Supplementary Results: additional results relating to the quantification of viral titres in donor and recipient bees of Experiments 1 and 2

Experiment 1, mimicking intracolony transmission

As explained in the main text, in the two treatments AA and AB in which honey bees were viral donors, viral titres in both donor and recipient bees were consistently high. In detail, viral titres in donor honey bees at 7 days of age were $>10^{12}$ genome equivalents (mean ± s.e.; treatment AA: $4.00^{12} \pm 1.73^{12}$, n = 6; treatment AB: $1.97^{13} \pm 4.23^{12}$ n = 6; Fig. 2B), over five orders of magnitude greater than the inoculum (10^7) injected into them and indicative of successful viral replication in donors (LMM, LR-Test: $\chi^2 = 56.907$, df = 3, p<0.001; post-hoc comparisons, donor in AA vs donor in AB: p = 0.177). Viral titres in recipient honey bees were one order of magnitude lower compared to *Apis* donors (recipients in treatment AA: $3.63^{11} \pm 6.49^{11}$, n = 9; post-hoc comparisons, donor in AA vs recipients in AA: p < 0.001), while titres in recipient bumble bees were five orders of magnitude lower than those of *Apis* donors (recipient in treatment AB: $3.44^8 \pm 5.86^8$; post-hoc comparisons, donor in AB vs recipient in AB: p < 0.001) and three orders of magnitude lower than those of recipient *Apis* in the AA treatment (post-hoc comparisons, recipient in AA vs recipient in AB: p < 0.001).

Viral titres in all donor bumble bees were consistently high (mean ± s.e.; treatment BA, $9.82^9 \pm 1.68^{10}$, n = 6; treatment BB: $1.36^{10} \pm 2.17^{10}$, n = 7) with no difference between treatments (LM, $F_{1,11} = 0.300$, p = 0.595 ; Fig. 2B) and greater than the 10^7 inoculum, indicating successful viral infection in donor bumble bees. As for honey bees, no bumble bee was infected in a control cage (Control: 5 of 5 *Bombus* individuals in 5 of 5 cages; Supplementary Table S1 and Supplementary Fig. S2A), confirming that our experimental paradigm functioned as expected.

Experiment 2, mimicking food-borne transmission

As explained in the main text, viral titres in donor honey bees inoculated with 10^7 genome equivalents of DWV were consistently high at day 7 (mean ± s.e.; *Apis* donors in treatment AA: $2.25^{13} \pm 4.59^{12}$, n = 6; *Apis* donors in treatment AB: $1.80^{13} \pm 3.22^{12}$, n = 6; difference

between treatments, LMM, LR-Test: $\chi^2 = 0.773$, df = 1, p = 0.379; Fig. 3B), demonstrating successful viral replication in donors.

Few recipient bumble bees in treatment AB were infected at day 7 (n = 3 of 18 bees) or at day 14 (n = 2 of 18 bees; Supplementary Table S2). Titres of infected recipient bumble bees were lower than those of their respective donor honey bees, but similar across sampling days (day 7: $2.45^7 \pm 2.96^6$, n = 3; day 14: $1.01^8 \pm 9.31^7$, n = 2; Fig. 3B). Interestingly, though recipient infected *Bombus* at day 7 (in treatment AB; $2.45^7 \pm 2.96^6$, n = 3) had a lower viral titre than recipient infected *Apis* at day 7 (in treatment AA; $5.36^{12} \pm 1.39^{12}$, n = 3), titres of infected recipient *Bombus* at day 14 (treatment AB; $1.01^8 \pm 9.31^7$, n = 2) were similar to those of infected recipient *Apis* on day 14 (treatment AA; $2.77^7 \pm 1.05^7$, n = 13).

Few recipient honey bees in treatment AA were infected at day 7 (n = 3 of 18 bees) but many more were infected by day 14 (n = 13 of 17 bees; Supplementary Table S2). Titres of infected recipient honey bees (day 7: $5.36^{12} \pm 1.39^{12}$, n = 3; day 14: $2.77^7 \pm 1.05^7$, n = 13) were lower than those of their respective donor honey bees (Fig. 3B), probably reflecting a delay in viral acquisition by recipients.

Inoculated donor bumble bees had high viral titres on day 7 (donor bumble bees in treatment BA: $1.74^{10} \pm 6.96^9$, n = 6; donor bumble bees in treatment BB: $5.18^9 \pm 2.78^9$, n = 6; difference between treatments were marginal, LM: $F_{1,10} = 5.126$, p = 0.047; Fig 3B), consistently higher than the 10^7 genome equivalents of DWV with which they had been inoculated seven days earlier.

Figure S1

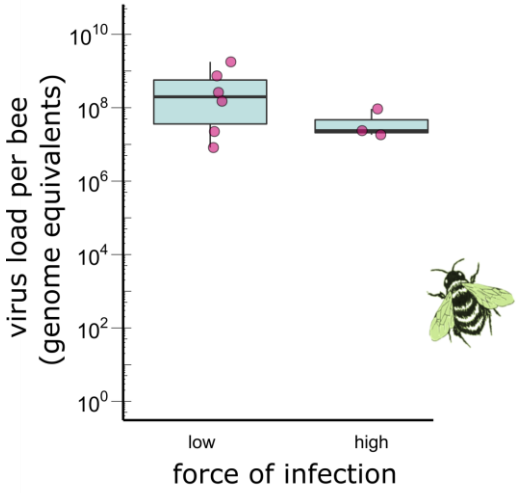

Figure S1. Viral titres in recipient *Bombus* in the treatment *Apis* to *Bombus* of Experiment 1, mimicking *intracolony transmission*, in relation to the force of infection. Viral (DWV-A) load of bumble bees from the *Apis* to *Bombus* treatment (in which honey bees were virus donors and bumble bees were virus recipients) at the end of the experiment (day 7) in relation to the force of infection at day 5, quantified as the number of surviving donor honey bees at day 5. Mortality of Apis was high in this treatment of this experiment in which Apis and Bombus were housed together, leading to variation in the force of infection (number of infected *Apis* donor bees) over the course of the experiment. Yet no difference in viral load was detectable at the end of the experiment between recipient bumble bees from cages with a low force of infection (five cages with 1 to 3 donor honey bees alive at day 5) and recipient bumble bees from cages with a high force of infection (two cages with 4 to 8 donor honey bees alive at day 5; LM: $F_{1,7} = 0.127$, p = 0.732; see Supplementary Table S2).

