Generation and characterization of *Wolbachia* transinfections and development of female-specific RIDL technology in *Aedes albopictus*

by Marcus Blagrove

Green Templeton College

DPhil Zoology

OXITEC

Acknowledgments

First and foremost, I would like to thank my supervisor Prof. Steve Sinkins for all of his invaluable advice and guidance throughout my DPhil.

I thank both Prof. Steve Sinkins and Dr Luke Alphey for giving me the opportunity to do this DPhil.

I would also like to thank my transfer panel, Dr Mike Bonsall and Dr Neil Morrison, as well as my examiners, Dr Alain Kohl and Dr Petros Ligoxygakis for their very helpful advice on improving this thesis.

I thank everyone in Steve Sinkins's lab and Oxitec for all the help they have given me during my DPhil, especially: Dr Isaac Black, Mr Josh Blight, Miss Cristina Di Genua, Dr Roya Haghighat-Khah, Dr Limb Hapairai, Dr Claire Harris, Mr Tim Harvey-Samuel, Dr Zakaria Kambris, Miss Jenny Molloy, Mrs Caroline Phillips, Dr Hoang Kim Phuc, Dr Sarah Scaife, Dr Kirsty Stainton, Mr Ed Sulston and Miss Beth Sutton.

On a more personal level, I'd like to thank my family and friends for all of their support throughout these four years, in particular, my mum and dad, and Amy Clare and Beth Sutton.

Cheers!

Abstract

Aedes albopictus is an important vector of dengue and chikungunya viruses, and, over recent decades, has resisted traditional control strategies allowing it to spread from its native Southeast Asia throughout the world. In this thesis, two alternative control methods are assessed and developed: transinfection with the inherited bacteria *Wolbachia*, for population replacement with a refractory strain; and a genetic equivalent to the sterile insect technique, RIDL (Release of Insects carrying a Dominant Lethal), for population suppression.

Wolbachia is a genus comprising maternally inherited intracellular α-proteobacteria which primarily infect arthropods. Certain strains of *Wolbachia* both have the ability to manipulate host reproduction through cytoplasmic incompatibility (CI) which allows *Wolbachia* to invade host populations by conferring a reproductive advantage on infected females, and have been shown to confer broad-spectrum pathogen resistance on their hosts. Here, a transinfection of *w*Mel in *Aedes albopictus* (Uju.wMel) was generated which shows complete bidirectional CI with the natural *Wolbachia* infection of *Ae. albopictus*, in the absence of any major fitness costs and (as shown by collaborators) completely abolishes dengue and chikungunya virus transmission. It was also shown that the pathogen inhibition in Uju.wMel occurs in the absence of immune stimulation. Evidence supporting cholesterol sequestration by *w*Mel as a mechanism for the pathogen inhibition observed in Uju.wMel was found.

Previous attempts to produce a conditionally inviable genetic sexing *Ae. albopictus* RIDL line have resulted in a sub-optimal strain in which the construct was not sufficiently specific or repressible, resulting in a high proportion of off-target inviable mosquitoes. Here, the mating competitiveness of RIDL males was shown to be not significantly different from wild-type, confirming the potential utility of the system. Multiple truncations of the promoter were made in an attempt to reduce the off-target expression.

Contents

Chapter 1: Introduction

1.1 *Aedes albopictus*

1.1.1 Disease transmission

The mosquito *Aedes albopictus* is an important vector of several important human diseases including dengue and chikungunya (Hawley, 1988). Dengue is endemic in over 110 countries (Ranjit, 2010) and infects between 50 and 390 million people per year worldwide (Whitehorn and Farrar, 2010; Bhatt *et al.,* 2013). The mortality rate is 1-5% but can rise to around 26% with severe disease resulting from secondary infection with a different serotype (Ranjit, 2010). Whilst *Ae. aegypti* is the most competent vector of dengue virus (Gubler, 1998), *Ae. albopictus* is considered a maintenance vector (Lambrechts *et al.,* 2010) which can be solely responsible for transmission in dengue epidemics in the absence of *Ae. aegypti* (Lounibos, 2002; Gratz, 2004) and serve as a bridge vector from sylvatic cycles in non-human primates and monkeys to 'urban' cycles in humans (Knudsen, 1977).

Chikungunya virus causes similar symptoms to dengue virus but has fewer outbreaks in more limited areas. The true extent of chikungunya may not be realized as it commonly co-circulates with dengue and it is often misdiagnosed as dengue fever (WHO, 2011). Similarly to dengue virus, chikungunya virus is primarily transmitted by *Ae. aegypti*; however, two mutations in the viral envelope genes E1 and E2, found during the 2005-06 Reunion Island outbreak, are believed to specifically enhance transmission of chikungunya virus by *Ae. albopictus* but not *Ae. aegypti* (Enserink, 2007; Tsetsarkin *et al.,* 2007;

Tsetsarkin *et al.,* 2011b). These mutations may both increase the frequency of infection as well as more readily expand the range to areas which contain *Ae. albopictus* but not *Ae. aegypti*, such as La Reunion island (Salvan and Mouchet, 1994).

Ae. albopictus is also implicated in the transmission of other veterinary and less serious arboviruses such as equine encephalitis virus (Moore and Mitchell 1997), LaCrosse encephalitis virus, St. Louis encephalitis virus and Cache Valley virus (Hawley, 1988), as well as *Dirofilaria immitis*, the etiological agent of heartworm in dogs and cats (Gratz, 2004).

1.1.2 Ecology

The relatively lower vectorial capacity of *Ae. albopictus* compared to *Ae. aegypti* may partly be explained by the difference in habitat and ecology of the two vectors. Whilst *Ae. aegypti* is closely associated with humans, being a primarily urban species and with a feeding preference for humans, *Ae. albopictus* is generally found in more rural areas with a more sparse human population (Lambrechts *et al.,* 2010), and has more catholic feeding habits, frequently taking blood meals from other mammals, birds and even reptiles (Hawley, 1988).

In accordance with its more rural habitat, the larval development sites of *Ae. albopictus* tend to be more natural reservoirs such as tree holes; as opposed to the predominantly man-made sites used by *Ae. aegypti*, such as waste containers and pot-holes in roads (Lambrechts *et al.,* 2010).

1.1.3 Global distribution and colonization

Ae. albopictus is native to south-east Asia (Gratz, 2004) but over recent decades has established populations throughout the world including the east USA (Gratz, 2004), south America (Forattini, 1986), throughout Africa (Cornel and Hunt, 1991; Savage *et al.,* 1992; Fontenille and Toto, 2001) and throughout Europe (Scholte and Schaffner, 2007). *Ae. albopictus* has previously entered Australia but has been prevented from colonizing the mainland by strict border controls (Russel *et al.,* 2005; Derraik, 2006); the species has however become established in the Torres Strait between Australia and Papua New Guinea (Ritchie, 2006).

The colonization of new areas with *Ae. albopictus* is believed to be expedited by increased human travel and goods transportation, in particular by the used tyre trade (Reiter, 1998), with the desiccation-resistant eggs being easily accidentally transported to a new area. The invasiveness of *Ae. albopictus* is also exacerbated by the ability of the species to tolerate extremes in climate, from its natural hot and humid south-east Asian environment to colder areas such as Italy and northern USA, where its eggs have been observed to overwinter (Hawley *et al.,* 1989; Hanson and Craig, 1995) and even survive sub-freezing temperatures in suitable microhabitats (Romi *et al.,* 2006).

An example of the rapid colonization ability of *Ae. albopictus* was seen during the widespread use of DDT in the 1960s. DDT was highly effective in eliminating local populations of *Anopheles* and *Ae. aegypti*, for example in Kolkata; however, *Ae. albopictus*

colonized this area, effectively displacing the former species. This was believed to be due primarily to the behaviour of the adults; DDT was mostly sprayed on the inner walls of properties whilst *Ae. albopictus*, unlike these other species, prefers to rest in the wider vicinity of the buildings (Gilotra, 1967). This, and the resulting reduction in larval competition, likely expedited the colonization of *Ae. albopictus*.

1.1.4 Control

So far, strict border controls as demonstrated by Australia (Russel *et al.,* 2005; Derraik, 2006) can be effective in preventing colonization; however, controlling an established population of *Ae. albopictus* has proven difficult, largely due to its adaptability and behaviour.

Control of *Ae. albopictus* has largely focused on removal of larval breeding sites such as removal of rubbish and unblocking of drains. In addition, other standing water such as pools in tyre stockpiles and long-lasting puddles are monitored and may be treated with insecticides. However, given the less urban and more natural habitat of *Ae. albopictus*, the identifying, monitoring and clearing of such sites can be more difficult than with *Ae. aegypti*.

As *Ae. albopictus* is a day biting species it is not susceptible to the widely used insecticide-treated bed nets which have proven effective against many other mosquito species. Adult traps have previously proven to be less efficient at catching *Ae. albopictus* than other species (Meeraus *et al.,* 2008), although, modern traps such as the BG sentinel trap which

use a ventilator to produce an upward air current of ammonia, fatty acids, and lactic acids resembling the smell of humans and are effective at trapping *Ae. albopictus*, especially when used in conjunction with carbon dioxide (Meeraus *et al.,* 2008). Such traps when used *en masse* have been shown to reduce the local population of *Ae. albopictus* but are prohibitively expensive and labour intensive for general use.

Recently, the sterile insect technique (SIT) has been explored as a means of population suppression, with somewhat encouraging results (Bellini *et al.,* 2007; Bellini *et al.,* 2013); however, low yield of male pupae and somatic damage to the adults caused by irradiation hinder the effectiveness of such a programme.

1.1.5 Need for alternative control methods

Given the emergence of new mutations in the chikungunya virus which raise the transmission potential of *Ae. albopictus*, combined with the lack of available vaccines and treatments for dengue, and chikungunya as well as the expanding range, day biting behaviour and difficult to manage rural habitat, there is clear need for the development of novel control methods to combat this important disease vector.

Two potential novel control methods are presented here. Firstly, the use of *Wolbachia* to replace a wild population with a refractory strain by utilizing the natural crossing sterility and pathogen resistance shown by this inherited bacterium is discussed. Secondly, 'release of insects carrying a dominant lethal' (RIDL®), a genetic equivalent to the sterile

insect technique is considered for its ability to suppress and potentially eradicate wild populations.

1.2 *Wolbachia*

1.2.1 Discovery and early research

Wolbachia pipientis are intracellular endosymbiotic alpha-proteobacteria, first discovered in 1924 in the ovaries of the mosquito *Culex pipiens* (Hertig and Wolbach, 1924). *Wolbachia* were largely ignored until 1967, when a crossing incompatibility, resulting in no offspring, was observed in *C. pipiens* between infected males and uninfected females (Ghelelovitch, 1952; Laven, 1967a; Yen & Barr, 1971; Yen & Barr, 1973). This discovery led to the suggestion that *Wolbachia* could be used for population control; some field testing followed but no large scale implementation was effected (Laven 1967b; Curtis and Adak, 1974). *Wolbachia* were later shown to cause the same crossing incompatibility in *Drosophila melanogaster* (Hoffmann and Turelli, 1988) – the quintessential model species of insect – which stimulated further investigation into these endosymbiots.

1.2.2 Phenotypic effects

Often described as 'reproductive parasites', *Wolbachia* utilize a variety of strategies to alter the reproduction of their hosts in order to give a selective advantage to infected females. These strategies include: parthenogenesis, in which virgin females produce daughters (Stouthamer *et al.,* 1990), feminization, in which genetic males reproduce as if they were female (Rousset *et al.,* 1992), male killing, infected male embryos die (Hurst,

1999; Dyson, 2002), and cytoplasmic incompatibility, in which uninfected females mated with infected males produce inviable embryos.

In addition to these reproductive effects, enhancement of fecundity and fertility (Giordano *et al.,* 1995; Dobson *et al.,* 2002; Dobson *et al.,* 2004) and parasite resistance (Hedges *et al.,* 2008; Osborne *et al.,* 2009) have been identified in natural infections, which may further increase the fitness advantage of infected individuals.

Despite failing to meet the criteria for Koch's postulates, *Wolbachia* has been identified as the causal agent for each of the phenotypes. The first evidence for this is that expression of the phenotype is limited to hosts possessing *Wolbachia*, whilst hosts with no *Wolbachia* do not exhibit the phenotype. Furthermore, curing a host of its infection using antibiotics or heat treatment results in the removal of the phenotype in subsequent generations, and transfecting *Wolbachia* into a novel host, or indeed reinfection of a cured natural host, causes the phenotype to be expressed.

1.2.3 Cytoplasmic incompatibility

Having been observed in every insect order (Johanowicz, 1998), cytoplasmic incompatibility (CI) is the most common of the aforementioned reproductive manipulations, and the only one to have been observed in mosquitoes (Stouthamer *et al.,* 1999).

1.2.3.1 Unidirectional CI

The simplest form of CI, unidirectional, occurs when an uninfected female mates with an infected male and produces inviable eggs, whilst infected females can produce viable offspring regardless of the infection status of the male parent (figure 1.1). This results in a frequency -dependent advantage for infected females, and thus the infection rapidly spreads into the population, as has been observed naturally in *Drosophila* in California, USA (Turelli and Hoffmann 1991) and in mosquito laboratory populations (e.g. Dobson *et al.,* 2002b; Walker *et al.,* 2011).

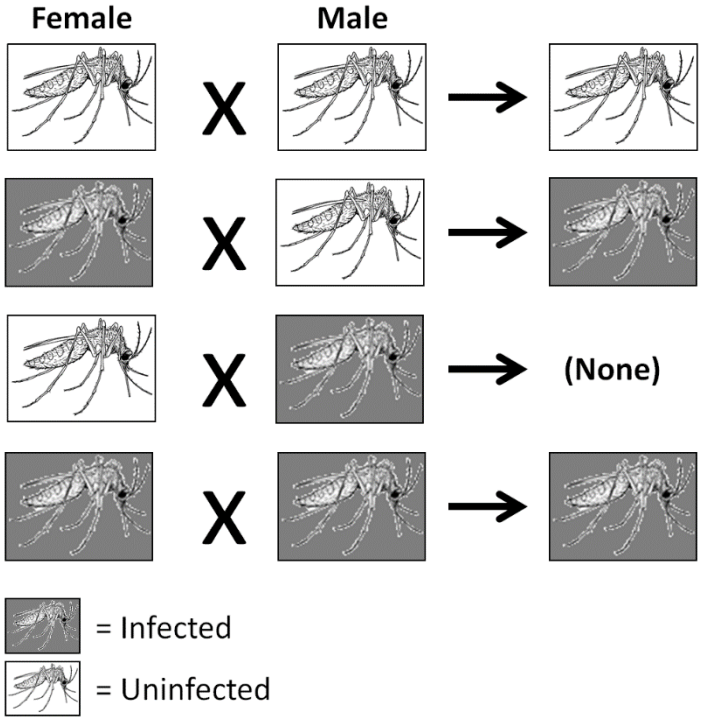

Figure 1.1: Unidirectional cytoplasmic incompatibility. Crosses between a *Wolbachia* uninfected female and infected male are incompatible; all other crosses yield viable embryos.

1.2.3.2 Bidirectional CI

Different strains of *Wolbachia* can also be incompatible with each other, leading to more complex crossing types. If individuals within a population are infected with different and incompatible strains of *Wolbachia,* bidirectional CI can occur between hosts carrying different stains. In this instance, if a male has a *Wolbachia* strain that the female does not, the offspring will be inviable (figure 1.2). Such patterns have been observed in the *C. pipiens* complex, where populations containing different strains of *Wolbachia* were crossed (Laven 1967). Bidirectional CI has also been observed in *Nasonia* wasps (Breeuwer and Werren 1993) and *Drosophila simulans* (O'Neill and Karr 1990; Nigro, 1991; Sinkins *et al.,* 1995).

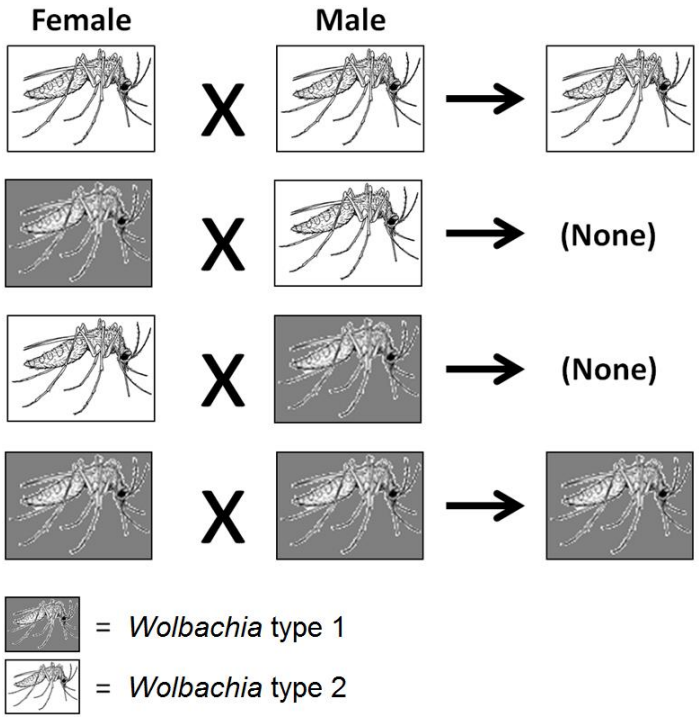

Figure 1.2: Bidirectional cytoplasmic incompatibility. Crosses between males and females possessing different bidirectionally incompatible *Wolbachia* strains result in no offspring. Only crosses where both individuals possess the same *Wolbachia* strain yield viable embryos.

When two *Wolbachia* strains producing bidirectional incompatibility exist in a freely interbreeding population, with all else being equal, hosts possessing the most prevalent *Wolbachia* would be expected to out-compete hosts possessing the other and ultimately reach fixation. This would occur as a female possessing the less prevalent *Wolbachia* would encounter an incompatible male more often than would a female possessing the more prevalent *Wolbachia* (Laven, 1967; Dobson *et al.,* 2002a). Exceptions to this could include significant fitness differences conferred by the two *Wolbachia*, speciation resulting from the incompatibility, and stochastic effects.

1.2.4 *Wolbachia*-mediated population manipulation

1.2.4.1 Manipulation with unidirectional CI

Due to the ability of *Wolbachia* to spread into a population from a low starting frequency via CI, wild populations could be manipulated by introducing novel *Wolbachia* strains with desirable phenotypes into an uninfected species. Additionally, unidirectional CI can be achieved by generating a superinfection in which the line contains both the natural infection and a bidirectionally incompatible *Wolbachia* (Sinkins *et al.,* 1995; Rousset *et al.,* 1999; Fu *et al.,* 2010); such infections would show similar spread dynamics as a single *Wolbachia* strain in an uninfected population as long as maternal transmission rates of both strains in the superinfection are high.

It is not possible to utilize this drive system to spread nuclear transgenes into a population as a result of successful mating between infected females and uninfected males allows continuous nuclear gene flow; therefor, after release, the frequency of nuclear genes

would remain unaffected in a population, stochastic effects notwithstanding (Sinkins and Godfray 2004). In principle, it would be possible to introduce a transgene into a wild population by inserting it into the *Wolbachia* genome (Sinkins and O'Neill 2000), however, despite attempts, a means of transformation of *Wolbachia* has not yet been developed.

1.2.4.2 Manipulation with bidirectional CI

Bidirectional CI can also be used for population replacement, although larger release numbers would be required. As males from each crossing type effectively sterilize females from the other, bidirectionally incompatible crossing types cannot stably co-exist (Laven, 1967) and the most prevalent *Wolbachia* would be expected to reach fixation. Additionally, it is also possible to introduce transgenes or selected traits into a population using this method as no gene flow should occur between the wild and the released populations; consequently any nuclear transgenes (or alternate natural alleles) in the release population would 'piggyback' on the *Wolbachia* population replacement.

Bidirectional CI is a potential source of sterile or more precisely, incompatible males for use in a sterile insect programme to suppress or eliminate a species (Laven, 1967). Whilst this is also possible with unidirectional CI, the accidental release of small numbers of females could result in population replacement and thus loss of the incompatibility; this is of much less concern with bidirectional CI as released females would be incompatible with wild males. Additionally, males from a bidirectionally incompatible line are more likely to be of a higher fitness than those from traditional irradiation SIT programmes given that irradiation often causes detrimental effects on male mating competitiveness

(Dame *et al.,* 2009); therefore, lower release numbers would likely be needed with the bidirectional CI approach.

1.2.4.3 Factors affecting the dynamics of CI

Wolbachia have the ability to invade (unidirectional) or replace (bidirectional) a population from a low starting frequency only if the maternal transmission efficiency is high, the penetrance of CI (percentage of inviable eggs from an incompatible cross) is high, there is sufficient out-crossing in the host population, and there are no or minimal negative fitness effects. If any of these parameters are unfavourable, the threshold needed for successful invasion or replacement increases (Hoffmann and Turelli 1997).

1.2.4.3.1 Maternal transmission

Maternal transmission of *Wolbachia* can be highly efficient: for example in *Drosophila simulans* a mean of 97% was observed in the field and 100% in the laboratory (e.g. Hoffmann *et al.,* 1998; Hoffmann *et al.,* 1990; Turelli and Hoffmann, 1995). However this is not always the case in other species such as *D. melanogaster* (Clancy *et al.,* 1997) or with newly transinfected lines, where the maternal transmission can be variable (McMeniman *et al.,* 2008), thus, if transmission rates do not improve with selection, higher starting frequencies would be needed and the equilibrium proportion of infected individuals would also be lower.

Paternal transmission of *Wolbachia* is very rare. *Wolbachia* are removed as sperm develop, but occasionally some *Wolbachia* persist and a low level of paternal transmission

(approximately 2%) has been shown (Bressac and Rousset 1993; Clark *et al.,* 2002; Hoffmann *et al.,* 1998; Turelli and Hoffmann 1995).

1.2.4.3.2 CI penetrance

In natural infections in mosquitoes, CI penetrance is often very high. Unidirectional CI crosses in *Ae. albopictus* have shown 100% sterility (Sinkins *et al.,* 1995), and bidirectional CI crosses in *C. pipiens* have shown 99% and 100% sterility (Laven, 1953; Laven, 1954) . In some other Dipteran species such as *D. melanogaster* CI has low penetrance or is observed only in crosses using very young males (Turelli and Hoffmann, 1995; Bourtzis *et al.,* 1996; Reynolds and Hoffmann, 2002). Penetrance in transinfections is somewhat more variable. Most transinfections show high (99-100%) penetrance (e.g. Xi *et al.,* 2005; Xi *et al.,* 2006; McMeniman *et al.,* 2009), whilst others show lower penetrance than for the natural host of the transinfected *Wolbachia* and/or the natural *Wolbachia* infection in that species; for example the *w*MelPop transinfection in *Ae. albopictus* shows lower penetrance than both *w*MelPop in *D. melanogaster* and *w*AlbA/B in *Ae. albopictus* (Suh *et al.,* 2009), and the *w*AlbA/B superinfection of *Ae. aegypti* showing 51% hatch in 'incompatible' crosses (Ruang-Areerate and Kittayapong 2006). The reasons for this are unknown but suggest a possible effect of the combination of both host and *Wolbachia*.

1.2.4.3.3 Limited mating range

Individuals are not typically free to mate with any other individual in a wild population as ranges of populations tend to be much larger than any individual can travel in a lifetime. This situation, termed population viscosity, would have an effect on *Wolbachia* population

invasion if the initial release is within a limited range. Whilst this has been difficult to study in a natural infection, because *Wolbachia* in most natural populations are at fixation or very high frequency, it has been observed in *D. simulans* in California, where the recently acquired *Wolbachia* was observed to spread at a rate of 100 km per year (Turelli and Hoffmann, 1991).

A further example of such restrictions on matings is the presence of local subpopulations between which no or very few matings occur (Sinkins and O'Neill 2000). *Wolbachia* spread in these scenarios would be limited, possibly requiring multiple releases in different areas.

1.2.5 Mechanism of CI

The mechanism of CI is poorly understood, but recent work on the effects of *Wolbachia* on the cell cycle during the first mitotic division provide some insight.

1.2.5.1 Disruption of embryonic cell cycle

In CI embryos (uninfected mother and infected father), significant disruption in the first mitotic division is seen. In the parasitic wasp *Nasonia vitripennis*, during metaphase the paternal chromatin was observed to be 'tangled' next to normal maternal chromatin (Ryan and Saul 1968; Breeuwer and Werren 1990). Additionally, extensive chromosome bridging during anaphase has been observed in *Drosophila* (Lassy and Karr, 1996; Callaini *et al.,* 1997; Tram *et al.,* 2006), indicative of damaged or incompletely replicated DNA, possibly due to failed separation of the sister chromatids (Serbus *et al.,* 2008). This DNA

damage is speculated to inhibit mitotic entry and hence delay cyclin-dependent kinase 1 activation.

The cytoplasm in CI embryos also contains an excess of centrosomes unassociated with the pronuclei (Lassy and Karr 1996; Callaini *et al.,* 1997). Such an excess of centrosomes has been associated with embryos lacking certain mitotic kinases which coordinate the cytoplasmic-driven centrosome replication cycle and the nuclear-driven mitotic cycle (Renault *et al.,* 2003). Dissociation of centrosomes from the nucleus also arises from faulty chromosome condensation and delays in mitosis (Takada *et al.,* 2003). These findings lead to the suggestion that in CI embryos male pronuclei lack the normal association with centrosomes required for early embryonic divisions (Robinson *et al.,* 1999) and that this defect may be the result of cell cycle timing misalignments and chromosome condensation anomalies (Serbus *et al.,* 2008). These large effects on the male pronucleus occur *in absentia* of *Wolbachia*, as the bacterium is not present in the mature sperm (Bressac and Rousset, 1993; Clark *et al.,* 2002).

1.2.5.2 Mod/Resc model

The effects on the early embryo described above must be reversible such that a female host possessing *Wolbachia* can produce viable offspring; an effect termed rescue. Indeed, it has been observed that when a *Wolbachia*-infected female is mated with an infected male, the paternal chromosomes condense and segregate normally leading to normal embryonic divisions and viable embryos (Callaini *et al.,* 1997; Lassy and Karr 1996; Reed and Werren 1995; Tram and Sullivan 2002). This observation has led to the

modification/rescue (Mod/Resc) model, whereby a *Wolbachia* capable of causing CI, i.e. modification, is termed Mod^+; similarly a *Wolbachia* capable of rescue is termed $Resc^+$. Whilst all possible combinations of Mod/Resc +/- have been observed (Charlat *et al.,* 2001; McGraw and O'Neill 1999; Werren 1997; Zabalou *et al.,* 2008), $Mod^+/Resc^+$ is by far the most common (Serbus *et al.,* 2008).

1.2.5.3 Mechanism of rescue

As described by Serbus *et al.,* (2008), there are three classes of model for rescue, all of which feature the maternal *Wolbachia* suppressing the paternal-*Wolbachia* induced chromosome defects.

In the first class of model the paternal *Wolbachia* either add or remove a component from the male pronucleus which prevents embryonic development; and that the maternal *Wolbachia* remove or replace this component.

The second class of rescue describes rescue occurring through compensatory changes in the female pronucleus such as the paternal *Wolbachia* altering the cell cycle timing of the paternal pronucleus; the maternal *Wolbachia* must then compensate by altering the timing in the female pronucleus and restoring cell cycle synchrony.

The third class hypothesizes that rescue occurs through compensatory changes in the male pronucleus. The male pronucleus is asynchronous with the female pronucleus but

the maternal *Wolbachia* slows or stops cell cycle events, giving the male pronucleus time to compensate for the changes.

There is very little evidence for which of these three classes is correct, or if different theories are correct for different *Wolbachia*. So far, studies have shown that rescue restores synchrony between nuclear envelope breakdown and Cdk1 activation (Tram and Sullivan 2002), supporting class two or three but cannot distinguish between them. On the other hand, *Wolbachia*-induced CI has been shown to dramatically reduce the frequency of androgenetic progeny (progeny developed solely from the male pronucleus) when mutations which disrupted pronuclear fusion were induced (Ferree and Sullivan 2006); this provides evidence that it is the male pronucleus which has been altered rather than timings misaligned, thus supporting the first rescue model.

1.2.6 Maternal transmission of *Wolbachia*

Maternal transmission of *Wolbachia* has been most extensively studied in *Drosophila*. Additionally, ovarian development has also been most extensively studied in *Drosophila* (described in detail in: King, 1970) making this model convenient for the study of *Wolbachia* transmission, as summarized here.

1.2.6.1 *Wolbachia* localization in the germarium

In *Drosophila* ovaries, the distal tip of each ovariole contains 2-3 germline stem cells; *Wolbachia* are present in these cells. As the stem cells divide, they create an identical cell and a differentiating germline cell known as the cystoblast. During mitosis, *Wolbachia* are

partitioned between these two cells, enabling both retention in the germline and passage into the differentiating cystoblasts (Serbus *et al.,* 2008). The maturing cystoblast undergoes four rounds of mitosis with incomplete cytokinesis resulting in a cyst of 16 interconnected germline cells, possessing an even distribution of *Wolbachia* (Ferree *et al.,* 2005). The cyst then contacts a group of somatic follicle stem cells, which may act as a secondary source of *Wolbachia* for the cyst (Frydman *et al.,* 2006); these encapsulate the cyst, creating the egg chamber (King, 1970).

1.2.6.2 *Wolbachia* localization during oogenesis

During early oogenesis, *Wolbachia* become asymmetrically arranged in the egg chamber through interactions with the cytoskeleton of the host. *Wolbachia* migrate towards the posterior germline cell – which becomes the oocyte – possibly using the cytoplasmic dynein of the host (Serbus and Sullivan 2007; Serbus *et al.,* 2008). This pattern has been reported for both *w*Mel in *D. malanogaster* and *w*Ri *D. simulans* (Ferree *et al.,* 2005; Serbus and Sullivan 2007).

The oocyte microtubule network undergoes significant reorganization during late oogenesis which coincides with the re-localization of *Wolbachia*, leading to a homogeneous distribution in the oocyte. Finally, a subset of *Wolbachia* concentrate towards the posterior pole of the oocyte (Serbus and Sullivan 2007).

1.2.6.3 Effect of posterior localization on transmission

Once an egg is fertilized its nuclei rapidly divide. Nuclei situated at the posterior pole of the embryo are surrounded by pole plasm, and become the germline cells of the developing individual (King, 1970). Through exploitation of this mechanism, the result of the posterior localization of *Wolbachia* is increased density in the next generation's germline.

Posterior localization, whilst being widespread and found in several mosquito and hymenopteran host species (Serbus *et al.,* 2008), is not universal. For example, *w*Ri of *D. simulans* remains homogeneously distributed in late oogenesis and instead appears to associate with embryonic nuclei (Kitzmiller 1959; Callaini *et al.,* 1994; Tram *et al.,* 2003), only about 3% of which become posterior germline cells; resulting in a low proportion of the total *Wolbachia* in the germline cells. Consistant with this less focused for maternal transmission, *w*Ri titre is approximately six-fold higher than that of *w*Mel in *D. melanogaster* (Veneti *et al.,* 2004). This untargeted strategy however, could have some advantages as detrimental influences on *Wolbachia* titre, such as overcrowding, increased host age and host genetic background, may be somewhat negated by the increased density of the *Wolbachia* (Serbus *et al.,* 2008).

1.2.6.4 *Wolbachia* in spermatogenesis

Despite not normally being paternally transmitted, *Wolbachia* are present throughout spermatogenesis up until the final stages of sperm maturation. *Wolbachia* are removed from spermatids towards the end of spermatogenesis as the spermatid volume is

reduced. Having already have modified the sperm, the *Wolbachia* are sequestered, along with other organelles and cytoplasm, into a 'waste bag' at the distal tip of the sperm tail (Bressac and Rousset 1993; Clark *et al.,* 2002; Fuller, 1993).

1.2.7 *Wolbachia* transinfections in *Aedes spp.*

To date many *Wolbachia* transinfections in *Aedes spp*. have been generated and characterized. Generally, *Wolbachia* have been transferred from their natural host via aspiration of cytoplasm from young embryos which is then microinjected into an embryo of the target species. Cytoplasm is both aspirated from and injected into the posterior pole of the embryo because, as described in section 1.2.6.2, in most cases this region of the embryo both contains the highest concentration of *Wolbachia* at this time point and develops into the germline of the host (Serbus *et al.,* 2008), increasing the chance of *Wolbachia* infection in the germline and consequently the generation of a heritable infection.

Alternatively, *Wolbachia* have been cultured in a cell line of the intended host, allowing for adaptation of the *Wolbachia* to the new host background with the assumption that this will increase the chance of a stable infection being generated (McGraw *et al.,* 2002). In this instance, after years of attenuation, samples of cells are lysed and the *Wolbachia* purified and injected into the posterior pole of the target embryo.

1.2.7.1 *Wolbachia* transinfections in *Ae. aegypti*

Ae. *aegypti* is the main vector of dengue virue and transmits other important arboviruses such as chikungunya. No natural population of *Ae. aegypti* has been found to be infected with *Wolbachia*; therefore, any transinfection would be able to rapidly spread throughout a natural uninfected population via unidirectional CI.

1.2.7.1 .1 Transfer of *w*AlbA/*w*AlbB into *Ae. aegypti*

In 2005, the first transinfection of *Ae. aegypti* was generated with *w*AlbB from *Ae. albopictus* (Xi *et al.,* 2005). The authors intended the transinfection to be used to spread "a desired genotype into the target field population". They found that the *Wolbachia* infection caused high rates of CI, and was thus able to spread through an uninfected population in seven generations from a starting frequency of 20%.

In 2010 it was discovered that this same transinfection suppressed dengue virus replication in the midgut, thorax and head of the mosquito, as well as significantly reducing transmission potential (Bian *et al.,* 2010). It was also noted that this *Wolbachia* transinfection increased longevity and basal immunity in mosquitoes when given a dengue virus infected blood meal.

Soon after the generation of the *w*AlbB transinfection into *Ae. aegypti*, a superinfection of both *w*AlbA and *w*AlbB from *Ae. albopictus* was established in *Ae. aegypti* (Ruang-Areerate and Kittayapong 2006). The fecundity of *Wolbachia* infected females was increased by approximately 50% compared to wild-type, and no other fitness effects were

identified. However, *Wolbachia* transmission efficiency was low (~25-75%), and CI was incomplete (51% hatch for incompatible crosses and 90% hatch for compatible crosses), making this line unattractive at present for field use.

1.2.7.1 .2 Transfer of *w*MelPop into *Ae. aegypti*

A transinfection of *Ae. aegypti* with *w*MelPop was generated in 2009 (McMeniman *et al.,* 2009) and was more thoroughly studied than its predecessors. The *w*MelPop strain of *Wolbachia* was serendipitously found in *D. melanogaster* whilst screening for mutations which cause brain degeneration. *w*MelPop was shown to shorten the lifespan of *D. melanogaster* by approximately 50% at 28°C (Min and Benzer, 1997). Although the mechanism of this life shortening is still unknown, *w*MelPop has been shown to cause degeneration of brain, retina and muscle tissue of its host (Min and Benzer, 1997).

This life shortening effect, unique amongst *Wolbachia* strains found to date, has potential applications in mosquito vectors as there is an extrinsic incubation periods of ~2 weeks for most pathogens they transmit, including dengue virus (Watts *et al.,* 1987) and malaria (Giles and Warrell, 2002). Mosquitoes must survive at least this amount of time to contribute to disease transmission; therefore the possibility of introducing a life-shortening strain of *Wolbachia* into disease vectors is attractive.

The life shortening phenotype of wMelPop was preserved in *Ae. aegypti*: its lifespan was roughly halved at 28°C. Similarly to the *Drosophila* infection, *w*MelPop exhibited complete unidirectional CI in this new host and egg hatch rates of compatible crosses were high

(McMeniman *et al.,* 2009). In addition, *w*MelPop transinfection was also shown to stimulate the immune system of its new host and confer resistance to the filarial worm *Brugia pahangi* (Kambris *et al.,* 2009) as well as *Plasmodium*, and dengue and chikungunya viruses (Moreira *et al.,* 2009).

However, further investigation of this line showed that wMelPop caused many additional fitness costs as the mosquito ages such as body 'shaking', increased probing time, a reduced blood meal volume and a 'bendy' proboscis (Turley *et al.,* 2009; Moreira *et al.,* 2009); as well as increased metabolic rate and locomotor activity (Evans *et al.,* 2009). Given the number and extent of these fitness costs, an argument was made that the *w*MelPop *Ae. aegypti* transinfection may be too uncompetitive for population replacement strategies (Turelli, 2010).

An unusual aspect of this transinfection is that it was generated after adapting the *Wolbachia* by continuous passage through *Ae. aegypti* cell culture for three years followed by microinjection of this mosquito-adapted *w*MelPop line into the embryos of *Ae. aegypti* (McMeniman *et al.,* 2009), rather than by direct cytoplasm transfer from donor embryos.

1.2.7.1.3 Transfer of *w*Mel into *Ae. aegypti*

Following the *w*MelPop transinfection, the related but non-pathogenic *w*Mel (Min *et al.,* 2009) was transinfected into *Ae. aegypti* after attenuation in a cell line (Walker *et al.,* 2011) with the goal of retaining the pathogen resistance of *w*MelPop but in the absence

of the fitness costs. The transinfected strain showed none of the above fitness costs associated with *w*MelPop whilst retaining the resistance to dengue (Walker *et al.,* 2011) and chikungunya viruses (van den Hurk *et al.,* 2012), albeit to a slightly lesser extent. Resistance to yellow fever virus was also discovered (van den Hurk *et al.,* 2012).

As predicted from the lack of fitness costs, *w*Mel was shown to invade caged *Ae. aegypti* populations (Walker *et al.,* 2011) and the transinfected line was later released into wild populations in Australia, reaching fixation in a few months (Hoffmann *et al.,* 2011) and has remained at fixation for 18 months (McGraw and O'Neill, 2013). Further field trials are planned in Vietnam for 2013, and regulatory approval is being sought for Indonesia and Brazil (McGraw and O'Neill, 2013).

1.2.7.2 *Wolbachia* transinfections in *Ae. albopictus*

Unlike in *Ae*. *aegypti*, all known natural *Ae*. *albopictus* populations are infected with two *Wolbachia*: *w*AlbA and *w*AlbB (Kittayapong *et al.,* 2002; Sinkins *et al.,* 1995). This superinfection causes complete CI in *Ae*. *albopictus* when crossed with *Wolbachia*-cured lines, and unidirectional CI with *Ae*. *albopictus* infected with only *w*AlbA (Dobson *et al.,* 2004). In species with natural infections, a novel *Wolbachia* strain can be transinfected into a line containing the natural infection, which in theory could generate a pattern of unidirectional CI analogous to transinfecting a naturally uninfected host. Alternatively, the host can be cured of its natural infection with antibiotics, generating an uninfected line into which *Wolbachia* can be transinfected. The latter option could yield a novel pattern of CI, possibly partial or complete unidirectional CI, or bidirectional CI in which

females of either line would not be able to produce fertile eggs with males of the other line.

1.2.7.2.1 Transfer of *w*Ri into *Ae. albopictus*

*w*Ri was transferred from its natural host *D. simulans* into tetracycline-cured *Ae*. *albopictus* (Xi *et al.,* 2006). The resulting line showed fairly strong bidirectional CI with the natural infection and both *w*AlbA and *w*AlbB single infections. The mating competitiveness of *w*Ri infected males was slightly reduced but remained sufficient for consideration as a population suppression tool.

Later, a stable triple infection was generated using *w*Ri, again from *D. simulans*, microinjected into a naturally double infected *Ae*. *albopictus* line (Fu *et al.,* 2010). The result was a stable triple infection of *w*AlbA, *w*AlbB and *w*Ri. As expected, strong unidirectional CI was observed. This was the first instance of such a crossing type, and, combined with minimal fitness effects, demonstrated that unidirectional CI is a viable option in this species.

1.2.7.2.2 Transfer of *w*MelPop into *Ae. albopictus*

In addition to *Ae. aegypti*, *w*MelPop has also been transferred into *Ae. albopictus*, but with less success (Suh *et al.,* 2009). For this transfer, *w*MelPop was taken directly from *D. melanogaster*, rather than being attenuated in a cell line. The *w*MelPop transinfection showed extreme pathogenicity, with significant reductions in both fecundity and egg hatch of *Ae. albopictus*. Very low egg hatch was observed in compatible crosses of

infected females and uninfected males, making it difficult to determine whether low egg hatch in incompatible crosses was due to CI or other effects. It was concluded that CI was occurring, but only after examination of embryonic development showed that more embryos were arrested in the early stages in crosses of uninfected females with *w*MelPop-infected males than compatible crosses, which is diagnostic of CI; however, CI was only moderate, with 20% hatch from incompatible crosses.

