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# Pathogen shifts in a honeybee predator following the arrival of the *Varroa* mite

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Emerging infectious diseases (EIDs) are a global threat to honeybees, and spillover from managed bees threaten wider insect populations. Deformed wing virus (DWV), a widespread virus that has become emergent in conjunction with the spread of the mite *Varroa destructor*, is thought to be partly responsible for global colony losses. The arrival of *Varroa* in honeybee populations causes a dramatic loss of viral genotypic diversity, favouring a few virulent strains. Here, we investigate DWV spillover in an invasive Hawaiian population of the wasp, *Vespula pensylvanica*, a honeybee predator and honey-raider. We show that *Vespula* underwent a parallel loss in DWV variant diversity upon the arrival of *Varroa*, despite the mite being a honeybee specialist. The observed shift in *Vespula* DWV and the variant-sharing between *Vespula* and *Apis* suggest that these wasps can acquire DWV directly or indirectly from honeybees. *Apis* prey items collected from *Vespula* foragers were positive for DWV, indicating predation is a possible route of transmission. We also sought cascading effects of DWV shifts in a broader *Vespula* pathogen community. We identified concurrent changes in a suite of additional pathogens, as well as shifts in the associations between these pathogens in *Vespula*. These findings reveal how hidden effects of the *Varroa* mite can, via spillover, transform the composition of pathogens in interacting species, with potential knock-on effects for entire pathogen communities.

## 1. Introduction

The movement of agricultural crops and animals, as well as the inadvertent introduction and establishment of non-native species, can drive the spread of emerging infectious diseases (EIDs), defined as diseases that involve a novel infectious agent or host, or are rapidly expanding in incidence or range [1]. Such EIDs can threaten economically important species, as well as the biodiversity of native communities interacting with managed populations. The western honeybee (*Apis mellifera*), an essential global source of pollination services, has recently experienced numerous EIDs affecting populations worldwide [2–6]. Several viral and fungal EIDs have spread rapidly, with serious consequences for apiculture. Many ‘honeybee’ pathogens, particularly viruses, infect a variety of other insects [7–9], and such EIDs have been recently recognized as a threat to native insect species [10–13]. Spillover of novel pathogens or pathogen strains from managed bee populations may be exposing naive species to virulent diseases against which they are poorly defended. Furthermore, managed and feral non-native honeybee populations can act as reservoirs for pathogens, increasing the exposure of native populations when these populations interact.

One such EID with the potential for spillover is deformed wing virus (DWV; Iflaviridae), a single-strand, positive-sense RNA virus that is widespread in honeybee populations globally and composed of several variants [4,14]. It also infects bumblebees [12,15] and has been detected in many other

insects [8,16]. In honeybee populations lacking the *Varroa* mite (*Varroa destructor*), the virus is transmitted orally, sexually, and vertically [17,18], yet it appears to remain relatively benign [19–21]. However, *Varroa* mites horizontally transmit the virus while feeding on bee haemolymph [14], dramatically increasing viral titres and shifting DWV variant communities toward more virulent strains [6,19,22,23], likely contributing to global colony losses [6,24]. The Hawaiian Islands recently provided an inadvertent experiment demonstrating the critical effect of *Varroa* mites on DWV prevalence and diversity. Honeybees have been widespread on the archipelago since their introduction in the late nineteenth century [25]. The arrival of the *Varroa* mite *ca* 2007–2008 rapidly transformed the DWV landscape within honeybees, sharply increasing viral prevalence and load, and reducing variant diversity, favouring only a few of the many pre-existing genotypes [19]. DWV strains vary in their virulence [19,20,26], therefore understanding the factors that shape DWV variant communities is critical to managing this disease. There is also evidence of viral spillover on Hawaii: a recent study found DWV in populations of a solitary bee and a paper wasp only on Oahu where *Varroa* occurs, not on Maui where *Varroa* is absent, suggesting a role of *Varroa* in mediating DWV spillover from honeybees [27].

Pathogens such as DWV influence host fitness directly, by using host resources, and indirectly, by altering the ‘pathosphere’, the larger community of host-sharing pathogens [23,28,29]. The honeybee pathosphere includes numerous viruses, bacteria, fungi, and trypanosomes, with varying distributions and degrees of virulence [28]. Such host-sharing pathogens may act independently, but commonly interact with each other, often via the host immune system [29]. Pathogens may facilitate each other; in honeybees, suites of viruses are positively correlated in colonies that collapse, suggesting pathogen synergy resulting in colony death [30]. *Nosema apis* fungal infections in honeybee workers can facilitate infection by filamentous virus, bee virus Y, and black queen cell virus (BQCV) [31], while *Nosema ceranae* may compete with and inhibit DWV in some situations [32], and not others [33]. Through interactions with other pathogens and the host immune system, pathogens like DWV have the potential to alter the pathosphere, and fitness, of alternative hosts in complex ways.

