The dynamics of biological Russian dolls: investigating the causes and consequences of variation in symbiont density in citrus mealybugs

Jasmine Frances Parkinson

Submitted in accordance with the requirements for the degree of

Doctor of Philosophy

The University of Sussex

School of Life Sciences

September 2015

The candidate confirms that the work submitted is her own, except where work which has formed part of a jointly authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others. This thesis, whether in the same or a different form, has not been previously submitted to this or any other university for a degree.

Chapter 4 contains work from a jointly authored publication:

PARKINSON, J. F., GOBIN, B. & HUGHES, W. O. H. 2014. Short-term heat stress results in diminution of bacterial symbionts but has little effect on life history in adult female citrus mealybugs. Entomologia Experimentalis et Applicata, 153, 1-9.

Author contributions are as follows: JFP & WOHH designed the study. JFP performed laboratory and molecular work, statistical analysis and manuscript drafting. WOHH assisted with statistical analysis. WOHH and BG supervised the work and assisted with manuscript drafting.

Chapter 6 contains work from a jointly authored manuscript:

Jasmine F. Parkinson, Thierry Gosselin, Julia Jones, Bruno Gobin & William O.H. Hughes (2015)

Author contributions are as follows: JFP, JJ & WOHH designed the study. JFP performed molecular work with assistance from JJ. TG performed initial bioinformatics analyses. JFP performed manuscript drafting and figure editing.

WOHH and BG supervised the work and assisted with manuscript drafting.

Signed:

Acknowledgements

The completion of this thesis would not have been possible without the generous professional and personal support which I have received over the past four years. I wish to firstly thank the BBSRC for funding my project, and the Universities of Leeds and Sussex, and the Proefcentrum voor Sierteelt Horticultural Research Centre for hosting my research. I would like to thank Professor Bill Hughes for his supervision, and Doctor Bruno Gobin and Doctor Alan Stewart for their co-supervision.

I extend my gratitude to Marc Vissers and Joachim Audenaert for providing my Belgian mealybug specimens, and for their help during my greenhouse trial, and Doctor Laura Ross for providing many of the mealybug specimens used for my metagenomics study. The Hughes Laboratory members have been my friends and colleagues throughout my PhD, and I would like send my thanks to Doctor Crystal Frost, Doctor Peter Graystock, Doctor Rowena Mitchell, Doctor Sophie Evison, Doctor Kirsten Foley and Doctor Katherine Roberts for their advice during my first year at the University of Leeds. I also wish to thank Victoria Norman, Christopher Tranter, Doctor Tobias Pamminger, David Treanor and Norma Neszi for their feedback on my manuscripts and oral presentations and stepping in to attend to my mealybugs in my absence.

I wish to thank my family, Jackie Parkinson, Tony Parkinson, Sarah Lawson and Matthew Lawson for their endless love and support when I needed it most.

And finally, I would like to extend my deepest gratitude to my love, James Lawrence.

“If you trust in yourself… and believe in your dreams… and follow your star… you'll still get beaten by people who spent their time working hard and learning things and weren't so lazy.”

— *Terry Pratchett*

Abstract

Endosymbiosis has been a major driver of evolutionary diversification of eukaryotes. However, symbiosis can create conflict between partners and symbiont density is often tightly regulated within hosts to ensure optimal functioning of the holobiont. The horticultural pest insects, citrus mealybugs, make an intriguing and potentially-powerful case study for endosymbiosis, harbouring two obligate, nutritional, vertically-transmitted bacteria: *Tremblaya princeps* and *Moranella endobia*, in a nested mutualism. In this thesis, I examine the variation in the density of each of these obligate symbionts in citrus mealybugs under controlled environmental conditions, using qPCR, as well as the diversity of facultative symbionts that infect the mealybugs using next-generation sequencing and conventional targeted PCR. Citrus mealybugs were found to harbour *Wolbachia*, *Spiroplasma*, *Cardinium* and *Rickettsia*, which have been found to impact the fitness of their hosts in other insect species, whereas long-tailed mealybugs were not found to harbour any of these bacteria, but the symbiont communities in both species were found to be dominated by their obligate symbionts. The density of the two obligate symbionts varied by up to six-fold between different populations kept under identical environmental conditions and a hybridisation experiment indicated that *M. endobia* and *T. princeps* density may be controlled by symbiont and host genotype respectively. However, symbiont density was not found to correlate with life-history traits in the laboratory, the ability of mealybugs to exploit different plant species, or the susceptibility of the mealybugs to insecticide and artificial reduction of symbiont density by heat-stress also had no effect on host fitness. Citrus mealybugs harbour seemingly superfluous symbionts with no clear fitness costs or benefits.

Table of Contents

“In the beginning the Universe was created. This has made a lot of people very angry and been widely regarded as a bad move.”

— *Douglas Adams*

1 General Introduction

1.1 Symbiosis as an evolutionary strategy and catalyst

Symbiosis, in its broadest sense, is defined as a relationship between two species, whereby one participant is either partially or wholly dependent upon the other, but it is more commonly understood as an intimate relationship between members of different species (Lewin, 1982). Such relationships may be mutualistic (benefiting both parties), such as flowers and pollinating bees, commensal (benefiting one party with negligible or no cost to the other), such as a bird nesting in a tree hollow, or parasitic (beneficial to one party whilst detrimental to the other), such as the intestinal worms of mammals. However, the relationships between hosts and symbionts are often not so neatly pigeon-holed into these three categories, and instead reside along a dynamic and often context-dependent spectrum (Swain, 2012, Gerardo, 2015). Symbiosis is now understood to be an essential, prevalent and hugely diverse feature of ecological functioning and structure, with an estimated 50% of all animal species being parasitic symbionts alone (Saffo, 1992, Price, 1980, Windsor, 1998).

Endosymbiosis, where one organism resides inside another, often intracellularly, takes this relationship to a more extreme level. The ramifications of this process to life as we know it became clear in the latter half of the 20th century, when it was discovered that mitochondria and chloroplast organelles are the descendants of free-living prokaryotes encapsulated in eukaryotic cells (Schwartz and Dayhoff, 1978). Endosymbiosis allows eukaryotes to gain novel genetic information and has paved

pivotal steps in eukaryote evolution, such as the acquisition of nitrogen fixation and bioluminescence capabilities (Giobel, 1926, Yasaki, 1928, Peix et al., 2015). Endosymbiosis has thus been a major driver of evolutionary diversification (Moran, 2007). Endosymbiosis within eukaryotic hosts holds several potential advantages to microbes, including a secure and homeostatic environment with a constant supply of nutrients, and thus such relationships have become abundant within the animal kingdom. Over 90% of cells in the human body, for example, are those of microbial symbionts, and the human gut is poor at extracting nutrition from food without its cohort of microbes (Hooper, 2002, Backhed, 2005, Ley, 2006).

Endosymbionts are often reliant on specific environments provided by their hosts that cannot be easily mimicked for culturing *in vitro*, thus hampering research for most of the 20th century (Moran, 2001). However, the increasing availability, reliability and sophistication of molecular techniques have allowed an explosion in research in this field, leading to fascinating and useful insights in symbiosis. The remainder of this chapter will review the evolutionary patterns, conflicts and consequences of symbiosis, focusing on the microbes of insects, in particular the aphids and mealybugs, with case examples. It will then discuss the potential application of symbiosis for the management of pest insects and finally outline the aims of this thesis.

1.2 The evolutionary consequences of endosymbiosis

Endosymbiosis is a specialised mode of survival, and thus symbiotic bacteria will face different evolutionary pressures from those faced by free-living microbes. The

nature of the symbiont will influence these pressures and their impact on its physiology. All symbionts can be assigned into two broad categories:

1. Obligate (a.k.a. primary), meaning a microbe where the relationship between it and its host is vital, either for the symbiont or for the host. These can be mutualistic, which tend to be vertically-transmitted, commensal, or parasitic, which can be horizontally and/or vertically-transmitted.
2. Facultative (a.k.a. secondary), meaning a microbe which requires a host, but is not essential to the survival of its host and can switch to novel hosts. They can thus be mutualistic, commensal or parasitic (Baumann, 2005).

Obligate, mutualistic symbionts tend to be transmitted vertically from parent to offspring, usually through the germline, although in some rarer cases they are efficiently transmitted horizontally to juveniles or acquired through ingestion (Frank, 1996c, Kikuchi et al., 2007). Facultative symbionts can be transmitted vertically or horizontally, depending upon the species.

The evolutionary impacts from these intimate associations, coupled with efficient vertical or horizontal transmission, can lead to coevolution of the partners, either as enemies in a Red Queen-style evolutionary arms race, or as allies (Moran and Telang, 1998). Mutualistic relationships can also lead to co-diversification, where speciation of the host is followed by speciation of the endosymbiont. Coevolution of a host and its mutualist endosymbiont can blur the boundaries between organism and organelle. Eventually, the relationship may become obligate, where both participants are unable to survive or reproduce without the other. Where the relationship is obligate for all members involved, it is increasingly being considered inappropriate

to consider a host and its endosymbiont/s as distinct organisms (Zilber-Rosenberg and Rosenberg, 2008), and the term “holobiont”, which intends to encapsulate the symbiotic partners as a single super organism, is gradually being adopted by some scientists. However, this term itself could be considered to be inappropriate when host-symbiont conflict is taken into account (discussed later), which can still occur even in obligate relationships.

Endosymbionts frequently experience dramatic genome reduction (Bennett and Moran, 2015, Moran and Bennett, 2014, McCutcheon and Moran, 2012, Moran and Wernegreen, 2000). This is a common consequence of the endosymbiotic lifestyle, which removes many of the selection pressures faced by free-living bacteria, and mutations in genes which would be required for independent survival may have accumulated and impeded their function. Furthermore, the bacteria are isolated from those residing in other hosts, preventing recombination and rendering them effectively asexual (Thao et al., 2002, Moran, 1996). This results in an irreversible process known as Muller’s Ratchet, which can “chip away” at the genome’s functionality (Lynch and Gabriel, 1990, Moran, 1996). The alterations to these genomes are drastic, as the ratchet has likely been exacerbated by bottleneck-caused selection. Bottlenecking is where only a small number of the bacteria cells are passed onto each new host generation. Noted changes include a high A+T content, increased rate of nucleotide substitution, amplification of the number of chromosomes residing in each cell from one to an average of 120, proliferation of plasmids and a loss of codon usage bias (the differences in the frequency of the occurrence of synonymous

amino acid-encoding codons in coding DNA) (Moran, 1996, Clark et al., 1999, Komaki and Ishikawa, 1999, Moya et al., 2002).

Despite the host and its symbionts living intimately as a "holobiont", they are still genetically separate entities. They will thus be exposed to different selection pressures and will act selfishly, even in mutualistic associations where the benefits of the relationship outweigh the cost (Bennett and Moran, 2015). The host will be selected to maximise its own fitness, whereas the symbiont will be selected to maximise its transmission rate to new hosts. These two objectives do not always result in the same outcome, and accommodating a symbiont will always incur some cost to the host (Bronstein, 2001). Increasing the transmission rate of the symbiont may involve competing for resources provided by the host with other symbiont species, or even strains, within the host, whether or not those additional symbionts are beneficial for the host. This was found in the pea aphid, *Acyrthosiphon pisum*, when superinfected with two facultative but mutualistic symbionts, who reduced the fitness of the host, likely through their hostile interactions or increased energetic demand (Oliver et al., 2006).

Much of this conflict can be resolved through efficient vertical transmission, which reduces the selection pressure to maximise horizontal transmission. Vertical transmission tends to result in a closer alignment of interests between host and symbiont, and more genetically homogenous symbionts within a host, both of which can select for lower virulence, such as is the case with organelles and the *Uroleucon ambrosiae* aphid symbionts (Frank, 1996a, Frank, 1996b, Smith, 2007, Birky et al., 1983, Funk et al., 2000). However, vertical transmission may not pacify this conflict

if it is only maternal. For symbionts which are only transmitted maternally, transmission into male offspring is likely to be an evolutionary dead-end without fitness benefits for the symbiont, even if the male still benefits from the arrangement. An exception to this could be found in the maternally-transmitted *Wolbachia*, which often manipulates the reproduction of the host via inducing cytoplasmic incompatibility. In this case, infected males can only successfully reproduce with infected females, so female hosts not harbouring the symbiont will be at a disadvantage when finding a mate (Engelstädter and Hurst, 2009). Thus, transmitting *Wolbachia* to male offspring indirectly favours the spread of the symbiont. *Wolbachia* has been found to also skew the sex ratio of their offspring towards females, and *Wolbachia*, *Spiroplasma* and *Rickettsia* have each been found to selectively kill male offspring (Fialho and Stevens, 2000, Jiggins et al., 2000, Lawson et al., 2001, von der Schulenburg et al., 2001).

1.3 Endosymbionts in the Insecta

Insects are rife with cases of bacterial symbiosis, which influence their evolution and daily ecology on a species and individual-level (Moran, 2001). The roles and functioning of these bacteria are as diverse as their hosts (examples discussed later), but one major theme is the acquisition of nutrition. The Insecta have extremely variable nutritional requirements across their taxa (Dadd, 1985, Douglas, 2009) and have evolved to occupy a remarkable variety of niches. This includes detritivores, carnivores and herbivores, the latter of which may specialise on particular plant species or tissues. The acquisition of endosymbionts has most likely been highly

influential in the diversification of insects. The understood role of obligate nutritional mutualistic endosymbionts is to provide new metabolic capacities that allow the hosts to exploit niches (Douglas, 2009). This may be to ease digestion or detoxify food material, or involve the synthesis of essential nutrients that are scarce in the available diet, such as is the case with wood-feeding termites and blood-feeding insects, such as tsetse flies (Breznak, 2000, Nakashima et al., 2002, Warnecke et al., 2007, Pais et al., 2008, Douglas, 2009). This is also the case for the endosymbionts of sap-feeding aphids (Hemiptera: Sternorrhyncha, Aphidoidea) and mealybugs (Hemiptera: Sternorrhyncha, Pseudococcidae), which have received much interest and are discussed in detail later.

The Sternorrhyncha are a suborder of the Hemiptera (true bugs), which includes the aphids (Aphidoidea), whiteflies (Aleyrodiodea), psyllids (Psylliodea) and scale insects (Coccoidea). These are sap-feeding insects, and their lifestyle has led to the association of each of these taxa with obligate endosymbionts that originate from the γ-3 subdivision of proteobacteria (Munson et al., 1991b, Thao et al., 2000, Thao et al., 2002, Thao and Baumann, 2004b, Gruwell et al., 2007, Matsuura et al., 2009). Aphids and mealybugs (Pseudococcidae members of the Coccoidea) are significant agricultural and horticultural crop pests. They feed upon host plants by inserting their specialised stylet mouthparts into the plant tissue in search of phloem vessels. They not only cause physical damage by probing plants in order to receive sap, and thus weakening it, but they also serve as vectors for a number of plant pathogens (discussed later) (Edwards, 1963). However, although plant sap is rich in carbohydrates, it is deficient in essential amino acids, which cannot be synthesised

de novo by animals (Douglas, 2006, Douglas, 1998, Shigenobu and Wilson, 2011). Thus, aphids and mealybugs have each independently formed relationships with bacteria that synthesise these nutrients for them. Obligate endosymbionts located in other Hemiptera groups are summarised in (Baumann, 2005). Nutritional obligate endosymbionts are not exclusive to the sap-feeding Hemiptera. Blood-feeding insects, for example bed bugs, sucking lice, wingless dipterans and triatomines, have a diet deficient in vitamin B and possess endosymbionts believed to be used for its synthesis (Dasch et al., 1984) (cited in (Beard et al., 2002)), but these are outside the scope of this review.

1.4 Ecology and etymology of aphids and *Buchnera*

Aphids (Fig. 1.4.1.) and *Buchnera* are model organisms for obligate endosymbiosis and are the focus for insect-endosymbiont research. Aphids are a group comprised of around 4,400 species worldwide, and together are highly polyphagous, feeding on a wide range of plant taxa (Dixon et al., 1987) and transmitting 50% of known insect-vectored viruses, including the damaging alfalfa mosaic virus, broad bean wilt virus-1 and strawberry mottle virus (Ng and Perry, 2004, Nault, 1997), and the bacterium *Pseudomonas syringae*, which attacks a variety of economically important crops (Stavrinides et al., 2009). They have a reproductive cycle which involves both sexual phases and parthenogenetic phases (where a female asexually produces clone female progeny), allowing populations to increase in numbers rapidly whilst still reaping the benefits of chromosomal recombination during sexual encounters (Moran, 1992). Located within the abdomen of each aphid resides an organ named the bacteriome,

which contains around 60 to 80 specialised cells named bacteriocytes (Baumann et al., 1995). It is these cells which house the obligate endosymbiont of aphids, *Buchnera*, tens of thousands of which will be encapsulated in vesicles. The great majority of aphid species harbour *B. aphidicola*, although this has been substituted with a yeast-like endosymbiont from the subphylum Ascomycotina in the Cerataphidini aphids (Munson et al., 1991b, Fukatsu and Ishikawa, 1996). Transmission for all obligate endosymbionts is strictly vertical, from mother to offspring, by the infection of eggs or embryos (Wernegreen, 2002).

Fig. 1.4.1. *Acyrthosiphon pisum* pea aphid adults and juveniles in California, USA, (Wild, 2015).

Aphids and *Buchnera* are one of the best studied cases of obligate mutualistic symbiosis in current literature. The role of *Buchnera* to assimilate essential amino acids from phloem sap has been demonstrated empirically. For example, it was found in two studies that infected pea aphids *A. pisum* are able to survive when fed on artificial diets that omitted individual amino acids, indicating that they were being synthesised from other available compounds, possibly the non-protein amino acid 5-methylmethionine, whereas cured *A. pisum* performed poorly in terms of growth (Akman Gündüz and Douglas, 2009, Febvay et al., 1999). In another study, the black bean aphid *Aphis fabae* cured of *Buchnera* infection showed stunted growth and a high mortality compared to infected *A. fabae* when fed on an amino-acid-poor artificial diet. Infected aphids in this study were able to assimilate essential amino acids from radiolabelled glutamic acid, whereas the uninfected aphids were not, signifying the role of this bacterium in nutrient synthesis (Douglas et al., 2001, Houk and Griffiths, 1980). The genomes of both *B. aphidicola* and *A. pisum* have now been sequenced (Shigenobu et al., 2000, Ra et al., 2010, The International Aphid Genomics Consortium, 2010), and the complementary nature of the symbiosis has become clearer. For example, *A. pisum* is unable to synthesise arginine, provided by *B. aphidicola*, yet remains able to produce some reactions in amino acid synthesis which are not covered by *B. aphidicola* (Wilson et al., 2010). The interlinking of metabolism reveals the antiquity and intimacy of the association, however, there is no evidence to suggest the horizontal transfer of functional genes from *B. aphidicola* to *A. pisum* (Ra et al., 2010, Nikoh et al., 2010). Horizontal gene transfer has been noted for the older mutualisms of aphids with mitochondria (Sunnucks and Hales, 1996), further suggesting that *B. aphidicola* may still reside in the grey boundary

between organism and organelle, rather than reaching full organelle status. As more is understood about endosymbiosis and its evolutionary consequences and processes, the precise distinction between symbiont and organelle (symbionts which have persisted and become not only obligate, but so genetically and evolutionarily entangled with their hosts that they were barely considered to be organisms in their own right, such as mitochondria and chloroplasts) is tenuous and still debated (Keeling et al., 2015). The horizontal transfer of genes into the host genome was previously considered the hallmark of organelles, but is no longer considered unique (Keeling et al., 2015, Wilson and Duncan, 2015, Hallam and McCutcheon, 2015). I would suggest that symbionts and organelles are instead considered to be points on a spectrum, rather than discrete categories.

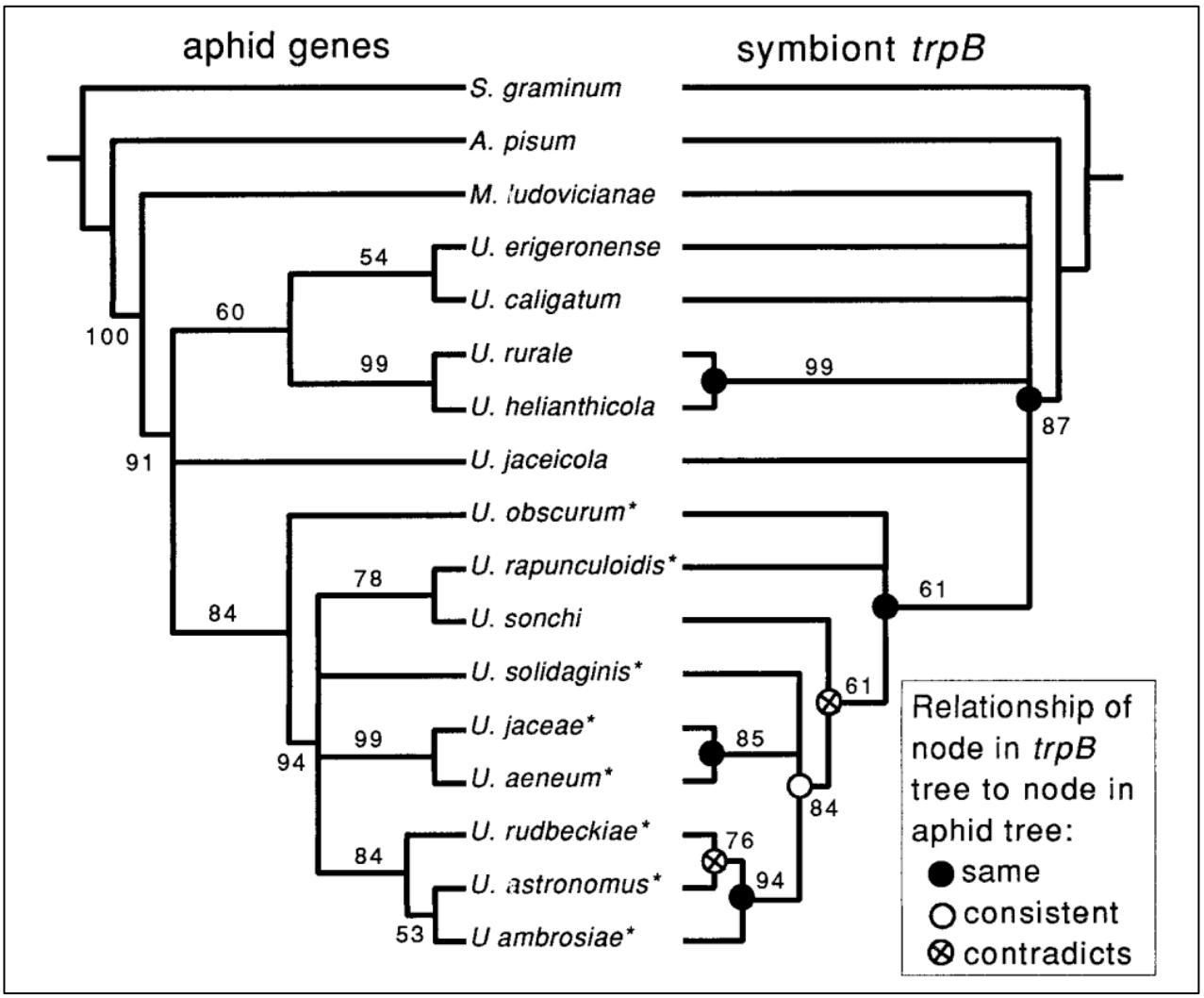
B. aphidicola is also unable to produce some cell-surface components, regulatory genes and genes involved in the defence of the cell (Shigenobu et al., 2000). Features such as these may allow *A. pisum* to remain in metabolic control over its endosymbiont (Shigenobu et al., 2000, Ra et al., 2010). However, *A. pisum* has lost several genes involved in the IMD immune pathway (The International Aphid Genomics Consortium, 2010, Gerardo et al., 2010). The suppression or adjustment of immune responses is common in symbiont hosts, which must amend their strategy for dealing with internal bacteria (Wang et al., 2009a, Ratzka et al., 2013, The International Aphid Genomics Consortium, 2010, Gerardo et al., 2010, McFall-Ngai et al., 2010). *A. pisum* has been found to employ distinct regulatory mechanisms for its obligate and facultative symbionts by varying its dietary nitrogen levels, revealing that it still maintains sophisticated control over its internal bacteria and can adjust

their density selectively (Wilkinson et al., 2007). The infection intensity/density of a symbiont, such as *B. aphidicola*, must be efficiently balanced within its host to ensure optimal performance, and the ideal density may vary depending upon the context and life stage of the host (Kono et al., 2008, Laughton et al., 2014). Too few symbionts would result in a shortage of amino acids, whereas too many symbionts could result in a surplus of amino acids which have no use to the host, but were still energetically costly to produce. Some indication of this has been found in the decreasing of *Buchnera* densities as the host ages (Lu et al., 2014). Maternal age also holds an influence on the density of *Buchnera* in the offspring when they reach adulthood (Laughton et al., 2014).

Buchnera may provide some additional non-nutritional benefits to aphids. A single nucleotide deletion in the *B. aphidicola* genome was found to disrupt a homopolymeric run within the transcriptional promoter for *ibpA*, a gene encoding a heat-shock protein (Dunbar et al., 2007). *A. pisum* individuals carrying the mutated bacterium suffered a complete reduction in fecundity following heat stress, compared to those carrying the wild type which did not. *B. aphidicola* also contributes to the pest status of aphids in a way other than nutrition aquisistion. The *Buchnera*-produced protein symbionin has been found to stabilise virus particles by preventing proteolytic degradation, and thus increase the efficiency of viral transmission by aphids into host plants. The application of the antibiotic chlortetracycline was found to counteract this by inhibiting bacterial protein synthesis and reducing viral transmission by over 70% (van den Heuvel et al., 1994).

Phylogenetic studies of aphids and their *Buchnera* strains indicate that this infection was a single event that occurred between 160 and 280 million years ago, around the same time as the origin of aphids, implying the significance of *Buchnera* in aphid ecology and evolution. The phylogenies show a mirroring of co-diversification with a strong congruence (Fig. 1.4.2.), reflecting the vertical transmission of this bacterium (Moran et al., 1993, Clark et al., 2000).

Fig. 1.4.2. Phylogenetic trees for aphids and corresponding *Buchnera* (here referred to as "*symbiont trpB*"), displaying the tight levels of congruence shared between host and symbiont. The *Buchnera* tree is based on partial sequences of *trpB*; the aphid tree is based on mitochondrial and nuclear sequences (Moran et al., 1999). Nodes resolved in the *Buchnera* tree are marked according to whether they match nodes on the aphid tree, are consistent, or contradict. Numbers on branches are bootstrap values for parsimony searches. Asterisk indicates taxa in which the *trpB* sequence contains an extra codon. Figure reproduced from (Clark et al., 2000).



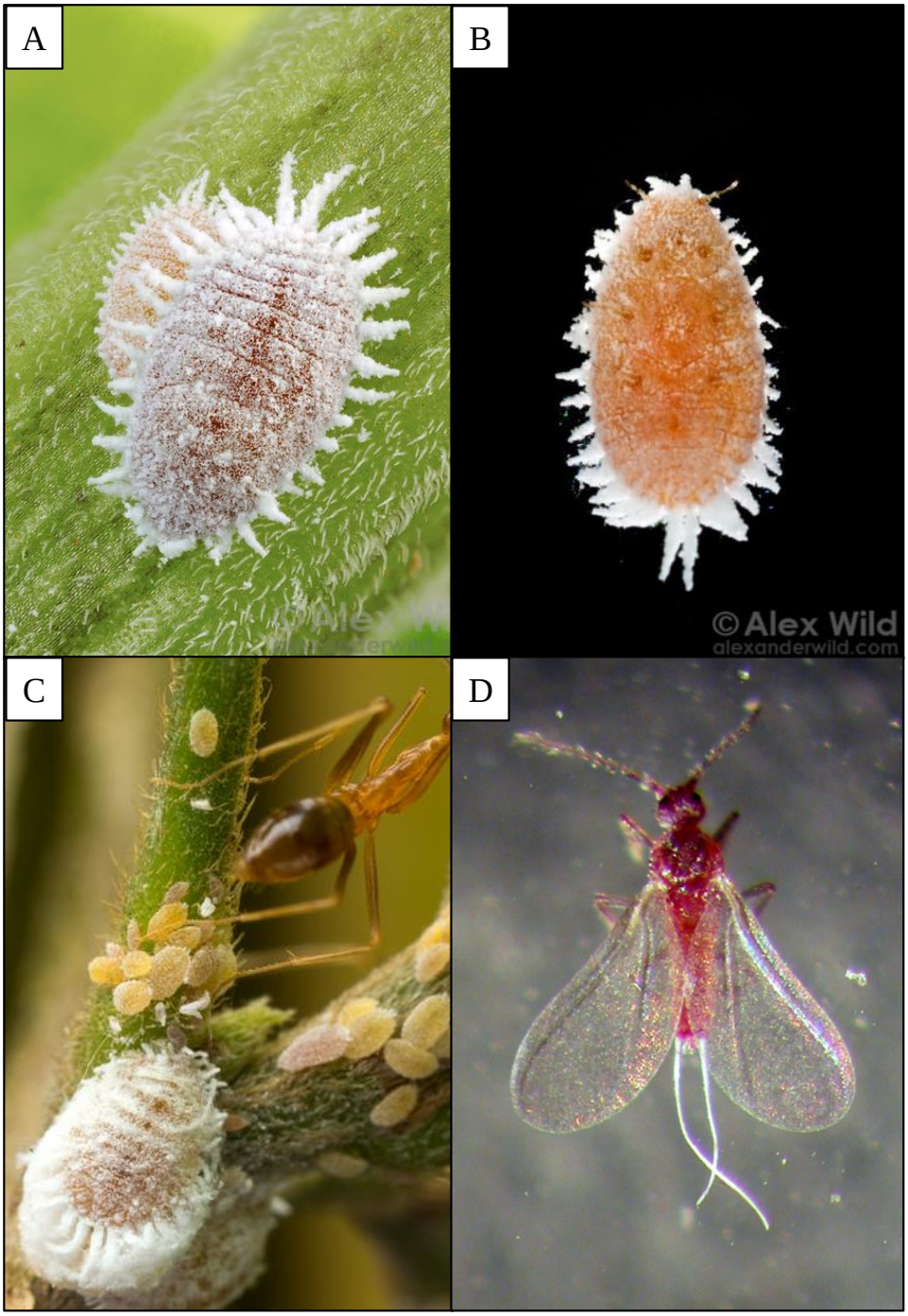
Hundreds of millions of years of intracellular symbiosis have made the *Buchnera* genome highly specialised. It is one of the smallest bacterial genomes known, comprising of 0.64 megabase pairs, one seventh the size of that of one of its closest free-living relatives, *Escherichia coli* (Wilson et al., 2010). However, the study of genomes of *Buchnera* from three different species of aphid host revealed that despite their extreme reduction in size, the chromosomes have maintained a conservation of gene order and composition, whilst some genes had transferred onto the two residential plasmids (Latorre et al., 2005). It has been suggested that polyploidy in *Buchnera*, believed to be a result the loss of ability to divide outside of the eukaryotic cell and genomic reduction, may in fact have then countered the ratchet by providing "back-up" copies of genes and slowed down what would have been a considerable rate of gene alteration (Komaki and Ishikawa, 1999). Either way, this genome reduction is irreversible and *Buchnera* may only become more specialised. It has been proposed that this shrinkage could lead to a loss of symbiotic capacity in *Buchnera* and the complementation, or potentially replacement, by the facultative symbiont *Serratia symbiotica*, which is later described (Pérez-Brocal et al., 2006).

1.5 Mealybugs and their ecology

Mealybugs (Fig. 1.5.1) are a division of the scale insects (Coccoidea) and are comprised of around 2,000 species worldwide (Thao et al., 2002, Ben Dov, 2015). They pose a similar threat to horticulture as aphids in that they probe plants with their stylets in search of phloem sap, causing mechanical damage to the plant, and transmitting a range of plant pathogens. The production of honeydew also

encourages the growth of black sooty moulds, which may render some crops worthless (Jelkmann, 1997, Sether, 1998, Charles, 2006). The citrus mealybug, *Planococcus citri* (Risso), is one of the most destructive species. Despite its name, it is a highly polyphagous pest that can feed upon plants originating from dozens of families, including citrus, cocoa (Ackonor, 2002), coffee (Staver et al., 2001), grapevine (Cid et al., 2006) and other horticultural and ornamental crops inside greenhouses and conservatories worldwide (Laflin and Parrella, 2004). *P. citri* can transmit plant pathogens from more than three genera of viruses, which include grapevine leafroll-associated virus 3 (a.k.a. Ampelovirus) (GLRaV-3) (Cid and Fereres, 2010, Martelli et al., 2002), *Badnavirus* (Phillips et al., 1999), including piper yellow mottle virus (Lockhart et al., 1997), and grapevine virus A, B and D (a.k.a. Vitivirus) (Adams et al., 2004). Adult females are particularly difficult to control as they produce a waxy secretion that coats their bodies (hence the name mealybugs) and effectively shields them from insecticides, which remain the most common form of control (Gullan and Kosztarab, 1997, Franco et al., 2009).

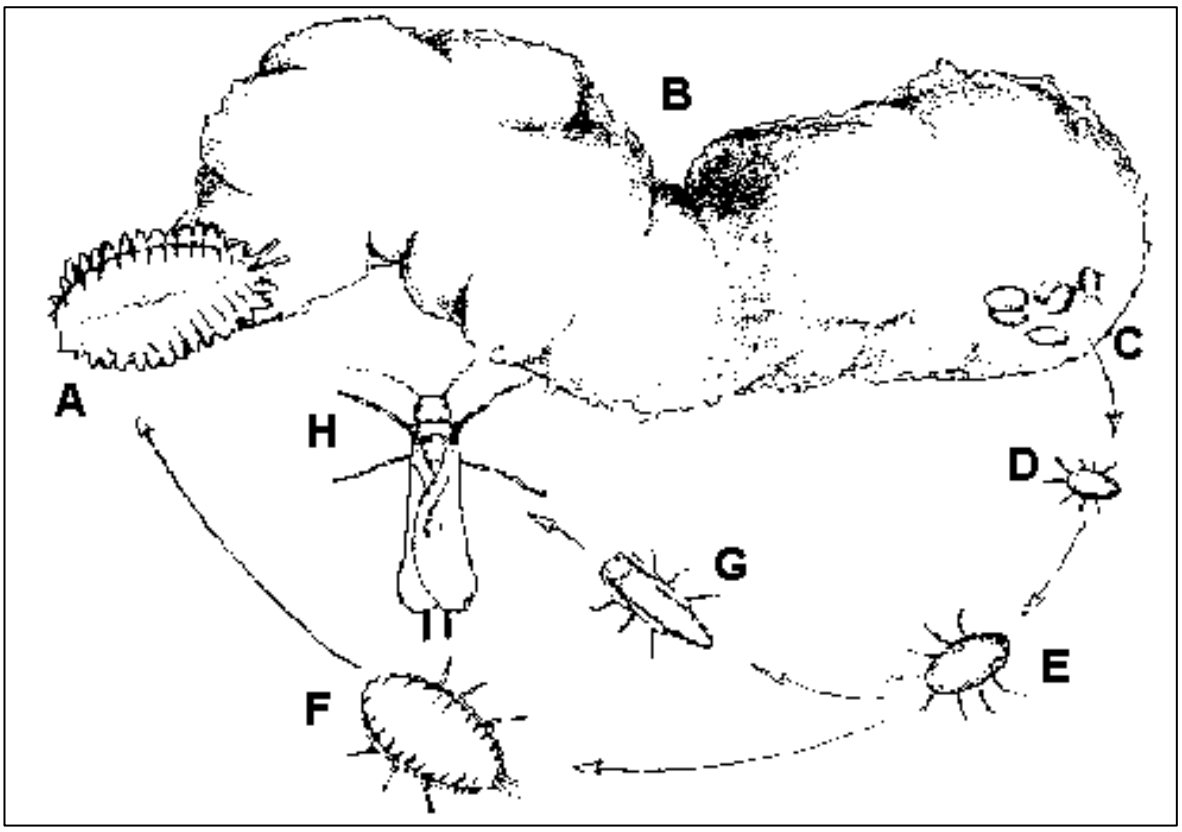
Fig 1.5.1. (A) *Planococcus citri* citrus mealybug adult female and juvenile (Wild, 2015); (B) ventral side of adult female *P. citri*, showing stylet and functional legs (Wild, 2015); (C) ovipositing adult female *P. citri* surrounded by juveniles and tended by yellow crazy ants (*Anopolepis gracilipes*) (Wild, 2015); (D) adult male *P. citri* (Osborne, 2010).


A
B
© Alex Wild
alexanderwild.com
C
D

Unlike aphids, the *P. citri* mealybug reproductive cycle includes only sexual phases and lasts for around 30 days when maintained at 25°C (Fig 1.5.2.), with instars perishing when maintained below 12°C or above 37°C (Arai, 1996, Goldasteh et al., 2009). Once hatched from their eggs, first instar nymphs begin life as mobile pink-orange wingless oval-shaped crawlers around 0.3 mm in length (Griffiths and Thompson, 1957), which disperse from the ovisac in search of food, tending to prefer the safety of plant crevices, or the fleshy undersides of leaves, or twigs and fruit. Once a suitable site is found, they settle, feed upon plant phloem and increase in body size, where sexual differentiation becomes more apparent and males elongate and become darkened grey in colour. There is dramatic sexual dimorphism at adulthood: the males go through three instars before entering a pre-pupal stage, where they produce a waxy cocoon and pupate. Adult males are around 4.5mm in length, brown-grey in colour with a pair of white wax threads protruding from their abdomen, winged and live only for a few days after pupation (having no mouthparts, they are unable to feed). During this short period, they fly and crawl in search of females and mate multiple times. In contrast, adult females go through 4 instars, but never pupate. They are peadomorphic, remaining in an enlarged 3mm long nymphal-like wingless state. They are grey in colour with a purple-grey dorsal stripe and coated with a thin layer of white wax. Adult females are mostly sessile and have relatively short legs, but will move when disturbed. Adult females can survive for up to several months whilst feeding upon the plant, waiting to be visited by males for mating. Once mated, females will commence oviposition, producing up to 600 orange eggs over several days, which are bundled in wax threads of an ovisac.

During the oviposition period, the female will reduce in size dramatically and finally die when her internal resources are depleted (Kerns et al., 2001).

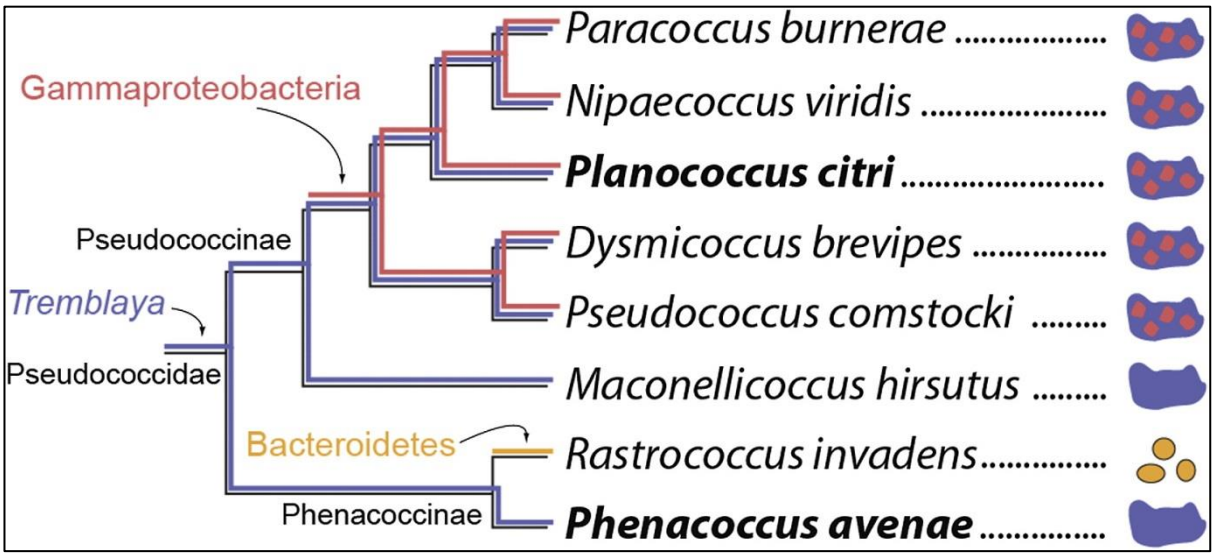
Fig 1.5.2. *Planococcus citri* citrus mealybug life cycle stages, displaying (A) paedomorphic adult female; (B) eggs which have been bundled together with wax threads during ovipoistion; (C-E) nymphs of both sexes; (F) female nymph; (G) male nymph; (H) winged adult male. Figure reproduced from (NCSU, 2015).



1.6 Citrus mealybugs and *Tremblaya princeps* and *Moranella endobia*

Citrus mealybugs are an interesting model system for the study of symbiosis. Like aphids, they house an obligate, nutritional, endosymbiotic bacterium species, *Candidatus* Tremblaya princeps, within a bacteriome organ surrounding the gut. *T. princeps* is a member of the β-proteobacteria and has the unusual feature of serving as host to an additional obligate nutritional bacterium, *Candidatus* Moranella endobia, a member of the γ-proteobacteria (Thao et al., 2002, Keeling, 2011, McCutcheon and von Dohlen, 2011, Von Dohlen et al., 2001, Baumann et al., 2002) (Fig 1.6.1.).

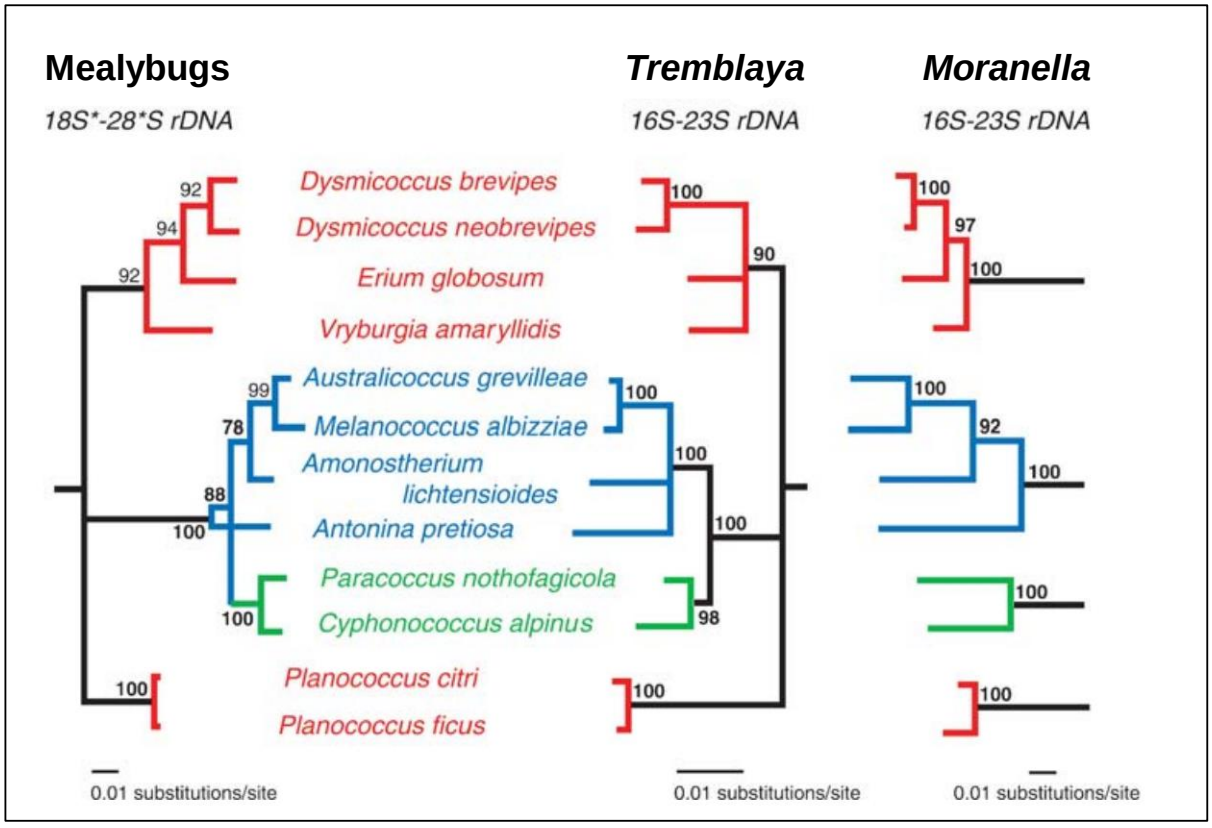
Fig. 1.6.1. Cladogram of mealybugs and their obligate symbionts. *Tremblaya* is the sole symbiont in some lineages of mealybugs (e.g., *P. avenae*); however it was replaced with a symbiont from the Bacteroidetes in some lineages (e.g., *Rastrococcus invadens*; yellow line) and was itself infected with gammaproteobacteria in other lineages of mealybugs (red lines; e.g., with *Moranella endobia* in *Planococcus citri*). This figure is a composite from previous work (Buchner, 1965, Gruwell et al., 2010, Hardy et al., 2008, Thao et al., 2002). Figure reproduced from (Husnik et al., 2013).



Together, *T. princeps* and *M. endobia* synthesise amino acids which are deficient in the diet of mealybugs. This is the only known example of a bacterium infecting another bacterium and is another illustration of how the concepts of organism, endosymbiont and organelle are not clear cut. The literature often refers to *M. endobia* as a "secondary" endosymbiont, e.g. (Thao et al., 2002), however the term "secondary" endosymbiont is usually applied to facultative bacteria. *M. endobia* is obligate, a characteristic of "primary" endosymbionts, and so this term may be no-longer appropriate in this case study. It could be argued that the categorisation of "primary" and "secondary" endosymbionts adds confusion to the understanding of symbiosis by failing to inform the function of the endosymbiont. For simplicity and a clearer understanding of the relationships between hosts and endosymbionts, it is suggested that the terms "obligate" and "facultative" would be more technically correct. Indeed, they are already widely used in the literature rather interchangeably with "primary" and "secondary" and so would not require a major transformation in terminology.

Phylogenetic studies indicate a strong congruence of co-diversification between mealybugs and *T. princeps*, reflecting strict vertical transmission into five major clusters following a unique infection event between 100 and 200 million years ago (Thao et al., 2002, Downie and Gullan, 2005). However, although *T. princeps* is monophyletic, the situation of *M. endobia* is not as simple. These bacteria are also grouped into five clusters, but they are distinct from each other. Thus, it has been concluded that different precursors of *M. endobia* infected *T. princeps* multiple times before co-diversifying with their hosts (Fig. 1.6.2.).

Figure 1.6.2. Comparisons of the phylogeny of mealybugs with *Tremblaya* and *Moranella*. *Tremblaya* is monophyletic, suggesting a single infection of a mealybug ancestor followed by co-speciation with the insect host. *Moranella* is polyphyletic; the different clusters are more closely related to a variety of sternorrhynchal insect secondary symbionts than to each other. Maximum likelihood analysis, numbers at nodes represent % bootstrap values after 500 replicates; only nodes supported by 70% or greater are shown. Host sequences are from (Downie and Gullan, 2004); *Tremblaya* and *Moranella* sequences are from (Thao et al., 2002). Figure reproduced from (Baumann, 2005).



The concentric arrangement of *T. princeps* and *M. endobia* has resulted in unusual physical and metabolic arrangements. Like other endosymbionts, these bacteria have reduced genomes, but *T. princeps* has taken this characteristic to the extreme. It possesses one of the smallest bacterial genomes known to science, at just under 139kb in length with only 120 protein coding genes (Husnik et al., 2013). Even the reduced genome of *M. endobia*, at 538kb, is still almost four-fold larger, although it codes for only half the number of essential amino acid gene homologs as *T. princeps* (McCutcheon and von Dohlen, 2011, Lopez-Madrigal et al., 2011, López-Madrigal et al., 2013). It is hypothesised that *T. princeps*' genome reduction may have been further exaggerated by it hosting a symbiont of its own and providing a second set of proteobacterial genes, rendering those in *T. princeps* redundant (McCutcheon and von Dohlen, 2011). The presence of functionally homologous *M. endobia* genes that complement the pseudogenes of *T. princeps* provide some evidence for this hypothesis (McCutcheon and von Dohlen, 2011). But much gene loss had already occurred before the acquisition of *M. endobia*, leading speculation that this arrangement may not be the major cause of the genomic reduction in *T. princeps* (Husnik et al., 2013). However, comparison of *T. princeps* against *Tremblaya phenacola*, the homologous symbiont of the Phenacoccinae mealybugs which lack *M. endobia*, found inconsistencies, with *T. princeps* having undergone concerted evolution of paralogous loci, suggesting that the atypical reductive evolution could be linked to *M. endobia* (López-Madrigal et al., 2015). Genetic comparison of *T. princeps* strains from five mealybug species of the *M. endobia*-harbouring Pseudococcinae revealed co-occurrence of concerted evolution, further supporting

the hypothesis that the acquisition of *M. endobia* is a major cause of this unusual evolution of *T. princeps*.

T. princeps has lost a number of functional genes, some of which are essential for the control of gene expression and still found in the genomes of other obligate insect symbionts, including those for translational release factors, aminoacyl-tRNA synthetases, ribosome recycling factor, elongation factor EF-Ts and peptide deformylase (Nakabachi et al., 2006, Husnik et al., 2013, McCutcheon and von Dohlen, 2011). *T. princeps* also lacks cell-envelope-related genes, and is hypothesised to depend on host-sourced membranes to construct its own cytoplasm (Husnik et al., 2013). The further genetic intimacy of *P. citri*, *T. princeps* and *M. endobia* was revealed through sequencing where it was found that the synthesis of all essential amino acids could only occur through a fusion of genetic pathways from *P. citri*, *T. princeps* and *M. endobia* (Keeling, 2011, McCutcheon and von Dohlen, 2011, Husnik et al., 2013). The *P. citri* genome also contains at least 22 functional horizontally-transferred bacterial genes from previous symbiotic relationships with a diverse array of bacteria, which may complement some of the genes lost in *T. princeps* and *M. endobia* (Husnik et al., 2013). Lysis of *M. endobia* may be the mechanism that allows the release of gene products out of the cell and into *T. princeps*, and it is predicted that facultative expression of the cell wall stability genes, *murABCDEF* and *mltD/amiD*, in the *P. citri* genome may control this event, with a reduction in expression leading to less stable *M. endobia* walls that are more prone to lysis (Koga et al., 2013, McCutcheon and von Dohlen, 2011, Husnik et al., 2013).

