DYNAMICS OF *WOLBACHIA* ON MALAYSIAN *AEDES ALBOPICTUS* WITH REFERENCE TO DENGUE VIRUS

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ABSTRACT

Dengue virus belonging to the family *Flaviviridae* is currently the major vector borne arboviral disease in the tropics. The virus is transmitted by *Aedes* mosquitoes. Both the primary vector *Aedes aegypti* and the secondary vector *Aedes albopictus* are capable of transmitting all four dengue virus serotypes. Vector control has been the hallmark for the surveillance and control of dengue. *Wolbachia pipientis* are vertically transmitted intracellular gram negative bacteria that have been associated with their capabilities to alter their host reproductive phenotypes. *Wolbachia*-based strategies have been proposed for control of vector population and pathogen transmission rate. *Aedes albopictus* is spreading at an alarming rate and may one day displace the population of *Ae. aegypti* in Malaysia as have been observed in a few other countries. In this study, the role of *Wolbachia* on Malaysian *Ae. albopictus* dynamics and on its susceptibility towards dengue virus have been elucidated. *Wolbachia* infection status was studied and was found that most (91.6%) of Malaysian *Ae. albopictus* tested were superinfected with *w*AlbA and *w*AlbB which each fell into two distinct clades. *Wolbachia* was found in all gonads and in some midguts of the mosquitoes. Colonies of naturally *Wolbachia* infected *Ae. albopictus* and antibiotic treated *Wolbachia* free *Ae. albopictus* was generated. Removal of *Wolbachia* from Malaysian *Ae. albopictus* caused reduction in their fecundity, longevity and egg viability. Unidirectional cytoplasmic incompatibility was expressed which could be used for *Wolbachia*-based *Ae. albopictus* population control strategies in the future. *Wolbachia* did not affect Malaysian *Ae. albopictus* dengue infection and dissemination rate on all four dengue virus serotypes. In addition, Malaysian *Ae. albopictus* was shown to be a better vector for dengue serotype one (DENV-1) compared to the rest.

ABSTRAK

Virus denggi dari keluarga *Flaviviridae* merupakan penyakit disebabkan oleh vektor yang utama di kawasan tropika. Virus tersebut disebarkan oleh nyamuk *Aedes*. Kedua-dua nyamuk, *Aedes aegypti* yang merupakan vektor utama dan *Aedes albopictus* yang merupakan vektor kedua, boleh menyebarkan kesemua empat serotaip virus denggi. Langkah-langkah yang paling berkesan untuk mengurangkan wabak denggi adalah dengan mengawal populasi vektor dan mengurangkan interaksi antara vektor dan patogen. *Wolbachia pipientis* merupakan bakteria gram negatif yang boleh dijumpai dalam sitoplasma haiwan dan pernah dikaitkan dengan kebolehannya untuk mengubah sistem reproduksi haiwan atau serangga yang dijangkitinya. Strategi untuk menggunakan *Wolbachia* telah dicadangkan untuk mengawal populasi vektor dan kadar penyebaran patogen. *Aedes albopictus* sedang menyebar pada kadar yang membimbangkan dan mungkin boleh mengambil alih *Ae. aegypti* di Malaysia, seperti yang dilihat di negara-negara lain. Dalam disertasi ini, saya akan membincangkan eksperimen-eksperimen yang telah dijalankan bagi mempelajari peranan *Wolbachia* dalam aspek pembiakan *Ae. albopictus* di Malaysia dan ke atas kecenderungan *Ae. albopictus* untuk dijangkiti dengan kesemua empat serotype virus denggi. Status jangkitan *Wolbachia* telah dikaji dan adalah didapati bahawa 91.6% nyamuk *Ae. albopictus* di Malaysia dijangkiti dengan *w*AlbA dan *w*AlbB. Kedua-dua kumpulan ini telah dikategorikan dalam kelompok taksonomi yang berasingan dalam analisa phylogenetik yang dibuat. Jangkitan *Wolbachia* telah dijumpai didalam semua gonad dan didalam beberapa perut nyamuk. Koloni *Ae. albopictus* yang secara asalnya memang dijangkiti dengan *Wolbachia* dan koloni *Ae. albopictus* yang telah dirawat dengan antibiotik untuk menyingkirkan jangkitan *Wolbachia* telah diperolehi dan distabilkan dalam makmal. Penyingkiran *Wolbachia* dari *Ae. albopictus* telah mengurangkan kesuburan nyamuk betina, hayat nyamuk dewasa dan kadar penetasan telur. *Wolbachia* menyebabkan ketidakserasian sitoplasma searah dan ini

membuka peluang untuk menggunakan *Wolbachia* dalam kaedah pengawalan populasi *Ae. albopictus* di Malaysia. Ia didapati bahawa kadar jangkitan virus denggi dan kadar penyebaran virus oleh *Ae. albopictus* tidak dipengaruhi oleh *Wolbachia*. Di samping itu, keputusan kajian juga menunjukkan bahawa *Ae. albopictus* di Malaysia merupakan vektor yang lebih berkesan bagi virus denggi serotaip satu apabila dibandingkan dengan serotaip-serotaip lain.

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LIST OF SYMBOLS AND ABBREVIATIONS

˚	Degree
µL	Microliter
mL	Millimetre
cm	Centimetre
mM	Millimolar
mg	Milligram
g	Gram
V	Volt
%	Percent
>	Greater than
<	Lesser than
˚C	Degree Celsius
et al.	et alia (“and others”)
Ae.	*Aedes*
Cx.	*Culex*
D.	*Drosophila*
Mn.	*Mansonia*
Ar.	*Armigeres*
MIR	Midgut infection rate
VDR	Viral dissemination rate
OIR	Ovary infection rate
wsp	*Wolbachia* surface protein
*w*AlbA	*Wolbachia* supergroup A from *Ae. albopictus*
*w*AlbB	*Wolbachia* supergroup B from *Ae. albopictus*
CI	Cytoplasmic incompatibility
WHO	World Health Organization
min	Minute

h	Hour
s	Second
rpm	Rate per minute
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
rDNA	Ribosomal deoxyribonucleic acid
PBS	Phosphate buffered saline
NaCl	Sodium chloride
DENV-1	Dengue virus serotype 1
DENV-2	Dengue virus serotype 2
DENV-3	Dengue virus serotype 3
DENV-4	Dengue virus serotype 4
DSS	Dengue shock syndrome
DHF	Dengue haemorrhagic fever
FBS	Foetal bovine serum
MEM	Minimum essential medium
CFU	Colony forming unit
RIDL	Release of Insects with Dominant Lethality

LIST OF PUBLICATIONS

Publications from this project

Joanne S, Vythilingam I, Yugavathy N, Doss JI (2014). **Modified technique of *Wolbachia* removal from Malaysian *Aedes albopictus*.** *Asian Pacific Journal of Tropical Biomedicine.* 4(7): 557-560

Joanne S, Vythilingam I, Yugavathy N, Leong C-S, Wong M-L, AbuBakar S (2015). **Distribution and dynamics of *Wolbachia* on Malaysian *Aedes albopictus*.** *Acta Tropica.*148: 38-45

Joanne S, Vythilingam I, Yugavathy N, Leong C-S, Wong M-L, AbuBakar S (2016). **Unidirectional cytoplasmic compatibility in Malaysian *Aedes albopictus* (Diptera: Culicidae).** *Annals of the Entomological Society of America.* 109 (3): 366-370

CHAPTER 1: GENERAL INTRODUCTION

1.1 Introduction

Insects are the most diverse group of animals on earth representing more than half of all known living organisms (Glenner et al., 2006). There are insects beneficial to environment and humans such as butterflies which pollinate flowering plants (Proctor et al., 1996) and honey bees (Ramos-Elorduy, 1997) or silkworms which provides honey and silk (Chen et al., 2006) but most insects are considered pests to humans due to their ability to transmit disease (Speight et al., 1999). Insects can behave as deadly potential vectors for several pathogens. Vectors are organisms that are capable of transmitting infectious disease between humans or between animals and humans. Vector-borne diseases account for over 17% of all infectious diseases (World Health Organization, 2015). Mosquitoes are the best known disease vectors. Mosquitoes which are commonly associated with disease transmission belong to the following genera: *Aedes*, *Anopheles*, *Culex* and *Mansonia*. *Aedes* mosquitoes are capable of transmitting chikungunya virus (Delatte et al., 2008), dengue (Gubler, 1989), Zika (Chouin-Carneiro et al., 2016), yellow fever (Mitchell et al., 1987) , West Nile (Hubálek & Halouzka, 1999; Sardelis et al., 2002) and Rift Valley viruses (Mitchell et al., 1987) whereas *Anopheles* causes malaria (Hoffman et al., 2002) and *Culex* mosquitoes cause Japanese encephalitis (Van den Hurk et al., 2009), lymphatic filariasis (Mak, 2007) and West Nile fever (Hubálek & Halouzka, 1999).