Many of the recent studies in pathogen spillover have focused on native bees [10–13,34]. However, few studies have examined predators of honeybees (but see [35,36]), such as the widespread social wasps of the genus *Vespula*. *Vespula* wasps are native to northern temperate climates but have become important invasive species around the world [37,38], including on Hawaii [39]. On Hawaii, *Vespula pensylvanica* workers prey on and scavenge adult honeybees at hive entrances and raid hives for honey [40,41], as well as share floral resources with bees [35]. *Vespula* can harbour several common pathogens also found in honeybees, including DWV [7,8,36,42–44]. Here, we ask whether the DWV variant community in invasive *Vespula* experienced a reduction in diversity in concert with the dramatic *Varroa*-induced drop in DWV diversity seen in honeybee populations on the Big Island of Hawaii [19]. Because interactions between different pathogens can shape pathogen communities and influence their abundance within hosts, we also predicted the cascading effects of perturbed DWV populations on the larger *Vespula* pathogen community.

## 2. Material and methods

### (a) Sample collection

We collected *Apis mellifera* and *Vespula pensylvanica* (hereafter *Apis* and *Vespula*, respectively) samples from Hawaii Volcanoes National Park on Hawaii’s Big Island, along Hilina Pali road and at Mauna Ulu. Our sites are natural areas greater than 10 km from managed bee hives and were first exposed to *Varroa* mites in 2009–2010 [19]. The mites are now common in feral bee hives at these sites (KJ Loope 2015, personal observation). Post-*Varroa* samples of *Vespula* and *Apis* were whole bodies of foragers collected in September 2015 at the nest entrance. Pre-*Varroa* *Vespula* samples were whole bodies of returning foragers collected from colony entrances during August–September 2006–2008. Unlike other samples, Pre-*Varroa* *Apis* samples were pieces of adult honeybees (typically heads or thoraces) that were carried by returning *Vespula* foragers captured at the nest entrance and were reported on in ref. [45]. Because pre-*Varroa* *Apis* samples were not whole bodies (no such samples were available when the study was conceived in 2015), we made no statistical comparisons between them and other samples and included them because they were indicative of the pathogens that were present in this population prior to *Varroa* arrival. All samples were immediately placed into 100% ethanol and frozen until they were returned to the laboratory and stored at  $-80^{\circ}\text{C}$ . Samples were collected into ethanol to avoid any degradation should they inadvertently thaw during transport back to the laboratory.

### (b) DWV diversity

#### (i) DWV variant identification

We successfully extracted RNA from 44 pre-*Varroa* and 41 post-*Varroa* individual *Vespula* workers (4–8 individuals from each of 6 pre-*Varroa* and 6 post-*Varroa* *Vespula* colonies). We also extracted 25 *Apis* foragers, 5 from each of 5 post-*Varroa* *Apis* colonies and 13 individual *Apis* parts collected at the nest entrance of 5 different *Vespula* colonies. RNA was extracted from the samples by bead-beating (BeadBeater 16, Biospec Products, USA) samples in GENEzol reagent (Geneaid, Taiwan) with 5%  $\beta$ -mercaptoethanol, with chloroform and isopropanol purification.

We analysed the variation in DWV strain diversity using a 100 bp region of the highly conserved *RdRp* gene [19,24], making our analyses comparable to the previous study on DWV variants in Hawaii [19]. High-resolution melting (HRM) analysis of DWV–reverse transcription polymerase chain reaction (RT-PCR) products was based on the method of Martin *et al.* [18]. cDNA was generated from 1  $\mu\text{g}$  RNA per sample using qScript XLT reverse transcriptase (Quantabio, Beverly, USA). Samples were then analysed in triplicate by qPCR with DWV\_Q\_F1 and R1 primers [19,24] and HRM ( $54$ – $95^{\circ}\text{C}$ ) using MeltDoctor HRM Master Mix and a QuantStudio 7 platform (Applied Biosystems/Thermo Fisher Scientific, USA).

Following the HRM assay, we sequenced representatives of the various HRM signature curves in order to deconvolute the information provided by the HRM analysis. PCR products were diluted 1:5 with water and combined with 0.2  $\mu\text{M}$  DWV\_Q\_F1 primer. Sequencing was provided by the Massey Genome Service (Massey University, NZ). Many samples had the same HRM curve so we deemed it unnecessary to sequence them all. Sanger sequencing was only performed with the forward primer because that provided all the information required and to reduce the number of sequencing reactions needing to be prepared. These sequences matched those obtained in the next-generation sequencing (NGS) dataset (see below), confirming their validity.

HRM analyses revealed 45 individuals with more than a single HRM peak, indicating infection with multiple variants. Using HRM, strains were not identifiable in mixtures, so we

chose to analyse these multiple-variant samples using NGS. For 43 of these individuals, we re-amplified the *RdRp* fragment for NGS with an Illumina MiSeq, allowing us to obtain sequences for multiple strains per sample. Two individuals of the original 45 failed to re-amplify and were not included. For details, see electronic supplementary material.