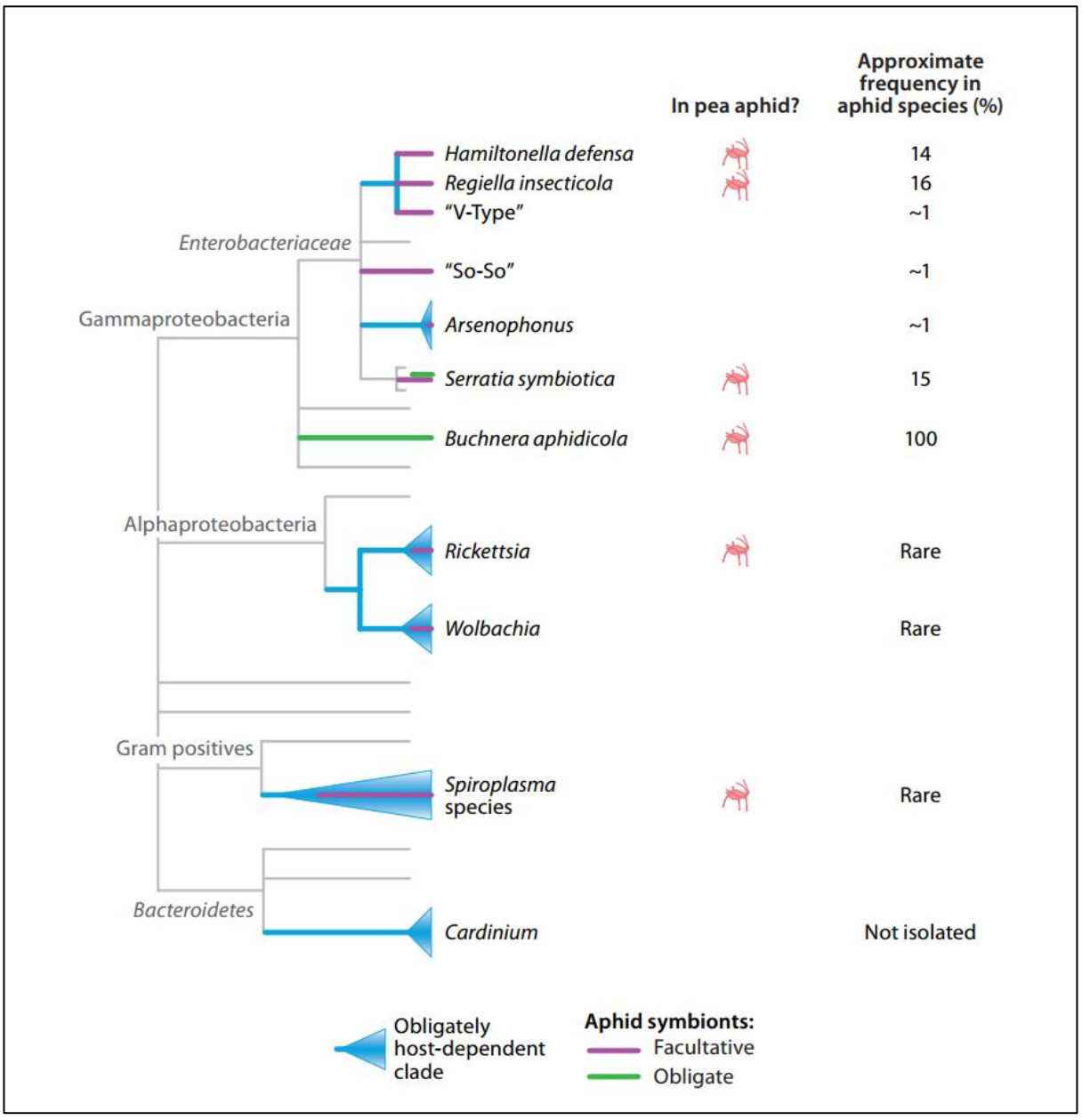
The density/infection intensity *T. princeps* and *M. endobia* within the bacteriome varies depending upon the age and gender of the host (Kono et al., 2008). A study into these dynamics with the mealybugs *Planococcus kraunhiae* and *Pseudococcus comstocki* found that whilst females maintain their endosymbionts until they are beyond reproductive age, males lose their endosymbionts after they pupate into an adult. As adult males have no mouthparts and thus cannot feed, nutritional endosymbionts serve no further purpose to them, and it is hypothesised that they are likely broken down for energy (Kono et al., 2008). Interestingly, this loss of symbionts is decoupled, with *M. endobia* disappearing more quickly than *T. princeps*. A similar absence of endosymbionts in males has been noted in some other arthropods, for example the social aphid *Colophina arma* (Fukatsu and Ishikawa, 1992), and the slender pigeon louse, *Columbicola columbae* (Fukatsu et al., 2007).

1.7 What are facultative symbionts?

Facultative endosymbionts differ from primary/obligate endosymbionts in that the relationship is not essential for the survival or reproduction of the host and the symbiont can survive in novel hosts, or even be free-living (Oliver et al., 2010). Facultative symbionts can be parasitic, commensal or mutualistic, however, in practise, they often do not slot into ridged categorical relationships, but instead fall on a multi-dimensional and context-specific spectrum. During particular circumstances, the symbionts may provide services that give the host an advantage over conspecifics without the relationship, or conversely, parasitic side-effects that maximise symbiont production to the detriment of the host or population. Facultative

symbionts are common in the Hemiptera, and one of the most intensely studied groups are the aphids, which harbour a wide range of species (Fig 1.7.1) (Oliver et al., 2010)**.** The facultative symbionts of mealybugs, on the other hand, have only received a fraction of the same investigative scrutiny. The known or common facultative symbionts of aphids and other Hemiptera and mealybugs will now be described.

Fig. 1.7.1. Phylogenetic distribution of vertically-transmitted facultative and obligate bacterial symbionts of aphids. Frequencies are based on 97 aphid species surveyed in (Haynes et al., 2003, Russell et al., 2003, Sandström et al., 2001). Figure reproduced from (Oliver et al., 2010).



1.8 Facultative symbionts in aphids and other Hemiptera

Facultative endosymbionts, including mutualistic, commensal and parasitic species, in aphids tend to reside in the haemolymph outside of the bacteriome in the syncytial or mycetocyte cells, although their location can also include several locations, such the ovaries and gut (Saridaki and Bourtzis, 2010, Koga et al., 2003, Sacchi et al., 2010). They are vertically transmitted maternally, although phylogenetic studies of the three major facultative endosymbionts of aphids, *Hamiltonella defensa* (Moran), *Regiella insecticola* (Moan) and *Serratia symbiotica* (Moran) have revealed a lack of congruence indicating widespread horizontal transfer of these bacteria amongst the aphids and psyllids (Russell et al., 2003). This occurs most commonly between closely related species, perhaps indicating some level of species-specific intimacy.

The interactions of facultative endosymbionts are more complex than that of *Buchnera*. Studies involving the curing of bacterial infection of individuals and transinfection of bacteria from infected into non-infected individuals are gradually and empirically demonstrating the roles of these endosymbionts in aphid ecology. Prokaryotes are able to produce a greater array of biologically active compounds, such as toxins, than eukaryotes, and thus can serve as a valuable asset in the defence against parasitoids and pathogens. Aphids have a limited encapsulation defence against parasitoids, particularly when under heat stress (Bensadia et al., 2006), and hence are especially vulnerable to parasitism. Genetic sequencing of *A. pisum* has identified the loss of genes integral to the IMD immune pathway, suggesting that *A. pisum* has a compromised immune system, and may be reliant on immune benefits from facultative endosymbionts (Ra et al., 2010).

Mealybugs have interacted with facultative symbionts in their evolutionary past, as the *P. citri* genome was found to contain several horizontally-transferred bacterial genes (Husnik et al., 2013). However, very little is known of their current associations with facultative bacteria. For example, the facultative bacterium *Rickettsia* (discussed later) has been observed in the mealybug, *P. solenopsis*, (Singh et al., 2013) and a *Spiroplasma*-like bacterium (discussed later) has previously been detected in the mealybug, *Antonina crawii*, (Fukatsu and Nikoh, 2000), but the ecological impacts of either of these relationships is hitherto unknown.

1.8.1 ***Hamiltonella defensa***

H. defensa occurs in aphids and the whitefly, *Bemisia tabaci* (Gennadius) (Yu-Feng et al., 2015, Oliver et al., 2014, Guay et al., 2009). It is a member of the γ-subdivision of the proteobacteria, previously named PABS (pea aphid *Bemisia*-like symbiont) or T-type symbiont, and can provide significant benefits to its host, but research into how it induces these phenotypic alterations has revealed a strong example of an illuminating aspect of endosymbiont evolution. *H. defensa* has been found to increase *A. pisum* resistance against the parasitoid wasp *Aphidius ervi* (Hymenoptera: Braconidae) (Oliver et al., 2003, Oliver et al., 2005, Ferrari et al., 2004). This was through higher levels of parasitoid larval mortality, in one experiment reducing mummification by 22.5%, and fecundity of parasitized individuals infected by *H. defensa* was also significantly higher than uninfected parasitized individuals. Horizontal gene transfer plays a crucial role in the relationship between *A. pisum* and *H. defensa*, and *A. pisum* has acquired genes from

several other facultative symbiont species, including *Wolbachia* and *Rickettsia* (Oliver et al., 2010). It is now understood that the presence of a lysogenic lambdoid bacteriophage named APSE (*A. pisum* viral facultative symbiont), which encodes eukaryote-targeting toxins, is essential to the efficacy of *H. defensa* (van der Wilk et al., 1999, Oliver et al., 2009). Across *A. pisum* reside different numbered variants and strains of APSE that possess slightly different properties in their toxins (van der Wilk et al., 1999, Moran et al., 2005b). Lab strains of *A. pisum* can often lose their APSE, and experiments found that *H. defensa*-infected, APSE-infected aphids were 90% more successful at countering the parasitoid *A. ervi* than *H. defensa*-infected, APSE-noninfected aphids (Oliver et al., 2009). Degnan and Moran (Degnan and Moran, 2008) hypothesise that APSE may allow the horizontal transfer of genetic material between endosymbionts *in vivo*. Indeed, genetic studies have uncovered evidence in APSE for horizontal gene transfer, recombination and transposition (Degnan and Moran, 2008). *H. defensa* has retained more free-living characteristics than *B. aphidicola* in its larger 2.1 megabase genome (Degnan et al., 2009). Despite being unable to produce several essential amino acids and being thus reliant on the obligate endosymbiont, it has maintained a greater ability to synthesise and regulate numerous cellular structures, possibly because its facultative nature requires a more robust genome.

Even a facultative endosymbiont relationship that appears specialised for mutualism can switch to parasitism under certain environmental conditions. Caged populations of *A. pisum* with a moderate starting frequency of *H. defensa* significantly increased the proportion of *H. defensa*-infected individuals following exposure to the *A. ervi*

parasitoid (Oliver et al., 2008). However, when populations of *A. pisum* were not exposed to *A. ervi*, the proportion of *H. defensa* and *S. symbiotica*-infected individuals was reduced. Harbouring this endosymbiont may come at a cost to *A. pisum* that is only outweighed by the benefits of increased protection when parasitoids are a significant threat, demonstrating the parasitic aspect of this symbiosis when selection pressures are not in favour of the relationship.

1.8.2 ***Regiella insecticola***

Facultative endosymbionts of aphids that provide parasite/parasitoid resistance can efficiently defend the host against a wide range of host-enemy species that will use diverse strategies and chemicals in their attacks. *R. insecticola*, another member of the γ-subdivision of the proteobacteria and previously named PAUS (pea aphid U-type symbiont), has been found to increase survival of *A. pisum* infected by the Entomophthorales fungus *Pandora (Erynia) neoaphidis*, in one experiment by around 30%, through reducing sporulation frequencies by around 60% (Ferrari et al., 2004, Scarborough et al., 2005). *R. insecticola* was also found to reside in a clone of the peach potato aphid, *Myzus persicae*, which was invulnerable to two parasitoid species, *Aphidius colemani* and *Diaeretiella rapae* (von Burg et al., 2008). *A. fabae* has been found to benefit from increased resistance to a third parasitoid species *Lysiphlebus fabarum* when infected with *H. defensa* (Vorburger et al., 2009). The relationship between host and endosymbiont may be complex and not revolve around the presence or absence of a single phenotypic trait. For example, *R. insecticola* has been additionally found to assist *A. pisum* host plant specialisation

(Tsuchida et al., 2004, Ferrari et al., 2007), perhaps fine-tuning the benefits provided by *B. aphidicola*.

1.8.3 ***Serratia symbiotica***

S. symbiotica, a member of the γ-subdivision of the proteobacteria and previously named S-symbiont, PASS (pea aphid secondary symbiont) or R-type, was found to improve *A. pisum* tolerance of a very different threat: heat stress. Individuals artificially infected with this bacterium retained 48% of their fecundity following heat stress, compared to non-infected individuals who retained just 7% of their fecundity (Montllor et al., 2002). This endosymbiont has also been found to provide some defence against *A. ervi* (Oliver et al., 2003). That this endosymbiont provides both heat stress and parasitoid resistance is of particular interest as *Hamiltonella defensa*-infected aphid defences are less effective following heat stress (Bensadia et al., 2006). It would be illuminating to examine whether *H. defensa* display a greater heat-stress integrity in the presence of *S. symbiotica*.

Sequencing could reveal the genes involved in these traits, and it would be enlightening to discover whether related genes are used for similar benefits exhibited by different endosymbionts. This would also uncover whether the traits have common evolutionary origins or have evolved independently, either using closely related genes in the same manner or developing unrelated genes to produce the same trait as forms of convergent evolution. The genome of *R. insecticola*, for example, has now been sequenced (Degnan et al., 2010). It was concluded that the common ancestor of this symbiont and its sister species, *H. defensa*, had evolved the

symbiotic lifestyle before these species diverged. This may suggest that genes involved in host defence may be homologous, although this is yet to be confirmed. Moreover, *R. insecticola* provides defence against a wide variety of parasites and parasitoids (Vorburger et al., 2010, Vorburger et al., 2009, Łukasik et al., 2013), but whether defence requires a single broad range immunity gene set or multiple gene sets specialised against specific enemies is still not yet known. As well as being of fundamental interest, a greater understanding of how and where these defences function may also assist more efficient biological control of pest species with parasites and parasitoids.

1.8.4 ***Rickettsia***

Rickettsia species, a member of the α-subdivision of the proteobacteria and previously named PAR (Pea Aphid *Rickettsia*) when in aphids, have expanded to pathogenically infect a wide diversity of animals through either vertical or horizontal transmission (Weinert et al., 2009, Caspi-Fluger et al., 2012). *Rickettsia* can have negative effects on *A. pisum* host fitness and suppress *B. aphidicola* (Sakurai et al., 2005). However, this endosymbiont still maintains a worldwide distribution in its hosts, and its prevalence may be due to effective horizontal/vertical transmission, or because of a not-yet understood advantage posed under certain situations (Sakurai et al., 2005). For example, they have been found to increase heat tolerance, parasitoid resistance and viral tolerance and resistance to insecticides in whiteflies (Brumin et al., 2011, HuiPeng and YouJun, 2012, Kontsedalov et al., 2008, Kliot et al., 2014). *Rickettsia* can also function as a reproductive manipulator, inducing parthenogenesis

in parasitoid wasps and male-killing in lady beetles (Lawson et al., 2001, von der Schulenburg et al., 2001, Giorgini et al., 2010), most likely because it is transmitted maternally and is being selected to maximise its own transmission at the expense of its hosts' fecundity. Although many parasites do have a high prevalence, so this feature is not unusual in itself, understanding how this apparently parasitic endosymbiont has remained so common in the host population may have potential value in the application of symbiont-host relationship manipulation for pest control.

1.8.5 ***Wolbachia***

Wolbachia is a facultative endosymbiont of the α-proteobacteria which is vertically maternally transmitted and tends to reside in the hosts' reproductive organs where it influences reproduction, although it can also be found across the body where it is involved in non-sexual traits, including in the salivary glands, gut, fat bodies, Malpighian tubules, haemocytes, brain, muscle and retina (Saridaki and Bourtzis, 2010). A meta-analysis predicted that around 66% of all insect species are infected by *Wolbachia* (Hilgenboecker et al., 2008), with a more recent independent study estimating it to be at a similar 61.9% (de Oliveira et al., 2015) and a maximum-likelihood approach estimated the incidence in terrestrial arthropods to be 52% (Weinert et al., 2015). One of these studies also pointed out that the prevalence of *Wolbachia* can vary within a species, and that it tends to either be near fixation (greater than 90% of individuals infected) or very rare (less than 10% of individuals infected) (Hilgenboecker et al., 2008). Typical sexual consequences of infection can include cytoplasmic incompatibility, where an infected male is only able to produce

viable offspring when mated with an infected female, parthenogenesis, genetic male feminisation and male progeny-killing; the latter three can skew population sex ratios towards females (Fialho and Stevens, 2000, Weeks and Breeuwer, 2001, Negri et al., 2006, Watanabe et al., 2010, Hu and Li, 2015).

Wolbachia has been of great interest for evolutionary and ecological research and pest management. Endosymbionts can persist in a host population either by providing fitness benefits to the host, or through parasitic adaptations that maximise symbiont production at the expense of the host population. This has been demonstrated in *Rickettsia bellii*, an endosymbiont which swept from rarity to near-fixation in *B. tabaci* populations across Arizona within 6 years due to fitness benefits and a female biased sex ratio in offspring (Himler et al., 2011). Likewise, *Wolbachia* has become possibly the most prevalent endosymbiont in the Insecta (Hilgenboecker et al., 2008). It has been identified across the Hempitera, for example, in the aphids *A. pisum*, *Cinara cedri* and *Sitobion miscanthi*, Cixiidae planthoppers, the Triatomine insect *Rhodnius pallescens*, predatory bug *Macrolophus pygmaeus*, *Drosicha* giant scale insects, the catkin bug *Kleidocerys resedae* and small brown planthopper *Laodelphax striatellus* to name a few (Matsuura et al., 2009, Gomez-Valero et al., 2004, Kikuchi and Fukatsu, 2003, Bressan et al., 2009, Espino et al., 2009, Machtelinckx et al., 2009, Küchler et al., 2010, Zhang et al., 2010, Wang et al., 2009b, Gauthier et al., 2015). *Wolbachia* has not yet been found in *P. citri* (Jeyaprakash and Hoy, 2000, Zchori-Fein and Perlman, 2004), but these searches have been quite limited and *Wolbachia* have been found in closely related species. For example, two studies each concluded that *Wolbachia* does not reside in *P. citri*

after screening a total of just five individual insects (Jeyaprakash and Hoy, 2000, Zchori-Fein and Perlman, 2004). Whether or not *Wolbachia* occurs naturally in these species, the possibility of exploring transinfection as a way to benefit from *Wolbachia*-based pest control remains. Attempts to induce parthenogenesis in *P. citri* have not been successful (Borges da Silva et al., 2010), although it has been reported in laboratory populations of the related *Phenacoccus solenopsis* (Vennila et al., 2010). It would be interesting to test whether *Wolbachia* is influential in mealybug reproductive biology.

Wolbachia is mostly transmitted maternally, and so has evolved to skew the sex ratios of its hosts' progeny towards females to increase its abundance. This can be achieved either by killing male progeny (for example, as found in black flour beetles *Tribolium madens* (Fialho and Stevens, 2000)), inducing parthenogenesis (as found in the phytophagous mite *Bryobia praetiosa* (Weeks and Breeuwer, 2001)*)* or by feminising genetically male embryos. The latter feature has been noted in the leafhoppers *Zyginidia pullula*, where the presence of *Wolbachia* transforms male embryos into intersex functional females by interfering with genomic imprinting, resulting in changes in the expression of genes involved in sexual differentiation and development (Negri et al., 2009). These feminised males resemble and reproduce like females, with the exception of characteristically male small chitinous structures which they retain on their abdomens. If sex skew or parthenogenesis-inducing *Wolbachia* spreads into a host population, theoretically it could cause population reduction, and in the long term host extinction, a potential evolutionary dead end (Charlat et al., 2003). This is an exciting prospect for pest management, although it is

predicted that in these situations there will be a strong selection pressure towards female hosts who can produce males, possibly preventing disaster for the species.

Cytoplasmic incompatibility can also increase the abundance of *Wolbachia* as it provides infected females with a reproductive advantage. Infected females can reproduce successfully with both infected and uninfected males, whereas uninfected females can only produce viable offspring with uninfected males, as *Wolbachia* modifies the males' sperm (Clark et al., 2003). When the modified sperm enters the egg, the paternal chromosomes fail to decondense unless *Wolbachia* infection is present to rescue the sperm (Lassy and Karr, 1996). *Wolbachia* infection density was found to be correlated with cytoplasmic incompatibility in two plant hopper species *Laodelphax striatellus* and *Sogatella furcifera* (Noda et al., 2001) and the predatory bug *Macrolophus pygmaeus* (Machtelinckx et al., 2009). Finally, transinfection of *Wolbachia* from *L. striatellus* into the brown planthopper *Nilaparvata lugens* was found to induce cytoplasmic incompatibility (Kawai et al., 2009), establishing a solid link between *Wolbachia* and this trait. By manipulating its hosts' reproductive system, *Wolbachia* has evolved into a successful parasite.

Phylogenetic studies have indicated that *Wolbachia* may have used bacteriophages to undergo intracellular recombination with co-inhabiting *Wolbachia* cells within the same host (Bordenstein and Wernegreen, 2004, Jiggins et al., 2001, Malloch and Fenton, 2005). This provides *Wolbachia* with an evolutionary advantage over endosymbionts which do not show signs of recombination, as it could potentially counteract Muller's Ratchet and retain a more viable genome. Although principally transmitted maternally, horizontal transfer of *Wolbachia* cells must occur in order to

invade new species of hosts, and indeed as already mentioned, this has been achieved artificially in the lab. Studies of the *Wolbachia* communities within *B. tabaci,* the planthopper *Nisia nervosa*, the flea beetle *Phyllotreta sp.* and the fleahopper *Halticus minutus* feeding upon the same pumpkin found evidence for horizontal transfer of the endosymbiont via the plant (Sintupachee et al., 2006), suggesting a mechanism by which horizontal transfer may occur in the wild.

1.8.6 ***Spiroplasma* and *Phytoplasma***

Spiroplasma and *Phytoplasma* are small genome-endowed members of the Mollicutes class of Eubacteria, related to the gram-positive bacteria (Weisburg et al., 1989). They include phytopathogens and occupy the sieve tube elements of plant hosts, vectored by Cicadellidae (leafhoppers), Fulgoridae (planthoppers) and, in some cases, Psyllidae (psyllids). *Phytoplasma* alone are causative of diseases across over 1,000 plant species in 98 families (McCoy et al., 1989, Gasparich, 2010)). The average lifecycle of the endosymbiont takes around 15-20 days. From the plant host, they invade the insect vector through ingestion where they multiply in the midgut to over 10^6 individuals and then spread into the haemolymph and organs, including the salivary glands, where they are re-injected into the plant. In these situations, the endosymbiont detrimentally impacts on the insect vector, reducing longevity by up to two days (Garnier et al., 2001).

Spiroplasma is a diverse and highly speciose group of actively motile and helical symbiotic bacteria and plant pathogens. They can be associated both extracellularly and intracellularly with a number of insect orders, including the Coleoptera, Diptera,

Hemiptera, Hymenoptera, Lepidoptera and Odonata, where they tend to occupy the epithelial cells of the gut lumen, but can also invade the haemolymph, ovaries, salivary glands, fat bodies and hypodermis (Gasparich, 2002, Regassa and Gasparich, 2006). *Spiroplasma* have also been associated with ticks and crustaceans (Taroura et al., 2005, Wang et al., 2005). They can be both horizontally and vertically transmitted, with transmission between plants and insects being mediated by the penetrating feeding mechanisms of sucking insects (Clark, 1982, Regassa and Gasparich, 2005).

Their impacts on hosts are varied, tending to be commensal, but with some cases of mutualism and parasitism (Ammar et al., 2011, Gasparich, 2002, Clark, 1977). For example, *Spiroplasma* has been found to induce a male-killing phenotype in *A. pisum*, fruit flies, butterflies, planthoppers and ladybeetles (Simon et al., 2011, Montenegro et al., 2005, Kageyama et al., 2007, Jiggins et al., 2000, Oliver et al., 2010). They can lead to reduced fitness in pea aphids (Fukatsu et al., 2001, Montenegro et al., 2005) but may be also correlated with increased resistance to the parasitoid *A. ervi* (Nyabuga et al., 2010). *Spiroplasma kunkelii* was found to increase the ability of the leafhopper, *Dalbulus maidis*, to survive cold temperatures (Ebbert and Nault, 1994). The shift in *Spiroplasma* from commensalism to parasitism tends to occur when *Spiroplasma* occupies host organs other than the gut lumen, such as in the case of bees (Regassa and Gasparich, 2006, Clark, 1977). Likewise, *Spiroplasma* is generally commensal when located on plant surfaces, but becomes pathogenic upon infection of internal plant tissues (Regassa and Gasparich, 2006).

1.8.7 *Other facultative endosymbionts in Hemiptera*

Facultative endosymbionts have been far less intensely studied in other members of the sap-feeding Hemiptera; however the more gradually emerging pictures appear to indicate that such relationships are common. They occur in planthoppers, leafhoppers, other whiteflies and giant scale insects (Crotti et al., 2009, Skaljac et al., 2010, Tang et al., 2010), although most of their functions are yet to be understood. Small pieces of information are being extracted; for example, the sweet potato whitefly *Bemisia tabaci*, one of the more studied members, also harbours *Rickettsia*, *H. defensa*, *Arsenophonus*, *Cardinium* and *Fritschea*, which correlate with biotype (Gottlieb et al., 2006, Chiel et al., 2007, Ahmed et al., 2010). This assortment also includes *Wolbachia*, a highly significant bacterium which is also the most prevalent facultative endosymbiont across all insect taxa.

1.9 The potential application of endosymbionts in microbial resource management

As more is understood about the ecology of agricultural pests and their endosymbionts, so potential chinks in their armour are exposed. These vulnerabilities can then be targeted by crop growers. By disrupting mutualistic relationships, or utilising new or existing parasitic relationships, a technique termed "Microbial Resource Management", research in this field holds great potential for effective and sustainable integrated pest management, and may prove to be revolutionary (Verstraete et al., 2007, Read, 2011, Douglas, 2007b).

Wolbachia, in particular, holds great potential for pest management and human disease eradication, and there are exciting discussions as to how this can be achieved (Brownstein et al., 2003, Zabalou et al., 2004, Cook and McGraw, 2010, Hancock et al., 2011). Models have predicted that the release of *Wolbachia*-infected male individuals that are either sterile or cytoplasmically incompatible could artificially reduce wild host populations (coined as the Cytoplasmic Incompatibility Management (CIM) strategy) (Dobson et al., 2002). It has been found that a shortened lifespan, cytoplasmic incompatibility and a reduced viral transmission efficacy can be induced in *Aedes aegypti* mosquitoes which serve as the vector for viral dengue fever, by transinfecting them with avirulent *w*Mel strains of *Wolbachia* (Walker et al., 2011). When released into wild populations, they will compete with uninfected wild-type males for matings with females and lead to a temporary reduction in offspring production during their lifetimes. Regular releases could serve as a novel form of long term pest management, with a reduced requirement for insecticides which necessitate management against the evolution of resistance (Elzen and Hardee, 2003, Atyame et al., 2015, Ferguson et al., 2015, Zhang et al., 2015, Ndii et al., 2015). Indeed, the CIM strategy is now being implemented in the field by Oxitec, although it is achieved by genetically engineering the mosquitoes rather than infecting them with *Wolbachia* (Lacroix et al., 2012).

If similar features such as reduced longevity, cytoplasmic incompatibility and sex ratio distortion can be induced in sap-feeding Hemiptera pests, then it may contribute to sustainable agricultural and horticultural pest management. Although *Wolbachia* has not been yet observed in *P. citri* or *A. pisum*, it has been located in species of the

same families for both these pests (Jeyaprakash and Hoy, 2000, Zchori-Fein and Perlman, 2004). As mentioned previously, cytoplasmic incompatibility was induced in the brown plant hopper *N. lugens* following successful transinfection from another plant hopper species *L. striatellus* (Kawai et al., 2009). If transinfection can be found to induce cytoplasmic incompatibility in *P. citri* or *A. pisum*, then the possibility of releasing infected males amongst crops as a form of CIM could be explored. The greatest threat against this strategy would be strong selection preference for any *Wolbachia*-infected females, which could lead to an increase in their prevalence and thus render the CIM method useless, or for mechanisms that overcome cytoplasmic incompatibility to develop. These risks should be evaluated and monitored accordingly. If research yields other parasitic bacteria of Hemiptera, then, depending upon their properties, they may also serve as potential biocontrol agents.

Non-*Wolbachia* based strategies may be a more viable option for growers with a rapid turnover of stock. Following my own discussions with greenhouse horticultural growers in Belgium, it is clear that the speed of pest control is essential. Pest populations are often introduced through new stock that has not been thoroughly inspected, a time-consuming process that may easily miss small or obscured insects. Infestations can spread rapidly across densely stored produce and individual plants may only be present for a relatively short period of time before being sold or transferred. Although offspring sex ratio-distorting strains of *Wolbachia* can serve as a long term strategy, it takes at least one generation of the pest insect to have passed before this bacterium's impact is actualised, by which point the infested plant may be worthless for retail or have spread its burden to surrounding stock. Furthermore,

storing *Wolbachia*-infected males may not be practical when pest populations come and go with moving stock. Targeting and disrupting obligate mutualistic symbioses, perhaps directly through antibiotic spraying (modelling could clarify whether the evolution of resistance would pose a considerable risk in this scenario) or indirectly via specific bacteriophages, may have a more immediate impact. Hosts without their obligate nutritional symbionts should rapidly struggle to survive. Facultative bacteria that provide resistance against parasitoids and pathogens, such as *H. defensa*, could also be aggressively targeted to reduce their efficacy, before or whilst applying the parasitoid/pathogen insect control.

Understanding the microbiota of pest insects in a given area may also offer indirect benefits to growers. For example, if *H. defensa*, alongside the APSE bacteriophage, is detected at moderate or high levels within pest aphids in a given field or greenhouse, the grower could be advised that parasitoid-based control would experience reduced efficacy. The grower could then focus their resources on implementing an alternative strategy that would not be hampered by the presence of *H. defensa*. As the world's population has recently surpassed 7 billion (Lutz and Samir, 2010), agricultural and horticultural growers are under ever-increasing stress to produce greater yields, whilst simultaneously being expected to improve methods to use fewer resources and pesticides. The green revolution solution to this crisis is unlikely to be found in a single silver bullet strategy, but will be created from a combination of approaches developed with expertise across multiple disciplines of science working together. Targeting pest endosymbiosis and manipulating their

relationships for their control may form a significant part of the future of food supply.

1.10 Aims

Bacterial endosymbiosis is a fascinating and fundamental evolutionary process, and is an essential component of insect adaptation and ecology. Citrus mealybugs present an intriguing and potentially highly informative model system, with a twist. They harbour a nutritional obligate symbiont, which itself contains a second symbiont. Rarely in animals have three genetically distinct organisms been so intimately associated. This raises questions in symbiosis evolution, such as whether two symbionts can be regulated independently within a host when one inhabits the other. Citrus mealybugs are also stubborn pests of horticulture, which are difficult to control. Pesticides are the most commonly applied control strategy, but these tools are becoming increasingly restricted and regulated in the EU and globally. Understanding the relationship and dynamics between mealybugs and their symbionts could help to comprehend symbiotic systems better and potentially pave the way for symbiont-based pest control strategies. These questions can be approached by investigating the variation in the density/infection intensity of each symbiont in citrus mealybugs, whether the symbionts are under independent regulatory mechanisms and how symbiont density impacts host fitness.

In my second chapter, I explore natural variation in the density of *T. princeps* and *M. endobia* in laboratory-reared citrus mealybug strains and whether this variation impacts life history. In my third chapter, I take this approach to a greenhouse setting,

where I investigate whether symbiont density impacts the hosts' ability to exploit different food sources and resist pesticide application. In my fourth chapter, I use heat stress to artificially reduce symbiont density in mealybugs and observe how their fitness is impacted. In my fifth chapter, I hybridise mealybug strains in order to investigate the heritability of symbiont density and separate the controlling mechanisms behind each symbiont. In my sixth chapter, I explore the facultative symbionts of citrus and long-tailed mealybugs, using Next Generation Sequencing. In my final chapter, I collate my findings and discuss as a whole their implications for the evolutionary ecology of endosymbionts, and microbe-based pest management.

“The entire universe has been neatly divided into things to (a) mate with, (b) eat, (c) run away from, and (d) rocks.”

― *Terry Pratchett*

2 The More, the Merrier? Population Variance in Symbiont Density Holds No Clear Fitness Benefits in an Obligate Host-Mutualist System

2.1 Abstract

Symbiotic bacteria are highly diverse, play an important role in ecology and evolution, and are also of applied relevance because many pest insects rely on them for their success. However, the dynamics and regulation of symbiotic bacteria within hosts is complex and still poorly understood outside of a few model systems. One of the most intriguing symbiotic relationships is the obligate, tripartite nutritional mutualism in sap-feeding, economically-destructive mealybugs (Hemiptera: Sternorrhyncha: Pseudococcidae), which involves γ-proteobacteria hosted within β-proteobacteria hosted within the mealybugs. Here, it is examined whether there is population variation in symbiont density (i.e. infection intensity, or titre) in the citrus mealybug, *Planococcus citri* (Risso), and how this impacts host life-history. Symbiont density is found to differ significantly between populations when reared under controlled environmental conditions, indicating that the density of symbiont infections is influenced by host or symbiont genotype. However, symbiont density changes in populations over multiple generations, indicating that symbiont densities are dynamic. Surprisingly, given that the symbionts are essential nutritional mutualists, the density of the symbionts does not correlate significantly with either host fecundity or development. Higher levels of symbionts had no clear benefit to hosts and therefore appear to be superfluous, at least under constant, optimised

environmental conditions. Excessive symbiont density may be an evolutionary artefact from a period of inefficient vertical transmission when the balance of conflict between host and symbiont was still being established.

2.2 Introduction

Symbiotic bacteria are now understood to be highly diverse and influential players in eukaryotic ecology and evolution (Saffo, 1992, Moran, 2001, Douglas, 2009). They are fundamental to many aspects of life, having given rise to mitochondria and chloroplasts, as well as numerous other pivotal evolutionary steps, such as nitrogen-fixation and bioluminescence (Yasaki, 1928, Peix et al., 2015, Giobel, 1926, Schwartz and Dayhoff, 1978). The impacts that symbionts have on their hosts can range from mutualistic, to commensal, to parasitic. However, rather than residing in strict categories, the relationships between hosts and symbionts exist on a dynamic spectrum, and may often be context-specific (Swain, 2012, Gerardo, 2015).

Each member in a symbiotic relationship will ultimately evolve to maximise its own fitness rather than that of its partner, so conflict between host and symbiont may often occur, even in mutualistic associations (Bennett and Moran, 2015). The maintenance of beneficial symbionts will still incur some cost to the host, and the host should reduce symbiont density (i.e. infection intensity) when it is in excess, whereas the symbiont should seek to optimise its density to maximise the likelihood of its own transmission to new hosts (Bronstein, 2001, Falkowski et al., 1993, Rio et al., 2006, Wilkinson et al., 2007, Cunning and Baker, 2014, Laughton et al., 2014). Symbionts may also increase their virulence to compete with other strains and species of symbionts that they encounter (Smith, 2007, Birky et al., 1983, Frank, 1996b, Funk et al., 2000). For example, superinfection of two facultative, mutualistic symbionts was found to impose substantial fecundity costs in pea aphids, *Acyrthosiphon pisum*, likely due to the increased bacteria load or interactions

between the bacteria (Oliver et al., 2006). Efficient vertical transmission of beneficial symbionts to host offspring may resolve this conflict for both partners and also reduces the chance of within-host competition between different strains of symbionts (Frank, 1996b).

Facultative changes in symbiont density, according to the sex and life stage of the host have been documented in many insects. For example, infection density of a nutritional symbiont increases in the cereal weevil *Sitophilus* during larval development, the period in which the symbiont is most required, and then decreases afterwards when high symbiont levels no longer hold a benefit to the host (Vigneron et al., 2014). Similarly, the infection density of the obligate, nutritional, *Buchnera* symbiont in pea aphids tends to decrease with host age, while those of the intracellular symbiont *Wolbachia* are lower in male *Aedes* mosquitos compared to females (Lu et al., 2014, Tortosa et al., 2010). Host genetics may also play a role, with *Wolbachia* infection intensity in adzuki bean beetles *Callosobruchus chinensis* varying depending on host genotype, being linked to host genes conveying insecticide resistance in the mosquito *Culex pipiens*, and influencing the cost to the host of infection (Kondo et al., 2005, Duron et al., 2006, Berticat et al., 2002). Similar results have been found in whiteflies, *Bemisia tabaci*, and their facultative symbionts (Ghanim and Kontsedalov, 2009).

One of the most intriguing examples of symbiosis is found in the mealybugs (Hemiptera: Sternorrhyncha: Pseudococcidae). They harbour a remarkable, nested symbiont set-up believed to be unique to the Pseudococcidae (Thao et al., 2002, Baumann et al., 2002). Most mealybugs harbour two maternally-transmitted,

obligate, nutritional, bacterial endosymbionts in bacteriocytes, which comprise their bacteriome organ surrounding the gut. These are *Candidatus Tremblaya princeps*, a β-proteobacterium, and *Candidatus Moranella endobia*, a γ-proteobacterium which resides inside *T. princeps* in a Russian doll-like fashion (Thao et al., 2002). It is the only example known to science of one bacterium residing inside another bacterium (Von Dohlen et al., 2001, Keeling, 2011).

The mealybugs *Planococcus kraunhiae* and *Pseudococcus comstocki* show changes in the density of both *T. princeps* and *M. endobia*, depending on host sex and life stage. The symbionts increase in infection intensity up until adulthood, and then become reduced in virgin females beyond reproductive age and lost in males completely post-pupation, probably because adult males do not feed and so break down the symbionts for energy (Kono et al., 2008).

Although the obligate nature of the two mutualistic symbionts in mealybugs means that they are probably key to host survival and fitness, there has been little investigation of whether the infection density of symbionts varies across mealybug species, populations or genotypes, or whether any variation in infection intensity affects host fitness. In this study it is therefore examined whether there is population variation in symbiont densities (i.e. infection intensities) in adult and juvenile citrus mealybug, *Planococcus citri* (Risso), females. It is also investigated whether symbiont density may affect host life history, including fecundity, development rate and size at adulthood.

2.3 Methods

2.3.1 *Sourcing and rearing of mealybugs*

Thirteen populations of citrus mealybug, *P. citri*, were obtained from commercial greenhouses in Belgium (see Table 1 for full list of locations, original host plants, population abbreviations and which populations were included in each experiment) and maintained for six months under standardised laboratory conditions on white organic potato sprouts at 25°C and 50% relative humidity in constant darkness.

2.3.2 *Population differences in obligate symbiont infection intensity*

To determine the intensities of *M. endobia* and *T. princeps* infections, quantitative PCR with symbiont-specific primers and protocols were used (see below; (Parkinson et al., 2014)) Newly eclosed adult females from 13 populations (20 individuals from each) and 2nd instar female juveniles from 12 of the populations (20 individuals from each) were randomly selected. Eight and 11 months after these samples were collected, additional sets of newly eclosed adult females were randomly selected from ten of the same populations (20 individuals from each) to examine whether symbiont densities were consistent over time. DNA was extracted by crushing individual mealybugs in 100µl of 5% Chelex and heating to 99°C for 15 min, before centrifuging the extract at 2,326 g for 20 min. The DNA supernatant was diluted to 1/10 in molecular grade water for use in qPCR reactions. Mean concentrations per mealybug of *T. princeps* and *M. endobia* were compared against the *P. citri* host control gene for three technical replicates using the comparative C_T method to produce relative ΔC_T values (Crotti et al., 2012) in a StepOnePlus^{TM} Real-Time PCR

System. Primers and probes for *P. citri* and *T. princeps* were designed using the software PRIMER3 (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) and analysed using the software NetPrimer (Primer Biosoft International, Palo Alto, CA, USA). Primers and probes for *M. endobia* were designed using the software Primer Express v.3.0 (Life Technologies, Foster City, CA, USA). The *GroEL* (AF476091) gene of *T. princeps* was amplified using the primers *TprincepsF* 5'-TCCAAGGCTAAATACCCACA-3' and *TprincepsR* 5'-ATACAAAAGGTACGCCGTCA-3' and the 6FAM florescent probe *TprincepsP* 5'-CGCGCATACGAACAGTCGGA-3'. The *16S* and *23S rDNA* (AF476107.1) region of *M. endobia* was targeted using the primers *MendobiaF* 5'-GAGCACCTGTTTTGCAAGCA-3', *MendobiaR* 5'-CCCCTAGAGTTGTGGAGCTAAGC-3' and the 6FAM florescent probe *MendobiaP* 5'-AGTCAGCGGTTCGATC-3'. The host control gene *28S rDNA* (AY179451.1) was amplified using the primers *PcitriF* 5'-TCCGAGGAGACGTGTAAAAGTTC-3', *PcitriR* 5'-CCTAGCCGCCGAAACGA-3' and the 6FAM florescent probe *PcitriP* 5'-ACGGCGCGTGTCGA-3'. Volumes of 10 µl were used for qPCR reactions with reagent final concentrations of 150 nM of each primer, 50 nM of probe, and 1× of ABI Taqman Universal Master Mix II with UNG (Life Technologies, Foster City, CA, USA). The cycle was 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and the annealing temperature (collection step) for 1 min. An annealing temperature of 60°C was used for *Tremblaya princeps* reactions and 64°C for *P. citri* and *Moranella endobia* reactions.

2.3.3 *Population differences in life history traits*

1) To determine if the fecundity of females differed between the populations, new adult females from all 13 populations (between 10 and 25 individuals per population) were placed with two males from the same population for 48 h to ensure mating. Females were then placed on individual potatoes for ten days, and the total number of eggs laid by each female during this time was counted.

2) To determine if the development rate of mealybugs differed between the populations, eggs were collected < 24 h after being laid by 3-4 females from all 13 populations (~150 eggs per population) using a fine paintbrush and transferred to a separate potato for observation. Emerging offspring were observed each day under a dissecting microscope. When an individual reached the adult instar (either as male or female), it was removed from the group and the day recorded (hatching day being designated as day 0). The number of days taken for surviving offspring to reach adulthood were analysed separately by sex.

3) Finally, to determine whether mealybugs differed in size between populations, the dorsal surface of new adult females from 11 of the populations (between 15 and 30 individuals per population; see Table 1 for which populations) were photographed under a dissecting microscope with a 1mm graticule. The dorsal surface area of each mealybug was then measured, using ImageJ software (Rasband, 2014).

2.3.4 *Statistical analysis*

qPCR data was processed using the comparative C_T method (Crotti et al., 2012). The C_T data were analysed for *T. princeps* and *M. endobia* separately using a generalized linear model with a Gamma distribution and log-link function and the likelihood ratio χ^2 statistic. This was to determine whether the 13 mealybug populations differed in symbiont density in adults at six months, and whether for ten of these populations, the density changed after eight and 11 months (with month and the interaction between month and population included in the model). We also determined whether symbiont density differed between 12 of the populations in juveniles at 6 months. The Spearman Rank Order Correlation was used to determine whether the symbiont densities in adults and juveniles were correlated across populations.

The number of eggs laid by females (fecundity) and the number of days taken to reach adult instar (development rate), were compared between populations using generalized linear models with a Poisson distribution and loglinear link function. The fecundity data were overdispersed, which was corrected by incorporating a scale weight variable of 0.02. The dorsal surface area of females (size) was analysed using general linear models with a gamma distribution with a log-link function and the Likelihood ratio χ^2 statistic**.**

The mean infection density of each symbiont in adult or juvenile females from each population after six months of lab rearing were correlated against the fecundity, development rate and size of mealybugs in that population using Spearman's correlations. All analyses were conducted in SPSS v.22 (Inc., 2013).

2.4 Results

2.4.1 *Population differences in obligate symbiont infection intensity*

The densities of *T. princeps* and *M. endobia* were found to differ significantly between the adult females of the 13 populations in the initial samples taken after six months under standard environmental conditions (χ^2 = 146.5, d.f. = 12, P < 0.001; χ^2 = 151.1, d.f. = 12, P < 0.001, respectively), with populations differing by as much as six-fold for both symbionts (Figure 2.1). Symbiont densities also differed significantly between 2nd instar juveniles of 12 of the populations, with populations differing by as much as 45- fold and 30-fold for *T. princeps* and *M. endobia* respectively (χ^2 = 58.1, d.f. = 11, P < 0.001; χ^2 = 296.1, d.f. = 11, P < 0.001, respectively). Symbiont density was also influenced by time, and the impact of this varied between populations (population*time interaction for *M. endobia*, χ^2 = 351.2, d.f. = 18, P <0.001, and for *T. princeps* χ^2 = 236.6, d.f. = 18, P <0.001) (Figure 2.2). However, neither the mean densities of *M. endobia* nor *T. princeps* correlated between adults and 2nd instars across populations (r_s = -0.231, P = 0.471; r_s = 0.014, P = 0.966, respectively).

2.4.2 *Population differences in life history traits*

The fecundity, development rate and size of mealybugs all differed significantly between populations (fecundity: χ^2 = 44.8, df = 12, P < 0.001; development rate: χ^2 = 96.0, df = 12, P < 0.001 for females and χ^2 = 105.7 df = 12, P < 0.001 for males; size: χ^2 = 29.5, d.f. = 10, P < 0.001). Fecundity varied between populations by up to

2.5-fold, development rate by up to 25% and 30% in females and males respectively and adult female size by up to 31% (Figure 2.3).

2.4.3 *Relationship between life-history and symbiont density*

There was no evidence of relationships between the densities of either symbiont and any of the host life-history traits measured (Supplementary Fig. S2.1). The mean densities of *M. endobia* and *T. princeps* in adults did not correlate across populations with the mean fecundity of females (r_s = 0.313, P = 0.297; r_s = 0.363, P = 0.224, respectively), the mean development rate for females or males (r_s = 0.049, P = 0.181 and r_s = -0.04, P = 0.276 for females; r_s = 0.027, P = 0.555 and r_s = -0.045, P = 0.284 for males, respectively), or the mean size of adult females (r_s = 0.071, P = 0.219, r_s = -0.501, P = 0.116, respectively). The mean densities of *M. endobia* and *T. princeps* in 2nd instar juveniles also did not correlate across populations with the mean fecundity of females (r_s = -0.178, P = 0.580, r_s = -0.16, P = 0.961, respectively), the mean development rate for females and males (r_s = 0.03, P = 0.926 and r_s = -0.395, P = 0.204 for females; r_s = 0.38, P = 0.223, r_s = -0.25, P = 0.938 for males, respectively), or the mean size of adult females (r_s = -0.101, P = 0.796, r_s = -0.43 P = 0.248, respectively).

2.5 Discussion

The results show that citrus mealybug populations reared under controlled environmental conditions differ substantially in multiple life-history traits and also in the densities of infections with their obligate mutualistic symbionts, both for adult

females and 2nd instar juveniles. However, symbiont infection density does not correlate significantly with any of the life-history traits measured, or between adults and juveniles, and was dynamic, changing in population-specific ways over an 8-11 month period.

The fact that the population differences are present when the mealybugs are kept under identical environmental conditions on the same host plant for six months (1 month is ~ 1 generation) suggests that they may be due to host or symbiont genotypes. However, symbiont density also significantly changes for 40% of the populations studied after a further 8 and 11 months in constant laboratory-rearing conditions, indicating that another factor may be involved, or that genetic drift has since occurred within the laboratory populations. This would reflect findings that the host genotype is associated with symbiont infection intensity in adzuki bean beetles and *Leptopilina heterotoma* parasitic wasps (Kondo et al., 2005, Mouton et al., 2007). Hybridisation experiments between citrus mealybug populations previously suggested a link between host genotype and symbiont density (Parkinson et al., 2016). The host genotype often undergoes mutations and other modifications to genes associated with immunity after symbiont acquisition, for example the pea aphids have been shown to lose genes central to the IMD immune pathway (Gerardo et al., 2010, The International Aphid Genomics Consortium, 2010). Changes to symbiont density following laboratory rearing are not unusual. For example, laboratory rearing for multiple generations was found to reduce the variation in symbiont density in *Drosophila* and mosquitos, whilst also resulting in genome-wide

selective sweeps as the host adapted to the new environment (Correa and Ballard, 2012, Dutton and Sinkins, 2004, Montgomery et al., 2010).

Symbiont densities in 2nd instar female juveniles are more variable within populations than is the case in adults. This indicates that symbiont density may be more sensitive to influencing factors, such as environment or epigenetics, during development, before reaching a common target density at adulthood. The density of the γ-proteobacterial symbiont in females of the *P. kraunhiae* and *P. comstock* mealybugs has also been found to show wider variation in 2nd instars compared to adults, although this did not appear to be the case for the β-proteobacterial symbiont in these species (Kono et al., 2008). *Buchnera* density also shows wide variation in pea aphid embryos with older mothers, with the impact of maternal age still influencing obligate symbiont intensity at adulthood (Laughton et al., 2014). Influences of host life stages such as these may partially explain why mean symbiont infection intensity did not significantly correlate across populations between juveniles and adults in the citrus mealybugs in this study.

Surprisingly, despite these symbionts being obligate nutritional mutualists, there is no evidence of mealybugs benefiting or suffering from variation in symbiont density. These results contrast with aphids, in which reductions in their obligate nutritional mutualist *Buchnera* has fitness costs for the host, and also with the theoretical prediction that some benefit must accrue from each symbiont cell hosted because accommodating even a mutualistic symbiont will incur some cost to the host, (Bronstein, 2001, Sakurai et al., 2005, Koga et al., 2007). Heat-stress-induced symbiont loss in adult citrus mealybugs was also not found to impact fecundity

(Parkinson et al., 2014). Possibly, variation in symbiont density in these mealybugs is compensated for by facultatively adjusting the metabolic activity of the obligate symbionts, with symbionts in low numbers creating more products per symbiont and vice versa.