Dengue virus has become a major threat in Malaysia as in other tropical and sub-tropical countries worldwide (Murrell et al., 2011). Number of deaths caused by dengue fever have been increasing yearly in all urban states of Malaysia (Pang & Loh, 2016). As of December 2015, there were 111 285 dengue cases with 301 deaths reported in Malaysia. This was 16.3% higher compared to the same period in 2014 where there were

103 610 dengue cases with 199 deaths (Samarasekera & Triunfol, 2016). The major vector for dengue in Malaysia is *Aedes aegypti* and the secondary vector is *Aedes albopictus*.

Both vectors are widespread all over the country (Lam, 1994). Populations of *Ae. albopictus* have been spreading more rapidly compared to *Ae. aegypti* (Dieng et al., 2010). Although *Ae. aegypti* is currently the major vector for dengue in Malaysia, chances of their population to be replaced by *Ae. albopictus* is quite high as it has been observed in three other countries namely Taiwan, Hawaii and Guam (Lambrechts et al., 2010). This triggers the necessity for more interest to be directed towards *Ae. albopictus* as most studies and vector control measures in Malaysia are focused towards *Ae. aegypti*.

Entomologists from all over the world have been researching various forms of vector control including biological pest control (Beard et al., 1998). Among strategies implemented are creating public awareness of *Aedes* control, fogging, use of larvicides (World Health Organization, 2009), release of genetically modified insects (Harris et al., 2012) and biological controls such as copepods (Kay et al., 2002) and *Wolbachia* (McGraw & O'Neill, 2013).

Wolbachia has become a major interest as a possible biological control of pests (Iturbe-Ormaetxe et al., 2011; Stouthamer et al., 1999). There has been a widespread interest in using *Wolbachia* as a biological control tool due to their massive abundance, effects on their hosts, which ranges from reproductive phenotype manipulation to mutualism, and potential applications in pest and disease vector control (Werren, 1997; Werren et al., 2008). *Wolbachia* can be used as a 'natural enemy' to either enhance a host population or to spread a desired genetic modification in an insect population (Bourtzis & O'Neill, 1998).

Aedes albopictus is naturally infected with *Wolbachia* (O'Neill et al., 1997). Thus, *Ae. albopictus* displays a great potential host to study the effect of *Wolbachia* on them and on how *Wolbachia* can be used to reduce *Ae. albopictus* populations in addition to their susceptibility to arboviruses.

Studies have indicated that *Wolbachia* may or may not affect the reproductive phenotype of their host and host susceptibility towards pathogens (Blagrove et al., 2012; Mousson et al., 2012; Werren, 1997). The mechanism underlying these relationships still remains unclear which makes their activity towards each different host unpredictable.

Wolbachia have been shown to alter fecundity, longevity and egg viability of the female host (Bourtzis & O'Neill, 1998; Dobson et al., 2004; Iturbe-Ormaetxe et al., 2011). Besides, they were able to express cytoplasmic incompatibility (CI) in most of their hosts (Blagrove et al., 2012; Calvitti et al., 2012; Dobson et al., 2004; Fu et al., 2010; Giordano et al., 1995; Hoffmann et al., 1998). Cytoplasmic incompatibility is when unviable offspring are produced when *Wolbachia* infected sperm fertilizes egg with different *Wolbachia* infection. Both these alterations combined, can be used as an effective tool in vector population control strategies.

Field trials and semi-field trials have been carried out in other countries and promising results were achieved. Field releases done in Australia have shown that it is possible to sustain artificially *Wolbachia* infected *Ae. aegypti* in field. *w*Mel infected *Ae. aegypti* successfully invaded two natural populations (Hoffmann et al., 2011). Similar results were reported by another semi-field trial conducted in Cairns, Australia in the same year. The latter also performed laboratory experiments and reported complete block of dengue virus transmission and reduced viral titre in whole mosquitoes (Walker et al., 2011). Two other semi-field trials were conducted by releasing incompatible male *Aedes polynesiensis* and both reported reduced egg hatch rate due to CI (Chambers et al., 2011;

O'Connor et al., 2012). To my knowledge, none has been conducted on native *Ae. albopictus*.

Malaysia has a large growing population of *Ae. albopictus* which should be naturally infected with *Wolbachia*. In order to examine the prospect of using *Wolbachia* to reduce population of *Ae. albopictus* in Malaysia, it is necessary to study the effect of *Wolbachia* on their fecundity, longevity and egg viability in addition to expression of cytoplasmic incompatibility.

According to a study conducted on naturally *Wolbachia*-infected *Ae. albopictus* from La Reunion island, *Wolbachia* did not affect dengue virus infection rate in them but reduced viral dissemination rate (Mousson et al., 2012). A few other studies on *Ae. aegypti* and *Drosophila melanogaster* reported that *Wolbachia* was able to reduce pathogen transmission rate in their respective hosts (Hedges et al., 2008; Moreira et al., 2009).

Although many studies have been carried out on the dynamics of *Wolbachia* in their native hosts and into newly transferred hosts, no conclusive pattern on their effects were obtained and to my knowledge no other studies have been conducted to study *Wolbachia* dynamics in Malaysian *Ae. albopictus*. Hence, it is necessary to study the effect of *Wolbachia* on Malaysian *Ae. albopictus* and their susceptibility to all four dengue serotypes.

1.2 Objectives

The objectives of this study are as follows:

(i) To determine the natural *Wolbachia* infection status in Malaysian *Ae. albopictus*.

(ii) To determine the distribution of *Wolbachia* in various organs of *Ae. albopictus* at different time points.

(iii) To establish *Ae. albopictus* colony with and without *Wolbachia* under laboratory condition.

(iv) To determine the dynamics of *Wolbachia* and cytoplasmic incompatibility (CI) in Malaysian *Ae. albopictus*.

(v) To determine the effect of *Wolbachia* on Malaysian *Ae. albopictus* susceptibility towards dengue virus.

These objectives will be discussed as separate chapters in this dissertation. Objective one and two will be combined into Chapter 1 while objectives three to five will be discussed in Chapters 4 to 6.

1.3 Justification of study

The rationale of this study are as follows:

(i) The distribution of different *Wolbachia* strains found in *Ae. albopictus* in different regions of Malaysia have not been resolved.

(ii) The effect of *Wolbachia* on Malaysian *Ae. albopictus* reproductive phenotypes are unknown

(iii) Cytoplasmic incompatibility status on Malaysian *Ae. albopictus* have not been established.

(iv) The effect of *Wolbachia* on virus susceptibility, midgut infection rate and viral dissemination rate in *Ae. albopictus* remains unclear

(v) No studies have been conducted on the effect of *Wolbachia* on all four dengue serotypes in *Ae. albopictus* in Malaysia.

CHAPTER 2: LITERATURE REVIEW

2.1 Dengue

2.1.1 Background

Dengue is currently the most important arthropod-borne disease worldwide (Gubler, 2006; Murray et al., 2013; World Health Organization, 2010). Dengue epidemics occur in subtropical and tropical regions in Southeast Asia, the Pacific, America, Africa and Eastern Mediterranean (World Health Organization, 2007). Millions of infections occur yearly including hospitalization due to dengue haemorrhagic fever and these incidences have been increasing at an alarming rate each year (Guzman et al., 2010). Dengue has spread to non-endemic countries in travellers (Wilder-Smith & Schwartz, 2005). At present all four dengue virus serotypes are circulating in Asia, Africa and America (Guzman & Istúriz, 2010). It was estimated that there were 96 million dengue infections worldwide in 2010. Asia contributed 70% (67 million infections) of this infections. India alone contributed 34% (33 million infections) of the global total. The Americas contributed 14% (13 million infections) which over half occurred in Brazil and Mexico. Their results showed that Africa's dengue burden is nearly equivalent to that of the Americas (16 million infections). The countries of Oceania contributed less than 0.2% of global infections (Bhatt et al., 2013).