In addition, when *w*MelPop-infected females were crossed with wMelPop-infected males (rather than uninfected males), the maternal transmission rate of *w*MelPop began to fall; a situation which reversed when uninfected males were again provided. Also, the longevity reduction typically associated with *w*MelPop was less pronounced in females and absent in males.

Whilst CI penetrance has been observed to change upon transfer of *Wolbachia* from one host to another, this range of phenotype differences was unexpected, and is to date unexplained; it appears that the combination of host and *Wolbachia* contributes to the final phenotype.

1.2.7.2.3 Transfer of *w*Pip into *Ae. albopictus*

*w*Pip was transferred from its natural host *C. pipiens* into *Ae. albopictus* (Calvitti *et al.,* 2010). Very strong bidirectional CI was observed between this transinfected line and the naturally superinfected *Ae. albopictus.* Maternal inheritance was close to 100% and larval and adult survival were not affected by the transinfection. However, fecundity and egg

hatch rates were significantly reduced as a result of the transinfection, limiting its potential use.

1.2.8 Lateral transfer of *Wolbachia* in nature

Based on phylogenetic evidence, it is believed that *Wolbachia* evolved 100 million years later than arthropods (Werren *et al.,* 1995); however, it is estimated that *Wolbachia* infect up to 76% of all insect species (Hilgenboecker *et al.,* 2008; Jayaprakash & Hoy, 2000). Given this, and considerable incongruities between host and *Wolbachia* phylogenies (O'Neill *et al.,* 1992; Warren *et al.,* 1996), it appears highly likely that lateral transfer of *Wolbachia* occurs in nature. However, such transfers are believed to be extremely rare except on an evolutionary time scale (Iturbe-Ormaetxe *et al.,* 2011) or require specific relationships between the hosts, such as parasitoidism (Heath *et al.,* 1999; Huigens *et al.,* 2004).

1.2.9 *Wolbachia* interference with pathogens

Various strains of *Wolbachia* have been shown to have pathogen inhibitory effects in their native hosts. So far this has included the inhibition of RNA viruses in *Drosophila*, such as *Drosophila* C virus, Cricket paralysis, and Flock House and Nora viruses (Hedges *et al.,* 2008; Osborne *et al.,* 2009; Teixeira *et al.,* 2008); as well as inhibition of the fungus *Beauveria bassiana* (Panteleev *et al.,* 2007). RNA viruses transmitted by mosquitoes have also been shown to be inhibited by native *Wolbachia* infections, such as West Nile virus in

C. quinquefasciatus (Glaser & Meola, 2010) and dengue virus, to a minor extent, in *Ae. albopictus* (Mousson *et al.,* 2012).

Transinfected *Wolbachia* have also been shown to inhibit pathogens. The *w*MelPop transinfection of *Ae. aegypti*, albeit possibly because it is arguably the most studied, has shown the strongest and widest range of pathogen inhibition which so far includes the gram-negative insect pathogen *Erwinia carotovora*, the filarial nematode *Brugia pahangi,* dengue and chikungunya viruses, and *Plasmodium gallinaceum* (Kambris *et al.,* 2009; Moreira *et al.,* 2009). The related *Wolbachia w*Mel also confreres resistance to dengue (Walker *et al.,* 2011), chikungunya and yellow fever viruses (van den Hurk *et al.,* 2012) in *Ae. aegypti*. In addition to these, *w*AlbB has been shown to cause some resistance to dengue virus in *Ae. aegypti* (Bian *et al.,* 2010), and reduces *Plasmodium falciparum* titre in the midgut and salivary glands of *An. stephensi* (Bian *et al.,* 2013). The *modes operandi* of *Wolbachia*-mediated pathogen inhibition is currently unknown, but several, non-mutually exclusive, hypotheses have been proposed.

1.2.9.1 Host immune upregulation

*w*MelPop has been shown to significantly upregulate the immune system of its newly transinfected *Ae. aegypti* host (Kambris *et al.,* 2009). Data obtained using whole genome gene expression microarrays showed upregulation of 199 transcripts by two-fold or more. Of these 199 transcripts, 78 (39.2%) have putative immune-related functionality. Furthermore, 15 of the 21 genes (71.4%) which were up regulated by 10-fold or more

have putative immune-related functionality, and, ignoring the genes of unknown function, all but one gene has a putative immune-related function.

Whilst one would expect *Wolbachia* to primarily activate an anti-bacterial rather than an anti-viral defence, some anti-microbial peptides produced by the Toll and IMD pathways (Toll - fungi and Gram-positive bacteria, IMD – Gram-negative bacteria) also have anti-viral functions, for example cecropins. Of the putative immune genes up regulated by *w*MelPop, six cecropin genes were up regulated by over 25-fold. Cecropins have wide spectrum anti-pathogen effects including anti-viral activity, for example inhibition of dengue virus replication and production of the NS3 viral protein (Carballar-Lejarazu *et al.,* 2008) and a strong inhibitory effect on *Plasmodium* by disrupting sporogenic development, thereby reducing the number of oocysts (Gwadz *et al.,* 1989; Kim *et al.,* 2004; Carballar-Lejarazu *et al.,* 2008). In addition, synthetic cecropin peptides are known to kill filarial nematodes by impeding motility (Chalk *et al.,* 1995).

Other notable genes up regulated in the presence of *w*MelPop include orthologs of *Rel2*, *TEP20* and *LRIM1*. *Rel2* is an NF-κB like gene expression regulator of Cecropins (Meister *et al.,* 2005), *TEP1* and *LRIM1* (Frolet *et al.,* 2006). RNAi silencing of *Rel2* decreases the resistance of *An. gambiea* to *Plasmodium falciparum* (Garver *et al.,* 2009). *TEP1* is a complement C3-like protein and is required for *Plasmodium* killing by melanization. *LRIM1* exists in the heamolymph as a complex with *APL1C*, this complex activates the aforementioned protein *TEP1* (Fraiture *et al.,* 2009; Povelones *et al.,* 2009) and allows its localization to the surface of *Plasmodium* thereby targeting it for destruction by melanization (Blandin *et al.,* 2004).

Up regulation of such a wide array of known pathogen inhibitory genes suggests a convincing mechanism for the broad spectrum pathogen resistance conferred by *Wolbachia*. However, to date, immune response has only been observed in hosts artificially infected with *Wolbachia*. Such immune responses may not be due to the presence of *Wolbachia* per se, rather the artificial combination of the two species which do not naturally interact. The immune response hypothesis fails to explain the pathogen resistance conferred in natural *Wolbachia*/host combinations such as *w*Ri in *D. simulans*, which does not illicit an immune response but still confers resistance to *Drosophila* C virus (Osborne *et al.,* 2009).

1.2.9.2 Resource competition

The second hypothesis for *Wolbachia*-mediated pathogen inhibition is a direct competition for resources between the pathogen and the already established *Wolbachia*. This hypothesis is consistent with the over-replicating *w*MelPop showing strong pathogen inhibition, as it would be expected to consume more resources. Furthermore, after injection with dengue virus, *Ae. aegypti* cells which contained *w*MelPop did not contain dengue virus; consistent with *w*MelPop depleting resources (Moreira *et al.,* 2009). One proposed explanation is that fatty acids essential to dengue virus replication are sequestered by *Wolbachia* (Moreira *et al.,* 2009; Heaton *et al.,* 2010; Heaton and Randall 2010; Iturbe-Ormaetxe *et al.,* 2011).

Further evidence for the resource competition hypothesis, albeit circumstantial, is that only *Wolbachia* which reach relatively higher titres, such as *w*Mel, *w*MelPop, and *w*Ri, provide strong resistance to pathogens; lower titre *Wolbachia,* such as *w*No and *w*Ha, provide no resistance (Osborne *et al.,* 2009). Variability of distribution within the host also seems important; in *Ae. aegypti*, *w*MelPop is present in a wide range of tissues, giving it a higher potential to interfere with pathogen replication, whereas natural infections, such as the *w*AlbA/*w*AlbB infection in *Ae. albopictus* and *w*Flu in *Ae. fluviatilis* (which can both still transmit pathogens despite high titres of *Wolbachia*) are more limited in their distribution (Iturbe-Ormaetxe *et al.,* 2011).

1.2.9.3 Modulation of autophagy

Autophagy is a lysosomal degradation pathway essential to eukaryotic cells as a means of organelle disposal and reclamation of cellular components. Autophagy has also been shown to act as an arm of the innate immune pathway, being used to dispose of viruses, bacteria and protozoa. Despite this, DENV requires the activation of autophagy for efficient replication. DENV induces autophagosomes which colocalize with lipid droplets, thus becoming autolysosomes and leading to the release of fatty acids, which are believed to be used for energy generation utilized by the DENV replication machinery (Heaton *et al.,* 2010; Heaton & Randall, 2010), and for DENV particle assembly (Samsa *et al.,* 2009). CHIKV has also been shown to activate autophagy, which promotes its replication (Krejbich-Trotot *et al.,* 2011).

There is some evidence that *Wolbachia* may also modulate or suppress autophagy, which may lead to the inhibition of such viruses. The related intracellular bacterium *Anaplasma phagocytophilum* is believed to acquire host nutrients by secreting a protein which hijacks the host autophagy initiation pathway (Niu *et al.,* 2012). Furthermore, there is evidence that *Wolbachia* in *Brugia* activates autophagy (Voronin *et al.,* 2012), and the activation of autophagy using rapamycin in *Ae. albopictus* cells reduces *Wolbachia* density (Voronin *et al.,* 2012).

The modulation of autophagy also supports the notion that a competition for resources may result in the inhibition of pathogens, particularly in the cases of DENV and CHIKV.

1.2.9.4 Reactive oxygen species production

Wolbachia have been shown to stimulate host production of reactive oxygen species (ROS) in an *Ae. albopictus* cell line (Brennan *et al.,* 2008). ROS are produced during aerobic respiration; high levels of ROS cause damage to lipids, nucleic acids and proteins (Fridell *et al.,* 2005) and are important in host immunity to microbes (Ha *et al.,* 2005; Hoffmann, 2003). For example, high levels of ROS are generated after *An. gambiae* takes a blood meal, and have been shown to confer resistance to *Plasmodium* (Kumar *et al.,* 2003).

ROS have also been implicated as a general defence against viruses in many other organisms (Skulachev, 1998). For example, ROS are stimulated by anti-viral antibodies in canine brain cells (Burge *et al.,* 1989), and in addition to general nucleic acid damage, ROS

have been shown to disrupt RNA replication in the human hepatitis C virus (Choi *et al.,* 2004). They are thus a prime candidate for *Wolbachia* induced protection against RNA viruses as shown by specific *Wolbachia* strains in *Drosophila* and *Ae. aegypti*.

Increased ROS production has been shown to shorten the lifespan of insects (Fridell *et al.,* 2005) which offers a possible explanation of how *w*MelPop may reduce the lifespan of its host, if a higher titre and wider tissue distribution of *Wolbachia* correlates with a higher titre of ROS.

Upregulation of genes related to immunity and redox reactions was found in the *w*AlbB transinfection of *Ae. aegypti* (Pan *et al.,* 2012). *w*AlbB was shown to increase oxidative stress and levels of ROS in the host which was linked to activation of the Toll pathway and subsequent expression of defensins and cecropins. Expression of these antimicrobial peptides was shown to be involved in the *w*AlbB-mediated DENV inhibition by RNAi knockdowns, demonstrating a convincing mechanism that ROS production and a linked activation of the immune system combine to effect pathogen resistance.

1.2.9.5 Other mechanisms

In addition to these mechanisms, *Wolbachia* has been shown to manipulate host miRNAs (Hussain *et al.,* 2011; Osei-amo *et al.,* 2012) which includes upregulation of aae-miR-2940 miRNA in *Ae. aegypti*, which downregulates the DNA methyltransferase gene *AaDnmt2* allowing for increased *Wolbachia* replication (Zhang *et al.,* 2013). In contrast, DENV

induces *AaDnmt2*, suggesting a possible causal link between *Wolbachia* manipulation of the host miRNA and DENV suppression.

The host RNAi response has been shown to play an important role in reducing DENV transmission in *Ae. aegypti* (Sánchez-Vargas *et al.,* 2009). However, the *Ae. albopictus* C6/36 cell line lacks functional expression of *Dicer-2* (Brackney *et al.,* 2010) yet shows significant inhibition of DENV (Frentiu *et al.,* 2010), suggesting that RNAi pathways are unlikely to play an important role in *Wolbachia*-mediated viral inhibition, at least in this species (Merkling *et al.,* 2013).

1.2.9.6 Lack of experimental consensus

So far, none of these mechanisms have been demonstrated to be active in all pathogen resistant *Wolbachia*/host combinations, and not present in non-refractory combinations. For example, given the lack of immune stimulation by *w*Mel and *w*Ri in *Drosophila spp.*, and the general host damaging effects of ROS, it seems likely that different mechanisms (or combinations of mechanisms) may be active in different *Wolbachia*/host combinations.

1.3 RIDL

Wolbachia population replacement strategies focus on introducing refractoriness or reducing vectorial capacity of a wild population; an alternative to this approach is population suppression or elimination using RIDL, a genetic equivalent to the sterile insect technique (SIT).

1.3.1 Sterile insect technique

The SIT uses male insects which have been treated with either γ-radiation or sterilizing chemicals which induce sterility by random damage to insect chromosomes and the induction of dominant-lethal mutations in the sperm (Alphey *et al.,* 2010). These males are then released in far larger numbers than males in the wild population, resulting in a high proportion of wild females mating with the released males, and hence producing fewer viable offspring.

The SIT has previously been successfully implemented against many insects leading to local elimination, which has been particularly effective in the case of the New World screw-worm (Lindquist *et al.,* 1992). The SIT targeting mosquitoes has also been successful in *Culex quinquefasciatus* (Patterson *et al.,* 1970) and *Anopheles albimanus* (Lofgren *et al.,* 1974). However, the Kenyan SIT program against *Ae. aegypti* proved unsuccessful (McDonald *et al.,* 1977). *Ae. albopictus* has also been targeted for the SIT; several pilot field studies have been conducted in Italy in which male pupae were separated by sieving, irradiated and then released (Bellini *et al.,* 2007; Bellini *et al.,* 2013). These studies found a significant increase in the sterility of eggs in treated areas, and suggested that the technique could potentially be used to suppress a population provided large enough numbers were used to result in a field egg sterility of over 81%.

Whilst such studies have achieved success using the SIT, the use of radiation can cause significant damage to the target insects, which can be observed in terms of reduced

longevity and/or male mating competitiveness. This negative side-effect can be ameliorated to a certain extent by increasing the release numbers, however, in some insects, particularly in many mosquito species, the somatic damage is more extreme (Dame *et al.,* 2009), thus increasing the desirability of alternatives to irradiation. One such approach is RIDL technology – a genetic equivalent to the SIT – which replaces the requirement for radiation or chemosterility by using a lethal transgene capable of being repressed for mass rearing of the insects, but is active in the environment as the repressor is absent resulting in the death of the offspring of the released insects.

1.3.2 BiSex RIDL technology

The first type of RIDL technology utilizes tetracycline-dependant repression of a dominant lethal gene in a two component system (Heinrich and Scott, 2000; Thomas *et al.,* 2000). The first component contains a promoter driving the expression of tetracycline-repressed transactivator tTA (Gossen and Bujard, 1992) whilst the second component contains a lethal gene under the control of tTA response element (tetO) and a minimal promoter. In the absence of tetracycline, tTA binds tetO and enhances the expression of the lethal gene; whereas, in the presence of tetracycline the system is suppressed by tetracycline binding tTA and preventing its binding to tetO, thus preventing the expression of the lethal gene. Consequently, insects are able to develop normally on a diet supplemented with tetracycline, but die without it (figure 1.3).

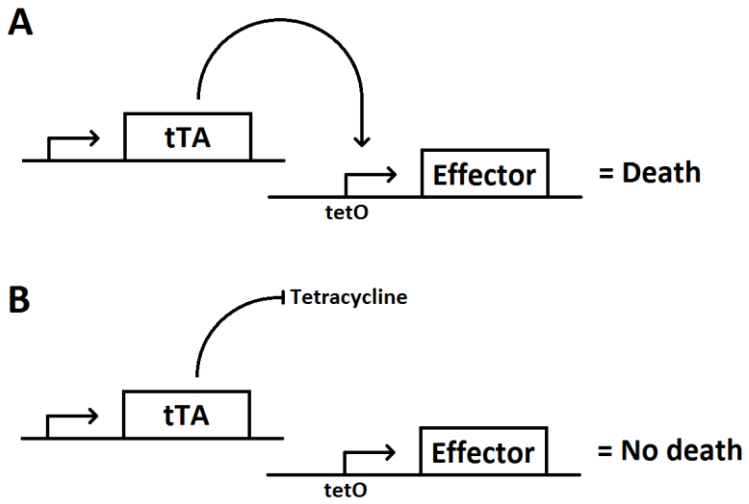

Figure 1.3: Two component RIDL. (A) In the absence of tetracycline a promoter drives the expression of tTA, which then binds tetO and enhances the expression of the lethal effector gene. (B) In the presence of tetracycline tTA is blocked and thus unable to bind tetO, preventing the expression of the lethal gene.

In addition, a simplified one component variant of this system has been developed in which tTA is under the control of a minimal promoter and tetO (Gong *et al.,* 2005). In the absence of tetracycline, the minimal promoter produces a basal amount of tTA which is sufficient to enhance its own expression by binding tetO in a positive feedback loop (figure 1.4). In this instance, tTA, which contains the transcriptional activator VP16, accumulates and effects toxicity through transcriptional squelching (Berger *et al.,* 1990; Gill and Ptashne, 1988); thus in this system tTA is both the transactivator and the effector (Gong *et al.,* 2005).

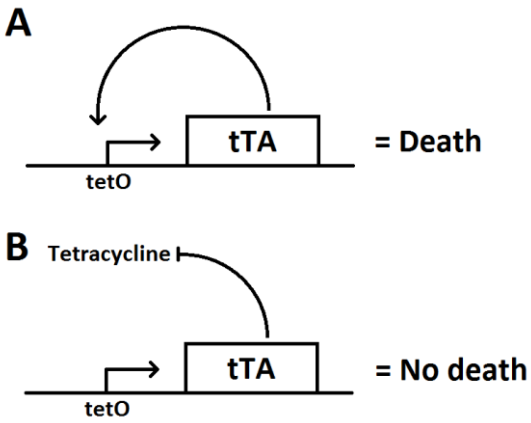

Figure 1.4: One component RIDL. (A) In the absence of tetracycline a minimal promoter produces small amounts of tTA, this tTA then binds tetO thus enhancing its own expression. A positive feedback cycle results in excess tTA which causes lethality. (B) In the presence of tetracycline tTA is blocked from binding tetO, preventing it from further enhancing its own expression.

Using this system in an SIT-like programme, insects homozygous for this cassette can be mass reared in the presence of tetracycline and released. After mating with wild insects, all of the offspring would inherit one copy of the dominant lethal system. As insufficient tetracycline for repression is found in natural breeding habitats, these offspring will die due to lethal effector gene expression (two component system) or VP16 accumulation (positive-feedback system) (Heinrich and Scott, 2000; Thomas *et al.,* 2000).

An additional advantage of RIDL is the ability to alter the stage at which the lethality occurs. In New World screw-worm, and many other agricultural pests, it is the larvae which are of concern and as such the irradiation form traditional SIT programmes – which cause early embryonic death – is ideal. However, in mosquitos, early lethality is not required as only the adult females transmit disease and pose a biting nuisance. Mathematical modelling has shown that late-acting lethality, such as during the pupal

stage, is more efficient as the RIDL larvae would still compete for food with wild-type, thus having an additional impact on the wild population before dying prior to adulthood (Atkinson *et al.,* 2007; Phuc *et al.,* 2007).

To this end, a transgenic *Ae. aegypti* RIDL line, OX513A, was developed using the one-component system to cause death at the pupal stage (Phuc *et al.,* 2007). In this case, the late-acting lethality of OX513 was not due to the construct design but rather a serendipitous epigenetic effect from the genomic context. Nonetheless, OX513A has had considerable success in suppressing wild *Ae. aegypti* populations recent field releases in Grand Cayman (Harris *et al.,* 2011; Harris *et al.,* 2012) and is currently being trialled in Brazil and Malaysia (Lacroix *et al.,* 2012). As has been observed in many SIT programmes, a reduction in the mating competitiveness of OX513A was observed in the field, however this can be abrogated by increasing the number of males released (Harris *et al.,* 2011).

1.3.3 Female-specific RIDL technology - genetic sexing

Both SIT and OX513A BiSex RIDL require only males be released because any females released would be capable of disease transmission, and, as has been observed in Mediterranean fruit fly SIT releases, 'distract' the sterile males from seeking out wild females (Rendon *et al.,* 2004). Various means of mechanical sex separation of mosquitoes have been devised but none are sufficiently scalable, and all require handling of the insect, increasing the chance of damage prior to release. Furthermore, whilst sex separation by size is fairly straightforward in some species, such as *Ae. aegypti*, it is much more difficult in others like *Anopheles spp.* and *Ae. albopictus* where the sexual

dimorphism can be less extreme; therefore further increasing the desirability of a genetic sexing system in such species. In an attempt to remedy this, a genetic sexing line of *An. stephensi* has been generated using a *Beta2-tubulin* promoter to dive expression of EGFP solely in the testes (Catteruccia *et al.,* 2005); consequently, efficient automated sex separation can be achieved through the use of a fluorescence detection device similar in principle to a flow cytometer. However, this mechanism still requires extra handling of the insects and would be expensive to implement at every trial site. An ideal sexing strain would impair the females automatically in the last rearing generation prior to release; such a system would eliminate any excess handling of the release males, increasing their fitness.

To this end, a two-component tetracycline-dependent RIDL system has been generated in *D.melanogaster* to produce female-specific lethality. This was achieved by using the female-specific promoter yolk protein 1 (*Yp1*) or yolk polypeptide 3 (*Yp3*), to drive expression the lethal genes *hid* or *Ras*V12A, and by using the female-specific lethal gene *msl-2*NOPU, under the control of tetO (Heinrich and Scott, 2000; Thomas *et al.,* 2000). In addition, female-specific lethality has been achieved in the Mediterranean fruit fly *Ceratitis capitata*, by the utilizing the sex-specific splicing of the sex-determination gene *transformer* to prevent tTA expression in males but not females (Fu *et al.,* 2007). More recently, sex-specific alternative splicing from the *doublesex* gene from pink Bollworm has been used to develop conditional lethal genetic sexing in both diamondback moth and pink bollworm (Jin *et al.,* 2013), and the *doublesex* gene from the silkworm, *Bombyx mori* has also been used to generate a genetic sexing system in this species (Tan *et al.,* 2013).

A genetic sexing strain, based on the RIDL technology, has also been produced in *Ae. aegypti,* showing female-specific conditional inviability in the form of flightlessness (Fu *et al.,* 2010). Female specificity is provided by the promoter and sex-specific splicing of the *Actin-4* gene which is naturally expressed in the female indirect flight muscles (Muñoz *et al.,* 2004). Further details of this system are provided in chapter 6.

1.4 Conclusion and aims

Given the increasing importance of *Ae. albopictus* as an emerging disease vector, and the difficulties current technologies face in the control of this species, there is clear need for the development of novel control technologies. The aims of this thesis are to develop a *w*Mel transinfection in *Ae. albopictus*, to perform selection to improve the fitness traits of the line and to assess range of fitness characteristics of this transinfection to study is potential utility as a means of DENV and CHIKV control in this species. In addition, the mechanism of *Wolbachia*-mediated viral inhibition is investigated, looking at immune stimulation and cholesterol sequestration of *w*Mel and other *Wolbachia* strains. The current female flightless RIDL technology in *Ae. albopictus* is also assessed and attempts are made to improve its characteristics in this species.

Chapter 2: Materials and methods

2.1 Mosquito husbandry

2.1.1 Egg hatching

Chapters 3-5: Mosquito eggs (all species) were hatched in water which had been left at 27^{o}C for approximately 1 month resulting in extensive algae growth. Yeast (~0.5g) and non-sterile liver powder solution (~0.25g) were added to one litre of this water. If required, larvae were separated at the second instar. More liver powder was added as needed.

Chapter 6: Eggs were submerged in deionised water and hatched overnight in a vacuum-hatcher. Larvae were fed on ground TetraMin® (Tetra GmbH, Germany) fish food flakes as required. If required, larvae were separated at the second instar.

2.1.2 Pupae picking

Larvae would pupate 6-7 days after hatching for *Aedes*, and 7-9 days after hatching for *Anopheles* mosquitoes. For important lines and individuals on which experiments were to be performed, pupae were picked using a cut Pasteur pipette into a 200 or 500mL pot containing a small quantity of tap water and placed into a 30cm x 30cm x 30cm BugDorm® cage (MegaView Ltd., Taiwan).

For colony maintenance on hardy wild type lines, water was gently sieved when ~80% of the mosquitoes were at pupae stage and the mosquitoes were poured into a 1L plastic pot and placed directly into a 30cm x 30cm x 30cm BugDorm® cage.

2.1.3 Adult mosquito maintenance

Adults were allowed to eclose from their pupae pots into 30cm x 30cm x 30cm BugDorm® cages.

Chapters 3-5: Adults were provided with a moist ball of cotton wool and a damp sucrose cube on top of each cage.

Chapter 6: Adults were provided with 10% sucrose with 14U/mL penicillin and 14µg/mL streptomycin inside the cage *ad libitum.*

2.1.4 Blood feeding and egg laying

Chapters 3-5: To obtain eggs, seven day old adults were blood-fed using a Hemotek® (Hemotek Ltd. UK) artificial feeder with defibrinated sheep blood and a stretched parafilm membrane.

Chapter 6: Adult females were blood-fed on defibrinated horse blood seven days post eclosion using a stretched parafilm membrane around an aluminium plate. The feeder was kept warm using a microwaved beanbag.

Eggs were collected by placing an egg bowl into the cages approximately three days after feeding. For most *Aedes* mosquitoes, a wet cotton wool ball with filter paper over the top was provided, for *Anopheles* mosquitoes, and *Ae. albopictus* TTI, a pot of water with a cone of filter paper dipped into the water was provided.

Anopheles eggs were transferred into the hatching water two days after laying. *Aedes* eggs were allowed to dry and stored for at least five days before hatching.

2.1.5 Individual female egg laying

Females were aspirated into small plastic vials (2.5cm diameter, 10cm long) two days after blood feeding. The vials contained moist cotton wool covered with filter paper at the bottom. The vial was sealed with cotton wool dipped in 10% sucrose to provide a sugar source.

2.2 Molecular techniques

2.2.1 DNA extraction

DNA was extracted using the Livak method. One to five mosquitoes were placed into a 1.7mL microcentrifuge tube and homogenized in 100µL of pH 9 Livak solution containing:

Reagent	Final concentration in solution
SDS	0.5%
NaCl	0.08M
sucrose	0.16M
EDTA	0.06M
Tris-HCl	0.12M

The mixture was incubated at 65^{o}C for 30 mins.

14µL of 8M potassium acetate was added and the homogenate was incubated on ice for 30 mins. The sample was then centrifuged at 10 000 RCF for 10 mins to pellet debris and precipitated SDS. The supernatant was removed using a P200 pipette, taking care not to pipette the surface layer of fat. The supernatant was transferred to a fresh 1.7mL microcentrifuge tube containing 1mL of 100% ethanol to precipitate the DNA.

The sample was then centrifuged at 20 000 RCF for 5 mins to pellet the DNA. The supernatant was removed and the pellet was washed twice in 1mL of 70% ethanol and centrifuged at 20 000 RCF.

2.2.2 RNA extraction

All equipment and gloves were sprayed with *RNAse away*® (Sigma-Aldrich™, UK) prior to use to minimize RNAse mediated RNA degradation.

Mosquitoes were anesthetized with CO_2, put into 1.5mL micro centrifuge tubes and incubated at -20°C for 5 mins to kill the mosquitoes. 0.5mL of TRIzol® (Invitrogen™, UK) was added to the tube and the mosquito was then homogenized with a plastic pestle.

Homogenized samples were incubated at 20°C for 5 mins to allow complete dissociation of nucleoprotein complexes from the RNA. 0.2mL chloroform was added to the samples and the tubes were capped, vigorously shaken for 15 seconds and incubated at 20°C for 3 minutes. Samples were then centrifuged at 12 000 RCF for 15 mins at 4°C. Following centrifugation, the mixture separated into a lower red, organic phase; an interphase; and

an upper aqueous phase containing the RNA, which was transferred into a fresh 1.5mL centrifuge tube.

To precipitate the RNA, 0.4mL of isopropyl alcohol was added to the aqueous phase. The samples were then incubated at 20°C for 10 mins and centrifuged at 12 000 RCF for 10 mins at 4°C.

The supernatant from the precipitation was removed; leaving a pellet of RNA which was washed with 1mL of 75% ethanol was added to the tube. The tube was vortexed to break up the pellet and then centrifuged at 7,500×g for 5 mins at 4°C.

The supernatant was removed and the pellet allowed to air dry for 10-20 mins before resuspension in 15µL of DEPC H_2O (1mL of 0.1% diethylepyrocarbonate per 1L of distilled and autoclaved H_2O, mixed and incubated at 20°C for 1 hour, and re-autoclaved) by gently pipetting up and down with a P20 Gilson pipette. The optical density of the RNA was measured using a Nanodrop® ND-1000 spectrophotometer (Thermo Scientific, DE, USA) to find the concentration; and the $A_{260/280}$ ratio was used to estimate the purity (1.8-2.0 being the acceptable range). The RNA was stored at -20°C.

2.2.3 Synthesizing cDNA

SuperScript® VILO™ cDNA synthesis kits (Invitrogen™, UK) were used for cDNA generation

Master mixes for 10µL Reactions were set up in, on ice, with the following reagents:

Reagent	1x
5X VILO Reaction Mix	2
10X SuperScript Enzyme Mix	1
300ng/µL RNA in dH20	7

The reactions were placed in a thermocycler set to and incubated at 25°C for 10 mins, 42°C for 60 mins and 85°C for 5 mins. cDNA was then stored at -20°C for later use.

2.2.4 Polymerase chain reaction

Chapter 3-5: Crimson *Taq*® DNA Polymerase (New England BioLabs™, UK) was used for PCR. Master mixes for 10µL Reactions were set up in, on ice, with the following reagents:

Reagent	1x
5x Crimson *Taq* Reaction Buffer	2µL
10mM dNTPs	0.2µL
10µM sense primer	0.2µL
10µM anti-sense primer	0.2µL
Template DNA	Variable
Crimson *Taq* DNA polymerase	0.05µL

dH_2O	to 10µL

Chapter 6: Herculase II fusion DNA Polymerase (Agilent Technologies™, UK) was used for PCR. Master mixes for 50µL reactions were set up in, on ice, with the following reagents:

Reagent	1x
5x Herculase II Reaction Buffer	10µL
25mM dNTPs	0.5µL
10µM sense primer	2.5µL
10µM anti-sense primer	2.5µL
Template DNA	Variable
Herculase II fusion DNA polymerase	1µL
dH_2O	to 50µL

Reactions protocols were dependent on the annealing temperature of the primers, whether or not a touchdown PCR was required, and for as many cycles as was deemed necessary (dependent upon concentration of target DNA).

2.2.5 Agarose gel electrophoresis

Agarose gels were used for electrophoretic analysis of PCR products. Dependent upon the size of the product, 0.5-2% agarose gels were used. The gels were made using TAE buffer containing:

Reagent	Amount
Tris base	10.8g
boric acid	5.5g
EDTA	4mL of 0.5M (pH 8)
dH_2O	upto 1L

For each gel, the required amount of agarose was added to the TAE buffer and heated in a microwave until the agarose had dissolved. 5µL of 10mg/mL ethidium bromide was added per 100mL of TAE. The gel was poured into a gel tank which was placed on ice until set.

The following loading solution was used to load DNA into agarose gels:

5-20µL PCR product

1µL 6× bromophenol blue loading dye per 5µL PCR product

The solution was pipetted up and down immediately prior to loading to mix. Gels were run at $5Vcm^{-1}$ for 30 mins.

2.2.6 PCR product purification

If PCR products were amplified with no non-specific amplification, the PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN, UK) according to the manufacturer's instructions. If additional products were amplified, the PCR products were run on a 0.5% agarose gel, visualized using a transilluminator, then excised using a clean scalpel and placed into 1.5 ml microcentrifuge tubes. DNA was then purified using a QIAquick Gel Extraction Kit (QIAGEN, UK), according to the manufacturer's instructions. DNA concentration and quality was assessed using a NanoDrop 1000 spectrophotometer.

2.2.7 Quantitative PCR

SYBR® Green (Invitrogen™, UK) was used for qPCR reaction chemistry. A mastermix, for each primer pair, was set up containing:

Reagent	1×
dH_2O	1µL
SYBR® green	5µL
Sense primer (10µM)	0.5µL
Anti-sense primer (10µM)	0.5µL

7µL of this mix was pipetted into 'Fast optical 96 well reaction plates' (Applied Biosystems™, UK). 3µL of cDNA, diluted to 1:20 with dH_2O, was added to each well.

Primer pair efficiencies were calculated using a standard curve (dilutions: 0.1, 0.05, 0.01 and 0.05), and the following formula:

$$10^{-1/\text{Gradient}}$$

Where 'gradient' is the regression gradient (as calculated by Microsoft® Excel®) of the dilution vs Ct value for the respective dilution.

$$\frac{\text{Control gene primer efficiency}^{(\text{Control gene Ct Value})}}{\text{Gene of interest primer efficiency}^{(\text{Gene of interest Ct value})}}$$

The 96 well plate was pulse centrifuged at 200 RCF then placed into an DNA Engine thermocycler (MJ Research, Canada) with a Chromo4 real-time PCR detection system (Bio-Rad, UK). The following programme was used:

95°C for 15 minutes

95°C for 10 seconds
59°C for 10 seconds
72°C for 20 seconds
} x 45

In addition to efficiencies, all primer specificities were tested using dissociation curve analysis: after amplification, the temperature was raised from 60°C to 90°C, with fluorescence acquisition every 2°C. qPCR data were analyzed in a Microsoft® Excel® spread sheet

2.2.8 DNA sequencing

Sequencing reactions were set up containing the following reagents in a 0.2µL PCR tube:

Reagent	1×
Sequencing buffer	1.5µL
Big Dye	1µL
Primer	3.2pmol in 1µL
DNA	20-100µg
dH_2O	to 10µL

This mixture was placed into the thermocycler for the following protocol:

Stage	temperature	duration	
Denaturation	96°C	2min	
Denaturation	96°C	10sec	x35
Annealing	50-60°C	10sec	
Extension	60°C	4min	
Termination	4°C	infinite	

The mixture was removed from the thermocycler and 40µL of H2O, 5µL of 3M sodium acetate and 125µL of 100% cold ethanol was added to precipitate the DNA, this mixture was incubated at room temperature for 1 hour.

The samples were centrifuged at 16 000 RCF for 30 mins, the supernatant removed and washed with 75% ethanol and again centrifuged at 16 000 RCF for 30 mins. The supernatant was removed and the samples were sent to the Zoology department of the University of Oxford for electrophoretic analysis. Sequence data was analysed using FinchTV (http://www.geospiza.com/Products/finchtv.shtml).

2.2.9 Restriction digests and ligation

Restriction enzyme digests were performed with New England BioLabs™ (UK) CutSmart™ restriction enzymes, according to the manufacturer's instructions. Ligation reactions were performed with New England BioLabs™ (UK) Quick Ligation™ kit, according to the manufacturer's instructions.

2.3 List of primers

Species/target	Gene name	Sense primer	Anti-sense primer	Use	Annealing	Reference/designer
*w*MelPop & *w*AlbA/B	*ftsZ*	TGATGCTGCAGCCAATAGAG	TCAATGCCAGTTGCAAGAAC	qPCR & qRT-PCR	59°C	
*w*Mel	*ftsZ*	GGGGCTTGGTGCTGGTGCTT	TGCCTCTCCGGTGCCGATCA	Sequencing	55°C	
*w*MelPop, *w*Mel & *w*AlbA/B	*Wsp*	TGGTCCAATAAGTGATGAAGAAAC	AAAAATTAAACGCTACTCCA	qPCR & qRT-PCR	55°C	Vronin et al. 2010
*w*Mel (specific)	*Wsp*	GGGTTGATGTTGAAGGAG	CACAGCTTTTACTTGACC	PCR & Sequencing	55°C	Zhou et al. 1998
An. gambiae	*CEC1*	CCAGAGACCAACCAACCACCAA	GCACTGCCAGCACGACAAAGA	qRT-PCR	59°C	Zakaria Kambris
An. gambiae	*DEF1*	CATGCCGCGCTGGAGAACTA	GATAGCGGCGAGCGATACAGTGA	qRT-PCR	59°C	Zakaria Kambris
An. gambiae	*LRIM1*	CATCCGCGATTGGGATATGT	CTTCTTGAGCCGTGCATTTTC	qRT-PCR	59°C	Zakaria Kambris
An. gambiae	*TEP1*	CGCCCAGGAGCGTACGTTGG	CCTGGCGAACAGACCCAAGCTG	qRT-PCR	59°C	Zakaria Kambris
An. gambiae	*CTL4*	ATCGGAATGTCGATCGCTAC	CTGTCCGGCGATCAAACTAT	qRT-PCR	59°C	Zakaria Kambris
An. gambiae	*CLIPB3*	CAGATTGTCGTCCACACTGG	GCTCAGGGGCAGACAGATAG	qRT-PCR	59°C	Zakaria Kambris
An. gambiae	*RS7R*	AGAACCAGCAGACCACCATC	GCTGCAAACTTCGGCTATTC	qRT-PCR	59°C	Zakaria Kambris
Ae. aegypti	*PGRPS1*	TGGAGCGACATTGGTTACAA	GCGATGCCAATCGACTTACT	qRT-PCR	59°C	Kambris et al. 2009

Ae. aegypti	*CECD*	GCTAGGTCAAACCGAAGCAG	TCCTACAACAACCGGGAGAG	qRT-PCR	59°C	Kambris et al. 2009
Ae. aegypti	*CLIPB37*	TTGGGGGAAAACAGAAACAG	GATCTGCTTCCCAGAGAACG	qRT-PCR	59°C	Kambris et al. 2009
Ae. aegypti	*CTL* galactose specific	GTCTCCGGGTGCAATACACT	CCCTATCGTTCCACTTCCAA	qRT-PCR	59°C	Kambris et al. 2009
Ae. aegypti	*TEP20*	ATTTTTGACGGCTTTTGTGG	TGGATTACTTGCCCCACTTC	qRT-PCR	59°C	Kambris et al. 2009
Ae. aegypti	*Actin 5C*	ATCGTACGAACTTCCCGATG	ACAGATCCTTTCGGATGTCG	qPCR & qRT-PCR	59°C	Zakaria Kambris
Ae. albopictus	*S17*	AAGCCCCTGCGTAACAAGAT	GTTATCTCTGCGCTCACGTTC	qRT-PCR	59°C	Zakaria Kambris
Ae. albopictus	*clip B37* orthologue	ACCCGAACCAGGTTGTGTAGCG	GGATGCAACCAGTACGCCGTCC	qRT-PCR	59°C	
Ae. albopictus	*CecD* orthologue	TTCACGAAGTTGTTCGCAAT	GGCATTGAAGACTCGTTTGC	qRT-PCR	59°C	
Ae. albopictus	*PGRPS1* orthologue	GCAACTTACTGGCCGCTCGC	CGTTGGAGCGCATACCCGTG	qRT-PCR	59°C	
Ae. albopictus	*TEP20* orthologue	TGCCCAGCGGATTTGTAGCAGAAG	AAACAGTCTGATTCGGGTCCCATGT	qRT-PCR	57°C	
Ae. albopictus	*DefD* orthologue	TGTTTCCTGGCTATGTGCCTC	CCAGCACCGGTTCCTGTGGG	qRT-PCR	59°C	Jenifer Molloy
Ae. albopictus	*PIAS* orthologue	TTTGTCCGTTGGGAAAGATG	ACAGTGACGCATCAAAGCAC	qRT-PCR	59°C	Jenifer Molloy
Ae. albopictus	*Casper* orthologue	AGAATGCGTAGCGGAGTGTC	GACCGGTGAGAACATAACGAA	qRT-PCR	59°C	Jenifer Molloy
Ae. albopictus	*Rel2* orthologue	TTTCGATACCAATCGGAGATG	CGGGAAGGTCTTCTTGCTTT	qRT-PCR	59°C	Jenifer Molloy
Ae. albopictus	*Rel1A* orthologue	TGGTGGTGGTGTCCTGCGTAAC	CTGCCTGGCGTGACCGTATCC	qRT-PCR	59°C	Xi et al. 2008
Ae. albopictus	*Cactus* orthologue	TGTTCAGCTCGTCTTCGTCA	GGACTGGTGGTACTGGTGCT	qRT-PCR	59°C	Jenifer Molloy

Ae. albopictus (OX4738)	*Actin4* promoter	GATCGAGGCGCGCCTGCTCAAA CACCATCCTCTGGC	GGTGTGAGATCTGCATGTCGTCA CACATTTTGGCGCCGCTTCCAGG TCCGTTGGGTCC	PCR	55°C	Anti-sense by Sarah Scaife
Ae. albopictus (OX4739)	*Actin4* promoter	GATCGAGGCGCGCCATTGGGAA GCGTGAGCATAGGTTC	GGTGTGAGATCTGCATGTCGTCA CACATTTTGGCGCCGCTTCCAGG TCCGTTGGGTCC	PCR	55°C	Anti-sense by Sarah Scaife
Ae. albopictus (MB5)	*Actin4* promoter	GATCGAGGCGCGCCTCAAACAA ATGCCCATCCGC	GGTGTGAGATCTGCATGTCGTCA CACATTTTGGCGCCGCTTCCAGG TCCGTTGGGTCC	PCR	55°C	Anti-sense by Sarah Scaife
Ae. albopictus (MB6)	*Actin4* promoter	GATCGAGGCGCGCCCACTTAGGG ATCACCCTGTGCAG	GGTGTGAGATCTGCATGTCGTCA CACATTTTGGCGCCGCTTCCAGG TCCGTTGGGTCC	PCR	55°C	Anti-sense by Sarah Scaife
OX4738, OX4739, MB5 & MB6 (screening)	OX4721 plasmid - *Actin4* promoter	GCAAGTGTAGCGGTCACGCTGC	GGTGTGCGTCTC CGGAATCACTA GACCGTATCGAACTGCACCAG	PCR	55°C	Sarah Scaife
OX4738, OX4739, MB5 & MB6 (screening)	OX4721 plasmid - *Actin4* promoter	GCAAGTGTAGCGGTCACGCTGC	GGTGTGCGTCTCTACCACCGCGC AGGCGCAG	Sequencing	55°C	Sarah Scaife

Chapter 3: Transfer of *Wolbachia* from *Drosophila* into *Aedes* mosquitoes

3.1 Introduction

3.1.1 *Aedes albopictus*

Ae. albopictus is an important vector of several human diseases such as dengue and chikungunya. Dengue is endemic in over 110 countries (Ranjit, 2010) and infects between 50 and 100 million people per year worldwide (Whitehorn and Farrar, 2010). Although *Ae. aegypti* is a more competent and important vector of the dengue virus (Gubler, 1998), *Ae. albopictus* is considered a maintenance vector (Lambrechts *et al.,* 2010) but can be solely responsible for dengue virus transmission in the absence of *Ae. aegypti* (Lounibos, 2002; Gratz, 2004), as well as serving as a bridge vector from sylvatic cycles in non-human primates and monkeys to 'urban' cycles in humans (Knudsen, 1977). *Ae. albopictus* is also often regarded as a secondary vector of chikungunya virus, however mutations which greatly increase the transmission of this virus by *Ae. albopictus* have recently been identified in the viral envelope genes (Enserink, 2007; Tsetsarkin *et al.,* 2007; Tsetsarkin *et al.,* 2011b).

