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Covert deformed wing virus infections have long-term deleterious effects on honeybee foraging and survival

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Several studies have suggested that covert stressors can contribute to bee colony declines. Here we provide a novel case study and show using radio-frequency identification tracking technology that covert deformed wing virus (DWV) infections in adult honeybee workers seriously impact long-term foraging and survival under natural foraging conditions. In particular, our experiments show that adult workers injected with low doses of DWV experienced increased mortality rates, that DWV caused workers to start foraging at a premature age, and that the virus reduced the workers' total activity span as foragers. Altogether, these results demonstrate that covert DWV infections have strongly deleterious effects on honeybee foraging and survival. These results are consistent with previous studies that suggested DWV to be an important contributor to the ongoing bee declines in Europe and the USA. Overall, our study underlines the strong impact that covert pathogen infections can have on individual and group-level performance in bees.

1. Introduction

Over the past decades, serious declines in both wild and managed bee pollinators have been recorded in many parts of the world [1–5], thereby threatening the ecosystem services they provide. The underlying cause of the recent wave of honeybee colony losses has been subject to much debate and the current consensus is that multiple stressors likely contribute to these declines, including malnutrition owing to a lack of flower diversity, exposure to agrochemicals or the spread of emerging pathogens and parasites [2,5–10]. Pinpointing these stressors, however, can be hard, especially when their effect becomes obvious only over extended periods of time, such as following exposure to sub-lethal doses of pesticides [9,11] or after contracting some seemingly harmless 'covert' pathogen infections [12,13].

Among pathogens, recent studies have suggested that deformed wing virus (DWV) represents an important long-term stressor, as it has been statistically associated with both winter mortality and colony collapse in many studies [14–24]. DWV has a near global distribution and is the most widespread of the currently described viruses that infect honeybees, often affecting between 50% and 75% of all honeybee hives [25,26]. In addition, the virus can spill over to other bees [27,28], thereby posing an additional threat. DWV is named after the characteristic wing deformities that can arise when honeybees are infected in

the larval or pupal stage via the ectoparasitic mite vector *Varroa destructor* [29]. In this case, virus infections result in bloated abdomens, miscolouring and shortened lifespans [29]. Typically, however, infections take on a more 'covert' form, resulting in no visible morphological symptoms, especially when infection occurs in the adult stage or when mites carry only low virus titres. Nevertheless, the fact that colonies with covert infections can suffer from weakness, depopulation and sudden collapse [22] and that the presence of the virus has been linked with both winter mortality and colony collapse [14–24] suggests that DWV exerts a significant amount of long-term stress.

Indeed, several recent studies using controlled artificial infection of adult honeybee workers have shown a number of important effects of DWV, including the impairment of sensory responsiveness, associative olfactory learning and memory formation [30] (possibly linked to replication of the virus in the mushroom bodies and antennal lobes [31]), an accelerated pace of behavioural transition through their age-linked task allocation [32] and reduced lifespans of infected adult honeybees [25]. Furthermore, two studies in which natural variation in infection levels of DWV was combined with experimentally manipulated variation in infection levels with the microsporidian parasite *Nosema* showed that DWV-infected bees displayed shortened flight distances and flight durations in flight mill experiments [33], but no differences in orientation flight behaviour in a harmonic radar tracking set-up [34], whereas the reverse pattern was seen for *Nosema* [33,34]. As yet, however, effects of DWV on honeybee flight behaviour and foraging patterns have not yet been investigated using controlled infection set-ups.

The aim of this study, therefore, was to determine the impact of DWV as a long-term stressor in honeybees, and test experimentally if inoculation of adult bees with the virus negatively affected honeybee foraging behaviour and performance. To this end, we used passively powered radiofrequency identification (RFID) transponder tags [9,11,35–40] as a key technology that enabled us to non-invasively monitor the long-term out-hive activity of honeybee workers that were or were not experimentally infected with the virus. Tracking out-hive activity is key in studies of the impact of pathogens on honeybee health, as the worker foraging force is responsible for all resource acquisition, and the foraging range, worker activity and the magnitude of resource influx are vital to colony growth and survival [41,42]. Previously, RFID technology has been successfully used to study sublethal effects of nutritional stress and pesticides on honeybee and bumblebee foraging behaviour [9,11,35–40]. Nevertheless, applications to the study of pathogen-induced stress on honeybee foraging behaviour are still rare, and are currently limited to one study on Israeli acute paralysis virus (IAPV) [43], which documented virus-induced differences in homing ability, but without taking full advantage of the technology to study the long-term impact on foraging behaviour, and one study that showed adverse effects of a microsporidian gut parasite, *Nosema apis*, on honeybee foraging and survival [44]. In addition, another tracking technology—harmonic radar—was recently used by one group to show that the emerging pathogen *Nosema ceranae* caused impaired homing behaviour in honeybees [45] and that *Nosema* infection also affected honeybee orientation flight behaviour [34]. The tracking method, however, was unable to reveal any correlation between DWV infection levels and orientation flight characteristic [34].

2. Material and methods

(a) Radiofrequency identification tracking set-up

Three three-frame observation hives with *Apis mellifera carnica* honeybees were installed at the laboratory's apiary in Leuven, Belgium to serve as host colonies for RFID-tagged bees that were or were not experimentally infected with DWV (400 of each treatment condition per host colony, see below). Each host colony contained two frames of brood, one frame with stored pollen and honey, a queen and around 3000 host colony workers (figure 1). The colonies were placed indoors at room temperature and were connected to the outside via a single entrance tunnel to allow free foraging. The end of the tunnel was outfitted with two iID® MAJA 4.1 RFID reader modules placed in series and connected to a MAJA 4.1 host computer (Microsensys, Germany) to record and log the timing of all RFID-tagged honeybees leaving or entering the hive. By setting up the reader modules in a serial set-up, successive signals from both readers gave information regarding the direction of movement of the detected bees. The readers were separated from each other by a 4 cm wooden tunnel block to prevent interference between the readers (figure 1).

(b) Introduction of control and experimentally infected bees

In each of the host colonies, we introduced 400 DWV-negative control bees and 400 DWV infection-positive honeybees. This was done by allowing bees to emerge from a single donor colony that based on a prior screen was confirmed to be free of DWV as well as of any of the major known honeybee viruses or pathogens [46], injecting newly eclosed workers with appropriate treatment solutions, and introducing these bees into one of three host colonies (see electronic supplementary material Methods for details). All colonies in our apiary, including the donor and host colonies, were treated with Thymovar for *Varroa* control according to the manufacturer's recommendations. The fact that only a single donor colony was used in our experiments was linked with the difficulty of finding a host colony that was free of the major known honeybee pathogens, but our experimental design partly compensated for this by incorporating replication across different host environments. Bees were allowed to emerge by placing brood frames of the donor colony in a MIR-253 incubator (Sanyo, Belgium) at 34°C and 60% humidity, after which newly eclosed workers were collected daily. Subsequently, 400 newly eclosed workers per treatment condition and host colony were injected with 3 µl of the appropriate treatment solution, using a 5 µl 26 s gauge Hamilton syringe inserted into the apical part of the thorax. Immediately afterwards, each of these bees were outfitted with a mic3® 64-bit read-only RFID transponder (Microsensys, Germany) by gluing the tag to the bee's thorax using Kombi Turbo two-component glue (Bison, The Netherlands). The tags measured 2.0 × 1.7 × 0.5 mm, weighed less than 5 mg and transmitted at 13.56 MHz. The RFID codes of all experimentally manipulated workers, together with the treatment condition, host colony and time of introduction, were added to a transponder information database by reading each code using a iID® PENmini USB pen (Microsensys, Germany). Up to 50 tagged individuals subjected to one of the two treatment conditions were kept in separate 15 × 10 × 7 cm cages kept at 34°C and 60% humidity, and contained a 10 × 8 cm piece of honey-filled comb and water, to allow the bees to settle down before introducing them into the host colonies (figure 1). Before introduction, the cages were placed on top of the observation hives, separated only by a wire mesh, for a 30 min period to increase acceptance rates [47]. Each of the 400 workers per host colony and treatment condition were introduced over the course of a period of 5 days, and foraging

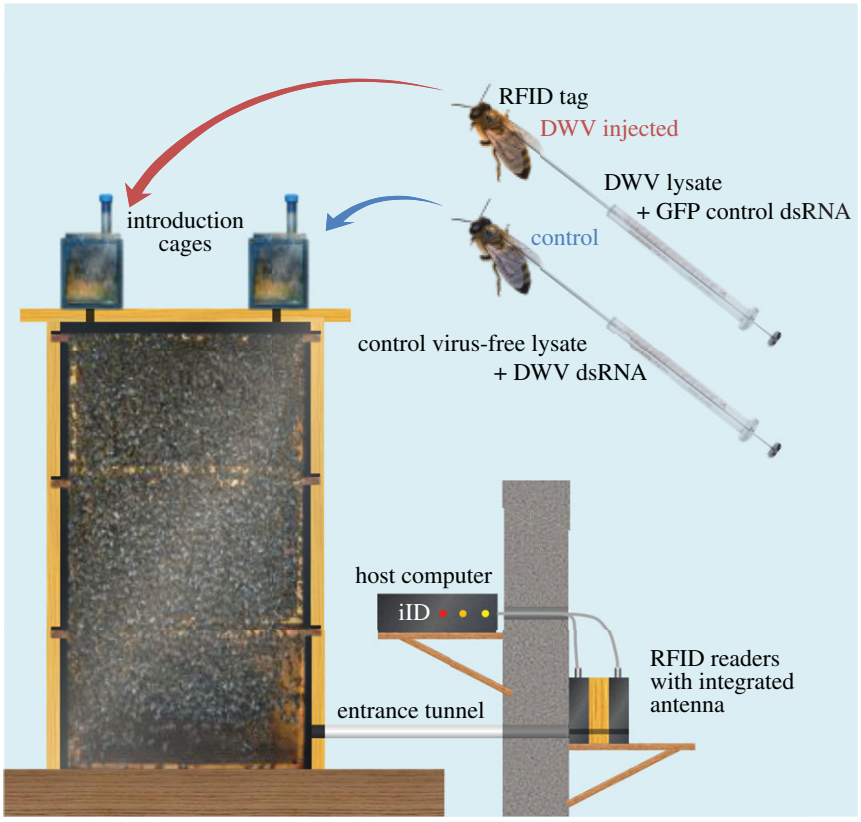

Figure 1. Experimental set-up. Observation hives were installed indoors with two RFID readers at the hive entrance to detect and log RFID-tagged bees entering or leaving the hive. The two RFID readers modules, connected to the host computer, were placed in series to determine the walking direction of detected bees. Tagged bees which were or were not experimentally infected with deformed wing virus were introduced into the host colony via separate introduction cages shown at the top ($n = 400$ bees per treatment and host colony). (Online version in colour.)

behaviour was monitored up to 40 days after introduction, in August–September 2012 for replicate host colonies A and B and September–October 2012 for host colony C.