Figure S2

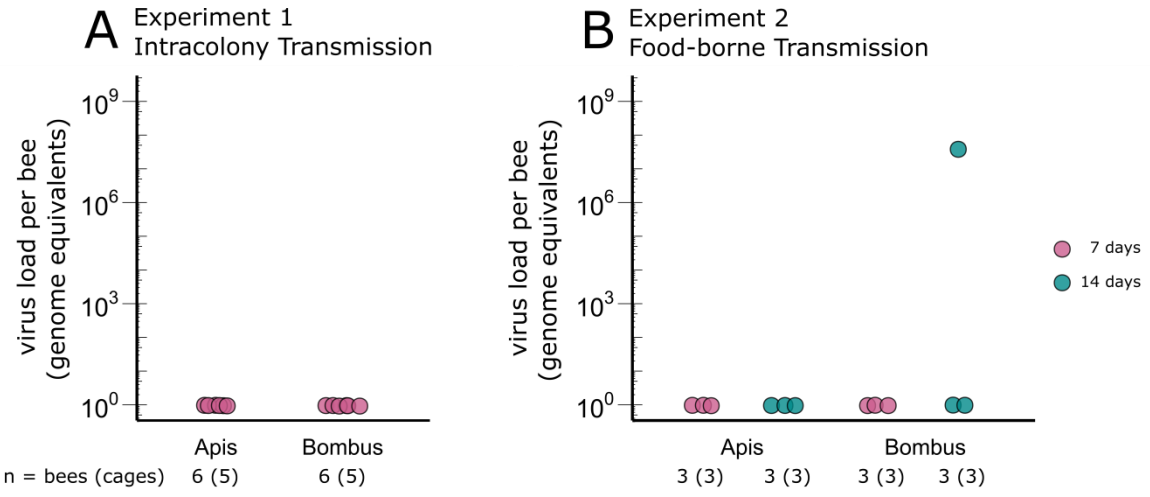

Figure S2 Control bee viral titres in Experiments 1 (mimicking intracolony transmission) and 2 (mimicking food-borne transmission). A: Viral (DWV-A) titre of untreated, control bees at day 7 (Experiment 1) and **B**: at days 7 and 14 (Experiment 2). One *Bombus terrestris* had a low DWV-A titre in one control cage of Experiment 2 after 14 days, probably due to a background infection in the colony (see Supplementary Table S3). Sample sizes given as bees (cages).

Figure S3A

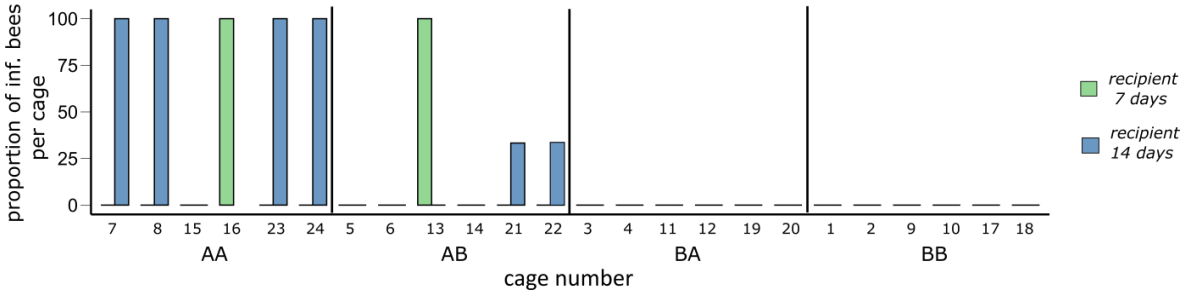

Figure S3B

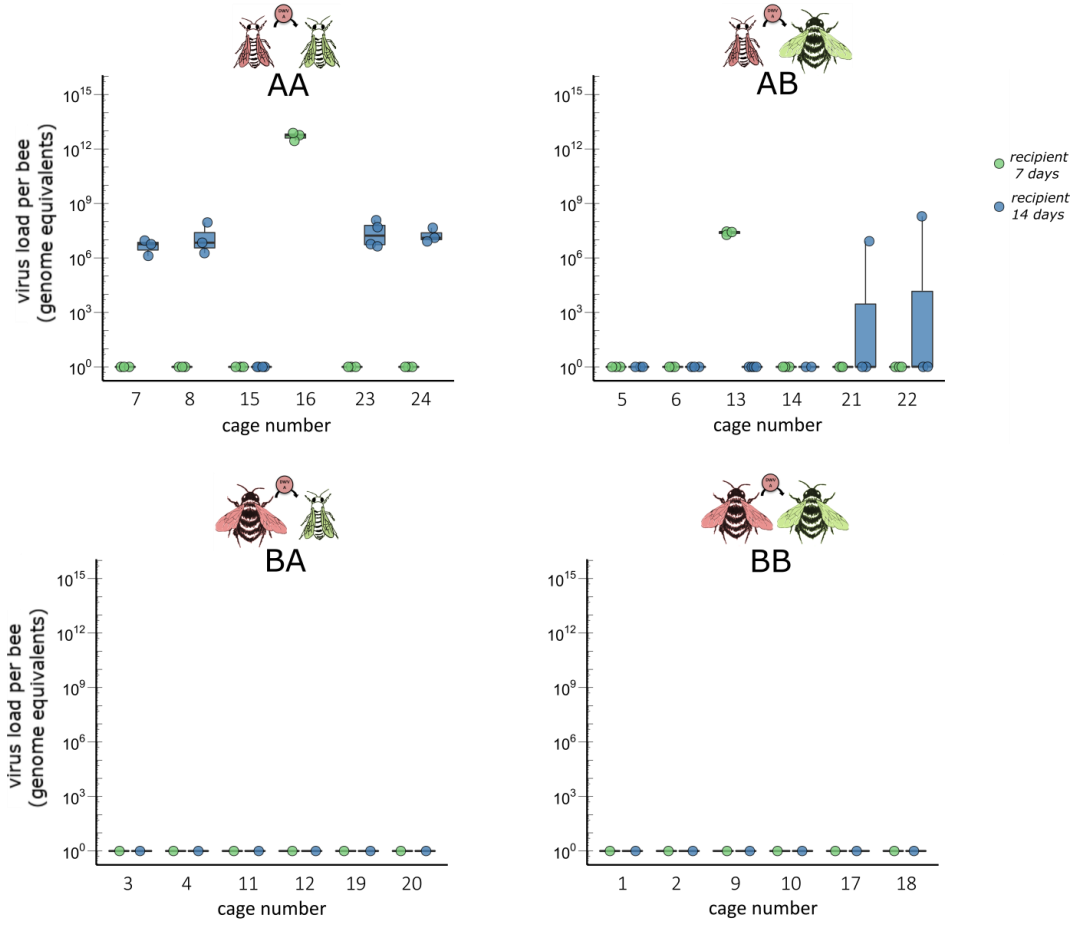

Figure S3 Viral prevalence and viral titres per bee for Experiment 2, mimicking food-borne transmission. A The proportion of recipient bees per cage that were infected with DWV by day 7 (2nd column: green) and day 14 (3rd column: blue) for all four treatments (AA: *Apis* to *Apis*; AB: *Apis* to *Bombus*; BA: *Bombus* to *Apis*; and BB: *Bombus* to *Bombus*). **B.** Viral (DWV-A) titres of recipient bees per cage at days 7 (green) and 14 (blue) for all four treatments (AA, AB, BA, BB); donor bee icons are in pink and recipient bee icons are in green. Raw data giving infection status are in Supplementary Table S2A.