Dengue virus belongs to the family *Flaviviridae,* genus *Flavivirus* (Rodenhuis-Zybert et al., 2010). There are four dengue virus serotypes which share approximately 65% of the genome with each other (Halstead, 2008). In 2013, a fifth dengue virus serotype was reported but this needs to be substantiated (Normile, 2013). Each serotype DENV-1, DENV-2, DENV-3 and DENV-4 have phylogenetically distinct genotypes and clades with 3-6% variation (Guzman et al., 2010).Virus were initially transmitted via sylvatic cycles between mosquito vectors and non-human primates. Each of them evolved

independently and entered the urban cycle at different time points (Holmes & Twiddy, 2003; Wang et al., 2000). Each serotype genome has a single open reading frame that translates three structural and seven non-structural proteins. Although the genome of each dengue virus serotype differs from each other, they have indistinguishable symptoms in human and circulate both sub-tropical and tropical regions (Halstead, 2008).

2.1.2 Signs and symptoms of dengue

Dengue virus causes several symptoms and the severity of each symptom depends on a number of factors such as age, gender, previous dengue infection, immunological status and race (Guzmán & Kourí, 2004; Guzmán et al., 1990). Infection in children causes mild non-specific febrile syndromes. Life-long immunity will be acquired against the same serotype through primary infection. Secondary infection in the same patient may result in dengue shock syndrome (DSS) (Halstead, 1970) or enhanced severity (Guzmán & Kouri, 2002). Dengue shock syndrome can also occur at the first infection. Infection in adults would cause a broad spectrum of symptoms after an incubation period of 4-8 days such as throbbing headache, acute febrile syndromes, retro-orbital pain, leukopenia, thrombocytopenia, body ache and rashes. Febrile syndromes includes high body temperature and spike fever (Gubler, 2006).

Dengue shock syndrome is a syndrome caused by dengue virus which may occur in both adults and children but affect kids below 10 years old the most (Halstead, 1970). DSS causes abdominal pain, haemorrhage and circulatory collapse. It is also known as dengue haemorrhagic fever (DHF). DSS starts with sudden onset of high continuous fever and headache with other symptoms such as sore throat, cough, nausea, vomiting and abdominal pain. There will be bloody bruises, blood spots on the skin and blood in the stool (Halstead, 1970; Thein et al., 1997). Higher number of death occurs among children and the most in infants under a year old (Halstead et al., 2002). Dengue becomes more

life threatening when they occur in individuals with asthma, diabetes and other chronic diseases (Guzman et al., 2010).

2.1.3 Dengue transmission cycle

Dengue virus is a single stranded RNA. The virus genome is within a capsid shell covered by envelope proteins surrounding lipid bilayer envelope (Hanley & Weaver, 2010). They tend to target the immune cells. Dengue virus transmission results from interactions between human and mosquitoes (Figure 2.1). A mosquito carrying the dengue virus is known as dengue vector. The transmission cycle starts with ingestion of dengue virus infected blood by mosquitoes. An extrinsic incubation period of 8-10 days is necessary after feeding on an infected human for viral replication in the mosquito. Within this period, virus penetrates the midgut barrier and infects the salivary glands, hence making the mosquito infectious (Rodenhuis-Zybert et al., 2010).

Once a dengue virus infected female mosquito bite a human, mature virus envelope protein binds to the cognate receptors of the immune cell surface. It triggers endocytosis and enters into the cell as endosomes. A proton pump within the endosome reduces the interior pH and changes the virus envelope protein to become hydrophobic. This allows them to bind to the endosome membrane and release the capsids into the cell cytoplasm. The capsid breaks and releases the RNA. It travels to rough endoplasmic reticulum and gets translated into proteins and RNA replication complex proteins which replicates the RNA. Each viral RNA binds to new capsid proteins and are packaged into new immature virus particles. These particles become mature in the Golgi apparatus and are released from the cell to infect other immune cells (Figure 2.1) (Rodenhuis-Zybert et al., 2010; van der Schaar et al., 2008)

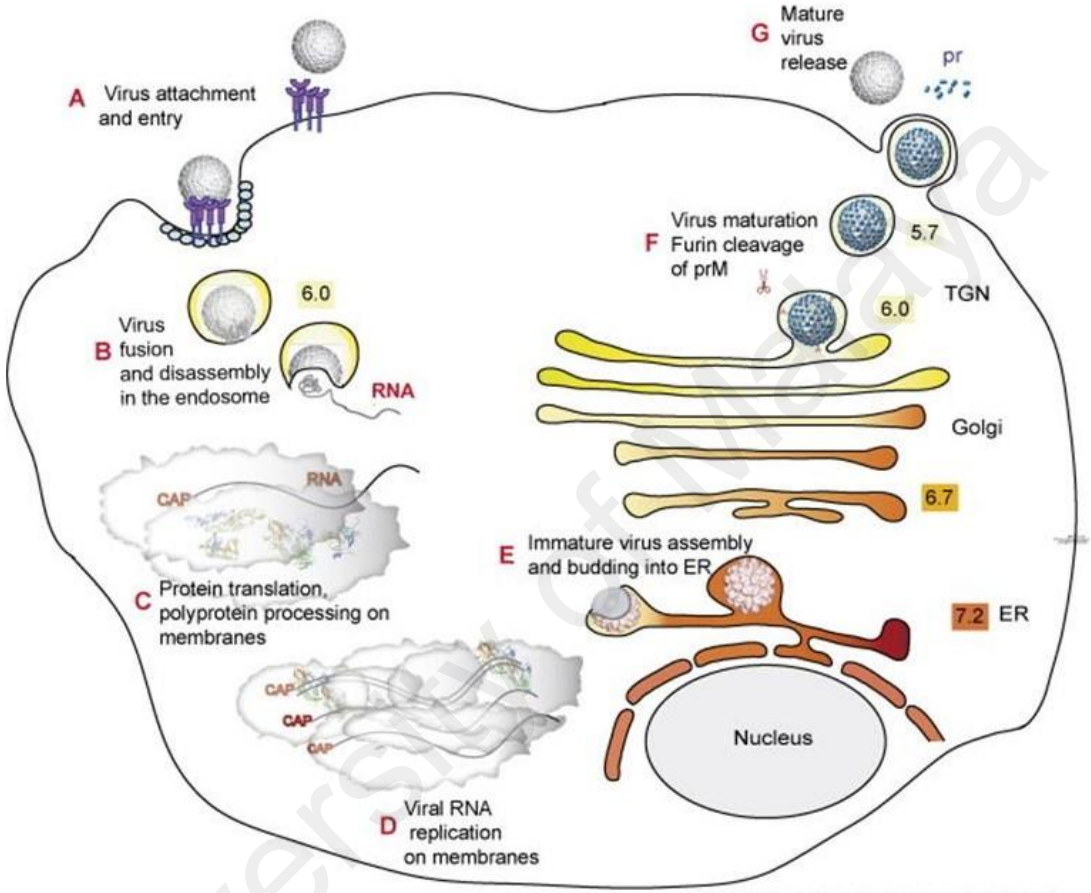

Figure 2.1: Dengue virus life cycle (Rodenhuis-Zybert et al., 2010).

Besides transmission through blood meal, transovarial transmission of the dengue virus has also been reported (Lee & Rohani, 2005). Dengue virus interaction with their host is complicated and unique. Distinctiveness of the interaction complicates vaccine development (Guzman et al., 2010). To date, there is only one licensed dengue vaccine which is being used in Mexico for individuals between nine to 45 years old. It is a live recombinant tetravalent dengue vaccine developed by Sanofi Pasteur (Vannice et al., 2016).

