AN ATTEMPTED TRANSINFECTION OF *WOLBACHIA* IN THE WESTERN HONEY BEE (*APIS MELLIFERA*)

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ABSTRACT

Wolbachia, an intracellular endosymbiont found in up to 60% of arthropods, has been celebrated for its highly varied host-phenotype interactions. These effects are diverse, ranging from reproductive manipulations to obligate mutualisms and facultative symbiosis. These facultative effects include increased resistance to, and reduction in the ability to vector, a number of RNA viruses in insects. Artificial transinfection to mediate human vector-borne diseases such as *Dengue* fever and *Zika virus* in *Aedes* mosquitoes has had considerable success globally. However, using *Wolbachia* to mediate zoonotic disease directly in threatened species has not been examined. The Western honey bee (*Apis mellifera*) has shown significant global population declines across the US and Europe, suffering from a diverse range of pathogens, including viral RNA and parasite vector networks. *Wolbachia* infection in honey bees has only been detected once and its effects have not been investigated. Here, I present the first attempted transinfection of *Wolbachia* in the Western honey bee using established transinfection protocols.

The natural, but rarely found, *Wolbachia* infection reported in *A. mellifera* was examined against a robust phylogeny of all existing *Wolbachia* supergroups, a feat that has not been updated in the literature since 2015. I discovered *Wolbachia* infection in *Ancistrocerus gazella*, the European tube wasp, where it has never been observed. I isolated the natural *Wolbachia* strain hosted by *Drosophila melanogaster* (*w*Mel) and more than 1200 individuals from a range of honey bee life stages (from eggs to adults) were used as potential *Wolbachia* recipients using sound microinjection protocols. Additionally, I present a novel transinfection avenue utilizing artificial insemination and honey bee breeding using *Wolbachia*-inoculated drone semen.

When no individuals were successfully infected with *Wolbachia* in F_0 or F_1, I investigated the expression of several antimicrobial peptides to characterize the immune response in young larvae to *Wolbachia* microinjection. There was a significant upregulation of apidaecin when injected with live *Wolbachia*, but not heat-treated bacteria, which has never been reported in host immune response to *Wolbachia* previously. The findings presented in this study highlight the importance of *Wolbachia* strain selection, immune response to *Wolbachia*, and the potential requirement for cell line culture in future transinfection attempts into *A. mellifera.* These findings will help inform future transinfection attempts, which are encouraged.

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The busy bee has no time for sorrow.

– William Blake

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CHAPTER 1

General Introduction

THE IMPORTANCE OF THE HONEY BEE

Pollination

Pollination is one of the most ecologically and economically important ecosystem services on Earth (Klein, *et al.*, 2007; Potts, *et al.*, 2010). Eighty-seven percent of angiosperm plants (approximately 308,000 species) depend, at least in part, on animal pollination for sexual reproduction (Ollerton, *et al*., 2011). Three-quarters of leading food crops, occupying 33-35% of all agricultural land in the world, benefit from animal pollination. Of the 107 global leading food crops, 85% rely on animal pollination (Williams, 1994; Gallai, *et al*., 2009) and the annual market value of the 5-8% of global food production directly linked with pollination services is estimated between US$235 billion and US$577 billion worldwide (Lautenbach, *et al*., 2012). Most pollinators of agricultural importance are insects, particularly the hymenopteran Apidae which are the most abundant and diverse pollinators, with over 20,000 species worldwide (Neff & Simpson, 1993; Klein, *et al*., 2007; Michener, 2007; Kleijn, *et al*., 2015).

Of these insects, the Western honey bee (*Apis mellifera* Linnaeus, and its subspecies) is thought to be the most important, with their management being the basis of global food security (Klein, *et al*., 2007). Of the leading 107 global crops, 91 of the leading global food crop commodities are dependent on them, generating over US$215 billion annually (Le Conte & Navajas, 2008; vanEnglesdorp & Meixner, 2010). Without honey bees, the yield of these crop species have been estimated to be reduced by up to 96% (Potts *et al*., 2010), with an associated significant reduction in seed set and recruitment (Klein, *et al*., 2007). The honey bee is now considered by some to be the "most important productive livestock" (Stoic, *et al*., 2016) and, due to its high pollination efficiency, is the primary choice of agricultural pollinator worldwide (Le Conte & Navajas, 2008; Klein, *et al*., 2007).

Pollinator-dependent agriculture has seen a 300% rise in line with globalized communities and human population growth over the last 50 years (Aizen & Harder, 2009). However, the rapid increase in agricultural production greatly exceeds that of the global stock of domesticated honey bees, which have only steadily risen by 45% since 1961 (Aizen & Harder, 2009). Long-term declines in bee populations in Europe and the United States, which are areas with some of the largest food consumption in the world, has

rung alarm bells regarding the pollination deficit that continues to grow in these areas. An evaluation by Gillai *et al*. (2009) concluded that in the event of a loss of insect pollination in highly populated areas, particularly in the US and Europe, the human consumption of fruit, vegetable, and stimulant crops (such as coffee) would exceed global production by up to 50%. Less densely populated areas would also be heavily impacted, including vegetable deficits throughout Africa and Oceania, and a shortfall of fruit to North America and West Africa. This scenario is particularly pertinent areas that produce more than their fair share of produce for export, such as East Asia, where the loss of honey bees would result in an overall deficit in fruit production of 26%, which would represent a considerable loss since the region produces 20% of global fruit output (Gillai, *et al*., 2009).

The human health implications resulting from honey bee losses through crop production reductions would be substantial, particularly in developing countries (Ellis, *et al*., 2015). Up to 56% of all people in Zambia, Uganda, Mozambique, and Bangladesh would be at risk of vitamin A, calcium, folate, iron, and zinc deficiencies in scenarios where honey bees and other pollinators are eliminated (Ellis, *et al*., 2015). Vitamin A deficiency causes an estimated 800,000 deaths worldwide in women and children currently, and roughly doubles the risk of death from common conditions such as measles, diarrhea, and malaria, and increases the risk of maternal mortality four-fold (Rice *et al*., 2004). Honey bee decline would, therefore, not only directly reduce food production, but would indirectly increase disease frequency, particularly in countries with limited medical resources. Additionally, the disappearance of bees will impact the 87.5% of wild angiosperms that are reliant on animal pollination, thus impacting plant diversity worldwide (Kevan & Phillips, 2001; Klein, 2007; Ollerton, *et al*., 2011).

Apiculture

Despite the slow increase in global honey bee stocks for pollination, apiculture is a commercial growth industry, with rapid global increases in natural honey export profit value averaging 10% profit growth between 2011 and 2015 (UN Comtrade Database, 2016). In 2015, the total global honey trade exceeded US$4.56 billion from over 140 countries (UN Comtrade Database, 2016), which is a fourfold increase from 2007. Producers of raw honey vary in quantity and quality. China is the world's largest mass producer with over 144,7586 tonnes exported in 2015, with an average wholesale value of US$1.99/kg. In the same year New Zealand produced an entire order of magnitude less honey for export but had the highest export value by over US$7.7/kg worldwide. New Zealand currently exports raw honey products for an average of US$20/kg and up to US$80/kg for wholesale honey (MPI NZ, 2015). High-value honey exports have driven a boom in New Zealand apiculture industries where exports are successful (MPI NZ, 2016, 2017).

In New Zealand, the growth in global apiculture is exemplified, with exports increasing from US$25 million to $200 million per annum and with an increase in price/kg of $6.25/kg to $18.71/kg between 2011

and 2016 (UN Comtrade Database, 2016). The drive in honey profits has promoted the increase of registered beekeeping enterprises. In New Zealand alone the number of registered apiaries almost doubled between 2010 and 2015, with an increase of approximately 200,000 registered hives in the same period. Manuka honey, produced from nectar of the New Zealand native Manuka plant, *Leptospermum scoparium*, has soared in popularity throughout global markets due to its antibacterial properties and proposed health benefits (Saikaly & Khachemoune, 2017). Today, New Zealand is the world's third-largest honey producer by value, but only 16th in the world by volume, highlighting the premium international prices of Manuka honey, that makes up the majority of the country's apiculture economy (MPI NZ, 2016).

The increased global demand for honey has led to increased hive densities in areas of high value, such as in Manuka scrubland. This increase in density has been suggested to malnourishment of honey bees due to inadequate floral resources (Decourtye, *et al*., 2010), fighting and robbing between hives, and the increased spread of pathogens. The combination of high density hive placement and the periodic movement of hives, often to follow seasonal crop pollination, has led to the widespread distribution of honey bee diseases (Berthoud, *et al*., 2010). Malnourishment, particularly of dietary protein (pollen), can lead to a range of problems for bees, such as insufficient essential amino acids needed for the synthesis of peptides in immune pathways (Grimble, 2001; Schmid-Hempel, 2005). Further, a lack of carbohydrates (nectar and honey) leads to reduced foraging in bees and energy-deficiency for metabolic processes related to immune response, causing increased vulnerability to microsporidian infection and disease (Schmid-Hempel, 2005).

HONEY BEES AT RISK

Western honey bees are vulnerable to, and are suffering from, a diverse range of pathogens and parasites. These range from bacterial and fungal infection, trypanosome protozoans, and DNA and RNA viruses (Cox-Foster, *et al*., 2007; Evans, *et al*., 2011). Extreme cases of honey bee loss has previously been seen in colony collapse disorder (CCD). Colony collapse disorder is a phenomenon wherein substantial honey bee hive losses in the US and Europe between 2005-2012 were ascribed to the sudden disappearance of adult worker bees despite the lack of evidence of dead workers near the hive, with adequate food and brood stores (vanEngelsdorp, *et al*., 2010). Multiple stressors appear responsible for CCD, with networks of pathogens being the main suspect, including the parasitic *Varroa* mite (*Varroa destructor)* and viruses it vectors (Schroeder & Martin, 2012; Cornman, *et al*., 2012). Of these viruses *Deformed wing virus* (DWV), that appears to be mutualistic with *Varroa*, is considered to be the most likely candidate for the majority of global honey bee colony loss worldwide over the last 50 years (Van Englesdorp, *et al*., 2009; Nazzi *et* al., 2012; Schroeder & Martin, 2012).

Varroa-vectored DWV plays an important role in 'parasitic mite syndrome' which causes *Varroa*-induced colony collapse, evidenced by field observation and through modelling approaches (Hung, *et al*., 1995, 1996; Martin, 2001). This syndrome describes the symptoms of an overt DWV outbreak at colony level, where fast brood turnover in spring can compensate for DWV-related bee losses until brood rearing slows in the autumn when virus epidemic accelerates, and the excessive loss of working bees causes the hive numbers to drop rapidly, and ultimately die (de Miranda & Genersch, 2010). The movement and distribution of the *Varroa* mite has been followed with elevated colony losses throughout the Northern Hemisphere (Neumann & Carreck, 2010). These losses included approximately 40% in the USA up to 53% and 85% losses in Europe and the Middle East, respectively and considerable losses throughout Japan and Taiwan compared to pre-*Varroa* levels (Crailsheim, *et al*., 2009; Gutierrez, 2009; Haddad *et al*., 2009; Soroker, *et al*., 2009; vanEnglesdorp, *et al*., 2009, 2010). Currently, the only viable methods to control these mites are chemicals and pesticides, and alternative miticides (De Miranda & Genersch, 2010, Ziegelmann, *et al*., 2017). However, mites quickly develop pesticide resistance and mite-killing pesticide residues have been found in honey, reducing sale value and exporter reputation (Johnson *et al*., 2010).

Insect sociality has a direct impact on disease transfer for insect colonies (Evans, *et al*., 2006). For example, in honey bee colonies thousands of individuals interact in close quarters in densities unparalleled in vertebrate groups. Combined with a homeostatic hive environment and presence of high-sugar food resources, bees are attractive targets for disease agents (Schmid-Hempel, 1998; Evans, *et al*., 2006). Bees have therefore developed "social immunity" strategies to combat many diseases, including grooming, hive hygiene and other behavioural traits to reduce the densities and impact of pathogen microsporidians and parasitic mites (Evans & Spivak, 2010). Social hygiene behaviours are very prominent in bees, particularly allogrooming, which is characterised by worker bees' detection of infected larvae and its removal and disposal from among healthy brood (eggs and larvae) (Spivak & Reuter, 2001). Additionally, auto-grooming exists, wherein bees will remove foreign pathogens, such as tracheal mites, from themselves (Evan & Spivak, 2010). However, these social behaviours are less effective when the bees are naïve to the pathogen, as is the case with the Western honey bee and its interactions with *Varroa* mite. Where the Eastern honey bee (*Apis cerana*) has evolved with *V. destructor*, it elicits effective allogrooming strategies to keep mite levels below a threshold level (Evans & Spivak, 2010) and prevents high levels of *Varroa*-vectored disease. However, as the mite is novel to the Western honey bee, these bees have not evolved such grooming behaviours and it is unable to remove the parasite (Rath & Drescher, 1990).

Breeding programs targeting *Varroa*-resistant bees exist, prioritizing stocks with desirable behavioural phenotypes, in particular bees that are able to detect, uncap and remove diseased brood (Spivak & Reuter, 2001). Auto-grooming, wherein the mites may be injured or killed (Ruttner & Hanel, 1992) or a promotion of behaviours of workers to attack free roaming mites (evidenced by dead mites on hive floors

with damage caused by bee mandibles is desirable (Fries, *et al*., 1996; Rinderer, *et al*., 2010). Indeed, attempts by apiarists at breeding brood that are unattractive to *Varroa* parasitism are currently being undertaken, as is selecting for unattractive larval food and comb properties (Nazzi, *et al*., 2001). Ultimately, however, it is unlikely that these approaches will make Western honey bees completely resistant to *Varroa* but they may eventually keep infestations to manageable levels as in *A. cerana* hives, (Peng, *et al*., 1987; Rinderer, *et al*., 2010).

Insect immune system

Individually, the honey bee immune system provides some degree of defense against pathogens (Casteels-Jossen, *et al*., 1994; Evans, 2004). Like most insects, honey bees are protected by a layer of antimicrobial secretion on the exoskeleton, and by a gut environment that is hostile to many potential pathogens (Evans, 2006). If pathogens move past the gut, the epithelium and peritrophic membrane lining the digestive tract are often sufficient to prevent further progress (DeGrandi-Hoffman & Chen, 2015). However, the Western honey bee's naivety to *Varroa* is illustrated by the mite's method of transmitting disease by piercing the bee's exoskeleton to get directly into the haemolyph, thereby bypassing the rigorous gut protection (Bowen-Walker & Gunn, 2001). When this barrier is breached, the pathogen is met by an cellular and humoral immune defense system that shares many parallels to the innate immune system found in vertebrates (Hoffman, 2003; Beutler, 2004; Evans, *et al*., 2006). The activation of the innate immune system relies of the recognition on highly conserved structural motifs on the surface of pathogens, which are not found on the host by pattern recognition receptor proteins (Brutscher & Flenniken, 2015; DeGrandi-Hoffman & Chen, 2015). Individual pathways within the honey bee immune system deal, with some cross-talk, with different pathogens. These pathways include the Jak/STAT and RNA interference (RNAi) pathways, both of which deal with only with viruses; the immune deficiency (Imd) pathway that processes virus particles and gram-negative bacteria; and the Toll pathway that is typically activated by fungi, gram positive bacteria and virulence factors (Evans, *et al*., 2006). Ultimately, these cascades regulate the transcription of target genes encoding effector molecules, including the rapid and transient synthesis of antimicrobial peptides (Hoffman & Reichhart, 1996).

Antimicrobial peptides (AMPs) are an important component of the humeral immune system (Giuliani, *et al*., 2007; Danihlik, *et al*., 2015) and over 200 have been described in insects (Li, *et al*., 2012). AMPs are secreted by insect fat bodies and hemocytes, and are secreted in response to microbial infection and septic wounding (Turner, 1994; Evans, *et al*., 2006; Laughton, *et al*., 2011). Most AMPs are classed as catatonic or amphipathic peptides, which allow them to interact with and disrupt negatively charged lipid membranes containing lipopolysaccharides, often found in microbes. AMPs interact with their target microbes in a number of ways, including the interaction of peptides resulting in the formation of channels

which enable the leakage of small ions and essential metabolites (Danihlik, *et al*., 2015). In some cases, this process allows the penetration of large molecules like peptides and small proteins with fatal effects for the target bacteria (Hancock, 1997; Shen, *et al*., 2010). Additionally, AMPs can interact directly with inner proteins, DNA, RNA or microbial cell compartments in their target (Maróti, *et al*., 2011). For instance, proline-rich peptides are able to pass through plasmatic membrane and bind to bacterial heat shock proteins and interact with chaperonins without lytic effect to the prokaryotic membrane (Otvos, *et al*., 2000; Kragol, *et al*., 2001; Scocchi, *et al*., 2009).

The insect microbiome is extensive and is a mixture of beneficial, pathenogenic and neutral resident bacteria (Engel, *et al*., 2012). In the case of internal symbionts, the insect immune system, while protecting its host from invading micro-organisms must also support this diverse microbiota that can contribute to various host functions, including immune function (Douglas, 2011). In the case of infection with endosymbionts, the host immune system function is challenged by distinct and conflicting selection pressures (Feldhaar & Gross, 2008, 2009). In the case of obligate symbionts, the host immune system may adapt via reduction, inhibiting the detection of specific bacterial species (Douglas, 2011). However, facultative or parasitic symbionts have not co-evolved with their host, and two alternative explanations exist for the lack of host detection: (i) the symbiont is being recognized as part of the host; (ii) lack of immune response is due to a mechanism of the symbiont evading immunity or causing the silencing of elicitors of immunity (Hurst, *et al*., 2003). Honey bees host a number of these naturally occurring facultative endosymbionts, including in the genera *Arensophononus*, *Spiroplasma* and *Rickettsia* (Yanez, *et al*., 2016).

WOLBACHIA

Not all microbes are pathogenic. In fact, gut microbiota is now considered to play a vital role in protecting hosts from infection (Sekirov, *et al*., 2010; Cryan & Dinan, 2012). For example, in humans, female vaginal microbes can provide resistance to sexually transmitted pathogens, such as HIV (Pyles *et al*, 2014). New treatments for human gastrointestinal disorders include fecal transplants to move bacterial microbiota from one human to another in order to fight disease (Pamer, 2014). Gut microbiota have been indicated to communicate with the central nervous system and has influence on brain function and behaviour (Cryan & Dinan, 2012).

Similarly, within honey bees, there is evidence to suggest that gastrointestinal bacteria play a key role in combating disease (Wu, *et al*., 2013). Like other species, the honey bee microbiome is made up of a mix of beneficial, commensal, and parasitic bacteria (Engel, *et al*., 2016). In honey bees, evidence has been found that certain species of *Firmicutes* bacteria can inhibit the growth of two principal pathogens,

Paenibacillus larvae and *Melissococcus plutonius*, responsible for American and European Foulbrood disease, respectively (Forsgren, *et al*., 2010; Vasquez, *et al*., 2012). In bumble bees, variation in gut community has been shown to either hinder or encourage the proliferation of the parasite *Crithidia bombi* (Koch, *et al*., 2011; Carvieau, *et al*., 2014). Some symbiotic species of bacteria have been shown to have a range of effects when hosted by different insect species across various orders. Of these, *Wolbachia* is the most prolific (Warren, *et al*., 2008; LePage & Borderstein, 2013).

Bacterial strains in the genus *Wolbachia* are Gram-negative cytoplasmically and maternally inherited within the Rickettsiaceae family that are present in the reproductive tissues of a plethora of invertebrates (Warren, 2008). These infections confer a variety of effects on their hosts, ranging from reproductive manipulation that benefits the transmission of the bacteria, to obligate mutualisms in nematodes, as well as facultative mutualisms in a variety of taxa. These facultative effects have various non-pathogenic effects, including increased virus resistance (Hoffmann, *et al*. 2011; Walker, *et al*. 2011). It is currently thought that *Wolbachia* is the most widely abundant intercellular bacterial species in insects (LePage & Borderstein, 2013). Present in all major insect orders, as well as arachnids and isopods, *Wolbachia* is being hailed as one of the biggest bacteria 'pandemics' in the history of life from a biodiversity perspective (LePage & Borderstein, 2013).

Wolbachia has attracted considerable attention for its ability to manipulate its hosts. Application potential of the conferred bacteria-host phenotypes effects are extensive. These applications range from increasing *Wolbachia* abundance in populations of mosquitoes to limit reproduction and lower population numbers through induction of cytoplasmic incompatibility (Walker, *et al*, 2011, Hoffman, *et al*., 2011), to transinfecting species of arthropods with strains of *Wolbachia* to prevent the vectoring of viruses (Dutra, *et al*., 2016; Aliota, *et al*. 2016), to the curing of *Wolbachia* where it is obligate to nematodes in order to reduce the cases of filariasis (Iturbe-Ormaetxe, *et al*., 2011; Tamarozzi, *et al*., 2014). Previously, these applications have only been used in order to mediate human diseases. However, there is scope for these *Wolbachia*-induced phenotypes to be applied to zoonotic diseases, particularly in threatened invertebrates, and perhaps honey bees (*Apis mellifera*). *Wolbachia* has been reported in very low densities in a common honey bee suspecies (*A. m. carnica*) (Pattabhiramaiah, *et al*., 2011). However, most authors consider it uncommon or a seasonal infection in these honey bees (Evison, *et al*., 2011; Yanez, *et al*., 2016).

Numerous studies have reported that *Wolbachia* infection confers anti-pathogenic effect in its hosts. Pathogens include several RNA viruses, *Plasmodium* species, fungi, and bacteria. Resistance is determined by an interaction between *Wolbachia* strain and host taxa (Zug & Hammerstein, 2016). *Drosophila melanogaster* wild-type flies infected with a strain of *Wolbachia* (*w*Mel, and its variants *w*MelCS and *w*MelPop) survive significantly longer when infected with RNA viruses, than do those without *Wolbachia*

(Hedges, *et al*. 2008). Moreover, *w*Mel-infected *Aedes aegypti* mosquitoes cannot carry viral dengue fever (Walker, *et al*., 2009, 2011). This has led to a population replacement strategy by the Eliminate Dengue Project (EDP) in Australia, South East Asia, and South America, with promising results. This success has encouraged the use of similar techniques to reduce the levels of malaria in a range of third world countries. The main carriers of *Plasmodium* malaria (Anopheline mosquitoes) do not naturally possess *Wolbachia* infection and are of particular interest. Anopheline mosquitoes transinfected with *Wolbachia* had few defects and caused refractoriness to *Plasmodium* infection (Bian, *et al*., 2013). Strains of *Wolbachia* naturally present in tsetse flies, the sole vectors of African trypanosomes, have the potential for the eradication of sleeping sickness and nagana (a livestock disease) (Doudoumis, *et al.*, 2012). Additionally, and of considerable recent interest, is the use of *Wolbachia* to reduce the spread of *Zika* virus (ZIKV). *Zika* is an arbovirus (spread by arthropods) that is responsible for an outbreak of febrile diseases in the Americas, and is vectored by *A. aegypti* mosquitoes. *Wolbachia*-infected *A. aegypti* are highly resistant to two currently circulating ZIKV strains (Dutra, *et al*., 2016; Aliota, *et al*. 2016). *Wolbachia*-harbouring individuals displayed lower viral prevalence and abundance, decreased disseminated infection, and, critically, did not carry ZIKV particles in the saliva, suggesting transmission had been blocked.