Table S1A Samples sizes and qPCR results (positive, Cq < 35; negative Cq > 35) for experiment 1 (*intracolony transmission*) as the number of bees alive at day 5 of the experiment and the number analysed at day 7 (end of experiment) for DWV (given as: 'positive' for DWV-A or 'negative' for DWV-A). Treatment AA: *Apis* (donor) to *Apis* (recipient); treatment AB: *Apis* (donor) to *Bombus* (recipient); treatment BA: *Bombus* (donor) to *Apis* (recipient); treatment BB: *Bombus* (donor) to *Bombus* (recipient). At the start of the experiment, there were n = 8 donor and 8 recipient bees per cage. Within a treatment, donors and recipients were housed in the same cage, mimicking intra-colony transmission. **B** Sample sizes of control cages in experiment 1.

A

Treatment	**Cage**	**Number of bees surviving to day 5**		**Number of bees analysed at day 7**			
				Donor DWV		**Recipient DWV**	
		Donor	**Recipient**	**positive**	**negative**	**positive**	**negative**
		Apis	***Apis***	***Apis***	***Apis***	***Apis***	***Apis***
AA	1	6	5	1	0	1	0
AA	2	5	8	1	0	1	0
AA	3	5	7	1	0	1	0
AA	4	0	0	-	-	-	-
AA	5	7	6	1	0	1	0
AA	6	5	5	2	0	2	0
AA	7	7	8	-	-	1	0
AA	8	8	8	-	-	-	-
AA	9	6	8	-	-	1	0
AA	10	7	7	-	-	-	-
AA	11	6	4	-	-	1	0
AA	12	7	4	-	-	-	-
		Apis	***Bombus***	***Apis***	***Apis***	***Bombus***	***Bombus***
AB	1	0	8	-	-	-	-
AB	2	0	8	-	-	-	-
AB	3	4	6	-	-	1	0
AB	4	1	8	-	-	-	-
AB	5	8	6	1	0	2	0
AB	6	1	8	1	0	1	0
AB	7	3	5	1	0	2	0
AB	8	3	8	1	0	1	0
AB	9	3	6	1	0	1	0
AB	10	1	5	1	0	1	0
		Bombus	***Apis***	***Bombus***	***Bombus***	***Apis***	***Apis***
BA	1	6	4	1	0	0	2
BA	2	4	5	-	-	-	-
BA	3	7	0	-	-	-	-
BA	4	6	3	1	0	0	1
BA	5	4	0	-	-	-	-
BA	6	8	5	-	-	0	1
BA	7	6	2	1	0	0	1
BA	8	7	3	1	0	0	1
BA	9	5	5	1	0	0	1
BA	10	3	1	-	-	-	-
BA	11	6	2	-	-	0	1
BA	12	7	5	1	0	0	1

		Bombus	***Bombus***	***Bombus***	***Bombus***	***Bombus***	***Bombus***
BB	1	8	8	1	0	0	1
BB	2	8	8	1	0	0	1
BB	3	7	7	1	0	0	1
BB	4	7	7	1	0	0	1
BB	5	6	6	1	0	0	1
BB	6	4	6	1	0	0	1
BB	7	8	7	1	0	-	-
BB	8	6	8	-	-	0	1
BB	9	3	3	-	-	-	-
BB	10	8	7	-	-	0	1
BB	11	4	6	-	-	-	-
BB	12	7	7	-	-	-	-

B

Treatment	**Cage**	**Number of bees surviving to day 7**		**Number of bees analysed at day 7**			
				***Apis* DWV**		***Bombus* DWV**	
		Apis	***Bombus***	**positive**	**negative**	**positive**	**negative**
Control	1	7	8	0	1	0	1
Control	2	1	6	0	1	0	1
Control	3	5	3	0	1	0	1
Control	4	7	2	0	1	0	1
Control	5	8	4	0	1	0	1

Table S2A Samples sizes and qPCR results (positive, Cq <35; negative, Cq >35) for experiment 2 (*floral transmission*) as the number of bees analysed for DWV on days 7 and 14 (given as 'positive' for DWV-A or 'negative' for DWV-A). Treatment AA: *Apis* (donor) to *Apis* (recipient); treatment AB: *Apis* (donor) to *Bombus* (recipient); treatment BA: *Bombus* (donor) to *Apis* (recipient); treatment BB: *Bombus* (donor) to *Bombus* (recipient). At the start of the experiment, there were n = 8 donor bees per donor cage and n = 8 recipient bees per recipient cage. Within a treatment, donors and recipients were housed in different cages and cages (donor cage, recipient cage) were paired for the duration of the experiment; the sucrose feeding tube of the donor cage was passed daily to the recipient cage, mimicking transmission at flowers. Visualisation of results per cage is in Supplementary Figure S3. **B** Sample sizes of control cages in experiment 2.

A

Treatment	**Cage pair**	**Number of donor bees**		**Number of recipient bees**			
		Screened for DWV day 7		**Screened for DWV day 7**		**Screened for DWV day 14**	
		Positive	**Negative**	**positive**	**negative**	**positive**	**negative**
		Apis	***Apis***	***Apis***	***Apis***	***Apis***	***Apis***
AA	7	-	-	0	3	3	0
AA	8	1	0	0	3	3	0
AA	15	2	0	0	3	0	4
AA	16	-	-	3	0	-	-
AA	23	1	0	0	3	4	0
AA	24	1	0	0	3	3	0
		Apis	***Apis***	***Bombus***	***Bombus***	***Bombus***	***Bombus***
AB	5	1	0	0	3	0	3
AB	6	1	0	0	3	0	3
AB	13	1	0	3	0	0	4
AB	14	1	0	0	3	0	2
AB	21	1	0	0	3	1	2
AB	22	1	0	0	3	1	2

		Bombus	***Bombus***	***Apis***	***Apis***	***Apis***	***Apis***
BA	3	1	0	0	1	0	1
BA	4	1	0	0	1	0	1
BA	11	1	0	0	1	0	1
BA	12	1	0	0	1	0	1
BA	19	1	0	0	1	0	1
BA	20	1	0	0	1	0	1
		Bombus	***Bombus***	***Bombus***	***Bombus***	***Bombus***	***Bombus***
BB	1	1	0	0	1	0	1
BB	2	1	0	0	1	0	1
BB	9	1	0	0	1	0	1
BB	10	1	0	0	1	0	1
BB	17	1	0	0	1	0	1
BB	18	1	0	0	1	0	1

B

Treatment	**Cage**	**Bees on day 7 Screened for DWV**		**Bees on day 14 Screened for DWV**	
		Positive	**Negative**	**Positive**	**Negative**
		Apis	***Apis***	***Apis***	***Apis***
Control: Apis	1	0	1	0	1
Control: Apis	2	0	1	0	1
Control: Apis	3	0	1	0	1
		Bombus	***Bombus***	***Bombus***	***Bombus***
Control: Bombus	1	0	1	0	1
Control: Bombus	2	0	1	0	1
Control: Bombus	3	0	1	1*	0

*, One Bombus terrestris had a low DWV-A titre in one control cage of experiment 2 after 14 days (see Supplementary Figure S2), probably due to a background infection in the colony (see Supplementary Table S1)

Table S3 Viral presence in four bumble bee and two honey bee colonies used as sources of bees for transmission experiments; qPCR Cq values for Deformed wing virus genotype A (DWV-A), Deformed wing virus genotype B (DWV-B), Black queen cell virus (BQCV), Sac brood virus (SBV), Chronic bee paralysis virus (CBPV) and Slow bee paralysis virus (SBPV); x: Cq value > 40; n.t.: not tested. Two *B. terrestris* colonies (T2, T3) had a Cq value <35 for DWV-A (values in red), suggesting slight background infection, while both honey bee colonies were devoid of DWV- A prior to experimentation.