### (ii) DWV variant diversity analyses

For NGS samples, we discarded strains with a relative copy abundance of less than 5% within individuals. This removed strains with fewer than 1000 copies, which could be artefacts or contamination, and also corrected the inflated strain number in an outlier sample with greater than 10× the average number of reads. The relative abundance of each strain within each sample was then calculated by dividing the number of reads by the total number of reads for that sample. We calculated the number of variants for each positive sample and compared diversity between DWV-positive pre- and post-*Varroa* samples within species using gamma-distributed generalized linear mixed models (GLMM) with date as a predictor and colony as a random effect. We compared the overall diversity of DWV variants in our pre- and post-*Varroa Vespula* and our post-*Varroa Apis* samples by using sample-based species accumulation curves (rarefaction) in the program EstimateS v.9.1 [46]. This is an occurrence-based calculation, only considering the presence or absence of each variant within each sample, and ignoring abundance information, which allowed us to combine the single-infection (HRM) and multiple-infection (NGS) datasets. We then compared the estimated diversity (S) among these three sample types (pre-*Varroa Vespula*, post-*Varroa Vespula*, post-*Varroa Apis*) by checking to see if the 84% confidence intervals overlapped, which is equivalent to a statistical comparison with  $\alpha = 0.05$  [47], using the rarified values. We also estimated the total variant diversity in each DWV population using the classic formula for the Chao2 estimator [48].

### (iii) DWV variant community composition changes

We compared the composition of DWV variant communities before and after the arrival of *Varroa* within *Vespula* samples using a permutational MANOVA analysis with the `adonis()` function in `vegan` [49]. We used occurrence (presence/absence) of each variant as the response variables. Date (pre- or post-*Varroa*) and colony were entered as fixed effects. We ran models using Bray–Curtis differences, though Euclidean distances gave qualitatively identical results.

### (iv) Viral replication

DWV replication was confirmed by the detection of the negative strand of the viral genome using reverse transcription and RT-PCR based on the standard method for detecting DWV replication [50], as recommended in [51]. Reverse transcription (SuperScript IV, Invitrogen/Thermo Fisher Scientific, Waltham, USA) of 1 µg RNA was performed with DWV Tag-F15 primer at a final concentration of 100 nM. PCR was then carried out on the cDNA or no-template negative controls using the Tag primer (agcctgcccaccgtgg) and DWV B23. The products were resolved by 2% agarose gel electrophoresis. Products were then sequenced, confirming DWV identity.

## (c) Broad pathogen screening

### (i) Sampling, RNA extraction, and cDNA synthesis

We extracted 2 pools of 5 workers each from 10 pre- and post-*Varroa Vespula* colonies, 10 post-*Varroa Apis* colonies, and 14 individual pre-*Varroa Apis* sample prey items (including the 13 analysed for DWV). All colonies analysed for DWV were also

used in this screen, though different individuals were extracted. Samples were homogenized by bead-beating in Genezol reagent plant (Geneaid). Chloroform and isopropanol were then used to extract and co-precipitate DNA and RNA, which was washed with 70% ethanol, and resuspended in water. SuperScript IV (Invitrogen/ThermoFisher) was used to synthesize cDNA from 2 µg RNA in 20 µl reactions following the manufacturer's instructions.

### (ii) Taqman array qPCR

We screened two pools of cDNA from 30 individual wasps for common pollinator pathogen targets using standard PCR and gel electrophoresis. We then selected a final set of pathogens, comprising three viruses (BQCV, Kashmir bee virus (KBV), and DWV), three fungal species (*Aspergillus fumigatus*, *Ascospaera apis*, *Nosema ceranae*), two bacteria (*Arsenophonus nasoniae*, *Enterococcus faecalis*), and the trypanosomes *Crithidia mellificae* and *Lotmaria passim*, which were not distinguishable using our primers (electronic supplementary material, table S1). Pathogen and gene targets were analysed using 384-well TaqMan Array Micro Fluidic Cards (Applied Biosystems) for these 9 pathogens and 4 control genes (electronic supplementary material, table S1). qPCR was repeated separately for KBV, which failed on the TaqMan array.

### (iii) Pathogen community changes

We compared the composition of pathogen communities before and after the arrival of *Varroa* within *Vespula* using a perMANOVA analysis with the `adonis()` function in `vegan` [49], with pathogen loads as response variables. Date (pre- or post-*Varroa*) and colony were entered as fixed effects. We dealt with pathogen absences in several alternative ways. In the primary analysis, we set the value for a 'negative' sample to half the minimum recorded abundance for that pathogen (approximating the lower detection threshold of our quantification method). These non-zero values allowed log transformation. We then used Bray–Curtis differences in the permutational MANOVA. We performed two similar, alternative analyses: one without the log transformation, and another with negatives as zeros and a Euclidean distance measure, giving qualitatively identical results to the primary analysis (electronic supplementary material, table S2).

