

Title: Evidence of *Varroa*-mediated Deformed Wing virus spillover in Hawaii

Authors:

Jessika Santamaria

Corresponding Author: jsantama@hawaii.edu

University of Hawaii at Mānoa, United States

3050 Maile Way, Honolulu, HI 96822

Ethel M. Villalobos

University of Hawaii at Mānoa, United States

3050 Maile Way, Honolulu, HI 96822

Laura E. Brettell

Hawkesbury Institute for the Environment at the West Sydney University,

Science Rd, Richmond, NSW 2753, Australia

Scott Nikaido

University of Hawaii at Mānoa, United States

3050 Maile Way, Honolulu, HI 96822

Jason R. Graham

University of Hawaii at Mānoa, United States

3050 Maile Way, Honolulu, HI 96822

Stephen Martin

The University of Salford

Manchester, UK M5 4WT

Abstract:

Varroa destructor, a parasitic mite of honey bees, is also a vector for viral diseases. The mite displays high host specificity and requires access to colonies of *Apis spp.* to complete its lifecycle. In contrast, the Deformed Wing Virus (DWV), one of the many viruses transmitted by *V. destructor*, appears to have a much broader host range. Previous studies have detected DWV in a variety of insect groups that are not directly parasitized by the mite. In this study, we take advantage of the discrete distribution of the *Varroa* mite in the Hawaiian archipelago to compare DWV prevalence on non-*Apis* flower visitors, and test whether *Varroa* presence is linked to a “viral spillover”. We selected two islands with different viral landscapes: Oahu, where *V. destructor* has been present since 2007, and Maui, where the mite is absent. We sampled individuals of *Apis mellifera*, *Ceratina smaragdula*, *Polistes aurifer*, and *Polistes exclamens*, to assess and compare the DWV prevalence in the Hymenoptera community of the two islands. The results indicated that, as expected, honey bee colonies on Oahu have much higher incidence of DWV compared to Maui. Correspondingly, DWV was detected on the Non-*Apis* Hymenoptera collected from Oahu, but was absent in the species examined on Maui. The study sites selected shared a similar geography, climate, and insect fauna, but differed in the presence of the *Varroa* mite, suggesting an indirect, but significant, increase on DWV prevalence in the Hymenoptera community on mite-infected islands.

Keywords:

Varroa mite, deformed wing virus, pollinator health, viral transmission, viral spillover, *Apis mellifera*

Abbreviations:

Deformed Wing Virus (DWV)

1. Introduction

In the last two decades, emerging diseases have caused extensive damage to crops and livestock (Morens and Fauci, 2013; Voyles et al., 2014,). Pathogens have been repeatedly shown to jump between species (Levitt et al., 2013; Li et al., 2011; Malmstrom and Alexander, 2016) and the Deformed Wing Virus (Iflaviridae; DWV) affecting honey bees is no exception (Villalobos, 2016). Recent molecular studies have shown that the DWV may have co-evolved with the European honey bee (*Apis mellifera*), and the original virus may have been a low prevalence pathogen with many variants and low virulence (Martin et al., 2012; Wilfert et al., 2016). Upon

contact with the Asian honey bee (*Apis cerana*), a new mite vector, *Varroa destructor*, jumped species from *A. cerana* to *A. mellifera* and with this new transmission route the prevalence and virulence of DWV in *A. mellifera* was amplified (Martin et al., 2012; Wilfert et al., 2016). Recent studies by Yanez et al. (2015) on sympatric colonies of the Asian honey bee, *Apis cerana*, and *A. mellifera* indicated that there are a large number of shared strains of DWV circulating in the Asian and the European honey bee populations, however the virus is more prevalent in the European honey bee colonies, suggesting a more efficient transmission route via the mite and/or greater susceptibility of *A. mellifera* to infection to the virus or the vector. A similar situation has been reported for the native Japanese honey bee *Apis cerana japonica*, which shares DWV infections with sympatric *A. mellifera* but at a much lower prevalence (Kojima et al., 2011).

