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Direct transmission by injection affects competition among RNA viruses in honeybees

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The arrival of the ectoparasitic mite *Varroa destructor* on the western honeybee *Apis mellifera* saw a change in the diversity and prevalence of honeybee RNA viruses. One virus in particular, deformed wing virus (DWV) has become closely associated with *V. destructor*, leading many to conclude that *V. destructor* has affected viral virulence by changing the mode of transmission. While DWV is normally transmitted via feeding and faeces, *V. destructor* transmits viruses by direct injection. This change could have resulted in higher viral prevalence causing increased damage to the bees. Here we test the effect of a change in the mode of transmission on the composition and levels of honeybee RNA viruses in the absence of *V. destructor*. We find a rapid increase in levels of two viruses, sacbrood virus (SBV) and black queen cell virus (BQCV) after direct injection of viral extracts into honeybee pupae. In pupae injected with high levels of DWV extracted from symptomatic adult bees, DWV levels rapidly decline in the presence of SBV and BQCV. Further, we observe high mortality in honeybee pupae when injected with SBV and BQCV, whereas injecting pupae with high levels of DWV results in near 100% survival. Our results suggest a different explanation for the observed association between *V. destructor* and DWV. Instead of *V. destructor* causing an increase in DWV virulence, we hypothesize that direct virus inoculation, such as that mediated by a vector, quickly eliminates the most virulent honeybee viruses resulting in an association with less virulent viruses such as DWV.

1. Introduction

The western honeybee *Apis mellifera* suffers from the negative effects of inappropriate use of pesticides [1] and a range of parasites and diseases [2]. By far the most important parasite today is the ectoparasitic mite *Varroa destructor*. The emergence of *V. destructor* is the result of a host shift that occurred when *A. mellifera* and the eastern honeybee, *Apis cerana*, were brought into contact by beekeepers in the 1930s [3].

When left untreated, *V. destructor* typically destroys the colonies of its host [4]. In Europe and the United States, managed honeybee colonies suffer greatly from *V. destructor* and require constant treatment with miticides to prevent colonies from dying. At the same time, wild or feral honeybee populations have been decimated or gone extinct [5].

Varroa destructor females feed on the haemolymph and fat body [6] of developing and adult bees and in doing so are thought to vector viruses carried therein [7,8]. Although a variety of viruses could potentially be transmitted by *V. destructor* [9], one in particular—deformed wing virus (DWV)—is strongly associated with *V. destructor* [10]. For example, as *V. destructor* sequentially invaded the islands of Hawaii, viral titres of DWV increased [11]. A similar phenomenon was seen in New Zealand where titres of DWV dramatically increased with the length of exposure to *V. destructor* [12]. While honeybees harbour a range of viruses, mainly positive-sense RNA viruses [13,14], in the absence

of *V. destructor*, these viruses are difficult to detect [11] and exist as subclinical infections with occasional seasonal outbreaks [15,16]. RNA viruses, such as DWV, are characterized by very rapid evolution, a combination of high rates of mutation and replication, which forms the basis of rapid adaptive evolution [17]. Clearly, the arrival of *V. destructor* had led to a change in the prevalence of DWV [18]. The question is how.

The arrival of a vector leads to a change in transmission, as pathogens are now transmitted directly instead of indirectly, via food or contact with other infected hosts. Such a change in the mode of transmission is predicted to lead to a change in virulence of the parasite (the damage incurred by the host owing to infection), although the direction of change is not necessarily easy to predict [19]. A simplistic view suggests that vector-based transmission can lead to an increase in virulence because it changes the evolutionary trade-off between virulence and transmission [20]. While an obligate parasite is selected to replicate quickly, so that it can infect as many hosts as possible, a high rate of replication may kill the host before the parasite is transmitted to its next host. Assuming high rate of replication equals virulence, selection will thus act against a pathogen that kills or immobilizes its host if this reduces its long-term transmission success [21,22]. The arrival of a vector, such as *V. destructor*, changes the dynamics of the transmission-virulence trade-off. If a pathogen can harness a mobile vector to facilitate its spread to new hosts, then it no longer relies on its current host for transmission and could become more virulent.

In addition to an evolutionary explanation for an increase in virulence after the arrival of a vector, vector-based transmission can also more directly lead to an increase in virulence simply because viral particles are now directly injected into a host, instead of having to pass through defensive barriers, such as the digestive system [23].

An increase in virulence after a change in route of transmission was recently documented in the obligate endosymbiont *Wolbachia* and one of its native hosts, the isopod *Armadillidium vulgare*. Because *Wolbachia* is normally transmitted vertically, via eggs, it requires its host to be alive and reach reproductive age. Hence, *Wolbachia* tends to form symbiotic relationships with its hosts. However, when the route of transmission was changed from vertical to horizontal, by injecting *Wolbachia* directly into the haemolymph of the host, *Wolbachia* titres quickly escalated and infections became highly virulent, resulting in the death of the hosts after only a few serial passages [24].