(c) Controlled infection

In order to obtain two groups of adult age-matched bees that were or were not infected with DWV, we injected bees with lysate of honeybees that were either infected with DWV (and none of the other common honeybee viruses) or with that of honeybees that were confirmed to be virus-free (for details, see electronic supplementary material). To reduce the likelihood of horizontal transmission from the DWV infected to the group of uninfected bees [48], we also added a double-stranded RNA treatment to our DWV-negative lysate [49,50] to try to keep those bees DWV-free, and added a control GFP-dsRNA treatment in the DWV positive lysate to control for the possible effects of foreign dsRNA injection. The amount of DWV injected was estimated at 1.2×10^4–4.6×10^5 DWV copies per bee, and was aimed at mimicking infection loads reported for bees with covert infections (1.4×10^3–2.4×10^9 copies per bee, [51]). Based on Illumina ultra-high throughput sequencing, the DWV strain used for inoculation was determined to belong to the type B DWV master variant [52], which has recently been found to be an emergent, slightly more virulent strain of the DWV virus [25] that is currently also the most common strain in Britain [25] (for details, see the electronic supplementary material).

To validate the effects of the two treatment solutions, 150 additional eclosed workers were injected with each treatment solution, paint-marked and introduced into a fourth observation. Every 4 days, 20 individuals of each treatment were sampled from this colony and subjected to MLPA analysis to determine their DWV infection state [46]. These analyses confirmed the establishment of stable DWV infections in the DWV-lysate-inoculated bees,

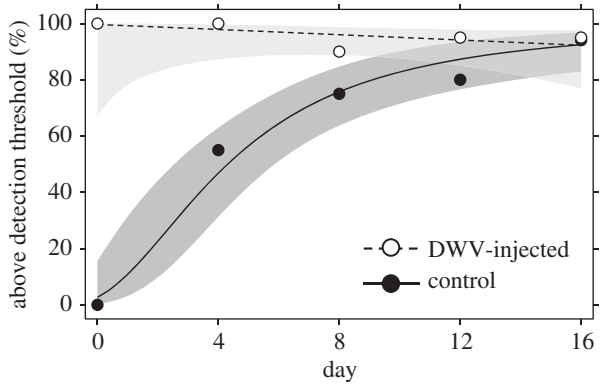

Figure 2. Treatment validation results. Evolution of the proportion of infected individuals over time in control and DWV treatment groups, based on MLPA analysis [46] of four daily sample sets of 20 individuals each from both treatments (fits and 95% CIs based on binomial GLM with treatment, $\log(x + 1)$ tranformed duration after introduction into the host colony and their interaction included as independent variables).

even if there was evidence that some control bees became infected during the later stages of the experiment as well (figure 2), though possibly at lower levels (see electronic supplementary material for a full discussion of the methods and results of these analyses).

(d) Data analysis

Raw RFID tracking data were analysed with the Track-A-Forager Java application [53], which filters out rapid-succession scans of the same scanner, labels ingoing and outgoing flights by tagged workers, and corrects occasional errors in the data, including the possible occurrence of missed scans (for details see electronic

supplementary material). To compare the foraging behaviour of DWV-infected and control bees, we quantified the number of trips performed by each individual, trip duration and the proportion of the introduced workers of each treatment group which survived up to foraging age, to gauge differential early-life mortality. In addition, we measured the age at onset of foraging, defined as the age of each individual at their first reconstructed trip, the foragers' life expectancy, measured as the age of each individual at their last scan, and forager activity span, i.e. the time difference in days between the first and last registered scan of each bee. We should note that our experiment could not distinguish between a DWV-induced reduction in direct mortality and a DWV-induced mortality owing to indirect effects, e.g. caused by a decrease in homing ability or an increase in the susceptibility to predation or other environmental stressors. Details of all statistical analyses performed are given in electronic supplemental material and the R script included on the Dryad repository.

3. Results

Visual analysis of foraging activity over the course of our experiment revealed clear disparities in the age at onset of foraging, forager life expectancy and forager activity span between the DWV-infected and control bees (figure 3). In addition, bees that survived to foraging age showed a clear deviation from the 50 : 50 ratio at which they were first introduced (figure 3), with control bees evidently having much better chances to survive to foraging age than DWV-infected ones. To thoroughly examine each of these effects as well as to look for other possible effects of DWV on foraging behaviour, we conducted a number of detailed statistical analyses. In particular, we tested for significant effects of DWV on the probability that bees would survive to foraging age, the onset of foraging, forager life expectancy and activity span as well as the number and trip duration of foraging trips carried out by individuals that survived to foraging age.

(a) Effect of deformed wing virus on the likelihood that bees would survive to foraging age

The biased representation of control bees among bees that started to forage relative to the 50 : 50 ratio at which they were first introduced (figure 3) indicates that the DWV-inoculated bees experienced greater mortality early on in their life. This is confirmed by the fact that a significantly greater proportion of the DWV-inoculated bees died before making it to foraging age than control bees in each of the three host colonies (277/400 versus 244/400, 324/400 versus 266/400 and 279/400 versus 175/400; binomial colony × treatment full factorial GLM, $z = 2.44$ and $p = 0.015$, $z = 4.61$ and $p = 4 \times 10^{-6}$, $z = 7.33$ and $p = 2 \times 10^{-13}$; figure 4*a*). We should note that this mortality also includes baseline mortality linked to occasional rejection of tagged bees by the host colony. To control for this baseline mortality, we also calculated the relative odds that bees would survive to foraging age. This showed that DWV-inoculated bees on average had 2.1 (s.e. 0.19) times lower odds to make it to foraging age than control bees.

(b) Effect of deformed wing virus on age at onset of foraging, life expectancy and activity span

We also found significant differences between the DWV-infected and non-infected treatments in the observed age at onset of foraging. In particular, bees that were inoculated with DWV and which survived until foraging age starting to forage 2.31 days earlier (s.e. 0.73), on average, than bees inoculated with the control solution (figure 4*b*; full factorial colony × treatment ANOVA, main effect of treatment: $p = 7 \times 10^{-9}$; LR $\chi^2_1 = 33.63$, sample sizes as shown in figure 3*a*). In addition, infected foragers had a life expectancy that was reduced by 4.74 days on average (s.e. 0.20) compared with control bees (figure 4*c*; ANOVA, main effect of treatment: $p = 0.01$; LR $\chi^2_1 = 28.7$) and a total activity span that was reduced by 2.60 days (s.e. 0.79) compared with control foragers (figure 4*d*; ANOVA, main effect of treatment: $p = 0.0003$; LR $\chi^2_1 = 13.26$; sample sizes for both are as given in figure 3*a*). Hence, DWV-infected bees had a reduced life expectancy and activity span also after they started to forage, even though the onset of foraging occurred 2.3 days earlier in the DWV-infected group than in the control group. Overall, the onset of foraging (at 12–17 days) was somewhat earlier and forager life expectancy shorter (11–26 days) than that observed in mature hives (onset at *ca* three weeks and forager life expectancy of *ca* six weeks [47,54]). Nevertheless, both figures were comparable to those found in other studies that used a comparable observation hive set-up (e.g. onset of foraging at *ca* 10 days and forager life expectancy of 14–40 days [55]). This discrepancy may be linked with stress induced by the RFID tags or the injection itself, as stress in honeybees is known to induce precocious foraging and shorten worker lifespan [32,56]. Even so, we expect our conclusions to be robust as our treatment effects are all measured relative to the control under identical conditions.

(c) Effect of deformed wing virus on number of foraging trips or trip duration

Although DWV infection could in principle also have affected the number of foraging trips and the duration of the trips carried out by individuals that survived to foraging age [33], our statistical analyses revealed that there was no strong evidence for this. For example, the effect of treatment on the number of trips performed was inconsistent and different across the replicate host colonies used (quasi-Poisson GLM, effects of treatment in colonies A, B and C: A. $z = 7.55$ and $p = 1 \times 10^{-7}$, B. $z = -2.49$ and $p = 0.04$; C. $z = -1.38$, $p = 0.42$). On average, across all three colonies, workers made 5.46 trips per bee per day (s.e. 0.50) in the DWV-inoculated group versus 5.85 trips per bee per day (s.e. 0.80) in the control (sample sizes as in figure 3*a*), which are figures consistent with those given in other studies [47,54]. Nevertheless, given that DWV infection strongly reduced the chances for workers to survive to foraging age and that DWV infection reduced the life expectancy and total activity span of foragers (cf. results above), it is clear that DWV still had a strongly negative overall effect on the net number of trips and amount of foraging performed. Similarly, the effect on trip duration was not consistent across host colonies, as DWV-inoculated bees made significantly longer trips than control bees in colony A (gamma GLMM, Tukey post-hoc test, 1.86 h versus 1.22 h, s.e. 0.17 and 0.08, $p = 0.0004$), whereas there was no significant effect on trip duration in colony C (2.89 h versus 2.50, s.e. 0.41 and 0.47, $p = 0.90$), and an opposite trend in colony B than in colony A (1.62 h versus 1.99 h, s.e. 0.12 and 0.27, $p = 0.39$). Inclusion of time or worker age as explicit linear or polynomial terms, either in interaction with treatment or not, did not

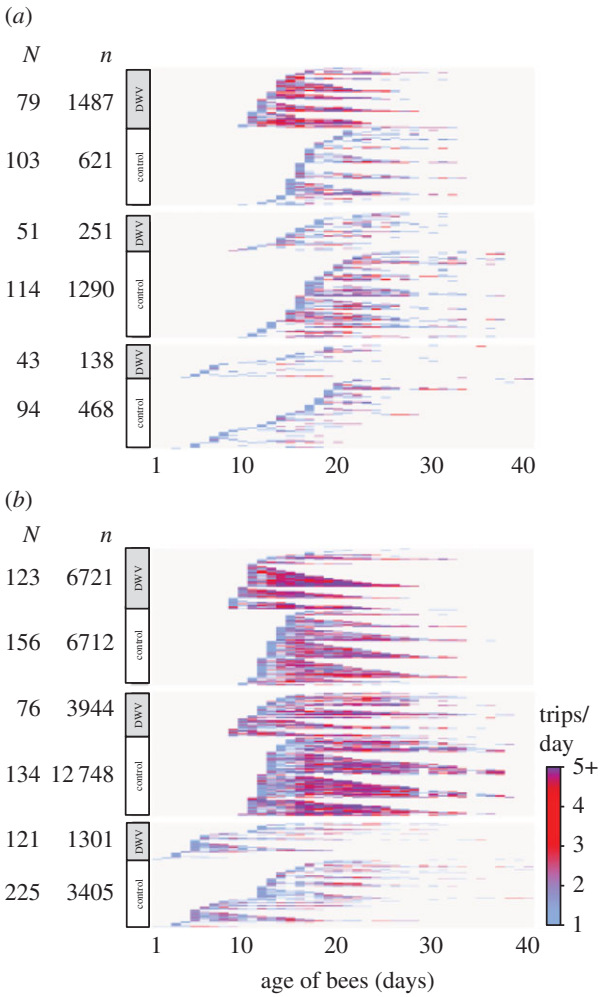

Figure 3. Total foraging activity of DWV-infected and control bees. The daily foraging activity over the course of the experiment is shown based on both the reconstructed foraging trips (*a*) and the unfiltered RFID scans (*b*) for colonies A (top), B (middle) and C (bottom) (N = total number of unique bees detected per treatment and host colony, n = total number of reconstructed foraging trips or scan events across all tracked bees). Individuals, represented as rows in the diagram, are sorted by treatment, age at onset of foraging and total activity span. The overrepresentation of control bees among observed foragers demonstrates that control workers had higher odds to survive to foraging age, and visual analysis of the data also indicate disparities between DWV and control bees in the age at onset of foraging, forager life expectancy and activity span (cf. figure 4). (Online version in colour.)