Computer modelling predicts that host protection will evolve in vertically transmitted parasites when they compete with horizontally transmitted pathogens in the same host (Zug & Hammerstein, 2012). As such, *Wolbachia* can maintain its position in the host population (Hedges, *et al*., 2008). *Wolbachia* possess a number of genetic abnormalities that are different from the rest of the Rickettsailles and these have been accredited to its success as an intracellular invader. Similar to the rest of the Ricketsailes, *Wolbachia* have small genomes (1.1 - 1.7 million base pairs) and are in accordance with the reductive trend following adaption to the host cell (Langridge, *et al*., 2015). However, *Wolbachia* genomes contain a high number of mobile and repetitive elements, with the latter comprising approximately 15% of the *w*Mel strain genome. These repeats undergo fast rates of recombination, allowing for quick adaptation into host cells. Most repeats transcribe Ankyrin domain proteins (ANK). These mediate the attachment of integral membrane proteins to the spectrin-actine based membrane cytoskeleton and are common in eukaryotes but unusual in bacteria. These repeats might mediate host-pathogen interactions (Wu, *et al*., 2004). Generally, the presence of repeats and mobile elements, is incongruent with the partitioning into parasitic or mutualistic strains among the Rickettsiales. Interestingly, *Wolbachia* strains that facilitate obligate nematodes symbiosis often have fewer repetitive elements and do not lack phage sequences (Fenn & Blaxter, 2006). Ankyrin domain proteins and prophage genes might be associated with *Wolbachia*-host cell interactions (Sinkins, *et al*., 2005). Direct effects of ANK and prophage genes within the bacteria phenotype remain unclear. However, their expression profiles appear to be associated with reproductive phenotypes (Walker, *et al*., 2007).

The mechanisms underlying the antiviral effects of *Wolbachia* are poorly understood. Antiviral effects are more common than antibacterial activity, suggesting their underlying mechanisms are independent as they do not occur together (Wong, *et al*., 2011; Rottschaefer & Lazzaro, 2012). To date, reported antiviral effects are currently known to be limited to RNA viruses. *Wolbachia* density is correlated with strength of these antiviral properties (Graham, *et al*., 2012). Two mechanistic explanations exist. Firstly, viral interference might result from the success of *Wolbachia* in competition for host resources (Osborne, *et al*., 2009, 2012; Frentiu, *et al*., 2010; Wong, *et al*., 2011; Lu, *et al*., 2012). Alternatively, several studies have suggested that *Wolbachia* might upregulate host immune response, particularly genes involved in the Toll and Immune Deficiency (IMD) pathway (Kambris, *et al*., 2009, 2010; Bian, *et al*., 2010; Pan, *et al*., 2012). While not essential for *Wolbachia*-mediated antiviral protection, the RNAi pathway plays a small part in blocking RNA viruses replication (Hedges, *et al*., 2012; Terradas, *et al*., 2017). However, while a mechanistic explanation of *Wolbachia*'s antiviral effects is lacking, successful host manipulations highlight its use (Hughes & Rasgon, 2014).

Strains vary markedly in their conferred antiviral effects within a single host species. For example, in *Drosophila simulans*, several strains of *Wolbachia* confer strong antiviral properties (*w*Mel, *w*Ri and *w*Au) while other common variants (*w*Ha and *w*No) produce no such effects (Osborne, *et al*., 2009). Male-killing native *Wolbachia* strains do not protect *D. bifasciata* flies from *Drosophila C Virus* or *Flock House Virus* (Longdon, *et al*., 2012) and several native African armyworm strains (*wExe1*, *wExe2*, and *wExe3*) significantly increase double stranded DNA baculovirus-induced mortality (Graham, *et al*., 2012). Similar strains also have different effects in different hosts. Recent studies have shown that *w*MelPop (a varient of the *w*Mel strain) protects *A. aegypti* mosquitoes from bacteria, RNA viruses, malaria parasites, and parasitic filarial nematodes (Kambris, *et al*., 2009; Moreira, *et al*., 2009). However, the *w*Mel strain in *D. melanogaster* and *D. simulans* confers protection from some RNA viruses but not bacterial pathogens, cellular parasites, or filarial nematodes (Teixerira, *et al*, 2008; Wong, *et al*., 2011; Martinez, *et al*., 2012; Rottschaefer & Lazzaro, 2012). Therefore, strain selection in transinfection experiments is critical to mediating diseases in the insects.

Wolbachia as a honey bee symbiont

It may be possible to manipulate increased viral resistance, potentially to key viruses like DWV, in honey bees through the manipulation of *Wolbachia*. The effects of reported natural *Wolbachia* infection in *Apis mellifera carnica* (the Carniolan honey bee, the most common managed subspecies; hence referred to as *Apis mellifera*) has not been examined and therefore the *Wolbachia*-host phenotypic is unknown. The investigation into the phenotype caused by *Wolbachia* infection in honey bees is reliant on artificial

infection of *Wolbachia* into *A. mellifera*. Samples of honey bees previously found positive for natural *Wolbachia* have since been destroyed and so cannot be further investigated (Pattabirhimaiah, pers. comm., 2017). No additional Wolbachia-positive bees have been found or are available. Intra-order transinfection between dipterans is common (Hughes, *et al*., 2014) but successful inter-order transinfection is less so, with few successful inter-order transinfections detailed to date (van Meer & Stouthamer, 1999; Zhong & Li, 2014; Kageyama, *et al*, 2017). To the best of my knowledge, transinfection into honey bees has never been attempted. However, natural infection of *Wolbachia* in the honey bee subspecies Cape bee (*Apis mellifera capensis*) and the Africanized bee (*A. m. scutellata*), and evidence of natural low density *Wolbachia* infection in *A. mellifera*, would suggest that artificial infection is plausible. Therefore, the novel approach used in dipteran hosts may be conferred to hymenopterans, using an appropriate strain of *Wolbachia* compatible with the honey bee immune system. Previous successful insertions of foreign strains of *Wolbachia* in arthropods with facultative results, including increased viral resistance (e.g., Walker, *et al*., 2011; Bain, *et al*, 2013; Dutra, *et al*., 2016), make *Wolbachia* a strong candidate against *Deformed wing virus* and other honey bee RNA viruses. Established transinfection methodologies make this concept potentially viable.

THESIS STRUCTURE

My goal in this study was to attempt a transinfection of *Wolbachia* into Western honey bees. This investigation was conducted to determine the phenotypic effect of *Wolbachia* in Western honey bees, by attempting novel transinfection of *Wolbachia* bacteria into honey bees with the ultimate goal of increasing resistance against pathogenic RNA viruses. Existing strategies to increase honey bee resistance to these pathogens and *Varroa* are limited to breeding programmes that select for beneficial behaviours such as heightened hygiene and the development of pesticides in order to reduce the impact of disease vectors. To the best of my knowledge, the infection of a bacteria found in the honey bee host, that is not hosted in the gut, has not before been considered as a strategy to confer resistance. This is, therefore, the first study to emply a number of previously successful transinfection methodologies used for dipteran studies in hymenopterans, as well innovative new protocols for *Wolbachia* infection utilizing honey bee insemination.

In Chapter 2, investigated the natural *Wolbachia* infection that was reported in honey bees by Pattabhiramaiah, *et al*. (2011), its relatedness to other *Wolbachia* supergroups and potential as an artificial infection strain source. I screened a number of hymenopteran species for *Wolbachia* and discovered *Wolbachia* infection in the European tube wasp *Ancistrocerus gazelle* where it has never been found before. I then attempted to infect honey bees with a stable infection of *Wolbachia* strain wMel in order to investigate the host-phenotype interaction induced. I trialed a variety of transinfection techniques on honey bees at

varying life stages (eggs, larvae, pupae and adults), including sound microinjection protocols and I developed a novel methodology utilizing artificial insemination in queens. I then investigated the possible cause of failed transinfection attempts. I looked at the expression of a range of immune response peptides over time, post-microinjection of live and dead *Wolbachia*, to determine the degree of immune response in honey bees to foreign bacteria.

In Chapter 3, I discuss my findings, their implications and future recommendations in this field.

Appendix 1 provides thorough information regarding honey bee larval rearing protocol.

Appendices 2 and 3 provide supplementary information on this study, including GenBank accession numbers and primer sequences used.

Research aims

Specifically, this thesis aims to:

1. Determine the strain of *Wolbachia* found in the reported natural infection of *Apis mellifera* as found by Pattabhiramaiah, *et al*. (2011) in context to a robust *Wolbachia* supergroup phylogeny.
2. Attempt to instigate a *Wolbachia* infection in *A. mellifera* through existing microinjection protocols that have been used in previously successful transinfection experiments in dipterans.
3. Produce a novel methodology of *Wolbachia* infection through artificial insemination in *A. mellifera*.
4. Characterise the immune upregulation of antimicrobial peptides expressed in *A. mellifera* when challenged with *Wolbachia* bacteria.

CHAPTER 2

Wolbachia transinfection viability in pre-pupated *Apis mellifera*

INTRODUCTION

Bacterial strains in the genus *Wolbachia* are gram-negative cytoplasmically and maternally inherited *Rickettsaiae*, which are present in the reproductive tissues of a plethora of invertebrates (Warren, 2008). Meta-analyses suggest that between 40% and 60% of all terrestrial arthropods are infected, making *Wolbachia* the mostly widely distributed endosymbiotic panzootic from a biodiversity perspective (Zug & Hammerstein, 2012; Frost, 2014; Wienert *et al*., 2015). Host specificity and phenotype changes between lineages of *Wolbachia*, which are classified into 16 supergroups named *A-F* and *H-Q* in order of their descriptions (Glowska, *et al*., 2015*;* Gerth, *et al*., 2016). These infections can confer a variety of effects on their hosts ranging from reproductive manipulations, obligate mutualism in nematodes, as well as facultative mutualisms in a variety of taxa with various non-pathogenic effects, including increased viral resistance and increased fecundity (Teixeira, *et al*., 2008; Warren, *et al*, 2008; Fast, *et al*., 2011; Walker, *et al*. 2011; Hoffmann, *et al*. 2011).

In *Drosophila* spp. flies, natural *Wolbachia* infection increases fecundity fourfold in *D. mauritania* compared to uninfected individuals (Fast, *et al*., 2011), and infection in *D. melanogaster* increases resistance to *Drosophila C Virus* and other RNA viruses (Hedges, *et al*, 2008). The artificial infection of *Wolbachia* native to *D. melanogaster* (*w*Mel strain) into *Aedes* sp. mosquito hosts has been successful, causing infected individuals to express cytoplasmic incompatibility as well as refractoriness to viral dengue fever infection (Walker, *et al*., 2011; Bian, *et al*., 2016). This phenotype combination has been the basis of the Eliminate Dengue Project which has now had successful controlled release of infected mosquitos in ten countries across the globe. This success has led to similar projects aimed at reducing the transmission of other insect-borne diseases, including *Zika* virus (ZIKV; (Dutra, *et al*., 2016; Aliota, *et al*. 2016), *Plasmodium* malaria (Bian, *et al*., 2013), and sleeping sickness spread by tsetse flies (Doudoumis, *et al*., 2012, 2013).

Manipulating antiviral effects of *Wolbachia* may also be applied in zoonotic fields. Instead of removing the vector's ability to carry viruses, where the emphasis is on ceasing the spread of infection, it

can be used to stop the carrying of viruses by the target directly, as has been witnessed in *Drosophila* spp. (Teixeira, *et al.,* 2008; Hedges, *et al*., 2004) and *Aedes* mosquitoes (Dutra, *et al*., 2016). Therefore, if transinfection into other Dipteran species has resulted in successful viral resistance, transinfection of *Wolbachia* into other species is plausible. One potential employment is in Western honey bees (*Apis mellifera*) to increase viral resistance against debilitating RNA viruses that have been suspected to be the main cause of worldwide honey bee decline over the last 50 years (De Miranda & Genersch, 2010; Schroeder & Martin, 2012).

Honey bees are considered the world's most economically important pollinator, with 104 leading global food crop commodities dependent on them (Le Conte & Navajas, 2008; vanEnglesdorp & Meixner, 2010). Now considered by some to be the "most important productive livestock" (Stoic, *et al*., 2016), the honey bee is the primary choice of agricultural pollinator worldwide (Klein, *et al*., 2007). Long term population decline throughout Europe and the United Sates has highlighted the importance of these insects, where bees are absent the yield of a number of fruits, vegetables, seeds, and nuts falls by up to 96% (Potts, *et al*., 2010). The decline in honey bees has been blamed on a number of pathogens, namely the network between the ectoparasite *Varroa destructor* and RNA viruses *Deformed wing virus* (DWV) and *Kashmir bee virus* (KBV) (Cornman, *et al,* 2012; Schroeder & Martin, 2012). Current strategies to reduce this pathogenic mutualism has been limited to the breeding of beneficial grooming behaviours such as varroa sensitive hygiene (Danka, *et al*., 2011) and miticide development (Mattos, *et al*., 2017; Rinkevich, *et al*., 2017). Therefore, the transinfection of bacteria with the intention of directly reducing viral loads is a novel approach for honey bee health management.

A natural infection of *Wolbachia* in *Apis mellifera* was reportedly found in low density by Pattabhiramaiah *et al*. (2011a), who amplified segments of the *Wolbachia* ribosomal *16S* gene in both workers and queens of a common strain of commercial bees (*A. m. carnica*) from hives in Bremen and Ploen, Germany. Honey bee subspecies, the Cape bee, *A. m. capensis* and the Africanized bee, *A.m. scutellata*, both native to South Africa, are also naturally infected with *Wolbachia* (Hoy, *et al*., 2003; Jeyaprakash, *et al*., 2003). The phenotypic effects and results of the *Wolbachia* infections in any of these species have never been examined. Unfortunately, further samples of the *Wolbachia*-infected Carniolan honey bees are no longer available (pers. comm, Pattabhiramaiah, 2017[1]) and so cannot be further analyzed. With the majority of honey bees testing negative for *Wolbachia* (Cox-Foster, *et al*., 2007; Martinson, *et al*.,

[1] Email correspondence with Mahesh Pattabhiramaiah and Dorothea Brueckner of the Honeybee Research Unit from the University of Bremen, Germany concluded that there are "no samples left from bees infected with *Wolbachia* in the laboratory". Therefore, no further research can be conducted on samples showing natural infection.

2007; Yanez, *et al*., 2017), highlighting the rarity of infection, assessments of *Wolbachia*-host phenotype will be limited to the ability to create a stable artificial infection within *Apis mellifera*.

A variety of approaches for bacterial transinfection exist, with varying levels of success dependent on the host species and age (reviewed by Hughes & Rasgon, 2014). The most common methodology is microinjection of *Wolbachia* cells into the host with the aim of proliferation into the germ line. The offspring of the F_0 are then tested for *Wolbachia* presence to determine infection of the ovaries and the capacity for maternal transmission. Microinjection can be operated on both embryos and older life stages including larvae, pupae and adults (Grenier *et al*., 1998; Kubota, *et al.*, 2005; Kageyama, *et al.*, 2008). Generally, embryonic microinjection of very young eggs has the highest rate of infection success due to the increased rate of bacterial cells reaching the germline and developing ovaries. However, eggs have a higher mortality rate than their more developed counterparts, creating a tradeoff between recipient survival and transinfection success (Hughes & Rasgon, 2014). *Wolbachia* injected intrathoracically in dipteran larvae and pupae has been shown to be somewhat successful, taking advantage of *Wolbachia*'s tendency to migrate to the germline (Frost, *et al*., 2014). In adult *Drosophila melanogaster*, injected *Wolbachia* are seen to localize within the stem cell niche within the germline (Frydman, *et al*., 2006). *Wolbachia* can often be successfully extracted from host egg cytoplasm and injected into recipient embryos or adults with successful infection, though in many cases *Wolbachia* must be reared in a cell culture of the recipient to reduce immune response against injected material (Walker, *et al*, 2009, 2011). Additional to microinjection, co-rearing of host and recipient has also shown to have limited success (Huigens, *et al*., 2004), and oral infection is inappropriate due to *Wolbachia*'s intracellular lifestyle (Rasgon, *et al*., 2006).

This study examines the viability of artificial *Wolbachia* infection through existing transinfection protocols. I first phylogenetically examine natural infecting strain of *Wolbachia* found in *A. mellifera* (Pattabhiramaiah *et al*., 2011a, b) against a robust *Wolbachia* phylogeny to determine the relationship between this and other *Wolbachia* strains. I screen a number of hymenopterans that are not known to naturally host *Wolbachia* and discover it in the European tube wasp, *Ancistrocerus gazella* where it has not previously been reported. Microinjection protocols are then trialed extensively in varying life stages of honey bees. I conceptualize and attempt a novel methodology utilizing artificial insemination as means by which to transinfect intracellular bacteria, which has never been attempted. I then infect honey bees with *Deformed wing virus* in order to determine if *Wolbachia* infection has an effect on survival in individuals honey bees. Lastly, I assess the expression of a number of antimicrobial peptides in first instar honey bee larvae following *Wolbachia* microinjection to determine the immune response of larvae exposed to *Wolbachia*.

METHODS AND MATERIALS

Phylogenic investigation

To determine the feasibility of artificial *Wolbachia* transinfection in *Apis mellifera,* the previously described naturally infecting *Wolbachia* strain found in *A. mellifera* (Pattabhiramaiah *et al*., 2011a) was examined against a robust phylogeny of *Wolbachia* strains from all described supergroups and classification of host-phenotype relationships was reviewed.

A robust phylogeny of existing *Wolbachia* was created using five genes, ribsomal *16S*, citrate synthase (*gltA*), a heat shock protein (*groEL*), an essential cell division protein (*ftsZ*) and a common surface protein (*wsp*) from the *Wolbachia* infection naturally found in the named host. Representatives from each of 16 of the 17 *Wolbachia* supergroups described so far were used to accurately evaluate *Wolbachia*-host phenotype and place the infection previously reported within bees (Pattabhiramaiah, *et al*., 2011a). Host taxa from supergroup *G* were excluded due to inadequate Multilocus Sequence Typing (MLST) evidence, as suggested by Baldo & Werren (2007). Fifty-seven independent host taxa were used, plus the Pattabhiramaiah, *et al*. (2011a) sequence, totaling 59 hosts. NCBI accession numbers for sequences of all five genes from all host taxa are provided in Appendix 2. Two species from the *Ehrlichia* genera were used as outgroups (Glowska, *et al*. 2015). All *Wolbachia* sequences were aligned in MEGA 7.0 (Kumar, *et al*., 2015) per gene and aligned with Clustal*W*. The ends of sequences were manually trimmed. Total lengths of alignments were 639bp for *gltA*, 876bp for *groEL*, 735bp for *ftsZ*, 592bp for *16S*, 452bp for *wsp*, and 3,297bp for a concatenated alignment that included taxa for which at least two of four gene sequences were available, with the exception of the *Apis* species, for which only one gene had been sequenced. All five sequences were run through Baysian Inference using the BEAST package (Drummond, *et al*., 2012) as a combined analysis. The genes' substitution models were independent as per diagnosis from jModelTest (Darriba, *et al*., 2012), with *16S*, *ftsZ* and *wsp* using the Tamura-Nei 93 model (TN93; Tamura & Nei, 1993) with gamma distribution at four levels, and *groEl* and *gltA* best suited to the HKY85 model (Hasegawa, *et* al., 1985) with gamma distribution at four levels. Strains were treated as species and a Yule Process tree prior was applied with a strict clock model (Almerão, *et al*., 2012). The concatenated tree was developed by linking individual gene trees in BEAUTi and were then processed through BEAST for Bayesian Inference using Markov chain Monte Carlo (MCMC) with 10,000,000 steps and 10% burn-in. This analysis was conducted three times to ensure consistency. The resulting tree was run through Tracer and then annotated in TreeAnnotator (Rambaut & Drummond, 2010a, b) and finally visualized using FigTree (Rambaut, 2009). Available literature was reviewed to determine established phenotypic effects of *Wolbachia* on host taxa. This information was then used to inform phenotypic trends within supergroups.

In order to find a reliable source of *Wolbachia*, the Victoria University of Wellington wildtype *Drosophila melanogaster* fly colony was screened using standard PCR in order to determine *Wolbachia* presence. Whole genomic DNA was extracted from flies by grinding 10 flies per sample using pestles with 5% β-mercaptoethanol, with chloroform and isopropanol purification. Concentrations of DNA were quantified with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, USA). To detect *Wolbachia*, the *wsp* gene was amplified using primers *wsp*81F and *wsp*691R (further information on primers used in this study can be found in Appendix 2). Polymerase Chain Reaction (PCR) was performed using MyTaq Red Mix (Bioline, UK) with each reaction at a final volume of 15µl (7µl of MyTaq Red Mix buffer, 1µl each of forward and reverse primers and 1µl of DNA sample and ddH_2O to the final volume). The thermal cycling conditions were an initial denaturation at 95°C for 1 minute; and 30 cycles of denaturation at 95°C for 15 seconds, annealing at 50°C and extension at 72°C for 10 seconds; with a final extension step at 72°C for 5 minutes and held at 4°C. Amplification was visualized in a 1.5% agarose gel stained with SyberSafe DNA Gel Stain (Thermo Fisher, USA). *Drosophila melanogaster* and *Ancistrocerus gazella* (see below) were used as independent positive controls and no template controls were used as negative controls. Positive sequences were sequenced by Massey Genome Service (Massey University, Palmerston North, NZ) and screened against similar sequences on the National Centre for Biotechnology's (NCBI) Basic Local Alignment Search Tool (BLAST).