The benefits of higher nutritional symbiont density may also be context-dependent, only becoming apparent under certain environmental conditions or when the host stressed. For example, the relative infection densities of facultative symbionts in whitefly *Bemisia tabaci* are influenced by the species of host plant on which the host is feeding (Pan, 2013), and the benefits of endophytic symbionts in *Lolium multiflorum* plants only become apparent when the plants are drought-stressed (Miranda et al., 2011). Mealybugs here are reared under standard conditions that do not expose them to environmental stresses, multiple host plants or the introduction of additional symbionts. Exposing mealybugs with different symbiont infection densities to these more stressful conditions could reveal their costs or benefits.

There is little evidence for horizontal transmission of *T. princeps* or *M. endobia* since the inception of their symbiosis and their vertical transmission is efficient (Baumann, 2005). Efficient vertical transmission tends to result in homogenous symbionts of low virulence as the selection pressure for horizontal transmission is relaxed (Smith, 2007, Birky et al., 1983, Funk et al., 2000, Frank, 1996b), and so high infection densities of *T. princeps* and *M. endobia* would not hold an obvious benefit to these symbionts in terms of increased transmission rate or more efficient transmission. However, it is possible that the higher symbiont infection intensities observed in some citrus mealybug populations may be an evolutionary relic from a

period where high intensity carried some transmission advantage or may have evolved to outcompete other facultative symbionts. As increased symbiont infection intensity appears to hold no clear fitness costs to the host, this virulence would then not necessarily be removed by selection.

The overall fitness of the holobiont should in theory require optimum regulation of the symbionts (Koga et al., 2003, Hoogenboom et al., 2010). Here, we have found that mealybug populations differ in the infection densities of their obligate symbionts, that this density can change dynamically even under standard environmental conditions, but that under standard conditions there is no clear fitness benefits to carrying more or fewer symbionts. The causes and consequences of high or low symbiont density therefore need to be investigated further to better understand the relationship between hosts and their symbionts. Not only will this be informative from an evolutionary ecology perspective, but also in terms of the potential application of microbial resource management to sustainable pest control.

2.6 Tables

2.6.1 ***Table 2.1.***

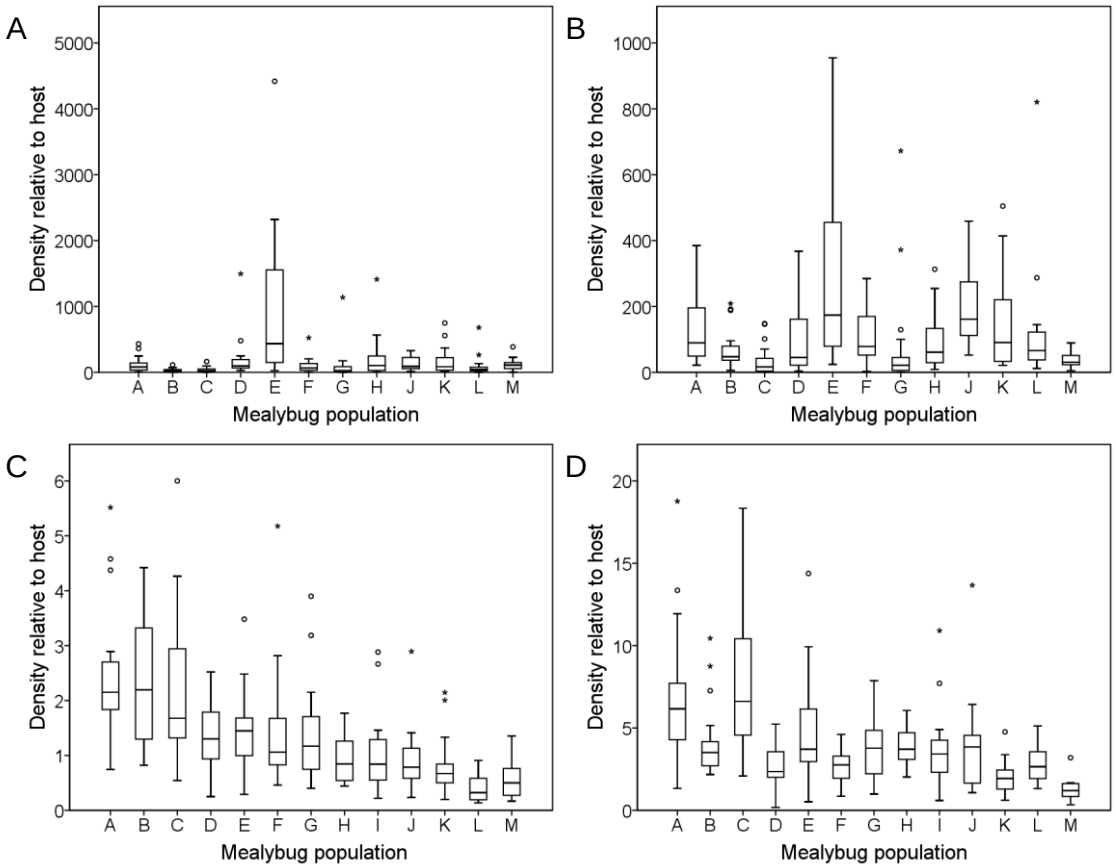
List of the 13 populations of citrus mealybugs used in the study, with the location and host plant they were originally collected from, and whether they were used in each part of the study.

			Used in observation?					
Population	**Sourced location**	**Original host plant**	**6 month qPCR**	**2nd instar qPCR**	**8 and 11 month qPCR**	**Fecundity**	**Developmental rate**	**Size**
A	Bloemisterji Bogaerts, Zoersel	*Gardenia* species	Yes	Yes	Yes	Yes	Yes	Yes
B	De Meyst Werner	*Aeschynanthus* species	Yes	Yes	Yes	Yes	Yes	Yes
C	Brico retailer	*Ficus benjamina*	Yes	Yes	Yes	Yes	Yes	No
D	Floraliën	*Croton* species	Yes	Yes	Yes	Yes	Yes	Yes
E	Proefcentrum voor Sierteelt	*Ficus benjamina*	Yes	Yes	No	Yes	Yes	Yes
F	De Meyst Werner	*Schefflera* species	Yes	Yes	Yes	Yes	Yes	Yes
G	Aquarella	*Ficus* species	Yes	Yes	No	Yes	Yes	Yes
H	Scheppersinstituut	*Ficus benjamina*	Yes	Yes	Yes	Yes	Yes	Yes
I	Aquarella	*Croton* species	Yes	No	No	Yes	Yes	Yes
J	Proefcentrum voor Sierteelt	*Crassula* species	Yes	Yes	Yes	Yes	Yes	Yes
K	Intratuin retailer	*Mandevilla* species	Yes	Yes	Yes	Yes	Yes	Yes
L	Proefcentrum voor Sierteelt	*Maranta* species	Yes	Yes	Yes	Yes	Yes	Yes
M	Thomas More Geel campus	*Ficus benjamina*	Yes	Yes	Yes	Yes	Yes	No

2.7 Figures

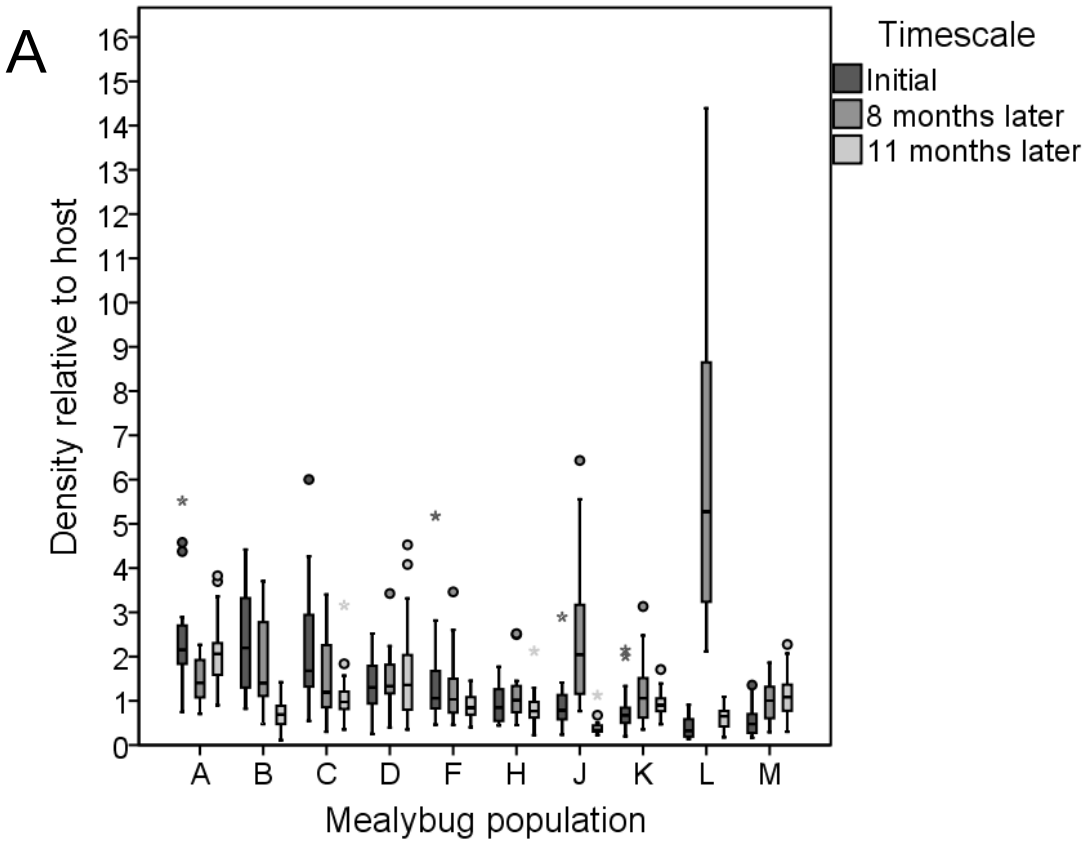
2.7.1 *Figure 2.1.*

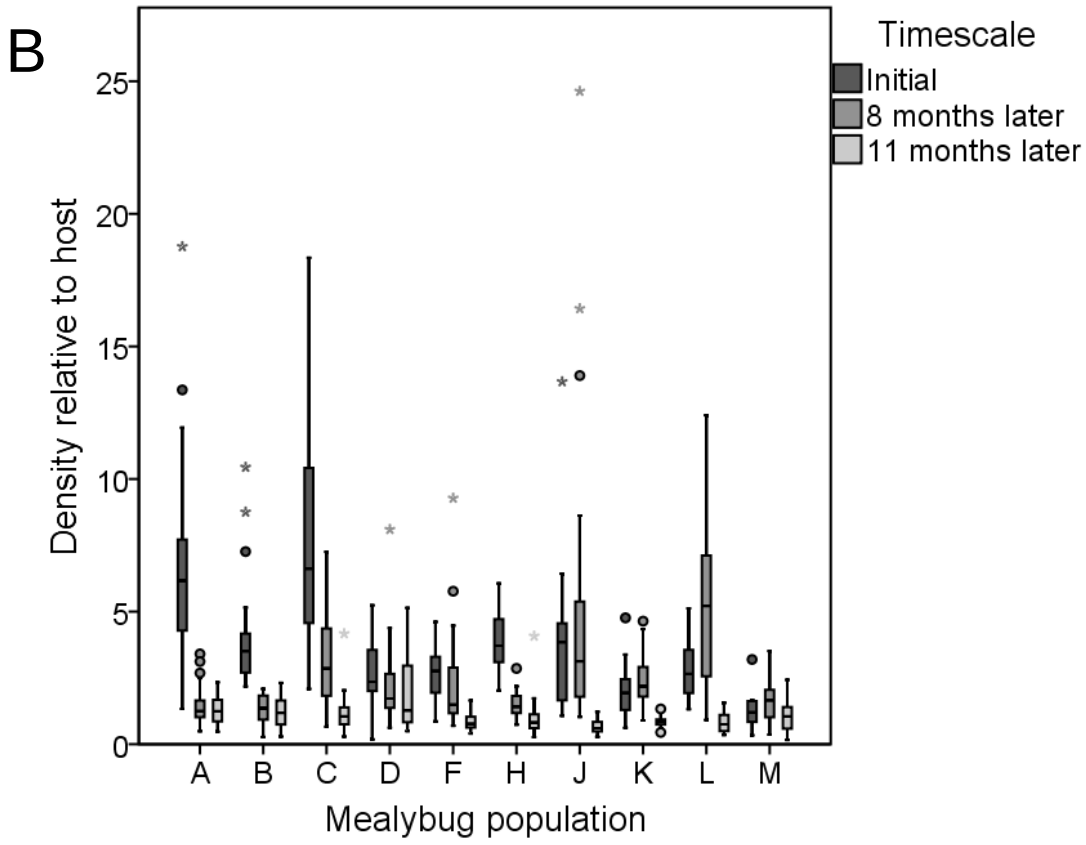
The infection densities of the M. endobia (A, C) and T. princeps (B, D) obligate mutualist symbionts in 12 populations of 2nd instar female citrus mealybugs (A, B), and 13 populations of adult females (C, D). Plots show mean infection densities (relative to the number of P. citri cells in each case), quartiles and 95th percentiles. Symbiont densities were calculated by qPCR.



2.7.2 ***Figure 2.2.***

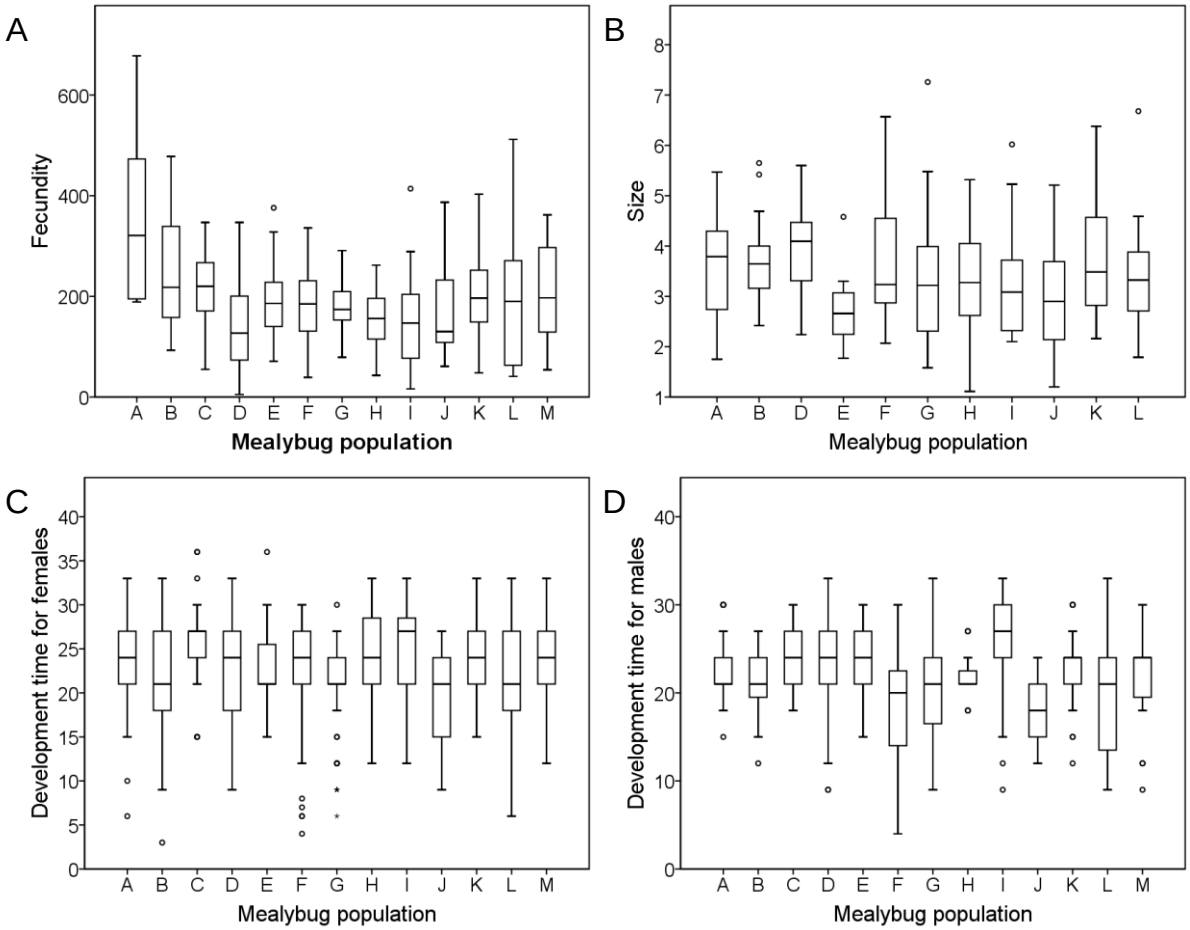
The infection densities of symbionts in 10 populations of adult female citrus mealybugs: initially and 8 and 11 months later. Plots show mean infection densities (relative to the number of *P. citri* cells in each case), quartiles and 95th percentiles of *M. endobia* (A) and *T. princeps* (B). Symbiont densities were calculated by qPCR.


A
Density relative to host
Mealybug population
A B C D F H J K L M
Timescale
Initial
8 months later
11 months later


B
Density relative to host
Mealybug population
A B C D F H J K L M
Timescale
Initial
8 months later
11 months later

2.7.3 ***Figure 2.3.***

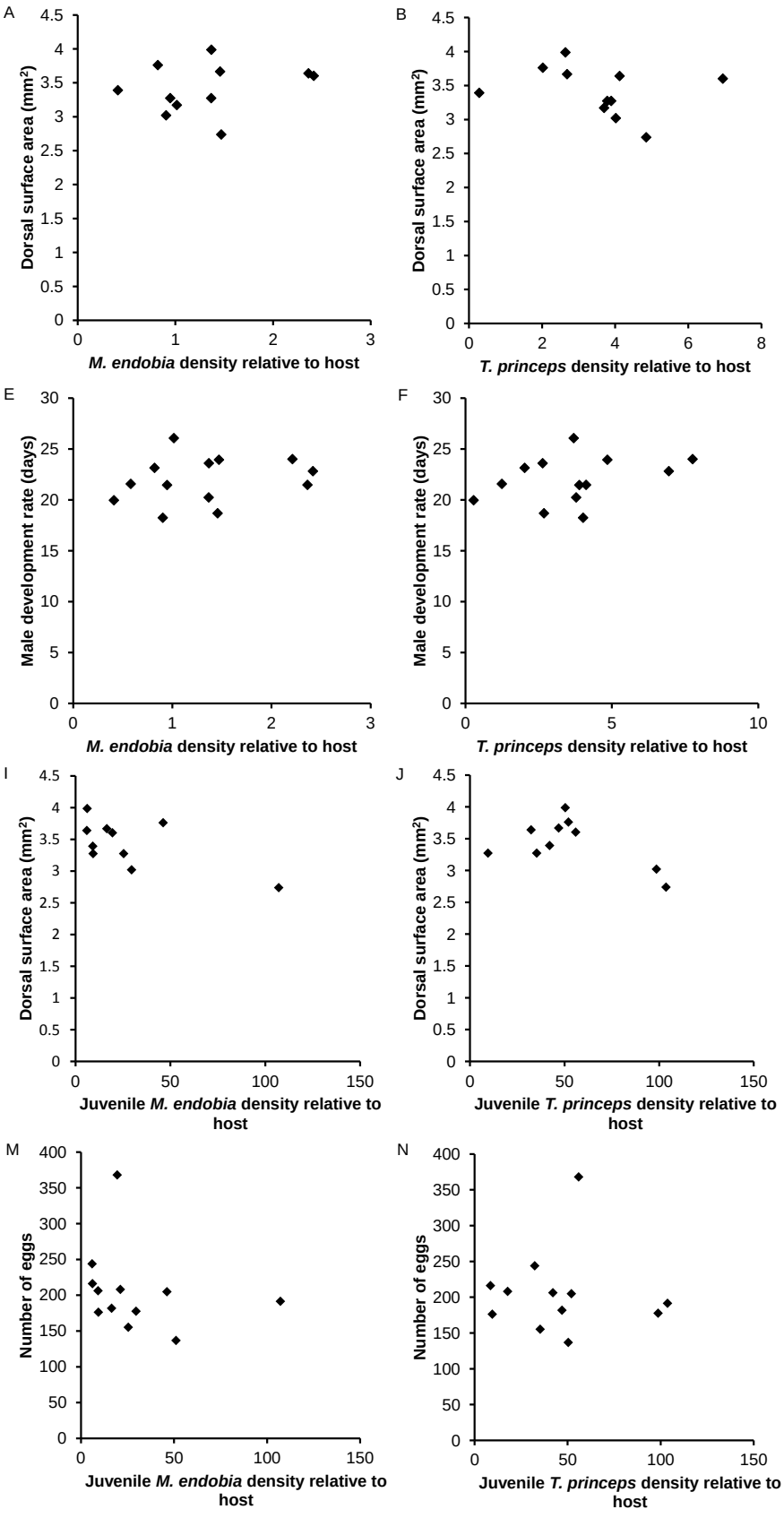
The mean, quartiles and 95th percentiles of (A) fecundity, (B) size, and development time of (C) females and (D) males from 12 populations of citrus mealybugs. Fecundity is measured as lifetime egg production of females, size as dorsal surface area of adult females in mm2, and development time as days from hatching to adulthood.

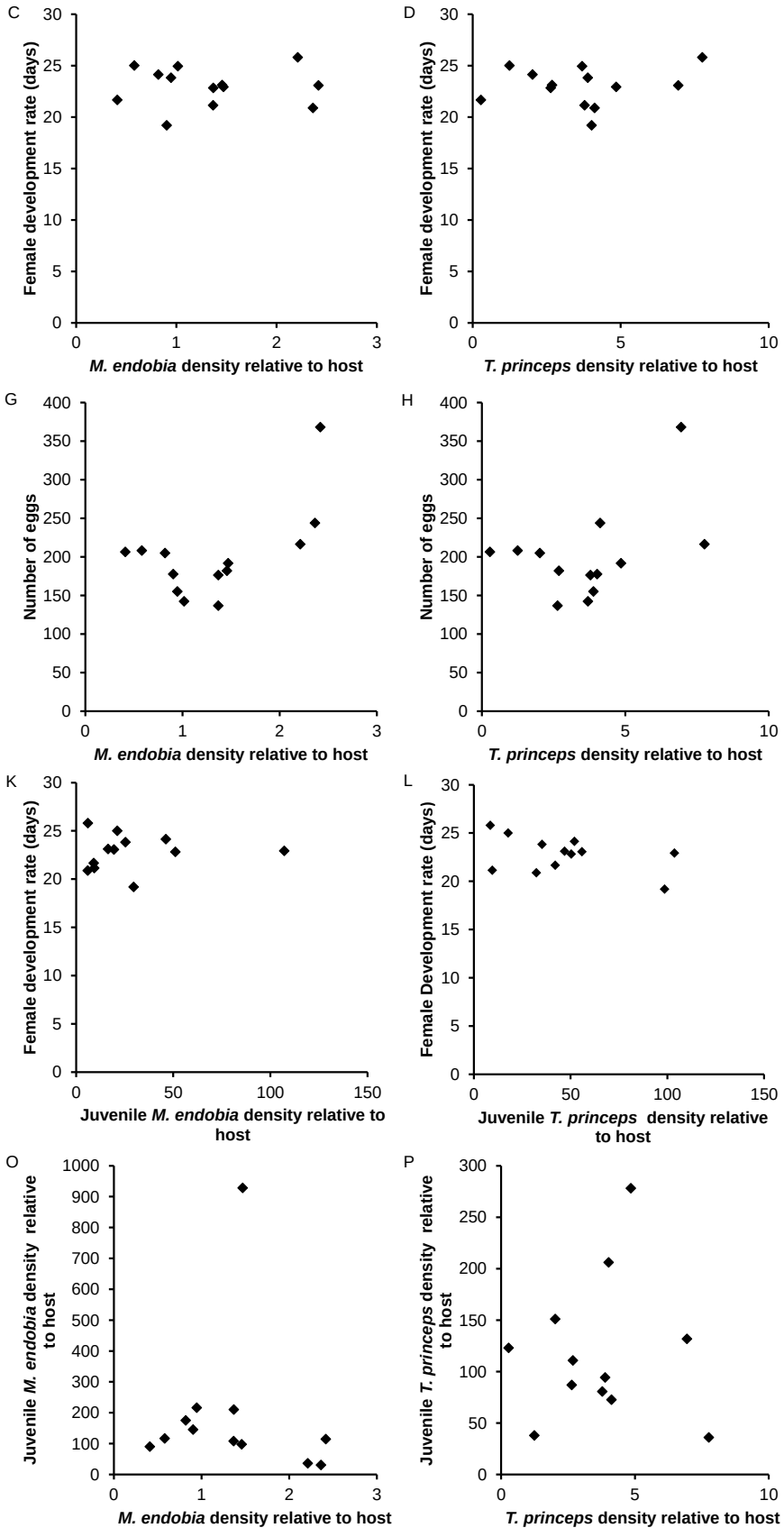


2.8 Supplementary information

2.8.1 ***Figure S.2.1***

The relationships between the mean densities (relative to the number of host mealybug cells) of the *M. endobia* (1st and 3rd columns) and *T. princeps* (2nd and 4th columns) obligate mutualist symbionts, in adult (A to H) and juvenile (I to P) females citrus mealybugs with the mean size (dorsal surface area), development rate of females, development rate of males, and fecundity of females (number of eggs laid across lifetime).


A
Dorsal surface area (mm²)
M. endobia density relative to host
B
Dorsal surface area (mm²)
T. princeps density relative to host
E
Male development rate (days)
M. endobia density relative to host
F
Male development rate (days)
T. princeps density relative to host
I
Dorsal surface area (mm²)
Juvenile M. endobia density relative to host
J
Dorsal surface area (mm²)
Juvenile T. princeps density relative to host
M
Number of eggs
Juvenile M. endobia density relative to host
N
Number of eggs
Juvenile T. princeps density relative to host


C
Female development rate (days)
M. endobia density relative to host
D
Female development rate (days)
T. princeps density relative to host
G
Number of eggs
M. endobia density relative to host
H
Number of eggs
T. princeps density relative to host
K
Female development rate (days)
Juvenile M. endobia density relative to host
L
Female Development rate (days)
Juvenile T. princeps density relative to host
O
Juvenile M. endobia density relative to host
M. endobia density relative to host
P
Juvenile T. princeps density relative to host
T. princeps density relative to host

“I love fools’ experiments. I am always making them.”

— *Charles Darwin*

3 Symbionts in excess? No effect of symbiont density on the ability of mealybug hosts to exploit plant species or tolerate insecticide stress

3.1 Abstract

The acquisition of obligate nutritional vertically-transmitted bacteria has been pivotal to the evolution and diversification of many insect taxa. Sap-feeding citrus mealybugs, *Planococcus citri*, pose a potentially powerful case study for obligate nutritional symbiosis, harbouring a pair of symbionts, *Tremblaya princeps* and *Moranella endobia*. Hosts can often vary in the densities of their symbionts and symbiont cells will inevitably carry some cost to the hosts, so it is hypothesised that a higher symbiont density will have some form of benefit to the host in order to outweigh this cost. Here, we examine whether populations of citrus mealybugs, with heritably different symbiont densities, differed in their abilities to exploit multiple plant species or to tolerate the stress from insecticide exposure. Plant species were found to significantly impact mealybug fitness, but higher symbiont densities did not compensate for reduced host-plant suitability and had no effect on susceptibility to insecticide treatment. *P. citri* harbour symbiont densities that do not appear to benefit the fitness of the host. This apparently sub-optimum symbiont density regulation in an otherwise intricate and tightly-knit tripartite symbiosis could be an evolutionary artefact of previous conflicts of interest.

3.2 Introduction

Symbiotic associations between organisms from separate kingdoms have allowed the joint exploitation of niches (Saffo, 1992). From respiration and photosynthesis, to sulphur oxidisation, to bioluminescence and nitrogen-fixation, bacterial alliances with eukaryotes have repeatedly sparked their expansion and diversification (Giobel, 1926, Yasaki, 1928, Schwartz and Dayhoff, 1978, Peix et al., 2015, Felbeck and Somero, 1982). In insects, an immensely diverse and ecologically important group of animals, the acquisition of a variety of specialised symbiotic bacteria with additional biosynthetic capacities have allowed them to gain sustenance from food sources that would otherwise be nutritionally-deficient (Moran, 2001, Douglas, 2009). For example, blood-feeding insects, such as the tsetse fly, use symbionts to acquire vitamins, symbionts of wood-feeding insects produce enzymes to degrade cellulose, and many phytophagous insects rely on bacteria to produce essential amino acids from plant sap (Akman et al., 2002, Wigglesworth, 1952, Tokuda and Watanabe, 2007, Carpenter et al., 2010, Clark et al., 2010, Buchner, 1965, Douglas, 2009, Akman Gündüz and Douglas, 2009).

Feeding upon plant material poses several challenges to insects. Plant tissues are generally nutritionally unbalanced for their requirements, and thus obligate and vertically-transmitted nutritional symbionts are often crucial to the survival of the host (Mattson Jr, 1980, Sandström and Moran, 1999, Douglas, 2009). The issue is complicated by the variation in chemical properties of plants across species and even the plant developmental stage (Wilkinson et al., 2001, Sandström and Moran, 1999, Karley et al., 2002, Fry et al., 2009). This variation impacts the fitness of the feeder

and selects for specialised digestive mechanisms which only target plant groups of a similar chemical composition. Thus, the symbionts of these insects have often experienced the preservation of essential nutrition-related genes particular to that host and plant type, whilst the rest of their genome becomes largely degraded and lost through the Muller's Ratchet-effects, creating bacteria with reduced and highly specialised genomes for their task (Bennett and Moran, 2015). Hence, phytophagous insects can generally only feed from a narrow range of plants (Clark et al., 2010), often developing and mating on an individual plant, creating inbreeding. Such host plant specialisation via symbiosis could trigger speciation events, as observed in Hawaiian leafhoppers (Drès and Mallet, 2002, Via, 2001, Bennett and Moran, 2015, Bennett and O'Grady, 2012).

Nutritional symbionts of different origins have adapted to meet the requirements of their particular host species. The *Blochmannia* symbiont of *Camponotus* ants has lost genes for the synthesis of arginine, possibly because this amino acid is not deficient in the host diet (Feldhaar et al., 2007). The weevil, *Sitophilus linearis*, lost its nutritional symbiont after a dietary shift from cereals to the more nutritious tamarind seed, reflecting that it was no longer required, and the ability of *Megacopta* spp stink bugs to exploit legumes is impacted by the genotype of its obligate gut bacteria (Delobel and Grenier, 1993, Zientz et al., 2004, Hosokawa et al., 2007). The most thoroughly studied example is *Buchnera* in the aphids. This genus of symbionts provides its hosts with essential amino acids that are deficient in phloem sap and is linked to a speciation event in these insects 200 million years ago (Moran et al., 1993, Lai et al., 1994). Genetic studies have found that different species of aphids

host distinct strains of *Buchnera* which differ in their biosynthetic capacities, reflecting the dissimilar nutritional requirements of the hosts (Shigenobu et al., 2000, Pérez-Brocal et al., 2006, van Ham et al., 2003, Moran et al., 2008). Indeed, it is not unusual for herbivorous insects to have sympatrically split into distinct populations or strains that are adapted to particular plant hosts (Mopper and Strauss, 1998, Drès and Mallet, 2002).

The genetic and epigenetic qualities of a symbiont thus impacts which plant species the insect can exploit, and an adaptable symbiont could potentially pose advantages to a host with a variable diet. However, despite its history of gene alteration to meet host requirements, *Buchnera* has a limited capability to alter gene expression over the lifetime of the host, being unable to adjust its biosynthesis rate to a shift in host diet or heat shock (Wilcox et al., 2003, Moran et al., 2003, Moran et al., 2005a, Wilson et al., 2006, Reymond et al., 2006). This is hypothesised to be due to its loss of transcriptional regulator genes, and is instead crudely compensated for by facultative alteration of symbiont cell quantity, as also observed in carpenter ants (Moran and Degnan, 2006, Bermingham et al., 2009, Stoll et al., 2009). For example, the density of *Buchnera aphidicola* was found to positively correlate with nitrogen quantity in the diet of its host, the pea aphid, *Acyrthosiphon pisum* (Wilkinson et al., 2007).

The presence of both obligate and facultative symbionts can in some cases impact the fitness of pest insects in an anthropogenic feeding environment by providing pesticide degradation and detoxification capabilities. For example, *Burkholderia* provides resistance to the insecticide Fenitrothion in stinkbugs, symbiotic yeast

detoxify xenobiotics in cigarette beetles and fungal symbionts allow insects such as bark beetles, ambrosia beetles, long-horned beetles, termites, leaf-cutting ants, wood wasps and drug store beetles to variously metabolise or detoxify lignins, tannins, terpenes, esters, chlorinated hydrocarbons and other toxins (Kikuchi et al., 2012, Dowd, 1992, Dowd, 1989). These capabilities may be an extension of the plant toxin-neutralising capabilities of some microbial symbionts of herbivores (Domínguez-Bello, 1996, Després et al., 2007, Karban and Agrawal, 2002). Conversely, the presence of *Candidatus* Liberibacter asiaticus in Asian citrus psyllids increases their susceptibility to a range of insecticides (Tiwari et al., 2011). The mixture of symbionts present in a host is also of importance, for example, the resistance of whitefly biotypes to several insecticides is influenced positively and negatively by the presence/absence, density and combination of *Arsenophonus*, *Rickettsia*, *Wolbachia*, *Hamiltonella* and *Portiera* (Ghanim and Kontsedalov, 2009, Pan, 2013).

Sap-feeding mealybugs pose a potentially powerful model system for understanding obligate nutritional symbiosis, harbouring a pair of nested symbionts, *Tremblaya princeps* and *Moranella endobia*, that work together in an entwined and complementary fashion in the biosynthetic pathways (Baumann et al., 2002, Husnik et al., 2013, Thao et al., 2002). The citrus mealybug, *Planococcus citri*, despite its name, is a highly polyphagous pest of horticulture, feeding upon dozens of plant species, indicating the versatility of their symbionts (Ben Dov, 2015). As is the case for aphids and carpenter ants, mealybug obligate symbionts are hypothesised to primarily control gene expression by altering symbiont density within the host (Kono

et al., 2008). However, a recent study found that laboratory populations of citrus mealybugs under identical environmental conditions differed from each other in their symbiont density, with this density being heritable, but there were no clear fitness benefits or costs associated with higher obligate symbiont density in terms of development, growth or fecundity (Parkinson et al. 2015; in prep).

As symbiont density is adjusted in mealybugs as they age, it would suggest that there may be a fitness benefit associated with higher or lower symbiont densities in adults that is not apparent when the mealybugs are kept in constant and optimised conditions (Kono et al., 2008). Here, we test this hypothesis by rearing mealybug populations, known to differ in their *T. princeps* and *M. endobia* densities, on four different species of host plant that vary in their susceptibility to mealybug infestation. We then treat the infestations with Teppeki insecticide. By exposing the mealybug populations to suboptimal and stressful conditions, we aim to test whether there is a previously unseen fitness benefit to harbouring nutritional symbionts at a particular density. This is of interest to the field of host-symbiont dynamics and the factors influencing symbiont density, and also from a potential applied perspective in determining whether manipulating symbionts in mealybugs could serve as a pest control tactic.

3.3 Methods

3.3.1 *Sourcing and rearing of mealybugs*

Ten citrus mealybug populations (labelled as 1 through to 10) were sourced from commercial greenhouses in Belgium and cultured in darkness at 25 °C and 20% r.h.

on white organic potato sprouts for 14 months until the start of the experiment. Adult females (virgin for qPCR reactions and gravid for host plant exploitation assessment) were randomly selected for use in experiments.

3.3.2 *Symbiont infection density*

The relative infection intensities of *T. princeps* and *M. endobia* were measured in individual newly-emerged adult virgin female mealybugs across the ten populations, using qPCR and the comparative C_T method (Schmittgen and Livak, 2008), which quantifies the ratio of symbiont cells to host cells. The primers and protocols used were first described in (Parkinson et al., 2014). DNA was extracted from twenty randomly selected individuals from each population, by crushing mealybugs in 100µL of 5% Chelex solution, heating the mixture to 99°C for 15min before centrifuging at 2,326 g for 20 min and pipetting off the extracted supernatant. All DNA supernatant was diluted to 10% in molecular grade water for use in qPCR reactions. Three technical replicates of each reaction were performed in a StepOnePlus™ Real-Time PCR System to generate relative ΔC_T values, which were processed and compared across populations. Primers and probes for host control *P. citri* (*28S rDNA*, AY179451.1) and symbiont *T. princeps* (*GroEL,* AF476091 gene) were designed using PRIMER3 software (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) and analysed for the presence of hairpin structures and dimers with NetPrimer software (Primer Biosoft International, Palo Alto, CA, USA). Primers and probe for the symbiont *M. endobia* (*16S and 23S rDNA*, AF476107.1) were designed using Primer Express v.3.0 software (Life

Technologies, Foster City, CA, USA). The primers and probes are as follows: *PcitriF* 5'-TCCGAGGAGACGTGTAAAAGTTC-3', *PcitriR* 5'-CCTAGCCGCCGAAACGA-3' and the 6FAM florescent probe *PcitriP* 5'-ACGGCGCGTGTCGA-3', *TprincepsF* 5'-TCCAAGGCTAAATACCCACA-3', *TprincepsR* 5'-ATACAAAAGGTACGCCGTCA-3' and the 6FAM florescent probe *TprincepsP* 5'-CGCGCATACGAACAGTCGGA-3' and MendobiaF 5'-GAGCACCTGTTTTGCAAGCA-3', MendobiaR 5'-CCCCTAGAGTTGTGGAGCTAAGC-3' and the 6FAM florescent probe MendobiaP 5'-AGTCAGCGGTTCGATC-3'. Volumes of 10 µl were used for qPCR reactions with reagent final concentrations of 150 nM of each primer, 50 nM of probe, and 1× of ABI Taqman Universal Master Mix II with UNG (Life Technologies, Foster City, CA, USA). The cycle was 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and the annealing temperature (collection step) for 1 min. An annealing temperature of 64°C used for the *P. citri* and *M. endobia* reactions, and 60°C for *T. princeps* reactions.

3.3.3 ***Experiment 1: effect of symbiont density on plant exploitative ability***

We set out to determine how successfully each of the ten mealybug populations could exploit different plant species. The experiment took place in a single research greenhouse at PCS, Ghent, maintained at 25°C at day and 20°C at night, commencing at the beginning of April 2015. Replicate host plant 'islands' were constructed, each containing a single potted plant of each of *Phalaenopsis* sp. (45 weeks old, single stem), *Aralia elegantissima* (10 weeks old), *Calathea roseopicta*

(10 weeks old) and *Fargesia murieliae* (5 weeks old). These plants were selected because of their commercial value in horticulture and known variation in susceptibility to mealybug infestation. These plants were sourced from *Microflor*, Erwin De Baere and *Denis-plants*. A single gravid adult female mealybug was placed on each plant (one mealybug per plant and 4 plants per island, with 1 plant of each species on each island). The islands were composed of a plastic crate, covered with a layer of rag-wadding, on top of which was place an irrigated plastic tray within which the four potted plants were positioned. These islands were randomly distributed across 8 tables (one replicate island for each mealybug population per table, 8 replicates per population in total). The tables were flooded with water to prevent the spread of mealybugs from island to island. Movement of insects between plants on the same island was not inhibited. Plants were irrigated at regular intervals, depending upon soil moisture content. Two months after the experiment was commenced (around two *P. citri* generations at 25°C), the number of mealybugs above 2nd instar were counted on each plant.

3.3.4 ***Experiment 2: effect of symbiont density on susceptibility to insecticide***

In order to determine whether the different symbiont densities of the ten mealybug populations may affect their ability to tolerate the stress from exposure to an insecticide, half of the tables were sprayed after Experiment 1 with 10 litres/m^2 of 0.14g/l of Teppeki insecticide mixed with 0.5ml/l of Trend 90 adjuvant; the other half of the tables were untreated as controls. One month after the treatment was administered, the number of mealybugs above 2nd instar were counted on each plant.

3.3.5 *Statistical analysis*

The C_T data of symbiont densities were converted into ΔCT values, as per the comparative C_T method (Schmittgen and Livak, 2008), and analysed with a generalized linear model, with mealybug population as the factor, a Gamma distribution with a log-link function, and the likelihood ratio χ^2 statistic.

The numbers of mealybugs on the plants in Experiments 1 and 2 were analysed using generalized linear models with Poisson distributions and a log-link function. Mealybug population, plant species and, in Experiment 2, insecticide exposure was included as factors in the model, as well as their interactions. Data were corrected for over dispersion using a scale parameter and nonsignificant interaction terms were removed based on AIC values to obtain the minimum adequate models. Pairwise comparisons were performed using the Sidak correction to the Wald test. Spearman's Rank-Order correlations were used to examine the relationships between symbiont density and number of mealybugs across the ten mealybug populations for each symbiont-plant species combination. All analyses were conducted in IBM SPSS 22.0.

3.4 Results

The infection density of the *M. endobia* and *T. princeps* symbionts differed significantly between the ten mealybug populations (χ^2 = 169.0, d.f. = 9, P < 0.001 and χ^2 = 111.2, d.f. = 9, P < 0.001, respectively). *M. endobia* and *T. princeps*

densities differed by up to 6.4-fold and 4.13-fold between populations, respectively, with Population 6 having a substantially higher density of *M. endobia* than the other populations, and Populations 4 and 6 having higher levels of *T. princeps* (Fig. 3.1).

In Experiment 1, plant species was a highly significant determinant of the mealybug infestation intensity on a plant (χ^2 = 530.0, d.f. = 3, P < 0.001), but mealybug population was not (χ^2 = 15.4, d.f. = 9, P = 0.081). There was no effect of the interaction between the mealybug population and plant species (χ^2 = 23.7, d.f. = 27, P = 0.649). The most prolific mealybug infestations were on *A. elegantissima*, followed by *Phalaenopsis* sp., *C. roseopicta* and *F. murieliae* (Fig. 3.2). No significant correlations were found between the density of either of the symbionts and the number of mealybugs on any plant species (Fig. 3.3).

In Experiment 2, there were no significant interactions between mealybug population and plant species, plant species and spray treatment, or mealybug population and spray treatment (χ^2 = 29.5, d.f. = 27, P = 0.337; χ^2 = 2.9, d.f. = 3, P = 0.413; χ^2 = 15.9, d.f. = 0.069, respectively). Overall, mealybug populations did not differ significantly in the number of mealybugs on plants (χ^2 = 11.6, d.f. = 9, P = 0.238). Although the spray treatment did tend to reduce the numbers of mealybugs on plants slightly, this effect was not found to be significant overall (χ^2 = 2.3, d.f. = 1, P = 0.145). However, plant species was a highly significant determinant of the number of mealybugs on a plant (χ^2 = 154.3, d.f. = 3, P < 0.001), with *A. elegantissima* again having most, and *F. murieliae* the least, mealybugs (Fig. 3.4).

3.5 Discussion

Overall, the ten mealybug populations used in the experiment differed significantly in the density of each of their obligate nutritional symbionts. However, there was no overall difference across the mealybug populations in their ability to exploit the different host plant species. Nor did symbiont density affect the impact of the application of Teppeki on mealybug infestation levels on each plant species. However, mealybugs showed a clear preference for *A. elegantissima* and performed the most poorly on *F. murieliae*.

It is surprising that mealybug populations with more symbionts, and hence in theory more nutritional resources at their disposal, did not perform better than those with fewer symbionts. This would imply that all of the mealybug populations harboured the maximum number of symbionts that could increase host fitness in these scenarios, and any additional symbionts were superfluous and benign, neither significantly benefiting nor costing the host. However, this result is supported by findings from a previous study which found that adult citrus mealybugs did not suffer reduced fecundity after their obligate symbiont density was halved using heat exposure (Parkinson et al., 2014). This contrasts with aphids, which suffer from lowered fitness if the density of their nutritional obligate symbiont, *Buchnera*, is reduced (Houk and Griffiths, 1980, Sakurai et al., 2005).

The mealybug populations performed considerably better and grew more quickly on *A. elegantissima* than the other plant species tested, with *F. murieliae* proving to be the least suitable for citrus mealybug infestations. *P. citri* is a known pest of *Aralia* species, vectoring *Schefflera* ringspot badnavirus (Lockhart and Olszewski, 1996),

and caused significant wilting and discolouration in this experiment. On *Phalaenopsis* and *C. roseopicta*, the mealybugs had relatively low infestations after two months, but dramatically increased their numbers by the end of the third month. At this point, the mealybugs had established infestations on *Phalaenopsis* and *C. roseopicta* that were around half the size of the *A. elegantissima* infestations. *Phalaenopsis* and *Calathea* are also known to be susceptible to citrus mealybugs, however neither are listed as hosts on the ScaleNet or Invasive Species Compendium (CABI) databases (Booth, 2014, Ben Dov, 2015, CABI, 2015, Goodwin et al., 2000). We would suggest that these plant species are formally considered as hosts for *P. citri*.

However, the mealybugs did not produce prolific infestations on *F. murieliae*, indicating that this plant species either does not meet the nutritional requirements of *P. citri* or contains physical or chemical defences that are effective against citrus mealybugs, in spite of the fact that *P. citri* is considered a pest of *F. murieliae* (Graciet, 2011). The mealybug populations with higher symbiont densities were not able to compensate for this unsuitable host plant and did not have greater infestations, demonstrating the limits of the benefits of this symbiotic association. The low quality of a host plant can hamper the ability of a nutritional symbiont to function; for example, the low concentration of amino acids in the phloem of *Lamium purpureum* reduces the ability of *Buchnera* to assimilate threonine for the aphid host (Wilkinson et al., 2001, Chandler et al., 2008).

The application of Teppeki insecticide did not reveal any significant differences in the resilience of mealybug populations to this treatment as a stressor, nor did it

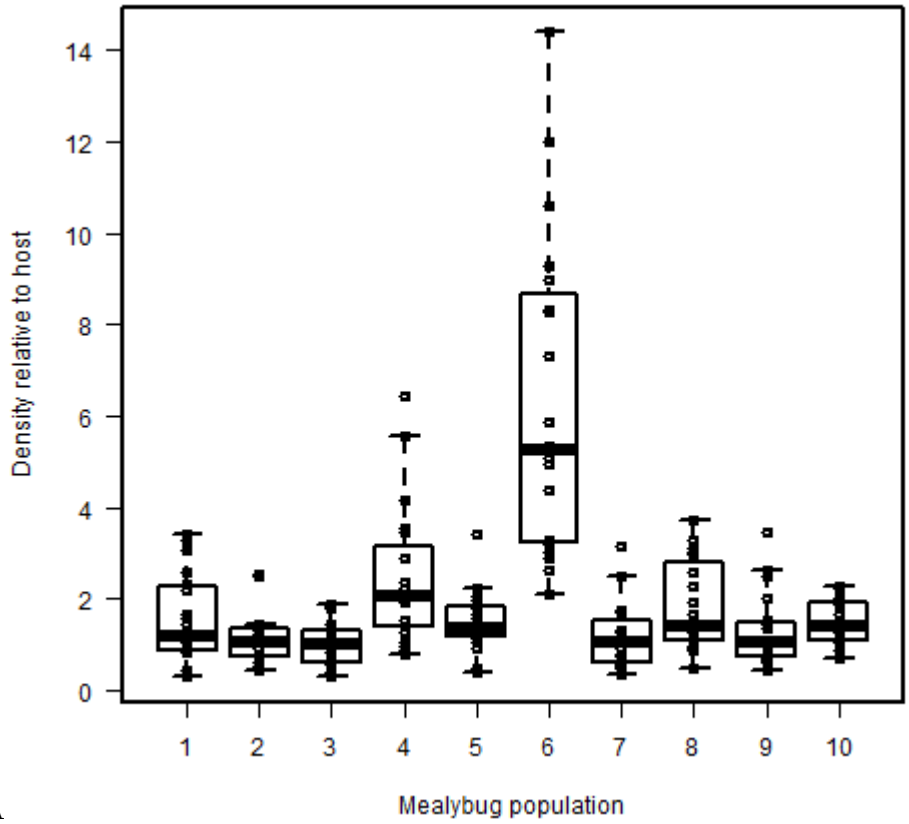
significantly impact the number of mealybugs on plants one month following treatment, except for on *A. elegantissima*. Facultative symbionts have been linked to insecticide resistance due to their compound-degrading capabilities in stinkbugs, and facultative symbiont density can increase in response to host insecticide resistance in whitefly and mosquito (Kikuchi et al., 2012, Pan, 2013, Duron et al., 2006, Echaubard et al., 2010, Berticat et al., 2002). Conversely, the presence of *Rickettsia* was correlated with insecticide susceptibility in whitefly (Kontsedalov et al., 2008). However, we are not aware of any examples of associations between obligate symbionts and insecticide resistance.

In conclusion, this study finds that citrus mealybugs harbour obligate nutritional symbionts with no clear fitness benefits, and that even mealybugs with high symbiont densities will vary in their fitness based on the host plant species. This indicates that the obligate symbionts of citrus mealybugs are likely to be poor targets for the symbiont-mediated pest control known as Microbial Resource Management (Read, 2011, Verstraete et al., 2007, Douglas, 2007b). It also raises evolutionary questions as to why these insect hosts harbour seemingly excessive densities of obligate symbiont and why this does not impact their fitness. RT-qPCR of the symbiont genes involved with nutrition could help to reveal how *T. princeps* and *M. endobia* are functioning, for example, whether mealybugs with low symbiont density compensate by increasing their biosynthetic activity. This would help to better understand the dynamics of this tripartite symbiosis.

