A Comparison of *Wolbachia* Infection Frequencies in *Varroa* With Prevalence of Deformed Wing Virus

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Received 5 July 2016; Editorial decision 27 March 2017

Subject Editor: Sara Goodacre

**Abstract**

*Wolbachia* are widely distributed bacterial endosymbionts of arthropods and filarial nematodes. These bacteria can affect host fitness in a variety of ways, such as protecting hosts against viruses and other pathogens. Here, we investigate the possible role of *Wolbachia* in the prevalence of the deformed wing virus (DWV), a highly virulent pathogen of honey bees (*Apis mellifera*) that is transmitted by parasitic *Varroa* mites (*Varroa destructor*). About 180 *Varroa* mites from 18 beehives were tested for infection with *Wolbachia* and DWV. We first screened for *Wolbachia* using two standard primers (*wsp* and 16S rDNA), and found 26% of the mites to be positive for *Wolbachia* using the *wsp* primer and 64% of the mites to be positive using the 16S rDNA primer. Using these intermediate *Wolbachia* frequencies, we then tested for statistical correlations with virus infection frequencies. The analysis revealed a significant positive correlation between DWV and *Wolbachia* using the *wsp* primer, but no significant association between DWV and *Wolbachia* using the 16S rDNA primer. In conclusion, there is no evidence for an anti-pathogenic effect of *Wolbachia* in *V. destructor*, but weak evidence for a pro-pathogenic effect. These results encourage further examination of *Wolbachia*-virus interactions in *Varroa* mites since an increased vector competence of the mites may significantly impact disease outbreaks in honey bees.

**Key words:** deformed wing virus, honey bee, protective symbiont, *Varroa destructor*, *Wolbachia*

Honey bees (*Apis mellifera L.*), important pollinators of wild plants and cultivated crops, are essential for ecosystem function and global agriculture (Fontaine et al. 2005, Bascompte et al. 2006, Klein et al. 2007). Over the past decade, there has been a serious decline in bee populations reported in the European Union and other parts of the world (EFSA 2008; van Engelsdorp et al. 2009; Potts et al. 2010a,b; van Engelsdorp and Meixner 2010; van der Zee et al. 2012, 2014; Goulson et al. 2015). While a multitude of causative factors, such as parasites, pathogens, diet, quantity, quality, diversity and the exposure to pesticides, is being discussed (Alaux et al. 2010; Brodschneider and Crailsheim 2010; Genersch et al. 2010; Blacquière et al. 2012; Di Pasquale et al. 2013; Schumacher et al. 2013; Goulson 2013, 2015; Sandrock et al. 2014), infestation with the invasive ectoparasitic mite *Varroa destructor* is now considered the most significant cause for colony losses (Anderson and Trueman 2000, Genersch et al. 2010, Rosenkranz et al. 2010, Dainat et al. 2012a, Martin et al. 2012). *Varroa destructor* is originally a parasite of the Asian *Apis cerana*, in which it inflicts only limited damage. In eastern Russia, this mite jumped hosts to *A. mellifera*, followed by near-global spread to *A. mellifera* populations worldwide (Rosenkranz et al. 2010, Martin et al. 2012, Mondet et al. 2014).

*Varroa* mites are known as effective vectors for several honey bee viruses (Bowen-Walker et al. 1999, Chen et al. 2004, Sumpter and Martin 2004, Berthoud et al. 2010). They are also hypothesized to downregulate honey bee immune genes (Nazzi et al. 2012), which may consequently activate covert virus infections (Yang and Cox-Foster 2005). Viral infections, as a consequence of high *Varroa* mite infestation rates, strongly correlate with the collapse of colonies (Cox-Foster et al. 2007, Highfield et al. 2009, Berthoud et al. 2010, Genersch et al. 2010, Le Conte et al. 2010, Dainat et al. 2012b, Francis et al. 2013). The association between *V. destructor* and Deformed Wing Virus (DWV) in particular is being discussed as one of the main causes for colony losses (de Miranda and Genersch 2010, Schroeder and Martin 2012, Mordecai et al. 2015a). This virus is widely prevalent and has a nearly worldwide distribution.
(Ellis and Munn 2005, Gauthier et al. 2007). Even though DWV can be found in Varroa-free colonies or bee populations, it rarely leads to overt disease in the absence of Varroa mites. Yet, the presence of Varroa mites has been shown to dramatically increase the prevalence of DWV (Martin et al. 2012). In addition to being vectored by the mite, DWV is also known to survive and successfully replicate in the mite (Yue and Genersch 2005). Moreover, Varroa mites influence the balance of different DWV strains with differing virulence, potentially leading to an increase of more virulent strains (Martin et al. 2012, Mondet et al. 2014, Ryabov et al. 2014, Mordecai et al. 2015b). Investigations of the viral composition of Varroa mites and factors that influence virus prevalence and the mite’s vector competence will contribute to understanding the dynamics of the Varroa-virus system and the potential threat it presents to honey bees.