While DWV evolved in close association with *Apis* bees, it also appears capable of infecting a broad range of non-*Apis* hosts (Genersch et al., 2006; Li et al., 2011; Melathopoulos et al., 2017). So far, DWV has been detected in 23 insect genera across Europe, North and South America (Guzman-Novoa et al., 2015; Levitt et al., 2013; Reynaldi et al., 2013; Singh et al., 2010), including social and non-social bees, wasps, ants, and a myriad of other insect groups. Not much is known about the impact of the virus in these host species (Tehel et al., 2016). Negative strands of DWV, suggestive of viral replication in the host, have been found only in 5 genera of non-*Apis* insects (Levitt et al., 2013; Tehel et al., 2016). However, the discovery of DWV among a wide range of species has created concerns about a possible “viral spillover” from honey bee colonies to other insect species, especially economically

important pollinators such as bumble bees (Graystock et al., 2016a, Graystock et al., 2016b). Work on viral spillover has been conducted, so far, in regions where *V. destructor* is present and DWV is prevalent in the honey bee population (Budge et al., 2015; Tehel et al., 2016; Traynor et al., 2016). In fact, the presence of *V. destructor* in honey bee colonies has been linked to increased viral loads, virulence, and prevalence of DWV in honey bee populations (Martin et al., 2012). Additionally, *Varroa* has also been associated with other viral diseases in honeybees including AKI, KBV, BQCV, and SBV (Francis et al., 2013; Martin, 2001; Shen et al., 2005).

The fragmented distribution of the *Varroa* mite on the Hawaiian archipelago makes for ideal study sites in which to examine pollinator communities with or without *Varroa* mites in the ecosystem. Honey bees first arrived to the Hawaiian archipelago in 1857 and became established across the eight islands by 1909 (Szalanski et al., 2016). In this study, we sampled local honey bees and non-*Apis* Hymenoptera species on the *Varroa*-positive island of Oahu and the *Varroa*-negative island of Maui. The selected study sites shared similar geography, floral resources, and insect communities; however, Oahu's honey bees have been in contact with *V. destructor* since 2007, and have high DWV prevalence and increased viral loads. In contrast, Maui remains mite free to this date, and the honey bee populations on that island have a much lower incidence of DWV (Martin et al., 2012). The non-*Apis* Hymenoptera species selected as representatives of the community were: *Ceratina smaragdula*, *Polistes aurifer*, and *Polistes exclamens*. *C. smaragdula*, commonly known as the small carpenter bee, is a mostly solitary bee abundant in garden environments in Hawaii, sharing nectar and pollen resources with honey bees.

Polistes spp. are common social wasps that hunt caterpillar prey, and visits flowers occasionally to feed on nectar.

Re-emerging viral diseases such as DWV represent one of the major threats to honey bee health, and the “spillover” of pathogens to wild bees and other insects may also contribute to the current global pollinator decline (Fürst et al., 2014; Genersch et al., 2006; Graystock et al., 2013a; Graystock et al., 2013b; Manley et al., 2015; Tehel et al., 2016). Here we carry out a preliminary comparison of the incidence of DWV on non-*Apis* insects in areas with and without *V. destructor*.

2. Methods

2.1 Specimen collection

We selected three species within two different Hymenoptera genera as representatives of the local community of flower visitors: the introduced small carpenter bee *Ceratina smaragdula* (Apidae) which was first recorded in Hawaii in 1999 (Magnacca and King, 2013), and introduced paper wasps *Polistes aurifer* and *Polistes exclamans* (Vespidae) first recorded in Hawaii in the 19th century and in 1952 respectively (Beggs et al., 2011). All samples were collected from five sites on Oahu (*Varroa*-positive island), and four sites on Maui, (*Varroa*-negative island). Collection sites on both islands consisted on a mix of agricultural fields, parks, gardens, and beach edge vegetation strips. The selected insect species are all relatively abundant and can be found in urban and agricultural environments, where they overlap in resource use with *A. mellifera*. Our selection of diverse habitats provided us with a preliminary bird’s eye view of the viral distribution on

each island, and represents the micro-climate diversity that characterizes the Hawaiian archipelago.

Polistes wasps collected on Oahu are *P. aurifer* and the specimens from Maui are *P. exclamans*. Consequently the comparisons between the paper wasps were at the genus level. Samples were collected from August 2014 to November 2015. Insects were collected while they were foraging in fields or flower patches, via a hand-held net. Paper wasps samples were also collected from around their nests. Each insect was stored individually and kept on ice in the field until transferred to a -80 °C freezer for long term storage.

2.2 RNA Extraction & Reverse Transcription PCR

Each individual was transferred to a nuclease free 1.5ml centrifuge tube, which was submerged in liquid nitrogen before the sample was crushed using a sterile mini pestle. Total RNA was then extracted from the resulting powder using the RNeasy Mini Kit (Qiagen) following manufacturer's conditions and resuspended in 30 µl of RNase-free water. RNA concentration was determined using a Nanodrop 2000c (Thermo Scientific) and samples were diluted to 25ng/µl. Reverse Transcription-PCR (RT-PCR) protocols adapted from Martin et al (2012) were carried out to determine whether samples contained DWV. Endogenous control reactions were also carried out to ensure RNA was intact. RT-PCR reactions contained 50ng RNA, 1x OneStep RT-PCR Buffer (QIAGEN), 400 µM each dNTP, 10units RNase Inhibitor (Applied Biosystems) and 0.6µM each primer. DWVQ_F1 and DWVQ_R1 primers (Highfield et al 2009) were used to amplify a conserved