2.1.4 Dengue in Malaysia

Dengue has been acknowledged as a national health threat in Malaysia (Er et al., 2010). The number of dengue cases in Malaysia has increased at an alarming rate over the decade (Mohd-Zaki et al., 2014). In December 2015, there were 111 285 dengue cases with 301 deaths reported in Malaysia. This was 16.3% higher compared to the same period in 2014 where there were 103 610 cases with 199 deaths according to World Health Organization (Samarasekera & Triunfol, 2016).

Three of the existing dengue serotypes have been circulating Malaysia in 2005 to 2015. DENV-1 predominated in 2005, DENV-1 and DENV-3 in 2006, DENV-1 and DENV-2 in 2007, and DENV-3 in 2008 and 2009 (Mohd-Zaki et al., 2014). In year 2015, the dominating serotype was DENV-1 (Mudin, 2015). Certain states are more endemic compared to others. Selangor has been reporting the highest number of cases, followed by Wilayah Persekutuan Kuala Lumpur, Perak and Johor. These four states are more populated compared to the other 10 states in Malaysia.

The climate in Malaysia plays a major role in spread of dengue infection throughout the country. Malaysia is situated at the equator which gives it an all year round hot, humid and at the same time rainy climate which is ideal for mosquito breeding and

virus development (Cheong et al., 2013). Female dengue vectors oviposit in stagnant clear water inside containers. Once the water level rises due to rain, the eggs hatch to become larvae. After four to five days, larva turns to pupa and becomes an adult mosquito. If the parent mosquito was dengue virus infected, the offspring produced may also be infected and it will be able to infect uninfected human. If that is not the case, the new offspring are prone to get the virus infection by feeding on an infected human (Guzman et al., 2010).

2.1.5 Mosquitoes involved in transmission of dengue and other arboviruses

Dengue is transmitted by the mosquitoes *Ae. aegypti* and *Ae. albopictus* (Hamady et al., 2013; Lambrechts et al., 2010). Besides dengue, other major diseases transmitted by this two vectors are chikungunya virus, Zika virus and Yellow Fever (Cadu & Harish, 2015; Charrel et al., 2007; Lee, 2016).

2.1.6 Dengue control

Dengue cases could be reduced by employing better outbreak prediction and detection through coordinated epidemiological and entomological surveillance, promoting the principles of integrated vector management, deploying effective urban household water management and prevention programmes whereas number of death caused by dengue can be decreased by executing early case detection and proper referral system for patients, managing severe cases with appropriate treatment, reorienting health services to cope with dengue outbreaks and training health personnel at all levels of the health system (World Health Organiation, 2012).

2.2 Other diseases transmitted by *Aedes* mosquitoes

2.2.1 Chikungunya

Chikungunya virus is transmitted primarily by *Ae. albopictus* and secondarily by *Ae. aegypti* (Reiter et al., 2006). Chikungunya virus was first described during an outbreak in southern Tanzania in 1952 (Robinson, 1955). It is a single stranded RNA virus that belongs to the genus *Alphavirus* and family *Togaviridae*. It has been found in Asia, Africa, Europe and America (Powers et al., 2000). During inter-epidemic periods, chikungunya virus may be maintained in a sylvatic cycle in non-human primates (Diallo et al., 1999). After a bite from an infected mosquito, symptoms are seen between 4 to 8 days. There is no specific vaccine for chikungunya virus. Major symptoms are joint pain, fever, muscle pain, nausea, fatigue and rash. Since 2005, more than a million cases has been reported in India, Maldives, Myanmar, Thailand and Indonesia (Sam et al., 2012). There have been three outbreaks in Malaysia since the 1960s. The first was in Klang, Selangor (1998) (Lam et al., 2001), followed by Bagan Panchor, Perak in 2006 (Ayu et al., 2010) and in Ipoh, Perak in 2007 (Noridah et al., 2007). *Aedes albopictus* was found to be a better laboratory vector for chikungunya virus in Malaysia compared to *Ae. aegypti* (Sam et al., 2012).

2.2.2 Zika virus

Zika virus is transmitted mainly by *Ae. aegypti* however, *Ae. albopictus* may also transmit the virus (Wong et al., 2013). Zika virus was originally identified in Zika forest of Uganda from a febrile sentinel monkey in 1947 (Dick, 1952). Human case was only identified in 1964 (Simpson, 1964). It is a positive single stranded RNA virus from the genus *Flavivirus* of the family *Flaviviridae* (Cadu & Harish, 2015). Outbreaks of Zika virus disease have been recorded in Africa, the Americas, Asia and the Pacific. Zika virus was declared a Public Health Emergency of International Concern in February 2016

(Cadu & Harish, 2015; Lee, 2016; Samarasekera & Triunfol, 2016). Symptoms shown by patient infected with Zika virus are almost identical to symptoms of dengue infection which includes spike fever, rashes, conjunctivitis, body ache and headache (Duffy et al., 2009; Simpson, 1964). These symptoms normally last for two to seven days and there is no vaccine nor cure for Zika virus infection.

2.3 *Aedes albopictus*

2.3.1 Background

Aedes albopictus is an invasive mosquito species belonging to the genus *Aedes*; subgenus *Stegomyia*. It was discovered by Skuse in 1895 and named as *Culex albopictus*. In 1932, Skuse renamed it to *Aedes albopictus* (Skuse, 1894). It was first found in tropical and subtropical areas of South East Asia. They are identified with the black and white stripes on their legs and body (Huang, 1968). They are called Asian tiger mosquito due to their stripes. They are morphologically quite similar to *Ae. aegypti*. *Aedes albopictus* have a white vertical line in the middle of the head that runs down their thorax whereas *Ae. aegypti* have two crescent shaped white marks on the right and left of their thorax (Rueda, 2004). Images of both mosquitoes are shown in Figure 2.2. In the larval stage, they can be differentiated by using the comb scales and hooks on thorax side. The comb-teeth in *Ae. albopictus* larvae is straight thorn-like whereas in *Ae. aegypti* is pitchfork shaped. *Ae. aegypti* have strong black hooks on the side of their thorax whereas *Ae. albopictus* have either small or no hooks (Figure 2.3).