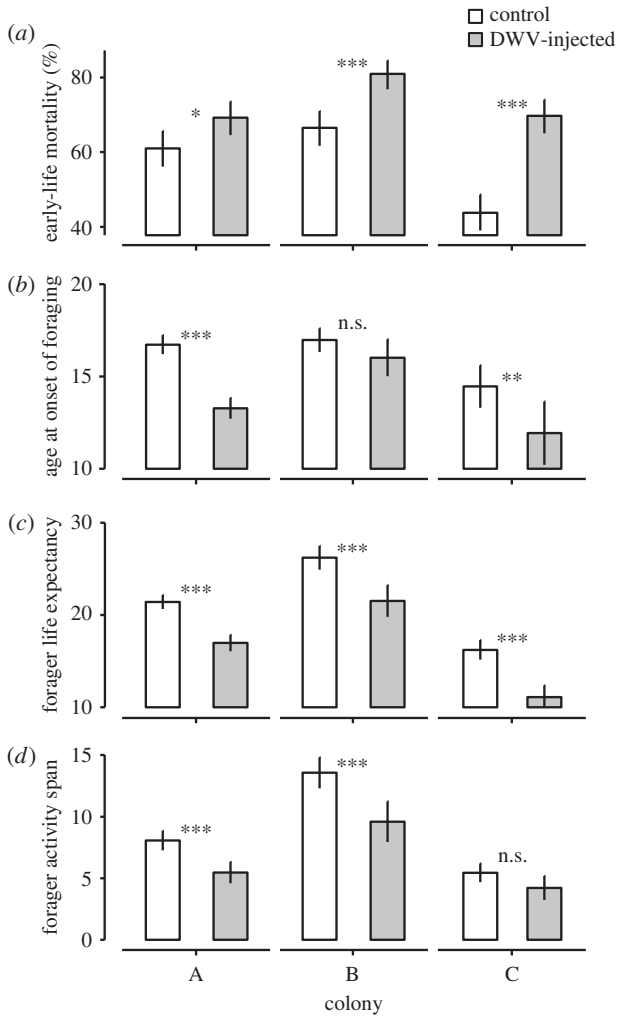

Figure 4. Effect of DWV infection on honeybee foraging and survival. Out of the 400 bees that were tagged per treatment and host colony, a significantly smaller proportion survived to foraging age (being detected at least once by the RFID scanners) in the DWV injected group than in the control group (panel *a*, binomial full factorial GLM, overall $p < 2 \times 10^{-16}$, LR $\chi^2_1 = 71.89$; means and 95% C.L.s are shown and significance levels per colony based on Tukey post-hoc Wald z tests shown by asterisks). In addition, honeybees that were artificially infected with DWV and which survived to foraging age started to forage significantly earlier than uninfected control bees (panel *b*, two-way full factorial ANOVA, $p = 7 \times 10^{-19}$, LR $\chi^2_1 = 33.63$; means and 95% C.L.s are shown and significance levels per colony based on Tukey post-hoc Wald z tests shown by asterisks; total number of tracked bees as in figure 3*a*), and DWV-infected foragers had a significantly reduced life expectancy (defined as age at last detection, panel *c*; ANOVA, $p = 0.01$, LR $\chi^2_1 = 28.7$) and activity span (defined as age at last foraging trip minus age at first foraging trip, panel *d*; ANOVA, $p = 0.0003$, LR $\chi^2_1 = 13.26$) (means and 95% C.L.s are shown and significance levels per colony based on Tukey post-hoc Wald z tests shown by asterisks; total number of tracked bees and foraging trips as in figure 3*a*).

improve the explanatory power of any of the fitted models, and hence such analyses were not further pursued.

4. Discussion

Overall, our results demonstrate that DWV infections have strongly deleterious effects on adult honey bees, with both mortality rates, and—to a lesser extent—foraging behaviour being clearly affected. In particular, DWV-infected bees started to forage at an earlier age and showed reduced lifespans and total activity spans than control bees. Finally, next-gen sequencing demonstrated that the DWV strain we used for inoculation belonged to the type B DWB master variant [52], which has recently been found to be an emergent, more virulent strain of the DWV virus [25] that currently appears to be the most common strain in Britain [25]. The fact that our DWV lysate was prepared from a randomly selected sample of bees with overt DWV infection symptoms suggest that this strain is now also common in Continental Europe.

Our finding that DWV-inoculated workers started foraging at an earlier age and experienced so-called precocious foraging was in line with expectation, as previous studies have also found that unhealthy or stressed honeybee workers start to perform risky foraging tasks at an earlier age compared with healthy individuals. For example, *Nosema* [13,57,58], sacbrood virus [59] and *Varroa* [60,61] have all been found to induce

precocious foraging in honeybees. From an ultimate perspective, diseased or health compromised workers have been suggested to benefit from starting to foraging earlier as a way to protect other individuals inside the nest from getting infected [58] or to make the most of their reduced lifetime [56]. In addition, it would be possible that the disease agent itself benefited from an earlier onset of foraging if this promoted its horizontal transmission to other host colonies [62]. Indeed, in the case of DWV, a direct influence on the behaviour of its host is not unlikely, given that DWV particles have previously been found in the mushroom bodies—a key higher brain centre of these insects [31] (but see [34] for a study where no behavioural effects were found). Irrespective of these possible adaptive causes, it is clear that precocious foraging would have a major effect on colony well-being, as premature foraging partially depletes the nurse bee population [63] and disrupts various activities inside the hive [64–66], and rapid behavioural maturation has been shown to strongly accelerate the failure of stressed honeybee colonies [63].

The strong evidence we found for a DWV-induced effect on mortality patterns and long-term survival was more unexpected. Traditionally, secondary DWV infections in adult workers are regarded as 'covert' and largely asymptomatic [29,67,68], but this proposition is clearly challenged by our findings, which document very clear and significant long-term effects of the virus. Although increased mortality has been documented in bees that display overt DWV infection symptoms and crippled wings [69–71], similar mortality in bees that acquired the virus in the adult stage has been demonstrated only recently in experimentally caged and non-foraging bees [25]. Our results now show that this mortality effect continues after the onset of foraging, and that the virus therefore acts as a long-term stressor on honeybee health and survival. DWV-induced mortality could have several causes. Given that DWV have been shown to occur in the honeybee brain, including in the mushroom bodies [31,62,72], which are involved in learning and memory, and that DWV infections have been shown to induce learning deficits [30], it is possible that increased mortality is caused by impaired orientation capabilities or predator avoidance or that it makes them more susceptible to other environmental stressors. Indeed, the DWV Kakugo strain has earlier been found to be associated with increased aggression and risk-taking behaviour [73,74]. Alternatively, it is possible that DWV directly results in increased mortality, e.g. owing to costly upregulation of the host's immune system [75]. However, given the well-documented effect on direct, early-life mortality, both in our study and that of McMahon *et al.* [25], we consider a direct mortality effect most likely. Furthermore, and regardless of the underlying causes, it is clear that the early disappearance of DWV-infected bees and their significantly shorter activity spans would have strongly deleterious effects on the total amount of pollen and nectar foraging performed by infected colonies. Additionally, a shorter activity span of workers would also cause fewer workers to engage in discovering novel food patches, thereby impacting the flow of information and causing further synergistic costs to global colony health [47].

The fact that in our RFID data, DWV inoculation did not affect trip duration or the number of trips performed by DWV-infected foragers went against the conclusions of [33], who concluded that DWV infection but not *Nosema ceranae* reduced average trip duration. As there was significant variation in the impact of the virus across our three replicate host environments, however, it is possible that the same effect would still have been found with a larger number of replicate donor and/or host colonies. Given that our donor bees all came from a single, rare uninfected colony, we had *a priori* not expected any large variation in the impact of the virus. Possible reasons for this variation could be linked with seasonal factors, variation in the genetic compatibility with the host colonies, or subtle differences in the performance or health of the host colonies, such as the possible presence of *Nosema* among the host workers, which we did not explicitly look at, but which is known to cause precocious foraging and affect longevity, activity and out-of-hive performance of honeybees [13,45,58,76,77]. Alternatively, it is possible that the variation in DWV impact is linked to some of the control bees having become infected during the later stages of our experiment, which our treatment validation results suggest may have been the case (though likely at lower levels, figure 2), and that the speed at which this occurred differed across host colonies. These results also suggest that a single dsRNA injection was not sufficient to fully protect bees for extended periods of time, and that continued oral administration would have to be used for effective long-term control via RNA interference [49,50,78]. Despite this variation in the effect of the virus, however, it was clear that overall, DWV had a strongly deleterious effect across all three colonies, with significant effects on early-life mortality (figure 4*a*), forager life expectancy (figure 4*c*) and forager activity span (figure 4*d*) in three replicate host colony environments, and significant effects on the onset of foraging in two out of three host colonies (figure 4*b*). These findings are consistent with studies showing that DWV is among the most important predictors implicated in honeybee colony declines in both Europe [14,16,17,24] and the USA [18] and hence an important contributory factor to the current pollination crisis.

Overall, our results highlight the impact of long-term stressors on bee health and survival, thereby reinforcing the conclusions of several recent tracking studies that have studied stress-induced changes in bees caused by either pathogens [33,43–45], nutritional stress [36] or pesticide exposure [9,11,35,37–40]. We hope that in the future, these approaches may continue to be used to further our understanding of the factors involved in the ongoing pollinator declines [1–5] and how they interact with each other in exerting long-term stress [2,5–10].

Data accessibility. All raw data files and code used in analyses are available in Dryad: http://dx.doi.org/10.5061/dryad.fm0r1. Illumina reads and the full genome sequence of the DWV inoculate are available from the Sequence Read Archive and GenBank (accession nos. PRJNA336281 and KX783225).

Authors' contributions. K.B., D.C., D.C.d.G., L.S. and T.W. conceived and designed the work; K.B., D.C. and L.D.S. performed the experiments; K.B., A.V.G. and T.W. analysed the data; L.D., D.C.d.G., L.D.S., L.B., S.J.M. and T.W. contributed reagents, materials, or analysis tools and K.B., M.H.D.L., L.B., S.J.M. and T.W. wrote the paper. All authors gave final approval for publication.

Competing interests. We have no competing interests.