Serial passage experiments such as the experiment mentioned above are a powerful experimental tool to study the change in virulence in the absence of confounding factors [25]. However, it is impossible to make any general predictions regarding the outcome of such studies as these are very system specific. In the absence of competition, serial passage experiments often lead to lower virulence, a phenomenon exploited in the development of vaccines (e.g. [26]); but in the presence of competitors, within-host competition tends to drive up virulence, as competing pathogens are selected to replicate as fast as possible [25]. When a pathogen has to be able to exploit multiple hosts, as in the case of vector-transmitted pathogens, then selection in one host via serial passage might lead to an inability to replicate in the alternate host (e.g. [27]).

The association between *V. destructor* and DWV could be explained by a vector-induced increase in virulence, either via selection on the virus or simply owing to a change in the way the virus enters the host. However, honeybees host many viruses that are both common and widespread [9,28] including viruses that, like DWV, are present in *V. destructor* and can also be vector-transmitted (e.g. viruses of the acute bee paralysis virus (ABPV) complex, such as Israeli acute paralysis virus (IAPV) and Kashmir bee virus (KBV) [29]). This raises the question: why has DWV become synonymous with *V. destructor* infestation, but not other honeybee viruses? An alternative explanation for the observed association is that more virulent viruses are eliminated from the population owing to excessive host mortality following vector-based transmission, thereby allowing less virulent viruses, such as DWV, to take the upper hand. We would then expect to see a succession of honeybee viruses after the arrival of *V. destructor* with an initial increase of the most virulent viruses, followed by more benign viruses once the competition has been eliminated [7,30]. Here, we test this alternative explanation empirically using a population of honeybees naive to both *V. destructor* and DWV and a serial passage protocol.

We injected extracts from bee pupa to bee pupa repeatedly for up to 30 transmission cycles. We thus changed the mode of transmission of bee viruses from oral-faecal to direct inoculation via injection. We found that the level of two viruses naturally present in our bee population, sacbrood virus (SBV) and black queen cell virus (BQCV), rapidly increased. We further investigated the effect DWV would have on our bees and on BQCV and SBV. DWV introduced via injection decreased after only a few transmission cycles, accompanied by a rapid increase in levels of SBV and BQCV. More importantly, DWV alone did not cause mortality in pupae, whereas injection with serially passaged bee extracts containing high levels of SBV and BQCV did. Our results suggest that the observed association between *V. destructor* and DWV may not necessarily be owing to *V. destructor* increasing the virulence of DWV, but could be explained by the change in the mode of transmission leading to the decline of more virulent viruses. We provide experimental support for the theoretical prediction that relatively benign viruses may become more abundant after the arrival of a vector because of the elimination of more virulent viruses that are better competitors in the absence of the vector [8].

2. Results

(a) Experimental overview

To mimic the effects of changing to a new transmission route based on direct inoculation, similar to what occurs upon arrival of a vector, we serially injected honeybee pupae with viruses and monitored the changes in virus levels. Injecting honeybee extracts into pupae has previously been used to incubate viruses prior to serological experiments [31] and to obtain standardized inoculum for injection experiments [32]. We adapted this protocol to conduct serial transmission of honeybee extracts by pupal injection for 20+ transmission cycles. We performed two independent transmission experiments with different starting inoculum: (i) extracts obtained from asymptomatic (DWV-naive) honeybees; and (ii) extracts obtained from symptomatic (DWV-infected) honeybees.

(b) Serial transmission of asymptomatic (deformed wing virus-naive) inoculum

In our first experiment (figure 1*a*; serial transmission 1), we took our starting inoculum from adults sampled from three