In order to determine the *Wolbachia* strain present in *Drosophila melanogaster* in the Victoria University colonies, a Maximum Likelihood tree was produced with a number of international *Wolbachia* strains, including *w*Mel to determine their relationship. *Wolbachia* surface protein (*wsp*) sequences were collected from Genbank, analysed in MEGA 7.0 (Kumar, *et al*., 2015) and aligned using Clustal*W* and manually trimmed. ML heuristic searches were conducted through the NNI (Nearest Neighbour Interchange) method under the TN93 model with gamma distribution at four levels.

Whilst screening *D. melanogaster* for *Wolbachia,* a number of hymenopterans were screened for *Wolbachia*. The Asian paper wasp (*Polistes chinensis*), the common wasp (*Vespula vulgaris*), the German wasp (*V. germanica*), the Western yellowjacket (*V. pensylvanica*), the European tube wasp (*Ancistrocerus gazella*), the invasive Argentine ant (*Linepithema humile*) and the New Zealand native ant (*Monomorium antarcticum*), as well as the honey bee mite, *Varroa destructor* were screened. In order to determine the native supergroup of *Ancistrocerus gazella*, Maximum Likelihood tree was produced with a number of international *Wolbachia* strains, including *w*Mel to determine their relationship. *Wolbachia* chaperonin gene *GroEL* gene sequences from a variety of supergroups were collected from Genbank and aligned in MEGA 7.0 (Kumar, *et al*., 2015) aligned using Clustal*W* and manually trimmed. ML heuristic searches

were conducted through the NNI method under the TN93 model with gamma distribution at four levels. Accession numbers can be found in Table 1.

Wolbachia isolation

Wolbachia was isolated from wildtype *Drosophila melanogaster*, which is naturally infected with the *w*Mel strain, from colonies at Victoria University of Wellington. Flies were grown and maintained at 25°C, with 60% relative humidity on standard apple juice/yeast/oatmeal medium. A standard 12 hour light:dark cycle was implemented (Ashburner, 1989). Flies were induced to lay by provision of yeast paste 12 hours prior to laying. Isolation methodologies from several *Drosophila* body components were examined in order to find the highest concentration of *Wolbachia*: whole female flies, dissected fly ovaries, pools of whole dechorionated eggs (50 eggs per pool), and cytoplasm extracted from eggs less than 60 minutes post-ovideposition.

Drosophila melanogaster eggs were collected within one hour of oviposition. Eggs that were to serve as *Wolbachia* donors were washed in 70% ethanol for 2 minutes and rinsed twice in double distilled water (ddH_2O). Eggs were dechorionized in 50% standard commercial bleach for 2 minutes followed by two sets of 2 minute ddH_2O rinses. Eggs were maneuvered with a sterile size 0 paint brush and lined up on a glass slide with strips of double sided tape to prevent movement during cytoplasm extraction. Cytoplasm was extracted using a glass capillary needle. Needles had approximately 10µm tips and were individually pulled using a laboratory duel-stage micropipette puller (Tritech Research Ltd., Los Angeles, USA). Cytoplasm was vacuumed from the dechorionated eggs using a digital microinjector and a direct drive mechanical micromanipulator (both Tritech Research Ltd., Los Angeles, USA). The extract from 100 eggs worth of cytoplasm was placed into a sterile 0.6ml microcentrifuge tube and kept on ice. This cytoplasm was then purified from an adapted protocol from Xi and Dobson (2005) and Rasgon *et al*. (2006). Cytoplasm and developmental cells were pelleted by centrifugation at 300 x g for 5 minutes to remove large debris. The supernatant was transferred into a new centrifuge tube and spun at 12,000 x g for 10 minutes to pellet *Wolbachia* cells. The pellet was resuspended in phosphate-buffered saline (PBS) buffer and kept on ice prior to use. Five microliters were aliquoted for cell viability staining using 50nM SYTO11 dye (ThermoFisher Ltd., Waltham, USA) to determine *Wolbachia* presence and 0.4% Trypan Blue to assess the percentage of non-viable cells.

To determine *Wolbachia* presence, pooled egg cytoplasm DNA was extracted using DNA Miniprep Kit (Zymo Research, USA) following manufacturer instructions. DNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, USA). The *Wolbachia* surface protein (*wsp*)

gene was amplified using primers *wsp*81F and *wsp*691R. PCR was conducted using protocols mentioned previously using *wsp* primers.

Samples with positive *wsp* bands (including positive controls to exclude sequencing error) had their PCR products purified using 1µl of ExoSAP-IT cleanup reagent (Thermo Fisher, USA) and following manufacturer's instructions, were incubated at 36°C for 15 minutes, then at 80°C for 15 minutes. Cleaned products sequenced with the forward *wsp*81F primer. Sequencing was completed by Massey Genome Service (Massey University, Palmerston North, NZ). Sequences were searched in the NCBI BLAST nucleotide database to determine identity. Those that matched at least 98% with a known *Wolbachia wsp* sequence were deemed to be positive for *Wolbachia* infection.

Microinjection assays

Egg microinjections

Honey bee (*Apis mellifera carnica*) eggs were collected from on-site beehives at Victoria University of Wellington, New Zealand. To collect eggs of a known age, queen bees were placed inside a queen cage (part of the Ezi-Queen queen rearing system, Auckland, New Zealand). Prior to queen containment, the cage was placed into the hive for one hour in place of a brood frame to allow the workers to acclimatize and clean out any existing honey or previously unsuccessful eggs. Queens were removed after three hours of containment. On a few occasions, when the queen was not able to be located, a fresh frame of pulled wax was placed in the brood box in place of a brood comb and left for three hours before removal. Laid eggs were transported in a warm (~28°C) box kept humid with a damp sponge. The viability of eggs is highly sensitive to disturbance, with movement of eggs from comb to plate having a significant effect on hatch rate (Collins, 2002). Therefore, eggs were kept in the EziQueen frame or within a section of cut comb to reduce direct contact during injection and laboratory handling. All eggs were injected immediately after removal before cell differentiation which occurs between 4-6 hours post oviposition.

Eggs were microinjected with one unit of resuspended *Wolbachia* (1psi for 0.1 second, Tritech Digital Microinjector) administered by a micromanipulated glass capillary needle. It is difficult to determine the exact quantity of each injection, but they were optimized to "inflate" the egg without overpressurizing it as explained by Xi and Dobson (2005). Optimal needle tip width was determined by trial and error. It was determined that a 10µm tip was the smallest tip that would both minimize trauma in the egg but prevent clogging (in any tip smaller than 10µm clogging of the needle occurred frequently). Injections were made into the posterior end of the egg, as illustrated in Figure 1. Eggs were then kept in comb/frame in standard larval rearing conditions, at 35.4°C with 80-90% relative humidity until hatching.

See Appendix section 1A Bee Larval Rearing Protocol for more detail. Approximately one quarter of the number of eggs that were injected with *Wolbachia* were injected with phosphate-buffered saline (PBS) as controls per trial.

A random sample of 20 intact microinjected eggs per treatment were taken per trial to be assessed for *Wolbachia* presence.

Larvae and pupae microinjections

Previous microinjection of *Wolbachia* into the larval stages of insects and isopods has proven successful, particularly in F_0 and has been shown to be an effective method to study *Wolbachia* somatic colonization (Grenier, *et al*., 1998; Kageyama, *et al.* 2008). I similarly attempted to infect honey bee larvae with *Wolbachia* via microinjection. *Apis mellifera* larvae were collected from brood combs by cutting sections of comb with a sharp knife. Larvae were aged by eye as per Human *et al*. (2014) and all instars (larval phases) were included in subsequent injections. Larvae were kept in comb to reduce contact prior to injection to reduce contact-induced mortality. Injections were conducted through the same methodology as with eggs but were targeted at the 5th and 6th abdominal segment of larvae where the germcell is located (Dearden, 2006).

White-eyed pupae were carefully removed from capped comb using blunt ended forceps (to reduce the likelihood of mechanical damage) and laid dorsal-side down onto a 72mm petri dish lined with a Kimwipe®. Pupae were covered and immobilized by placing them in -20°C for 60 seconds. Pupae were injected between the 3-4th abdominal segment, which is a common feeding place for *V. destructor* (Kanbar & Engels, 2003). Approximately one third of the quantity of larvae and pupae that were injected with *Wolbachia* were injected with phosphate-buffered saline (PBS) as controls. Replicates varied dependent on the number of individuals of the required life stage found in the comb in any given trial.

Both larvae and pupae were kept in standard rearing conditions (34.5 (±1)°C with 80-90% relative humidity) on comb for 12 hours. After 12 hours all dead larvae were removed to eliminate individuals killed from injection-related damage. Individuals still alive after 12 hours were transferred from comb to 72mm petri dishes and fed *ad-libtum* standard larvae diet (53% royal jelly, 3% glucose, 3% fructose, 1% yeast extract; see Appendix 1). Larvae and pupae were checked for mortality at 12, 24, 48 and 56 hours post-microinjection. Larvae and pupae were euthanized 72 hours post-injection and kept at -80°C prior to DNA extraction and analysis.

Survival analyses for eggs, larvae and pupae were conducted on R (R Development Core Team, 2008) using the "survival" (Therneau, 2015), "survminer" (Kassambara & Kosinski, 2016) "simPH"

(Gandrud, 2017) packages. Data was partitioned by treatment, a variable with two factors, *Wolbachia* injections and control injections (PBS injections). Within treatment groups, individuals were classed into life stages at four levels: egg, young larvae (between instars 1-3), older larvae (between instars 3-6), and pupae. Analyses were conducted within and between groups. Between groups, Chi-squared tests was conducted to compare survival curves associated with each treatment, and confirmed with Wald and Log Rank tests, both with a single degree of freedom. Within treatment groups, to determine the impact of life stage on survival rate, Log-likelihood ratio models were expressed with an exponential distribution, to produce Chi-square ratios and pairwise comparisons made between life stages. Confidence intervals were constructed using a robust nonparametric method by Brookmeyer & Crowley (1982).

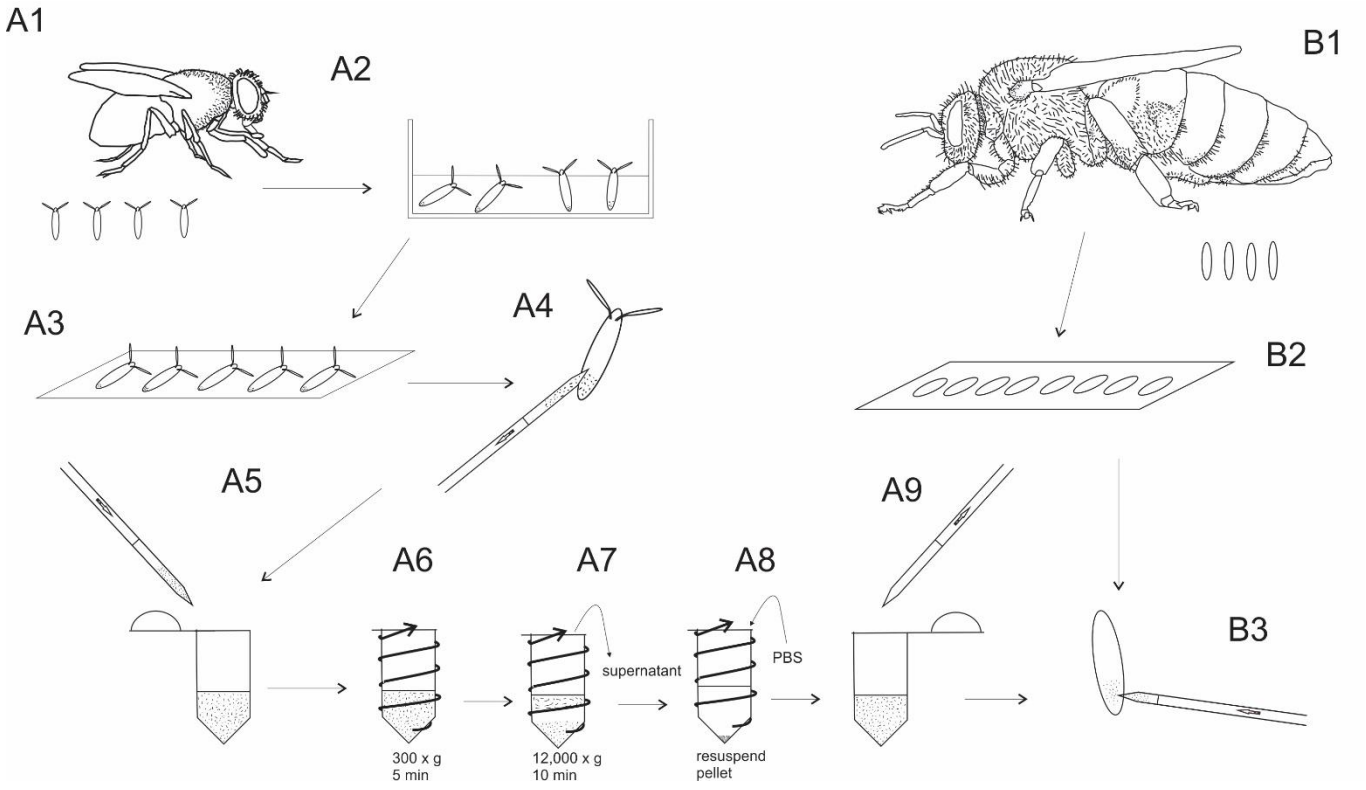

Figure 1 Overview of the microinjection process modified from Xi & Dobson (2005) and Rasgon, *et al*. (2006). A1, *Wolbachia*-infected *Drosophila melanogaster* and her eggs; A2, *D. melanogaster* eggs being dechorionated in 1:1 diluted bleach solution for two minutes followed by two water rinses (not shown); A3, dechorionated eggs laid out on a glass slide; A4; *Wolbachia*-positive cytoplasm vacuumed from eggs via 10µm needle; A5; cytoplasm ejected into eppendorf tube for short term storage and kept on ice. A6, centrifugation of cytoplasm at 300 x g for 5 minutes to separate large debris. A7, Supernant is removed and then spun for 10 minutes at 12,000 x g to pellet *Wolbachia*. A8, Pellet resuspended with phosphate-buffered saline (PBS). A9, isolated suspended *Wolbachia vac*uumed into needle. B1; *Apis mellifera* queen and her eggs; B2, eggs laid out on slide (eggs were kept in the comb to reduce egg disturbance); B3, *Apis mellifera* eggs injected in the posterior end with resuspended *Wolbachia*. Source: author's own.

Artificial insemination assay

Transinfected *Wolbachia* requires access to the ovaries of the recipient to integrate into the ovarian tissue and allow maternal transmission (Werren *et al.*, 2008; Hughes, *et al*., 2014). An alternative to thoracic or embryonic injections is to infect the F_0's ovaries directly so that eggs produced in the F_1 are infected without the need for further intervention (Hughes & Rasgon, 2014). In honey bees, artificial insemination is common industry practice to breed queens (and therefore colonies) with desirable traits and for genetic studies (Hunt, *et al*., 1998; Cobey, 2007). Here, I attempted a *Wolbachia* transinfection simultaneously with an artificial insemination event. Utilizing such methods have never before been attempted in *Wolbachia* transinfection studies, nor has the purposeful bacterial infection in bees through artificial insemination.

Honey bee virgin queens were artificially inseminated with *Wolbachia* using a modified artificial insemination protocol used in the New Zealand commercial apiculture industry (BettaBees New Zealand, Ltd.), following protocols similar to Cobey *et al* (2013). Five-day old virgin queens were supplied by Beaut Bees Ltd. (Auckland, NZ). Virgin queens were couriered as per standard industry practice in plastic vented containers with up to seven nurse bees and supplied with industry-grade "queen candy" (50% icing sugar, 50% honey) as a food supplement. Queens were placed into dequeened hives 48 hours prior to insemination to allow the hive workers to be exposed to queen pheromones and prevent negative or defensive interaction post-insemination. Twenty-four hours prior to inseminations, queens were removed from their hives and anesthetized for 5 minutes under CO_2, transferred into queen cell cages with a queen excluder opening to allow workers to access her and then placed back into their respective hives.

Semen was collected from approximately 150 drones to the total of 50µl. Semen was diluted and activated with 10% industry-standard insemination buffer (0.11% NaCl, 0.06% tris, 1% glucose, 10% lysine, 10% argenine, 90% distilled autoclaved water with the antibiotic component left out). *Wolbachia* was isolated from approximately 300 *Drosophila melanogaster* eggs as previously described. *Wolbachia* cells were kept in cell-free Schneider's *Drosophila* media supplemented with 10% heat-inactivated fetal bovine serum as described by Ragson *et al*. (2006) and Gamston & Ragson (2007), and kept at 4°C prior to insemination. In a sub-sample, bacteria viability was checked using an 10µL aliquot of isolated cells stained with SYTO11 to visualize *Wolbachia* and 0.4% Trypan blue dye to determine the quantity of unviable bacterial cells. Viable and dead cells were counted under 510nm fluorescent microscopy.

During insemination, queens were briefly anesthetized with CO_2 for approximately 15 seconds until major movements ceased before being placed into a plastic queen holder, orientated so the end of her abdomen was sticking out of the hold, and her head was facing downward into a low flow of CO_2. A small pair of specially designed pressure-controlled forceps were used to hold and gently pull the queen's stinger outwards and a special hook on the ventral side used to open the queen's oviduct passage (Fig. 6B). Ten

microlitres of semen-buffer mix containing *Wolbachia* was injected using a glass capillary needle after *Wolbachia* was identified post-staining. The queens were then returned to their respective hives in the same queen cell container with queen candy to eat through to encourage acclimatization post insemination.

Hives were kept under strict containment. As the potential effects of *Wolbachia* have not been evaluated in *A. mellifera*, interaction between infected queens and naive bees was strictly controlled and we were careful to not release any transinfected bees into the environment. Therefore all hives were kept indoors with no access to the outdoors. Sample size in this experiment was consequently limited by quarantine requirements and minimization of interactions between bees belonging to different queens. A single control queen was inseminated with semen containing no additional *Wolbachia*. Only one control queen was used due to restricted availability of queens within the same cohort by the supplier, and budget and quarantine constraints. Ovaries from one additional queen were taken 24 hours after insemination to screen for ovarian infection as we were confident of no pre-existing *Wolbachia* infection.

Hives were kept indoors and isolated from one another (for quarantine purposes) for the next five weeks. Queens were sampled 2 weeks and 5 weeks post-inseminations when a brood frame was removed and replaced with a fresh frame with additional pollen supplements. Worker bees were collected from frames within the hive. Samples were immediately frozen at -80°C to test for *Wolbachia* as described above.

Deformed Wing Virus infection and survival assay

In the event of *Wolbachia* infection in the progeny of inseminated queens, it was important to utilize these individuals to determine the phenotypic influence of *Wolbachia*. To do evaluate *Wolbachia*'s influence on RNA viruses in *A. mellifera*, individual adult bees that were suspected to be infected with *Wolbachia* were infected with *Deformed wing virus* (DWV).

Deformed wing virus was extracted from a pool of red-eyed *A. mellifera* pupae (approximately 19-20 days post egg oviposition) from *Varroa destructor* mite-infested hives and homogenized in potassium phosphate buffer (PPS; pH8) with 10% diethyl ether and chloroform purification before pelleting remaining cell debris for 2 minutes at 13,000 x g. The homogenate was passed through a 0.22µm bacteria filter and then serially diluted (Remnant, pers. comm. 2018). Extracted DWV was kept at -20°C prior to injection. It was likely that this extract contained other viral particles other than DWV. *Deformed wing virus* viral RNA was extracted with the Quick gDNA Miniprep Kit (Zymo Research, USA) following manufacturer instructions. RNA was reverse transcribed into cDNA using Superscript III Reverse Transcriptase (Thermofisher, USA) using specific oligonucleotide primers and dNTPs, following manufacturer's instructions. *Deformed wing virus* presence was diagnosed through standard PCR using standard Platinum Taq (Thermofisher, USA). Each 15µL reaction was made up of 2.5µL of 10X buffer, 0.75µL 50mM $MgCl_2$,

0.5µL 10mM dNTPs, 0.5µL of each forward and reverse primer, approximately 100ng of template, 0.1 Platinum taq, and ddH_2O to 15µL. A standard thermocycling protocol was followed, comprised of: initial denaturation at 94°C for 2 minutes, 30 cycles of 94°C for 30 seconds, 55° for 30 seconds and 72°C for 1 minute, followed by a final elongation step of 72°C for 5 minutes and held at 4°C. Products were visual on a 1.5% agarose gel and stained with SYBR Safe DNA stain (Invitrogen, USA).

Prior to microinjection, adult worker bees were anesthetized, immobilized in groups of ten, by cooling them at -20°C for two minutes (Human, *et al*., 2013). Anesthetized bees were then injected with DWV through a glass capillary needle with a 10µm tip. Each bee received approximately the same volume of extract to ensure consistency, with settings of 1 psi pressure for 0.1 seconds on a digital microinjector (MINJ-D; Tritech Research, Ltd.). This manipulation was done manually, with DWV suspensions injected ventrally through the intersegmental membrane between the 3rd and 4th abdominal segment (the typical feeding place of *V. destructor* (Kanbar & Engels, 2003)). Control pupae were injected with the same quantity of DWV-free potassium phosphate buffer.

Bees were kept in standardized incubation conditions at 34.5°C and 80% RH in vented containers and fed with 50% sucrose solution. Bees were kept with bees from the same hive to prevent mixing progeny from inseminated queens and control queens. Survival was monitored evert 6 hours up to 64 hours post-injection. Once dead, bees were frozen at -80°C, and bees that survived up to 64 hours were euthanized and also kept at -80°C.

PCR Screening for infection

Wolbachia infection in larvae injected with isolated *Wolbachia* was diagnosed 72 hours post-injection. DNA and RNA were extracted from pools of three larvae in the same instar by homogenizing samples using pellet pestles (Sigma-Alrdrich, USA) in GENEzol reagent (Geneaid, Taiwan) with 5% β-mercaptoethanol, with chloroform and isopropanol purification (Gruber, *et al*., 2017). For adult bees, pools of three bees were homogenized by bead-beating (BeadBeater 16, Biposec products, USA). Due to limited tissue in injected eggs, egg DNA was extracted using DNA Miniprep Kit (Zymo Research, USA), following manufacturer instructions, instead of the GENEzol protocol. Concentrations of DNA were quantified with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, USA).