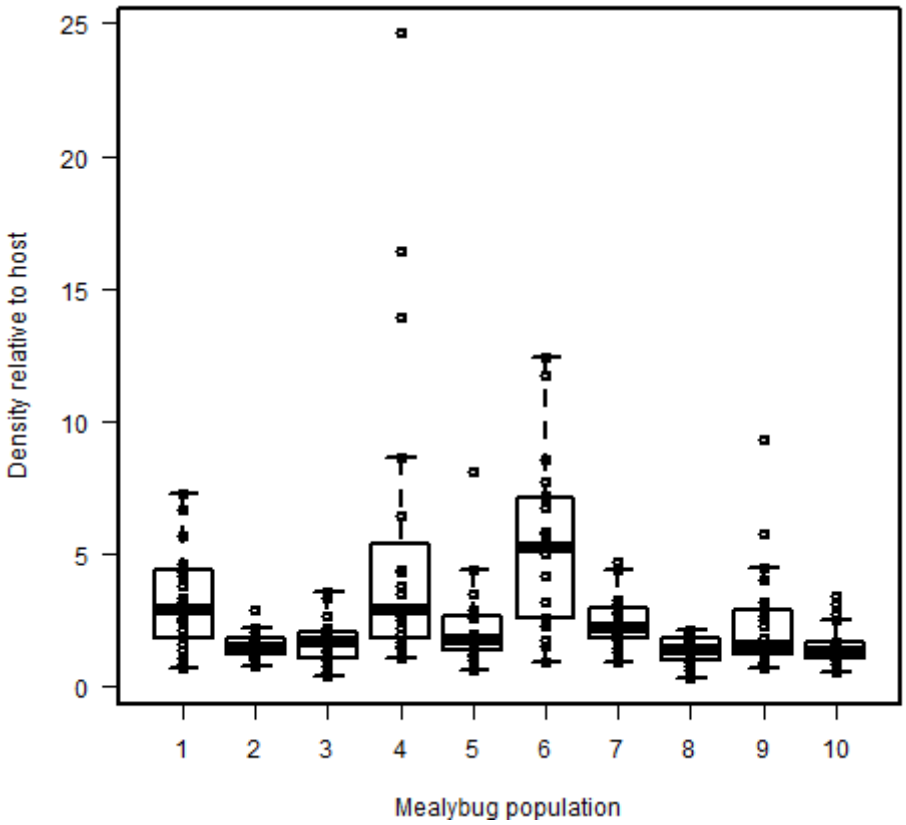
3.6 Figures

3.6.1 ***Figure 3.1.***

The mean, quartiles, 95th percentiles and individual data points of the densities of the (A) *M. endobia* and (B) *T. princeps* bacterial symbionts in adult citrus female mealybugs from populations 1 to 10. Symbiont density was measured using qPCR, calculated as relative to *P. citri* host control gene using the comparative C_T method.


Density relative to host
0
2
4
6
8
10
12
14
1
2
3
4
5
6
7
8
9
10
Mealybug population

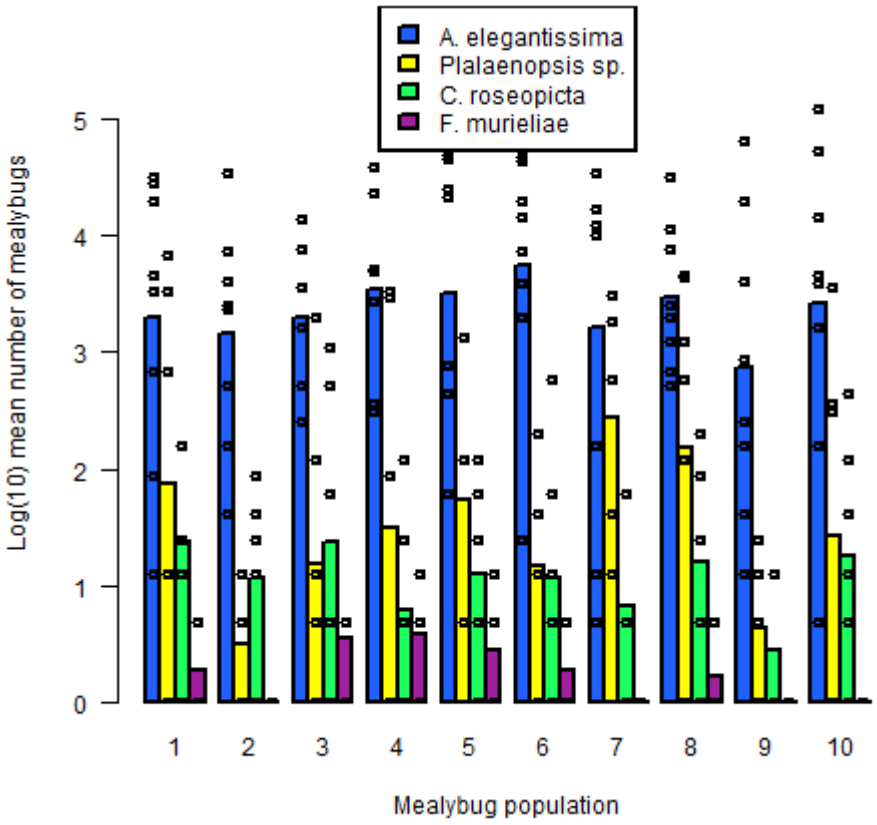
A


Density relative to host
0
5
10
15
20
25
1
2
3
4
5
6
7
8
9
10
Mealybug population

B

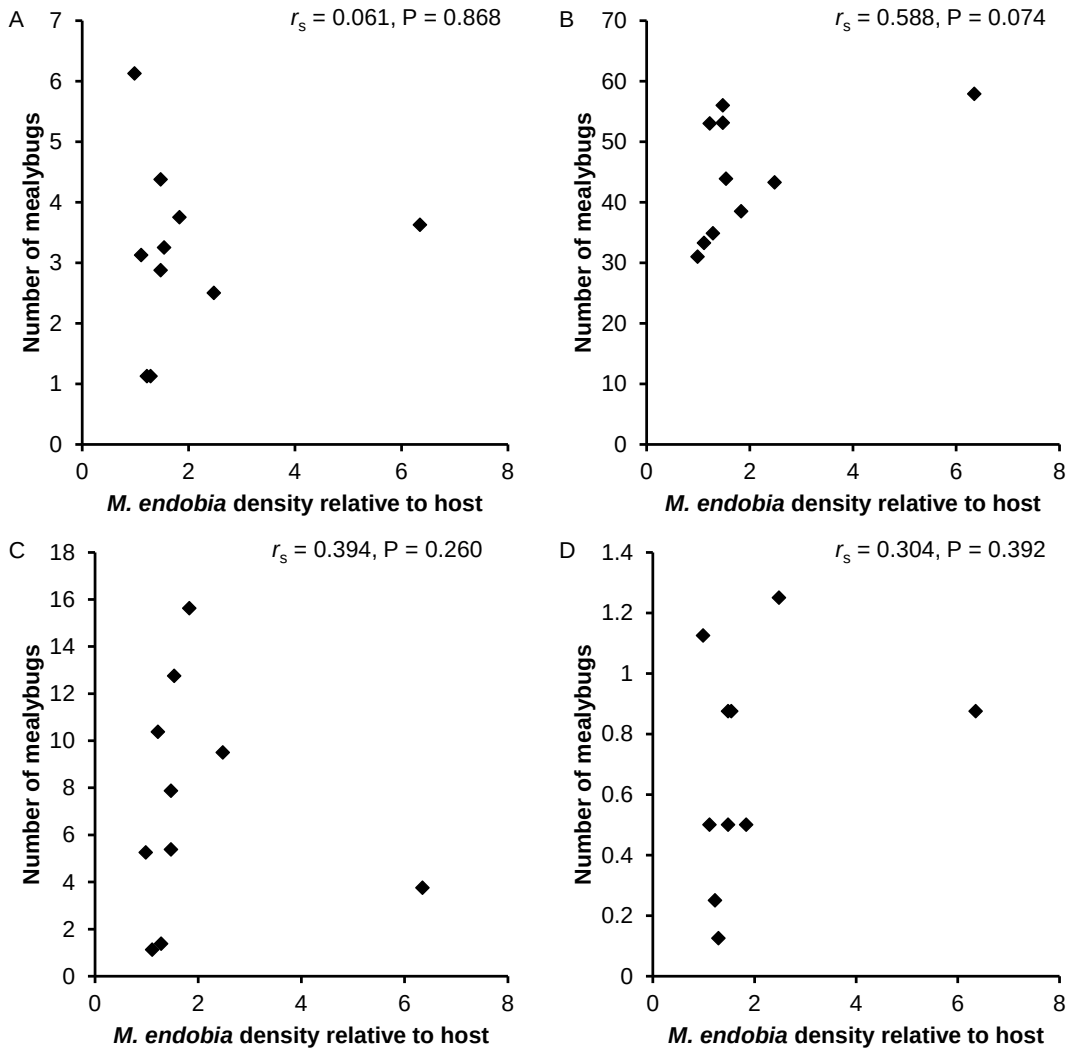
3.6.2 ***Figure 3.2.***

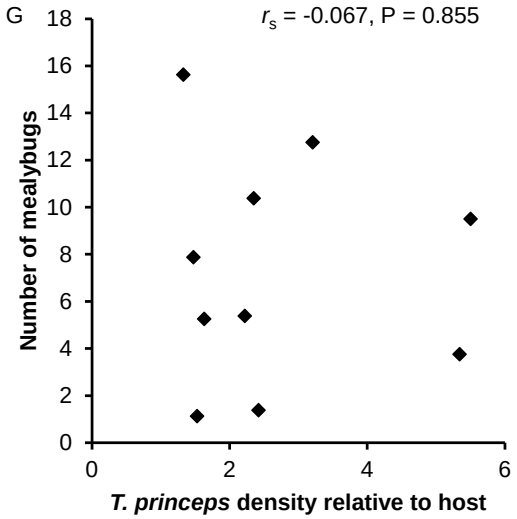
The Log_{10} mean and individual data points of the number of mealybugs on four plant species, *Aralia elegantissima*, *Phalaenopsis* sp. *Calathea roseopicta* and *Fargesia murieliae*. Data are for ten mealybug populations that differed in the densities of their obligate symbionts, after two months (two generations) on the plants.

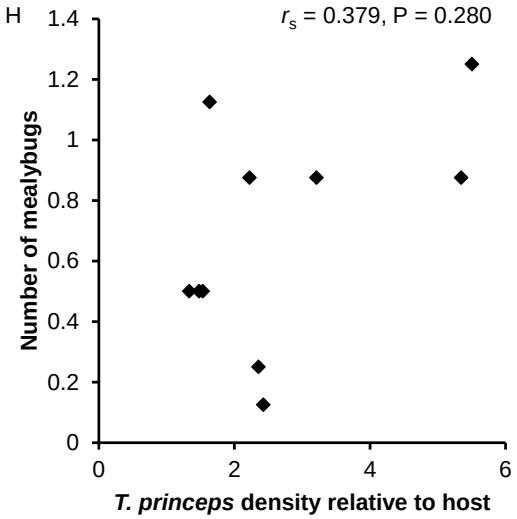


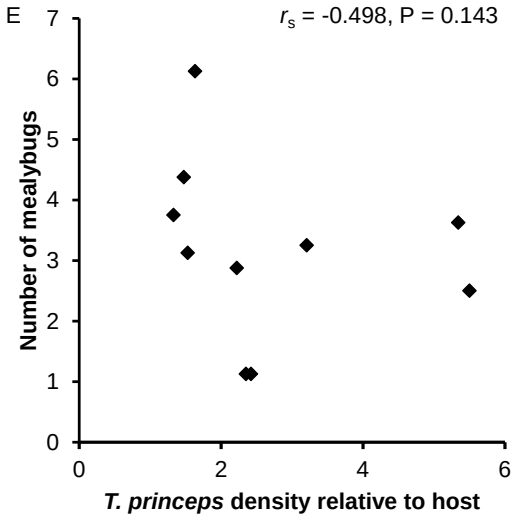
3.6.3 ***Figure 3.3.***

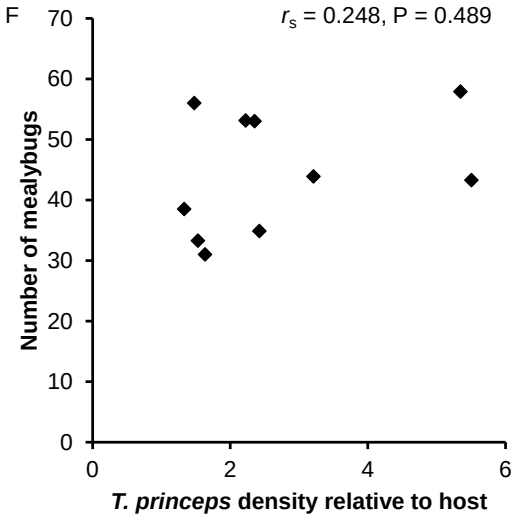
Relationships between the mean number of mealybugs on four plant species, (A, E) *Calathea roseopicta*, (B, F) *Aralia elegantissima*, (C, G) *Phalaenopsis* sp. and (D, H) *Fargesia murieliae*, with the mean densities of the (A, B, C, D) *M. endobia* and (E, F, G, H) *T. princeps* bacterial symbionts relative to the host in adult citrus female mealybugs from populations 1 to 10. Symbiont density was measured using qPCR, calculated as relative to *P. citri* host control gene using the comparative C_T method.




G
r_s = -0.067, P = 0.855
Number of mealybugs
0
2
4
6
8
10
12
14
16
18
0
2
4
6
T. princeps density relative to host

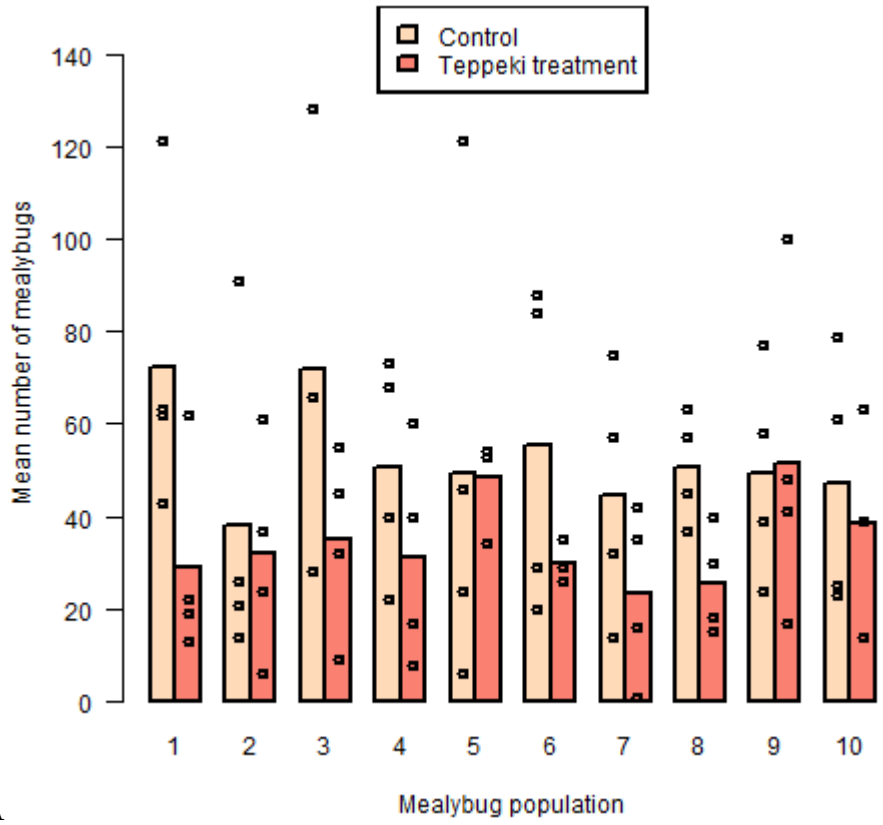

H
r_s = 0.379, P = 0.280
Number of mealybugs
0
0.2
0.4
0.6
0.8
1
1.2
1.4
0
2
4
6
T. princeps density relative to host


E
r_s = -0.498, P = 0.143
Number of mealybugs
0
1
2
3
4
5
6
7
0
2
4
6
T. princeps density relative to host

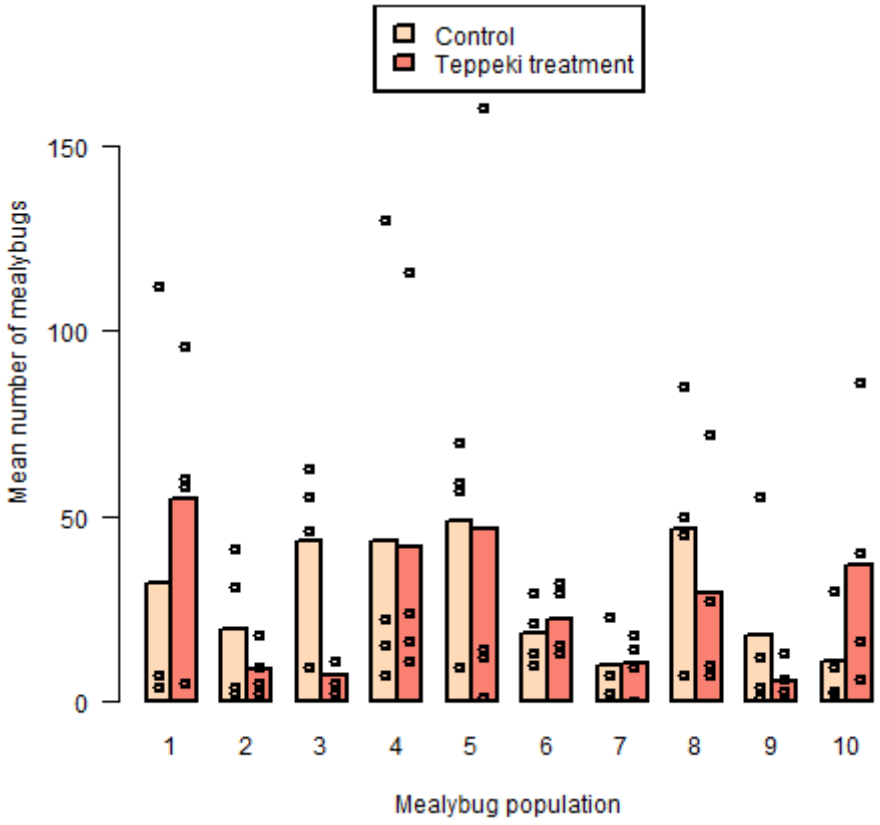

F
r_s = 0.248, P = 0.489
Number of mealybugs
0
10
20
30
40
50
60
70
0
2
4
6
T. princeps density relative to host

3.6.4 ***Figure 3.4.***

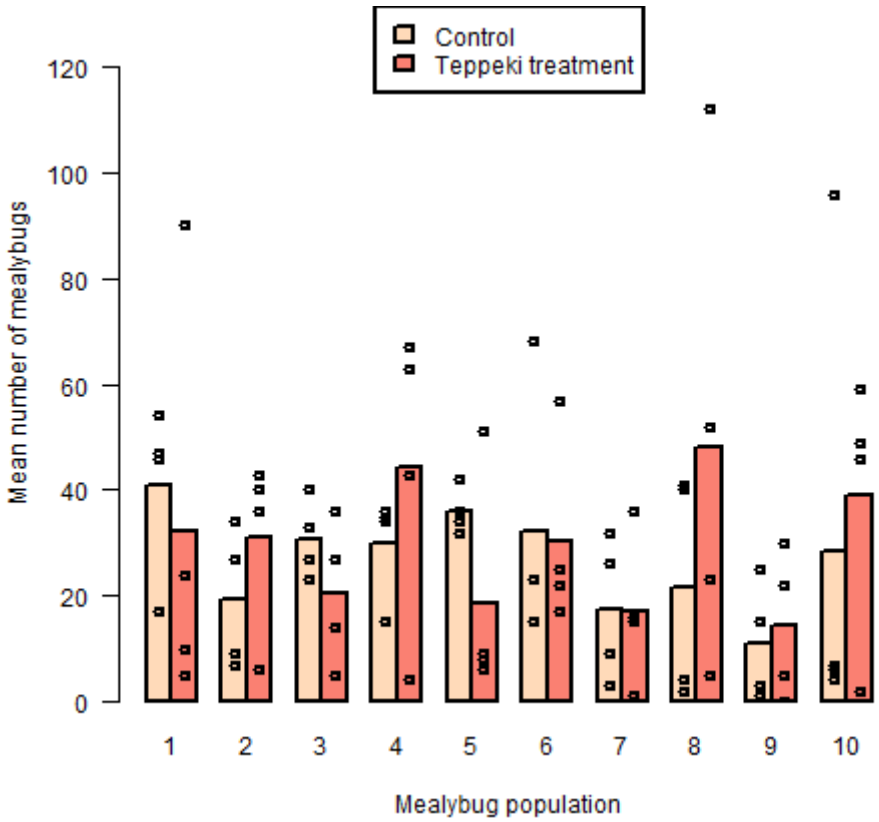
The mean and individual data points of the number of mealybugs across all ten mealybug populations on four plant species, (A) *Aralia elegantissima*, (B) *Phalaenopsis* sp., (C) *Calathea roseopicta* and (D) *Fargesia murieliae*, after three months of development with or without (control) Teppeki treatment.


Control
Teppeki treatment
Mean number of mealybugs
0
20
40
60
80
100
120
140
1
2
3
4
5
6
7
8
9
10
Mealybug population

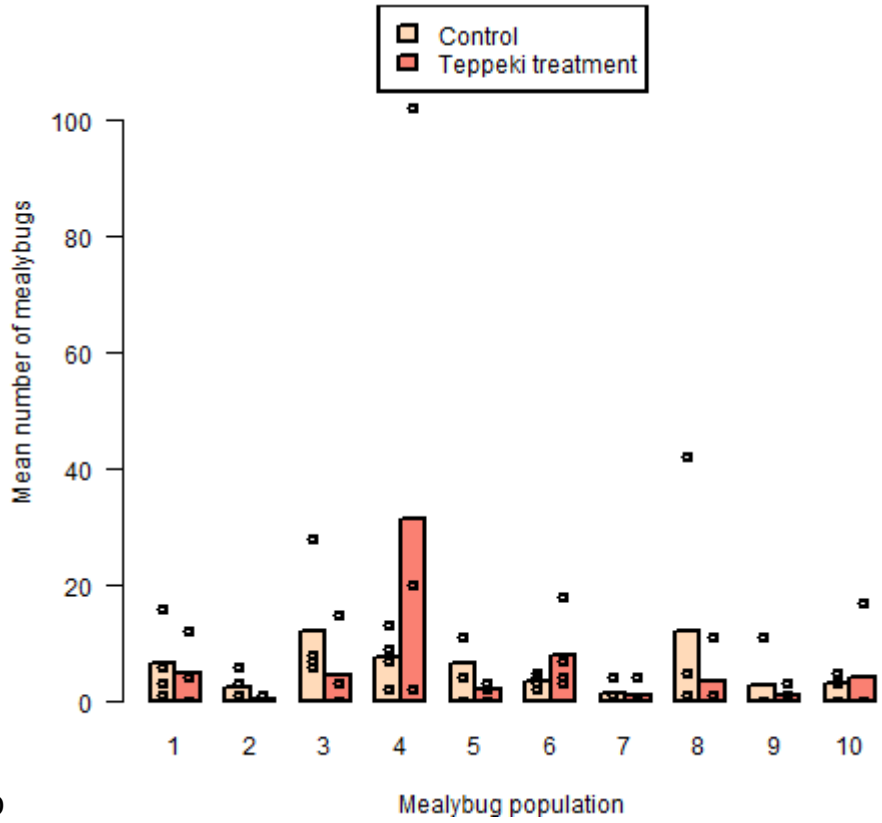
A


Control
Teppeki treatment
Mean number of mealybugs
0
50
100
150
1
2
3
4
5
6
7
8
9
10
Mealybug population

B


Control
Teppeki treatment
Mean number of mealybugs
0
20
40
60
80
100
120
1
2
3
4
5
6
7
8
9
10
Mealybug population

C


Control
Teppeki treatment
Mean number of mealybugs
0
20
40
60
80
100
1
2
3
4
5
6
7
8
9
10
Mealybug population

D

“It is paradoxical, yet true, to say, that the more we know, the more ignorant we become in the absolute sense, for it is only through enlightenment that we become conscious of our limitations. Precisely one of the most gratifying results of intellectual evolution is the continuous opening up of new and greater prospects.”

— *Nikola Tesla*

4 Short-term heat stress results in diminution of bacterial symbionts but has little effect on life-history in adult female citrus mealybugs

4.1 Abstract

Mealybugs are sap-feeding insect pests that pose a serious threat to horticulture. The citrus mealybug, *Planococcus citri* (Risso) (Hemiptera: Pseudococcidae), like most other mealybug species, harbours two obligate maternally-transmitted bacterial endosymbionts, which are essential for nutrient acquisition and host survival. These are *Tremblaya princeps*, a member of the β-Proteobacteria, and *Moranella endobia*, a member of the γ-Proteobacteria. The density of symbionts in the hosts is now understood to be dynamic, being influenced by the age and gender of the host, and by environmental conditions during development. Here we examine the impact of short-term heat stress treatment on the obligate symbionts and life-history parameters of *P. citri*, using qPCR to measure changes in symbiont density. Heat stress killed juveniles and adult males, and significantly reduced levels of *M. endobia* and *T. princeps* in adult females. However, adult females were resilient to this and it did not affect their fecundity or brood survival, although the sex ratio of their brood was slightly, but significantly, more female biased. Our results suggest that *T. princeps* and *M. endobia* are not as essential to the fitness of adult mealybugs as they are to immature mealybugs and that heat treatment alone is unlikely to be effective as a disinfestation tactic.

4.2 Introduction

Bacterial endosymbiosis is now appreciated to be a diverse, integral and influential aspect of insect ecology and evolution (Saffo, 1992), which has potential applications in sustainable pest management, known as "microbial resource management" (Douglas, 2007a, Verstraete et al., 2007, Read, 2011, Crotti et al., 2012). Many insects harbour obligate bacterial symbionts which are essential for their survival, but the prevalence and density of symbionts is often dynamic, being influenced by the age and gender of the host and environmental conditions (Burke, 2010, Chiel et al., 2007, Kono et al., 2008, Moran et al., 2008).

Mealybugs (Hemiptera: Sternorrhyncha: Pseudococcidae) comprise around 2000 species worldwide (Thao et al., 2002). These sap-feeding pests pose a persistent threat to horticulture due to mechanical damage, the transmission of a range of plant pathogens, and the excretion of honeydew which encourages the growth of black sooty mould (Jelkmann, 1997, Sether, 1998, Charles, 2006). The citrus mealybug, *Planococcus citri* (Risso), is one of the most economically destructive species of mealybug, being a polyphagous and cosmopolitan pest that can feed upon plants from dozens of families (Ben Dov, 2015) including citrus, cocoa (Ackonor, 2002), coffee (Staver et al., 2001), grapevine (Cid et al., 2006), and other horticultural and ornamental crops in greenhouses and conservatories (Brødsgaard and Albajes, 2000, Laflin and Parrella, 2004). *P. citri* is an international pest, native to Asia, but occurring across the tropics, Europe, Oceania, USA and Mexico, at outside temperatures ranging from 20-32 °C or in greenhouses (CABI/EPPO, 1999).

P. citri transmits plant pathogens such as grapevine leafroll-associated virus 3 (GLRaV-3) (a.k.a. Ampelovirus), Grapevine Virus A, B and C (a.k.a. Vitivirus) and *Badnavirus*, including piper yellow mottle virus (Martelli et al., 2002, Cid and Fereres, 2010, Phillips et al., 1999, Adams et al., 2004, Lockhart et al., 1997). Chemical application is the most common control strategy of mealybugs (Franco et al., 2009); however, they are difficult to eliminate due to their cryptic behaviour and waxy secretions which shield them from pesticides. Biological control strategies have been explored, including parasitoids, predators, nematodes and fungi, with mixed results (Odindo, 1992, Stuart, 1997, Davies et al., 2004, Ceballo and Walter, 2005, Afifi et al., 2010, Demirci, 2011, van Niekerk, 2012). More effective and reliable strategies are needed.

P. citri, like most mealybug species, harbours two obligate maternally-transmitted bacterial endosymbionts within the bacteriome. These are *T. princeps* and *M. endobia*, the latter residing within the former, a feature believed to be unique to the Pseudococcidae (Thao et al., 2002, Keeling, 2011, McCutcheon and von Dohlen, 2011, Von Dohlen et al., 2001, Baumann et al., 2002). The mutualistic relationship between *P. citri* and these symbionts likely evolved because of the restricted diet of the host, a common characteristic in insect-endobacteria relationships (Douglas, 2006). Mealybugs feed solely upon plant sap, which is deficient in essential amino acids that the insect cannot assimilate. Endosymbionts can compensate for these shortfalls with their wider metabolic capacity and thus provide nutrients for the hosts, allowing them to exploit otherwise impenetrable niches (Douglas, 2009). *T. princeps* and *M. endobia* are capable of synthesising the full range of required

essential amino acids through a fusion of genetic pathways (Keeling, 2011, McCutcheon and von Dohlen, 2011, Husnik et al., 2013). This biochemical complementation demonstrates the evolutionary specificity of these partners and why no successful *in vitro* culturing, nor aposymbiotic mealybugs (those lacking symbionts), have been reported.

The ecological function of *T. princeps* and *M. endobia* may lead to variations in the density of infection based upon host requirements. The abundance of *T. princeps* and *M. endobia* alters, depending upon the age and gender of the host (Kono et al., 2008). A qPCR study into these dynamics with the mealybugs *Planococcus kraunhiae* (Kuwana) and *Pseudococcus comstocki* (Kuwana) found that although females maintain their endosymbionts after maturity, they are at reduced levels and males lose their endosymbionts entirely after pupation, most likely because adult males do not feed (Miller, 1999) and therefore do not require nutritional symbionts. Despite their physical and biochemical connections, this loss of symbionts is decoupled, with *M. endobia* disappearing more quickly than *T. princeps* in males (Kono et al., 2008).

Rearing temperature influences the life-history parameters of mealybugs. *P. citri* instars died below 12°C and above 37 °C, and the longevity of adult females is greatest at 18 °C, whereas fecundity is highest at 23 °C (Goldasteh et al., 2009). A constant temperature of 30 ºC as opposed to 25 ºC led to female-biased sex ratios in *P. citri* (Ross et al., 2011), whereas another study found sex ratios to be female-biased at 15 – 30 ºC, but male-biased at 32 ºC (Goldasteh et al., 2009). Older mating ages and starvation also triggered this male bias (Varndell and Godfray, 1996, Ross

et al., 2011). Other species of mealybug show similar life-history patterns, with fecundity, longevity and adult weight peaking at species-specific optimum temperatures in *Maconellicoccus hirsutus* (Green) (Patil et al., 2011), *Pseudococcus citriculus* (Green) and *P. kraunhiae* (Arai, 1996), *Paracoccus marginatus* (Williams & Granara de Willink) (Amarasekare et al., 2008) and *Pseudococcus longispinus* (Targioni Tozzeti) (Santa-Cecília et al., 2011). Long-term exposure of juvenile and adult *P. citri* to 39 ºC led to dismantling of the mycetocytes, ultimately leading to the death of the hosts (Köhler and Schwartz, 1962). This demonstrates temperature as a limiting factor in mealybug growth and reproduction, and as an influential factor in sex determination.

Short-term heat stress treatment has been found to lead to dramatic reductions in obligate symbiont density in the pea aphid *Acyrthosiphon pisum* (Harris), with an observed 80% loss of the bacterium *Buchnera aphidicola* Munson et al., which did not recover 96 h following treatment, unless the host was co-infected with the facultative symbiont *Serratia symbiotica* Moran et al. (Burke, 2010). It has yet to be studied whether short-term temperature stress could lead to the reduction of symbionts in mealybugs, affect life history or distort the sex ratio of offspring, which could potentially be applied as a pest control tactic. Here we examine the impact of short-term heat stress on the symbionts and life-history parameters of *P. citri*, using qPCR to measure changes in symbiont density.

4.3 Materials and methods

4.3.1 *Sourcing and rearing of mealybugs*

Individual *P. citri* were collected from the horticultural research centre Proefcentrum voor Sierteelt, Ghent, Belgium. These were sourced from a variety of host ornamental plants which had been brought in from commercial greenhouses from across Belgium and pooled into a single community. Mealybugs were reared in darkness at 25 °C and 50% r.h. on white organic potato sprouts. Offspring from this established 16 month-old laboratory stock were used in the experiment. Mealybug eggs laid by multiple females were collected and reared for 29 days until females had reached maturity, with pupating males being separated from females to ensure female virginity.

4.3.2 *Heat stress treatment*

At the end of the rearing period, half of the virgin adult females were maintained at 25 ºC and 50% r.h. as controls, whilst the remaining females were exposed to heat stress treatment. This involved a 2 h period of gradually increasing the environmental temperature from 25 up to 50ºC, followed by a 2 h period at 50 ºC and finally a 2 h period of gradual reduction of environmental temperature from 50 ºC back to 25 ºC, the r.h. was maintained at 50% throughout in a humidity-controlled incubator. Fifty degrees was chosen as the heat stress temperature because preliminary studies with this culture had found that 55 ºC caused mass mortality (JF Parkinson, unpubl.), and the aim was to test a sub-lethal treatment here. Virgin females were flash frozen in liquid nitrogen 48 or 72 h after treatment to examine

short and longer changes in symbiont density, and stored in absolute ethanol at -20ºC until use for qPCR analysis. A hundred second-instar juvenile mealybugs of mixed sex and 30 newly emerged adult male mealybugs were also exposed to the heat stress treatment. After treatment, the surviving individuals were counted.

4.3.3 *Life-history study*

Immediately following treatment, a subset of 40 adult virgin females from the treated group and 34 from the control group were separated out and mated with virgin males taken from the reared population. These females were exposed to two males each to ensure mating. The eggs laid by these females were counted, along with the offspring which then reached adulthood themselves under normal rearing conditions, and their sex ratio at adulthood was assessed.

4.3.4 *Symbiont infection intensity study*

We quantified the infection intensity of the two symbionts in heat-stressed and control mealybugs using qPCR with the comparative C_T method and a host gene to control for DNA quantity (Schmittgen and Livak, 2008). qPCR primers and probes for the variable housekeeping 28S rDNA region of the host *P. citri*, and the 16S rDNA and 23S rDNA intergenic spacer region of the γ-proteobacterial symbiont *M. endobia* (Thao et al., 2002), were designed using the software Primer Express v.3.0 (Life Technologies, Foster City, CA, USA). Primers and probe for the *GroEL* gene were developed for the *P. citri* strain of the β-proteobacterial symbiont, *Tremblaya*

princeps (Thao et al., 2002). These were designed using the software PRIMER3 (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) and analysed using the software NetPrimer (Premier Biosoft International, Palo Alto, CA, USA) (Table 4.1). DNA was extracted from 25 individual adult mealybugs per treatment at 48 h after treatment, and 26 mealybugs at 72 h after treatment, by soaking each mealybug in distilled water before crushing in 100 µl of 10% Chelex and heating to 99 ºC. The resulting product was centrifuged at 2326 g for 20 min and the supernatant was pipetted off. Inhibitors from this supernatant were removed using the OneStep96™ PCR Inhibitor Removal Kit as per manufacturer's instructions (Zymo Research, Irvine, CA, USA). DNA from individual mealybugs was diluted to 1/10 in molecular grade water for use in qPCR reactions. Triplett qPCR reactions for individual mealybugs were performed in a StepOnePlus™ Real-Time PCR System. Volumes of 10 µl were used for qPCR reactions with reagent final concentrations of 150 nM of each primer, 50 nM of probe, and 1× of ABI Taqman Universal Master Mix II with UNG (Life Technologies, Foster City, CA, USA). The cycle was 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and the annealing temperature (collection step) for 1 min. An annealing temperature of 64 °C was used for *P. citri* and *M. endobia* reactions and 60 °C for *T. princeps* reactions. Mean concentrations of *T. princeps* and *M. endobia* were compared against the *P. citri* host control using the comparative C_T method to produce relative ΔC_T values. These were compared between control and treatment groups to produce $\Delta\Delta C_T$ values, which were used to calculate fold differences.

4.3.5 *Statistical analysis*

The numbers of eggs laid in the two treatments were tested for normality and homogeneity of variance, found to fit these assumptions, and then analysed using a General Linear Model. The percentages of surviving offspring and female offspring between treatments were analysed using a Generalized Linear Model with gamma distribution and log link function, using the likelihood ratio χ^2 test statistic. The numbers of females in each treatment which failed to oviposit were analysed using a Fisher's Exact Test. qPCR data were processed using the comparative C_T method (Schmittgen and Livak, 2008), which calculates the relative density between target gene and host control gene. Data for symbiont density were tested for normality and homogeneity of variance. The data were not found to fit these assumptions and were analysed using a Generalized Linear Model, again using a gamma distribution, log link function, and the likelihood ratio χ^2 test statistic. All analyses were conducted in SPSS 20 (IBM-SPSS Statistics, Armonk, NY, USA).

4.4 Results

4.4.1 *Life-history*

Both second-instar mealybugs of mixed sex and adult male mealybugs experienced 100% mortality when exposed to the heat stress treatment. Two of 40 adult female mealybugs in the treated group died 1 h following the treatment. No further premature mortality was observed in this group, nor was any mortality observed in the 34 adult female mealybugs used in the control group. In the control group, two of 34 females failed to oviposit and one female produced an egg sac devoid of eggs. In

the treated group, six of 38 surviving females failed to oviposit and one female produced an egg sac devoid of eggs (Fig. 4.1). These females which did not lay eggs were discounted further from the experiment. Neither the proportion of females failing to lay eggs, the number of eggs laid, nor the brood survival (%) to adulthood differed significantly between treatment and control mealybugs (Fisher's Exact Test, $P = 0.32$; $F_{1,60} = 0.539$, $P = 0.47$; and $\chi^2 = 0.054$, d.f. = 60, $P = 0.88$, respectively), However, the sex ratio of offspring produced did differ significantly ($\chi^2 = 5.37$, d.f. = 60, $P = 0.020$), with treated females producing progeny with a more female-biased sex ratio at adulthood (Fig. 4.1).

4.4.2 *Symbiont infection intensity*

Heat-stressed mealybugs had significantly reduced levels of *M. endobia* DNA relative to control mealybugs 48 h ($\chi^2 = 5.447$, d.f. = 49, $P = 0.020$) and 72 h following treatment ($\chi^2 = 11.332$, d.f. = 49, $P = 0.001$), with heat-stressed densities of *M. endobia* being reduced by 52% after 48 h and 50% after 72 h (Fig. 4.2). Heat stress treatment was not found to cause a statistically significant difference in levels of *T. princeps* DNA 48 h following treatment ($\chi^2 = 2.71$, d.f. = 49, $P = 0.10$), although it did follow the same trend as *M. endobia*, being reduced by 40%. However, levels of *T. princeps* DNA 72 h following treatment were significantly reduced ($\chi^2 = 8.338$, d.f. = 50, $P = 0.004$), with a 58% decrease (Fig. 4.2).

4.5 Discussion

The qPCR results showed that short-term heat stress at 50 °C led to reduced density of '*Ca*. Moranella endobia' and '*Ca*. Tremblaya princeps' DNA in *P. citri*. Absence of DNA indicates that the bacteria were digested or excreted by the host. This reflects a previous study, in which long-term heat stress at 39 °C physically damaged the symbiont system (Köhler and Schwartz, 1962). This may suggest that the heat is associated with cell death, or perhaps triggers an internal molecular mechanism or molecular cascade in *P. citri* which caused a host response to eradicate the symbiotic bacteria. However, in a previous study, the long-term heat treatment of 39 °C for 20 days resulted in the premature mortality of the adult mealybugs (Köhler and Schwartz, 1962), which was not observed for our short-term intense treatment. Decoupling of the symbiont reduction reflects the results observed (Kono et al., 2008), with *M. endobia* reducing more rapidly. It may be that *M. endobia* is of lesser importance, or that *T. princeps* may digest or eject *M. endobia* before rupturing itself. The first suggestion is unlikely and the other appears maladaptive, as the biochemical dependency of these partners is obligate (McCutcheon and von Dohlen, 2011). Cell lysis has been suggested as a mechanism for the exportation of proteins from *M. endobia* to *T. princeps* (Husnik et al., 2013), and stressful conditions may disrupt this controlled event.

Although previous studies have found that constant rearing temperatures, varying typically across studies between 12 and 37 °C (Varndell and Godfray, 1996, Goldasteh et al., 2009, Ross et al., 2011), are greatly influential to the life-history parameters and survivorship of mealybugs, adult virgin female *P. citri* displayed

strong physical resilience to the short-term intense heat stress treatment of 50 °C. This is despite this temperature killing 100% of second-instar and adult male mealybugs, and being only 5 °C less than the lethal temperature for adult females. Short-term heat stress did not impact the fecundity of the females, which suggests that key factors which determine the reproductive success of an individual occur during its development, and are only altered by environmental temperature experienced in immature stages. As the symbionts are necessary for amino acid synthesis (McCutcheon and von Dohlen, 2011), they are probably most needed during the growth stages of the host, and are of lesser importance in adults, remaining present for transmission to the next generation. It would be of interest to know whether symbionts remain at reduced levels in treated virgin mealybugs for the remainder of their life span compared to control mealybugs or whether the offspring of females with reduced symbiont levels also have fewer symbiont cells. Adult male mealybugs naturally lose their symbionts post-pupation (Kono et al., 2008), so the loss of symbionts via heat stress is unlikely to be the cause of their mortality. Both adult males and juveniles are smaller than adult females and will have a larger surface area to volume ratio, thus likely rendering them more vulnerable to desiccation, which may explain their higher mortality rates.

Previous studies have shown that long-term exposure to raised temperatures during development can alter the sex ratio of mealybugs (Varndell and Godfray, 1996, Goldasteh et al., 2009, Ross et al., 2011). Our experiment has demonstrated that even a short transient exposure to higher temperatures can cause an effect. Females from both the control and heat stress treatment produced brood with a female-biased sex

ratio. However, the bias was slightly, but significantly, greater for treated females. This finding is in concordance with a previous study which found that hotter and more stressful conditions increased the prevalence of females in brood (Ross et al., 2011). Crowded females are more likely to produce male-biased brood, and age at mating is a complex interacting factor (Ross et al., 2010a). Mealybugs can facultatively adjust the sex ratio of their offspring through paternal genome elimination in males (Schrader, 1921, Brown and Nelson-Rees, 1961, Ross et al., 2010b, Ross et al., 2012), and is likely related to heterochromatic proteins (Buglia et al., 2009). The adult sexes are dimorphic, males being winged and dispersing and females being paedomorphic and sessile. There may be adaptive reasons for adjusting sex ratios following heat stress, or temperature may non-adaptively alter the determination mechanisms. Conversely, male brood of heat-stressed females may have suffered a higher mortality rate than those of non-heat-stressed females, although we do not have any data to confirm this hypothesis.

These results, along with the findings that symbiont density is reduced in post-reproductive females (Kono et al., 2008), indicate that host physical deterioration, perhaps triggered by senescence or stress, sways the relationship between host and bacteria. Although these symbionts are essential for the overall survival of the host, cost is incurred with maintaining a symbiont, and some environmental conditions may initiate a purge. Conversely, stressful conditions and physical deterioration may render the host incapable of housing symbionts and meeting their requirements. Symbiont degradation caused by heat stress and that caused by host senescence may not necessarily occur via the same mechanism and it would be interesting to

investigate whether other environmental factors, such as food supply, cold exposure, and host plant species, can also alter the density of symbionts in mealybugs. This experiment provides only a snapshot of the dynamic relationship between mealybugs and their obligate symbionts, and it is possible that females could have recovered their symbionts after the qPCR measurements were taken. Such a recovery mechanism would imply that adult mealybugs are adapted to cope with symbiont fluctuation; hence, their reproductive fitness was unaffected. However, although fecundity was not affected, other fitness traits, such as immunocompetence or the ability to exploit different environments and host plants of other species, were not investigated in this study and may serve as significant factors when incorporated.

High temperatures have been tested in combination with other short-term disinfestation treatments, such as hot water immersion and ozone fumigation, as control strategies for mealybug pests on horticultural plants (Hansen et al., 1992, Lester et al., 1995, Hara et al., 1996, Dentener et al., 1997, Hollingsworth and Armstrong, 2005). Although often effective, high-heat treatments usually involve another element and may not be practical methods for some plants. Our results indicate that short-term, sub-lethal heat stress alone would not be an effective control strategy against mealybug infestations populated with many adults, although it would be highly effective against immature mealybug stages and does provide a potential experimental method for manipulating symbiont densities. It would be of great interest to observe whether other aspects of fitness were impacted, and whether other stressors also result in diminished symbiont densities.

4.6 Tables

4.6.1 ***Table 4.1.***

PCR primers and probes used in the study for *Planococcus citri* host control, β-proteobacterial symbiont *T. princeps*, and γ-proteobacterial symbiont *M. endobia*.

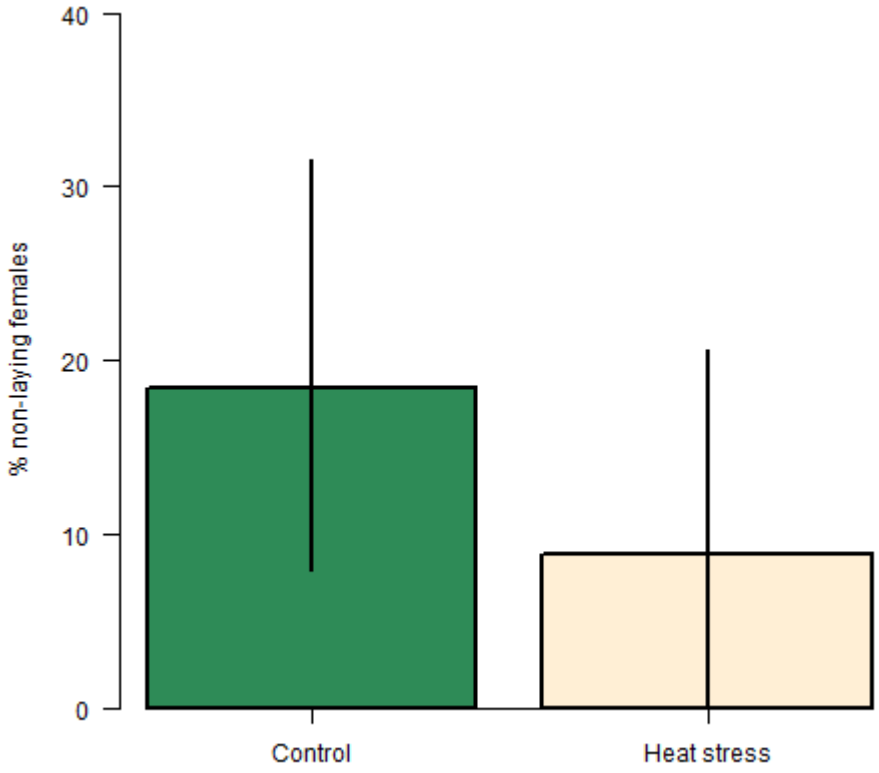
Target organism	**Target gene**	**Olgio name**	**Function**	**Fluorescence[a]**	**Oligo sequence 5'-3'**	**Product size (bp)**
P. citri	28S rDNA (AY179451.1)	*PcitriF*	Forward primer	-	TCCGAGGAGACGTGTAAAA GTTC	56
		PcitriR	Reverse primer	-	CCTAGCCGCCGAAACGA	
		PcitriP	Probe	FAM	ACGGCGCGTGTCGA	
T. princeps	*GroEL* (AF476091)	*TprincepsF*	Forward primer	-	TCCAAGGCTAAATACCCAC A	155
		TprincepsR	Reverse primer	-	ATACAAAAGGTACGCCGTC A	
		TprincepsP	Probe	FAM	CGCGCATACGAACAGTCGG A	
M. endobia	16S and 23S rDNA (AF476107.1)	MendobiaF	Forward primer	-	GAGCACCTGTTTTGCAAGCA	64
		MendobiaR	Reverse primer	-	CCCCTAGAGTTGTGGAGCTA AGC	
		MendobiaP	Probe	FAM	AGTCAGCGGTTCGATC	

[a] 6FAM, 6-fluorescein amidite 5′ dye.