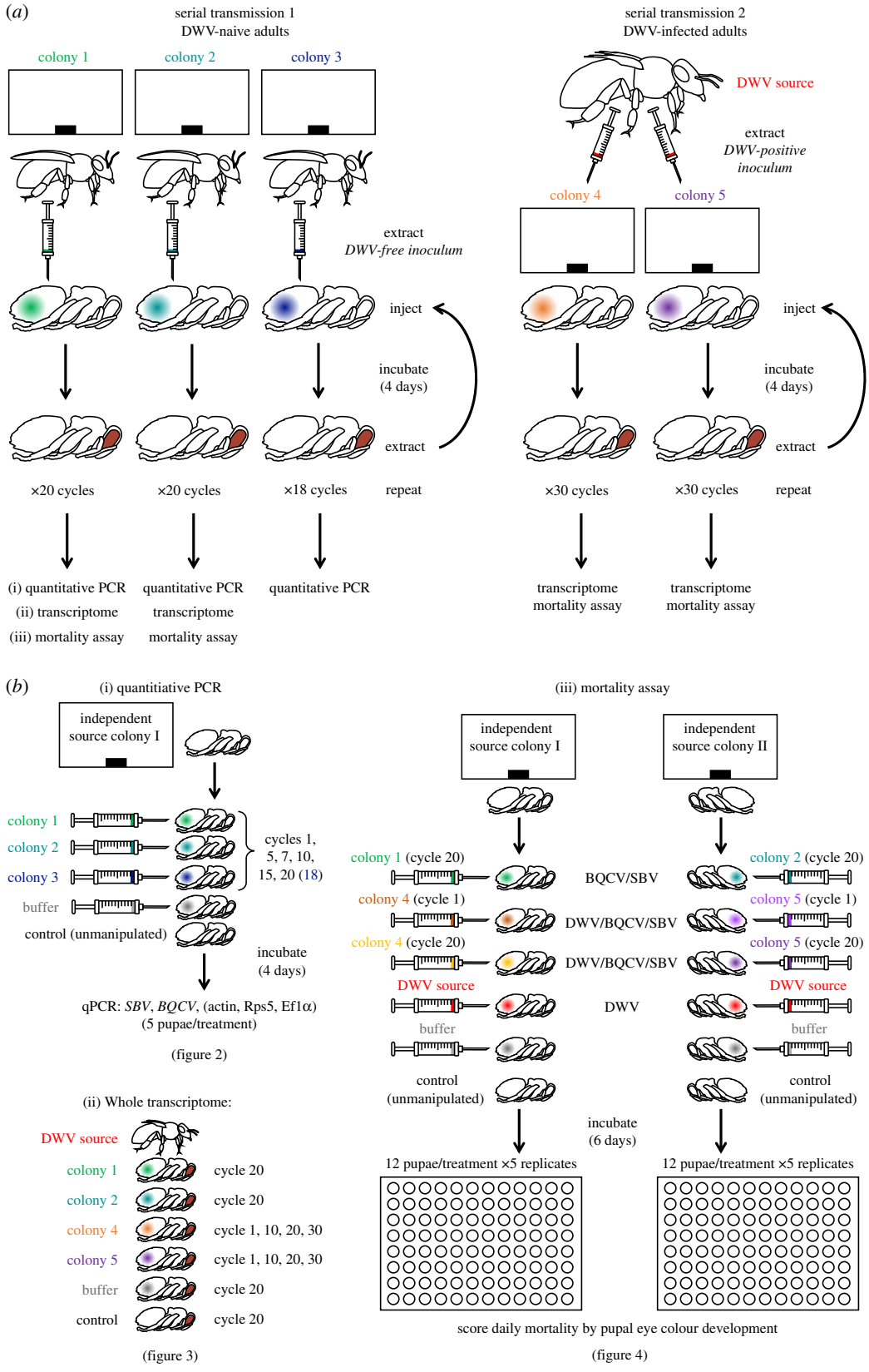

Figure 1. Experimental design of serial transmission experiments. (*a*) Serial transmission 1, with starting inoculum derived from DWV-naive adults, and injected into pupae from colonies 1–3 for 20 serial transmission cycles. The number of cycles differed for colony 3 as this colony lost its queen after 18 cycles. Serial transmission 2, with starting inoculum derived from DWV-positive adults from New Zealand, injected into pupae from colonies 4–6. Colony 6 lost its queen early on in the experiment; hence this colony was not included in any further analyses. (*b*) Resulting virus levels and virulence were determined by quantitative polymerase chain reaction (PCR) (see Results in figure 2), whole transcriptome sequencing (figure 3) and mortality assays (figure 4, and text for further details). (Online version in colour.)

asymptomatic honeybee colonies from Sydney, Australia (lacking DWV and naive to *Varroa*, referred to hereafter as colonies 1, 2 and 3). We subjected white-eyed pupae from the same three colonies to each of three treatments: (i) pupae injected with an inoculum containing viruses; (ii) pupae injected with extraction buffer as a procedural control

