DIVERSITY OF ARTHROPOD-BORNE VIRUSES AND IMPLICATIONS FOR *WOLBACHIA*-BASED BIOCONTROL

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Arthropod-borne viruses; arboviruses; *Aedes aegypti*; *Aedes albopictus*; dengue virus; extrinsic incubation period; genetic diversity; vector mosquito; *Wolbachia.*

Abstract

Arthropod-borne viruses (arboviruses) pose a major threat to both human and animal health and many are responsible for emerging and re-emerging diseases. Dengue viruses (DENV) comprise four antigenically distinct serotypes (DENV-1, 2, 3 and 4) and the disease they cause, dengue, has re-emerged over the past 50 years resulting in greater morbidity and mortality in humans than that due to any other arbovirus. In the absence of effective vaccines and/or antiviral therapies for many arboviruses, outbreak prevention has relied, primarily, on vector control through application of insecticides. The bacteria *Wolbachia* has been proposed as an alternative biocontrol agent in order to avoid the evolution of resistance to insecticides by mosquitoes and environmental pollution from these potentially harmful chemicals.

There is extensive genetic diversity within and between DENV serotypes but little is known about the implications of this diversity on the extrinsic incubation period (the time it takes for a pathogen to replicate within a mosquito) in vector mosquito populations or for the ability of strains of *Wolbachia* to inhibit their replication. The EIP is a critical parameter in understanding the potential of different strains of DENV to cause outbreaks. *Wolbachia* are maternally inherited, obligate, intracellular bacteria that are present in many arthropod hosts. When introduced into mosquito vectors, they are able to block the replication of some arboviruses. Different strains of *Wolbachia* vary in their ability to inhibit replication of arboviruses and the fitness cost they impose when transfected to *Aedes* mosquitoes.

To evaluate the effect of serotype and strain diversity on DENV transmission, differences in EIP were assessed in Australian populations of *Aedes aegypti* and *Aedes albopictus* mosquitoes in laboratory experiments. Mosquitoes were fed blood meals containing similar titres of each strain of each of the four DENV serotypes and tested for the presence of viral RNA in bodies, and in wings and legs, as a proxy for disseminated virus at 3, 6, 10 and 14 days post exposure. The EIP varied between different strains of DENV, as well as between the two species of *Aedes* mosquitoes, suggesting that EIP can be influenced by both viral and mosquito genotypes.

Wolbachia strains *w*AlbB and *w*MelPop blocked replication of each DENV strain to similar extents but the magnitude of the blocking was different between strains of DENV. This study raises the possibility that *w*AlbB may be a sustainable

alternative to remedy the inadequacies of both *w*MelPop and *w*Mel as biocontrol agents to reduce the transmission of DENV under diverse field conditions.

In addition to DENV and other flaviviruses (Zika and Kunjin viruses), *Wolbachia* strain *w*AlbB inhibited the replication of alphaviruses (Barmah Forest, Ross River and Sindbis viruses) in C6/36 cells with the magnitude of inhibition varying with each virus suggesting that *w*AlbB may be a candidate for broadly based vector-centred arbovirus control.

More broadly, these findings highlight the variation within and between arboviruses and strains of arboviruses in their ability to be transmitted by both uninfected and *Wolbachia*-infected, *Ae. aegypti* and *Ae. albopictus* mosquitoes and the likelihood that a particular strain of virus might be involved in an outbreak of disease. Additionally, *Wolbachia* strain *w*AlbB promises to have utility in the field for the control of all serotypes of DENV and a broad range of other arboviruses, especially in hot climates, thus adding to the pool of *Wolbachia* strains available to inhibit transmission of arboviruses by *Ae. aegypti* and *Ae. albopictus* mosquitoes.

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List of Abbreviations

ADE	antibody dependent enhancement
BSA	bovine serum albumin
BFV	Barmah Forest virus
CHIKV	Chikungunya virus
CI	cytoplasmic incompatibility
CO_2	carbon dioxide
CPE	cytopathic effect
Ct	threshold cycle number
DENV	dengue virus
d.p.e.	days post exposure
d.p.i.	days post infection
DF	dengue fever
DSS	dengue shock syndrome
DHF	dengue haemorrhagic fever
E. coli	*Escherichia coli*
EIP	extrinsic incubation period
ELISA	enzyme-linked immunosorbent assay
FBS	foetal bovine serum
FFU	focus forming unit
FISH	fluorescent *in situ* hybridization
m.o.i.	multiplicity of infection
QIMRB	Queensland Institute of Medical Research Berghofer
QUT	Queensland University of Technology
KUNV	Kunjin virus
PCR	polymerase chain reaction
PBS	phosphate buffered saline
PFU	plaque forming unit
Qld	Queensland
RH	relative humidity
r.p.m.	revolutions per minute
RRV	Ross River virus

SINV	Sindbis virus
USA	United States of America
WHO	World Health Organization
WNV	West Nile virus
YF	Yellow fever virus

Statement of Original Authorship

The work contained in this thesis has not been submitted previously to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

Signature: QUT Verified Signature

Date: February 2019

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"Now to Him who is able to do immeasurably more than all we ask or imagine, according to His power that is at work within us, to Him be glory in the church and in Christ Jesus throughout all generations forever and ever! Amen." *Ephesians 3:20-21*

Chapter 1: Introduction

1.1 ARBOVIRUSES

Arthropod-borne viruses (arboviruses) (WHO, 1967) are zoonotic viruses transmitted to vertebrate hosts by blood-feeding insects including biting midges, sandflies, ticks and mosquitoes. These viruses replicate to a reasonably high viraemia, sufficient for a susceptible arthropod to become infected after taking a blood meal from their host. Arboviruses then replicate within arthropod tissues and may be transmitted to another vertebrate when next the arthropod feeds next (Karabatsos, 2001). They are responsible for disease in millions of humans and animals annually (S. B. B. J. Higgs, 2005), and the past few decades have seen an increase in the number of epidemics of arbovirus infection, mostly from viruses that were barely of any public health significance (Mayer, Tesh, & Vasilakis, 2017). Increased epidemic activity has been attributed to increased human travel, unregulated urbanisation and a failure of vector control programs.

The International Catalogue of Arboviruses maintained by the United States Centers for Disease Control and Prevention has more than 500 virus entries (https://wwwn.cdc.gov/arbocat/). Most arboviruses have RNA genomes and belong to seven families of viruses including *Bunyaviridae, Flaviviridae, Orthomyxoviridae, Togaviridae, Reoviridae* and *Rhabdoviridae* (Baltimore, 1971). The *Asfaviridae* (e.g. African swine fever virus) are the only arboviruses with DNA genomes (Calisher & Karabatsos, 1988; Dixon, 2005).

1.2 *FLAVIVIRIDAE*

The viruses of the *Flaviviridae* family comprise a large number of important human and animal pathogens and are divided into four genera: *Flavivirus, Pestivirus, Pegivirus* and *Hepacivirus* (Simmonds et al., 2017). The *Flavivirus* genus contains more than 50 species of arboviruses, most of which employ mosquito vectors. The mosquito-borne viruses that are important human pathogens include Yellow fever virus (YFV), Japanese encephalitis virus (JEV), dengue virus (DENV), West Nile virus (WNV) and Zika virus (ZIKV). In Australia, Murray Valley encephalitis virus (MVEV), which can cause fatal encephalitis, and West Nile Kunjin virus (WNV_{KUN}),

which causes a febrile illness with less severe encephalitis than MVE, are among the most important flaviviruses.

Members of the genus *Flavivirus* have a non-segmented, single-strand, positive-sense RNA genome containing 9.2 to 11 kilo-bases. The genome has a single, open reading frame (ORF) adjoined by 5'- and 3'-terminal non-coding regions, which make specific secondary structures essential for genome replication (Simmonds et al., 2017). The proteins required for virus replication are similar among all members of the genus *Flavivirus* and are encoded at similar locations in the genome (Simmonds et al., 2017). Initially, *Flaviviruses* (Group B arboviruses) were classified with *Alphaviruses* (Group A arboviruses) in the *Togaviridae* family with regards to structural similarities but differences in genome organisation, virion structure and modes of replication resulted in these two groups of viruses being assigned to different families (Strauss & Strauss, 2001).

1.2.1 Dengue virus (DENV)

DENV is the cause of dengue, the most important arthropod-borne viral disease of humans, and is responsible for an estimated 390 million infections annually (Bhatt et al., 2013). Prior to 1970, only nine countries had experienced DENV epidemics (Gubler, 1998). Now, the disease is endemic in more than 100 countries and DENV has emerged as an important human pathogen globally.

There are four serologically distinct groups of DENV (serotypes) designated DENV-1, DENV-2, DENV-3 and DENV-4 (Calisher & Karabatsos, 1988; Katzelnick et al., 2017). The first DENV isolates were recovered from military personnel in Japan (Hotta, 1951) and New Guinea (Sabin, 1950). Earliest medical literature from China indicates that DENV may have been responsible for disease in China for more than a thousand years (Gubler, 1998). DENV was believed to have evolved in non-human primates in the forests of Africa (Gubler, 1998) but strains of DENV recovered in Southeast Asia are basal in all phylogenetic trees suggesting an origin in that region (Liu, Pickering, Duchene, Holmes, & Aaskov, 2016; A. T. Pyke et al., 2016; S. C. Weaver & Vasilakis, 2009).

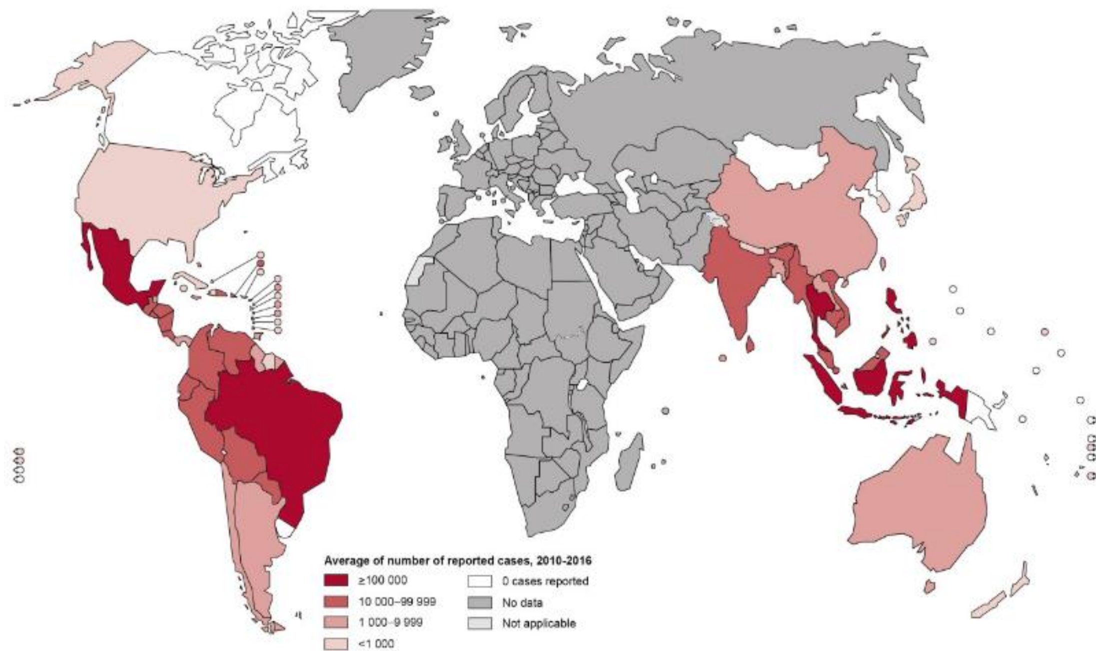

Figure 1.1 Distribution of suspected and confirmed cases of dengue reported WHO, 2010 – 2016 (WHO, 2016a).

The Americas, Southeast Asia and the Western Pacific (Figure 1.1) are the regions most seriously affected by dengue with significant increases in the number of areas becoming hyperendemic for multiple DENV serotypes (WHO, 2015). The emergence of the disease has been attributed to increased human travel, unplanned urbanisation, decreased public health control and increased human populations (Petersen & Marfin, 2005).

1.2.1.1 Virus structure, genome organisation and replication

The DENV virion consists of an RNA genome of approximately 11,000 nucleotides enclosed in a capsid composed of core (C) proteins and an outer lipid bilayer derived from host cells in which membrane (M) protein, pre-Membrane protein (prM) and envelope proteins (E) are anchored. Most prM protein is cleaved to a smaller membrane protein (M) by furin immediately before release from the host cell (Kuhn et al., 2002; Leitmeyer et al., 1999). The genome is made up of the protein-coding genes C, prM, E, NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5, with the non-coding regions at the 5' and 3' ends (Figure 1.2).

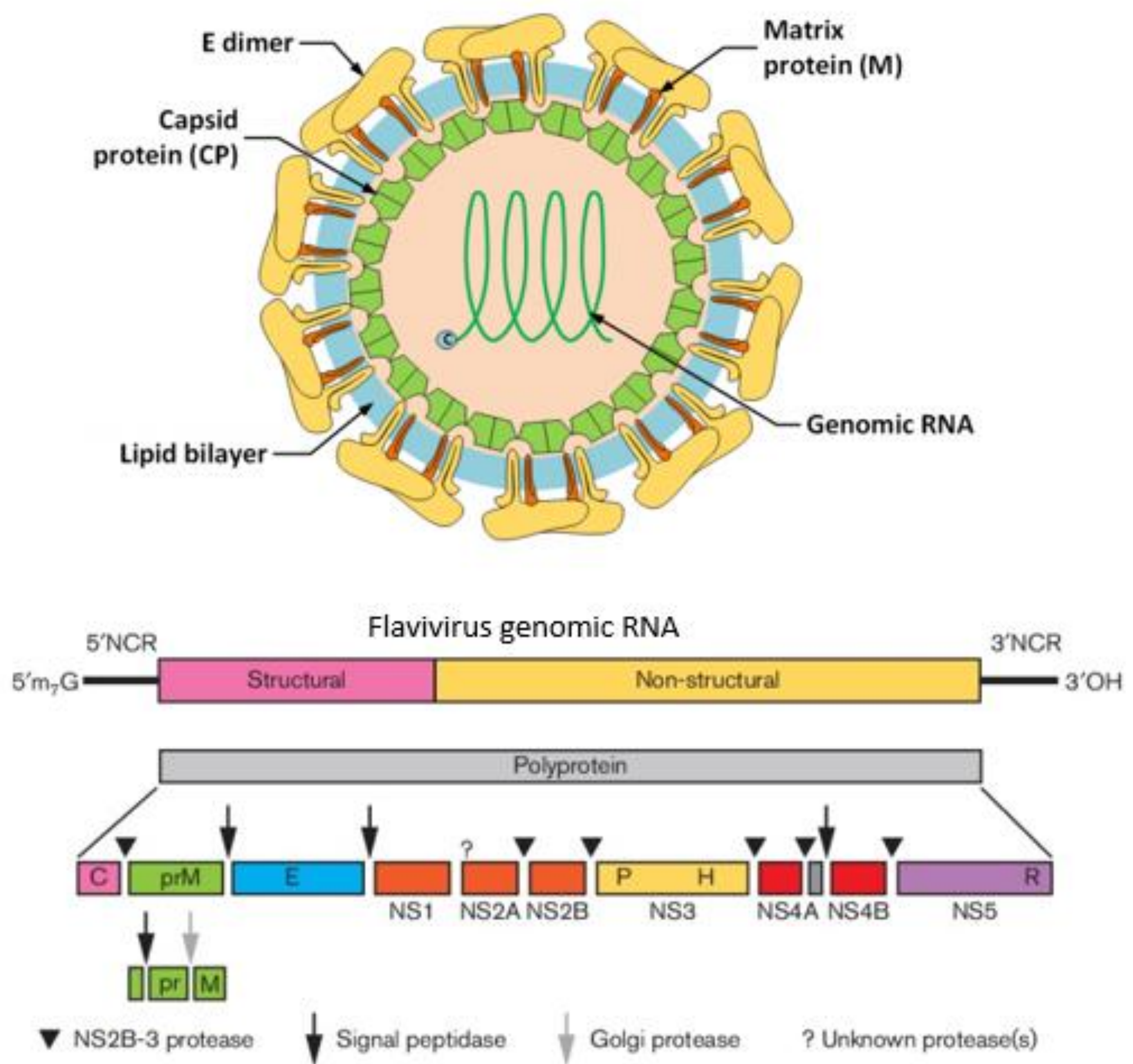

Figure 1.2 Schematic representation of the virion, genome organization and polyprotein processing of members of the genus *Flavivirus.* Coloured boxes show viral proteins generated by proteolytic processing. NCR, non-coding region (Simmonds et al., 2017).

The E protein plays a key role in cell entry with potential glycosylation sites at N-67 and N-153. Apart from viral fusion and receptor binding, other functions attributed to E glycoprotein include the induction of antibody responses and viral assembly (Kuhn et al., 2002). The DENV enters the host cell by receptor-mediated endocytosis by attaching, via the E protein, to the glycosaminoglycan and other specific receptors (Y. Chen, Maguire, & Marks, 1996). The low pH of the endosome results in major conformational changes in the E protein, exposing a fusion peptide which embeds itself in the lipid membrane of the host cell. Non-structural proteins form a replication complex that replicates the viral RNA on the ER-derived membranes. C protein molecules enclose the viral RNA, and the immature virus, containing prM and E proteins, buds from the ER. The immature virion is processed in the trans-Golgi network, with the cleavage of prM by furin to produce a mature (infectious) virus which is released from the infected cell by exocytosis (Figure 1.3) (Acosta, Talarico, & Damonte, 2008; Alcaraz-Estrada, Yocupicio-Monroy, & de Angel, 2010; Clyde, Kyle, & Harris, 2006; Mukhopadhyay, Khun, & Rossmann, 2005).

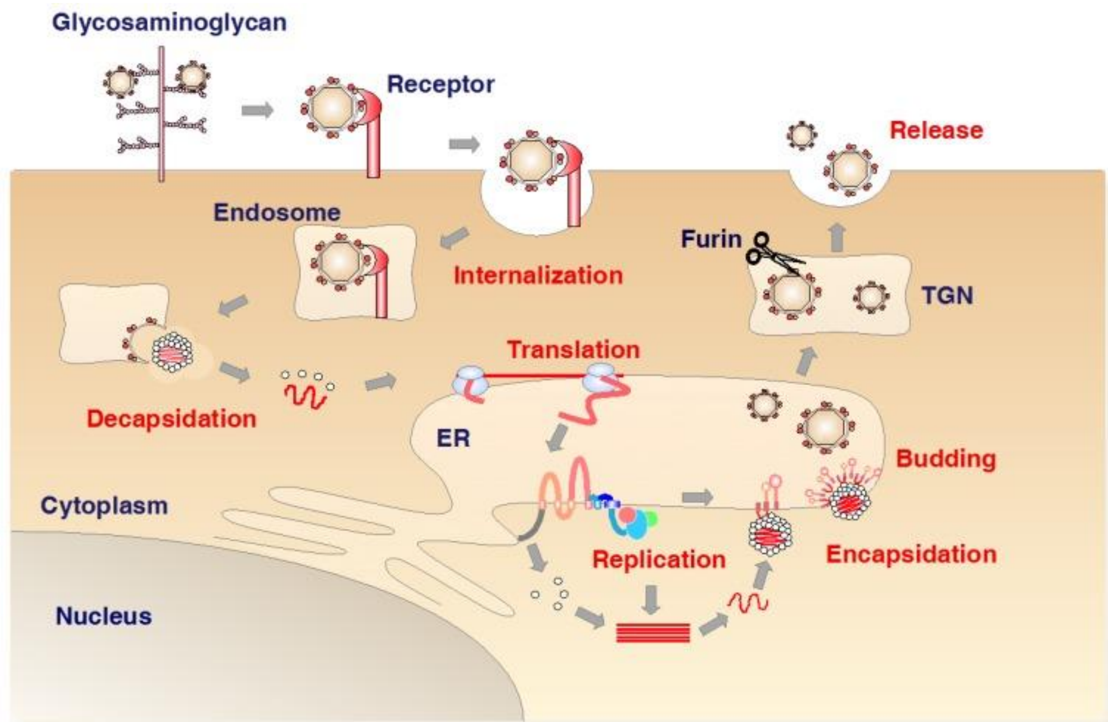

Figure 1.3 Dengue virus replication schematic (Okamoto et al., 2017).

1.2.1.2 Symptoms and diagnosis of dengue

Before 2009, dengue cases were classified as dengue fever (DF) or dengue haemorrhagic fever (DHF grades 1 to 4). The most recent WHO classification for dengue severity is divided into the following: a) dengue without warning signs, b) dengue with warning signs and c) severe dengue (WHO, 2009). Dengue is characterised by sudden onset of high fever, severe headaches, pain behind the eyes, fatigue, nausea, joint pain and occasionally a rash. Usually it is a sub-clinical, self-limiting illness (Gubler, 1998; H. Zhang et al., 2014). The most recent WHO classification (WHO, 2009) defines "dengue without warning signs" as a patient with a fever and at least two of the following – nausea, vomiting, rash, aches/pain, leucopoenia, or a positive tourniquet test. "Dengue with warning signs" is defined as abdominal pain or tenderness, persistent vomiting, ascites/pleural effusion, mucosal bleeding, lethargy/restlessness and liver enlargement greater than 2 cm. Patients with severe dengue can present with plasma leakage that may lead to hypovolemic shock and fluid accumulation resulting in respiratory distress. Thrombocytopenia, haemorrhage and shock due to excessive plasma leakage are the clinical outcomes of severe dengue (SD), which is rare but often fatal (Halstead, 2014). Death can occur within 12 to 24 h unless appropriate treatment is given (WHO, 2009).

Many cases of dengue are misclassified because of the wide spectrum of disease signs and symptoms and lack of effective case definitions (WHO, 2013). Although the global strategy for dengue prevention and control states that most cases of dengue are diagnosed on clinical grounds alone, the actual numbers of dengue cases are probably under-reported (WHO, 2015). In areas where the disease is endemic, most cases are diagnosed on clinical grounds alone. This approach has proved sufficient for effective clinical management where clinical support is available. However, even skilled infectious disease physicians may misdiagnose 30 to 60 per cent of dengue patients. Most laboratory diagnoses of dengue are made by detecting anti-DENV IgM antibody in acute phase serum samples from patients with dengue-like symptoms using commercial, or in-house, IgM capture Enzyme-Linked Immunosorbent Assays (ELISA) (Peeling et al., 2010). Alternatively, diagnoses may be made by detecting anti-DENV IgM or high titre anti-DENV IgG in acute phase sera using "rapid tests" immunochromatographic assays ("rapid tests") (Tricou et al., 2010; Tuan et al., 2015). More sophisticated laboratories may diagnose dengue by detecting DENV or its RNA

or protein derivatives in patient sera. The development of commercial ELISA and immunochromatographic tests to detect DENV non-structural protein 1 (NS 1) in acute phase sera from some dengue patients has provided a simpler mechanism for detection of DENV antigens (Young, Hilditch, Bletchly, & Halloran, 2000).

1.2.1.3 Dengue treatment and prevention

There are no antivirals for the treatment of dengue. The WHO has developed guidelines and recommended supportive care, especially careful fluid management, to treat the most common clinical manifestations of dengue (WHO, 2009). Even though there is no cure, mortality rates due to severe dengue can be reduced from more than 20 %, when untreated, to 1 % with proper medical care. Following infection, the incubation period is approximately 4 to 7 days. The symptomatic period consists of a febrile phase, a critical phase, and a recovery phase (WHO, 2009). During the febrile phase, rest, liberal oral fluid administration and antipyretic treatment with paracetamol as required is recommended (WHO, 2009). Due to the risk of Reyes Syndrome in children and its anticoagulation effects, aspirin is not recommended for dengue patients (Hayes & Gubler, 1992; Rigau-Perez et al., 1998). Fluid leakage indicates the beginning of the critical phase and signs of more severe disease including haemorrhage and shock. Hospitalization and admission to intensive care is recommended (Rajapakse, Rodrigo, & Rajapakse, 2012; WHO, 2009).

The first dengue vaccine Dengvaxia® is currently licensed in twenty countries but has recently been withdrawn. It was recommended for use only in populations above 9 years of age (Guy et al., 2017). In the first 25 months after the first dose of vaccine, the efficacy against virologically confirmed symptomatic dengue in seropositive participants above 9 years of age was 76 % but was much lower, 38 %, in seronegative persons. Overall, there is an increased risk of hospitalized and severe dengue in seronegative individuals (WHO, 2018).

1.2.1.4 Evolution and genetic diversity of DENV

The emergence and re-emergence of DENV has been a threat to human health for centuries. DENV has ancestral transmission cycles in rural settings being maintained by mosquito vectors from the enzootic cycle to a human-mosquito-human cycle in urban settings. The four serotypes of DENV are thought to be have evolved independently from a common sylvatic (i.e. forest-dwelling) ancestor (E. C. Holmes

& Twiddy, 2003; Rico-Hesse, 1990). Each of the four serotypes of DENV can be further subdivided into distinct genotypes based on phylogenetic analysis of the envelope, or other genes (Figure 1.4). It is believed that DENV was maintained in sylvatic cycles with non-human primates as hosts and sylvatic mosquitoes as vectors until the global dissemination of *Aedes aegypti* along trade routes, principally during the last 100 to 300 years. This also coincided with a large increase in the human population, particularly in urban settings (reviewed in Holmes and Twiddy, 2003). The recent recovery of two ancestral genotypes of DENV from travellers returning from Borneo (Liu et al., 2016; A. T. Pyke et al., 2016) suggests that sylvatic transmission of DENV is still occurring in Southeast Asia.

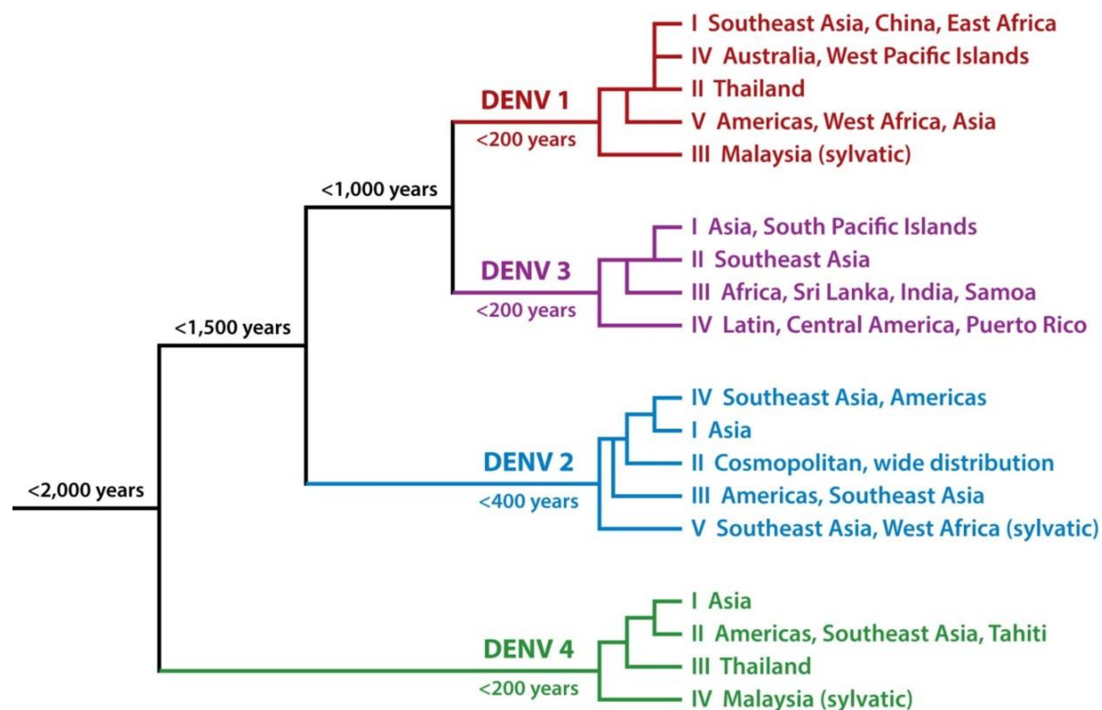

Figure 1.4 Dengue virus serotypes and genotypes with origin and time to most recent common ancestor (Tabachnick, 2016).

The original classification of DENV, based on serology, was relatively imprecise and even gave rise to the suggestion of two additional serotypes, DENV 5 and 6 (Rico-Hesse et al., 1997). The capacity to genotype viruses demonstrated that each serotype could be further divided into clades and strains based on the consensus nucleotide sequence of viral genes or genomes (Eddie C. Holmes & Burch, 2000;

Rico-Hesse, 1990, 2003). Finally, it has been possible to demonstrate the presence of extensive within-host genetic diversity in DENV populations (J. Aaskov et al., 2007; J. Aaskov, Buzacott, Thu, Lowry, & Holmes, 2006; Craig et al., 2003; Thai et al., 2012; W.-K. Wang, Lin, Lee, King, & Chang, 2002; W.-K. Wang, Sung, Lee, Lin, & King, 2002). Much of this diversity results in defective genomes that cannot be replicated (J. Aaskov et al., 2006; Li et al., 2011).

Intra-host populations of DENV, like many other RNA viruses, are very diverse genetically. This genetic diversity arises from the error-prone nature of RNA virus replication, where the RNA-dependent RNA polymerase (RdRp) lacks the proof-reading capacity of DNA polymerases (Steinhauer, Domingo, & Holland, 1992). This results in RNA viruses having very high mutation rates estimated to be from 10^3 to 10^5 per site per round of replication (Drake, 1993; Holland et al., 1982). These estimates correspond to, on average, 0.1 to 10 mutations in the genome of each progeny virus (Domingo & Holland, 1997). Despite the slower evolutionary rate of DENV (Jenkins & Holmes, 2003), there is evidence that genetic diversity ensures there are variants that have higher transmission potential (Hanley, Nelson, Schirtzinger, Whitehead, & Hanson, 2008; Louis Lambrechts et al., 2012; Thu et al., 2004), enhancing adaptation to new environments.

DENV diversity and evolution also is thought to be affected by the size of the human population. The increase in and availability of new and susceptible hosts, together with increasing global movement of humans, has led to an increase in DENV population size. In turn, this has led to an increase in the number of viral lineages. The maintenance of a sustainable transmission network, therefore, seems to be tied to the presence of a large enough host population (Zanotto, Gould, Gao, Harvey, & Holmes, 1996). There is evidence that novel lineages of virus strains can emerge through selection and proliferate (Bennett et al., 2003; Twiddy et al., 2002) and multiple introductions of DENV from a variety of locations may be followed by local transmission (A-Nuegoonpipat et al., 2004; Barrero & Mistchenko, 2004). However, there is little evidence for positive (diversifying) selection of DENV genomes in nature. Analyses of DENV typically detect strong negative (purifying) selection (Lequime, Fontaine, Ar Gouilh, Moltini-Conclois, & Lambrechts, 2016).

Recombination also may contribute to genetic diversity in virus populations, although in arboviruses the contribution is believed to be less than that due to mutation

(AbuBakar, Wong, & Chan, 2002; Lai, 1992; Tolou et al., 2001; Worobey, Rambaut, & Holmes, 1999). Genotypes of DENV within the same serotype have been demonstrated, through phylogenetic analyses, to undergo recombination (J. Aaskov et al., 2007; Bharaj et al., 2008; Craig et al., 2003; E. C. Holmes, Worobey, & Rambaut, 1999; W. K. Wang, Chao, Lin, King, & Chang, 2003; Wenming et al., 2005). This is thought to be due to the RdRp swapping templates in the course of replication within a cell that contains genomes from more than one DENV genotype. There is limited information on the frequency of co-infection with multiple genotypes of DENV and only a small fraction of DENV-infected patients are ever analysed so it has been impossible to estimate how common recombination in DENV is. There is no evidence of recombination between serotypes and this may reflect how uncommon infection with multiple serotypes is.

The requirement for arboviruses to replicate in alternating cycles between vertebrate and arthropod host is thought to significantly constrain genetic diversity among some alphaviruses and flaviviruses (S. C. Weaver, Brault, Kang, & Holland, 1999). The consequences of genetic diversity in DENV may include differences in virulence between strains (Rico-Hesse, 1990), vector-driven selection of strains for increased transmissibility (Louis Lambrechts et al., 2012), and different responses to interventions to stop virus transmission (Ferguson et al., 2015).

1.2.1.5 Vectors of DENV

A vector is defined as "an organism that transmits pathogens from one infected entity (animal or person) to another, causing serious diseases in human populations" (WHO, 1985). The ability of viruses, protozoa and helminths to replicate in mosquitoes makes them the most important vectors of human diseases (Tolle, 2009). Members of the *Aedes* genus of mosquitoes are significant vectors of arboviruses, including DENV.

1.2.1.5.1 Aedes aegypti

Ae. aegypti is a diurnal, peri-domestic mosquito species that lays 30 to 60 eggs 3 to 4 days after every blood meal (Bacot, 1916). It is widely distributed in tropical and sub-tropical regions of the world. It prefers to feed on humans and has adapted to living close to them, propagating in domestic water containers (Trpis & Hausermann, 1978). *Ae. aegypti* is the primary vector of DENV, yellow fever virus (YFV) (Reed, Carroll, Agramonte, & Lazear, 1900) and Zika virus (ZIKV) (G. W. A. Dick, 1952).