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References

1. Ellis JD, Evans JD, Pettis J. 2010 Colony losses, managed colony population decline, and colony collapse disorder in the United States. *J. Apic. Res.* **49**, 134–136. (doi:10.3896/ibra.1.49.1.30)
2. McMenamin AJ, Genersch E. 2015 Honey bee colony losses and associated viruses. *Curr. Opin. Insect Sci.* **8**, 121–129. (doi:10.1016/j.cois.2015.01.015)
3. Lee K *et al.* 2015 A national survey of managed honey bee 2013–2014 annual colony losses in the USA. *Apidologie* **46**, 292–305. (doi:10.1007/s13592-015-0356-z)
4. Potts SG, Biesmeijer JC, Kremen C, Neumann P, Schweiger O, Kunin WE. 2010 Global pollinator declines: trends, impacts and drivers. *Trends Ecol. Evol.* **25**, 345–353. (doi:10.1016/j.tree.2010.01.007)
5. Neumann P, Carreck N. 2010 Honey bee colony losses. *J. Apic. Res.* **49**, 1–6. (doi:10.3896/ibra.1.49.1.01)
6. Goulson D, Nicholls E, Botías C, Rotheray EL. 2015 Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science* **347**, 1255957. (doi:10.1126/science.1255957)
7. Barron AB. 2015 Death of the bee hive: understanding the failure of an insect society. *Curr. Opin. Insect Sci.* **10**, 45–50. (doi:10.1016/j.cois.2015.04.004)
8. Manley R, Boots M, Wilfert L. 2015 Emerging viral disease risk to pollinating insects: ecological, evolutionary and anthropogenic factors. *J. Appl. Ecol.* **52**, 331–340. (doi:10.1111/1365-2664.12385)
9. Gill RJ, Ramos-Rodriguez O, Raine NE. 2012 Combined pesticide exposure severely affects individual-and colony-level traits in bees. *Nature* **491**, 105–108. (doi:10.1038/nature11585)
10. Whitehorn PR, O'Connor S, Wackers FL, Goulson D. 2012 Neonicotinoid pesticide reduces bumble bee colony growth and queen production. *Science* **336**, 351–352. (doi:10.1126/science.1215025)
11. Henry M, Béguin M, Requier F, Rollin O, Odoux J-F, Aupinel P, Aptel J, Tchamitchian S, Decourtye A. 2012 A common pesticide decreases foraging success and survival in honey bees. *Science* **336**, 348–350. (doi:10.1126/science.1215039)
12. Bryden J, Gill RJ, Mitton RAA, Raine NE, Jansen VAA. 2013 Chronic sublethal stress causes bee colony failure. *Ecol. Lett.* **16**, 1463–1469. (doi:10.1111/ele.12188)
13. Kralj J, Fuchs S. 2010 *Nosema* sp. influences flight behavior of infected honey bee (*Apis mellifera*) foragers. *Apidologie* **41**, 21–28. (doi:10.1051/apido/2009046)
14. Dainat B, Evans JD, Chen YP, Gauthier L, Neumann P. 2012 Predictive markers of honey bee colony collapse. *PLoS ONE* **7**, e32151. (doi:10.1371/journal.pone.0032151)
15. Highfield AC, El Nagar A, Mackinder LCM, Noel L, Hall MJ, Martin SJ, Schroeder DC. 2009 Deformed wing virus implicated in overwintering honeybee colony losses. *Appl. Environ. Microbiol.* **75**, 7212–7220. (doi:10.1128/aem.02227-09)
16. Bacandritsos N, Granato A, Budge G, Papanastasiou I, Roinioti E, Caldon M, Falcaro C, Gallina A, Mutinelli F. 2010 Sudden deaths and colony population decline in Greek honey bee colonies. *J. Invert. Pathol.* **105**, 335–340. (doi:10.1016/j.jip.2010.08.004)
17. Genersch E *et al.* 2010 The German bee monitoring project: a long term study to understand periodically high winter losses of honey bee colonies. *Apidologie* **41**, 332–352. (doi:10.1051/apido/2010014)
18. Cornman RS, Tarpy DR, Chen Y, Jeffreys L, Lopez D, Pettis JS, Vanengelsdorp D, Evans JD. 2012 Pathogen webs in collapsing honey bee colonies. *PLoS ONE* **7**, e43562. (doi:10.1371/journal.pone.0043562)
19. Berthoud H, Imdorf A, Haueter M, Radloff S, Neumann P. 2010 Virus infections and winter losses of honey bee colonies (*Apis mellifera*). *J. Apic. Res.* **49**, 60–65. (doi:10.3896/ibra.1.49.1.08)
20. Lanzi G, De Miranda JR, Boniotti MB, Cameron CE, Lavazza A, Capucci L, Camazine SM, Rossi C. 2006 Molecular and biological characterization of deformed wing virus of honeybees (*Apis mellifera* L.). *J. Virol.* **80**, 4998–5009. (doi:10.1128/JVI.80.10.4998-5009.2006)
21. Cox-Foster DL *et al.* 2007 A metagenomic survey of microbes in honey bee colony collapse disorder. *Science* **318**, 283–287. (doi:10.1126/science.1146498)
22. Berenyi O, Bakonyi T, Derakhshifar I, Koglberger H, Nowotny N. 2006 Occurrence of six honeybee viruses in diseased Austrian apiaries. *Appl. Environ. Microbiol.* **72**, 2414–2420. (doi:10.1128/aem.72.4.2414-2420.2006)
23. Kielmanowicz MG, Inberg A, Lerner IM, Golani Y, Brown N, Turner CL, Hayes GJ, Ballam JM. 2015 Prospective large-scale field study generates predictive model identifying major contributors to colony losses. *PLoS Pathog.* **11**, e1004816. (doi:10.1371/journal.ppat.1004816)
24. Evison SE, Roberts KE, Laurenson L, Pietravalle S, Hui J, Biesmeijer JC, Smith JE, Budge G, Hughes WO. 2012 Pervasiveness of parasites in pollinators. *PLoS ONE* **7**, e30641. (doi:10.1371/journal.pone.0030641)
25. McMahon DP, Natsopoulou ME, Doublet V, Fürst M, Weging S, Brown MJF, Gogol-Döring A, Paxton RJ. 2016 Elevated virulence of an emerging viral genotype as a driver of honeybee loss. *Proc. R. Soc. B* **283**, 20160811. (doi:10.1098/rspb.2016.0811)
26. Ravoet J, Maharramov J, Meeus I, De Smet L, Wenseleers T, Smagghe G, de Graaf DC. 2013 Comprehensive bee pathogen screening in Belgium reveals *Crithidia mellificae* as a new contributory factor to winter mortality. *PLoS ONE* **8**, e72443. (doi:10.1371/journal.pone.0072443)
27. Ravoet J, De Smet L, Meeus I, Smagghe G, Wenseleers T, de Graaf DC. 2014 Widespread occurrence of honey bee pathogens in solitary bees. *J. Invert. Pathol.* **122**, 55–58. (doi:10.1016/j.jip.2014.08.007)
28. Fürst MA, McMahon DP, Osborne JL, Paxton RJ, Brown MJF. 2014 Disease associations between honeybees and bumblebees as a threat to wild pollinators. *Nature* **506**, 364–366. (doi:10.1038/nature12977)
29. de Miranda JR, Genersch E. 2010 Deformed wing virus. *J. Invert. Pathol.* **103**, S48–S61. (doi:10.1016/j.jip.2009.06.012)
30. Iqbal J, Mueller U. 2007 Virus infection causes specific learning deficits in honeybee foragers. *Proc. R. Soc. B* **274**, 1517–1521. (doi:10.1098/rspb.2007.0022)
31. Shah KS, Evans EC, Pizzorno MC. 2009 Localization of deformed wing virus (DWV) in the brains of the honeybee, *Apis mellifera* Linnaeus. *Virol. J.* **6**, 182. (doi:10.1186/1743-422x-6-182)
32. Natsopoulou ME, McMahon DP, Paxton RJ. 2016 Parasites modulate within-colony activity and accelerate the temporal polyethism schedule of a social insect, the honey bee. *Behav. Ecol. Sociobiol.* **70**, 1019–1031. (doi:10.1007/s00265-015-2019-5)
33. Wells T, Wolf S, Nicholls E, Groll H, Lim KS, Clark SJ, Swain J, Osborne JL, Haughton AJ. 2016 Flight performance of actively foraging honey bees is reduced by a common pathogen. *Envrion. Microb. Rep.* **8**, 728–737. (doi:10.1111/1758-2229.12434)
34. Wolf S, Nicholls E, Reynolds AM, Wells P, Lim KS, Paxton RJ, Osborne JL. 2016 Optimal search patterns in honeybee orientation flights are robust against emerging infectious diseases. *Sci. Rep.* **6**, 32612. (doi:10.1038/srep32612)
35. Schneider CW, Tautz J, Gruenewald B, Fuchs S. 2012 RFID tracking of sublethal effects of two neonicotinoid insecticides on the foraging behavior of *Apis mellifera*. *PLoS ONE* **7**, e30023. (doi:10.1371/journal.pone.0030023)
36. Molet M, Chittka L, Stelzer RJ, Streit S, Raine NE. 2008 Colony nutritional status modulates worker responses to foraging recruitment pheromone in the bumblebee *Bombus terrestris*. *Behav. Ecol. Sociobiol.* **62**, 1919–1926. (doi:10.1007/s00265-008-0623-3)
37. Decourtye A, Devillers J, Aupinel P, Brun F, Bagnis C, Fourrier J, Gauthier M. 2011 Honeybee tracking with microchips: a new methodology to measure the effects of pesticides. *Ecotoxicology* **20**, 429–437. (doi:10.1007/s10646-011-0594-4)
38. Gill RJ, Raine NE. 2014 Chronic impairment of bumblebee natural foraging behaviour induced by sublethal pesticide exposure. *Funct. Ecol.* **28**, 1459–1471. (doi:10.1111/1365-2435.12292)
39. Thompson H, Coulson M, Ruddle N, Wilkins S, Harkin S. 2016 Thiamethoxam: assessing flight activity of honeybees foraging on treated oilseed rape using radio frequency identification technology. *Environ. Toxicol. Chem.* **35**, 385–393. (doi:10.1002/etc.3183)
40. Fischer J, Müller T, Spatz A-K, Greggers U, Gruenewald B, Menzel R. 2014 Neonicotinoids interfere with specific components of navigation in honeybees. *PLoS ONE* **9**, e91364. (doi:10.1371/journal.pone.0091364)

