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Endosymbiotic bacteria in honey bees: Arsenophonus spp. are not transmitted transovarially

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One sentence summary: Arsenophonus endosymbiotic bacteria are not transmitted transovarially in honey bees.

ABSTRACT

Intracellular endosymbiotic bacteria are common and can play a crucial role for insect pathology. Therefore, such bacteria could be a potential key to our understanding of major losses of Western honey bees (Apis mellifera) colonies. However, the transmission and potential effects of endosymbiotic bacteria in A. mellifera and other Aps spp. are poorly understood. Here, we explore the prevalence and transmission of the genera Arsenophonus, Wolbachia, Spiroplasma and Rickettsia in Aps spp. Colonies of A. mellifera (N = 33, with 20 eggs from worker brood cells and 100 adult workers each) as well as mated honey bee queens of A. cerana, A. dorsata and A. florea (N = 12 each) were screened using PCR. While Wolbachia, Spiroplasma and Rickettsia were not detected, Arsenophonus spp. were found in 24.2% of A. mellifera colonies and respective queens as well as in queens of A. dorsata (8.3%) and A. florea (8.3%), but not in A. cerana. The absence of Arsenophonus spp. from reproductive organs of A. mellifera queens and surface-sterilized eggs does not support transovarial vertical transmission. Instead, horizontal transmission is most likely.

Keywords: Endosymbionts; Arsenophonus; honey bees; Apis mellifera; Apis dorsata; Apis florea

INTRODUCTION

Endosymbiotic bacteria are widespread in arthropods (Hilgenboeker et al. 2008; Duron et al. 2008). Their interactions with hosts are highly variable ranging from obligate (primary) to facultative (secondary) symbiosis and from parasitic to mutualistic symbiosis (Werren, Skinner and Huger 1986; Perotti et al. 2007). The bacteria of the genera Wolbachia (Alphaproteobacteria, Rickettsiales), Spiroplasma (Mollicutes, Entomoplasmatales), Rickettsia (Alphaproteobacteria, Rickettsiales) and Arsenophonus (Gammaproteobacteria, Enterobacteriales) are, in general, facultative endosymbionts and exhibit an extensive host range, including arthropods, nematodes, plants and vertebrates (Bové et al. 2003; Perlman, Hunter and Zchori-Fein 2006; Kozek and Rao 2007; Séméty et al. 2007; Bressan et al. 2009; Nováková, Hypša and Moran 2009; Wilkes et al. 2011).

Transmission of endosymbiotic bacteria to novel hosts is an apparent key element to understand their biological and potential benefits for their hosts. The endosymbionts are usually transmitted vertically. Their spread into the host population can be achieved, in some cases, by manipulating host reproduction, and may cause feminization, cytoplasmic incompatibility and male killing (Werren, Skinner and Huger 1986; Breeuwer and Werren 1990; Werren and O’Neill 1997; Hurst et al. 1999; Hurst and Jiggins 2000; Wilkes et al. 2011).

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and and Helmholtz 2009). Transmission may be transovarial, in which the bacteria are already present within the eggs (Rollend, Fish and Childs 2013) or transovum, in which the bacteria are present on the eggshells (Prado, Rubinoff and Almeida 2006). Endosymbiotic bacteria can also be transmitted horizontally through contact with infected individuals (Thao and Baumann 2004; Gehrer and Vorburger 2012; Ahmed et al. 2013) and from the environment (Bright and Bulguresci 2010), which is thought to enhance spread to distantly related host species (Russell and Moran 2005; Gehrer and Vorburger 2012).