1.2.1.5.2 Aedes aegypti in Australia and dengue outbreaks

Dengue outbreaks in Australia predate the 20th century (Hare, 1898) but the past two decades have seen increased importations of DENV , frequent outbreaks and local transmission (Hanna & Ritchie, 2009a; Viennet, Ritchie, Faddy, Williams, & Harley, 2014; David Warrilow, Northill, & Pyke, 2012). This recent pattern of dengue transmission is restricted to north Queensland, where there is an established presence of *Aedes aegypti* (Russell et al., 2009; Viennet et al., 2014). Regulatory enforcement coupled with mosquito surveillance and the removal of domestic water tanks which serve as larval habitats may have accounted for the disappearance of *Ae. aegypti* in southeast Queensland especially Brisbane (Trewin et al., 2017). Viremic travellers, mainly from nearby countries in Southeast Asia where DENV is endemic, carry DENV into north Queensland (Gubler, 2002; Hanna & Ritchie, 2009b; Hanna et al., 1998; Leggat, 2009) and local transmission can be initiated. Although DENV is not endemic to Queensland, the vector is and the consequence is local transmission and outbreaks that have been recorded in the urban areas of north Queensland. Since 1900, all four DENV serotypes have been associated with outbreaks in Australia. DENV-2 is the most common and DENV-4 the least frequent (Figure 1.5, C). The frequent importation of different serotypes of DENV increases both the chances of dengue epidemics and the probability of humans being exposed to multiple serotypes of DENV and therefore of developing potentially life-threatening, severe dengue.

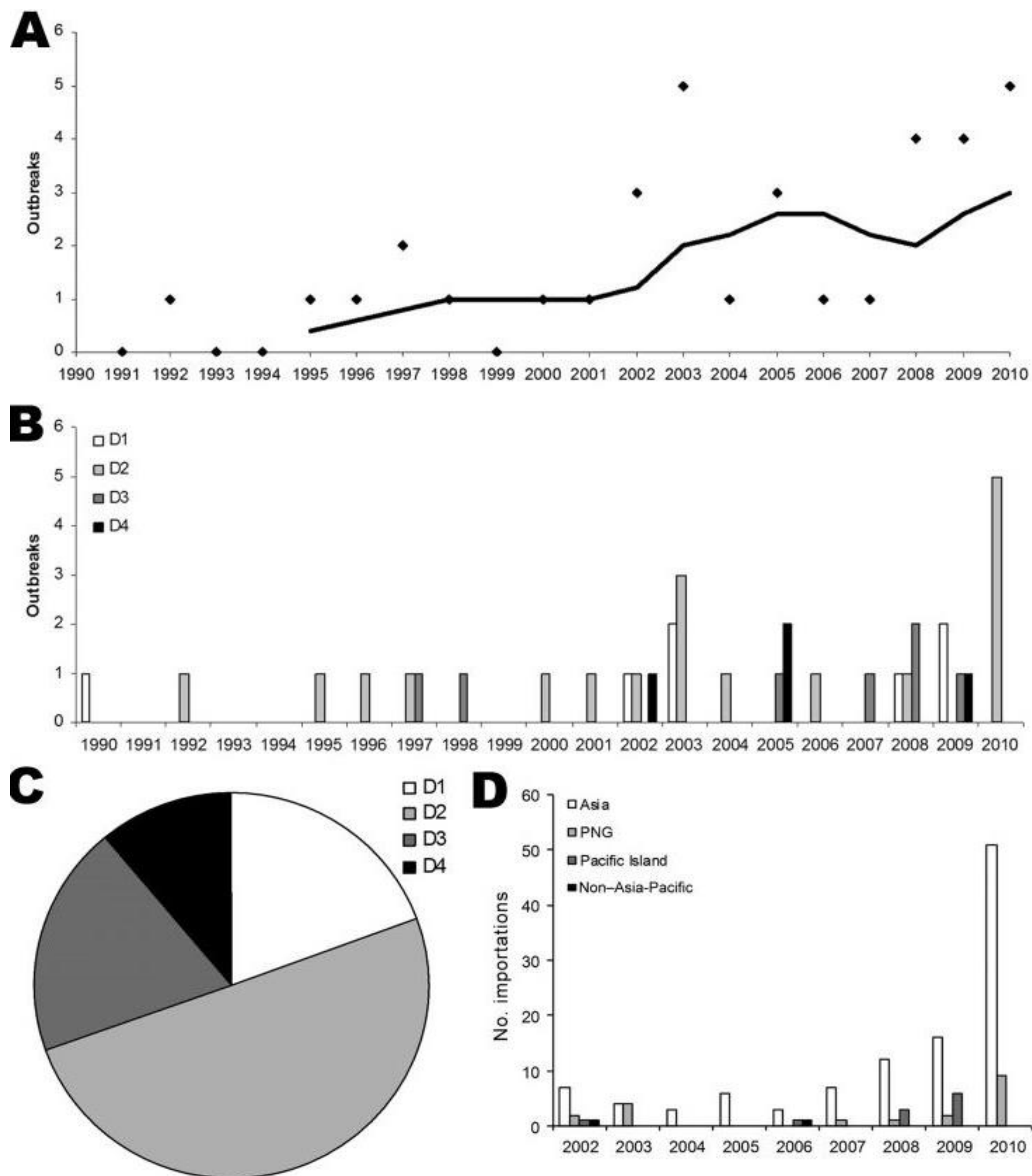

Figure 1.5 DENV serotypes responsible for dengue outbreaks in North Queensland, Australia since 1990. (A) Number of outbreaks each year. (B) DENV serotypes involved. (C) Proportion of dengue virus serotypes responsible for the outbreaks shown in A and B. (D) Geographic origins of imported dengue cases (David Warrilow et al., 2012).

1.2.1.5.3 Aedes albopictus

Ae. albopictus is probably the most invasive mosquito species in the world (Medlock et al., 2012). It is active all day, aggressive, will bite any exposed skin (Caglioti et al., 2013; McClelland, Hartberg, & Courtois, 1973) and will feed on a wider variety of vertebrate hosts than *Ae. aegypti* i.e. other mammals in addition to humans (Ponlawat & Harrington, 2005; Sullivan, Gould, & Maneechai, 1971). Depending on the environmental conditions, it has a lifespan of 5 to 8 weeks (Hartman, 2015), which is longer than most mosquito species (Charrel, de Lamballerie, & Raoult, 2007). It is both zoophilic and anthropophilic, and can lay eggs in natural and artificial containers (Charrel et al., 2007; Pardigon, 2009). *Ae. albopictus* is widely distributed in Europe and the United States of America (Tsetsarkin, Vanlandingham, McGee, & Higgs, 2007), and there is evidence in Japan that the eggs are able to overwinter at temperatures much lower than had been earlier estimated (Kobayashi, Nihei, & Kurihara, 2002).

Ae. albopictus has colonized the Torres Strait islands in northern Australia and could find suitable habitats all around Australia. However, while it does not inhabit mainland Australia (Ritchie et al., 2006), it has been intercepted on many occasions at ports around the country (Foley, Hemsley, Muller, Maroske, & Ritchie, 1998; Kay et al., 1990; Lamche & Whelan, 2003). Strict control measures by the Australian Quarantine and Inspection Service of the Department of Agriculture and Water Resources have prevented the establishment of *Ae. albopictus* on mainland Australia (Muzari et al., 2017).

Figure 1.6 Torres Strait and the projected distribution of *Ae. albopictus* in mainland Australia (Ritchie et al., 2006)

Ae. albopictus has played a significant role in recent CHIKV outbreaks in the Indian Ocean Islands (Ligon, 2006). It is the sole vector of DENV in China (Sang et al., 2015) and is capable of transmitting many arboviruses endemic to Australia, such as Ross River virus (RRV) and Barmah Forest virus (BFV) (Nicholson, Ritchie, & Van Den Hurk, 2014). It was formerly associated with thickets and arboreal vegetation but now also has a significant urban presence (Caglioti et al., 2013; Tsetsarkin et al., 2007; Scott C. Weaver, 2014). In Europe, *Ae. albopictus* eggs survive and hatch at temperatures as low as -7°C (Thomas, Obermayr, Fischer, Kreyling, & Beierkuhnlein, 2012). With these traits, *Ae. albopictus* could play a more prominent role in the transmission of arboviruses in temperate areas of Australia (Nicholson et al., 2014), should incursion result in establishment of the mosquito.

1.2.2 Zika virus (ZIKV)

ZIKV was isolated in Uganda in 1947 (G. W. Dick, Kitchen, & Haddow, 1952) and the first reported infections in humans were in 1954 with patients presenting with mild flu-like symptoms (Weinbren & Williams, 1958). It was believed to be a zoonotic pathogen transmitted by *Aedes* mosquitoes including *Ae. aegypti* and *Ae. albopictus* (Diallo et al., 2014; G. W. A. Dick, 1952; Macnamara, 1954). Small numbers of cases were identified outside Africa between 1960 and 2007 (Hayes, 2009). The first major outbreak of ZIKV infection occurred on Yap Island in the Pacific in 2007 (Duffy et al., 2009). Following a subsequent outbreak in French Polynesia in 2013 (Cao-Lormeau et al., 2014), large epidemics of ZIKV infection associated with microcephaly in new-born babies and Guillain-Barré syndrome in adults were reported from south America, beginning in Brazil in 2015 (Baud, Gubler, Schaub, Lanteri, & Musso, 2017; Hennessey, Fischer, & Staples, 2016; Schuler-Faccini et al., 2016; Simon et al., 2018; WHO, 2016c). In 2016, microcephaly and neurological disorders were declared by the World Health Organization to be a global public health emergency due to their association with ZIKV infection (WHO, 2016b).

Although the principal vector of ZIKV is a mosquito, it also can be transmitted sexually (D'Ortenzio et al., 2016; Foy et al., 2011; Musso et al., 2015), from mother to foetus (Besnard, Lastere, Teissier, Cao-Lormeau, & Musso, 2014; Oliveira Melo et al., 2016), with a blood transfusion (Musso et al., 2014) and by animal bite (Leung, Baird, Druce, & Anstey, 2015). Like other flaviviruses, ZIKV is a positive-sense single-strand RNA virus with a similar replication cycle to that described above for DENV (Section 1.3.2). ZIKV can be classified into one Asian and two African lineages (Gong, Gao, & Han, 2016; Shen et al., 2016) which may vary in their pathogenicity for humans (Yannick Simonin et al., 2016; Y. Simonin, van Riel, Van de Perre, Rockx, & Salinas, 2017). While African-lineage ZIKV strains may produce acute, self-limiting infections, the Asian ZIKV strains give rise to persistent infections in the central nervous system of foetuses because of their lower yield, poor induction of early cell death and relatively lower infection rate (Anfasa et al., 2017).

There is no specific antiviral therapy or vaccine to prevent or treat ZIKV infection. Disease management is mostly supportive and includes analgesics, rest and lots of fluids. In addition to numerous vaccine candidates being developed (Kim et al.,

2016; Lipsitch & Cowling, 2016; Xu et al., 2018), vector control is a major focus for preventing ZIKV infection.

1.2.3 West Nile virus (Kunjin subtype)

West Nile virus (WNV) is an encephalitic flavivirus that is transmitted by mosquitoes. Unlike DENV and ZIKV, WNV replicates to high titres within avian hosts and is transmitted primarily by mosquitoes of the *Culex* genus (Kramer, Styer, & Ebel, 2008). Symptoms in humans vary from mild headache, fever, sore muscles and joints to more severe meningitis or encephalitis (Goldblum, Sterk, & Paderski, 1954). In Australia, a subtype of WNV called Kunjin virus (WNV_{KUN}) has been isolated most frequently from *Culex annulirostris* mosquitoes (Kay, Fanning, & Carley, 1984; Prow et al., 2016). This mosquito has been demonstrated to be a competent vector in laboratory experiments (van den Hurk et al., 2014). KUNV is not thought to be as pathogenic for humans as WNV (Phillips, Aaskov, Atkin, & Wiemers, 1992). As there is no licensed vaccine against WNV infections in humans, control options are limited to, and concentrate on, reducing mosquito vector populations.

1.3 *TOGAVIRIDAE*

The *Togaviridae* family comprises two genera, *Alphavirus* and *Rubivirus.* Rubella virus is the only member of the genus Rubivirus that is not vector-borne. The *Alphavirus* genus is made up of more than thirty viruses, most of which are vector-borne. Alphaviruses are enveloped, single-strand positive-sense RNA viruses with genomes containing 11 to 12 kb (Figure 1.7). The genome contains two open reading frames (ORFs) which separate the translation of the structural from that of the non-structural proteins. The structural proteins include the capsid protein (C), three envelope glycoproteins (E1, E2 and E3) and a small protein, 6K, with a yet – to – be identified role (Firth, Chung, Fleeton, & Atkins, 2008). The non-structural proteins (nsP1-4) constitute two thirds of the coding region from the 5’end of the genome (Jose, Snyder, & Kuhn, 2009). Figure 1.7 is a schematic of the genome organization and polyprotein processing of the genus *Alphavirus.*

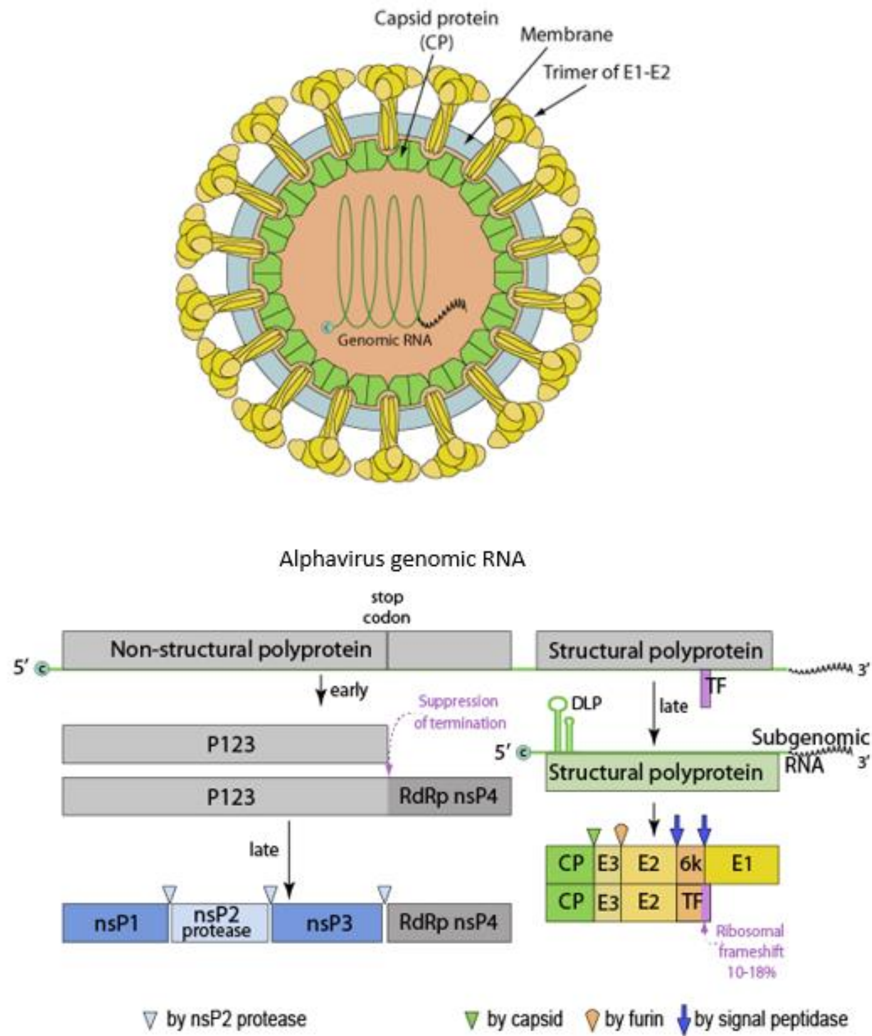

Figure 1.7 Schematic representation of virion, genome organization and polyprotein processing of members of the genus *Alphavirus.* Coloured boxes show viral proteins generated by proteolytic processing (SIB, 2017).

Alphaviruses are transmitted by mosquitoes and infect a variety of mammals and birds. Even though humans can be infected with alphaviruses, they are not the

preferred hosts as they are mostly enzootic. They are maintained and amplified through cycles involving a mosquito vector and a mammalian or marsupial vertebrate host. Most pathogenic alphaviruses cause febrile illness in humans and animals that may progress to either encephalitis or arthralgia/arthritis (Forrester et al., 2012). Although some overlap exists, alphaviruses that cause encephalitic disease have been referred to as New World viruses while Old World viruses result in chronic arthritic diseases (Zacks & Paessler, 2010). Alphaviruses of medical importance include: Chikungunya virus (CHIKV), Barmah Forest virus (BFV), Ross River virus (RRV), Western equine encephalitis virus (WEEV) and Sindbis virus (SINV).

While both alphaviruses and flaviviruses may be vector-borne, the alphaviruses employ a more extensive range of vectors (Russell, 2002). Many alphaviruses use both *Aedes* and *Culex* species as primary vectors, as well as other mosquito genera. For example, RRV has been isolated from more than twenty different species of mosquitoes and at least ten of these have been shown to be competent vectors in the laboratory (Russell, 2002). It is thought that this wide range of vectors extends their geographic boundaries to change and spread their host range and makes them potential emerging or re-emerging pathogens (Forrester et al., 2012). Arboviruses are a significant public health concern in Australia, with 13 of the 75 known arboviruses being implicated in human disease (Russell & Dwyer, 2000). Among the alphaviruses, RRV and BFV are of greatest concern. They cause approximately 5500 and 1000 cases respectively, of clinical disease, each year. Epidemic polyarthritis patients (RRV infection) have arthritic symptoms which commonly last 30 to 40 weeks (Fraser, 1986). Patients with BFV infections have milder and less prolonged symptoms.

SINV is found in Africa and parts of northern Europe where it causes an acute polyarthritis similar to that caused by RRV (Skogh & Espmark, 1982).

1.3.1 Ross River virus

Ross River virus was named after Ross River in Townsville Queensland after the virus was isolated from *Aedes vigilax* mosquitoes collected near the river in 1959. It is both enzootic and endemic in Papua New Guinea and Australia (Hii, Dyke, Dagoro, & Sanders, 1997). With rheumatic manifestations and a characteristic syndrome of rash, Ross River virus disease is the most common disease of

arboviruses in Australia (Fraser, 1986). Even though RRV causes disease only in humans and horses, its transmission cycles involve several mosquito and vertebrate hosts in urban and rural settings (Russell, 2002). In the past, RRV has caused large epidemics in the Pacific nations of Cook Islands, New Caledonia, Samoa, and Fiji (J. G. Aaskov et al., 1981; Fauran, Donaldson, Harper, Oseni, & Aaskov, 1984; Rosen, Gubler, & Bennett, 1981). Disease treatment is palliative given that there are no approved vaccines or antiviral therapies. In Australia, the disease is estimated to cost US$10 million annually (J. G. Aaskov, Chen, Hanh, & Dennington, 1998). RRV has been recovered from more than twenty species of mosquitoes (Russell, 2002) but the vectors most important to RRV transmission cycles involving humans are believed to be *Ae. vigilax, Ae. camptorhynchus* and *Culex annulirostris* (Harley, Sleigh, & Ritchie, 2001).

1.3.2 Barmah Forest virus

Barmah Forest virus (BFV), a member of the *Alphavirus* genus, causes the second most common mosquito-borne disease in Australia and is transmitted primarily by *Aedes* and *Culex* mosquitoes (Russell, 1998). It has been recovered from mosquitoes caught, principally in coastal regions in Australia where tropical temperatures and expanding urban population create ideal habitats for vector proliferation (Naish, Mengersen, & Tong, 2013).

1.3.3 Sindbis virus

Sindbis virus (SINV) is an alphavirus that is transmitted by mosquitoes of the *Culex* genus from avian hosts to humans. It is the causative agent of Ockelbo and Pogosta disease in Europe and the symptoms include fever, arthralgia, and rash. Human infection with SINV has been described in parts of Australia and is most common in North, South and Eastern Africa, Israel, the Philippines, and in northern Europe, particularly in Finland (Lundstrom, 1999). SINV has been used as the prototype alphavirus for studies examining virus replication and protein function (Atkins, 2013). The ability of SINV to initiate and sustain persistent infections in mosquitoes and mosquito cells has been documented (Karpf, Blake, & Brown, 1997).

1.4 VECTOR COMPETENCE

Vector competence refers to the capacity of some arthropods to acquire, amplify and eventually transmit a pathogen to a susceptible host (Beerntsen, James, &

Christensen, 2000). Though often used interchangeably with vectorial capacity, vector competence is a component of vectorial capacity which encompasses behavioural, environmental, cellular and biochemical factors that affect the interaction between vector and pathogen (Beerntsen et al., 2000).

In the laboratory, vector competence experiments attempt to simulate what happens in the field and expose mosquitoes to an arbovirus either in the blood of a viraemic animal, in an artificial blood meal or by intrathoracic inoculation (Smith, Carrara, Aguilar, & Weaver, 2005). This is followed by a period, termed the extrinsic incubation period (EIP), when the virus replicates in the mosquito. Transmission is evaluated by either allowing potentially viraemic mosquitoes to bite a susceptible animal or by quantifying the virus titre in the saliva from infected mosquitoes (Mitchell, 1983). However, it has been difficult to compare results of laboratory-based vector competence experiments undertaken by different groups or at different times because of different means of rearing mosquitoes, different modes of exposure to viruses and variations in methods of quantifying viruses e.g. infectious titres or genome copy number.

Vector competence differs between and within species of mosquitoes (Severson, Brown, & Knudson, 2001) and can vary between laboratory-adapted and field-derived strains (Armstrong & Rico-Hesse, 2001). Vector competence is largely defined by intrinsic factors such as genetics, which in turn influence susceptibility (Woodring, 1996). The susceptibility of a vector to a pathogen refers to the vector's ability to support the development of the pathogen to its infective stage. Following the ingestion of a viraemic blood meal, the transmission of an arbovirus by a mosquito initially involves infecting the midgut and then disseminating into the haemocoel before viral replication occurs in secondary organs (M.J. Turell, 1988). The intrinsic barriers that may need to be overcome for transmission of an arbovirus to occur include: midgut infection barrier, midgut escape barrier, salivary gland infection barrier and salivary gland escape barrier (Hardy, Houk, Kramer, & Reeves, 1983; Mitchell, Miller, & Gubler, 1987). The virus then migrates into the salivary glands before transmission can occur, following an appropriate EIP (M.J. Turell, 1988).

1.4.1 Extrinsic incubation period

In arbovirology, the EIP is defined as the period required for a pathogen to complete its development within an intermediate host involving replication and dissemination within the vector. EIP covers the period from ingestion of an infective blood meal until the vector becomes infectious (i.e., the salivary glands become infected) (Hartemink, Cianci, & Reiter, 2015; Tjaden, Thomas, Fischer, & Beierkuhnlein, 2013). The EIP for DENV is estimated to be between 8 and 12 days (WHO, 2009), based on laboratory observations where no blood-fed mosquitoes were infectious until after 8 days of exposure to virus and all were infectious by 12 days post exposure (T. L. Bancroft, 1906; Schule, 1928; Siler, 1926). However, viral, vector and climatic factors may cause variation in the EIP. Variation in EIP has been observed with different strains of DENV (Sabin, 1952) and distinct genotypes, serotypes and mosquito populations (Armstrong & Rico-Hesse, 2001; Gubler, Nalim, Tan, Saipan, & Sulianti Saroso, 1979; Louis Lambrechts et al., 2012). Longer EIPs also have been observed with attenuated strains of DENV (W. H. Bancroft et al., 1982; Miller, Beaty, Aitken, Eckels, & Russell, 1982). There is increased virus replication and a reduced EIP (i.e. shorter time to transmission) at higher temperatures within the temperature range at which vectors remain viable (Brady et al., 2014; McLean et al., 1974; Watts, Burke, Harrison, Whitmire, & Nisalak, 1987). The dose of ingested virus and route of exposure also affect EIP (Gubler et al., 1979; Louis Lambrechts et al., 2012).

1.5 VECTOR CONTROL

The transmission of arboviruses is dependent on the availability of competent vectors. Therefore, traditional control strategies for eliminating diseases such as dengue have been targeted at reducing vector populations. Moreover, the absence of antiviral drugs or effective vaccines against some arboviruses has made vector control and eradication a core strategy for reducing disease burden. However, the extensive use of insecticides has led to resistance in target species (Ranson et al., 2011; Somwang et al., 2011). Coupled with concerns over the impact of chemical insecticides on non-target species including humans, there is growing interest in finding alternative vector control methods. The use of biological agents such as copepods (Marten, Bordes, & Nguyen, 1994), *Bacillus thuringiensis* (Harwood, Farooq, Turnwall, & Richardson, 2015) and entomopathogenic fungi, as natural predators of mosquito larvae, has proved successful in some cases (Kay et al., 2002).

Currently, research outside that involving insecticides and insecticide resistance is focused on genetic manipulation of mosquitoes (Alphey & Bonsall, 2014; Pfeiler, Flores-López, Mada-Vélez, Escalante-Verdugo, & Markow, 2013; D. Zhou et al., 2014) and modification of insect vector microbiota to block the replication of pathogens (McGraw & O'Neill, 2013). An example of this strategy is found in *Wolbachia*-based biocontrol.

1.5.1 Wolbachia

Wolbachia are endosymbiotic alphaproteobacteria that inhabit many arthropod and filarial nematode hosts (Taylor & Hoerauf, 1999; Werren & Windsor, 2000; Werren, Zhang, & Guo, 1995; Zug & Hammerstein, 2012). *Wolbachia* are considered among the most widespread parasitic bacteria of arthropods (Kittayapong, Baisley, Baimai, & O'Neill, 2000; Werren & Windsor, 2000). They reside in Golgi-associated vacuoles in the cytoplasm, mainly associated with microtubules, and establish themselves in the germ-line of insect hosts to ensure maternal transmission (Yen & Barr, 1971). They possess limited metabolic capacities, lacking biosynthetic pathways to produce amino acids, and therefore acquire most of their resources from their host (Wu et al., 2004). *Wolbachia* are obligate parasites but appear not to be required for host survival. *Wolbachia* survive and replicate by manipulating the reproduction of their hosts. This may lead to sex ratio distortion, parthenogenesis, different survival rates for offspring, feminization of males, reciprocal-cross sterility and cytoplasmic incompatibilities (CI) (K. Bourtzis & Braig, 1999; K. Bourtzis, Braig, & Karr, 2003; Kostas Bourtzis, Dobson, Braig, & O'Neill, 1998; McGraw & O'Neill, 2013; J. H. Werren, 1997; John H. Werren, 1997).

CI is an mechanism that confers a reproductive advantage on *Wolbachia*-infected females, enabling them to reproduce after mating with any male, regardless of *Wolbachia* infection status (Turelli & Hoffmann, 1999). It prevents, or limits, the production of viable offspring after females without *Wolbachia,* or with a different variant of *Wolbachia,* mate with *Wolbachia*-infected males. CI was proposed several decades ago as a means for the eradication of mosquito vectors of pathogens (Laven, 1967). Turelli and Hoffmann (1999) suggested that this phenomenon could be exploited to expedite the spread of *Wolbachia* in order to suppress or eradicate vector mosquito populations. *Wolbachia* has been established in Australian *Aedes* populations (Cairns) with the aim of suppressing DENV transmission (Hoffmann et

al., 2011; Ritchie, Townsend, Paton, Callahan, & Hoffmann, 2015). *Wolbachia* has also has been used in attempts to suppress the mosquito population in French Polynesia (Brelsfoard & Dobson, 2012). Other effects of *Wolbachia* include the ability to shorten the lifespan of its mosquito hosts (McMeniman et al., 2009) and inhibition of virus replication in mosquitoes, termed pathogen blocking.

1.5.1.1.1 Wolbachia-mediated inhibition of pathogen replication

Wolbachia has been shown to reduce the replication of DENV, CHIKV and ZIKV in mosquitoes (Bian, Joshi, et al., 2013; Dutra et al., 2016; Moreira et al., 2009; Walker et al., 2011).

Wolbachia infected *Ae. aegypti* mosquitoes have longer EIPs when infected with DENV than mosquitoes not carrying *Wolbachia* (L. B. Carrington et al., 2018; Ye et al., 2015). However, there are conflicting reports of the effect of *Wolbachia* strains on the replication and transmission of West Nile virus (WNV) (Dodson et al., 2014; Hussain et al., 2013).

The mechanisms by which *Wolbachia* interferes with viral replication and transmission are not yet clear. However, increasing the intracellular density of *Wolbachia* resulted in decreased viral replication in cell lines and in mosquitoes (F. D. Frentiu, Robinson, Young, McGraw, & O'Neill, 2010; Joubert et al., 2016; Lu, Bian, Pan, & Xi, 2012; Martinez et al., 2015). The distribution and density of *Wolbachia* in mosquitoes varies from strain to strain of *Wolbachia* (Dobson et al., 1999) and the proximity to, or co-localization of, *Wolbachia*-infected tissues with sites of viral replication may be a critical determinant of interference with viral replication (Bian, Xu, Lu, Xie, & Xi, 2010). *Wolbachia* infection also may stimulate innate immune responses in mosquitoes, which may have bystander effects on viral replication (Rances et al., 2013; G. Zhang, Hussain, O'Neill, & Asgari, 2013). More recently, a role for the insulin receptor and competition for host amino acids and other resources, such as cholesterol, have been proposed to underlie blocking of pathogen replication by *Wolbachia* (Caragata, Rances, O'Neill, & McGraw, 2014; Geoghegan et al., 2017; Haqshenas et al., 2019).

1.5.1.1.2 Genetic and phenotypic diversity in Wolbachia and implications for anti-viral activity

There is enormous genetic diversity in the genus *Wolbachia,* with sub-division into eight phylogenetic units termed “supergroups” A to H based on genetic

similarities in 16S rRNA and the three protein-coding genes – *gltA, groEL* and filamenting temperature-sensitive Z (*ftsz*) (Casiraghi et al., 2005; Lo, Casiraghi, Salati, Bazzocchi, & Bandi, 2002; Rowley, Raven, & McGraw, 2004; Werren & Windsor, 2000; W. Zhou, Rousset, & O'Neill, 1998). All the supergroups are thought to be members of one species, *Wolbachia pipentis* (Lo et al., 2007). Supergroups A and B are the most common among arthropods (Werren & Windsor, 2000), with supergroups C and D restricted to filarial nematodes (Bandi, Anderson, Genchi, & Blaxter, 1998). The members of the supergroups E, F, G and H occur less frequently in arthropods (Bordenstein & Rosengaus, 2005; Czarnetzki & Tebbe, 2004; Panaram & Marshall, 2007; Rowley et al., 2004). There is no rigorous definition of the term supergroup, but the *Wolbachia* surface protein (*wsp*) has been used regularly for supergroup description (Haine & Cook, 2005; Kyei-Poku, Colwell, Coghlin, Benkel, & Floate, 2005; Malloch & Fenton, 2005; Sintupachee, Milne, Poonchaisri, Baimai, & Kittayapong, 2006; Zeh, Zeh, & Bonilla, 2005).

Wolbachia strains *w*MelPop and *w*Mel, originally from *Drosophila melaganoster* (Min & Benzer, 1997), belong to supergroup A (Baldo et al., 2006) and have been stably transinfected into *Ae. aegypti* mosquitoes (McMeniman et al., 2009; Walker et al., 2011). They partially, or completely, block the replication of a range of flaviviruses such as DENV (Bian et al., 2010; Moreira et al., 2009; Walker et al., 2011), YFV(van den Hurk et al., 2012), WNV (Hussain et al., 2013), ZIKV (Dutra et al., 2016) and the alphavirus CHIKV (Moreira et al., 2009; van den Hurk et al., 2012) *in vitro* and or in mosquitoes. However, field trials in Australia and Vietnam with the *w*MelPop strain of *Wolbachia* indicated it was not possible to replace wild type mosquito vector populations with *Wolbachia*-infected ones. This was attributed to reduced life span, fecundity and egg hatch rates in *Wolbachia*-infected mosquitoes (Nguyen et al., 2015). On the other hand, while the *w*Mel strain of *Wolbachia* invades and persists in mosquito populations with little or no fitness costs (M. S. Blagrove, Arias-Goeta, Di Genua, Failloux, & Sinkins, 2013; M. S. C. Blagrove, Arias-Goeta, Failloux, & Sinkins, 2012; Ferguson et al., 2015) it is not associated with the same reduction in DENV titres in mosquito tissues as *w*MelPop (F. D. Frentiu et al., 2014; Walker et al., 2011; Ye et al., 2015), ZIKV (Aliota, Peinado, Velez, & Osorio, 2016; Dutra et al., 2016) and CHIKV (Aliota, Peinado, et al., 2016; Tan et al., 2017).