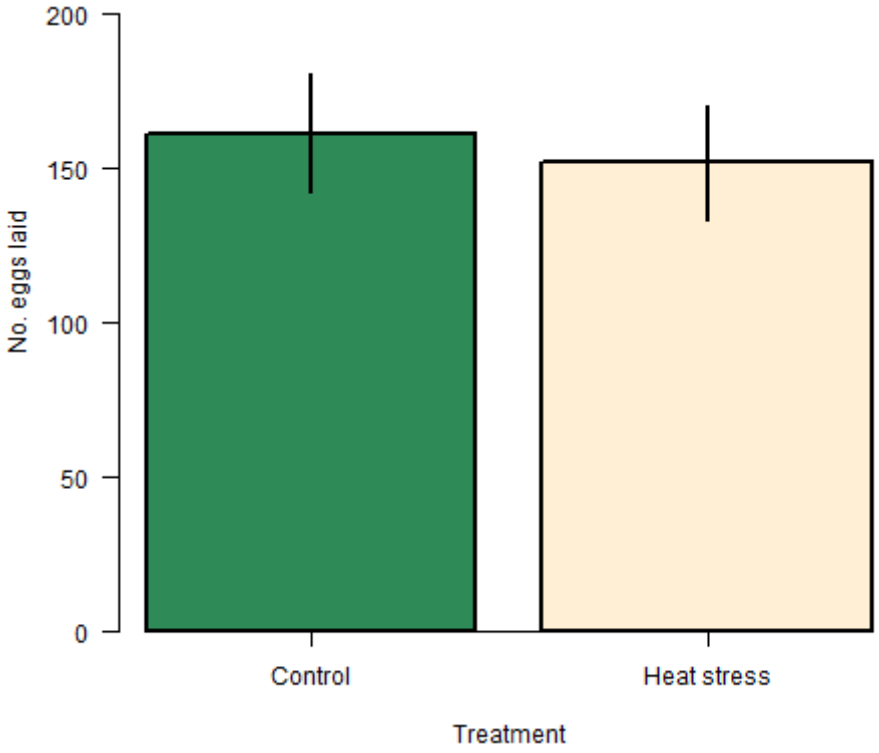
4.7 Figures

4.7.1 ***Figure 4.1.***

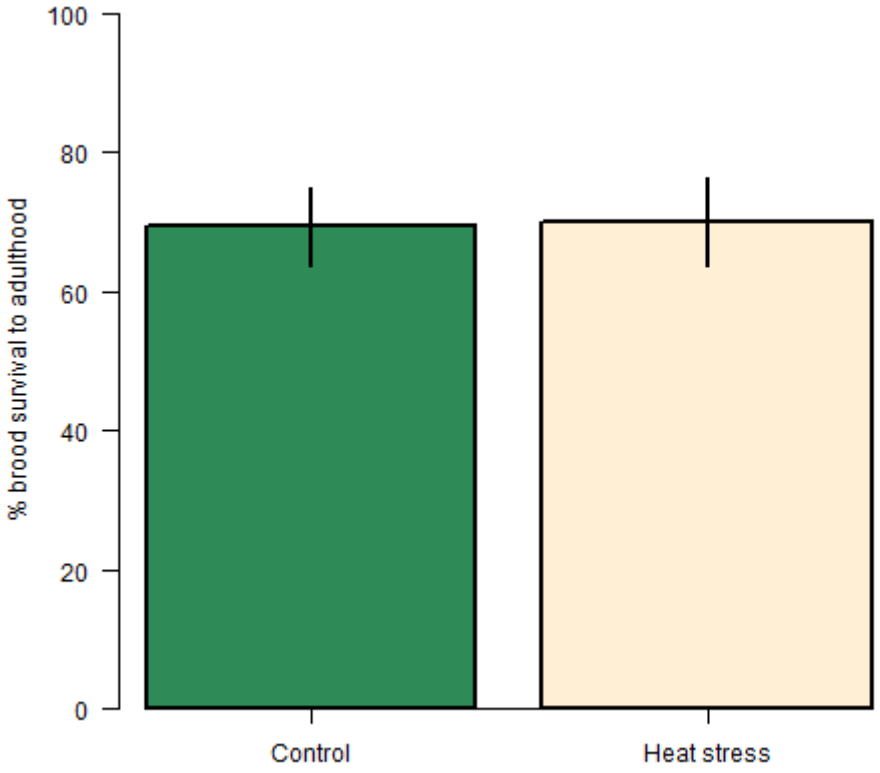
Mean and 95% confidence intervals of life-history parameters of adult female citrus mealybugs that were either exposed to short-term heat stress (50 °C) or control conditions: (A) Females (%) which failed to oviposit; (B) fecundity; and (C) brood survivorship to adulthood. The mean, quartiles, 95th percentiles and individual data points of the sex ratios of adult brood laid by treated females (% female prevalence) (D).


40
30
20
10
0
% non-laying females
Control
Heat stress
Treatment

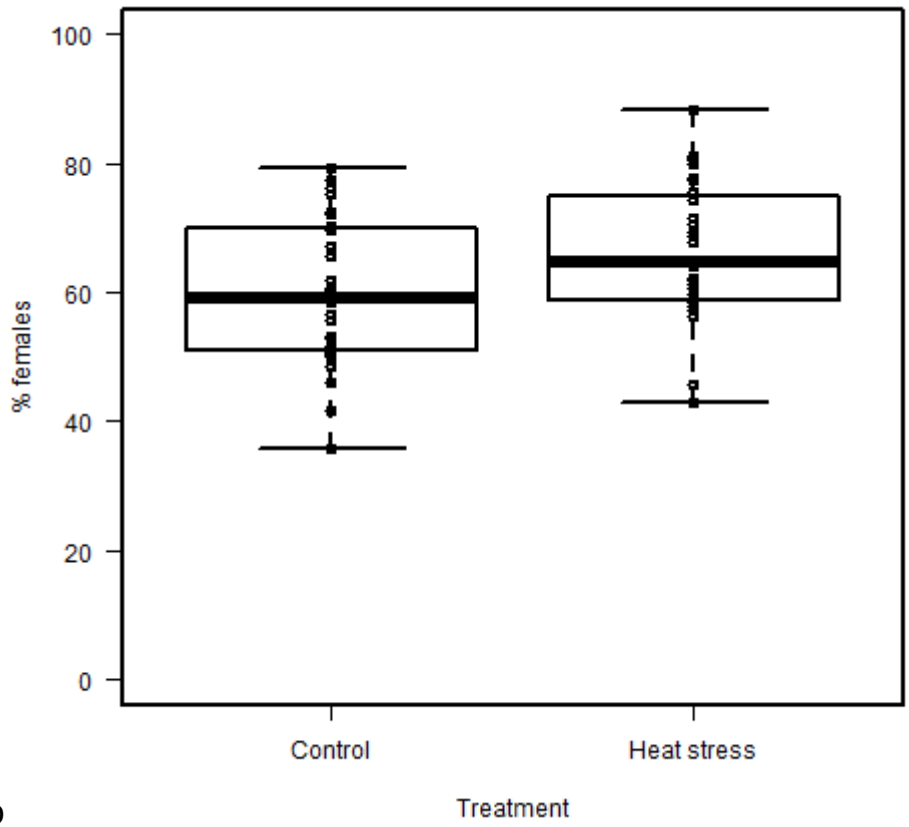
A


200
150
100
50
0
No. eggs laid
Control
Heat stress
Treatment

B


100
80
60
40
20
0
% brood survival to adulthood
Control
Heat stress
Treatment

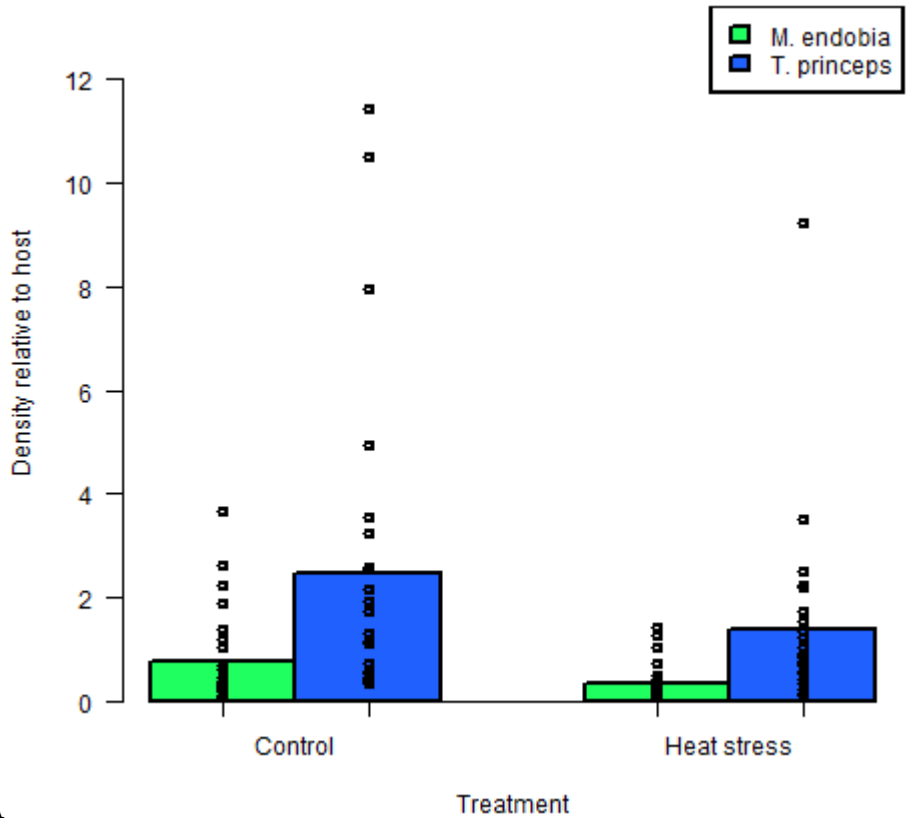
C


100
80
60
40
20
0
% females
Control
Heat stress
Treatment

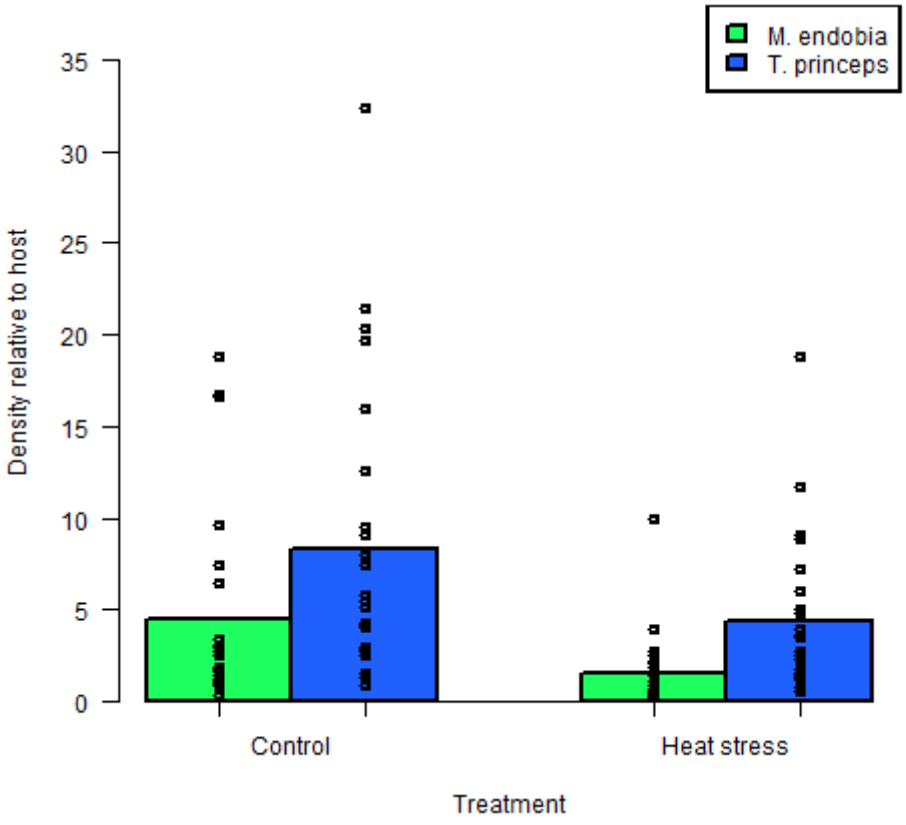
D

4.7.2 ***Figure 4.2.***

Mean and individual data points of densities relative to host control gene of the *M. endobia* and *T. princeps* endosymbionts in adult female citrus mealybugs that were either exposed to short-term heat stress (50 °C) or control conditions, at (A) 48 h following treatment (both n = 25), or (B) 72 h following treatment (n = 25 for heat stressed, n = 26 for control).


M. endobia
T. princeps
Density relative to host
0
2
4
6
8
10
12
Control
Heat stress
Treatment

A


M. endobia
T. princeps
Density relative to host
0
5
10
15
20
25
30
35
Control
Heat stress
Treatment

B

“Wisdom comes from experience. Experience is often a result of lack of wisdom.”

— *Terry Pratchett*

5 Heritability of symbiont density reveals distinct regulatory mechanisms in a tripartite symbiosis

5.1 Abstract

Beneficial eukaryotic-bacterial partnerships are integral to animal and plant evolution. Understanding the density regulation mechanisms behind bacterial symbiosis is essential to elucidating the functional balance between hosts and symbionts. Citrus mealybugs, *Planococcus citri* (Risso), present an excellent model system for investigating the mechanisms of symbiont density regulation. They contain two obligate nutritional symbionts, *Moranella endobia*, which resides inside *Tremblaya princeps*, which has been maternally transmitted for 100-200 million years. We investigate whether host genotype may influence symbiont density by crossing mealybugs from two inbred laboratory-reared populations that differ substantially in their symbiont density to create hybrids. The density of the *M. endobia* symbiont in the hybrid hosts matched that of the maternal parent population, in keeping with density being determined either by the symbiont or the maternal genotype. However, the density of the *T. princeps* symbiont was influenced by the paternal host genotype. The greater dependency of *T. princeps* on its host may be due to its highly reduced genome. The decoupling of *T. princeps* and *M. endobia* densities, in spite of their intimate association, suggests that distinct regulatory mechanisms can be at work in symbiotic partnerships, even when they are obligate and mutualistic.

5.2 Introduction

Symbiotic associations are extremely widespread in nature, and beneficial eukaryotic-bacterial partnerships have shaped the very foundations of plant and animal evolution (Schwartz and Dayhoff, 1978). Symbiosis creates an overlap of selective interests between partners, which will increase with the degree to which symbiont transmission is vertical rather than horizontal, and will be strongest in the hosts that vertically transmit the symbiont (usually females). However, even mutualistic symbiotic associations are inherently selfish, with benefits given only so long as they are reciprocated and, as well as selection for cooperation, there is also selection pressure to cheat and exploit the partnership (Bennett and Moran, 2015). As they coevolve, hosts will be selected to increase their own fecundity, with or without symbionts, whereas symbionts will be selected to maximise their transmission to new hosts, whilst simultaneously outcompeting other strains and species of symbiont for the limited resources provided by the host (Frank, 1996a).

This conflict of selective interests between hosts and symbionts can in part be resolved by vertical transmission of the symbionts, and consequent dependency of the symbiont upon the host. Guaranteed vertical transmission to the next generation relaxes selection for horizontal transmission, leads to genetic homogeneity within hosts, and thus favours decreased virulence of symbionts (Smith, 2007, Frank, 1996a). Evidence for this tendency to transition to avirulence and homogeneity within hosts can be observed in organelles (Birky et al., 1983), and the *Uroleucon ambrosiae* symbionts of aphids (Funk et al., 2000). Symbiont dependency upon the

host increases following symbiont genome reduction- a common result of the symbiont lifestyle (Bennett and Moran, 2015, Moran and Bennett, 2014).

Even in the case of vertically-transmitted symbionts, strict regulation of symbiont density within the host is essential for the efficient functioning of the partnership (Rio et al., 2006, Laughton et al., 2014, Cunning and Baker, 2014, Falkowski et al., 1993, Wilkinson et al., 2007). Too few symbiont cells will cause a deficiency of gene products for the host and inefficient vertical transmission for the symbiont, whilst too many cells will incur some cost to the host without a proportionate benefit. As accommodating a symbiont, even when it is beneficial, will always incur some cost to the host in terms of energy or resources (Bronstein, 2001), an excess of symbionts could also be metabolically-demanding to the host. Costs to the host could lead to long-term costs to the symbionts through reduced host fecundity and hence reduced vertical transmission. In terms of host fitness, the "optimum" within-host symbiont density will be complex and dynamic, being unlikely to be constant throughout the lifecycle of the host, or in every environmental situation that the host encounters, but will instead change depending on context, and be subject to multiple, possibly conflicting, selection pressures and host requirements. Facultatively manipulating symbiont density may prove to be costly to the host. Additionally, the symbiont will be selected to maintain at minimum the threshold density required to ensure vertical transmission, which may in itself vary throughout the life of the host. There may then be selection on both host and symbiont to maintain a compromised symbiont density across environmental and physiological conditions (Kono et al., 2008, Laughton et al., 2014, Rio et al., 2006).

Regulation of symbiont density can occur via the host or the symbiont. Symbionts may change their density by varying their replication rate to maintain or increase their density, whereas hosts can control symbionts using several mechanisms. Depending upon the method of transmission, a screening process can prevent unwanted symbionts from entering the host (Nyholm and McFall-Ngai, 2004). Antimicrobial peptides, in some cases symbiont-specific, can be deployed (Hooper et al., 2012, Balmand et al., 2011). Superfluous bacteria can in some cases be simply evicted (Ruby and Asato, 1993, Dimond and Carrington, 2008). Nutrient acquisition by the host is positively correlated with symbiont density in pea aphids and some corals, which may be a limiting factor in the proliferation of symbionts (Wilkinson et al., 2007, Falkowski et al., 1993, Muller-Parker et al., 1994, Snyder et al., 2010). Regulatory mechanisms may be linked, rather than acting in isolation, for example, the rates of degradation and expulsion of zooxanthellae by the coral, *Stylophora pistillata*, are both triggered by starvation of the host (Titlyanov et al., 2000).

Immune mechanisms in some host species still provide a sophisticated form of symbiont density control (Hinde, 1971, Falkowski et al., 1993, Bennett and Moran, 2015). Indeed, maintaining a symbiont requires that the host amends its approach to dealing with internal bacteria, and suppresses or adjusts its immune responses (Wang et al., 2009a, Ratzka et al., 2013, McFall-Ngai et al., 2010). For example, the pea aphid *Acyrthosiphon pisum*, has lost genes involved in the IMD immune pathway (The International Aphid Genomics Consortium, 2010, Gerardo et al., 2010).

Citrus mealybugs, *Planococcus citri* (Risso), are an intriguing and potentially powerful model system for investigating the roles of host and symbiont in regulating

symbiont density. Citrus mealybugs contain two maternally, vertically-transmitted obligate nutritional symbionts, a β-proteobacterium, *Tremblaya princeps*, and a γ-proteobacterium, *Moranella endobia*, which reside in bacteriocytes in the bacteriome organ surrounding the host gut (Thao et al., 2002). These two symbionts have co-evolved intimately, with *M. endobia* actually residing inside *T. princeps*, which was first acquired by the Pseudococcidae 100-200 million years ago (Thao et al., 2002, Baumann et al., 2002, Husnik et al., 2013).

Both symbionts have reduced genomes (Husnik et al., 2013, Baumann et al., 2002), which could potentially compromise their ability to self-regulate their density within the host. Genome reduction is a common Muller's Ratchet-type consequence of the relieved natural selection pressures experienced by intracellular bacteria (Moran and Bennett, 2014, McCutcheon and Moran, 2012). *T. princeps* holds one of the smallest bacterial genomes known to science, at just under 139kb (Husnik et al., 2013), whilst *M. endobia* carries a larger, yet still reduced, genome of 538kb (McCutcheon and von Dohlen, 2011). It is hypothesised that the dramatic gene loss experienced by *T. princeps* is partly due to it harbouring its own symbiont which can compensate for loss of genetic function (Husnik et al., 2013).

T. princeps relies on both the mealybug host and *M. endobia* to counteract its loss of genes and their functions, which could render it dependent on these partners to regulate its density (McCutcheon and von Dohlen, 2011, Husnik et al., 2013, Sloan et al., 2014, Lopez-Madrigal et al., 2011). For example, genes involved in the construction of cell wall components are found horizontally transferred from other bacterial species into the mealybug genome and are highly expressed in the

bacteriocytes where *T. princeps* resides (Husnik et al., 2013), and translation-related genes no longer present in *T. princeps* are expressed in *M. endobia* (McCutcheon and von Dohlen, 2011).

There is some evidence for genotypic differences in symbiont density within *P. citri*. Citrus mealybug populations have been found to differ in the density of both of their bacterial symbionts by over six-fold, even when cultured under standard laboratory conditions (JFP, BG & WOHH, unpubl. data). The consistency of differences in symbiont density between mealybug populations supports the case for genotypic variation in the propensity to harbour a high or low symbiont density in citrus mealybugs. However, it is not clear whether the differences between populations are caused by the genotype or epigenetics of the host or of the symbiont. In this study, we disentangle the effects of host genome from symbiont genome by crossing mealybugs from two inbred laboratory-reared mealybug populations that differ substantially in their symbiont density in order to create F_1 hybrid daughters. These hybrid mealybugs host the symbionts from their maternal population because symbiont transmission is entirely maternal (Thao et al., 2002), but will have a genome that is derived from both paternal and maternal parents. Any significant deviation in symbiont density from the maternal population would therefore be attributable to the paternal genotype, and indicative of host genotype influencing symbiont density. Alternatively, a non-significant deviation in symbiont density from the maternal population would indicate that symbiont density is determined only by symbiont genotype (or maternally-specific genotypic effects such as via imprinting).

5.3 Methods

Two mealybug populations (A and B) were used which had been obtained from commercial greenhouses in Belgium and cultured in darkness at 25°C and 20% RH on white organic potato sprouts for eight months (approximately eight generations). These populations had been found previously to differ approximately two-fold in the densities of both the *M. endobia* and *T. princeps* symbionts (Parkinson, Gobin & Hughes, unpubl. data). Newly emerged adult females from these populations were separated from their populations of origin and maintained on potato sprouts for five days. Any females which commenced oviposition in this time period were discarded (ca. 20% of females) to ensure virginity. Adult males from the other population were then placed with the females for 48 h to allow for mating (males from Population A were placed with females from Population B and vice versa). This hybridisation process created two F1 generation hybrid populations: A♀B♂ and A♂B♀. When each female commenced oviposition, she was placed on an individual potato to lay eggs in isolation. The F1 hybrid offspring from each female were allowed to hatch and mature on these isolated potatoes, with all male offspring being removed to ensure the virginity of their sisters. F1 females were allowed to grow to maturity (~ 30 days post hatching).

5.3.1 ***Symbiont quantification***

Newly emerged adult females from Populations A (n = 39) and B (n = 40) and the hybrid populations A♀B♂ and A♂B♀ (20 offspring per mother, n = 28 mothers for

A♀B♂, n = 29 mothers for A♂B♀) were crushed individually in 100µL 5% Chelex solution, heated to 99°C for 15 min and centrifuged at 2,326g for 20min. The supernatant was pipetted off and diluted 1:10 with molecular grade water for use in qPCR reactions. DNA from multiple offspring were pooled to create a single DNA sample per mother.

Symbiont infection intensity was quantified by measuring gene copy number using qPCR with the comparative C_T method, using the host *28S* gene to control for DNA quantity (Schmittgen and Livak, 2008), as per (Parkinson et al., 2014). Primers and probes for the *P. citri* control gene, 28S rDNA and *T. princeps GroEL* gene were designed using PRIMER3 software (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) and analysed using NetPrimer software (Primer Biosoft International, Palo Alto, CA, USA). Primers and probes for *M. endobia 16S* and *23S* rDNA were designed using Primer Express v.3.0 software (Life Technologies, Foster City, CA, USA) (Table 1). To ensure that only a single PCR product would be amplified for *M. endobia*, the forward primer for *M. endobia* was checked against the *M. endobia* complete genome (Accession number CP003881.1), which was isolated from the citrus mealybug PCVAL strain, and found to match at only a single site (López-Madrigal et al., 2013). The forward primer also only matched a single site for the *M. endobia* complete genome (Accession number CP002243.1), which was isolated from the citrus mealybug PCIT strain (McCutcheon and von Dohlen, 2011). To ensure that only a single PCR product would be amplified for *P. citri*, the forward primer for *P. citri* was checked against *28s rDNA* GenBank sequences (Accession numbers GU134660.1, JF714181.1, JQ651165.1, JQ651169.1,

JQ651170.1, JQ651171.1, JQ651362.1, JQ651363.1, JQ651364.1, JQ651365.1) and found to match at only a single site (Sethusa et al., 2013, Beltrà et al., 2012, Malausa et al., 2011). The *28S rDNA* gene has been used for several phylogenetic studies in mealybugs, with being present as a single copy in citrus mealybugs (Hardy et al., 2008, Downie and Gullan, 2004). 10 µl reaction volumes were used for qPCR in a StepOnePlus^{TM} Real-Time PCR System (Applied Biosystems), with 150nM of each primer, 50 nM of probe, and 1× of ABI Taqman Universal Master Mix II with UNG (Life Technologies, Foster City, CA, USA). The cycle was 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and the annealing temperature (collection step) for 1 min. An annealing temperature of 64°C was used for *P. citri* and *M. endobia* reactions, and 60°C was used for *T. princeps* reactions.

The densities of *T. princeps* and *M. endobia* in individual mealybugs were determined by comparing symbiont gene copy number against the *P. citri* host control gene, using the comparative C_T method, which standardises for differences in tissue quantities (Crotti et al., 2012, Schmittgen and Livak, 2008). All samples were run in triplicate and non-concordant replicates and samples were re-run or excluded. The C_T values of all three target genes were measured for each mealybug. Then the difference in C_T value between the symbiont genes and the host control gene for each mealybug were calculated and expressed as fold differences in the symbiont genes relative to the host genes by $2^{-(\text{symbiont CT} - \text{host CT})}$.

5.3.2 *Statistical analysis*

Analysis was conducted by converting relative ΔC_T values into host-symbiont ratios. Symbiont densities in the different populations were analysed using a generalized linear model with a Gamma distribution and log-link function and the Likelihood ratio χ^2 statistic. The sequential Bonferroni correction to the Wald test was used for pairwise comparisons of populations. Data for *T. princeps* and *M. endobia* were analysed separately. Differences in extraction and quantification efficacies for the two symbionts mean that the quantites cannot be compared between the symbionts.

5.4 Results

The qPCR data gave us the relative infection intensity of the two bacterial symbionts in the two parent populations of mealybugs and their hybrid daughters. The relative infection intensity of the *M. endobia* symbiont differed significantly between the mealybug populations ($\chi^2 = 56.4$, d.f. = 3, $P < 0.001$). Population B had on average 58% fewer *M. endobia* cells per host cell than Population A (Fig. 1A). Pairwise comparisons reveal that the F_1 hybrid populations differed significantly from their paternal populations ($P < 0.001$ in both instances), but not their maternal population ($P = 0.892$ for A♀B♂ and $P = 0.141$ for A♂B♀).

The same pattern did not follow for the *T. princeps* symbiont (Fig. 1B). Symbiont density again differed significantly between mealybug populations ($\chi^2 = 85.3$, d.f. = 3, $P < 0.001$), and Population B had on average 71% fewer *T. princeps* cells per host cell than Population A. However, pairwise comparisons revealed that both F_1 hybrid populations differed significantly from not only their paternal populations ($P < 0.001$

in both instances), but also both their maternal populations (P = 0.010 for A♀B♂ and P < 0.001 for A♂B♀). Population A♀B♂ had a *T. princeps* density that was higher than either of its parent populations (185% greater than that of Population A), while Population A♂B♀ had a *T. princeps* density intermediate between those of its parent populations (51% of that of Population A; Fig 1B).

5.5 Discussion

In order to separate the effects of bacterial-derived versus host-derived regulation of symbiont density, we crossed two laboratory strains of citrus mealybug with consistently different infection intensities of the *T. princeps* and *M. endobia* symbionts to create two new hybrid strains. *M. endobia* densities in adult females from these hybrid strains were not significantly different from those of the maternal populations, indicating that *M. endobia* density was not affected by host paternal genotype. However, *T. princeps* densities in adult females from these hybrid strains were significantly higher than from those of their maternal populations, indicating that the paternal host genotype influenced the density of the symbiont, possibly in a non-additive way as the hybrid strain A♀B♂ had a *T. princeps* density that was higher than either of the parental populations. This may also have been a result of heterosis of the host genome, which may have enabled the host to harbour more *T. princeps* cells. Despite this, the hybrid strains still held a *T. princeps* density that was more similar to the maternal than the paternal line, so *T. princeps* may to some degree control its own density.

P. citri holds a logistical advantage for regulating its symbionts' densities. *T. princeps* and *M. endobia* reside in specialised bacteriocytes which compose the bacteriome organ surrounding the gut of the host, a prime location for nutritional symbionts to function (Thao et al., 2002). Cordoning symbionts into a single location also eases organised density control and bacteriocytes often express high levels of antimicrobial peptides, such as observed in the rice weevil, *Sitophilus oryzae*, (Login et al., 2011). *Bemisia tabaci* whiteflies are less capable of effectively regulating symbionts that are situated outside of their bacteriocytes (Su et al., 2014).

The decoupling of *T. princeps* and *M. endobia* densities suggests that, despite their intimate evolutionary association, distinct regulatory mechanisms are at work for the two symbionts. Decoupling of the two symbionts has been observed in adult male mealybugs, who lose *M. endobia* at a faster rate than *T. princeps* as they approach their aposymbiotic stage (Kono et al., 2008). Differential regulation pathways for obligate versus facultative symbiont density have also been found in the pea aphid, revealed by varying dietary nitrogen levels (Wilkinson et al., 2007), reflecting the distinct relationships that aphids share with different types of symbiont. However, *T. princeps* and *M. endobia* are both obligate nutritional mutualists and, moreover *M. endobia* resides inside *T. princeps*, so their inconsistent responses to hybridisation are surprising.

The nested relationship of *M. endobia* inside *T. princeps* and their discrepancies in genome size may account for their different density regulatory mechanisms. *T. princeps* has a dramatically reduced genome, one of the smallest known to science with only 120 protein-coding genes, and relies on *M. endobia* and the host for much

of its function (Husnik et al., 2013). It is argued that in terms of gene number and genome size, *T. princeps* is more similar to an organelle than a symbiont (McCutcheon and Moran, 2012, Husnik et al., 2013). It could be argued that such dependence and efficient vertical transmission will mean that *T. princeps* may thus behave as a part of *P. citri*, rather than a separate organism within *P. citri* with its own conflicting evolutionary interests. However, even intra-genome conflict can occur, and the fitness requirements of one individual in a symbiotic relationship is unlikely to align flush with that of its partner (Herre et al., 1999, Eberhard, 1980). Even organelles can still conflict with their hosts, for example the Cytoplasmic Male Sterility (CMS) induced by mitochondria in some plant species (Chase, 2007). Uniparental transmission benefits hosts by preventing competition between unrelated organelles, but deems one of the sexes to be an evolutionary dead-end for the organelles (Law and Hutson, 1992, Hurst, 1995).

T. princeps has lost functional genes for bacterial translational release factors, aminoacyl-tRNA synthetases, ribosome recycling factor, elongation factor EF-Ts and peptide deformylase (McCutcheon and von Dohlen, 2011). It is common for symbionts to lose genes associated with cell wall structure, for example, *T. princeps* lacks cell-envelope-related genes and relies on its host for the creation of a cell membrane (Husnik et al., 2013, McCutcheon and Moran, 2012, McCutcheon and von Dohlen, 2011). It could therefore be the case that the larger and more functionally complete genome of *M. endobia* gives it more control of its own regulation, than *T. princeps*. However, the expression of *murABCDEF* and *mltD/amiD* genes in the host genome are believed to control the cell wall stability

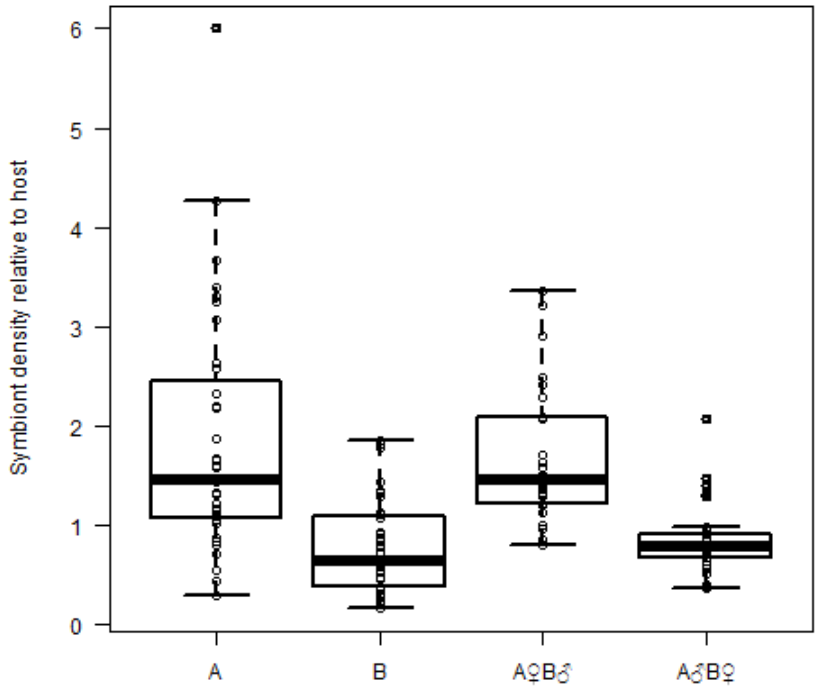
and lysis of *M. endobia*, so even this symbiont may still be partially influenced by its host's genotype (Husnik et al., 2013, Koga et al., 2013, McCutcheon and von Dohlen, 2011).

In summary, the decoupling of *M. endobia* and *T. princeps* densities following crossing of mealybug lines with different symbiont infection intensities reveals that even nested intracellular symbionts can have different regulatory mechanisms. *T. princeps* provides an example of how the defined boundary between organism and organelle can be blurred, and, despite their antiquity, it may be more appropriate to consider organelles as part of the same evolutionary spectrum as symbionts rather than a discrete functional category (McCutcheon and Keeling, 2014). Understanding the density regulatory mechanisms behind bacterial symbiosis will be essential to understanding the functional balance between hosts and symbionts and how they have evolved to overcome their conflicts of interests.

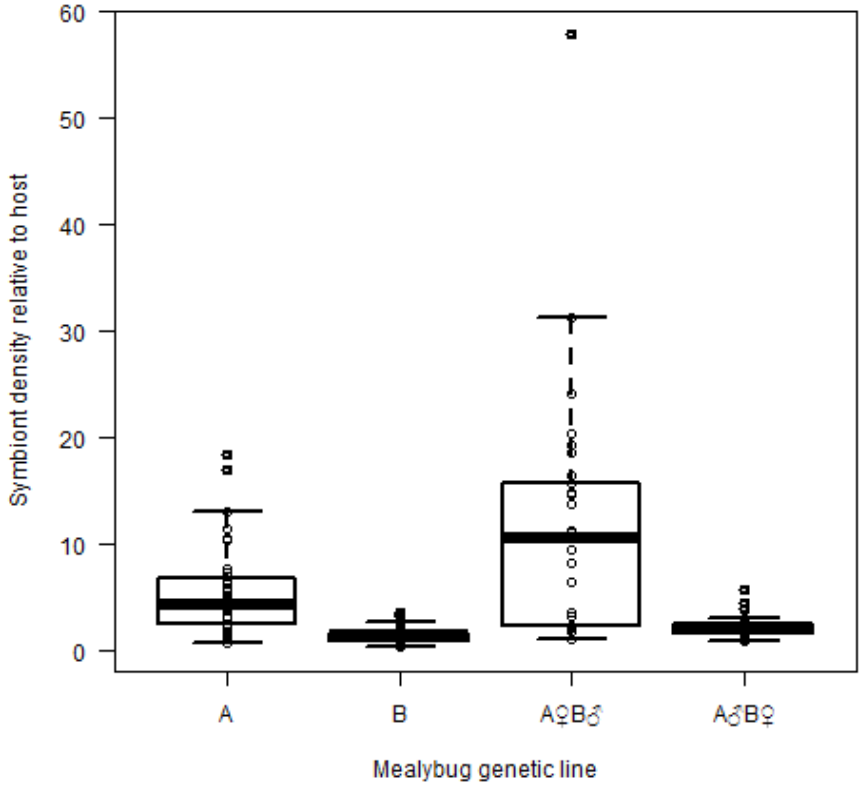
5.6 Figures

5.6.1 ***Figure 5.1.***

The mean, quartiles, 95th percentiles and individual data points of the densities (relative to the host control gene) of the (A) *M. endobia* and (B) *T. princeps* bacterial symbionts in adult citrus female mealybugs from parental Populations A and B, and the hybrid offspring Populations A♀B♂ and B♀A♂. Symbiont density was measured using qPCR, calculated as relative to *P. citri* host control gene using the comparative C_T method.


Symbiont density relative to host
0
1
2
3
4
5
6
A
B
A♀B♂
A♂B♀
Mealybug genetic line

A.


Symbiont density relative to host
0
10
20
30
40
50
60
A
B
A♀B♂
A♂B♀
Mealybug genetic line

B.

“If nothing else works, a total pig-headed unwillingness to look facts in the face will see us through.”

— *General Melchett*

6 Community-Profiling of the Symbiotic Microbiota of Citrus and Long-tailed Mealybugs

6.1 Abstract

Bacterial symbiosis is pivotal to the early evolution of eukaryotes, having given rise to mitochondria and chloroplasts. Our understanding of host-symbiont evolutionary ecology is largely based on obligate relationships, but the vast majority of symbiotic relationships are facultative. These facultative symbionts can nevertheless have profound effects on host biology, and so the characterisation of their occurrence and effects is essential for a full understanding of symbiosis and host evolution more broadly. Here, we characterise the communities of facultative symbionts in mealybugs, a group which serve as models for understanding obligate symbiosis, and which are also economically important pests. We examine citrus mealybugs *Planococcus citri* and long-tailed mealybugs *Pseudococcus longispinus* (Pseudococcidae), using metagenomic sequencing and targeted conventional PCR of specific symbionts. The symbiont communities of *P. citri* mealybugs differed significantly in their structure from those of *P. longispinus*, as did *P. citri* samples that came from different countries. The Alpha diversity for each library varied from 1.4 to 2.4 in the Inverse Simpson Diversity Index and the number of number of OTUs varied from 27 to 69. *Spiroplasma* was found at low levels in all of the samples examined with metageonimcs, and conventional PCR-sequencing also

revealed the presence of *Rickettsia* in *P. citri*. This systematic census of facultative symbionts provides a deeper insight into the diverse bacterial biomes of mealybugs.

6.2 Introduction

Bacterial symbiosis is now understood to be one of the fundamental pillars of the ecology and evolution of eukaryotes, (Saffo, 1992, Moran, 2001, Douglas, 2009). For example, mitochondria and chloroplasts are the descendants of once free-living prokaryotes engulfed and encapsulated by host eukaryotic cells, the human body contains ten-fold more cells of microbial origin than its own, and our gut bacteria are essential for healthy food digestion (Schwartz and Dayhoff, 1978, Ley, 2006, Backhed, 2005, Hooper, 2002). Symbiosis is such a successful strategy that it is estimated that more than 50% of all animal species are parasitic symbionts alone (Price, 1980, Windsor, 1998). Symbiotic interactions are also diverse. They can range from mutualistic relationships which benefit both partners, through commensal relationships which benefit one partner with negligible impact on the other, to parasitic relationships which benefit one partner at a cost to the other, but in practise symbiotic relationships often tend to fall along this continuum in a context-dependent way rather than necessarily in neat and rigid categories (Swain, 2012, Gerardo, 2015).

Although many fascinating and important symbionts form obligate relationships, facultative symbiotic relationships are also of great importance. Facultative symbionts are not essential for the survival of the host, and have evolved a diversity of strategies to maintain and expand their prevalence, such as horizontal transfer, sex-ratio distortion of the host, and induced cytoplasmic incompatibility (Russell and Moran, 2005, Russell et al., 2003, Weeks et al., 2003, Negri et al., 2006, Gotoh et al., 2007). The diverse ecology of facultative symbionts has been studied in particular

depth in the aphids, most notably the pea aphid *Acyrthosiphon pisum*. Within this system, three major facultative symbiont species, amongst several others, have been identified. Infection by *Hamiltonella defensa*, in combination with a bacteriophage, is associated with parasitoid resistance (Oliver et al., 2003, Jiggins et al., 2000, Oliver et al., 2005, Wilcox et al., 2003), *Regiella insecticola* is associated with fungal resistance, parasitoid resistance and host plant specialisation (von Burg et al., 2008, Vorburger et al., 2009, Jiggins et al., 2000, Scarborough et al., 2005, Tsuchida et al., 2004, Ferrari et al., 2007), and *Serratia symbiotica* is associated with heat-shock resistance, and parasitoid resistance and in some cases may complement or replace the obligate symbiont *Buchnera aphidicola* (Oliver et al., 2003, Montllor et al., 2002, Moran et al., 1999). Understanding the dynamics of facultative symbiont infections in insects such as aphids is of applied, as well as fundamental, importance. As each of the three main facultative symbionts in pea aphids is associated with parasitoid resistance, their presence could negatively impact the efficacy of agricultural parasitoid biocontrol agents against aphids. Thus, information on the infection status of pest aphids in a field or greenhouse could prove crucial for a grower's decision on pest control tactics.

However, our understanding of the occurrence, dynamics and impact of facultative symbiont communities in most insects is still very limited. Here, we characterise the composition of facultative symbiont communities in mealybugs (Pseudococcidae), a group which are an important model in host-symbiont evolutionary biology, and which are also economically-important pests of agriculture and horticulture (Franco et al., 2004). Mealybugs possess a unique nested hierarchy of obligate symbionts.

The γ-proteobacterium *Moranella endobia* is nested within the β-proteobacterium *Tremblaya princeps*, which is in turn nested within the bacteriome organ surrounding the mealybug gut (Thao et al., 2002). The citrus mealybug genome contains several genes horizontally-transferred from facultative symbionts, revealing associations with these bacteria in its evolutionary past (Husnik et al., 2013), and *Rickettsia* and *Spiroplasma*-like bacterium have been detected in some other mealybugs (Singh et al., 2013, Hardy et al., 2008). However, what, if any, facultative symbionts infect mealybugs and what effect the facultative symbionts may have on the ecology of the host, is otherwise unknown. Here, we explore the microbiome of the citrus mealybug *Planococcus citri* (Risso) and long-tailed mealybug *Pseudococcus longispinus* (Targioni-Tozzetti) (Pseudococcidae), both common, polyphagous, globally distributed species (Ben Dov, 2015) using metagenomic sequencing of selected mealybug populations and larger-scale conventional PCR-screening of targeted symbionts.

6.3 Methods

P. citri citrus mealybugs and *P. longispinus* long-tailed mealybugs were collected for metagenomic analysis from Belgian commercial greenhouses, and the PCS Ornamental Plant Research Centre in Ghent (Belgium) in 2011, Lisbon (Portugal), Cantania (Italy), and Bet Dagan (Israel) in 2012, and Oxford (UK) in 2013, for sequencing (Table 6.1). Adult female *P. citri* and *P. longispinus* individuals were collected for conventional PCR screening from Belgian commercial greenhouses in 2012 and stored in absolute ethanol at -20°C until DNA extraction. Mealybugs were

cultured in darkness at 25°C and 50% relative humidity on white organic potato sprouts.

6.3.1 *DNA isolation, PCR and gene library preparation for metagenomic analysis*

DNA was isolated using a Zymo Research Tissue and Insect DNA Microprep™ kit. DNA from mealybugs (15 populations, 8-20 mealybugs per population) was pooled into a single sample for each population. Three technical replicate 50 µL PCR reactions were conducted using 24µL molecular grade H_2O, 10µL Promega GoTaq Flexi green buffer, 6.25µL $MgCl_2$, 2.5µL dNTPs (2.5 mM each), 1µL forward primer, 1µL reverse primer, 0.25µL Promega GoTaq Polymerase and 5µL DNA. Forward primer *F515* and reverse primer *R806* targeted the *V4* region of bacterial *16S rRNA* genes to produce a ~250bp amplicon (Munson et al., 1991a). As per the Earth Microbiome Project 16S rRNA amplification protocol version 4_13 (Wilson et al., 2006), the *F515* was incorporated with a 5' Illumina adapter, a forward primer pad and a forward primer linker and *R806* was incorporated with a reverse complement of the 3' Illumina adapter, a unique Golay barcode for each population, a reverse primer pad and a reverse primer linker (see Table 6.1 for details).

The thermal cycle was as follows: 95°C 10 min, followed by 35 cycles of 95°C for 30 s, 59°C for 30 s and 72°C for 30 s, followed by a final stage of 72°C for 7 min. PCR technical replicates were pooled and DNA concentration was measured using an Invitrogen Qubit® Fluorometer 2.0. PCR products were purified using an

Ambion^{R} Magnetic Stand-96 as per manufacturer's instructions. PCR products were measured for DNA concentration again and proportionally pooled in relation to concentration into a single library sample so that each population was equally represented. The library was run using gel electrophoresis on a 2% agarose gel at 35V for 10 minutes, followed by 100V for 2 hours. For gel electrophoresis, 400µL of library, 80µL of New England Biolabs gel loading dye and 400µL of glycerol were used in combination in a single enlarged well. Gel was viewed under UV light and library band was removed using razor blades. Band was purified using a QIAquick^{R} Gel Extraction kit, as per manufacturer's instructions. DNA concentration was measured using an Invitrogen Qubit^{R} Fluorometer 2.0 and 260/280 ratio using a Nanodrop 2000 Spectrophotometer, until it met the requirements specified by the University of Oxford Wellcome Trust Centre for Human Genetics. There sequences were read with an Illumina MiSeq platform (Martinson, 2011).

6.3.2 *DNA isolation and conventional PCR-screening for targeted symbionts*

310 *P. citri* and 72 *P. longispinus* adult females from Belgian commercial greenhouses in 2012 were randomly selected and screened for specific bacterial symbionts *Wolbachia*, *Rickettsia*, *Spiroplasma*, *Cardinium*, *Arsenophonus* and *Asaia*. DNA was extracted by crushing individual mealybugs in 10% Chelex solution, boiling at 99°C for 15 min and centrifuging at 2,204 g for 20 min before pipetting off the supernatant for use in PCR reactions. Host control PCR reactions were performed to ensure the quality of DNA extraction, using the primers *TL2-N-3014* and *C1-J-2183* (Simon et al., 1994); annealing temperature 55°C. PCR

reactions were performed with 4.8µL molecular grade H_2O, 2µL Promega GoTaq Flexi green buffer, 1.25µL $MgCl_2$, 2.5µL dNTPs (2.5 mM each), 0.2µL forward primer, 0.2µL reverse primer, 0.05µL Promega GoTaq Polymerase and 1µL DNA solution. The thermal cycle was 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, the annealing temperature for 30 s and 72°C for 1 min, followed by a final stage of 72°C for 7 min. PCR products were run on an agarose gel and viewed under a UV lamp.

The following bacterial groups were screened for each mealybug alongside positive controls: *Wolbachia* (primers *WSPF* and *WSPR* (Kondo et al., 2002), annealing temperature 55°C), *Spiroplasma* (primers *SpoulF* and *SpoulR* (Montenegro et al., 2005), annealing temperature 50°C), *Rickettsia* (primers *16SA1* (Fukatsu and Nikoh, 1998) and *Rick16SR* (Fukatsu et al., 2001), annealing temperature 55°C), *Cardinium* (primers *CF* (Weeks et al., 2003) and *CR* (Singh et al., 2013), annealing temperature 49°C), *Arsenophonus* (primers *Ars23S-1* and *Ars23S-2* (Thao and Baumann, 2004a), annealing temperature 62°C) and *Asaia* (primers *Asafor* and *Asarev* (Crotti et al., 2009), annealing temperature 59°C).

6.3.3 ***Data processing and statistical analysis***

We created *V4* region *16S rRNA* libraries for each of the mealybug populations studied. Sequences were read from each library from an Illumina MiSeq platform. Raw fastq files were processed with MORTHUR software (Windsor, 1998) into contigs files. Duplicate sequences and sequences longer than 275 bp were removed. The remaining sequences were aligned, filtered and pre-clustered into groups with a

maximum of two bp differences. Chimeras were removed using the UCHIME algorithm (Edgar et al., 2011). Sequences were then classified using a Bayesian classifier, and any sequences outside the scope of this study, i.e. those classed as chloroplast, mitochondria, archaea, eukaryote or unknown, were removed. The error rate was assessed against a mock group. Sequences were clustered into Operational Taxonomic Units (OTUs) to the level of order and classified (Table 6.2). Phinch software (Wild, 2015) was used to visualise the taxonomic structure of the samples. The total number of filtered reads and number of reads for major taxonomic composition (after the removal of γ-proteobacteria which made up 33.3% of reads across *P. citri* samples and 29.9% across *P. longispinus* samples) was recorded.

A rarefaction curve was generated to display how sample diversity related to sampling effort as a collector's curve; the figure was constructed in R v3.1.1, using the phyloseq package (Supplementary Fig. S.6.1) (Verstraete et al., 2007). The alpha diversity of samples was calculated using Inverse Simpson's Diversity Index. A dendrogram was generated to display similarities in membership and structure across samples, using the jclass and thetayc calculators, and displayed in FigTree (http://tree.bio.ed.ac.uk/software/figtree/). In order to determine whether structure varied between subsets of the populations which varied in an aspect of interest (see Table 6.3 for selected samples), quantitative Unifrac weighted and qualitative Unifrac unweighted analyses were conducted. Sequence data was converted into biom format for further use (Gauthier et al., 2015).

6.4 Results

The number of OTUs plateaued with increasing number of read sequences, indicating that our sampling depth was sufficient to provide a complete profile of the bacterial communities in the mealybugs (Supplementary Fig. S.6.1). The number of OTUs for each library varied from 27-69. The Alpha diversity for each library varied from 1.4 to 2.4 in the Inverse Simpson Diversity Index (Fig. 6.1). A dendrogram calculated at a 0.03 cut-off to display the clustering of community similarity across samples showed that clustering in membership and structure of library communities did not seem to be influenced by mealybug host species, host plant or location of origin. Most the populations cluster closely, except for three out-groups: PCK, PLD with PLN, and PCB (Fig. 6.2). Bar charts depicting the microbial community composition of each library showed that populations were mostly comprised of either Actinobacteria or Mollicutes, with the exception of PCF, which was dominated by Bacilli (Fig. 6.3). Following the exclusion of γ-proteobacteria, the most common bacteria found across the samples included *Pseudomonas*, *Staphylococcus*, *Corynebacterium*, *Staphylococcaceae*, *Leucobacter*, *Acinetobacter*, *Nesterenkonia*, *Ochrobactrum*, *Tsukamurella*, *Oceanobacillus* and *Delftia* (Table 6.2). Venn diagrams calculated at a 0.03 cut-off showed that many of the bacterial OTUs were shared between *P. longispinus* collected from the same location with different host plant species, *P. citri* sourced from the same host plant species from different locations, *P. citri* sourced from different countries and wild versus laboratory-reared *P. citri* (Fig. 6.4).

P. citri microbial communities differed significantly in structure from *P. longispinus* microbial communities in a quantitative weighted Unifrac analysis (0.187, P <0.001), but not in a qualitative unweighted analysis (0.614, P = 0.156; Table 6.3). Pairwise comparisons did not find significant differences in either analysis for *P. longispinus* samples from different host plants at the same location, nor between *P. citri* samples from the same host plant from different locations (Table 6.3). Significant differences between *P. citri* samples from different countries were not found in unweighted analyses but were found in half of the weighted pairwise analyses (Table 6.3). The laboratory-reared *P. citri* library, when compared to wild-collected *P. citri* samples from the same country of origin, was not found to differ significantly in weighted or unweighted analyses.

None of the *Wolbachia*, *Cardinium*, *Spiroplasma*, *Rickettsia*, *Arsenophonus* or *Asaia* symbionts targeted in the conventional PCR-screening were detected in *P. longispinus* mealybugs. *Arsenophonus* and *Asaia* were also not found in *P. citri* mealybugs, however, *Wolbachia* and *Cardinium* were observed in *P. citri* at very low levels (0.96% and 2.25% of individuals respectively), whilst *Spiroplasma* and *Rickettsia* were detected in *P. citri* at higher levels of prevalence (5.2% and 11.3% respectively, Table 6.4, Fig. 6.5).