Species	**Date screened**	**colony**	**DWV-A**	**DWV-B**	**BQCV**	**SBV**	**CBPV**	**SBPV**
Bombus terrestris	03.09.2019	T1	35.9	36.4	X	n.t.	n.t.	n.t.
		T2	32.7	x	X	n.t.	n.t.	n.t.
		T3	34.9	x	38.8	n.t.	n.t.	n.t.
		T4	x	x	x	n.t.	n.t.	n.t.
	date	colony	DWV-A	DWV-B	BQCV	SBV	CBPV	SBPV
Apis mellifera	14.08.2019	A	x	38.2	x	x	x	x
		R7	x	x	x	x	x	x

Table S4 List of qPCR primers used in this study

Target	Name	Sequence	Reference
DWV-A	DWVq-F2	TGTCTTCATTAAAGCCACCTGGAA	(McMahon *et al*.,
	DWVq-R2a	TTTCCTCATTAACTGTGTCGTTGAT	2015)
DWV-B	VDVq-F2	TATCTTCATTAAAACCGCCAGGCT	(McMahon *et al*.,
	VDVq-R2a	CTTCCTCATTAACTGAGTTGTTGTC	2015)
*DWV-A for standard	DWV-F1a	GGAAACATCTGGAATTAGCGACAAA	(McMahon *et al*.,
*DWV-A for standard	DWVDV-7A-R	AATCCGTGAATATAGTGTGAGG	2015)
BQCV	BQCV-qF7893	AGTGGCGGAGATGTATGC	(Locke *et al*., 2012)
	BQCV-qB8150	GGAGGTGAAGTGGCTATATC	
CBPV	CBPV1-qF1818	CAA CCT GCC TCA ACA CAG	(Locke *et al*., 2012)
	CBPV1-qB2077	AAT CTG GCA AGG TTG ACT GG	
SBPV	SBPV-F3177	GCGCTTTAGTTCAATTGCC	(De Miranda *et al*.,
	SBPV-B3363	ATTATAGGACGTGAAAATATAC	2010)
SBV	SBV-qF3164	TTGGAACTACGCATTCTCTG	(Locke *et al*., 2012)
	SBV-qB3461	GCTCTAACCTCGCATCAAC	
β-actin	Am-actin2-qF	CGTGCCGATAGTATTCTTG	(Locke *et al*., 2012)
	Am-actin2-qB	CTTCGTCACCAACATAGG	

*DWV-A for standard: primers used to genenate PCR template for absolute quantification of DWV-A

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Chapter III:

Contents:

Supplemental Methods: Additional information to molecular biological (qPCR) and experimental methods used in this study.

Quantification of viral titre

Most known viruses of honey bees are positive single stranded (+)ssRNA viruses (Yan Ping Chen & Siede, 2007; Grozinger & Flenniken, 2019), a class of virus know to have extremely high rates of mutation (Holmes, 2009). DWV, a (+)ssRNA virus, is a picorna-like virus in the family Iflaviridae that often leads to crippled wings and high mortality in honey bees; it is efficiently vectored by the mite *V. destructor* (Brettell *et al*., 2017; de Miranda & Genersch, 2010; Francis *et al*., 2013; Grozinger & Flenniken, 2019; Martin & Brettell, 2019; Tehel *et al*., 2019). It comprises at least three distinct genotypes (Kevill *et al*., 2017; Mordecai *et al*., 2016), two of which (DWV-A and DWV-B) are widespread and differentially virulent in adult honey bees (McMahon *et al*., 2016) though not in honey bee pupae (Tehel *et al*., 2019). BQCV, another (+)ssRNA virus (family Dicistroviridae), kills honey bee queen pupae, leaving them mottled black (Bailey & Woods, 1977), but is frequently found in *A. mellifera* workers (McMahon *et al*., 2015). Together with DWV, it is one of the most prevalent viruses detected in non-*Apis* bees (McMahon *et al*., 2015; Murray *et al*., 2019; Radzevičiūtė *et al*., 2017).

To check that bumble bees (12 source colonies: labelled B1 to B12) and honey bees (2 source colonies, labelled 5.1 and G) as well as the fresh-frozen pollen pellets were devoid of viral pathogens, we tested them by real-time quantitative PCR (qPCR) for seven common honey bee viral targets: DWV-A, DWV-B, BQCV, acute bee paralysis virus (ABPV), chronic bee paralysis virus (CBPV), sacbrood virus (SBV), and slow bee paralysis virus (SBPV) using primers given in (McMahon *et al*., 2015, Supplementary Table S1 for colonies). We additionally screened colonies for the Microsporidia *Nosema apis*, *Nosema bombi* and *Nosema ceranae* using methods in Fries *et al.* (2013); all PCRs were negative, suggesting no background microsporidial infection.

For viral screening, we collected 10-20 adult worker bees per colony or 2 x 0.3g of pollen pellets (i.e. two samples), crushed them in a plastic RNAse-free mesh bag (BioReba, Reinach, Switzerland) with ultrapure diethylpyrocarbonate (DEPC)-treated water (500µl per honey bee, 1000µl per bumble bee, 1000µl per 0.3g of pollen) after snap-freezing them on dry ice, and then recovered 100µL of homogenate from beyond the BioReba mesh for RNA extraction.

We ran quality control checks on each 96-well qPCR reaction plate. To check that the correct template had been amplified, PCR products were denatured for one minute at 95°C, cooled to 55°C for one minute, and then a melting profile was generated from 55°C to 95°C (0.5°C per second increment) to ensure the expected product had been amplified, namely a single peak at the product's dissociation temperature (Tm). In addition, a virus-free (negative control) and a virus-infected (positive control) sample were included on each 96-well plate. A host housekeeping gene, *Apis mellifera*-β-actin, was also amplified for all samples as an internal reference marker using the primers given in Locke *et al.* (2012). All PCRs showed the expected signals for the negative (no signal) and positive ($Ct < 35$) controls and β-actin gave Ct values for all samples between 16 and 26, suggesting no

contamination or error in pipetting, no RNA degradation, no error in RNA extraction, and no failure in cDNA synthesis.

Experimental inoculation

Bumble bees – fed inoculum, satiated.

