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

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Abstract:
The spread of disease between closely related species is often times exacerbated by a vector. In this study, the *Varroa* mite, a pest exclusive to honeybees, has been shown to amplify the natural levels of the Deformed Wing virus, resulting in a spillover effect. The virus was found in non-Apis species on Oahu, and island where the mite is established. However, the virus was not found in non-Apis species on Maui, and island where the mite has not been introduced.

Keywords:
Varroa mite, spill over, deformed wing virus, European honeybee, pollinator health, viral transmission

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 be able to jump between species (Engering et al., 2013) and the Deformed Wing virus (Iflaviridae; DWV) affecting honeybees is no exception (Wilfert et al., 2016). An inconsistency exists were DWV appears to be a “generalist” RNA virus - detected in a broad range of hosts - yet interspecific transfer of viruses are infrequent between *Apis* species (Yanez et al., 2016). Furthermore, although DWV has frequently been detected among Hymenoptera it has always been in regions were a high prevalence of DWV exists, due to the presence of the *Varroa* mite (Budge et al., 2015; Traynor et al., 2016). The mite and the DWV appeared to have co-evolved with bees in the genus *Apis* (Wilfert et al., 2016); however, recent surveys have detected DWV in 21 insect genera (Levitt et al., 2013; Singh et al., 2010). Re-emerging viral diseases such as DWV represent one of the major threats to honeybee health, and the “spillover” of pathogens to wild bees also contribute in the current global pollinator decline

(Fürst et al., 2014, Genersch et al., 2006; Graystock et al., 2013a; Graystock et al., 2013b).

The Hawaiian Islands make for an ideal site to examine the role of *V. destructor* in the spillover of DWV to the wider hymenopteran community, since it is the only known location in the world where there are paired locations (islands) which are either *Varroa*-free or where the mite is established and that have similar geography, floral resources and insect community including the honeybee *A. mellifera,* which became established on the Hawaiian Islands in 1857 (Martin 2010). The aim of this study was to compare the prevalence of DWV in the Hymenopteran community on Oahu, an island where *Varroa* became established in 2007 and Maui, which remains free from *Varroa*. It has previously been established that DWV prevalence and load in honeybees is very high in Oahu and very low in Maui (Martin et al., 2012). We selected five species within two different Hymenoptera genera as representatives of the local community of flower visitors: *Ceratina smaragdula* (Apidae), *Polistes aurifer* and *Polistes exclamans* (Vespidae). *Ceratina* and *Polistes* were collected from *Varroa* free and *Varroa* infected islands. This study presents crucial evidence on the role the *Varroa* mite has played not only in the spread of DWV among honeybee populations, but also on the distribution and abundance of DWV in the non-*Apis* community in Hawaii.

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 invasive small carpenter bee *Ceratina smaragdula* (Apidae) which was first recorded in Hawaii in 1999 (Magnacca and King, 2013), and invasive 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*-infested island), and four sites on Maui, (*Varroa* free 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 introduced, abundant, and commonly found in urban and agricultural environments, where they overlap in resource use with *A. mellifera.* 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 wasp 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 an PRC clean? ependorf, which was submerged in liquid nitrogen before the sample was crushed using a mini pestle. Total RNA was then extracted from the resulting powder using the RNeasy Mini Kit (Qiagen) following manufacturers 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 RNA as well as endogenous control reactions to ensure RNA was intact. RT-PCR reactions contained 50ng RNA, 1x OneStep RT-PCR Buffer (QIAGEN), 400 µM each dNTP, 10units RNaseInhibitor (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 ran using a T100 Thermocycler (Bio-Rad) with an initial reverse transcription step 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. 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.

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 the significance of the data.

3. Results

We confirmed via RT-PCR that DWV prevalence was significantly higher ($p<0.0001$, Fisher's exact test) among honeybees from Oahu (83%) than Maui (7%) Whereas, the prevalence of DWV in the small carpenter bee (*Ceratina smaragdula*) and *Polistes spp* was 27% and 45% respectively on Oahu, but not detected in any of the 42 samples tested from Maui (Table 1).

4. Discussion

We confirmed that the presence of the *Varroa* mite in a honey bee population increases the prevalence of DWV (Fig. 1). This is indirectly due to the feeding habits of *Varroa* which has established a new viral transmission route, and selected for a particular virulent strain of DWV (Martin et al., 2012; Ryabov et al., 2014). Furthermore, the presence of the mites importantly also increases the viral load within the infested honeybee population by many orders of magnitude. The low prevalence of DWV on Maui supports the results from large-scale surveys conducted by Martin et al. (2012), where only four out of 33 colonies in Maui tested positive for DWV, all of which were very low level. This supports the theory that DWV arrived to the islands of Hawaii with the European honeybee, without the mite intervention, and remains a low prevalence pathogen in *Varroa*-free areas of Hawaii (Martin et al., 2012;) and a Scottish Island (Ryabov et al., 2014).

The prevalence of DWV in *Ceratina smaragdula* was strongly associated to the presence of the mite on that island; one out of four of the small carpenter bees sampled from Oahu tested positive for the virus, while those from Maui were all tested negative. Direct mite parasitism is not involved in the transmission of viral diseases to non-*Apis* species so it is unclear how solitary bees may acquire the virus. Floral resources are a potential contact point between species, since viable DWV particles have been found in pollen (Mazzei et al, 2014); both honeybees and *C. smaragdula* were found foraging on the same common garden herbs, crops, and ornamentals on both Oahu and Maui (personal observations). *Ceratina* were collected foraging from the exact same plants, and florets as honey bees were visiting, either *Scaevola sericea* (Naupaka) or *Heliotropium foertherianum*. Thus, the potential DWV transmission route via pollen or flowers existed on both islands. That is, the difference in DWV prevalence in *Ceratina* between Oahu and Maui is unlikely to be due to differences in habitat or floral resources.