In honey bees (Apis spp.), Wolbachia has been detected in workers of *Apis mellifera* capensis and *A. m. scutellata* (Jeyaprakash, Hoy and Allsopp 2003, 2009). Some Wolbachia strains have been characterized for *A. m. capensis* (Jeyaprakash, Hoy and Allsopp 2009), but in general virtually nothing is known about effects on host bees. It has been suggested that one of those strains may be responsible for thelytokous parthenogenesis (Hoy et al. 2003), but this hypothesis was later rejected (Latort, Moritz and Fuchs 2005). Two species of Spiroplasma, Spiroplasma apis (Mouches et al. 1983) and *S. melliferum* (Clark et al. 1985), have been characterized, and *S. apis* may be the causal agent of ‘May disease’ (Mouches, Bové and Albisetti 1984). Ricketsia in honey bees has been associated with milky hemolymph of infected workers (Wille and Pinter 1961), but later it was shown that the causal agent was filamentous virions (Clark 1978). *Arsenophonus* spp. have been detected in the gut microbiota (Babendreier et al. 2007; Corman et al. 2012) and in the hemolymph (Gauthier et al. 2015) of *A. mellifera* workers and seems to be abundant in the bees’ body surface (Aizenberg-Gershtein, Izhaki and Halpern 2013). Interestingly, *Arsenophonus* spp. appear to be more abundant in colonies displaying clinical symptoms of Colony collapse disorder (CCD; Corman et al. 2012). *Arsenophonus* spp. have also been recently found in Varroa destructor (Hubert et al. 2015), an ectoparasitic mite, which feeds on the honey bee hemolymph.

Since many endosymbionts may be beneficial for their hosts (Hansen et al. 2007; Oliver et al. 2010) and may also play a role in honey bee pathology (Evans and Armstrong 2006), it is important to better understand the role of endosymbionts in honey bees in light of *A. mellifera* colony losses (Neumann and Carreck 2010; Aebi and Neumann 2011). Indeed, depending on the strain, Wolbachia can protect other hosts against several vector-borne RNA viruses (Teixeira, Ferreira and Ashburner 2008) and can be regarded as part of host immunity (Zindel, Gottlieb and Hurst 2009). Since even bacterial strains may differ in their effects on hosts, e.g. strains of endosymbiont *Regiella insecticola* differ in their ability to protect pea aphids from parasitoid wasps (Hansen, Vorburger and Moran 2012), it is crucial to also investigate the phylogenetic relationship of the bacteria associated with different species of honey bees. In addition, this may reveal pattern on how these bacteria are interspecifically transmitted. Here, we explore the transmission, prevalence and phylogeny of the endosymbiotic genera *Arsenophonus*, Wolbachia, Spiroplasma and Rickettsia in honey bees *Apis* spp. and focus on transmission of the only detected *Arsenophonus* spp.

**MATERIALS AND METHODS**

**Sampling of Asian honey bee queens**

Twelve mated queens of *Apis cerana*, *A. dorsata* and *A. florea* each were collected from managed (*A. cerana*) or wild colonies in Chiang Mai and Phatthalung (Thailand), kept in 95% EtOH and stored at −80°C until further analyses.

**Sampling and screening of *Apis mellifera* colonies**

Mated *A. mellifera* queens were sampled from colonies that were tested positive for either Wolbachia, Spiroplasma, Rickettsia or *Arsenophonus*. For the screening of local *A. mellifera* colonies (*N* = 33, predominantly *A. m. carnica*), 20 eggs from worker brood cells and 100 adult workers from the middle frames were collected at three apiaries in Bern, Switzerland. All egg samples were homogenized with a sterile plastic pestle in 50μl of Chelex® solution (Bio-Rad, Hercules, CA, USA) for DNA extraction. Samples were incubated at 95°C for 20 min and centrifuged at 12,000 rpm for 2 min. Twenty fold dilutions were used for PCR reactions. DNA was extracted from the pooled worker samples following standard procedures (Evans et al. 2013) using the NucleoSpin® Tissue kit (Macherey-Nagel, Dueren, Germany) following the supplier’s guidelines. PCR was performed using the high-fidelity Kapa HiFi DNA Polymerase Kit (Kapa Biosystems, Woburn, MA, USA) following the manufacturer’s recommendations. Primers and PCR conditions were obtained from previous publications (Table S1, Supporting Informaiton). Parallel amplification of the honey bee Lys-1 gene (Harpur and Zayed 2013) was used to verify the DNA quality. Negative and positive controls were included in the analyses. PCR products were stained using GelRed for 30 min after electrophoresis in 1.2% agarose gel. Bands were visualized under UV light.