Recent progress in microbiome studies reveals that host microbial composition has a significant impact on both host susceptibility to pathogen infection, and pathogen performance in infected hosts (Kamata et al. 2013, Dennison et al. 2014, Vogt et al. 2015, reviewed in Bämmler and Sperrando 2016). Not only the complete microbiota but also symbiotic microorganisms, have been shown to impact host’s susceptibility and resistance. Of particular interest are intracellular bacteria of the genus Wolbachia (Werren et al. 2008). Wolbachia are widely distributed in terrestrial arthropods and filarial nematodes, with an estimated 20–70% of insect species infected (Hilgenboecker et al. 2008, Weinert et al. 2015). Empirical studies show that Wolbachia interferes with viruses and other pathogens inside the arthropod host, thereby either impeding or promoting the pathogen’s replication and survival (reviewed in Zug and Hammerstein 2015) as well as the host’s survival (Wong et al. 2011, Shokal et al. 2016). Anti-pathogenic effects of Wolbachia were demonstrated for Dengue virus (Moreira et al. 2009, Ban et al. 2010), West Nile virus (Hussain et al. 2013), and Plasmodium falciparum (Moreira et al. 2009), whereas neutral or pro-pathogenic effects of Wolbachia were shown for Brugia pahangi (Dutton and Sinkins 2005), Japanese encephalitis virus (Tsai et al. 2006), Drosophila C virus (Osborne et al. 2009) and Plasmidium gallinaceum (Baton et al. 2013).

Although Wolbachia has been previously detected in V. destructor (Pattabhiramaiah et al. 2010), information about possible inter-actions of Wolbachia with the mite’s virome is lacking. Here, taking a correlative approach, we compare infection frequencies of Wolbachia and DWV in V. destructor to investigate whether the presence of Wolbachia correlates with virus prevalence in the mites. We hypothesize that an anti-pathogenic effect of Wolbachia will result in lower virus frequencies among the Wolbachia-infected mites compared to Wolbachia-free mites, and a pro-pathogenic effect will result in the opposite pattern.

Materials and Methods

Mite Collection
Apis mellifera samples were collected in October 2011 from 18 hives in 9 different apiaries across Hesse (Germany) (2 colonies per apiary). From each hive, bees were shaken from a comb onto a plastic sheet, immediately transferred to a labeled vial, and frozen at −20 °C until analysis (Genersch et al. 2010). To collect the mites, individual bees of each sample were visually inspected and mites were removed manually with forceps.

DNA/RNA Extraction
Both DNA for Wolbachia detection and RNA for DWV detection were extracted from 10 individual mites per hive (180 total samples) according to the NucleoSpin TriPrep protocol (Macherey & Nagel, Düren, Germany).

PCR for Wolbachia Detection
Wolbachia infection rate is commonly measured by using primers for 16S rDNA, or primers for the outer surface-protein coding gene wsp (Marcon et al. 2011, Beckmann and Fallon 2012, Zha et al. 2014). Specifically, we used the wsp81f and wsp691r (Zhou et al. 1998) primers and the 16s rDNA76f and 16s rDNA1012r (O’Neill et al. 1992) primers (Supp Table 1 [online only]). PCR products were analyzed on an agarose gel. Approximately 10% positive amplicons of both primer pairs were sent for sequencing to confirm identity (Macrogen, Amsterdam, Netherlands) and sequences were deposited in GenBank (Supp Table 2 [online only]).

One-step RT-PCR for DWV Detection
DWV infection was detected with RT-PCR according to the protocol of OneStep-RT-PCR Kit and as previously described in Genersch (2005), using the primer pair F7, B11 (Supp Table 1 [online only]) for DWV detection. Around 10% positive amplicons were sent for sequencing to confirm identity and sequences were deposited in GenBank (Supp Table 2 [online only]). Only a short RNA region was used for DWV detection, so that even degraded DNA caused by long-term storage at −20 °C would provide a suitable template (Dainat et al. 2011).