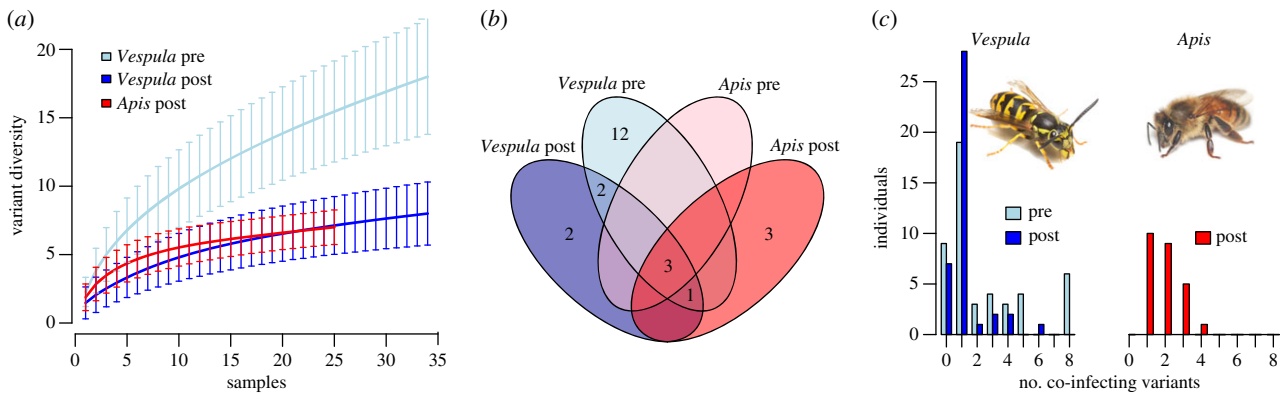
Individual pathogen occurrences for *Vespula* samples were compared with exact logistic regression at the colony level, with date (pre- or post-*Varroa*) as the predictor. Pathogen loads for positive samples were compared with mixed effect models (`lme()` function [52]) on log-transformed relative abundance, with date as a fixed effect and colony as a random effect. We used the `anova()` function to compare to an identical model lacking the date predictor to determine significance.

We looked for associations between pathogens using Spearman rank correlations of relative pathogen load across all samples for all pairs of pathogens with greater than 25% occurrence with each species and time period. *p*-values were corrected using a False Discovery Rate (FDR) procedure (function `corr.test` [53]).

## 3. Results

### (a) DWV variant diversity

We first examined how the diversity of DWV variants changed due to the *Varroa* introduction. Combining data from HRM qPCR and Illumina amplicon sequencing, we detected 23 variants at a 100 bp region of the highly conserved *RdRp* gene, all of DWV Type A (figure 1b and electronic supplementary material, figure S1). Type A DWV variants in



**Figure 1.** DWV diversity in *Vespula* and *Apis* before and after *Varroa* arrival. (a) Rarefaction curves for population DWV variant diversity, showing the expected number of variants (*y*-axis) for a given number of samples (*x*-axis). Error bars represent 84% confidence intervals; non-overlapping intervals indicate significant differences in diversity for  $\alpha = 0.05$  (b) Venn diagram of the distribution of the 23 variants among sample types. (c) Distributions of the number of co-infecting DWV variants per individual insect for *Vespula* and *Apis* pre- and post-*Varroa* mite arrival on the Big Island of Hawaii. Photo credit: Sean McCann (*Vespula*).

honeybees have been commonly associated with *Varroa* parasitism and subsequent colony collapse [4,19]. Three common variants were shared between *Apis* and *Vespula* and persisted following *Varroa* arrival, while a clade of variants only detected in *Vespula* was virtually extinguished following *Varroa* arrival (electronic supplementary material, figures S1 and S2). Overall, 12 variants were observed in *Vespula* prior to *Varroa* arrival but were absent from these wasps after *Varroa* establishment (figure 1b). The three common variants found in all 4 types of samples were by far the most prevalent, with genotypes 1–3 being detected in 75%, 38%, and 18% of the 104 samples sequenced, respectively (electronic supplementary material, figure S3). The remaining rarer genotypes were detected in 1–7 individuals. Estimated DWV variant diversity was higher in *Vespula* pre-*Varroa* samples than in other sample sets, as indicated by non-overlapping confidence intervals, and diversity was similar for post-*Varroa* *Vespula* and *Apis* samples (figure 1a). The Chao2 estimator similarly predicted higher variant diversity in the *Vespula* pre-*Varroa* population (37.6; 95% CI = 21.8–118.2) than in the *Vespula* and *Apis* post-*Varroa* populations (*Vespula*: 12.9, 95% CI = 9.5–36.9, *Apis*: 8.0, 95% CI = 7.1–20.3). Overall, pre-*Varroa* and post-*Varroa* DWV variant communities differed within *Vespula* (permutational MANOVA on occurrence data with Bray–Curtis distances:  $F_{\text{date}} = 8.18$ ,  $R^2_{\text{date}} = 0.11$ ,  $p_{\text{date}} < 0.001$ ).