6.5 Discussion

Together, our metagenomic and conventional PCR screening revealed that citrus and long-tailed mealybugs contain diverse microbiomes, and harbour bacteria known to be common symbionts of insects, including *Spiroplasma* and *Rickettsia*, with

Wolbachia and *Cardinium* occurring at low levels. Some components of the microbiomes were affected by mealybug species, the plant they were feeding on, or their geographical origin, while other components of the microbiome appeared to be unaffected by these factors.

The mealybug samples used in this study were found to have a far greater number of OTUs than a previous study of whole whitefly, aphid and psyllid-generated samples analysed by *16S rRNA V6-V7* pyrosequencing analysis, which observed just 3-7 OTUs (Jing et al., 2014). This is despite mealybugs also being members of the Sternorrhyncha and phloem-feeders, and thus sharing very similar niches to these insects. Low microbial diversity is not unusual in insect studies, and has been observed in mosquito guts, tsetse flies, whole common bed bugs, and the abdominal tissues and guts of honeybees and bumblebees (Aksoy et al., 2014, Meriweather et al., 2013, Martinson, 2012, Moran, 2012, Martinson, 2011). However, another study examining pea aphid microbiota using the *V4-5* region found 21 bacterial OTUs (Gauthier et al., 2015). It may be that these discrepancies are at least partly due to differences in the protocols used, and highlights the need for caution when comparing seemingly like-for-like microbiome studies. PCR bias in metagenomic studies can impact the overall results. Variation in protocols such as DNA extraction have been found to result in different PCR biases (and thus different reported compositions in microbiome communities) for identical original samples (Brooks et al., 2015). The samples in this study may have been subject to PCR bias, however, this is not a concern when comparing their microbiotas because all samples were

subject to the same protocols, and so any PCR bias would have been consistent between them.

In our metagenomic study, the use of both Unifrac weighted and unweighted analyses can reveal interesting differences in population microbiome structure. In this case, *P. citri* and *P. longispinus* are not significantly different from each other in terms of which microbial taxa were present, but did differ in their relative abundances within their communities. *P. citri* samples located from different countries did not differ significantly in the presence/absence of OTUs but did differ significantly in composition in half of cases. Originating from the same host plant for *P. citri,* or the same geographical location for *P. longispinus,* seemed to result in microbial communities which were similar qualitatively and quantitatively in composition. It could be that inhabiting the same country provides sufficient shared ancestry, opportunities for horizontal transfer, or exposure to similar environmental microbes for mealybugs to maintain similar communities of facultative symbionts. This echoes a previous study in mosquitoes which found that gut microbiota would alter with diet, and a study in pea aphids which found bacterial community to be influenced by both host biotype and host plant specialisation (Martinson, 2011, Martinson, 2012, Gauthier et al., 2015).

P. citri mealybugs in this study which had been reared in the laboratory over two years did not differ significantly from *P. citri* which were freshly collected from commercial greenhouses, indicating that lab-rearing had not lead to a significant loss of symbionts. Jing et al. (2014) also found that laboratory-reared and wild populations of whiteflies, aphids and psyllids did not differ significantly in their

richness or diversity. In fruit flies, in contrast, the microbial gut community composition can alter after periods of captivity (Chandler et al., 2011). It may be that the relatively constant plant sap diet of mealybugs results in them retaining their initial symbiont communities when moved to the laboratory, whereas the more substantial change in diet when other insects are moved to the laboratory results in their microbial communities changing more markedly.

The metagenomics method used in this study holds the advantage of capturing a diverse range of bacterial taxa. However, caution should be taken when applying ecological diversity indices to 16S barcode reads, as bacteria are known to vary in gene copy number (Klappenbach et al. 2001). The presence of a bacterial taxon with high 16S rRNA gene copy number will skew the results of a diversity index. In order to compare the abundances of different bacterial taxa confidently, gene copy number must thus be taken into account and the data adjusted accordingly.

Spiroplasma was found at low levels in all of the metagenomic samples studied, and in some of the conventional PCR samples. *Spiroplasma* are diverse bacteria found in several orders of insects, which can be horizontally and vertically transmitted, and can be insect symbionts and plant pathogens vectored by sap-sucking insects (Clark, 1982, Gasparich, 2010, Gasparich, 2002, Regassa and Gasparich, 2005). *Spiroplasma* are generally commensal, usually occupying the gut lumen of insects, but can prove parasitic when they invade other tissues (Regassa and Gasparich, 2006, Gasparich, 2002). They can lead to reduced fitness in pea aphids and male-killing in insects across different orders, such as fruit flies, butterflies, planthoppers and ladybeetles (Fukatsu et al., 2001, Montenegro et al., 2005, Kageyama et al.,

2007, Jiggins et al., 2000). However, there have also been examples of *Spiroplasma* in mutualistic associations, including parasitoid resistance in pea aphids (Nyabuga et al., 2010). Thus, there are a variety of possible fitness impacts of *Spiroplasma* on *P. citri* and *P. longispinus* mealybugs, and further work will be needed to determine the effects of the symbiont in these hosts.

The conventional PCR-screening revealed the presence of *Rickettsia* in just over 11% of *P. citri* from Belgian greenhouse populations. *Rickettsia* have diversified to pathogenically infect a wide variety of animals with either vertical or horizontal transmission (Weinert et al., 2009, Caspi-Fluger et al., 2012). They negatively impact host fitness in pea aphids, induce male-killing in buprestid and lady beetles and parthenogenesis in parasitoid wasps (Sakurai et al., 2005, Lawson et al., 2001, von der Schulenburg et al., 2001, Giorgini et al., 2010). However, *Rickettsia* can also have beneficial effects, increasing heat tolerance in whiteflies, and parasitoid tolerance and resistance to viruses and insecticides in whiteflies (Brumin et al., 2011, HuiPeng and YouJun, 2012, Kontsedalov et al., 2008, Kliot et al., 2014). As with *Spiroplasma*, the wide-ranging effects of *Rickettsia* means that comparative life-history experiments or experimental manipulations of the symbiont will be needed to determine whether it causes any fitness costs (or benefits) to mealybugs.

This study provides the first in-depth bacterial community profiling of *P. citri* and *P. longispinus* mealybugs. Microbial communities varied quantitatively but not qualitatively, across mealybug species, host plant and geographic distribution. The reasons behind this warrant further study. The presence of *Spiroplasma*, *Rickettsia*, *Wolbachia* and *Cardinium* is of interest, but their presence alone does not necessarily

indicate they have a significant impact on these insect hosts, and further experimentation will be required to establish this. *Spiroplasma* in particular should be studied further in mealybugs, given its importance in plant health and insect ecology, and the prevalence of it across mealybug populations. This research will be important not only for understanding symbiont and mealybug ecology, but also for developing the potential for microbial resource management in integrated pest management. For microbial resource management to be implemented, a comprehensive knowledge of symbiont communities is needed, and this study provides the first step towards such an insight for two widespread and economically-destructive mealybug species.

6.6 Tables

6.6.1 ***Table 6.1.***

Mealybug populations used to generate libraries in this study, their species, location of origin, host plant (where known), number of individuals analysed and the reverse primer used (with a unique Golay barcode for each population) and the complete oligo sequence. The same forward primer, *F515*, was used for each population. The complete oligo sequence for *F515* is 5'-AATGATACGGCGACCACCGAGATCTACAC TATGGTAATT GT GTGCCAGCMGCCGCGGTAA-3'.

Library number	**Mealybug species**	**Location**	**Host plant**	**Library abbreviation**	**N° individuals used**	**Reverse primer and Golay barcode**	**Complete oligo sequence with unique Golay barcode 5'-3'**
1	*P. citri*	Lisben, Portugal	-	PCP	20	R806(AD 001)	CAAGCAGAAGACGGCATACGAG AT TCGTGAT AGTCAGTCAG CC GGACTACVSGGGTATCTAAT
2	*P. citri*	University of Oxford Botanic Garden, UK	-	PCO	20	R806(AD 002)	CAAGCAGAAGACGGCATACGAG AT TACATCG AGTCAGTCAG CC GGACTACVSGGGTATCTAAT
3	*P. citri*	Bet Dagan, Israel	-	PCI	20	R806(AD 003)	CAAGCAGAAGACGGCATACGAG AT TGCCTAA AGTCAGTCAG CC GGACTACVSGGGTATCTAAT
4	*P. citri*	Cantania, Sicily	-	PCS	20	R806(AD 004)	CAAGCAGAAGACGGCATACGAG AT TTGGTCA AGTCAGTCAG CC GGACTACVSGGGTATCTAAT
5	*P. citri*	Proefcentrum Voor Sierteelt Belgium (laboratory stock)	-	PCL	20	R806(AD 005)	CAAGCAGAAGACGGCATACGAG AT TCACTGT AGTCAGTCAG CC GGACTACVSGGGTATCTAAT
6	*P. longispin*	Ghent commercia	*Dracaena* species	PLD	15	R806(AD 006)	CAAGCAGAAGACGGCATACGAG AT TATTGGC AGTCAGTCAG

	us	l grower (Roggeman), Belgium					CC GGACTACVSGGGTATCTAAT
7	*P. longispinus*	Ficoplant-Konaplant, Belgium	*Nolina* species	PLN	20	R806(AD007)	CAAGCAGAAGACGGCATACGAG AT TGATCTG AGTCAGTCAG CC GGACTACVSGGGTATCTAAT
8	*P. longispinus*	De Meyst Werner, Belgium	*Hoya* species	PLH	8	R806(AD008)	CAAGCAGAAGACGGCATACGAG AT TTCAAGT AGTCAGTCAG CC GGACTACVSGGGTATCTAAT
9	*P. citri*	Brico, Belgium	*Ficus benjamina*	PCB	20	R806(AD009)	CAAGCAGAAGACGGCATACGAG AT TCTGATC AGTCAGTCAG CC GGACTACVSGGGTATCTAAT
10	*P. citri*	Scheppersinstituut, Belgium	*Ficus benjamina*	PCF	8	R806(AD010)	CAAGCAGAAGACGGCATACGAG AT TAAGCTA AGTCAGTCAG CC GGACTACVSGGGTATCTAAT
11	*P. citri*	Thomas More Kempen, Belgium	*Ficus benjamina*	PCK	20	R806(AD011)	CAAGCAGAAGACGGCATACGAG AT TGTAGCC AGTCAGTCAG CC GGACTACVSGGGTATCTAAT
12	*P. longispinus*	Meise National Botanic Garden, Belgium	*Phytolaca* species	PLP	20	R806(AD012)	CAAGCAGAAGACGGCATACGAG AT TTACAAG AGTCAGTCAG CC GGACTACVSGGGTATCTAAT
13	*P. longispinus*	Meise National Botanic Garden, Belgium	*Calliandra* species	PLC	20	R806(AD013)	CAAGCAGAAGACGGCATACGAG AT GTTGACT AGTCAGTCAG CC GGACTACVSGGGTATCTAAT
14	*P. longispinus*	Meise National Botanic Garden, Belgium	*Guava* species	PLM	20	R806(AD014)	CAAGCAGAAGACGGCATACGAG AT CGGAACT AGTCAGTCAG CC GGACTACVSGGGTATCTAAT
15	*P. longispinus*	Meise National Botanic Garden, Belgium	*Lophostemon* species	PLL	20	R806(AD015)	CAAGCAGAAGACGGCATACGAG AT CTGACAT AGTCAGTCAG CC GGACTACVSGGGTATCTAAT

6.6.2 ***Table 6.2.***

Total number of filtered sequence reads and OTUs (calculated with a 0.03 cut-off) for bacterial symbiont communities from 15 mealybug populations (PC = *Planococcus citri* and PL = *Pseudococcus longispinus*; the third letter indicates the geographical location or host plant), and the number of sequence reads for the most prevalent classified bacterial taxa after the removal of gamma proteobacteria, which made up the majority of readings.

Library	Sequence reads	OTUs	*Pseudomonas*	*Spiroplasma*	*Staphylococcus*	*Corynebacterium*	Staphylococcaceae	*Leucobacter*	*Acinetobacter*	*Nesterenkonia*	*Ochrobactrum*	*Tsukamurella*	*Oceanobacillus*	*Delftia*
PCB	753700	27	12	151	0	8	0	0	60	1	0	0	0	7
PCF	501347	27	4	96	391	33	0	0	35	0	0	0	0	4
PCI	693303	69	285	94	297	3213	519	383	67	173	191	177	25	4
PCK	597846	36	8	113	5	8	2	2	77	0	0	0	0	25
PCL	692452	54	19	143	583	3481	178	312	60	18	93	107	13	2
PCO	740533	52	138	167	240	2439	189	97	78	11	25	17	9	4
PCP	665817	51	1,326	120	110	2130	113	82	67	20	47	41	7	9
PCS	642697	47	19	129	121	12390	181	99	58	23	6	9	9	3
PLC	693841	43	6	141	406	980	147	33	81	27	0	0	38	15
PLD	706968	41	10	154	5	24	6	3	103	0	1	0	1	16
PLH	818458	52	33	168	5	13	1	1	122	0	0	0	0	53
PLL	610566	46	43	115	106	349	75	16	65	8	2	1	10	7
PLM	616690	40	9	119	538	1025	195	184	38	66	0	7	129	3
PLN	757649	46	22	175	13	20	0	0	125	1	0	0	0	39
PLP	386094	37	16	81	121	1123	211	135	36	44	0	0	20	4

6.6.3 ***Table 6.3.***

Metagenomic library groups for *P. citri* and *P. longispinus* (PC = *Planococcus citri* and PL = *Pseudococcus longispinus*; third letter indicates geographical location or host plant) selected for comparative quantitative Unifrac weighted and qualitative Unifrac unweighted analyses of microbial community composition.

		Unifrac unweighted		Unifrac weighted	
Groups	Feature of interest	Test statistic- UW Score	P-value	Test statistic- W score	P-value
All groups	Different species	0.614007	0.156	0.187084	<0.001
PLP, PLC, PLM, PLL	Same location, different host plants				
	PLC-PLL	1	1.00001	1	0.235
	PLC-PLM	1	1.00001	1	0.119
	PLLL-PLM	1	1.00001	1	0.184
	PLC-PLP	1	1.00001	1	<0.001
	PLLL-PLP	1	1.00001	1	<0.001
	PLM-PLP	1	1	1	<0.001
PCB, PCF, PCK	Different location, same host plant species				
	PCB-PCF	1	1.00001	1	0.596
	PCB-PCK	1	1.00001	1	0.666
	PCF-PCK	1	1	1	0.477
PCP, PCO, PCI, PCS	Different country of origin				
	PCI-PCO	1	1.00001	1	0.069
	PCI-PCP	1	1.00001	1	0.024
	PCO-PCP	1	1.00001	1	0.084
	PCI-PCS	1	1.00001	1	0.049
	PCO-PCS	1	1.00001	1	0.236
	PCP-PCS	1	1	1	0.073
PCL, PCB, PCF, PCK	Wild versus laboratory-reared populations				
	PCB-PCF	1	1.00001	1	0.631
	PCB-PCK	1	1.00001	1	0.696
	PCF-PCK	1	1.00001	1	0.480
	PCB-PCL	1	1.00001	1	0.247
	PCF-PCL	1	1.00001	1	0.173
	PCK-PCL	1	1	1	0.182

6.6.4 ***Table 6.4.***

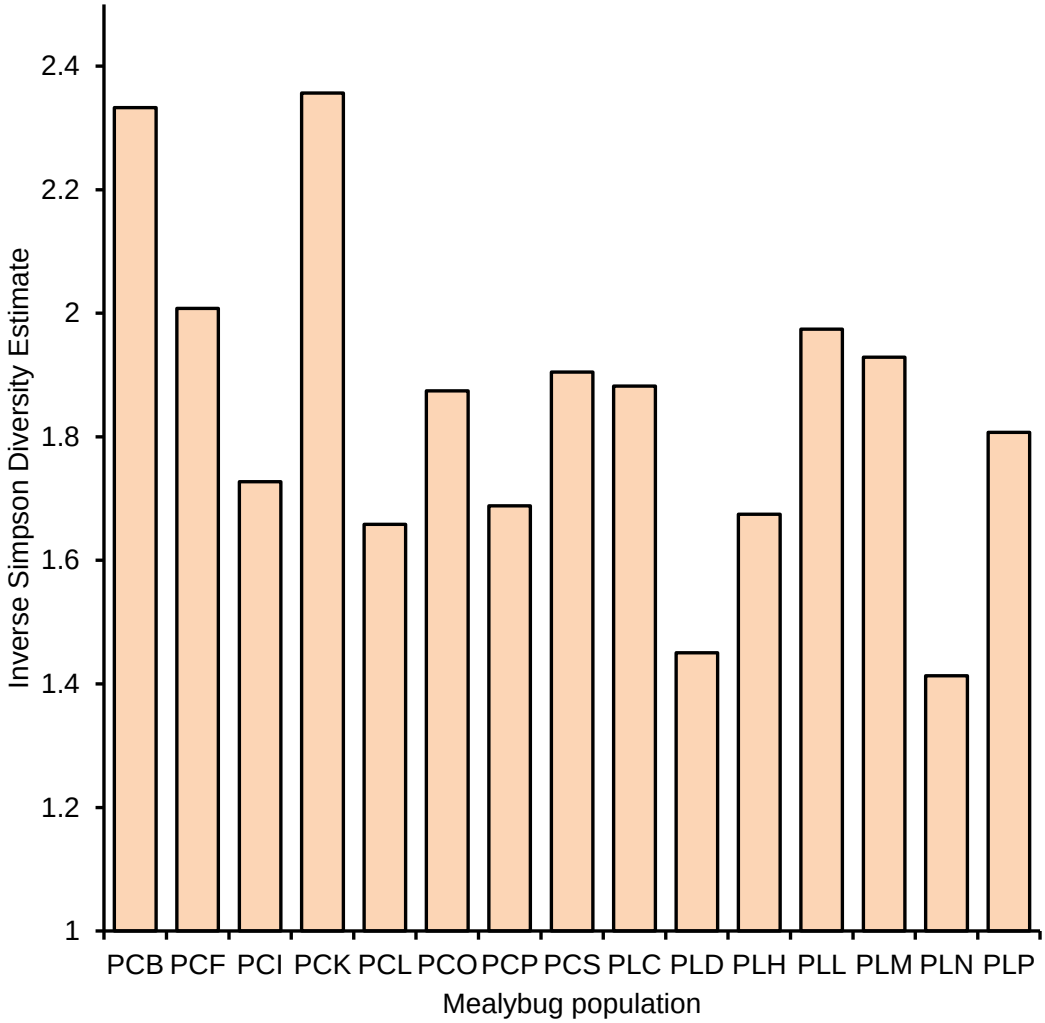
Percentage infection rate of *Wolbachia*, *Spiroplasma*, *Rickettsia*, *Cardinium*, *Arsenophonus* and *Asaia* in 310 *Planococcus citri* and 72 *Pseudococcus longispinus* adult female individuals from commercial Belgian greenhouses based on targeted conventional PCR screening.

	Infection rate (%)	
Bacterial groups	*Planococcus citri*	*Pseudococcus longispinus*
Wolbachia	0.96	0
Spiroplasma	5.16	0
Rickettsia	11.29	0
Cardinium	2.25	0
Arsenophonous	0	0
Asaia	0	0

6.7 Figures

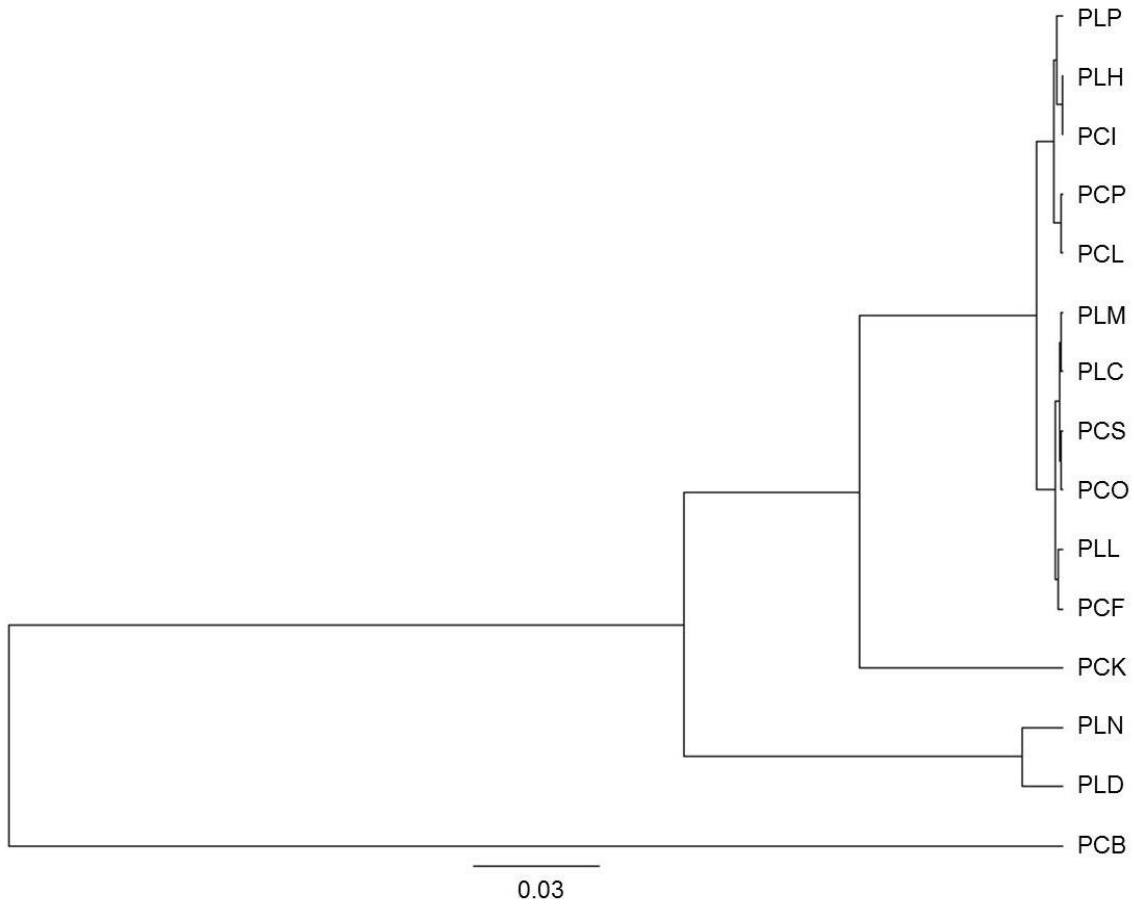
6.7.1 ***Figure 6.1.***

Inverse Simpson Diversity Estimates of the microbial communities for each mealybug population used in metagenomic symbiont screening (PC = *Planococcus citri* and PL = *Pseudococcus longispinus*; third letter indicates geographical location or host plant).



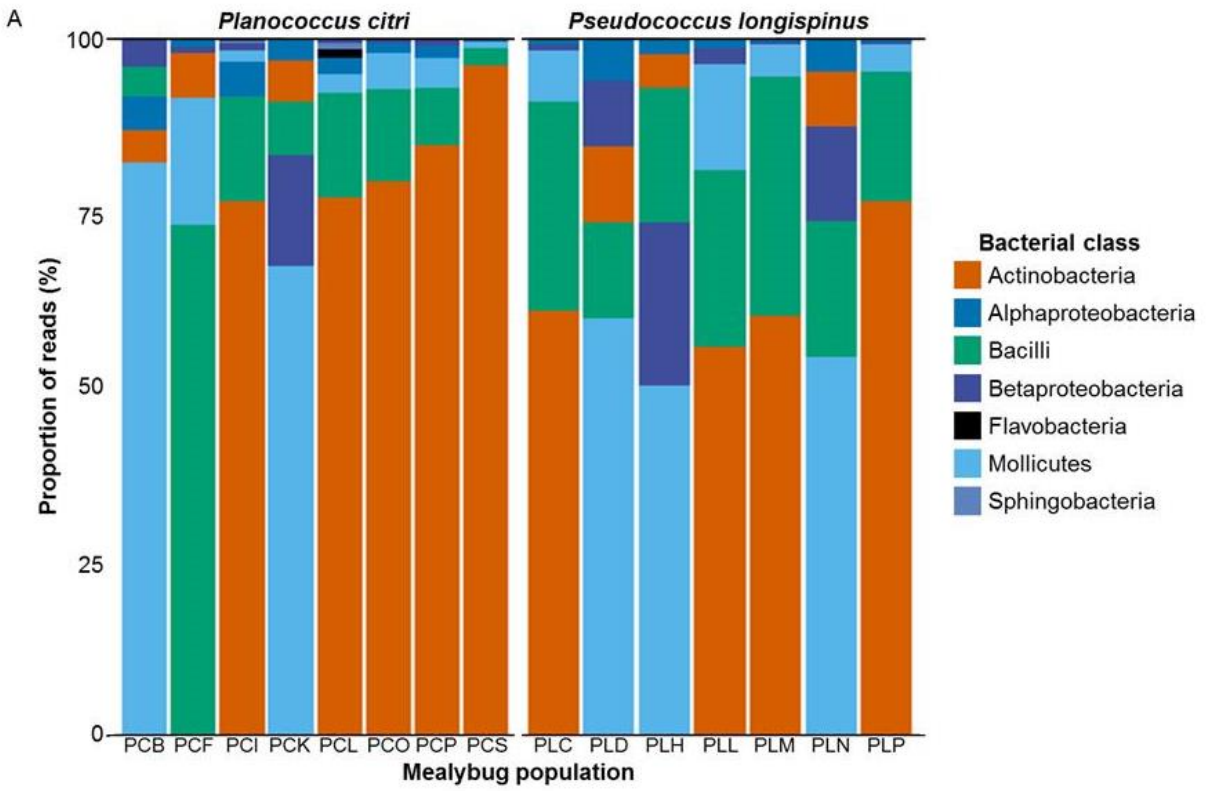
6.7.2 ***Figure 6.2.***

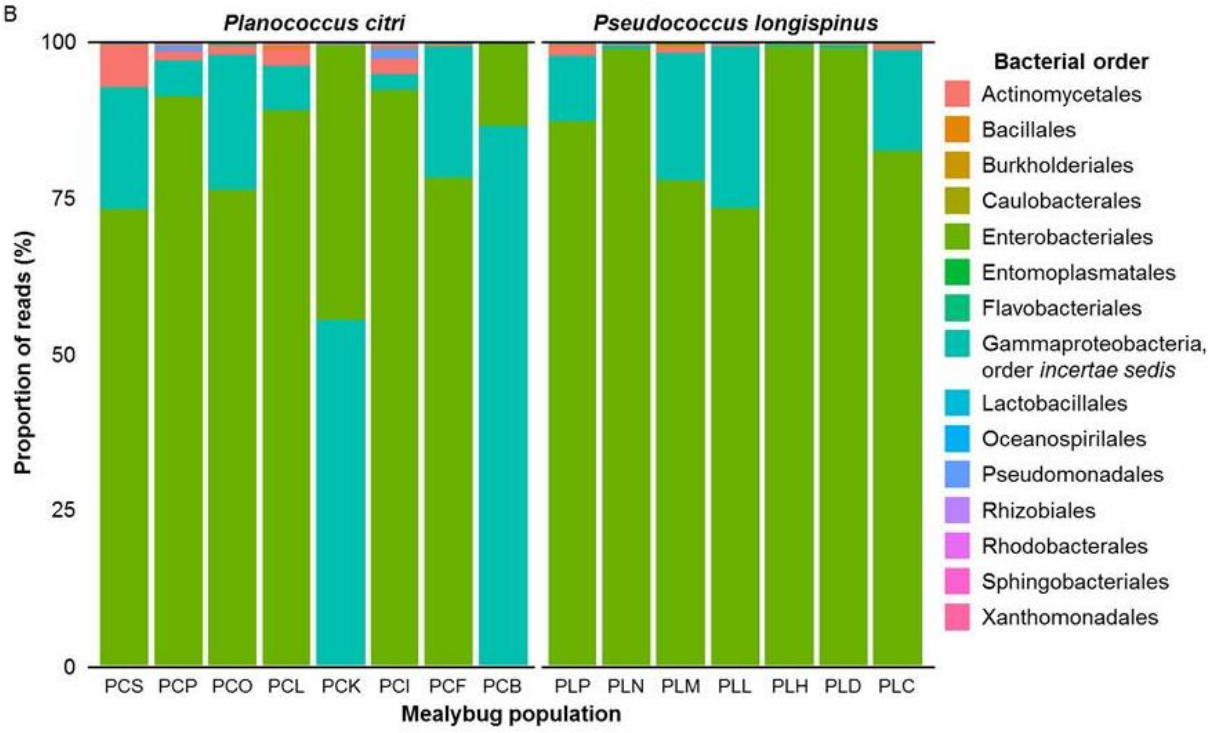
Dendrogram of the microbial communities for each mealybug population used in metagenomic symbiont screening (PC = *Planococcus citri* and PL = *Pseudococcus longispinus*; third letter indicates geographical location or host plant) with 0.03 cut-off distance displaying clustering of microbial communities across mealybug populations, using the jclass and thetayc calculators.



6.7.3 ***Figure 6.3.***

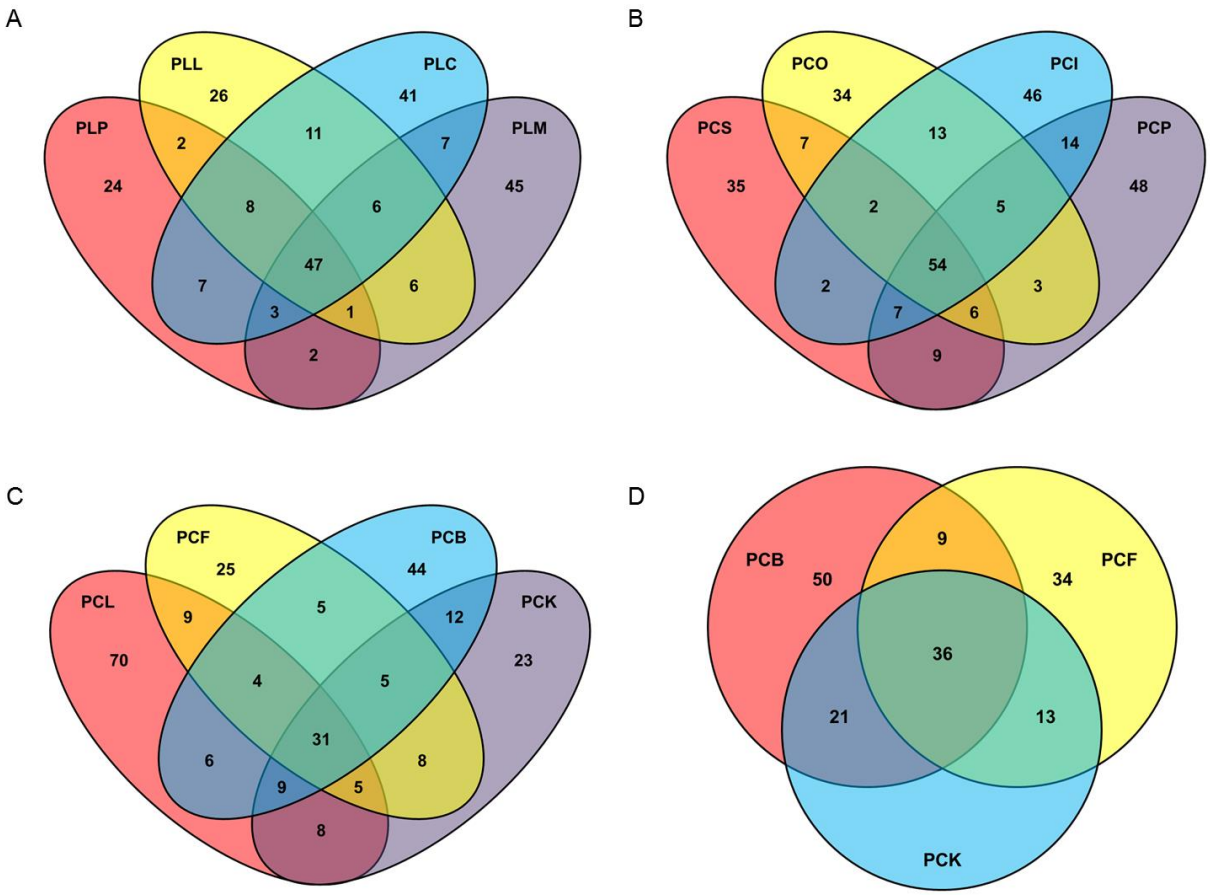
Composition of the bacterial symbiont communities composition of 15 mealybug populations used in metagenomic symbiont screening (PC = *Planococcus citri* and PL = *Pseudococcus longispinus*; third letter indicates geographical location or host plant). (A) class level, with gamma-proteobacteria omitted, which consisted of the majority of reads, and unidentified bacterial groups removed, and (B) order level with only unidentified bacterial groups removed.


A
Planococcus citri
Pseudococcus longispinus
100
75
50
25
0
Proportion of reads (%)
PCB PCF PCI PCK PCL PCO PCP PCS
PLC PLD PLH PLL PLM PLN PLP
Mealybug population
Bacterial class
Actinobacteria
Alphaproteobacteria
Bacilli
Betaproteobacteria
Flavobacteria
Mollicutes
Sphingobacteria


B
Planococcus citri
Pseudococcus longispinus
100
75
50
25
0
Proportion of reads (%)
PCS PCP PCO PCL PCK PCI PCF PCB
PLP PLN PLM PLL PLH PLD PLC
Mealybug population
Bacterial order
Actinomycetales
Bacillales
Burkholderiales
Caulobacterales
Enterobacteriales
Entomoplasmatales
Flavobacteriales
Gammaproteobacteria, order incertae sedis
Lactobacillales
Oceanospirilales
Pseudomonadales
Rhizobiales
Rhodobacterales
Sphingobacteriales
Xanthomonadales

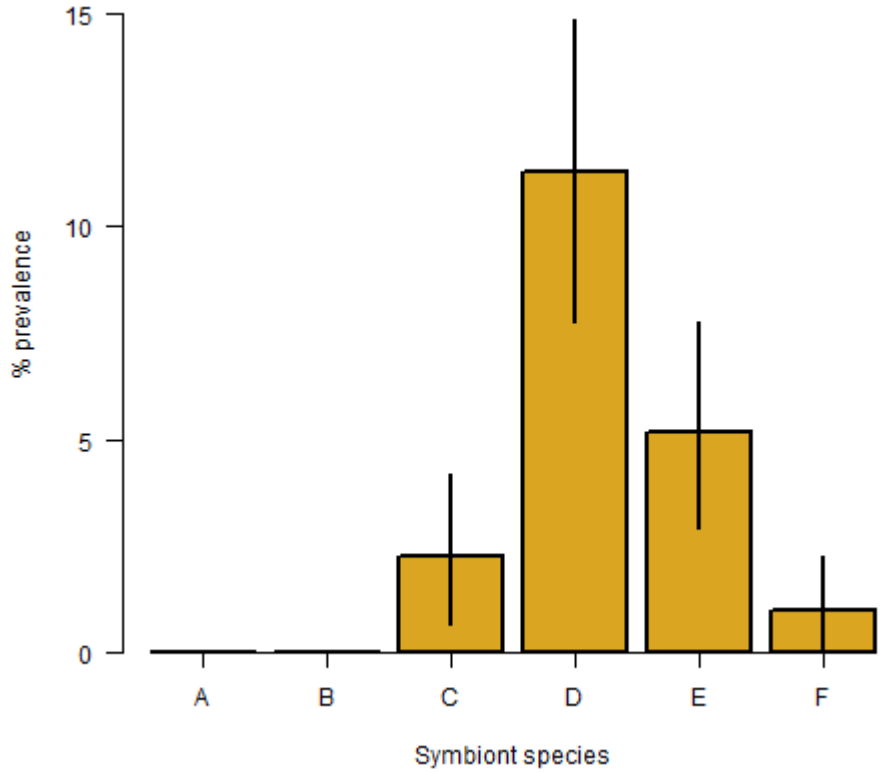
6.7.4 ***Figure 6.4.***

Venn diagrams displaying shared and unique microbial OTUs between mealybug populations: (A) PLP, PLL, PLC, PLM (*Pseudococcus longispinus* collected from different host plant species at the same geographical location); (B) PCS, PCO, PCI, PCP (*P. citri* collected from different countries); (C) PCL, PCF, PCB, PCK (wild versus laboratory-reared (PCL) *P. citri*); (D) PCB, PCF, PCK. (*P. citri* collected from the same host plant species but different geographical locations).



6.7.5 ***Figure 6.5.***

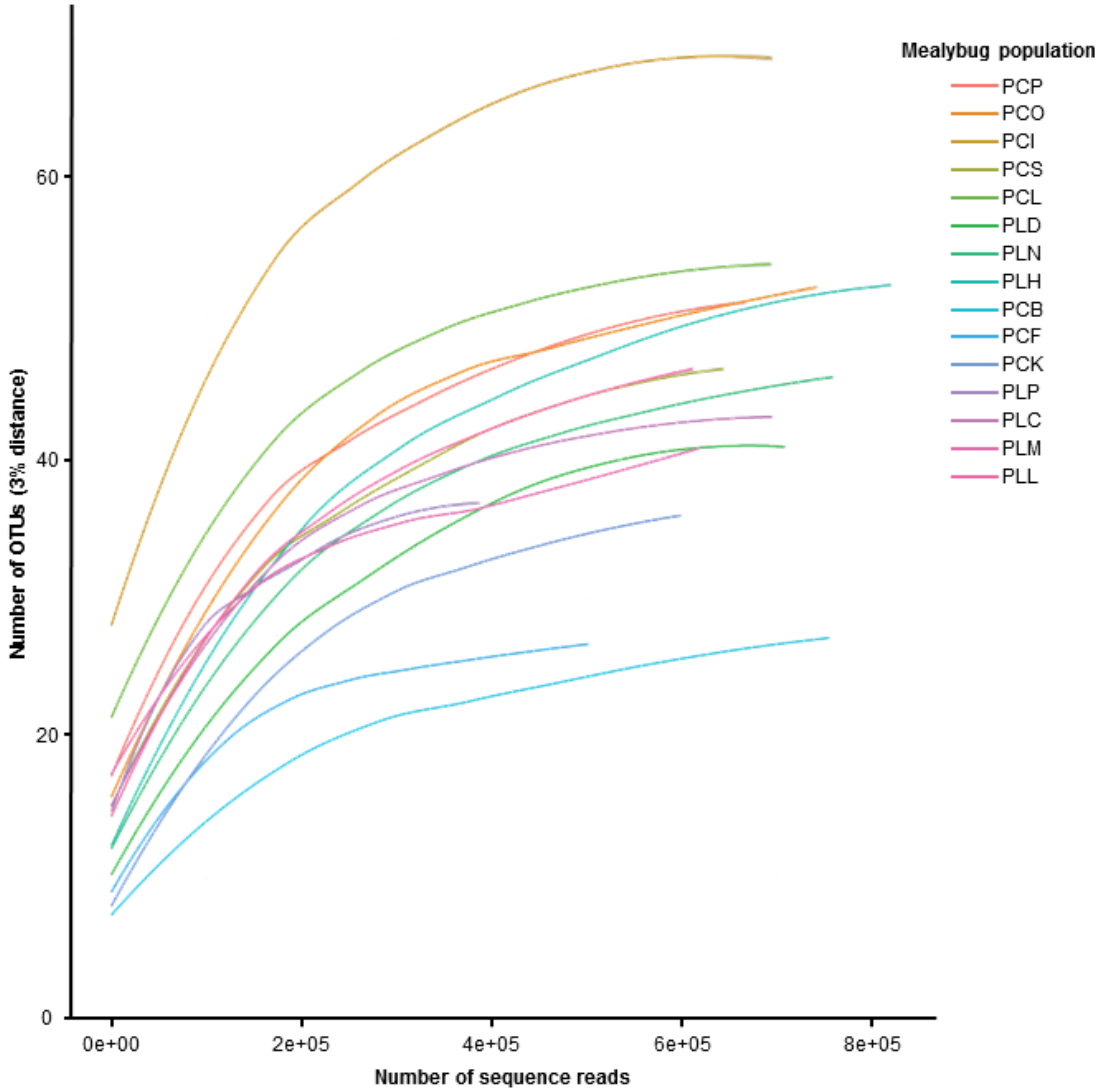
Percentage and 95% confidence intervals of the prevalence of: (A) *Asaia spp.*; (B) *Arsenophonus spp.*; (C) *Cardinium spp.*; (D) *Rickettsia spp.*; (E) *Spiroplasma spp.*; and (D) *Wolbachia spp.* bacterial symbionts in 310 *Planococcus citri* adult females.



6.8 Supplementary Figures

6.8.1 *Supplementary Figure S.6.1.*

Rarefaction curve displaying number of sequence reads against number of OTUs obtained of the microbial communities for each mealybug population used in the metagenomic symbiont screening (PC = *Planococcus citri* and PL = *Pseudococcus longispinus*; third letter indicates geographical location or host plant) with a 0.03 distance.



“I may not have gone where I intended to go, but I think I have ended up where I needed to be.”

— *Douglas Adams*

7 General Discussion

This thesis aimed to investigate both the evolutionary ecology of endosymbiosis and the potential application of symbiont manipulation for pest control. I set out to examine the factors influencing, and fitness impacts of, variation in obligate symbiont density in citrus mealybug hosts, and determine whether disrupting the association between *Planococcus citri* and its symbionts, *Moranella endobia* and *Tremblaya princeps*, would reduce host fitness. I also explored the microbiota of *P. citri* in detail in search of common insect facultative symbionts that are known to affect host ecology.

The density of *T. princeps* and *M. endobia* within *P. citri*, i.e. the number of symbiont cells relative to host cells, was chosen as a factor to examine the dynamics between this host and its symbionts. The quantity of mutualistic symbiont cells that a host requires will vary depending upon the situation, and regulation of symbiont density is thus essential for optimum host functioning (Rio et al., 2006, Laughton et al., 2014, Cunning and Baker, 2014, Falkowski et al., 1993, Wilkinson et al., 2007). Several host species, some of which are insects, have been found to adaptively adjust beneficial microbial endosymbiont density in certain scenarios or life stages (Vigneron et al., 2014, Rio et al., 2006, Hooper et al., 2012, Balmand et al., 2011, Ruby and Asato, 1993, Dimond and Carrington, 2008, Hinde, 1971, Falkowski et al., 1993). Moreover, two mealybug species, *Planococcus kraunhiae* and *Pseudococcus comstocki*, were found to facultatively adjust their obligate symbiont density during their lifetime; for example, males lost their symbionts after pupation, most likely

because adult males are unable to feed and so assimilate the symbionts for sustenance (Kono et al., 2008).

It is worth considering the suitability, consistency, accuracy and precision of qPCR, and the protocols used in this thesis, for the measurement of symbiont density within mealybugs and other insect hosts. It could be argued that the lack of correlation between mealybug fitness and symbiont density in this thesis was due to inaccurate and imprecise measurement of symbiont density by qPCR. However this is highly unlikely to be the case for two main reasons: first, qPCR is a widely used and accepted method of measuring gene copy number and second, if the qPCR measurements in this thesis were unreliable, then this would have created noise in the results, which was not the case (both discussed below).

The measurement of gene copy number by qPCR (not to be confused with the measurement of gene expression by RT qPCR/qRT PCR) is a standard and commonly-used method for measuring bacterial symbiont density in insect and other eukaryotic hosts, whether fluorescence is emitted by a gene-specific molecular probe (used in this thesis) or a non-specific fluorescent dye, such as SYBR Green, (Kono et al., 2008, Correa et al., 2009, Luyten et al., 2006, Goffredi et al., 2007, Zhong et al., 2007, Lu et al., 2012). Indeed, the qPCR assays used in this thesis were inspired by a qPCR assay used on other mealybug species (Kono et al., 2008). Studies have found that the data of symbiont gene copy number measurement by qPCR is well-correlated with that by FISH and are often used or considered together, further supporting the suitability and reliability of qPCR (Loram et al., 2007, Mahadav et al., 2008, Kumar et al., 2015, Turnbaugh and Gordon, 2009, de Souza et al., 2009,

Epis et al., 2013). A study on human stool microbiota found that FISH was able to detect higher median levels of bifidobacteria than qPCR, however, this should not be of major concern to this thesis as the main interest was symbiont quantities in mealybugs relative to symbiont quantities in other mealybugs, rather than absolute symbiont numbers (titre) (Nakamura et al., 2009). In this thesis, qPCR also held the advantage over FISH in its ease of use when examining large numbers of samples. I optimised the qPCR assay with serial dilutions to ensure that amplification efficiency lay within the accepted boundaries of 95-105%. I also found symbiont densities to be consistent within laboratory populations of mealybugs, and there were consistent differences across mealybug populations for them to differ significantly from each other. Finally, an imprecision in the qPCR assays would affect all populations or treatments being compared similarly and result in increased noise in the data, but significant differences in symbiont density between mealybug populations were detected in Chapters 2 and 3, and a significant effect of heat-stress on symbiont densities in Chapter 4. This demonstrates that if the qPCR protocol resulted in any experimental noise, then the work had sufficient statistical power to handle it.

Mealybug populations with higher symbiont densities did not perform significantly better than those with lower symbiont densities (either naturally or artificially reduced), which suggests that the majority of citrus mealybugs harbour mutualistic symbionts that do not increase or decrease host fitness (hence excessive *T. princeps* and *M. endobia* are commensal rather than beneficial). This raises the question as to why symbiont density varies so much in citrus mealybugs. It could be that it is non-adaptive, and variation has persisted simply because it is unrelated to host fitness,

allowing excessive symbiont quantities to go unchecked by the host. Another possibility is that excessively high symbiont density is an evolutionary artefact. A key difference between selection on the host and selection on the symbiont is that the host is selected to maximise its fecundity, whereas the symbiont is selected to increase its transmission rate to new hosts, perhaps competing with other symbiont species or strains for the limited resources provided by the host (Frank, 1996a). In horizontally-transmitted symbionts, this could select for symbionts to maximise their density, but efficient vertical transmission resolves this conflict and leads to genetic homogeneity of symbionts (Smith, 2007, Frank, 1996a). Following the transition from horizontal to vertical transmission, the tendency of a symbiont to maximise its density could remain if it does not harm the fitness of the host. Although mealybugs differ in the density of their bacteria, the rate of synthetic activity of *M. endobia* and *T. princeps* was not investigated. It could be, for example, that mealybugs with low symbiont density compensate with bacteria with higher gene expression rates. This would be a worthwhile aspect to explore in the future by measuring the expression of *T. princeps* and *M. endobia* genes involved with amino acid synthesis, using RT qPCR.

The results would seem to indicate that attempts to control citrus mealybugs in a field or greenhouse setting by targeting their obligate symbionts would likely prove ineffective, as mealybugs are resilient to even substantial reductions in symbiont densities. However, these symbionts are obligate to citrus mealybugs and do perform essential functions, so their complete removal should be fatal, or have significant fitness costs. I did not examine aposymbiotic mealybugs, and I am not aware of any

cases of mealybugs being completely purged of symbionts. It would be interesting to find the genetic link to symbiont density, and explore the possibility of symbiont oppression by using RNAi to target the host genome. For this, I would recommend targeting mRNA of the *P. citri murABDEF* genes with siRNA (Husnik et al., 2013). These genes are believed to be associated with the regulation of the cell wall stability of *M. endobia*, with a decrease in activity being expected to result in increased lysis of *M. endobia* (Husnik et al., 2013). It would also be interesting to confirm and clarify the role and activity level of these genes, as the results from the hybridisation experiment of Chapter 5 would suggest that it is the *M. endobia* genotype, rather than the *P. citri* genotype, that influences the density of this symbiont. It could be the case that the expression levels of *murABDEF* are not usually a limiting factor for *M. endobia*. If RNAi of these genes is successful, then the juveniles of *P. citri* would likely experience the greatest fitness costs, as essential amino acids will possibly be most needed by growing insects.

Facultative symbionts can be highly influential in insect ecology and relevant to pest management, for example, *Hamiltonella defensa* and *Serratia symbiotica* in aphids (Russell et al., 2003, Degnan et al., 2009, Oliver et al., 2003, Oliver et al., 2005, Ferrari et al., 2004, Montllor et al., 2002). In Chapter 6, we used Next Generation Sequencing to identify the facultative bacteria present in citrus and long-tailed, *Pseudocoocus longispinus*, mealybugs. *Wolbachia*, *Cardinium*, *Spiroplasma* and *Rickettsia* were not found in *P. longispinus*, but were found with varying prevalence in *P. citri*. These bacteria have been found to have diverse impacts on their hosts, but their effects on mealybugs has not yet been established (Saridaki and Bourtzis, 2010,

Zchori-Fein and Perlman, 2004, Gasparich, 2002, Regassa and Gasparich, 2006, Fialho and Stevens, 2000, Jiggins et al., 2000, Lawson et al., 2001, von der Schulenburg et al., 2001). It would be interesting to examine the incidence of these symbionts in mealybugs further and their interactions with these hosts.

In this thesis, *T. princeps* was consistently measured as being at a higher density than *M. endobia*. As all *T. princeps* cells will contain at least one *M. endobia* cell, and often more, this suggests that absolute *M. endobia* densities may have been consistently underestimated. The likely cause of this was the DNA extraction method, boiling crushed *P. citri* bodies and cadavers in Chelex, which was used in all the qPCR experiments in this thesis. Chelex DNA extraction protocols are widely used in insects and other arthropods to examine endosymbionts (Hansen et al., 2007, Benson et al., 2004, White et al., 2009, Graystock et al., 2013, Roberts and Hughes, 2014). Adult female mealybugs are also soft-bodied, so breaking down a hard exoskeleton was not an issue, and Chelex-based extraction protocols also held the advantage that they are economical and simple to perform. The issue may have lain with the fact that two layers of bacterial cell wall would need to be punctured in order to extract *M. endobia* DNA: the cell wall of *T. princeps* and that of *M. endobia*. Chelex-based extraction may not have been sufficient to break 100% of *M. endobia* cell walls. With this information now apparent, I would recommend that additional measures are taken to extract DNA from mealybugs when intending to measure absolute densities of *M. endobia*. However, it is important to recognise that the underestimation of *M. endobia* would have been consistent, as the same protocol was used each time. The focus of this thesis was to determine how mealybugs varied

from each other in their symbiont density, rather than comparing the absolute densities (titre) of the two symbionts, and so the consistent underestimation of *M. endobia* density would not have impacted any of the conclusions in this thesis.