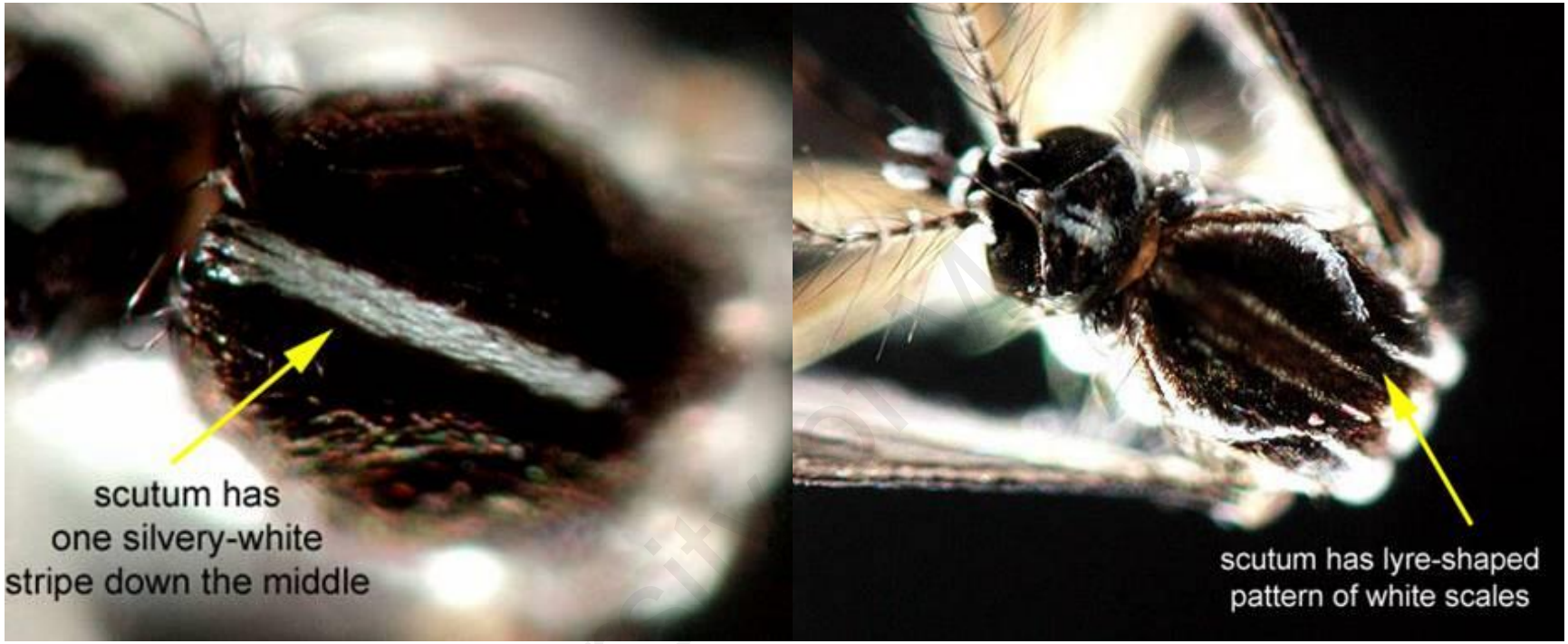

Figure 2.2: Thorax of adult *Aedes* mosquitoes. *Aedes aegypti* (right); *Aedes albopictus* (left).

(Source: http://fmel.ifas.ufl.edu/key/quick_larval/larval_02.shtml)

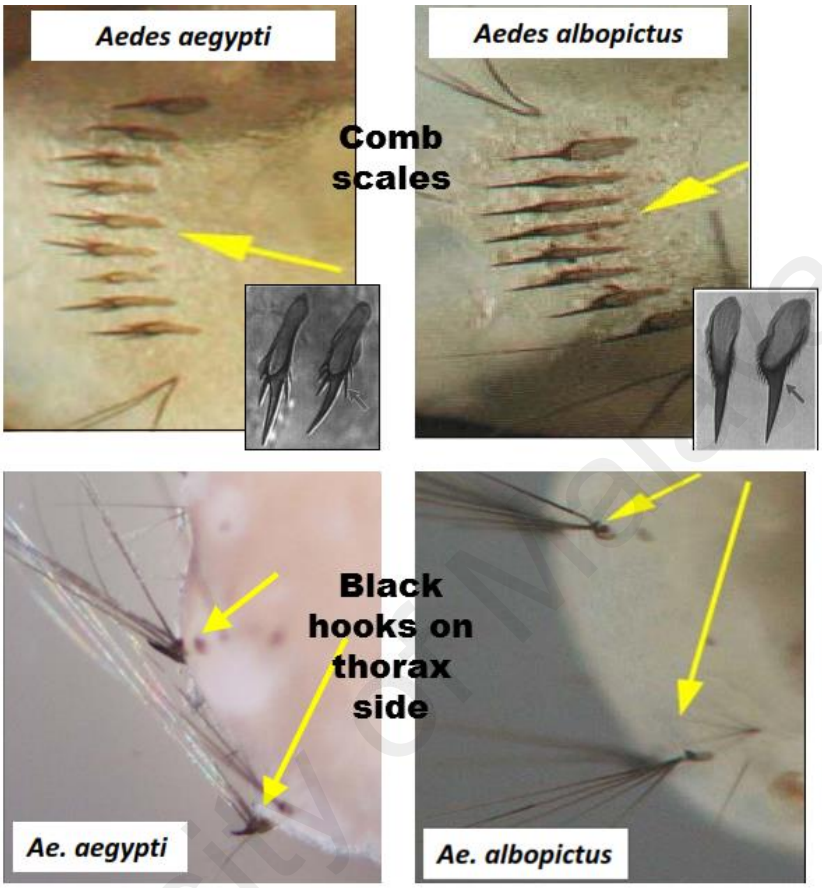

Figure 2.3: Identification keys for *Aedes* larvae. Comb scales (up) and thorax (down) side hooks of *Ae. aegypti* (left) and *Ae. albopictus* (right) (Rueda, 2004).

2.3.2 Life cycle

Aedes mosquitoes have four main stages. Eggs develop into larvae, then to pupae and finally to adults. After mating, female mosquitoes lay its eggs on the sides of containers with water following a blood meal. Eggs develop into larvae in the water. Larvae are divided into four stages namely the first, second, third and fourth instar. Each instar is bigger than the previous instar. All four larval stages are ravenous eaters. They eat bacteria, fungal spores, algae and microscopic particles in the water. After the fourth instar larvae, they develop into pupae. They do not feed at pupae stage. Finally, three days later, they emerge as adults and the whole cycle begins again (Figure 2.4).

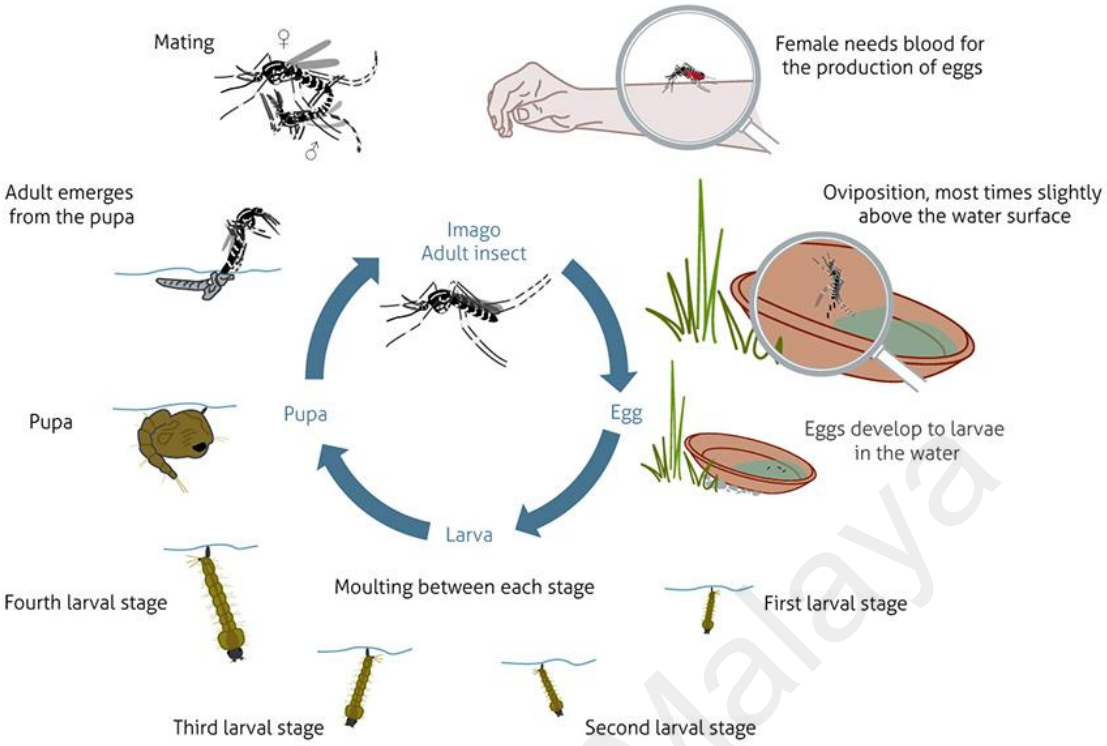

Figure 2.4: Life cycle of *Aedes* mosquitoes.

(Source:

http://www.biogents.com/cms/website.php?id=/en/traps/mosquitoes/tiger_mosquitoes.htm)

2.3.3 Ecology and host preference

Aedes albopictus breeds in stagnant clear water. The female lay eggs in near clean water collected in tyres, dark holes, blocked drains, flower pot holders, water tanks and tree trunk holes(Paupy et al., 2009). *Aedes albopictus* is a day biting mosquito that was originally a zoophilic forest species from Asia. They are generalists that easily adjust to different environmental conditions in both tropical and temperate regions (Rai, 1991). *Aedes albopictus* are catholic feeders which feed on variety of animals which made them potentially dangerous bridge vector between human and animal pathogens (Richards et al., 2006). In the last decade, caused by environmental changes due to deforestation and developments, they are no longer found only in areas with high vegetation. These species are currently found is most regions including urbanised areas alongside *Ae. aegypti*. Recent studies have reported that *Ae. albopictus* have a higher preference towards feeding on humans compared to animals (Delatte et al., 2010) and a study conducted in Thailand reported that 95% *Ae. albopictus* fed on human blood which was similar to *Ae. aegypti* feeding rate (Ponlawat & Harrington, 2005). *Aedes albopictus* have been reported to feed on single host multiple times if given the chance (Kek et al., 2014).