The reduced vectorial capacity of *Ae. albopictus* compared to *Ae. aegypti* may partly be the result of *Ae. albopictus* being less closely associated with humans, preferring a more

rural habitat and having a more catholic feeding habit than *Ae. aegypti* (Lambrechts *et al.,* 2010; Hawley, 1988).

Ae. albopictus is a day-biting species and therefore not susceptible to the widely used protection method of insecticide-treated bednets. Instead, control of *Ae. albopictus* is largely focused on removal of larval breeding sites by e.g. removal of rubbish and unblocking of drains, which is very time-consuming and labour intensive; and insecticide spraying, which is also time-consuming as well as expensive, and affects non-target insects. Given the lack of treatments and vaccines for dengue and chikungunya, the expanding range of *Ae. albopictus*, and that no effective and efficient large scale mosquito control system exists for this species, there is a clear need for novel methods of vector and disease control.

3.1.2 *Aedes polynesiensis*

Ae. polynesiensis is a vector of the most important aetiological agent of human lymphatic filariasis, *Wuchereria bancrofti*, as well as dengue virus and dog heartworm (*Dirofilaria immitis*) (Rosen, 1954; Lee *et al.,* 1987). Its range is limited to the South Pacific region (Lee *et al.,* 1987). The behaviour and habitat of *Ae. polynesiensis* are very similar to that of *Ae. albopictus*; it has been found to breed in semi-domestic and natural containers (Lee *et al.,* 1987), its eggs resist desiccation (Ingram, 1954), and it is a day-biting species, biting both humans and a range of animals (Jachowski, 1954; Lee *et al.,* 1987).

3.1.3 Transinfection concept

Wolbachia can be transferred from one species to another by aspiration of embryo cytoplasm from the native host and injection of this cytoplasm into a novel host. Cytoplasm is aspirated from the posterior of the donor species embryo, and injected into the posterior of the recipient species. The posterior of the embryo is used because after cellularization it becomes the germline of the insect and also has a high concentration of *Wolbachia.* Provided sufficient *Wolbachia* are transferred, survive and replicate in the germline of a G_0 female, the transinfection may be passed on to the next generation. In many previous *Wolbachia* transinfections, the phenotype which the *Wolbachia* conferred on its native host has been transferred to its new host. This has included cytoplasmic incompatibility (Xi *et al.,* 2005; Xi *et al.,* 2006), life span reduction (McMeniman *et al.,* 2009) and inhibition of RNA viruses (Osborne *et al.,* 2009; Moreira *et al.,* 2009), as well as new phenotypes (or phenotypes which are untestable in the original host), such as inhibition of filarial worms (Kambris *et al*. 2009).

3.1.4 Aims

The aim of this chapter was to generate a novel *Wolbachia* transinfection in *Aedes* species which could be used for disease control. A transinfection of *Ae. aegypti* with the *Wolbachia* strain *w*MelPop (McMeniman *et al.,* 2009) showed inhibition of DENV and CHIKV dissemination (Moreira *et al.,* 2009), A *w*MelPop transinfection in *Ae. aegypti* produced an inhibitory effect against the filarial nematode worm *Brugia pahangi (*Kambris *et al*., 2009). *Ae. aegypti* does not transmit human filarial nematodes in the wild, whereas *Ae. polynesiensis* is a regionally important vector of *Wuchereria bancrofti;* it was therefore

chosen as a recipient of *w*MelPop transfer to examine whether the same filarial inhibition phenotype would be produced.

The *w*MelPop strain has already been stably transferred into *Ae. albopictus* (Suh *et al*., 2009), where it exhibited greater pathogenicity in the form of very low egg hatch and this fitness cost would be too high for CI to drive it into a population. The *Wolbachia* strain *w*Mel is a very similar but non-pathogenic strain of *Wolbachia,* also from *D. melanogaster*. *w*Mel has been shown to confer resistance against RNA viruses in its natural host and in *D. simulans* via a transinfection (Osborne *et al.,* 2009). Consequently it was predicted that that the viral inhibition conferred by *w*Mel in *Drosophila* spp. may also be conferred on *Ae. albopictus* following transinfection.

All known wild populations of *Ae. albopictus* are naturally superinfected with two *Wolbachia*, *w*AlbA and *w*AlbB (Kittayapong *et al.,* 2002; Sinkins *et al.,* 1995). A line of the Uju strain of *Ae. albopictus* has previously been cured of its *Wolbachia* infection with tetracycline (Otsuka and Takaoka, 1997). This cured strain is an ideal recipient of a transinfected *Wolbachia* as there will be no competition with an established *Wolbachia* strain. Given that other transinfections of *Wolbachia* into *Aedes* spp. have produced CI phenotypes (Xi *et al.,* 2005; Xi *et al.,* 2006), it was predicted that *w*Mel may cause CI with the natural superinfection of *Ae. albopictus*, and therefore provide a mechanism for population replacement.

The aims of this chapter are to generate novel transinfections of *w*MelPop in *Ae. polynesiensis* and *w*Mel in *Ae. albopictus*. and carry out selection to increase the maternal

inheritance rate and improve the hatch and fecundity of the line. The CI crossing type of transinfections will be assessed.

3.2 Chapter specific methods

3.2.1 Embryo microinjection method (*w*MelPop and *Ae. polynesiensis*)

*w*MelPop was transferred from *Ae. aegypti* (Ref^{m}, Ae_Pop; Kambris *et al.,* 2009) embryos into *Wolbachia*-free *Ae. polynesiensis* (APMT) embryos by cytoplasm transfer. Approximately seven days after blood feeding, ~15 females were aspirated into a small (3cm diameter, 10cm height) plastic tube with moist filter paper on the bottom to encourage oviposition. Eggs were collected approximately 30mins post-oviposition. Both donor and recipient eggs were laid out on the side of a moist nitrocellulose membrane and allowed to partially desiccate. Two pieces of filter paper were used as blotters to keep the nitrocellulose moist (figure 3.1).

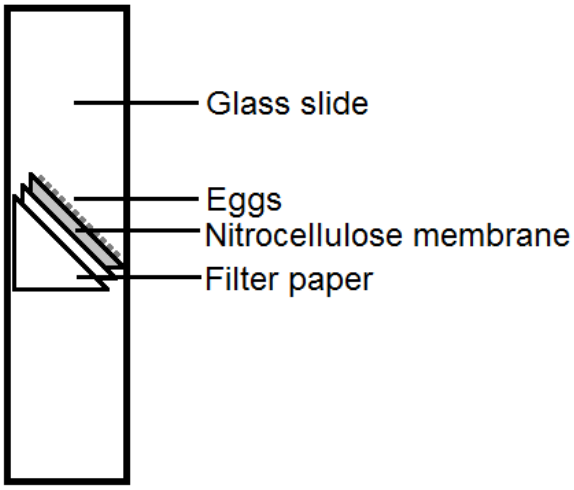

Figure 3.1: Embryo microinjection method 1. Approximately 30min old eggs were aligned against the side of a moist nitrocellulose membrane which was kept moist using two pieces of filter papers as blotters. Eggs were injected from the right hand side.

Cytoplasm was aspirated from the posterior of the donor eggs using a FemtoJet microinjector and injected into the posterior of recipient eggs. After a short incubation time eggs were transferred onto wet filter paper, stored at 100% humidity and 27^{O}C for five days, and then hatched in deoxygenated water. G_0 larvae were reared using standard conditions.

3.2.2 Embryo microinjection method (*w*Mel and *Ae. albopictus*)

*w*Mel was transferred from *D. melanogaster* (y*w*^{67c23}) embryos into *Wolbachia*-free *Ae. albopictus* (UjuT) by the transfer of cytoplasm. Adult *D. melanogaster* females were encouraged to oviposit using apple juice agar plates and yeast paste. Eggs were collected approximately 30mins post-oviposition. *Ae. albopictus* were encouraged to lay eggs by aspirating ~15 females, blood-fed ~7 days prior, into a plastic tube with moist filter paper on the bottom. Eggs were collected approximately 30mins post-oviposition. *Ae. albopictus* eggs were lined up on a slightly damp nitrocellulose membrane and allowed to slowly desiccate whilst the *Drosophila* eggs were lined up on the membrane. Both donor and recipient eggs were then transferred to a small glass slide using double sided tape and covered with Voltalef oil. The lining up of the *Ae. albopictus* eggs first and allowing them to slowly desiccate ensured that they were more desiccated than the *Drosophila*

eggs, expediting aspiration from the latter and injection into the former. Following this preparation, eggs were injected following the same protocol as in section 3.2.1.

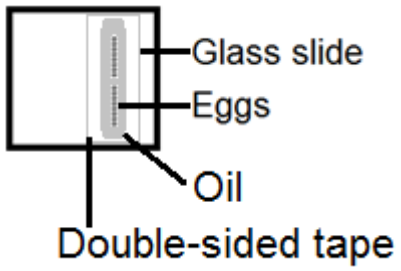

Figure 3.2: Embryo microinjection method 2. Approximately 30min old eggs were aligned with the posterior side facing right on a piece of moist filter paper, the eggs were then transferred to the glass slide using double sided tape and then covered in Voltalef oil. Eggs were injected from the right hand side.

3.2.3 Rearing and breeding of newly transinfected lines

Newly transinfected lines were sexed at the pupal stage and all males removed. Males from the tetracycline cured lines used for the transinfections were provided to outcross the lines to prevent population bottlenecking. Females were separated after a blood meal and allowed to lay eggs individually in plastic tubes. After egg laying the females were PCR-screened for *Wolbachia* using primers 81F and 691R (Zhou *et al.,* 1998); only eggs from infected females were hatched. In some generations, where the number of females was low, male pupae were also screened using PCR to give a better estimate of *Wolbachia* inheritance.

3.2.4 Selection for *Wolbachia* presence, and high hatch rates and fecundity

Selection was applied to Uju.wMel females for presence of *Wolbachia*, high hatch rates and large egg batches. This was achieved by separating females after blood feeding and allowing them to lay in plastic tubes. Once eggs had been laid the females were sacrificed, their DNA extracted and then PCR-screened for *Wolbachia*. Following PCR analysis, the egg batches were counted and hatched. Hatch rates were calculated by counting second instar larvae. Only larvae from *Wolbachia*-positive females with large egg batches and high hatch rates were used to continue the line.

3.2.5 CI crosses

Crossing experiments designed to characterize the crossing type of *w*Mel in *Ae. albopictus* were performed using UjuT, Ascoli (naturally infected with *w*AlbA and *w*AlbB), and Uju.wMel lines. Every combination of cross between individual lines was tested (figure 3.3). All individuals were sexed at the pupal stage. Males and females were then placed in cages and allowed to eclose. Adults were given a constant supply of water and sucrose and blood fed at 6 days old, two days later the females were separated into plastic tubes for individual laying. Eggs were dried and allowed to mature at 27^{O}C and 70% RH for 5 days before being counted and hatched in deoxygenated water. Larvae were fed with dried liver powder. Second instar larvae from each female were counted to give hatch rates.

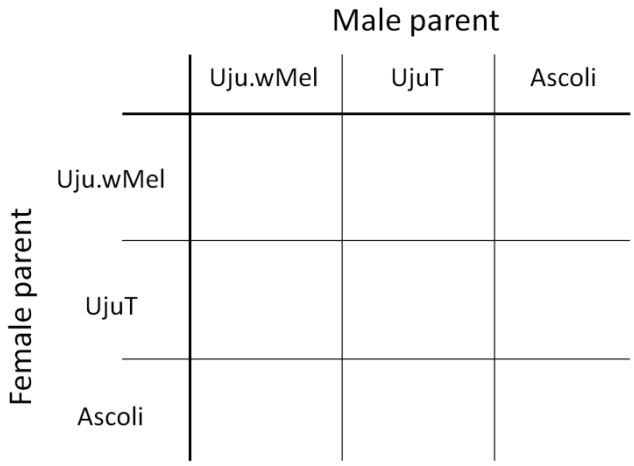

Figure 3.3: Crossing scheme for CI experiments. All combinations of male and female infection types were crossed together, yielding a total of nine crosses.

3.3 Results

3.3.1.1 *w*MelPop transfer into *Ae. polynesiensis*

*w*MelPop-infected *Ae. aegypti* eggs were used as cytoplasm donors for the transinfection of tetracycline-cured *Ae. polynesensis* (APMT). A total of 88 APMT embryos were injected, which yielded a single positive female (figure 3.4) from which a line was established.

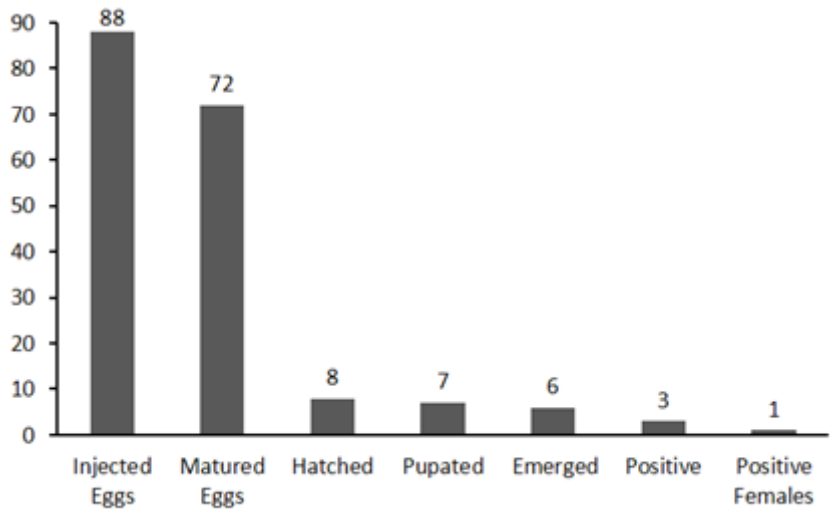

Figure 3.4: Survival of APMT after injection of *w*MelPop. 88 APMT eggs were injected with cytoplasm containing *w*MelPop aspirated from *Ae. aegypti,* eggs were allowed to mature for five days at 27°C and 100% humidity before hatching. Larvae were reared under standard conditions. Of the surviving adults, two males and one female tested positive for *Wolbachia* using PCR. The female was tested after laying the first batch of eggs.

3.3.1.2 APM.wMelPop

The surviving *w*MelPop-positive female was blood fed and an isofemale line was established. In each generation male pupae were removed and female APM.*w*MelPop were outcrossed to APMT males to introduce nuclear genetic diversity. Females were for egg laying and only eggs from females which tested PCR-positive for *Wolbachia* were hatched.

After G_2 the egg hatch rate and successful feeding of the females rapidly declined and by G_4 the infection was no longer present in any individual (figure 3.5). Given these data, and the observation that no APM.wMelPop female survived for longer than 14 days after

eclosion, it was concluded that *w*MelPop is too pathogenic to form a stable and useful transinfection in this host.

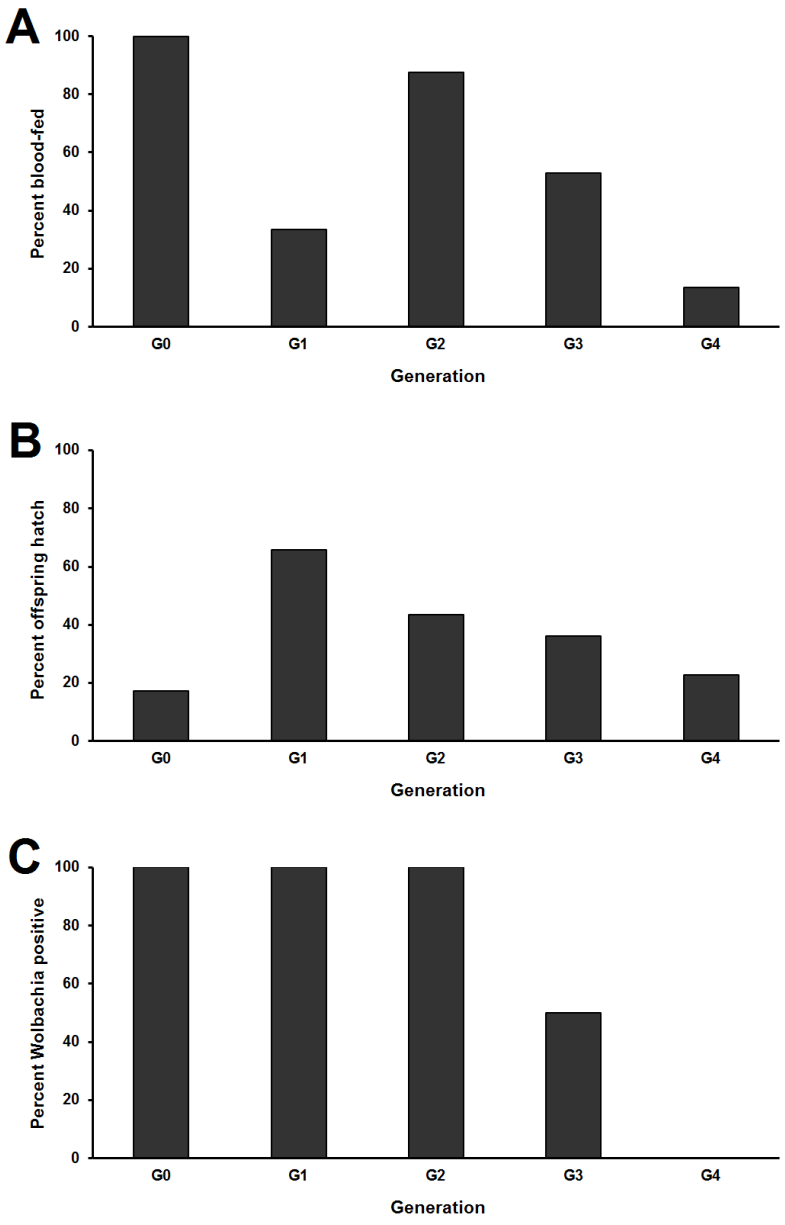

Figure 3.5: Blood feeding, hatch rate and infection rate of APM.*w*MelPop. (A) Proportion of females which blood-fed was determined immediately after feeding. (B) Proportion of eggs hatched determined by counting eggs and second instar larvae. (C) APM.wMelPop infection status of male pupae and adult females was determined with PCR.

3.3.2 *w*Mel transfer into *Ae. albopictus*

3.3.2.1 Generation of Uju.wMel

A stable infection of *w*Mel into previously tetracycline-cured *Ae. albopictus* (UjuT) was generated. Cytoplasm from *D. melanogaster* y*w*^{67c23} was injected into ~100 UjuT embryos resulting in four surviving G_0 females. Of these four, one was positive for *w*Mel and sufficient progeny was obtained in order to generate an isofemale line. This line was outcrossed by addition of UjuT males each generation and selected for stability of maternal inheritance by hatching eggs from only *w*Mel PCR-positive females. 100% maternal inheritance was achieved from G_4 (figure 3.6). In addition to selection for presence of *w*Mel, selection was also applied for high wMel concentration – measured using qPCR (figure 3.7); only individuals with relatively higher concentrations of *Wolbachia* were selected to contribute to the next generation. The *w*Mel concentration in G_1 was ~2-fold higher than the combined concentration of *w*AlbA and *w*AlbB in the naturally infected Ascoli line. The concentration dropped to almost 50-fold lower than in the natural infection by G_3 but then began to increase again as the infection became stable. The concentration stabilized at between approximately 6- and 10-fold higher than the natural wAlbA/B infection in Ascoli (figure 3.7).

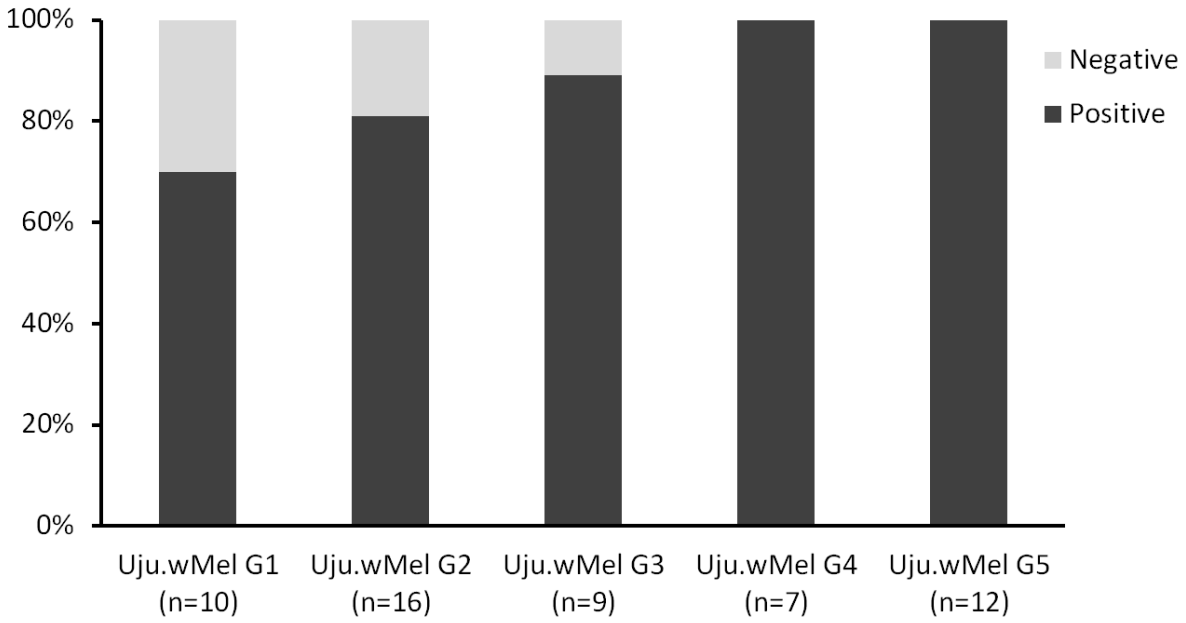

Figure 3.6: Maternal inheritance of *w*Mel in *Ae. albopictus*. Female Uju.wMel who oviposited were assayed for *Wolbachia* using PCR and only eggs from infected females were hatched. G_1 Uju.wMel males were also PCR-analysed and their data added because of the low number of females in the first generation.

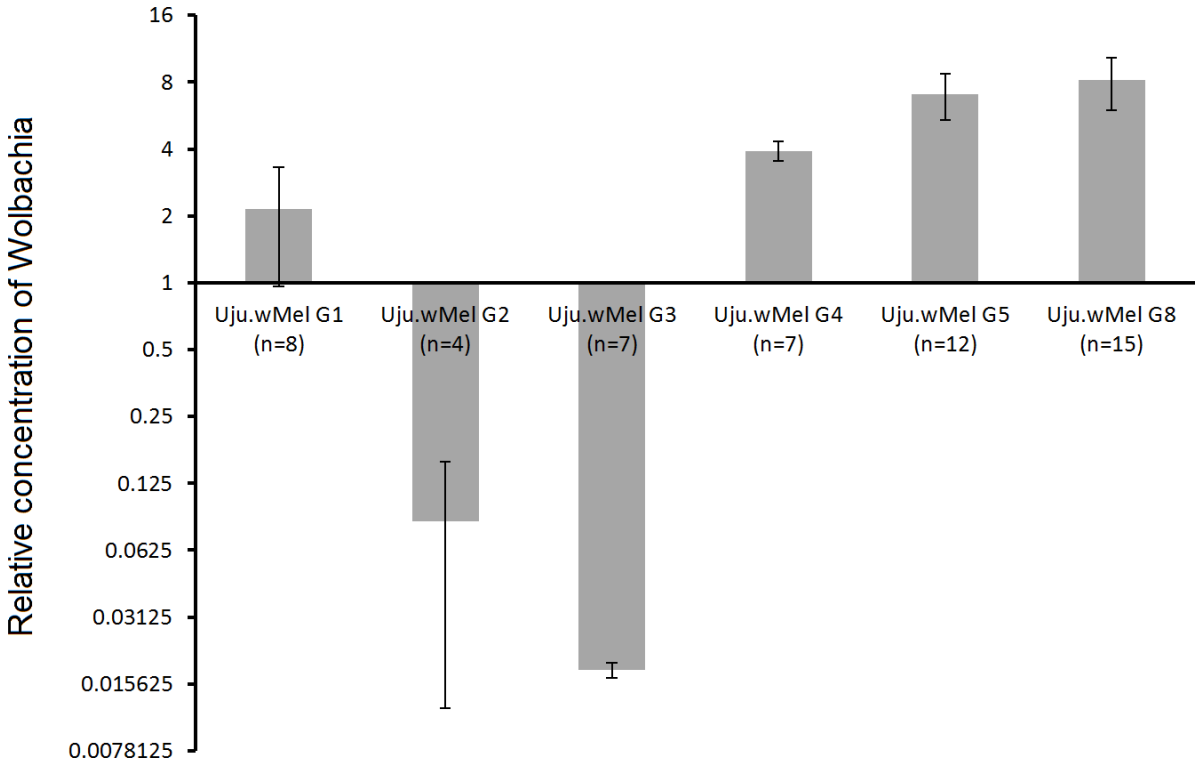

Figure 3.7: Density of *Wolbachia* in Ascoli and Uju.wMel lines. DNA was extracted from adult Uju.wMel and Ascoli females 11 days after eclosion. qPCR was used to quantify *Wolbachia wsp* DNA and host *S17* DNA; the ratio of these two genes gave an estimate of the density of *Wolbachia*. The combined density of *w*AlbA and *w*AlbB in the Ascoli strain was arbitrarily designated 1, and the concentration of *w*Mel in Uju.wMel was plotted relative to this. Error bars show the SEM.

3.3.2.1.1 Confirmation of *Wolbachia* strain identity

The *Wolbachia* was confirmed to be *w*Mel, and not residual *w*AlbA/B leftover from curing or contamination, by sequencing the *Wolbachia* surface protein gene (*wsp*). This yielded 497 bases of high quality sequence, which was then compared to the *wsp* sequence of *w*Mel from *D. melanogaster,* and *w*AlbA and *w*AlbB from *Aedes albopictus* obtained from GenBank. No mismatches were found between the Uju.wMel sequence described here

and the *w*Mel sequence stored in GenBank; 57 and 110 nucleotide differences were found compared the database *w*AlbA and *w*AlbB sequences respectively (figure 3.8).

The *ftsZ* gene from Uju.wMel and from the *Drosophila* line used as a cytoplasm donor for the generation of Uju.wMel was also sequenced to determine that the *Wolbachia* was *w*Mel and not contamination from the related strain *w*MelCS. *w*MelCS is found in certain strains of *D. melanogaster* and has an identical *wsp* sequence to *w*Mel, but shows less consistency in CI penetrance with male age and other factors (Yamada *et al.,* 2007). The *ftsZ* sequencing showed that both Uju.*w*Mel and the *D. melanogaster* stock used for the line generation contained *w*Mel not *w*MelCS (figure 3.9).

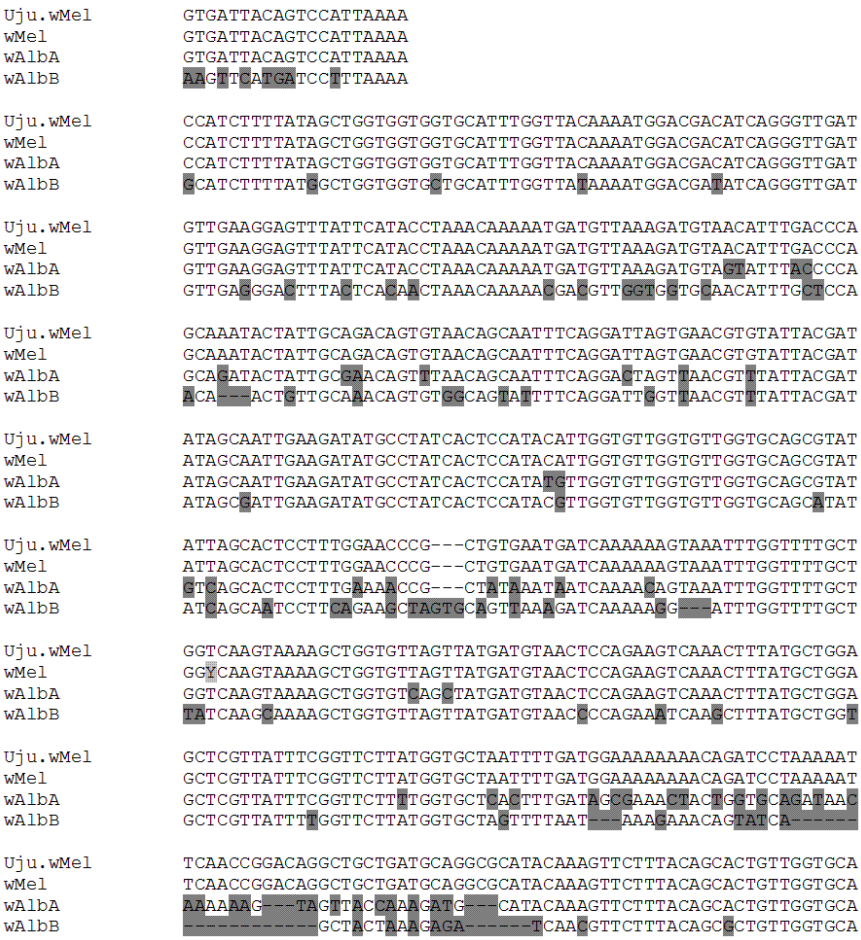

Figure 3.8: Comparison of *wsp* sequences. ClustalW was used to compare the *wsp* sequence obtained from the *Wolbachia* in the Uju.wMel line to the GenBank records for *w*Mel, *w*AlbA and *w*AlbB. The Uju.wMel sequence was identical to the GenBank *w*Mel sequence.

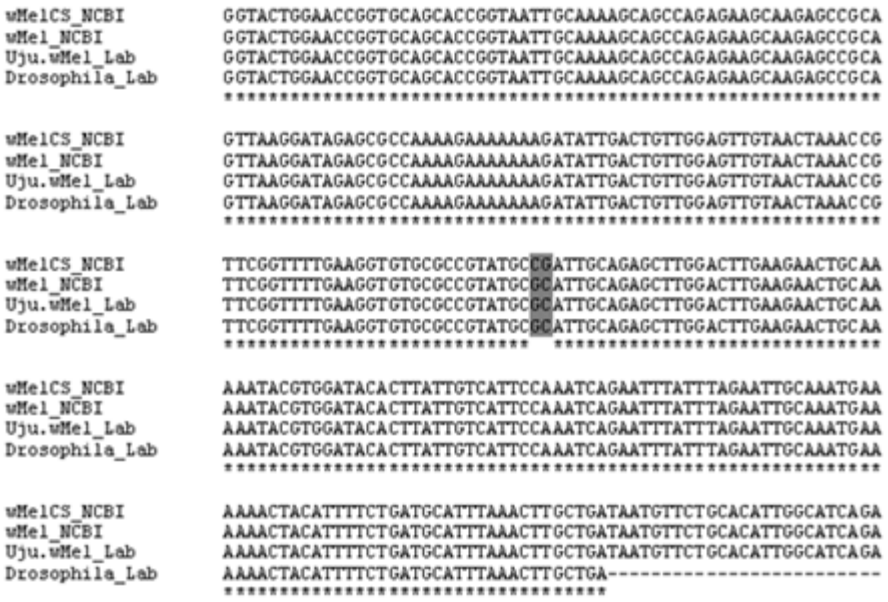

Figure 3.9: Comparison of *ftsZ* sequences. ClustalW was used to compare the *ftsZ* sequence obtained from the *Wolbachia* in the Uju.wMel line and the *D. melanogaster* donor line to the GenBank records for *w*Mel and *w*MelCS. Both the Uju.wMel and *Drosophila* sequence were identical to the *w*Mel GenBank sequence.

3.3.2.2 Selection for high hatch rate in Uju.wMel

In the early generations of the Uju.wMel line the hatch rate was lower than that of UjuT and Ascoli. Selection was therefore performed to increase the hatch rate of the Uju.wMel line. Females were separated to lay eggs and only larvae from the 50-75% of clutches with the highest hatches were used for the next generation; egg hatch increased steadily over the first 10 generations (figure 3.10).

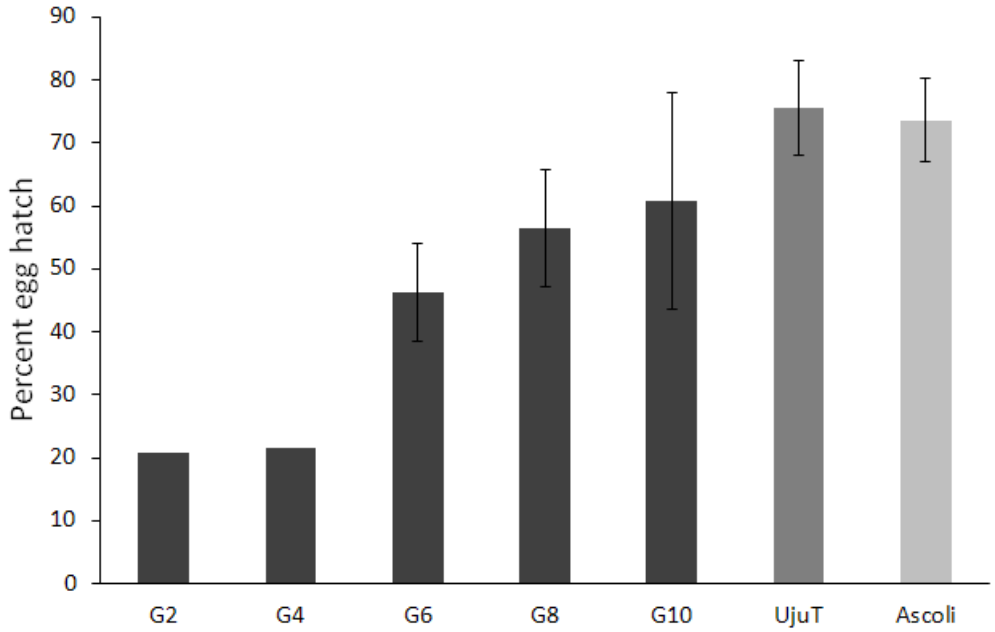

Figure 3.10: Hatch rate of Uju.wMel over first 10 generations. Hatch rates are shown for eggs laid by female Uju.wMel, UjuT and Ascoli strains. G_2 and G_4 Uju.wMel females were mated with UjuT males, all other crosses were between males and females from the same line. All eggs laid were counted (including eggs which were 'selected out' from forming the following generation) G_2 n = 406; G_4 n = 374; G_6 n = 663; G_8 n = 528; G_{10} n = 494; UjuT n = 858; Ascoli n = 447; where n = the number of eggs scored. Error bars show SEM, no error bars are present for G_2 and G_4 hatch rates because eggs were pooled prior to hatching

3.3.2.3 Selection for increased fecundity in Uju.wMel

Beginning at G_6, selection for increased fecundity was applied by using eggs from only females with the largest egg batches, in tandem with selection for high hatch rates. Between G_6 and G_{10}, when fecundity plateaued, the average clutch size increased by 36.6% from 60.27±6.97 to 82.33±12.61. There was no significant difference in fecundity between G_{10} Uju.wMel and Ascoli ($p = 0.575$, two-tailed Wilcoxon rank sum test) (figure 3.11).

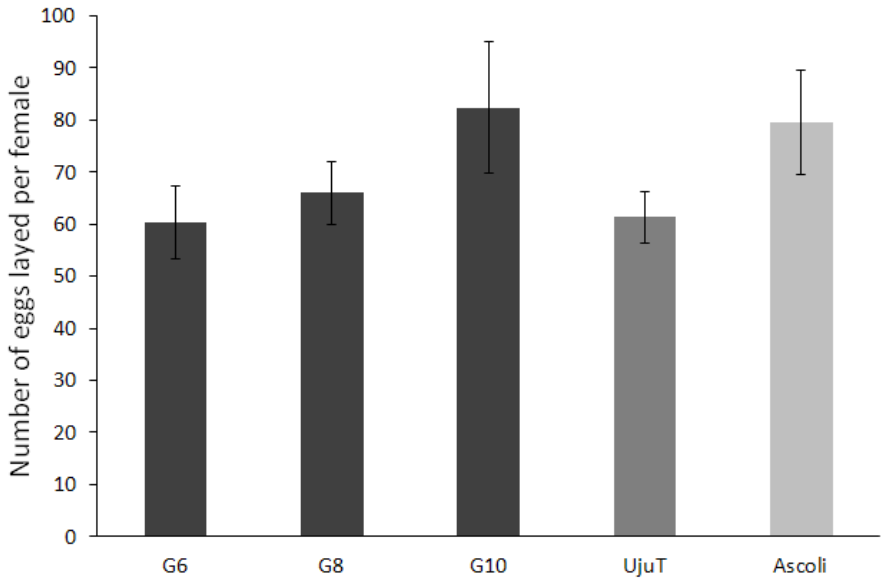

Figure 3.11: Effect of *w*Mel on the fecundity of *Ae. albopictus*. Females were separated after blood feeding into small plastic tubes and allowed to lay eggs on wet filter paper. G_6 n = 11; G_8 n = 8; G_{10} n = 6; UjuT n = 14; Ascoli n = 6, where n = the number of egg batches counted.