region of the RNA dependant RNA polymerase (RdRp) gene. For the endogenous controls actin primers were used (Highfield et al 2009). Reactions were run using a T100 Thermocycler (Bio-Rad) starting with reverse transcription at 50°C for 30 minutes, followed by an initial denaturation step at 94°C for 30 seconds. This was followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for DWVQ primers (58°C for actin) for 30 seconds, extension at 72°C for 1 minute, and a final extension step at 72°C for 10 minutes. Agarose gel electrophoresis was used to determine the results. RT-PCR products were ran on a 2% agarose gel stained with SYBR Safe DNA gel stain (Invitrogen) with a 100bp TrackIt ladder (Invitrogen), and visualised under ultraviolet light. All samples were determined, via the detection of a bright band at 120bp on an agarose gel, to contain sufficient intact RNA, confirming the absence of DWV in those samples that failed to amplify a DWV fragment.

2.3 Statistical Analysis

To compare the DWV prevalence between species across islands, data was arranged across a contingency table and Fisher's Exact Test was used to test for significance. Statistical test selection was based on the small sample size, and the large number of zeros in the data counts for Maui.

3. Results

We established via RT-PCR that DWV was present in the honey bee population on both islands; however, virus prevalence was significantly higher ($p < 0.0001$, Fisher's Exact Test) among *Apis mellifera* from Oahu (83%, $n=58$) compared to individuals from Maui (7%, $n=29$) (Fig.1). The RT-PCR results for the non-*Apis* insects showed a distinct dichotomy based on island; DWV was found on both of our non-*Apis* study species on Oahu, while the virus was completely absent from both of the non-*Apis* Maui samples (Fig. 1). Within the Oahu samples, the prevalence of DWV in *Ceratina smaragdula* and *Polistes aurifer* was 27% ($n=61$) and 45% ($n=20$) respectively (Fig.1).

4. Discussion

We confirmed, as expected, that the presence of the *Varroa* mite on Oahu greatly increased the prevalence of DWV in honey bees (Table 1). In this study, eight out of 10 forager honey bees collected on Oahu were positive for DWV, compared to a DWV detection rate of 0.7 out 10 bees in Maui. The low prevalence of DWV in Maui's bees concurs with a previous survey by Martin et al. (2012) in which four out of 33 Maui colonies tested positive for DWV. The detection of DWV on *Varroa*-negative islands also agrees with the theory that this virus arrived in Hawaii along with the European honey bee prior to the global spread of the mite, and that it remains present in the *Varroa*-negative honey bee population as a low prevalence pathogen (Martin et al., 2012; Ryabov et al., 2014; Wilfert et al., 2016).

According to the review by Tehel et al. (2016), 17 species of bees, including one species in the genus *Ceratina*, have been described as positive for DWV. In our

study, detection of DWV in *C. smaragdula* was associated only with *Varroa*-positive areas, where one out of four small carpenter bees sampled tested positive for the virus. Singh et al (2010) reported DWV infection in *Ceratina dupla*, where two out of three individuals sampled were positive. DWV has also been detected in several wasp species, including yellow jackets (*Vespula spp*) (Levitt et al., 2013) and several *Polistes spp* (Singh et al., 2010). Our study shows that, as with the small carpenter bees, the presence of DWV in paper wasps was limited to the samples from Oahu where 45% of the *P. aurifer* specimens collected were positive for DWV.

Our results suggest a possible DWV spillover from honey bees to flower visitors that is indirectly linked to *Varroa* presence in the region. However, there are still large gaps of knowledge with regard to cross species transfer of DWV, in particular: the routes of virus transmission, the range of species that are susceptible, and the potential impact, if any, of the virus on the non-*Apis* hosts. Tehel et al. (2016) argue that simple PCR detection of DWV at a single location does not provide enough information to make inferences about viral spillover from honey bees to the rest of the insect community. The confirmation of a higher prevalence of DWV in non-*Apis* insects from a *Varroa*-positive island compared to a *Varroa*-free island suggests the need for more in-depth studies that include multiple locations, samples from a wide range of insect species, and confirmation of viral replication in the hosts. In addition, there is a need for comparative studies of the virulence of the different DWV genotypes and the susceptibility of honeybees and other potential insect hosts to each of these variants (McMahon et al., 2016).