To detect *Wolbachia*, the *wsp* gene was amplified using primers *wsp*81F and *wsp*691R. PCR was performed using MyTaq Red Mix (Bioline, London, UK) with each reaction at a final volume of 15µl (7µl of MyTaq Red Mix buffer, 1µl each of forward and reverse primers and 1µl of DNA sample and ddH_2O to the final volume). The thermal cycling conditions were an initial denaturation at 95°C for 1 minute; and 30

cycles of denaturation at 95°C for 15 seconds, annealing at 50°C and extension at 72°C for 10 seconds; with a final extension step at 72°C for 5 minutes and held at 4°C. Amplification was visualized in a 1.5% agarose gel stained with SyberSafe DNA Gel Stain (Thermo Fisher, USA). *Drosophila melanogaster* and *Ancistrocerus gazella* were used as independent positive controls and no template controls were used as negative controls.

Immune response

In order to determine the honey bee immune response to *Wolbachia* microinjection, the expression of a number of downstream antimicrobial peptides (AMP) were assessed in first instar larvae. First instar larvae were microinjected instead of eggs in this experiment in order to accurately determine mortality in injected individuals and eliminate cases of inhibited or downregulated peptide expression confounded by death. In eggs, mortality is unable to be determined apart from observed lack of development. It is difficult to determine egg development without the use of a microscope, whereas in larvae, development can be monitored by eye.

The antimicrobial peptides, abaecin, apidaecin, defensin and hymenoptaecin were chosen for their previously determined affinity to gram-negative bacteria (Table 2; Evans, 2004; Evans, *et al*., 2006). The housekeeping gene Proteasome 54kD subunit (Pros54) was used for reference (Cameron, *et al*., 2013) and *Wolbachia* titre was determined by the *w*Mel primer (Chrostek, *et al*., 2013). Information on all primers can be found in Appendix 3. *Wolbachia* is a Gram-negative bacterium and in order to rule out potential upregulation of other non-specific bacteria, abaecin, an AMP that targets Gram-positive bacteria, was included in these experiments.

First instar honey bee larvae were randomly assigned one of three treatments: *Wolbachia* microinjection, heat-treated *Wolbachia* microinjection (where isolated *Wolbachia* was incubated at 95°C for 10 minutes (Rasgon, *et al*., 2006), or no microinjection (control). Larvae were microinjected with one unit of either resuspended *Wolbachia* or heat-treated *Wolbachia* (1psi for 0.1 second, Tritech Digital Microinjector) administered by a micromanipulated glass capillary needle as described above. It was determined that a 10µm tip was the smallest tip that would minimize trauma in the egg but prevent clogging (in any tip smaller than 10µm clogging of the needle occurred frequently). Individuals were then kept in standard conditions for either 0, 12, 24, 36, or 48 hours and then frozen at -80°C for genetic analysis.

DNA and RNA were extracted from pools of three larvae by homogenizing samples using pellet pestles (Sigma-Alrdrich, USA) in GENEzol reagent (Geneaid, Taiwan) with 5% β-mercaptoethanol, and chloroform and isopropanol purification (Gruber, *et al*., 2017). DNA was removed using the PerfeCta DNase I (Quanta Bio Inc.) following manufacture's instruction. cDNA was prepared by reverse

transcription of 8µL RNA (approximately 80ng) with random hexamers and oligo(dT) primers using qScript XLT cDNA SuperMix (Quanta Bio Inc.) and incubated for 5minutes at 25°C, 60 minutes at 42°C and 5 minutes at 85°C. The resulting cDNA was diluted 1:10 with nuclease-free water.

Drosophila melanogaster egg DNA used for confirmation of *Wolbachia* presence was extracted using DNA Miniprep Kit (Zymo Research, USA), following manufacturer instructions. Concentrations of DNA were quantified with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, USA).

Gene expression analyses

Quantitative real-time PCR (qPCR) was performed to detect expression of the four nominated antimicrobial peptides, a reference gene, and *Wolbachia* with specific oligio(dT) primers run in duplicate using a magnetic induction cycler (Mic qPCR Cycler, Bio Molecular Systems). Reaction mixes of 20µL contained 10µL PerfeCta SYBR Green Fastmix, 2µL cDNA template, and 2µL of each forward and reverse primer at 10nM. Thermal protocol followed that of Evans *et al*. (2006) consisting of 5 minutes at 95°C, then 40 cycles of a four step protocol of 94°C for 20 seconds, 60°C for 30 seconds, 72°C for 1 minute, and 78°C for 20 seconds. Amplification was followed by a melt-curve dissociation program to confirm expected product size. Results were screened for appropriate dissociation values to check for and eliminate primer-dimer artifacts.

Antimicrobial peptide and *Wolbachia* primer expression were standardized against the reference gene Proteasome 54kD subunit (Pros54; Cameron, *et al*., 2013) to find ΔCt scores for each gene. Microinjection treatments (*Wolbachia* or heat-treated *Wolbachia*) were then standardized against control treatment values for each gene using the ΔΔCt method to determine the expression fold change (2^- ΔΔCt) (Schmittgen and Livak, 2008). Differences in expression between genes were tested using two-way ANOVAs, with gene and treatment as factors using R (R Core Development Team, 2013).

Table 1 Information about the honey bee antimicrobial peptides examined in this study: abaecin, apidaecin, hymenoptaecin, and defensin.

Peptide	Target	Characteristics	Reference
Abaecin	Gram-positive bacteria	Proline-rich. Composed of 34 amino acids. Abaecin-like peptides also found in bumble bee hemolymph.	Xu, *et al*., 2009 Hara & Yamakawa, 1995 Evans, 2004
Apidaecin	Gram-negative bacteria but can affect gram-positive at high enough concentrations. Permeate bacterial membrane without lytic effect. Inhibit bacterial DnaK heat shock proteins and chaperonin GroEL-GroES complex.	Proline-rich Composed of 18 amino acids. Four natural forms, but only apidaecins 1a, 1b and 2 detected in bees *in vivo.*	Casteels, *et al*., 1989 Casteels, *et al.*, 1993 Casteels-Josson, *et al.*, 1993 Casteels & Tempst, 1994 Li, *et al*., 2006
Hymenoptaecin	Gram-positive and gram-negative bacteria. Accesses bacterial periplasmic space by creating small lesions in the outer membrane.	Glycin-rich. Linear, composed of 93 amino acids. Single form found in *A. mellifera*, while 13 forms found in *A. cerana*. Low basal levels in honey bee brood than in adult workers.	Casteel *et al*., 1993 Chan, *et al*., 2006 Xu, *et al*., 2009
Defensin	Gram-positive and some gram-negative bacteria, fungi, protozoa and some viruses.	Cysteine-rich. Two natural forms found in honey bees, d*efensin-1* and *defensin-2*. Key antimicrobial component of honey.	Casteels-Josson *et al*., 1994 Mandrioli, *et al*., 2003 Bulet & Stocklin, 2005 Evans *et al*., 2006 Kwakman *et al*., 2011, 2012 Reviewed in Ilyasov *et* al, 2012

RESULTS

Phylogeny

The Bayesian Inference (BI) *Wolbachia* phylogeny produced was unique in that it is the first tree (to date) to include representatives of all 16 known supergroups (*A-F*, *H-Q*; Figure 2) and include information on phenotypic traits. Topology was congruent with existing phylogenies that have looked at individual or few supergroups, including that produced by Glowska, *et al.* (2015), Gerth, *et al.* (2014) and Casiraghi, *et al*. (2005). The tree had good support. Basal support was high, with posterior probabilities of 0.7-1. Support for the placement of the supergroups in general was well supported, however positions between taxa within these groups were less resolved. Multiple BI runs resulted in identical trees, and similar posterior probabilities, implying convergence among runs. Effective sample sizes (ESS) ranged from 2232 to 2420 between tree runs (an estimate of how many truly independent samples as a parameter of the output of Markov chain Monte Carlo runs, which determines the accuracy of topology placements (Drummond, *et al*., 2006; Lenfear, *et al*., 2016)).

Tree topology distinguishes supergroups in agreement with other studies (Glowska, *et al*., 2015). Supergroups *A* and *B* include *Wolbachia* strains from arthropods only (Werren, *et al*., 1995), while supergroup *C* and *D* are restricted to obligate filarial nematodes (Taylor, *et al*. 2013). Supergroup *E* is made up of Collemobolla *Mesaphoriura italica* and *Folsomia candida* (Timmermans, *et al*., 2004), and supergroup *F* is individual in that it contains both arthropod and nematode hosts (Ros *et al*, 2009). Supergroup *H* contains only isopterans in the *Zootermopsis* genus (Wang, *et al*., 2014), while supergroup *I* infect siphonapteran fleas (Gorham, *et al*., 2003), Supergroup *J* holds only the animal parasitic nematode *Dipetalonema gracile* (Ros, *et al*., 2009)*;* while *K* infects spider mites (e.g. *Bryobia* spp.), and *L* holds the plant-boring *Radopholus similis* (Spirurda) (Ros, *et al.,* 2009). Supergroups *M* and *N* contain hemipteran aphids (Augustinos *et al*., 2011), while *O* contains the lepidopteran pest *Bemisia tabaci;* and groups *P* and *Q* contain quill mites and pseudoscorpians (Glowska, *et al*., 2015). *Wolbachia* that was previously reported to be found in found in *A. mellifera* (Pattabhiramaiah, *et al*., 2011a) was found clustered closely to *Drosophila melanogaster*'s native *w*Mel strain.

Phenotypic review found that the effects of *Wolbachia* on different taxa could be split into four categories: reproductive manipulation, obligate mutualism, facultative mutualism, and undetermined effects. This information is summarized in Table 2 below. Of all *Wolbachia* taxa, 34.5% caused some sort of reproductive manipulation, 29% possessed obligate mutualisms with their hosts, 10.3% shared facultative mutualisms to some extent, and 27.5% had undetermined effects. These effects are only the known and empirically tested traits that populate the literature. It is highly likely that these strains have additional undetermined effects beyond what has been tested. Some strains had multiple effects, such as the *wCon* strain occurring in *Tribolium confusum* which can be beneficial despite incurring weak cytoplasmic incompatibility in its hosts. The natural *Wolbachia* infection present in all filarial nematodes in groups *C, D* and *F* are known as obligate mutualists; many arthropod hosts in supergroups *B* and *F* were victims of reproductive manipulation, with the majority thought to express cytoplasmic incompatibility. Outside of supergroup *A*, facultative mutualism was found only in plant parasites, aphids *Brevicoryne brassicae* and *Aphis fabae*, and the burrowing nematode *Radopholus similis* (Augustinos, *et al*, 2011). Phenotypic effects in supergroup *A* are varied, with a number possessing facultative effects as well as inducing cytoplasmic incompatibility.

The Maximum Likelihood phylogeny of several common *Wolbachia* strains was produced (Fig. 3A). *Wolbachia* sourced from the Victoria University colonies was placed neatly in the *w*Mel group, showing strong similarities with *w*Mel from around the world and backed up with very strong bootstrap support (>0.8).

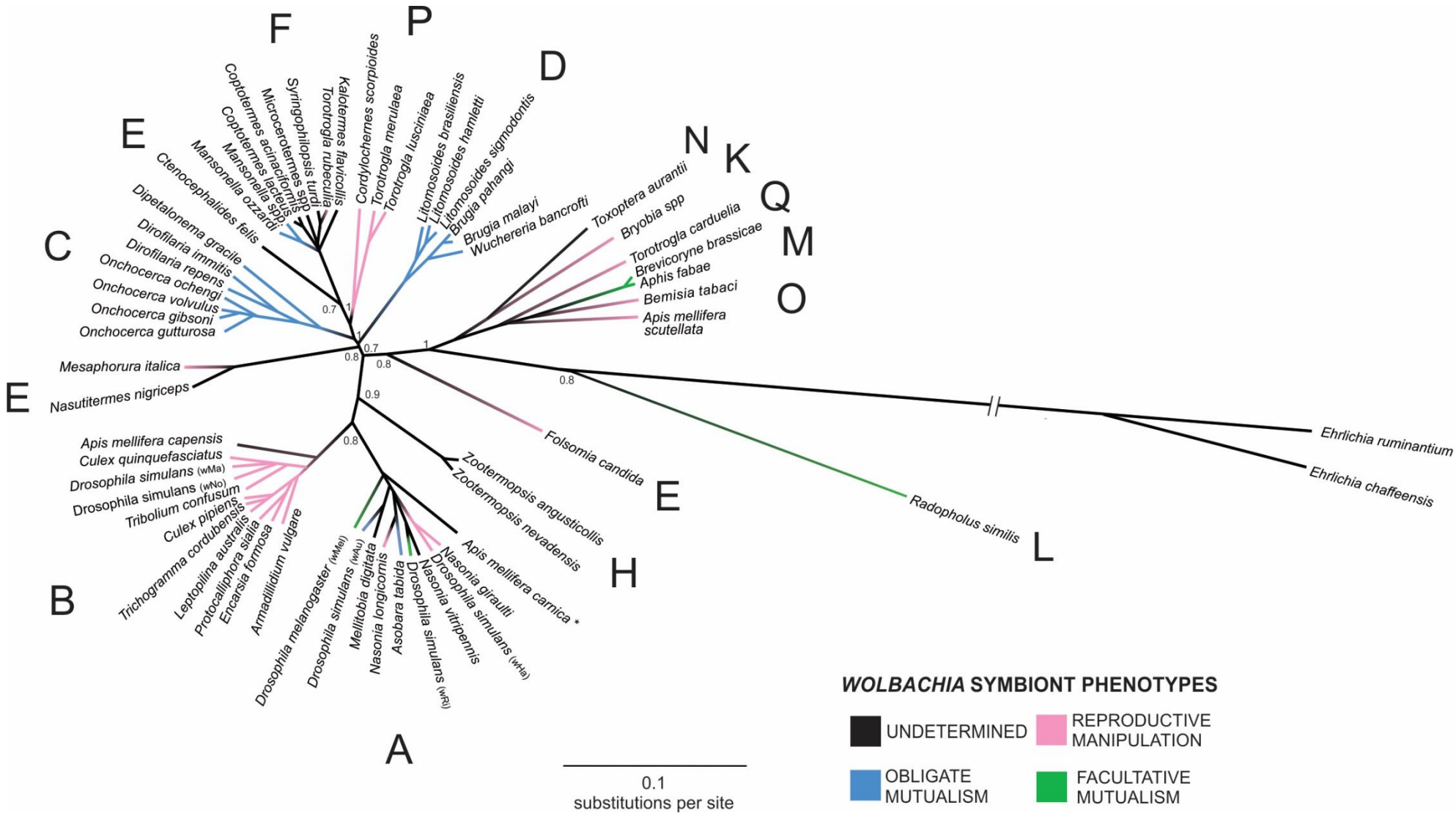

Figure 2 Concatenated Bayesian phylogenic tree of *Wolbachia* infections of 57 host taxa and outgroup (*Ehrlichia spp.* – a related group of bacteria in Rikettsiales). Sequence concatenated from rRNA 16S gene, *gltA, groEL* and *ftsZ* gene sequences (2,845bp). Topology labels represent *Wolbachia* host taxa from which *Wolbachia* was identified and posterior probabilities are labeled on nodes. Colouration key represents phenotypic effects of *Wolbachia* on presented host taxa (refer to Table 2), showing facultative mutualism (green), obligate mutualism (blue), reproductive manipulation (pink) and undetermined effects (black).Supergroup clades are shown by capital letters outside the corresponding supergroup. Suspected *Apis mellifera Wolbachia* sequence (Pattabhiramaiah, e*t al*.,2011) is indicated by * and is placed within the A supergroup. For accession numbers please refer to Appendix 2.

Of the screened hymenopterans, *Wolbachia* was not present in any of the wasp species. *Wolbachia* has been found in low abundance in *Vespula germanica* and *V. vulgaris* previously (Evison, *et al*., 2012). *Wolbachia* was also not found in *Monomorium antarcticum* or *Linepthema humile*, congruent with other studies (Reuter, *et al*., 2004). A *Wolbachia* infection was discovered in *Ancistrocerus gazella* from which it has not been found before. The produced phylogeny places the solitary parasitoid within the *A* supergroup (Figure 3B) along with *Asobara fabidae*. Bootstrap support was highly supportive and produced topology was congruent with similar phylogenies (eg Glowska, *et al.*, 2015; Ma, *et al*., 2017)

Wolbachia isolation

Wolbachia was successfully extracted from *Drosophila melanogaster* in a number of forms. *Wolbachia* presence was visualized under fluorescent microscopy (Fig 4 a) of whole flies, egg cytoplasm, dechorionated eggs, and fly ovaries. All contained and showed positive for *Wolbachia* (Fig 4C). Egg cytoplasm was chosen as the candidate for host *Wolbachia* cells as cytoplasm was extracted at age prior to cell division and specialization, thus reducing the likelihood of excess host cells being included in subsequent microinjections.

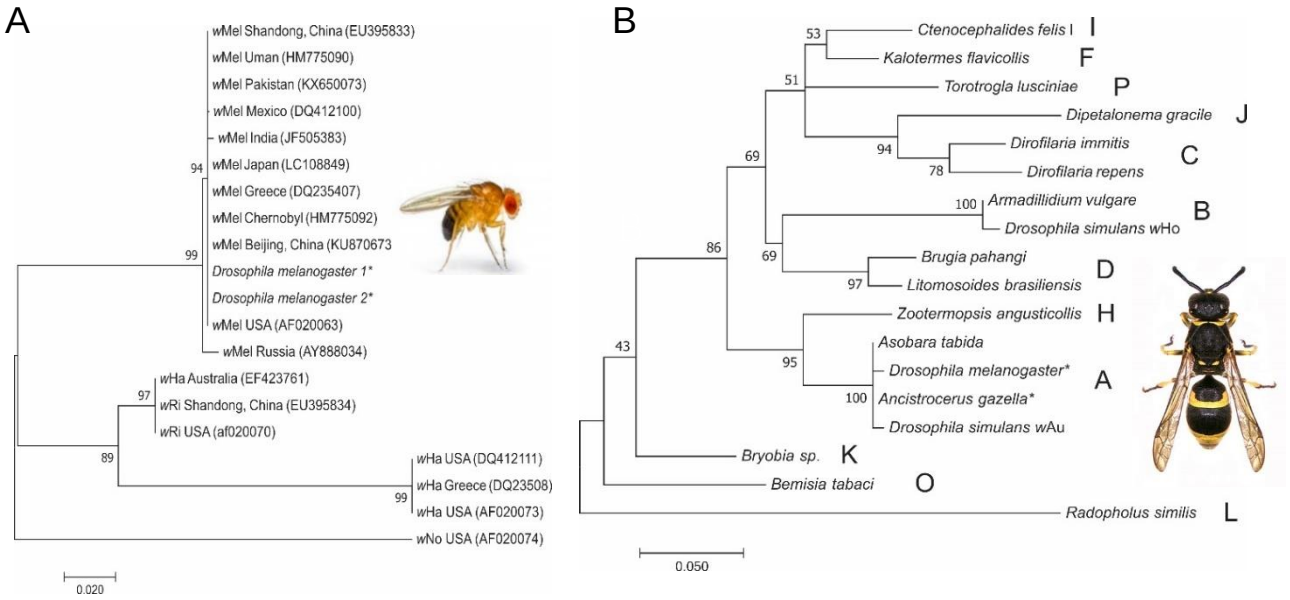

Figure 3 A) Maximum likelihood phylogeny of *Wolbachia pipientis* strains of the *Wolbachia* surface protein (*wsp*) found in *Drosophila melanogaster* in the Victoria University *Drosophila* colony against international *D melanogaster* strains. Sequence accession numbers are in brackets. Numbers on nodes show bootstrap support. * indicates sequences from this study. Scale bar represents nucleotide seInsert: *Drosophila melanogaster*, https://kxci.org/podcast/drosophila-melanogaster/

B) Maximum likelihood phylogeny of *Ancistrocerus gazella* placement within the *Wolbachia* supergroup syndrome. Accession numbers as per Table 1. Numbers on nodes show bootstrap support. * indicates sequences from this study. *D. melanogaster* sequence identical to *Drosophila melanogaster* 1* in A. Insert: *Ancistrocerus gazelle*, Landcare Research, New Zealand

Table 2 Taxa information for the phylogeny presented in Figure 2. Information includes the *Wolbachia* host species, the strain of naturally infecting *Wolbachia* if known, *Wolbachia* strain's respective supergroup for that taxa, *Wolbachia*-host phenotype and sources for phenotypic information. CI, cytoplasmic incompatibility; PI, parthenogenesis induction; undetermined. Ordered by Supergroup. Dotted lines separate Supergroups.