3.3.2.4 Uju.wMel crossing type experiments

Crossing experiments designed to characterize the crossing type of *w*Mel were performed using UjuT, Ascoli (a naturally superinfected line containing *w*AlbA and *w*AlbB), and G_6 Uju.wMel lines (figure 3.12). As expected, UjuT males were compatible with all females. Males of both Uju.wMel and Ascoli lines caused strong CI when mated to UjuT females (0.26% and 0% hatch for Uju.wMel and Ascoli males respectively). Uju.wMel and Ascoli lines showed strong bidirectional incompatibility, with 0% hatch when males of one strain were mated with females of the other. However, incomplete rescue was shown in Uju.wMel male x female crosses, with only 46.1±7.8% hatch compared to 60.3±10.7% for Uju.wMel female x UjuT male.

A second round of crossing experiments was performed after two generations of selection for high hatch rate when the *w*Mel line reached G_8. These crosses were limited to (F x M): Uju.wMel x Uju.wMel, Ascoli x Uju.wMel, Uju.wMel x Ascoli and Uju.wMel x UjuT. In this second round of crossing experiments the hatch rates for eggs laid by Uju.wMel females raised from 46.1±7.8% to 56.4±9.4% for the Uju.wMel x Uju.wMel cross and from 60.3±10.7% to 79.2±6.0% points for the Uju.wMel x UjuT cross (figure 3.12).

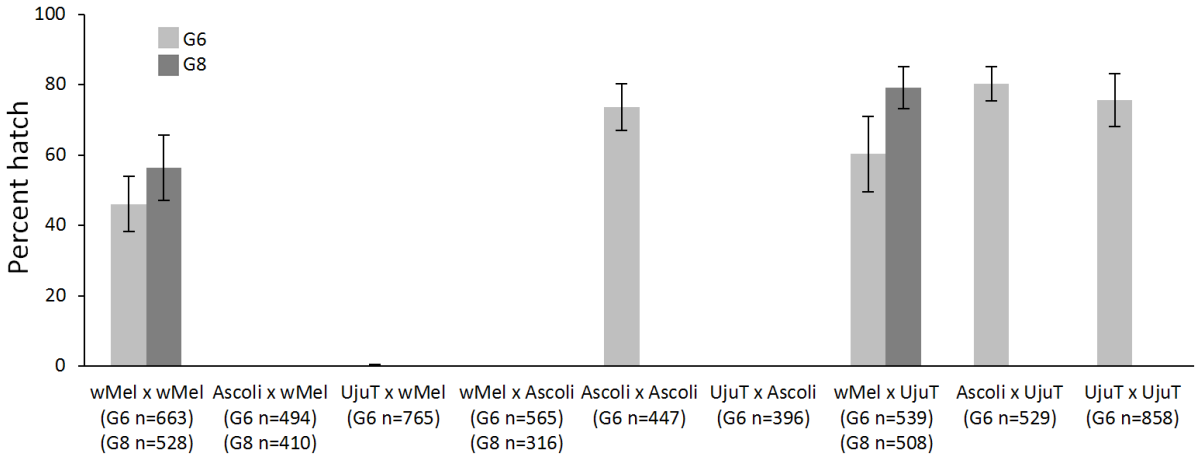

Figure 3.12: Uju.wMel crossing type. Crossing experiments designed to characterize the crossing type of *w*Mel were performed using UjuT, Ascoli, and G_6 Uju.wMel lines (female × male); some crosses were repeated using G_8 Uju.wMel. All individuals were sexed at pupae stage. Males and females were then placed in their respective cages (total of nine crosses), and allowed to eclose. Adults were blood fed at six days old, and the females separated into plastic vials for individual laying. Eggs were dried and allowed to mature at 27^{O}C and 80% RH for five days. Eggs were counted and hatched in deoxygenated water containing algae and yeast. Larvae were fed with dried liver powder. Second instar larvae from each female were counted to give hatch rates. The spermathecae of females with no egg hatch were dissected to check for successful mating; egg hatch rates from females who were unmated were disregarded. Error bars represent the SEM. No statistically significant difference was found between the same crosses in G_6 and G_8 using unpaired Wilcoxon tests.

3.4 Discussion

3.4.1 Establishment of APM.*w*MelPop

The initial transfer of *w*MelPop into *Ae. polynesiensis* with was successful but the feeding efficiency, egg hatch and *Wolbachia* transmission fell until G_4, when the infection was lost (figure 3.5). This may be explained by the virulence of *w*MelPop, especially considering its highly pathogenic effects in the closely related host *Ae. albopictus* (Suh *et al.,* 2009). The gradual reduction of fitness over successive generations may be explained by the over-replication of *w*MelPop; it is likely that a low concentration of *Wolbachia* was initially transferred and that this increased over successive generations, with culminating in too high pathogenicity to allow line maintenance and very strong selection for individuals that did not carry the *w*MelPop *Wolbachia*. Alternatively, the original G_0 female may have had a permissive genetic background, and with outcrossing this permissiveness was lost leading to the reduced fitness of the line. In both scenarios there is an incompatibility between the *Wolbachia* and the host; therefore it was decided to attempt a different *Wolbachia*/host combination. Given the success of the *w*Mel transinfection in *Ae. albopictus* (discussed later), and the similarity between *Ae. albopictus* and *Ae. polynesiensis*, a transinfection of *Ae. polynesiensis* with *w*Mel could be considered in future.

3.4.2 Generation of Uju.wMel

The focus changed to the related *w*Mel for transinfection. *w*Mel has also been shown to have anti-viral effects (Osborne *et al.,* 2009) but without the virulence of *w*MelPop. Transfer of *w*Mel from *D. melanogaster* to *Ae. albopictus* was achieved by transfer of embryo cytoplasm. Maternal transmission of the infection reached 100% after four generations (figure 3.6).

3.4.3 Selection for increased hatch and fecundity of Uju.wMel

The hatch rate and fecundity of Uju.wMel were low in early generations but both responded well to selection and after eight to 10 generations were comparable to UjuT and Ascoli lines (figures 3.10 and 3.11). Uju.wMel therefore compares well to other transinfections in *Ae. albopictus*; the hatch rate is much higher than the 10-20% previously observed for a *w*MelPop strain transinfection (Suh *et al.,* 2009), and does not show the fecundity cost associated with *w*Pip transinfecton (Calvitti et al., 2010).

At G_1, the concentration of *w*Mel in Uju.wMel was ~2-fold higher than the concentration of both *w*AlbA and *w*AlbB in the Ascoli line but dropped over the next two generations to 50-fold lower (figure 3.7). During these generations there was imperfect maternal transmission (figure 3.6). By G_4, the concentration of wMel began to stabilize, maternal transmission reached 100%, and the hatch and fecundity of the line began to steadily improve with selection pressure (figures 3.10 and 3.11). It seems likely that these variables are linked and that their simultaneous improvement was the result of both the selection pressure applied and co-adaption between *Wolbachia* and host.

A thorough investigation of fitness was conducted in the next chapter using a *w*AlbA/B line backcrossed into the Uju genetic background to enable more direct comparisons of the specific effects of *w*Mel.

3.4.4 Crossing type of Uju.wMel

Uju.wMel shows bidirectional incompatibility with Ascoli, with 0% hatch in incompatible crosses between these two lines (figure 3.12). As one might have expected, this is consistent with the *w*Mel crossing type shown previously in *D. melanogaster* (Hoffman, 1998); however, it somewhat unexpectedly differs from the crossing type of a *w*MelPop transinfection into *Ae. albopictus* (Suh *et al.,* 2009), given the phylogenetic similarity between *w*Mel and *w*MelPop (Suh *et al.,* 2003). However, there was low egg hatch in the *w*MelPop transinfection which masked CI and this could have been the result of the over-replication and pathogenicity of *w*MelPop, which is not shown in *w*Mel.

The complete bidirectional CI shown by Uju.wMel with the wild-type Ascoli strain provides a method for stably introducing *wMel* into *Ae. albopictus* populations, because bidirectionally incompatible crossing types cannot stably coexist (Laven, 1967b). Whichever strain is at a local majority has a reproductive advantage, as females with that *Wolbachia* will more frequently encounter and mate with males with whom they are compatible.

At G_{6-8} there were lower hatch rates from the Uju.wMel male and female crosses than the hatch rates of Uju.wMel females and UjuT males (figure 3.12), suggesting incomplete rescue of the modified sperm. A similar effect has however previously been observed in newly transinfected *D. simulans* (McGraw *et al.,* 2002) and *Ae. aegypti* (Xi *et al.,* 2005), and the hatch rates gradually improved over successive generations. Hatch rate selection was continued after this experiment and the hatch rate continued to improve (figure 3.10).

3.4.5 DENV and CHIKV inhibition

Alongside the selection and characterization work, the ability of *w*Mel to inhibit the RNA viruses DENV and CHIKV in this host were assessed by Camilo Arias-Goeta at the Institut Pasteur. *Ae. albopictus* is a major global vector of dengue and chikungunya viruses, both of which are RNA viruses. *w*Mel has previously been shown to inhibit RNA viruses in *Drosophila* spp. (Osborne *et al.,* 2009); CHIKV and DENV challenges were conducted to determine whether this RNA virus inhibitory phenotype is found in its new host.

Uju.wMel, UjuT and wild-type lines were amplified and 2000-5000 eggs collected from each line in Oxford and sent to the Institut Pasteur, firstly for assessment of DENV transmission competence and then again for CHIKV competence. The results showed that *w*Mel infection completely blocks transmission of both DENV-2 (Blagrove *et al.,* 2012) and CHIKV (Blagrove *et al.,* 2013) (see appendix 1 and 2); this is the first time *Wolbachia*-mediated virus inhibition has been demonstrated in *Ae. albopictus*.

The viral inhibition appears to be limited to certain combinations of host and *Wolbachia*. *w*AlbB, one of the natural *Wolbachia* strains found in *Ae. albopictus*, has been shown to moderately inhibit DENV in an *Ae. aegypti* transinfection (Bian *et al.,* 2010), and whilst a recent study has shown tentative evidence that DENV may be inhibited by a very small degree by the natural *w*AlbA/B infection in *Ae. albopictus* (Mousson *et al.,* 2012), no evidence of inhibition of DENV transmission was seen by the natural *Wolbachia* infection of *Ae. albopictus* in this study (Blagrove *et al.,* 2012).

*w*Mel completely abolished transmission of CHIKV possessing the recently identified E1-A226V mutation, which has been shown to increase its transmission potential in *Ae. albopictus* (Tsetsarkin *et al.,* 2007; Tsetsarkin *et al.,* 2011a; Tsetsarkin *et al.,* 2011b; Tsetsarkin *et al.,* 2011c). It would be interesting to further test whether *w*Mel causes inhibition of CHIKV without this mutation and CHIKV possessing the E2-L210Q mutation, although given that the E1-A226V variant has the strongest fitness advantage in *Ae. albopictus* (Tsetsarkin *et al.,* 2011b) it is expected that inhibition of these other variants would be at least as strong as with the E1-A266V seen here.

Given both this viral inhibition and the complete bidirectional CI shown by Uju.wMel, this work demonstrates that *w*Mel transinfection can provide a viable new option for dengue and chikungunya virus control in this species. In support of this, a *w*Mel transinfection was independently generated in *Ae. aegypti*, showing strong DENV inhibition, complete

CI, and has been used in field releases with the ultimate goal of suppressing DENV in Queensland, Australia (Walker *et al.,* 2011; Hoffmann *et al.,* 2011). Taken together with the data shown here, there is now a potential method of control of DENV and CHIKV in both *Ae. aegypti* and *Ae. albopictus*, the two main vectors of these viruses.

To follow on from this work it is important to know whether *w*Mel has any fitness effects on its host that may affect the viability of this line as a control option, either by increasing the required release threshold or, if the fitness costs are too great, potentially making the strategy unfeasible. It will also be important in future studies, prior to field releases, to determine whether 100% viral inhibition, of both CHIKV and DENV, is maintained long-term as *w*Mel and the host attenuate to each other.

Chapter 4: Assessment of the comparative fitness of Uju.wMel and methods of *Wolbachia* curing for introgression

4.1 Introduction

The generation of a *w*Mel transinfection in *Ae. albopictus*, Uju.wMel, was described in chapter 3. Uju.wMel showed complete bidirectional CI with the wildtype infection, high fecundity and hatch rate compared to previous transinfections, 100% maternal inheritance, and total abolition of DENV and CHIKV transmission capacity. This unique combination of phenotypes in *Ae. albopictus* gives Uju.wMel the potential to be used as a tool for reducing the DENV and CHIKV transmission capacity of wild *Ae. albopictus* populations.

If Uju.wMel is to be used for such purpose it is critical that the *Wolbachia* does not impose major fitness penalties on its host. It has previously been reported that the natural *w*AlbA/B superinfection in *Ae. albopictus* provides a significant fitness advantage to its host in increased longevity and fecundity compared to a *Wolbachia*-free line (Dobson *et al.,* 2002; Dobson *et al.,* 2004); given that Uju.wMel does not possess *w*AlbA/B, and that *w*Mel and *Ae. albopictus* are not specifically adapted to each other, fitness costs compared to the wild-type infection are possible. For example a similar transinfection with the related *Wolbachia* strain *w*MelPop caused a greatly reduced egg

hatch from intra-strain mating (Suh *et al.,* 2009) and a transinfection with *w*Pip caused a reduction in both fecundity and egg hatch (Calvitti *et al.,* 2010).

Mathematical models of population dynamics have highlighted the importance of fitness parameters in CI based population replacement strategies (Hancock *et al.,* 2011a; Hancock *et al.,* 2011b). Any fitness costs associated with *w*Mel in this host, particularly any costs related to fecundity, egg hatch rates, longevity and male mating competitiveness, need to be assessed in order to determine whether the transinfection of *w*Mel is a viable method for population replacement.

Whilst ideally these fitness parameters would be tested in conditions as close to natural as possible, due to the difficulty in setting up such experiments under semi-field conditions, as well as potential containment issues, fitness assessment in a climate controlled insectary was deemed most practical for this stage in the characterization of the line. Such small cage lab assays have been shown to be useful indicators of field success in previous *Aedes* release programs (Harris *et al.,* 2011; Hoffmann *et al.,* 2011).

An additional hurdle to overcome in the use of a bidirectionally incompatible line for population replacement is the lack of introgression following release. Unlike in a unidirectional system, there is no nuclear gene flow between the two incompatible populations which raises some concerns: firstly the wild population is likely to have local

adaptations, increasing its fitness; secondly, if the behaviour of the two strains is sufficiently different they may not interbreed and a release may result in two sympatric populations of the same species; and thirdly, there is a risk of introducing new traits from different genetic backgrounds, such as the ability to diapause. Given that all known wild populations of *Ae. albopictus* possess the *w*AlbA/B superinfection, a means of introgressing *w*Mel into local strain backgrounds is required. Traditionally, this would be achieved by using antibiotics to cure a colony of the desired local strain; however, curing *Ae. albopictus* using this method has previously been shown to be difficult (Dobson & Rattanadechakul, 2001). This is likely due to a combination of the high *Wolbachia* concentration of the natural infection and a protective effect from the host; increasing antibiotic concentration in an attempt to overcome this proves lethal to the larvae. Therefore, an alternative method of reliably curing *Ae. albopictus* is needed.

Wolbachia-free *Ae. albopictus* lines are also preferable for use with Oxitec's RIDL technology as it utilizes a tetracycline-repressible dominant lethal system. Injection of the construct with tetracycline and continual rearing on tetracycline-treated water can cause curing of some individuals, leading to cytoplasmic incompatibility which hinders the development and analysis of the line.

The aims of this chapter are to assess the potential of Uju.wMel to be used for population replacement by comparing its fitness to a newly backcrossed Uju.wt line generated by introgressing *w*AlbA/B into the Uju host background; to assess important fitness characteristics such as hatch, fecundity, longevity, and male mating competitiveness; and

to attempt novel methods of curing *Ae. albopictus* and introgression between bidirectionally incompatible lines in order to generate an efficient protocol for introgressing *w*Mel into new host backgrounds.

4.2 Chapter specific methods

4.2.1 Backcrossing *w*AlbA/B into the Uju genetic background

The *w*AlbA and *w*AlbB strains of *Wolbachia* were introgressed into the UjuT by removing all male pupae from one colony of the *w*AlbA/B infected Ascoli strain and providing an approximately equal number of UjuT males for seven generations. After seven generations the resulting line, Uju.wt, thus had over 99% UjuT nuclear background, and contained both *w*AlbA and *w*AlbB.

4.2.2 Longevity assay

Longevity was assessed in 30 × 30 × 30cm BugDorm cages (MegaView Ltd., Taiwan) each containing approximately 100 male and 100 female mosquitoes (which were sexed as pupae). The mosquitoes were provided with moist cotton and supplied with sucrose *ad libitum*. A blood meal was provided at seven days, and subsequently every 14 days; damp filter paper used as oviposition substrates were provided three days after blood-feeding. Dead mosquitoes were counted and removed every four days.

4.2.3 Male mating competitiveness

Male mating competitiveness was assessed using three independent replicates of 50 male Uju.wMel : 50 male Uju.wt : 50 females (either Uju.wMel or Uju.wt), sexed at the pupal stage. Adults were allowed to emerge in 30 × 30 × 30cm BugDorm cages and were left to mate for 10 days. Females were then blood-fed, and two days later were aspirated into small plastic vials for individualized laying. Eggs were hatched five days after laying; given the complete CI (zero egg hatch) observed in both directions in crosses between wild-type and *w*Mel-transinfected parents, the male parent was determined according to whether or not embryos hatched – hatching indicating that it contained the same *Wolbachia* strain as the female parent. For all females that produced eggs with zero hatch, spermathecae were dissected and examined under a microscope for the presence of stored sperm, and data from any females without sperm were disregarded.

4.2.4 Hatch and fecundity assay

Females were blood-fed at six days post-eclosion, and aspirated into small tubes for individualized laying two days later. Eggs were matured for five days before being counted to give fecundity data and then hatched in deoxygenated water in small plastic vials. Second instar larvae were counted to give hatch rate.

4.2.5 Egg longevity

The ability of Uju.wMel and Uju.wt eggs to survive for long periods was tested by hatching 50 eggs, every 10 days from five different egg papers from Uju.wt and Uju.wMel colonies. These eggs were hatched in deoxygenated water and the larvae counted at the second instar to give hatch rates.

4.2.6 Larval development time

Larvae reared at a density of 100 larvae/L and given 1 mg liver powder per larva per day; the adults were counted and sacrificed daily until all pupae had eclosed.

4.2.7 Curing *Ae. albopictus*

Two *Ae. albopictus* lines were used in curing attempts: a Malaysian line (KLP) provided by Oxitec which is used for RIDL injections, and a recently colonized line from Thursday Island in the Torres Strait (TTI) provided by Andrew van den Hurk.

4.2.7.1 Injection of antibiotics into *Ae. albopictus* eggs

Eggs were collected from female *Ae. albopictus* (KLP) approximately seven days after blood-feeding. Eggs aged 30-60 minutes were laid out on a damp nitrocellulose membrane and injected with antibiotics using a Femtojet microinjector. Eggs were stored

at 27°C and 100% humidity for five days before hatching. In later experiments adults were provided with rifampicin at 30µg/mL in 10% sucrose.

4.2.7.2 Antibiotic treatment of larvae water and adult sugar water

For curing experiments *Ae. albopictus* (TTI) mosquitoes were treated with tetracycline at the larval stage and/or rifampicin at the adult stage. TTI larvae were given tetracycline at a concentration of 0.1mg/mL throughout the larval stage or 2.5mg/mL for 24 hours at the second instar stage. Adult TTI mosquitoes were given rifampicin at 0.5mg/mL or 2.5mg/mL in 10% sucrose solution for two weeks immediately after eclosion. The antibiotic sucrose solution was then removed and replaced with moist cotton and a sucrose cube for five days. Females were then blood-fed, and eggs collected on damp filter paper and hatched seven days later.

4.2.7.3 Introgression using partial *Wolbachia* curing

Introgression from TTI.wt (containing *w*AlbA/B) into Uju.wMel was attempted using 19 day old antibiotic-treated TTI.wt males with the hypothesis that the *Wolbachia* in these males may be at a sufficiently low concentration that CI penetrance would be reduced (both antibiotics and increased male age (Raynolds *et al.,* 2002) should reduce CI penetrance). Around 50 antibiotic treated males aged 19 days post-eclosion from two of the curing experiments in section 4.2.6.2 (0.5mg/mL rifampicin; or 0.1mg/mL tetracycline and 0.5mg/mL rifampicin) were aspirated into cages containing female Uju.wMel or (Uju.wMel x TTI.wt) F_1 & F_2 pupae respectively, and left to mate for 10 days prior to blood

feeding and egg collection. The *Wolbachia* infection status of male offspring at the pupal stage was determined by PCR.

4.3 Results

4.3.1 Generation of Uju.wt

In order to accurately measure fitness characteristics of *w*Mel in this host, a line is needed which has the natural *w*AlbA/B superinfection in the same nuclear background. Uju.wMel was generated from the cured UjuT line and then backcrossed to it for seven generations. Therefore, an infected line named Uju.wt was generated by backcrossing Ascoli females to UjuT males for seven generations; the nuclear background of the resulting line was >99% the same as that of UjuT [(1 – 0.5^7) × 100 = 99.22] and Uju.wMel, and the line contained the natural *w*AlbA/B superinfection. Uju.wt was used to compare to Uju.wMel and UjuT in all characterization experiments in this chapter to minimize the effect of any strain differences between the hosts.

4.3.2 Longevity of Uju.wMel

Neither *w*Mel nor the natural *w*AlbA/B infection appear to have an effect on the longevity of female *Ae. albopictus* in the lab cage conditions used (figure 4.1 A). However, the longevity of Uju.wMel males was significantly increased compared to both the uninfected UjuT ($p = 0$, log rank test) and the naturally superinfected Uju.wt ($p = 0$, log rank test)

(figure 4.1 B). The average male lifespan was increased from 33.4 days (UjuT) or 33.6 days (Uju.wt) to 52.5 days (Uju.wMel).

The lack of a significant difference in longevity in either sex at 27°C between Uju.wt and UjuT contrasts with a previous report (Dobson *et al.,* 2002b). However, Dobson *et al.,* (2002b) did not perform introgression on the lines after curing, so the observed difference there may be an artefact of different genetic backgrounds due to bottlenecking.

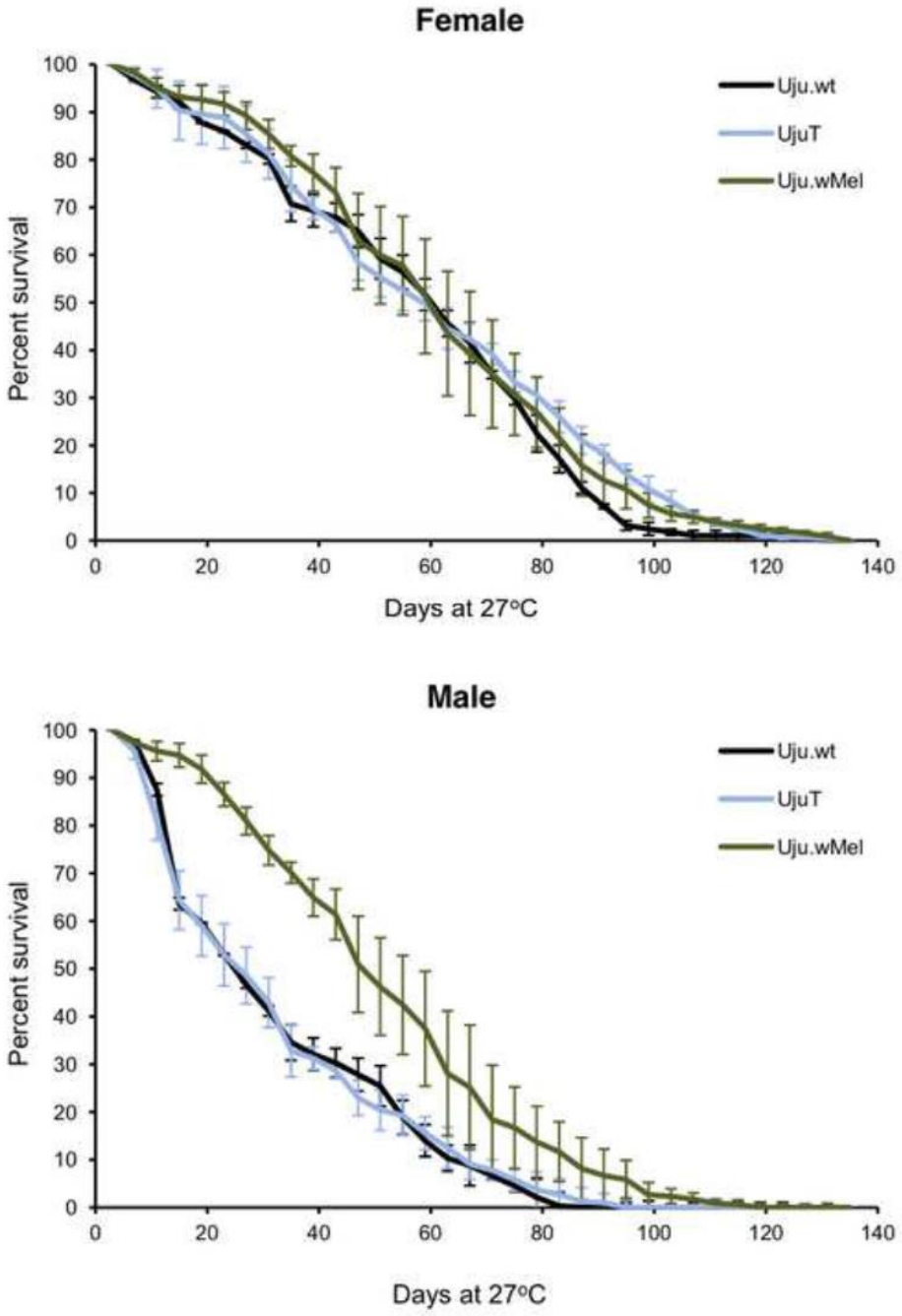

Figure 4.1: Longevity of Uju.wt, UjuT and Uju.wMel at 27°C. The longevity of three *Ae. albopictus* lines was assessed in three independent replicates of cages containing approximately 100 male and 100 female mosquitoes sexed at the pupal stage (total of 1600 mosquitoes in eight 30 × 30 × 30cm cages, due to equipment failure resulting in the loss of one Uju.wt replicate). The mosquitoes were provided with moist cotton and supplied with sucrose. A blood meal was provided at seven days, and again every 14 days. Dead mosquitoes were counted and removed every four days. Error bars show the SEM for each time point over the three replicates. Male Uju.*w*Mel

longevity was significantly increased compared to UjuT (p = 0, log rank test) and Uju.wt (p = 0, log rank test).

4.3.3 Male mating competitiveness

Mating competitiveness of Uju.wMel males was compared to that of the naturally infected Uju.wt males using three replicates of 50 male Uju.wMel : 50 male Uju.wt : 50 females (either Uju.wMel or Uju.wt. Hatching eggs from an individual female indicated that a compatible mating had occurred with a male with the same *Wolbachia* strain, whilst no hatch indicated an incompatible cross (data from females with empty spermathecae were disregarded). No significant difference was found between the competitiveness of the wild-type and transinfected males under these conditions (p = 0.652, Chi-squared analysis using a likelihood framework) (figure 4.3). *The author thanks Timothy Harvey-Samuel for his assistance with the statistical analysis for this experiment, and Cristina Di Genua for her assistance in performing the experiment*.

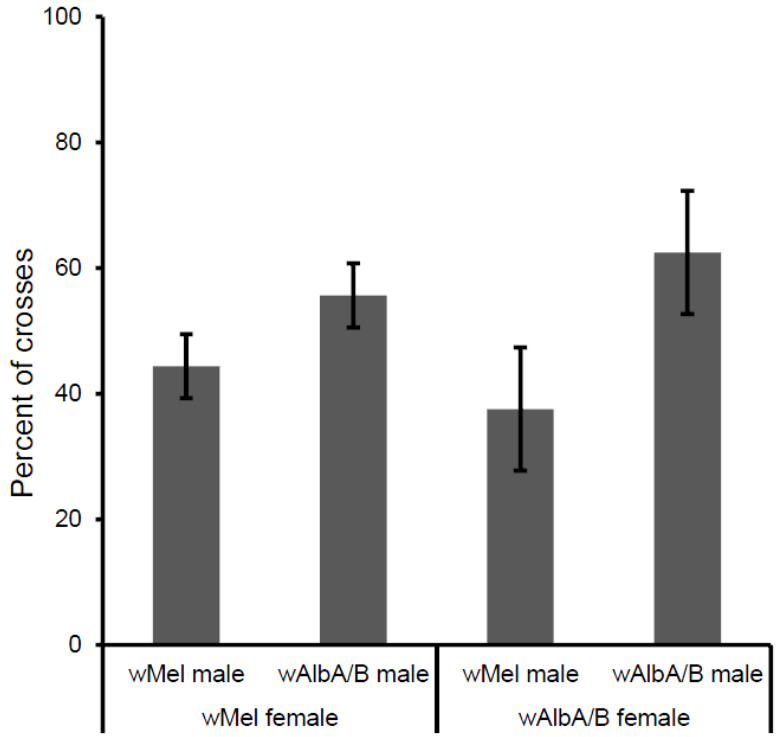

Figure 4.2: Mating competitiveness of Uju.*w*Mel males. Competitiveness of Uju.*w*Mel males was assessed using three independent replicates of 50 male *w*Mel : 50 male *w*AlbA/B : 50 females (either Uju.*w*Mel or Uju.wt), sexed at the pupal stage (total of 300 females in six $30cm^3$ cages). Adults were allowed to emerge in the 30cm x 30cm x 30cm cage after sexing and were left to mate for 10 days. Females were then blood fed and two days later were aspirated into small plastic vials for individualized laying. Eggs were hatched five days after laying; any hatch indicated a compatible cross (i.e. both male and female possessed the same *Wolbachia*). Spermathecae were dissected from females with no egg hatch to ensure mating; results from non-mated females were disregarded. Error bars show the SEM for the three replicates. No significant differences were found between the data from a Chi-squared analysis using a likelihood framework.

4.3.4 Egg hatch and fecundity

The egg hatch rate and the fecundity per single gonotrophic cycle observed in Uju.wMel were not significantly different to those in Uju.wt (hatch rate, $p = 0.369$, Wilcoxon rank sum test; fecundity, $p = 0.738$, Wilcoxon rank sum test) (figure 4.4). This is consistent with

observations in other novel transinfections in later generations (McGraw *et al.,* 2002; Xi *et al.,* 2005; Boyle *et al.,* 1993; Calvetti *et al.,* 2010). The fecundity of Uju.wt was significantly higher than UjuT ($p = 0.028$, Wilcoxon rank sum test), supporting previous reports of increased fecundity in the presence of the natural *Ae. albopictus Wolbachia* superinfection compared to a cured *Wolbachia*-uninfected line (Dobson *et al.,* 2002; Dobson *et al.,* 2004); Uju.wMel was also significantly more fecund than UjuT ($p = 0.0261$, Wilcoxon rank sum test). The fecundity of the Uju.wMel line contrasts with the significant fecundity reduction previously observed for the *w*Pip transinfection of *Ae. albopictus* (Van den Hurk *et al.,* 2012), and the hatch rate is much higher in Uju.wMel than that previously observed for a *w*MelPop transinfection in *Ae. albopictus* (Suh *et al.,* 2009).

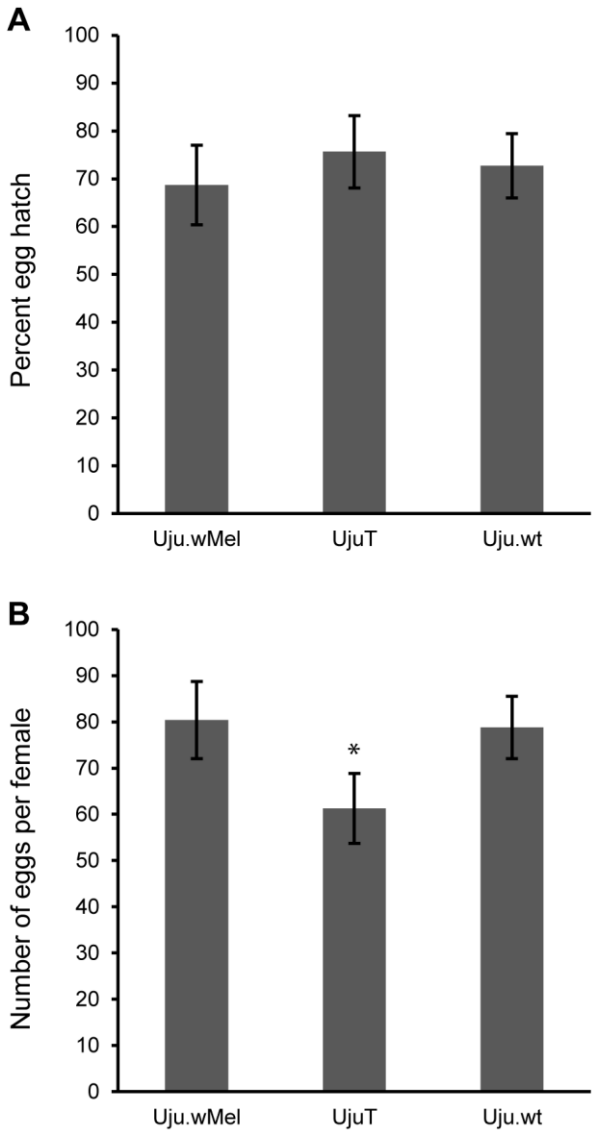

Figure 4.3. Hatch rate and fecundity of Uju.wMel. Egg hatch (A) and fecundity (B) of Uju.wMel were assessed at G_{16}. Females were blood fed at six days post-eclosion, individualized for laying, and eggs hatched after five days. Second instar larvae were counted to calculate percent egg hatch (A) and eggs per batch per female counted to give fecundity (B). A: Uju.wMel n = 452, UjuT n = 858, Uju.wt n = 508. B: Uju.wMel n = 16, UjuT n = 14, Uju.wt n = 20. Error bars represent the SEM. * = $p < 0.05$ using Wilcoxon rank sum test between UjuT and both Uju.wMel and Uju.wt.

4.3.5 Uju.wMel egg longevity

Egg longevity was assessed by hatching 50 eggs, every 10 days from five different egg papers, larvae were counted at the second instar to give hatch rates. At days 20, 40 and 50 the hatch rates of Uju.wMel was significantly lower than Uju.wt, p = 0.016, 0.009 and 0.47 respectively, using Wilcoxon rank sum tests. For all remaining time points there was no significant difference in hatch rates.

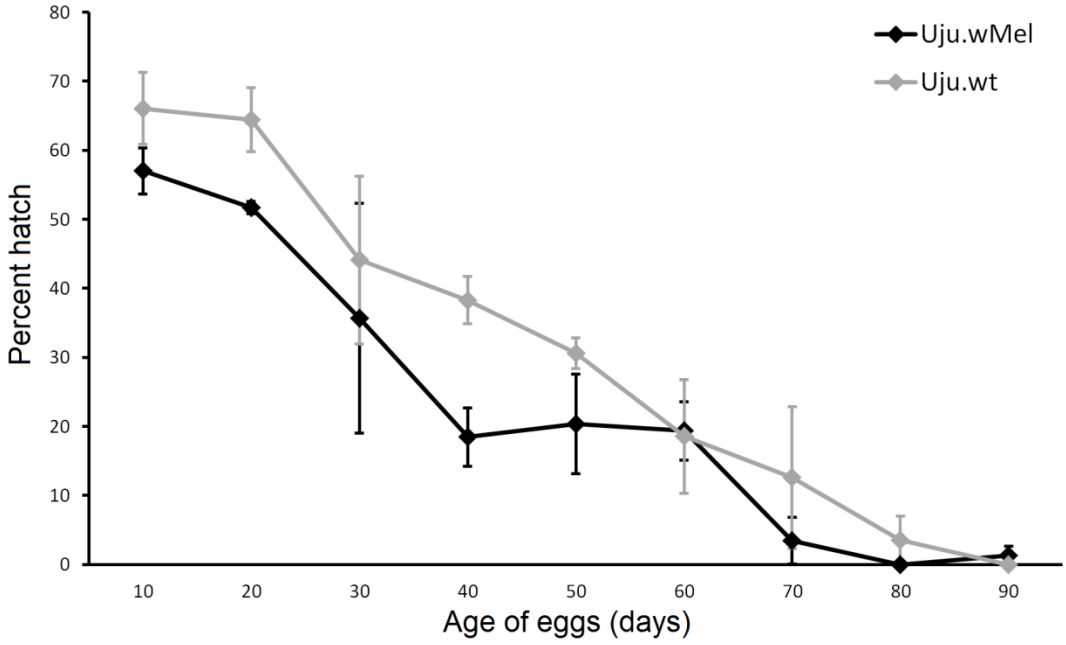

Figure 4.4. Egg longevity. Egg papers were collected from five colonies of Uju.wt and Uju.wMel: every 10 days 50 eggs from each paper were counted, hatched and then the second instar larvae counted to give hatch rates.

4.3.6 *w*Mel effect on larval development time

The effect of *w*Mel on the development time of larvae was tested by hatching eggs from UjuT, Uju.wMel and Uju.wt lines and rearing them under standardized conditions.

Individuals were considered developed once the pupae had eclosed, the experiment was replicated twice (figure 4.5).

No significant difference was found between the larval/pupal development times of Uju.wMel and Uju.wt using data from both replicates; the mean time taken to eclose from egg hatch was 10.85±1.18 days (n = 505) and 10.87±1.52 days (n = 951) for Uju.wMel and Uju.wt respectively (p = 0.931 log rank test; error bars represents one standard deviation).

The first replicate (figure 4.5 A) showed a significant increase in the development time of UjuT compared to the other lines. In the second replicate (4.5B) no such increase was found and the development time of UjuT was almost identical to the other two lines. Given the striking similarity between development times it was concluded that the increase in UjuT development time in the first replicate was probably the result of using older eggs (all other egg batches were seven days old).

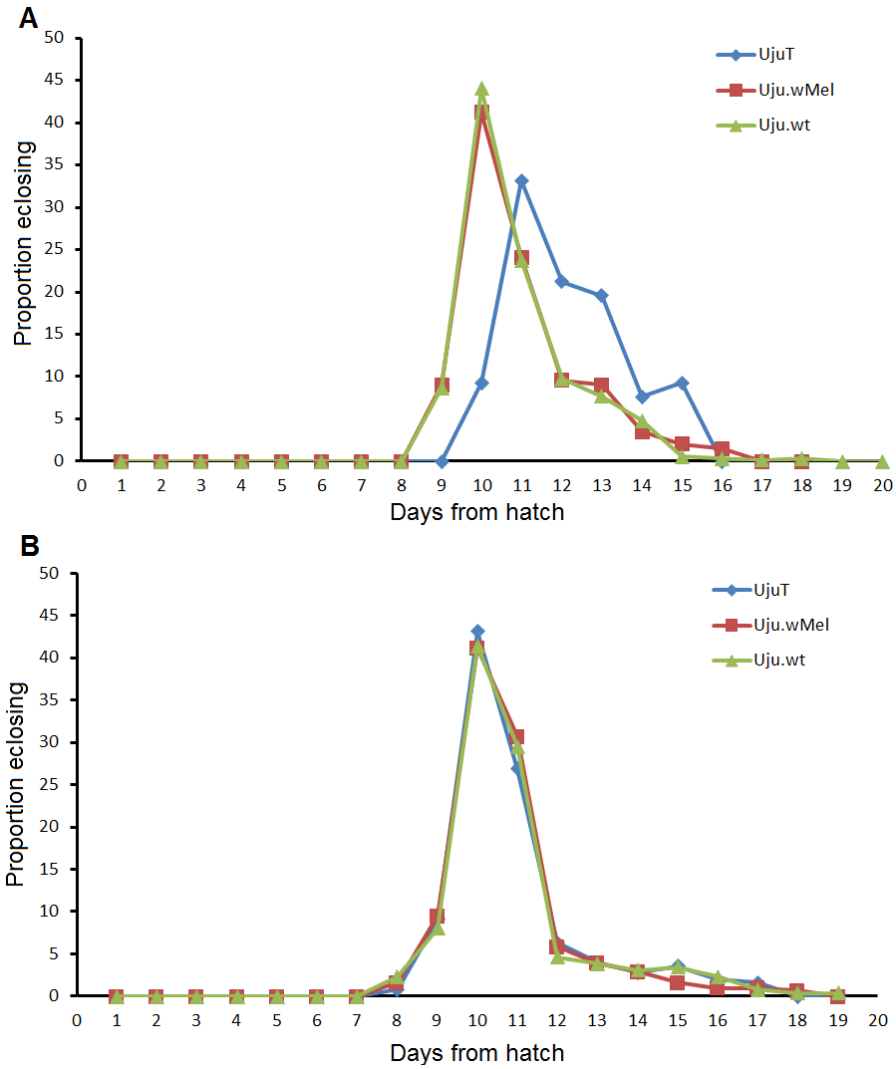

Figure 4.5. Larval development time of Uju.wMel, UjuT and Uju.wt. Eggs were hatched and larvae reared at 100 larvae/litre and 1 mg liver powder per larva per day. The time taken from hatching to eclosion was scored for each larvae. All egg papers were seven days old except the UjuT egg paper in the first replicate which was older.