Freshly emerged (24-48h after eclosion) bumble bee workers, collected as described in 2.3 "Bumble bees general handling", were transferred individually to an inverted plastic cup on a plastic lid (one bee per cup) then starved for 4 - 5 hours. Thereafter, bees were individually fed with 10^9 viral genome equivalents (or the equivalent control solution devoid of virus) in 10µL of 50% (w/v) sucrose solution pipetted to the bottom of the cup. Food intake was observed; bees that did not consume the entire inoculum within 15min were excluded from the experiment. After an additional 1 – 2h to ensure that bees did not regurgitate food, they were transferred to a new, autoclaved metal cage in small groups (5 to 10 bees per cage grouped according to treatment).

Figure S1

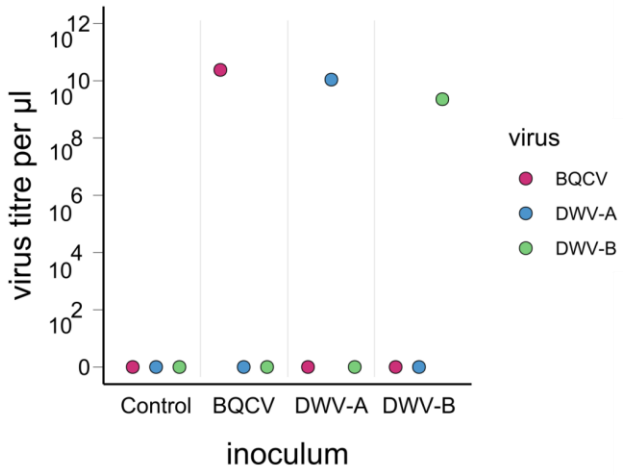

Figure S1. Viral titre of 1µl of the inocula that were used for all experiments. All inocula were screened for the presence of DWV-A, DWV-B and BQCV as well as ABPV, CBPV, SBPV and SBV; contaminant virus was not detected. To propagate DWV-A and DWV-B for experimental inocula, we used the inocula from Tehel *et al.* (2019), a propagation from the genotype-specific inocula of McMahon *et al.* (2016), which had originally been extracted from an adult, heavily virus-infected honey bee with normal wings from Great Britain (DWV-A) or Germany (DWV-B). Our BQCV inoculum was prepared by propagating the BQCV inoculum of Doublet *et al.* (2015), originally derived from infected *A. mellifera* collected in Harpenden, England (Bailey & Woods, 1977). Viral propagation in honey bee pupae followed precisely methods in Tehel *et al.* (2019). We crushed three honey bee pupae in 500µL of 0.5 M of potassium phosphate buffer (PPB) (pH 8.0) using a plastic pestle, 50µL of which was used for RNA isolation. Viral detection by qPCR followed methods described above. We always generated the correct virus inoculum from the original inoculum, which was devoid of other viruses. Batches of pupal homogenates cross-contaminated with another virus (either BQCV or DWV-B were occasional cross-contaminants) were not used as inocula. Un-injected white-eyed pupae devoid of virus by qPCR were used to generate a control inoculum that was identical to viral inocula, but for the lack of virus. Absolute quantification of virus in inocula followed methods in Tehel *et al.* (2019) using a dilution series (10^{-1}–10^{-8}) of an external DNA standard to generate calibration curves. Duplicate qPCRs were run for each sample and the mean Ct value was used; Ct values always differed by < 0.5 between duplicates. Primer efficiencies were 96% for DWV-A, 95% for a DWV-B and 98% for BQCV, with correlation coefficients (R^2) ≥ 0.9.

Figure S2

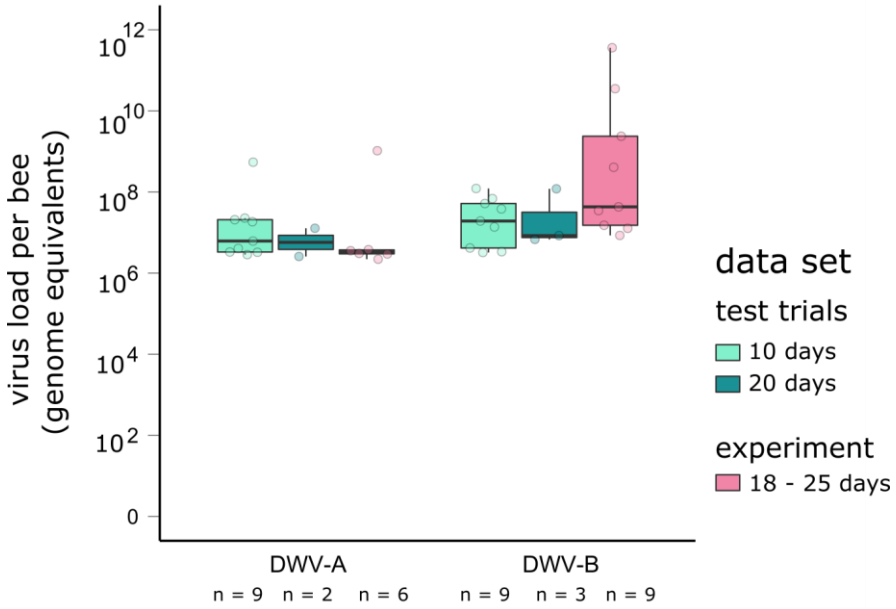

Figure S2. Viral genome equivalents in bumble bee worker abdomens after feeding with 10^9 viral genome equivalents of DWV-A or DWV-B in initial trials, then freeze killed at 10 or 20 days post infection (d.p.i.); in the reported starvation experiment, bees were freeze killed 18-25 d.p.i.. Viral titres did not differ across treatments and experiments (LM: DWV-A $F_{2,14} = 0.135$, $p = 0.875$; DWV-B $F_{2,18} = 2.727$, $p = 0.092$). Two bees were excluded (one DWV-A inoculated bee in the test trial at 20 d.p.i.; one DWV-A inoculated bee in the reported experiment) because inoculation failed (the bee was devoid of virus).

Figure S3

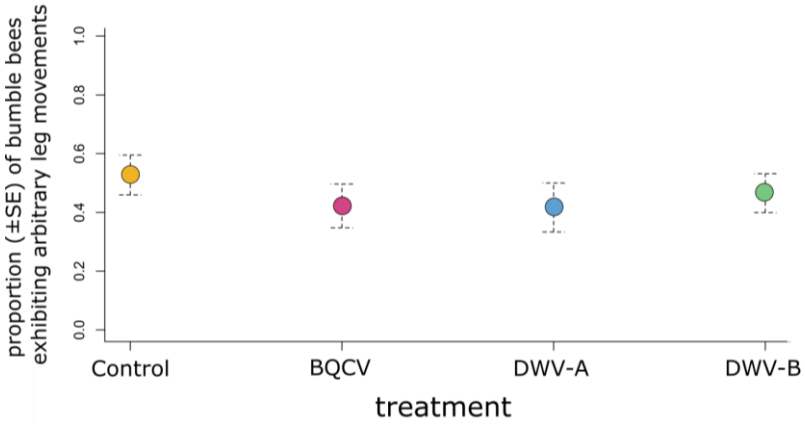

Figure S3. Proportion of bees lying on their backs and exhibiting arbitrary movements with their legs when starved during the starvation experiment. Time of death was difficult to determine because, after 10-40 hours, bees fell on their backs and arbitrarily waved their legs. We therefore measured the time when bees first fell on their backs as well as 2 hours after continuously remaining on their backs, waiving legs arbitrarily. Arbitrary leg movement was independent of treatment. Moreover, use of the first time point (first time on the back) versus the second time point (continuously on back for over two consecutive hours) made no qualitative difference to statistical analyses and so results from only the first time point are reported. Treatments did not differ significantly from each other (LM: $F_{3,190} = 0.502$, $p = 0.681$).