**Queen dissections and screening assays**

Laying queens were sampled from *A. mellifera* colonies, which were found positive for any of the tested bacteria (see above). The ovaries and digestive tracts of five *A. mellifera* queens were dissected following standard procedures (Carreck et al. 2013). The remains of the queen’s bodies were preserved for further analyses. The ovaries, spermathecae, digestive tracts, thoraces and heads from additional three *A. mellifera* queens were also dissected. DNA was extracted from the dissected queen’s body parts and the whole bodies of surface sterilized *A. cerana*, *A. dorsata* and *A. florea* queens (Table S2, Supporting Informaiton). Samples were homogenized using a Mixer Mill MM 300 (RETSCHE GmbH, Haan, Germany) machine in TN buffer with 3-mm metal beads. DNA extraction and PCR reactions were performed as detailed before. Positive PCR products were Sanger sequenced to ascertain the endosymbiont identity based on 98%–99% BLAST similarity. The *Arsenophonus* spp. sequences derived from ten queens (eight *A. mellifera*, one *A. dorsata* and one *A. florea*) were submitted to the European Nucleotide Archive (ENA) under the accession numbers LN555525-29 and LN890581-85.

**Quantification of *Arsenophonus* spp.**

Quantitative real-time PCR (qPCR) was used for the quantification of *Arsenophonus* sp. in *A. mellifera* queens. Reactions were performed in triplicate, in a total 12 μl final volume containing 20 ng of template DNA, 6 μl of 2X qPCR Master Mix and 0.2 μM of the forward and reverse primers, using the Kapa SYBR® Fast qPCR kit (Kapa Biosystems, Woburn, MA, USA). Primers were designed from the outer membrane protein assembly factor (yaeT) gene (Table S1, Supporting Informaiton). The cycling profile of the real-time qPCR consisted of 30 s incubation at 95°C, 40 cycles of 3 s at 95°C and 30 s at 57°C for annealing, extension and
Transmission pathway of Arsenophonus spp.

To test if Arsenophonus spp. can be transmitted vertically in honey bees, twenty additional queen-laid eggs were taken from each of three Arsenophonus spp. positive Apis mellifera colonies, and subjected to two treatments as follows: (i) ten eggs were surface sterilized in 3% sodium hypochlorite for 1 min and rinsed three times in distilled water for 1 min (Vaughn 1971), (ii) the 10 remaining eggs were not treated prior to DNA extraction (= control). In addition, to test if there is a relation between the presence of Arsenophonus spp. in queen’s bodies and eggs, ten non-surface-sterilized eggs from all eight Arsenophonus-positive colonies were individually analyzed. Ex extractions with Chelex® solution from individual eggs, PCR and gel electrophoresis as well as sequencing, were performed under the same conditions as described above.

Phylogenetic analyses

To get a first approach to the phylogenetic relationship of Arsenophonus spp. and the Apis hosts, the 16S rRNA sequences obtained from queens screening were aligned using the MUSCLE program (Edgar 2004a,b) and compared using the maximum likelihood method with the MEGAS5.2 program (Tamura et al. 2011), under the Kimura two-parameter with a discrete gamma distribution model (K2 + G), because this model was the best suited one for our dataset by using the model testing option implemented in the MEGAS5.2 program. The tree topology was evaluated by bootstrap resampling (1000 times).

RESULTS

Arsenophonus spp. were the only endosymbiont tested positive in our samples consisting of eggs, workers and queens. It was detected in 24.2% of the surveyed Apis mellifera colonies (eight out of 33). This result is based on egg screening, in which Arsenophonus identity was confirmed by Sanger sequencing (see below). Since the worker screening was leading to false positive detections due to unspecific amplification of other gammaproteobacteria, e.g. Gilliamella apicala, it was not taken into consideration for the frequency analyses. All queens sampled from those Arsenophonus-positive colonies tested positive as well. Arsenophonus spp. were also detected in a single A. dorsata and A. florea queen (8.3%), but not in the 12 A. cerana queens.