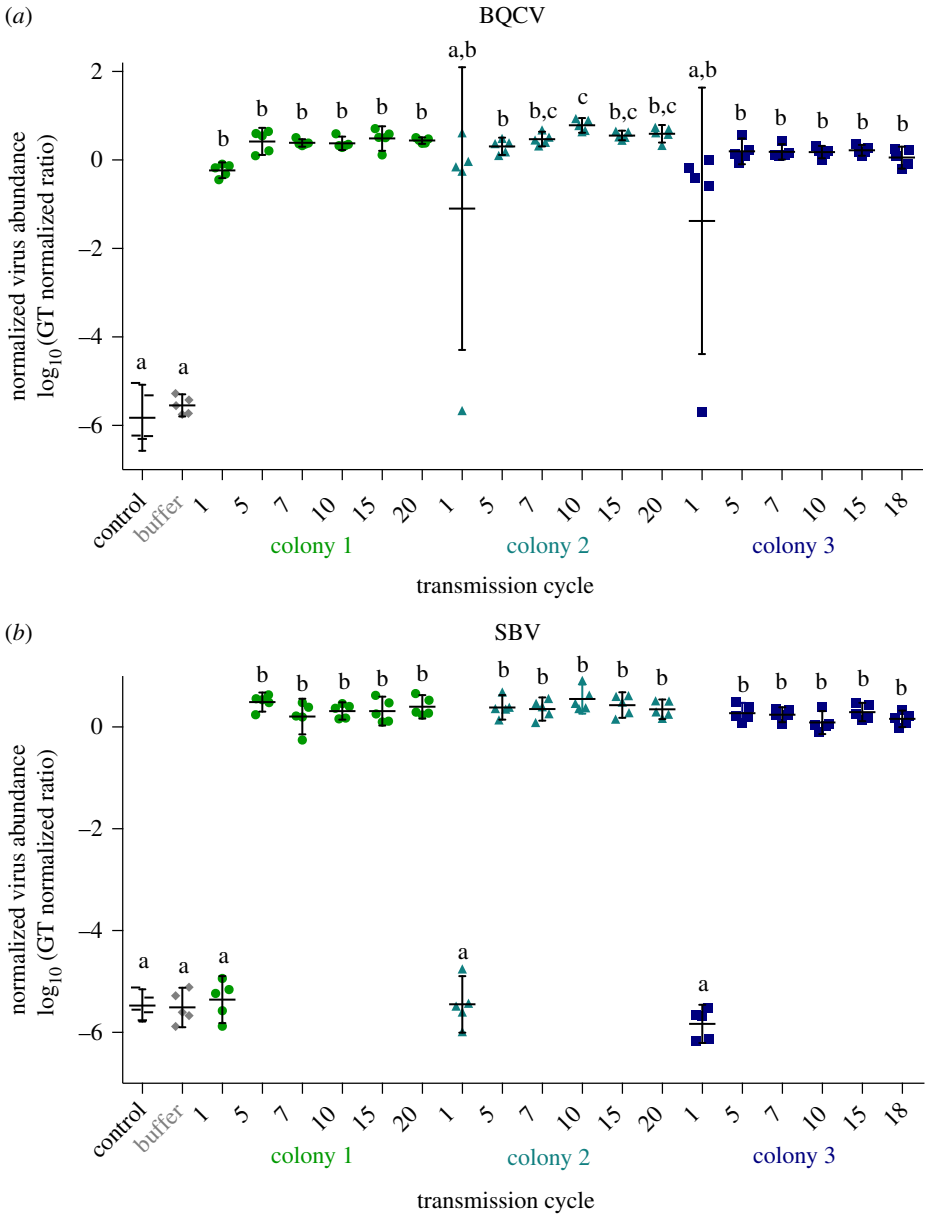

Figure 2. Dot plots showing the normalized virus abundance ($\log_{10}$(GT normalized ratio), mean $\pm$ 95% confidence interval) of (*a*) BQCV and (*b*) SBV compared to two internal honeybee control genes (*Actin* and *Rps5*) in pupae sourced from an independent colony and injected with serially transmitted inoculum from colonies 1–3 (transmission cycles 1, 5, 7, 10, 15 and 18 or 20), and control and buffer-injected pupae. Letters indicate which groups differed statistically. See the electronic supplementary material, table S2 for details of the statistical analyses. (Online version in colour.)

(buffer); and (iii) pupae left unmanipulated (control). After 4 days, we harvested pupae for extraction to generate inoculum for the next transmission cycle. We passaged inoculum for 20 transmission cycles (18 for colony 3; see Material and methods in the electronic supplementary material).

We used endpoint polymerase chain reaction (PCR) to screen for the presence of the eight common honeybee viruses, including the five known viruses present in Australia [33] in our initial adult workers and in pupae sampled at regular intervals during the 18–20 serial transmission cycles (electronic supplementary material, table S1). We detected just two viruses: SBV and BQCV. Control pupae did not test positive for SBV and BQCV. By contrast, buffer-injected procedural controls occasionally tested positive for SBV and BQCV. It has been well documented that the effect of injection procedure alone can cause the irruption of latent viral diseases in bees [31], in line with our observations of SBV and BQCV in our buffer, but not unmanipulated, control pupae.

(c) Serial transmission results in a rapid increase in virus levels

To determine whether serial transmission resulted in increased virus levels, we assessed levels of SBV and BQCV using quantitative PCR (qPCR) and compared those to the expression levels of two endogenous control genes, *Actin* and *Rps5* (see Material and methods). We standardized between the three independent colonies and transmission cycles by re-injecting bee extract from colonies 1–3, transmission cycles 1, 5, 7, 10, 15 and 18 (colony 3) or 20 (colonies 1 and 2) into pupae sourced from an independent colony and performed qPCR on these samples, together with buffer-injected and unmanipulated controls.