Statistics
All analyses were performed using R 3.1.1. We performed the analysis with the infection frequency of each of the 18 hives, testing 10 individual mites per hive. To determine the relationship between Wolbachia prevalence and virus presence in Varroa mites, we used a completely balanced experimental design, i.e., equal sample numbers. In a correlative approach, we used the 16S r DNA, wsp and an additive combination of both primers, where there were 16 degrees of freedom left.

Results
Using the wsp primer, we detected Wolbachia in only 26% of V. destructor. Less than 70% of mites from one hive were found infected, and 44.4% of hives were Wolbachia free (Fig. 1A). In contrast, when using the 16S rDNA primer, 64% of V. destructor were positive for Wolbachia and all hives were found to be infected (Fig. 1B). Overall, Wolbachia prevalence in our samples varied from 30% to 70% in the majority of hives. Notably, not all mites with a positive signal for wsp were also positive with the 16S rDNA primer pair. For example, hive 5 showed higher infection frequencies with wsp than with 16S rDNA. DWV presence differed substantially between mites from different hives (Fig. 1C). We found a total DWV infection frequency of 61%. Only 5 out of 18 hives had a 100% DWV infection, while in two hives no DWV was detected. Sequencing of positive samples did not reveal false positive Wolbachia signals.

To test our hypothesis of the anti- or pro-pathogenic properties of Wolbachia, we ran a multivariate correlational analysis between the Wolbachia and DWV infection frequencies based on the two Wolbachia primers, wsp and 16S rDNA. The analysis suggests that Wolbachia has a pro-pathogenic function in Varroa, indicated by the positive linear correlation between Wolbachia prevalence and DWV presence, when Wolbachia infection was determined using the wsp primer (t = 3.774, P = 0.002, Fig. 2A). When Wolbachia

infection was determined using the 16S rDNA primer there was no significant correlation ($t = 0.050, P = 0.961$, Fig. 2B). The additive combination of both primers result in no significant correlation ($t = 0.127, P = 0.901$, Fig. 2C).

**Discussion**

*Wolbachia* infections have been recently reported in *Varroa* (Pattabhiramaiah et al. 2010), a vector of DWV (de Miranda and Genersch 2010). To explore the potential of *Wolbachia* bacteria mediating virus transfer, we investigated the *Wolbachia* frequency in *V. destructor*. Our results gave a first indication of *Wolbachia* having an effect on the prevalence of DWV in *Varroa*. The results were remarkably different depending on whether *wsp* or 16S rDNA primers were used. Most notably, mites tested for *Wolbachia* infection using the *wsp* primer resulted in far lower frequencies of infection than those tested with the 16S rDNA primer. This is in line with previous studies showing that *wsp* produces false negatives in certain *Wolbachia*-host systems, particularly when *Wolbachia* titers are low (Schneider et al. 2014). Therefore, the lower percentage of *Varroa* mites that tested positive for *Wolbachia* using the *wsp* primer was not unexpected. However, surprisingly, >20% of the *wsp* positive mites (10 out of 47) were negative for 16S rDNA. A possible explanation for this finding is that *Varroa* harbors more than one *Wolbachia* strains and that the different strains are detected differentially well by the two primers (de Oliveira et al. 2015). Alternatively, *wsp* may have detected, at least in some samples, a closely related species from the order of Rickettsiales (Simek et al. 2011). Whatever the reason for the difference between the primers, the results suggest that *Wolbachia* frequencies in our sampling of *V. destructor* are at intermediate levels.