The role that A. mellifera queens may play in the transmission of Arsenophonus spp. was investigated by analyzing (i) the location of Arsenophonus spp. in different queen body parts, (ii) the relation between the Arsenophonus spp. density in the queen’s bodies and the number of Arsenophonus spp.-positive eggs and (iii) the location of bacteria within or on the surface of the eggs. First, the PCR-based diagnostics of the 16S rRNA gene sequences did neither detect Arsenophonus spp. in the ovaries of the queens (N = 8), nor in the spermathecae, thoraces and heads of surface-sterilized queens (N = 3). Arsenophonus spp. was only detected in the digestive tracts of the queens. Second, the qPCR-based assays (yaT gene) indicate a poor relationship between the Arsenophonus spp.-positive eggs and respective loads in the queen’s bodies (Pearson r = 0.066, df = 6, two tailed P = 0.88; Table S3, Supporting Information). Third, in order to investigate whether Arsenophonus spp. could be transovarially transmitted, the presence of Arsenophonus spp. was PCRs diagnosed in surface-sterilized eggs (N = 30) and in untreated ones (N = 30), collected from three A. mellifera queens (Table S3, Supporting Information). While 41% of untreated eggs were positive for Arsenophonus spp., the bacteria were not detected in any of the surface-sterilized eggs (Pearson Chi Square test, χ² = 15, df = 1, P < 0.001).

The amplicons originating from the 16S rRNA gene from the eight A. mellifera queens and from the single positive queens of A. dorsata and A. florea were sequenced. The Arsenophonus spp. identity was confirmed by high similarity (98%–99%) to sequences deposited in GenBank (accession numbers: FN545282, DQ837612). The phylogenetic tree shows that all sequences from the honey bee queens cluster together with previous sequences from honey bee workers and other insect hosts of Arsenophonus (Fig. 1). Interestingly, despite the low bootstrap support inside the Arsenophonus clade, the Arsenophonus spp. sequences originating from the Asian A. dorsata and A. florea are grouped together (bootstrap value 70%).

DISCUSSION

The absence of Wolbachia, Spiroplasma and Rickettsia in our samples supports a low and/or seasonal prevalence of these bacteria in the genus Apis. Arsenophonus spp. was found in A. mellifera (24.2% of colonies) and also in queens of A. dorsata (8.3%) and A. florea (8.3%), but not in A. cerana. The phylogenetic analyses and low prevalence in sympatric A. cerana suggest that horizontal transmission from other honey bees is unlikely to be the source of Arsenophonus spp. in A. dorsata and A. florea. Instead, it appears as if Arsenophonus spp. are repeatedly acquired from the environment. The data also show that transovarial transmission of Arsenophonus spp. is unlikely in A. mellifera.

The low incidence of Arsenophonus and the non-detection of Wolbachia, Spiroplasma and Rickettsia in the four honey bee species confirm the sporadic presence of those bacteria in the genus Apis. The non-detection of Wolbachia and Rickettsia is in line with the most comprehensive microbial surveys in A. mellifera (Cox-Foster et al. 2007; Martinson et al. 2011). The only report of Wolbachia in European A. mellifera is based on PCR results, but unfortunately without confirmation by sequencing (Pattah-hiramia et al. 2011). Wolbachia has been reported in African honey bees (Jeyaprakash, Hoy and Allsopp 2003; Jeyaprakash, Hoy and Allsopp 2009); nevertheless, its occurrence in European and Asian honey bees is still uncertain. The absence of Spiroplasma spp. in our tested colonies and queens may in part be explained by strong seasonal and regional variation of Spiroplasma species in honey bees as documented in Brazil and the USA (Schwarz et al. 2014).