Overall, this thesis has shown that citrus mealybugs will often harbour apparently excessive quantities of mutualistic symbionts at varying densities, that two distinct regulatory mechanisms exist for *M. endobia* and *T. princeps*, and that common insect facultative symbionts can infect *P. citri*. All the work presented here invites the prospect of further investigation, which I have described earlier, including the monitoring of gene expression rates in these symbionts, the targeting of *P. citri* genes to suppress *M. endobia* and the characterisation of *Wolbachia*, *Cardinium*, *Spiroplasma* and *Rickettsia* in *P. citri*. From an evolutionary ecology perspective, these results have revealed unusual dynamics in a bizarre tripartite symbiosis, and other symbiotic associations. From a pest control perspective, it shows that targeting *M. endobia* and *T. princeps* in a symbiont disruption method will likely be ineffective unless it can reduce these symbionts to below the critical threshold for efficient functioning. This does not close the door on Microbial Resource Management of citrus mealybugs, but means that further investigation will be needed to determine where this threshold lies.

8 Literature Cited

ACKONOR, J. B. 2002. Current levels of incidence of parasitism and predation in *Planococcus citri* Risso (Homoptera: Pseudococcidae) in Ghanaian cocoa (*Theobroma cacao L.*) farms. *Insect Science and its Application,* 22**,** 105-112.

ADAMS, M. J., ANTONIW, J. F., BAR-JOSEPH, M., BRUNT, A. A., CANDRESSE, T., FOSTER, G. D., MARTELLI, G. P., MILNE, R. G. & FAUQUET, C. M. 2004. Virology Division News: The new plant virus family Flexiviridae and assessment of molecular criteria for species demarcation. *Archives of Virology,* 149**,** 1045-1060.

AFIFI, A. I., EL ARNAOUTY, S. A., ATTIA, A. R. & ABD, A. A.-M. 2010. Biological control of citrus mealybug, *Planococcus citri* (Risso) using coccinellid predator, *Cryptolaemus montrouzieri* Muls. *Pakistan journal of biological sciences: PJBS,* 13.

AHMED, M. Z., REN, S., XUE, X., LI, X. X., JIN, G. & QIU, B. L. 2010. Prevalence of endosymbionts in *Bemisia tabaci* populations and their in vivo sensitivity to antibiotics. *Current Microbiology,* 61**,** 322-328.

AKMAN GÜNDÜZ, E. & DOUGLAS, A. E. 2009. Symbiotic bacteria enable insect to use a nutritionally inadequate diet. *Proceedings of the Royal Society B: Biological Sciences,* 276**,** 987-991.

AKMAN, L., YAMASHITA, A., WATANABE, H., OSHIMA, K., SHIBA, T., HATTORI, M. & AKSOY, S. 2002. Genome sequence of the endocellular

obligate symbiont of tsetse flies, *Wigglesworthia glossinidia*. *Nature Genetics,* 32**,** 402-407.

AKSOY, E., TELLERIA, E. L., ECHODU, R., WU, Y., OKEDI, L. M., WEISS, B. L., AKSOY, S. & CACCONE, A. 2014. Analysis of multiple tsetse fly populations in Uganda reveals limited diversity and species-specific gut microbiota. *Applied and Environmental Microbiology,* 80**,** 4301-4312.

AMARASEKARE, K. G., CHONG, J.-H., EPSKY, N. D. & MANNION, C. M. 2008. Effect of temperature on the life history of the mealybug *Paracoccus marginatus* (Hemiptera: Pseudococcidae). *Journal of Economic Entomology,* 101**,** 1798-1804.

AMMAR, E.-D., GASPARICH, G. E., HALL, D. G. & HOGENHOUT, S. A. 2011. *Spiroplasma*-like organisms closely associated with the gut in five leafhopper species (Hemiptera: Cicadellidae). *Archives of Microbiology,* 193**,** 35-44.

ARAI, T. 1996. Temperature-dependent development rate of three mealybug species, *Pseudococcus citriculus* Green, *Planococcus citri* (Risso), and *Planococcus kraunhiae* (Kuwana)(Homoptera: Pseudococcidae) on Citrus. *Japanese Journal of Applied Entomology and Zoology,* 40**,** 25-34.

ATYAME, C. M., CATTEL, J., LEBON, C., FLORES, O., DEHECQ, J.-S., WEILL, M., GOUAGNA, L. C. & TORTOSA, P. 2015. *Wolbachia*-based population control strategy targeting *Culex quinquefasciatus* mosquitoes proves efficient under semi-field conditions. *PLoS ONE,* 10**,** e0119288.

BACKHED, F., LEY, R. E., SONNENBURG, J. L., PETERSON, D. A. & GORDON, J. I. 2005. Host-bacterial mutualism in the human intestine. *Science,* 307**,** 1915–1920.

BALMAND, S., VALLIER, A., VINCENT-MONÉGAT, C., VIGNERON, A., WEISS-GAYET, M., ROCHAT, D. & HEDDI, A. 2011. Antimicrobial peptides keep insect endosymbionts under control. *Science,* 334**,** 362-365.

BAUMANN, L., THAO, M. L. L., HESS, J. M., JOHNSON, M. W. & BAUMANN, P. 2002. The genetic properties of the primary endosymbionts of mealybugs differ from those of other endosymbionts of plant sap-sucking insects. *Applied and Environmental Microbiology,* 68**,** 3198.

BAUMANN, P. 2005. Biology of bacteriocyte-associated endosymbionts of plant sap-sucking insects. *Annual Review of Microbiology,* 59**,** 155-189.

BAUMANN, P., BAUMANN, L., LAI, C., ROUHBAKHSH, D., MORAN, N. A. & CLARK, M. A. 1995. Genetics, physiology, and evolutionary relationships of the genus *Buchnera*: intracellular symbionts of aphids. *Annual Reviews in Microbiology,* 49**,** 55-94.

BEARD, C. B., CORDON-ROSALES, C. & DURVASULA, R. V. 2002. Bacterial symbionts of the Triatominae and their potential use in control of chagas disease transmission 1. *Annual Review of Entomology,* 47**,** 123-141.

BELTRÀ, A., SOTO, A. & MALAUSA, T. 2012. Molecular and morphological characterisation of Pseudococcidae surveyed on crops and ornamental plants in Spain. *Bulletin of Entomological Research,* 102**,** 165.

BEN DOV, Y. 2015. *ScaleNet* [Online]. Available: sel.barc.usda.gov/scalenet/scalenet.htm [Accessed July 2015].

BENNETT, G. M. & MORAN, N. A. 2015. Heritable symbiosis: The advantages and perils of an evolutionary rabbit hole. *Proceedings of the National Academy of Sciences of the U.S.A.*, 112**,** 10169-10176.

BENNETT, G. M. & O'GRADY, P. M. 2012. Host–plants shape insect diversity: Phylogeny, origin, and species diversity of native Hawaiian leafhoppers (Cicadellidae: Nesophrosyne). *Molecular Phylogenetics and Evolution,* 65**,** 705-717.

BENSADIA, F., BOUDREAULT, S., GUAY, J. F., MICHAUD, D. & CLOUTIER, C. 2006. Aphid clonal resistance to a parasitoid fails under heat stress. *Journal of Insect Physiology,* 52**,** 146-157.

BENSON, M. J., GAWRONSKI, J. D., EVELEIGH, D. E. & BENSON, D. R. 2004. Intracellular symbionts and other bacteria associated with deer ticks (*Ixodes scapularis*) from Nantucket and Wellfleet, Cape Cod, Massachusetts. *Applied and Environmental Microbiology,* 70**,** 616-620.

BERMINGHAM, J., RABATEL, A., CALEVRO, F., VIÑUELAS, J., FEBVAY, G., CHARLES, H., DOUGLAS, A. & WILKINSON, T. 2009. Impact of host developmental age on the transcriptome of the symbiotic bacterium *Buchnera aphidicola* in the pea aphid (*Acyrthosiphon pisum*). *Applied and Environmental Microbiology,* 75**,** 7294-7297.

BERTICAT, C., ROUSSET, F., RAYMOND, M., BERTHOMIEU, A. & WEILL, M. 2002. High *Wolbachia* density in insecticide–resistant mosquitoes. *Proceedings of the Royal Society of London B,* 269**,** 1413-1416.

BIRKY, C. W., MARUYAMA, T. & FUERST, P. 1983. An approach to population and evolutionary genetic theory for genes in mitochondria and chloroplasts, and some results. *Genetics,* 103**,** 513-527.

BOOTH, C. 2014. *Orchid Plant Insects Overview* [Online]. OrchidPlantCare.info. Available: http://www.orchidplantcare.info/ [Accessed July 2015].

BORDENSTEIN, S. R. & WERNEGREEN, J. J. 2004. Bacteriophage flux in endosymbionts (*Wolbachia*): infection frequency, lateral transfer, and recombination rates. *Molecular Biology and Evolution,* 21**,** 1981-1991.

BORGES DA SILVA, E., MENDEL, Z. & FRANCO, J. C. 2010. Can facultative parthenogenesis occur in biparental mealybug species? *Phytoparasitica,* 38**,** 19-21.

BRESSAN, A., ARNEODO, J., SIMONATO, M., HAINES, W. P. & BOUDON PADIEU, E. 2009. Characterization and evolution of two bacteriome inhabiting symbionts in cixiid planthoppers (Hemiptera: Fulgoromorpha: Pentastirini). *Environmental Microbiology,* 11**,** 3265-3279.

BREZNAK, J. A. 2000. Ecology of prokaryotic microbes in the guts of wood-and litter-feeding termites. *Termites: Evolution, Sociality, Symbioses, Ecology.* Springer.

BRØDSGAARD, H. F. & ALBAJES, R. 2000. Insect and mite pests. *In:* ALBAJES, I. R., LODOVICA GULLINO, M., VAN LENTEREN, J. C. & ELAD, Y. (eds.) *Integrated Pest and Disease Management in Greenhouse Crops.* Dordrecht, The Netherlands: Kluwer Academic Publishers.

BRONSTEIN, J. L. 2001. The costs of mutualism. *American Zoologist,* 41**,** 825-839.

BROWN, S. W. & NELSON-REES, W. A. 1961. Radiation analysis of a lecanoid genetic system. *Genetics,* 46**,** 983.

BROWNSTEIN, J. S., HETT, E. & O'NEILL, S. L. 2003. The potential of virulent *Wolbachia* to modulate disease transmission by insects. *Journal of Invertebrate Pathology,* 84**,** 24-29.

BRUMIN, M., KONTSEDALOV, S. & GHANIM, M. 2011. *Rickettsia* influences thermotolerance in the whitefly *Bemisia tabaci* B biotype. *Insect Science,* 18**,** 57-66.

BUCHNER, P. 1965. *Endosymbiosis of animals with plant microorganims,* New York, John Wiley & Sons, Inc.: Interscience Publishers.

BUGLIA, G. L., DIONISI, D. & FERRARO, M. 2009. The amount of heterochromatic proteins in the egg is correlated with sex determination in *Planococcus citri* (Homoptera, Coccoidea). *Chromosoma,* 118**,** 737-746.

BURKE, G., FIEHN, O, & MORAN, N, 2010. Effects of facultative symbionts and heat stress on the metabolome of pea aphids. *ISME,* 4**,** 242-252.

CABI. 2015. *Planococcus citri (citrus mealybug)* [Online]. CABI. Available: cabi.org/isc/datasheet/45082 [Accessed July 2015].

CABI/EPPO 1999. *Planococcus citri. Distribution of Plant Pests no. 43,* Wallingford, UK, CAB International.

CARPENTER, K. J., HORAK, A. & KEELING, P. J. 2010. Phylogenetic position and morphology of Spirotrichosomidae (Parabasalia): new evidence from *Leptospironympha* of *Cryptocercus punctulatus*. *Protist,* 161**,** 122-132.

CASPI-FLUGER, A., INBAR, M., MOZES-DAUBE, N., KATZIR, N., PORTNOY, V., BELAUSOV, E., HUNTER, M. S. & ZCHORI-FEIN, E. 2012. Horizontal transmission of the insect symbiont *Rickettsia* is plant-mediated. *Proceedings of the Royal Society B: Biological Sciences,* 279**,** 1791-1796.

CEBALLO, F. A. & WALTER, G. H. 2005. Why is *Coccidoxenoides perminutus*, a mealybug parasitoid, ineffective as a biocontrol agent--Inaccurate measures of parasitism or low adult survival? *Biological Control,* 33**,** 260-268.

CHANDLER, J. A., LANG, J. M., BHATNAGAR, S., EISEN, J. A. & KOPP, A. 2011. Bacterial communities of diverse *Drosophila* species: ecological context of a host–microbe model system. *PLoS Genetics,* 7**,** e1002272.

CHANDLER, S., WILKINSON, T. & DOUGLAS, A. 2008. Impact of plant nutrients on the relationship between a herbivorous insect and its symbiotic bacteria. *Proceedings of the Royal Society B: Biological Sciences,* 275**,** 565.

CHARLAT, S., HURST, G. D. D. & MERCOT, H. 2003. Evolutionary consequences of *Wolbachia* infections. *Trends in Genetics,* 19**,** 217-223.

CHARLES, J., COHEN, D., WALKER, J., FORGIE, S., BELL, V., & BREEN, K. 2006. A review of the ecology of grapevine leafroll associated virus type 3 (GLRaV-3). *New Zealand Plant Protection* 59**,** 330-337.

CHASE, C. D. 2007. Cytoplasmic male sterility: a window to the world of plant mitochondrial–nuclear interactions. *Trends in Genetics,* 23**,** 81-90.

CHIEL, E., GOTTLIEB, Y., ZCHORI-FEIN, E., MOZES-DAUBE, N., KATZIR, N., INBAR, M. & GHANIM, M. 2007. Biotype-dependent secondary symbiont communities in sympatric populations of *Bemisia tabaci*. *Bulletin of Entomological Research,* 97**,** 407-413.

CID, M. & FERERES, A. 2010. Characterization of the probing and feeding behavior of *Planococcus citri* (Hemiptera: Pseudococcidae) on grapevine. *Annals of the Entomological Society of America,* 103**,** 404-417.

CID, M., PEREIRA, S., SEGURA, A. & CABALEIRO, C. 2006. Seguimiento de la población de *Planococcus citri* (Risso) Homoptera: Pseudococcidae) en un viñedo de las Rías Baixas (Galicia). *Boletín de Sanidad Vegetal. Plagas,* 32**,** 339-344.

CLARK, E. L., KARLEY, A. J. & HUBBARD, S. F. 2010. Insect endosymbionts: manipulators of insect herbivore trophic interactions? *Protoplasma,* 244**,** 25-51.

CLARK, M. A., MORAN, N. A. & BAUMANN, P. 1999. Sequence evolution in bacterial endosymbionts having extreme base compositions. *Molecular Biology and Evolution,* 16**,** 1586-1598.

CLARK, M. A., MORAN, N. A., BAUMANN, P. & WERNEGREEN, J. J. 2000. Cospeciation between bacterial endosymbionts (*Buchnera*) and a recent radiation of aphids (*Uroleucon*) and pitfalls of testing for phylogenetic congruence. *Evolution,* 54**,** 517-525.

CLARK, M. E., VENETI, Z., BOURTZIS, K. & KARR, T. L. 2003. *Wolbachia* distribution and cytoplasmic incompatibility during sperm development: the cyst as the basic cellular unit of CI expression. *Mechanisms of Development,* 120**,** 185-198.

CLARK, T. 1977. *Spiroplasma* sp., a new pathogen in honey bees. *Journal of Invertebrate Pathology,* 29**,** 112-113.

CLARK, T. B. 1982. *Spiroplasmas*: diversity of arthropod reservoirs and host-parasite relationships. *Science,* 217**,** 57-59.

COOK, P. E. & MCGRAW, E. A. 2010. *Wolbachia pipientis*: an expanding bag of tricks to explore for disease control. *Trends in Parasitology,* 26**,** 373-375.

CORREA, A. M., MCDONALD, M. D. & BAKER, A. C. 2009. Development of clade-specific *Symbiodinium* primers for quantitative PCR (qPCR) and their application to detecting clade *D* symbionts in Caribbean corals. *Marine Biology,* 156**,** 2403-2411.

CORREA, C. C. & BALLARD, J. W. O. 2012. *Wolbachia* gonadal density in female and male *Drosophila* vary with laboratory adaptation and respond differently to physiological and environmental challenges. *Journal of Invertebrate Pathology,* 111**,** 197-204.

CROTTI, E., BALLOI, A., HAMDI, C., SANSONNO, L. & MARZORATI, M., ET AL. 2012. Microbial symbionts: a resource for the management of insect-related problems. *Microbial Biotechnology,* 5.

CROTTI, E., DAMIANI, C., PAJORO, M., GONELLA, E., RIZZI, A., RICCI, I., NEGRI, I., SCUPPA, P., ROSSI, P. & BALLARINI, P. 2009. *Asaia*, a versatile acetic acid bacterial symbiont, capable of cross-colonizing insects of phylogenetically distant genera and orders. *Environmental Microbiology,* 11**,** 3252-3264.

CUNNING, R. & BAKER, A. C. 2014. Not just who, but how many: the importance of partner abundance in reef coral symbioses. *Frontiers in Microbiology,* 5.

DADD, R. H. 1985. Nutrition: organisms. *Comprehensive insect physiology, biochemistry and pharmacology,* 4**,** 313-390.

DASCH, G. A., WEISS, E. & CHANG, K. P. 1984. Endosymbionts of insects. *Bergey's Manual of Systematic Bacteriology,* 1**,** 811-833.

DAVIES, A. P., CEBALLO, F. A. & WALTER, G. H. 2004. Is the potential of *Coccidoxenoides perminutus*, a mealybug parasitoid, limited by climatic or nutritional factors? *Biological Control,* 31**,** 181-188.

DE OLIVEIRA, C., GONÇALVES, D., BATON, L., SHIMABUKURO, P., CARVALHO, F. & MOREIRA, L. 2015. Broader prevalence of *Wolbachia*

in insects including potential human disease vectors. *Bulletin of Entomological Research,* 105**,** 305-315.

DE SOUZA, D. J., BÉZIER, A., DEPOIX, D., DREZEN, J.-M. & LENOIR, A. 2009. *Blochmannia* endosymbionts improve colony growth and immune defence in the ant *Camponotus fellah*. *BMC Microbiology,* 9**,** 29.

DEGNAN, P. H., LEONARDO, T. E., CASS, B. N., HURWITZ, B., STERN, D., GIBBS, R. A., RICHARDS, S. & MORAN, N. A. 2010. Dynamics of genome evolution in facultative symbionts of aphids. *Environmental Microbiology,* 12**,** 2060-2069.

DEGNAN, P. H. & MORAN, N. A. 2008. Diverse phage-encoded toxins in a protective insect endosymbiont. *Applied and Environmental Microbiology,* 74**,** 6782-6791.

DEGNAN, P. H., YU, Y., SISNEROS, N., WING, R. A. & MORAN, N. A. 2009. *Hamiltonella defensa*, genome evolution of protective bacterial endosymbiont from pathogenic ancestors. *Proceedings of the National Academy of Sciences of the U.S.A.*, 106**,** 9063-9068.

DELOBEL, B. & GRENIER, A. 1993. Effect of non-cereal food on cereal weevils and tamarind pod weevil (Coleoptera: Curculionidae). *Journal of Stored Products Research,* 29**,** 7-14.

DEMIRCI, F., MUŞTU, M., KAYDAN, M. B., & ÜLGENTÜRK, S. 2011. Laboratory evaluation of the effectiveness of the entomopathogen; *Isaria farinosa*, on citrus mealybug, *Planococcus citri*. *Journal of Pest Science,* 84**,** 337-342.

DENTENER, P. R., BENNETT, K. V., HOY, L. E., LEWTHWAITE, S. E., LESTER, P. J., MAINDONALD, J. H. & CONNOLLY, P. G. 1997. Postharvest disinfestation of lightbrown apple moth and longtailed mealybug on persimmons using heat and cold. *Postharvest Biology and Technology,* 12**,** 255-264.

DESPRÉS, L., DAVID, J.-P. & GALLET, C. 2007. The evolutionary ecology of insect resistance to plant chemicals. *Trends in Ecology & Evolution,* 22**,** 298-307.

DIMOND, J. & CARRINGTON, E. 2008. Symbiosis regulation in a facultatively symbiotic temperate coral: zooxanthellae division and expulsion. *Coral Reefs,* 27**,** 601-604.

DIXON, A. F. G., KINDLMANN, P., LEPS, J. & HOLMAN, J. 1987. Why there are so few species of aphids, especially in the tropics. *American Naturalist,* 129**,** 580-592.

DOBSON, S. L., FOX, C. W. & JIGGINS, F. M. 2002. The effect of *Wolbachia*-induced cytoplasmic incompatibility on host population size in natural and manipulated systems. *Proceedings of the Royal Society of London. Series B: Biological Sciences,* 269**,** 437-445.

DOMÍNGUEZ-BELLO, M. Detoxification in the rumen. Annales de Zootechnie, 1996. 323-327.

DOUGLAS, A. 2007a. Symbiotic microorganisms: untapped resources for insect pest control. *Trends in Biotechnology,* 25**,** 338-342.

DOUGLAS, A. E. 1998. Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria *Buchnera*. *Annual Review of Entomology,* 43**,** 17-37.

DOUGLAS, A. E. 2006. Phloem-sap feeding by animals: problems and solutions. *Journal of Experimental Botany,* 57**,** 747-754.

DOUGLAS, A. E. 2007b. Symbiotic microorganisms: untapped resources for insect pest control. *Trends in Biotechnology,* 25**,** 338-342.

DOUGLAS, A. E. 2009. The microbial dimension in insect nutritional ecology. *Functional Ecology,* 23**,** 38-47.

DOUGLAS, A. E., MINTO, L. B. & WILKINSON, T. L. 2001. Quantifying nutrient production by the microbial symbionts in an aphid. *Journal of Experimental Biology,* 204**,** 349-358.

DOWD, F. 1989. In situ production of hydrolytic detoxifying enzymes by symbiotic yeasts in the cigarette beetle (Coleoptera: Anobiidae). *Journal of Economic Entomology,* 82**,** 396-400.

DOWD, P. F. 1992. Insect fungal symbionts: a promising source of detoxifying enzymes. *Journal of Industrial Microbiology,* 9**,** 149-161.

DOWNIE, D. A. & GULLAN, P. J. 2004. Phylogenetic analysis of mealybugs (Hemiptera: Coccoidea: Pseudococcidae) based on DNA sequences from three nuclear genes, and a review of the higher classification. *Systematic Entomology,* 29**,** 238-259.

DOWNIE, D. A. & GULLAN, P. J. 2005. Phylogenetic congruence of mealybugs and their primary endosymbionts. *Journal of Evolutionary Biology,* 18**,** 315-324.

DRÈS, M. & MALLET, J. 2002. Host races in plant–feeding insects and their importance in sympatric speciation. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences,* 357**,** 471-492.

DUNBAR, H. E., WILSON, A. C. C., FERGUSON, N. R. & MORAN, N. A. 2007. Aphid thermal tolerance is governed by a point mutation in bacterial symbionts. *PLoS Biology,* 5**,** 1006-1015.

DURON, O., LABBÉ, P., BERTICAT, C., ROUSSET, F., GUILLOT, S., RAYMOND, M., WEILL, M. & PROMISLOW, D. 2006. High *Wolbachia* density correlates with cost of infection for insecticide resistant *Culex pipiens* mosquitoes. *Evolution,* 60**,** 303-314.

DUTTON, T. & SINKINS, S. 2004. Strain-specific quantification of *Wolbachia* density in *Aedes albopictus* and effects of larval rearing conditions. *Insect Molecular Biology,* 13**,** 317-322.

EBBERT, M. A. & NAULT, L. R. 1994. Improved overwintering ability in *Dalbulus maidis* (Homoptera: Cicadellidae) vectors infected with *Spiroplasma kunkelii* (Mycoplasmatales: Spiroplasmataceae). *Environmental Entomology,* 23**,** 634-644.

EBERHARD, W. G. 1980. Evolutionary consequences of intracellular organelle competition. *Quarterly Review of Biology,* 55**,** 231-249.

ECHAUBARD, P., DURON, O., AGNEW, P., SIDOBRE, C., NOËL, V., WEILL, M. & MICHALAKIS, Y. 2010. Rapid evolution of *Wolbachia* density in insecticide resistant *Culex pipiens*. *Heredity,* 104**,** 15-19.

EDGAR, R. C., HAAS, B. J., CLEMENTE, J. C., QUINCE, C. & KNIGHT, R. 2011. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics,* 27**,** 2194-2200.

EDWARDS, A. R. 1963. A non-colonizing aphid vector of potato virus diseases. *Nature,* 200**,** 1233 - 1234.

ELZEN, G. W. & HARDEE, D. D. 2003. United States Department of Agriculture - agricultural research service research on managing insect resistance to insecticides. *Pest Management Science,* 59**,** 770-776.

ENGELSTÄDTER, J. & HURST, G. D. 2009. The ecology and evolution of microbes that manipulate host reproduction. *Annual Review of Ecology, Evolution, and Systematics,* 40**,** 127-149.

EPIS, S., MANDRIOLI, M., GENCHI, M., MONTAGNA, M., SACCHI, L., PISTONE, D. & SASSERA, D. 2013. Localization of the bacterial symbiont *Candidatus Midichloria mitochondrii* within the hard tick *Ixodes ricinus* by whole-mount FISH staining. *Ticks and Tick-Borne Diseases,* 4**,** 39-45.

ESPINO, C. I., GOMEZ, T., GONZALEZ, G., DO SANTOS, M. F., SOLANO, J., SOUSA, O., MORENO, N., WINDSOR, D., YING, A. & VILCHEZ, S. 2009. Detection of *Wolbachia* bacteria in multiple organs and feces of the Triatomine insect *Rhodnius pallescens* (Hemiptera, Reduviidae). *Applied and Environmental Microbiology,* 75**,** 547-550.

FALKOWSKI, P. G., DUBINSKY, Z., MUSCATINE, L. & MCCLOSKEY, L. 1993. Population control in symbiotic corals. *Bioscience,* 43**,** 606-611.

FEBVAY, G., RAHBE, Y., RYNKIEWICZ, M., GUILLAUD, J. & BONNOT, G. 1999. Fate of dietary sucrose and neosynthesis of amino acids in the pea

aphid, *Acyrthosiphon pisum*, reared on different diets. *Journal of Experimental Biology,* 202**,** 2639-2652.

FELBECK, H. & SOMERO, G. N. 1982. Primary production in deep-sea hydrothermal vent organisms: roles of sulfide-oxidizing bacteria. *Trends in Biochemical Sciences,* 7**,** 201-204.

FELDHAAR, H., STRAKA, J., KRISCHKE, M., BERTHOLD, K., STOLL, S., MUELLER, M. J. & GROSS, R. 2007. Nutritional upgrading for omnivorous carpenter ants by the endosymbiont *Blochmannia*. *BMC Biology,* 5**,** 48.

FERGUSON, N. M., KIEN, D. T. H., CLAPHAM, H., AGUAS, R., TRUNG, V. T., CHAU, T. N. B., POPOVICI, J., RYAN, P. A., O'NEILL, S. L. & MCGRAW, E. A. 2015. Modeling the impact on virus transmission of *Wolbachia*-mediated blocking of dengue virus infection of *Aedes aegypti*. *Science Translational Medicine,* 7**,** 279ra37-279ra37.

FERRARI, J., DARBY, A. C., DANIELL, T. J., GODFRAY, H. C. J. & DOUGLAS, A. E. 2004. Linking the bacterial community in pea aphids with host-plant use and natural enemy resistance. *Ecological Entomology,* 29**,** 60-65.

FERRARI, J., SCARBOROUGH, C. L. & GODFRAY, H. C. J. 2007. Genetic variation in the effect of a facultative symbiont on host-plant use by pea aphids. *Oecologia,* 153**,** 323-329.

FIALHO, R. F. & STEVENS, L. 2000. Male-killing *Wolbachia* in a flour beetle. *Proceedings of the Royal Society of London. Series B: Biological Sciences,* 267**,** 1469-1473.

FRANCO, J. C., SUMA, P., DA SILVA, E. B., BLUMBERG, D. & MENDEL, Z. 2004. Management strategies of mealybug pests of citrus in Mediterranean countries. *Phytoparasitica,* 32**,** 507-522.

FRANCO, J. C., ZADA, A. & MENDEL, Z. 2009. Novel approaches for the management of mealybug pests. *In:* ISHAAYA, I. & HOROWITZ, A. R. (eds.) *Biorational Control of Arthropod Pests.* Dordrecht, The Netherlands: Springer.

FRANK, S. A. 1996a. Host-symbiont conflict over the mixing of symbiotic lineages. *Proceedings of the Royal Society of London. Series B: Biological Sciences,* 263**,** 339-344.

FRANK, S. A. 1996b. Host-symbiont conflict over the mixing of symbiotic lineages. *Proceedings of the Royal Society of London B,* 263**,** 339-344.

FRANK, S. A. 1996c. Host control of symbiont transmission: the separation of symbionts into germ and soma. *American Naturalist,* 148**,** 1113-1124.

FRY, H. R., QUIRING, D. T., RYALL, K. L. & DIXON, P. L. 2009. Influence of intra-tree variation in phenology and oviposition site on the distribution and performance of *Ennomos subsignaria* on mature sycamore maple. *Ecological Entomology,* 34**,** 394-405.

FUKATSU, T. & ISHIKAWA, H. 1992. Soldier and male of an eusocial aphid *Colophina arma* lack endosymbiont: implications for physiological and evolutionary interaction between host and symbiont. *Journal of Insect Physiology,* 38**,** 1033-1042.

FUKATSU, T. & ISHIKAWA, H. 1996. Phylogenetic position of yeast-like symbiont of *Hamiltonaphis styraci* (Homoptera, Aphididae) based on 18S rDNA sequence. *Insect Biochemistry and Molecular Biology,* 26**,** 383-388.

FUKATSU, T., KOGA, R., SMITH, W. A., TANAKA, K., NIKOH, N., SASAKI-FUKATSU, K., YOSHIZAWA, K., DALE, C. & CLAYTON, D. H. 2007. Bacterial endosymbiont of the slender pigeon louse, *Columbicola columbae*, allied to endosymbionts of grain weevils and tsetse flies. *Applied and Environmental Microbiology,* 73**,** 6660-6668.

FUKATSU, T. & NIKOH, N. 1998. Two intracellular symbiotic bacteria from the mulberry psyllid *Anomoneura mori* (Insecta, Homoptera). *Applied and Environmental Microbiology,* 64**,** 3599-3606.

FUKATSU, T. & NIKOH, N. 2000. Endosymbiotic microbiota of the bamboo pseudococcid *Antonina crawii* (Insecta, Homoptera). *Applied and Environmental Microbiology,* 66**,** 643.

FUKATSU, T., TSUCHIDA, T., NIKOH, N. & KOGA, R. 2001. *Spiroplasma* symbiont of the pea aphid, *Acyrthosiphon pisum* (Insecta: Homoptera). *Applied and Environmental Microbiology*, 67**,** 1284-1291.

FUNK, D. J., HELBLING, L., WERNEGREEN, J. J. & MORAN, N. A. 2000. Intraspecific phylogenetic congruence among multiple symbiont genomes. *Proceedings of the Royal Society of London B,* 267**,** 2517-2521.

GARNIER, M., FOISSAC, X., GAURIVAUD, P., LAIGRET, F., RENAUDIN, J., SAILLARD, C. & BOVÉ, J. M. 2001. Mycoplasmas, plants, insect vectors: a matrimonial triangle. *Comptes Rendus de l'Académie des Sciences-Series III-Sciences de la Vie,* 324**,** 923-928.

GASPARICH, G. E. 2002. Spiroplasmas: evolution, adaptation and diversity. *Frontiers in Bioscience,* 7**,** 19-40.

GASPARICH, G. E. 2010. Spiroplasmas and phytoplasmas: microbes associated with plant hosts. *Biologicals,* 38**,** 193-203.

GAUTHIER, J.-P., OUTREMAN, Y., MIEUZET, L. & SIMON, J.-C. 2015. Bacterial communities associated with host-adapted populations of pea aphids revealed by deep sequencing of 16S ribosomal DNA. *PLoS ONE,* 10**,** e0120664.

GERARDO, N. M. 2015. Harnessing evolution to elucidate the consequences of symbiosis. *PLoS Biology,* 13**,** e1002066-e1002066.

GERARDO, N. M., ALTINCICEK, B., ANSELME, C., ATAMIAN, H., BARRIBEAU, S. M., DE VOS, M., DUNCAN, E. J., EVANS, J. D., GABALDÓN, T. & GHANIM, M. 2010. Immunity and other defenses in pea aphids, *Acyrthosiphon pisum*. *Genome Biology,* 11**,** R21.

GHANIM, M. & KONTSEDALOV, S. 2009. Susceptibility to insecticides in the Q biotype of Bemisia tabaci is correlated with bacterial symbiont densities. *Pest Management Science,* 65**,** 939-942.

GIOBEL, G. 1926. Relation of the soil nitrogen to nodule development and fixation of nitrogen by certain legumes. *New Jersey Agricultural Experiment Stations, Bulletin,* 436**,** 125.

GIORGINI, M., BERNARDO, U., MONTI, M., NAPPO, A. & GEBIOLA, M. 2010. *Rickettsia* symbionts cause parthenogenetic reproduction in the parasitoid wasp *Pnigalio soemius* (Hymenoptera: Eulophidae). *Applied and Environmental Microbiology,* 76**,** 2589-2599.

GOFFREDI, S. K., JOHNSON, S. B. & VRIJENHOEK, R. C. 2007. Genetic diversity and potential function of microbial symbionts associated with newly discovered species of *Osedax* polychaete worms. *Applied and Environmental Microbiology,* 73**,** 2314-2323.

GOLDASTEH, S., TALEBI, A. A., FATHIPOUR, Y., OSTOVAN, H., ZAMANI, A. & SHOUSHTARI, V. R. 2009. Effect of temperature on life history and population growth parameters of *Planococcus citri* (Homoptera, Pseudococcidae) on coleus [*Solenostemon scutellarioides* (L.) Codd.]. *Archives of Biological Sciences,* 61**,** 329-336.

GOMEZ-VALERO, L., SORIANO-NAVARRO, M., PEREZ-BROCAL, V., HEDDI, A., MOYA, A., GARCIA-VERDUGO, J. M. & LATORRE, A. 2004. Coexistence of *Wolbachia* with *Buchnera aphidicola* and a secondary symbiont in the aphid *Cinara cedri*. *Journal of Bacteriology,* 186**,** 6626-6633.

GOODWIN, S., STEINER, M., PARKER, R., TESORIERO, L., CONNELLAN, G., KESKULA, E., COWPER, B., MEDHURST, A. & RODRIGUEZ, C. 2000. Integrated pest management in ornamentals: information guide. Horticulture series: Agrilink,your growing guide to better farming. *In:* QAL0004, A. S. (ed.). Queensland Horticulture Institute. Brisbane, Queensland.

GOTOH, T., SUGASAWA, J., NODA, H. & KITASHIMA, Y. 2007. *Wolbachia*-induced cytoplasmic incompatibility in Japanese populations of *Tetranychus urticae* (Acari: Tetranychidae). *Experimental and Applied Acarology,* 42**,** 1-16.

GOTTLIEB, Y., GHANIM, M., CHIEL, E., GERLING, D., PORTNOY, V., STEINBERG, S., TZURI, G., HOROWITZ, A. R., BELAUSOV, E. & MOZES-DAUBE, N. 2006. Identification and Localization of a *Rickettsia sp.* in *Bemisia tabaci* (Homoptera: Aleyrodidae). *Applied and Environmental Microbiology,* 72**,** 3646-3652.

GRACIET, D. 2011. *Horticulture - Pépinières* [Online]. Agricultures et Territoires Cahmbres d'Agriculture. Available: chambres-agriculture.fr 2015].

GRAYSTOCK, P., YATES, K., DARVILL, B., GOULSON, D. & HUGHES, W. O. 2013. Emerging dangers: deadly effects of an emergent parasite in a new pollinator host. *Journal of Invertebrate Pathology,* 114**,** 114-119.

GRIFFITHS, J. T. & THOMPSON, W. L. 1957. Insects and mites found on Florida citrus. *University of Florida Agricultural Experiment Station Bulletin,* 591**,** 30-33.

GRUWELL, M. E., HARDY, N. B., GULLAN, P. J. & DITTMAR, K. 2010. Evolutionary relationships among primary endosymbionts of the mealybug subfamily Phenacoccinae (Hemiptera: Coccoidea: Pseudococcidae). *Applied and Environmental Microbiology,* 76**,** 7521-7525.

GRUWELL, M. E., MORSE, G. E. & NORMARK, B. B. 2007. Phylogenetic congruence of armored scale insects (Hemiptera: Diaspididae) and their primary endosymbionts from the phylum Bacteroidetes. *Molecular Phylogenetics and Evolution,* 44**,** 267-280.

GUAY, J. F., BOUDREAULT, S., MICHAUD, D. & CLOUTIER, C. 2009. Impact of environmental stress on aphid clonal resistance to parasitoids: role of *Hamiltonella defensa* bacterial symbiosis in association with a new

facultative symbiont of the pea aphid. *Journal of Insect Physiology,* 55**,** 919-926.

GULLAN, P. J. & KOSZTARAB, M. 1997. Adaptations in scale insects. *Annual Review of Entomology,* 42**,** 23-50.

HALLAM, S. J. & MCCUTCHEON, J. P. 2015. Microbes don't play solitaire: how cooperation trumps isolation in the microbial world. *Environmental Microbiology Reports,* 7**,** 26-28.

HANCOCK, P. A., SINKINS, S. P. & GODFRAY, H. C. J. 2011. Strategies for Introducing *Wolbachia* to Reduce Transmission of Mosquito-Borne Diseases. *PLoS Neglected Tropical Diseases,* 5**,** e1024.

HANSEN, A. K., JEONG, G., PAINE, T. D. & STOUTHAMER, R. 2007. Frequency of secondary symbiont infection in an invasive psyllid relates to parasitism pressure on a geographic scale in California. *Applied and Environmental Microbiology,* 73**,** 7531-7535.

HANSEN, J. D., HARA, A. H. & TENBRINK, V. L. 1992. Vapor heat: a potential treatment to disinfest tropical cut flowers and foliage. *HortScience,* 27**,** 139-143.

HARA, A. H., HATA, T. Y., TENBRINK, V. L., HU, B. K.-S. & KANEKO, R. T. 1996. Postharvest heat treatment of red ginger flowers as a possible alternative to chemical insecticidal dip. *Postharvest Biology and Technology,* 7**,** 137-144.

HARDY, N. B., GULLAN, P. J. & HODGSON, C. J. 2008. A subfamily-level classification of mealybugs (Hemiptera: Pseudococcidae) based on integrated molecular and morphological data. *Systematic Entomology,* 33**,** 51-71.

HAYNES, S., DARBY, A. C., DANIELL, T. J., WEBSTER, G., VAN VEEN, F. J. F., GODFRAY, H. C. J., PROSSER, J. I. & DOUGLAS, A. E. 2003. Diversity of bacteria associated with natural aphid populations. *Applied and Environmental Microbiology,* 69**,** 7216-7223.

HERRE, E., KNOWLTON, N., MUELLER, U. & REHNER, S. 1999. The evolution of mutualisms: exploring the paths between conflict and cooperation. *Trends in Ecology & Evolution,* 14**,** 49-53.

HILGENBOECKER, K., HAMMERSTEIN, P., SCHLATTMANN, P., TELSCHOW, A. & WERREN, J. H. 2008. How many species are infected with *Wolbachia*?–a statistical analysis of current data. *Federation of European Microbiological Societies Microbiology Letters,* 281**,** 215-220.

HIMLER, A. G., ADACHI-HAGIMORI, T., BERGEN, J. E., KOZUCH, A., KELLY, S. E., TABASHNIK, B. E., CHIEL, E., DUCKWORTH, V. E., DENNEHY, T. J. & ZCHORI-FEIN, E. 2011. Rapid spread of a bacterial symbiont in an invasive whitefly is driven by fitness benefits and female bias. *Science,* 332**,** 254-256.

HINDE, R. 1971. The control of the mycetome symbiotes of the aphids *Brevicoryne brassicae*, *Myzus persicae*, and *Macrosiphum rosae*. *Journal of Insect Physiology,* 17**,** 1791-1800.

HOLLINGSWORTH, R. G. & ARMSTRONG, J. W. 2005. Potential of temperature, controlled atmospheres, and ozone fumigation to control thrips and mealybugs on ornamental plants for export. *Journal of Economic Entomology,* 98**,** 289-298.

HOOGENBOOM, M., BERAUD, E. & FERRIER-PAGÈS, C. 2010. Relationship between symbiont density and photosynthetic carbon acquisition in the temperate coral *Cladocora caespitosa*. *Coral Reefs,* 29**,** 21-29.

HOOPER, L. V., LITTMAN, D. R. & MACPHERSON, A. J. 2012. Interactions between the microbiota and the immune system. *Science,* 336**,** 1268-1273.

HOOPER, L. V., MIDTVEDT, T. & GORDON, J. I. 2002. How host-microbial interactions shape the nutrient environment of the mammalian intestine. *Annual Review of Nutrition,* 22**,** 283–307

HOSOKAWA, T., KIKUCHI, Y., SHIMADA, M. & FUKATSU, T. 2007. Obligate symbiont involved in pest status of host insect. *Proceedings of the Royal Society B: Biological Sciences,* 274**,** 1979-1984.

HOUK, E. J. & GRIFFITHS, G. W. 1980. Intracellular symbiotes of the Homoptera. *Annual Review of Entomology,* 25**,** 161-187.

HU, H.-Y. & LI, Z.-X. 2015. A novel *Wolbachia* strain from the rice moth *Corcyra cephalonica* induces reproductive incompatibility in the whitefly *Bemisia tabaci*: sequence typing combined with phenotypic evidence. *Environmental Microbiology Reports,* 7**,** 508-515.

HUIPENG, P. & YOUJUN, Z. 2012. Progress in the insect symbiont *Rickettsia*. *Acta Entomologica Sinica,* 55**,** 1103-1108.

HURST, L. D. 1995. Selfish genetic elements and their role in evolution: the evolution of sex and some of what that entails. *Philosophical Transactions of the Royal Society B: Biological Sciences,* 349**,** 321-332.

HUSNIK, F., NIKOH, N., KOGA, R., ROSS, L., DUNCAN, R. P., FUJIE, M., TANAKA, M., SATOH, N., BACHTROG, D. & WILSON, A. C. 2013.

Horizontal gene transfer from diverse bacteria to an insect genome enables a tripartite nested mealybug symbiosis. *Cell,* 153**,** 1567-1578.

INC., I. S. 2013. IBM SPSS Statistics for Windows, Version 22.0. . Armonk, NY: IBM Corp.

JELKMANN, W., FECHTNER B & AGRANOVSKY AA 1997. Complete genome structure and phylogenetic analysis of little cherry virus, a mealybug-transmissible closterovirus. *Journal of General Virology,* 78**,** 2067-2071.

JEYAPRAKASH, A. & HOY, M. A. 2000. Long PCR improves *Wolbachia* DNA amplification: *wsp* sequences found in 76% of sixty-three arthropod species. *Insect Molecular Biology,* 9**,** 393-405.

JIGGINS, F., HURST, G., JIGGINS, C. & MAJERUS, M. 2000. The butterfly *Danaus chrysippus* is infected by a male-killing *Spiroplasma* bacterium. *Parasitology,* 120**,** 439-446.

JIGGINS, F. M., SCHULENBURG, J. H. G., HURST, G. D. D. & MAJERUS, M. E. N. 2001. Recombination confounds interpretations of *Wolbachia* evolution. *Proceedings of the Royal Society of London. Series B: Biological Sciences,* 268**,** 1423-1427.

JING, X., WONG, A. C. N., CHASTON, J. M., COLVIN, J., MCKENZIE, C. L. & DOUGLAS, A. E. 2014. The bacterial communities in plant phloem-sap-feeding insects. *Molecular Ecology*, 23**,** 1433-1444.

KAGEYAMA, D., ANBUTSU, H., SHIMADA, M. & FUKATSU, T. 2007. *Spiroplasma* infection causes either early or late male killing in *Drosophila,* depending on maternal host age. *Naturwissenschaften,* 94**,** 333-337.

KARBAN, R. & AGRAWAL, A. A. 2002. Herbivore offense. *Annual Review of Ecology and Systematics,* 33**,** 641-664.

KARLEY, A., DOUGLAS, A. & PARKER, W. 2002. Amino acid composition and nutritional quality of potato leaf phloem sap for aphids. *Journal of Experimental Biology,* 205**,** 3009-3018.

KAWAI, S., MATSUMOTO, Y., GOTOH, T. & NODA, H. 2009. Transinfection of *Wolbachia* in planthoppers: Nymphal Injection of cultured *Wolbachia* and infection dynamics. *Environmental Entomology,* 38**,** 1626-1633.

KEELING, P. J. 2011. Endosymbiosis: bacteria sharing the load. *Current Biology,* 21**,** R623-R624.

KEELING, P. J., MCCUTCHEON, J. P. & DOOLITTLE, W. F. 2015. Symbiosis becoming permanent: survival of the luckiest. *Proceedings of the National Academy of Sciences,* 112**,** 10101-10103.

KERNS, D., WRIGHT, G. & LOGHRY, J. 2001. Citrus mealybug (*Planococcus citri*). *College of Agriculture Cooperative Extension.* University of Arizona.

KIKUCHI, Y. & FUKATSU, T. 2003. Diversity of *Wolbachia* endosymbionts in heteropteran bugs. *Applied and Environmental Microbiology,* 69**,** 6082-6090.

KIKUCHI, Y., HAYATSU, M., HOSOKAWA, T., NAGAYAMA, A., TAGO, K. & FUKATSU, T. 2012. Symbiont-mediated insecticide resistance. *Proceedings of the National Academy of Sciences of the U.S.A.,* 109**,** 8618-8622.

KIKUCHI, Y., HOSOKAWA, T. & FUKATSU, T. 2007. Insect-microbe mutualism without vertical transmission: a stinkbug acquires a beneficial gut symbiont from the environment every generation. *Applied and Environmental Microbiology,* 73**,** 4308-4316.

KLIOT, A., CILIA, M., CZOSNEK, H. & GHANIM, M. 2014. Implication of the bacterial endosymbiont *Rickettsia* spp. in interactions of the whitefly *Bemisia tabaci* with tomato yellow leaf curl virus. *Journal of Virology,* 88**,** 5652-5660.

KOGA, R., NIKOH, N., MATSUURA, Y., MENG, X.-Y. & FUKATSU, T. 2013. Mealybugs with distinct endosymbiotic systems living on the same host plant. *FEMS Microbiology Ecology,* 83**,** 93-100.

KOGA, R., TSUCHIDA, T. & FUKATSU, T. 2003. Changing partners in an obligate symbiosis: a facultative endosymbiont can compensate for loss of the essential endosymbiont *Buchnera* in an aphid. *Proceedings of the Royal Society of London. Series B,* 270**,** 2543-2550.

KOGA, R., TSUCHIDA, T., SAKURAI, M. & FUKATSU, T. 2007. Selective elimination of aphid endosymbionts: effects of antibiotic dose and host genotype, and fitness consequences. *FEMS Microbiology Ecology,* 60**,** 229-239.

KÖHLER, M. & SCHWARTZ, W. 1962. Untersuchungen über die Symbiose von Tieren mit Pilzen und Bakterien. IX. Über die Beziehungen zwischen Symbionten und Wirtsorganismus bei *Pseudococcus citri*, *Ps. maritimus* und *Orthezia insignis*. *Zeitschrift für Allgemeine Mikrobiologie,* 2**,** 190-208.

KOMAKI, K. & ISHIKAWA, H. 1999. Intracellular bacterial symbionts of aphids possess many genomic copies per bacterium. *Journal of Molecular Evolution,* 48**,** 717-722.

KONDO, N., IJICHI, N., SHIMADA, M. & FUKATSU, T. 2002. Prevailing triple infection with *Wolbachia* in *Callosobruchus chinensis* (Coleoptera: Bruchidae). *Molecular Ecology,* 11**,** 167-180.

KONDO, N., SHIMADA, M. & FUKATSU, T. 2005. Infection density of *Wolbachia* endosymbiont affected by co-infection and host genotype. *Biology Letters,* 1**,** 488-491.

KONO, M., KOGA, R., SHIMADA, M. & FUKATSU, T. 2008. Infection dynamics of coexisting beta-and gammaproteobacteria in the nested endosymbiotic system of mealybugs. *Applied and Environmental Microbiology,* 74**,** 4175-4184.

KONTSEDALOV, S., ZCHORI-FEIN, E., CHIEL, E., GOTTLIEB, Y., INBAR, M. & GHANIM, M. 2008. The presence of *Rickettsia* is associated with increased susceptibility of *Bemisia tabaci* (Homoptera: Aleyrodidae) to insecticides. *Pest Management Science,* 64**,** 789-792.

KÜCHLER, S. M., DETTNER, K. & KEHL, S. 2010. Molecular characterization and localization of the obligate endosymbiotic bacterium in the birch catkin bug *Kleidocerys resedae* (Heteroptera: Lygaeidae, Ischnorhynchinae). *Federation of European Microbiological Societies Microbiology Ecology,* 73**,** 408-418.

KUMAR, H., WACKLIN, P., NAKPHAICHIT, M., LOYTTYNIEMI, E., CHOWDHURY, S., SHOUCHE, Y., MÄTTÖ, J., ISOLAURI, E. & SALMINEN, S. 2015. Secretor status is strongly associated with microbial alterations observed during pregnancy. *PLoS ONE,* 10**,** e0134623.

LACROIX, R., MCKEMEY, A. R., RADUAN, N., WEE, L. K., MING, W. H., NEY, T. G., AA, S. R., SALMAN, S., SUBRAMANIAM, S. & NORDIN, O. 2012. Open field release of genetically engineered sterile male *Aedes aegypti* in Malaysia. *PLoS ONE,* 7**,** e42771.

LAFLIN, H. M. & PARRELLA, M. P. 2004. Developmental biology of citrus mealybug under conditions typical of California rose production. *Annals of the Entomological Society of America,* 97**,** 982-988.