The *w*AlbB strain of *Wolbachia,* which naturally co-infects *Ae. albopictus* along with *w*AlbA (Steven P. Sinkins, 2004) belongs to supergroup B (Baldo et al., 2006). Although *w*AlbB has yet to be tested in the field, it has invaded caged populations of *Ae. aegypti* (Joubert et al., 2016; Xi, Khoo, & Dobson, 2005). Importantly, it exhibited complete cytoplasmic incompatibility, maintaining a high and stable density and high maternal transmission fidelity in *Ae. aegypti* when tested at high temperatures (26°–27°C) (Ant, Herd, Geoghegan, Hoffmann, & Sinkins, 2018; P. A. Ross et al., 2017). The cyclical heat stress simulated the field temperature setting in the tropics where mosquitoes harbouring *Wolbachia* are expected to thrive. The resistance of *w*AlbB to this heat fluctuation conferred an advantage on *w*AlbB over *w*MelPop and *w*Mel, which exhibit loss of maternal transmission fidelity and eventual reduction in *Wolbachia* density when exposed to cyclical heat (P. A. Ross et al., 2017; Ulrich, Beier, Devine, & Hugo, 2016). In laboratory-reared mosquitoes, *w*AlbB exerted a fitness cost intermediate between *w*Mel and *w*MelPop. *w*AlbB has been reported to restrict replication of DENV (Lu et al., 2012; Mousson et al., 2012), CHIKV (Raquin et al., 2015), and ZIKV (Schultz et al., 2017). DENV replicated less well in *Ae. aegypti* co-infected with *w*Mel and *w*AlbB than in insects infected with *w*Mel alone (Joubert et al., 2016). Given these potential advantages, *w*AlbB has been proposed as a potential biocontrol candidate.

1.6 THESIS OBJECTIVES

There is little information on variation in EIP for Australian *Ae. aegypti* and *Ae. albopictus* mosquitoes infected with different strains of DENV and other regional arboviruses that pose a threat to human health. Native Australian *Ae. aegypti* populations are being replaced by *Wolbachia*-infected ones and field trials of *Wolbachia*-infected mosquitoes are occurring in parallel in more than ten countries. An assessment of how various *Wolbachia* biocontrol candidates are able to block replication of different DENV serotypes and strains is urgently needed. Furthermore, there has been no estimate of possible benefits of different *Wolbachia* candidates in controlling the transmission of other regional arboviruses.

Therefore, the aims of this study are:

1. To determine the impact of serotype and strain diversity on the EIP of DENV in Australian mosquito populations;
2. To compare the effect of *Wolbachia* infection on the replication of different serotypes and strains of DENV;
3. To determine the effect of *Wolbachia* strain *w*AlbB on the replication of the flaviviruses DENV, ZIKV and KUNV, and alphaviruses BFV, RRV and SINV in mosquito cells *in vitro*.

Chapter 2: Effect of serotype and strain diversity on extrinsic incubation period and vector competence of Australian vector mosquitoes for dengue virus

2.1 ABSTRACT

Dengue viruses (DENV) are the most important arthropod-borne viral pathogens of humans, and they comprise four serotypes which can be further subdivided into genotypes. The time that elapses between a mosquito taking a DENV-infected blood meal and that mosquito becoming infectious is known as the extrinsic incubation period (EIP). EIP is an important parameter that influences the transmission potential of vector mosquitoes. The EIP of DENV is poorly characterized, despite its epidemiological significance, and data on the effect of serotype and strain diversity of DENV on EIP are scant. In this chapter, the impact of DENV serotype and strain diversity on EIP in *Aedes aegypti* and *Aedes albopictus* using virus dissemination as a proxy for EIP is reported. Adult mosquitoes were fed eight strains of DENV representing all four serotypes and assayed for infection with, and dissemination of, virus following 3, 6, 10 and 14-day incubation periods using real-time quantitative RT-PCR. Dissemination rates varied depending on the strain of virus but there were no differences between infection or dissemination rates between *Ae. aegypti* and *Ae. albopictus* mosquitoes. Despite the higher genome copy numbers in *Ae. albopictus* than *Ae. aegypti* bodies, DENV-2 and DENV-4 disseminated faster in *Ae. aegypti.* All DENV strains appeared in at least some *Ae. albopictus* bodies by day 14 and in *Ae. aegypti* bodies by day 10. These data suggest that DENV strain diversity may affect EIP. These results provide the most comprehensive evaluation to date of the vector competence of Australian vector mosquitoes for different serotypes and strains of DENV, with a particular focus on strains circulating in Southeast Asia and the Pacific, and from where most dengue cases are imported into Australia.

2.2 INTRODUCTION

Dengue is caused by four antigenically distinct serotypes of virus, each of which exhibit substantial genetic variation (Calisher & Karabatsos, 1988; R. Chen & Vasilakis, 2011). Dengue viruses (DENV) are among the most important arthropod-borne viruses affecting humans. DENV are members of the Flavivirus genus of the positive-sense RNA viruses. Dengue outbreaks in Australia predate the 20th century (Hare, 1898), but the past two decades have seen an increasing number of outbreaks related to increased international air travel and the consequent importation of viruses with travellers returning to Australia from endemic countries (Hanna & Ritchie, 2009a; Viennet et al., 2014; David Warrilow et al., 2012). While most reports of imported dengue cases have come from Western Australia, local virus transmission is restricted to north Queensland, where there is an established presence of *Aedes aegypti,* the primary vector of DENV in Australia (Russell et al., 2009; Viennet et al., 2014). The increasing number of DENV importations and frequency of dengue epidemics will increase the chances of exposing local human populations to multiple serotypes of DENV and potentially life-threatening, severe dengue (Halstead, 2007). *Aedes albopictus*, a secondary vector of DENV, is a highly successful invasive species and also a competent vector of DENV and other arboviruses (Bonizzoni, Gasperi, Chen, & James, 2013; Gratz, 2004). It thrives in both temperate and tropical climates (Thomas et al., 2012), suggesting that almost all of mainland Australia would be a suitable habitat should it invade (Nicholson et al., 2014). Seasonal transmission of dengue in urban centres like Brisbane or Perth will then become a significant risk and have associated public health costs (Darbro et al., 2017). Established populations of *Ae. albopictus* in the Torres Strait are thought to have been introduced by human maritime traffic from Indonesia rather than by a range expansion out of Papua New Guinea (Beebe et al., 2013; Nicholson et al., 2014) and the species is commonly intercepted at Australia's mainland air and seaports (Sly & Mack, 2018). In 2016, *Ae. albopictus* was implicated as the vector of a dengue outbreak affecting residents on two islands of the Torres Strait (Muzari et al., 2017).

Variation in the competence of mosquito vectors to transmit DENV has been demonstrated through differences in susceptibilities of mosquito populations from different geographic locations to infection with different strains of DENV (Gubler & Rosen, 1976; Tesh, Gubler, & Rosen, 1976). Variation between DENV serotypes and

lineages within genotypes also have been associated with differences in mosquito vector competence (Gubler et al., 1979; Vazeille-Falcoz, Mousson, Rodhain, Chungue, & Failloux, 1999; S. C. Weaver & Vasilakis, 2009). Genetic diversity among human hosts also may be correlated with severity of disease (Descloux, Cao-Lormeau, Roche, & De Lamballerie, 2009). With no antiviral therapy or effective vaccines, dengue prevention efforts have focused on mosquito control strategies which integrate the use of physical barriers, chemical insecticides and biocontrol (Parasites and VectorsHoffmann, Ross, & Rasic, 2015; WHO, 2009). So far, these efforts have been unsustainable and at times ineffective due, in part, to the limited understanding of the optimal entomological targets and the thresholds that regulate DENV transmission.

The time it takes for DENV to develop in the mosquito, from the ingestion of an infected blood meal to the point at which the virus can be detected in the salivary glands (indicating that the mosquito is infectious), is the extrinsic incubation period (EIP) (Schule, 1928). The EIP is a key determinant influencing the intensity and temporal dynamics of transmission since, in association with the life span of a mosquito and vector competence (i.e. the intrinsic susceptibility of a vector to infection, replication and transmission of a virus) (Hardy et al., 1983), it determines the proportion of mosquitoes that may become infectious. Previous studies have reported variation in DENV EIPs in single colonies of *Ae. aegypti*. For example, differences in the dissemination rates of DENV-2 strains have been observed within a laboratory colony (Christofferson & Mores, 2011) and a Southeast Asian genotype of DENV-2 has been shown to have shorter EIP than the American strain which it displaced (Rico-Hesse et al., 1997). Most vector competence studies have tended to focus on the susceptibility of mosquito populations to infection with diverse strains of DENV (da Moura et al., 2015; Poole-Smith et al., 2015) rather than the EIPs associated with each strain.

Considering that most vector competence studies have tended to focus on the susceptibility of mosquito populations to infection with diverse strains of DENV (da Moura et al., 2015; Poole-Smith et al., 2015) rather than the EIPs associated with each strain, it is of great interest to understand the role of EIP in the transmission of DENV. This will be vital in predicting risks and improving future control strategies.

Therefore, this study aimed to determine the impact of serotype and strain diversity on the EIP of DENV. We hypothesized that the EIP of DENV is variable and is influenced by serotype and strain diversity. Hence, the infection and dissemination of eight strains of DENV from Southeast Asia and the Pacific were quantified in Australian *Ae. aegypti* and *Ae. albopictus mosquitoes.* These strains represented all 4 DENV serotypes.

2.3 MATERIALS AND METHODS

2.3.1 Viruses

Eight strains representing the four DENV serotypes (Table 2.1), with two strains per serotype, were used in this study. The strains were obtained from the WHO Collaborating Centre for Arbovirus Reference and Research at the Queensland University of Technology Australia, Dr Alyssa Pyke (Forensic and Scientific Services, Queensland Health) and Myrielle Dupont-Rouzeyrol (Institut Pasteur, New Caledonia). Most of the strains were within 3 to 7 passages post isolation. All viruses were propagated at 27°C in C6/36 cells following infection at a multiplicity of infection (m.o.i.) of ~ 0.01. The m.o.i. is the average number of virus particles infecting each cell. Supernatants containing infectious virus were harvested 5 days post infection for DENV-1 NC-483, DENV-2 55763, DENV-4 NC-39 and DENV-4 MY1261, at 6 days post infection for DENV-2 (VN-130604) and DENV-3 (ET-3) and finally at 7 days post infection for DENV-1 ET-243 and DENV-3 31298. The approach ensured that maximum virus titres were harvested, according to previously determined growth curves for each strain. Virus stocks were then concentrated by ultrafiltration in 100 kDa Amicon filters (Merck Millipore) according to the manufacturer's instructions and aliquoted into sterile 2ml tubes before freezing at – 80°C.

2.3.2 Immuno-focus assay

Titres of infectious virus were quantified by performing an immuno-focus assay based on the immuno-detection of infectious foci developing in cell monolayers. Vero (green monkey kidney) cells were grown in 175-cm^2 flasks in Dulbecco's Modified Eagle Medium with 1 g/l of D-Glucose, 110 mg/l sodium pyruvate and L-glutamine (DMEM, Invitrogen) supplemented with 10% FBS. Twenty-four well plates were seeded with Vero cells at 2.0 x 10^5 cells/ml per well and incubated for 24

h at 37°C and 5% CO_2 to produce confluent monolayers. The growth media was removed, and resultant confluent monolayers of cells were rinsed with sterile PBS and inoculated with 200 µl of serial ten-fold dilutions of virus samples. Viral adsorption was allowed to proceed for 2 h at 37°C with gentle rocking of the flasks every 15 min. Overlay medium (750µl of 8% w/v carboxy-methyl cellulose [CMC, Sigma-Aldrich] in Medium 199 [Sigma-Aldrich]) was added to each well after 2 h and plates were incubated at 37°C in an atmosphere of 5% v/v CO_2/air. After the desired length of incubation, as described above, the CMC overlay was discarded, and the cell monolayers washed in PBS, air dried and fixed with ice-cold (1:1 v/v) acetone-methanol (200µl/well) at room temperature for 5 min. The fixative was then aspirated and the plates air dried again for 1 h. Plates were stored inverted and covered for at least 8 h at 4°C to avoid condensation. Non-specific binding of antibodies to the cell monolayer was blocked by the addition of 200µl of 5% w/v skim milk powder (SMP; Woolworths) in PBS for 1 h at 37°C. DENV-infected cells were detected by the addition of 200µl anti-flavivirus envelope reactive monoclonal antibody 4G2 (TropBio) (Henchal, Gentry, McCown, & Brandt, 1982) diluted 1:1000 in 5% (w/v) SMP in PBS to each well for 1 h at 37°C. After six washes with PBS, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Invitrogen) diluted 1:2000 v/v in 5% w/v SMP/PBS was added to each cell monolayer for 1 h at 37°C. The antibody solution then was discarded, the cell monolayers washed six times with PBS and 200µl of substrate/chromogen (urea hydrogen peroxide/3,3'Diaminobenzidine, Sigma-Aldrich) added and incubated in the dark for 15 minutes.

Table 2.1 Strains of DENV used in vector competence experiments

	Strain	Country of origin	Date of isolation
DENV-1	NC483	New Caledonia	2008
	ET243*	Timor-Leste	2013
DENV-2	VN130604	Vietnam	2002
	55763	Timor-Leste	1985
DENV-3	ET-3*	Timor-Leste	2000
	31298	Cook Islands	1988
DENV-4	MY1261	Myanmar	2000
	NC-39	New Caledonia	2009

*Strains were isolated in Australia from patients infected in Timor-Leste

2.3.3 Mosquitoes

Ae. aegypti and *Ae. albopictus* used in infection experiments were sourced from established colonies at QIMRB. The *Ae. albopictus* colony originated from eggs collected on Hammond Island, Torres Strait in June 2014. The *Ae. aegypti* colony was established from eggs collected from Cairns, Queensland in 2015. Both mosquito colonies were established and maintained in the QIMR Berghofer insectary at 27 (± 1) °C and 75 (± 5) % relative humidity (RH), with a photoperiod of 12h: 12h light: dark (L: D) cycles. Eggs were hatched by submerging in aged de-chlorinated tap water (*Ae. aegypti*) or rain water (*Ae. albopictus*). Larvae were reared at a density of 250 mosquitoes in 3 L of water in 45 x 62.5 cm larval development trays. Larvae were provided daily with 2 ml of 0.05g/ml of fish food (Tetramin) per ~3 L of water. Pupae were transferred to 500ml bowls inside 30 x 30 x 30 cm BugDorm-1 insect rearing cages (Megaview) where adults emerged and mated freely. Adults were maintained on 10% w/v sugar (Woolworths) water and provided with blood meals. The blood meal consisted of 1:1 mix of defibrinated sheep blood (Equicell) and virus (~ 10^7 PFU/ml). Adenosine Triphosphate (ATP) (Sigma-Aldrich) was added to the mixture to a final concentration of 5 mM. Moist filter paper (Whatman No.1) was used to line water-filled containers that were placed in the cages to facilitate oviposition. These papers were checked daily for the presence of eggs and replaced every 2 to 3 days. The filter papers were removed with the attached eggs and allowed to partially dry, placed into a zip-lock bag and stored in a damp container for at least three days. Prior to use in vector competence experiments, eggs were hatched and larvae fed with 2 ml of 0.05 g/ml of fish food (Tetramin) per 3 L of water under the insectary conditions above. Upon pupation, groups of approximately 200 pupae were placed into each BugDorm-1 cage. Females were aspirated into 750 ml gauze-covered containers (Figure 2.1a) and maintained until offered a virus-infected blood meal via a membrane feeding system (Figure 2.1b).

2.3.4 Infection of mosquitoes with DENV

Mosquito infection with DENV occurred in a Biosafety level 3 insectary at QIMR Berghofer. Groups of approximately 100 female mosquitoes (5 to 7 days old) were starved of sugar water for 12 h and deprived of water for 6 h prior to being offered a viraemic blood meal for 1 h via a membrane. The feeding apparatus consisted of a

series of glass membrane feeders with inner blood-filled chambers covered with pig intestinal membrane (sausage casing) and outer chambers connected by pipes circulating water from a 37°C water bath (Figure 2.1c). After 1 h of feeding, mosquitoes were anaesthetized with CO_2 and sorted on a cold table within a Perspex glove box (Figure 2.1d). Mosquitoes that did not feed or that were not completely engorged were discarded. Fully engorged mosquitoes were transferred to 250 ml plastic cups and held for 14 days in environmental chambers at 27 (± 1) °C under a 12h:12h light: dark (L: D) cycle and provided with 10% w/v sugar water *ad libitum*.

Given that *Ae. albopictus* do not engorge as much as *Ae. aegypti* in artificial blood-feeding systems (personal observations), preliminary blood-feeding trials were performed to optimize the protocol and to determine how many mosquitoes needed to be offered a blood meal in order to obtain the required number of engorged insects for the whole experiment. To ensure that the titre of virus in the blood meal had not declined substantially over the feeding period, sub-samples of the blood meal were saved at the completion of feeding for subsequent viral titration by immuno-focus assay in C6/36 cells. Preliminary trials found no substantial decrease in blood meal virus titre over a period of 1 h (Appendix A).

2.3.5 DENV localization in mosquito tissues

Random samples of whole mosquitoes from each DENV strain / mosquito combination were fixed in 4% paraformaldehyde (PFA), dehydrated and embedded in paraffin according to standard procedures. Sections (3 to 4 µM) were fixed to adhesive slides and air dried overnight before being deparaffinated using xylol and dilutions of ethanol in water (100%, 90% and 70%). Slides were incubated in antigen retrieval solution (Biocare Medical) in a decloaking chamber at 125°C for 4 min and allowed to cool at room temperature for 20 min. Indirect immunofluorescence was performed by the QIMR Berghofer HistoTechnology Facility using 4G2-monoclonal antibody (undiluted hybridoma culture supernatant) for the primary antibody and Alexa Fluor 488 labelled donkey anti-mouse antibody diluted at 1:300 in phosphate buffered saline (PBS) for the secondary antibody. Slide sections were incubated in 4’6’-diamidino-2-phenylindole stain (DAPI) at 0.5 µg/ml in wash buffer (1: 20,000) for staining of nuclei. Images were obtained by scanning slides on an Aperio FL fluorescent slide scanner using a x20 objective. DAPI was detected using an excitation wavelength of 345 nm, an emission wavelength of 455 nm and exposure time of 200 ms. Alexa Fluor

488 was detected using an emission wavelength of 495 nm, excitation wavelength of 519 nm and an exposure time of 200 ms.

Figure 2.1 Pictorial representation of the processes involved in feeding *Aedes* mosquitoes blood meals.

2.3.6 Mosquito tissue collection

For each mosquito-virus combination, 20 to 30 mosquitoes were sampled at 3, 6, 10 and 14 days post exposure (d.p.e.). These times were selected in order to capture the EIP of the strains. Mosquitoes were anaesthetised with CO_2 and dissected on a chill plate (4°C) in a glove box. Individual legs and wings were removed using sterile scalpel blades and transferred separately into pre-labelled 1.5 ml microfuge tubes containing four 2.3 mm Zirconia silica beads (Daintree Scientific). In between dissections, blades were sterilized with 80% ethanol to prevent cross-contamination of virus between the bodies, legs and wings of each mosquito-virus group. Mosquito samples, leg/wings and bodies were transferred from – 80°C to RNA Later (Thermo Fisher) to be released from the BSL-3 facility and processed in BSL-2. All samples were stored at – 80°C until tested.

2.3.7 Nucleic acid extraction and quantitative RT-PCR to detect DENV

Samples were thawed on ice and total RNA isolated according to Terradas et al. (2017). Briefly, each sample was homogenized with 100-µl extraction buffer (10mM Tris pH 8.2, 1 mM EDTA, 50 mM NaCl) and 60 µl proteinase K (15mg/ml Bioline) in a mini-beadbeater (Biospec) for 1.5 min and then incubated in a heat block for 5 min at 56°C. The sample was then heated at 98°C for 5 min (to inactivate proteinase K). One-step qRT-PCR was carried out in 384-well Hard-Shell® thin-wall plates (Bio-Rad) with the CFX384™ real-time PCR detection system (Bio-Rad). The reaction mix was prepared using Taqman® Fast Virus One-Step RT-PCR Master Mix Reagents (Applied Biosystems), following the manufacturer's instructions. Each sample was assayed in a 10 µl reaction volume that had 2.5 µl of Taqman® Fast Virus One-Step mix and contained 3 µl of RNA extract, 400 nM of forward primer, 250 nM of probe and 400 nM of reverse primer (Table 2.2) able to amplify and detect all DENV serotypes. The thermal cycling profile consisted of an RT step at 50°C for 6 min, 20 s of RT inactivation and initial denaturation at 95°C followed by 45 cycles of PCR with 30 s of annealing and extension at 60°C and 72°C for 1 s with single fluorescence acquisition. Plasmids of known concentrations were used as cDNA standards. The copy numbers of the plasmid standards were calculated from their concentration and molecular weight. Ten-fold dilution series (10^7 to 10^1 DNA

copies/µl) of the purified linearized plasmids were prepared to generate standard curves and to determine the limit of detection across qPCR runs.

Table 2.2 Nucleotide sequence of the universal DENV primers and probe used in the qRT-PCR assay

	Sequence
Forward primer	5’-AAGGACTAGAGGTTAKAGGAGACCC-3’
Probe	5’-FAM-TCTGGTCTTCAGCGTCAATATGCTGTT-BHQI-3’
Reverse primer	5’-CGWTCTGTGCCTGGAWTGATG-3’

The primers and probe have been described previously (D. Warrilow, Northill, Pyke, & Smith, 2002).

Fresh dilutions of the plasmids were made in triplicate for each qPCR run. To determine the threshold cycle (Ct), the threshold level of fluorescence was optimized so that the standard curve gradient was close to the theoretical value of – 3.30, which indicates 100% PCR efficiency. For all plates, the determination of a successful run was a slope between – 3.0 and – 3.6 for the standard curve and a correlation coefficient (r^2) value above 0.95. The detection limit was 10 genome copies/µl. All samples were analysed in triplicate with each plate containing the following: positive and negative controls, H_20 non-template control (NTC) and a seven-dilution standard curve of DNA standard (10^7 to 10^1 DNA copies). Ct values ≤ 31 were scored as "positive" indicating the presence of DENV.

2.3.8 Analysis

In total, 1260 mosquitoes were processed. The time interval between virus exposure and dissemination to the legs and wings was used as a proxy for EIP. Dissemination was deemed to have occurred if more than 10 DENV RNA copies were detected in legs/wings. The infection rate was calculated as the number of mosquitoes with positive bodies divided by the total number of mosquitoes exposed to DENV. The dissemination rate was calculated as the number of mosquitoes with positive legs/wings samples divided by the number of mosquitoes engorged. Significant differences in infection and dissemination rates within each serotype were determined using Fisher's exact test. The effects of serotype, mosquito species, and their interactions were included as explanatory variables in a general linear model. Multiple comparisons of all pair-wise means with 95% confidence intervals were conducted on statistically significant effects using two-way ANOVA. Post hoc comparisons were conducted using Tukey's test. All statistical tests and graphs were conducted with STATA version 15 and GraphPad Prism 7. Differences were considered statistically significant if $P<0.05$.

2.4 RESULTS

2.4.1 Blood feeding rates

The blood feeding rate was defined as the number of fully engorged mosquitoes divided by the total number of mosquitoes exposed to the virus blood meal. The mean blood-feeding rate was not significantly different for *Ae. aegypti*

mosquitoes (63%, N = 200) and *Ae. albopictus* mosquitoes (57%, N = 200) (Table 2.3).

Table 2.3 Blood feeding rates for *Ae. aegypti* and *Ae. albopictus* for eight DENV strains.

Serotype /strain	Number of engorged mosquitoes (%)	
	Ae. aegypti	*Ae. albopictus*
DENV-1 NC-483	412 (67.9)	119 (59.2)
DENV-1 ET-243	105 (49.5)	105 (49.5)
DENV-2 VN-130604	131 (64.8)	135 (66.5)
DENV-2 55763	149 (68.6)	130 (61.3)
DENV-3 31298	133 (61.9)	110 (55.0)
DENV-3 ET-3	142 (68.6)	108 (52.4)
DENV-4 MY1261	138 (64.9)	128 (61.2)
DENV-4 NC-39	128 (60.3)	112 (56.0)

2.4.2 Infection and dissemination

2.4.2.1 Incubation periods and mosquito species

Ae. aegypti and *Ae. albopictus* mosquitoes were susceptible to infection by all DENV strains tested and exhibited detectable infection of bodies as early as 3 days post exposure (d.p.e.) However, there was no detectable dissemination to legs and wings at 3 d.p.e. regardless of mosquito species or virus strain. In *Ae. aegypti,* more than 60% (5/8) DENV strains used in this study resulted in detectable body infections by 3 d.p.e. By 10 d.p.e., all the virus strains had infected *Ae. aegypti* mosquito bodies. Dissemination was detected at 6 d.p.e. in the five virus strains for which infection was detected at day 3. All remaining virus strains had disseminated by day 10 (Figure 2.2, A, C). In *Ae. albopictus,* the percentage of virus strains that had detectable infection in mosquitoes increased from 37.5% (3/8) to 100% (8/8) at 3 and 14 d.p.e respectively. Virus dissemination in *Ae. albopictus* was first detected at 6 d.p.e., and by 14 d.p.e. all remaining virus strains were detectable in the legs and wings (Figure 2.2, B, D). To test for an overall interaction between mosquito species and infection or dissemination, DENV strains were aggregated and post-exposure incubation periods were divided into simplified intervals: (i) Day 0 to 3, (ii) Day 4 to 6, (iii) Day 7 to 10 and (iv) Day 11 to 14. No significant interactions were found ($P>0.05$, Fisher's exact test of association; Table 2.4) but it was generally true that most virus strains appeared to replicate more slowly in *Ae. albopictus* (Figure 2.2).

2.4.2.2 Serotypes and incubation periods

To test for an association between DENV serotype and incubation period for either infection or dissemination, data for both mosquito species were pooled and post-exposure incubation periods were divided into simplified intervals: (i) Day 0 to 3 vs Day 4+ and (ii) Day 0 to 6 vs. Day 7+. There was no general association between infection or dissemination rates and serotype ($P>0.05$, Fisher's exact test of association; Table 2.5).

Differences in serotypes were also assessed separately for each mosquito species. In *Ae. aegypti,* infection was significantly affected by serotype at every time point except 3 d.p.e. ($P<0.05$, Fisher's exact test of association). Dissemination to legs and wings was significantly faster with a greater proportion of mosquitoes affected at 6 and 10 d.p.e. Serotypes 1 and 3 had the highest and lowest dissemination rates respectively at day 6 (Table 2.5). For *Ae. albopictus* mosquitoes, significant

differences were found in proportions infected at 6, 10 and 14 d.p.e. among serotypes but the proportion of disseminated infections only differed at 6 d.p.e. DENV-1 consistently resulted in higher infection and dissemination rates in both mosquito species except at 14 d.p.e when serotype 3 had the highest dissemination rate (Table 2.6).

2.4.2.3 DENV strains and incubation periods

There were significant differences in the infection and dissemination rates between the eight strains of DENV. In both mosquito species, the proportions infected were significantly different at 6, 10 and 14 d.p.e. while dissemination differed significantly at 6 and 10 d.p.e. ($P<0.05$, Fisher's exact test of association) (Table 2.6). While DENV-2 VN 130604 and DENV-3 31298 disseminated in higher proportions in *Ae. aegypti* and *Ae. albopictus* respectively, DENV-1 NC-483 infected the highest proportions of both mosquito species.

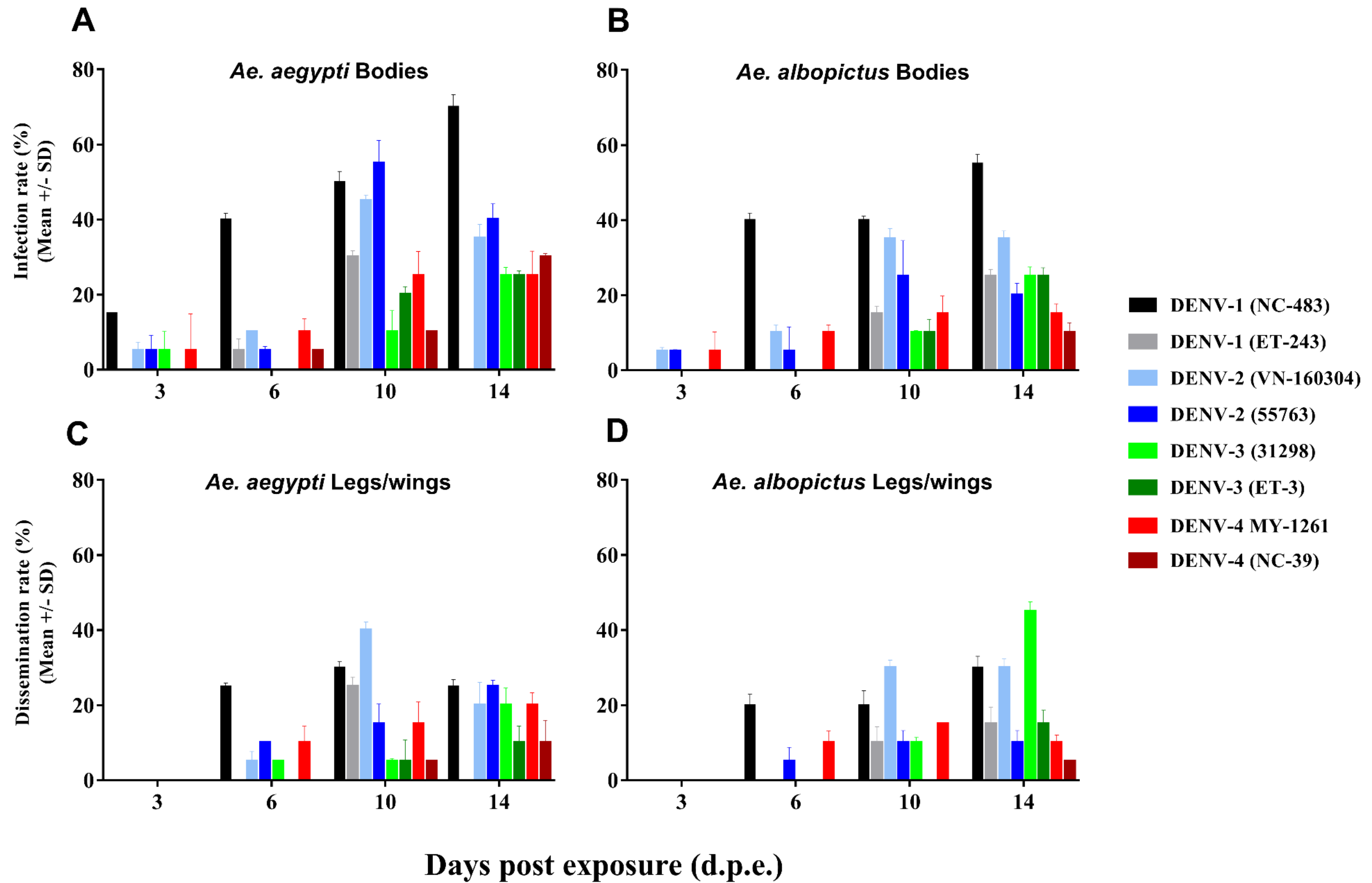

Figure 2.2 Proportion of bodies and legs/wings of *Ae. aegypti* and *Ae. albopictus* infected with DENV. Infection rates (A and B) and dissemination rates (C and D). Strains of DENV are represented by different colours. The standard deviation for each group is shown as bars.

Table 2.4 Number (percentage) of the 8 DENV strains at the time of first appearance of virus in the body and legs/wings aggregated across mosquito species. P-values were calculated using Fisher's exact test of association.

	Incubation period*	**Mosquito species**		**Chi-square value**	**P-value**
		Ae. Aegypti	*Ae. Albopictus*		
Body (Infection)	0 – 3 Days	5 (62.5%)	3 (37.5%)	χ_3^2=2.76	p=0.52
	4 – 6 Days	2 (66.7%)	1 (33.3%)		
	7 – 10 Days	1 (25.0%)	3 (75.0%)		
	11 – 14 Days	0 (0%)	1 (100%)		
Legs/wings (Dissemination)	0 – 3 Days	-	-	χ_2^2=1.61	p=0.62
	4 – 6 Days	5 (62.5%)	3 (37.5%)		
	7 – 10 Days	3 (42.9%)	4 (57.1%)		
	11 – 14 Days	0 (0%)	1 (100%)		

*Incubation period is the time interval of first appearance of virus after exposure to virus blood meal.

Table **2.5** Number (percentage) of DENV serotypes at the time of first appearance of virus in the body and legs/wings aggregated across mosquito species. P-values were calculated using Fisher's exact test of association.