41. Naug D. 2009 Nutritional stress due to habitat loss may explain recent honeybee colony collapses. *Biol. Conserv.* **142**, 2369–2372. (doi:10.1016/j.biocon.2009.04.007)
42. Feigenbaum C, Naug D. 2010 The influence of social hunger on food distribution and its implications for disease transmission in a honeybee colony. *Insect. Soc.* **57**, 217–222. (doi:10.1007/s00040-010-0073-6)
43. Li Z *et al.* 2013 Viral infection affects sucrose responsiveness and homing ability of forager honey bees, *Apis mellifera* L. *PLoS ONE* **8**, e77354. (doi:10.1371/journal.pone.0077354)
44. Lach L, Kratz M, Baer B. 2015 Parasitized honey bees are less likely to forage and carry less pollen. *J. Invert. Pathol.* **130**, 64–71. (doi:10.1016/j.jip.2015.06.003)
45. Wolf S, McMahon DP, Lim KS, Pull CD, Clark SJ, Paxton RJ, Osborne JL. 2014 So near and yet so far: harmonic radar reveals reduced homing ability of *Nosema* infected honeybees. *PLoS ONE* **9**, e0103989. (doi:10.1371/journal.pone.0103989)
46. De Smet L, Ravoet J, de Miranda JR, Wenseleers T, Mueller MY, Moritz RFA, de Graaf DC. 2012 BeeDoctor, a versatile MLPA-based diagnostic tool for screening bee viruses. *PLoS ONE* **7**, e47953. (doi:10.1371/journal.pone.0047953)
47. Seeley TD. 2009 *The wisdom of the hive: the social physiology of honey bee colonies*. Cambridge, MA: Harvard University Press.
48. Möckel N, Gisder S, Genersch E. 2011 Horizontal transmission of deformed wing virus: pathological consequences in adult bees (*Apis mellifera*) depend on the transmission route. *J. Gen. Virol.* **92**, 370–377. (doi:10.1099/vir.0.025940-0)
49. Desai SD, Eu YJ, Whyard S, Currie RW. 2012 Reduction in deformed wing virus infection in larval and adult honey bees (*Apis mellifera* L.) by double-stranded RNA ingestion. *Insect Mol. Biol.* **21**, 446–455. (doi:10.1111/j.1365-2583.2012.01150.x)
50. Maori E, Paldi N, Shafir S, Kalev H, Tsur E, Glick E, Sela I. 2009 IAPV, a bee-affecting virus associated with colony collapse disorder can be silenced by dsRNA ingestion. *Insect Mol. Biol.* **18**, 55–60. (doi:10.1111/j.1365-2583.2009.00847.x)
51. Martin SJ, Highfield AC, Brettell L, Villalobos EM, Budge GE, Powell M, Nikaido S, Schroeder DC. 2012 Global honey bee viral landscape altered by a parasitic mite. *Science* **336**, 1304–1306. (doi:10.1126/science.1220941)
52. Mordecai GJ, Wilfert L, Martin SJ, Jones IM, Schroeder DC. 2016 Diversity in a honey bee pathogen: first report of a third master variant of the deformed wing virus quasispecies. *ISME J.* **10**, 1264–1273. (doi:10.1038/ismej.2015.178)
53. Van Geystelen A, Benaets K, de Graaf DC, Larmuseau MHD, Wenseleers T. 2015 Track-a-Forager: a program for the automated analysis of RFID tracking data to reconstruct foraging behaviour. *Insect. Soc.* **63**, 175–183. (doi:10.1007/s00040-015-0453-z)
54. Winston ML. 1987 *The biology of the honey bee*. Cambridge, MA: Harvard University Press.
55. Tenczar P, Lutz CC, Rao VD, Goldenfeld N, Robinson GE. 2014 Automated monitoring reveals extreme interindividual variation and plasticity in honeybee foraging activity levels. *Anim. Behav.* **95**, 41–48. (doi:10.1016/j.anbehav.2014.06.006)
56. Tofilski A. 2009 Shorter-lived workers start foraging earlier. *Insect. Soc.* **56**, 359–366. (doi:10.1007/s00040-009-0031-3)
57. Goblirsch M, Huang ZY, Spivak M. 2013 Physiological and behavioral changes in honey bees (*Apis mellifera*) induced by *Nosema ceranae* infection. *PLoS ONE* **8**, e0058165. (doi:10.1371/journal.pone.0058165)
58. Dussaubat C, Maisonnasse A, Crauser D, Beslay D, Costagliola G, Soubeyrand S, Kretzchmar A, Le Conte Y. 2013 Flight behavior and pheromone changes associated to *Nosema ceranae* infection of honey bee workers (*Apis mellifera*) in field conditions. *J. Invert. Pathol.* **113**, 42–51. (doi:10.1016/j.jip.2013.01.002)
59. Bailey L, Fernando EFW. 1972 Effects of sacbrood virus on adult honey-bees. *Ann. Appl. Biol.* **72**, 27–35. (doi:10.1111/j.1744-7348.1972.tb01268.x)
60. Downey DL, Higo TT, Winston ML. 2000 Single and dual parasitic mite infestations on the honey bee, *Apis mellifera* L. *Insect. Soc.* **47**, 171–176. (doi:10.1007/pl00001697)
61. Janmaat AF, Winston ML. 2000 The influence of pollen storage area and *Varroa jacobsoni* Oudemans parasitism on temporal caste structure in honey bees (*Apis mellifera* L.). *Insect. Soc.* **47**, 177–182. (doi:10.1007/pl00001698)
62. Cardoen D, Ernst UR, Boerjan B, Bogaerts A, Formesyn E, de Graaf DC, Wenseleers T, Schoofs L, Verleyen P. 2012 Worker honeybee sterility: a proteomic analysis of suppressed ovary activation. *J. Proteom Res.* **11**, 2838–2850. (doi:10.1021/pr201222s)
63. Perry CJ, Søvik E, Myerscough MR, Barron AB. 2015 Rapid behavioral maturation accelerates failure of stressed honey bee colonies. *Proc. Natl Acad. Sci. USA* **112**, 3427–3432. (doi:10.1073/pnas.1422089112)
64. Khoury DS, Myerscough MR, Barron AB. 2011 A quantitative model of honey bee colony population dynamics. *PLoS ONE* **6**, e18491. (doi:10.1371/journal.pone.0018491)
65. Woyciechowski M, Moroń D. 2009 Life expectancy and onset of foraging in the honeybee (*Apis mellifera*). *Insect. Soc.* **56**, 193–201. (doi:10.1007/s00040-009-0012-6)
66. Schmickl T, Crailsheim K. 2004 Inner nest homeostasis in a changing environment with special emphasis on honey bee brood nursing and pollen supply. *Apidologie* **35**, 249–263. (doi:10.1051/apido:2004019)
67. Yue C, Schroder M, Gisder S, Genersch E. 2007 Vertical-transmission routes for deformed wing virus of honeybees (*Apis mellifera*). *J. Gen. Virol.* **88**, 2329–2336. (doi:10.1099/vir.0.83101-0)
68. de Miranda JR, Fries I. 2008 Venereal and vertical transmission of deformed wing virus in honeybees (*Apis mellifera* L.). *J. Invert. Pathol.* **98**, 184–189. (doi:10.1016/j.jip.2008.02.004)
69. Chen YP, Higgins JA, Feldlaufer MF. 2005 Quantitative real-time reverse transcription-PCR analysis of deformed wing virus infection in the honeybee (*Apis mellifera* L.). *Appl. Environ. Microbiol.* **71**, 436–441. (doi:10.1128/aem.71.1.436-441.2005)
70. Tentcheva D, Gauthier L, Bagny L, Fievet J, Dainat B, Cousserans F, Colin ME, Bergoin M. 2006 Comparative analysis of deformed wing virus (DWV) RNA in *Apis mellifera* and *Varroa destructor*. *Apidologie* **37**, 41–50. (doi:10.1051/apido:2005057)
71. Yue C, Genersch E. 2005 RT-PCR analysis of deformed wing virus in honeybees (*Apis mellifera*) and mites (*Varroa destructor*). *J. Gen. Virol.* **86**, 3419–3424. (doi:10.1099/vir.0.81401-0)
72. Fujiyuki T, Matsuzaka E, Nakaoka T, Takeuchi H, Wakamoto A, Ohka S, Sekimizu K, Nomoto A, Kubo T. 2009 Distribution of kakugo virus and its effects on the gene expression profile in the brain of the worker honeybee *Apis mellifera* L. *J. Virol.* **83**, 11 560–11 568. (doi:10.1128/jvi.00519-09)
73. Fujiyuki T, Takeuchi H, Ono M, Ohka S, Sasaki T, Nomoto A, Kubo T. 2004 Novel insect picorna-like virus identified in the brains of aggressive worker honeybees. *J. Virol.* **78**, 1093–1100. (doi:10.1128/jvi.78.3.1093-1100.2004)
74. Fujiyuki T, Takeuchi H, Ono M, Ohka S, Sasaki T, Nomoto A, Kubo T. 2005 Kakugo virus from brains of aggressive worker honeybees. *Adv. Virus Res.* **65**, 1–27. (doi:10.1016/s0065-3527(05)65001-4)
75. Shen MQ, Yang XL, Cox-Foster D, Cui LW. 2005 The role of varroa mites in infections of Kashmir bee virus (KBV) and deformed wing virus (DWV) in honey bees. *Virology* **342**, 141–149. (doi:10.1016/j.virol.2005.07.012)
76. Mayack C, Naug D. 2009 Energetic stress in the honeybee *Apis mellifer*a from *Nosema ceranae* infection. *J. Invert. Pathol.* **100**, 185–188. (doi:10.1016/j.jip.2008.12.001)
77. Naug D, Gibbs A. 2009 Behavioral changes mediated by hunger in honeybees infected with *Nosema ceranae*. *Apidologie* **40**, 595–599. (doi:10.1051/apido/2009039)
78. Hunter W *et al.* 2010 Large-scale field application of RNAi technology reducing Israeli acute paralysis virus disease in honey bees *(Apis mellifera*, Hymenoptera: Apidae). *PLoS Pathog.* **6**, e1001160. (doi:10.1371/journal.ppat.1001160)

SUPPLEMENTAL METHODS

Controlled infection

In order to obtain two groups of adult age-matched bees that were or were not infected with DWV, we first prepared two treatment solutions, which upon injection were designed to increase or keep down DWV virus titers. This required preparing lysates of honeybees that were infected with DWV but none of the other common honeybee viruses, as well as of honeybees that were confirmed to be virus-free. To this end, we produced a lysate of a pool of 10 bees, which based on multiplex ligation-dependent probe amplification (MLPA) analysis and the use of specific PCR primers were confirmed to be entirely virus and pathogen-free or infected only with DWV (see below) [1]. These pools of bees were obtained following an initial screening of 10 individuals each from a set of 10 *A. mellifera carnica* colonies available in our apiary in Leuven, as well as based on the screening of individuals with overt symptoms of DWV-infection (i.e. displaying crippled wings). This screening also allowed the selection of the previously mentioned virus and pathogen-free donor colony. DWV lysate and virus-free control lysate were extracted from the selected set of DWV-infected and virus-free bees by homogenizing the mass equivalent of five bees (around 500 mg) of the selected samples (stored at -80°C) after immersion in liquid nitrogen, and mixing the homogenate with 5 ml phosphate-buffered saline (PBS, pH 7.4). Subsequently, the samples were centrifuged at 3000 r.p.m. for 30 min at 4°C and the supernatant was stored in aliquots at -80°C for future use [2].

The artificial inoculation with a DWV lysate that was used in this study has previously been shown to be effective in short-term, individual-based setups [2]. In our present study, however, the bees stayed in close contact with each other in the observation hive, throughout their adult lives, so that the virus could still be transmitted horizontally during the experiment. To try to mitigate the effects of horizontal transmission from the DWV infected to the group of uninfected bees [3] we therefore opted to also add a double-stranded RNA treatment to our DWV-negative lysate [4, 5]. In particular, DWV-specific dsRNA was added to the control lysate to try to keep these bees DWV-free and prevent DWV cross-infection inside the host colony, whereas control GFP-dsRNA was added to the DWV lysate to control for the possible effects of foreign dsRNA injection on honeybee physiology. DWV and GFP-dsRNA were provided by Beeologics (Israel) and were dissolved in nuclease free water at 3 mg/ml. Prior to injection, both DWV and control lysates were diluted 1:1000 (cf. Iqbal and Mueller [2] in insect saline buffer (ISB, containing 150 mM NaCl, 10 mM KCl, 4 mM CaCl2, 2 mM MgCl2, and 10 mM HEPES; pH 7.0), after which dsRNA was added at a dosage of 5 µg per bee to obtain the final treatment solutions. Given that bees with overt symptoms have been reported to contain between 1.8×10^{10} and 6.9×10^{11} DWV genome equivalents per bee [6], we estimate that our protocol resulted in the injection of between 1.2×10^{4} and 4.6×10^{5} DWV copies per bee, which is well within the infection loads reported for bees with covert infections (1.4×10^{3} – 2.4×10^{9} copies per bee, [6]). Hence, our DWV inoculation treatment adequately mimicked covert DWV infection levels.