Nevertheless, the absence of the mite on Maui provided us with the opportunity to completely exclude the effects of *Varroa* parasitism from one site, while comparing the prevalence of the DWV on two geographically close regions. The information collected in this study can be considered preliminary evidence in support of directionality of transfer of DWV from Oahu's honey bees to other insect species in this island, as mediated by the presence of *Varroa* and the associated higher viral titers in *A. mellifera*.

One of the proposed routes of DWV transmission involves ingestion of contaminated hive products such as, pollen and honey, and/or consumption of larvae, pupae, or adult bees (Chen et al., 2006; Genersch et al., 2006; Möckel et al., 2010; Singh et al., 2010). Insects that rob colony resources, or those that feed directly on live or dead bees, may take in viral particles with the food they ingest. This mechanism of infection has been suggested for yellow jackets, possibly ants, and for hive parasites (Evinson et al., 2012; Sébastien et al., 2015). This transmission route however, is not a likely explanation for our study species. Small carpenter bees feed solely on flowers, and, although carnivorous, *Polistes* exclusively hunt caterpillars to feed their young and do not rob honey bee colonies. A more likely transmission route in our study is through the flowers shared by the insects. Floral resources have been identified as a potential contact point between species and viable DWV particles have been found in pollen (Mazzei et al., 2014; McArt et al., 2014; Singh, 2011). Both honey bees and *C. smaragdula* were found foraging on the same common garden herbs, crops, and ornamentals – such as *Scaevola sericea* (Naupaka) and *Heliotropium foertherianum* - on both Oahu and Maui (pers. obs.).

Bees require pollen and nectar to rear their young, and it is possible that either of those resources could have been contaminated with DWV. *Polistes* spp. are active foragers that move quickly among the vegetation looking for caterpillar prey, but they occasionally pause to feed on nectar from a variety of flowers during a foraging bout (pers. obs.). Consequently, the shared floral use could also be an alternative route of viral transmission in predatory or parasitic wasps.

The honey bee colonies on Maui, as well as those on other *Varroa*-negative Hawaiian islands, showed a much lower DWV prevalence (Martin et al., 2012), thus the number of infected individuals, and the viral titer of the infected bees foraging in that community is expected to be much lower. In contrast, forager honey bees on Oahu are more likely to be DWV positive and to carry an elevated viral load, which could translate into a higher rate of floral contamination on this island and a higher prevalence of DWV in non-*Apis* flower visitors (Fig. 1).

The pathogenicity of DWV and the relationship between the virus, the mite, and the honey bee continue to be the focus of much research in honey bee pathology (Di Prisco et al., 2016; Möckel et al., 2010; Ryabov et al., 2014). The known DWV strains (Type A, B, and C), and recombinants thereof, may be linked to differences in DWV virulence in honey bee colonies (Martin et al., 2012; McMahon et al., 2016; Ryabov et al., 2014; Zioni et al., 2011), however, there is no evidence that strains may be specifically linked to wing deformities on bees, rather it appears that viral loads of DWV play a significant role in the expression of this phenotype in honey bees (Brettell et al., 2017). By comparison to the existing work on honey bees, our

understanding about DWV transmissibility and its effect on the fitness of non-*Apis* bees, and other insects, is much more limited. Based on the summary presented by Tehel et al. (2016) 17 species of non-*Apis* bees carry DWV, 7 species show evidence of DWV replication (via a negative RNA strand), and the pathogenicity of DWV has been confirmed for two species of bumble bee, *Bombus terrestris*, and *Bombus pascuorum* (Genersch et al., 2006; Graystock et al., 2016b). DWV replication in non-bee species has also been reported; Levitt et al. (2012) found evidence of RNA replication in paper wasps, *Vespula spp.*, and Eyer et al., (2009) reported negative RNA strands in the small hive beetle, *Aethina tumida*. Research on alternative commercial pollinators such as, the alkali bee and the alfalfa leafcutter bee; have shown that food stores, eggs, and larvae, may be infected with numerous viruses shared with honey bees including DWV, IAPV, and BQCV (McArt et al., 2014; Singh, 2011). However, quantifying the impact of DWV infection on non-*Apis* insects can be difficult since for many species we only have access to the non-symptomatic adults. In depth research is needed to examine fitness impacts to non-*Apis* bees and to survey wild hosts that could become reservoirs of DWV leading to a complex web of infections.

5. Conclusion

1-In this study, a higher rate of DWV detection in non-*Apis* insects was associated to *Varroa*-positive areas.

2-Across-species transmission of DWV in our study was likely the result of shared flower resources (pollen and nectar) between honey bees and non-*Apis* insects.