	Incubation Period	**Serotype**				**Chi-square**	**P-value**
		DENV-1	DENV-2	DENV-3	DENV-4		
Body (Infection)	0 – 3 Days	1(12.5%)	4 (50%)	1 (12.5%)	2 (25%)	χ^2_3=5.51	p=0.15
	4 + Days	3(37.5%)	0 (0%)	3 (37.5%)	2 (25%)		
	0 – 6 Days	3(27.3%)	4(36.4%)	1 (9%)	3 (27.3%)	χ^2_3=5.19	p=0.18
	7+ Days	1 (20%)	0 (0%)	3 (60%)	1 (20%)		
Legs/wings (Dissemination)	0 – 3 Days	0	0	0	0	-	NA
	4 + Days	4	4	4	4		
	0 – 6 Days	2 (25%)	3(37.5%)	1 (12.5%)	2 (25%)	χ^2_3=4.12	p=0.33
	7+ Days	2 (25%)	1(12.5%)	3 (37.5%)	2 (25%)		

Table 2.6 Infection with, and dissemination of DENV in *Ae. aegypti* and *Ae. albopictus.* Number (percentage) of mosquitoes either infected or disseminated across the four time points for each mosquito species and virus serotype combination.

Mosquito species	Virus Serotype	n	Days post exposure							
			Day 3		Day 6		Day 10		Day 14	
			Infection	*Dissemination*	*Infection*	*Dissemination*	*Infection*	*Dissemination*	*Infection*	*Dissemination*
Ae. aegypti	DENV-1	40	3 (7.5%)	0 (0%)	9 (22.5%)	5 (12.5%)	16 (40%)	11 (27.5%)	14 (70%)†	5 (25%)†
	DENV-2	40	2 (5%)	0 (0%)	8 (20%)	3 (7.5%)	20 (50%)	11 (27.5%)	15 (37.5%)	9 (22.5%)
	DENV-3	40	1 (2.5%)	0 (0%)	1 (2.5%)	1 (2.5%)	6 (15%)	2 (5%)	10 (25%)	6 (15%)
	DENV-4	40	1 (2.5%)	0 (0%)	4 (10%)	2 (3.33%)	7 (17.5%)	4 (10%)	11 (27.5%)	6 (15%)
	p-value		0.10	NA	<0.001	0.013	0.012	0.029	0.005	0.66
Ae. albopictus	DENV-1	40	0 (0%)	0 (0%)	8 (40%)	4 (20%)	11 (27.5%)	4 (20%)	16 (40%)	9 (22.5%)
	DENV-2	40	2 (5%)	0 (0%)	5 (12.5%)	1 (2.5%)	12 (30%)	8 (20%)	12 (30%)	8 (20%)
	DENV-3	40	0 (0%)	0 (0%)	0 (0%)	0 (0%)	4 (10%)	2 (5%)	17 (42.5%)	11 (27.5%)
	DENV-4	40	2 (5%)	0 (0%)	2 (5%)	2 (5%)	3 (7.5%)	1 (2.5%)	5 (12.5%)	3 (7.5%)
	p-value		0.57	NA	<0.001	0.007	0.003	0.10	0.003	0.17

† Number of mosquitoes per virus strain per time point = 20; Two virus strains per serotype = 40

2.4.3 Virus replication kinetics

The levels of DENV RNA accumulation in bodies and legs/wings harvested after the four incubation periods were quantified by qRT-PCR. Virus titres, defined here as DENV RNA copies/mosquito, are presented in Figure 2.3 for *Ae. aegypti* and Figure 2.4 for *Ae. albopictus*. DENV strains in both mosquito species exhibited typical DENV growth dynamics with mean viral RNA titres peaking at 6 and 10 d.p.e. for bodies and 14 d.p.e. for the legs/wings (data given in Appendices C to J). In *Ae. aegypti*, the highest virus titres detected in bodies was at 10 d.p.e. for DENV-3 (ET-3) with a mean of 6.10 ± (1.2) log_{10} RNA copies/mosquito. In legs/wings, DENV-2 55763 displayed the peak virus titre of 4.62 ± 1.2 log_{10} RNA copies/mosquito at 14 d.p.e. In *Ae. albopictus*, DENV-2 VN130604 and DENV-1 ET243 were present at their highest titres in bodies at 10 d.p.e. with a mean of 5.91 ± (0.8) log_{10} RNA copies/mosquito and 5.90 ± 0.4 log_{10} RNA copies/mosquito, respectively. For dissemination, DENV-1 (NC-483) displayed the highest virus titre at 14 d.p.e. (4.27 ± 1.2 log_{10} RNA copies/mosquito). Overall, DENV strains replicated to higher titres in the bodies of *Ae. albopictus* than *Ae. aegypti* despite having lower infection and dissemination rates for most virus strains. The lowest titres of virus in both mosquito species at 14 d.p.e. followed infection with DENV-4 (NC-39).

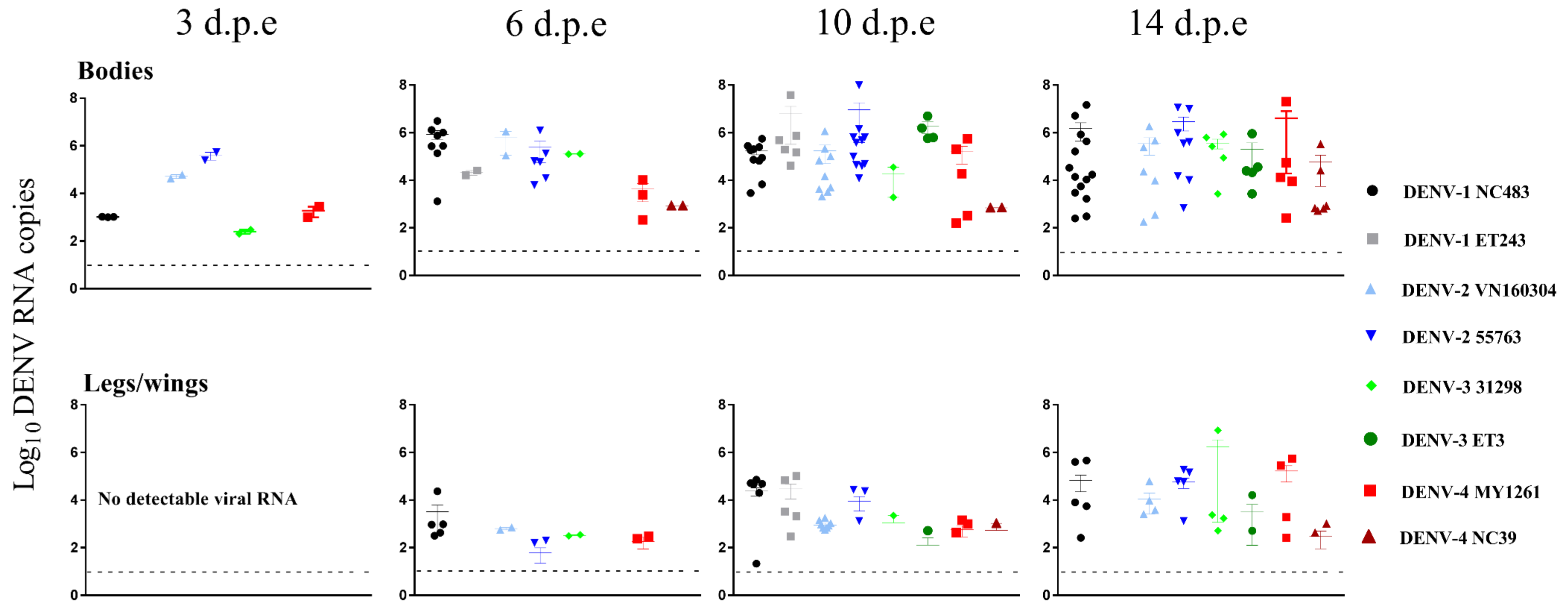

Figure 2.3 Copies of DENV RNA in bodies and legs/wings of *Ae. aegypti* at the intervals indicated after taking an infected blood meal. Twenty samples per virus strain at each time point. Symbols represent individual bodies or legs/wings. The standard deviation for each group is shown as error bars. Dotted lines indicate limit of detection of the qRT-PCR. **Ae. aegypti* exposed to ET-243 died prior to 14 d.p.e. hence no data.

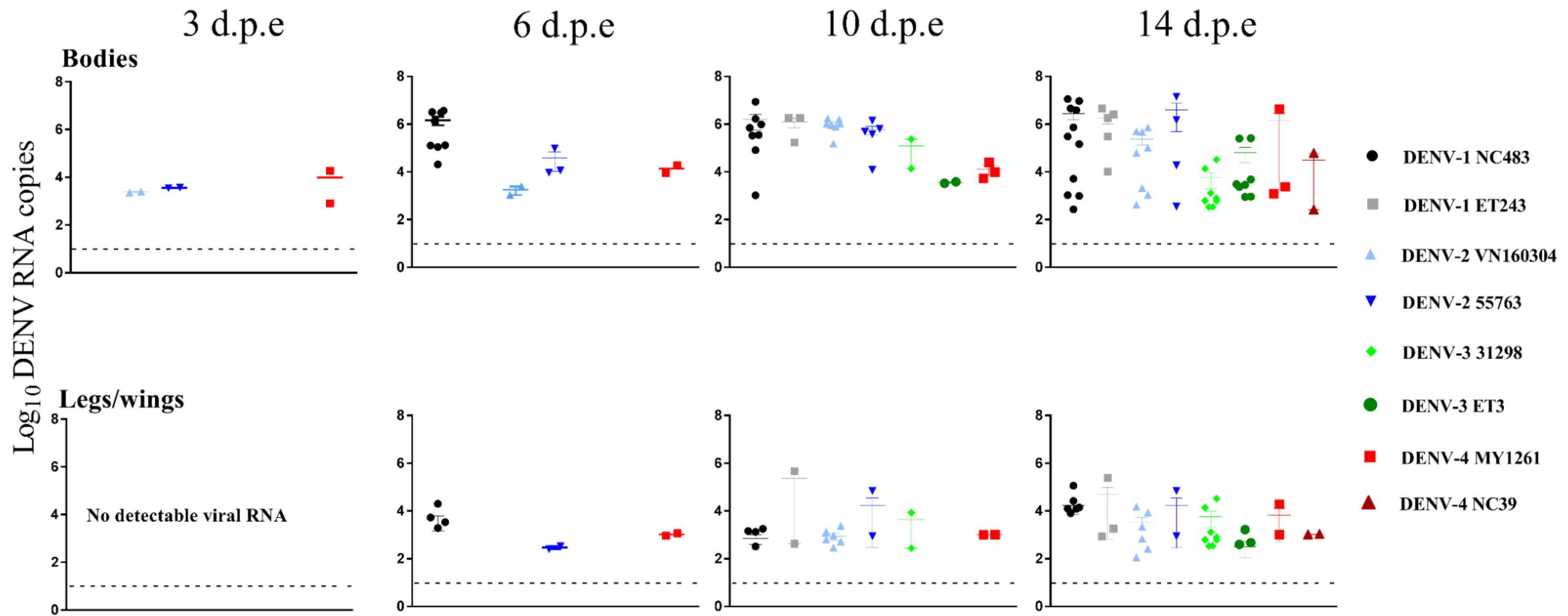

Figure 2.4 Copies of DENV RNA in bodies and legs/wings of *Ae. albopictus* at the intervals indicated after taking an infected blood meal. Twenty samples per virus strain at each time point. Symbols represent individual bodies or legs/wings. The standard deviation for each group is shown as error bars. Dotted lines indicate limit of detection of the qRT-PCR.

2.4.4 Localization of DENV in mosquito tissues

DENV E protein was detected in the midgut and head and thorax in *Ae. albopictus* with considerable staining of the salivary glands 10 d.p.e. to a blood meal containing 1 x 10^7 PFU/ml of DENV-2 (55673). This illustrates that the virus had escaped the midgut barriers and reached the salivary glands (Figure 2.5).

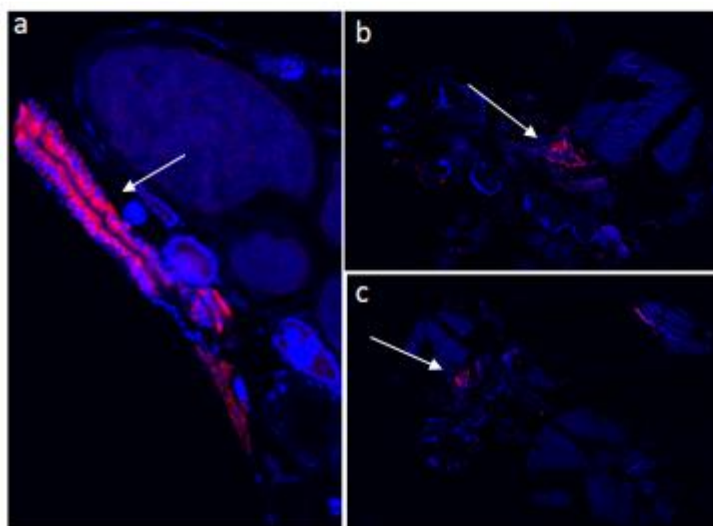

Figure 2.5 Detection of DENV in *Ae. albopictus* 10 d.p.e. to blood meal containing 1 x 10^7 PFU/ml of DENV-2 (55763). Immunofluorescence staining of mosquito sections showing DENV E protein (red). Sections were probed with anti-DENV antibody 4G2 followed by Alexa 594 (red) conjugated secondary antibody. DNA (blue) is stained with DAPI. (a) Midgut (b) Head and thorax (c) Salivary glands. Scale bars: 50 µm

2.5 DISCUSSION

We found significant variation in the interval between the ingestion of a blood meal and the appearance of detectable virus in the legs and wings of both mosquito species, a proxy for EIP, among eight DENV isolates representing the diversity of all DENV serotypes circulating in Southeast Asia and the Pacific. There were no associations found between these intervals and mosquito species when aggregated across the eight strains of DENV. This variation among DENV strains could potentially affect the risk and magnitude of dengue outbreaks. All DENV strains could be detected in at least some *Ae. aegypti* by day 10 and in some *Ae. albopictus* by day 14. Our data suggested that the temporal variation in dissemination reflects viral genetic variation in the transmission kinetics among DENV strains.

Consistent with earlier studies that showed EIP variations within DENV serotype 2 for *Ae. aegypti* (Anderson & Rico-Hesse, 2006; Watts et al., 1987), we further demonstrated that variations in EIP exist in all DENV serotypes. Although it is well established that other factors including temperature (Carrington, Armijos, Lambrechts, & Scott, 2013; Tjaden et al., 2013; Watts et al., 1987) and mosquito genes (Ye et al., 2015) influence the duration of the EIP, the impact of variation in specific strain characteristics is often ignored in models studying vector-borne pathogens (Reiner et al., 2013). Our data support the conclusion that these differences stem from strain diversity and contribute to heterogeneity in DENV transmission dynamics. While it may be impossible to consider individual strain attributes, an attempt to capture the evolutionary and epidemiological potential of different strains should be considered in future dengue control strategies and models.

In a previous report, differences in EIP duration were associated with fitness in virus populations (Anderson & Rico-Hesse, 2006). Variation in EIP could instigate the evolution of a reduced EIP which would be able to confer an advantage by enhancing the probability of transmission. For instance, multiple outbreaks in Australia are due to hundreds of imported cases and local transmissions. Ritchie et al. (2013) previously reported an explosive outbreak of dengue which was caused by a strain of DENV-3 with a relatively short EIP and which was imported from Indonesia to Cairns in 2009. Having evaluated multiple regionally relevant strains of DENV (Hanna & Ritchie, 2009b; Hanna et al., 2006; A. Pyke, 2018) in regionally relevant colonies of mosquitoes, this study concludes that the strain of DENV partly determines the speed

of dissemination of virus in Australian populations of both *Ae. aegypti* and *Ae. albopictus.*

There is limited literature on the influence of serotype on the EIP of DENV and little conclusive evidence of any significant differences (Chan & Johansson, 2012). Most previous studies of the EIP for DENV have employed one or multiple strains of virus in a single species of mosquito or a small number of strains in several mosquito species (Armstrong & Rico-Hesse, 2001; Gubler et al., 1979; Rohani, Wong, Zamre, Lee, & Zurainee, 2009). The comparative susceptibility of both mosquito species has been previously suggested for DENV (da Moura et al., 2015; Whitehorn et al., 2015), and herein we provide evidence of variations in the dissemination rates in Australian populations of *Ae. aegypti* and *Ae. albopictus*. The current study showed that significant variations in infection and dissemination rates were mostly independent of serotype. For example, while DENV-1 NC483 out-performed most of the other DENV strains in both infection and dissemination, DENV-1 ET243 disseminated at a relatively slower rate, especially in *Ae. albopictus.* In general, DENV-2 VN130604 had the highest dissemination rate in *Ae. aegypti* at day 10 while DENV-3 ET3 disseminated most extensively in *Ae. albopictus* at 14 days post infection. This may be due to differences in virus replication kinetics that reflect intrinsic differences between virus strains. It suggests that individual strains will have significant but unpredictable impacts on the speed of transmission by vector populations.

Ae. albopictus has been implicated in DENV transmission in the Torres Strait (Muzari et al., 2017) and should it become established on the Australian mainland, it would present a serious risk of DENV transmission in any urban centres that experience large numbers of viraemic visitors or returning residents (Nicholson et al., 2014). Key to gauging that risk is an understanding of the competence of *Ae. albopictus* for commonly circulating strains of DENV. Our findings that the dissemination rates at 14 days post infection varied from 10 to 50% in *Ae. aegypti* and from 5 to 45 % in *Ae. albopictus*, indicated that both species were equally likely to transmit DENV, albeit different strains. This observation underscores the potential for a comparable vector competence with *Ae. aegypti* in Australia.

Despite exhibiting higher titres of virus in their bodies, fewer *Ae. albopictus* disseminated DENV-2 55763 or DENV-4 NC39 infections. Although not statistically significant, this trend is consistent with previous observations demonstrating that *Ae.*

albopictus was more susceptible to infection but that dissemination of virus took longer than in *Ae. aegypti* (L. Lambrechts, Scott, & Gubler, 2010; Whitehorn et al., 2015). Even though *Ae. albopictus* has been associated with arbovirus transmission since the mid-nineteenth century, it is considered to play a secondary role in transmission of DENV, especially in places where *Ae. aegypti* exists (Bonizzoni et al., 2013; Gratz, 2004). This interspecies difference will have epidemiological consequences. Early infection can shorten the length of the extrinsic incubation period of the pathogen, which can lead to an abrupt increase in virus replication rate. Any reduction in the EIP improves the chances of mosquitoes surviving long enough to incubate the virus and infect a new host. The variation in infection and dissemination rates observed in both mosquito species during this study is in general agreement with previous reports (Whitehorn et al., 2015).

The Pacific strain of DENV-4 NC39 was detected in legs/wings from *Ae. albopictus* less frequently than in legs/wings from *Ae. aegypti.* Although other explanations are possible, these observations are compatible with the infrequent outbreaks of DENV-4 infection in Australia despite the constant introductions of this serotype by travellers (David Warrilow et al., 2012).

DENV-3 31298 was poorly disseminated in blood-fed *Ae. aegypti* mosquitoes. However, it is interesting to note that the dissemination of DENV-3 31298 in *Ae. albopictus* occurred more rapidly and frequently than any other strain at 14 days post infection. This finding again suggests that *Ae. albopictus* will be more than a marginal contributor to DENV transmission. It is possible that DENV strain-specific diversity may largely contribute on what defines primary or secondary vectorship in mosquitoes.

The appearance of detectable virus in the legs and wings of both mosquito species was used as a proxy for EIP due to the logistical constraints of saliva expectoration in large numbers of mosquitoes. The presence of virus in the legs and wings was assumed to indicate potential transmission based on previous evidence that dissemination of DENV to legs and wings of mosquitoes is a reliable indicator of whether mosquitoes are capable of transmitting DENV (Gubler et al., 1979; M. J. Turell, Beaman, & Tammariello, 1992; Vazeille, Rosen, Mousson, & Failloux, 2003). Although the infectious titre of the disseminated virus is positively correlated with the likelihood of detecting DENV in saliva (Louis Lambrechts et al., 2012), calculations for the length of EIP based on dissemination alone may possibly be overestimates. We

cannot exclude that some virus titres from legs/wings samples that were too low to be detected may have been high enough to result in virus release in saliva. Another feature of this study which may affect how the outcome is compared to other transmission potential systems is the use of qRT-PCR to quantify virus titre in mosquito tissues. Even though this protocol is well established in our laboratory, immuno-plaque assay is better used to detect live virus and/or measure the amount of live virus in tissue samples. However, this method was not employed because of the large numbers of mosquitoes analysed. A third issue in our study is that the mosquitoes were exposed to a single infectious dose of DENV. While the dose used in the virus blood meal, 10^7 PFU/ml, may be considered high enough and comparable to human viraemia, a range of infectious doses may have presented a different set of parameter estimates given that infectious dose is one of the powerful determinants of EIP duration and DENV infection probability in *Ae. aegypti* (Duong et al., 2015; Ferguson et al., 2015; Nguyen et al., 2015). Lastly, given that virus stocks were generated through a few cell culture passages, there may have been adaptation to mosquito cells. However, there is no known evidence that adaptation to mosquito cell culture would interfere with the speed of virus dissemination *in vivo.*

One important characteristic of our results is the significance of the observed variation in transmission kinetics among the eight DENV isolates. There is no evidence that there is a difference between the duration of the EIP of all the virus strains tested in both mosquito species. Put in a loose epidemiological perspective, this implies that EIP duration which is an influential factor underlying DENV transmission, may not be different for *Ae. aegypti* and *Ae. albopictus.* However, with our sample size, caution must be applied, as the findings might not be generally applicable to other populations. Further studies will validate this lack of difference by sampling more populations of *Ae. aegypti* and *Ae. albopictus* with diverse strains of DENV.

This is the first parallel evaluation of infection and dissemination of multiple strains of DENV in mosquitoes from colonies of Australian *Ae. aegypti* and *Ae. albopictus.* The study found that EIP varies significantly from one strain of DENV to the next but not as much between *Ae. aegypti* and *Ae. albopictus*. Furthermore, it highlighted the heterogeneity in DENV transmission dynamics. As EIP is a parameter of operational importance, these data confirm the public health hazards associated with

Ae. albopictus, and provide empirical data for dengue modelling efforts to confirm the response parameters for vector management operations.

Chapter 3: Effect of *Wolbachia* on the replication of different strains of dengue in mosquito cells

3.1 ABSTRACT

DENV causes more morbidity and mortality in humans than any other arbovirus. A biocontrol strategy pioneered in Australia, and currently being trialled in several other countries, utilizes *Wolbachia,* a bacterial endosymbiont of arthropods, to restrict DENV transmission. Recent studies have shown that leading *Wolbachia* strains, *w*Mel and *w*MelPop, proposed for biocontrol may be compromised by cyclical heat stress in the field. It is not clear how much variation there is in the magnitude of the inhibition of replication of different strains of DENV by alternative *Wolbachia* strains. Using paired *Wolbachia*-infected and uninfected *Aedes albopictus*-derived cell lines and nine DENV isolates, our results suggest that a heat-resistant strain, *w*AlbB, was associated with similar, and significant, reductions in the yield of DENV such as *w*MelPop following infection with all four serotypes. However, there were significant differences between the magnitudes of the inhibition of replication of different strains of DENV. In this study we have shown that the reduction in DENV titre by *w*AlbB in mosquito cell culture is similar to that of *w*MelPop, and that the magnitude of reduction varied substantially with virus strain, indicating genetic contribution from individual virus strain attributes and suggesting that *Wolbachia*-mediated blocking may eventually be expressed differentially across virus strains.

3.2 INTRODUCTION

Dengue is the most important arthropod-borne viral disease of humans and is caused by dengue virus (DENV), an RNA virus of the genus *Flavivirus.* More than 2 billion people are at risk of DENV infections (Bhatt et al., 2013). This is due, in a large part, to the global expansion of its mosquito vectors, *Aedes aegypti* and *Aedes. albopictus.* There are no effective vaccines or antiviral drugs to prevent or treat this disease. Attempts to prevent dengue by controlling the mosquito vectors have been ineffective, unsustainable or both. However, a new vector-biocontrol method has been developed which employs mosquitoes transinfected with strains of *Wolbachia* bacteria to block the replication of DENV in these vectors.

Wolbachia are maternally-transmitted, obligate, intracellular, bacterial endosymbionts of arthropods and an estimated 40% of all insects are thought to be infected with various strains of these bacteria (J. H. Werren, 1997; Zug & Hammerstein, 2012). They invade and spread rapidly through *Wolbachia*-free insect populations by cytoplasmic incompatibility (CI). CI is a phenotype that enables *Wolbachia* to manipulate their host's reproduction and so increase the proportion of infected females over successive generations (Hoffmann et al., 2011; Steven P. Sinkins, 2004; Werren & Jaenike, 1995). *Wolbachia* inhibit the replication of a range of flaviviruses including DENV (Ant et al., 2018; Bian et al., 2010; McMeniman, Hughes, & O'Neill, 2011; Moreira et al., 2009; Walker et al., 2011), YFV (van den Hurk et al., 2012), WNV (Hussain et al., 2013), ZIKV (Dutra et al., 2016; Schultz et al., 2017) and the alphavirus CHIKV (Moreira et al., 2009; van den Hurk et al., 2012) in mosquitoes and *in vitro.*

Different strains of *Wolbachia* vary in their capacity to inhibit replication of viruses in mosquitoes (Ant et al., 2018; Joubert et al., 2016). Some of this variation is linked to the intracellular density of *Wolbachia* with a high density in host tissues being associated with greater inhibition of virus replication (M. S. Blagrove et al., 2013; M. S. C. Blagrove et al., 2012; F. D. Frentiu et al., 2010; Joubert et al., 2016; Lu et al., 2012). For example, the *w*MelPop strain of *Wolbachia*, originally derived from *Drosophila melanogaster,* grows to high density in host cells and has been associated with the strongest inhibition of DENV replication observed to date (Ferguson et al., 2015; Moreira et al., 2009; van den Hurk et al., 2012).

Field trials with the *w*MelPop strain of *Wolbachia* in Australia and Vietnam did not result in replacement of wild vector populations with *w*MelPop-infected ones, despite the high density of *w*MelPop in the *Ae. aegypti* mosquitoes that were introduced. This was believed to be due to reduced life spans, fecundity and egg hatch rates in the introduced mosquitoes (Nguyen et al., 2015). *Ae. aegypti* mosquitoes infected with another strain of *Wolbachia, w*Mel, also are being evaluated in field trials for their ability to introduce this strain of *Wolbachia* into dengue endemic areas (Hoffmann et al., 2014; Hoffmann et al., 2011). Although *w*Mel is able to inhibit the replication of a number of arboviruses, it is not as efficient as *w*MelPop in inhibiting the replication of DENV (F. D. Frentiu et al., 2014; Walker et al., 2011; Ye et al., 2015), ZIKV (Aliota, Peinado, et al., 2016; Dutra et al., 2016) and CHIKV (Aliota, Peinado, et al., 2016; Tan et al., 2017). However, *w*Mel can invade and persist in mosquito populations and it imposes less fitness costs on host mosquitoes than does *w*MelPop (Hoffmann et al., 2014; Walker et al., 2011).

Protocols for the evaluation of *Wolbachia* infection in mosquitoes, and the effect of these infections on the replication of arboviruses, have had to be reassessed following the observation that the densities of *w*Mel and *w*MelPop in larvae and adult mosquitoes are reduced when exposed to the fluctuations in temperature experienced in the field. These temperature fluctuations (26°C to 37°C) also reduce the rate of maternal transmission of *w*Mel and *w*MelPop (P. A. Ross et al., 2017; Ulrich et al., 2016).

A third *Wolbachia* strain being considered for vector biocontrol is *w*AlbB. It is a natural symbiont of *Ae. albopictus* mosquitoes, which are also are co-infected with the *w*AlbA strain of *Wolbachia* (Steven P. Sinkins, 2004). Although natural *Wolbachia* symbionts have been found to be less effective in inhibiting arbovirus replication than transinfected ones (Bian et al., 2010; Bian, Zhou, Lu, & Xi, 2013), *w*AlbB has been observed to reduce replication of DENV (Lu et al., 2012; Mousson et al., 2012), CHIKV (Raquin et al., 2015) and ZIKV (Schultz et al., 2017) in *Ae. albopictus* mosquitoes. *w*AlbB has an effect on the fitness of host mosquitoes intermediate between that of *w*MelPop and *w*Mel (Axford, Ross, Yeap, Callahan, & Hoffmann, 2016; P. A. Ross, Endersby, & Hoffmann, 2016). In contrast to *w*Mel and *w*MelPop, the intracellular density and rate of maternal transmission of *w*AlbB is relatively stable when host mosquitoes are exposed to levels of heat stress observed in nature (P. A. Ross et al., 2017).

Although the magnitude of inhibition of virus replication is influenced by the strain of *Wolbachia* present (Joubert et al., 2016; Moreira et al., 2009; Walker et al., 2011), the extent to which DENV replication is affected also may be influenced by the extensive genetic diversity between DENV strains (S. C. Weaver & Vasilakis, 2009). For instance, when the replication of DENV from fresh viraemic blood from dengue patients was compared in *w*Mel-infected and uninfected *Ae. aegypti,* that of DENV-1 was reduced less than that of DENV 2, 3 and 4.

The hypothesis tested here was that the replication of each strain of DENV would be affected to a different extent by the presence of *Wolbachia* in host cells. To test the hypothesis, the ability of *w*AlbB to inhibit the replication of nine strains of DENV, representing the four serotypes, was compared with that of *w*MelPop.

3.3 MATERIALS AND METHODS

3.3.1 Cells

The *Aedes albopictus* cell line – C6/36 (Igarashi, 1978) was maintained in RPMI-1640 media containing 25 Mm HEPES (Sigma-Aldrich), supplemented with 10% v/v heat-inactivated foetal bovine serum (FBS, Gibco) and 1% v/v L-glutamine (Invitrogen) at 28°C. The *w*MelPop-infected cell line, C6/36-*w*MelPop, was obtained from Prof. Scott O'Neill (Monash University, Australia). The *w*AlbB-infected cell line, designated C6/36-*w*AlbB, was generated by introducing *Wolbachia* strain, *w*AlbB from RML12 *Ae. albopictus* (a gift from Prof. Jason Rasgon, Pennsylvania State University) into C6/36 cells using the shell vial technique (Dobson, Marsland, Veneti, Bourtzis, & O'Neill, 2002; F. D. Frentiu et al., 2010). C6/36-*w*AlbB cells were maintained in a 2:1 mixture of RPMI-1640 media buffered with HEPES (Sigma-Aldrich) and Schneider's Drosophila's Modified media (Lonza) supplemented with 10% v/v FBS and 1% v/v L-glutamine. All insect cells were maintained at 28°C and subcultured once each week.

3.3.2 Viruses

Nine strains belonging to the four DENV serotypes (Table 2.1) were described in Chapter 2, Section 2.3.1.

3.3.3 Fluorescent *in situ* hybridization

C6/36, C6/36-*w*MelPop and C6/36-*w*AlbB cells (600 µl of 2.5 x 10^6 cells/ml) were cultured in sterile 8 chamber slides (Bio-Basic, Canada) using RPMI-1640

supplemented with 10% v/v FBS (Gibco). Cells were seeded into duplicate wells and incubated for 24 h at 28°C. Media then was aspirated from the wells and the well chamber detached from the slide. Cells on the slides were washed with sterile phosphate buffered saline (PBS), fixed with ice-cold 4% paraformaldehyde (PFA) (VWR Alfa) at 4°C for 30 min and the slides washed three times in 0.1M phosphate buffer. The cells were dehydrated by sequential immersion of the slides, at 2 min intervals, in 70%, 95% and 100% v/v ethanol/water at room temperature. Hybridization was conducted overnight at 37°C in a humidified container with hybridization cocktail II+50% formamide (Astral Scientific) containing 100ng/µl of *Wolbachia*-specific 16S rRNA W2 oligonucleotide probe (5'-CY5-CTTCTGTGAGTACCGTCATTATC-3', (Moreira et al., 2009). The W2 probe was synthesized by Integrated DNA Technologies. After hybridization, the slides were rinsed, at room temperature, in wash buffer 1 (1X SSC-10mM DTT [AppliChem]), washed twice in wash buffer 1 and then twice in wash buffer 2 (0.5X SSC + 10mM DTT). All washes were performed at 55°C for 15 min each. Cells then were stained with 0.5 µg/ml 4'6'-diamidino-2-phenylindole (DAPI Sigma-Aldrich) / wash buffer 2 for 10 min at room temperature in the dark followed by two rinses in wash buffer 2 and two rinses in distilled water. ProLong Gold anti-fade solution (Invitrogen) and coverslips were added to the slides and images captured on an epifluorescent microscope (Zeiss).The same microscope settings were used for all samples. The combination of oligonucleotide probe W2 labelled with carbocyanine (red) at its 5'end and 4'6'-diamidino-2-phenylindole stain (DAPI) to stain DNA allowed simultaneous *in situ* detection of both *Wolbachia* (red) and cell nuclei (blue). Signals from five separate microscope fields from three independent samples were analysed.