RFID data analysis

Raw RFID tracking data were analyzed with the Track-A-Forager Java application, which filters out rapid-succession scans of the same scanner, labels ingoing and outgoing flights by tagged workers, and corrects occasional errors in the data, including the possible occurrence of missed scans [7]. The

setup options were adjusted based on the foraging type and entrance/exit system that was used in the experimental setup, namely 'natural foraging' and 'joined two scanners'. In the data filtering phase, the default time constraint settings were applied to filter out rapid-succession scans of the same scanner, determine 'IN' and 'OUT' events of tagged foragers and reconstruct forager trips. The output of the application generated the complete list of all the scans of each RFID tag at each scanner with their corresponding time stamp, the reconstructed trips per tag with their corresponding time stamp and the durations of reconstructed trips with the age of each individual at the time of the trip. The resulting data was then joined with the transponder information database consisting of the unique RFID tag code, the treatment, colony and date of introduction of each individual.

To test for differences in the proportion of workers of each treatment group that survived to foraging age, we used a binomial GLM in which 'colony', 'treatment' and their interaction effect were included as fixed factors. ANOVAs with 'colony', 'treatment' and their interaction effect included as fixed factors were used to assay the difference in the age at onset of foraging, life expectancy and activity span. To assess the number of reconstructed trips made by individuals of each group we used a quasipoisson generalized linear model (GLM) with 'colony', 'treatment' and their interaction effects included as fixed factors and 'activity span' included as a covariate. Finally, the duration of the reconstructed trips was analysed with a gamma distributed generalized linear mixed model (GLMM) with the unique RFID tag specified as a random factor and 'colony' and 'treatment' plus their interaction effect specified as fixed factors. All statistical analyses were carried out using *R* v. 3.2.2 and the *lme4* 1.1-9 (for generalized mixed models) and *effects* 2.3-0 packages (to produce effect plots).

MLPA-based screening for viral infections and treatment validation

Multiplex ligation-dependent probe amplification (MLPA) was used to identify virus-free donor colony candidates, select honeybee samples that were best suited for preparing the lysates for the two treatments and to validate the effects of these treatments. MLPA was performed as described in De Smet *et al.* [1], using MLPA probes and RT-primers designed for six virus targets, covering the ten most common honeybee viruses: chronic bee paralysis virus (CBPV), deformed wing virus (DWV, including A, B, i.e. VDV-1, and C type virus, [8]), acute bee paralysis virus (ABPV)/Kashmir bee virus (KBV)/Israeli acute paralysis virus (IAPV), black queen cell virus (BQCV), slow bee paralysis virus (SBPV) and sacbrood virus (SBV) (Table S1). The amplified MLPA products were analyzed by electrophoresis on a 4% high resolution agarose gel with a specific MLPA ladder.

Table S1. Primers and half-probes used for detecting the positive strand of different honeybee viruses and virus species complexes through RT-MLPA. Adapted from De Smet *et al. [1]*.

VIRUS	FUNCTION	SEQUENCE (5'-3')	SIZE (bp)
CBPV	(-)cDNA	GCCCCGATCATATAAGCAAA	88
	(+)MLPA-LPO	gggttccctaagggttggaCCGTAGCTGTTTCTGCTGCGGT	
	(+)MLPA-RPO	P-ACTCAGCTCAGCTCGACGCTAGAtctagattggatcttgctggcac	
DWV	(-)cDNA	TCACATTGATCCCAATAATCAGA	95
(A+B+C type)	(+)MLPA-LPO	gggttccctaagggttggaTGACCGATTCTTTATGAGCGAGCTCT	
	(+)MLPA-RPO	P-TACGTGCGAGTCGTACTCCTGTGACAtctagattggatcttgctggcac	
ABPV	(-)cDNA (ABPV)	CAATGTGGTCAATGAGTACGG	104
KBV	(-)cDNA (KBV&IAPV)	TCAATGTTGTCAATGAGAACGG	
IAPV	(+)MLPA-LPO	gggttccctaagggttggaCTCACTTCATCGGCTCGGAGCATGGATGAT	
	(+)MLPA-RPO	P-ACGCACAGTATTATTCAGTTTTTACAACGCCCtctagattggatcttgctggcac	
BQCV	(-)cDNA	CGGGCCTCGGATAATTAGA	122
	(+)MLPA-LPO	gggttccctaagggttggaCTTCATGTTGGAGACCAGGTTTGTTTGCCGACTTACGGAA	
	(+)MLPA-RPO	P-TGTCGTTAAACTCTAGGCTTTCCGGATGGCTTCTTCATGGtctagattggatcttgctggcac	
SBPV	(-)cDNA	CGCAAACACGACGAATTTTA	131
	(+)MLPA-LPO	gggttccctaagggttggaCGTTCAATGGTCGAGATAGAAGCCACAGTAGAAGTATTACGCGCT	
	(+)MLPA-RPO	P-TCTTGTGTTTTGGCTTATGGGCGTGGGCCTGATCTTCATTCAGCtctagattggatcttgctggcac	
SBV	(-)cDNA	TGGACATTTCGGTGTAGTGG	140
	(+)MLPA-LPO	gggttccctaagggttggaCGTTGATCCAATGGTCAGTGGACTCTTATACCGATTTGTTTAATGGTTGG	
	(+)MLPA-RPO	P-GTTTCTGGTATGTTTGTTGACAAGAACGTCCACCTTCAGCCATTCAGCtctagattggatcttgctggcac	

Samples to derive DWV lysates from were selected for further use if they tested DWV positive but negative for the other tested viruses. To identify suitable honeybee samples, RNA was first extracted from collected bees with and without deformed wings and screened them for pathogens using MLPA. 20 sets of ten adult bees from different colonies without and where possible with overt symptoms of DWV were pooled and whole bodies were homogenized in liquid nitrogen. 80-100 mg of the homogenized tissues were then mixed with 1 ml Qiazol reagent in MagNA Lyser Green Beads sample tubes (Roche, Belgium) for RNA-extraction with the RNA lipid tissue mini kit (Qiagen, Germany). Leftover tissue was kept and stored at -80°C for lysate preparation. Pooled tissues in Qiazol were further disrupted and homogenized on a MagNa Lyser Instrument (Roche, Belgium) for 30 sec at 6500 Hz. The total RNA was isolated according to the kit's instructions and eluted in a final volume of 50 µl. The quality and concentrations of RNA was checked using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Belgium).

To validate the effects of the two treatment solutions, 150 additional eclosed workers were injected with each treatment solution, paint-marked and introduced into a fourth observation hive without the RFID setup. Every four days, 20 individuals of each treatment were sampled from this colony and frozen in liquid nitrogen for storage at -80°C. Subsequently, RNA extraction and MLPA analysis was carried out as described above. We should note that this method produces binary data and scores bees as containing DWV titers that are above or below the MLPA detection threshold. Several quantitative qPCR-based approaches were also tried, but unfortunately failed – likely due to a mismatch in the primer sites.

Confirmation of disease-free status of donor colony using specific PCR primers

To test that our DWV-free donor colony identified before was also free of most other common honeybee pathogens, we collected three sets of ten bees each, which were each homogenised in 5 ml PBS in MagNA Lyser Green Beads sample tubes (Roche, Belgium) on a MagNa Lyser (30 sex at

6500 Hz), extracted their DNA from 120 µl supernatant using the DNeasy Blood & Tissue Kit (Qiagen), and used the specific PCR primer sets given in Ravoet *et al.* [9, 10] to detect the possible presence of for the microsporidian parasites *Nosema apis* and *N. ceranae*, the fungal parasite *Ascosphaera*, the Mollicute pathogen *Spiroplasma* spp. and the Trypanosomatid parasites *Lotmaria passim* and *Crithidia mellificae*. None of the PCR reactions resulted in a PCR product of the expected size, thereby demonstrating that our DWV-free donor colony was not only free of DWV, but that it was also free of most other major known honeybee pathogens (although evidently we cannot exclude their presence at a low rate in a small subset of the workers). We should note that the presence of pathogens in any of the host colonies was not determined, as with *n*=3 host colonies, the statistical power to relate the presence of particular pathogens to variation in the impact of DWV would have been too low, and that such differences would also have been intrinsically confounded with genetic and environmental differences between those colonies.

Characterization of experimental inocula via ultra-deep sequencing

Five bees that were inoculated with DWV lysate, collected 12 days post injection, were subjected to ultra-deep sequencing to determine the DWV strain type the bees were injected with as well as to confirm the MLPA and PCR-based results showing that the bees were free of any other major known honeybee pathogens. For these analyses, total RNA was conducted as described above with an additional on-column DNase digestion with the RNase-free DNase set (Qiagen, Germany) to remove DNA contamination. The quality and concentration of the RNA samples was assessed using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Belgium). Subsequently, the libraries were subjected to 90 bp pair-end RNA sequencing on the Illumina Hiseq2000 platform at BGI, using random hexamer primed cDNA synthesis and a 200 bp short insert library. Adaptors, contamination and low-quality reads were removed by BGI and quality control was performed using FastQC [11]. This produced between between 9 and 10 million filtered, high-quality reads per sample, and a total of 88 936 091 reads from our DWV inoculated bees.

To determine that only a single strain of DWV was present in our inoculated bees, and that no other major pathogens were present, we used Bowtie v. 2.2.6 [12] to align the pooled 88.9 million reads to the reference genome sequences of a set of major honeybee pathogens, which included deformed wing virus types A, B and C (accession nrs. NC_004830.2, AY251269.2 and CEND01000001), acute bee paralysis virus (NC_002548.1), aphid lethal paralysis virus (NC_004365.1), black queen cell virus (NC_003784.1), chronic bee paralysis virus-1 (NC_010711.1), chronic bee paralysis virus-2 (NC_010712.1), Israeli acute paralysis virus (NC_009025.1), Kashmir bee virus (NC_004807.1), sacbrood virus (NC_002066.1), the Trypanosomatid parasite *Lotmaria passim* (GCA_000635995.1), the microsporidian parasites *Nosema apis* (GCA_000447185.1) and *Nosema ceranae* (GCA_000182985.1) and the Mollicute pathogen *Spiroplasma apis* (GCA_000500935.1) and *Spiroplasma melliferum* (GCA_000236085.3) as well as the *Apis mellifera* genome, in order to filter out host RNA (using the latest version 4.5 NCBI assembly). Out of the obtained 88.9 million sequence fragments, 78% (69.2 million) did not map onto the host genome and out of these non-host fragments, 73% (50.7 million) mapped onto one of the included pathogens. Out of all the pathogen-mapped fragments, 99.91% mapped to deformed wing virus type B, whereas the remaining reads (0.09%) mapped onto DWV type A. Nevertheless, the latter had such low counts that they were likely caused by either sequence mismatches between our inoculate and the reference DWV type B strain,

sequencing error, or mapping mistakes. In addition, no reads mapped onto any of the other pathogens, thereby confirming our MLPA and PCR results that the donor colony was indeed free of any of the major known honeybee pathogens.