The occurrence of Arsenophonus spp. in colonies/queens of A. mellifera (24.2%), A. dorsata (8.3%), A. florea (8.3%) and A. cerana (0 %) is in agreement with previous reports showing an irregular pattern of incidence of this bacterium among honey bee colonies (Babendreier et al. 2007; Cormnan et al. 2012). In general, the incidence of the genus Arsenophonus in field collected insects is estimated to be around 5% (Duron et al. 2008), and its prevalence

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Figure 1. Maximum likelihood tree of Arsenophonus spp. isolates from A. mellifera (filled triangle), A. dorsata (filled circle) and A. florea (filled square) queens. The 587 bp 16SrRNA alignment from the sampled queens and isolates from other Arsenophonus were retrieved from the NCBI-GenBank. Morganella morganii and Pantoea agglomerans (Enterobacteriaceae) were used as outgroups. The bar indicates the genetic distance scale (number of nucleotide differences per site). Bootstrap values above 50 are shown in the corresponding nodes.

can also vary between populations of the same host species. For instance, in the yellow crazy ant (Anoplolepis gracilipes) the incidence of Arsenophonus spp. varies from 0%–50.8% between different populations (Sebastien, Gruber and Lester 2012). Therefore, the differential prevalence levels of Arsenophonus spp. in the four studied Apis species are well within the previously reported variation.

The detection of Arsenophonus spp. in mated queens highlights a possible vertical transmission pathway mediated by the queen reproductive organs. However, the absence of Arsenophonus spp. from the ovaries, the spermathecae and sodium hypochlorite treated eggs, taken together with the non-significant correlation between the Arsenophonus spp. loads of queens and the number of positive eggs, do not support a transovarial transmission pathway that supposes the acquisition of the bacteria during oogenesis (Burgdorfer and Varma 1967). For secondary (facultative) endosymbionts, the colonization of the host’s ovaries is frequent (Kose and Karr 1995; Goto, Anbutsu and Fukatsu 2006; Matsuura et al. 2012; Genty et al. 2014), but not exclusive, as secondary endosymbionts could also freely circulate in the hemolymph of the insect host (Cheng and Aksoy 1999; Goto, Anbutsu and Fukatsu 2006). Therefore, the detection of Arsenophonus spp. in the digestive tract of A. mellifera queens and on the surface of non-sterilized eggs suggests that, if vertically transmitted, the transmission might occur, for instance, during the oviposition (transovum) (Andreadis 1987). However, exclusive horizontal transmission may also be possible as in case of A. nasoniae (Werren, Skinner and Huger 1986). Indeed, the presence of Arsenophonus spp. on the egg surface can be also explained by the horizontal transmission through contaminated combs and/or contact with nurse bees. Then, one may consider Arsenophonus spp. as part of the honey bee gut microbiota, which seems to be exclusively horizontally transmitted through contact with nestmates after emergence (Martinson, Moy and Moran 2012; Powell et al. 2014). There is also evidence that this bacterium can cross the gut barrier and circulate in the honey bee hemolymph, as it was previously found associated with milky white hemolymph symptoms traditionally attributed to A. mellifera filamentous virus infections (Gauthier et al. 2015).

The finding of Arsenophonus spp. in V. destructor also implies that this mite might play a role as a vector in the horizontal transmission of Arsenophonus spp. between honey bees at both individual and colony level (Hubert et al. 2015). Indeed, horizontal transmission might also involve other bee species. Phylogenetic analyses of the Arsenophonus spp. from honey bees clustered together with those isolated from solitary bees such as Megachile rotundata (McFrederick, Mueller and James 2014), Colletes cucullatus and C. halophilus (Gert et al. 2015). The phylogenetic analysis of our Arsenophonus spp. isolates from A. mellifera (Fig. 1) also supports these previous results. The findings of the bacterium
on the body surface of honey bees (Aizenberg-Gershtein, Izhaki and Halpern 2013), as well as in corbicular pollen (Corby-Harris, Maes and Anderson 2014) support the potential for transmission between bees when collecting nectar and pollen from shared flowers.