LAI, C.-Y., BAUMANN, L. & BAUMANN, P. 1994. Amplification of trpEG: adaptation of *Buchnera aphidicola* to an endosymbiotic association with aphids. *Proceedings of the National Academy of Sciences of the U.S.A.,* 91**,** 3819-3823.

LASSY, C. W. & KARR, T. L. 1996. Cytological analysis of fertilization and early embryonic development in incompatible crosses of *Drosophila simulans*. *Mechanisms of Development,* 57**,** 47-58.

LATORRE, A., GIL, R., SILVA, F. J. & MOYA, A. 2005. Chromosomal stasis versus plasmid plasticity in aphid endosymbiont *Buchnera aphidicola*. *Heredity,* 95**,** 339-347.

LAUGHTON, A. M., FAN, M. H. & GERARDO, N. M. 2014. The combined effects of bacterial symbionts and aging on life history traits in the pea aphid, *Acyrthosiphon pisum*. *Applied and Environmental Microbiology,* 80**,** 470-477.

LAW, R. & HUTSON, V. 1992. Intracellular symbionts and the evolution of uniparental cytoplasmic inheritance. *Proceedings of the Royal Society of London B: Biological Sciences,* 248**,** 69-77.

LAWSON, E. T., MOUSSEAU, T. A., KLAPER, R., HUNTER, M. D. & WERREN, J. H. 2001. *Rickettsia* associated with male-killing in a buprestid beetle. *Heredity,* 86**,** 497-505.

LESTER, P. J., DENTENER, P. R., PETRY, R. J. & ALEXANDER, S. M. 1995. Hot-water immersion for disinfestation of lightbrown apple moth (*Epiphyas postvittana*) and longtailed mealy bug (*Pseudococcus longispinus*) on persimmons. *Postharvest Biology and Technology,* 6**,** 349-356.

LEWIN, R. A. 1982. Symbiosis and parasitism: definitions and evaluations. *Bioscience,* 32**,** 254-260.

LEY, R. E., PETERSON, D. A. & GORDON, J. I. 2006. Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell,* 124**,** 837–848.

LOCKHART, B. & OLSZEWSKI, N. *Schefflera* ringspot virus, a widely distributed mealybug-transmitted badnavirus occurring in *Schefflera* and *Aralia*. IX International Symposium on Virus Diseases of Ornamental Plants 432, 1996. 196-203.

LOCKHART, B. E. L., KIRATIYA-ANGUL, K., JONES, P., ENG, L., DE SILVA, P., OLSZEWSKI, N. E., LOCKHART, N., DEEMA, N. & SANGALANG, J. 1997. Identification of Piper yellow mottle virus, a mealybug-transmitted badnavirus infecting *Piper spp.* in Southeast Asia. *European Journal of Plant Pathology,* 103**,** 303-311.

LOGIN, F. H., BALMAND, S., VALLIER, A., VINCENT-MONÉGAT, C., VIGNERON, A., WEISS-GAYET, M., ROCHAT, D. & HEDDI, A. 2011.

Antimicrobial peptides keep insect endosymbionts under control. *Science,* 334**,** 362-365.

LÓPEZ-MADRIGAL, S., LATORRE, A., MOYA, A. & GIL, R. 2015. The link between independent acquisition of intracellular gamma-endosymbionts and concerted evolution in *Tremblaya princeps*. *Frontiers in Microbiology,* 6.

LOPEZ-MADRIGAL, S., LATORRE, A., PORCAR, M., MOYA, A. & GIL, R. 2011. Complete genome sequence of" *Candidatus* Tremblaya princeps" strain PCVAL, an intriguing translational machine below the living-cell status. *Journal of Bacteriology,* 193**,** 5587.

LÓPEZ-MADRIGAL, S., LATORRE, A., PORCAR, M., MOYA, A. & GIL, R. 2013. Mealybugs nested endosymbiosis: going into the 'matryoshka'system in *Planococcus citri* in depth. *BMC Microbiology,* 13**,** 74.

LORAM, J. E., BOONHAM, N., O'TOOLE, P., TRAPIDO-ROSENTHAL, H. G. & DOUGLAS, A. E. 2007. Molecular quantification of symbiotic dinoflagellate algae of the genus *Symbiodinium*. *The Biological Bulletin,* 212**,** 259-268.

LU, P., BIAN, G., PAN, X. & XI, Z. 2012. *Wolbachia* induces density-dependent inhibition to dengue virus in mosquito cells. *PLoS Neglected Tropical Diseases,* 6**,** e1754-e1754.

LU, W.-N., CHIU, M.-C. & KUO, M.-H. 2014. Host life stage-and temperature-dependent density of the symbiont *Buchnera aphidicola* in a subtropical pea aphid (*Acyrthosiphon pisum*) population. *Journal of Asia-Pacific Entomology,* 17**,** 537-541.

ŁUKASIK, P., VAN ASCH, M., GUO, H., FERRARI, J. & GODFRAY, C. J. 2013. Unrelated facultative endosymbionts protect aphids against a fungal pathogen. *Ecology Letters,* 16**,** 214-218.

LUTZ, W. & SAMIR, K. C. 2010. Dimensions of global population projections: what do we know about future population trends and structures? *Philosophical Transactions of the Royal Society B: Biological Sciences,* 365**,** 2779-2791.

LUYTEN, Y. A., THOMPSON, J. R., MORRILL, W., POLZ, M. F. & DISTEL, D. L. 2006. Extensive variation in intracellular symbiont community composition among members of a single population of the wood-boring bivalve *Lyrodus pedicellatus* (Bivalvia: Teredinidae). *Applied and Environmental Microbiology,* 72**,** 412-417.

LYNCH, M. & GABRIEL, W. 1990. Mutation load and the survival of small populations. *Evolution,* 44**,** 1725-1737.

MACHTELINCKX, T., VAN LEEUWEN, T., VANHOLME, B., GEHESQUIÈRE, B., DERMAUW, W., VANDEKERKHOVE, B., GHEYSEN, G. & DE CLERCQ, P. 2009. *Wolbachia* induces strong cytoplasmic incompatibility in the predatory bug *Macrolophus pygmaeus*. *Insect Molecular Biology,* 18**,** 373-381.

MAHADAV, A., GERLING, D., GOTTLIEB, Y., CZOSNEK, H. & GHANIM, M. 2008. Parasitization by the wasp *Eretmocerus mundus* induces transcription of genes related to immune response and symbiotic bacteria proliferation in the whitefly *Bemisia tabaci*. *BMC Genomics,* 9**,** 342.

MALAUSA, T., FENIS, A., WAROT, S., GERMAIN, J.-F., RIS, N., PRADO, E., BOTTON, M., VANLERBERGHE-MASUTTI, F., SFORZA, R. & CRUAUD, C. 2011. DNA markers to disentangle complexes of cryptic taxa in mealybugs (Hemiptera: Pseudococcidae). *Journal of Applied Entomology,* 135**,** 142-155.

MALLOCH, G. & FENTON, B. 2005. Super-infections of *Wolbachia* in byturid beetles and evidence for genetic transfer between A and B super-groups of *Wolbachia*. *Molecular Ecology,* 14**,** 627-637.

MARTELLI, G. P., AGRANOVSKY, A. A., BAR-JOSEPH, M., BOSCIA, D., CANDRESSE, T., COUTTS, R. H. A., DOLJA, V. V., FALK, B. W., GONSALVES, D. & JELKMANN, W. 2002. The family Closteroviridae revised. *Archives of Virology,* 147**,** 2039-2044.

MARTINSON, V. G., DANFORTH, B. N., MINCKLEY, R. L., RUEPPELL, O., TINGEK, S., & MORAN, N. A. 2011. A simple and distinctive microbiota associated with honey bees and bumble bees. *Molecular Ecology,* 20**,** 619-628.

MARTINSON, V. G., MOY, J., & MORAN, N. A. 2012. Establishment of characteristic gut bacteria during development of the honeybee worker. *Applied and Environmental Microbiology,* 78**,** 2830-2840.

MATSUURA, Y., KOGA, R., NIKOH, N., MENG, X. Y., HANADA, S. & FUKATSU, T. 2009. Huge symbiotic organs in giant scale insects of the genus *Drosicha* (Coccoidea: Monophlebidae) harbor flavobacterial and enterobacterial endosymbionts. *Zoological Science,* 26**,** 448-456.

MATTSON JR, W. J. 1980. Herbivory in relation to plant nitrogen content. *Annual Review of Ecology and Systematics***,** 119-161.

MCCOY, R. E., CAUDWELL, A., CHAN, C. J., CHEN, T. A., CHIYKOWSKI, L. N., COUSIN, M. T., DALE, J. L., DE LEEUW, G. T. N., GOLINO, D. A., HACKETT, K. J., KIRKPATRICK, B. C., MARWITZ, R., PETZOLD, H., SINHA, R. C., SUGIURA, M., WHITCOMB, R. E., YANG, I. L., ZHU, B. M. & SEEMIILLER, E. 1989. Plant diseases associated with mycoplasma-like organisms. *Acholeplasmas, and Mycoplasmas of Plants and Arthropods.* New York: Academic Press.

MCCUTCHEON, J. P. & KEELING, P. J. 2014. Endosymbiosis: protein targeting further erodes the organelle/symbiont distinction. *Current Biology,* 24**,** R654-R655.

MCCUTCHEON, J. P. & MORAN, N. A. 2012. Extreme genome reduction in symbiotic bacteria. *Nature Reviews Microbiology,* 10**,** 13-26.

MCCUTCHEON, J. P. & VON DOHLEN, C. D. 2011. An interdependent metabolic patchwork in the nested symbiosis of mealybugs. *Current Biology,* 21**,** 1366-1372.

MCFALL-NGAI, M., NYHOLM, S. V. & CASTILLO, M. G. 2010. The role of the immune system in the initiation and persistence of the *Euprymna scolopes*–*Vibrio fischeri* symbiosis. *Seminars in Immunology,* 22**,** 48-53.

MERIWEATHER, M., MATTHEWS, S., RIO, R. & BAUCOM, R. S. 2013. A 454 Survey Reveals the Community Composition and Core Microbiome of the Common Bed Bug (*Cimex lectularius*) across an Urban Landscape. *PLoS ONE*.

MILLER, D. 1999. Identification of the pink hibiscus mealybug, *Maconellicoccus hirsutus* (Green) (Hemiptera: Sternorrhyncha: Pseudococcidae). *Insecta Mundi* 13**,** 189-203.

MIRANDA, M. I., OMACINI, M. & CHANETON, E. J. 2011. Environmental context of endophyte symbioses: interacting effects of water stress and insect herbivory. *International Journal of Plant Sciences,* 172**,** 499-508.

MONTENEGRO, H., SOLFERINI, V., KLACZKO, L. & HURST, G. 2005. Male killing *Spiroplasma* naturally infecting *Drosophila* melanogaster. *Insect Molecular Biology,* 14**,** 281-287.

MONTGOMERY, M. E., WOODWORTH, L. M., ENGLAND, P. R., BRISCOE, D. A. & FRANKHAM, R. 2010. Widespread selective sweeps affecting microsatellites in *Drosophila* populations adapting to captivity: implications for captive breeding programs. *Biological Conservation,* 143**,** 1842-1849.

MONTLLOR, C. B., MAXMEN, A. & PURCELL, A. H. 2002. Facultative bacterial endosymbionts benefit pea aphids *Acyrthosiphon pisum* under heat stress. *Ecological Entomology,* 27**,** 189-195.

MOPPER, S. & STRAUSS, S. Y. 1998. Genetic structure and local adaptation in natural insect populations. *In:* MOPPER, S. & STRAUSS, S. Y. (eds.) *Effects of ecology, life history, and behaviour.* London: Chapman & Hall 1998.

MORAN, N. A. 1992. The evolution of aphid life cycles. *Annual Review of Entomology,* 37**,** 321-348.

MORAN, N. A. 1996. Accelerated evolution and Muller's rachet in endosymbiotic bacteria. *Proceedings of the National Academy of Sciences,* 93**,** 2873-2878.

MORAN, N. A. 2001. Bacterial menageries inside insects. *Proceedings of the National Academy of Sciences of the U.S.A.,* 98**,** 1338-1340.

MORAN, N. A. 2007. Symbiosis as an adaptive process and source of phenotypic complexity. *Proceedings of the National Academy of Sciences,* 104**,** 8627-8633.

MORAN, N. A. & BENNETT, G. M. 2014. The tiniest tiny genomes. *Annual Review of Microbiology,* 68**,** 195-215.

MORAN, N. A. & DEGNAN, P. H. 2006. Functional genomics of *Buchnera* and the ecology of aphid hosts. *Molecular Ecology,* 15**,** 1251-1261.

MORAN, N. A., DUNBAR, H. E. & WILCOX, J. L. 2005a. Regulation of transcription in a reduced bacterial genome: nutrient-provisioning genes of the obligate symbiont *Buchnera aphidicola*. *Journal of Bacteriology,* 187**,** 4229-4237.

MORAN, N. A., HANSEN, A.K., POWELL, J.E., SABREE, Z.L. 2012. Distinctive gut microbiota of honey bees assessed using deep sampling from individual worker bees. *PLoS ONE*.

MORAN, N. A., KAPLAN, M. E., GELSEY, M. J., MURPHY, T. G. & SCHOLES, E. A. 1999. Phylogenetics and evolution of the aphid genus *Uroleucon* based on mitochondrial and nuclear DNA sequences. *Systematic Entomology,* 24**,** 85-93.

MORAN, N. A., MCCUTCHEON, J. P. & NAKABACHI, A. 2008. Genomics and evolution of heritable bacterial symbionts. *Annual Review of Genetics,* 42**,** 165-190.

MORAN, N. A., MUNSON, M. A., BAUMANN, P. & ISHIKAWA, H. 1993. A molecular clock in endosymbiotic bacteria is calibrated using the insect hosts. *Proceedings of the Royal Society B: Biological Sciences***,** 167-171.

MORAN, N. A., PLAGUE, G. R., SANDSTRÖM, J. P. & WILCOX, J. L. 2003. A genomic perspective on nutrient provisioning by bacterial symbionts of insects. *Proceedings of the National Academy of Sciences of the U.S.A.,* 100**,** 14543-14548.

MORAN, N. A., RUSSELL, J. A., KOGA, R. & FUKATSU, T. 2005b. Evolutionary relationships of three new species of Enterobacteriaceae living as symbionts of aphids and other insects. *Applied and Environmental Microbiology,* 71**,** 3302-3310.

MORAN, N. A. & TELANG, A. 1998. Bacteriocyte-associated symbionts of insects. *Bioscience,* 48**,** 295-304.

MORAN, N. A. & WERNEGREEN, J. J. 2000. Lifestyle evolution in symbiotic bacteria: insights from genomics. *Trends in Ecology & Evolution,* 15**,** 321-326.

MOUTON, L., HENRI, H., CHARIF, D., BOULÉTREAU, M. & VAVRE, F. 2007. Interaction between host genotype and environmental conditions affects bacterial density in *Wolbachia* symbiosis. *Biology Letters,* 3**,** 210-213.

MOYA, A., LATORRE, A., SABATER-MUÑOZ, B. & SILVA, F. J. 2002. Comparative molecular evolution of primary (*Buchnera*) and secondary symbionts of aphids based on two protein-coding genes. *Journal of Molecular Evolution,* 55**,** 127-137.

MULLER-PARKER, G., MCCLOSKEY, L., HOEGH-GULDBERG, O. & MCAULEY, P. 1994. Effect of ammonium enrichment on animal and algal biomass of the coral *Pocillopora damicornis*. *Pacific Science,* 48**,** 273-283.

MUNSON, M., BAUMANN, P., CLARK, M., BAUMANN, L., MORAN, N., VOEGTLIN, D. & CAMPBELL, B. 1991a. Evidence for the establishment of aphid-eubacterium endosymbiosis in an ancestor of four aphid families. *Journal of Bacteriology,* 173**,** 6321-6324.

MUNSON, M. A., BAUMANN, P. & KINSEY, M. G. 1991b. *Buchnera gen. nov.* and *Buchnera aphidicola sp. nov.*, a taxon consisting of the mycetocyte-associated, primary endosymbionts of aphids. *International Journal of Systematic Bacteriology,* 41**,** 566-568.

NAKABACHI, A., YAMASHITA, A., TOH, H., ISHIKAWA, H., DUNBAR, H. E., MORAN, N. A. & HATTORI, M. 2006. The 160-kilobase genome of the bacterial endosymbiont *Carsonella*. *Science,* 314**,** 267.

NAKAMURA, N., GASKINS, H. R., COLLIER, C. T., NAVA, G. M., RAI, D., PETSCHOW, B., RUSSELL, W. M., HARRIS, C., MACKIE, R. I. & WAMPLER, J. L. 2009. Molecular ecological analysis of fecal bacterial populations from term infants fed formula supplemented with selected blends of prebiotics. *Applied and Environmental Microbiology,* 75**,** 1121-1128.

NAKASHIMA, K., WATANABE, H. & AZUMA, J.-I. 2002. Cellulase genes from the parabasalian symbiont *Pseudotrichonympha grassii* in the hindgut of the wood-feeding termite *Coptotermes formosanus*. *Cellular and Molecular Life Sciences CMLS,* 59**,** 1554-1560.

NAULT, L. 1997. Arthropod transmission of plant viruses: a new synthesis. *Annals of the Entomological Society of America,* 90**,** 521-541.

NCSU. 2015. *Citrus Mealybugs* [Online]. NC State University. Available: ipm.ncsu.edu/AG136/mealy1.html 2015].

NDII, M. Z., HICKSON, R., ALLINGHAM, D. & MERCER, G. 2015. Modelling the transmission dynamics of dengue in the presence of *Wolbachia*. *Mathematical Biosciences,* 262**,** 157-166.

NEGRI, I., FRANCHINI, A., GONELLA, E., DAFFONCHIO, D., MAZZOGLIO, P. J., MANDRIOLI, M. & ALMA, A. 2009. Unravelling the *Wolbachia* evolutionary role: the reprogramming of the host genomic imprinting. *Proceedings of the Royal Society B: Biological Sciences,* 276**,** 2485-2491.

NEGRI, I., PELLECCHIA, M., MAZZOGLIO, P. J., PATETTA, A. & ALMA, A. 2006. Feminizing *Wolbachia* in *Zyginidia pullula* (Insecta, Hemiptera), a leafhopper with an XX/X0 sex-determination system. *Proceedings of the Royal Society B: Biological Sciences,* 273**,** 2409-2416.

NG, J. C. K. & PERRY, K. L. 2004. Transmission of plant viruses by aphid vectors. *Molecular Plant Pathology,* 5**,** 505-511.

NIKOH, N., MCCUTCHEON, J. P., KUDO, T., MIYAGISHIMA, S., MORAN, N. A. & NAKABACHI, A. 2010. Bacterial genes in the aphid genome: absence of functional gene transfer from *Buchnera* to its host. *PLoS Genetics,* 6**,** e1000827-e1000827.

NODA, H., KOIZUMI, Y., ZHANG, Q. & DENG, K. 2001. Infection density of *Wolbachia* and incompatibility level in two planthopper species, *Laodelphax*

striatellus and *Sogatella furcifera*. *Insect Biochemistry and Molecular Biology,* 31**,** 727-737.

NYABUGA, F. N., OUTREMAN, Y., SIMON, J. C., HECKEL, D. G. & WEISSER, W. W. 2010. Effects of pea aphid secondary endosymbionts on aphid resistance and development of the aphid parasitoid *Aphidius ervi*: a correlative study. *Entomologia Experimentalis et Applicata,* 136**,** 243-253.

NYHOLM, S. V. & MCFALL-NGAI, M. 2004. The winnowing: establishing the squid–*Vibrio* symbiosis. *Nature Reviews Microbiology,* 2**,** 632-642.

ODINDO, M. 1992. Future prospects for application of insect pathogens as a component of integrated pest management in tropical root crops. *Biocontrol Science and Technology,* 2**,** 179-191.

OLIVER, K. M., CAMPOS, J., MORAN, N. A. & HUNTER, M. S. 2008. Population dynamics of defensive symbionts in aphids. *Proceedings of the Royal Society B: Biological Sciences,* 275**,** 293-299.

OLIVER, K. M., DEGNAN, P. H., BURKE, G. R. & MORAN, N. A. 2010. Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. *Annual Review of Entomology,* 55**,** 247-266.

OLIVER, K. M., DEGNAN, P. H., HUNTER, M. S. & MORAN, N. A. 2009. Bacteriophages encode factors required for protection in a symbiotic mutualism. *Science,* 325**,** 992-994.

OLIVER, K. M., MORAN, N. A. & HUNTER, M. S. 2005. Variation in resistance to parasitism in aphids is due to symbionts not host genotype. *Proceedings of the National Academy of Sciences of the United States of America,* 102**,** 12795-12800.

OLIVER, K. M., MORAN, N. A. & HUNTER, M. S. 2006. Costs and benefits of a superinfection of facultative symbionts in aphids. *Proceedings of the Royal Society of London B,* 273**,** 1273-1280.

OLIVER, K. M., RUSSELL, J. A., MORAN, N. A. & HUNTER, M. S. 2003. Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *Proceedings of the National Academy of Sciences of the United States of America,* 100**,** 1803-1807.

OLIVER, K. M., SMITH, A. H. & RUSSELL, J. A. 2014. Defensive symbiosis in the real world -advancing ecological studies of heritable, protective bacteria in aphids and beyond. *Functional Ecology,* 28**,** 341-355.

OSBORNE, L. S. 2010. *Mealybugs* [Online]. Mid-Florida Research and Education Center. Available: mrec.ifas.ufl.edu/lso/mealybugs.htm 2015].

PAIS, R., LOHS, C., WU, Y., WANG, J. & AKSOY, S. 2008. The obligate mutualist *Wigglesworthia glossinidia* influences reproduction, digestion, and immunity processes of its host, the tsetse fly. *Applied and Environmental Microbiology,* 74**,** 5965-5974.

PAN, H. P., CHU, D., LIU, B. M., XIE, W., WANG, S. L., WU, Q. J., XU, B. Y., ZHANG, Y. J. 2013. Relative amount of symbionts in insect hosts changes with host-plant adaptation and insecticide resistance. *Environmental Entomology,* 42**,** 74-78.

PARKINSON, J. F., GOBIN, B. & HUGHES, W. O. H. 2014. Short-term heat stress results in diminution of bacterial symbionts but has little effect on life history in adult female citrus mealybugs. *Entomologia Experimentalis et Applicata,* 153**,** 1-9.

PARKINSON, J. F., GOBIN, B. & HUGHES, W. O. H. 2016. Heritability of symbiont density reveals distinct regulatory mechanisms in a tripartite symbiosis. *Ecology and Evolution,* 6**,** 2053–2060.

PATIL, S. V., PATIL, C. D., SALUNKHE, R. B., MAHESHWARI, V. L. & SALUNKE, B. K. 2011. Studies on life cycle of mealybug, *Maconellicoccus hirsutus* (Green)(Hemiptera: Pseudococcidae), on different hosts at different constant temperatures. *Crop Protection,* 30**,** 1553-1556.

PEIX, A., RAMÍREZ-BAHENA, M. H., VELÁZQUEZ, E. & BEDMAR, E. J. 2015. Bacterial associations with legumes. *Critical Reviews in Plant Sciences,* 34**,** 17-42.

PÉREZ-BROCAL, V., GIL, R., RAMOS, S., LAMELAS, A., POSTIGO, M., MICHELENA, J. M., SILVA, F. J., MOYA, A. & LATORRE, A. 2006. A small microbial genome: the end of a long symbiotic relationship? *Science,* 314**,** 312-313.

PHILLIPS, S., BRIDDON, R. W., BRUNT, A. A. & HULL, R. 1999. The partial characterization of a badnavirus infecting the greater asiatic or water yam (*Dioscorea alata*). *Journal of Phytopathology,* 147**,** 265-269.

PRICE, P. W. 1980. *Evoluntionary Biology of Parasites,* Princeton (NJ), Princeton University Press.

RA, G., NM, G., DENIS, T., AC, W., MN, C., HH, D., SN, J., LR, L. & YS, L. 2010. Genome sequence of the pea aphid *Acyrthosiphon pisum*. *Plos Biology,* 8**,** e1000313-e1000313.

RASBAND, W. S. 2014. ImageJ. U. S. National Institutes of Health, Bethesda, Maryland, USA,.

RATZKA, C., GROSS, R. & FELDHAAR, H. 2013. Gene expression analysis of the endosymbiont-bearing midgut tissue during ontogeny of the carpenter ant *Camponotus floridanus*. *Journal of Insect Physiology,* 59**,** 611-623.

READ, S., MARZORATI M, GUIMARÃES BC & BOON N 2011. Microbial resource management revisited: successful parameters and new concepts. *Applied Microbiology and Biotechnology,* 90**,** 861-871.

REGASSA, L. & GASPARICH, G. 2005. *Spiroplasmas*: evolutionary relationships and biodiversity. *Frontiers in Bioscience,* 11**,** 2983-3002.

REGASSA, L. B. & GASPARICH, G. E. 2006. Spiroplasmas: evolutionary relationships and biodiversity. *Frontiers Biosciences,* 11**,** 2983-3002.

REYMOND, N., CALEVRO, F., VIÑUELAS, J., MORIN, N., RAHBÉ, Y., FEBVAY, G., LAUGIER, C., DOUGLAS, A., FAYARD, J.-M. & CHARLES, H. 2006. Different levels of transcriptional regulation due to trophic constraints in the reduced genome of *Buchnera aphidicola* APS. *Applied and Environmental Microbiology,* 72**,** 7760-7766.

RIO, R. V., WU, Y.-N., FILARDO, G. & AKSOY, S. 2006. Dynamics of multiple symbiont density regulation during host development: tsetse fly and its microbial flora. *Proceedings of the Royal Society B,* 273**,** 805-814.

ROBERTS, K. E. & HUGHES, W. O. H. 2014. Immunosenescence and resistance to parasite infection in the honey bee, *Apis mellifera*. *Journal of Invertebrate Pathology,* 121**,** 1-6.

ROSS, L., DEALEY, E. J., BEUKEBOOM, L. W. & SHUKER, D. M. 2011. Temperature, age of mating and starvation determine the role of maternal

effects on sex allocation in the mealybug *Planococcus citri*. *Behavioral ecology and sociobiology***,** 1-11.

ROSS, L., LANGENHOF, M. B. W., PEN, I., BEUKEBOOM, L. W., WEST, S. A. & SHUKER, D. M. 2010a. Sex allocation in a species with paternal genome elimination: the roles of crowding and female age in the mealybug *Planococcus citri*. *Evolutionary Ecology Research,* 12**,** 89-104.

ROSS, L., PEN, I. & SHUKER, D. M. 2010b. Genomic conflict in scale insects: the causes and consequences of bizarre genetic systems. *Biological Reviews,* 85**,** 807–828.

ROSS, L., SHUKER, D. M., NORMARK, B. B. & PEN, I. 2012. The role of endosymbionts in the evolution of haploid-male genetic systems in scale insects (Coccoidea). *Ecology and evolution,* 2**,** 1071-1081.

RUBY, E. & ASATO, L. 1993. Growth and flagellation of *Vibrio fischeri* during initiation of the sepiolid squid light organ symbiosis. *Archives of Microbiology,* 159**,** 160-167.

RUSSELL, J. A., LATORRE, A., SABATER MUÑOZ, B., MOYA, A. & MORAN, N. A. 2003. Side stepping secondary symbionts: widespread horizontal transfer across and beyond the Aphidoidea. *Molecular Ecology,* 12**,** 1061-1075.

RUSSELL, J. A. & MORAN, N. A. 2005. Horizontal transfer of bacterial symbionts: heritability and fitness effects in a novel aphid host. *Applied and Environmental Microbiology,* 71**,** 7987.

SACCHI, L., GENCHI, M., CLEMENTI, E., NEGRI, I., ALMA, A., OHLER, S., SASSERA, D., BOURTZIS, K. & BANDI, C. 2010. Bacteriocyte-like cells

harbour *Wolbachia* in the ovary of *Drosophila melanogaster* (Insecta, Diptera) and *Zyginidia pullula* (Insecta, Hemiptera). *Tissue and Cell,* 42**,** 328-333.

SAFFO, M. B. 1992. Invertebrates in endosymbiotic associations. *American Zoologist,* 32**,** 557-565.

SAKURAI, M., KOGA, R., TSUCHIDA, T., MENG, X. Y. & FUKATSU, T. 2005. *Rickettsia* symbiont in the pea aphid *Acyrthosiphon pisum*: novel cellular tropism, effect on host fitness, and interaction with the essential symbiont *Buchnera*. *Applied and Environmental Microbiology,* 71**,** 4069-4075.

SANDSTRÖM, J. & MORAN, N. How nutritionally imbalanced is phloem sap for aphids? Proceedings of the 10th International Symposium on Insect-Plant Relationships, 1999. Springer, 203-210.

SANDSTRÖM, J. P., RUSSELL, J. A., WHITE, J. P. & MORAN, N. A. 2001. Independent origins and horizontal transfer of bacterial symbionts of aphids. *Molecular Ecology,* 10**,** 217-228.

SANTA-CECÍLIA, L., PRADO, E., DE SOUSA, M., DE SOUSA, A. & CORREA, L. 2011. Effects of temperature in the development and survival of the mealybug *Pseudococcus longispinus* (Targioni Tozzeti, 1867)(Hemiptera: Pseudococcidae) in coffee plants. *Coffee Science,* 6**,** 91-97.

SARIDAKI, A. & BOURTZIS, K. 2010. *Wolbachia*: more than just a bug in insects genitals. *Current Opinion in Microbiology,* 13**,** 67-72.

SCARBOROUGH, C. L., FERRARI, J. & GODFRAY, H. C. J. 2005. Aphid protected from pathogen by endosymbiont. *Science,* 310**,** 1781.

SCHMITTGEN, T. D. & LIVAK, K. J. 2008. Analyzing real-time PCR data by the comparative CT method. *Nature Protocols,* 3**,** 1101-1108.

SCHRADER, F. 1921. The chromosomes of *Pseudococcus nipae*. *Biological Bulletin,* 40**,** 259-270.

SCHWARTZ, R. M. & DAYHOFF, M. O. 1978. Origins of prokaryotes, eukaryotes, mitochondria, and chloroplasts. *Science,* 199**,** 395-403.

SETHER, D., ULLMAN D & HU J 1998. Transmission of pineapple mealybug wilt-associated virus by two species of mealybug (*Dysmicoccus* spp.). *Phytopathology,* 88.

SETHUSA, M. T., VAN DER BANK, M., VAN DER BANK, H. F. & MILLAR, I. M. 2013. DNA barcoding scale insects of economic importance in South Africa. University of Johannesburg.

SHIGENOBU, S., WATANABE, H., HATTORI, M., SAKAKI, Y. & ISHIKAWA, H. 2000. Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera sp.* APS. *Nature,* 407**,** 81-86.

SHIGENOBU, S. & WILSON, A. C. C. 2011. Genomic revelations of a mutualism: the pea aphid and its obligate bacterial symbiont. *Cellular and Molecular Life Sciences,* 68**,** 1297-1309.

SIMON, C., FRATI, F., BECKENBACH, A., CRESPI, B., LIU, H. & FLOOK, P. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America,* 87**,** 651-701.

SIMON, J. C., BOUTIN, S., TSUCHIDA, T., KOGA, R., LE GALLIC, J. F., FRANTZ, A., OUTREMAN, Y. & FUKATSU, T. 2011. Facultative symbiont infections affect aphid reproduction. *PloS one,* 6**,** e21831.

SINGH, S. T., KUMAR, J., THOMAS, A., RAMAMURTHY, V. & RAJAGOPAL, R. 2013. Detection and Localization of *Rickettsia* sp in Mealybug. *Environmental Entomology,* 42**,** 711-716.

SINTUPACHEE, S., MILNE, J. R., POONCHAISRI, S., BAIMAI, V. & KITTAYAPONG, P. 2006. Closely related *Wolbachia* strains within the pumpkin arthropod community and the potential for horizontal transmission via the plant. *Microbial Ecology,* 51**,** 294-301.

SKALJAC, M., ZANIC, K., BAN, S., KONTSEDALOV, S. & GHANIM, M. 2010. Co-infection and localization of secondary symbionts in two whitefly species. *BMC Microbiology,* 10**,** 142.

SLOAN, D. B., NAKABACHI, A., RICHARDS, S., QU, J., MURALI, S. C., GIBBS, R. A. & MORAN, N. A. 2014. Parallel histories of horizontal gene transfer facilitated extreme reduction of endosymbiont genomes in sap-feeding insects. *Molecular Biology and Evolution,* 31**,** 857-871.

SMITH, J. 2007. A gene's-eye view of symbiont transmission. *American Naturalist,* 170**,** 542-550.

SNYDER, A. K., DEBERRY, J. W., RUNYEN-JANECKY, L. & RIO, R. V. 2010. Nutrient provisioning facilitates homeostasis between tsetse fly (Diptera: Glossinidae) symbionts. *Proceedings of the Royal Society of London B: Biological Sciences,* 277**,** 2389-2397.

STAVER, C., GUHARAY, F., MONTERROSO, D. & MUSCHLER, R. G. 2001. Designing pest-suppressive multistrata perennial crop systems: shade-grown coffee in Central America. *Agroforestry Systems,* 53**,** 151-170.

STAVRINIDES, J., MCCLOSKEY, J. K. & OCHMAN, H. 2009. Pea aphid as both host and vector for the phytopathogenic bacterium *Pseudomonas syringae*. *Applied and Environmental Microbiology,* 75**,** 2230-2235.

STOLL, S., FELDHAAR, H. & GROSS, R. 2009. Transcriptional profiling of the endosymbiont *Blochmannia floridanus* during different developmental stages of its holometabolous ant host. *Environmental Microbiology,* 11**,** 877-888.

STUART, R., POLAVARAPU S, LEWIS EE & GAUGLER R 1997. Differential susceptibility of *Dysmicoccus vaccinii* (Homoptera: Pseudococcidae) to entomopathogenic nematodes (Rhabditida: Heterorhabditidae and Steinernematidae). *Journal of Economic Entomology* 90**,** 925-932.

SU, Q., XIE, W., WANG, S., WU, Q., GHANIM, M. & ZHANG, Y. 2014. Location of symbionts in the whitefly *Bemisia tabaci* affects their densities during host development and environmental stress. *PloS one,* 9**,** e91802.

SUNNUCKS, P. & HALES, D. F. 1996. Numerous transposed sequences of mitochondrial cytochrome oxidase I-II in aphids of the genus *Sitobion* (Hemiptera: Aphididae). *Molecular Biology and Evolution,* 13**,** 510-524.

SWAIN, T. D. 2012. Context-dependent effects of symbiosis: *Zoanthidae* colonization generally improves *Demospongiae* condition in native habitats. *Marine Biology,* 159**,** 1429-1438.

TANG, M., LV, L., JING, S., ZHU, L. & HE, G. 2010. Bacterial symbionts of the brown planthopper, *Nilaparvata lugens* (Homoptera: Delphacidae). *Applied and Environmental Microbiology,* 76**,** 1740-1745.

TAROURA, S., SHIMADA, Y., SAKATA, Y., MIYAMA, T., HIRAOKA, H., WATANABE, M., ITAMOTO, K., OKUDA, M. & INOKUMA, H. 2005. Detection of DNA of '*Candidatus* Mycoplasma haemominutum' and *Spiroplasma* sp. in unfed ticks collected from vegetation in Japan. *Journal of Veterinary Medical Science,* 67**,** 1277-1279.

THAO, M. L. & BAUMANN, P. 2004a. Evidence for multiple acquisition of *Arsenophonus* by whitefly species (Sternorrhyncha: Aleyrodidae). *Current Microbiology,* 48**,** 140-144.

THAO, M. L. L. & BAUMANN, P. 2004b. Evolutionary relationships of primary prokaryotic endosymbionts of whiteflies and their hosts. *Applied and Environmental Microbiology,* 70**,** 3401-3406.

THAO, M. L. L., GULLAN, P. J. & BAUMANN, P. 2002. Secondary (ɣ-proteobacteria) endosymbionts infect the primary (β-proteobacteria) endosymbionts of mealybugs multiple times and coevolve with their hosts. *Applied and Environmental Microbiology,* 68**,** 3190-3197.

THAO, M. L. L., MORAN, N. A., ABBOT, P., BRENNAN, E. B., BURCKHARDT, D. H. & BAUMANN, P. 2000. Cospeciation of psyllids and their primary prokaryotic endosymbionts. *Applied and Environmental Microbiology,* 66**,** 2898-2905.

THE INTERNATIONAL APHID GENOMICS CONSORTIUM 2010. Genome sequence of the pea aphid *Acyrthosiphon pisum*. *PLoS Biology,* 8**,** e1000313.

TITLYANOV, E., TSUKAHARA, J., TITLYANOVA, T., LELETKIN, V., VAN WOESIK, R. & YAMAZATO, K. 2000. Zooxanthellae population density and physiological state of the coral *Stylophora pistillata* during starvation and osmotic shock. *Symbiosis,* 28**,** 303-322.

TIWARI, S., PELZ-STELINSKI, K. & STELINSKI, L. L. 2011. Effect of *Candidatus* Liberibacter asiaticus infection on susceptibility of Asian citrus psyllid, *Diaphorina citri*, to selected insecticides. *Pest Management Science,* 67**,** 94-99.

TOKUDA, G. & WATANABE, H. 2007. Hidden cellulases in termites: revision of an old hypothesis. *Biology Letters,* 3**,** 336-339.

TORTOSA, P., CHARLAT, S., LABBÉ, P., DEHECQ, J.-S., BARRÉ, H. & WEILL, M. 2010. *Wolbachia* age-sex-specific density in *Aedes albopictus*: a host evolutionary response to cytoplasmic incompatibility? *PLoS ONE,* 5**,** e9700.

TSUCHIDA, T., KOGA, R. & FUKATSU, T. 2004. Host plant specialization governed by facultative symbiont. *Science,* 303**,** 1989.

TURNBAUGH, P. J. & GORDON, J. I. 2009. The core gut microbiome, energy balance and obesity. *Journal of Physiology,* 587**,** 4153-4158.

VAN DEN HEUVEL, J. F. J. M., VERBEEK, M. & VAN DER WILK, F. 1994. Endosymbiotic bacteria associated with circulative transmission of potato leafroll virus by *Myzus persicae*. *Journal of General Virology,* 75**,** 2559-2565.

VAN DER WILK, F., DULLEMANS, A. M., VERBEEK, M. & VAN DEN HEUVEL, J. F. J. M. 1999. Isolation and characterization of *AP*SE-1, a

bacteriophage infecting the secondary endosymbiont of *Acyrthosiphon pisum*. *Virology,* 262**,** 104-113.

VAN HAM, R. C., KAMERBEEK, J., PALACIOS, C., RAUSELL, C., ABASCAL, F., BASTOLLA, U., FERNÁNDEZ, J. M., JIMÉNEZ, L., POSTIGO, M. & SILVA, F. J. 2003. Reductive genome evolution in *Buchnera aphidicola*. *Proceedings of the National Academy of Sciences of the U.S.A.,* 100**,** 581-586.

VAN NIEKERK, S., & MALAN, AP, 2012. Potential of South African entomopathogenic nematodes (Heterorhabditidae and Steinernematidae) for control of the citrus mealybug, *Planococcus citri* (Pseudococcidae). *Journal of Invertebrate Pathology,* 111**,** 166-174.

VARNDELL, N. & GODFRAY, H. 1996. Facultative adjustment of the sex ratio in an insect (*Planococcus citri*, Pseudococcidae) with paternal genome loss. *Evolution***,** 2100-2105.

VENNILA, S., DESHMUKH, A. J., PINJARKAR, D., AGARWAL, M., RAMAMURTHY, W., JOSHI, S., KRANTHI, K. R. & BAMBAWALE, O. M. 2010. Biology of the mealybug, *Phenacoccus solenopsis* on cotton in the laboratory. *Journal of Insect Science,* 10**,** 1-9.

VERSTRAETE, W., WITTEBOLLE, L., HEYLEN, K., VANPARYS, B., DE VOS, P., VAN DE WIELE, T. & BOON, N. 2007. Microbial resource management: the road to go for environmental biotechnology. *Engineering in Life Sciences,* 7.

VIA, S. 2001. Sympatric speciation in animals: the ugly duckling grows up. *Trends in Ecology & Evolution,* 16**,** 381-390.

VIGNERON, A., MASSON, F., VALLIER, A., BALMAND, S., REY, M., VINCENT-MONÉGAT, C., AKSOY, E., AUBAILLY-GIRAUD, E., ZAIDMAN-RÉMY, A. & HEDDI, A. 2014. Insects recycle endosymbionts when the benefit is over. *Current Biology,* 24**,** 2267-2273.

VON BURG, S., FERRARI, J., MÜLLER, C. B. & VORBURGER, C. 2008. Genetic variation and covariation of susceptibility to parasitoids in the aphid *Myzus persicae*: no evidence for trade-offs. *Proceedings of the Royal Society B: Biological Sciences,* 275**,** 1089-1094.

VON DER SCHULENBURG, J. H. G., HABIG, M., SLOGGETT, J. J., WEBBERLEY, K. M., BERTRAND, D., HURST, G. D. & MAJERUS, M. E. 2001. Incidence of male-killing *Rickettsia* spp.(α-Proteobacteria) in the ten-spot ladybird beetle *Adalia decempunctata* L.(Coleoptera: Coccinellidae). *Applied and Environmental Microbiology*, 67**,** 270-277.

VON DOHLEN, C. D., KOHLER, S., ALSOP, S. T. & MCMANUS, W. R. 2001. Mealybug β-proteobacterial endosymbionts contain γ-proteobacterial symbionts. *Nature,* 412**,** 433-436.

VORBURGER, C., GEHRER, L. & RODRIGUEZ, P. 2010. A strain of the bacterial symbiont *Regiella insecticola* protects aphids against parasitoids. *Biology Letters*, 6**,** 109.

VORBURGER, C., SANDROCK, C., GOUSKOV, A., CASTAÑEDA, L. E. & FERRARI, J. 2009. Genotypic variation and the role of defenive endosymbionts in an all parthenogenetic host-parasitoid interaction. *Evolution,* 63**,** 1439-1450.

WALKER, T., JOHNSON, P. H., MOREIRA, L. A., ITURBE-ORMAETXE, I., FRENTIU, F. D., MCMENIMAN, C. J., LEONG, Y. S., DONG, Y., AXFORD, J. & KRIESNER, P. 2011. The *w*Mel *Wolbachia* strain blocks dengue and invades caged *Aedes aegypti* populations. *Nature,* 476**,** 450-453.

WANG, J., WU, Y., YANG, G. & AKSOY, S. 2009a. Interactions between mutualist *Wigglesworthia* and tsetse peptidoglycan recognition protein (PGRP-LB) influence trypanosome transmission. *Proceedings of the National Academy of Sciences of the U.S.A.,* 106**,** 12133-12138.

WANG, W., GU, W., DING, Z., REN, Y., CHEN, J. & HOU, Y. 2005. A novel *Spiroplasma* pathogen causing systemic infection in the crayfish *Procambarus clarkii* (Crustacea: Decapod), in China. *FEMS Microbiology Letters*, 249**,** 131-137.

WANG, Z., SHEN, Z., SONG, Y., LIU, H. Y. & LI, Z. X. 2009b. Distribution and diversity of *Wolbachia* in different populations of the wheat aphid *Sitobion miscanthi* (Hemiptera: Aphididae) in China. *European Journal of Entomology,* 106**,** 49-55.

WARNECKE, F., LUGINBÜHL, P., IVANOVA, N., GHASSEMIAN, M., RICHARDSON, T. H., STEGE, J. T., CAYOUETTE, M., MCHARDY, A. C., DJORDJEVIC, G. & ABOUSHADI, N. 2007. Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. *Nature*, 450**,** 560-565.

WATANABE, M., MIURA, K., HUNTER, M. S. & WAJNBERG, E. 2010. Superinfection of cytoplasmic incompatibility-inducing *Wolbachia* is not

additive in *Orius strigicollis* (Hemiptera: Anthocoridae). *Heredity,* 106**,** 642-648.

WEEKS, A. R. & BREEUWER, J. A. J. 2001. *Wolbachia*–induced parthenogenesis in a genus of phytophagous mites. *Proceedings of the Royal Society of London. Series B: Biological Sciences,* 268**,** 2245-2251.

WEEKS, A. R., VELTEN, R. & STOUTHAMER, R. 2003. Incidence of a new sex–ratio–distorting endosymbiotic bacterium among arthropods. *Proceedings of the Royal Society of London B: Biological Sciences,* 270**,** 1857-1865.

WEINERT, L. A., ARAUJO-JNR, E. V., AHMED, M. Z. & WELCH, J. J. 2015. The incidence of bacterial endosymbionts in terrestrial arthropods. *Proceedings of the Royal Society of London B: Biological Sciences,* 282.

WEINERT, L. A., WERREN, J. H., AEBI, A., STONE, G. N. & JIGGINS, F. M. 2009. Evolution and diversity of *Rickettsia* bacteria. *BMC Biology,* 7**,** 6.

WEISBURG, W. G., TULLY, J. G., ROSE, D. L., PETZEL, J. P., OYAIZU, H., YANG, D., MANDELCO, L., SECHREST, J., LAWRENCE, T. G. & VAN ETTEN, J. 1989. A phylogenetic analysis of the mycoplasmas: basis for their classification. *Journal of Bacteriology,* 171**,** 6455-6467.

WERNEGREEN, J. J. 2002. Genome evolution in bacterial endosymbionts of insects. *Nature Reviews Genetics,* 3**,** 850-861.

WHITE, J. A., KELLY, S. E., PERLMAN, S. J. & HUNTER, M. S. 2009. Cytoplasmic incompatibility in the parasitic wasp *Encarsia inaron*: disentangling the roles of *Cardinium* and *Wolbachia* symbionts. *Heredity,* 102**,** 483-489.

WIGGLESWORTH, V. B. 1952. Symbiosis in blood-sucking insects. *Tijdschr. Entomol,* 95**,** 63-68.

WILCOX, J. L., DUNBAR, H. E., WOLFINGER, R. D. & MORAN, N. A. 2003. Consequences of reductive evolution for gene expression in an obligate endosymbiont. *Molecular Microbiology,* 48**,** 1491-1500.

WILD, A. 2015. *Alex Wild Photography* [Online]. Available: http://www.alexanderwild.com.

WILKINSON, T., ADAMS, D., MINTO, L. & DOUGLAS, A. 2001. The impact of host plant on the abundance and function of symbiotic bacteria in an aphid. *Journal of Experimental Biology,* 204**,** 3027-3038.

WILKINSON, T., KOGA, R. & FUKATSU, T. 2007. Role of host nutrition in symbiont regulation: impact of dietary nitrogen on proliferation of obligate and facultative bacterial endosymbionts of the pea aphid *Acyrthosiphon pisum*. *Applied and Environmental Microbiology,* 73**,** 1362-1366.

WILSON, A. C., DUNBAR, H. E., DAVIS, G. K., HUNTER, W. B., STERN, D. L. & MORAN, N. A. 2006. A dual-genome microarray for the pea aphid, *Acyrthosiphon pisum*, and its obligate bacterial symbiont, *Buchnera aphidicola*. *BMC Genomics,* 7**,** 50.

WILSON, A. C. & DUNCAN, R. P. 2015. Signatures of host/symbiont genome coevolution in insect nutritional endosymbioses. *Proceedings of the National Academy of Sciences,* 112**,** 201423305.

WILSON, A. C. C., ASHTON, P. D., CALEVRO, F., CHARLES, H., COLELLA, S., FEBVAY, G., JANDER, G., KUSHLAN, P. F., MACDONALD, S. J. & SCHWARTZ, J. F. 2010. Genomic insight into the amino acid relations of

the pea aphid, *Acyrthosiphon pisum*, with its symbiotic bacterium *Buchnera aphidicola*. *Insect Molecular Biology,* 19**,** 249-258.

WINDSOR, D. A. 1998. Most of the species on Earth are parasites. *International Journal for Parasitology,* 28**,** 1939-1941.

YASAKI, Y. 1928. On the nature of the luminescence of the knight-fish (*Monocentris japonicus* (Houttuyn)). *Journal of Experimental Zoology,* 50**,** 495-505.

YU-FENG, Q., YONG-TENG, L., XIANG-DONG, L., JI-CHAO, F. & HUI-FANG, G. 2015. Relationships between infection with facultative symbionts and sex ratio of Bemisia tabaci on different host plants. *Chinese Journal of Applied Entomology,* 52**,** 89-95.

ZABALOU, S., RIEGLER, M., THEODORAKOPOULOU, M., STAUFFER, C., SAVAKIS, C. & BOURTZIS, K. 2004. *Wolbachia*-induced cytoplasmic incompatibility as a means for insect pest population control. *Proceedings of the National Academy of Sciences of the United States of America,* 101**,** 15042–15045.

ZCHORI-FEIN, E. & PERLMAN, S. J. 2004. Distribution of the bacterial symbiont *Cardinium* in arthropods. *Molecular Ecology,* 13**,** 2009-2016.

ZHANG, F., GUO, H., ZHENG, H., ZHOU, T., ZHOU, Y., WANG, S., FANG, R., QIAN, W. & CHEN, X. 2010. Massively parallel pyrosequencing-based transcriptome analyses of small brown planthopper (*Laodelphax striatellus*), a vector insect transmitting rice stripe virus (RSV). *Biomedical Central Genomics,* 11.

ZHANG, X., TANG, S. & CHEKE, R. A. 2015. Birth-pulse models of *Wolbachia*-induced cytoplasmic incompatibility in mosquitoes for dengue virus control. *Nonlinear Analysis: Real World Applications,* 22**,** 236-258.

ZHONG, J., JASINSKAS, A., BARBOUR, A. G. & ROMESBERG, F. 2007. Antibiotic treatment of the tick vector *Amblyomma americanum* reduced reproductive fitness. *PloS ONE,* 2**,** e405-e405.

ZIENTZ, E., DANDEKAR, T. & GROSS, R. 2004. Metabolic interdependence of obligate intracellular bacteria and their insect hosts. *Microbiology and Molecular Biology Reviews,* 68**,** 745-770.

ZILBER-ROSENBERG, I. & ROSENBERG, E. 2008. Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution. *Federation of European Microbiological Societies Microbiology Reviews,* 32**,** 723-735.