The interpretation that only a single strain of DWV was present in the inoculate was confirmed based on a de-novo assembly of all the fragments that mapped onto any of the RNA viruses, obtained using the Vicuna viral assembler version 1.3, which was developed to characterize possibly heterogeneous virus populations [13]. As expected if only a single DWV strain was present, the Vicuna assembly returned only one single 5941 nt long contig that mapped with very high fidelity (99.0% high sequence similarity) to the 5' end of the DWV type B reference strain. As coverage across the complete DWV genome was highly variable, however, the Vicuna pipeline was not able to recover the full DWV genome. To obtain the full genome sequence, and given the very high sequence similarity of the obtained fragment with the type B reference strain, we therefore used Bowtie v. 2.2.6 [12] instead to map all virus-mapping reads to the DWV type B reference genome (using option –very-sensitive-local, to allow for a maximum number of sequence mismatches), after which *samtools mpileup* and *bcftools call* was used to obtain the consensus sequence of the position-sorted BAM alignment. The resulting full DWV genome of our inoculated strain (accession number KX783225) was 10112 nts long and contained a 2893 AA long polyprotein, which had sequence similarities of 99.28% at the nucleotide level (73/10112 nts substituted) and 99.76% at the AA level (7/2893 AAs substituted) with the DWV type B reference strain (accession number AY251269.2), as well as an identical length, but greater sequence divergence with reference types A or C (Fig. S1). There was no evidence of our strain being a recombinant between strain types [14]. The DWV type B strain was formerly known as *Varroa destructor virus*-1, but is now classified into the deformed wing virus complex as one of three master variants [8], and has recently been found to be an emergent, more virulent strain of the DWV virus [14], which currently appears to be the most common deformed wing virus strain in Britain. As our inoculate was prepared from bees with overt DWV symptoms from a randomly selected hive, our results suggest that this strain is now also common in Continental Europe. Overall per-nucleotide coverage, calculated using *bedtools coverage*, was 902k on average, with a range of 19 to 15 million. Coverage, together with the tentative positions of polyprotein cleavage sites and their resulting products and other genomic features, annotated following Lanzi *et al.* [15], and the overall structure of the virus, based on what is known from other Picornaviridae, are shown in Fig. S2.

Treatment validation results

MLPA analysis of 4-daily sets of samples of 20 individuals per treatment shows that, as expected, there was a strong main effect of treatment on DWV infection rates (binomial GLM, $p = 0.0006$, $z = 3.4$) (Fig. 2), but that DWV infection rates also increased and that the difference in infection rates between the two treatment groups decreased over the course of the experiment (main effect of log(x+1) transformed duration after introduction into the host colony: $p = 3.4E\text{-}6$, $z = 4.6$; interaction effect of log(time+1) and treatment: $p = 0.003$, $z = -3.0$). Based on the calculated 95% confidence limits, however, the difference in infection rate between the two treatments remained statistically significant until the 12th day of the experiment (Fig. 2).

These treatment results imply that in the beginning of our experiment, infection rates strongly differed between the two treatment groups, but that the control workers also may have horizontally

acquired new DWV infections at the later stages of our experiment or perhaps fed on DWV infected pollen or nectar leading to a sizeable virus load in the gut. It is important to note, however, that MLPA is a qualitative technique that only signals the presence or absence of virus particles in the analyzed samples but does not yield any information on the virus titers. Hence, it is possible, and indeed likely, that the DWV titers in control bees that became infected over the later stages of our experiment were still significantly lower than those in the group which were experimentally infected with DWV, even if we did not succeed in formally demonstrating this using a qPCR-based approach. Nevertheless, the fact that we find statistically significant behavioural effects between our two treatment groups up until late into our experiment, when the proportion of DWV positive bees in the control group approached that in the DWV inoculated group, only strengthens our conclusion that DWV has strongly deleterious effects on honeybee foraging behaviour. That is, the total effect could in fact have been even greater if the control group had remained completely uninfected for the full duration of our experiment, and our estimates of the effects on foraging behaviour and mortality should therefore be interpreted as minimum estimates.

That the dsRNA RNAi treatment [4] in the control bees was not fully effective at keeping bees virus-free may have several causes. First and foremost, our setup combined a mix of uninfected and artificially inoculated workers, and this led to an unusually high potential for horizontal transmission inside the colony. Second, in our experiment we only treated the bees one single time, using a single dsRNA injection, to ensure a standardized approach with equal amounts of dsRNA provided to each bee. In real-life applications, by contrast, dsRNA could be administered orally over extended periods in the food [4, 5, 16], and colonies are treated pro-actively, so that colonies may be able to fight off the virus at a much earlier stage than in our experiments. Hence, the potential of dsRNA treatments to combat DWV infections and help to mitigate its associated costs still deserves further study.

References

[1] De Smet, L., Ravoet, J., de Miranda, J.R., Wenseleers, T., Mueller, M.Y., Moritz, R.F.A. & de Graaf, D.C. 2012 BeeDoctor, a versatile MLPA-based diagnostic tool for screening bee viruses. *PLoS ONE* **7**, e47953. (doi:10.1371/journal.pone.0047953).

[2] Iqbal, J. & Mueller, U. 2007 Virus infection causes specific learning deficits in honeybee foragers. *Proc R Soc Lond B* **274**, 1517-1521. (doi:10.1098/rspb.2007.0022).

[3] Möckel, N., Gisder, S. & Genersch, E. 2011 Horizontal transmission of deformed wing virus: pathological consequences in adult bees (*Apis mellifera*) depend on the transmission route. *J Gen Virol* **92**, 370-377. (doi:10.1099/vir.0.025940-0).

[4] Desai, S.D., Eu, Y.J., Whyard, S. & Currie, R.W. 2012 Reduction in deformed wing virus infection in larval and adult honey bees (*Apis mellifera* L.) by double-stranded RNA ingestion. *Insect Mol Biol* **21**, 446-455. (doi:10.1111/j.1365-2583.2012.01150.x).

[5] Maori, E., Paldi, N., Shafir, S., Kalev, H., Tsur, E., Glick, E. & Sela, I. 2009 IAPV, a bee-affecting virus associated with colony collapse disorder can be silenced by dsRNA ingestion. *Insect Mol Biol* **18**, 55-60. (doi:10.1111/j.1365-2583.2009.00847.x).

[6] Martin, S.J., Highfield, A.C., Brettell, L., Villalobos, E.M., Budge, G.E., Powell, M., Nikaido, S. & Schroeder, D.C. 2012 Global honey bee viral landscape altered by a parasitic mite. *Science* **336**, 1304-1306. (doi:10.1126/science.1220941).

[7] Van Geystelen, A., Benaets, K., de Graaf, D.C., Larmuseau, M.H.D. & Wenseleers, T. 2015 Track-a-Forager: a program for the automated analysis of RFID tracking data to reconstruct foraging behaviour. *Insect Soc* **63**, 175-183. (doi:10.1007/s00040-015-0453-z).

[8] Mordecai, G.J., Wilfert, L., Martin, S.J., Jones, I.M. & Schroeder, D.C. 2016 Diversity in a honey bee pathogen: first report of a third master variant of the Deformed Wing Virus quasispecies. *ISME J* **10**, 1264-1273. (doi:10.1038/ismej.2015.178).
[9] Ravoet, J., De Smet, L., Meeus, I., Smagghe, G., Wenseleers, T. & de Graaf, D.C. 2014 Widespread occurrence of honey bee pathogens in solitary bees. *J Invert Pathol* **122**, 55-58. (doi:10.1016/j.jip.2014.08.007).
[10] Ravoet, J., Schwarz, R.S., Descamps, T., Yañez, O., Tozkar, C.O., Martin-Hernandez, R., Bartolomé, C., De Smet, L., Higes, M., Wenseleers, T., et al. 2015 Differential diagnosis of the honey bee trypanosomatids *Crithidia mellificae* and *Lotmaria passim*. *J Inv Pathol* **130**, 21-27. (doi:10.1016/j.jip.2015.06.007).
[11] Andrew, S. 2010 FastQC: A quality control tool for high throughput sequence data. Version 0.11.5. Available at http://www.bioinformatics.babraham.ac.uk/projects/fastqc/
[12] Langmead, B. & Salzberg, S.L. 2012 Fast gapped-read alignment with Bowtie 2. *Nat Meth* **9**, 357-U354. (doi:10.1038/nmeth.1923).
[13] Yang, X., Charlebois, P., Gnerre, S., Coole, M.G., Lennon, N.J., Levin, J.Z., Qu, J., Ryan, E.M., Zody, M.C. & Henn, M.R. 2012 De novo assembly of highly diverse viral populations. *BMC Genom* **13**, 1-13. (doi:10.1186/1471-2164-13-475).
[14] McMahon, D.P., Natsopoulou, M.E., Doublet, V., Fürst, M., Weging, S., Brown, M.J.F., Gogol-Döring, A. & Paxton, R.J. 2016 Elevated virulence of an emerging viral genotype as a driver of honeybee loss. *Proc R Soc Lond B* **283**, in press. (doi:10.1098/rspb.2016.0811).
[15] Lanzi, G., De Miranda, J.R., Boniotti, M.B., Cameron, C.E., Lavazza, A., Capucci, L., Camazine, S.M. & Rossi, C. 2006 Molecular and biological characterization of deformed wing virus of honeybees (*Apis mellifera* L.). *J Virol* **80**, 4998-5009. (doi:10.1128/JVI.80.10.4998-5009.2006).
[16] Hunter, W., Ellis, J., Vanengelsdorp, D., Hayes, J., Westervelt, D., Glick, E., Williams, M., Sela, I., Maori, E. & Pettis, J. 2010 Large-scale field application of RNAi technology reducing Israeli acute paralysis virus disease in honey bees *(Apis mellifera*, Hymenoptera: Apidae). *PLoS Pathog* **6**, e1001160. (doi:10.1371/journal.ppat.1001160).

