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Short communication

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

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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; Spleen et al. 2013; Steinhauer et al. 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; 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 (Kamada et al. 2013, Dennision et al. 2014, Vogt et al. 2015, reviewed in Bäumler and Sperandio 2016). Not only the complete microbiota but also symbiotic microbes, 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, Bian 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 *Plasmodium gallinaceum* (Baton et al. 2013).

Although *Wolbachia* has been previously detected in *V. destructor* (Pattabhiramaiah et al. 2010), information about possible interactions 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 *wsp*81f and *wsp*691r (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 *Wobachia* 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*

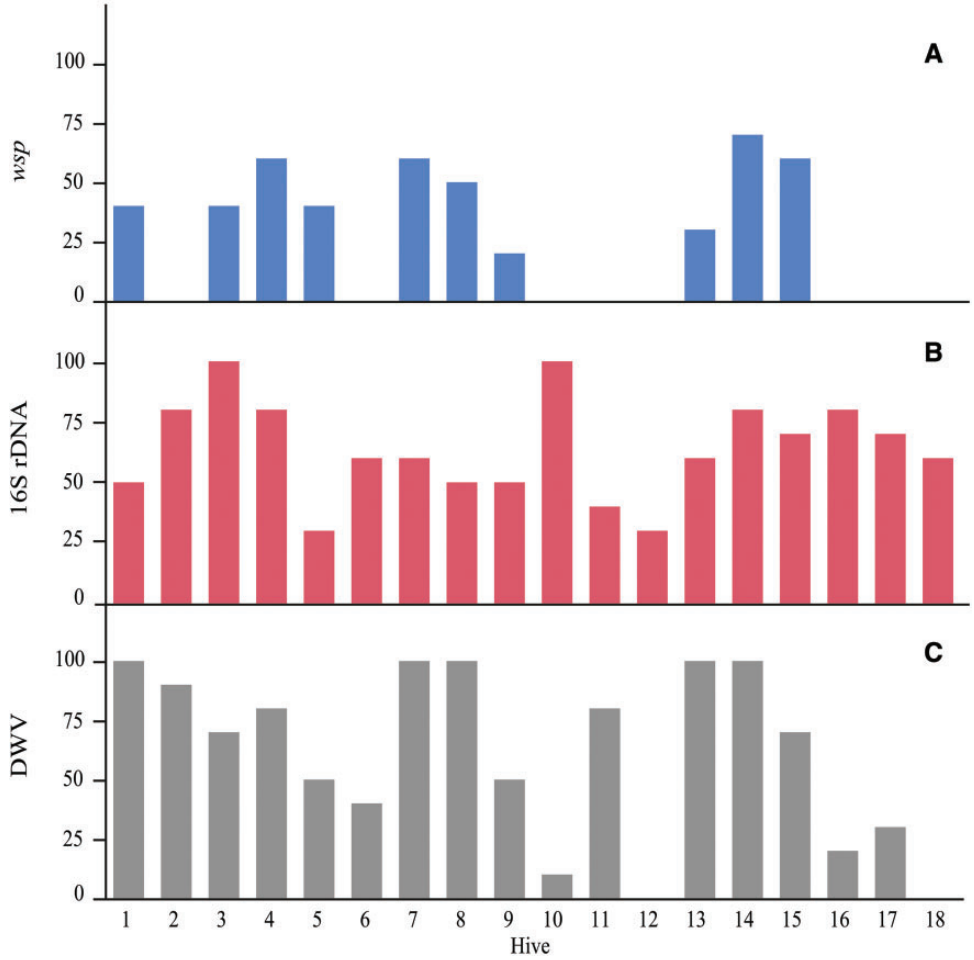

Fig. 1. Infection frequencies of *Wolbachia* (A) using *wsp* primer (B) using 16S rDNA primer and Deformed Wing Virus (C) using DWV primer in *Varroa* mites collected from 18 different hives from across Hesse, Germany in 2011. DNA and RNA from 10 mites from each hive were sampled, identical mites were used with all three primers.

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 (Simoes 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 (Engelstädter 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

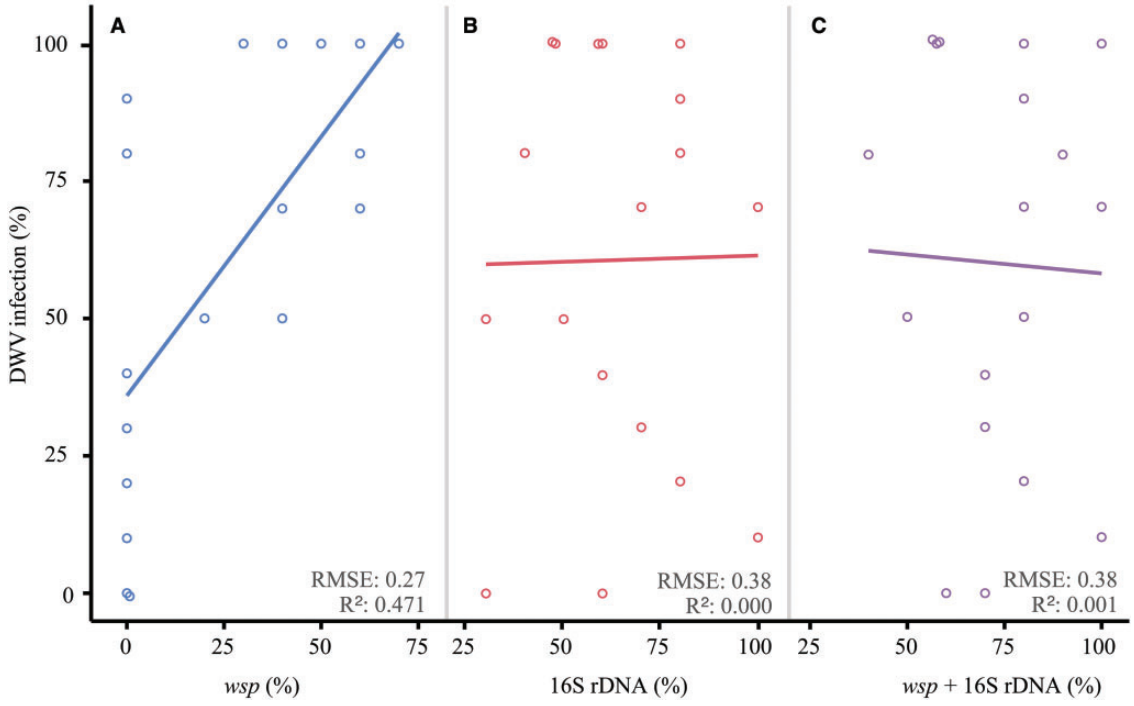

Fig. 2. Relationship between *Wolbachia* prevalence and Deformed Wing Virus (DWV) load in *Varroa* mites. When using the *wsp* primer (A) a positive linear correlation between *Wolbachia* prevalence and DWV load can be detected, in accordance with our hypothesis on pro-pathogenic function of *Wolbachia* infection in *Varroa*. No such relation can be detected upon using the 16S rDNA primer (B) and the additive combination of *wsp* with 16S rDNA primer (C). R^2 and RSME (root-mean-square error) given as estimates for goodness of fit. For better visual presentation overlapping points were jittered.

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 ~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* infection 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 the epidemiology of virus infections in bees and possibly influence colony losses?

Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

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