### (b) Number of co-infecting DWV variants

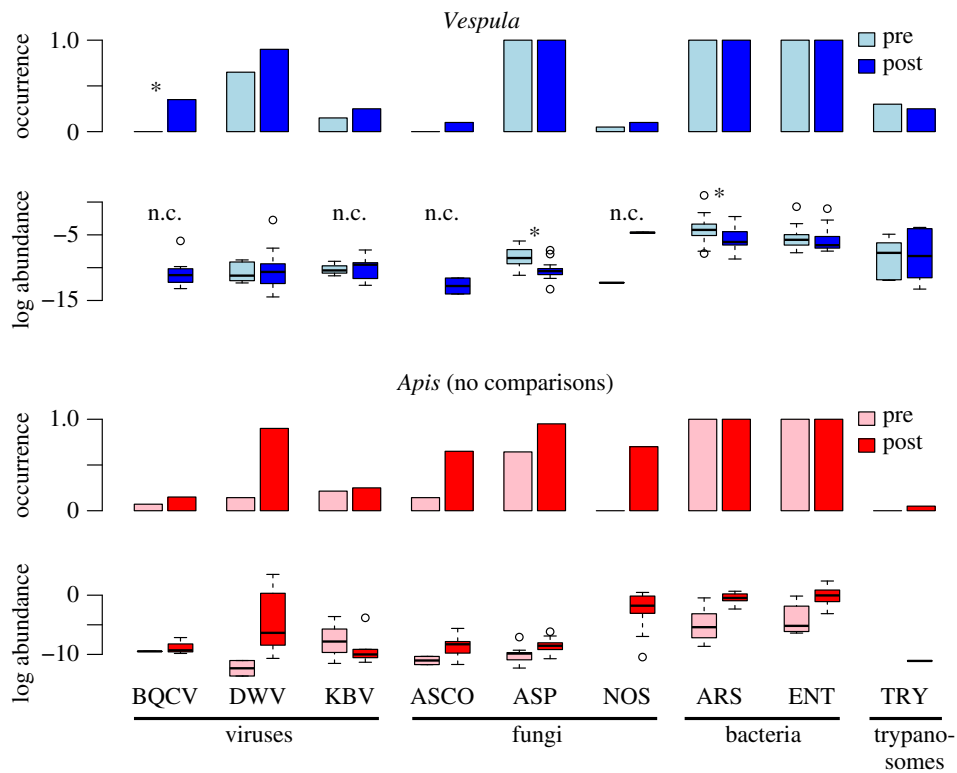
We next examined how the number of DWV variants changed for samples of both *Vespula* and their honeybee prey after the introduction of *Varroa*. For *Vespula* samples, the proportion of individuals positive for DWV screened with HRM qPCR of the *RdRp* gene was statistically similar pre- and post-*Varroa* introduction [*Vespula*: 80% (35/44) versus 83% (34/41), respectively, Fisher's exact test  $p > 0.05$ ]. However, the proportion of infected *Vespula* individuals with multiple variants decreased following *Varroa* arrival [46% (16/35), versus 18% (6/34), respectively, Fisher's exact test  $p < 0.019$ ]. Samples with multiple variants were subsequently analysed using amplicon sequencing on an Illumina MiSeq. *Vespula* samples collected prior to *Varroa* introduction had a greater number of co-infecting variants than post-*Varroa* *Vespula* (figure 1c; variant number: gamma GLMM; estimate =  $-0.24 \pm 0.09$ ;  $t = -2.73$ ,  $p = 0.006$ ).

### (c) Viral replication

To determine if DWV was parasitizing wasps, we examined samples for evidence of viral replication. We detected negative-strand DWV in pooled samples of both *Vespula* and *Apis* (electronic supplementary material, figure S4), indicating viral replication in both species. Sanger sequencing of the PCR products confirmed that the fragments were amplified from DWV. This method of detection carries the risk of detecting false-positives. However, two lines of evidence support our finding of an active infection in wasps: (1) not all samples were positive in the strand-specific RT-PCR assay despite being positive for virus by standard PCR, indicating that the viral replication assay was able to differentiate between inactive and active virus; (2) previously published papers have demonstrated that honeybee viruses, including DWV, replicate in a variety of insects, including *Vespula* wasps [8,44,54]; thus, our finding of replication is not surprising.