Levels of SBV and BQCV virus rapidly increased after direct transmission of bee extracts, compared to virus levels in control and buffer-injected pupae (figure 2 and electronic supplementary material, table S2). BQCV levels increased in

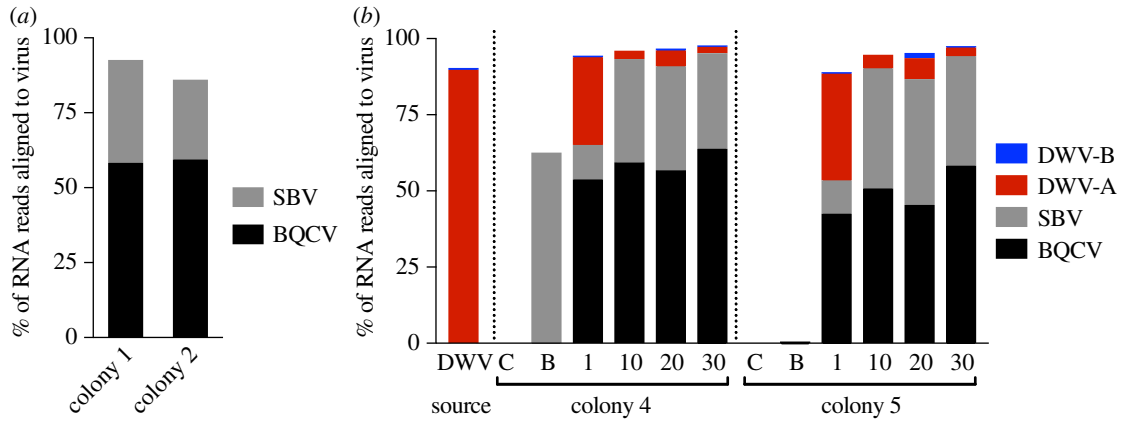

Figure 3. Change in viral abundance during (*a*) serial transmission experiment 1 (DWV-naive); and (*b*) serial transmission experiment 2 (DWV-positive). (*a*) Levels of SBV (grey) and BQCV (black) in pupae from colony 1 and 2, 4-days post injection with inoculum after 20 serial transmission cycles. Virus levels reached 92 and 86% as a percentage of total RNA, respectively. (*b*) Levels of SBV, BQCV and DWV strain A (red) and strain B (blue) in our original inoculum obtained from DWV-positive adults (DWV source), and pupae from colonies 4 and 5, 4-days post injection with inoculum after 1, 10 and 20 serial transmission cycles. Also shown are control and buffer pupae from transmission cycle 20. Although our original inoculum ('source (DWV)') contained exclusively DWV, DWV levels dropped dramatically after injection into pupae, while the levels of SBV and BQCV increased. While our original inoculum mainly contained DWV strain A; the contribution of strain B had increased after 20 transmission cycles, particularly in colony 5, but decreased again after 30 transmission cycles. Data used to produce the figure are presented in the electronic supplementary material, table S3. Electronic supplementary material, table S4 indicates the estimated virus abundance levels (see Material and methods in the electronic supplementary material for details). (Online version in colour.)

pupae injected with bee extract after only one transmission cycle, after which levels remained high (figure 2*a*). Levels of SBV remained low after one transmission cycle but had increased by transmission cycle 5 and remained high thereafter (figure 2*b*).

To correlate virus abundance as measured by qPCR to total RNA content, we examined the amount of viral RNA in pupae injected with bee extract after 20 transmission cycles (colonies 1 and 2, as colony 3 was no longer available owing to the loss of the colony's queen) using HiSeq (Illumina) total RNA sequencing. BQCV and SBV levels made up the vast majority of non-ribosomal RNA in pupae, collectively accounting for 92.6% and 86% of total RNA in colony 1 and 2 pupae, respectively. BQCV levels comprised 58–60% of total RNA, equivalent to 250 000–300 000 virus genomes for every million RNA molecules per sample. SBV levels ranged between 26 and 35%, approximately 100 000–125 000 virus genomes per million RNA molecules (figure 3*a* and the electronic supplementary material, tables S3 and S4).

(d) Serial transmission of deformed wing virus results in a decrease in deformed wing virus levels

We then repeated our serial transmission experiment (figure 1*a*, serial transmission 2) using inoculum obtained from five symptomatic, DWV-infected adult bees from New Zealand (see Material and methods, including details of quarantine permits), and injected into pupae obtained from two independent recipient honeybee colonies (referred to as colonies 4 and 5; naive to both DWV and *Varroa*). It is known that injecting DWV into white-eyed pupae results in an overt infection [34]. We passaged inoculum for 30 transmission cycles. We quantified the total amount of viral RNA in the initial adults (DWV source) and after 1, 10, 20 and 30 transmission cycles in pupae 4 days post injection, along with buffer-injected and control pupae taken from cycle 20 using HiSeq (Illumina) sequencing (figure 3*b* and the electronic supplementary material, table S3).