***Wolbachia* host species**	**Order**	***Wolbachia* strain**	**Supergroup**	**Wolbachia phenotype**	**Phenotype references**
Apis mellifera	Hymenoptera	-	A	Undetermined	Pattabhiramaiah, *et al*. 2011
Asobara tabida	Hymenoptera	wAtab1	A	Obligate mutualist	Dedeine, *et al*., 2005
Drosophila melanogaster	Diptera	*wMel*	A	Facultative mutualism; CI	Werren, *et al* 2008; Fry, *et al*. 2004
Drosophila simulans	Diptera	*wAu*	A	Facultative mutualism; CI	Sutton, *et al*., 2014
Drosophila simulans	Diptera	wHa	A	CI	Zabalou, *et al*. 2008
Drosophila simulans	Diptera	*wRi*	A	Facultative mutualism	Hoffmann, *et al*., 2015
Encarsia formosa	Hymenoptera	wEfo	A	PI	Stouthamer, *et al*. 2002
Mellitobia digitata	Hymenoptera	*wDig*	A	Undetermined	-
Nasonia giraulti	Hymenoptera	wNigPa	A	CI	Bordenstein, *et al*., 2000
Nasonia vitripennis	Hymenoptera	wNvit	A	CI	Bordenstein, *et al*., 2000
Tribolium confusum	Coleoptera	*wCon*	A	Facultative; CI	Wade *et al*., 1995
Apis mellifera capensis	Hymenoptera	wCap-B1	B	Undetermined	-
Apis mellifera scutellata	Hymenoptera	wCap-B2	B	Undetermined	-
Armadillidium vulgare	Isopod	wVulC	B	Feminization	Valette *et al*, 2013
Culex pipiens	Diptera	*wPip*	B	CI	Rasgon & Scott, 2003
Culex quinquefasciatus	Diptera	*wPip*	B	CI	de Almedia, *et al*., 2011
Drosophila simulans	Diptera	*wNo*	B	CI	Zabalou, *et al*. 2008
Leptopilina australis	Hymenoptera	*wAus*	B	PI	Cook & Butcher, 1999
Nasonia longicornis	Hymenoptera	wNLonCA1	B	CI	Bordenstein, *et al*., 2000
Protocalliphora sialia	Hymenoptera	wProtSi	B	-	Baudry, *et al*., 2003; Whitworth, *et al*., 2007
Trichogramma cordubensis	Hymenoptera	*wTco*	B	PI	Pintureau, *et al*., 2002; Grenier, *et al*., 2002
Dirofilaria immitis	Spirurida	*wDi*	C	Obligate mutualist	Bandi, *et al*., 1999
Dirofilaria repens	Spirurida		C	Obligate mutualist	Taylor, *et al*., 2000
Onchocerca gibsoni	Spirurida	*wOg*	C	Obligate mutualist	Tamarozzi, *et al*., 2011

Onchocerca gutturosa	Spirurida	-	C	Obligate mutualist	Tamarozzi, *et al*., 2011
Onchocerca ochengi	Spirurida	*wOo*	C	Obligate mutualist	Tamarozzi, *et al*., 2011
Onchocerca volvulus	Spirurida	-	C	Obligate mutualist	Hise & Gillette-Ferguson, 2004
Brugia malayi	Spirurida	-	D	Obligate mutualist	Foster, *et al*., 2005
Brugia pahangi	Spirurida	-	D	Obligate mutualist	Andrews, *et al.*, 2012
Litomosoides brasiliensis	Spirurida	-	D	Obligate mutualist	Chagas-Moutinho, *et al*., 2015
Litomosoides hamletti	Spirurida	-	D	Obligate mutualist	Hoerauf, *et al*., 2000
Litomosoides sigmodontis	Spirurida	-	D	Obligate mutualist	Hoerauf, *et al*., 2000
Wuchereria bancrofti	Spirurida	*wWb*	D	Obligate mutualist	Gayen, *et al*. 2010
Folsomia candida	Collembola	-	E	PI	Pike & Kingcomb, 2009
Coptotermes acinaciformis	Dictyoptera	-	F	Undetermined	Salunke *et al*. 2010
Coptotermes lacteus	Dictyoptera	-	F	Undetermined	-
Kalotermes flavicollis	Dictyoptera	-	F	Undetermined	Panaram & Marshall, 2006
Mansonella ozzardi	Spirurida	-	F	Obligate mutualist	Kairser, *et al*., 2008
Mansonella sp.	Spirurida	-	F	Obligate mutualist	Kairser, *et al*., 2008
Microcerotermes sp	Dictyoptera	-	F	Undetermined	-
Syringophilopsis turdi	Parasitiformes	-	F	Undetermined	Wang, *et al*., 2011
Mesaphorura italica	Isoptera	*w*Ita	H	PI	Czarnetzki & Tebbe, 2003
Nasutitermes nigriceps	Isoptera	-	H	Undetermined	Salunke *et al*., 2010
Zootermopsis angusticollis	Isoptera	-	H	Undetermined	Salunke *et al*., 2010
Zootermopsis nevadensis	Isoptera	-	H	Undetermined	Salunke *et al*., 2010
Ctenocephalides felis	Siphonaptera	-	I	Unknown	Rolain, *et al*., 2003
Dipetalonema gracile	Spirurida	-	J	Obligate mutualist	Ferri *et al*., 2011
Bryobia sp.	Spider mite	-	K	Feminization	Ros, *et al*., 2012
Radopholus similis	Spirurida	-	L	Facultative	Haegeman, *et al*, 2009
Aphis fabae	Hemiptera	-	M	Faculative mutualism	Augustinos, *et al*., 2011
Brevicoryne brassicae	Hemiptera	*wBraKMM1*	M	Undetermined	-
Toxoptera aurantii	Hemiptera	*w*AuGLM	N	Undetermined	-
Bemisia tabaci	Lepidoptera	*wTab*	O	CI	Nirgianaki *et al*, 2003; Ahmed, *et al*., 2010
Cordylochernes scorpioides	Pseudoscorpian	*wCsc2*	O	Male killing	Koop, *et al*., 2009

Torotrogla lusciniaea	Parasitiformes	EG074	P	CI/PI	Glowska, *et al*., 2015
Torotrogla merulaea	Parasitiformes	EG035	P	CI/PI	Glowska, *et al*., 2015
Torotrogla rubeculia	Parasitiformes	EG169	P	CI/PI	Glowska, *et al*., 2015
Torotrogla carduelia	Parasitiformes	EG121	Q	CI/PI	Glowska, *et al*., 2015
Outgroup					
Ehrlichia chaffeensis	Rickettsiales				Glowska, *et al*., 2015
Ehrlichia ruminantium	Rickettsiales				Glowska, *et al*., 2015

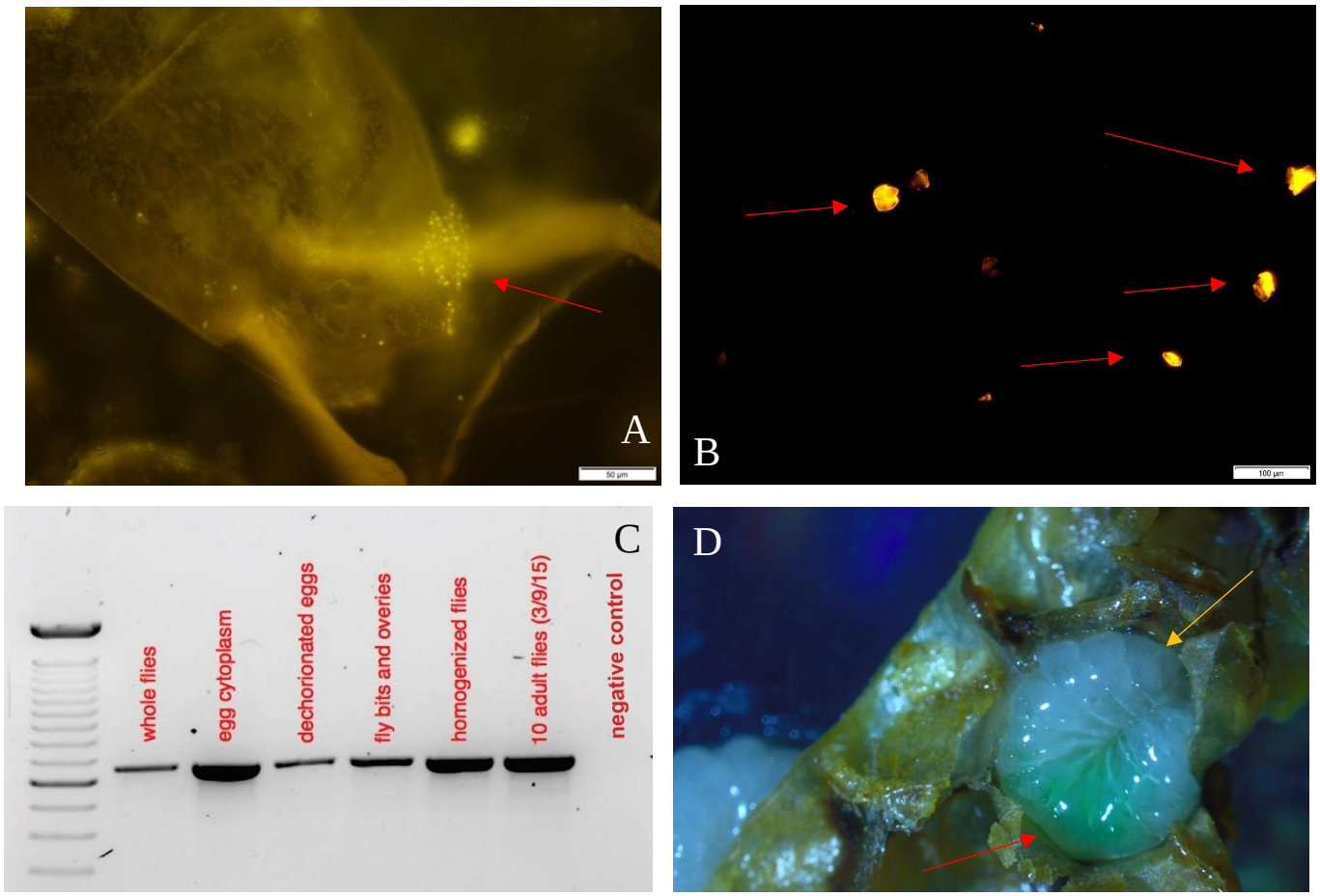

Figure 4 A) *Wolbachia* in the ventral-anterior of a 10 minute-old Drosophila melanogaster egg stained with SYTO11 dye. B) Wolbachia present in honey bee drone semen mixed before insemination stained with SYTO11 dye. *Wolbachia* presence was confirmed with PCR amplification and subsequent sequences. C) Visualized PCR products of Wolbachia surface protein (wsp) on a 1.5% agarose gel, sourced from Victoria University's *Drosophila melanogaster* colonies of varying body parts and eggs. D) Fifth instar A. mellifera larvae injected with Phosphate-buffered saline (PBS) and inert green dye to show injection location between the 5-6 body segment (red arrow). Yellow arrow indicates larvae head for reference. Larvae were kept in honey bee comb to reduce mortality. Source: Jess Russell

Microinjection assays

In total, 1207 individuals were injected with either *Wolbachia* or control phosphate-buffered saline (PBS) containing no *Wolbachia*. These individuals were comprised of 882 eggs, 89 young larvae (between instars 1-3), 96 older larvae (between instars 4-6), and 40 pupae. Of these, no eggs injected with *Wolbachia* hatched after 72 hours post-injection, however, 22% of the embryos in the control treatment of injection with PBS injected hatched successfully.

Larvae and pupae that died 12 hours post-injection were considered to have died of mechanical injury from injection. Seventy-two hours after being injected only 6.4% of *Wolbachia*-injected individuals were alive (n = 953), compared to 20% of control-injected individuals (n = 200) (Figure 5B). Survival

probability was significantly lower in individuals injected with *Wolbachia* than those control injected (χ^2 = 47.5, 1df, $p < 0.001$). There were significant differences in the survival of eggs ($t = 12.5$, $p < 0.001$) and older larvae ($t = 10.6$, $p < 0.001$) between treatments (Fig. 5A). Complete mortality of eggs in *Wolbachia* injected individuals may be responsible for the clear differentiation in survival between treatment groups (Fig 5B).

Larval life stage had significant influence on survival probability irrespective of whether individuals were injected with *Wolbachia* or control PBS (*Wolbachia*: $\chi^2 = 464.65$, $p < 0.001$; Control: χ^2 = 729.72, $p < 0.001$). Life stages each had different survival probability from one another (overall, Likelihood ratio test = 55.4, $p < 0.0001$) but with varying degrees of statistical significance when paired against one another (Table 2). Earlier life stages - eggs in particular - had very low survival followed by a general increase in survivorship with age. Of all microinjected individuals screened for *Wolbachia* 72 hours post-injection via PCR amplification, none produced positive bands.

High mortality in eggs was expected. However, in order to determine whether *Wolbachia* infection was possible in eggs, only a few eggs needed to survive. However, the complete mortality of all eggs injected with *Wolbachia*, but not those injected with PBS, implies an effect of *Wolbachia* that may explain the mortality.

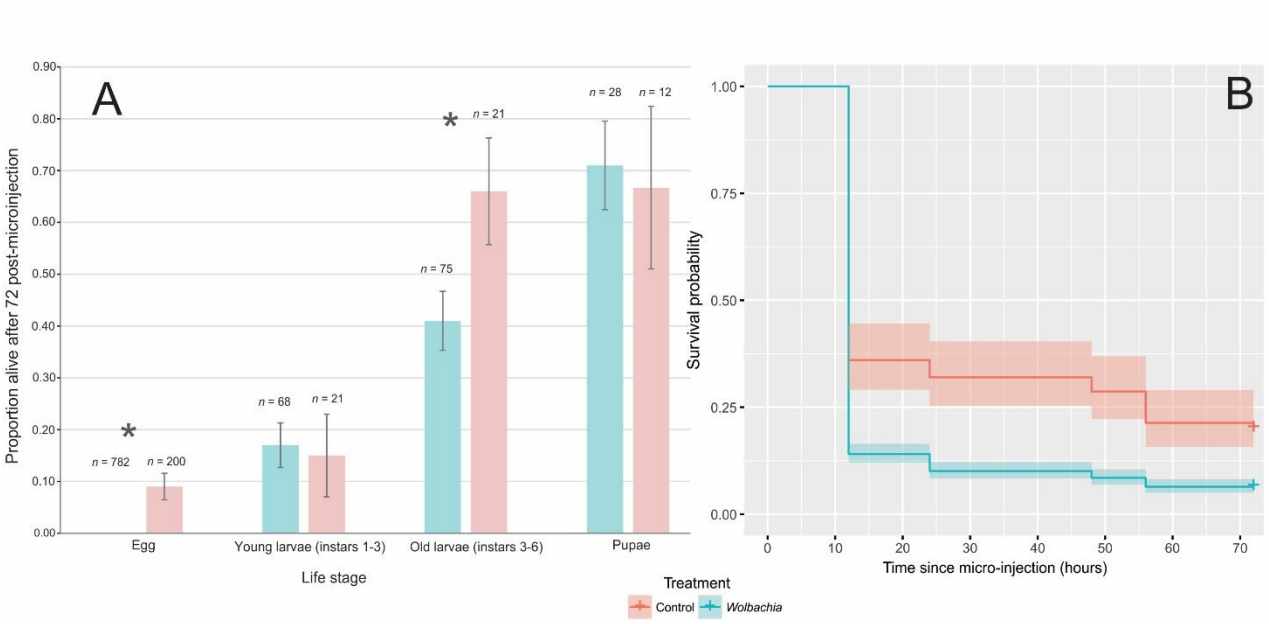

Figure 5 A) Proportion of *Apis mellifera* individuals of varying life stages (egg, young larvae, older larvae, and pupae) alive after 72 hours post micro-injection with either *Wolbachia* or control phosphate-buffered saline (PBS) (±SE). Statistically significant differences between groups indicated by *. B) Kaplein-Meyer survival graph of cumulative survival of all life stages (eggs, young larvae, older larvae, and pupae) post microinjection with *Wolbachia* or PBS controls. Shading around the lines indicate 95% confidence intervals.

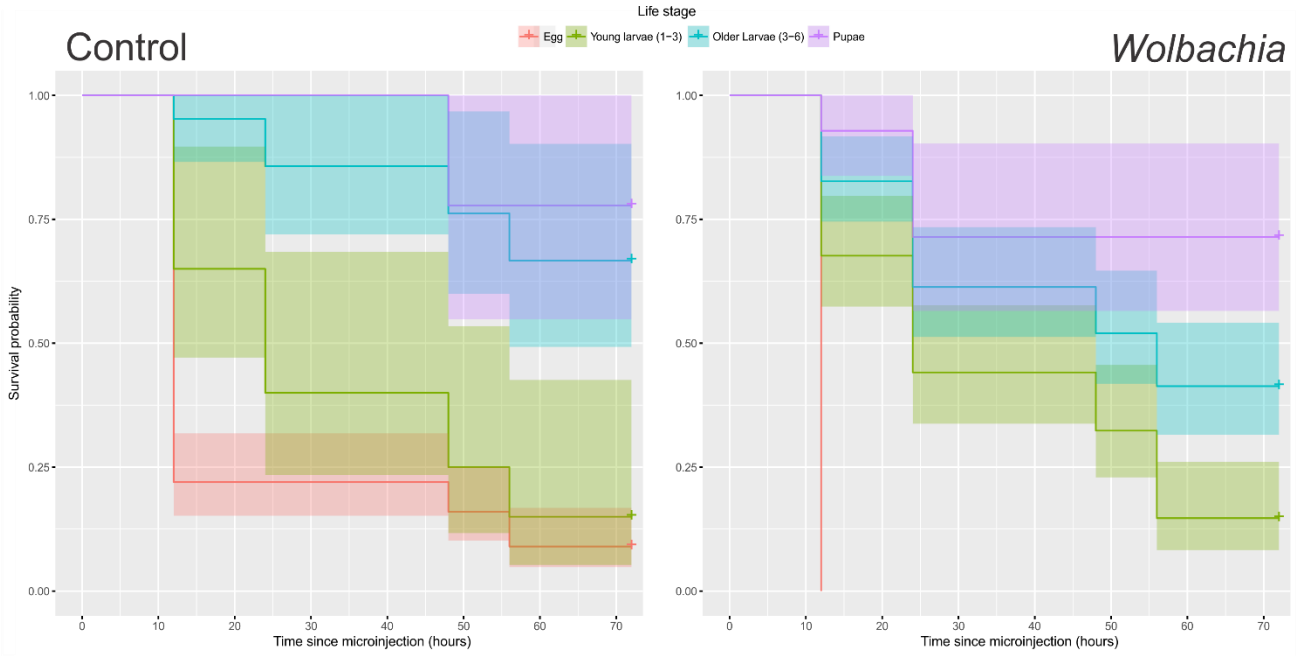

Figure 6 Kaplain Meyer survival curves of pre-pupated *Apis mellifera* microinjected with either *Wolbachia* or phosphate-buffered saline (control). Colours represent life stage: Red: egg; green: young larvae (instars 1-3); blue: old larvae (instars 3-6); purple: white-eyed pupae. Shading around lines represent 95% confidence intervals.

Table 2 Pairwise comparisons between survival curves of pre-pupated *Apis mellifera* microinjected with either *Wolbachia* or phosphate-buffered saline (control). Each value is comparing the survival curve between life stage as per Figure 6. Analysis by Long-rank tests with p-value adjustment using the Benjamini and Hochberg method (1995) to account for false errors in multiple testing. All comparisons were significant at the 5% significance level in the *Wolbachia* treatment. In the control treatment, all comparisons were significant also, apart from the difference between young larvae and egg survival, and between pupae and older larvae survival.

***Wolbachia*-injected**

	Egg	Larvae (1-3)	Larvae (3-6)	Pupae
Egg	-	-	-	-
Larvae (1-3)	<0.0001	-	-	-
Larvae (3-6)	<0.0001	0.0007	-	-
Pupae	<0.0001	<0.0001	0.0132	-

Control

	Egg	Larvae (1-3)	Larvae (3-6)	Pupae
Egg	-	-	-	-
Larvae (1-3)	0.2041	-	-	-
Larvae (3-6)	<0.0001	0.0004	-	-
Pupae	<0.0001	0.0002	0.5174	-

Artificial insemination

The semen that was inoculated with *Wolbachia* used for insemination was deemed positive for *Wolbachia* through visual analysis under 510 nm fluorescent microscopy (Fig 3B) and less than 10% of bacterial cells were unviable when stained under 10% trypan blue. The definitive presence of *Wolbachia* in the semen-cytoplasm mix was also assessed using PCR amplification of the *wsp* gene, which also proved positive (Fig 8A).Of the 5 inseminated queens, three were successfully fertilized, queens laying viable fertilized eggs after two weeks. Eggs and larvae of fertilized queens that were tested for *Wolbachia* through PCR screening were not positive after two weeks. After five weeks, eggs and larvae showed a faint band when PCR products were visualized (Fig 7). These bands were approximately one third of the length of the expected *wsp* products and were sent for sequencing. The sequences were non-conclusive for *wsp* with unspecific peaks, and when searched in the BLAST database showed no resemblance to any other nucleotide sequence in the GenBank database. It was concluded that these samples did not contain *Wolbachia* and that partial bands present were due to non-specific binding.

To determine *Wolbachia* ovarian infection post insemination, ovaries of inseminated queens were dissected and screened for *Wolbachia*. Ovaries 24 hours-post infection showed a positive band for *wsp*, evidence that *Wolbachia* reached the ovaries within 24 hours. However, ovaries of laying queens taken 6 weeks-post insemination produced no bands in the same PCR reaction. Figure 8 summarizes all microinjection and artificial insemination results.

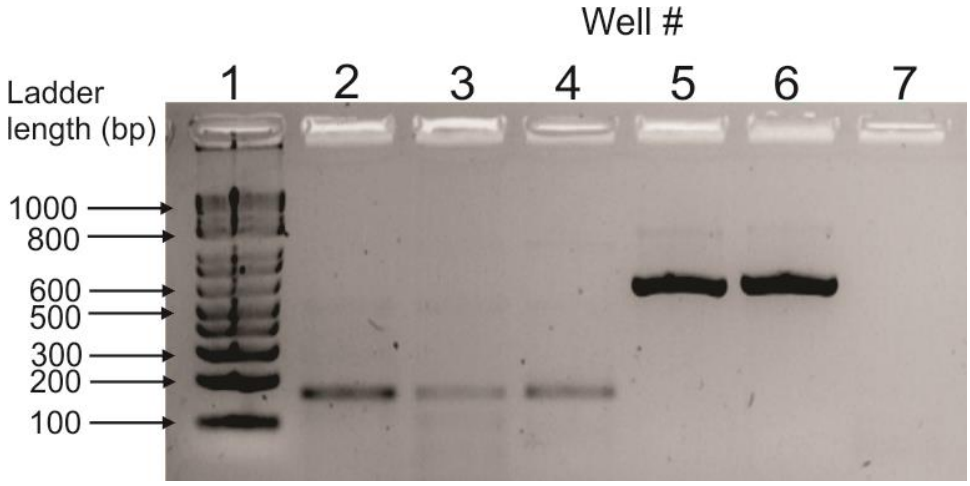

Figure 7 Visualised PCR products of *wsp* gene on 1.5% agarose gel. Lanes: L, Hyperladder 1kb; 1, fertilized queen #1 pooled larvae sample five weeks-post insemination; 2, fertilized queen #2 pooled larvae sample five weeks-post insemination; 3, fertilized queen #3 pooled larvae sample five weeks-post insemination; 4, positive control (*D. melanogaster*); 5, positive control (*A. gazella*); 6, negative control (inseminated queen #4 no *Wolbachia*); 7, no DNA template control.