3.3.4 Growth kinetics of DENV in mosquito cell cultures

C6/36 cells and *Wolbachia*-infected C6/36 cells (C6/36-*w*MelPop and C6/36-*w*AlbB) were seeded into 24-well plates at 2.5 x 10^5 cells per well and allowed to attach for 24 h at 28°C. Cells were infected, in triplicate, with virus at m.o.i. of 0.1, 1, 10 and 20 using serum-free RPMI-1640 media (Sigma-Aldrich), with virus-free media as control. Each "treatment" comprised of one cell line, one virus strain and one m.o.i., harvested at each time point (Figure 3.1). Following adsorption for 2 h, the virus inoculum was removed and the cell monolayer washed twice with sterile PBS and incubated at 28°C in fresh maintenance media (RPMI-1640 containing 25 mM HEPES

[Sigma Aldrich] supplemented with 2% v/v FBS [Gibco] and 1% v/v Glutamax [Sigma Aldrich]) in an atmosphere of 5% v/v CO_2/air. At each time point (2, 4, 6 and 8 days post infection) all supernatants from each of three culture plate wells per treatment were collected into separate tubes and stored at – 80°C. Cells were scraped from each well, collected in separate tubes and frozen at – 80°C in order to estimate intracellular *Wolbachia* densities. Virus titres in culture supernatants were determined by immuno-focus assay (Section 3.3.5 below).

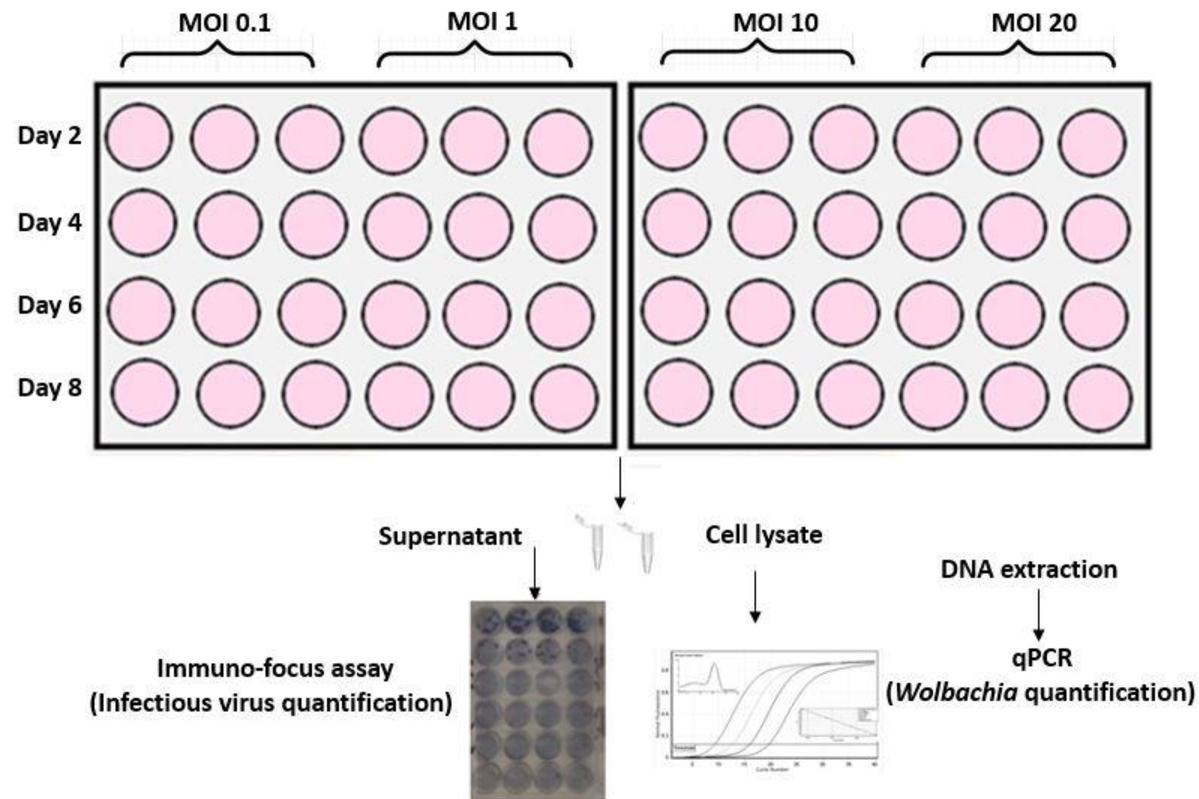

Figure 3.1. Schematic of viral growth curve determinations. C6/36 cells with and without *w*AlbB or *w*MelPop were infected with DENV. Following infection, cells were washed and fresh growth media added. Cell culture supernatant and cell lysates were harvested at the time points indicated to quantify yields of virus and to determine *Wolbachia* titres.

3.3.5 DENV immuno-focus assay

Virus titres were determined by immuno-focus assay on Vero cells infected with serial ten-fold dilutions of virus. Vero (African green monkey kidney) cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) L-glutamine (Sigma-Aldrich) supplemented with 10% v/v heat-inactivated foetal bovine serum (FBS, Gibco) at 37°C. Twenty-four well plates were seeded with Vero cells at 2.5 x 10^5 cells per well and incubated overnight at 37°C in an atmosphere of 5% v/v CO_2/air to produce confluent monolayers of cells. The growth media was removed and monolayers of cells were rinsed with sterile PBS pH 7.2 before addition of 200 µl of serial ten-fold dilutions of virus samples. Virus was allowed to adsorb for 2 h at 37°C with gentle rocking of plates every 15 min. Overlay media (750 µl of 8% w/v carboxy-methyl cellulose [CMC, Sigma-Aldrich] in Medium 199 [Sigma-Aldrich]) was added to each well at the conclusion of the adsorption and plates were incubated at 37°C in an atmosphere of 5% v/v CO_2/air. After the desired length of incubation (5 days for DENV-1 and 2, 6 days for DENV-3 and 7 days for DENV-4), the CMC overlay was removed and the cell monolayers washed twice in PBS, air dried and fixed with ice-cold (1:1 v/v) acetone-methanol (Thermo Fisher) (200µl/well) at room temperature for 5 min. The fixative then was aspirated and the plates air dried again for 1 h. Plates were stored inverted and covered for at least 8 h at 4°C to prevent condensate forming on the monolayers. Non-specific binding of antibodies to the cell monolayer was blocked by the addition of 200 µl of 5% w/v skim milk powder (SMP, Woolworths) in PBS for 1 hr at 37°C. DENV-infected cells were detected by the addition of 200 µl anti-flavivirus monoclonal antibody 4G2 (Henchal et al., 1982; TropBio Cairns) diluted 1:1000 in 5% w/v SMP/PBS to each well for 1 h at 37°C. After six washes with PBS, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Invitrogen) diluted 1:2000 v/v in 5% w/v SMP/PBS was added to each cell monolayer for 1 h at 37°C. The antibody solution then was discarded, the cell monolayers washed six times with PBS and 200µl of substrate/chromogen (urea hydrogen peroxide/3, 3'Diaminobenzidine, Sigma-Aldrich) added and the plates incubated in the dark for 15 min at room temperature. Plaques of infected cells appeared as brown-black spots.

3.3.6 Real-time PCR to quantify *Wolbachia*

DNA was extracted from C6/36, C6/36-*w*AlbB and C6/36-wMelPop cell lysates using the Promega ReliaPrep™ gDNA Tissue Miniprep System (Promega) according

to the manufacturer's instructions. *Wolbachia* DNA was detected using primers targeting the *Wolbachia* surface protein (*wsp*) in a real-time SYBR-Green based assay and was used to estimate the *Wolbachia* genome copy, which was normalized against that of the *Ae. albopictus* mosquito host gene *Rps*7. The primer sequences for both genes are shown in Table 3.1.

Table 3.1 Sequences of oligonucleotide primers used in the *Wolbachia* qPCR assay

Primer	Forward	Reverse
wsp-	CATTGGTGTTGGTGTTGGTG	ACACCAGCTTTTACTTGACCAG
TM2	CTCGACCGCTGTGTACGAT	CAATGGTGGTCTGCTCTGGTTC
rps7		

The primers have been described previously (F. D. Frentiu et al., 2014)

The SYBR qPCR assays were performed in a 384-well plate in a final volume of 15µl per reaction. Each reaction contained 10 µl of 1 X SYBR mastermix (PowerUp™ SYBR Green Master Mix, Applied Biosystems, Thermo Fisher Scientific), 1 µl each of the forward and reverse primers and 3 µl water. DNA recovered from cells (4 µl) was added to each reaction, amplified and the cDNA detected in real time on the Bio-Rad CFX384™ real-time PCR detection system (Bio-Rad, USA) using the following conditions for cycling and temperature regimes: 1 cycle at 50°C followed by one at 95°C, each for 2 min, and then 40 cycles at 95°C for 15 s and 60°C for 15 s. The final extension step was for 1 min at 72°C. qPCRs were performed in triplicate. Differences in the crossing point (Cp) of the two genes (*wsp* and *rps7*) were averaged to obtain an estimate of *Wolbachia* density. These values were transformed by $2^{-\Delta\Delta Ct}$ to obtain relative *Wolbachia* densities.

3.4 RESULTS

3.4.1 *Wolbachia* infection in mosquito cell lines

Wolbachia infection in C6/36 cell lines could be detected by FISH (Figure 3.2). The proportion of cells containing *Wolbachia* 16S RNA ranged from 70 to 100 per cent (Table 3.2). The CY5 label associated with detection of *Wolbachia* was evenly distributed throughout C6/36 cells infected with *w*MelPop and *w*AlbB throughout the course of the experiment (Figure 3.2, top panels).

Even though the proportion of cells with *w*AlbB fluctuated from passage to passage, an increase in the proportion of cells infected with *w*AlbB was observed from passage 28 when Schneider's Drosophila's Modified media (Lonza, 04-351Q) was added to RPMI-1640 (Sigma) (2:1) for routine cell maintenance (Figure 3.2). The proportion of C6/36 cells infected with *w*AlbB stabilized at passage 40 and was comparable to that of C6/36 cells infected with *w*MelPop throughout the course of the subsequent experiments in which the cells were infected with DENV.

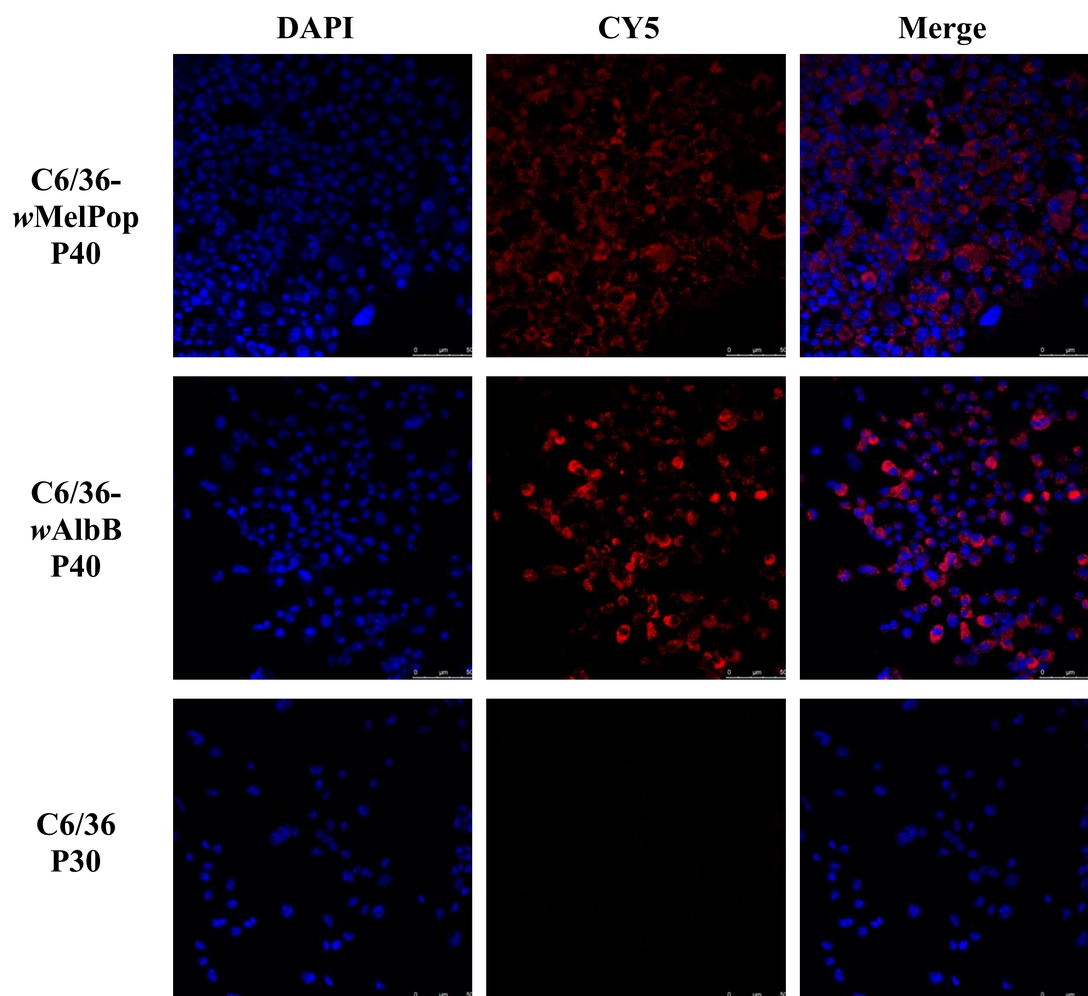

Figure 3.2 *Wolbachia* in C6/36 cells detected by Fluorescent *in situ* hybridization (FISH). CY5-labelled oligonucleotide probe (red) detected *Wolbachia* 16S rRNA in the cytoplasm of C6/36 cells (transinfected with *w*MelPop) at passage 40 and in C6/36 cells (transinfected with *w*AlbB) at passage 40. Cell nuclei (blue) were labelled with 4,6-diamidino-2-phenylindole (DAPI). Scale bar, 50 µm.

Table 3.2 Proportion of C6/36 cells infected with the *Wolbachia* strains shown.

Cell line	Passage No.	No. of cells infected / total cells counted (%)*
C6/36	44	0/98 (0)
C6/36-*w*AlbB	31	56/80 (70)
C6/36-*w*AlbB	40	72/76 (95)
C6/36-*w*MelPop	40	97/97 (100)

*Mean of five independent microscope fields from three independent samples.

3.4.2 Growth kinetics of DENV in *Wolbachia*-infected C6/36 cells

The titres of virus in culture supernatant of C6/36 cells infected with *Wolbachia* were lower than those for the corresponding cells not infected with *Wolbachia,* in almost all experiments with all DENV strains and serotypes and at every time point and m.o.i tested (Figures 3.3 to 3.11). The kinetics of DENV production in *Wolbachia*-infected cells varied from virus to virus and with m.o.i. There was no consistent pattern of one strain of *Wolbachia* inhibiting DENV replication more effectively than another and in some instances (e.g. DENV1 NC-483 and DENV1 ET243) the strain having the strongest effect on DENV replication varied with the m.o.i. However, at day 8, there was no significant difference in the reduction of virus titres due to infection with either strain of *Wolbachia* except at m.o.i. of 1 where DENV 2 ET300 grew to approximately the same titre in *Wolbachia*-uninfected C6/36 and C6/36-*w*AlbB cells, and DENV-4 MY1261 had higher titre in C6/36-*w*AlbB than C6/36 cells (Table 3.3). There were significant differences in the effect of *Wolbachia* infection on the replication of different strains of DENV by day 8 at each m.o.i. The largest differences in the effect of *Wolbachia* on DENV replication were observed at the lowest m.o.i. with the reduction in day 8 viral yields ranging from 0.7 to 6.7 log_{10} at m.o.i. 0.1, to 0.7 to 5.5 log_{10} at m.o.i. 1, to 2.0 to 5.3 log_{10} at m.o.i.10, and 1.7 to 4.9 log_{10} at m.o.i. 20.

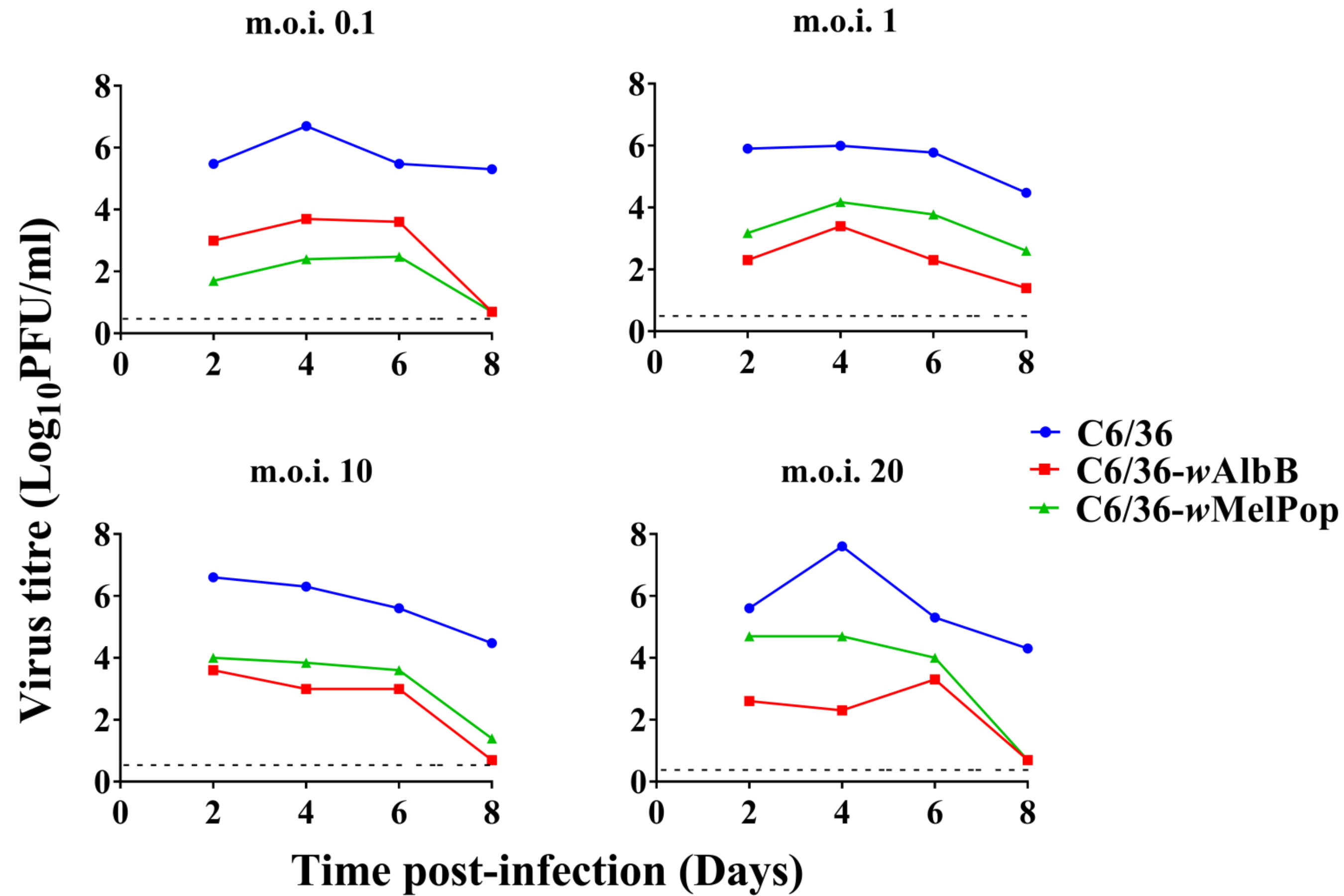

Figure 3.3 Kinetics of production of DENV-1 NC-483 in C6/36 (blue), C6/36-*w*AlbB (red) and C6/36-*w*MelPop (green) cells infected at m.o.i. of 0.1, 1, 10 or 20. Limit of detection shown as a dashed line.

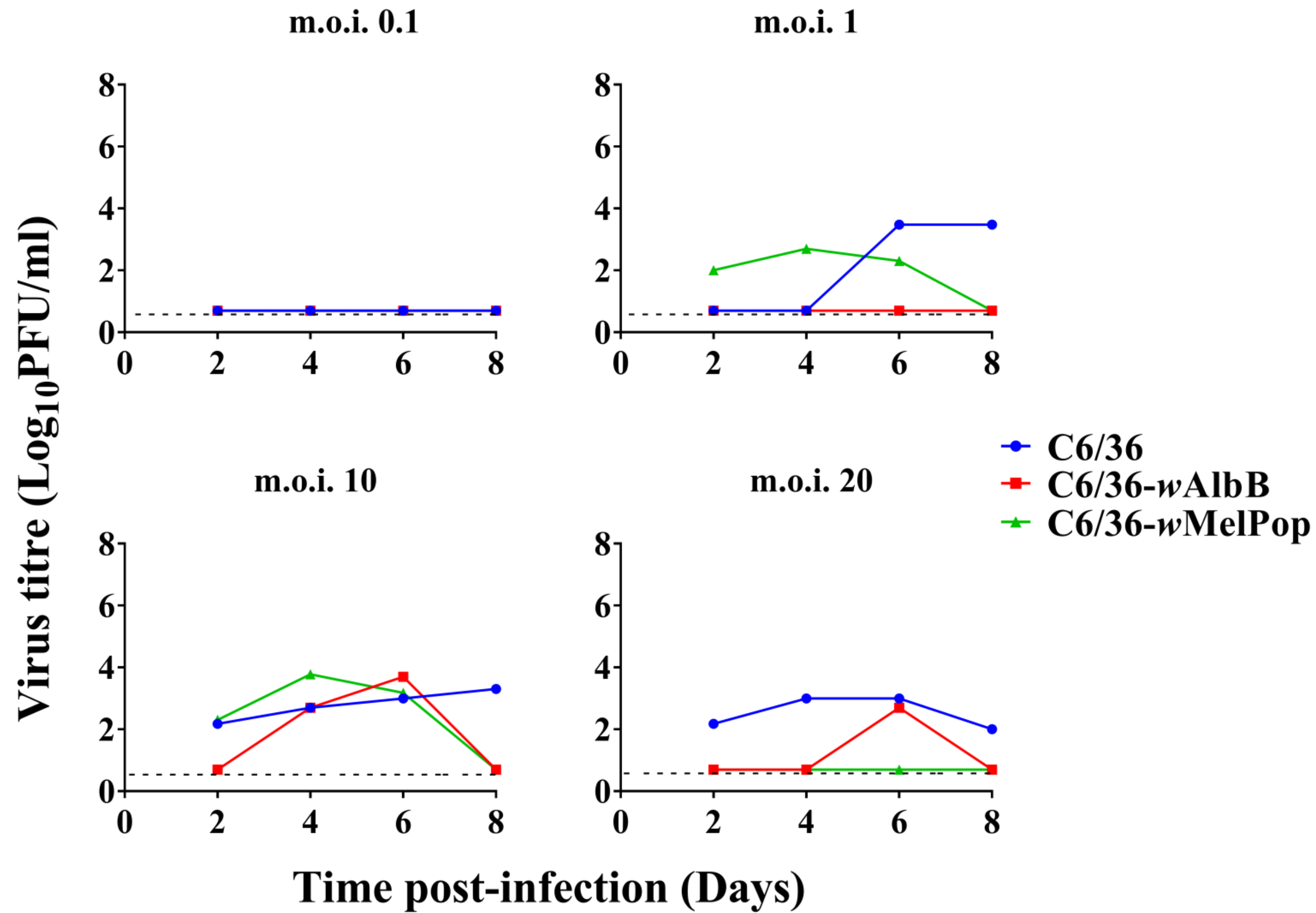

Figure 3.4 Kinetics of production of DENV-1 ET-243 in C6/36 (blue), C6/36-*w*AlbB (red) and C6/36-*w*MelPop (green) cells infected at m.o.i. of 0.1, 1, 10 or 20. Limit of detection shown as a dashed line.

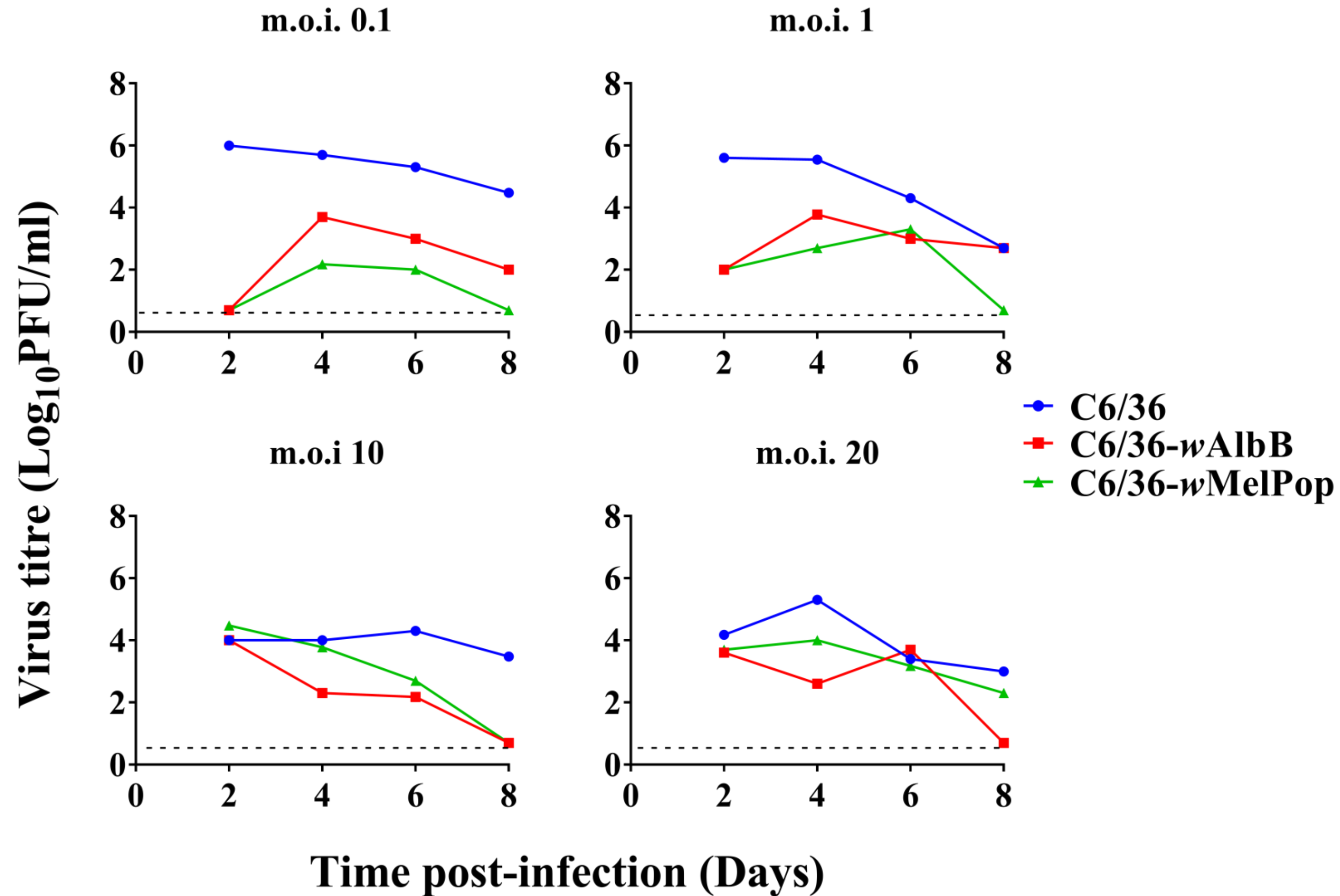

Figure 3.5 Kinetics of production of DENV-2 ET-300 in C6/36 (blue), C6/36-*w*AlbB (red) and C6/36-*w*MelPop (green) cells infected at m.o.i. of 0.1, 1, 10 or 20. Limit of detection shown as a dashed line.

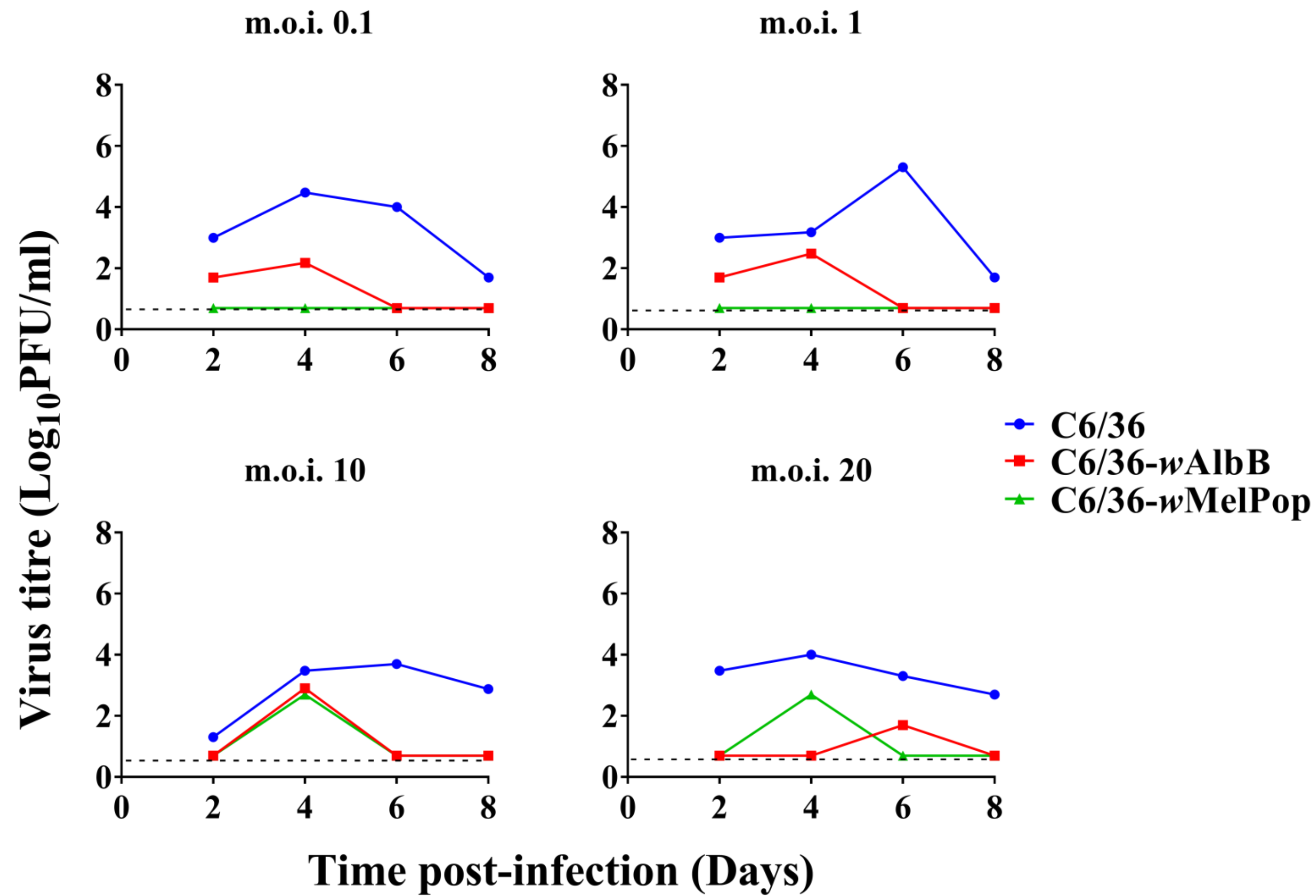

Figure 3.6 Kinetics of production of DENV-2 (VN-130604) in C6/36 (blue), C6/36-*w*AlbB (red) and C6/36-*w*MelPop (green) cells infected at m.o.i. of 0.1, 1, 10 or 20. Limit of detection shown as a dashed line.