Almost 90% of non-ribosomal RNA came from DWV in our original inoculum, equating to 311 261 virus genomes per million RNA molecules present in the sample, indicating that the viral load of symptomatic adult honeybees can reach extreme levels (figure 3*b*: 'DWV source'; electronic supplementary material, tables S3 and S4). After one transmission cycle, DWV levels reached 25–32% of total non-ribosomal RNA in injected pupae from both colonies (77 000–79 000 virus genomes per million RNA molecules). Thereafter, DWV levels decreased rapidly until only a small amount (less than 10%, or 8000–10 000 genomes per million RNA molecules) could be attributed to DWV after 10 transmission cycles (figure 3*b* and the electronic supplementary material, tables S3 and S4). The decrease in DWV was accompanied by an increase in BQCV and SBV abundance (figure 3*b*), similar to the increase seen in our serial transmission experiment without the inclusion of DWV (figure 3*a* and the electronic supplementary material, tables S3 and 4). In the buffer-injected pupae from colony 4, we also saw high levels of SBV, indicating that the injection procedure alone can result in an increase in endogenous virus levels, in line with previous observations [31]. The total amount of RNA attributable to virus ranged between 88 and 97% in pupae injected with virus inoculum at all cycles tested, in contrast with control (0.3–0.4% virus) and buffer samples (62% in colony 4 (mentioned above), and 0.18% in colony 5).

We also saw a shift in DWV strain composition. DWV is known to comprise three main master variants: strain DWV-A, DWV-B and DWV-C [35,36]. Strain A is globally associated with increased viral titres and colony decline [11,37]. Strain B is an emerging DWV genotype that has increased virulence compared to DWV-A in laboratory experiments [32,35,36,38] (the effect of strain C is currently unknown). Our original inoculum contained low amounts of strain B (0.34% of total viral RNA) which had increased after 20 transmission cycles, particularly in colony 5 (1.66%), only to drop again after 30 cycles (figure 3*b* and the electronic supplementary material, table S3). In addition, we saw a shift in DWV-A sequence variation. Phylogenetic analysis of

consensus sequences in each sample showed that DWV from New Zealand source bees and cycle 1 clustered together, whereas strains present in cycles 10, 20 and 30 clustered separately (electronic supplementary material, figure S2a). Closer inspection of total variation indicated a reduction in sequence polymorphisms in later cycles compared to the original DWV source (electronic supplementary material, figure S3). By contrast, no sequence changes were observed in SBV and BQCV genomes from cycles 1 to 30 (electronic supplementary material, figure S2b,c).

(e) Injecting pupae with serially transmitted sacbrood virus and black queen cell virus results in high mortality while deformed wing virus alone does not

To compare the virulence of our serially passaged extracts, we injected white-eyed pupae with inoculum extracted from our DWV source adults, inoculum from serial transmission experiment 1, cycle 20 (containing BQCV/SBV, without DWV), and inoculum from serial transmission experiment 2, cycle 1 and 20 (containing DWV/BQCV/SBV). The composition of viruses present in our inoculum is presented in figure 3. We performed two independent survival experiments, testing inoculum from colonies 1 and 4 in one independent source colony (colony I), and colonies 2 and 5 in a second independent source colony (colony II; figure 1*b* for schematic).

Overall survival was significantly affected by treatment in both assays ($\chi^2_5 = 79.77$, $p < 0.00001$, $n = 360$ in colony I and $\chi^2_5 = 128.70$, $p < 0.00001$, $n = 360$ in colony II; electronic supplementary material, table S5). Mortality of pupae when injected with DWV alone was not statistically different from buffer-injected controls (both $p > 0.194$; figure 4 and the electronic supplementary material, table S5). When pupae were injected with inoculum from both serial transmission experiments, mortality between inoculum with and without DWV was not statistically different in colony I (all $p > 0.677$; figure 4 and the electronic supplementary material, table S5). The same trend held in colony II, although in this case pupae injected with cycle 1 inoculum with DWV showed statistically lower mortality than pupae injected with cycle 20 inoculum without DWV ($p = 0.020$; figure 4 and the electronic supplementary material, table S5). In both assays, mortality was much higher in all cycle 1 and 20 inoculum-injected pupae compared to buffer-injected pupae and pupae injected with DWV alone (all $p < 0.00001$; figure 4 and the electronic supplementary material, table S5). When testing the effect of 'source colony' on pupae survival, we found that our first source colony had a significantly higher survival than the second ($\chi^2_1 = 6.86$, $p = 0.0088$, $n = 720$). However, the overall result was the same for both colonies.

3. Discussion

We aimed to investigate the effect of changing the route of transmission, from faecal–oral to direct transmission via injection, to determine if such a change in route of transmission alone is sufficient to change the prevalence, abundance and damage caused by RNA viruses of honeybees. We found that the level of two viruses naturally present in our honeybee population, SBV and BQCV, rapidly increased when transmitted via injection into white-eyed