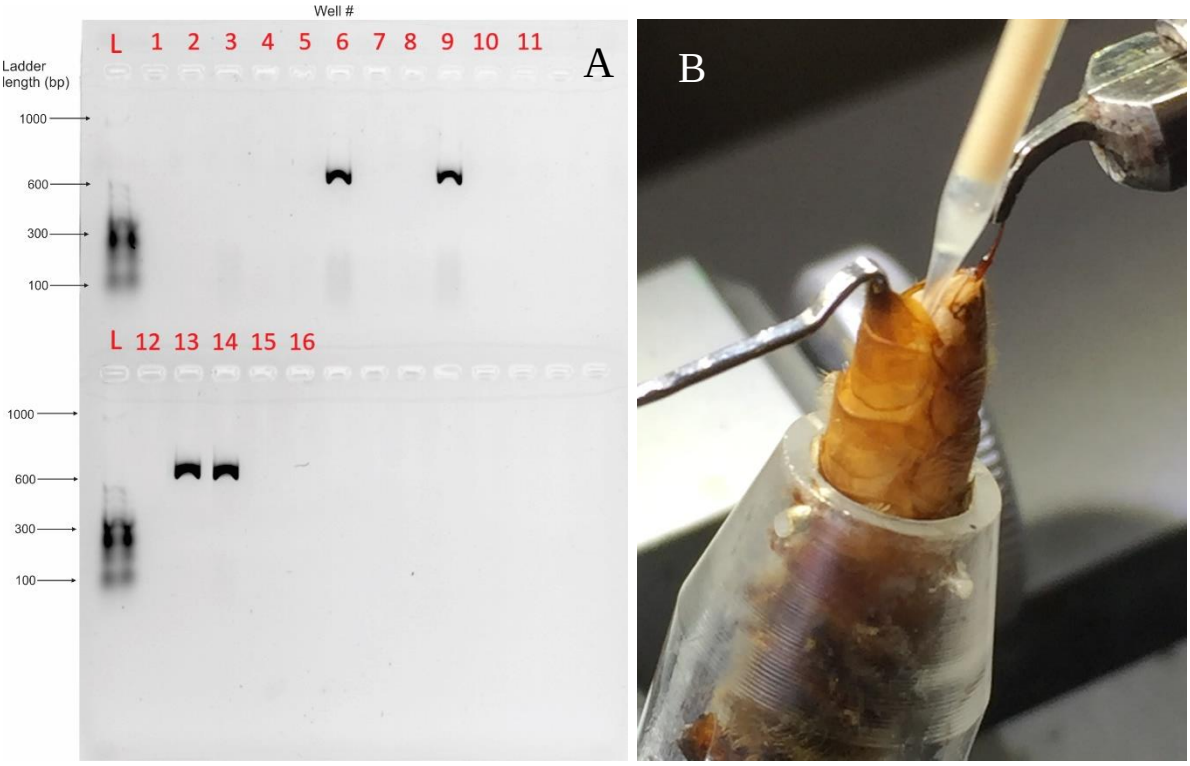

Figure 8 A) Summary of all visualised PCR products of *wsp* gene (to show presence of *Wolbachia*) on 1.5% agarose gel. All life stages belong to *Apis mellifera* unless otherwise mentioned and all were PCR screened 72 hours-post treatment unless otherwise stated.

L: Hyperladder 100kb, top half is very faint. Lane 1: Control adult *A. mellifera* (no treatment); 2: *A. melifera* eggs injected with *Wolbachia*,; 3: Larvae injected with *Wolbachia*; 4: Pupae injected with *Wolbachia*; 5: Adults injected with *Wolbachia*; 6: *A. mellifera* drone semen mixed with isolated *Wolbachia*; 7: Control drone semen with no added *Wolbachia*; 8: Control queen ovaries (no artificial insemination); 9: Artificially inseminated ovaries with semen containing *Wolbachia*, 24 hours post insemination event; 10: Artificially inseminated ovaries with semen containing *Wolbachia*, 6 weeks post insemination event; 11: Fertilized AI queen eggs; 12: Fertilized AI queen larvae; 13: Isolated *Wolbachia* used for injections and inseminations sourced from *Drosophila melanogaster*; 14: Positive control (*Drosophila melanogaster*); 15: AI queen stomatic cells; 16: NTC

B) *A. mellifera* queen being artificially inseminated with 15µL of drone semen mixed with live *Wolbachia* using a glass capillary needle as per industry practice.

Deformed wing virus infection and assay

As the insemination event was limited to a single round of artificial inseminations, it was important to conduct any subsequent assays on the inseminated queen's eggs and larvae even if *Wolbachia* hadn't been yet diagnosed. In order to utilize progeny from artificially inseminated queens that were potentially infected with *Wolbachia*, this assay was conducted prior to sequencing confirmation of *Wolbachia* infection. During the time of sequencing of the PCR band of larvae from queens inseminated with *Wolbachia*, 269 adult

progeny of inseminated queens were injected with DWV. *Deformed wing* was found present in bees from both *Wolbachia* inseminated queens and control bees based on PCR diagnosis.

After 64 hours, only four individuals remained alive between both treatments. There was a very high level of mortality between 0 and 12 hours-post-injection, which levelled off after 12 hours (Fig. 9). Treatment (DWV or control) had no significant influence on mortality (Long rank = 0.6325, p =0.4586). However, as the survival of bees without any kind of injection was not measured, it is not possible to conclude that mechanical injury alone explains the mortality rate.

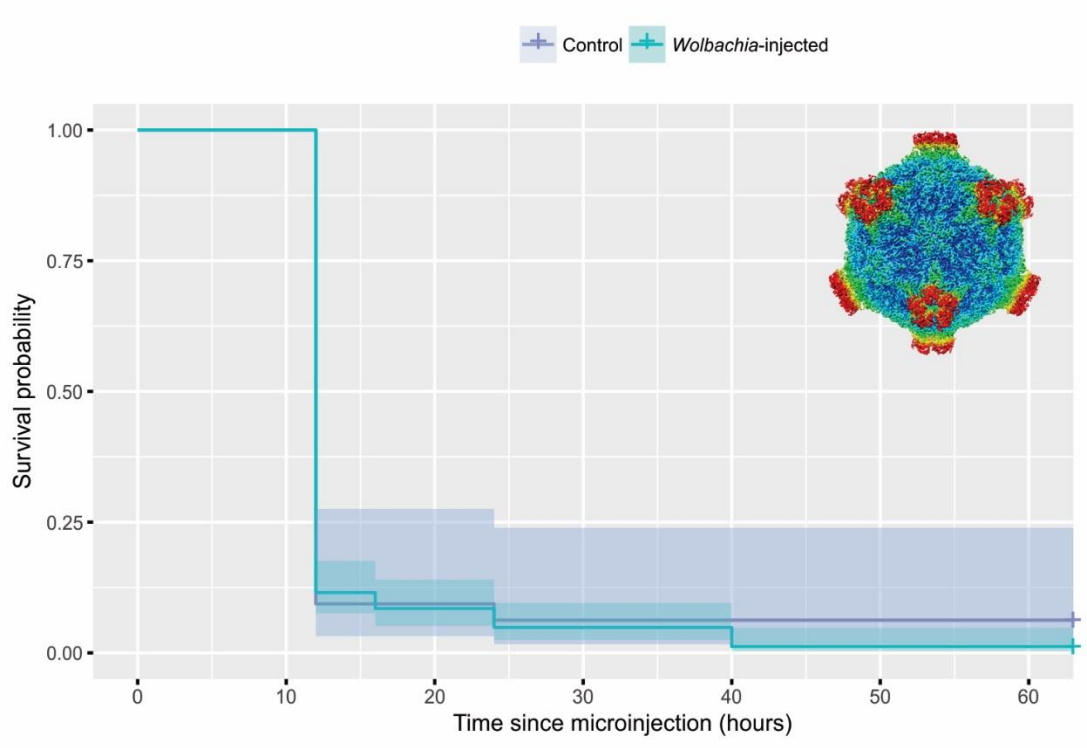

Figure 9 Kaplain-meyer survival graph of honey bees injected with DWV suspected to be infected with *Wolbachia* after F_0 artificial insemination. Individuals were monitored every six hours up to 64 hours post-injection. Shadows indicate 95% confidence intervals. Insert is a visual representation of the molecular surface of a *Deformed wing virus* particle under cryo-electron microscopy by Skubnik *et al*. (2017).

Antimicrobial peptide expression

Of the four antimicrobial peptides examined, only apidaecin was significantly upregulated post-*Wolbachia* microinjection compared to control treatments (F = 6.425, p = 0.0129; Figure 10). No AMP was upregulated in response to being injected with heat-treated *Wolbachia*. In individuals injected with *Wolbachia*, *Wolbachia* expression is high immediately after injection but decreases with increasing acceleration over

the course of 48 hours. Apidaecin was significantly upregulated 8-fold in comparison to control individuals who were not injected ($F = 2.271$, $p = 0.0439$). By 24 hours this expression increased to a maximum mean relative expression of 12 times that of control individuals. Between 24 and 48 hours, *Wolbachia* and apidaecin both fall to near control expression levels. In this time frame there was an increase in hymenoptaecin from 0.28 to 3.13 times that of control treatments.

In the heat-treated treatments, *Wolbachia* expression was nearing identical to control treatments. Its presence was still recorded, however the 2^(ΔΔCt) method does not allow 0 expression as 2^(0) = 1, meaning that an expression of *Wolbachia* in control or heat-treated treatments does not equate to experimental contamination. Small expressional increases in abaecin and hymenoptaecin were recorded in heat-treated treatments but never surpassing twice that of the control treatment.

The correlation of highly upregulated apidaecin and falling *Wolbachia* suggests immune response in *A. mellifera* larvae in response to experimental infection. The slow rise in apidaecin between 0 and 24 hours correlates with the steepest fall in *Wolbachia* expression. *Wolbachia* expression reaches negligible levels by 48 hours post-injection and apidaecin follows this trend. Error bars may be explained by variation in the quantities of *Wolbachia* during microinjection and variation between individual bees immune response.

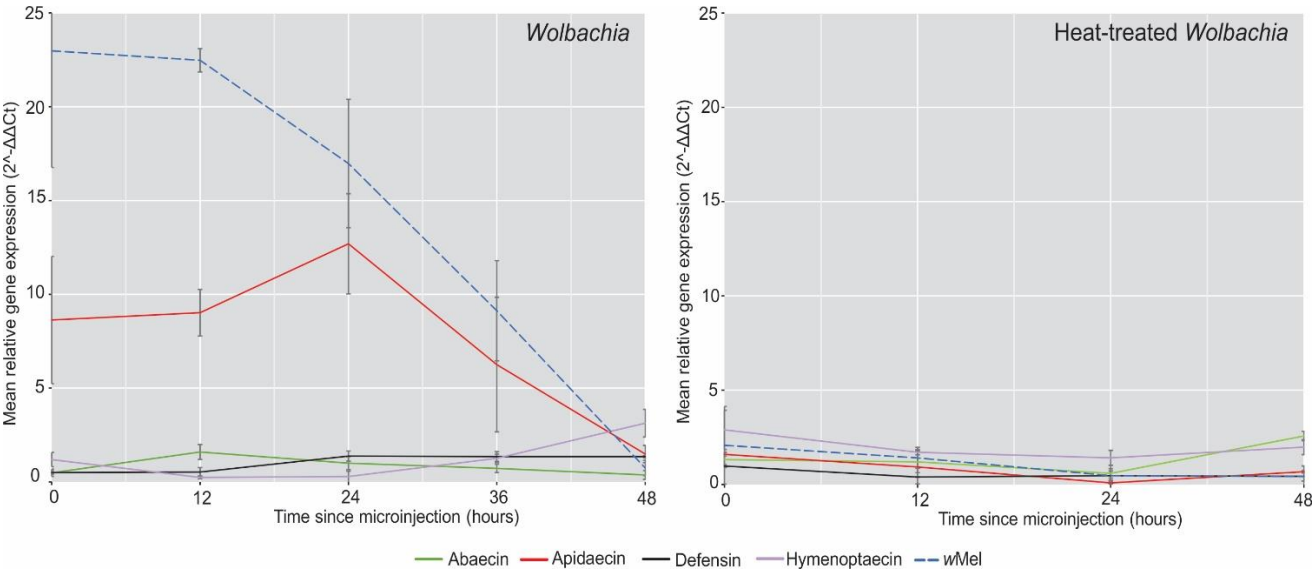

Figure 10 Mean relative antimicrobial expression in first instar *Apis mellifera* larvae following microinjection with *Wolbachia* or *Wolbachia* heat-treated for 30 minutes at 85°C over 48 hours. Treatment expression of antimicrobial peptide genes (abaecin, apidaecin, defensin, and hymenoptaecin) and *Wolbachia* (*w*Mel) is recorded as 2^(ΔΔCt), with treatment expression standardized against housekeeping gene *prost54* and standardized against control treatment (no microinjection). Error bars are ±1 SE.

DISCUSSION

Microinjection

Transinfection of *Wolbachia* into *Apis mellifera* did not prove successful via microinjection techniques that have been successfully used in *Drosophila* species (Xi *et al*., 2005; Walker, *et al*., 2009). Younger honey bee life stages were of higher risk of mortality, with zero eggs microinjected with *Wolbachia* hatching, only 22% of control embryos hatching and a lower proportion of young larvae suviving than older larvae in both treatments. Handling of young lifestages, particularly eggs, is precarious and high mortality in pre-pupated *Apis mellifera* has been noted in other studies (DuPraw, 1961; Collins, 2002, 2004). Based on previous studies, it appears that honey bee eggs have lower hatch rates than seen in other model insects, including *Drosophila*, which is reported to have a minimum hatch rate around 85% (Prout & Clark, 2000; Rashed, *et al*., 2008) *Aedes* mosquitoes of 87.5% (Edgerly, *et al*., 1993; McMeniman, *et al*., 2009), and the flour beetle *Tribolium castaneum* at approximately 80% in favourable conditions (Howe, 1957). Honey bees however, particularly carniolan bees (*A. m. carnica*, the species used in this study) hatch almost half as consistently, with a minimum hatching rate as low as 60%, dependant on queen characteristics, and further reduced by any change in ambient humidity (Collins, 2004; Al-Ghamdi, *et al*., 2014). Moreover, Collins (2002) found that any movement of honey bee eggs from the comb to plate has significant effect on hatch rate. Further manipulation, including lifting of the egg, further reduced the hatch rate by almost 50%. Older eggs appear to tolerate more handling than younger eggs. Previous successful honey bee microinjection experiments have found considerably lowered hatch rates in injected bees compared to those found in control treatments (Milne, *et al*., 1988). Eggs 24 hours old or younger have been considered too readily damaged to collect them from the comb safely to use for further experiments (Collins & Mazur, 2006).

The significant difference in hatch rate between *Wolbachia*-injected and control eggs, or more, the lack of any *Wolbachia*-injected eggs hatching at all, suggests that there is something the *Wolbachia* isolate that prevents hatching. The isolation protocol used in this study was somewhat rudimentary, and it is possible that left over constituent from *D. melanogaster* egg cytoplasm may have had a cytotoxic effect in *A. mellifera* eggs. However, this protocol has been successfully used to infect *Wolbachia*-cured *Drosophila* and mosquito cell lines (Xi & Dobson, 2005; Rasgon *et al*., 2011). It is also possible that the *Wolbachia* itself is causing the increase in immune response.

The signficant differences between life stages over all microinjection experiments indicates that resilience is strongly correlated with age, as is to be expected with the growth of individuals and the development of the immune system and defensive pathways (Evans, *et al*., 2006). However, the tradeoff between successful infection and survival exists (Hughes & Rasgon, 2014) and needs to be addressed when

considering future *Wolbachia* infection avenues utlizing microinjection. Compounding effects influencing egg hatch rate (including intrinsically low hatch rates) including: ambient stress, changes in temperature and humidity during injection, and mechanical trauma increase of egg turgidity and increase the likelihood of cytoplasm spill, all create harsh conditions for the embryo to overcome in its development. Therefore, additional effort needs to be placed on older life stages where mortality is reduced. Alternatively, isolating the bacteria through a more specific protocol and suspending in a differently media may be beneficial.

A potential limitiation in this study was the abiguity of liquid injected into the recipient individual. The Tritech digital microinjector (Tritech, USA) was always set on the minimum settings (1psi for 0.1 seconds) but the quantity of liquid expelled from the needle changed visibly between individuals and when expelled onto an empty slide. Therefore, it is difficult to say that the differing quantities of isolate injected did not have an influence onsurvival, particularly between *Wolbachia* and control treatments in eggs. It was clear however, that older individuals responded better to larger quantities, likely due to it being a lesser percentage of overall body volume. Previous successful honey bee microinjection experiments use very fine needles (~5µm) but utilize either innate oils (Milne, *et al*., 1988) or very small particles, such as interference RNAs (RNAi) (Dearden, *et al*., 2009) and so did not experience clogging of the needle point. However, in this study, due to clogging difficulties, perhaps to large quantities of suspended bacteria, a minimum needle point width of 10µm was required, potentially leading to further trauma of the eggs and young larvae.

Wolbachia strain

Phylogenetic analysis showed that the strain reported by Pattabhiramaiah (2011) was very closely related to the *w*Mel strain native to some *Drosophila melanogasteri* populations. It was found that the *D. melanogaster* colony at Victoria University of Wellington was naturally infected with *w*Mel. It is possible that the *w*Mel strain, while naturally infecting *D. melanogaster*, is not compatibile as an artificial infectant in *A. mellifera*. Alternative strains from closely related *A. m. capensis* or *A. m. scutellata* (*w*Cap-B1 and *w*Cap-B2) may be more appropriate. However, importation of South African bees into New Zealand is unlawful, posing considerable biosecurity risk (Biosecurtiy Act (2015)) and is therefore not a logistical possibility here. This may be a promising avenue for research in other countries, however.

It has been shown that closely related *Wolbachia* strains can infect evolutionarily distant host species, while the same host species can carry diverse *Wolbachia* strains (Werren, *et al*., 1995, 2008). This investigation found a clear example of this, seen in the strains originating from closely-related honey bee subspecies *A. m. capensis* and *A. m. scutellata* (*w*Cap-B1 and *w*Cap-B2) that were placed into distant

supergroups *B* and *O* respectively within our phylogenetic analysis. The *w*Cap-B2 formed a monophyly with Lepidopteran *Bremisia tabaci* with a divergence less than 2.5% over all genes, distant from *w*Cap-B1.

The phylogenetic analysis in this study (Fig. 2) highlights that naturally occurring strains from closely related hosts do not have predictable phenotypic interaction with their given hosts and that it cannot be assumed that closely related strains will behave in the same way. The lack of congruence between strain relatedness makes the choice of candidate strain for transinfection less simple than picking the strain from a species closely related to the recipient host. In this case, while it may be beneficial to examine strains found naturally in Hymenoptera, there is no way to predict their effects. The *w*Mel strain native to *D. melanogaster* has been well-studied (e.g. Harcombe & Hoffman, 2004; Thomas, *et al.,* 2011*;* Walker, *et al*., 2011; Ross, *et al*., 2017) and while it provides pathogen blocking and increased lifespan in some hosts, its cytoplasmic incompatibility-inducing properties need to be taken into consideration when using *Wolbachia* as a means of conservation (Blagrove, *et al*., 2012). Therefore, careful quarantine protocols should be followed in all future studies due to the variety of unknown (and potentially unpredictable effects) of *Wolbachia.*

That being said, there is evidence of successful transinfection between orders of insects in a number of successful inter-order transinfections, which generally experiment with cytoplasmic-incompatibility inducing strains. These include transinfection from *Muscidifurax uniraptor* (Hymenoptera: Pteromalidae) to *Drosophila simulans* (Meer & Stouthamer, 1999), transinfection of a known CI strain from the parasitic wasp *Scleroderma guani* (*w*SguBJ) to the whitefly *Bemisia tabaci* (Zhong & Li, 2014); and from lepidopteran butterfly (*Eurema mandarina*) to the hymenopteran saw fly (*Athalia rosae*) (Kageyama, *et al.,* 2017). All of these studies and the majority of successful transinfection studies have utlized host or recipient cell lines inoculated with *Wolbachia* in order to improve infection rates. In this study, I was not able to explore the avenue of *Apis mellifera* cell lines due to logistical, permitting, and funding issues. In future, however, this approach may be required in order to have successful transinfection as well as examine *in-vitro* effects of *Wolbachia* in honey bee cells.

Artificial insemination

This study is the first to attempt the novel approach of artificial insemination as a transinfection methodolgy using semen containing *Wolbachia*. The methodology was unsuccessful in this instance, but remains a unique avenue. Reasons for failure may include insufficient *Wolbachia* concentration in semen solution, or misrepresentative sampling of *Wolbachia* in viability testing resulting in unexpected quantities of dead bacteria. Queen ovaries were positive for *Wolbachia* 24 hours post-insemination, implying that the bacteria

was effective in reaching the ovaries, and the migration implies that it was viable. However, it is possible that the queen immune system may have played a role in it not being present 6-weeks post-insemination.

As *Wolbachia*'s effect on *A. mellifera* is unknown, queens inseminated with *Wolbachia* were kept under strict containment protocols, inside an enclosed bee room with no outdoor access as was required to reduce biosecurity risk. High densities of bees belonging to different hives in close proximity over a six week duration may have caused increased stress for both queens, workers and drones. Increased stress has been shown to increase the likelihood of bacterial infections in insects (Boucias & Pendland, 2012) by altering the internal mirohabitat or resulting in nutrient deficiency and thereby reducing immunocompetancy. However intra-ceullar infections rely on the survival of their hosts and therefore this stress may have been detrimental to invasion. The positive PCR presence of *Wolbachia* in inseminated queens 24 hour post-insemination highlights that insemination is an efficient pathway by which to place *Wolbachia* in the oviaries.

Deformed wing virus assay

Due to the injected inseminated progeny not being infected with *Wolbachia*, the *Deformed wing virus* assay was not informative. However, the development of methodologies for this assay will be beneficial for future experiments. As *Deformed wing virus* is made up of multiple closely-related viral variants (de Miranda & Genersch, 2012) with several master variants (DWV-A and DWV-B), it will be important to select an appropriate strain for further infection studies. It has been found that both DWV-A and DWV-B have been detected in honey bees in the absence of *Varroa* (Yue & Genersch, 2005; Zioni, *et al*., 2011; Martin, *et al*., 2012). However, DWV-A is the only strain that has been detected in honey bee colonies which, in the presence of *Varroa* mites, leads to colony death (Di Prisco, *et al*., 2011; Martin, *et al*., 2012; Mordecai, *et al*., 2015). There have been no reported instances of DWV-B being directly linked to colony death.

Immune response

The signficant upregulation of apideaecin in first instar larvae microinjected with *Wolbachia*, but not in those injected with heat-treated *Wolbachia*, suggests live *Wolbachia* elicits an immune response in immature honey bee larvae. High initial *Wolbachia* expression was expected due to prior microinjection. Early initial apidaecin expression in this assay is supported by Casteels *et al.* (1993), who found that apidaecin expression can be detected early post infection in honey bees inoculated with *E. coli* with highest levels being reached approximately 12 hours post-incoculation and remaining up-regulated and steady for the next 24 hours. In the assay presented here, the highest apidaecin levels were reached at 24 hours post injection but had an initial, potentially latent, expression at 0 hours relative to control treatments. This initial expression of apidaecin is consistent in all trials, and may be explained by stress caused during the lifting

process from the comb to microscope plate and exacerbated by a drop in ambient temperature, from 34.5°C in the hive down to room temperature (approximately 22°C) during the microinjection phase, which is close to the optimum temperature for proline-rich peptide activity (Zufelato, *et al.*, 2004*;* Danihlik, *et al*., 2015).