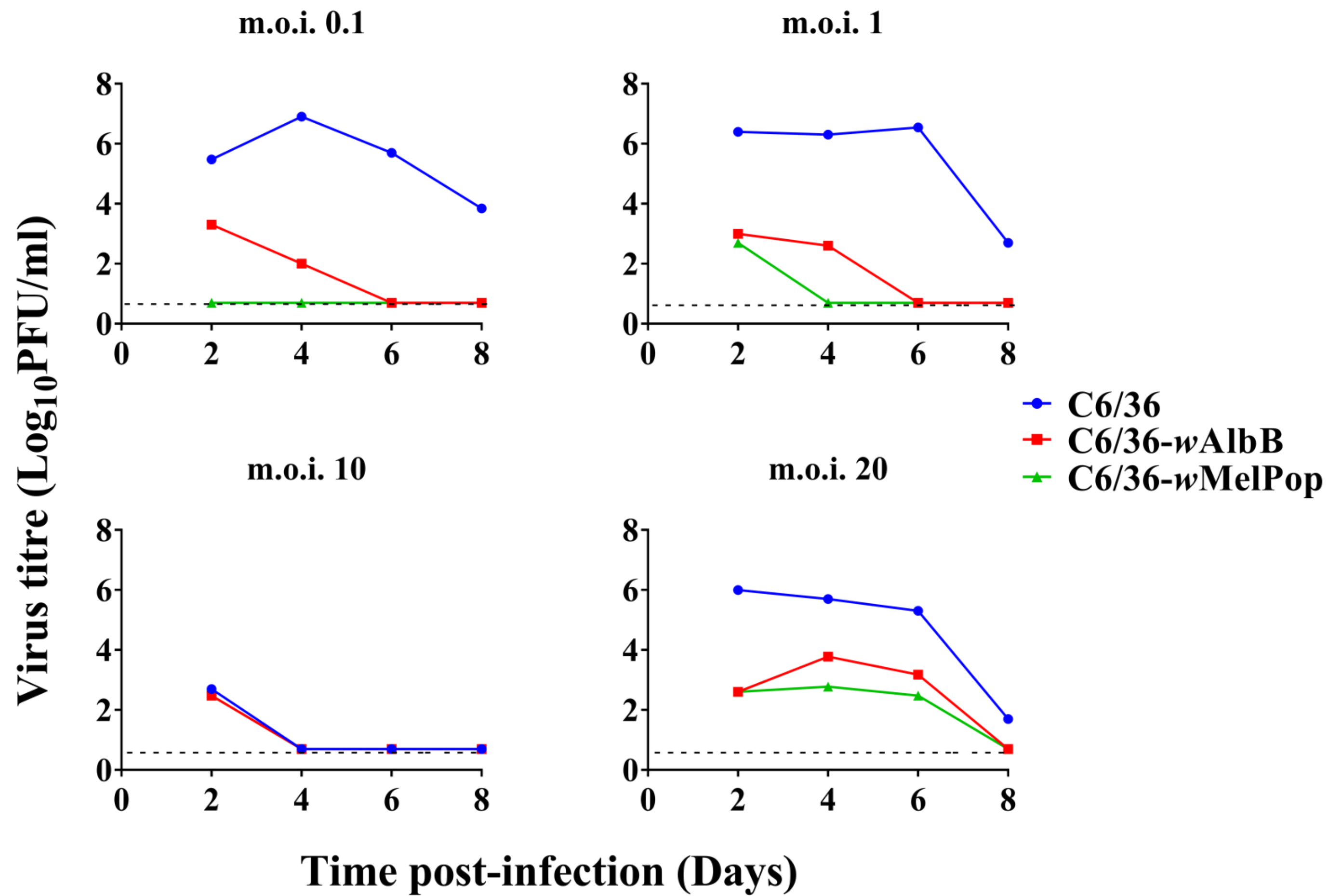

Figure 3.7 Kinetics of production of DENV-2 55763 in C6/36 (blue), C6/36-*w*AlbB (red) and C6/36-*w*MelPop (green) cells infected at m.o.i. of 0.1, 1, 10 or 20. Limit of detection shown as a dashed line.

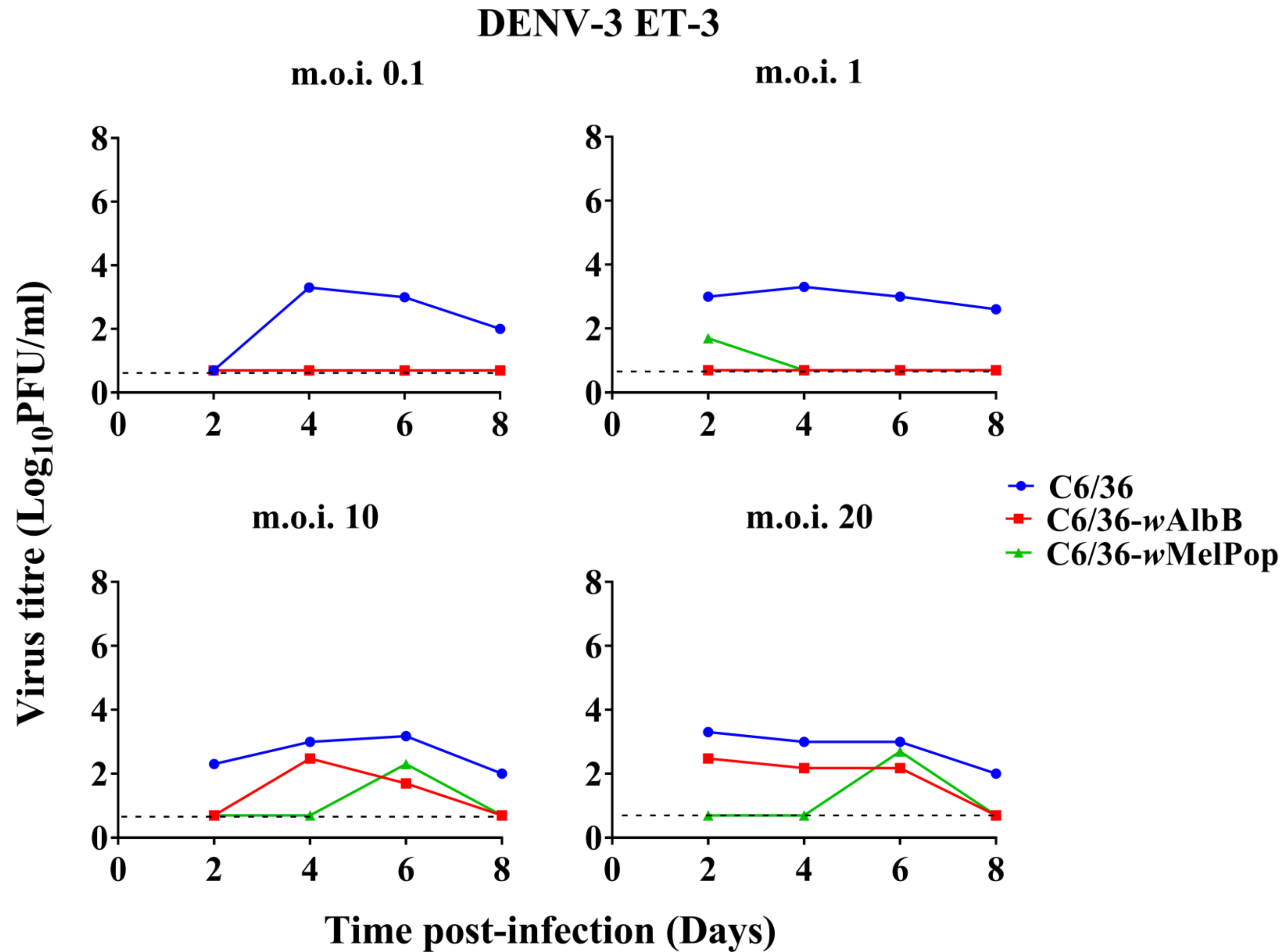

Figure 3.8 Kinetics of production of DENV-3 ET-3 in C6/36 (blue), C6/36-*w*AlbB (red) and C6/36-*w*MelPop (green) cells infected at m.o.i. of 0.1, 1, 10 or 20. Limit of detection shown as a dashed line.

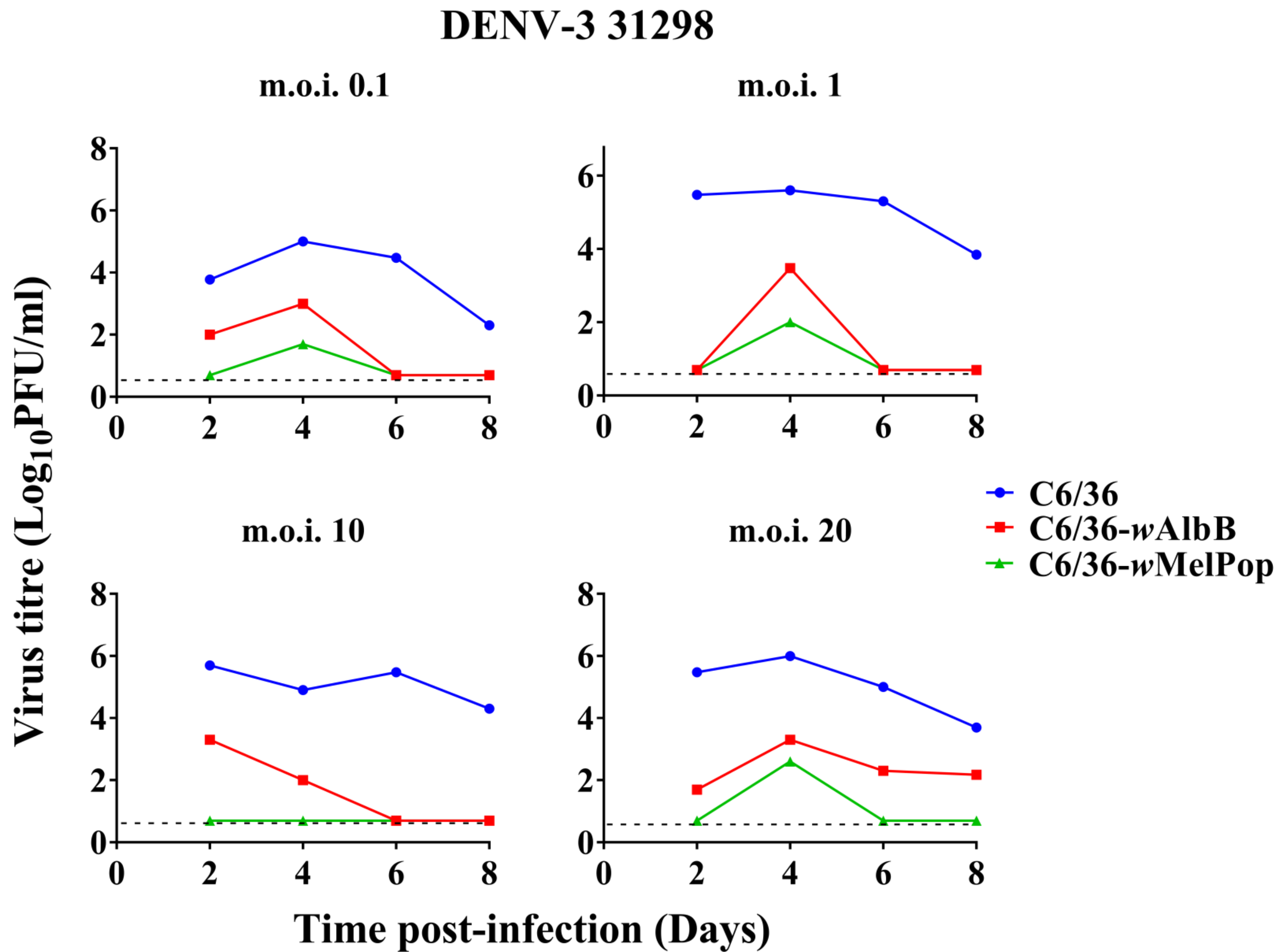

Figure 3.9 Kinetics of production of DENV-3 31298 in C6/36 (blue), C6/36-*w*AlbB (red) and C6/36-*w*MelPop (green) cells infected at m.o.i. of 0.1, 1, 10 or 20. Limit of detection shown as a dashed line.

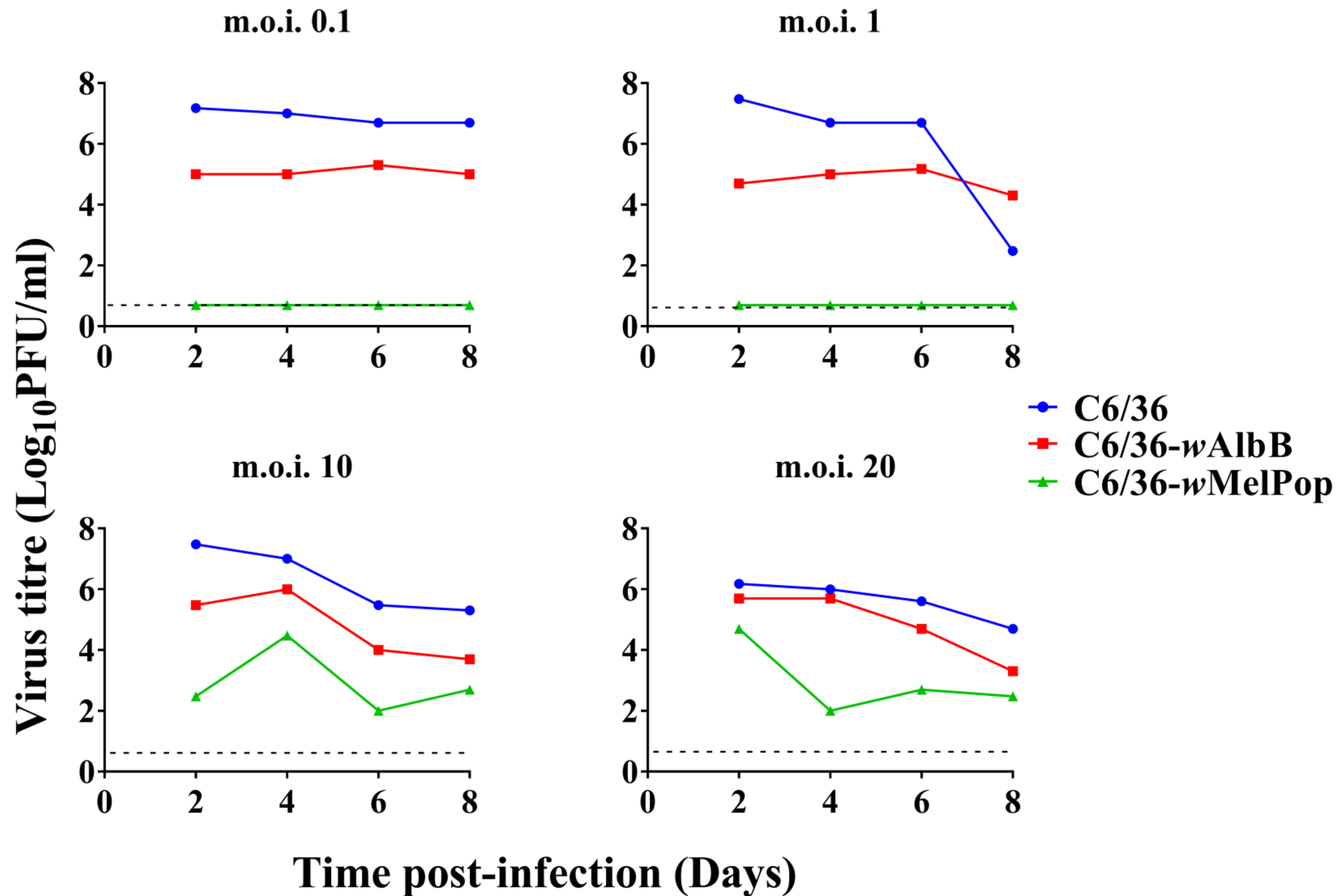

Figure 3.10 Kinetics of production of DENV-4 MY1261 in C6/36 (blue), C6/36-*w*AlbB (red) and C6/36-*w*MelPop (green) cells infected at m.o.i. of 0.1, 1, 10 or 20. Limit of detection shown as dashed line.

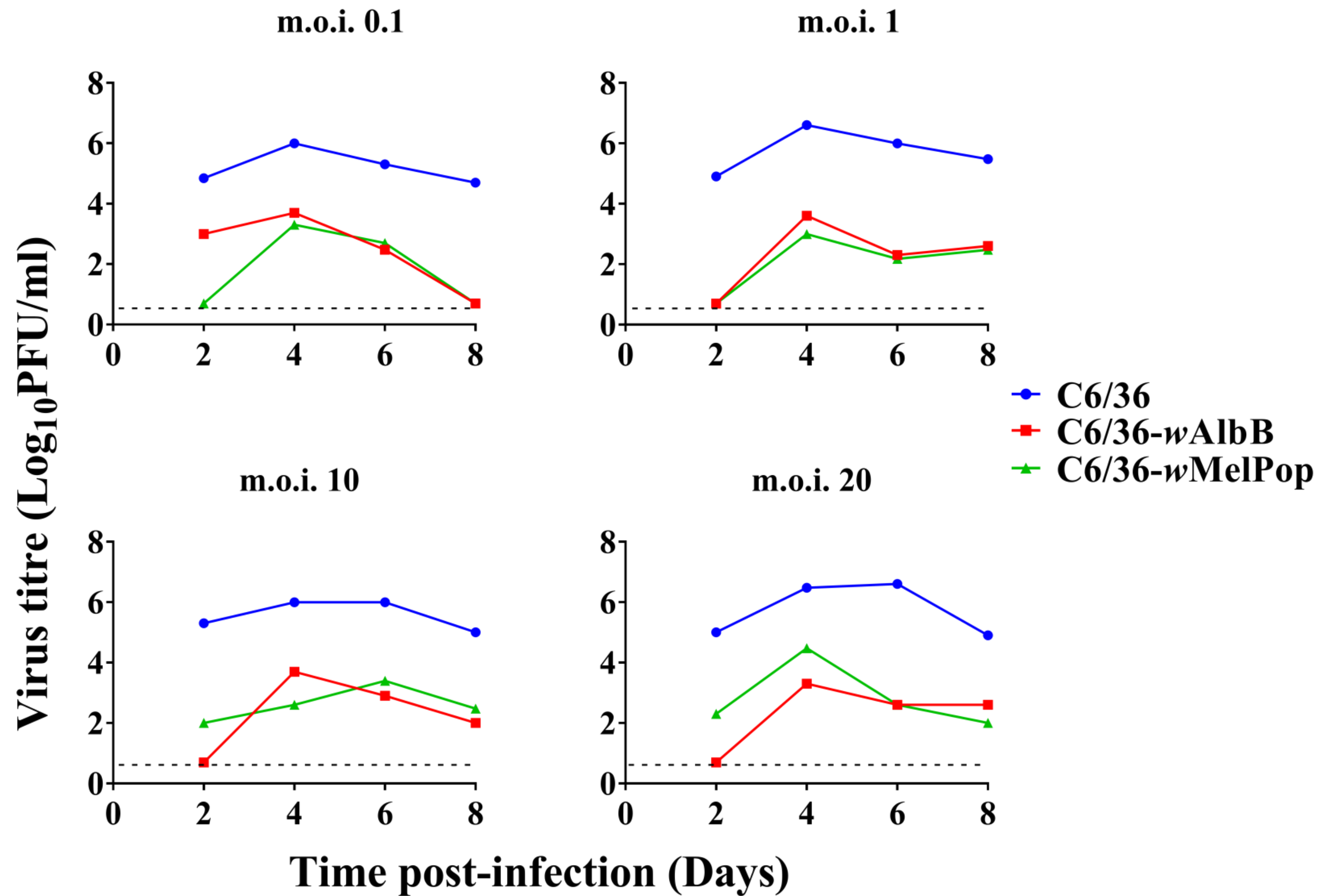

Figure 3.11 Kinetics of production of DENV-4 (NC-39) in C6/36 (blue), C6/36-*w*AlbB (red) and C6/36-*w*MelPop (green) cells infected at m.o.i. of 0.1, 1, 10 or 20. Limit of detection shown as a dashed line.

Table 3.3 Reduction in the titre of DENV in cultures of C6/36-*w*MelPop and C6/36-*w*AlbB compared to the titre in *Wolbachia*-free C6/36 cells, 8 days after infection.

Serotype	Strain	m.o.i.							
		0.1		1		10		20	
		*w*MelPop	*w*AlbB	*w*MelPop	*w*AlbB	*w*MelPop	*w*AlbB	*w*MelPop	*w*AlbB
1	NC-483	5.3	5.3	4.5	4.5	4.5	4.5	4.3	4.3
	ET-243	0.7*	0.7*	3.5	3.5	3.3	3.3	2.0	2.0
2	ET-300	4.5	4.5	2.7	0.7*	3.5	3.5	2.9	3.0
	VN-130604	1.7	1.7	2.7	2.7	2.9	2.9	2.7	2.7
	55673	3.9	3.9	2.7	2.7	4.0	4.0	1.7	1.7
3	ET-3	2.0	2.0	2.6	2.6	2.0	2.0	2.0	2.0
	31298	2.3	2.3	3.9	3.9	4.3	4.3	3.7	3.7
4	MY-1261	6.7	6.7	2.5	0.7*	5.3	5.3	4.7	4.7
	NC-39	4.7	4.7	5.5	5.5	5.0	5.0	4.9	4.9

*Limit of detection $Log_{10}5 = 0.7$.

3.4.3 *Wolbachia* density during virus infection

The effect of DENV infection on the density of *Wolbachia* in wMelPop-C6/36 and C6/36-*w*AlbB infected C6/36 cells was evaluated using two strains of DENV, DENV-3 31298 and DENV-4 NC39 (Figure 3.12). The density of *Wolbachia* in DENV-infected and uninfected C6/36 cells did not change significantly during the 8 days of the experiment and there were no significant differences between the densities of *Wolbachia* in DENV-infected and uninfected cells. ($p = 0.5330$, Kruskal-Wallis test) (Figure 3.12).

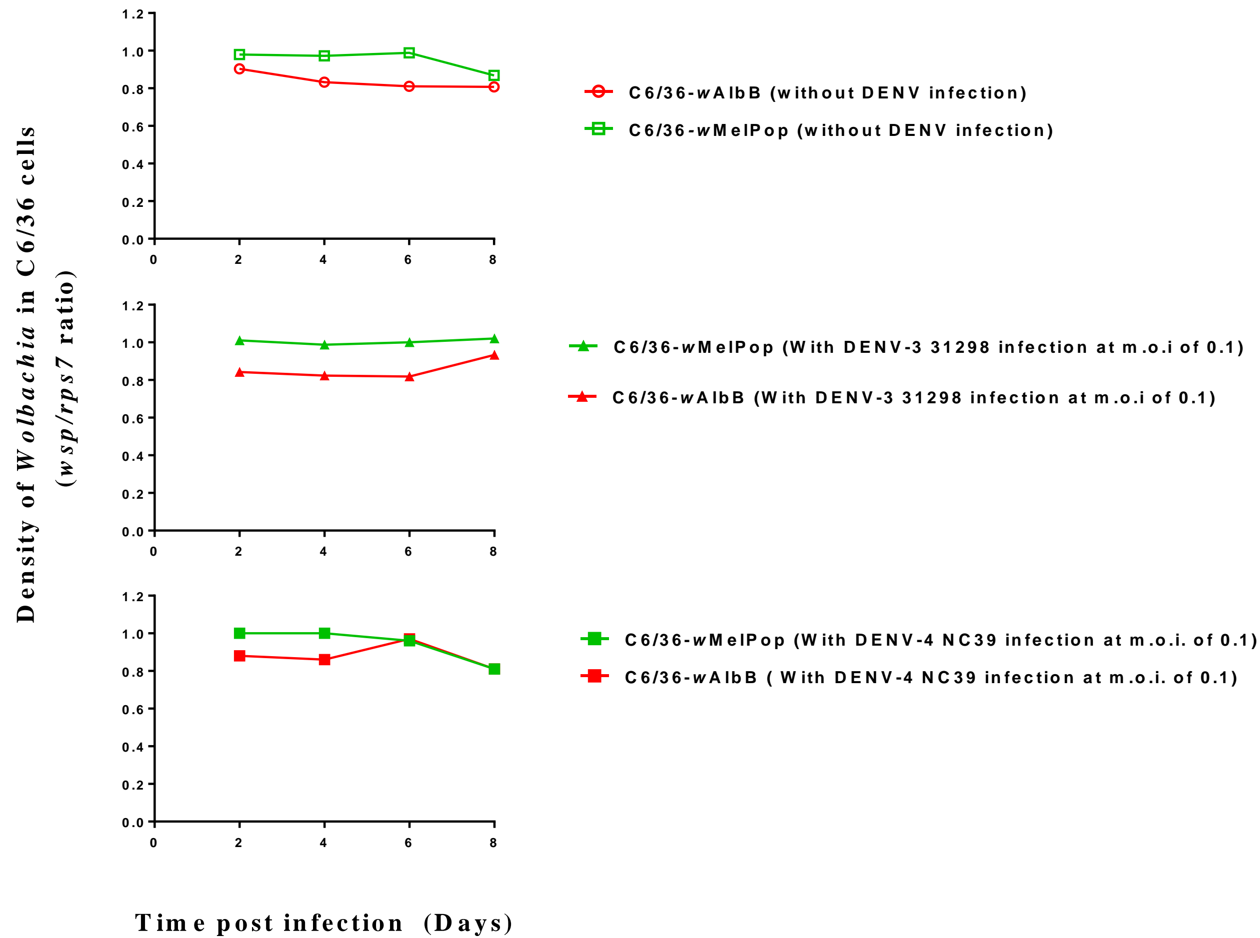

Figure 3.12 Relative density of *Wolbachia* during DENV infection expressed as fold increase of *wsp* gene copy numbers obtained from the crossing points of mosquito specific and *Wolbachia*-specific markers in qPCR for DENV-uninfected *Wolbachia* cells and *Wolbachia* cells infected with DENV-3 31298 and DENV-4 NC39.

3.5 DISCUSSION

In this study, we aimed to measure strain-level variation present in *Wolbachia*-mediated DENV blocking in cell lines infected with *Wolbachia* strains, *w*MelPop and *w*AlbB. To do so, we used paired *Wolbachia*-infected and uninfected *Ae. albopictus*-derived C6/36 cell lines to obtain growth curves for nine DENV isolates. These isolates, comprising at least two strains from each serotype, were then used to test for variations in the magnitude of reduction in virus yield across the isolates.

The experiments demonstrate that the presence of *w*AlbB or *w*MelPop in *Aedes albopictus* C6/36 cells was associated with similar reductions in the yield of DENV following infection with all four serotypes. In the culture supernatant of both C6/36-*w*AlbB and C6/36-*w*MelPop cells, virus titres were significantly reduced compared to the *Wolbachia*-uninfected C6/36 cells. While these results are consistent with previous studies that showed reduced virus yield by *w*AlbB and *w*MelPop in mosquito cells (F. D. Frentiu et al., 2010; Lu et al., 2012), the similarity in the reduction of virus yield by *w*AlbB and *w*MelPop was unexpected because *w*AlbB is a natural endosymbiont of *Ae. albopictus* (S. P. Sinkins, Braig, & O'Neill, 1995) and natural symbionts have been reported to be less effective in suppressing viral replication than strains of *Wolbachia* (e.g. *w*Mel, *w*MelPop) that have been transinfected into hosts or cells (Glaser & Meola, 2010; Joubert et al., 2016). The choice of *Wolbachia* strain, *w*MelPop, for comparison with an alternative strain, *w*AlbB, is because it is associated with much higher density and stronger inhibition of DENV replication in the primary dengue mosquito, *Ae. aegypti* than other *Wolbachia* strains (F. D. Frentiu et al., 2010; Moreira et al., 2009; Yeap et al., 2014). It is possible that the similarity in reduction of virus yield recorded for the two *Wolbachia* strains was due to the comparable densities of *Wolbachia* in both cell lines prior to experimental infection with DENV.

These results extend the observation that *w*AlbB inhibits the replication of DENV-2 in an *Ae. albopictus*-derived cell line (Lu et al., 2012) to include all DENV serotypes and multiple strains of each serotype. Here, the magnitude of the reduction in virus yield varied from strain to strain of DENV, suggesting that the replication of different strains of DENV was affected to different degrees by *Wolbachia* infection. Given the growing evidence that *Wolbachia* pathogen interference is influenced by the interactions between host, virus and *Wolbachia* (Bhattacharya, Newton, & Hardy,

2017) it seemed likely that the variation in magnitude of reduction in virus yield stems from genetic contributions of individual virus strains. Another factor that may possibly contribute to why the replication of different strains of DENV was affected to various degrees is the rate of replication among virus strains. It is believed that slow-replicating viruses are bound to be more prone to RNA degradation (Balistreri et al., 2014; Nagy & Pogany, 2011; Salonen, Ahola, & Kaariainen, 2005), therefore, viral RNA is more likely to be susceptible to decay due to slowed replication in the presence of *Wolbachia.* Thomas et al. (2018), using *Wolbachia* strain *w*Mel, suggested that *Wolbachia*-mediated virus blocking is highest with slower replicating viruses. While this partly agrees with our findings, there were exceptions. For example, a fast replicating strain like DENV-4 MY1261 was as efficiently blocked as the slow-replicating DENV-3 ET3. Nonetheless, it is not unlikely that *Wolbachia* strains employ different mechanisms of action to inhibit different strains of DENV.

Supporting the variation in *Wolbachia*-mediated inhibition of virus replication, our results are not inconsistent with those of another study employing blood from dengue patients as a source of virus (Ferguson et al., 2015) which observed strong resistance to DENV infection and significant differences in the time that DENV serotypes replicated to high enough titre to be transmitted from the salivary glands of *w*Mel-infected *Ae. aegypti* mosquitoes (Lauren B. Carrington et al., 2018). The data from these studies (Ferguson et al. 2015 and Carrington et al. 2018a) also suggested there were significant differences in the time required for each DENV serotype to replicate to high enough titre in *w*Mel-infected *Ae. aegypti* mosquitoes to be transmitted in their saliva. These studies support the possibility of differential inhibition to replication of different DENV serotypes by *Wolbachia.* But given that one strain per serotype was used, it is difficult not to confound strain with serotype. By the same token, in the absence of data for the genotypes of DENV being studied here, it is difficult to determine whether the variation in the magnitude of virus yield reduction was strictly serotype-dependent or genotype-specific. Further studies, which take genotypes into account, will need to be undertaken.

Previous studies have shown that the density of *Wolbachia* plays a key role in determining the strength of inhibition of virus replication (Lu et al., 2012; Moreira et al., 2009; Walker et al., 2011). Therefore, after determining the magnitude of inhibition to virus replication, we also compared the *Wolbachia* load for cells infected and uninfected with DENV in the course of virus infection. We observed that the density

of *Wolbachia* in C6/36-*w*AlbB and C6/36-*w*MelPop with or without DENV infection did not change significantly during the 8 days of virus infection. This suggests that the presence of virus within the cells does not modulate the density of *Wolbachia.* It remains to be determined whether *w*AlbB have similar effects on DENV replication *in vivo* and if it would be necessary to reach the same, extremely high, levels of intracellular *Wolbachia* infection in mosquitoes to achieve this (F. D. Frentiu et al., 2010).

Wolbachia has continued to be trialled in the field for its ability to limit DENV transmission from vector mosquitoes to humans (Ferguson et al., 2015; Hoffmann et al., 2011; Nguyen et al., 2015). The ability of *Wolbachia* to establish in wild populations and the stability of expression of the inhibition phenotype under field-approximating conditions will contribute to the long-term success of the *Wolbachia* biocontrol strategy. Understanding how much variation, among the strains, there is for blocking is therefore critical. *Wolbachia* strains differ substantially in their susceptibility to the fluctuations in temperature seen in nature (P. Ross & Hoffmann, 2018; P. A. Ross et al., 2017) and the relative temperature resistance of *w*AlbB may make it a more suitable alternative to *w*MelPop if it is able to reduce DENV replication in mosquitoes as effectively as *w*MelPop. Here, we highlight the utility of *w*AlbB, which has a similar level of virus blocking to *w*MelPop and greater temperature stability than *w*Mel (P. Ross & Hoffmann, 2018; P. A. Ross et al., 2017), suggesting it may be more effective at blocking virus transmission in tropical climates.

It was beyond the scope of this study to determine whether each strain of DENV was evolving towards, or away from, complete resistance to the effect of *Wolbachia* or whether there are random fluctuations around some mean susceptibility that was being driven by the within-population genetic diversity of DENV strains. However, subsequent work on this will experimentally evolve diverse DENV strains in the present mosquito cell lines hosting *w*AlbB and any adaptive variants that may have arisen during experimental evolution will be identified using next generation sequencing. This study may be limited in interpretation due to the use of mosquito cell lines instead of whole mosquitoes. However, previous studies have shown that cell lines parallel observations from whole insects and thus provide useful models to examine *Wolbachia*-mediated virus inhibition (F. D. Frentiu et al., 2010; Lu et al., 2012).

In summary, our results have shown that *Wolbachia* strain *w*AlbB can reduce the yield of DENV in mosquito cell culture as much as *w*MelPop, with similar densities of *Wolbachia* in both cells prior to infection, but that the magnitude of reduction varied from strain to strain and that there were no significant changes in density of *Wolbachia* through the course of virus infection. This suggests that *Wolbachia*-mediated blocking may be expressed differentially across diverse strains of DENV. Understanding the magnitude of variation in the reduction of virus titres by *Wolbachia* will aid the development of strategies that optimize the choice of *Wolbachia* strains being developed for biocontrol. These findings provide a functional platform to initiate *in vivo* evaluation of *w*AlbB as a robust alternative in *Wolbachia*-mediated control of dengue.