4.3.7 Curing *Ae. albopictus* of *Wolbachia*

An additional consideration for field releases, beyond the fitness effects of *w*Mel on *Ae. albopictus,* is the differences in host genetic background between the *w*Mel-infected population and the local strain.

Two *Ae. albopictus* lines were used for assessing curing methods: KLP from Malaysia and TTI from the Torres Strait. Previous studies have shown that *Ae. albopictus* is more resistant than other mosquito species to curing by using tetracycline-treated larvae water (Dobson & Rattanadechakul, 2001), therefore other different methods of curing were also assessed for their ability to quickly and easily cure *Ae. albopictus* of *w*AlbA/B.

4.3.7.1 Curing *Ae. albopictus* of *Wolbachia* by egg injection of antibiotics

A preliminary experiment of 625 KLP eggs injected with two different concentrations of tetracycline produced no egg-laying cured females (table 4.1). However, qPCR analysis showed that the concentration of *Wolbachia* had been reduced to an average of 8±1.5% of the original concentration, indicating that the antibiotic was suppressing the *Wolbachia*.

The experiment was repeated using two concentrations of tetracycline, as well as rifampicin, in addition, the surviving adults were given rifampicin in 10% sucrose (table 4.2). These injections produced 29 egg-laying females, 17 of which were PCR-negative for *Wolbachia*. Eggs from 15 of these 17 females hatched indicating that most or all of the males were also cured.

Condition	Number injected	Number hatched	Number of adults	Number of females	Number of fed females	Number of egg-laying females	Number of egg-laying *Wolbachia*-negative females
Tetracycline 60µg/mL	100	8	8	3	0	0	0
Tetracycline 100µg/mL	525	13	13	5	0	0	0

Table 4.1. Curing of *Ae. albopictus* (KLP) using tetracycline injections into eggs. Injections of 60µg/mL or 100µg/mL of tetracycline were performed on ~1 hour old *Ae. albopictus* embryos. Eggs were then hatched and reared under standard conditions. Data from two experiments are pooled. The low hatch rate is believed to be partially due to excessive desiccation of the eggs in the second experiment.

Condition	Number injected	Number hatched	Number of adults	Number of females	Number of fed females	Number of egg-laying females	Number of egg-laying *Wolbachia*-negative females
Tetracycline 30µg/mL	87	13	12	6	1	1	1
Tetracycline 100µg/mL	279	39	39	21	16	13	5
Rifampicin 30µg/mL	80	3	3	2	2	2	2
Rifampicin 100µg/mL	144	37	34	17	14	13	9

Table 4.2. Curing of *Ae. albopictus* (KLP) using antibiotic injections into eggs and antibiotic sucrose water for adults. Injections of 30µg/mL or 100µg/mL of tetracycline or rifampicin were

performed on ~1 hour old *Ae. albopictus* embryos. Eggs were then hatched, reared under standard conditions and adults given rifampicin at 30 µg/mL in 10% sucrose.

Injections of 100µg/mL rifampicin, followed by 30µg/mL rifampicin in 10% sucrose provided to the adults, produced the most cured adults. With the help of Dr Geneviève Labbé and Siân Morgan at Oxitec, 1800 KLP embryos were therefore injected with 100µg/mL of rifampicin with the hope of generating a genetically diverse *Wolbachia*-free line. However, despite good survival from these eggs, the experiment failed to produce any *Wolbachia*-free egg-laying females (table 4.3). The inconsistency seems likely to result from the injection volume being highly variable and largely uncontrollable.

Condition	Number injected	Number hatched	Number of adults	Number of females	Number of fed females	Number of egg-laying females	Number of egg-laying *Wolbachia*-negative females
Rifampicin 100µg/mL	1800	274	231	117	104	72	0

Table 4.3. Large scale curing of *Ae. albopictus* (KLP) using rifampicin. Injections of 100µg/mL rifampicin were performed on ~1 hour old *Ae. albopictus* embryos. Eggs were then hatched, reared under standard conditions and adults given rifampicin at 30µg/mL in 10% sucrose.

4.3.7.2 Curing *Ae. albopictus* by antibiotic-treated larvae water and sugar water

Given that antibiotic injection into *Ae. albopictus* embryos proved to be too time-consuming and inconsistent a method of curing, a range of larval and adult antibiotic treatments were tested in the hope of finding an efficient curing regimen.

In addition to this, an alternate strategy was designed in which the concentration of *Wolbachia* in wild-type males was suppressed with the aim of reducing the penetrance of CI sufficiently to result in some egg hatch, thus allowing for genetic introgression. Previous studies have shown a correlation between *Wolbachia* density and the penetrance of CI in a number of mosquito species (Ruang-areerate and Kittayapong, 2006; Calvitti *et al.,* 2010). Increasing male age and number of matings has also been shown to reduce the penetrance of CI (Raynolds *et al.,* 2002).

Treating second instar larvae with 2.5mg/mL tetracycline for 24 hours resulted in complete larval mortality soon after antibiotic treatment. Subsequent experiments therefore used lower concentrations of antibiotics: 0.1mg/mL tetracycline throughout the larval stage and/or 0.5mg/mL or 2.5mg/mL rifampicin in 10% sucrose solution for 14 days immediately after eclosion for 14 days, followed by five days of no antibiotic prior to blood-feeding. Very low fecundity and egg hatch rates were recorded from these experiments (table 4.4). In addition, PCR results from treated females showed the presence of *Wolbachia* in all individuals except two females from the 0.1mg/mL tetracycline and 0.5mg/mL rifampicin cohort; from which no eggs hatched (table 4.4). These results are consistent with (Dobson *et al.,* 2001) who showed that *Ae. albopictus* is

resistant to curing with antibiotics and increasing the dosages to overcome this results in larval mortality or adult infertility.

Larvae treatment	Adult treatment	Fecundity	Hatch rate	Offspring PCR result
2.5mg/mL tetracycline for 24 hours	None	NA	NA	NA
None	2.5mg/mL rifampicin for 14 days	11 eggs from ~100 females	0%	NA
0.1mg/mL tetracycline throughout	0.5mg/mL rifampicin for 14 days	19 eggs from ~100 females	0%	2 cured TTI females
None	0.5mg/mL rifampicin for 14 days	69 eggs from ~100 females	4.3%	All *Wolbachia*-positive

Table 4.4. Curing TTI of *w*AlbA/B using antibiotic larval and/or adult antibiotic treatment. TTI eggs were hatched in deoxygenated water. Where applicable antibiotics were added to the larvae water at the first instar (for 0.1mg/mL) or second instar for (2.5mg/mL). Adults were provided with 10% sucrose containing rifampicin immediately for 14 days; this was replaced with 10% sucrose and no antibiotic for five days prior to blood-feeding. Eggs were collected, counted and hatched after seven days. Adult females were sacrificed and after laying and their *Wolbachia* infection status was determined by PCR.

4.3.7.3 Introgression of *w*Mel by reducing CI penetrance

In an attempt to backcross *w*Mel into the incompatible TTI line, approximately 50 19-day old males from the 0.5mg/mL rifampicin adult treatment curing experiment were transferred into a cage containing 20 female Uju.wMel pupae. Approximately one third of the eggs hatched from this experiment (table 4.5), demonstrating that CI can be suppressed sufficiently to allow for direct backcrossing into bidirectionally incompatible lines.

A second generation of backcrossing was attempted using TTI males treated with 0.1mg/mL tetracycline larvae water and 0.5mg/mL rifampicin in adult 10% sucrose water in an attempt to improve the hatch rate. However, considerably fewer eggs were obtained from this treatment and approximately the same hatch rate was achieved (table 4.5). A third generation of backcrossing was then performed using the same regimen as the first; egg yield increased to similar levels as in the first generation, indicating that adult 0.5mg/mL rifampicin treatment alone was more efficient for this method.

Larvae treatment	Adult treatment	Fecundity	Hatch rate	PCR result
None	0.5mg/mL rifampicin for 14 days	259 eggs from 20 Uju.wMel females	33%	*w*Mel-positive
0.1mg/mL tetracycline throughout	0.5mg/mL rifampicin for 14 days	78 eggs from 20 1st generation backcrossed females	32%	*w*Mel-positive
None	0.5mg/mL rifampicin for 14 days	314 eggs from 16 2nd generation backcrossed females	29%	*w*Mel-positive

Table 4.5. Introgression of *w*Mel into a TTI background using antibiotic-treated older males. TTI eggs were hatched in deoxygenated water. Where applicable, antibiotics were added to the larvae water at first instar. Adults were provided with 10% sucrose containing rifampicin immediately for 14 days; this was replaced with 10% sucrose and no antibiotic for five days prior to blood feeding. Males were then aspirated into cages containing female Uju.wMel or backcrossed pupae and allowed to mate for 10 days. Females were then blood-fed and allowed to lay eggs. Eggs were collected, counted and hatched after seven days. Larvae were counted at the second instar to give hatch rates. Male pupae (not used for backcrossing) were sacrificed and their *Wolbachia* infection status determined by PCR.

4.4 Discussion

4.4.1 Fitness parameters

4.4.1.1 Longevity and mating competitiveness

No difference was found between the longevity of Uju.wMel, UjuT and Uju.wt females (figure 4.1. This contrasts with a previous report showing greater longevity in *Ae. albopictus* harbouring the natural *w*AlbA/B infection (Dobson *et al.,* 2002b). However, in that report the authors did not perform introgression on the lines after curing; therefore bottlenecking and a founder effect on alleles present in cured individuals may have biased the result. Interestingly, male Uju.wMel longevity was significantly increased compared to both Uju.wt and UjuT (figure 4.1); the reason(s) for this increase remain unknown.

Uju.wMel and Uju.wt males were not significantly different in their mating competitiveness (figure 4.2) in the cage conditions used here, though, Uju.wMel males did mate with fewer (41%) females which could become significant over larger field populations. However, in the event that *w*Mel-infected males had reduced competitiveness in field conditions, a sufficiently higher ratio of released to wild-type males could be used to abrogate the problem.

4.4.1.2 Hatch and fecundity

Following the selection for increased hatch and fecundity in chapter 3, there appears to be no effect of *w*Mel compared to the wild-type *w*AlbA/B infection on either hatch rate or fecundity in the Uju genetic background (figure 4.3). Interestingly, both Uju.wMel and Uju.wt showed increased fecundity compared to UjuT, suggesting that *w*Mel is able to provide this same fitness advantage conferred by *w*AlbA/B to *Ae. albopictus*.

Both hatch and fecundity are extremely important factors in fitness and therefore in population replacement dynamics. Unlike previous transinfections with wRi (Xi *et al.,* 2006), wPip (Calvitti *et al.,* 2010), and wMelPop (Suh *et al.,* 2009), which all showed either reduced hatch, fecundity or both, wMel imposes no such fitness costs and is therefore a potentially more effective transinfection for population replacement. However, the longevity of Uju.wMel eggs appears somewhat reduced compared to Uju.wt eggs which may affect the ability of this line to survive longer periods of drought before hatching. This slight reduction in egg longevity is much less extreme than the egg survival cost found in *w*MelPop transinfection of *Ae. aegypti* (McMeniman & O'neill, 2010). Additionally, the results presented here are from an earlier generation of eggs and, like fecundity and egg hatch rate, the longevity of eggs would likely be increased by imposed selection or simply by co-adaption between host and symbiont over time.

4.4.2 Population replacement potential of Uju.wMel

The data collected here give a broad lab fitness characterization for many important traits relating to field release of a bidirectionally incompatible transinfected strain. All fitness traits measured here have shown that *w*Mel causes no or relatively small fitness costs compared to the natural *Wolbachia* infection in *Ae. albopictus*. There will obviously be numerous differences in the conditions encountered in the wild compared to cage populations so extrapolation to the field from lab insectary studies should always be approached with care. In previous field releases of *Aedes* mosquitoes however, small-cage lab assays have been shown to be a fairly reliable indicator of field fitness and success (Harris *et al.,* 2011; Hoffmann *et al.,* 2011). Taken together with the complete bidirectional CI and 100% maternal transmission shown in chapter 3, as well as the total abolition of DENV and CHIKV transmission capability, this *w*Mel transinfection may provide a valuable new tool for DENV and CHIKV control.

A recently published mathematical model (Hancock *et al.,* 2011) gives an example of a method to introduce *Wolbachia* into discrete seasonal populations using a male-biased release strategy. Whilst female releases are required because of the maternal inheritance of *Wolbachia*, it is desirable to minimize the number of biting females released. If large numbers of males are released they will mate with a large proportion of wild-type females, rendering them effectively infertile and thus reducing the release threshold for females. Male pupae can be separated from female pupae by size from females and the majority emerge before females making such a sex separation relatively simple. If this strategy is implemented at the start of the rainy season when the natural population size

is low and continued over a period of months the release threshold can be further reduced.

Using the data generated in this chapter, chapter 3, and a modified mathematical model from Hancock *et al.,* (2011), Penny Hancock (unpublished, personal communication) demonstrated that using a release ratio of 98% males every three days over two months should result in fixation well before the end of a six-month rainy season. Once established at 100% frequency, the transinfection would be resistant to moderate levels of immigration of wild-types from surrounding populations, since immigrating wildtype females would be incompatible with the majority of the males they encountered.

The release of a bidirectionally incompatible line has advantages over that of a unidirectional crossing type as the former is less able to spread beyond the initial release area due to the requirement for a population majority to replace the natural infection. Furthermore, it would also be possible to reverse the infection by mass-release of the wild-type line should an unexpected negative outcome occur.

Studies published whilst this work was being completed reported the generation of a *w*Mel transinfection in *Ae. aegypti* (Walker *et al.,* 2011), which also showed DENV and CHIKV inhibition (Walker *et al.,* 2011; van den Hurk *et al.,* 2012), as well as successful field trials of this line in Queensland, Australia (Hoffman *et al.,* 2011), supporting the findings here and demonstrating the feasibility of field application of a *Wolbachia*-based population replacement strategy. Taken together, these transinfections show that both

major vectors of DENV and CHIKV are amenable to *Wolbachia*-based population replacement and virus inhibition.

There will be many fluctuating and unknown variables encountered in field conditions which make extrapolation of results from lab assays to the wild uncertain. Therefore semi-field trials are an important next step in better understanding the fitness characteristics of the *w*Mel transinfection; for example semi-field contained greenhouse trials would give a better estimate for field longevity and mating competitiveness than insectary cages. Such semi-field trials should be completed and estimates for release size requirements updated before open field releases are effected.

4.4.3 Potential of Uju.wMel as a source of incompatible males

In addition to its use as a population replacement mechanism, Uju.wMel also has potential field application as a source of incompatible males, analogous to the RIDL OX513 technology in *Ae. aegypti* (Phuc *et al.,* 2007) but with the advantage of not being genetically modified and therefore subject to fewer regulations and restrictions. Similarly to OX513, the mosquitoes would need to be mechanically separated by sex; whilst any females that were inadvertently released would be refractory they could potentially effect population replacement if released in large enough numbers so great care would need to be taken in the sex separation process.

For such an application, the mating competitiveness of the males is the most important consideration. No significant difference was found between the male mating

competitiveness of Uju.wMel and Uju.wt (figure 4.2); therefore this line represents a viable source of males for an incompatible insect technique application.

4.4.4 Introgression of *w*Mel into different lines of *Ae. albopictus*

In naturally uninfected species, such as *Ae. aegypti*, *Wolbachia* can be readily introgressed into a target population, allowing for fitness to be maximized by acquisition of local adaptations and preventing the introduction of new traits into target populations. However, in naturally infected species which show bidirectional CI with transinfected strains, this process is considerably less efficient, traditionally requiring the target population to be cured of *Wolbachia* prior to introgression.

This is a particular problem with *Ae. albopictus* given the difficulty in curing the species (Dobson & Rattanadechakul, 2001); in some cases several generations are required to cure a line (Geneviève Labbé, personal communication).

The development of a fast and efficient curing regimen for *Ae. albopictus* was attempted by injecting embryos with antibiotics (tables 5.1 and 5.2). Initially, tetracycline was injected into the embryos, but no completely cured individuals were generated (table 5.1). Later experiments used varying concentrations of rifampicin or tetracycline, and adults were provided with rifampicin-treated sugar water. This method produced some fertile cured females (table 5.2) but this result was not reproduced when the method was replicated on a larger scale (table 5.3), and so was concluded to be too time-consuming and inconsistent for an efficient curing method. A second curing method was attempted

using various regimens of larval and/or adult antibiotic treatment but again failed to produce any fertile cured individuals.

An alternate method was successfully attempted in which the CI penetrance of wild-type males was reduced by partial *Wolbachia* knockdown resulting from each-generation antibiotic treatment of wild-type 19-day old males. This reduction in CI penetrance resulted in approximately one third of the offspring eggs successfully hatching (table 5.4) enabling crossing between bidirectionally incompatible lines.

This protocol will considerably expedite the introgression of *w*Mel into different strains of *Ae. albopictus* by removing the need to wait for curing and amplification of the target line. More broadly, this technique may also be applicable to other naturally infected species where transinfected strains are bidirectionally incompatible with wild-type strains.

4.4.5 Conclusion

The fitness parameters which have been measured here are generally favourable with respect to the aim of introducing the *w*Mel transinfection into natural *Ae. albopictus* populations for the purposes of disease prevention. The additional hurdle of local strain introgression in a species with a bidirectionally incompatible wild-type infection has also been addressed by the generation of a technique allowing for backcrossing using partially cured males. Taken together, the *w*Mel transinfection appears, at this stage of

characterization, to be a viable tool for population replacement of *Ae. albopictus* with a refractory strain.

Chapter 5: Investigation into the mechanisms of *Wolbachia*-mediated pathogen resistance

5.1 Introduction

The specific mechanism(s) by which *Wolbachia* mediates pathogen inhibition in its host are currently unknown. As described in chapter 1, there are multiple (not mutually exclusive) theories that could explain the resistance, including: immune stimulation prior to pathogen acquisition (Kambris *et al.,* 2009); competition for resources, e.g. cholesterol (Moreira *et al.,* 2009); and an increase in the concentration of reactive oxygen species, which may be linked to immune activation (Brennan *et al.,* 2008; Pan *et al.,* 2012); and modulation of autophagy (Niu *et al.,* 2012; Voronin *et al.,* 2012). Elucidation of this mechanism(s) would enable a more informed choice when generating a novel transinfection and increase our understanding of insect immunity and pathogen susceptibilities.

At the time this work started *Wolbachia*-mediated immune stimulation and pathogen inhibition had recently been shown in *Ae. aegypti* by *w*MelPop (Kambris *et al.,* 2009). This pathogen inhibition was presumed to be caused by the observed strong upregulation of anti-microbial effectors, particularly defensins and cecropins. Furthermore, many components of the Toll pathway, which has been shown to play a role in DENV dissemination control (Zambon *et al.,* 2005; Xi *et al.,* 2008), were also upregulated by

*w*MelPop. It was not known whether this immune response would be limited to the pathogenic *w*MelPop, or whether other *Wolbachia* strains would elicit the same response. Furthermore, it was also unknown whether the immune response would be limited to *Ae. aegypti* and other *Wolbachia*-naïve hosts, or would be found in any novel *Wolbachia* transinfection.

In order to study *Wolbachia*-induced immune stimulation, multiple strains of *Wolbachia* would ideally need to be transinfected into multiple hosts. However, as the generation of stable *Wolbachia* transinfections is both time consuming and technically challenging, a somatic model of these infections using purified *Wolbachia* to inoculate adult mosquitoes would be more efficient and allow for greater number of *Wolbachia*/host combinations to be studied. Such a technique has been demonstrated before: the ability of purified *Wolbachia* to survive, albeit without replication, in a cell-free environment has been shown (Rasgon *et al.,* 2006); and it was later shown that *Wolbachia* purified in this way could be intrathoracically injected into adult *An. gambiae* mosquitoes, where they were able to replicate and establish a somatic infection (Jin *et al.,* 2009).

In addition to study of the immune system, somatic models would allow desirable combinations of *Wolbachia* and host to be identified prior to the generation of stable transinfections as some novel transinfections have unexpected phenotypes. For example, *w*AlbB causes DENV inhibition in *Ae. aegypti* (Bian *et al.,* 2010), whilst its natural host, *Ae.*

*albopictus, w*AlbB appears to have little or no effect on DENV transmission capacity (Blagrove *et al.,* 2012).

In addition to immune stimulation, competition for resources has also been suggested as a potential mechanism for pathogen inhibition. Moreira *et al.,* (2009) suggested that competition for cholesterol could cause pathogen inhibition, as the mosquito host, *Wolbachia,* and many pathogens, such as DENV and CHIKV, all require cholesterol but none are capable of synthesizing it. For example, cholesterol biosynthesis modulates DENV replication (Rothwell *et al.,* 2009) and is required by alphaviruses, suck as CHIKV for optimal endosomal fusion (Keilian *et al.,* 2010). Competition for such a resource could create a bottleneck for pathogen replication as the only sources would be the larval diet and blood meals. Furthermore, as the *Wolbachia* would have been established since the embryo stage, it may have sequestered the cholesterol needed by pathogens before they infect the mosquito, rendering them unable to complete their life cycle.

The aims of this chapter are to generate and assess the utility of a somatic model of *Wolbachia* infection on the host immune system, and use this model to determine which combinations of *Wolbachia* and host result in immune upregulation. Host immune upregulation and cholesterol sequestration in Uju.wMel and Aα23 cell lines are also assessed to investigate the possible mechanism(s) of RNA-virus inhibition in Uju.wMel.

5.2 Materials and methods

5.2.1 Mosquito cell culture maintenance

Cells were grown in 75cm^2 culture flasks to ~50% confluence in Schneider's medium containing 10% foetal bovine serum (FBS) and 140 units of penicillin per mL and 140µg of streptomycin per mL. Cells were examined under a microscope and passaged every 3-5 days.

5.2.2 *Wolbachia* purification

Wolbachia was purified from cell lines using a modified version of the procedure found in (Rasgon *et al.,* 2006). Aα23, Rml12 & MOS55 lines were used. Cells were harvested by vigorous shaking of the flasks to dislodge the cells, then 50mL of this suspension was poured into a Falcon tube. Cells were pelleted by centrifugation at 2,500 x *g* and 4°C for 10 mins. The supernatant was removed and the cells were resuspended in 10 mL of antibiotic-free Schneider's medium containing 10% FBS in a 50mL centrifuge tube. Cells were lysed by vortexing for 3 mins with approximately 100 sterile 3mm borosilicate glass beads. The lysate was then centrifuged at 2,500 x *g* and 4°C for 10 mins to pellet large cellular debris. The supernatant was decanted, passed through a 5µm syringe filter, and centrifuged at 20,800 x *g* at 4°C for 5 mins to pellet *Wolbachia*.

5.2.3 Intrathoracic inoculation

The *Wolbachia* pellet was resuspended in Schneider's media with 10% FBS (without antibiotics) to an optical density of OD = 0.09 at 400nm wavelength. 69 nL of this

Wolbachia suspension (or 69 nL Schneider for the controls) were microinjected into the thorax of *An. gambiae* (G3), *Ae. aegypti* (Ref^{m}) and *Ae. albopictus* (UjuT) females using a Nanoject microinjector (Drummond).

5.2.4 Adult mosquito dissection

Dissections of adult female *Ae. albopictus* were carried out under a light microscope. Prior to dissection, females were sacrificed by freezing and the wings and legs removed by hand. The mosquitoes were then placed on a glass slide and covered with 1x phosphate buffered saline (PBS) (137mM NaCl, 2.7 mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4).

Midguts were dissected by stabbing the thorax with a probe and pulling the second-to-last abdominal segment with forceps to expose the midgut which was removed from the thorax. Salivary glands were dissected by removing the head with forceps and gently pushing on the thorax. Ovaries were dissected by holding the thorax with forceps and pulling the second-to-last abdominal segment with forceps. Fat body was dissected by crushing the thorax with a probe and removing the fat tissue from the exoskeleton with forceps.

5.2.5 Cell harvesting for cholesterol assay

A tetracycline cured *Ae. albopictus* Aα23 cell line (Aα23 W-) was infected with *w*AlbB to create the line Aα23 *w*AlbB and, separately, with *w*Mel to create the line Aα23 *w*Mel, by Dr Sofia Pinto (unpublished). Cells from these three lines were grown to 100% confluence and stored in the 27°C incubator for a further 48 hours in an attempt to ensure media

resources were depleted. Cells were harvested by vigorous shaking of the flasks to dislodge the cells; 50mL of this suspension was poured into a Falcon tube. Cells were pelleted by centrifugation at 1,000 x *g* and 4°C for 10 mins. The supernatant was removed and the cells were washed twice in 500µL PBS and pelleted by centrifugation at 1,000 x *g* and 4°C for 10 mins.

5.2.6 Cholesterol assay

Free and total cholesterol were assayed using the Amplex® Red Cholesterol Assay kit (Invitrogen™, Carlsbad, California) using manufacturer's protocols Following dissection or cell pelleting, 110µL 1X reaction buffer was added to each sample. Two borosilicate beads were added and the sample was vortexed for five minutes to lyse cells. 50µL of this suspension was added to two wells of a microplate.

A cholesterol esterase positive master mix of 300µM Amplex® Red reagent was prepared to detect total cholesterol; this contained 2 U/mL horseradish peroxidase, 2 U/mL cholesterol oxidase, and 0.2 U/mL cholesterol esterase in 1X reaction buffer to 2.5mL. A second master mix not containing cholesterol esterase was prepared to only detect free cholesterol; this contained 2 U/mL HRP and 2 U/mL cholesterol oxidase in 1X reaction buffer to 2.5mL.

The reactions were initiated by adding 50µL of the cholesterol esterase positive master mix solution to one half of the wells, and adding 50µL of the cholesterol esterase negative

master mix solution to the other half such that each sample had two reactions, one with each master mix.

The reactions were incubated for 30 mins at 37°C and the fluorescence measured in a FLUOstar Omega® microplate reader (BMG Labtech™ , Offenburg, Germany) using excitation in the range of 530–560 nm and emission detection at ~590 nm. For each point, the background fluorescence was corrected for by subtracting the values derived from a no cholesterol control.

5.3 Results

5.3.1 Assessment of the utility of somatic infection models

The utility of a somatic model of *Wolbachia* transinfection was assessed using qRT-PCR to compare the effects on host immunity of the stable inherited transinfection of *w*MelPop in *Ae. aegypti* (Ref^m), the line in which immune stimulation was first identified (Kambris *et al.,* 2009), to a cured (*Wolbachia*-free) Ref^m line injected with *w*MelPop purified from the same cell line (RML12) as was used for the original transinfection (McMeniman *et al.,* 2009) by qRT-PCR. The use of the same host line and *Wolbachia* source minimized any effect of different host genetic background or *Wolbachia* lines.

The somatic *Wolbachia* infection induced upregulation of four immune genes (a peptidoglycan recognition protein, *PGRPS1*; cecropin D, *CECD*; a CLIP-domain serine

protease, *CLIPB37*; and a C-type galactose-specific lectin) (figure 5.1), selected for qRT-PCR based on their upregulation in the stable *w*MelPop *Ae. aegypti* transinfection (Kambris *et al.,* 2009), and for a broad range of function. The scale of upregulation was, however, considerably lower than observed in the stable transinfection (Kambris *et al.,* 2009). This reduced effect may be explained by there being approximately 176±70 times more *w*MelPop per host cell in the stable infection compared to the somatic infection – estimated using qPCR on the single copy genes *ftsZ* (*Wolbachia*), and *Actin5C* (*Ae. aegypti*) for normalization (three replicates of four adult mosquitoes per condition, p = 0.495, Wilcoxon rank sum test).

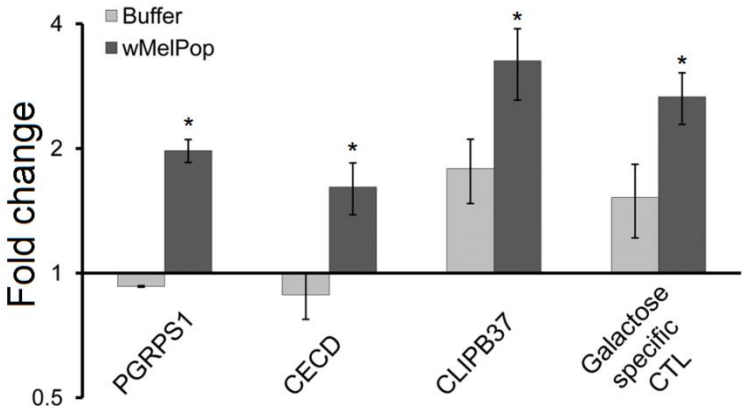

Figure 5.1: Immune gene expression in *Ae. aegypti* somatically infected with *w*MelPop. The expression of four immune genes, *PGRPS1*, *CECD*, *CLIPB37* and *galactose specific CTL,* was analyzed by qRT-PCR. Adult females were injected with *w*MelPop or the buffer alone, approximately seven days post-eclosion. RNA was extracted from these adults eight days after injection. Expression was normalized to non-injected adult females of the same age from the same colony. Error bars show the SEM of three biological replicates, each containing eight adult females (total of 24 mosquitoes per condition). * = $p < 0.05$ using Wilcoxon rank sum test between injected samples and non-injected controls.

Given that immune upregulation was shown in all four genes tested, it was concluded that intrathoracic inoculation is a valuable method for testing the effects of *Wolbachia* on host immunity. Using this method, extrapolations to different species is possible although they must be made with care. However, it is likely that any effects observed on host immunity using this methodology will be conservative.

5.3.2 Effect of *w*MelPop on *An. gambiae* immune gene expression

Using this intrathoracic model of *Wolbachia* transinfections, a *w*MelPop somatic infection of *An. gambiae* (G3) was generated by intrathoracic inoculation with *Wolbachia* purified from an *An. gambiae* cell line (MOS55). The gene expression of six immune genes (leucine-rich repeat immune protein, *LRIM1*; thioester-containing protein, *TEP1*; cecropin, *CEC1*; defensin, *DEF1*; a C-type lectin, *CTL4*; and clip-domain serine protease, *CLIPB3*) from these transinfected females was then quantified using qRT-PCR. *The An. gambiae injections were completed jointly with Dr Zakaria Kambris.*

As was shown for *Ae. aegypti*, *w*MelPop also caused significant immune upregulation in five out of six immune genes in adult *An. gambiae* females compared to uninjected controls (see figure 4.2). Interestingly, two *E. coli*-injected controls showed significant downregulation of two immune genes; this is likely the result of a negative feedback pendulum effect following the immune response and clearing of the *E. coli.*

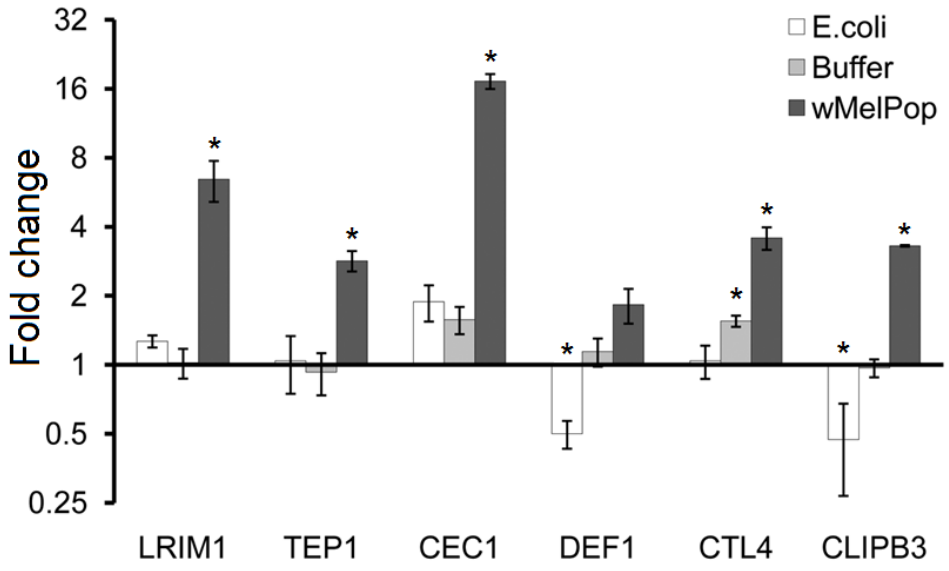

Figure 5.2: Immune gene expression in *An. gambiae* somatically infected with *w*MelPop. The expression of six immune genes (*LRIM1*, *TEP1*, *CEC1*, *DEF1*, *CTL4* and *CLIPB3*) was analyzed by qRT-PCR. Adult *An. gambiae* females were injected with *E. coli*, *w*MelPop or buffer alone, 2–3 days post-eclosion, and RNA was extracted from these adults eight days after injection. Expression was normalized to non-injected adult females of the same age from the same colony. Error bars show the SEM of three biological replicates, each containing eight adult females (total of 24 mosquitoes per condition). * = $p < 0.05$ using Wilcoxon rank sum test between injected samples and non-injected controls.

In order to provide further evidence that this immune stimulation is not an artefact of the intrathoracic injection process, immune gene expression in *w*MelPop-infected *An. gambiae* MOS55 cells was compared to the expression in tetracycline cured MOS55 cells. Again, significant upregulation of five out of six immune genes was observed in the infected cell line (figure 4.3), adding confidence to the results shown by intrathoracic inoculations.

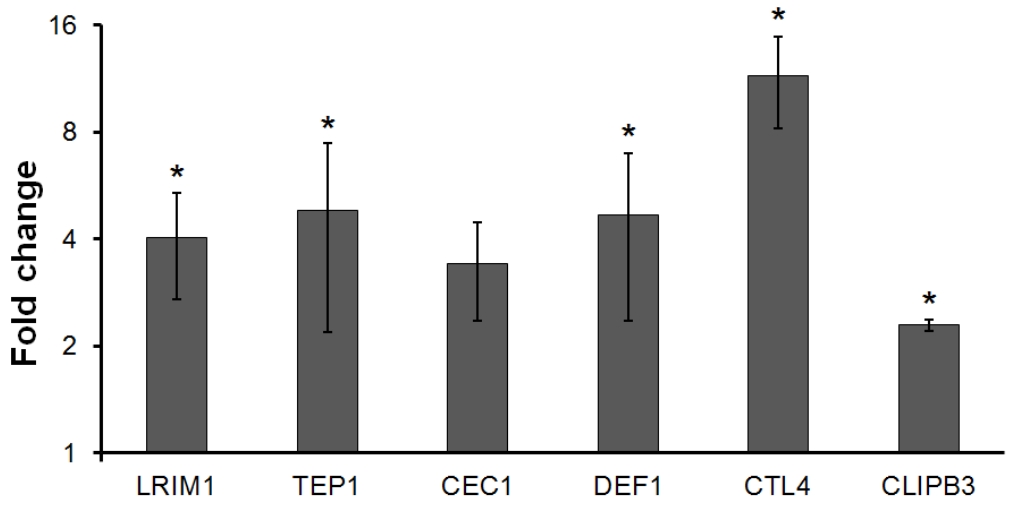

Figure 5.3: Immune gene expression in the *An. gambiae* *w*MelPop-infected MOS55 cell line. The expression of six immune genes (as described in figure 5.2) were analyzed, by qRT-PCR, in *An. gambiae* MOS55 cell line infected with *w*MelPop. Expression was normalized to a tetracycline-cured, *w*MelPop-free, genetically identical MOS55 cell culture. Three samples of cells were taken from the cultures at different times (3, 5 and 7 days after fresh media had been added); error bars show the SEM of these three samples. * = $p < 0.05$ using Wilcoxon rank sum test between infected and uninfected cells.

5.3.3 Immune stimulation by *Wolbachia* in *Ae. aegypti* and *Ae. albopictus*

Given the significant immune stimulation found by somatic injection of *w*MelPop in both *Ae. aegypti* and *An. gambiae*, different *Wolbachia* and host combinations were tested in order determine whether this effect is limited to *w*MelPop, and/or naïve host species. To this end, somatic infections of *w*MelPop and *w*AlbB in *Ae. aegypti* and *Ae. albopictus* were generated. Adult females were injected with a suspension of *Wolbachia* purified from *Ae. albopictus* cell lines (Aα23 and Rml12 for *w*AlbB and *w*MelPop respectively) approximately three days post-eclosion. Expression of selected immune genes was tested

at five and nine (figure 5.4) or one and nine (figure 5.5) days after injection. Presence of live *Wolbachia* was also confirmed for all *w*MelPop and *w*AlbB injected samples using RT-PCR, as presence of RNA indicates the *Wolbachia* is live. Again, a range of immune gene types were selected (a peptidoglycan recognition protein, *PGRPS1*; cecropin D, *CECD*; CLIP-domain serine protease, *CLIPB37*; and a thioester-containing protein, *TEP20, for Ae. aegypti*, and their respective orthologs for *Ae. albopictus*) which includes important antimicrobial genes and is based on their previously identified upregulation in the presence of *w*MelPop (Kambris *et al.,* 2009).

Immune stimulation was observed in *Ae. aegypti* with both *w*MelPop and *w*AlbB when compared to non-injected, buffer and heat-killed *E. coli* controls. However, no significant immune stimulation was observed in *Ae. albopictus* in the presence of either *Wolbachia*. A spike in immune gene expression was observed at one day after injection for all injections, presumably due to septic shock (figure 5.5), but by days five and nine, the immune expression had returned to near-normal levels for *Ae. aegypti* injected with *E. coli* and for all *Ae. albopictus* injections; only *Ae. aegypti* infected with *Wolbachia* (either strain), retained an immune response at days five and nine. The data between the nine day time points in both experiments were largely consistent, adding confidence to the results. The lack of immune gene stimulation at day nine in *Ae. albopictus,* when injected with either *Wolbachia*, also indicates that no residual peptidoglycan (which might be immune stimulating) from the *Wolbachia* purification is present at this time point, further adding confidence that immune stimulation in other *spp.* is not an artefact of injection.