The absence of upregulated abaecin expression confirms that other inherited Gram-positive bacteria, that were not controlled for, such as *Spiroplasma*, were not responsible for the increase of apidaecin which is reported to effect Gram-positive bacteria at high concentrations (Cisak, *et al*., 2015; Danihlik, *et al*., 2015). As causal effect has not been determined, however, it cannot be confirmed that other gram-negative bacteria besides *Wolbachia* are not influencing expression.

By 48 hours post-injection, *Wolbachia* expression was comparable to that of control treatments. In microinjection trials, eggs and larvae were euthenized and diagnosed for *Wolbachia* infection via PCR 72 hours post-microinjection. If trends found in this assay are consistent, they would explain why larvae in the microinjection trials appeared negative via PCR for *Wolbachia* presence despite direct microinjection of the bacteria. Additionally, false negative reactions with insect material have been documented (Jayaprakash & Hoy, 2000). Beckmann and Fallon (2012) discovered, after inconsistencies between *Wolbachia* infection in adult *Culex pipiens* mosquitoes observed through microscopy and subsequent PCR results, that DNA templates prepared from whole insect bodies contains an inhibitor of the PCR reaction. Decapitation of the mosquitoes prior to DNA extraction restored reliability of PCR reactions. Due to the early developmental stages of the honey bee larvae microinjected, decapitation was not considered in DNA extraction protocols. Therefore, there is evidence to suggest that *Wolbachia* may have been present in microinjected individuals despite seemingly negative PCR results. Additionally, this would explain why the ovaries from the queen euthenized 24 hours post-artificial insemination reported positive for *w*Mel via PCR. Moreover, this increased immune response may provide explanation for why no honey bee eggs injected with *Wolbachia* hatched but those injected with only PBS did.

Apidaecin, a proline-rich family of peptides, are found in a number of social hymenoptera, including bumblebees (*Bombus terrestris*), *Vespula* wasps (*V. germanica*, *V. vulgaris*), and the bald-faced hornet (*Dolichovespula maculata*). Positively charged apidaecins are attracted to the highly negatively charged outer membrane found on Gram-negative bacteria. Unlike conventional antibacterial peptides, apidaecins are not amiphipathic, allowing them to enter into the periplasmic space of their target without forming pores (Casteels, *et al*., 1994). This enables them to directly target the heat shock protein DnaK and the bacterial chaperonin GroEL, inhibiting their ATPase activity and preventing protein folding, leading to bacterial death. Apidaecin's antibacterial activity is suspected to be influenced by the metabolic activity of target bacteria, exemplified with this activity being reduced 10-fold when bacteria is depleted in PBS for

20 hours prior to apidaecin exposure. Therefore, this heightened expression may confirm that *Wolbachia* cells microinjected were metabolically active.

Few investigations into *Wolbachia*'s effect on the immune expression of antimicrobial markers have been conducted, but the results of these studies have been shown to vary dependent on *Wolbachia* strain and host. In dipterans (*Drosophila simulans* and *Aedes albopictus* there was no evidence induced or suppressed peptide expression (Bourtzis *et al*., 2000). However, this study examined the influence of *Wolbachia* on infected and cured populations of species naturally infected by *Wolbachia*. Therefore, this lack of immune response is not surprising as the presence of *Wolbachia* as a secondary symbiont in these species is long withstanding (Poinsot & Merçot, 1997; Dobson, *et al*., 2002). Conversely, the virulent *w*Melpop strain, which has pathogenic effects in *Drosophila simulans* has been shown to upregulate the expression of the AMPs Cecropin C and lysozyme (McGraw & O'Neill, 2004). This result is thought to be due to the strain's uncontrolled replication lysing host cells, signalling receptors and initiating host immune response (McGraw & O'Neill, 2004; Siozios, *et al*., 2008). A recent study by Pan, *et al*. (2018) found that when *Aedes egypti* are artificially infected with *w*AlbB, it boosts basal immune response and upregulates the IMD and Toll pathways, and induces peptidoglycan recognition proteins expression. When these pathways are silenced there is significant reduction in *w*AlbB titre. Therefore, the upregulation of immune response in *A. mellifera* is not unexpected and does not rule out potential successful *Wolbachia* infection in the future.

CHAPTER 3

Conclusions and future recommendations

CONCLUSIONS

This thesis investigated the feasibility of transinfecting a strain of the endosymbiont *Wolbachia*, sourced from *Drosophilia melanogaster*, into the Western honey bee *Apis mellifera*. I conducted a thorough investigation on the phylogeny and relationships between all described *Wolbachia* supergroups in order to determine an appropriate *Wolbachia* strain for transinfection based from a previously described natural infection in *A. mellifera*. This phylogeny encompassed 59 representatives from all *Wolbachia* supergroups and *Wolbachia*-host phenotypes were reviewed and correlated with each supergroup. It was found that the natural infection found in honey bees by Pattabhiramaiah *et al*. (2011) was phylogenetically clustered with the *Drosophila melanogaster* native strain *w*Mel. Additionally, *Wolbachia* from supergroup *A* was discovered in *Ancistrocerus gazella*, the European tube wasp, where it has never been found before. Using *Wolbachia* isolated from *D. melanogaster*, I attempted the first ever transinfection in *A. mellifera* using established transinfection protocols. I extensively trialed existing microinjection protocols in over 1200 individual bees over various life stages, ranging from freshly laid eggs to red-eyed pupae. I developed and attempted a novel methodology of *Wolbachia* transinfection through the artificial insemination of queen bees with *Wolbachia*-inoculated drone semen. When these transinfection attempts proved unsuccessful, I investigated the expression of several antimicrobial peptides in response to *Wolbachia* microinjection and determined a significant upregulation of the peptide apidaecin in response to the presence of this foreign endosymbiont.

FUTURE RECOMMENDATIONS

Transinfection of *Wolbachia* has proven successful via microinjection in a number of dipteran species, including in the Drisiophilidae, Tephritidae and Culicidae families (reviewed by Hughes & Rasgon, 2014). Included in these microinjection experiments are those into arboviral reservoir *Aedes egypti* by Walker *et al*. (2011) to reduce its ability to vector viral dengue fever (DENV). This successful project highlighted *Wolbachia's* ability to manipulate the ability of its host's immune system to prevent the vectoring of a number of different human mosquito-vectored viruses. This movement has since progressed from solely DENV treatment, to a broad spectrum attempt to reduce the spread of other mosquito-vector diseases,

including *Zika* virus, *Chikungunya* (CHIKV), *West Nile* and bacterial malaria (Hughes, *et al*., 2011; Hussain, *et al*., 2012; Aliota *et al*., 2016; Dutra, *et al*., 2016). On the other hand, *Wolbachia*'s negative effects on its hosts have also encouraged research utilizing *Wolbachia* as a natural form of biocontrol in pest species such as crop pests (Zabalou, *et al*., 2004, reviewed in Bourtzis, 2008; Blackwood, *et al*., 2018). With up to 65% of arthropods estimated to be infected with *Wolbachia* (Zug & Hammerstein, 2012; LePage & Bordenstein, 2013), the literature is flooded with publications on the diagnosis of new species infected with the endosymbiont (Krstić, *et al.,* 2018*). Wolbachia* has sparked new curiosity regarding the interaction of endosymbionts with their hosts and the immune response (or lack thereof) in response to reproductive manipulations (Siozios, *et al*., 2008; Herbert & McGraw, 2018). In fact, *Wolbachia* has become a research hotspot, with over 3500 peer reviewed publications on the topic being produced since 2000 (Web of Science database, 2018). Despite this research boom, there is no evidence that *Wolbachia* transinfection has been reported in insects where it does not naturally occur in order to support their conservation.

Cell culturing of *Wolbachia*

The transinfection of *Wolbachia* into the honey bee is a novel concept. However, while current transinfection strategies work effectively between diptertan families, it is possible that they require tweaking to enable successful infection in *Apis mellifera*. This thesis utilized cytoplasm transfer from eggs of the natural host *Drosophila melanogaster* to *A. mellifera* recipient. While this methodology has proven successful previously, particularly in earlier studies (Boyle, *et al*., 1993; Poinsot & Mercot, 2001; McGraw, *et al*., 2001; Xi & Dobson, 2005), more modern studies, including almost all successful inter-order transinfections, passage *Wolbachia* in cell culture prior to microinjection (Dobson, *et al*., 2002; Walker, *et al*., 2011; Zhong & Li, 2014; Kageyama, *et al*., 2017). The shell-vial technique has proven very successful in infecting existing cell lines with *Wolbachia* (Dobson, *et al*., 2002). Understandably, the recipient appears more likely to accept a microinjection of self-cells than of non-self (Royet, 2004). The significant relative upregulation of apidaecin found in this study to the presence of *Wolbachia*, but not to dead, heat-treated *Wolbachia*, highlights that live foreign bacteria elicits an immune response in first instar larvae. The immature honey bee immune system is substantial (Evans, 2006; Gätschenberger, *et al*., 2013) and it has been shown that insect eggs contain immune elicitors. The tobacco hornworm (*Maduca sexta*), for instance, shows upregulation of the embryonic immune system when eggs are challenged (Adbel-latief & Hiker, 2008) and it has been proven that honey bee eggs inherit vitellogenin, a protein yolk precursor and pathogen recognition receptor (Zhang, *et al*., 2011; Salmela, *et al*., 2015). Therefore, it is possible that, when injected into honey bee eggs, *Wolbachia* elicits an immune response in the eggs, similar to that in larvae, that eventually results in the mortality of the bacteria, egg, or both.

It appears that *Wolbachia* retained in cell lines evolves substantially during passaging (McMeniman, *et al*., 2008; Woolfit, *et al.*, 2013). When a strain of *Wolbachia* native to *D. melanogaster* was passaged in an *Aedes albopictus* cell line for ~300 serial passages and then reintroduced to the original host, it showed significant loss of infectivity, grew to reduced densities and had limited phenotypic influences compared to the unpassaged strain. (McMeniman, *et al*., 2008). Further research on genomic evolution of these passaged strains found rapid bursts of genomic changes during cell line passaging, but these alterations do not occur if transinfected prior to culturing (Woolfit, *et al*., 2013). It appears that very small changes in the genome between strains has significant differences in phenotypic outcome. For instance, genomic differences between variants of the pathogenic *w*MelPop and the closely related nonpathogenic *w*MelCS are minor. No SNPs, indels, transposable or mobile elements have been identified between strains, only very minor substitution changes (Woolfit, *et al*., 2013). Therefore, the adaptation of *Wolbachia* to the host cell system is important to both the acceptance of the bacteria by the host but also to the evolution of the symbiont. However, the feat of culturing *Wolbachia* in host cell lines for the purpose of adaption is very time intensive. To develop *Wolbachia* into a biocontrol agent (Walker, *et al*., 2011), the *w*Mel derivative *w*MelPop was cultured in *A. egypti* culture for 3.5 years to allow adaptation to the mosquito intracellular environment to produce *w*MelPop-CLA (Woolfit, *et al*., 2013). Therefore, cell line development to this degree would not have been plausible in the time frame of this thesis. However, it is highly recommended to inoculate *Apis mellifera* cells with *Wolbachia* prior to microinjection in further research efforts to reduce immune response and lower the chance of rejection of *Wolbachia* in the recipient.

Apis mellifera life stage

Wolbachia's ability to migrate from somatic tissues to the ovaries and germ line is well established (Grenier, *et al*., 1998; Kang, *et al*., 2003; Frydman, *et al*., 2006). To do this, the bacteria must cross a number of tissues, a process that takes up to 15 days in mosquitoes (Frydman, *et al*., 2006). The total larval stage for the honey bee, from ovideposition to pupation, takes 21 days (Rembold, *et al*., 1980; Winston, 1991) and they are very sensitive during this time. Collins (2002) found that any handling of eggs reduced their hatch rate by up to 50%. Deviations in relative humidity can cause the shriveling of eggs and significant reduction in the hatching of healthy larvae, with a drop from 90% to 80% RH, lowering healthy larvae production by 30%. *In-situ*, larval rearing conditions are complex. Worker bee larvae is regulated at significantly higher temperatures than that of drone brood irrespective of location of the brood frame within the hive (Levin & Collison, 1989). Worker eggs and young larvae are kept at higher relative humidity than that of older worker larvae pupae (Li, *et al*., 2016), with both kept at a higher precision than immature drones, varying, on average, less than 1.5°C. However, pupae are much more loosely regulated within the hive, and are able to

withstand greater fluctuations in both temperature and humidity without significant influence on survival (Li, *et al*., 2016). The honey bee germline is developed by 48 hours post oviposition (Dearden, 2006) and so direct microinjection is required prior to this time, when the egg is at its most sensitive (Collins, 2004). Therefore, microinjection either needs to occur before 48 hours of age, or at a life-stage where the individual will cope with the Wolbachia's 15-day migration. Given the tradeoff between survival and infection, combined with results found in this study, it would be advisable to shift experimental efforts towards older life stages where survival is higher.

Artificial insemination as a novel transinfection route

The insemination of the F_0 as a means of *Wolbachia* transinfection has never before been attempted. This methodology could cut experimental time substantially if successful, as a single queen with *Wolbachia*-infected ovaries could produce up to 2000 eggs per day (Winston, 1987), with a single queen typically living 2-3 years. Positive infection of *Wolbachia* in queen ovaries 24 hours post-artificial inseminations found in this study, suggest that transport of *Wolbachia* artificially through the sperm duct is a viable and novel approach to transport *Wolabchia* to the ovaries. The disappearance of *Wolbachia* in sampling after this time frame is suggestive of an immune response, backed up by the upregulation of apidaecin found in larvae. Insects have been shown to have trans-generational immune priming and cumulative evidence shows that maternal exposure to dead or alive bacterial cells leads to increased immunocompetence in offspring (Moret, 2006; Roth, *et al*., 2009; Hernandez-Martinez, *et al*., 2010; Moreau, *et al*., 2012). Hence, immune upregulation in queens bees to *Wolbachia* (leading to the potential rejection of *Wolbachia*) may influence her offspring to resist bacterial infection also. Therefore, bees from multiple hives acting as potential *Wolbachia* recipients will need to be independent from previously *Wolbachia*-inseminated queens in future attempts of this methodology.

Honey bee immune response to *Wolbachia*

The immune response in insects to *Wolbachia* is varied and complex. In hosts where *Wolbachia* is naturally occurrent, such as *Drosophila* sp., it has been shown that several genes related with both humeral and cellular host immune response are upregulated in the presence of *w*Mel, including for two AMPs (Zheng, *et al.*, 2011). Additionally, when in *Drosophila* S2 culture, *Wolbachia* upregulates several genes involved in the Imd, Toll and JNK (Lemaitre & Hoffman, 2007), including the NF- κB transcription factors *Relish, Dorsal* and *dJun* (Bohmann, *et al*., 1994; Hetru & Hoffman, 2009). However, an *Anopheles gambiae* culture

infected with *w*Ri and *w*AlbB showed downregulation of over 75% of the immune-related genes involved in pathogen recognition that encode for effector molecules including those associated with the production of AMPs (Hughes, *et al*., 2011). Meanwhile, the infection of *w*Str in the small brown planthopper (*Laodelphax striatellus*) did not influence the expression of any immune genes whatsoever (Nakamura, *et al*., 2011). Kremer, *et al*., (2012) showed that in the parasitoid wasp *Asobara tabida*, the regulation of immune genes by native *Wolbachia* strains were tissue and sex-specific. In male wasps harbouring *Wolbachia*, numerous upstream genes associated with immune Imd, Toll, JNK and RNAi pathways were significantly upregulated while downstream genes coding for AMPs were downregulated. Immune genes were transcribed at a lower rate in the ovaries of female wasps. Therefore, it is important to further investigate the immune response of honey bees to *Wolbachia* at a finer scale. It is likely that if one AMP is upregulated, then *Wolbachia* may be influencing transcription elsewhere. It is recommended to assess this immune response in honey bees at varying life stages, including in queens, to determine *Wolbachia*'s influence.

It may also be beneficial to attempt to quell the honey bee immune system temporarily to increase the likelihood of *Wolbachia* infection. Modern methodologies such as RNA interference using double stranded RNAs (dsRNAs) have had success in the control of a number of pest insects (Baum, *et al*., 2007; Tomoyasu, *et al*., 2008). This process utilizes the enzymes which cleave long artificially supplied dsRNA of a target gene, into short interfering RNA (siRNAs) fragments which bind to the naturally occurring version of the sequence, hindering its translation (Huvenne & Smagghe, 2010). Utilizing this system, it may be possible to inhibit the transcription factors such as *Relish* that regulate the expression of target AMPs (Schluns & Crozier, 2007), lower immune response and thus increase the likelihood of *Wolbachia* infection.

Wolbachia strain in honey bees

Wolbachia's effects on its hosts are impossible to predict and therefore the selection of a "suitable" strain is difficult. A lack of suitable outgroup has prevented a satisfactory resolution of its phylogeny (Lo, *et al*., 2007), and evidence of horizontal transmission within arthropods, as well as between nematodes and arthropods, further confuses its origins and the evolution of phenotypic-effects (Bandi, *et al*., 1998; Glowska, *et al*., 2015). The lack of congruency between hosts and symbiont phylogenies (O'Niell, *et al*., 1992; Stouthamer, *et al*., 1999), together with closely related *Wolbachia* present in taxonomically distant hosts as seen in Supergroup *F* (Baldo, *et al*., 2006b; Raychoudhury, *et al*., 2009), and disparate *Wolbachia* strains in the same host all imply that lateral transmission is frequent and ongoing in *Wolbachia* (Vavre, *et al*., 1999; Duron & Hurst., 2013). However, based on current phylogenies, strains of *Wolbachia* that have

been shown to have facultative effects on arthropod hosts are limited to Supergroup *A*, with outliers in plant parasites where *Wolbachia* may supplement diet (Antonis, *et al*., 2012). Therefore, examining lab-rearable hymenopterans with natural *Wolbachia* infection in Supergroup *A*, such as *Nasonia vitripennis* may be advantageous. There is no guarantee that effects found in one host will be transferable to another (Warren, *et al*., 2008). Given that the large majority of *Wolbachia*'s described effects on insects involve reproductive manipulations, strict quarantine protocols should be undertaken.

Future work

Considering the large quantity of *Wolbachia*-centred research that continues to be published annually, it is surprising that there is no evidence of *Wolbachia* transinfection in honey bees, especially given their consistent media attention and the known consequences of global honey bee colony loss. In fact, it seems unlikely that studies similar to the one presented in this thesis have not been conducted previously. The current paradigm within the industry to not publish negative results (Matosin, *et al*., 2014) makes it difficult to know if similar trials have been conducted with results consistent (or perhaps contrasting) with what is presented in this thesis. Given that science is, by nature, a collaborative discipline, it seems a great waste if multiple parties are following the same routes of investigation and end up with similar negative results that go unpublished when said research could be otherwise informative.

There is nothing to imply that transinfection of *Wolbachia* into the Western honey bee is not possible. Given the global importance of honey bees to the world's food security, the influence of RNA viruses on their health and past success of *Wolbachia* as an artificial symbiont, future transinfection attempts in honey bees would not go wasted.

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APPENDIX 1

Protocol for the rearing of *Apis melliera* larvae from eggs through to pupation

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BACKGROUND

Honey bee development

Apis mellifera is a eusocial colonial hymenopteran, with three definitive caste divisions. These castes are divided into the reproductive queen, female workers and male drones (Winston, 1991; Matherson & Reid, 2011). Their development, regardless of caste, is fully holometabolous (undergoes full metamorphosis) and is broken down into egg, larvae, pupae and adult stages, but development time varies between caste (Fig 1). All bees, irrespective of caste, take three days to hatch out of their eggs, but from hatching vary from 16 days in queens, to 19 in workers to up to 23 days for drones. This time is split between larval and pupal stages. Larvae go through four moults, coinciding with transition from first instar larva through to 4th instar, when their cell is capped. They then spend up to nine days as pupae before chewing through the capped cell and emerging as adults (Mathieson & Reid, 2011).

Honey bee eggs are laid vertically in the comb well (Figure 2, cell 2). The posterior end of the egg is narrower than the top and is connected to the wax by a glue-like substance secreted by the queen during oviposition. The egg is positioned with the anterior (head) end facing upward. Over the course of approximately three days (which is somewhat dependent on temperature), the egg slowly tilts onto its side on comb cell's base. The embryo is nourished by yolk within the egg package, made up of vitellogenin (Salmela, *et al.* 2015). To hatch, the larvae flexes within the egg, releasing fluid along the dorsal midline which begins the digestion of the egg shell.

During their development, honey bee larvae undergo five instars, each lasting varying lengths of time (Bertholf, 1925). Larvae hatch from their eggs 66-93 hours after oviposition (Collins, 2004). There are a number of morphological features by which to characterize the age of the five instars of larval development, all viewable under a dissecting microscope (Human, *et al*., 2013). These include the head diameter, development of the wing buds, leg buds, mouth pieces and gonapophyses. It is important to be aware that the first instar lasts between 14-20 hours. Once the larvae has reached the age of one day (24 hours post hatching from the egg), it has already reached the second instar (Bertholf, 1925; Rembold, *et al.*,1980). In a case where eggs rather than larvae are desired, it is important to frequently check when

oviposition has begun and to check regularly over the next few hours until the queen has laid enough eggs for the given experimental design. The honey bee embryonic germline develops 48 hours post-oviposition (Dearden, 2006). Therefore, egg age will need to be considered for experiments where this is a crucial component, such as embryological studies.