Figure S4

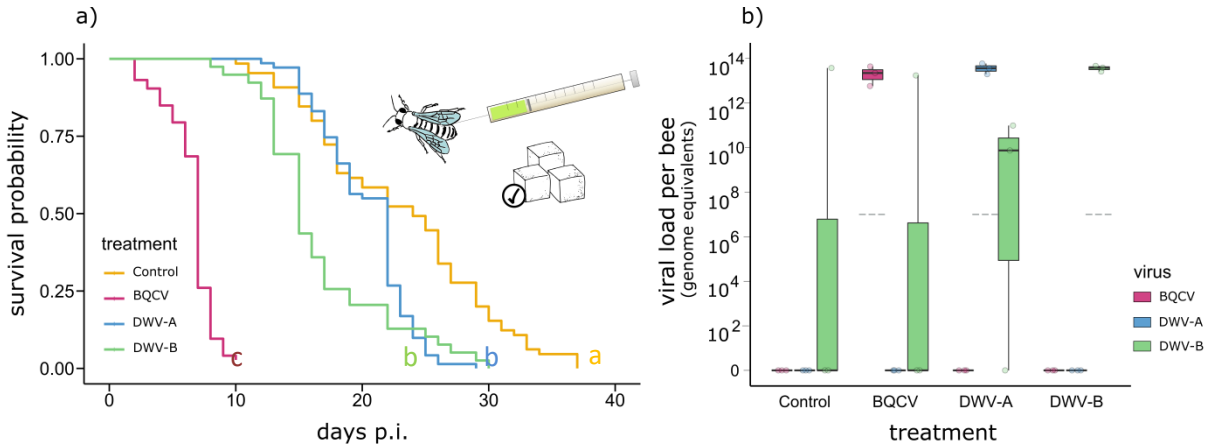

Figure S4. (a) Survival in days post infection (p.i.) of honey bee workers when inoculated by injection with 10^7 viral genome equivalents of BQCV, DWV-A or DWV-B then fed *ad libitum* (Cox proportional hazards survival curves: Control, n = 66; BQCV, n = 73; DWV-A, n = 71; DWV-B n = 39); virus treatments followed by a different lower case letter, $P < 0.05$ (details in Supplementary Table S2); (b) viral titre per honey bee at 10 days p.i. (box and whiskers plot of n = 3 bees per treatment, dashed lines represent viral genome equivalents initially injected per bee); one honey bee source colony had a background infection of DWV-B (see Supplementary Material S1), hence the background infection of DWV-B in the control and across all treatments. To obtain freshly emerged bees for the experiment, frames of sealed brood from our two honey bee colonies were kept in an incubator (35°C) overnight and, the next morning, the freshly eclosed workers were collected.

Figure S5

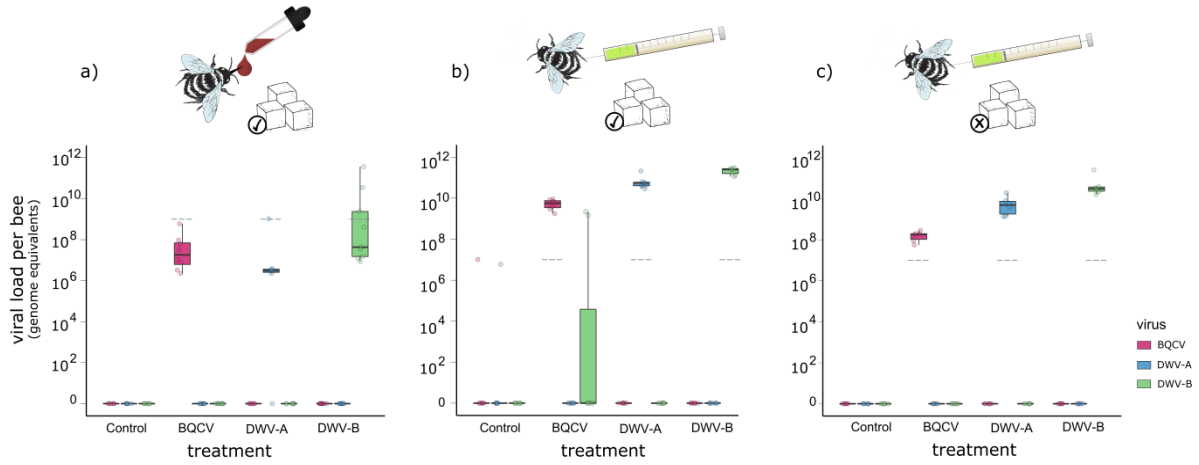

Figure S5. Viral titre of bumble bee workers (a) when inoculated by feeding with 10^9 viral genome equivalents of BQCV, DWV-A or DWV-B then fed *ad libitum;* values per bumble bee abdomen at 18-25 days p.i. (box and whiskers plot of n = 7 bees per treatment, dashed lines represent viral genome equivalents initially fed per bee); (b) when inoculated by injection with 10^7 viral genome equivalents of BQCV, DWV-A or DWV-; values per bumble bee abdomen at 10 days p.i. (box and whiskers plot of n = 6, 8, 9 and 7 bees per respective treatment, dashed lines represent viral genome equivalents initially injected per bee); one bumble bee source colony had a background infection of DWV-B (see Supplementary Material S1); (c) when inoculated by injection with 10^7 viral genome equivalents of BQCV, DWV-A or DWV-B, fed *ad libitum* for 13 days then starved at hour 0; values per bumble bee abdomen at 13 days post infection (box and whiskers plot of n = 6, bees per treatment, dashed lines represent viral genome equivalents initially injected per bee). These data for are reported in Figure 2 of the main manuscript only for the virus used in each treatment. Here we show background contamination with other viruses, which was negligible across experiments. Symbols are representing the way of infection and the availability of food in form of sugar.