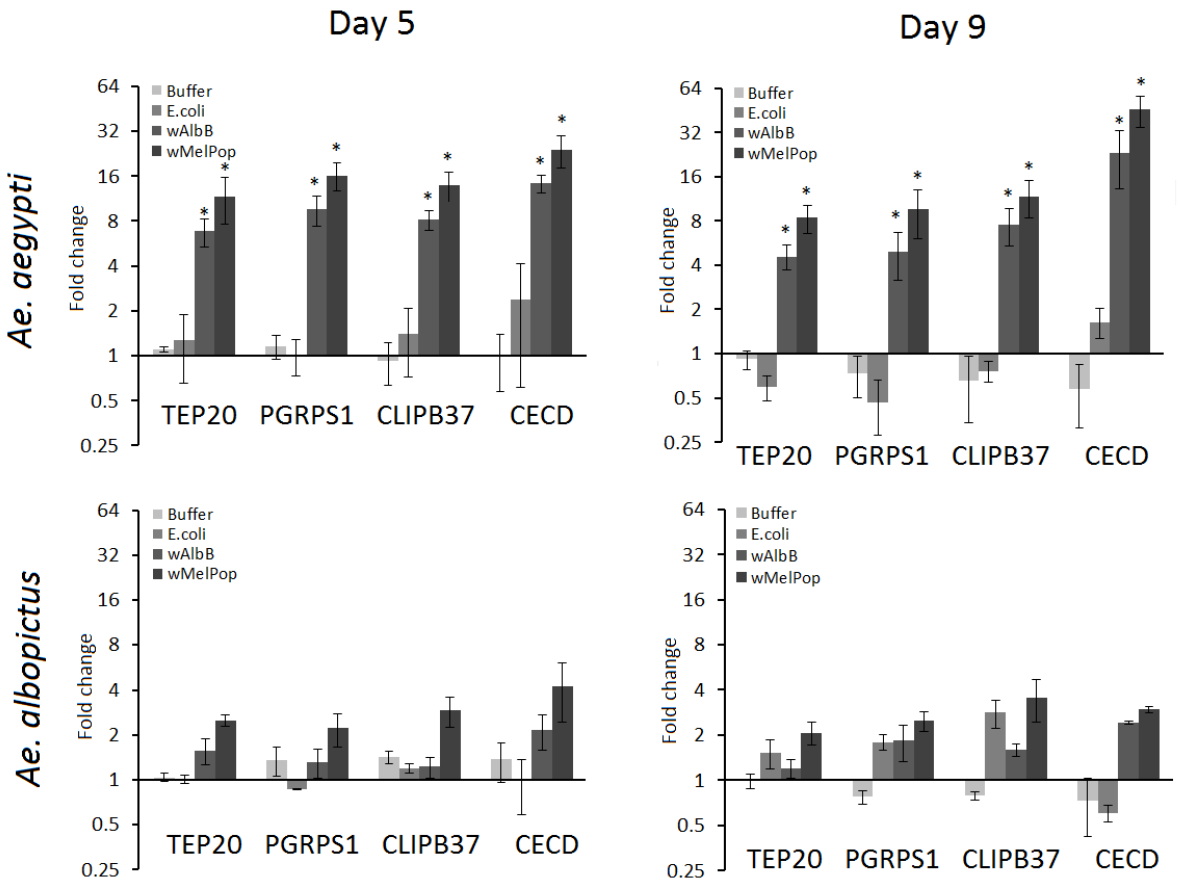

Figure 5.4: Immune gene expression in *Ae. aegypti* and *Ae. albopictus* somatically infected with *w*MelPop or *w*AlbB. Adult females were injected with *w*MelPop, *w*AlbB, heat killed *E. coli*, or injection buffer alone, approximately three days post-eclosion. RNA was extracted from half of these adults five days after injection, and from the remaining half nine days after injection. The expression of four *Ae. aegypti* immune genes (*PGRPS1*, *CECD*, *CLIPB37* and *TEP20*), and their orthologs *Ae. albopictus*, were analyzed by qRT-PCR**.** Expression was normalized to non-injected adult females of the same age from the same colony. Error bars show the SEM of three biological replicates, each containing five adult females (total of 30 mosquitoes per condition over two time points). * = $p < 0.05$ using Wilcoxon rank sum tests between injected samples and non-injected controls.

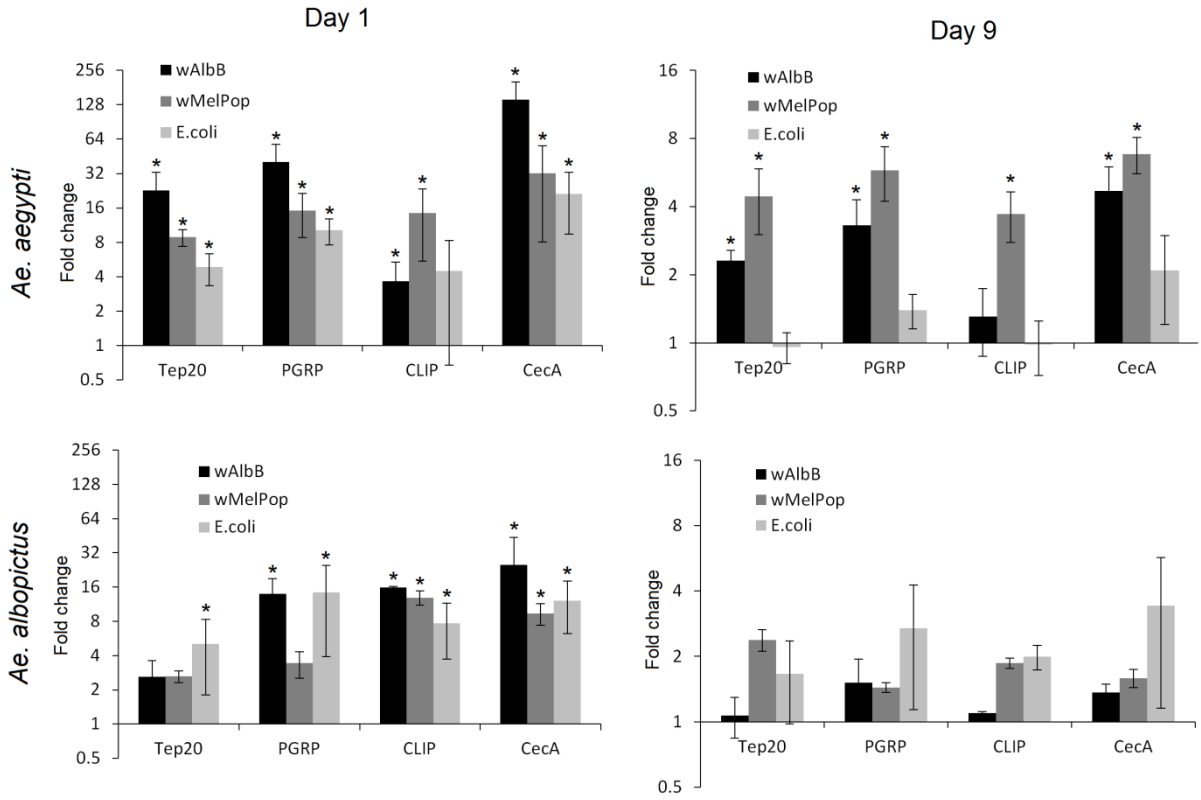

Figure 5.5: Immune gene expression in *Ae. aegypti* and *Ae. albopictus* somatically infected with *w*MelPop or *w*AlbB. Adult females were injected with *w*MelPop, *w*AlbB, or *E. coli*, approximately three days post-eclosion. RNA was extracted from half of these adults one day after injection, and from the remaining half nine days after injection. The expression of four immune genes was analyzed by qRT-PCR as described in figure 3.4A. Expression was normalized to non-injected adult females of the same age from the same colony. Error bars show the SEM of three biological replicates, each containing five adult females (total of 30 mosquitoes per condition over two time points). * = $p < 0.05$ using Wilcoxon rank sum tests between injected samples and non-injected controls.

The change in concentration of *Wolbachia* between days one and nine of the replicate shown in figure 5.5 was also estimated using qRT-PCR. The concentration of *Wolbachia* increased in three of the four combinations, indicating not only survival but replication of the *Wolbachia*, demonstrating that this method produces a viable infection, which is

consistent with previous findings (Jin *et al.,* 2009). *Ae. albopictus* injected with *w*AlbB showed a non-significant reduction in *w*AlbB concentration (figure 5.6).

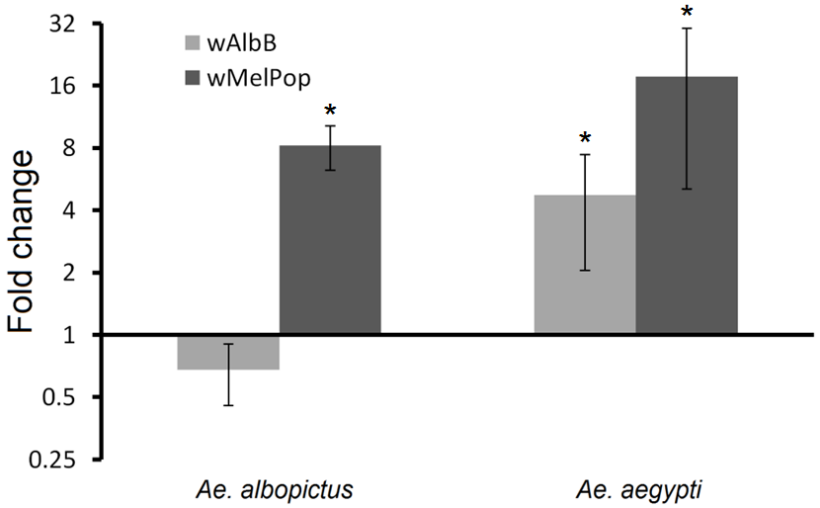

Figure 5.6: Fold change in *Wolbachia* concentration between days 1 and 9 post injection. Adult females were injected with *w*MelPop or *w*AlbB, approximately three days post-eclosion. RNA was extracted from half of these adults one day after injection, and from the remaining half nine days after injection. *Wolbachia* concentration change between days one and nine was estimated using qRT-PCR of *ftsZ* (*Wolbachia*), and *Actin5C* (*Ae. aegypti*) or *S17* (*Ae. albopictus*) for normalization. Error bars show the SEM of three biological replicates, each containing five adult females (total of 30 mosquitoes per condition over two time points). * = $p < 0.05$ using Wilcoxon rank sum test between results from days one and nine.

5.3.4 Immune stimulation in Uju.wMel

Following the generation of a *w*Mel transinfection in *Ae. albopictus* described in chapter 3, the effect of *w*Mel on *Ae. albopictus* immune gene expression was investigated. G_5 and G_{10} Uju.*w*Mel, UjuT and wild-type (Ascoli or Uju.wt) females were used, 11 days-post eclosion (figure 5.7). There was no significant difference in immune gene transcription

between the cured UjuT line and the wild-type lines. There was however, slight immune upregulation in G_5 Uju.wMel.

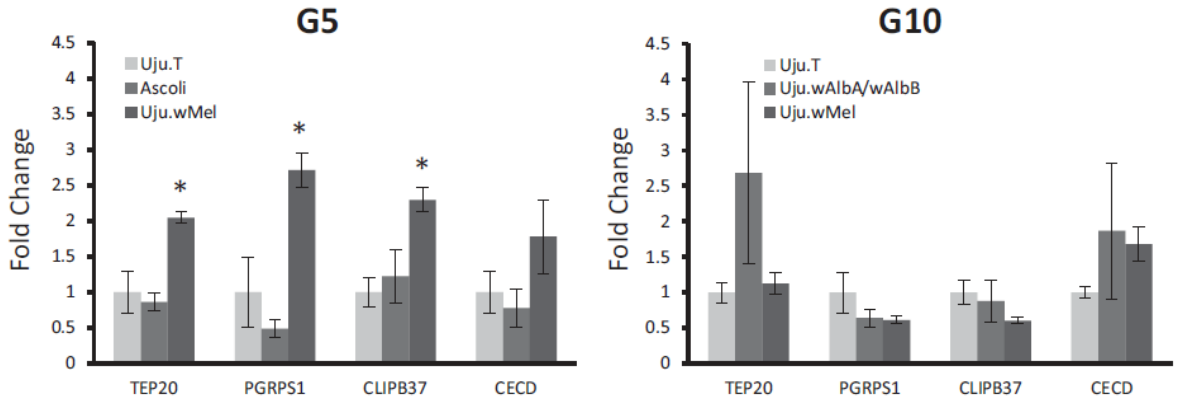

Figure 5.7: Immune gene expression in Uju.*w*Mel G_5 and G_{10}. RNA was extracted from adult females of Uju.*w*Mel, UjuT and Ascoli or Uju.wt lines at 11 days post-eclosion. The expression of four *Ae. albopictus* orthologs for *Ae. aegypti* immune genes *PGRPS1*, *CECD*; *CLIPB37* and *TEP20* was analyzed by qRT-PCR**.** Expression was normalized to the UjuT adult females. Error bars show the SEM of three biological replicates, each containing four adult females (total of 12 mosquitoes per condition). * = $p < 0.05$ using Wilcoxon rank sum tests.

Following the finding of little or no immune stimulation in Uju.wMel, a larger selection of immune genes selected from the Toll, IMD and STAT pathways were tested with G_{12} Uju.wMel to more thoroughly examine whether any of the immune system arms were activated or suppressed. The genes tested were the antimicrobial peptide Defensin D, the negative regulators of the Toll, IMD and STAT pathways *Cactus*, *Caspar* and *PIAS*, plus two NF-κβ Relish-like transcription factors *Rel2* and *Rel1A*, which are expressed in the Toll and IMD pathways respectively. No significant up or down regulation of any tested gene was found (figure 5.8).

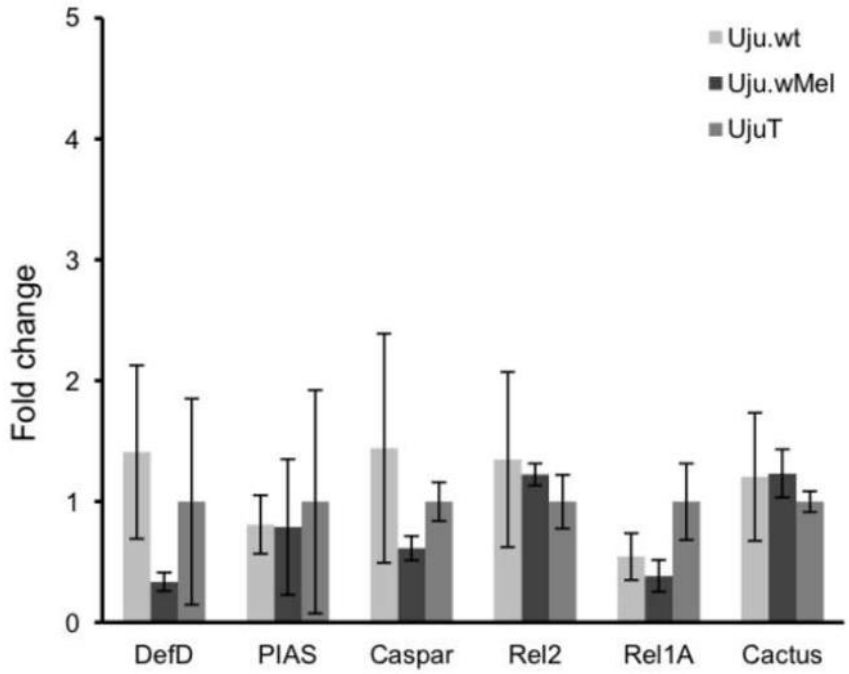

Figure 5.8: Further immune gene expression in Uju.*w*Mel G_{12}. RNA was extracted from UjuT, Uju.wt and G_{12} Uju.*w*Mel at 11 days post-eclosion. The expression of six *Ae. albopictus* orthologs of *Ae. aegypti* immune genes, *DefD*, *PIAS*, *Casper*, *Rel2 Rel1A* and *Cactus*, were analyzed by qRT-PCR. Expression was normalized to UjuT. Error bars show the SEM of three biological replicates each containing six adults (total of 18 mosquitoes per condition). No significant differences were found using Wilcoxon rank sum tests.

In order to examine whether the immune gene transcription patterns in *Ae. albopictus* seen in figure 5.7 is the result of the line quickly becoming attenuated to *Wolbachia w*Mel TTI.wMel backcross generation three (TTI.wMel BC3) mosquitoes were tested using the same genes as for Uju.wMel (with the exception of *Rel1 A*, as the primers would not amplify TTI cDNA). TTI.wMel BC3 is 87.5% TTI background and acquired *w*Mel through this backcrossing (see chapter 4), thus are only minimally attenuated to the presence of *w*Mel. Again, no immune gene upregulation was observed (figure 5.9), suggesting that the

presence of *w*Mel.in *Ae. albopictus* does not have significant effects on the host immune system.

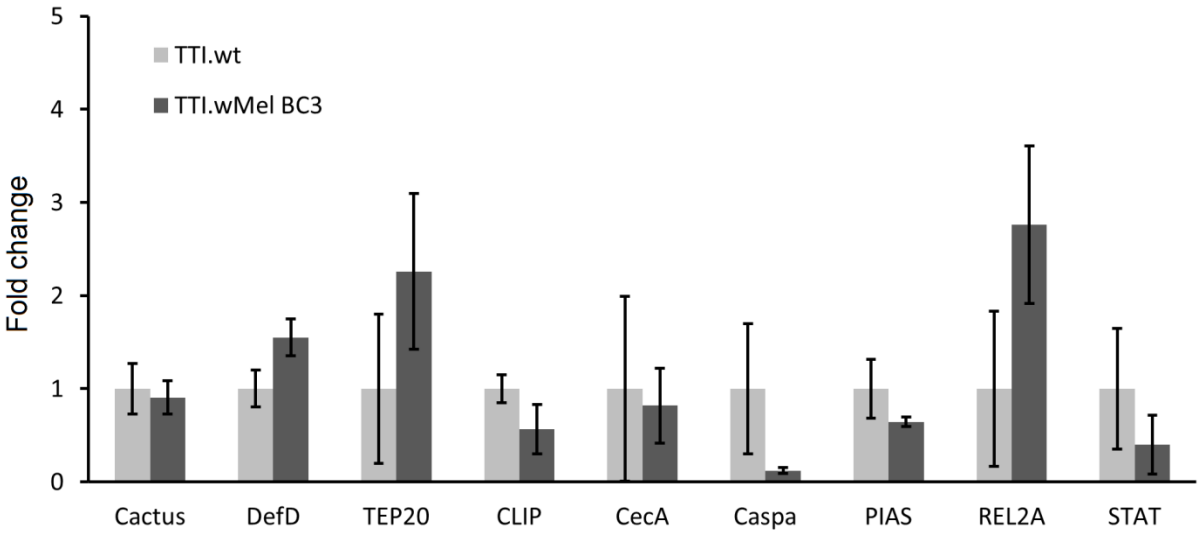

Figure 5.9: Immune gene expression in TTI.wMel BC3. RNA was extracted from adult females TTI.wMel BC3 and TTI.wt at 11 days post-eclosion. The expression of nine *Ae. albopictus* orthologs for *Ae. aegypti* immune genes was analyzed by qRT-PCR. Expression was normalized to the TTI.wt adult females. Error bars show the SEM of three biological replicates, each containing four adult females (total of 12 mosquitoes per condition). No significant differences were found using Wilcoxon rank sum tests.

5.3.5 Concentration of *w*Mel in host tissues

The concentration of *Wolbachia* was measured in different tissues to identify any areas of high concentration which are more likely to be of importance for effects on pathogen replication. No significant difference between the concentration of *w*Mel in Uju.wMel and of *w*AlbA/B in Uju.wt was found in the fat body or midgut (figure 5.10). The concentration of *w*Mel in Uju.wMel eggs was approximately 11-fold higher than wAlbA/B in Uju.wt (p =

0.0495, Wilcoxon rank sum test). The concentration of *w*Mel in the ovaries of Uju.wMel was not statistically significantly different to that in wildtypes due to very high variance, although mean density was approximately 23-fold higher. Whilst these tissues are not involved in pathogen transmission, the concentration of *Wolbachia* is expected to be highest in germ-line cells and this helps explain the high concentration of *w*Mel found in chapter 3 (figure 3.4). In addition to the tissues tested in figure 5.10, salivary glands were also dissected; however, there was insufficient DNA for *Wolbachia* qPCR amplification (host *S17* was amplified). The lack of amplification is likely the combined result of the small amount of tissue in salivary glands and low concentrations of *Wolbachia* present.

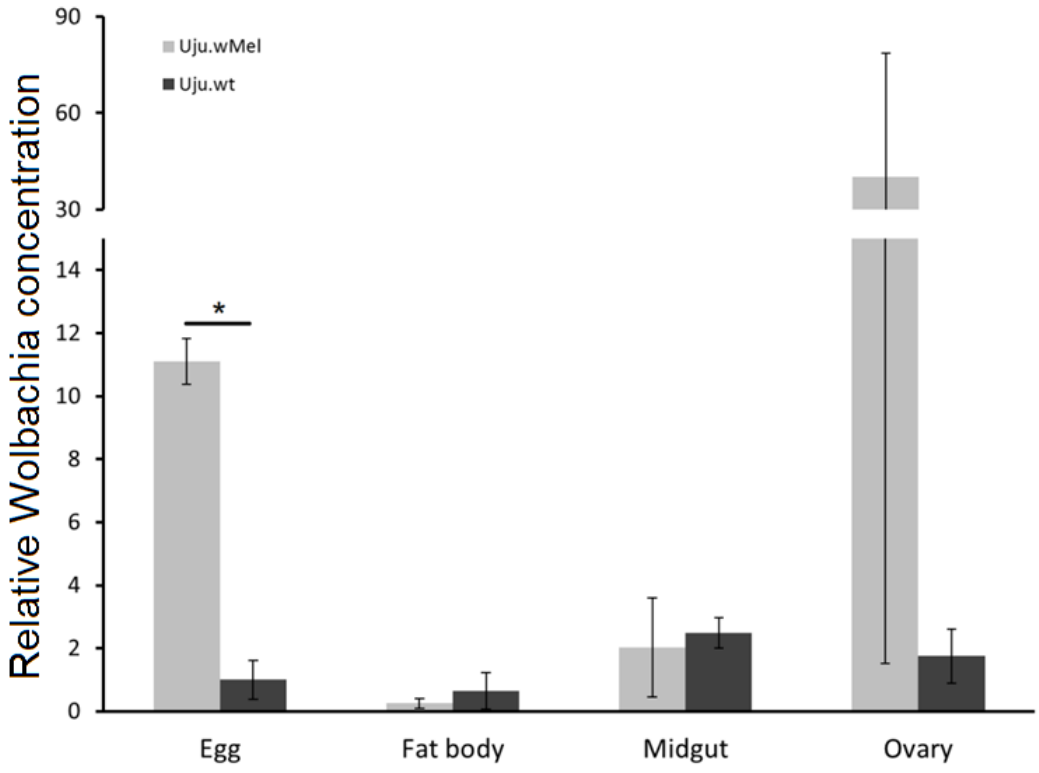

Figure 5.10: Concentration of *Wolbachia* in Uju.wMel and Uju.wt tissues. DNA was extracted from eggs and tissues of adult Uju.wMel and Uju.wt females 11 days after eclosion. qPCR was used to quantify *Wolbachia wsp* DNA and host *S17* DNA; the ratio of these two genes gave an estimate of the concentration of *Wolbachia*. The combined concentration of *w*AlbA and *w*AlbB in

the eggs of Uju.wt was arbitrarily designated 1; all other data were plotted relative to this. Error bars show the SEM. * = $p < 0.05$ using Wilcoxon rank sum test.

5.3.5 Sequestration of cholesterol by *w*Mel

The ability of *w*Mel to sequester cholesterol was measured in *Ae. albopictus* cell lines. A tetracycline cured Aα23 cell line (Aα23 W-) was infected with *w*AlbB (Aα23 *w*AlbB) and *w*Mel (Aα23 *w*Mel) by Dr Sofia Pinto (unpublished) to generate three cell lines comprised of the same host cells but different *Wolbachia* infections. The amount of free cholesterol in these cells was measured as a proportion of the total cholesterol (figure 5.11). The proportion of free cholesterol in Aα23 *w*Mel cells was significantly less than was found in Aα23 *w*AlbB cells, suggesting that *w*Mel sequesters a higher proportion of the cholesterol in the Aα23 cell line.

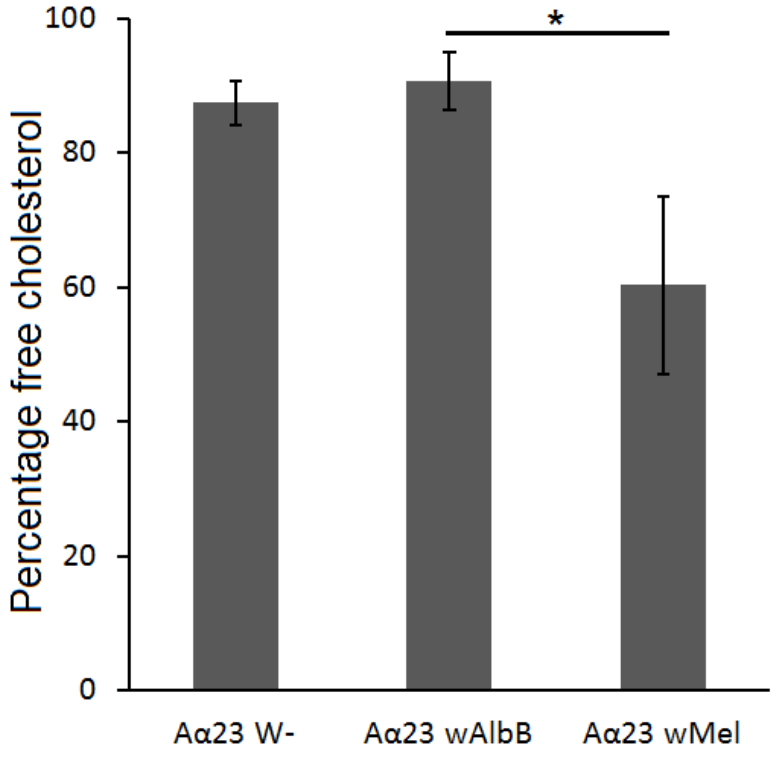

Figure 5.10: Percentage free cholesterol in *Wolbachia*-infected and uninfected Aα23 cell lines. Aα23 W-, Aα23 *w*Mel and Aα23 *w*AlbB cells were harvested two days after 100% confluence had been reached. Percentage free cholesterol was estimated using the Invitrogen™ Amplex® Red Cholesterol Assay kit both with and without cholesterol esterase to give total and free cholesterol levels respectively. Three replicates per cell line were performed from independent aliquots at the last passage. Error bars represent SEM of these three samples. * = $p < 0.05$ using Wilcoxon rank sum test.

Following the finding that *w*Mel sequesters cholesterol in the Aα23 cell line, the proportion of free cholesterol was measured in the midgut, fat body and remaining carcass of Uju.wMel, Uju.wt and UjuT adult females (figure 5.11). The proportion of free cholesterol in all tissues from all lines was approximately 90% with the exception of the midgut of Uju.wMel, which had a significantly reduced free cholesterol proportion of 51% ($p = 0.495$, Wilcoxon rank sum test).

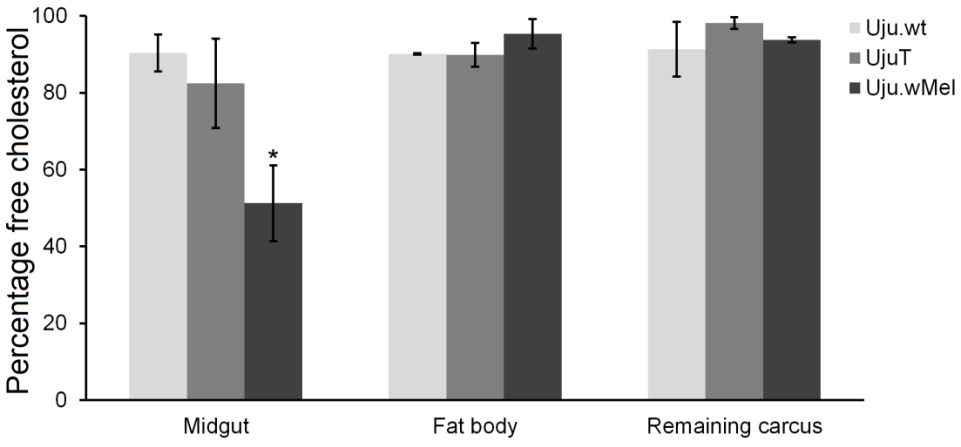

Figure 5.11: Percentage free cholesterol in Uju.wMel, Uju.wt and UjuT. The midgut and fat body of three-day old Uju.wMel, Uju.wt and UjuT females was dissected and the remaining carcasses collected. Percentage free cholesterol in these tissues was estimated using the

Invitrogen™ Amplex® Red Cholesterol Assay kit both with and without cholesterol esterase to give total and free cholesterol levels respectively. Error bars show the SEM of three biological replicates, each containing six adult females (total of 18 mosquitoes per condition). * = $p < 0.05$ using Wilcoxon rank sum test.

5.4 Discussion

5.4.1 Utility of the somatic infection model

Somatic infections of *Wolbachia* into adult female mosquitoes appear to be an effective means of identifying which host/*Wolbachia* combinations cause host immune stimulation. Based on comparison to previous microarray data (Kambris *et al.,* 2009), immune stimulation caused by somatic infection of *Wolbachia* in *Ae. aegypti* (figure 5.1) seems to be a conservative estimation of that caused by a stably inherited infection; this is possibly due to the titre of *Wolbachia* being much lower (approximately a 176±70-fold reduction) in the somatic model. This development will expedite the study of *Wolbachia*-mediated host immune stimulation by removing the need to generate large numbers of stably transinfected lines.

It was also noted that immune stimulation was much higher in the second and third round of *w*MelPop injection into *Ae. aegypti* (figures 5.4 and 5.5) than in the first (figure 5.1). This could be due to higher confluence of the cells from which the *w*MelPop was purified; the older and more confluent cells are more likely to be in the stationary phase thus with limited resources, and thus *Wolbachia* extracted from these cells is likely to be

in poorer metabolic condition compared to *Wolbachia* extracted from log phase cells and as such less able to make a strong infection or any infection at all.

5.4.2 Effect of *w*MelPop on the immune system of *An. gambiae*

The *An. gambiae* immune system was activated when somatically infected with *w*MelPop (figure 5.2), demonstrating that this effect is not limited to *Ae. aegypti* and provides a good indication that should a stably infected *An. gambiae* line be generated, its immune system will also be activated.

Immune stimulation was also shown in the *w*MelPop infected MOS55 cell line compared to uninfected cells (figure 5.3). These data add confidence to the hypothesis that it is the *Wolbachia* infection that is causing this effect and not an artefact of the intrathoracic injection process. The degree of stimulation differed between cell line infection and adult somatic infection; however differences are to be expected given that many immune genes are expressed in specific cell types or organs which would not be present in the cell line (whose cellular composition is unknown), for example fat body cells or in the case of *TEP1*, haemocytes (Blandin *et al.,* 2004).

In parallel to this work, Dr Zakaria Kambris, Dr Andrew Blagborough and Dr Sofia Pinto conducted a study which showed that *w*MelPop inhibits the development of *Plasmodium* using the same intrathoracic inoculation model shown here (Kambris *et al.,* 2010). The *Plasmodium* inhibition was presumably the result of the immune stimulation, which as

described here, included the upregulation of anti-pathogen effector genes which have been established to inhibit *Plasmodium* (Moreira *et al.,* 2009). Further evidence for this was provided by an RNAi knockdown of TEP1, which caused a reversal of the *Wolbachia*-induced *Plasmodium* inhibition (Kambris *et al.,* 2010).

5.4.3 Effects of different *Wolbachia* strains and host natural infection status

Immune stimulation was observed when *Ae. aegypti* was somatically infected with *w*AlbB (figure 5.4 & 5.5), and there was no significant difference between the immune stimulation caused by *w*MelPop and *w*AlbB in *Ae. aegypti,* demonstrating that immune stimulation is not limited to *w*MelPop. No significant immune stimulation was shown by *Ae. albopictus* when infected with *w*AlbB, one of its natural *Wolbachia*, adding further confidence that the increased stimulation is not an artefact of the injection process. Additionally, no significant immune stimulation was exhibited by *Ae. albopictus* when somatically infected with *w*MelPop, indicating that it is the natural infection status of the host that is the most important factor in the level of immune response. As the cell lines in which the *Wolbachia* were cultured were *Ae. albopictus* cell lines, it is possible the reduced immune stimulation in *Ae. albopictus* adults may be partly due to *Wolbachia* attenuating specifically to the *Ae. albopictus* cell environment; however, any confounding effect as a result of this is likely to be minimal given the phylogenetic similarity between these two *Aedes* species.

Minor immune stimulation was observed in G_5 Uju.wMel (figure 5.7) but not in G_{10} or G_{12} (figure 5.8) Uju.wMel, nor was it observed in the newly backcrossed TTI.wMel BC3 (figure

5.9). Given that the immune stimulation in G_5 Uju.wMel was very low compared to the immune stimulation shown in *Ae. aegypti* stably infected with *w*MelPop (Kambris *et al.,* 2009), and not reproducible, it may be that it is simply an artefact of that experiment such as larval rearing or cage conditions, and that *w*Mel does not cause a significant immune response in *Ae. albopictus*.

Recently, a stable transinfection of *An. stephensi* with *w*AlbB was generated and characterized (Bian *et al.,* 2013). Although the authors did not assess whether *w*AlbB caused immune stimulation, *w*AlbB was found to inhibit *Plasmodium* in this species supporting the data shown here and in Kambris *et al.,* (2010).

When taken together these immune data are all consistent with the hypothesis that it is the natural infection status of the host species which determines whether significant immune stimulation is exhibited. This hypothesis is also consistent with other published work showing that *w*AlbB initiates an immune response in *An. gambiae* (Hughes *et al.,* 2011) and in *Ae. aegypti* (Pan *et al.,* 2012).

5.4.4 Cholesterol sequestration by *w*Mel

As described in chapter 3, transmission of DENV and CHIKV is completely abolished in Uju.wMel; however, as shown here, this happens in the apparent absence of prior immune priming and therefore there may be additional mechanisms causing the inhibition. One possible mechanism is a competition for resources between the host, *Wolbachia* and virus; cholesterol has previously been proposed as such a resource as it

required by all three, but none are capable of synthesizing it (Moreira *et al.,* 2009). The data presented here show that *w*Mel sequesters cholesterol both in Aα23 cells (figure 5.10) and in the midgut of Uju.wMel females (figure 5.11). This sequestration of cholesterol in the midgut of adult females may be a mechanism by which *w*Mel inhibits DENV and CHIKV, as both are RNA viruses, many of which require host cholesterol to enter cells (Mackenzie *et al.,* 2007; Cater *et al.,* 2009). Cholesterol biosynthesis has been shown to modulate DENV replication (Rothwell *et al.,* 2009) and is required by alphaviruses, suck as CHIKV for optimal endosomal fusion (Keilian *et al.,* 2010); it is possible that *w*Mel may reduce the amount of free cholesterol sufficiently to block the viruses.

The concentration of *w*Mel was not significantly higher than the concentration of *w*AlbA/B in the midgut of Uju lines (figure 5.11); and this increased sequestration of cholesterol by *w*Mel provides a possible explanation of the observed viral inhibition without the need for increased local concentration.

These data and hypotheses are supported by recent studies which show that increased dietary cholesterol reduces the ability of *w*MelPop and *w*MelCS to inhibit the RNA virus *Drosophila* C virus in *D. melanogaster* (Caragata *et al.,* 2013). Additionally, high experimental CHIKV titres have been shown to correspond to a reduction in the concentration of *w*AlbA/B in *Ae. albopictus*, supporting the notion of a competition for resources (Mousson *et al.,* 2010).

5.4.5 Combinatorial hypothesis of anti-pathogen effects

When all available data is taken together, it appears likely that there are multiple mechanisms by which *Wolbachia* inhibits such a range of pathogens in a range of hosts, some of which may be active in some hosts but not others. For example, here, *w*AlbB shows reduced sequestration of cholesterol compared to wMel (figures 5.10 & 5.11) and confers little or no resistance to DENV in *Ae. albopictus* (Blagrove *et al.,* 2012), but causes DENV inhibition in *Ae. aegypti* (Bian *et al.,* 2010), which has been attributed to immune stimulation (figures 5.1, 5.4 & 5.5; Pan *et al.,* 2012). Conversely, *w*Mel confers DENV resistance in *Ae. albopictus* (Blagrove *et al.,* 2012) in the absence of any detected immune stimulation (figures 5.4, 5.5, 5.7, 5.8 & 5.9) but with significant sequestration of cholesterol (figures 5.10 & 5.11). Furthermore, it seems unlikely that constitutive immune stimulation is the primary mechanism for pathogen inhibition given the obvious metabolic and resource costs to host.

In addition to these effects, *Wolbachia* has also been shown to manipulate host miRNAs (Hussain *et al.,* 2011; Osei-amo *et al.,* 2012) which includes upregulation of aae-miR-2940 miRNA in *Ae. aegypti*, which downregulates the DNA methyltransferase gene *AaDnmt2* allowing for increased *Wolbachia* replication (Zhang *et al.,* 2013). In contrast, DENV induces *AaDnmt2*, suggesting a causal link between *Wolbachia* manipulation of the host miRNA and DENV suppression.

Direct production of reactive oxygen species (ROS) by *Wolbachia* is an alternate hypothesis for viral inhibition. Direct production of ROS by other bacteria has been

observed to inhibit *Plasmodium* (Cirimotich *et al.,* 2011), and ROS have also been shown disrupt RNA replication in other viruses (Choi *et al.,* 2004).

From these studies it is clear that the exact mechanism(s) of *Wolbachia*-induced pathogen resistance is not fully understood. It seems likely that a combination of one or more of immune stimulation, sequestration of cholesterol and possibly other lipids and limited metabolic resources in the cell, along with other previously proposed mechanisms (discussed in section 1.2.9) such as miRNA manipulation, RNAi activation, autophagy modulation, possibly direct ROS production, and other undiscovered mechanisms are involved. Which mechanisms are active in any particular transinfection may also affect the strength of inhibition, given the wide range of resistances seen in different transinfections. In order to study the relative importance of these effects, a comprehensive set of *Wolbachia* and host combinations would likely be needed; given the ability of the somatic model of *Wolbachia* infection described in this chapter to predict immune stimulation and pathogen inhibition, it could potentially be used to expedite such an investigation should it be a reliable predictor of other *Wolbachia*-induced effects.

Chapter 6: Development of RIDL female flightless technology in *Ae. albopictus*

6.1 Introduction

In addition to the *Wolbachia*-based population replacement strategy, the use of RIDL technology (described in detail in chapter 1) is under development for population suppression or elimination of *Ae. albopictus*.

A genetic-sexing strain, based on the RIDL technology, has been produced in *Ae. aegypti* which shows female specific conditional inviability (Fu *et al*., 2010). Female specificity is provided by the promoter and sex-specific splicing of the *Actin-4* gene which is naturally expressed in the female indirect flight muscles (Muñoz *et al*., 2004). Conditional inviability is achieved through this promoter driving expression of the tetracycline-repressible transactivator (tTA) protein. tTA is comprised of a tetracycline repressor (tetR) and VP16 transcription activator. In the absence of tetracycline, tTA binds a tetracycline response element (tetO) (elsewhere in the construct), via tetR; the VP16 component of tTA then allows an hsp70 minimal promoter to drive the expression of a separate VP16 protein, excessive levels of which are toxic (Berger *et al*., 1990; Gill & Ptashne, 1988).

Due to the tissue specificity of the *Actin-4* promoter, this toxic effect is limited to female indirect flight muscles and results in females which are unable to fly and therefore unable to escape predation or mate. Conversely, in the presence of tetracycline, tetR

preferentially binds tetracycline, is therefore unable to bind tetO; additional VP16 is not expressed, resulting in females capable of flight. Males carrying this construct are capable of flight regardless of the presence of tetracycline.

The practical application of this is the ability to mass rear *Ae. aegypti* homozygous for this construct in tetracycline-supplemented larval water. Tetracycline is removed for the final generation, resulting in a release population containing only homozygous males, eliminating the requirement for time-consuming and damaging mechanical sex separation of pupae. All the offspring of these males in the field will be heterozygous for this construct, and thus the female offspring will be inviable as in the field tetracycline levels do not meet the required threshold for tTA-mediated repression (Liu *et al*., 2009; Labbé, 2011). Furthermore, half of the offspring from the male progeny of such a cross would also possess the construct, and so on, theoretically extrapolating to an increased number of inviable females over time.

Ae. albopictus has been transformed with two female-specific RIDL constructs, one based on the construct used in *Ae. aegypti*, OX3688, which uses the *Ae. aegypti Actin 4* gene, and the other, OX4358, which uses the *Ae. albopictus Actin-4* gene (Labbé *et al*., 2012). Both constructs induced the desired flightless phenotype in females reared in the absence of tetracycline, with no females able to fly. However, for both constructs, there was a marked reduction in the proportion of flying females reared in 60µg/mL tetracycline: 31 ± 3% of female pupae producing flightless adult females in G_6 OX3688A-Aal and ~50% for OX4358, compared to ~17% for wild type (Labbé *et al*., 2012; Labbé, 2011); increased concentrations of tetracycline did not ameliorate the problem and resulted in a reduced

eclosion rate (Labbé, 2011). A reduction in the proportion of viable females, as well as the low hatch rate observed for OX3688A-Aal eggs (Labbé, 2011) would greatly reduce the efficiency of mass rearing and thus the feasibility for application of these lines in the field. Furthermore, a proportion of flightless males are observed in both OX3688A-Aal and OX4358 lines when reared both with and without tetracycline; whilst it is unlikely that this would have a large effect on mass rearing generations due to males being able to mate with multiple females, it would reduce efficiency in the field as a proportion of males in the release generation would be flightless. In addition, it seems likely that a proportion of the flying males would have sustained sub-lethal damage and thus might be less able to compete for females, which would further reduce the effectiveness of a release programme. The mating competitiveness of OX3688A-Aal males was concluded to not be statistically significantly different to wild-type, but the relative sterility index (RSI) of the line was 0.36 (with 0.5 indicating equal competitiveness with wild-type) (Labbé, 2011). Any reduction in mating competitiveness would need to be abrogated by increased numbers in a release programme, again compounding the reduction in efficiency.