In this scenario, the occurrence of Arsenophonus spp. in the digestive tract of honey bees may constitute an oral-faecal route of transmission for this bacterium. Similarly, the oral-faecal transmission has been suggested for Wolbachia in the leaf-cutting ant Acromyrmex echinatior (Frost et al. 2014). Oral-faecal transmission has particular potential in the social insects because of the high population density and hygienic behavior in their colonies. Further, research should be undertaken to clarify the impact of Arsenophonus spp. infections on honey bee health.

Regarding the honeybee’s Arsenophonus from A. dorsata and A. florea, the phylogenetic analysis from the 16S rRNA gene sequences shows some degree of divergence with Arsenophonus from A. mellifera (Fig. 1). This might be explained by the large distance between geographical origins of the samples. In general, there does not appear to be a relation between Arsenophonus diversification and the social habits of the host as bees, ants or aphids. However, since enterobacteria can carry several variable rRNA copies (Moran, McCutcheon and Nakabachi 2008; Sorfova, Skerikova and Hyspa 2008), those results should be confirmed with the use of additional phylogenetic markers.

In conclusion, this study shows for first time the presence of Arsenophonus spp. in queens, belonging to three different honey bee species. Taken together, the data do not support the vertical transmission of these bacteria through the queen, but the occurrence in the bees’ guts rather support a horizontal transmission following contact with nest mates or contaminated wax combs.

**SUPPLEMENTARY DATA**

Supplementary data are available at FEMSLE online.

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**REFERENCES**

Aebi A, Neumann P. Endosymbionts and honey bee colony losses? Trends Ecol Evol 2011;26:494.

Ahmed Z, De Barro J, Ren X et al. Evidence for horizontal transmission of secondary endosymbionts in the Bemisia tabaci cryptic species complex. PLoS One 2013;8:e53084.

Aizenberg-Gershtein Y, Izhaki I, Halpern M. Do honeybees shape the honeybee community composition in floral nectar? PLoS One 2013;8:e67556.

Andreadis, TG. Transmission. In: Fuxa JR, Tanada Y (eds). Epizoology Of Insect Diseases. New-York: John Wiley and Sons, 1987, 159–76.

Babendreier D, Joller D, Romeis J et al. Bacterial community structures in honeybee intestines and their response to two insecticidal proteins. FEMS Microbiol Ecol 2007;59:600–10.

Bove JM, Renaudin J, Saillard C et al. Spiroplasma citri, a plant pathogenic mulligote: relationships with its two hosts, the plant and the leafhopper vector. Annu Rev Phytopathol 2003;41:483–500.

Breeuwer AJ, Werren J. Microorganisms associated with chromosome destruction and reproductive isolation between two insect species. Nature 1990;346:558–60.

Bressan A, Sémétoy O, Arneodo J et al. Vector transmission of a plant-pathogenic bacterium in the Arsenophonus clade sharing ecological traits with facultative insect endosymbionts. Phytopathology 2009;99:1289–96.

Bright M, Bulgheresi S. A complex journey: transmission of microsymbionts. Nat Rev Microbiol 2010;8:218–30.

Burgdorfer W, Varma G. Trans-stadial and transovarial development of disease agents in arthropods. Ann Rev Entomol 1967;12:347–76.

Carreck N, Andre M, Brent C et al. Standard methods for Apis mellifera anatomy and dissection. J Apic Res 2013;52:1–40.

Cheng Q, Aksoy S. Tissue tropism, transmission, and expression of foreign genes in vivo in midgut symbionts of tsetse flies. Insect Mol Biol 1999;8:125–32.

Clark T, Whitcomb R, Tully J et al. Spiroplasma melliferum, a new species from the honeybee (Apis mellifera). Int J Syst Bacteriol 1985;35:296–308.

Clark T. A filamentous virus of the honey bee. J Invertebr Pathol 1978;32:332–40.

Corby-Harris V, Maes P, Anderson K. The bacterial communities associated with honey bee (Apis mellifera) foragers. PLoS One 2014;9:e95056.

Cormann R, Tarpy D, Chen Y et al. Pathogen webs in collapsing honey bee colonies. PLoS One 2012;7:e43562.