Figure S6

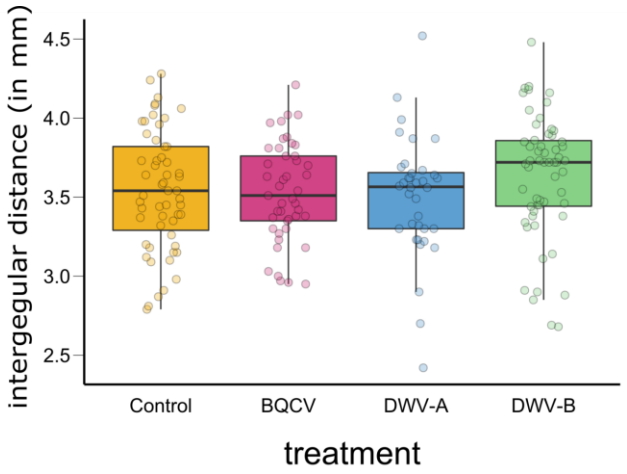

Figure S6. Distribution of bumble bee intertegular distances in mm (a measure of body size) across treatments within the starvation experiment. Treatments did not differ significantly from each other (LM: $F_{3,190}$ = 1.172, p = 0.322). Additionally bees were weighed directly after death. As weight and intertegular distance were highly correlated (Spearman rank correlation rho = 0.762, p < 0.001), intertegular distance alone was used in further analyses.

Figure S7

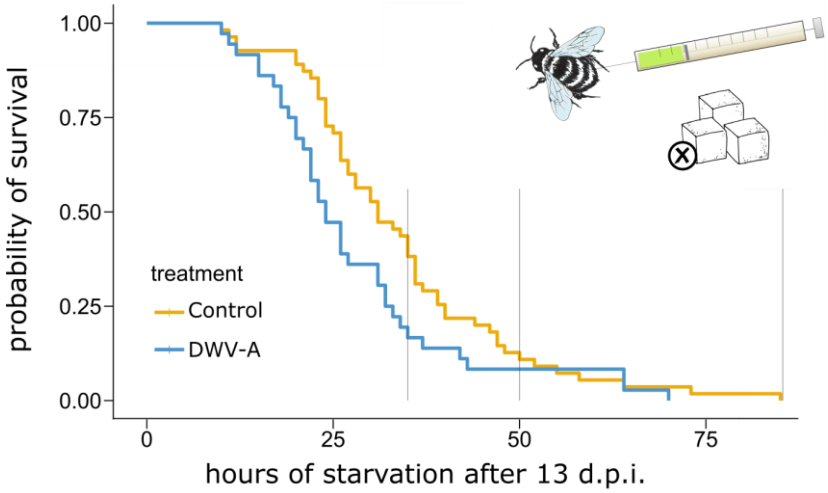

Figure S7. DWV-A virulence when injected into virus-free, 24-48h old bumble bee workers that were fed *ad libitum* till day 13 p.i. then kept without food in the starvation experiment. Fitted Cox proportional hazards survival curves in hours post-initiation of starvation till death of all individuals. Vertical lines represent time points where significance was tested using a Cox proportional hazards model (after 35h and death of 70% of bumble bees, Exp. (β) = 2.192, p = 0.016; after 50h when the lifespans of treatment and control nearly converge (see Fig. 1 of Fürst *et al.* 2014) and death of 90% of bumble bees, Exp. (β) = 1.808, p = 0.050; and at the end of the experiment, after death of all bumble bees, Exp. (β) = 1.725, P = 0.060; treatment DWV-A, n = 36 bumble bees; Control n = 55 bumble bees). DWV-B and BQCV (see Fig. 1c) did not cause significantly shorter lifespan when checked at the same time points (35h after initiation of starvation: Cox proportional hazards for DWV-B: Exp. (β) = 1.332, p = 0.230; BQCV: Exp. (β) = 1.441, p = 0.260; 50h after initiation of starvation: Cox proportional hazards for DWV-B: Exp. (β) = 1.191, p = 0.380; BQCV: Exp. (β) = 1.098, p = 0.750).

Table S1 Viral presence in 12 bumble bee and 2 honey bees colonies used as sources of bees for experiments; qPCR Ct values for DWV-A, DWV-B, BQCV, sac brood virus (SBV), chronic bee paralysis virus (CBPV) and slow bee paralysis virus (SBPV); x: Ct value > 40; n.t.: not tested. One *B. terrestris* colony had a Ct value of 27 for DWV-B and three had a Ct value of 33 - 35 for BQCV, suggesting slight background infection while both honey bee colonies and both pollen samples were devoid of virus prior to experimentation

Experiment	**date**	**colony**	**DWV-A**	**DWV-B**	**BQCV**	**SBV**	**CBPV**	**SBPV**
***Bombus* fed.sat.**	06.03.2018	B1	x	x	37	n.t.	n.t.	n.t.
		B2	x	x	38	n.t.	n.t.	n.t.
		B3	x	x	38	n.t.	n.t.	n.t.
		B4	27	x	39	n.t.	n.t.	n.t.
		B5	x	x	37	n.t.	n.t.	n.t.
***Bombus* inj.sat.**	31.05.2018	B6	x	x	37	x	x	x
		B7	x	x	37	x	x	x
		B8	x	x	35	x	x	x
		B9	x	x	33	x	x	x
***Bombus* inj.starv.**	18.01.2019	B10	x	x	36	x	n.t.	n.t.
		B11	x	x	37	x	n.t.	n.t.
		B12	x	x	34	x	n.t.	n.t.
	date	**colony**	**DWV-A**	**DWV-B**	**BQCV**	**SBV**	**CBPV**	**SBPV**
***Apis* inj.sat.**	14.06.2018	G	x	27	38	x	x	x
		5.1	27	33	36	36	x	x
	28.06.2018	G	x	x	x	x	x	x
		5.1	x	x	x	x	x	x
	25.07.2018	G	x	x	x	x	x	x
		5.1	x	x	x	x	x	x
	03.08.2018	G	x	36	x	x	x	x
		5.1	x	34	x	x	x	x

Table S2 Final Cox proportional hazards model of worker bee mortality following experimental infection (s.e., standard error); within an experiment, p values show significance differences of β (the standardised effect size) of treatment versus control whilst different lower case letters show statistical differences among β by a *posteriori* Tukey test with Westfall correction for multiple comparisons; exp (β) is equivalent to the hazard ratio, the instantaneous probability of death (a high value is equivalent to a high probability of death)

	Coefficients						**model testing**		
	β	**s.e. (β)**	**exp. (β)**	**z**	**p**		**Chi²**	**df**	**p**
Honey bees									
Injection - benign						**treatment**	46.144	3	<0.001
Control	0 a		1						
BQCV	6.331 b	0.683	562.259	9.28	<0.001				
DWV-A	0.912 c	0.329	2.489	2.77	0.006				
DWV-B	1.495 c	0.351	4.461	4.26	<0.001				
Bumble bees									
Feeding - benign						**treatment**	3.313	3	0.371
Control	0		1						
BQCV	-0.061	0.193	0.940	-0.32	0.75				
DWV-A	0.218	0.194	1.244	1.12	0.26				
DWV-B	0.198	0.191	1.218	1.03	0.30				
Injection - benign						**treatment**	5.090	3	0.165
Control	0		1						
BQCV	-0.473	0.313	0.623	-1.51	0.13				
DWV-A	0.214	0.294	1.240	0.73	0.47				
DWV-B	-0.080	0.295	0.923	-0.27	0.79				
Injection - starved						**treatment**	2.880	3	0.412
Control	0 a		1						
BQCV	0.044 a	0.272	1.059	0.16	0.87				
DWV-A	0.462 a	0.282	1.589	1.64	0.10				
DWV-B	0.141 a	0.248	1.167	0.57	0.57				
Age	-0.010 a	0.020	0.988	-0.51	0.61				
size	0.542 b	0.234	1.665	2.32	0.03				