Chapter 4: *In vitro* assessment of *Wolbachia* strain *w*AlbB as a biocontrol agent for flaviviruses and alphaviruses

4.1 ABSTRACT

Wolbachia pipentis is an endosymbiotic bacterium of insects that has the ability to inhibit the replication of arboviruses such as DENV or ZIKV in vector mosquitoes, and has been proposed as a vector-biocontrol agent to reduce the global burden of virus transmission to humans. Two *Wolbachia* strains, *w*Mel and *w*MelPop, have been released into wild mosquito populations to test their virus-blocking potential. However, experiments have shown reduction in maternal transmission of *w*Mel under the range of ambient temperatures found in nature, and large fitness costs on mosquito hosts, leading to the failure of *w*MelPop to establish in wild populations. Consequently, it is important to examine an alternative *Wolbachia* strain. Using a cell line model, growth curves were generated for six arboviruses. We show that *w*AlbB reduced the titres of flaviviruses (ZIKV, DENV and WNV [Kunjin]) and alphaviruses (BFV, RRV and SINV) produced following infection of C6/36 mosquito cells with these viruses. The extent of reduction of virus titre varied with each virus and the variation may stem from genetic contributions from the viruses. We observed that for RRV, BFV, SINV and ZIKV, virus production in the *w*AlbB infected C6/36 cells fell to undetectable levels. Our results expand the potential use of *w*AlbB to block arboviruses and suggest that *w*AlbB could be deployed as a biocontrol agent for a broad range of viruses. The broad anti-viral effect of *w*Alb infection in *Aedes albopictus* cells justifies further study of the effect of this agent *in vivo*. For the *in vivo* evaluations to be of relevance to vector control, they must be extended to those mosquitoes that are the principal vector(s) of each of these viruses.

4.2 INTRODUCTION

Vector control still remains the main option to control mosquito-borne diseases despite evidence that it is becoming less and less effective A new vector-biocontrol initiative utilizes intracellular bacteria (*Wolbachia)* that have the potential to inhibit virus replication in mosquitoes (F. D. Frentiu et al., 2014; Walker et al., 2011; Ye et al., 2015). *Wolbachia* are maternally-inherited obligate intracellular bacteria that have evolved diverse ways to manipulate vital reproductive processes of their arthropod hosts and perpetuate themselves. The bacteria invade and rapidly spread through *Wolbachia*-free insect populations by cytoplasmic incompatibility (CI) which enables them to manipulate their host's reproduction and to increase the proportion of infected females over successive generations (Hoffmann et al., 2011; Steven P. Sinkins, 2004; Werren & Jaenike, 1995). In *Aedes* mosquitoes, strains of *Wolbachia* also inhibit the replication of some arboviruses which, in turn, limits the transmission of these viruses (Bian et al., 2010; F. D. Frentiu et al., 2014; Hussain et al., 2013; Moreira et al., 2009; Mousson et al., 2012; Walker et al., 2011).

Inhibition of virus replication in mosquitoes is influenced by the virus, the strain of *Wolbachia,* and the mosquito host (Ferguson et al., 2015; Parasites and VectorsHoffmann et al., 2015). Stable infections of *Ae. aegypti* with *Wolbachia* have been achieved with *w*MelPop (McMeniman et al., 2009), *w*Mel (which is native to *Drosophila melanogaster*) (Walker et al., 2011)*, w*AlbB (naturally superinfected with *w*AlbA in *Ae. albopictus*) (Xi et al., 2005) and by *w*Mel/*w*AlbB superinfection (Joubert et al., 2016).

*w*MelPop is a highly invasive strain of *Wolbachia* that restricts replication and transmission of Yellow fever virus (van den Hurk et al., 2012), DENV (Bian et al., 2010; Moreira et al., 2009), CHIKV (Moreira et al., 2009; van den Hurk et al., 2012) and WNV (Kunjin subtype) (Hussain et al., 2013). However, due to the severe longevity and fertility costs it imposes on its mosquito hosts, *w*MelPop is unable to persist in wild populations (McMeniman & O'Neill, 2010; Nguyen et al., 2015; P. A. Ross et al., 2016; Yeap et al., 2011). In contrast, the closely related *w*Mel strain is able to invade naïve mosquito populations (Hoffmann et al., 2011). It does not reduce the fitness of host mosquitoes and it limits DENV (F. D. Frentiu et al., 2014; Walker et al., 2011; Ye et al., 2015), ZIKV (Aliota, Peinado, et al., 2016; Dutra et al., 2016), and CHIKV (Aliota, Walker, et al., 2016) replication in these insects. Although field

releases aimed at spreading *Wolbachia* in wild *Ae. aegypti* populations have focused primarily on *w*Mel, concerns have been raised about the constraints *w*Mel may encounter in the field. These include the reduced expression of cytoplasmic incompatibility when exposed to cyclical heat stress, loss of maternal transmission fidelity and eventual reduction in *Wolbachia* density (P. A. Ross et al., 2017; Ulrich et al., 2016). *w*AlbB has spread through caged populations of *Ae. aegypti* but it has yet to be tested in the field. In contrast to *w*Mel, *w*AlbB exhibit complete cytoplasmic incompatibility and high maternal transmission fidelity in *Ae. aegypti* populations at elevated temperatures (Ant et al., 2018; P. A. Ross et al., 2017).

Given that *w*AlbB have features that may facilitate invasion of mosquito populations, this study was undertaken to test whether *w*AlbB is also able to inhibit the replication of a range of human arboviruses including the flaviviruses (DENV, ZIKV and KUNV), and the alphaviruses (BFV, RRV and SINV) using a cell line model. Here, the hypothesis was that *w*AlbB would inhibit the replication of representative examples of both alpha- and flaviviruses that are pathogenic to humans.

4.3 MATERIALS AND METHODS

4.3.1 Mosquito cells and transinfection of *w*AlbB

The *Ae. albopictus* cell line C6/36 (Igarashi, 1978) and its *w*AlbB-infected counterpart were maintained as previously described (Section 3.3.1, Chapter 3).

4.3.2 Fluorescent *in situ* hybridization for *w*AlbB detection

FISH and microscope imaging for the detection of *w*AlbB in cultured cells were carried out as previously described (Section 3.3.3, Chapter 3).

4.3.3 Viruses

DENV-2 (ET-300) was a gift from Professor Paul Young at the University of Queensland, Australia. WNV_{KUN}, RRV, BFV and SINV were obtained from the WHO Collaborating Centre for Arbovirus Reference and Research at the Queensland University of Technology Australia. Brazilian Zika virus (Genbank accession number KU365780) and French Polynesian Zika virus isolate P13F/251013-18 (Genbank accession number KX369547) were courtesy of Dr Pedro Vasconcelos and Dr Van-Mai Cao-Lormeau respectively. African Zika virus MR766 was the third ZIKV strain used. All viruses were propagated by infecting C6/36 cells at an m.o.i. of 0.01. Culture

supernatant was harvested 2 days following SINV infection, 3 days for RRV and BFV infections and 4 days for WNV_{KUN}. Supernatants were harvested 4 days post-infection with ZIKV strain KU365780 and 5 days post-infection with ZIKV strains (MR766 and H/PF/2013) and DENV-2. Cell debris was removed from culture supernatants by centrifugation at 4000*g* for 10 min at 4°C and the supernatant concentrated by ultrafiltration through a 100kDa filter in an Amicon filter device (Merck Milipore) according to the manufacturer's instructions. The concentrate was aliquoted into sterile 2 ml cryovials before freezing at -80°C.

4.3.4 Kinetics of virus production in mosquito cells

C6/36 and C6/36-*w*AlbB cells were seeded into 24-well plates at 2.5 x 10^5 cells per well and allowed to attach for 24 h, at 28°C. Infection with each virus strain was performed at an m.o.i. of 0.1, 1 or 10 in serum-free RPMI-1640 media (Sigma-Aldrich). Virus-free medium was employed as a control. Triplicate wells were employed for each treatment. The virus was allowed 2 h to adsorb to the cell monolayers before the inoculum was removed, the cell monolayers washed twice with sterile PBS and the cells incubated at 28°C in fresh maintenance media (RPMI-1640 containing 25mM HEPES [Sigma-Aldrich] supplemented with 2% FBS [Gibco] and 1% Glutamax [Sigma-Aldrich]). Culture supernatants were harvested from three replicate wells every 24 h for eight days from cultures infected with flaviviruses. This experimental protocol was employed in order to avoid repeat sampling of each well. Because alphaviruses replicate much faster than flaviviruses, supernatants were sampled every 8 h up to 48 h post infection (8, 16, 24, 32, 40, 48 h), then every 24 h until day 6 (72, 96, 120, 144 h) and finally at day 8 (192 h). Titres of virus in culture supernatants were determined by plaque and immuno-focus assay.

4.3.5 Plaque and immuno-focus assay

Titres of infectious virus were quantified using an immuno-focus assay on Vero (green monkey kidney) cells maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) containing 5% v/v foetal bovine serum (FBS, Gibco) and 1% L-glutamine (Invitrogen) at 37°C in an atmosphere of 5% v/v CO_2/air. Vero cells were seeded in 24-well plates at 2.0 x 10^5 cells per well and incubated overnight at 37°C. The growth medium was removed and the confluent monolayers of cells were rinsed with sterile PBS and infected with 200 µl of serial ten-fold dilutions of virus for 2 h at 37°C with gentle rocking every 15 min. Overlay medium (750 µl of 8% w/v

carboxy-methyl cellulose [CMC, Sigma-Aldrich] in Medium 199 [Sigma-Aldrich]) was added to each well and plates were incubated at 37°C in an atmosphere of 5% v/v CO_2/air. After the desired length of incubation, i.e. 2 days for SINV, 3 days for RRV and BFV, 4 days for KUNV and ZIKV [KU365780] and 5 days for ZIKV [MR766 and SN6P], the overlay medium was removed and the cell monolayers washed twice in PBS. The cells then were stained with 300 µl of 0.05% w/v Crystal violet in 1% v/v formaldehyde and PBS for 1 h, rinsed with water, dried and plaques counted.

As DENV did not produce plaques reliably with the protocol above, plaques of DENV-infected cells were identified by immuno-focus assay as described in Chapter 3 (Section 3.3.5 above).

4.3.6 Analysis

Virus titres were log_{10}-mean transformed and comparisons were performed using a general linear model. Chi-square test of association, Fisher's exact test and the general linear model were used to compare the results from cell lines separately for each time point and for each multiplicity of infection. All the statistical analyses were performed using IBM SPSS 23 and GraphPad 7.

4.4 RESULTS

4.4.1 Stable transinfection of C6/36 cells with *Wolbachia* strain *w*AlbB

FISH and microscopy identified *Wolbachia* in the cytoplasm of C6/36-*w*AlbB cells (Figure 4.1 A). No *Wolbachia* were detected in uninfected C6/36 control cells (Figure 4.1 B, lower three panels). The density of *w*AlbB in the cytoplasm of infected C6/36 cells was consistently low in early cell passages. However, the use of media consisting of RPMI-1640 and Lonza Schneider's medium in a 2:1 ratio for cell maintenance increased the percentage of *w*AlbB-infected cells from approximately 60% in passage 28 to more than 95% in passage 40 (Figure 4.1 C). qPCR of DNA from C6/36 and C6/36-*w*AlbB cells detected the *Wolbachia*-specific protein (*wsp*) gene only in the C6/36-*w*AlbB cells.

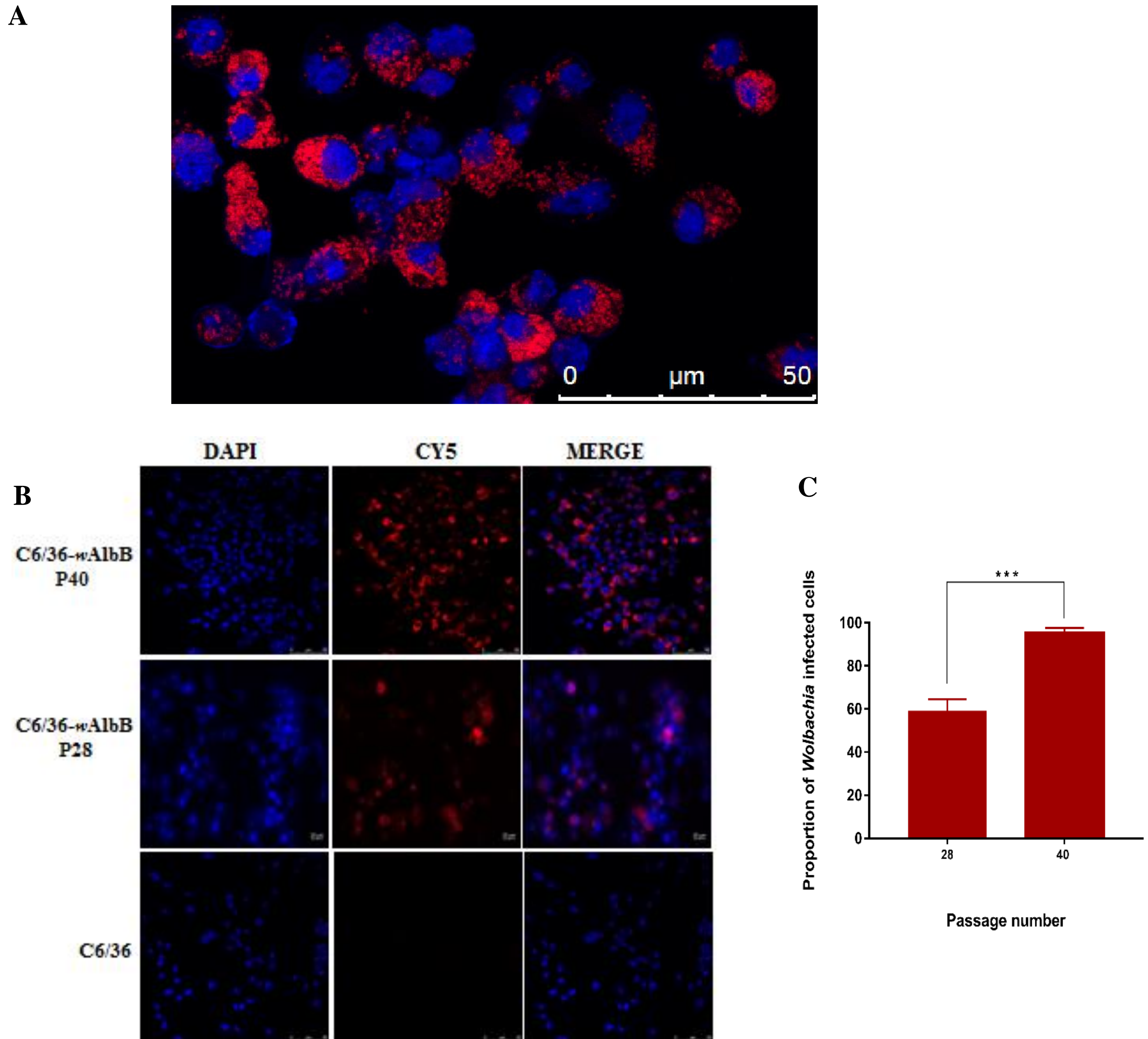

Figure 4.1 Detection of *Wolbachia w*AlbB by Fluorescent *in situ* hybridization of C6/36-*w*AlbB cells. (A) CY5-labelled oligonucleotide probe corresponding to nucleotide sequences in *Wolbachia* 16S rRNA within the cytoplasm of the host cell (red). Cell nuclei stain blue with DAPI. (B) Increasing intracellular density of *w*AlbB in C6/36 cells between passages 28 (P28) and 40 (P40). Data for C6/36 and C6/36-*w*Alb P40 shown previously in Figure 3.2. (C) Proportion of cells containing *Wolbachia w*AlbB detectable by FISH. Error bars represent the standard deviation of the mean of 10 independent samples.

4.4.2 Comparison of alphavirus and flavivirus production in C6/36 and *w*AlbB infected C6/36 cells.

The titres of alphaviruses (RRV, BFV and SINV) and flaviviruses (DENV 2, WNV_{KUN} and ZIKV) in cultures of C6/36 cells were consistently higher than those for the same viruses in cultures of *w*AlbB infected C6/36 cells. The inhibition of virus replication in *w*AlbB was not overcome by increasing the m.o.i. from 0.1 to 10, i.e. a 100-fold increase. Within eight days of infection with alphaviruses or ZIKV, no virus could be detected in cultures of *w*AlbB infected C6/36 cells. DENV 2 and WNV_{KUN} replication was not affected to the same degree, i.e. the virus could still be detected in 4/6 experiments eight days after infection (Figure 4.2). Also, at higher m.o.i., the inhibitory effects of *w*AlbB infection in C6/36 cells on alphavirus and ZIKV replication became less pronounced, i.e. there were fewer time points at which the virus could not be detected in the supernatant of cultures from *w*AlbB C6/36 cells infected with these viruses (Figures 4.3 and 4.4).

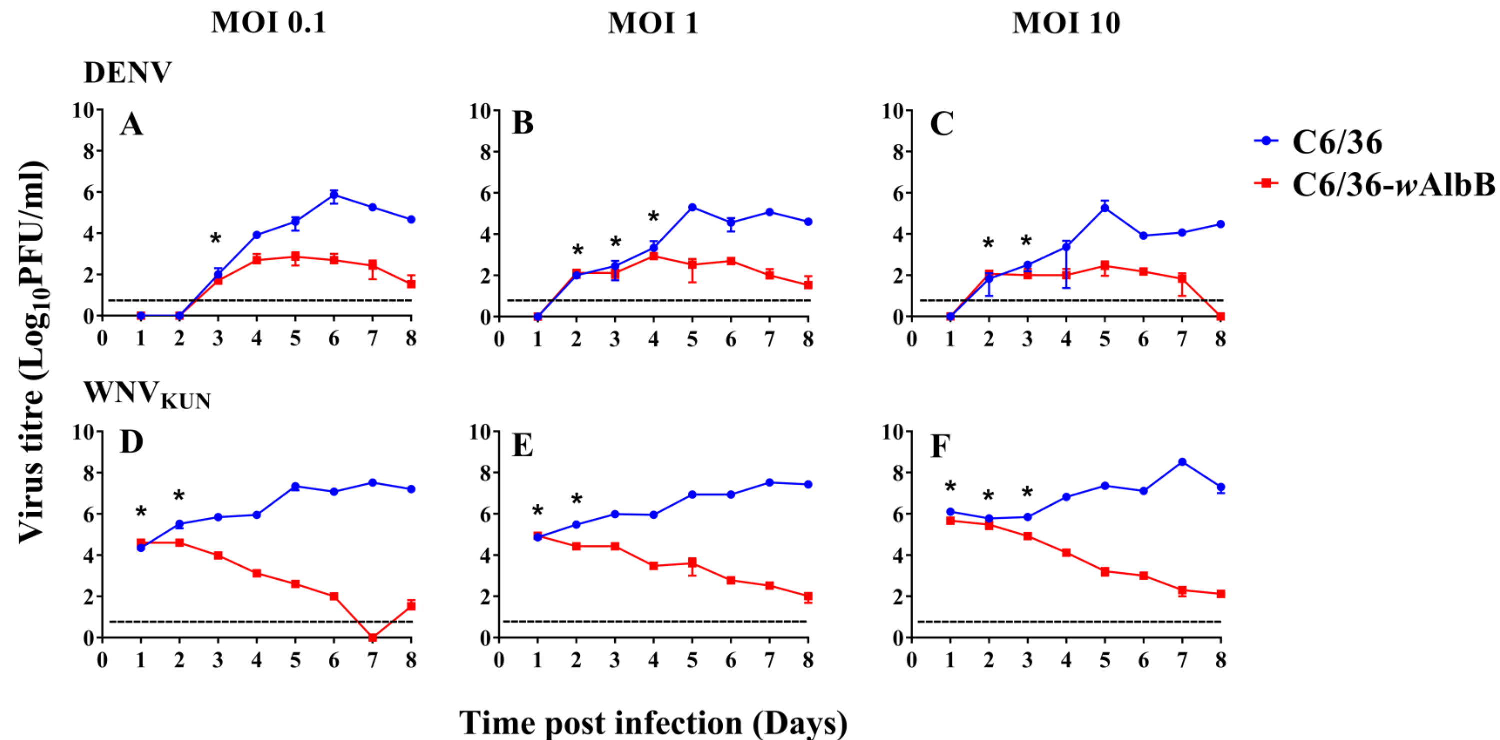

Figure 4.2 Kinetics of virus production following infection of C6/36 and C6/36-*w*AlbB cells with DENV 2 (A to C) and WNV$_{KUN}$ (D to F) at m.o.i. of 0.1, 1 and 10. Means and standard deviations (error bars) for each time point are shown. N = 3 wells per time-point; PFU = plaque forming unit. Virus yields are significantly different for both cell lines at all time points apart from those shown with an asterisk *. The limit of detection is shown as a dotted line.

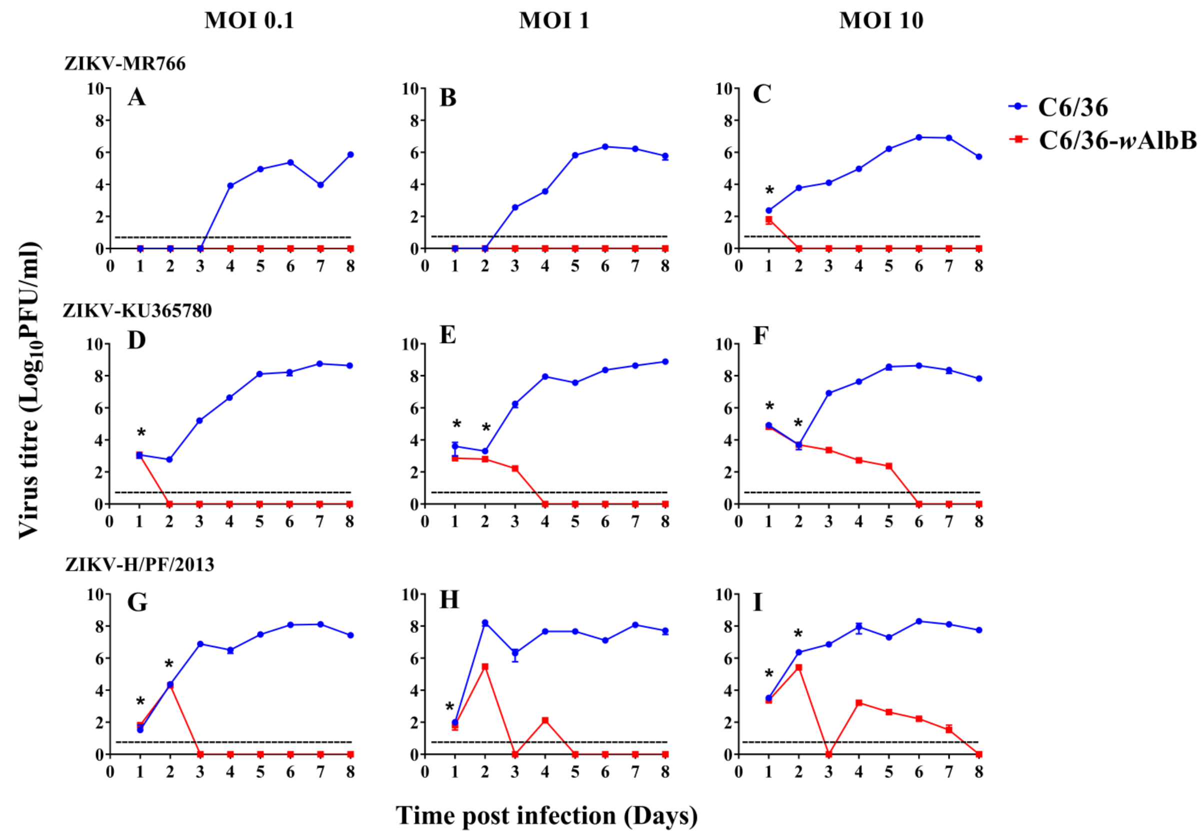

Figure 4.3 Kinetics of virus production following infection of C6/36 and C6/36-*w*AlbB cells with three strains of ZIKV at m.o.i. of 0.1, 1 and 10. African strain MR766 (A to C), Brazilian strain KU365780 (D to F), and French Polynesian strain H/PF/2013 (G to I). Means and standard deviations (error bars) for each time point are shown. N = 3 wells per time-point; PFU = plaque forming unit. Virus yields are significantly different for both cell lines at all time points apart from those shown with an asterisk *. The limit of detection is shown as dotted line.

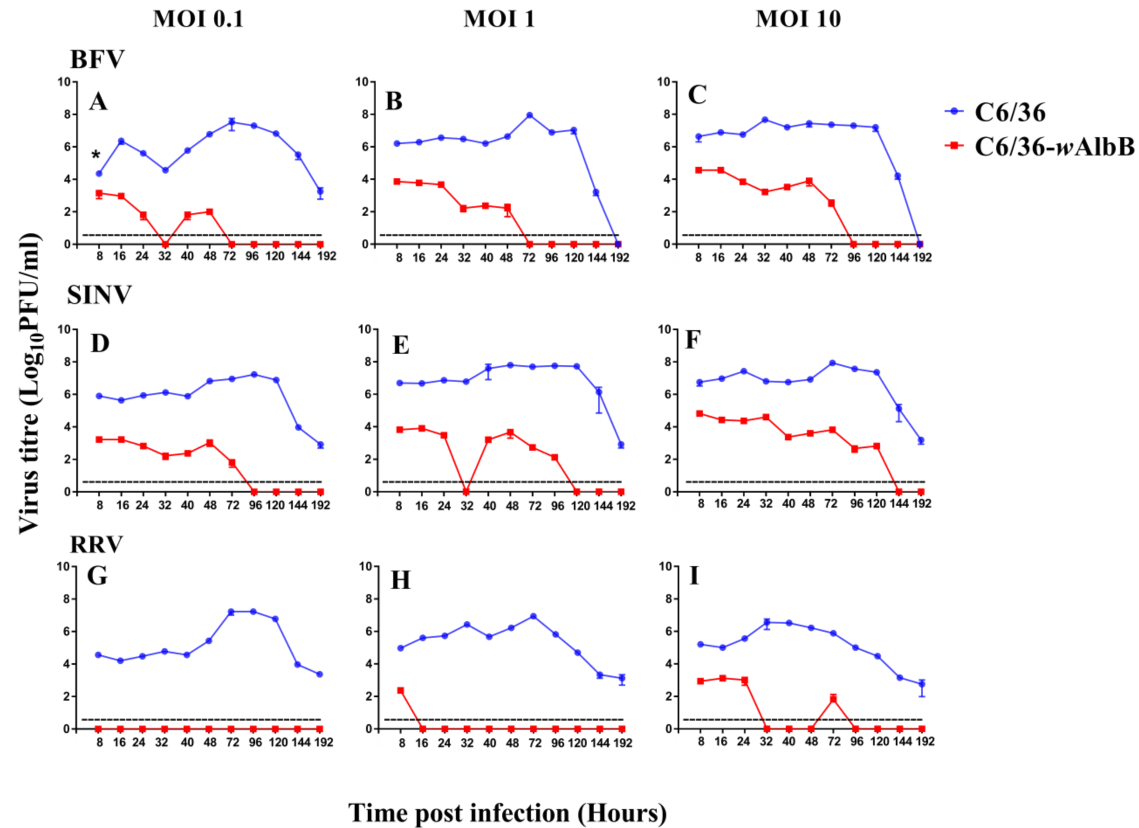

Figure 4.4 Kinetics of virus production following infection of C6/36 and C6/36-*w*AlbB cells with the alphaviruses BFV (A to C), SINV (D to F) and RRV (G to I) in C6/36 at m.o.i. of 0.1, 1 and 10. Means and standard deviations (error bars) for each time point are shown. N = 3 wells per time-point; PFU = plaque forming unit. Virus yields are significantly different for both cell lines at all time points apart from those shown with an asterisk *. The limit of detection is shown as dotted line.

4.5 DISCUSSION

The data presented here show that the yields of infectious virus from C6/36 cells infected with of a range of alphaviruses and flaviviruses were consistently higher than when these viruses were used to infect *w*AlbB infected C6/36 cells. In the case of RRV, BFV, SINV and ZIKV, virus production in the *w*AlbB infected C6/36 cells fell to undetectable levels. Whether "undetectable" means the *absence* of virus is not clear because of the limit of detection for the assay employed. In Figures 4.2D, 4.3H, 4.3I, 4.4A, 4.4E and 4.4I, levels of virus fell to undetectable levels only to rebound. Had time permitted, these virus populations would have been examined to determine whether the rebound reflected the viral populations, which were likely to be very genetically diverse, evolving around the block imposed by *w*AlbB. This could have profound significance for the use of *w*AlbB as a biocontrol agent.

Even though there is considerable uncertainty on evolutionary consequences of both *Wolbachia* and arboviruses, an alternative hypothesis for the comeback of infectious viruses from undetectable levels is that, as expected, there will be a strong selection for viruses to avoid blocking. This tendency could be supported by the few pockets of *Wolbachia*-free cells (Table 3.2). On the other hand, there may be a limit to viral evolutionary superiority which is predicted over *Wolbachia* (Bull & Turelli, 2013) given that viral replication can occur within the cell even when transmission is blocked (Mousson et al., 2012). This also suggests that there is a possibility that evolution may ultimately reverse blocking, even temporarily.

While comparisons of reduction in yields of virus within families probably are valid, comparisons between viral families may not be, because of variations in sensitivity to infection with different viruses of the Vero cells used for plaque assays to quantify viral titres. Another note of caution when interpreting these results is the source of the cell line. C6/36 cells were derived from *Ae. albopictus* mosquitoes (Igarashi, 1978; Singh, 1967) and despite this mosquito being a competent vector for a number of the viruses studied (Nicholson et al., 2014; Russell, 1998), it is not the principal vector for any of them. However, no *Wolbachia*-infected cell lines corresponding to the principal vectors were available. None-the-less, *w*AlbB was observed to have effects on ZIKV and DENV transmission in *Ae. aegypti* mosquitoes (Ant et al., 2018) similar to those observed in this study, i.e. transmission of DENV

was affected less than that of ZIKV. Similarly, *w*AlbB also reduced virus growth of ZIKV when transinfected into other *Ae. albopictus* cell lines, C710 and Aa23 (Schultz et al., 2017).

There is an extensive literature demonstrating that the intracellular density of *Wolbachia* is an important determinant of the degree of inhibition of viral replication (Ant et al., 2018; F. D. Frentiu et al., 2010; Lu et al., 2012). In this study, the issue of variations in intracellular density of *w*AlbB was resolved by changing the cell culture conditions. This was associated with an increase in the proportion of *w*AlbB infected C6/36 cells to approximately ninety-five per cent. The magnitude of the reduction in virus yield observed using these cells may reflect the extremely high proportion of *w*AlbB infected C6/36 cells, or perhaps more importantly, the very small proportion that were uninfected with *Wolbachia*. A significant proportion of *w*AlbB-free C6-36 in the C6-36-*w*AlbB cell stocks would reduce the magnitude of the reduction in virus replication observed.

At all multiplicities of infection, a downward trend of virus titre was observed in the supernatant of WNV_{KUN} 24h after infection of C6/36-*w*AlbB cells. Even though the reason for this pattern of reduction is unclear, it suggests that *Wolbachia*-mediated antiviral activity is independent of virus concentration and appears to occur at the early stages of infection, probably interfering with virus entry which subsequently leads to decay of the adsorbed virus over time. *Wolbachia*-mediated reduction in titre of WNV agrees with published data for *Culex quinquefasciatus* (Glaser & Meola, 2010), *Ae. aegypti* cell line (Aag2) and mosquitoes transinfected with *Wolbachia* strain *w*MelPop (Hussain et al., 2013). However, a previous study found that *w*AlbB increased the infection rates of *Cx. tarsalis* for WNV (Dodson et al., 2014). *Wolbachia*-mediated effects manifest a range of phenotypes depending on the *Wolbachia* strain-pathogen-vector system (Glaser & Meola, 2010; Hughes, Vega-Rodriguez, Xue, & Rasgon, 2012).

The titres of three different strains of ZIKV (MR766, KU365780, and H/PF/2013) were significantly reduced in C6/36-*w*AlbB cells. While these results are similar to those reported for ZIKV in *Ae. albopictus* Aa23 cells (Schultz et al., 2017), here, the African strain MR766 was consistently reduced below detection. It is possible that this could be a viral-strain-specific effect.

Our data show that differences in the virus growth phenotype between the alphaviruses and flaviviruses trends with individual virus strains and not families. *w*AlbB-induced reduction of virus yields inhibition. For example, among the alphaviruses, the reduction in the yield of RRV was mostly undetectable for most time points while BFV was largely reduced mostly at later time points. This suggests that *Wolbachia* may inhibit members of the same virus family at different stages of their growth cycle. Previous study suggests that Semliki Forest virus, an alphavirus, was shown to be inhibited early in viral replication (Rainey et al., 2016).

In summary, our results have implications for using *Wolbachia* to control arboviruses. With the failure of *w*MelPop to establish in wild mosquito populations (Nguyen et al., 2015), current *Wolbachia*-enabled strategy for arbovirus control relies solely on *w*Mel (Dutra et al., 2016). Nevertheless, as the capacity for *w*Mel to adapt to high temperatures in field appears limited (P. Ross & Hoffmann, 2018) and a recent study suggests that it may increase susceptibility of *Ae. aegypti* to DENV (King, Souto-Maior, Sartori, Maciel-de-Freitas, & Gomes, 2018), alternative strains are sought to remedy this deficiency. Our study emphasizes the robust inhibition to virus replication afforded by *w*AlbB to some wide range arboviruses in mosquito cell lines. Therefore, the broad antiviral effect of *w*AlbB infection in *Aedes albopictus* cells justifies further study of the effect of this agent *in vivo* and predicts that *w*AlbB may have utility in field releases for the control of arboviruses. For the *in vivo* evaluations to be of relevance to vector control, they must be extended to include those mosquitoes that are the principal vector(s) of each of these viruses.