Overall, these results suggest that *Actin4*-tTA/tetO-VP16 constructs are too toxic in *Ae. albopictus* resulting in incomplete and likely inadequate specificity and repressibility by tetracycline. The aims of this chapter were to address this in two ways. Firstly, to determine the RSI of OX4358 males reared in the absence of tetracycline, in order to assess whether this construct has a significant detrimental effect. Secondly, a range of truncations of the *Ae. albopictus Actin-4* promoter were made in an attempt to reduce the expression, and hence the toxicity, of this construct in this species. These truncated promoters were inserted in place of the *Actin-4* promoter used in OX4358 *Actin4*-tTA/tetO-VP16 transgene as well as an *attP* site for specific integration into *Ae. albopictus;*

this enables direct comparison of the expression from the resulting lines. Two *Ae. albopictus* lines containing *attB* integration sites have previously been generated (Labbé *et al*., 2010) and a comparison of the fitness of these two lines was undertaken in order to identify which line is optimal for injection.

6.2 Materials and methods

6.2.1 Insect strains and rearing

An *Ae. albopictus* Malaysian wild-type (MWT) strain was colonised in 2006 from Malaysia (Institute of Medical Research, Kuala Lumpur). *Ae. albopictus* KLP is a non-diapausing Malaysian strain colonised by Georgetown University, Washington (USA).

All mosquito strains were reared at 27°C (± 1°C) and 70% (± 10%) relative humidity. Larvae were fed on dry fish food (TetraMin® flake food from Tetra GmbH, Germany) and adults *ad libitum* on 10% sucrose with 14U/mL penicillin and 14µg/mL streptomycin. Adult females were blood-fed on defibrinated horse blood.

6.2.2 Mating competitiveness

Ae. albopictus KLP and homozygous OX4358F were vacuum-hatched in deionised tetracycline-free water. 24 hours later, larvae were aliquoted into 1L round pots at a density of 1 larva/mL (300 larvae per pot). Larvae were fed ground TetraMin fish food according to the following regimen:

Day	Tetramin(g)/300 larvae
1	0
2	0.012
3	0.012
4	0.0124
5	0.048
6	0.096
7	0.096
8	0.096
9	0.048
10	0
11	0.024
12	0.024

The water was replaced on days six and 11.

Pupae were separated by sex and the cephalothorax of wild-type and OX4358F males were measured from digital photographs of the dorsal view, with a 0.1mm graticule in view. The width of the cephalothorax was measured across its widest section using ImageJ 1.47v for 64bit Windows 7 (http://rsbweb.nih.gov/ij/download.html). Pupae were placed into cages to eclose. Adults were provided *ad libitum* a 10% sucrose solution containing 14 U/mL penicillin and 14 µg/ml streptomycin and allowed to mature until the start of the experiment.

Following maturation, 10 sexually mature males (2 to 8 days old) of each line were transferred into a cage (30 × 30 × 30 cm) and allowed to settle for five minutes before 10

virgin KLP females were added. Males were removed after 48 hours. After this the females were fed on defibrinated horse blood daily for three days before being transferred into individual egg laying tubes. Eggs were vacuum-hatched four days after oviposition. Paternity of the offspring was scored by checking 3rd-4th instar larvae for full body AmCyan expression; no fluorescent larvae indicated the male parent was KLP, all fluorescent larvae indicated the male parent was OX4358F, and a mix of fluorescent and non-fluorescent indicated that both a KLP and a OX4358F male had inseminated the female, mixed florescence was scored as a success for both KLP and OX4358F. Three repeats of six cages were carried out.

6.2.3 Transcription factor binding site prediction

Putative transcription factor binding sites (TFBSs) were predicted using PROMO (http://alggen.lsi.upc.es/) with the search limited to dipteran sites and factors. The *Actin-4* promoter regions of *Ae. aegypti*, *Ae. albopictus* and *An. gambiae* were input and only TFBSs present at least once in all three sequences were identified and displayed.

6.2.4 Construct generation

Truncated *Ae. albopictus Actin-4* promoters were made by PCR from plasmid OX4358, with AscI and BglII sites added to the 5' and 3' ends respectively. The full *Actin-4* promoter in plasmid OX4721 (described later) was replaced with these truncated promoters by digestion of the plasmid with AscI and BglII followed by ligation of the truncated promoters into these sites.

6.2.5 Fitness investigation of OX3860 lines

Three *attB* docking site containing OX3860 lines, OX3860B, OX3860C and OX3860B + full body DsRed2 insertion, were assessed for fitness costs of their construct. Larvae were reared as in section 6.2.2, except that pots contained 200 rather than 300 larvae and the quantity of food and water was reduced to reflect this.

For each line, four cages of 200 hemizygotes were allowed to breed freely and eggs collected. These eggs were pooled, hatched and aliquoted into five pots each containing 200 larvae which were scored at the pupa stage by fluorescent microscopy (generation one). Wild-type pupae were removed and individuals allowed to mature and eclose in five separate cages and mate. Eggs were again collected, pooled, hatched, aliquoted and pupae screened with wild-type pupae removed (generation two). This was repeated for one more generation.

6.2.6 Microinjection

Pre-blastoderm embryos were injected following a modified version of the protocol described by Morris *et al.* (Morris *et al*., 1989). In brief, approximately 10 to 15 females were transferred four days after blood-feeding into small plastic tubes (8 x 3 cm) with moist filter paper for oviposition. When eggs were 30-60mins old they were oriented with posterior poles aligned on damp filter paper. Eggs were transferred to a glass slide using double sided tape, allowed to dry briefly, and then covered in halocarbon oil. Glass capillary needles were loaded with injection mix containing purified plasmid DNA (300 ng/µL) and ΦC31 mRNA (600 ng/µL) in 1X injection buffer (0.1mM sodium phosphate,

5mM KCl, pH 6.8). The ΦC31 mRNA was transcribed and purified by Andrea Miles using the mMESSAGE mMACHINE® T7 and MEGAclear™ kit (Ambion).

Once injected, slides were placed vertically in water to drain the oil for >1 hour, and then placed vertically in a sealed humid box for four days. G_0 eggs were vacuum-hatched and larvae reared at low density in water containing 60 µg/mL tetracycline. Pupae were separated by sex and allowed to eclose in cages, two male G_0 pupae per cage with 20 wild-type females and all G_0 females in a single cage with a roughly equal number of wild-type males. Resulting adults were blood-fed and their offspring reared at low density in water containing 60 µg/mL tetracycline, and screened at the pupa stage for fluorescence using a Leica MZ95 microscope with filter sets from Chroma Technology (Rockingham, VT) (filters: ECFP: exciter D436/20x, emitter D480/40m, DsRed2: exciter HQ545/30x, emitter HQ620/60m).

6.3 Results

6.3.1 Mating competitiveness of OX4358F males

As the size of adult males can affect their mating ability, a sample of 50 KLP and OX4358F male pupae were measured in ImageJ 1.47v. There was no significant difference between the average width of the cephalothorax of KLP (0.989 ± 0.045mm) and OX4358F (1.007 ± 0.045mm) male pupae in this experiment ($p = 0.0643$, Wilcoxon rank sum test), therefore the males were suitable for use in the mating competitiveness experiment.

Mating competitiveness of OX4358F males was compared to wild-type KLP males using three repeats of six cages each containing 10 male OX4358F homozygotes : 10 male KLP : 10 female KLP; mosquitoes were left to mate for two days before removal of males and blood feeding of females. Females were individualized for egg laying and resultant larvae screened for fluorescence to determine paternity. OX4358F males were found to be significantly less competitive than the wild-type KLP males and under these conditions ($p = 0.0222$ Fisher's exact test) with 41% of matings in this experiment by an OX4358F male, corresponding to an RSI of 0.41 (table 6.1).

Repeat 1				Repeat 2				Repeat 3			
Cage	KLP	4358F	Both	Cage	KLP	4358F	Both	Cage	KLP	4358F	Both
1	7	3	2	1	4	7	1	1	3	6	
2	8	3	1	2	7	5	2	2	7	2	1
3	5	4	1	3	4	4		3	4	4	
4	5	3		4	7	5	1	4	6	5	2
5	4	4		5	4	6	2	5	7	1	
6	6	3	1	6	7	3		6	7	3	1
Total	35	20		Total	33	30		Total	34	21	
Prop.	0.636	0.363		Prop.	0.523	0.476		Prop.	0.618	0.381	

Table 6.1: Mating competitiveness of OX4358F males. OX4358F and KLP larvae, reared in the absence of tetracycline, were aliquoted 24 hours after hatching and fed according to a standardized regimen. Pupae were separated by sex and placed into cages to emerge and mature. Following maturation, 10 sexually mature males (2 to 8 days old) of each line were transferred into a cage (30 × 30 × 30 cm) and allowed to settle for a few minutes before 10 virgin KLP females were added. Males were removed after 48 hours. Females were fed on defibrinated horse blood daily for three days before being transferred into individual egg laying tubes. Eggs were vacuum-hatched four days after oviposition and larvae screened for fluorescence to determine paternity. Batches of larvae with both fluorescent and non-fluorescent individuals were scored as a success for both OX4358F and KLP (multiple matings are noted). Three experiments of six cages each were carried out.

6.3.2 Identification of putative TFBS clusters in *Ae. albopictus Actin-4* promoter

In order to reduce the strength of the *Ae. albopictus Actin-4* promoter in OX4358, it was decided to delete sections of the promoter from the 5' end. The *Ae. albopictus*, *Ae. aegypti* and *An. gambiae Actin-4* promoters were analysed together using PROMO set to identify clusters of putative dipteran TFBSs present at least once in all three sequences which may represent important regulatory regions. The *Ae. albopictus Actin-4* promoter in OX4358 is only 746 bp long because of a close upstream gene in the opposite orientation, therefore, only the first 746bp of the *Ae. aegypti* and *An. gambiae* promoters was used for this analysis. PROMO identified a cluster of putative TFBSs between 290bp and 410bp of the start of the *Ae. albopictus* promoter sequence (figure 6.1); although current understanding of promoter regions is limited, this is a likely candidate for an important regulatory region in the promoter.

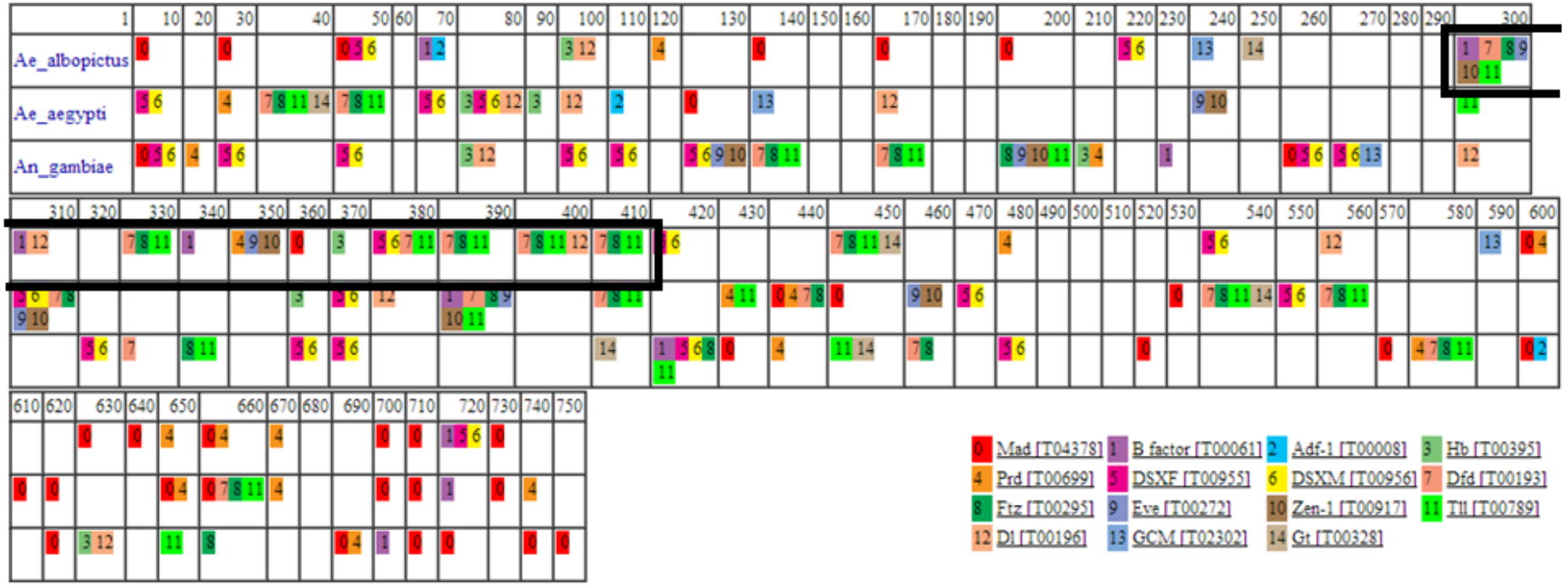

Figure 6.1: Identification of putative TFBSs in *Actin-4* promoter region. 746bp of the *Actin-4* promoter regions of *Ae. albopictus*, *Ae. aegypti* and *An. gambiae* were input into PROMO (http://alggen.lsi.upc.es/), all dipteran TFBS sequences present in all three sequences were identified. The putative TFBS cluster in *Ae. albopictus* is highlighted.

6.3.3 Generation of promoter truncation constructs

In order to accurately determine the effect of different partial promoter deletions, site specific integration of promoter truncation constructs is extremely helpful in order to minimise by positional effects observed in *piggyBac* transformation. To this end, Dr Sarah Scaife designed and built OX4721, a construct identical to OX4358 apart from having the *piggyBac* integration system removed and replaced with a Lambda phage *attP* site. *Ae. albopictus* acceptor lines (OX3860) containing Lambda phage *attB* have previously been generated (Labbé *et al*., 2010).

In addition to OX4721, two constructs with 5' truncations of the *Actin-4* promoter were designed and constructed with the help of Dr Sarah Scaife, one upstream (and therefore including) the TFBS cluster, OX4738; and one downstream of (and therefore not including) the TFBS cluster, OX4739 (figure 6.2). Two further constructs, MB5 and MB6, were designed with the help of Dr Sarah Scaife but not completed. Dr Sarah Scaife also designed and made construct OX4733, which is the OX4721 construct with the 5'UTR (including the sex-specific intron) removed.

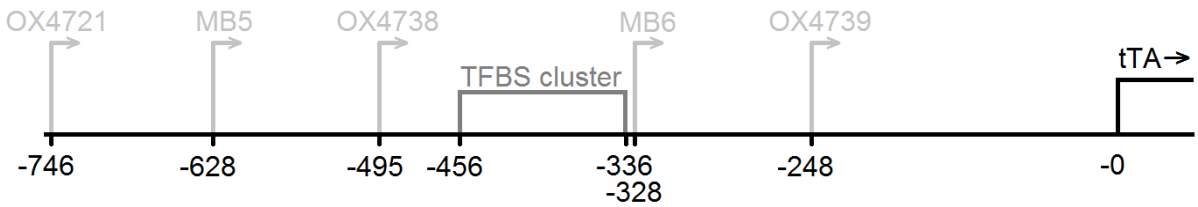

Figure 6.2: Diagram of *Ae. albopictus Actin 4* promoter deletion constructs. The 746bp promoter region is represented with -746 being the 5' end of the full length promoter and -0 as the start of the tTA gene. The predicted TFBS cluster is shown in dark grey, and the starting points of the promoters for OX4721 and four truncations are shown in light grey.

6.3.4 Fitness assessment of OX3860 *attB* docking lines

Two *Ae. albopictus attB* docking lines, OX3860B and OX3680C, have been made previously by inserting the *attB* acceptor site into the genome with the *piggyBac* construct OX3680 (Labbé *et al*., 2010). Whilst either line would be suitable for the injection of *attP*-containing constructs, OX3860B is anecdotally believed to be more efficient for transformation (Sian Morgan, personal communication) and has a more easily screened marker, with AmCyan fluorescence in both eyes and anal papillae compared with only weak fluorescent eyes for OX3860C (Labbé *et al*., 2010) which expedites the continued enrichment of the line. However, evidence for a fitness penalty has previously been observed in OX3860B, but not in OX3860C when offspring ratios from hemizygous x wild-type cross were analysed (Labbé, 2011).

The possible fitness cost of the construct in the OX3860 lines was investigated by measuring the frequencies of transgenic phenotypes over multiple generations to give a more accurate estimation of any detrimental effect of the insertion over all life stages. The fitness of three lines was compared: OX3860B, OX3860C and OX3860B + full body DsRed2 insertion in the *attP* site. Beginning with hemizygotes, individuals were allowed to mate freely and their offspring screened and compared to those expected according to the Hardy-Weinberg theorem. Wild-type pupae were removed and this was continued until generation three. The expected ratio of transgenics under these conditions, assuming no fitness costs, according to the Hardy-Weinberg theorem is 0.75, 0.82 and 0.87 for generations one, two and three respectively (figure 6.3).

At generation three, no statistically significant fitness cost was found in OX3860C (Fisher's exact test [1x2] (P1<>P2), p = 0.0652), whilst highly significant fitness costs were observed in both OX3860B and OX3680B + full body DsRed2 insertion (Fisher's exact test [1x2] (P1<>P2), p = 0.000, for both). These results are consistent with the previous findings in Labbé, (2011).

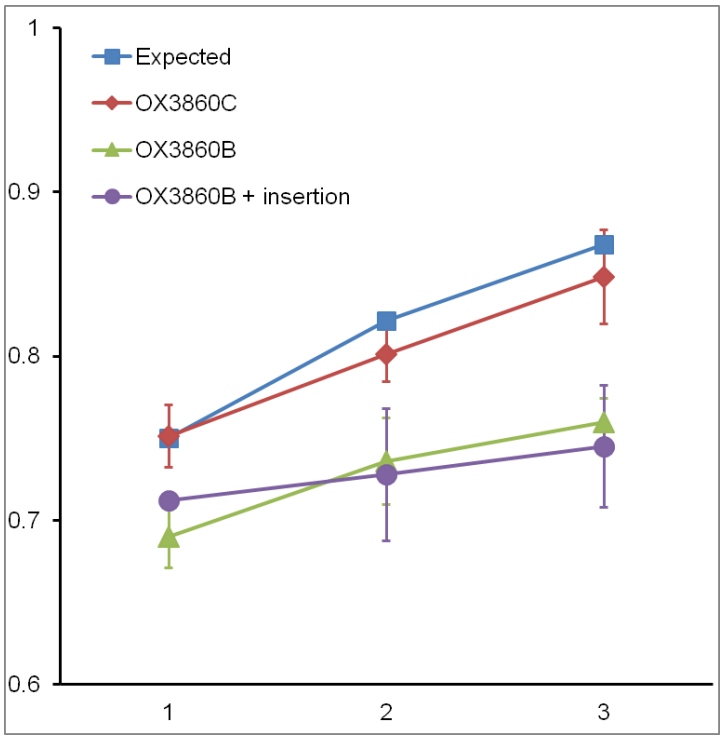

Figure 6.3: Fitness costs of OX3860 lines in *Ae. albopictus*. Three hemizygous OX3860 lines, OX3860B, OX3860C and OX3860B + full body DsRed2 insertion, were allowed to breed freely and the phenotypes of their offspring scored by fluorescent microscopy (generation one). Pupae were screened, counted, and wild-type pupae removed. Eclosing adults were allowed to mature and mate freely. This was continued until generation three. Results show the frequencies of transgenic (dominant) phenotype at each generation of four repeats of 200 pupae (generation one) and five repeats of 200 pupae (generations two and three) compared to the expected frequencies according to the Hardy-Weinberg theorem. Error bars represent standard error of the mean between these four or five repeats.

Whilst the fitness cost in OX3860B was highly statistically significant (by virtue of a high 'n'), the fitness cost was not considered to be sufficiently strong to use OX3860C as the acceptor line for microinjection of the promoter truncation constructs, given the weak marker expression and the anecdotal evidence of reduced transformation efficiency in OX3860B. Whilst not significantly different, the fitness of OX3860B + full body DsRed insertion appears to be reduced compared to OX3860B in generations two and three. This fitness effect was considered to be an artefact of the experiment; wild-type larvae seemed to develop faster in this line than transgenic larvae (and hence presumably hemizygotes developed faster than homozygotes) so it is likely that hemizygote males eclosed before homozygote males and thus had a competitive advantage in mating. This explains the observed higher fitness in the first generation as in this generation virgin adults were aspirated into cages, whereas in generations two and three, all pupae were allowed to eclose at their own pace into the same cage; hence the disadvantage from decreased development speed of transgenic larvae was negated in the first generation.

6.3.5 Microinjection of *Actin-4* promoter deletion constructs

The constructs OX4721, OX4733, OX4738 and OX4739 were injected into OX3860B eggs. A total of 10439 eggs were injected, and of these, 886 hatched (table 6.2).

Construct	Number injected	Number hatched
OX4721	2214	162
OX4733	2807	246
OX4738	3461	305
OX4739	1957	173

Table 6.2: Microinjection of *Actin-4* promoter deletion constructs. 30-60 min old eggs from *Ae. albopictus* OX3860B were collected, orientated and injected with an injection mix containing purified plasmid DNA and ΦC31 mRNA. Eggs were stored in a humidity chamber for four days then hatched in a vacuum-hatcher.

From fluorescence microscopy, a total of five G_1 pupae were found to have the 3xP3 DsRed2 marker, indicating successful integration of the construct; however, no lines could be generated from any of these individuals. One positive G_1 female was generated from OX4738 injection but would not blood-feed. The remaining four G1s were from OX4721, and consisted of one male who died during eclosion, one male and one female both of whom were flightless, and one female who was able to fly and blood-feed but unable to lay eggs.

6.4 Discussion

6.4.1 Mating competitiveness of OX4358F males

OX4358F males were found to have an RSI of 0.41; whilst this was significantly reduced compared to KLP, this score is higher than the RSI of 0.36 for OX3688A-Aal measured previously (Labbé, 2011). The RSI for OX4358F is similar to the RSI of OX513, which was found to be 0.55 using a similar methodology (Harris *et al.,* 2011) and significantly higher than the threshold for medfly (*Ceratitis captiata*) which is 0.2 (FAO/IAEA/USDA, 2003). It therefore appears that female flightless RIDL technology does not have sufficient major negative effects on the mating competitiveness of *Ae. albopictus* males to preclude considering it for population control.

and, in terms of this attribute, makes OX4358F a more attractive line for an SIT-like programme and shows that the female flightless RIDL technology

6.4.2 *Ae. albopictus Actin-4* promoter deletion constructs

Both OX3688A-AaI and OX4358F produce increased numbers of flightless females in the presence of tetracycline and flightless males with and without tetracycline; these undesirable phenotypes were attributed to the *Actin-4* promoters not being sufficiently repressible by tetracycline. Given that the *Ae. albopictus Actin-4* promoter, as used in OX4358F, appears to have slightly better attributes, including a reduced detrimental effect on males reared in the absence of tetracycline, constructs containing truncations of this promoter were generated in an attempt to reduce the expression, and hence the toxicity, of this construct in *Ae. albopictus*. A cluster of putative TFBSs was identified between bases -456 and -336 in the promoter, which may represent an important regulatory region; truncations were designed around this region in order to test this hypothesis. Unfortunately, no transgenic lines were generated by microinjection of constructs containing these deletions.

There are a number of possible reasons for the lack of transgenic lines generated by microinjection of these constructs. The transformation efficiency of the site-specific integration system is much lower than that of *piggyBac* in *Ae. albopictus* (Labbe, 2011; Dr Kelly Matzen, personal communication) and the OX3860B lines were not fully homozygous, leading to a lower average number of docking sites. Furthermore, the

constructs all contained a toxic transgene which can result in off-target flightlessness and other fitness costs, as seen in OX4358F and OX3688A-Aal; given the hypothesis that this is caused by an over-active promoter, and that the AmCyan marker in OX3860B is relatively highly expressed (compared to OX3860C), indicating it may be in an enhancing genomic context, over-expression of the *Actin4*-tTA/tetO-VP16 transgene may account for increased mortality of transgenic individuals at G_0 and G_1. This hypothesis is supported by all of the surviving putative transgenic G_1 individuals being inviable, including two of the five being flightless. These factors, as well as possible sub-optimal injection technique, may have combined to reduce the odds of generation of a transgenic line.

6.4.3 Conclusion and future work

The relatively high RSI for OX4358F demonstrates that when reared in the absence of tetracycline, as for a release population, the construct does not have a large effect on the male mating competitiveness for flying males. However, the off-target effects, including flightless males and flightless females in the presence of tetracycline, would greatly reduce the efficiency of mass rearing of this strain, which, given the general difficulties in mass rearing of *Ae. albopictus* which have been found in recent field trials (Bellini *et al*., 2007; Bellini *et al*., 2013), may prove too detrimental for large-scale application.

With the goal of reducing these off-target effects by reducing the strength of the *Actin-4* promoter, future work could test the *Ae. albopictus Actin-4* promoter truncation constructs generated here in *Ae. aegypti*. Previous studies have demonstrated that constructs containing the *Ae. albopictus Actin-4* promoter produce the desired phenotype

in *Ae. aegypti*, with reduced off-target flightlessness and fitness costs (Labbé *et al.*, 2012). Given this, and the increased transformation efficiency in the *Ae. aegypti* site-specific system (Prof. Luke Alphey and Dr Kelly Matzen, personal communication), the effect of promoter truncations could be assessed using *Ae. aegypti* as a model. A small number of such injections were performed (not reported) but time constraints meant that no transgenic individuals were generated. Once a desirable promoter truncation has been selected/generated, this could be inserted into the *Ae. albopictus* genome using the more efficient *piggyBac* system, with the goal of developing a more suitable *Ae. albopictus* female flightless RIDL strain for field trials.

One more general concern with the use female-flightless RIDL strains is the possibility of strong selection pressure leading to the development of resistance to the construct, especially in lines showing incomplete penetrance, as appears to have happened with OX3688A-Aal (Labbe, 2011). In the short-term, individuals more resistant are more likely to survive in lab rearing, potentially leading to the spread of resistance alleles through their descendants. The chance of such resistance alleles developing/spreading can be reduced by reducing the selection pressure as has been done by increasing the tetracycline concentration during the rearing of *Ae. albopictus* RIDL lines (Labbe, 2011).

Other potential possibilities for investigating the effectiveness of sex-specific RIDL technology in *Ae. albopictus* include the potential use of constructs based on the sex-specific splicing of the gene *doublesex*, as has been successfully achieved in both diamondback moth and pink bollworm (Jin *et al*., 2013) as well as the silkworm, *Bombyx mori* (Tan *et al*., 2013). Whilst the benefit of increased larval competition would be lost

using a construct based on *doublesex* that led to death relatively early in development, the strategy could be more efficient if the construct showed greater specificity and repressibility by tetracycline. An additional possibility could also be to attempt a large-scale series of piggyBac insertions of OX4358, OX3688 and potentially any other constructs designed for female specific lethality, in the hope of a construct being integrated into a more desirable genomic context for female flightlessness/specific lethality.

Chapter 7: General discussion

7.1 Summary

Aedes albopictus is an important vector of DENV and CHIKV. It has resisted traditional control strategies, allowing it to spread over recent decades from its native Southeast Asia throughout the world. In this thesis, two alternative control methods for *Ae. albopictus* were developed and assessed: transinfection with the inherited bacteria *Wolbachia*, for population replacement with a refractory strain; and RIDL (Release of Insects carrying a Dominant Lethal), a genetic equivalent to the sterile insect technique, for population suppression.

Chapter 3 describes the generation of a stable transinfection of *w*Mel in *Ae. albopictus* (Uju.wMel), which showed complete bidirectional CI with the natural *Wolbachia* infection of *Ae. albopictus*, thus providing a mechanism of introducing *w*Mel into natural populations. In addition to this, Uju.wMel was shown – by collaborators in the Institut Pasteur – to completely abolish both DENV and CHIKV transmission, providing the first evidence of pathogen resistance in a naturally infected mosquito species.

Chapter 4 details an assessment of important fitness characteristics of Uju.wMel. No significant detrimental effects on hatch rate, fecundity, longevity and male mating competitiveness were found, and as such the characteristics were generally favourable with respect to potential released *w*Mel reaching a high population frequency. In addition, a novel means of backcrossing a bidirectionally incompatible strain of *Wolbachia*

was developed to expedite the process of introgression into a local genetic background prior to any future release.

In chapter 5 it was shown that the pathogen inhibition observed in Uju.wMel occurs in the absence of immune stimulation, which was previously believed to play a key role in *Wolbachia*-mediated pathogen resistance. Additional evidence from transient somatic infections, using multiple host and *Wolbachia* combinations, showed that immune stimulation is limited to *Wolbachia*-naïve host species. Cholesterol sequestration by *w*Mel was proposed as a mechanism for the pathogen inhibition observed in Uju.wMel; experimental evidence supported this hypothesis, and suggested that inhibitory phenotypes may be produced by combinations of different mechanisms.

The final results chapter focused on the development of female flightless RIDL technology in *Ae. albopictus,* which currently suffers from off-target expression of the RIDL construct, causing increased numbers of inviable adults in the presence of tetracycline and inviable males in the absence of tetracycline. Males with the female flightless RIDL construct OX4358F were shown to have high mating competitiveness compared to wild type, confirming the potential utility of the system. A potentially important regulatory region of the promoter was identified and multiple constructs containing truncations of the promoter around this region were made in an attempt to reduce the off-target expression.

7.2 Future research and considerations

7.2.1 *Wolbachia*-based strategies

As discussed in chapter 4, the next stage for investigating the potential application of the *w*Mel transinfection in *Ae. albopictus* is to assess the relative fitness of the line in semi-field and open field trials. In addition to this, further study is required on the transinfection, and in particular the mechanism of pathogen inhibition.

Future experiments are needed to determine whether it is possible to generate a stable triple infection of *w*Mel and *w*AlbA/B in *Ae. albopictus*. Based on previous work (Fu *et al.*, 2010), it is expected that the crossing type of such a transinfection would show unidirectional CI with the wild type infection and thus have the potential to spread through larger populations more efficiently.

Additionally, the mechanism of *Wolbachia*-mediated pathogen resistance is still to be fully elucidated. The results shown in chapter 5 and other studies (Pan *et al.,* 2012; Zhang *et al.,* 2013) indicate that it is likely that there are multiple mechanisms by which *Wolbachia* inhibit such a range of pathogens in a range of hosts, some of which may be active in some hosts but not others. It may also be useful to examine whether the inhibition effect in Uju.wMel operates in the midgut, haemocel, salivary glands or all three. Further study on elucidating these mechanisms may improve our understanding of mosquito immunity and also aid in predicting which *Wolbachia*/host combinations would likely be most effective prior to time-consuming transinfection experiments.

In addition to these areas of future research, existing laboratory colonies and especially lines released into the field should be monitored for any changes in *Wolbachia*/host/pathogen interactions. For example, hosts could develop resistance to *Wolbachia*, reducing the density of *Wolbachia* and potentially the desirable phenotypes; this type of resistance has previously been observed in *Drosophila* (McGraw *et al.,* 2002). Such a change in the host/*Wolbachia* relationship could reduce the effectiveness of strategies which aim to replace a natural population with a refractory one, such as the *w*Mel transinfection in *Ae. aegypti* (Hoffmann *et al.,* 2011), and any future application of the *w*Mel transinfection of *Ae. albopictus* developed here. Additionally, as pathogens have evolved resistance to vaccines and treatments, they could also evolve resistance to the mechanism(s) by which *Wolbachia* inhibits them. Continual monitoring of pathogens should be effected in order to identify if any such resistance is developing, and where possible, *Wolbachia*-based disease control should be integrated with other disease control strategies such as vaccines and treatments to reduce the risk of pathogens developing resistance.

7.2.1 RIDL-based strategies

As discussed in chapter 6, more research is needed to increase the specificity of the *Actin4* promoter in order to improve the female flightless RIDL technology in *Ae. albopictus*; possibly through further investigation of promoter truncations or the use of alternate, less toxic effectors. The sex-specific splicing of the gene *doublesex* could also be investigated as an alternate means of achieving sex specific conditional lethality, as has

been done in diamondback moth, pink bollworm (Jin *et al*., 2013) and the silkworm, *Bombyx mori* (Tan *et al*., 2013).

For the female flightless RIDL technology, strong selection pressure could lead to the development/spread of resistance alleles to the construct, especially in lines showing incomplete penetrance, as appears to have happened with OX3688A-Aal (Labbé, 2011). Such selection pressure could also lead to the spread of resistance alleles throughout wild populations, especially given the survival of male offspring in the field and therefore the potential for introgression into wild populations. Attempts have been made to overcome this by using higher doses of tetracycline for lab rearing (Labbé, 2011), but this remains an important concern for this type of technology and should be monitored.

7.3 Conclusions

This thesis has discussed two potential methods of controlling the important disease-transmitting mosquito *Ae. albopictus*. The transinfection of *Ae. albopictus* with the *Wolbachia w*Mel has provided a means of potentially replacing a wild pathogen-transmitting population with a refractory one. Although untested in the field, this novel control method appears promising, and together with the *w*Mel transinfection of *Ae. aegypti* (Walker *et al.,* 2011), shows that both main vectors of DENV and CHIKV could potentially be controlled with *Wolbachia* population replacement strategies.

In contrast, RIDL technology in *Ae. albopictus* in is its infancy, likely requiring considerable further research and development to generate a line with sufficiently low off-target

effects to allow for efficient mass rearing. However, the demonstration that male mating competitiveness is not greatly reduced by the transgene shows that the technology has potential utility as a genetic equivalent to the sterile insect technique for population suppression, provided that the specificity and repressibility of the system can be improved.

Chapter 8: References

Alphey L, Benedict M, Bellini R, Clark GG, Dame DA, Service MW, Dobson SL (2010) Sterile-insect methods for control of mosquito-borne diseases: an analysis.*Vector Borne Zoonotic Dis* **10:** 295-311

Atkinson MP, Su Z, Alphey N, Alphey LS, Coleman PG, Wein LM (2007) Analyzing the control of mosquito-borne diseases by a dominant lethal genetic system. *Proc Natl Acad Sci U S A* **104:** 9540-9545

Bellini R, Medici A, Puggioli A, Balestrino F, Carrieri M (2013) Pilot field trials with Aedes albopictus irradiated sterile males in Italian urban areas. *J Med Entomol***50:** 317-325

Berger SL, Cress WD, Cress A, Triezenberg SJ, Guarente L (1990) Selective inhibition of activated but not basal transcription by the acidic activation domain of VP16: evidence for transcriptional adaptors. *Cell* **61:** 1199-1208

Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, Drake JM, Brownstein JS, Hoen AG, Sankoh O, Myers MF, George DB, Jaenisch T, Wint GR, Simmons CP, Scott TW, Farrar JJ, Hay SI (2013) The global distribution and burden of dengue. *Nature* **496:** 504-507

Bian G, Xu Y, Lu P, Xie Y, Xi Z (2010) The endosymbiotic bacterium Wolbachia induces resistance to dengue virus in Aedes aegypti. *PLoS Pathog* **6:** e1000833

Blagrove MS, Arias-Goeta C, Di Genua C, Failloux AB, Sinkins SP (2013) A Wolbachia wMel transinfection in Aedes albopictus is not detrimental to host fitness and inhibits Chikungunya virus. *PLoS Negl Trop Dis* **7:** e2152

Blagrove MS, Arias-Goeta C, Failloux AB, Sinkins SP (2012) Wolbachia strain wMel induces cytoplasmic incompatibility and blocks dengue transmission in Aedes albopictus. *Proc Natl Acad Sci U S A* **109:** 255-260

Blandin S, Shiao SH, Moita LF, Janse CJ, Waters AP, Kafatos FC, Levashina EA (2004) Complement-like protein TEP1 is a determinant of vectorial capacity in the malaria vector Anopheles gambiae. *Cell* **116:** 661-670

Bourtzis K, Nirgianaki A, Markakis G, Savakis C (1996) Wolbachia infection and cytoplasmic incompatibility in Drosophila species. *Genetics* **144:** 1063-1073

Brackney DE, Scott JC, Sagawa F, Woodward JE, Miller NA, Schilkey FD, Mudge J, Wilusz J, Olson KE, Blair CD, Ebel GD (2010) C6/36 Aedes albopictus cells have a dysfunctional antiviral RNA interference response. *PLoS Negl Trop Dis* **4:** e856

Breeuwer JA, Werren JH (1990) Microorganisms associated with chromosome destruction and reproductive isolation between two insect species. *Nature* **346:** 558-560

Breeuwer JA, Werren JH (1993) Cytoplasmic incompatibility and bacterial density in Nasonia vitripennis. *Genetics* **135:** 565-574

Brennan LJ, Keddie BA, Braig HR, Harris HL (2008) The endosymbiont Wolbachia

pipientis induces the expression of host antioxidant proteins in an Aedes albopictus cell line. *PLoS One* **3:** e2083

Bressac C, Rousset F (1993) The reproductive incompatibility system in Drosophila simulans: DAPI-staining analysis of the Wolbachia symbionts in sperm cysts. *J Invertebr Pathol* **61:** 226-230

Bürge T, Griot C, Vandevelde M, Peterhans E (1989) Antiviral antibodies stimulate production of reactive oxygen species in cultured canine brain cells infected with canine distemper virus. *J Virol* **63:** 2790-2797

Callaini G, Dallai R, Riparbelli MG (1997) Wolbachia-induced delay of paternal chromatin condensation does not prevent maternal chromosomes from entering anaphase in incompatible crosses of Drosophila simulans. *J Cell Sci* **110 (Pt 2):** 271-280

Callaini G, Riparbelli MG, Dallai R (1994) The distribution of cytoplasmic bacteria in the early Drosophila embryo is mediated by astral microtubules. *J Cell Sci* **107 (Pt 3):** 673-682

Calvitti M, Moretti R, Lampazzi E, Bellini R, Dobson SL (2010) Characterization of a new Aedes albopictus (Diptera: Culicidae)-Wolbachia pipientis (Rickettsiales: Rickettsiaceae) symbiotic association generated by artificial transfer of the wPip strain from Culex pipiens (Diptera: Culicidae). *J Med Entomol* **47:** 179-187

Carballar-Lejarazú R, Rodríguez MH, de la Cruz Hernández-Hernández F, Ramos-Castañeda J, Possani LD, Zurita-Ortega M, Reynaud-Garza E, Hernández-Rivas R,

Loukeris T, Lycett G, Lanz-Mendoza H (2008) Recombinant scorpine: a multifunctional antimicrobial peptide with activity against different pathogens. *Cell Mol Life Sci* **65:** 3081-3092

Carter GC, Bernstone L, Sangani D, Bee JW, Harder T, James W (2009) HIV entry in macrophages is dependent on intact lipid rafts. *Virology* **386:** 192-202

Catteruccia F, Benton JP, Crisanti A (2005) An Anopheles transgenic sexing strain for vector control. *Nat Biotechnol* **23:** 1414-1417

Chalk R, Townson H, Ham PJ (1995) Brugia pahangi: the effects of cecropins on microfilariae in vitro and in Aedes aegypti. *Exp Parasitol* **80:** 401-406

Charlat S, Calmet C, Merçot H (2001) On the mod resc model and the evolution of Wolbachia compatibility types. *Genetics* **159:** 1415-1422

Choi J, Lee KJ, Zheng Y, Yamaga AK, Lai MM, Ou JH (2004) Reactive oxygen species suppress hepatitis C virus RNA replication in human hepatoma cells.*Hepatology* **39:** 81-89

Cirimotich CM, Dong Y, Clayton AM, Sandiford SL, Souza-Neto JA, Mulenga M, Dimopoulos G (2011) Natural microbe-mediated refractoriness to Plasmodium infection in Anopheles gambiae. *Science* **332:** 855-858

Clancy DJ, Hoffmann AA (1997) Behavior of Wolbachia endosymbionts from Drosophila simulans in Drosophila serrata, a novel host. *Am Nat* **149:** 975-988

Clark ME, Veneti Z, Bourtzis K, Karr TL (2002) The distribution and proliferation of the

intracellular bacteria Wolbachia during spermatogenesis in Drosophila. *Mech Dev* **111:** 3-15

Collins SR, Weldon CW, Banos C, Taylor PW (2009) Optimizing irradiation dose for sterility induction and quality of Bactrocera tryoni. *J Econ Entomol* **102:** 1791-1800

Cornel AJ, Hunt RH (1991) Aedes albopictus in Africa? First records of live specimens in imported tires in Cape Town. *J Am Mosq Control Assoc* **7:** 107-108

Curtis CF, Adak T (1974) Population replacement in Culex fatigens by means of cytoplasmic incompatibility. Laboratory experiments with non-overlapping generations. *Bull World Health Organ* **51:** 249-255

Dame DA, Curtis CF, Benedict MQ, Robinson AS, Knols BG (2009) Historical applications of induced sterilisation in field populations of mosquitoes. *Malar J* **8 Suppl 2:** S2

Dame DA, Lofgren CS, Ford HR, Boston MD, Baldwin KF, Jeffery GM (1974) Release of chemosterilized males for the control of Anopheles albimanus in El Salvador. II. Methods of rearing, sterilization, and distribution. *Am J Trop Med Hyg* **23:** 282-287

Delatte H, Gimonneau G, Triboire A, Fontenille D (2009) Influence of temperature on immature development, survival, longevity, fecundity, and gonotrophic cycles of Aedes albopictus, vector of chikungunya and dengue in the Indian Ocean. *J*

Med Entomol **46:** 33-41

Derraik JG (2006) A scenario for invasion and dispersal of Aedes albopictus (Diptera: Culicidae) in New Zealand. *J Med Entomol* **43:** 1-8

Dobson SL, Fox CW, Jiggins FM (2002a) The effect of Wolbachia-induced cytoplasmic incompatibility on host population size in natural and manipulated systems.*Proc Biol Sci* **269:** 437-445

Dobson SL, Marsland EJ, Rattanadechakul W (2002b) Mutualistic Wolbachia infection in Aedes albopictus: accelerating cytoplasmic drive. *Genetics* **160:** 1087-1094

Dobson SL, Rattanadechakul W (2001) A novel technique for removing Wolbachia infections from Aedes albopictus (Diptera: Culicidae). *J Med Entomol* **38:** 844-849

Dobson SL, Rattanadechakul W, Marsland EJ (2004) Fitness advantage and cytoplasmic incompatibility in Wolbachia single- and superinfected Aedes albopictus.*Heredity (Edinb)* **93:** 135-142

Dyson EA, Kamath MK, Hurst GD (2002) Wolbachia infection associated with all-female broods in Hypolimnas bolina (Lepidoptera: Nymphalidae): evidence for horizontal transmission of a butterfly male killer. *Heredity (Edinb)* **88:** 166-171

Enserink M (2007) Epidemiology. Tropical disease follows mosquitoes to Europe. *Science* **317:** 1485

Evans O, Caragata EP, McMeniman CJ, Woolfit M, Green DC, Williams CR, Franklin CE,

O'Neill SL, McGraw EA (2009) Increased locomotor activity and metabolism of Aedes aegypti infected with a life-shortening strain of Wolbachia pipientis. *J Exp Biol* **212:** 1436-1441

FAO/IAEA/USDA (2003) Manual for product quality control and shipping procedures for sterile mass-reared tephritid fruit flies v5.0. *Vienna, Austria, IAEA*

Ferree PM, Frydman HM, Li JM, Cao J, Wieschaus E, Sullivan W (2005) Wolbachia utilizes host microtubules and Dynein for anterior localization in the Drosophila oocyte. *PLoS Pathog* **1:** e14

Ferree PM, Sullivan W (2006) A genetic test of the role of the maternal pronucleus in Wolbachia-induced cytoplasmic incompatibility in Drosophila melanogaster.*Genetics* **173:** 839-847

Focks DA (1980) An improved separator for the developmental stages, sexes, and species of mosquitoes (Diptera: Culicidae). *J Med Entomol* **17:** 567-568

Fontenille D, Toto JC (2001) Aedes (Stegomyia) albopictus (Skuse), a potential new Dengue vector in southern Cameroon. *Emerg Infect Dis* **7:** 1066-1067

Forattini OP (1986) Identification of Aedes (Stegomyia) albopictus (Skuse) in Brazil. *Rev Saude Publica* **20:** 244-245

Fraiture M, Baxter RH, Steinert S, Chelliah Y, Frolet C, Quispe-Tintaya W, Hoffmann JA, Blandin SA, Levashina EA (2009) Two mosquito LRR proteins function as complement control factors in the TEP1-mediated killing of Plasmodium. *Cell*

Host Microbe **5:** 273-284

Frentiu FD, Robinson J, Young PR, McGraw EA, O'Neill SL (2010) Wolbachia-mediated resistance to dengue virus infection and death at the cellular level. *PLoS ONE* **5:** e13398

Fridell YW, Sánchez-Blanco A, Silvia BA, Helfand SL (2005) Targeted expression of the human uncoupling protein 2 (hUCP2) to adult neurons extends life span in the fly. *Cell Metab* **1:** 145-152

Frolet C, Thoma M, Blandin S, Hoffmann JA, Levashina EA (2006) Boosting NF-kappaB-dependent basal immunity of Anopheles gambiae aborts development of Plasmodium berghei. *Immunity* **25:** 677-685

Frydman HM, Li JM, Robson DN, Wieschaus E (2006) Somatic stem cell niche tropism in Wolbachia. *Nature* **441:** 509-512

Fu G, Lees RS, Nimmo D, Aw D, Jin L, Gray P, Berendonk TU, White-Cooper H, Scaife S, Kim Phuc H, Marinotti O, Jasinskiene N, James AA, Alphey L (2010a) Female-specific flightless phenotype for mosquito control. *Proc Natl Acad Sci U S A* **107:** 4550-4554

Fu Y, Gavotte L, Mercer DR, Dobson SL (2010b) Artificial triple Wolbachia infection in Aedes albopictus yields a new pattern of unidirectional cytoplasmic incompatibility. *Appl Environ Microbiol* **76:** 5887-5891

Fuller MT (1993) Spermatogenesis. In: The Development of Drosophila melanogaster,

In: Bate M & Arias AM, editors. New York: Cold Spring Harbor Lab. Press. 71–147.