Population of *Ae. albopictus* have been growing rapidly (Benedict et al., 2007). There have been reports of areas previously co-inhabited by both *Ae. albopictus* and *Ae. aegypti*, are now only colonised by *Ae. albopictus* (Gratz, 2004).

2.3.4 Vector Competence

The recent dramatic global expansion in geographic distribution of *Ae. albopictus* has triggered considerable concern among scientists and public health officials over the possibility of an increased risk of arthropod-borne virus transmission.

Aedes albopictus is able to transmit most viruses including eight *alphaviruses*, four *bunyaviruses* and eight *flaviviruses* (Paupy et al., 2009). It is the primary vector of chikungunya virus worldwide and besides, it also transmit major pathogens such as dengue virus, Zika virus (Wong et al., 2013), yellow fever (Mitchell et al., 1987) and West Nile virus (Sardelis et al., 2002). *Aedes albopictus* was reported to be responsible for the dengue epidemics that occurred in Japan and Taiwan in the 1940s. More recent epidemics caused by this species were in La Reunion Island (1977), China (1978) and Macau (2001) (Lambrechts et al., 2010). Though, the majority of the cases had only classical dengue fever, very few severe and fatal cases were observed. All dengue haemorrhagic fever have only occurred in areas where *Ae. aegypti* was also found (Gubler, 1998). The population of *Ae. aegypti* was replaced by *Ae. albopictus* in Taiwan, Hawaii and Guam (Lambrechts et al., 2010). Although dengue cases were observed in these regions, they have not experienced major epidemics in the recent years.

Meta-analysis carried out in 2010 reported that *Ae. albopictus* have higher midgut dengue infection rate compared to *Ae. aegypti* whereas the opposite for salivary gland dengue dissemination rate (Lambrechts et al., 2010). Transovarial transmission of dengue virus to its offspring were also compared between these two species in three different studies in between 1983 to 1997. Two of the studies reported *Ae. albopictus* to have higher dengue virus vertical transmission rate (Bosio et al., 1992; Rosen et al., 1983) while one of them reported *Ae. aegypti* to have higher transmission rate (Lee et al., 1997).

2.4 *Aedes aegypti*

2.4.1 Background

Aedes aegypti also belong to the genus *Aedes* and subgenus *Stegomyia.* It transmits four dangerous arboviruses; dengue, chikungunya virus, yellow fever and Zika virus. It bites during the day and is found in tropical and temperate regions. It mates, feeds, lays eggs and spreads around human habitation (Christophers, 1960).

They are the primary vector for yellow fever that prevails in South America and Africa, thus they were called the yellow fever mosquitoes (Christophers, 1960). The yellow fever mosquito was a common vector in Florida until the invasion of the Asian tiger mosquito, *Ae. albopictus* (Skuse) (Lounibos et al., 2001). Since the introduction of the Asian tiger mosquito in 1985, the population of *Ae. aegypti* in Florida has declined dramatically, but still thrives in urban areas of South Florida. *Aedes albopictus* larvae out competes *Ae. aegypti* larvae for food, and develop at a faster rate (Barrera, 1996).

2.4.2 Ecology and host preference

Similar to *Ae. albopictus*, *Ae. aegypti* are container dwelling mosquitoes. They often breed in unused flowerpots, spare tires, untreated swimming pools, and drainage ditches. Since they live in urban areas, they have constant close contact with human which makes them very good vector.

Aedes aegypti adults have white scales on the thorax that form the shape of a lyre (Carpenter & LaCasse, 1955). Flight range of *Ae. aegypti* are relatively low (less than 200m, rarely up to 500m), suggesting that people rather than mosquitoes are the primary mode of dengue virus dissemination within and among communities (Fonzi et al., 2015; Harrington et al., 2005).

2.4.3 Vector competence

Transmission of dengue virus by *Ae. aegypti* depends on environmental and intrinsic factors associated with virus-vector interaction (Hardy, 1988). Naturally, dengue virus is ingested through a blood meal and the viral load found in the midgut is dose dependent. However, the ability to overcome the physical barriers in the mosquito depends on the vector's susceptibility.

After feeding on dengue virus infected blood meal, the virus has to cross the midgut epithelium and enter the midgut. It then multiplies within the midgut and escapes the midgut through midgut infection barrier. Only then, it invades other tissues and organs. It only infects the salivary gland once it has successfully passed the salivary gland infection barrier (Tabachnick, 2013). The first study that assessed vector competency of *Ae. aegypti* towards dengue virus was executed in 2002 and they reported that mosquito population from different regions had a significant difference in their dengue virus susceptibility and transmission rate (Bennett et al., 2002).

2.5 Vector control

Ministry of Health Malaysia (MOH) has been implementing numerous existing measures and studying possible new methods to reduce the number of cases of mosquito borne diseases. The major target has been to reduce the vector population and host-vector interaction. Among strategies that are being implemented or explored are fogging, netting (World Health Organization, 2009), lethal ovitraps (Paz-Soldan et al., 2016), larviciding biological control such as larvivorous fish (Nam et al., 2000), copepods (Kay et al., 2002), ovitraps and *Wolbachia* (McGraw & O'Neill, 2013), repellents (World Health Organization, 2009), insecticides and release of genetically modified mosquitoes (RIDL) (Harris et al., 2012). Lately, *Wolbachia* have become an interest for vector population

control and in reducing transmission of pathogens (Dobson et al., 2002; Iturbe-Ormaetxe et al., 2011).

Chemical insecticides are no longer a robust method as mosquitoes tend to develop resistance towards all insecticides used over time (Chen et al., 2005; Koou, Chong, Vythilingam, Lee, et al., 2014; Koou, Chong, Vythilingam, Ng, et al., 2014; Low et al., 2013). Even larvicides face the same issue of development of resistance. The more frequently an insecticide or a larvicide is being used, the faster the target develops resistance (Taylor et al., 1983). Biological control using *Wolbachia* is a relatively new control measure being studied in Malaysia although *Wolbachia* have been associated with vector population control in many studies previously. Limited number of studies are being conducted to explore this hypothesis in Malaysia.

Most of the control methods are targeted to reduce only *Ae. aegypti* population. This is because *Ae. albopictus* was typically more rural whereas *Ae. aegypti* more urban. However, since this is longer the case, once *Ae. aegypti* population have been reduced, *Ae. albopictus* may become a major threat in Malaysia due to its rapidly growing population and its ability to transmit dengue, Zika virus and chikungunya virus.

Recent study on dengue virus and vector control have described use of long-lasting formulations of synthetic pyrethroids applied to walls, curtains, window screens, and water container covers as possible vector control tools. Besides those, reduction of larval sources through either container removal or applications of insecticides or biological agents was also suggested. To be most effective, larval control needs to be combined with methods targeting adult mosquitoes (Reiner Jr et al., 2016).

2.6 *Wolbachia*

2.6.1 Background

Wolbachia pipientis species of *Wolbachia* genus is an intracellular maternally inherited alpha proteobacteria that is found in most arthropods and in few nematodes. It was first found in *Culex pipiens* in 1924 (Hertig, 1936). It is a gram negative bacteria with symbiotic features. Over the years, phylogenetic studies have been performed to classify *Wolbachia* strains from different hosts using 16S rDNA (O'Neill et al., 1997), 23S rDNA, surface protein-coding genes (*wsp*) (Armbruster et al., 2003; O'Neill et al., 1997) and cell-cycle gene (*fts*Z) (Werren et al., 1995). The 16S rDNA gene evolved too slowly, hence instigated introduction of *wsp* and *fts*Z genes. *Wsp* gene has been commonly used over recent years as they exhibit increased recombination rate which accelerates genetic and functional diversity making them a fast evolving region and suitable for classification of the supergroups (Werren et al., 2008). *Wsp* gene possess 10 times the variability of the *fts*Z gene and hence more suitable to elucidate evolutionary relationship of *Wolbachia* isolates (Werren et al., 1995; Zhou et al., 1998).