Paper wasps (*Polistes* spp.) represent a common species often found to co-exist with honeybees. Our results indicate that at least 40% of wasps sampled from Oahu contained DWV. This high prevalence of DWV in species of social wasps is not uncommon; the prevalence DWV in yellow jackets (*Vespula* spp.) ranges from 30% to 57% (Evison et al. 2012, Levitt et al, 2013; Manley et al., 2013).

There are multiple possible transmission routes for DWV to spread from honey bees to wasps including: direct consumption of adult bees or bee larvae, raiding the colony for honey, and/or feeding on nectar from flowers where DWV positive bees may have fed (Narbona and Dirzo, 2010; Richter and Tisch, 1999). It has been proposed that the yellow jackets' tendencies to rob stored honey from honeybee hives may expose them to DWV (Evison et al. 2012). Unlike

Vespula, the *Polistes* workers prey on Lepidopteran caterpillars and are rarely if ever seen invading honeybee colonies, thus potentially limiting their point of contact with DWV to floral resources (Manley, Boots, and Wilfert, 2015).

There is a large gap in our knowledge with respect to transmission routes, virulence and impact of DWV on the health of wild bees, wasps, and other insects. Previous work on *Osmia cornuta* (Mazzei et al, 2014) showed solitary bees to be susceptible to horizontal infection of DWV via pollen. Hoover (2015) reported greater mortality on larvae and pupae of the alfalfa leafcutter bee, *Megachilidae rotundata*, that tested positive for viruses, indicating a possible effect during larval stages

The degree of cross species transmission of DWV has been described exclusively in geographical areas where the Varroa mite is already well established (Evison, et al., 2012; Levitt et al., 2013; Genersch et al., 2006; Fürst et al 2014; Graystock et al., 2013a, b) so DWV prevalence in the honey bee population is always very high (Traynor et al., 2016; Budge et al., 2015). The Hawaiian system provides the best possible study system. So, where DWV prevalence and load is very low in the honey bee population, (e.g. Maui) no DWV was detected in the two other Hymenoptera species studied. However, in a region (e.g. Oahu), where DWV prevalence and load is very high, spill over into these two species has occurred. Neither species has any direct contact with honey bees or the Varroa mite, so viral transmission must be indirect. This study indicates that the impact of Varroa extends well beyond the honey bees they infect. Studies are now required to determine the impact of DWV on the health of species into which DWV has spilled over from honeybees since there is increasing evidence of virus-induced behaviour change in insects (Han et al., 2015).

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Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

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DWV Positive Prevalence		
Species	**Oahu**	**Maui**
A. mellifera	83 % (n=58)	7% (n=29)
C. smaragdula	27% (n=61)	0% (n=24)
Polistes aurifer	45% (n=20)	NA
Polistes exclamans	NA	0% (n=18)

Table 1. –Comparison of DWV prevalence in several species of Hymenoptera on Oahu an island where the Varroa mite is well established and DVW prevalence and load among honeybees is very high and the island of Maui were the absence of the mite means the prevalence and load of DWV is very low.

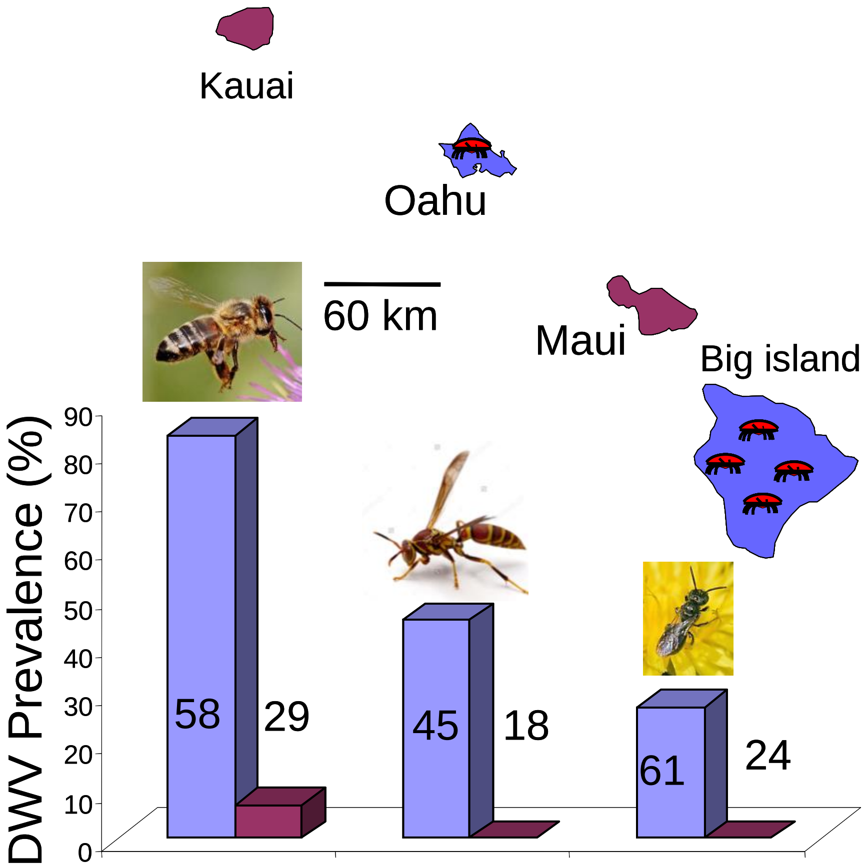

Figure 1- Comparative prevalence of DWV on Oahu (DWV+) (a), Maui (DWV-) (b), USA mainland based on Levitt et al. and Singh et al. (c), and (d).