Meta-analysis studies of terrestrial arthropods show that *Wolbachia* infection frequencies are usually either high (>90%) or low (<20%) (Hilgenboecker et al. 2008, Weinert et al. 2015). High *Wolbachia* infected arthropods include *Culex pipiens* (Rasgon and Scott 2003), *Aedes albopictus* (Kitrayapong et al. 2002, Joanne et al. 2015), *Drosophila simulans* (Kriesner et al. 2013), as well as the *Nasonia* species complex (Bordenstein et al. 2001). These examples of high infection frequencies are explained by the joint effects of high maternal transmission rates (95–100%) and *Wolbachia*-induced cytoplasmic incompatibility (Engelstaedter and Telschow 2009). Systems with low *Wolbachia* frequencies are less well understood. Low infection rates can be caused by low levels of maternal inheritance (80–90%) and by *Wolbachia* strains that cause male-killing of the insect, as reported in *Drosophila innubila* (Dyer and Jaenike 2004, Unckless and Jaenike 2012). Paternal and horizontal transmission of *Wolbachia* is considered to be negligible in all
mentioned species (Hoffmann and Turelli 1988, Schuler et al. 2016). Our observed intermediate Wolbachia frequencies in V. destructor differ from this general pattern and resemble the frequencies of the two-spotted spider mite Tetranychus urticae. This plant herbivore has been extensively studied for Wolbachia infection and showed a remarkable temporal and local variation in infection frequencies that ranged between 2.5% and 77.5%, with a median of /C24 30% (Chen et al. 2009, Yu et al. 2011, Su et al. 2012). Although the factors that drive Wolbachia infection dynamics in T. urticae are not well understood, this case suggests that Wolbachia frequencies can fluctuate around intermediate levels without spreading to fixation or going to extinction. This may also be the case in V. destructor, especially when considering the relatively young age of this system. Currently, the system is most likely not evolutionary stable, and instead, strong coevolutionary dynamics are dominating.

In this study, we give a first indication of whether Wolbachia infections in V. destructor correlates in a pro-pathogenic, neutral, or anti-pathogenic manner with DWV. Our analysis revealed a significant positive correlation between Wolbachia prevalence and DWV load measured by the wsp primer and DWV (Fig. 2). The results based on 16S rDNA and the additive combination of both primers, however, revealed no significant correlation, suggesting that the presence of Wolbachia is not correlated to DWV infection (Fig. 2). These results are puzzling at a first sight. A possible explanation is that the Wolbachia-infected mites differ with respect to Wolbachia titer, and possibly also with respect to the number of present Wolbachia strains. The positive correlation between wsp and DWV may then be the result of an increased susceptibility to the virus in mites with high Wolbachia titer and/or the presence of certain Wolbachia strains. However, a more in depth quantitative analysis is needed to answer whether high Wolbachia titers and/or certain Wolbachia strains really have a pro-pathogenic effect on DWV. Furthermore, the results strongly suggest careful choice of Wolbachia-primers in future studies, especially in systems with presumably low titers.

With our approach, we cannot rule out whether the Wolbachia found in mites results from a true infection of the mite or rather from ingested honey bee hemolymph, which was infected with Wolbachia. For example, in Metaseiulus occidentalis mites, mite starvation reduced and eliminated Wolbachia detection (Wu and Hoy 2012). Therefore, the question remains whether Wolbachia is a true endosymbiont of V. destructor. Future studies should consider starving mites or collecting the respective hosting bee to add to our understanding. Nevertheless, in this study we give a first indication of whether Wolbachia infections in V. destructor correlates in a pro-pathogenic, neutral, or anti-pathogenic with DWV.

In conclusion, we found strong evidence for intermediate Wolbachia frequencies in V. destructor, but based on the presented data, we can neither conclude nor disprove that Wolbachia affects DWV. We suggest that additional quantitative information on Wolbachia and the internal virus titers of corresponding mites will contribute to a better understanding of the role of Wolbachia in the composition of the V. destructor virome. Furthermore, future studies should consider artificial infection experiments in mite backgrounds with and without Wolbachia, as well as differing Wolbachia titers and DWV concentrations. These data may reveal the answer to the important question: Do Wolbachia infections in Varroa mites, and their temporal and spatial variation, play a role in the epidemiology of virus infections in bees and possibly influence colony losses?

Supplementary Data
Supplementary data are available at Journal of Insect Science online.
Acknowledgments
We gratefully thank H. Strasser, E. Leider, and U. Hubbe for sample collection. This project was funded by the Volkswagen Foundation’s Initiative on Evolutionary Biology with grants to G.J. and A.T. S.D., T.G., and G.J. are funded within the LOEWE Center for Insect Biotechnology and Bioresources (ZBi), granted by the German state of Hessen’s excellence initiative. G.J. as well as J.F.S. and A.T. were supported by the German Science Foundation (SPP 1399, JO 962/1-1 and TE 976/2-1).

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