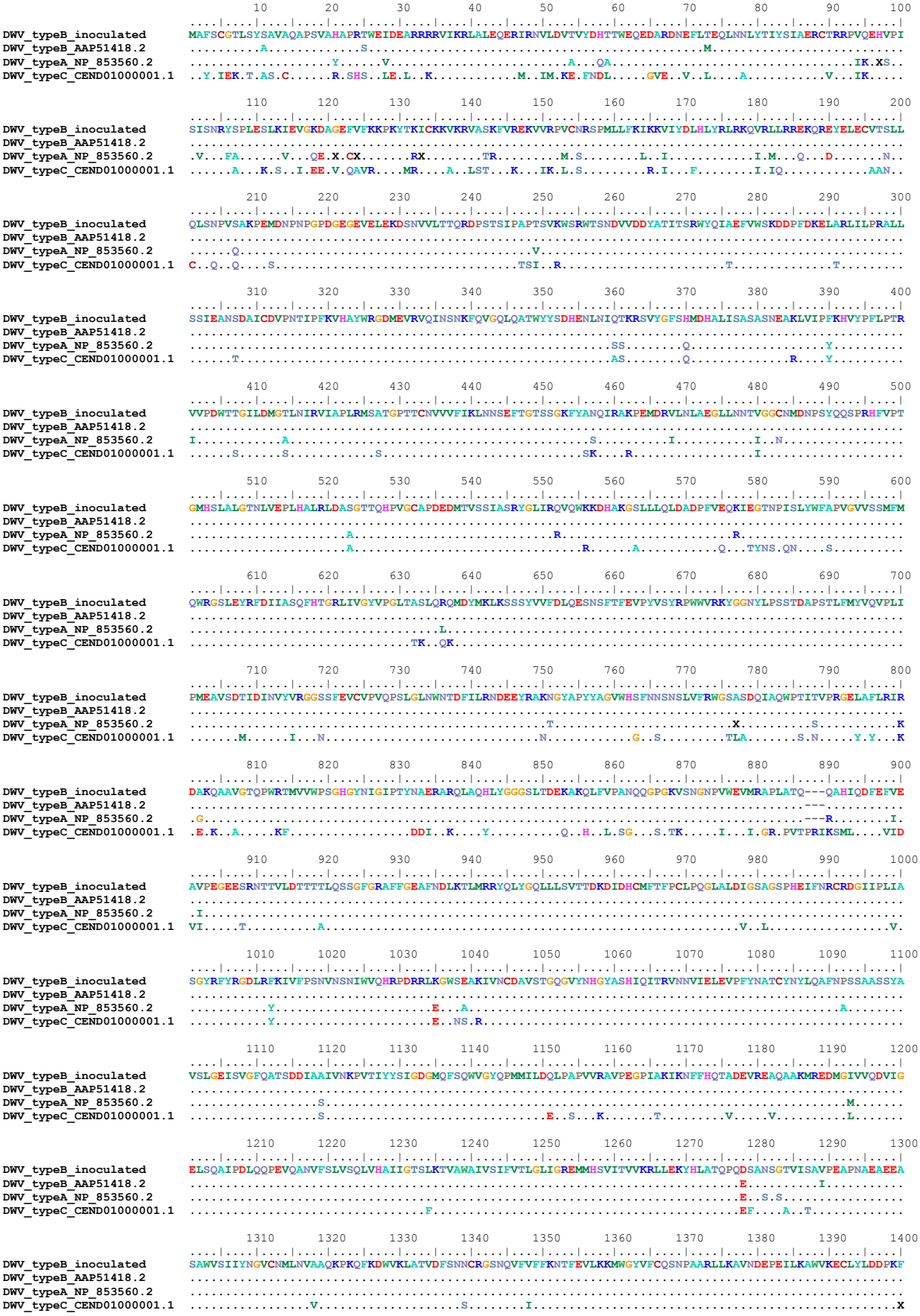
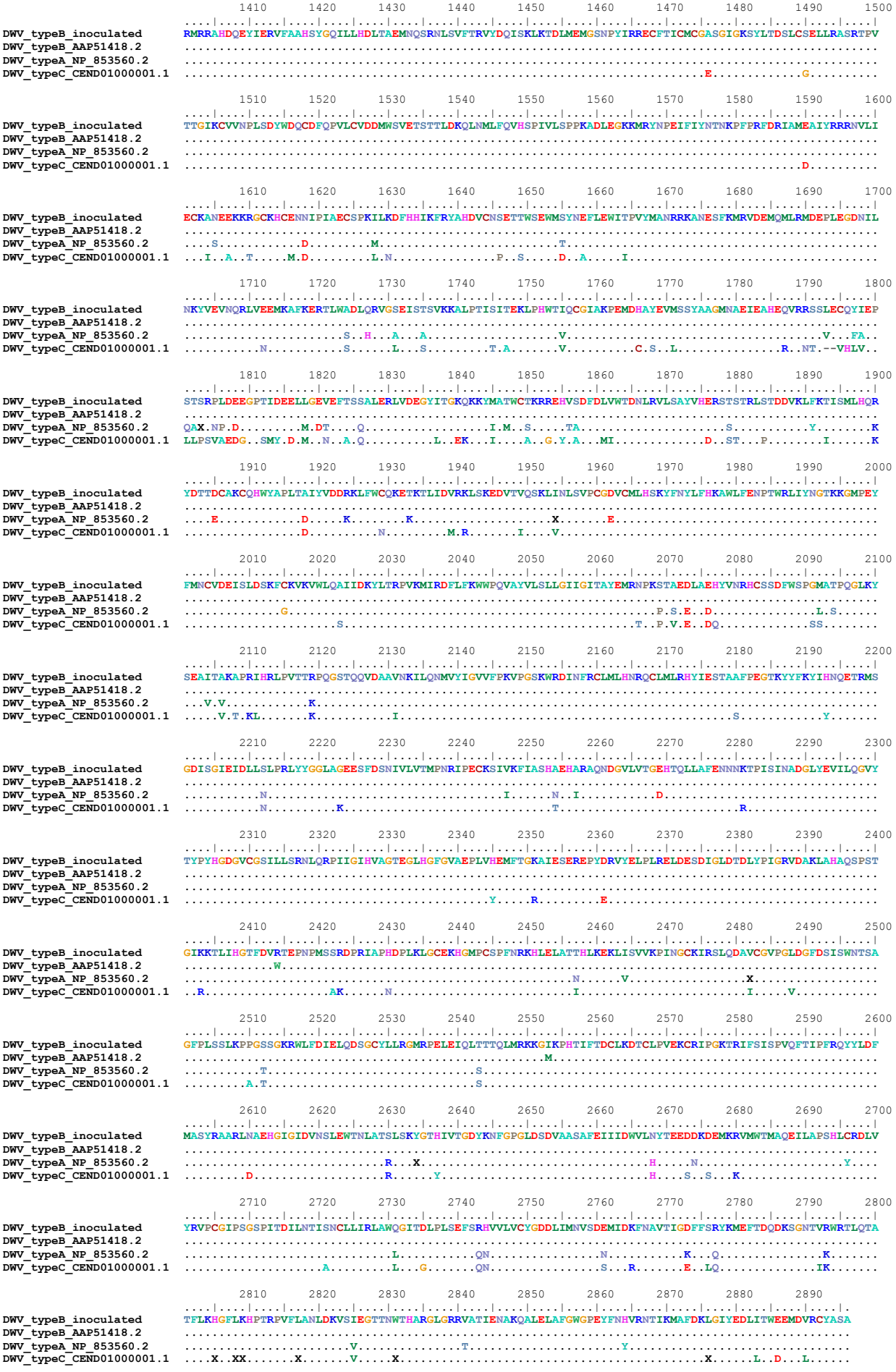


Fig. S1. Amino-acid sequence of the polyprotein encoded by the DWV type B strain that we used to inoculate our bees and comparison to the reference DWV master variants of type B, A and C.



(Fig. S1 continued)

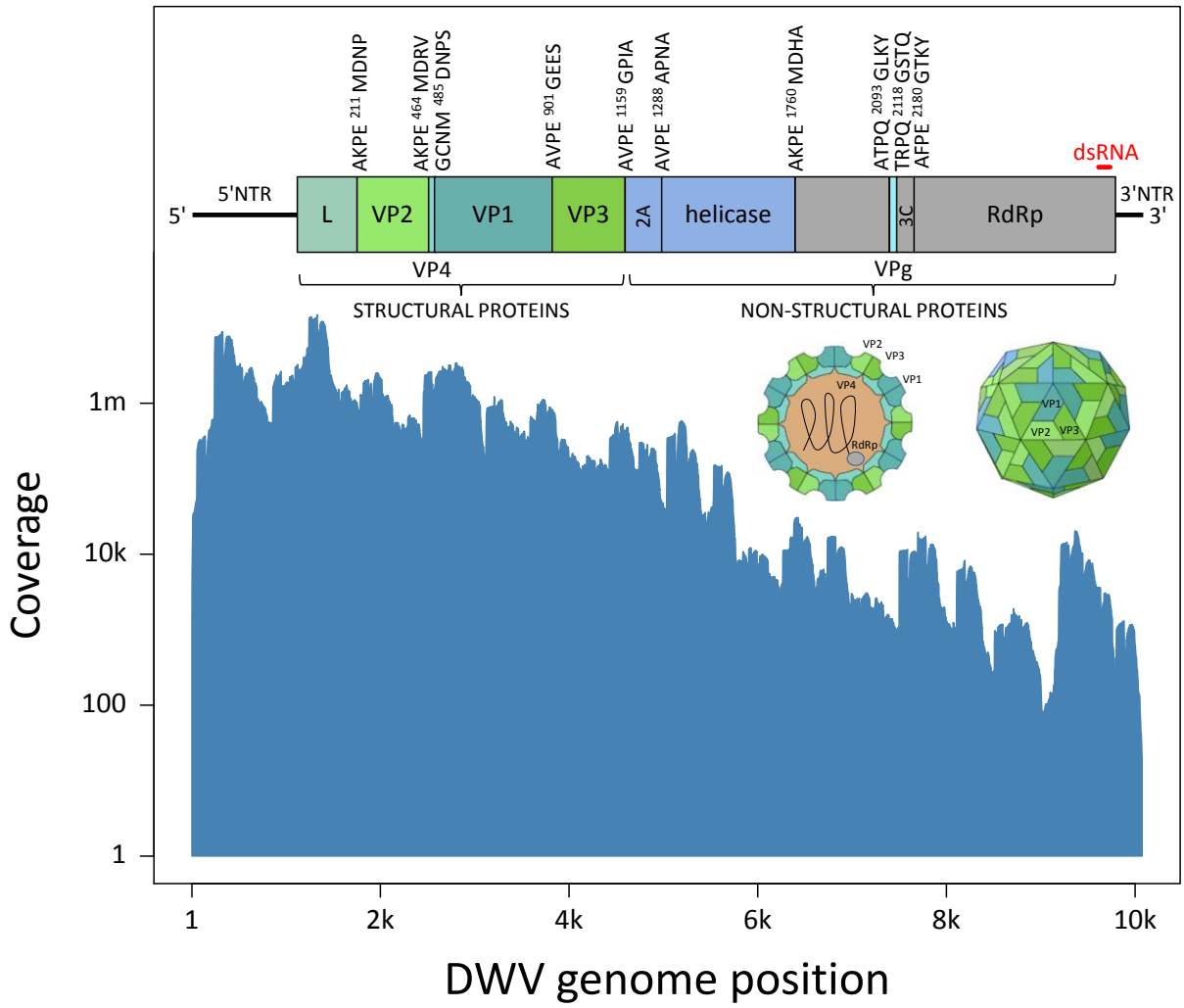


Fig. S2. Fragment coverage over the DWV genome reached in our Bowtie mapping shown together with inferred cleavage sites in the virus's polyprotein and the resulting products, and other genomic features (5'NTR and 3'NTR = non-translated regions, L = L-protein, VP1 to VP4 = capsid proteins, 2A = 2A-like protease site, VPg = VPg protein, 3C = 3C-protease, RdRp = RNA-dependent RNA polymerase, dsRNA = position of dsRNA fragment used in our RNA interference protocol [4], cf. [15]).