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B. Curriculum vitae

Personal information

Name:	Anja Tehel
Date and location of birth:	12.01.1990, Karl-Marx-Stadt, Germany
Nationality:	German

Education

Since 08/2015	**Ph.D. candidate** *Martin-Luther-University Halle-Wittenberg* (Germany) Institute of Biology/ General Zoology *Dissertation title: "*Pathogen spill-over: directionality and impact on biodiversity and the ecosystem service of pollination“
10/2011 - 01/2015	**M.Sc. in Biology** *Martin-Luther-University Halle-Wittenberg* **Master's degree** (grade: 1.6) *Master thesis*: "Insights into the social organisation and patterns of spatial genetic structure in the primitively eusocial sweat bee, Lasioglossum laticeps (Hymenoptera, Halictidae)" (grade 1.3) Supervised by: Dr. Antonella Soro, Prof. Dr. Robert J. Paxton
10/2008 - 10/2011	**B.Sc. in Biology** *Martin-Luther-University Halle-Wittenberg* **Bachelor's degree** (grade: 2.4) *Bachelor thesis*: "Zum Einfluss von Licht-Dunkel-Zyklen unterschiedlicher Amplitude und Frequenz auf den Aktivitätsrhythmus von Phodopus sungorus“ (grade 1.3) Supervised by: PD. Dr. Dietmar Weinert

Conferences

2022 - 69. Jahrestagung der Arbeitsgemeinschaft der Institute für Bienenforschung e.V. (Hohenheim) - **talk:** *Experimental transmission of DWV-A and DWV-B between honey bees and bumble bees*

2021 - 68. Jahrestagung der Arbeitsgemeinschaft der Institute für Bienenforschung e.V. online (Göttingen) - **talk***: Experimental infection reveal viral spill-over from honey bees to bumble bees but no spill-back*

2019 - Central European Meeting of the IUSSI Wien/Klosterneuburg - p**oster:** *Experimental infection of B. terrestris with DWV and BQCV*

- Entomologentagung der DGaaE Halle - **talk:** *Experimental infection of B. terrestris with DWV and BQCV*

- 66. Jahrestagung der Arbeitsgemeinschaft der Institute für Bienenforschung e.V. Frankfurt am Main - **talk***: Honey bee associated viruses in different bee families*

2018 - *iDiv* Annual Conference in Leipzig - **talk:** *Experimental infection of bumble bees (Bombus terrestris) with DWV and BQCV*

- Eurbee 8 *Ghent* - **poster:** *Experimental infection of bumble bees (Bombus terrestris) with DWV and BQCV*

- *65. Jahrestagung* der Arbeitsgemeinschaft der Institute für Bienenforschung e.V. Koblenz - **poster:** *Screening of different wild bee species for honey bee-associated viruses*

2017 - 64. Jahrestagung der Arbeitsgemeinschaft der Institute für Bienenforschung e.V. Celle - **talk:** *Assessing the virulence of deformed wing virus and Varroa destructor virus-1 on the development of honey bee pupae (Apis mellifera)*

2016 - iDiv Annual Conference in Leipzig - **poster:** *Assessing the virulence of deformed wing virus and Varroa destructor virus-1 on development of honey bees (Apis mellifera)*

2014 - 11. Hymenopterologen-Tagung Stuttgart - **talk:** *Insights into the social organisation and patterns of spatial genetic structure in the primitively eusocial sweat bee, Lasioglossum laticeps*

Reasearch stay abroads

31.10.2018 - 04.12.2018	Adelaide (Australia)
04.10.2019 - 18.11.2019	Adelaide (Australia)

Sholarships

07.11.2019	Scholarship of the Fazitstiftung granted for 6 months (6.600€)
22.05.2015	Scholarship of the Deutsche Bundesstiftung Umwelt granted for 3 years (approx. 55.000€)

Anja Tehel, Halle, 30.09.2022

C. Publication list

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

2. **Tehel, A**., Vu, Q., Bigot, D., Gogol-Döring, A., Koch, P., Jenkins, C., Doublet, V., Theodorou, P., & Paxton, R. (2019). The two prevalent genotypes of an emerging infectious disease, deformed wing virus, cause equally low pupal mortality and equally high wing deformities in host honey bees. *Viruses*, *11*(2), 1–18. https://doi.org/10.3390/v11020114

3. **Tehel, A**., Streicher, T., Tragust, S., & Paxton, R. J. (2020). Experimental infection of bumble bees with honey bee-associated viruses: no direct fitness costs but potential future threats to novel wild bee hosts. *Royal Society Open Science*, *7*, 200480. https://doi.org/10.1098/rsos.200480rsos200480

4. Dosch, C., Manigk, A., Streicher, T., **Tehel, A**., Paxton, R. J., & Tragust, S. (2021). The gut microbiota can provide viral tolerance in the honey bee. *Microorganisms*, *9*(4). https://doi.org/10.3390/microorganisms9040871

5. **Tehel, A**., Streicher, T., Tragust, S., & Paxton, R. J. (2022). Experimental cross species transmission of a major viral pathogen in bees is predominantly from honey bees to bumble bees. *Proceedings of the Royal Society B: Biological Sciences*, *289*, 20212255. https://doi.org/10.1098/rspb.2021.2255

Anja Tehel, Halle, 11.04.2022

D. Declaration of own contribution to the original articles presented in this thesis

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

Literature review:	50%
Visualisation:	100%
Writing the paper:	50%

II. **Tehel, A**., Streicher, T., Tragust, S., & Paxton, R. J. (2022). Experimental cross-species transmission of a major viral pathogen in bees is predominantly from honey bees to bumble bees. *Proceedings of the Royal Society B: Biological Sciences*, *289*, 20212255. https://doi.org/10.1098/rspb.2021.2255

Design of the project:	60%
Experimental work:	65%
Data analysis:	90%
Visualisation:	100%
Writing the paper:	80%

III. **Tehel, A**., Streicher, T., Tragust, S., & Paxton, R. J. (2020). Experimental infection of bumble bees with honey bee-associated viruses: no direct fitness costs but potential future threats to novel wild bee hosts. *Royal Society Open Science*, *7*, 200480. https://doi.org/10.1098/rsos.200480rsos200480

Design of the project:	60%
Experimental work:	95%
Data analysis:	90%
Visualisation:	100%
Writing the paper:	80%

Anja Tehel, Halle, 11.04.2022 Simon Tragust, Halle, 11.04.2022

E. Eidesstattliche Erklärung

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

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

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

Anja Tehel, Halle, 11.04.2022