Garver LS, Dong Y, Dimopoulos G (2009) Caspar controls resistance to Plasmodium falciparum in diverse anopheline species. *PLoS Pathog* **5:** e1000335

Ghelelovitch S (1952) [Genetic determinism of sterility in the cross-breeding of various strains of Culex autogenicus Roubaud]. *C R Hebd Seances Acad Sci* **234:**2386-2388

Gill G, Ptashne M (1988) Negative effect of the transcriptional activator GAL4. *Nature* **334:** 721-724

Gilles HM & Warrell DA (2002) Essential Malariology, Arnold, London, ed. 4

Gilotra SK, Rozeboom LE, Bhattacharya NC (1967) Observations on possible competitive displacement between populations of Aedes aegypti Linnaeus and Aedes albopictus Skuse in Calcutta. *Bull World Health Organ* **37:** 437-446

Giordano R, O'Neill SL, Robertson HM (1995) Wolbachia infections and the expression of cytoplasmic incompatibility in Drosophila sechellia and D. mauritiana.*Genetics* **140:** 1307-1317

Glaser RL, Meola MA (2010) The native Wolbachia endosymbionts of Drosophila melanogaster and Culex quinquefasciatus increase host resistance to West Nile virus infection. *PLoS One* **5:** e11977

Gong P, Epton MJ, Fu G, Scaife S, Hiscox A, Condon KC, Condon GC, Morrison NI, Kelly

DW, Dafa'alla T, Coleman PG, Alphey L (2005) A dominant lethal genetic system for autocidal control of the Mediterranean fruitfly. *Nat Biotechnol* **23:** 453-456

Gossen M, Bujard H (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci U S A* **89:** 5547-5551

Gratz NG (2004) Critical review of the vector status of Aedes albopictus. *Med Vet Entomol* **18:** 215-227

Gubler DJ (1998) Dengue and dengue hemorrhagic fever. *Clin Microbiol Rev* **11:** 480-496

Gwadz RW, Kaslow D, Lee JY, Maloy WL, Zasloff M, Miller LH (1989) Effects of magainins and cecropins on the sporogonic development of malaria parasites in mosquitoes. *Infect Immun* **57:** 2628-2633

Ha EM, Oh CT, Ryu JH, Bae YS, Kang SW, Jang IH, Brey PT, Lee WJ (2005) An antioxidant system required for host protection against gut infection in Drosophila.*Dev Cell* **8:** 125-132

Hancock PA, Sinkins SP, Godfray HC (2011a) Population dynamic models of the spread of Wolbachia. *Am Nat* **177:** 323-333

Hancock PA, Sinkins SP, Godfray HC (2011b) Strategies for introducing Wolbachia to reduce transmission of mosquito-borne diseases. *PLoS Negl Trop Dis* **5:** e1024

Hanson SM, Craig GB (1995) Aedes albopictus (Diptera: Culicidae) eggs: field

survivorship during northern Indiana winters. *J Med Entomol* **32:** 599-604

Harris AF, McKemey AR, Nimmo D, Curtis Z, Black I, Morgan SA, Oviedo MN, Lacroix R, Naish N, Morrison NI, Collado A, Stevenson J, Scaife S, Dafa'alla T, Fu G, Phillips C, Miles A, Raduan N, Kelly N, Beech C, Donnelly CA, Petrie WD, Alphey L (2012) Successful suppression of a field mosquito population by sustained release of engineered male mosquitoes. *Nat Biotechnol* **30:** 828-830

Harris AF, Nimmo D, McKemey AR, Kelly N, Scaife S, Donnelly CA, Beech C, Petrie WD, Alphey L (2011) Field performance of engineered male mosquitoes. *Nat Biotechnol* **29:** 1034-1037

Hawley WA (1988) The biology of Aedes albopictus. *J Am Mosq Control Assoc Suppl* **1:** 1-39

Hawley WA, Pumpuni CB, Brady RH, Craig GB (1989) Overwintering survival of Aedes albopictus (Diptera: Culicidae) eggs in Indiana. *J Med Entomol* **26:** 122-129

Heath BD, Butcher RD, Whitfield WG, Hubbard SF (1999) Horizontal transfer of Wolbachia between phylogenetically distant insect species by a naturally occurring mechanism. *Curr Biol* **9:** 313-316

Heaton NS, Perera R, Berger KL, Khadka S, Lacount DJ, Kuhn RJ, Randall G (2010) Dengue virus nonstructural protein 3 redistributes fatty acid synthase to sites of viral replication and increases cellular fatty acid synthesis. *Proc Natl Acad Sci U S A* **107:** 17345-17350

Heaton NS, Randall G (2010) Dengue virus-induced autophagy regulates lipid metabolism. *Cell Host Microbe* **8:** 422-432

Hedges LM, Brownlie JC, O'Neill SL, Johnson KN (2008) Wolbachia and virus protection in insects. *Science* **322:** 702

Hedges LM, Yamada R, O'Neill SL, Johnson KN (2012) The small interfering RNA pathway is not essential for Wolbachia-mediated antiviral protection in Drosophila melanogaster. *Appl Environ Microbiol* **78:** 6773-6776

Heinrich JC, Scott MJ (2000) A repressible female-specific lethal genetic system for making transgenic insect strains suitable for a sterile-release program. *Proc Natl Acad Sci U S A* **97:** 8229-8232

Hertig M, Wolbach SB (1924) Studies on Rickettsia-Like Micro-Organisms in Insects. *J Med Res* **44:** 329-374.327

Hilgenboecker K, Hammerstein P, Schlattmann P, Telschow A, Werren JH (2008) How many species are infected with Wolbachia?--A statistical analysis of current data. *FEMS Microbiol Lett* **281:** 215-220

Hoffmann AA, Hercus M, Dagher H (1998) Population dynamics of the Wolbachia infection causing cytoplasmic incompatibility in Drosophila melanogaster. *Genetics***148:** 221-231

Hoffmann AA, Montgomery BL, Popovici J, Iturbe-Ormaetxe I, Johnson PH, Muzzi F, Greenfield M, Durkan M, Leong YS, Dong Y, Cook H, Axford J, Callahan AG,

Kenny N, Omodei C, McGraw EA, Ryan PA, Ritchie SA, Turelli M, O'Neill SL (2011) Successful establishment of Wolbachia in Aedes populations to suppress dengue transmission. *Nature* **476:** 454-457

Hoffmann AA, Turelli M (1988) Unidirectional incompatibility in Drosophila simulans: inheritance, geographic variation and fitness effects. *Genetics* **119:** 435-444

Hoffmann AA, Turelli M (1997) Cytoplasmic incompatibility in insects, In: O'Neill RV, Hoffmann AA, Werren JH, editors. Influential Passengers. Oxford: Oxford University Press. 42–80

Hoffmann AA, Turelli M, Harshman LG (1990) Factors affecting the distribution of cytoplasmic incompatibility in Drosophila simulans. *Genetics* **126:** 933-948

Hoffmann JA (2003) The immune response of Drosophila. *Nature* **426:** 33-38

Hughes GL, Koga R, Xue P, Fukatsu T, Rasgon JL (2011) Wolbachia infections are virulent and inhibit the human malaria parasite Plasmodium falciparum in Anopheles gambiae. *PLoS Pathog* **7:** e1002043

Huigens ME, de Almeida RP, Boons PA, Luck RF, Stouthamer R (2004) Natural interspecific and intraspecific horizontal transfer of parthenogenesis-inducing Wolbachia in Trichogramma wasps. *Proc Biol Sci* **271:** 509-515

Hurst GD, Bandi C, Sacchi L, Cochrane AG, Bertrand D, Karaca I, Majerus ME (1999) Adonia variegata (Coleoptera: Coccinellidae) bears maternally inherited flavobacteria that kill males only. *Parasitology* **118 (Pt 2):** 125-134

Hussain M, Frentiu FD, Moreira LA, O'Neill SL, Asgari S (2011) Wolbachia uses host microRNAs to manipulate host gene expression and facilitate colonization of the dengue vector Aedes aegypti. *Proc Natl Acad Sci U S A* **108:** 9250-9255

INGRAM RL (1954) A study of the bionomics of Aedes (Stegomyia) polynesiensis marks under laboratory conditions. *Am J Hyg* **60:** 169-185

Iturbe-Ormaetxe I, Walker T, O' Neill SL (2011) Wolbachia and the biological control of mosquito-borne disease. *EMBO Rep* **12:** 508-518

Jahanowicz DL, Hoy MA (1998) The manipulation of arthropod reproduction by Wolbachia endosymbionts. *Florida Entomologist* **81**: 310-317

Jeyaprakash A, Hoy MA (2000) Long PCR improves Wolbachia DNA amplification: wsp sequences found in 76% of sixty-three arthropod species. *Insect Mol Biol* **9:**393-405

Jin C, Ren X, Rasgon JL (2009) The virulent Wolbachia strain wMelPop efficiently establishes somatic infections in the malaria vector Anopheles gambiae. *Appl Environ Microbiol* **75:** 3373-3376

Jin L, Walker AS, Fu G, Harvey-Samuel T, Dafa'alla T, Miles A, Marubbi T, Granville D, Humphrey-Jones N, O'Connell S, Morrison NI, Alphey L (2013) Engineered female-specific lethality for control of pest Lepidoptera. *ACS Synth Biol* **2:** 160-166

Kambris Z, Blagborough AM, Pinto SB, Blagrove MS, Godfray HC, Sinden RE, Sinkins SP

(2010) Wolbachia stimulates immune gene expression and inhibits plasmodium development in Anopheles gambiae. *PLoS Pathog* **6:** e1001143

Kambris Z, Cook PE, Phuc HK, Sinkins SP (2009) Immune activation by life-shortening Wolbachia and reduced filarial competence in mosquitoes. *Science* **326:**134-136

Kielian M, Chanel-Vos C, Liao M (2010) Alphavirus Entry and Membrane Fusion. *Viruses* **2:** 796-825

King RC. (1970) Ovarian Development in Drosophila melanogaster. New York: Academic

Kim W, Koo H, Richman AM, Seeley D, Vizioli J, Klocko AD, O'Brochta DA (2004) Ectopic expression of a cecropin transgene in the human malaria vector mosquito Anopheles gambiae (Diptera: Culicidae): effects on susceptibility to Plasmodium. *J Med Entomol* **41:** 447-455

Kitrayapong P, Baimai V, O'Neill SL (2002) Field prevalence of Wolbachia in the mosquito vector Aedes albopictus. *Am J Trop Med Hyg* **66:** 108-111

Kitzmiller JB (1959) Parthenogenesis in Culex fatigans. *Science* **129:** 837-838

Krejbich-Trotot P, Gay B, Li-Pat-Yuen G Hoarau JJ, Jaffar-Bandjee MC, Briant L, Gasque P, Denizot M (2011) Chikungunya triggers an autophagic process which promotes viral replication. *Virol J* **8:** 432

Kumar S, Christophides GK, Cantera R, Charles B, Han YS, Meister S, Dimopoulos G,

Kafatos FC, Barillas-Mury C (2003) The role of reactive oxygen species on Plasmodium melanotic encapsulation in Anopheles gambiae. *Proc Natl Acad Sci U S A* **100:** 14139-14144

Knudsen AB (1977) The silent jungle transmission cycle of dengue virus and its tenable relationship to endemic dengue in Malaysia. *Malayan Nature Journal* **31**: 41-47

Labbé GM, Nimmo DD, Alphey L (2010) piggybac- and PhiC31-mediated genetic transformation of the Asian tiger mosquito, Aedes albopictus (Skuse). *PLoS Negl Trop Dis* **4:** e788

Labbé GM, Scaife S, Morgan SA, Curtis ZH, Alphey L (2012) Female-specific flightless (fsRIDL) phenotype for control of Aedes albopictus. *PLoS Negl Trop Dis* **6:**e1724

Lacroix R, McKemey AR, Raduan N, Kwee Wee L, Hong Ming W, Guat Ney T, Rahidah A A S, Salman S, Subramaniam S, Nordin O, Hanum A T N, Angamuthu C, Marlina Mansor S, Lees RS, Naish N, Scaife S, Gray P, Labbé G, Beech C, Nimmo D, Alphey L, Vasan SS, Han Lim L, Wasi A N, Murad S (2012) Open field release of genetically engineered sterile male Aedes aegypti in Malaysia. *PLoS One* **7:** e42771

Lambrechts L, Scott TW, Gubler DJ (2010) Consequences of the expanding global distribution of Aedes albopictus for dengue virus transmission. *PLoS Negl Trop Dis***4:** e646

Lassy CW, Karr TL (1996) Cytological analysis of fertilization and early embryonic development in incompatible crosses of Drosophila simulans. *Mech Dev* **57:** 47-

58

Laven H (1953) [Reciprocally differentiable crossing of mosquitoes (Culicidae) and its significance for plasmatic heredity]. *Z Indukt Abstamm Vererbungsl* **85:** 118-136

Laven H (1954) [Crossing experiments with European and American strains of the Culex pipiens complex]. *Z Tropenmed Parasitol* **5:** 317-323

Laven H (1967a) Eradication of Culex pipiens fatigans through cytoplasmic incompatibility. *Nature* **216:** 383-384

Laven H (1967b) in Genetics of Insect Vectors of Disease. Wright R, Pal R, editors. Amsterdam: Elsevier. 251–275

Lee DJ, Hicks MM, Griffiths M, Debenham ML, Bryan JH, Russel RC, Geary M & Marks EN (1987) The Culicidae of the Australasian region. Volume 4. *Canberra, Australian Government Publishing Service*

Lindquist DA, Abusowa M, Hall MJ (1992) The New World screwworm fly in Libya: a review of its introduction and eradication. *Med Vet Entomol* **6:** 2-8

Lofgren CS, Dame DA, Breeland SG, Weidhaas DE, Jeffery G, Kaiser R, Ford HR, Boston MD, Baldwin KF (1974) Release of chemosterilized males for the control of Anopheles albimanus in El Salvador. 3. Field methods and population control. *Am J Trop Med Hyg* **23:** 288-297

Lounibos LP (2002) Invasions by insect vectors of human disease. *Annu Rev*

Entomol **47:** 233-266

Lu P, Bian G, Pan X, Xi Z (2012) Wolbachia induces density-dependent inhibition to dengue virus in mosquito cells. *PLoS Negl Trop Dis* **6:** e1754

Mackenzie JM, Khromykh AA, Parton RG (2007) Cholesterol manipulation by West Nile virus perturbs the cellular immune response. *Cell Host Microbe* **2:** 229-239

McDonald PT, Hausermann W, Lorimer N (1977) Sterility introduced by release of genetically altered males to a domestic population of Aedes aegypti at the Kenya coast. *Am J Trop Med Hyg* **26:** 553-561

McGraw EA, Merritt DJ, Droller JN, O'Neill SL (2002) Wolbachia density and virulence attenuation after transfer into a novel host. *Proc Natl Acad Sci U S A* **99:**2918-2923

McGraw EA, O'Neill SL (1999) Evolution of Wolbachia pipientis transmission dynamics in insects. *Trends Microbiol* **7:** 297-302

McGraw EA, O'Neill SL (2013) Beyond insecticides: new thinking on an ancient problem. *Nat Rev Microbiol* **11:** 181-193

McMeniman CJ, Lane RV, Cass BN, Fong AW, Sidhu M, Wang YF, O'Neill SL (2009) Stable introduction of a life-shortening Wolbachia infection into the mosquito Aedes aegypti. *Science* **323:** 141-144

McMeniman CJ, O'Neill SL (2010) A virulent Wolbachia infection decreases the viability of the dengue vector Aedes aegypti during periods of embryonic

quiescence.*PLoS Negl Trop Dis* **4:** e748

Meeraus WH, Armistead JS, Arias JR (2008) Field comparison of novel and gold standard traps for collecting Aedes albopictus in Northern Virginia. *J Am Mosq Control Assoc* **24:** 244-248

Meister S, Kanzok SM, Zheng XL, Luna C, Li TR, Hoa NT, Clayton JR, White KP, Kafatos FC, Christophides GK, Zheng L (2005) Immune signaling pathways regulating bacterial and malaria parasite infection of the mosquito Anopheles gambiae. *Proc Natl Acad Sci U S A* **102:** 11420-11425

Merkling SH, van Rij RP (2013) Beyond RNAi: antiviral defense strategies in Drosophila and mosquito. *J Insect Physiol* **59:** 159–170

Min KT, Benzer S (1997) Wolbachia, normally a symbiont of Drosophila, can be virulent, causing degeneration and early death. *Proc Natl Acad Sci U S A* **94:**10792-10796

Moore CG, Mitchell CJ (1997) Aedes albopictus in the United States: ten-year presence and public health implications. *Emerg Infect Dis* **3:** 329-334

Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, Lu G, Pyke AT, Hedges LM, Rocha BC, Hall-Mendelin S, Day A, Riegler M, Hugo LE, Johnson KN, Kay BH, McGraw EA, van den Hurk AF, Ryan PA, O'Neill SL (2009) A Wolbachia symbiont in Aedes aegypti limits infection with dengue, Chikungunya, and Plasmodium. *Cell* **139:** 1268-1278

Morris AC, Eggleston P, Crampton JM (1989) Genetic transformation of the mosquito Aedes aegypti by micro-injection of DNA. *Med Vet Entomol* **3:** 1-7

Mousson L, Zouache K, Arias-Goeta C, Raquin V, Mavingui P, Failloux AB (2012) The native Wolbachia symbionts limit transmission of dengue virus in Aedes albopictus. *PLoS Negl Trop Dis* **6:** e1989

Muñoz D, Jimenez A, Marinotti O, James AA (2004) The AeAct-4 gene is expressed in the developing flight muscles of female Aedes aegypti. *Insect Mol Biol* **13:**563-568

Nigro L (1991) The effect of heteroplasmy on cytoplasmic incompatibility in transplasmic lines of Drosophila simulans showing a complete replacement of the mitochondrial DNA. *Heredity (Edinb)* **66 (Pt 1):** 41-45

Niu H, Xiong Q, Yamamoto A, Hayashi-Nishino M, Rikihisa Y (2012) Autophagosomes induced by a bacterial Beclin 1 binding protein facilitate obligatory intracellular infection. *Proc Natl Acad Sci U S A* **109:** 20800–20807

Norikuni K. Futoshi K. Dai H & Tsuguo K (2008) Irradiation does not affect field dispersal ability in the West Indian sweetpotato weevil, Euscepes postfasciatus. *Entomologia Experimentalis et Applicata* **130**: 63-72

O'Neill SL, Giordano R, Colbert AM, Karr TL, Robertson HM (1992) 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *Proc Natl Acad Sci U S A* **89:** 2699-2702

O'Neill SL, Karr TL (1990) Bidirectional incompatibility between conspecific populations of Drosophila simulans. *Nature* **348:** 178-180

Osborne SE, Leong YS, O'Neill SL, Johnson KN (2009) Variation in antiviral protection mediated by different Wolbachia strains in Drosophila simulans. *PLoS Pathog***5:** e1000656

Osei-Amo S, Hussain M, O'Neill SL, Asgari S (2012) Wolbachia-induced aae-miR-12 miRNA negatively regulates the expression of MCT1 and MCM6 genes in Wolbachia-infected mosquito cell line. *PLoS One* **7:** e50049

Otsuka Y, H Takaoka (1997) Elimination of Wolbachia pipientis from Aedes albopictus. *Med. Entomol. Zool*. **48**:257-260

Panteleev DIu, Goriacheva II, Andrianov BV, Reznik NL, Lazebnyĭ OE, Kulikov AM (2007) [The endosymbiotic bacterium Wolbachia enhances the nonspecific resistance to insect pathogens and alters behavior of Drosophila melanogaster]. *Genetika* **43:** 1277-1280

Patterson RS, Weidhaas DE, Ford HR, Lofgren CS (1970) Suppression and elimination of an island population of Culex pipiens quinquefasciatus with sterile males.*Science* **168:** 1368-1370

Phuc HK, Andreasen MH, Burton RS, Vass C, Epton MJ, Pape G, Fu G, Condon KC, Scaife S, Donnelly CA, Coleman PG, White-Cooper H, Alphey L (2007) Late-acting dominant lethal genetic systems and mosquito control. *BMC Biol* **5:** 11

Povelones M, Waterhouse RM, Kafatos FC, Christophides GK (2009) Leucine-rich repeat protein complex activates mosquito complement in defense against Plasmodium parasites. *Science* **324:** 258-261

Presgraves DC (2000) A genetic test of the mechanism of Wolbachia-induced cytoplasmic incompatibility in Drosophila. *Genetics* **154:** 771-776

Ritchie SA, Moore P, Carruthers M, Williams C, Montgomery B, Foley P, Ahboo S, van den Hurk AF, Lindsay MD, Cooper B, Beebe N, Russell RC (2006) Discovery of a widespread infestation of *Aedes albopictus* in the Torres Strait, Australia. *J Am Mosq Control Assoc* **22:** 358-365

Ranjit S, Kissoon N, Jayakumar I (2005) Aggressive management of dengue shock syndrome may decrease mortality rate: a suggested protocol. *Pediatr Crit Care Med* **6:** 412-419

Rasgon JL, Gamston CE, Ren X (2006) Survival of Wolbachia pipientis in cell-free medium. *Appl Environ Microbiol* **72:** 6934-6937

Reed KM, Werren JH (1995) Induction of paternal genome loss by the paternal-sex-ratio chromosome and cytoplasmic incompatibility bacteria (Wolbachia): a comparative study of early embryonic events. *Mol Reprod Dev* **40:** 408-418

Reiter P (1998) Aedes albopictus and the world trade in used tires, 1988-1995: the shape of things to come? *J Am Mosq Control Assoc* **14:** 83-94

Renault AD, Zhang XH, Alphey LS, Frenz LM, Glover DM, Saunders RD, Axton JM (2003)

giant nuclei is essential in the cell cycle transition from meiosis to mitosis.*Development* **130:** 2997-3005

Rendón P, McInnis D, Lance D, Stewart J (2004) Medfly (Diptera: Tephritidae) genetic sexing: large-scale field comparison of males-only and bisexual sterile fly releases in Guatemala. *J Econ Entomol* **97:** 1547-1553

Reynolds KT, Hoffmann AA (2002) Male age, host effects and the weak expression or non-expression of cytoplasmic incompatibility in Drosophila strains infected by maternally transmitted Wolbachia. *Genet Res* **80:** 79-87

Ritchie SA, Moore P, Carruthers M, Williams C, Montgomery B, Foley P, Ahboo S, van den Hurk AF, Lindsay MD, Cooper B, Beebe N, Russell RC (2006) Discovery of a widespread infestation of Aedes albopictus in the Torres Strait, Australia. *J Am Mosq Control Assoc* **22:** 358-365

Robinson JT, Wojcik EJ, Sanders MA, McGrail M, Hays TS (1999) Cytoplasmic dynein is required for the nuclear attachment and migration of centrosomes during mitosis in Drosophila. *J Cell Biol* **146:** 597-608

Romi R, Severini F, Toma L (2006) Cold acclimation and overwintering of female Aedes albopictus in Roma. *J Am Mosq Control Assoc* **22:** 149-151

Rosen L (1954) Observations on Dirofilaria immitis in French Oceania. *Ann Trop Med Parasitol* **48:** 318-328

Rothwell C, Lebreton A, Young Ng C, Lim JY, Liu W, Vasudevan S, Labow M, Gu F,

Gaither LA (2009) Cholesterol biosynthesis modulation regulates dengue viral replication. *Virology* **389:** 8-19

Rousset F, Bouchon D, Pintureau B, Juchault P, Solignac M (1992) Wolbachia endosymbionts responsible for various alterations of sexuality in arthropods. *Proc Biol Sci* **250:** 91-98

Ruang-Areerate T, Kittayapong P (2006) Wolbachia transinfection in Aedes aegypti: a potential gene driver of dengue vectors. *Proc Natl Acad Sci U S A* **103:**12534-12539

Russell RC, Williams CR, Sutherst RW, Ritchie SA (2005) Aedes (Stegomyia) albopictus--a dengue threat for southern Australia? *Commun Dis Intell Q Rep* **29:** 296-298

Ryan SL, Saul GB (1968) Post-fertilization effect of incompatibility factors in Mormoniella. *Mol Gen Genet* **103:** 29-36

Salvan M, Mouchet J (1994) [Aedes albopictus and Aedes aegypti at Ile de la Réunion]. *Ann Soc Belg Med Trop* **74:** 323-326

Samsa MM, Mondotte JA, Iglesias NG, Assunção-Miranda I, Barbosa-Lima G, Da Poian AT, Bozza PT, Gamarnik AV (2009) Dengue virus capsid protein usurps lipid droplets for viral particle formation. *PLoS Pathog* **5:** e1000632

Sánchez-Vargas I, Scott JC, Poole-Smith BK, Franz AW, Barbosa-Solomieu V, Wilusz J, Olson KE, Blair CD (2009) Dengue virus type 2 infections of Aedes aegypti are modulated by the mosquito's RNA interference pathway. *PLoS Pathog* **5:**

e1000299

Savage HM, Ezike VI, Nwankwo AC, Spiegel R, Miller BR (1992) First record of breeding populations of Aedes albopictus in continental Africa: implications for arboviral transmission. *J Am Mosq Control Assoc* **8:** 101-103

Scholte JE, Schaffner F (2007) Waiting for the tiger: establishment and spread of the Aedes albopictus mosquito in Europe, In: Takken W & Knols BGJ, editors. Emerging pests and vector-borne diseases in Europe. Wageningen: Academic Publishers

Serbus LR, Casper-Lindley C, Landmann F, Sullivan W (2008) The genetics and cell biology of Wolbachia-host interactions. *Annu Rev Genet* **42:** 683-707

Serbus LR, Sullivan W (2007) A cellular basis for Wolbachia recruitment to the host germline. *PLoS Pathog* **3:** e190

Sinkins SP, Braig HR, O'Neill SL (1995) Wolbachia superinfections and the expression of cytoplasmic incompatibility. *Proc Biol Sci* **261:** 325-330

Sinkins SP, Godfray HC (2004) Use of Wolbachia to drive nuclear transgenes through insect populations. *Proc Biol Sci* **271:** 1421-1426

Sinkins SP, Gould F (2006) Gene drive systems for insect disease vectors. *Nat Rev Genet* **7:** 427-435

Sinkins SP, O'Neill SL (2000) Wolbachia as a vehicle to modify insect populations. In: Hander AM, James AA, editors. *Insect Transgenesis: Methods And Applications*.

Boca Raton: CRC Press. 271–87

Skulachev VP (1998) Possible role of reactive oxygen species in antiviral defense. *Biochemistry (Mosc)* **63:** 1438-1440

Stouthamer R, Breeuwer JA, Hurst GD (1999) Wolbachia pipientis: microbial manipulator of arthropod reproduction. *Annu Rev Microbiol* **53:** 71-102

Stouthamer R, Luck RF, Hamilton WD (1990) Antibiotics cause parthenogenetic Trichogramma (Hymenoptera/Trichogrammatidae) to revert to sex. *Proc Natl Acad Sci U S A* **87:** 2424-2427

Suh E, Mercer DR, Fu Y, Dobson SL (2009) Pathogenicity of life-shortening Wolbachia in Aedes albopictus after transfer from Drosophila melanogaster. *Appl Environ Microbiol* **75:** 7783-7788

Sun LV, Riegler M, O'Neill SL (2003) Development of a physical and genetic map of the virulent Wolbachia strain wMelPop. *J Bacteriol* **185:** 7077-7084

Takada S, Kelkar A, Theurkauf WE (2003) Drosophila checkpoint kinase 2 couples centrosome function and spindle assembly to genomic integrity. *Cell* **113:** 87-99

Tan A, Fu G, Jin L, Guo Q, Li Z, Niu B, Meng Z, Morrison NI, Alphey L, Huang Y (2013) Transgene-based, female-specific lethality system for genetic sexing of the silkworm, Bombyx mori. *Proc Natl Acad Sci U S A* **110:** 6766-6770

Teixeira L, Ferreira A, Ashburner M (2008) The bacterial symbiont Wolbachia induces

resistance to RNA viral infections in Drosophila melanogaster. *PLoS Biol* **6:** e2

Thomas DD, Donnelly CA, Wood RJ, Alphey LS (2000) Insect population control using a dominant, repressible, lethal genetic system. *Science* **287:** 2474-2476

Tram U, Ferree PM, Sullivan W (2003) Identification of Wolbachia--host interacting factors through cytological analysis. *Microbes Infect* **5:** 999-1011

Tram U, Fredrick K, Werren JH, Sullivan W (2006) Paternal chromosome segregation during the first mitotic division determines Wolbachia-induced cytoplasmic incompatibility phenotype. *J Cell Sci* **119:** 3655-3663

Tram U, Sullivan W (2002) Role of delayed nuclear envelope breakdown and mitosis in Wolbachia-induced cytoplasmic incompatibility. *Science* **296:** 1124-1126

Tsetsarkin KA, Chen R, Leal G, Forrester N, Higgs S, Huang J, Weaver SC (2011a) Chikungunya virus emergence is constrained in Asia by lineage-specific adaptive landscapes. *Proc Natl Acad Sci U S A* **108:** 7872-7877

Tsetsarkin KA, Chen R, Sherman MB, Weaver SC (2011b) Chikungunya virus: evolution and genetic determinants of emergence. *Curr Opin Virol* **1:** 310-317

Tsetsarkin KA, Vanlandingham DL, McGee CE, Higgs S (2007) A single mutation in chikungunya virus affects vector specificity and epidemic potential. *PLoS Pathog***3:** e201

Tsetsarkin KA, Weaver SC (2011c) Sequential adaptive mutations enhance efficient vector switching by Chikungunya virus and its epidemic emergence. *PLoS*

*Pathog***7:** e1002412

Turelli M (2010) Cytoplasmic incompatibility in populations with overlapping generations. *Evolution* **64:** 232-241

Turelli M, Hoffmann AA (1991) Rapid spread of an inherited incompatibility factor in California Drosophila. *Nature* **353:** 440-442

Turelli M, Hoffmann AA (1995) Cytoplasmic incompatibility in Drosophila simulans: dynamics and parameter estimates from natural populations. *Genetics* **140:** 1319-1338

Turley AP, Moreira LA, O'Neill SL, McGraw EA (2009) Wolbachia infection reduces blood-feeding success in the dengue fever mosquito, Aedes aegypti. *PLoS Negl Trop Dis* **3:** e516

van den Hurk AF, Hall-Mendelin S, Pyke AT, Frentiu FD, McElroy K, Day A, Higgs S, O'Neill SL (2012) Impact of Wolbachia on infection with chikungunya and yellow fever viruses in the mosquito vector Aedes aegypti. *PLoS Negl Trop Dis* **6:** e1892

Veneti Z, Clark ME, Karr TL, Savakis C, Bourtzis K (2004) Heads or tails: host-parasite interactions in the Drosophila-Wolbachia system. *Appl Environ Microbiol* **70:**5366-5372

Voronin D, Tran-Van V, Potier P, Mavingui P (2010) Transinfection and growth discrepancy of Drosophila Wolbachia strain wMel in cell lines of the mosquito

Aedes albopictus. *J Appl Microbiol* **108:** 2133-2141

Voronin D, Cook DA, Steven A, Taylor MJ (2012) Autophagy regulates Wolbachia populations across diverse symbiotic associations. *Proc Natl Acad Sci U S A* **109:** E1638 –E1646

Walker T, Johnson PH, Moreira LA, Iturbe-Ormaetxe I, Frentiu FD, McMeniman CJ, Leong YS, Dong Y, Axford J, Kriesner P, Lloyd AL, Ritchie SA, O'Neill SL, Hoffmann AA (2011) The wMel Wolbachia strain blocks dengue and invades caged Aedes aegypti populations. *Nature* **476:** 450-453

Watts DM, Burke DS, Harrison BA, Whitmire RE, Nisalak A (1987) Effect of temperature on the vector efficiency of Aedes aegypti for dengue 2 virus. *Am J Trop Med Hyg* **36:** 143-152

Werren JH, Zhang W, Guo LR (1995) Evolution and phylogeny of Wolbachia: reproductive parasites of arthropods. *Proc Biol Sci* **261:** 55-63

Werren JH (1997) Biology of Wolbachia, *Annu Rev Entomol* **42**: 587-609

West SA, Cook JM, Werren JH, Godfray HC (1998) Wolbachia in two insect host-parasitoid communities. *Mol Ecol* **7:** 1457-1465

Whitehorn J, Farrar J (2010) Dengue. *Br Med Bull* **95:** 161-173

World Health Organization (2011), Chickungunya fact sheet, URL: http://www.searo.who.int/LinkFiles/Chikungunya_feve_fact_sheet.pdf.

Accessed 13 september 2011.

Xi Z, Khoo CC, Dobson SL (2005) Wolbachia establishment and invasion in an Aedes aegypti laboratory population. *Science* **310:** 326-328

Xi Z, Khoo CC, Dobson SL (2006) Interspecific transfer of Wolbachia into the mosquito disease vector Aedes albopictus. *Proc Biol Sci* **273:** 1317-1322

Xi Z, Ramirez JL, Dimopoulos G (2008) The Aedes aegypti toll pathway controls dengue virus infection. *PLoS Pathog* **4:** e1000098

Yamada R, Floate KD, Riegler M, O'Neill SL (2007) Male development time influences the strength of Wolbachia-induced cytoplasmic incompatibility expression in Drosophila melanogaster. *Genetics* **177:** 801-808

Yen JH, Barr AR (1971) New hypothesis of the cause of cytoplasmic incompatibility in Culex pipiens L. *Nature* **232:** 657-658

Yen JH, Barr AR (1973) The etiological agent of cytoplasmic incompatibility in Culex pipiens. *J Invertebr Pathol* **22:** 242-250

Zabalou S, Apostolaki A, Pattas S, Veneti Z, Paraskevopoulos C, Livadaras I, Markakis G, Brissac T, Merçot H, Bourtzis K (2008) Multiple rescue factors within a Wolbachia strain. *Genetics* **178:** 2145-2160

Zambon RA, Nandakumar M, Vakharia VN, Wu LP (2005) The Toll pathway is important for an antiviral response in Drosophila. *Proc Natl Acad Sci U S A* **102:**7257-7262

Zhang G, Hussain M, O'Neill SL, Asgari S (2013) Wolbachia uses a host microRNA to regulate transcripts of a methyltransferase, contributing to dengue virus inhibition in Aedes aegypti. *Proc Natl Acad Sci U S A* **110:** 10276-10281

Zhou W, Rousset F, O'Neil S (1998) Phylogeny and PCR-based classification of Wolbachia strains using wsp gene sequences. *Proc Biol Sci* **265:** 509-515

Chapter 9: Appendix