### (d) Pathogen community changes

We examined changes in the abundance and interactions between nine known pathogens of honeybees and wasps, prior to and after *Varroa* introduction. The pathogen targets were determined from an initial screen of a pooled sample, and the final set comprised three viruses (BQCV, KBV, and DWV), three fungal species (*Aspergillus fumigatus*, *Ascospaera apis*, *Nosema ceranae*), two bacteria (*Arsenophonus nasoniae*, *Enterococcus faecalis*), and the trypanosomes *Crithidia mellificae* and *Lotmaria passim*, which were not distinguishable using our primers (electronic supplementary material, table S1). We found significant pathogen community compositional changes due to date and colony in *Vespula* (permutational MANOVA:  $F_{\text{date}} = 4.97$ ,  $R^2_{\text{date}} = 0.07$ ,  $p_{\text{date}} < 0.002$ ,  $F_{\text{colony}} = 2.25$ ,  $R^2_{\text{colony}} = 0.62$ ,  $p_{\text{colony}} < 0.001$ ). Alternative analyses gave qualitatively similar results (electronic supplementary material, table S2). *Apis* samples were not compared given the difference in sampling procedures between pre- and post-*Varroa* samples in bees. We found significantly higher occurrences of BQCV in *Vespula* following *Varroa* arrival (figure 2). We only detected *Ascospaera* and BQCV in post-*Varroa* *Vespula* samples, and only detected *Nosema* and trypanosomes in post-*Varroa* *Apis* samples, possibly because samples for pre-*Varroa* *Apis* were only partial bodies, not whole-body samples. For positive samples, *Arsenophonus* and *Aspergillus* loads decreased in *Vespula* (figure 2).



**Figure 2.** Pathogen community shifts in *Vespula* and *Apis* following *Varroa* arrival in Hawaii Volcanoes National Park. Significant occurrence and abundance differences before and after *Varroa* arrival are indicated by \* ( $p < 0.05$ ). n.c. indicates no quantitative comparison was made due to an insufficient number of positive samples. No comparisons were made between pre- and post-*Varroa* *Apis* samples because they were not collected in the same way; pre-*Varroa* *Apis* samples were small pieces of *Apis* tissue brought back by returning *Vespula* foragers. BQCV, black queen cell virus; DWV, deformed wing virus; KBV, Kashmir bee virus; ASCO, *Ascospaera apis*; ASP, *Aspergillus fumigatus*; NOS, *Nosema ceranae*; ARS, *Arsenophonus nasoniae*; ENT, *Enterococcus faecalis*; TRY, trypanosomes (*Crithidia mellificae*/ *Lotmaria passim*). Lower (and upper) whiskers represent first (and third) quartiles minus (plus) 1.5 times the interquartile range (or extreme values).

Within each host and time period, we found positive and negative correlations of relative pathogen loads across pooled samples of five individuals (figure 3). For *Vespula*, most correlations prior to *Varroa* arrival were positive but non-significant; following *Varroa* arrival, several of the same correlations were stronger and significant among *Arsenophonus*, *Enterococcus*, trypanosomes, and *Aspergillus*. Pre-*Varroa*, DWV load was not correlated with any other pathogens (Spearman's  $\rho < 0.14$ ). However, following *Varroa* arrival, DWV was moderately positively associated with KBV ( $\rho = 0.51$ ;  $p = 0.02/0.09$  before/after FDR correction) and *Aspergillus* ( $\rho = 0.58$   $p = 0.01/0.06$  before/after FDR correction) in *Vespula*. Pathogen associations in post-*Varroa* *Apis* samples differed from those of *Vespula*, with positive associations between *Ascospaera* and both *Arsenophonus* and *Enterococcus* in post-*Varroa* *Apis*, as well as a negative association between KBV and *Arsenophonus*.