Chapter 5: General discussion

The overarching aim of this study was to evaluate the effect of *Wolbachia* on the replication of DENV and a range of other arboviruses. The first step in this process was to determine the range of EIPs for regional (Australia, Southeast Asia and the Pacific) strains of DENV, of all serotypes, in Australian *Aedes aegypti* and *Aedes albopictus* mosquitoes which had not been infected, artificially, with *Wolbachia*. The second was to evaluate two strains of *Wolbachia* for their ability to interfere with the replication of the same regional strains of DENV. The third component was intended to inform future efforts to control not only dengue, but as wide a range as possible of other arboviral diseases of concern to humans including local alphaviruses. The enormous genetic diversity both within and between populations of DENV (Choudhury et al., 2015; Sessions et al., 2015) suggested that it would be unlikely that the replication of each would be equally susceptible to the effect of *Wolbachia* infection of their host (cells).

This study found no associations between EIP and mosquito species when aggregated across the eight strains of DENV fed to *Ae. aegypti* and *Ae. albopictus*. However, both mosquito species showed similar responses to infection and dissemination by most virus strains (Figure 2.2). Similar results have been reported for previous studies examining inter- and intra-serotype variations in infection and dissemination of DENV (Armstrong & Rico-Hesse, 2001; Cologna, Armstrong, & Rico-Hesse, 2005; Rosen, Roseboom, Gubler, Lien, & Chaniotis, 1985). However, most did not report the time required for viral dissemination, and one used only one strain of DENV per serotype in both vector species (Whitehorn et al., 2015). Expanding on earlier studies that examined EIP variations in DENV, our study (Chapter 2) tested two strains from each of the four DENV serotypes in the same population of *Ae. aegypti* or *Ae. albopictus* in order to increase the potential for association analysis among virus strains. It is possible that differences in virus growth phenotypes may be due to translational differences between viral strains. These differences have been demonstrated in human cell cultures (Edgil, Diamond, Holden, Paranjape, & Harris, 2003). Surprisingly, given their genetic diversity, the EIP for the eight strains of DENV ranged from 6 to 14 days in both *Ae. aegypti* and *Ae. albopictus.*

Thus, although infection rates of DENV were higher in *Ae. aegypti*, both mosquito species could potentially transmit each strain within the same time frame.

There is conflicting literature on whether *Ae. albopictus* is less susceptible than *Ae. aegypti* to infection with the same isolates of DENV (Alto, Reiskind, & Lounibos, 2008; W. J. Chen, Wei, Hsu, & Chen, 1993; S. Higgs et al., 2006; Jumali et al., 1979; Rosen et al., 1985; Vazeille et al., 2003; Whitehead, Yuill, Gould, & Simasathien, 1971; Whitehorn et al., 2015). The factors that determine the ability of DENV to infect and be disseminated in *Aedes* mosquitoes are not known. Phylogenetic studies suggest that DENV E protein may be essential for the adaptation of the virus to vectors (E. Wang et al., 2000). Even though *Ae. albopictus* is known to be a secondary vector of DENV and has not been associated with the explosive outbreaks that *Ae. aegypti* can initiate (L. Lambrechts et al., 2010), our study reported that *Ae. albopictus* and *Ae. aegypti* were equally likely to develop disseminated infection when fed concurrently with the same DENV strains in the laboratory. Other laboratory studies carried out with different populations of *Ae. albopictus* and DENV serotypes or strains have shown high variability with regards to susceptibility to DENV infection (Gubler & Rosen, 1976; Moore, Johnson, Smith, Ritchie, & Van Den Hurk, 2007; Nicholson et al., 2014). Apart from DENV, *Ae. albopictus* is also capable of transmitting CHIKV (van den Hurk, Hall-Mendelin, Pyke, Smith, & Mackenzie, 2010) and other major Australian arboviruses such as BFV and RRV (Johnson et al., 2009; Nicholson et al., 2014) at comparable rates to the known primary vectors and at short EIP (Kay & Jennings, 2002) representative of both tropical and temperate Australian climates. The comparable infection rate and similar length of time taken for virus dissemination, coupled with the wide range of viruses which it can vector suggest that *Ae. albopictus* may have a greater role to play, either as a secondary epidemic vector or maintenance vector, should it become established in mainland Australia. While control programs are continually mounted to curb the expansion of *Ae. albopictus* into mainland Australia (Muzari et al., 2017), it is vital to continuously assess the vector competence of local mosquito populations in order to generate updated baseline risk assessment data for vector control agencies (Nicholson et al., 2014; van den Hurk et al., 2010).

The failure to detect an association between EIP and DENV serotype may have been due to the relatively small number of strains within each serotype that could be evaluated in this study. While it is difficult to imagine how the EIP for all strains of

DENV circulating in a region could be determined in an operational setting, there is evidence that a strain of DENV 3 with a short EIP contributed to the explosive epidemic of DENV-3 in Cairns in 2009 (Ritchie et al., 2013) and a number of studies have observed that the EIP of DENV is lengthened in *Wolbachia*-infected *Ae. aegypti* resulting in reduced potential for transmission of virus by the mosquitoes (Lauren B. Carrington et al., 2018; Ferguson et al., 2015; Ye et al., 2015).

In vitro experiments took these observations one step further by measuring the ability of *Wolbachia* to interfere with the replication of these strains of DENV in a mosquito cell line (*Ae. albopictus,* C6/36). While the replication of all strains of DENV was inhibited by *w*AlbB and *w*MelPop, the magnitude of the inhibition again was dependent on the strain of DENV being studied. Because *w*AlbB may be more robust over the range of temperatures encountered in nature and may impose less fitness costs on its mosquito host, *in vitro* studies with this strain of *Wolbachia* were extended to include other flaviviruses which cause significant disease in humans (ZIKV and WNV) as well as the alphaviruses RRV, BFV and SINV. *w*AlbB infection of C6/36 cells resulted in a significant reduction in the replication of all viruses studied. These results, combined with the observations that *w*AlbB appears to be able to withstand the fluctuations in ambient temperatures in areas where *Ae. aegypti* are found and to maintain relatively high maternal transmission fidelity (Ant et al., 2018; Bian et al., 2010; Joubert et al., 2016; P. A. Ross et al., 2017) suggest it may be a more viable control agent than *w*Mel or *w*MelPop (McMeniman et al., 2009; P. A. Ross et al., 2016; P. A. Ross et al., 2017).

There is a growing body of literature describing proposed mechanisms of *Wolbachia*-mediated blocking of virus replication (Bhattacharya et al., 2017; Brennan, Keddie, Braig, & Harris, 2008; Caragata et al., 2013; Geoghegan et al., 2017; Molloy, Sommer, Viant, & Sinkins, 2016; Pan et al., 2012; Rances, Ye, Woolfit, McGraw, & O'Neill, 2012) but establishing a unified and conserved mode of action behind *Wolbachia*'s virus blocking phenotype is difficult and often proves inconclusive in different *Wolbachia*-host-virus systems. Of these, however, two concepts prevail and have been consistently reported across different *Wolbachia*-host associations. The first results from competition between *Wolbachia* and virus for intracellular space and metabolic resources like macronutrients and lipids; the second is from the stimulation of cellular stress which makes the cell environment unconducive for viral replication

(Caragata et al., 2014; Caragata, Rezende, Simoes, & Moreira, 2016; Carro & Damonte, 2013; Francesca D. Frentiu, 2017; Harding et al., 2003; Molloy et al., 2016; Pan et al., 2012; Sasao, Igarashi, & Fukai, 1980; Wu et al., 2004). While it was outside the scope of our study to investigate the mechanism of action of *Wolbachia,* the substantial overlap in the obligate intracellular nature of both *Wolbachia* and the viruses appears to suggest that the mechanism of virus blocking is likely broad-spectrum and not independently evolved to target different viruses. However, further research is required to reconcile the differential reduction in the virus yield of individual virus strains as previous work involving *Wolbachia*-induced host factors shows that different viruses may be affected differently by host factors that may be elevated or down-regulated in response to *Wolbachia* (Bhattacharya et al., 2017). Given that the densities of *Wolbachia* were comparable in the cell lines at the start of the experiment, it will be challenging to explain inhibition in a density dependent manner.

Our approach of testing the same *Wolbachia* (*w*AlbB) strain in a common host genetic background against a panel of different viruses allows us to detect any pattern or correlations between genotypes, serotypes, or families and infer whether the magnitude of reduction in virus yield is linked. The results demonstrate that the replication of different strains of DENV was affected to different degrees by *Wolbachia* infection, thus leading to a wide range of virus yield between C6/36-*w*AlbB and *Wolbachia*-uninfected C6/36 cells. The titre of DENV-4 MY1261 in C6/36-*w*AlbB was reduced by up to 10^7 PFU/ml compared to the less than 10^2 PFU/ml seen in DENV-2 VN-130604. This suggests that the differential reduction in virus titre may stem from contributions from the virus genome or influenced by the genotype:genotype interactions between *Wolbachia* and virus, as well as the genetic background of the cell line (host). Variations in the impact of *Wolbachia* due to differences in DENV genotypes or serotypes have been demonstrated previously with *w*Mel in *Ae. aegypti* (Lauren B. Carrington et al., 2018). It is suggested that the large variation observed in DENV's response to *Wolbachia* demands that *Wolbachia* strains are carefully selected for introduction into wild mosquito populations to prevent the transmission of prevalent DENV genotypes circulating in an area.

The hypothesis that replication of each strain of DENV would be affected to a different extent by the presence of *Wolbachia* in host cells was supported by the data

obtained. No association was observed between the EIP of each strain of DENV and the magnitude of reduction in virus titre due to the presence of *Wolbachia*.

The observation that *w*AlbB and *w*MelPop reduced the yield of DENV in cultures of *Aedes albopictus* C6/36 cells by similar amounts was unanticipated. Natural symbionts (e.g. *w*AlbB, which is a natural endosymbiont of *Ae. albopictus* (S. P. Sinkins et al., 1995)), have been reported to be less effective in inhibiting viral replication (Bian, Zhou, et al., 2013; Joubert et al., 2016), than strains of *Wolbachia* (e.g. *w*MelPop) that have been transinfected into hosts or cell lines (Moreira et al., 2009). Given that the magnitude of inhibition of DENV replication is related to the density of *Wolbachia* in host cells (F. D. Frentiu et al., 2010; Lu et al., 2012), it is possible that the similar levels of inhibition of viral replication observed in this study were due to similar densities of *Wolbachia* in the cells employed in this study (Table 3.2)

There are several caveats to the results observed in the *in vitro* experiments reported here. The first is that it was possible to infect a very high proportion of the C6/36 cells with *Wolbachia* and the infected cells all contained very high densities of *Wolbachia. Wolbachia*-mediated virus inhibition studies consistently present a strong positive correlation between *Wolbachia* density and the extent of the virus blocking phenotype (Lu et al., 2012; Schultz et al., 2017). The second was that *Wolbachia* did not prevent all virus replication. While it is likely that more virus in mosquito saliva may be more likely to cause an infection in a human host, this association may apply only when there are reductions in existing small amounts of virus in the saliva e.g. < 5 to 10 PFU, so reducing virus replication may not prevent infection of the host bitten by the mosquito.

This study has provided clear evidence that differences in strain and serotype affect the EIP of DENV and by extension may affect the risk and magnitude of dengue outbreaks. *Ae. albopictus* populations which are currently established in the Torres Strait have been shown to have similar capacity to transmit multiple strains of DENV such as *Ae. aegypti* should it get into mainland Australia. With a strong virus blocking phenotype in mosquito cells, this study has provided indication that *Wolbachia* strain *w*AlbB has broad antiviral effects and is associated with similar strength of inhibition compared to *w*MelPop, and this justifies further consideration in mosquito feeding

experiments using viraemic blood as it promises to be an effective candidate for vector-biocontrol trials.

Chapter 6: Conclusions

I have established that there exist significant variations in the interval between the ingestion of a DENV blood meal and the appearance of detectable virus in the legs and wings of Australian *Ae. aegypti* and *Ae. albopictus,* a proxy for EIP, among DENV isolates representing the diversity of all DENV serotypes circulating in Southeast Asia and the Pacific. The heterogeneity in DENV transmission dynamics highlighted here by the significant differences in EIP, even within the same serotype, could potentially affect the risk and magnitude of dengue outbreaks. Should *Ae. albopictus* get established in mainland Australia, I have demonstrated the ability of this species to transmit multiple strains of DENV as much as *Ae. aegypti.* This work represents the first parallel evaluations of multiple regionally relevant strains of DENV in regionally relevant colonies of *Ae. aegypti* and *Ae. albopictus* mosquitoes and raises important questions about the epidemiological implications of strain and serotype diversity in DENV given the observed variation in the duration of the EIP. It also underscores the potential role of *Ae. albopictus* in DENV transmission in Australia. Previous studies have shown that the EIP of DENV is lengthened in *Wolbachia*-infected *Ae. aegypti* resulting in reduced potential for transmission of DENV by the mosquitoes (Lauren B. Carrington et al., 2018; Ferguson et al., 2015; Ye et al., 2015).

While the replication of all strains of DENV was similarly inhibited by *Wolbachia* strains, *w*AlbB and *w*MelPop, *w*AlbB infection of C6/36 cells resulted in a significant reduction in the yield of other representatives of flaviviruses (WNV and ZIKV) and alphaviruses (BFV, RRV and SINV) studied. I have demonstrated that *w*AlbB could exert virus blocking capacity similar to *w*MelPop in *Ae. albopictus*-derived C6/36 cells for flavi- and alphaviruses. However, the magnitude of the inhibition again was dependent on the strain of virus being studied. Although further research is required, the results of these studies combined with the observations that *w*AlbB appears to be able to withstand fluctuations in ambient temperatures in tropical areas and to maintain relatively high maternal transmission fidelity (Ant et al., 2018; Bian et al., 2010; Joubert et al., 2016; P. A. Ross et al., 2017) suggest it may be a more viable control agent than *w*Mel or *w*MelPop (McMeniman et al., 2009; P. A. Ross et al., 2016; P. A. Ross et al., 2017). These make a strong case for the evaluation of

Wolbachia strain *w*AlbB as a biocontrol agent wherever *Ae. aegypti* or *Ae. albopictus* are vectors of viruses of significant concern to humans. While this study underscores the potential utility of a single *Wolbachia* strain *w*AlbB for a broad range of arboviruses, it also shows *w*AlbB-induced virus inhibition is expressed differentially across diverse strains of DENV and other arboviruses. It remains to be determined whether *w*AlbB and *w*MelPop have similar effects on DENV replication *in vivo* and whether it would be necessary to achieve the same, extremely high, levels of intracellular *Wolbachia* infection in mosquitoes to achieve this

This study has extended our understanding of the implications and complexity of strain and serotype diversity of DENV and other arboviruses, and their interactions with *Wolbachia.* Our results show the potential significance of virus strain diversity – an often-ignored certainty in projections of vector competence and *Wolbachia*-mediated strategy which deserves attention when designing *Wolbachia*-based strategies for disease control.

6.1 FUTURE DIRECTIONS

The results of this study require some further investigations to clarify some of the outcomes and enhance our understanding of the implications of strain and serotype diversity in vector competence and *Wolbachia*-mediated vector-biocontrol strategy, which are as follows

1. Evaluate intraspecific variations and elucidate other potential roles of Torres Strait *Ae. albopictus* populations on transmission cycles of all DENV serotypes and other arboviruses in Australia.
2. Experimentally evolve any adaptive virus variants, determine the viral sequence diversity of the rebound virus samples, determine whether there are consistent nucleotide differences, and compare the virus inhibiting phenotypes.
3. Evaluate and compare the impact of *w*AlbB on the vector status and vector competence of *Ae. albopictus* and *Ae. aegypti* mosquitoes for DENV in addition to the primary vectors of other arboviruses.

Appendices

Appendix A

Preliminary blood feeding experiment with *Ae. albopictus* mosquitoes with pre- and post-feeding virus titres

DENV serotype	Strain	Initial concentration (PFU/ml)	Final concentration (PFU/ml)
DENV-1	NC-483	2.5×10^6	1×10^6
DENV-2	ET-300	2.5×10^6	1.0×10^6
	ET-300*	8.5×10^6	6.0×10^6
DENV-3	31298	2.5×10^6	1.0×10^6
DENV-4	NC-39	2.5×10^6	8.0×10^5

Appendix B

Numbers (percentage) of mosquitoes either infected or disseminated across the four time-points for each mosquito species and virus strain combination. P-values were calculated using Fisher's exact test of association.

Mosquito species	Virus strain	Days post-exposure							
		Day 3		Day 6		Day 10		Day 14	
		Infection	Dissemination	Infection	Dissemination	Infection	Dissemination	Infection	Dissemination
Ae. aegypti	DENV-1 NC-483	3/20 (15)	0/20 (0)	8/20 (40)	5/20 (25)	10/20 (50)	5/20 (25)	14/20 (70)	5/20 (25)
	DENV-4 MY-1261	1/20 (0)	0/20 (0)	3/20 (15)	2/20 (10)	5/20 (25)	3/20 (15)	5/20 (25)	4/20 (20)
	DENV-2 55763	1/20 (5)	0/20 (0)	6/20 (30)	2/20 (10)	6/20 (30)	3/20 (15)	8/20 (40)	5/20 (25)
	DENV-2 VN130604	1/20 (5)	0/20 (0)	2/20 (10)	1/20 (5)	9/20 (45)	8/20 (40)	7/20 (35)	4/20 (20)
	DENV-3 31298	1/20 (5)	0/20 (0)	1/20 (5)	1/20 (5)	2/20 (10)	1/20 (5)	5/20 (25)	4/20 (20)
	DENV-3 ET-3	0/20 (0)	0/20 (0)	0/20 (0)	0/20 (0)	4/20 (20)	1/20 (5)	5/20 (25)	2/20 (10)
	DENV-1 ET-243	0/20 (0)	0/20 (0)	1/20 (5)	0/20 (0)	6/20 (30)	5/20 (25)	- -	- -
	DENV-4 NC-39	0/20 (0)	0/20 (0)	1/20 (5)	0/20 (0)	2/20 (10)	1/20 (5)	6/20 (30)	2/20 (10)
	p-value	0.41	NA	0.002	0.042	0.037	0.035	0.043	0.79
Ae. albopictus	DENV-1 NC-483	0/20 (0)	0/20 (0)	8/20 (40)	4/20 (20)	8/20 (40)	4/20 (20)	11/20 (55)	6/20 (30)
	DENV-4 MY-1261	2/20 (10)	0/20 (0)	2/20 (10)	2/20 (10)	3/20 (15)	2/20 (10)	3/20 (15)	2/20 (10)
	DENV-2 55763	1/20 (5)	0/20 (0)	3/20 (15)	1/20 (5)	5/20 (25)	2/20 (10)	4/20 (20)	2/20 (10)
	DENV-2 VN130604	1/20 (5)	0/20 (0)	2/20 (10)	0/20 (0)	7/20 (35)	6/20 (30)	8/20 (40)	6/20 (30)
	DENV-3 31298	0/20 (0)	0/20 (0)	0/20 (0)	0/20 (0)	2/20 (10)	2/20 (10)	9/20 (45)	4/20 (20)
	DENV-3 ET-3	0/20 (0)	0/20 (0)	0/20 (0)	0/20 (0)	2/20 (10)	1/20 (5)	8/20 (40)	2/20 (10)
	DENV-1 ET-243	0/20 (0)	0/20 (0)	0/20 (0)	0/20 (0)	3/20 (15)	2/20 (10)	5/20 (25)	3/20 (15)
	DENV-4 NC-39	0/20 (0)	0/20 (0)	0/20 (0)	0/20 (0)	0/20 (0)	0/20 (0)	2/20 (10)	1/20 (10)
	p-value	0.57	NA	<0.001	0.017	0.015	0.15	0.019	0.27

Appendix C

Mean virus titres for bodies of mosquitoes harvested at various time points post exposure to virus meal (**Day 3**)

Serotype (strain)	Mosquito species	Infected (%)	Mean titre ± SD log_{10} RNA copies/mosquito
DENV-1 (NC-483)	*Ae. aegypti*	3/20 (15)	3.02 ± 0.0
	Ae. albopictus	0/20 (0)	0
DENV-1 (ET-243)	*Ae. aegypti*	0/20 (0)	0
	Ae. albopictus	0/20 (0)	0
DENV-2 (VN-130604)	*Ae. aegypti*	1/20 (5)	4.71 ± 0.11
	Ae. albopictus	1/20 (5)	5.00 ± 0.04
DENV-2 (55763)	*Ae. aegypti*	1/20 (5)	5.56 ± 0.23
	Ae. albopictus	1/20 (5)	5.00 ± 0.02
DENV-3 (31298)	*Ae. aegypti*	1/20 (5)	2.39 ± 0.12
	Ae. albopictus	0/20 (0)	0
DENV-3 (ET-3)	*Ae. aegypti*	0/20 (0)	0
	Ae. albopictus	0/20 (0)	0
DENV-4 MY-1261	*Ae. aegypti*	1/20 (5)	3.22 ± 0.32
	Ae albopictus	2/20 (10)	3.58 ± 2.65
DENV-4 (NC-39)	*Ae aegypti*	0/20 (0)	0
	Ae albopictus	0/20 (0)	0

Appendix D

Mean virus titres for bodies of mosquitoes harvested at various time points post exposure to virus meal **(Day 6)**

Serotype (strain)	Mosquito species	Infected (%)	Mean titre ± SD log_{10} RNA copies/mosquito
DENV-1 (NC-483)	*Ae. aegypti*	8/20 (40)	5.45 ± 0.08
	Ae. albopictus	8/20 (40)	5.64 ± 0.10
DENV-1 (ET-243)	*Ae. aegypti*	1/20 (5)	4.31 ± 0.14
	Ae. albopictus	0/20 (0)	0
DENV-2 (VN-130604)	*Ae. aegypti*	2/20 (10)	5.55 ± 0.01
	Ae. albopictus	2/20 (10)	3.21 ± 0.06
DENV-2 (55763)	*Ae. aegypti*	6/20 (30)	4.80 ± 0.06
	Ae. albopictus	3/20 (15)	3.93 ± 0.26
DENV-3 (31298)	*Ae. aegypti*	1/20 (5)	2.39 ± 0.12
	Ae. albopictus	0/20 (0)	0
DENV-3 (ET-3)	*Ae. aegypti*	0/20 (0)	0
	Ae. albopictus	0/20 (0)	0
DENV-4 (MY-1261)	*Ae. aegypti*	3/20 (15)	3.68 ± 0.14
	Ae. albopictus	2/20 (10)	4.11 ± 2.14
DENV-4 (NC-39)	*Ae. aegypti*	1/20 (5)	2.93 ± 0.00
	Ae. albopictus	0/20 (0)	0

Appendix E

Mean virus titres for bodies of mosquitoes harvested at various time points post exposure to virus meal **(Day 10)**

Serotype (strain)	**Mosquito species**	**Infected (%)**	**Mean titre ± SD log_{10} RNA copies/mosquito**
DENV-1 (NC-483)	*Ae. aegypti*	10/20 (50)	4.89 ± 0.14
	Ae. albopictus	8/20 (40)	5.50 ± 0.06
DENV-1 ET-243	*Ae. aegypti*	6/20 (30)	5.68 ± 0.10
	Ae. albopictus	3/20 (15)	5.90 ± 0.11
DENV-2 (VN-130604)	*Ae. aegypti*	9/20 (45)	4.39 ± 0.06
	Ae. albopictus	7/20 (35)	5.91 ± 0.16
DENV-2 (55763)	*Ae. aegypti*	6/20 (30)	5.38 ± 0.29
	Ae. albopictus	5/20 (25)	5.32 ± 0.42
DENV-3 (31298)	*Ae. aegypti*	2/20 (10)	3.89 ± 0.21
	Ae. albopictus	2/20 (10)	4.75 ± 0.03
DENV-3 (ET-3)	*Ae. aegypti*	4/20 (20)	6.10 ± 0.13
	Ae. albopictus	2/20 (10)	3.55 ± 0.13
DENV-4 MY1261	*Ae. aegypti*	5/20 (25)	3.98 ± 0.20
	Ae. albopictus	3/20 (15)	4.01 ± 0.19
DENV-4 (NC-39)	*Ae. aegypti*	2/20 (10)	2.84 ± 0.01
	Ae. albopictus	0/20 (0)	0

Appendix F

Mean virus titres for bodies of mosquitoes harvested at various time points post exposure to virus meal **(Day 14)**

Serotype (strain)	Mosquito species	Infected (%)	Mean titre ± SD log_{10} RNA copies/mosquito
DENV-1 (NC-483)	*Ae. aegypti*	14/20 (70)	4.46 ± 0.14
	Ae. albopictus	11/20 (55)	4.79 ± 0.04
DENV-1 ET-243	*Ae. aegypti*	*	-
	Ae. albopictus	5/20 (25)	5.75 ± 0.11
DENV-2 (VN-130604)	*Ae. aegypti*	7/20 (35)	4.33 ± 0.15
	Ae. albopictus	8/20 (40)	4.49 ± 0.12
DENV-2 (55763)	*Ae. aegypti*	8/20 (40)	5.23 ± 0.16
	Ae. albopictus	4/20 (20)	5.02 ± 0.11
DENV-3 (31298)	*Ae. aegypti*	5/20 (25)	5.09 ± 0.13
	Ae. albopictus	9/20 (45)	3.19 ± 0.09
DENV-3 (ET-3)	*Ae. aegypti*	5/20 (25)	4.53 ± 0.06
	Ae. albopictus	8/20 (40)	3.83 ± 0.07
DENV-4 MY1261	*Ae. aegypti*	5/20 (25)	4.40 ± 0.33
	Ae. albopictus	3/20 (15)	4.35 ± 0.08
DENV-4 (NC-39)	*Ae. aegypti*	6/20 (30)	3.53 ± 0.04
	Ae. albopictus	3/20 (15)	3.60 ± 0.07

*No mosquitoes were tested

Appendix G

Mean virus titres for legs/wings of mosquitoes harvested at various time points post exposure to virus meal (**Day 3**).

Serotype (strain)	Mosquito species	Disseminated (%)	Mean titre ± SD log_{10} RNA copies/mosquito
DENV-1 (NC-483)	*Ae. aegypti*	0	0
	Ae. albopictus	0	0
DENV-1 (ET-243)	*Ae. aegypti*	0	0
	Ae. albopictus	0	0
DENV-2(VN-130604)	*Ae. aegypti*	0	0
	Ae. albopictus	0	0
DENV-2 (55763)	*Ae. aegypti*	0	0
	Ae. albopictus	0	0
DENV-3 (31298)	*Ae. aegypti*	0	0
	Ae. albopictus	0	0
DENV-3 (ET-3)	*Ae. aegypti*	0	0
	Ae. albopictus	0	0
DENV-4 MY-1261	*Ae. aegypti*	0	0
	Ae albopictus	0	0
DENV-4 (NC-39)	*Ae aegypti*	0	0
	Ae albopictus	0	0

Appendix H

Mean virus titres for legs/wings of mosquitoes harvested at various time points post exposure to virus meal (**Day 6**).

Serotype (strain)	Mosquito species	Disseminated (%)	Mean titre ± SD log_{10} RNA copies/mosquito
DENV-1 (NC-483)	*Ae. aegypti*	5/20 (25)	3.09 ± 0.03
	Ae. albopictus	4/20 (20)	3.70 ± 0.07
DENV-1 (ET-243)	*Ae. aegypti*	0	0
	Ae. albopictus	0	0
DENV-2 (VN-130604)	*Ae. aegypti*	2/20 (10)	2.80 ± 0.08
	Ae. albopictus	0	0
DENV-2 (55763)	*Ae. aegypti*	2/20 (10)	2.20 ± 0.29
	Ae. albopictus	2/20 (10)	2.46 ± 0.09
DENV-3 (31298)	*Ae. aegypti*	1/20 (5)	2.52 ± 0.03
	Ae. albopictus	0	0
DENV-3 (ET-3)	*Ae. aegypti*	0	0
	Ae. albopictus	0	0
DENV-4 (MY-1261)	*Ae. aegypti*	2/20 (15)	2.43 ± 0.11
	Ae. albopictus	2/20 (10)	3.01 ± 0.09
DENV-4 (NC-39)	*Ae. aegypti*	0	0
	Ae. albopictus	0	0

Appendix I

Mean virus titres for legs/wings of mosquitoes harvested at various time points post exposure to virus meal (**Day 10**).

Serotype (strain)	Mosquito species	Disseminated (%)	Mean titre ± SD log_{10} RNA copies/mosquito
DENV-1 (NC-483)	*Ae. aegypti*	6/20 (30)	4.09 ± 0.04
	Ae. albopictus	4/20 (20)	3.00 ± 0.12
DENV-1 ET-243	*Ae. aegypti*	5/20 (25)	3.83 ± 2.49
	Ae. albopictus	2/20 (10)	4.09 ± 0.23
DENV-2 (VN-130604)	*Ae. aegypti*	8/20 (40)	2.97 ± 0.06
	Ae. albopictus	6/20 (30)	2.91 ± 0.06
DENV-2 (55763)	*Ae. aegypti*	3/20 (15)	3.95 ± 5.31
	Ae. albopictus	2/20 (10)	3.88 ± 0.11
DENV-3 (31298)	*Ae. aegypti*	2/20 (10)	3.35 ± 0.82
	Ae. albopictus	2/20 (10)	3.18 ± 0.04
DENV-3 (ET-3)	*Ae. aegypti*	2/20 (10)	2.70 ± 5.80
	Ae. albopictus	0/20 (0)	0
DENV-4 MY1261	*Ae. aegypti*	3/20 (15)	2.90 ± 0.17
	Ae. albopictus	3/20 (15)	3.00 ± 0.10
DENV-4 (NC-39)	*Ae. aegypti*	2/20 (10)	3.02 ± 0.10
	Ae. albopictus	0/20 (0)	0

Appendix J

Mean virus titres for legs/wings of mosquitoes harvested at various time points post exposure to virus meal (**Day 14**).

Serotype (strain)	Mosquito species	Disseminated (%)	Mean titre ± SD $\log_{10}$ RNA copies/mosquito
DENV-1 (NC-483)	*Ae. aegypti*	5/20 (250)	4.26 ± 1.79
	Ae. albopictus	11/20 (55)	4.27 ± 0.13
DENV-1 ET-243	*Ae. aegypti*	*	-
	Ae. albopictus	5/20 (25)	4.15 ± 0.16
DENV-2 (VN-130604)	*Ae. aegypti*	4/20 (20)	3.91 ± 0.25
	Ae. albopictus	6/20 (30)	2.96 ± 0.06
DENV-2 (55763)	*Ae. aegypti*	5/20 (25)	4.62 ± 0.07
	Ae. albopictus	2/20 (10)	2.92 ± 0.11
DENV-3 (31298)	*Ae. aegypti*	4/20 (20)	4.05 ± 0.14
	Ae. albopictus	9/20 (45)	2.99 ± 0.09
DENV-3 (ET-3)	*Ae. aegypti*	2/20 (10)	3.44 ± 0.13
	Ae. albopictus	3/20 (15)	2.89 ± 0.12
DENV-4 MY1261	*Ae. aegypti*	4/20 (20)	4.21 ± 0.13
	Ae. albopictus	2/20 (10)	3.01 ± 0.09
DENV-4 (NC-39)	*Ae. aegypti*	2/20 (10)	2.80 ± 0.15
	Ae. albopictus	2/20 (10)	3.02 ± 0.01

*No mosquitoes were tested at 14 d.p.e.

Appendix K

Number (percentage) of viruses blocked aggregated for m.o.i. levels

m.o.i.	Day 2	Day 4	Day 6	Day 8
0.1	13 (48.1%)	8 (29.6%)	12 (44.4%)	17 (63.0%)
1	9 (33.3%)	7 (25.9%)	10 (37.0%)	12 (44.4%)
10	7 (25.9%)	4 (14.8%)	6 (22.2%)	13 (48.1%)
20	7 (25.9%)	5 (18.5%)	3 (11.1%)	13 (48.1%)

Appendix L

Appendix 10 Number (percentage) of viruses blocked aggregated for cell line.

Cell line	Day 2	Day 4	Day 6	Day 8
C6/36	3 (8.3%)	2 (5.6%)	1 (2.8%)	2 (5.6%)
C6/36-*w*AlbB	14 (38.9%)	7 (19.4%)	13 (36.1%)	25 (69.4%)
C6/36-*w*MelPop	19 (52.8%)	15 (41.7%)*	17 (47.2%)	28 (77.8%)

*Significant pair-wise difference between *Wolbachia* cell lines ($p<0.05$), chi-square test of association

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