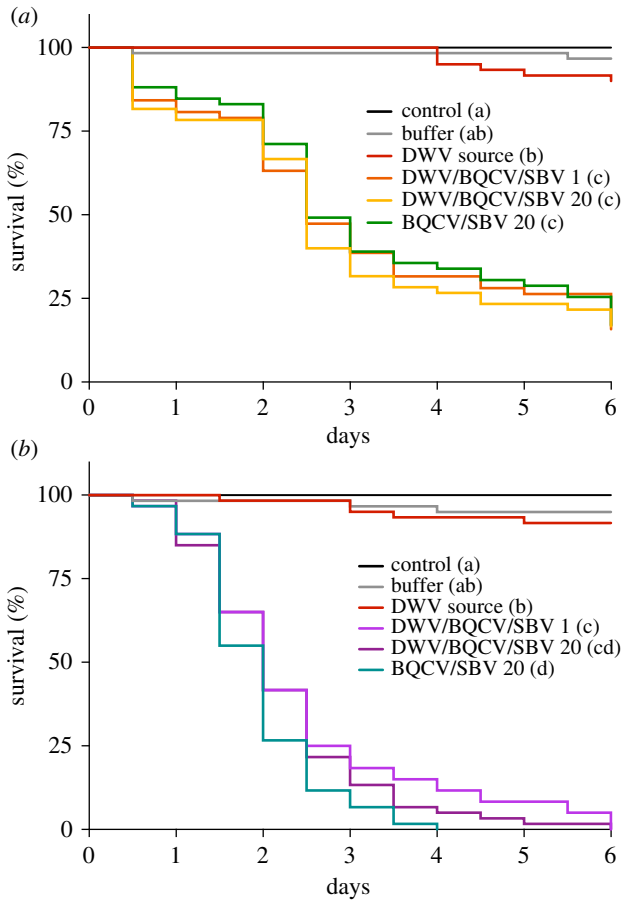

Figure 4. Survival of pupae after injections with inoculum obtained from our original source adults (DWV source) containing mainly DWV strain A, inoculum extracted after 1 transmission cycle containing DWV, BQCV and SBV, and inoculum extracted after 20 transmission cycles in the absence and presence of DWV, from serial transmission experiment 1 (BQCV/SBV) and serial transmission experiment 3 (DWV/BQCV/SBV). Treatments with the same letter do not differ significantly. Virus levels present in the different inoculums are given in figure 3. 'Control': unmanipulated pupae; 'buffer': pupae injected with buffer only. (*a*) BQCV/SBV inoculum from cycle 20 of colony 1; DWV/BQCV/SBV inoculum from cycle 1 and 20 of colony 4, injected into independent source colony I; (*b*) BQCV/SBV inoculum from cycle 20 of colony 2; DWV/BQCV/SBV inoculum from cycle 1 and 20 of colony 5, injected into independent source colony II. See the electronic supplementary material, figure S2 for photographs depicting the eye colour change observed in a control and bee extract-injected pupa, used to determine time of mortality in the survival analysis. See electronic supplementary material, table S5 for details of the statistical analyses. (Online version in colour.)

pupae. By contrast, when we injected inoculum containing high levels of DWV strain A, DWV viral levels rapidly decreased, most likely owing to competition with SBV and BQCV. Interestingly, injecting high levels of DWV strain A into pupae did not result in the death of the pupae, indicating that this strain of DWV does not kill developing brood despite its association with *V. destructor*. Injecting high levels of SBV and BQCV did result in high mortality. Clearly, increased mortality is owing to the increased levels of BQCV particularly, not to the presence of DWV, as survival was already low after one transmission cycle when levels of DWV were still relatively high and levels of BQCV had already reached their peak (figure 3*b*).

Both SBV and BQCV cause brood diseases; young larvae normally become infected early on via feeding by adult bees

[39]. When brood dies from either virus, nurse bees will remove and partially cannibalize the dead brood, thus themselves accumulating the virus. Because both viruses end up in the bees' hypopharyngeal gland (in which brood food is produced), nurse bees transmit the viruses when feeding young larvae [39]. Under natural conditions, and in the absence of *V. destructor*, both SBV and BQCV were found to occur at a frequency of around 10% in summer in Britain using immunodiffusion tests [40]. Both viruses are easily detected when bee extract from adult bees is injected into pupae [41], indicating that both viruses are present at low incidences without causing overt infections and readily amplify upon injection into pupae. In Australia, BQCV was found in 65% and SBV in 35% of colonies using more sensitive molecular detection methods, further indicating high viral prevalence in the absence of overt infections [33]. Our results suggest that vector-mediated transmission of bee extract containing SBV or BQCV could rapidly lead to such high viral levels that the brood never develops to adulthood, most likely because the viruses can now circumvent the bee's digestive tract [23]. As the vector will also die in the process, strains of BQCV and SBV that replicate to high titres when injected directly through the cuticle will be selected against, allowing viruses that are less damaging, such as DWV, to increase in abundance.

While we found no evidence of evolutionary change of SBV and BQCV over the course of our experiment, we did see a shift in DWV sequence variation (electronic supplementary material, figure S2). We observed a reduction in polymorphic sites across the DWV genome during serial transmission (electronic supplementary material, figure S3). Such a change could be owing to a genetic bottleneck, or selection. While we cannot distinguish between the two different explanations, a genetic bottleneck seems less likely as we would then also expect the other two viruses to be subjected to the same bottleneck. If indeed DWV replication is negatively affected by competition with SBV and BQCV, then the reduction in DWV strain diversity could be owing to selection against the worse performing DWV strains. Alternatively, in the absence of *V. destructor*, the serial passage could select against DWV variants that normally replicate within the mite. Ryabov *et al*. [42] similarly found a change in DWV variants depending on the mode of transmission of the virus.