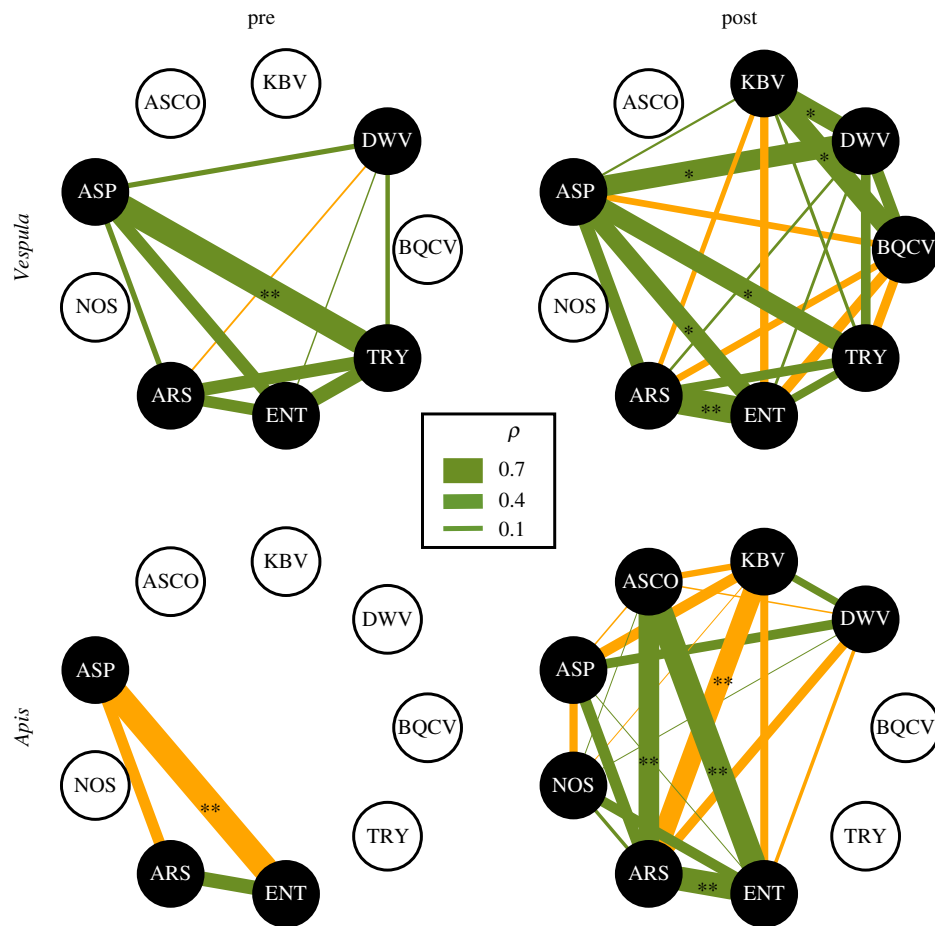
## 4. Discussion

Our results show that the arrival of the *Varroa* mite in *Apis* populations in Hawaii produced a parallel reduction in DWV diversity in an invasive *Vespula* wasp population, despite the mite being an *Apis*-specialist parasite. DWV variant diversity in *Vespula*, both at the individual and population level, declined following *Varroa* arrival, with the same single genotype dominating both *Apis* and *Vespula* samples. All variants we observed, before and after *Varroa*, were in the DWV type A group associated with symptomatic infections and colony losses [4,19], indicating the potential for

*Vespula* to experience and transmit a virulent form of this virus. These findings support the hypothesis that honeybees and their species-specific parasite play a central role in shaping generalist hymenopteran pathogen dynamics in insect communities. Although most studies examining the spillover effects of bee pathogens examine native bees [10–13,15], our results show that predators may be influenced by, and could participate in, pathogen spillover as well.

We did not observe an increase in DWV abundance or prevalence in *Vespula* individuals with the arrival of *Varroa*, in contrast to recent work on *Polistes* wasps, which only possess DWV on Hawaiian islands where *Varroa* is present [27]. Instead, we observed a marked change in strain diversity, demonstrating that spillover may result in subtle effects on pathogen diversity, rather than overall pathogen load. The importance of variant composition has been recognized in *Apis* [19–21,26], as has the importance of pathogen spillover for non-*Apis* species [11,12], but the consequences of variant diversity changes in spillover to other hosts are poorly understood. Subtle changes in pathogen variant composition could have potentially important consequences in other species, given the clear variation in virulence among DWV variants in honeybees. Although lethality of DWV infections has been demonstrated in laboratory-reared bumblebee workers (*Bombus* spp.) [12,55], we know of no studies examining the effects of different strains of DWV in non-*Apis* insects. Experimental infection of *Vespula* with different viral strains would elucidate the importance of viral diversity shifts in this species.

The sharing of pathogen variants between sympatric host populations can indicate interspecific pathogen transmission



**Figure 3.** Pathogen load correlation networks. Nodes indicate the nine pathogens quantified in this study ( $n = 20$  samples (pools of 5 individuals) for each time period, except pre-*Varroa Apis*, which were 14 sampled prey items). White nodes indicate pathogens omitted from correlation analysis (less than 25% occurrence within each category). Edge width shows Spearman rank correlation coefficients ( $\rho$ ). Samples that did not amplify were analysed as zero (lowest rank) abundance. Green edges show positive correlations, orange show negative correlations. \* indicates  $p < 0.05$ ; \*\* indicates significance after FDR correction. BQCV, black queen cell virus; DWV, deformed wing virus; KBV, Kashmir bee virus; ASCO, *Ascosphaera apis*; ASP, *Aspergillus fumigatus*; NOS, *Nosema ceranae*; ARS, *Arsenophonus nasoniae*; ENT, *Enterococcus faecalis*; TRY, trypanosomes (*Crithidia mellificae*/Lotmaria passim).