Cox-Foster D, Conlan S, Holmes E et al. A metagenomic survey of microbes in honey bee colony collapse disorder. Science 2007;318:283–7.

Duron O, Bouchon D, Boutin S et al. The diversity of reproductive parasites among arthropods: Wolbachia do not walk alone. BMC Biol 2008;6:27.

Edgar R. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics 2004a;5:113.

Edgar R. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 2004b;32:1792–7.

Engelstädter J, Hurst G. The ecology and evolution of microbes that manipulate host reproduction. Ann Rev Ecol Evol System 2009;40:127–49.

Evans J, Armstrong T. Antagonistic interactions between honey bee bacterial symbionts and implications for disease. BMC Ecol 2006;6:4.

Evans J, Schwarz R, Chen Y et al. Standard methods for molecular research in Apis mellifera. J Apic Res 2013;52:1–54.

Frost C, Pollock S, Smith J et al. Wolbachia in the flesh: Symbiont intensities in germ-line and somatic tissues challenge the conventional view of Wolbachia transmission routes. PLoS One 2014;9:e95122.

Gauthier L, Cornman S, Hartmann U et al. The Apis mellifera filamentous virus genome. Viruses 2015;7:3798–815.

Gehrer L, Vorburger C. Parasitoids as vectors of facultative bacterial endosymbionts in aphids. Biol Lett 2012;8:613–5.

Genty L, Bouchon D, Boutin S et al. Wolbachia infect ovaries in the course of their maturation: last minute passengers and priority travellers? PLoS One 2014;9:e94577.
Genomic basis of endosymbionts reveals taxon-specific distribution patterns among bees (Hymenoptera, Anthophila). FEMS Microbiol Ecol 2015;91:e00471.

Goto S, Anbutusu H, Fukatsu T. Asymmetrical interactions between Wolbachia and Spiroplasma endosymbionts coexisting in the same insect host. Appl Environ Microb 2006;72:4805–10.

Hansen A, Jeong G, Paine T et al. Frequency of secondary symbiont infection in an invasive psyllid relates to parasitism pressure on a geographic scale in California. Appl Environ Microb 2007;73:7531–5.

Hansen A, Vorburger C, Moran N. Genomic basis of endosymbiont-conferring protection against an insect parasitoid. Genome Res 2012;22:106–14.

Harpur B, Zayed A. Accelerated evolution of innate immunity proteins in social insects: adaptive evolution or relaxed constraint? Mol Biol Ecol 2013;30:1665–74.

Hilgenboecker K, Hammerstein P, Schlattmann P et al. How many species are infected with Wolbachia? – a statistical analysis of current data. FEMS Microbiol Lett 2008;281:215–20.

Hubert J, Erban T, Kamler M et al. Bacteria detected in the honeybee parasitic mite Varroa destructor collected from bee hive winter debris. J Ap I micr o 2015;119:640–54.

Hurst G, Jiggins F, von der Schellenburg J et al. Male–killing Wolbachia in two species of insect. P Roy Soc Lond B Bio 1999;266:735–40.

Hurst G, Jiggins F. Male-killing bacteria in insects: mechanisms, incidence, and implications. Emerg Infect Dis 2000;6:329–36.

Jaenike J, Unckless R, Cockburn S et al. Adaptation via symbiosis: recent spread of a Drosophila defensive symbiont. Science 2010;329:212–5.

Jeyaprakash A, Hoy M, Allsopp M. Bacterial diversity in worker Wolbachia pipientis. Mol Ecol 2009;18:165–74.

Kose H, Karr T. Organization of Spiroplasma and other spiroplasmas for honey-bees in southwestern France. Ann Inst Pasteur Microbiol 1984;135:151–5.

Kozek W, Rao R. The discovery of Wolbachia in arthropods and nematodes – a historical perspective. In: Hoerauf A; Rao R (eds). Wolbachia Endosymbiont in the workers of European honeybee. Apis mellifera carnica. EJ Bio 2011;7:81–5.