Our experimental conditions were restricted to pupae, as our quarantine permits required injected pupae to be terminated prior to eclosion. Therefore, our results reflect conditions that are favourable to replication in brood, as we harvested injected pupae randomly, regardless of whether they would have successfully eclosed. Considering that *V. destructor* parasitizes brood initially, the process of vector-mediated transmission similarly begins in brood. However, in contrast to our experimental conditions, only those surviving to eclosion will harbour the viruses that are selected for. This suggests that *V. destructor* selects against high replication of viruses causing brood mortality, whereas our selective regime did the opposite.

Another virus commonly found in honeybees, ABPV, cannot replicate when injected into pupae that already contain either SBV or BQCV [40], showing that indeed SBV and BQCV are highly competitive, probably owing to their ability to replicate rapidly. The outcome of competition appears to depend on the exact mixture of viruses present. Both when fed to adult bees and in cell culture (in an embryonic honeybee cell line, AmE-711), IAPV (a dicistrovirus like BQCV) outcompetes SBV even when SBV is initially present in much higher titres [43]. However, when in combination with KBV (also a dicistrovirus), IAPV is unable to replicate to high levels. Interestingly cell line AmE-711 contains a covert DWV infection but DWV could only replicate to high levels in the absence of other viruses [43], confirming its low competitiveness. Hence, while in our specific circumstances, DWV seemed to be outcompeted by BQCV and SBV, in other bee populations, competition might be more severe among a different set of RNA viruses. Moreover, BQCV in particular comprises a large number of different strains [44] which may differ in their competitiveness and virulence.

ABPV, and the closely related KBV and IAPV, are often the first viruses to be associated with the arrival of *V. destructor* before they are gradually displaced by DWV [11,16]. A study documenting the change in viral landscape as *V. destructor* invaded the islands of New Zealand found negative associations between KBV and DWV and between DWV and SBV in both bee and mite samples, while SBV and BQCV were positively associated in both bees and mites [12]. As the time since the arrival of *V. destructor* increased, the prevalence of KBV and SBV decreased, BQCV levels remained similar and DWV levels increased [12]. The positive association between *V. destructor* and DWV was most strong in the first few years after the arrival of the mite. In later years, the prevalence of DWV less closely followed mite infestation rates. These results are consistent with the hypothesis that the succession of honeybee viruses after the arrival of *V. destructor* is due to the most virulent viruses being selected against, if *V. destructor* transmission facilitates an increase in replication rate [7,30]. Our results are the first to provide experimental evidence for this hypothesis.

The last few years have seen a surge in publications that link the arrival of *V. destructor* to the emergence of specific strains of DWV [11,12,35–37]. Initially, it was thought that DWV strain A was the most virulent strain while strain B was considered to be more benign [35,45]. However, this simple interpretation now seems questionable, as recently strain B has been associated with colony losses [38] and appears to be more virulent in an experimental setting [32,46]. Regardless, there is a clear association between *V. destructor* and DWV worldwide. Our experiment offers an explanation for the association between *V. destructor* and DWV that does not imply a change in virulence of DWV, an explanation that was proposed in an earlier modelling study [7] and supported by the documented change in viral landscape as *V. destructor* invaded the islands of New Zealand [12]. In the presence of more virulent viruses, DWV is outcompeted and, if present at all, often below detection level in the absence of *V. destructor*. The arrival of *V. destructor* quickly selects for an increase in the prevalence of the most virulent viruses until they become so virulent their transmission grinds to a halt owing to the death of the brood and thus the mites. Now more benign viruses such as DWV can make their appearance. Hence, instead of *V. destructor* directly causing a change in virulence of DWV, DWV is simply more favourable to the mite's lifecycle and therefore given the upper hand after more virulent species have been selected against.

While our results provide one explanation to support the observed succession of honeybee viruses as documented by Mondet *et al*. [12], our experimental protocol excluded one important player: *V. destructor*. If, for example, DWV could

replicate in *V. destructor* but other viruses cannot, then the dynamics of the viruses present in the host would be different from the dynamics seen in the absence of the vector. Further, we cannot conclude whether DWV virulence has increased owing to changes at the sequence level without directly comparing the virulence of DWV strains from before and after the arrival of *V. destructor*. Regardless, our results show that the known association between *V. destructor* and DWV is more complex than initially thought.

4. Material and methods

See the electronic supplementary material for detailed Material and methods.

Data accessibility. The raw sequencing reads from this project have been deposited to GenBank under the Bioproject ID PRJNA397460 at the Sequence Read Archive (SRA Study ID: SRP114989). The reference SBV, BQCV and source DWV sequences used in this study have been deposited to GenBank under accession numbers MF623170, MF623171 and MF623172. All data generated or analysed during this study are included in the manuscript and supporting files.

Competing interests. We declare we have no competing interests.

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