[12]. The observed shift in *Vespula* DWV diversity in response to *Varroa* arrival and the variant-sharing between *Vespula* and *Apis* suggest that *Vespula* may acquire DWV directly or indirectly from *Apis*. This acquisition could occur via flowers [56], as both *Vespula* and *Apis* collect nectar from a variety of blooming plants at our field site. These predators also hunt and scavenge *Apis* adults and brood [40]. We detected DWV in *Apis* prey items being carried back to the nest by *Vespula* foragers; such predation may expose adults and the larvae they feed to *Apis*-derived DWV. DWV and other viruses can be transmitted orally through food and feeding [18]. These wasps are frequent honey-raiders inside hives [40,41]; this carbohydrate feeding could be another route of DWV transmission. Our samples were whole *Vespula* foragers, raising the possibility of contamination from exposure to infected *Apis* tissue prior to capture, rather than actual infection in the wasps. However, we think this is unlikely for two reasons. First, *Vespula* foragers do not ingest protein prey but instead carry prey back to the nest, where it is fed to larvae, limiting the possibilities for contamination. Second, we found two pathogens, *Nosema* and *Ascosphaera*, that were common in *Apis* but rare in *Vespula*, which is inconsistent with forager contamination from *Apis* tissue (figure 3).

We also found significant differences in the structure of *Vespula* pathogen communities following *Varroa* arrival, and

the associations of pathogen abundance within hosts also changed. DWV variants compete with one another, and the outcome of that competition can have important consequences for host survival [20,26], and therefore could also influence the susceptibility of hosts to other pathogens. One important way pathogens alter the success of other pathogens is via the host immune system [57]. In honeybees, DWV may alter host immunity [22,58,59], with one study suggesting that viral immune suppression promotes parasitism by *Varroa* [59]. Such immunosuppression by virulent DWV strains could similarly facilitate infection from other pathogens, though whether such effects exist likely depend on the details of the host species immune response and the variety of interacting pathogens. It is unknown if DWV similarly affects *Vespula* immune systems, but KBV infection increases expression of honeybee immune gene orthologues in *Vespula* [44], suggesting some conserved aspects of immunity between bees and wasps.

While our results do not prove that the *Varroa* mite, and associated changes in DWV diversity, caused the observed changes in *Vespula* pathogen webs, several pathogen association changes are suggestive of a DWV/*Varroa* influence. DWV became positively associated with KBV in *Vespula* following *Varroa* arrival (figure 3). KBV is transmissible between honeybees via *Varroa* [60], though no association

between *Varroa* and KBV was previously detected in other populations of honeybees in Hawaii [19]. The nascent association with DWV in our *Vespula* population could result from DWV variant shifts affecting pathogen interactions within isolated *Vespula* pathogen communities, or it could also be influenced by changes in pathogen communities in *Apis* (though we saw no such positive association between DWV and KBV in *Apis*). Similarly, DWV became positively associated with *Aspergillus*, which may have benefited from host immunosuppression [61]. Finally, BQCV was only detected in post-*Varroa* *Vespula* samples; the increase in BQCV prevalence in *Vespula* could thus be an effect of the change in DWV variant composition, though we saw no correlation between DWV and BQCV load in post-*Varroa* samples within sampling periods. More generally, we found several non-DWV pathogen associations, mostly positive correlations, that also differed between species and between time periods. This indicates that pathogen competition and facilitation can be dynamic and host specific and sets the stage for further experimental investigation of these novel associations of the pathogens in both *Vespula* and *Apis*.

The apparently wide overlap in pathogen communities means that *Vespula* populations could be adversely affected by spillover from managed *Apis*. While this may not be viewed as detrimental in invasive populations like the one studied here, in their native range, *Vespula* can be effective pollinators [62] and are important generalist predators that likely play a large role in food webs. *Vespula* abundance varies tremendously between years [63–65], with strong density dependence effects that could be driven by pathogens [65]. How pathogen spillover from *Apis* populations contributes to these dynamics is completely unexplored. Although pathogens have been considered as a control agent against

invasive *Vespula* [38], pathogen sharing between *Vespula* and *Apis* means that using such pathogens as control agents for *Vespula* may be difficult, given the need to avoid exacerbating threats to native and commercial pollinators.

## 5. Conclusion

*Varroa* has been introduced around the globe, nearly everywhere there are honeybees (except Australia). The direct mortality effects on honeybees have been abundantly clear. Our work suggests that alongside these direct effects there has been a hidden, yet remarkable, change in the genetic diversity of a key virus, perhaps influencing larger pathogen webs, in both honeybees and in an associated predatory insect. We confirm that the effects of *Varroa* introduction have cascaded through entire communities [27].

**Data accessibility.** Data are available from the Dryad Digital Repository at: <http://dx.doi.org/10.5061/dryad.17371h3> [66].

**Authors' contributions.** E.E.W.R. and P.J.L. conceived the study, and all authors contributed to design of data collection. J.W.B., E.E.W.R., and K.J.L. collected data. K.J.L. and E.E.W.R. analysed data and drafted the manuscript. All authors contributed substantially to editing the manuscript and approved the final version.

**Competing interests.** We declare we have no competing interests.

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