Wsp genes were classified into 9 supergroups namely A, B, C, D, E, F, G, H (Werren et al., 2008) and I. Supergroup I was only identified in 2009 (Haegeman et al., 2009). All the supergroups are monophyletic unlike other Rickettsiales (Werren et al., 2008). Supergroup A, B, E, G and H have been found in arthropods (Casiraghi et al., 2005; Haegeman et al., 2009; Rowley et al., 2004) (most commonly supergroup A and B) (O'Neill et al., 1992), supergroup C, D and I have been found in nematodes (Bandi et al., 1998; Foster et al., 2005; Haegeman et al., 2009) whereas supergroup F have been found in both arthropods and nematodes (Casiraghi et al., 2005). A list of supergroups, phylum and few species infected with each supergroup are shown in Table 2.1.

Table 2.1: Supergroups with their respective phylum and species.

Supergroup	Phylum	Example of species
A	Arthropoda	*Cx. pipiens* (Hertig, 1936) *Ae. albopictus* (O'Neill et al., 1992) *Drosophila sechellia* (Giordano et al., 1995) *D. melanogaster* (O'Neill et al., 1992) *Nasonia spp.* (Bandi et al., 1998)
B	Arthropoda	*Cx. pipiens* (Hertig, 1936) *Ae. albopictus* (O'Neill et al., 1992) *D. melanogaster* (O'Neill et al., 1992) *Drosophila mauritania* (Giordano et al., 1995) *Nasonia spp.* (Werren, 1997) *Trichogamma deion* (Huigens & Stouthamer, 2003)
C	Nematode	*Dirofibria repens* (Bandi et al., 1999) *Onchocerca volvulus* (Higazi et al., 2005)
D	Nematode	*Brugia pahangi* (Bandi et al., 1999) *Brugia malayi* (Foster et al., 2005) *Wuchereria bancrofti* (Fenn & Blaxter, 2006)
E	Arthropoda	*Folsomia candida* (Vandekerckhove et al., 1999) *Mesaphorura macrochaeta* (Vandekerckhove et al., 1999)
F	Arthropoda and Nematode	*Rhinocyllus conicus* (Lo et al., 2002) *Microcero termes* *Mansonella spp.* (Casiraghi et al., 2001)
G	Arthropoda	Australian spiders (Rowley et al., 2004)
H	Arthropoda	*Zootermopsis angusticollis* (Bordenstein & Rosengaus, 2005)
I	Nematode	*Radopholus similis* (Haegeman et al., 2009)

Wolbachia is estimated to infect more than 65% of insect species making it the most copious intracellular bacteria genus learnt so far in infecting at least 1, 000, 000 insect species. *Wolbachia* infecting nematodes have displayed mutualisms with their host, whereas *Wolbachia* infecting arthropods have displayed both mutualism and parasitism (Fenn & Blaxter, 2006). Two full genomes of *Wolbachia* are available at present. The *w*Mel strain from *D. melanogaster* and the mutualistic *w*Bm strain from filarial nematode host *B. malayi*.

Numerous studies have reported identification of *Wolbachia* in several species. A study in Taiwan showed that 51.7% of their mosquito species collected were infected with *Wolbachia* and 26.7% were superinfected with *w*AlbA and *w*AlbB. Among mosquitoes with superinfection were *Armigeres omissus, Ae. albopictus*, *Malaya genurostris* and *Mansonia uniformis*. Of the nine genera tested, only two did not harbour *Wolbachia*, *Anopheles* and *Heizmannia* (Tsai et al., 2004). *Aedes albopictus* singly infected with *w*AlbA only have been reported in two locations, in Koh Samui and Mauritius (Kambhampati et al., 1993; Sinkins et al., 1995).

2.6.2 Reproductive phenotype alterations

Wolbachia exhibits a wide range phenotypic effects on their hosts and behaves as reproductive symbionts (Dobson et al., 2002). They have the unique capability to live within the host and manipulate both cellular and reproductive processes. Another arthropod symbiont that have shown similar manipulative properties as *Wolbachia* is *Candidatus cardinium hertigii* (Zchori-Fein & Perlman, 2004). They have shown to induce feminization of genetic males, parthenogenetic induction, killing of male progeny from infected females and cytoplasmic incompatibility (O'Neill et al., 1997).

2.6.2.1 Feminization

Feminization is when male offspring develop to females. Most of the offspring will be females and a minority of them males. This alteration have been identified in isopods and insects. It has been observed in *Eurema hecabe* (Hiroki et al., 2002) and *Zyginidia pullula* (Negri et al., 2008). Graphical representation of feminization is shown in Figure 2.5.

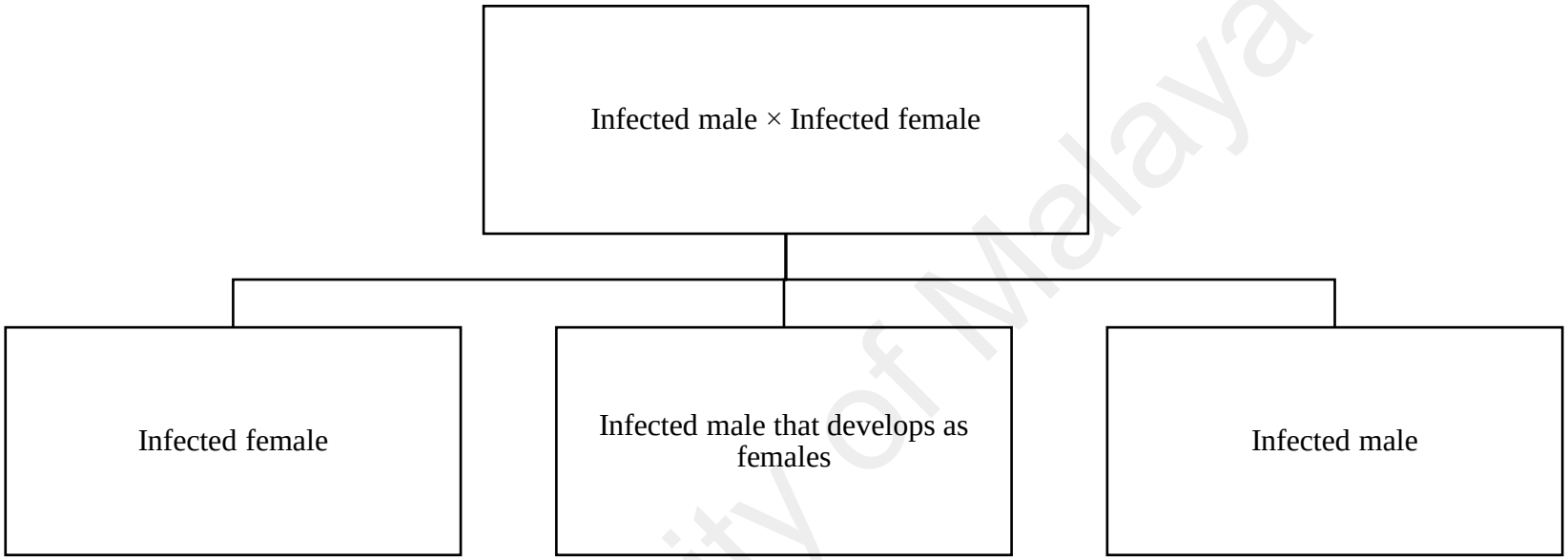

Figure 2.5: Feminization alteration by *Wolbachia*

2.6.2.2 Parthenogenesis induction

Parthenogenesis causes female offspring to develop from non-fertilized eggs. Only female adults will be produced asexually. This phenotype has only been observed in organism with haplodiploidy sex determination system where all unfertilized eggs develops to become male and all fertilized eggs become females. Parthenogenesis has been reported in the wasps *Telenomus nawai* (Jeong & Stouthamer, 2005) and *Trichogramma* (Rousset et al., 1992).