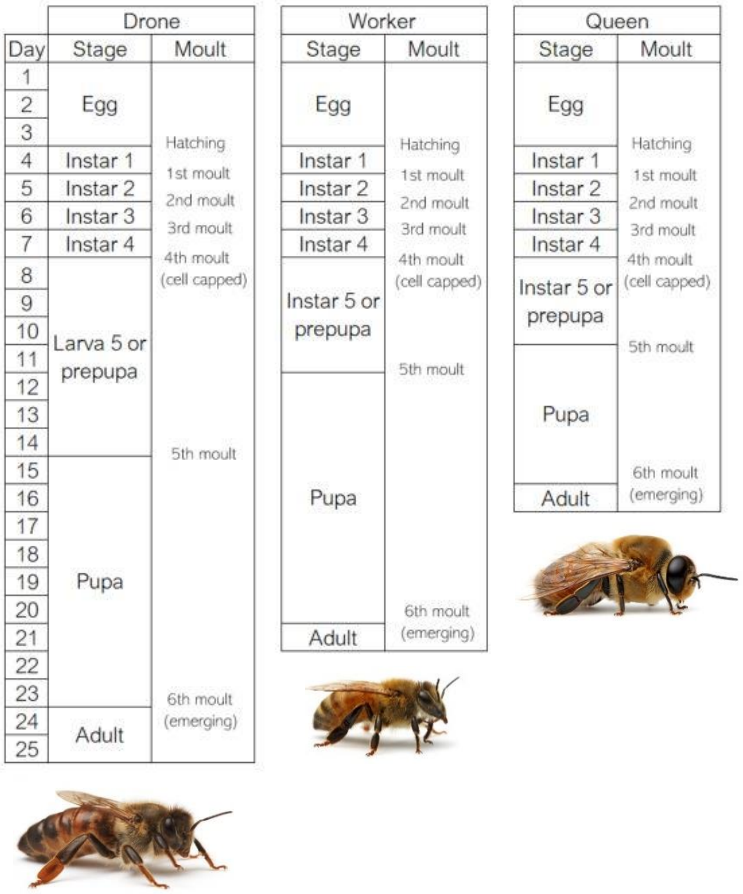

Figure 3 Honey bee (*Apis mellifera*) developmental stages per caste. Photos: Alexander Wild

PROTOCOL

Obtaining eggs

Naturally, eggs are not laid in chronological order along the comb, so eggs and larvae within the comb are not ordered by age and must be visually identified (Figure 2). To streamline this process, the queen can be manipulated to oviposit eggs on comb that is convenient to access, remove from the hive and handle in the laboratory. To obtain eggs (or 1st instar larvae), a queen should be confined using a queen excluder cage (where workers can pass through the cage grid while the queen remains in the matrix) for 24-36 hours on worker comb. The queen excluder should be placed towards the centre of the hive to ensure the queen is

well fed by nurse bees (Crailsheim, *et al*., 2013). The length of time the queen remains in the excluder box is dependent on the age of eggs or larvae desired and the speed of oviposition initiation, duration and continuation which are dependent on the given queen. The first instar lasts 14-20 hours after hatching from the egg, which occurs approximately three days after ovideposition (Winston, 1987; Matherson & Reid, 2011). Queens do not start oviposition immediately after being placed in an excluder and this needs to be confirmed visually after a few hours of caging (Crailsheim, *et al*., 2013).

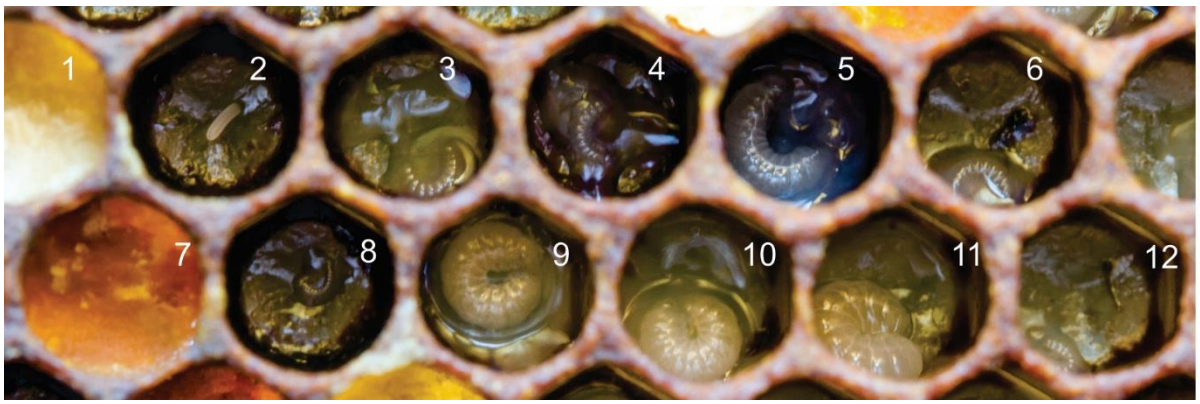

Figure 4 Honey bee eggs and larvae laid *in-situ*. Egg laying is not chronological by cell within the comb and so larvae found is not ordered by age. By cell: 1 and 7, bee bread – honey and pollen; 2, egg between 1-2 days old; 3-4, 6 and 12, early 2nd instar larvae; 5, 3rd instar larvae; 8, 1st instar larvae, recently hatched; 9-10, early 3rd instar larvae; 11, late 3rd instar larvae.

Collins (2002) found that young eggs are incredibly sensitive to disturbance and that any handling can reduce their hatch rate by up to 50%. Deviations in relative humidity can cause shriveling of the eggs and significantly reduce the proportion of eggs that hatch into healthy larvae. The movement of comb to plate or petri dish is precarious and in order to reduce impact it is advisable to keep eggs within the comb until hatching. Microinjection applications can often be conducted with eggs remaining on the comb if the excess height of the wax walls is cut down with a sharp scalpel or scissors.

If eggs are to hatch successfully, they need to be grafted from the comb onto the desired plate in the same position. That is, the end of the egg in contact with the wax comb will develop into the anus-end of the larvae and to promote healthy hatching, the end of the egg that comes into contact with the plate needs to be consistent with that in contact with the wax in-situ.

Incubation environment

The inside of a beehive is regulated to vary between no more than 2°C in brood frames (Li, *et al*., 2016) and the relative humidity (RH) is retained at approximately 90-95% RH during egg oviposition and larval development up to pupation. Reduced RH can result in the shriveling of eggs and reduced hatch rate. A drop from 90% to 80% RH has been shown to lower healthy larvae production by 30% (Collins, 2002)

Honey bee larvae may be kept in air tight plastic containers (such as Tupperware) and placed inside incubators to facilitate humidity regulation. It is important to maintain an optimum temperature of 34.5°C with minimal variation (±1°C) as suboptimal temperatures have been shown to affect adult bee longevity and can induce malformed wings (Vojviodic, *et al.* 2010). Humidity can be maintained and adjusted by including a dish of saturated K_2SO_4 solution to maintain 95%RH. After the 6^{th} day with the onset of pupation, the K_2SO_4 may be replaced with a saturated NaCl solution to achieve 80% RH. This adjustment has proved to be appropriate in a number of studies (eg. Rembold & Lackner (1981) Vandenberg & Shimanuki (1987), Peng *et al*. (1992), Aupinel, *et al.* (2005)) but was not found to have any noticeable effect during this protocol. Alternatively, if using a separated container with a ventilated division (eg Sistema deli storers) the bottom of the tray can be lined with sterile tissues dampened with autoclaved distilled water. However, if this technique is chosen, it is important to avoid saturation of the tissues as this causes the moisture in the air to condense and drown the larvae. During the egg stage, the effect of humidity is related to the ability of an egg to maintain its membrane integrity. Therefore, inadequate humidity will cause disruption of the chorion/vitelline membrane within the eggs and cause damage or leakage and therefore a lowered hatch rate.

Preparation of diet

There are many diet recipes used by different labs and they vary in their ratios of sugars, water and royal jelly. In general, it is important that the larval diet does not contain *less* than 50% (v/v) royal jelly or *more than* 33.3% (v/v) aqueous solutions. If these requirements are not met, the larval diet will contain too much water and young larvae will drown in the diet or have problems with digestion (Crailsheim, 2013). Studies by Kaftanoglu *et al.* (2010a, 2010b) have shown that variation in sugar ratios (glucose : fructose) had impact on the number of adults emerged, and on the number of queens and intercastes produced. Lower quantities of sugars enable growth but cause death during pupation, while higher sugar quantities increase the chance of queens and intercastes.

The diet used in this protocol used the following recipe ratio:

- 6% D-glucose
- 6% D-frucotse
-1% yeast extract
-53% royal jelly
-33% distilled H_2O

It is recommended to make the diet in bulk for ease of labour. Diet stored at 4°C should be kept and used for 3 days only. Alternatively, diet made for the whole exposure period may be prepared at rearing initiation, aliquoted, and stored at -20°C for the duration.

Keep royal jelly frozen at -20°C and thaw by placing it at 4°C overnight or at room temperature for an hour dependent on the size of the container. Make up the aqueous solution (glucose, fructose, yeast and distilled H_2O) in a beaker with warm water cool enough for hands to touch (45-55°C) and ensure everything is dissolved before decanting into the royal jelly and mixing with a spatula. This solution can then be aliquoted for daily feeding dependent on the number of larvae being reared. It is important to warm the diet to 35.4°C before feeding to avoid chilling the larvae.

Mortality

The presence of dead larvae in the feeding plates increases the likelihood of bacterial or fungal infection and increases the risk of mortality in the rest of larval cohort. Therefore, it is vital to remove dead larvae as soon as possible to prevent decomposition by bacterial or fungal saprophytes and contamination of the rest of the larvae. Dead larvae can be recognized by the following symptoms, either by eye or under a dissecting microscope: absence of movement (particularly in older instars), lack of turgidity, flattened body, discolouration or necrosis of tissue (Genersch *et al.*, 2005, 2006). (Figure 3B) Additionally, any eggs that have not hatched after 90 hours post-oviposition should be removed.

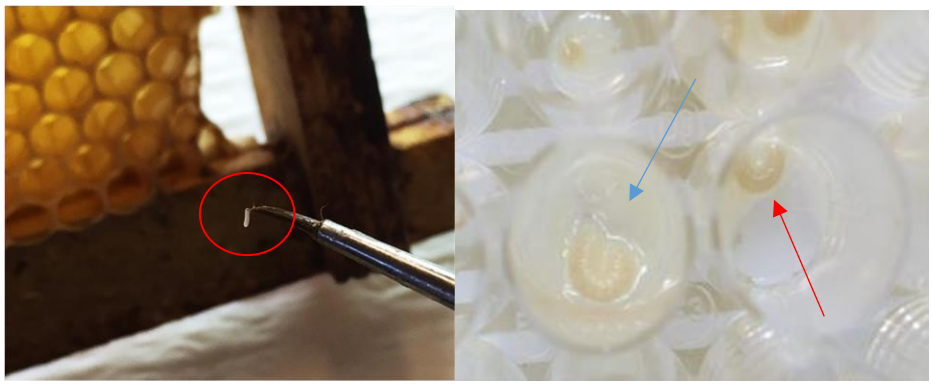

Figure 5 A, honey bee egg removed from comb using a size 0 paint brush with damp bristles tapered to a point. B, Visual difference between alive larvae (blue arrow) and dead larvae (red), distinguishable by discolouration.

Customized protocol

- Honey bee eggs are kept in-comb until hatching. If using a removable queen excluder comb, the excluder should be kept upright in a large air tight container, as occurs in the hive. The eggs must be checked multiple times per day, and once a few eggs are seen to be hatching, the comb should be placed flat so there is no sliding of the first instar larvae.
- If the eggs are required to be moved prior to hatching, eggs should be grafted as groups of 3 into the wells of 24-well cell culture plates using the end of a size 1 paint brush with dampened and tapered bristles (Fig 4a). A light dissecting microscope may help with this. Alternatively, resting the comb with the light source positioned behind the wax frame may help to visualize the eggs. Eggs are very easily damaged (Collins, 2004) and are prone to being bent, squashed and pierced. In order to minimize trauma, eggs should be placed into their respective wells by rotating the paint brush during the placement to reduce pressure or smearing of the egg. Wegner, *et al*. (2009) provide additional options for moving eggs.
- Dependent on the frequency of visits affordable each day, if the eggs are close to hatching (<48 hours since ovideposition) a small quantity of diet (see section 'Preparation of diet' above) can be placed below the eggs to ensure adequate food for the larvae upon hatching. This is done *ad lib* using a small spatula, placed in the centre of the well and spread to the base of the eggs in a thin layer to prevent potential drowning of the larvae upon hatching. Larvae should be placed with the same side up as they hatched, as their spiracles used for respiration are found here. Turning them will result in suffocation, and in the presence of liquid food, drowning.
- Once hatched, 1st instar larvae are transferred with a paint brush in the same manner as eggs, with a thick smear of diet placed near the head using a paintbrush. Alternatively, the diet can be aliquoted onto the plate and the larvae gently placed on top. This increases the risk of drowning but there is a

smaller likelihood of the diet drying out, so the larvae need to be checked less frequently during this stage.

- Larvae below instar three should be fed using a paint brush. The diet should be smeared in the well with the majority of diet placed near the larva's head. If the head is unable to be determined from the posterior end of the larvae (as young larvae are relative homogenous looking, and mouthparts are difficult to distinguish without microscopy), equal amounts can be placed on each end.
- Larvae should be checked preferably every 4 hours to replace food. It is important to provide adequate diet when the larvae are being left unattended overnight.
- In general, the larvae should receive approximately 10µL of diet each on the first day (Aupinel, *et al*., 2005), and increase as development continues. However, as long as a balance can be reached between providing adequate quantities of food without drowning, the exact quantities of food does not matter. This can be measured by eye within reason. If they have eaten their allotted food, provide more.
- If there is diet remaining from the previous day, siphon it away using a pipette before supplying fresh diet as the water component of the diet will eventually separate and increasing the risk of drowning.
- As the larvae grow and moult, they can have their food carefully pipetted next to their mouthparts. As the larva grows the risk of drowning decreases.

Pupation

- On the 6th day the larvae may start depositing uric acid crystals on the dorsal side of the body. This is an indication of their reaching the defecation stage and predicts the beginning of the spinning stage. The larvae should now be removed from the feeding dishes and transferred to larger petri dishes (100 x 15mm), lined with Kimwipes® tissue paper.
- The next day, the old Kimwipes® tissue paper containing feces should be removed and the larvae should be moved to fresh tissue and transferred to 80% RH. Once defecation ends, spinning will begin and the larval will initiate pupation. For workers, pupation lasts approximately 9 days.
- Any dead or non-pupated larvae should be removed after 24 hours.

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APPENDIX 2

Phylogenetic analysis accession numbers

Table S1 Accession numbers for taxa examined in the phylogenetic analysis in Figure 2. Ordered by host species in alphabetical order for all available sequences for *16S*, *gltA*, *groEL*, *ftsZ*, and *wsp* for each species.

			Accession numbers				
***Wolbachia* host species**	**Order**	**Supergroup**	***16S***	***gltA***	***groEL***	***ftsZ***	***wsp***
Aphis fabae	Hemiptera	M	JX296462	JN316248	JN316284	HQ843854	EU822302
Apis mellifera	Hymenoptera	A	EF032158	-	-	-	-
Apis mellifera capensis	Hymenoptera	B	-	-	-	-	FJ438823
Apis mellifera scutellata	Hymenoptera	B	-	-	-	-	AF510085
Armadillidium vulgare	Isopod	B	AJ133196	FJ390331	FJ390366	DQ778102	AJ419987
Asobara tabida	Hymenoptera	A	FJ603467	-	AY714809	-	AY581189.1
Bemisia tabaci	Lepidoptera	O	KF454771	KF587270	KF452543	-	KX650070
Brevicoryne brassicae	Hemiptera	M	JX296429	JN316250	JN316284	JN316233	-
Brugia malayi	Spirurida	D	AJ010275	AJ609643	AF373870	AJ010269	AY52702
Brugia pahangi	Spirurida	D	AJ012646	AJ609642	AJ609654	AJ010270	-
Bryobia sp.	Spider mite	K	EU499316	EU499326	EU499331	EU499321	JN572881
Coptotermes acinaciformis	Dictyoptera	F	DQ837197	-	AJ627384	-	AJ833931
Coptotermes lacteus	Dictyoptera	F	DQ837199	-	AJ627385	-	AJ833930
Cordylochernes scorpioides	Pseudoscorpian	O	-	FJ390336	FJ390370	AY916134	AY916133
Ctenocephalides felis	Siphonaptera	I	AY157504	AJ609650	AJ609659	AJ628415	KY363325
Culex pipiens	Diptera	B	KJ512994	AY714785	-	-	-
Culex quinquefasciatus	Diptera	B	KX611381	AY714789	AY714804	-	-
Dipetalonema gracile	Spirurida	J	KY255233	AJ609648	AJ609658	-	-
Dirofilaria immitis	Spirurida	C	Z49261	AJ609641	AJ558023	AJ010272	KF359767

Dirofilaria repens	Spirurida	C	AJ276500	-	AJ609653	AJ010273	AJ232176
Drosophila melanogaster	Diptera	A	AB360385	AE017260	AE017257	U28189	KX650072
Drosophila simulans	Diptera	A	DQ235280	AY714792	AY714807	AY227739	DQ235409
Drosophila simulans	Diptera	A	DQ235279	AY714790	AY714805	AY508998	AF020068
Drosophila simulans	Diptera	A	DQ235278	AY714791	AY714806	U28178	AF020070
Drosophila simulans	Diptera	B	DQ235288	AY714787	AY714800	AY509001	AF020074
Encarsia formosa	Hymenoptera	A	AF045189	AY714783	AY714797	U28196	FJ222455
Folsomia candida	Collembola	E	KU255239	AJ609649	-	AJ344216	KT799615
Kalotermes flavicollis	Dictyoptera	F	Y11377	AJ609651	AJ609660	AJ292345	-
Leptopilina australis	Hymenoptera	B	-	-	AY714802	-	AF071920
Litomosoides brasiliensis	Spirurida	D	KU255241	AJ609646	AJ609655	-	AF409112
Litomosoides hamletti	Spirurida	D	KU255244	-	AJ609656	-	-
Litomosoides sigmodontis	Spirurida	D	FR827944	AJ609645	AF409113	AJ010271	-
Mansonella ozzardi	Spirurida	F	AJ279034	AJ609647	AJ609657	-	-
Mansonella sp.	Spirurida	F	AJ279034	AJ628413	AJ628412	AJ628414	-
Mellitobia digitata	Hymenoptera	A	-	-	AY714808	-	KS308227
Mesaphorura italica	Isoptera	H	AJ575104	-	-	AJ575103	-
Microcerotermes sp	Dictyoptera	F	AJ292347	-	AJ628411	AJ292346	-
Nasonia giraulti	Hymenoptera	A	M84690	AY714793	AY714810	U28182	AY622512
Nasonia longicornis	Hymenoptera	B	M84692	AY714794	AY714811	-	AF448385
Nasonia vitripennis	Hymenoptera	A	-	AY714795	AY714812	U28188	DQ508544
Nasutitermes nigriceps	Isoptera	H	DQ837204	FJ390333	-	FJ390318	-
Onchocerca gibsoni	Spirurida	C	-	AJ609639	AJ609652	AJ010267	AJ252178
Onchocerca gutturosa	Spirurida	C	AF172401	-	-	AJ010268	-
Onchocerca ochengi	Spirurida	C	AJ276499	AJ609640	-	AJ010266	AJ252178
Onchocerca volvulus	Spirurida	C	AJ276498	-	Y09416	AJ276501	-
Protocalliphora sialia	Hymenoptera	B	X62247	AY714788	AY714801	U28202	AY622511
Radopholus similis	Spirurida	L		-	EU833484	EU833483	-
Syringophilopsis turdi	Parasitiformes	F	KP114103	KP114117	-	-	-
Torotrogla carduelia	Parasitiformes	Q	KP114100	-	-	-	--

Torotrogla lusciniaea	Parasitiformes	P		KP114118	KP114120	-	-
Torotrogla merulaea	Parasitiformes	P	KP114099	-	KP114119	KP114113	-
Torotrogla rubeculia	Parasitiformes	P	AY764280	AY714784	KP114123	-	-
Toxoptera aurantii	Hemiptera	N	JN384094	-	-	-	-
Tribolium confusum	Coleoptera	A	LO2883	AY714784	AY714798	U28194	KJ152796
Trichogramma cordubensis	Hymenoptera	B	AJ292347	-	AY714803	-	AF245164
Wuchereria bancrofti	Spirurida	D	AF093510	AJ609644	-	AF081198	DQ235409
Zootermopsis angusticollis	Isoptera	H	AY764280	AY764281	AY764278	AY764283	-
Zootermopsis nevadensis	Isoptera	H	-	AY764282	AY764278	AY764284	-
Outgroup							
Ehrlichia chaffeensis	Rickettsiales		CP007480	AF304142	-	AF221944	-
Ehrlichia ruminantium	Rickettsiales		NR074155	DQ513390	DQ647013	DQ647000	-

APPENDIX 3

Primers

Table S2 Primers used throughout this thesis

Locus	Primer (F/R)	Primer sequence (5'-3')	PCR type	Reference
16S	Wspecf/Wspecr	CATACCTATTCGAAGGGATAG	Standard	Werren et al., 2000
		AGCTTCGAGTGAAACCAATTC		
FtsZ	ftsZ_F1/ftsZ_R1	ATYATGGARCATATAAARGATAG	Standard	Baldo et al., 2006
		TCRAGYAATGGATTRGATAT		
groEL	WgroF1/ WgroRev1	CAACRGTRGSRRYAACTGCDGG	Standard	Casiraghi et al., 2005
		GATADCCRCGRTCAAAYTGC		
FbpA	FbpA_F1/fbpA_R1	GCTGCTCCRCTTGGYWTGAT	Standard	Baldo et al., 2006
		CCRCCAGARAAAAYYACTATTC		
wsp	81F/691R	TGGTCCAATAAGTGATGAAGAAAC	Standard	Zhou et al., 1998
		AAAAATTAAACGCTACTCCA		
Deformed wing virus	DWV_F/DWV_R	TCCATCAGGTTCTCCAATAACGGA	RT-PCR	Yue & Genersch, 2005
		CCACCCAAATGCTAACTCTAAGCG		
abaecin	Abaec_F1/Abaec_R1	CAGCATTCGCATACGTACCA	RT-qPCR	Evans, 2004
		GACCAGGAAACGTTGGAAAC		
apidaecin	apidF1/ApidR1	TAGTCGCGGTATTTGGGAAT	RT-qPCR	Evans *et al* 2006
		TTTCACGTGCTTCATATTCTTCA		
defensin 1	Defen1F/DefenR	TGCGCTGCTAACTGTCTCAG	RT-qPCR	Evans, 2004
		AATGGCACTTAACCGAAACG		
hymenoptaecin	hymen1_F/hymen1_R	CTCTTCTGTGCCGTTGCATA	RT-qPCR	Evans *et al* 2006
		GCGTCTCCTGTCATTCCATT		
wMel	w1_f/w1_R	GCTCCTCTGGTTGCTACTGG	RT-qPCR	Lee, *et al*., 2012
		ACTATGCCATCGCGTTTTGC		
Pros54	pros54_F/pros54_R	TCGAACCAAGATGGTACTGGAA	RT-qPCR	Cameron *et al*., 2013
		TTGTTGTGCTTGCAGTCGTG		