3-The prevalence of DWV in *C. smaragdula* and *P. aurifer* in Hawaii is comparable to that of other species of bees and wasps from the mainland US, where the mite has been present for about 30 years.

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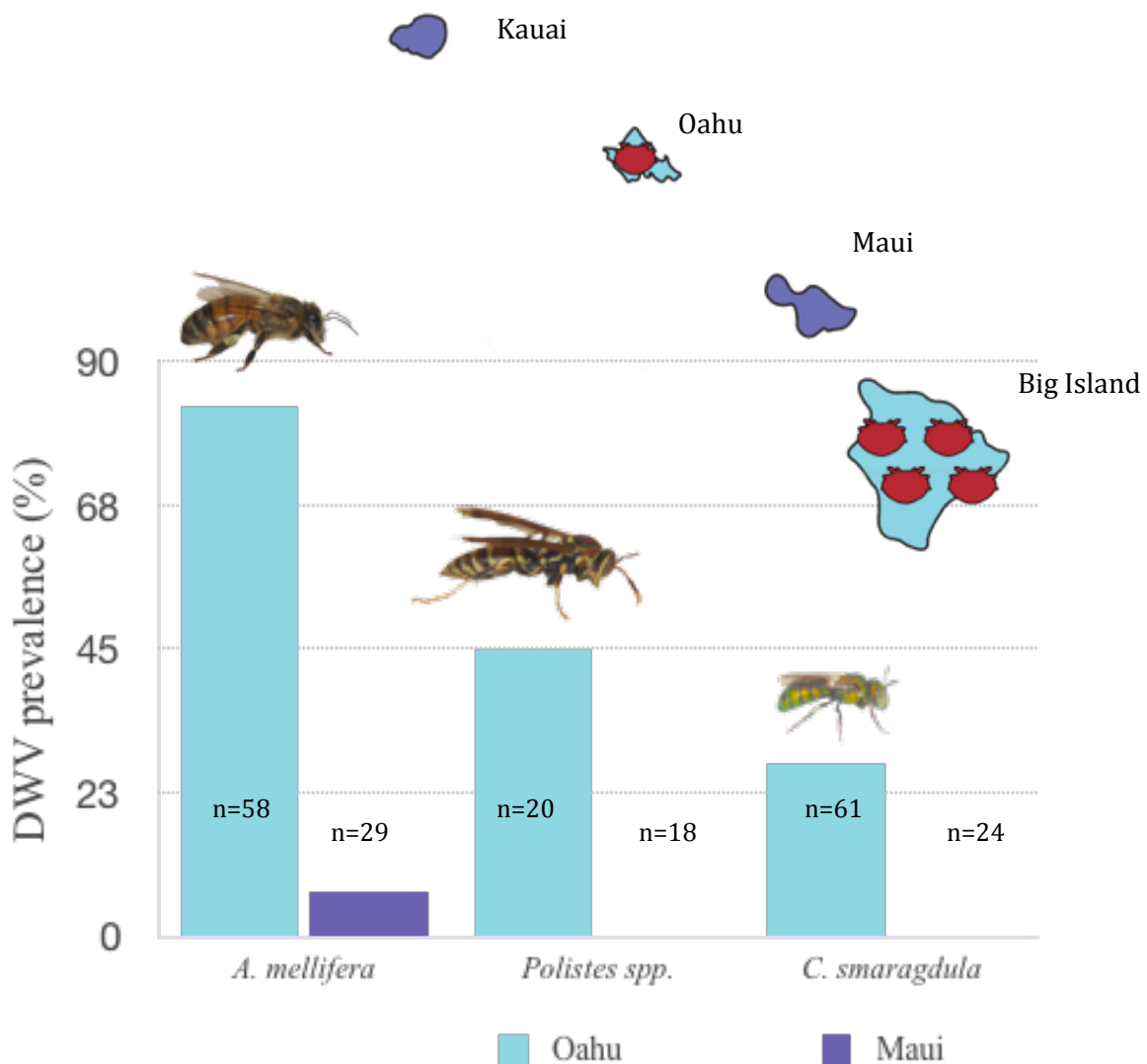


Fig 1. –Comparison of DWV prevalence in several species of Hymenoptera on Oahu, (light blue bars) and Maui (purple bar). The *Varroa* mite is well established on Oahu, and the DVW prevalence and viral load among honey bees is high compared to Maui where the mite is absent, and the prevalence and load of DWV is very low. Each column includes sample size for the group. Map shows the current distribution of *V. destructor* in the Hawaiian archipelago using mite icons and the same color-codes as the histogram bars; Oahu and Big Island, where the mite is present are light blue, and *Varroa*-free Kauai and Maui, purple.