Perlman SJ, Hunter MS, Zchori-Fein E. The emerging diversity of Rickettsia. P Roy Soc Lond B Bio 2006;273: 2097–106.

Perotti M, Allen J, Reed D et al. Host-symbiont interactions of the primary endosymbiont of human head and body lice. FASEB J 2007;21:1058–66.

Powell J, Martinson V, Urban-Mead K et al. Routes of acquisition of the gut microbiota of the honey bee Apis mellifera. Appl Environ Microb 2014;80:7378–87.

Prado SS, Rubinson D, Almeida RPP. Vertical transmission of a pentatomid caeca-associated symbiont. Ann Entomol Soc Am 2006;99:577–85.

Rollend L, Fish D, Childs J. Transovarial transmission of Borrelia spirochetes by ixodes scapularis: a summary of the literature and recent observations. Ticks Tick Borne Dis 2013;4:46–51.

Russell J, Moran N. Horizontal transfer of bacterial symbionts: Heritability and fitness effects in a novel aphid host. Appl Environ Microbiol 2005;71:7987–94.

Schwarz R, Teixeira E, Tauber J et al. Honey bee colonies act as reservoirs for two Spiroplasma facultative symbionts and incur complex, multiyear infection dynamics. MicrobiologyOpen 2014;3:341–55.

Sebastien A, Gruber M, Lester P. Characterization of a c-3 Proteobacteria responsible for the syndrome “basses richesses” of sugar beet transmitted by Pentatistiridius sp. (Hemiptera: Cixiidae). Phytopathology 2007; 97: 72–78.

Sémétoy O., Gatineau F., Bressan A. et al. Characterization of a c-3 Proteobacteria responsible for the syndrome “basses richesses” of sugar beet transmitted by Pentatistiridius sp. (Hemiptera: Cixiidae). Phytopathology 2007; 97: 72–78.

Sorfova P, Skerikova A, Hyspa V. An effect of 16S rRNA intercristronic variability on coevolutionary analysis in symbiotic bacteria: molecular phylogeny of Arsenophonus triatominem. Syst Appl Microbiol 2008;31:88–100.

Tamura K, Peterson D, Peterson N et al. MEGAS: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 2011;28:2731–9.

Teixeira L, Ferreira A, Ashburner M. The bacterial symbiont Wolbachia induces resistance to RNA viral infections in Drosophila melanogaster. PLoS Biol 2008;6:e1000002.

Thao ML, Baumann P. Evidence for multiple acquisition of Arsenophonus by whitely species (Sternorrhyncha: Aleyrodiidae). Curr Microbiol 2004;48:140–4.
Vaughn J. Cell culture media and methods. In: Vago C. (ed). Invertebrate Tissue Culture. New York: Academic Press, 1971, 4–40.
Ward L, Waite R, Boonham N et al. First detection of Kashmir bee virus in the UK using real-time PCR. *Apidologie* 2007;38: 181–90.
Werren J, Baldo L, Clark M. *Wolbachia*: master manipulators of invertebrate biology. *Nat Rev Microbiol* 2008;6:741–51.
Werren J, O’Neill S. Evolution of heritable symbionts. In: O’Neill S, Hoffmann A, Werren J (eds). *Influential Passengers: Inherited Microorganisms and Arthropod Reproduction*. New York: Oxford Univ. Press, 1997, 1–41.
Werren JH, Skinner SW, Huger AM. Male-killing bacteria in a parasitic wasp. *Science* 1986;231:990–2.
Wilkes T, Duron O, Darby A et al. The genus *Arsenophonus*. In: Bourtzis EZ-FK (ed). *Manipulative Tenants*. Florida: CRC Press, 2011, 225–44.
Wille H, Pinter L. Untersuchungen über bakterielle Septikämien der erwachsenen Honigbienen in der Schweiz. *Bull Apic* 1961;4:141–61.
Zindel R, Gottlieb Y, Aebi A. Arthropod symbioses: a neglected parameter in pest- and disease-control programmes. *J Appl Ecol* 2011;48:864–72.