2.6.2.3 Male killing

Male killing is when all male offspring fail to become adults. *Wolbachia* kills off all the male eggs during embryogenesis. This phenotype alteration have been observed in the ladybird *Adalia bipunctata* and the butterfly *Acraea encedon* (Hurst et al., 1999).

2.6.2.4 Cytoplasmic incompatibility

Cytoplasmic incomapatibility (CI) is observed when both male and female have different type of *Wolbachia* infection. It is the most commonly observed reproductive alteration by *Wolbachia* and is by far given the highest interest as it has been associated with host population control. There are two types of CI which are unidirectional CI and bidirectional CI. Cytoplasmic incompatbility has been described as modify-rescue system (Telschow et al., 2005). When the male is infected with *Wolbachia* , the sperm is modified. If the female is also infected with the same type of *Wolbachia* infection, it would be able to rescue the sperm and viable eggs will be produced. If the female is not infected or infected with different type of *Wolbachia* strain, the sperm would not be rescued and thus no viable eggs will be produced (Werren, 1997). Unidirectional CI is when no viable offspring is obtained when infected male mates with uninfected female but viable offspring are obtained when uninfected male mates with infected female (Dobson et al., 2004; Laven, 1967) (Figure 2.6). Unidirectional CI has been reported in countless hosts. Bidirectional CI is when no viable offspring are obtained when mating male and females are infected with different type of *Wolbachia* infection (Telschow et al., 2005) (Table 2.2). However, the bidirectional CI have only been described in very few cases in mosquitoes and parasitic wasp.

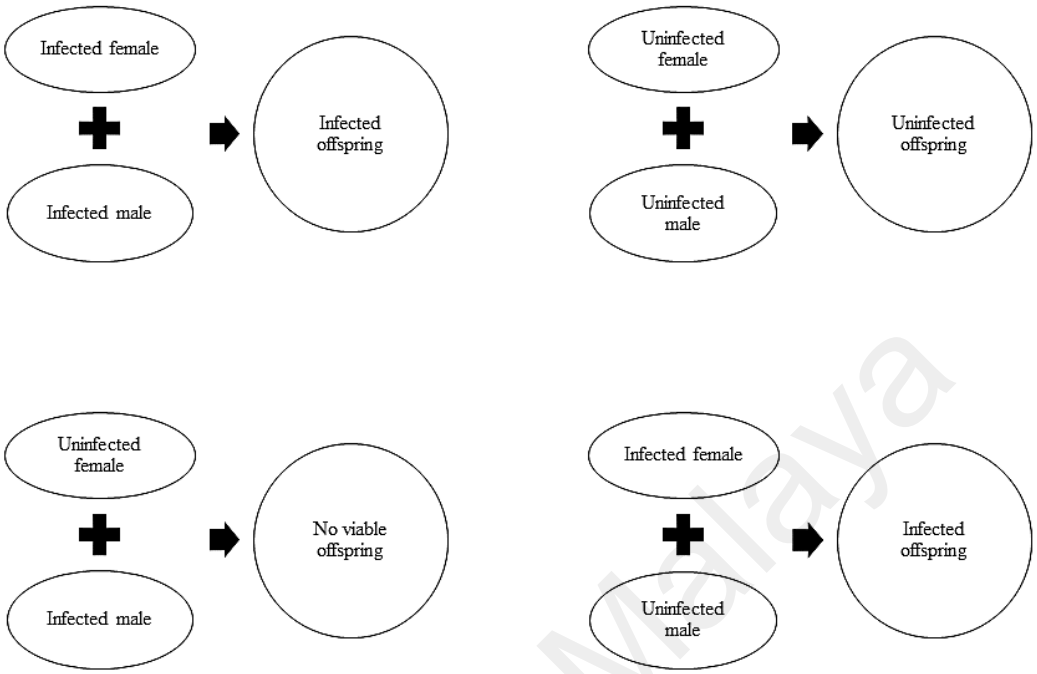

Figure 2.6: Unidirectional cytoplasmic incompatibility.

Table 2.2: Bidirectional CI.

		***Wolbachia* supergroup A infected female**	***Wolbachia* supergroup B infected female**
Bidirectional CI	***Wolbachia* supergroup A infected male**	*Wolbachia* supergroup A infected offspring	No viable offspring
	***Wolbachia* supergroup B infected male**	No viable offspring	*Wolbachia* supergroup B infected offspring

2.7 Cytoplasmic incompatibility by *Wolbachia* on different hosts

Cytoplasmic incompatibility ensures the same type of *Wolbachia* infection to be passed from one generation of the host to following generations by means of vertical transmission. It ensures *Wolbachia* continuation in the host without the necessity for horizontal transmission. Only females having the matching *Wolbachia* infection type as in the male will be able to produce viable offspring which would also have the same infection type. As it is an advantage mechanism for *Wolbachia*, this trait can be used against the host harbouring *Wolbachia*. Cytoplasmic incompatibility can be used to reduce host populations and may be used to reduce host susceptibility towards pathogen infection as well as transmission rate (Blagrove et al., 2012; Calvitti et al., 2015; Dobson et al., 2004).

Among the earliest studies conducted to explore CI in *Ae. albopictus* was in 2001 using naturally superinfected Houston strain (Hou) and tetracycline treated *Wolbachia* uninfected strain (HT1). Unidirectional cytoplasmic incompatibility was observed. Egg hatching rate was highest in the cross between Hou strains followed by between Hou female and HT1 male and lower in cross between HT1 strains. Hatching rate was zero in the cross in between Hou male and HT1 female (Dobson et al., 2002). Similar results were observed in naturally *Wolbachia* infected *D. melanogaster* (Hoffmann et al., 1998; Merçot & Poinsot, 1998) and in spider mites *Tetranychus urticae* and *T. turkestani* (Breeuwer, 1997).

Wolbachia's ability to induce cytoplasmic incompatibility differs from one host to another. Among factors that seem to affect is the host, type of *Wolbachia* infection and nativeness to the host. *Wolbachia* did not express any CI in a study conducted on *Drosophila simulans* in 1996 (Hoffmann et al., 1996).

The first report on inter-population unidirectional CI was done in 1992 between Mauritius strains with other five strains from different locations. No viable eggs were obtained in crosses between Mauritius female and male from other locations. All other crosses between the six locations including between Mauritius males and other females yielded viable eggs. All strains used were superinfected with *w*AlbA and *w*AlbB. They also mentioned that tetracycline treatment was not able to give an absolutely *Wolbachia* free colony (Kambhampati et al., 1993).

Unidirectional CI was reported in several other studies where *Wolbachia* was artificially introduced into another host. *Aedes aegypti* are not naturally infected with *Wolbachia*. In order to use *Wolbachia* as a population control measure, many researchers have introduced *Wolbachia* from other hosts such as *D. melanogaster* and *Cx. pipiens* into *Ae. aegypti* using microinjection methods and studied the establishment of CI (Walker et al., 2011; Yeap et al., 2014). They have also introduced *Wolbachia* from other host into *Ae. albopictus* in certain studies (Blagrove et al., 2012; Calvitti et al., 2015).

A stable triple infection was generated by introducing *Wolbachia w*Ri from *D. simulans* into a naturally superinfected *Ae. albopictus* strain. The triple-infected strain displayed a pattern of unidirectional incompatibility with the naturally infected strain. This unidirectional CI, combined with a high fidelity of maternal inheritance and low fecundity effects, suggests that the artificial cytotype could serve as an appropriate vehicle for gene drive (Fu et al., 2010).

Bidirectional CI is often only observed when *Wolbachia* strain from a different host is introduced into a new host. Complete bidirectional CI was reported when crossing was made between wild type superinfected *Ae. albopictus* with tetracycline treated *Wolbachia* free *Ae. albopictus* infected with *Wolbachia* (*w*Mel) from *D. melanogaster* (Blagrove et al., 2012).

Another strain that has given promising result was *w*MelPoP, also from *D. melanogaster* but an over replicating strain. CI caused by *w*MelPoP halved the lifespan of both its native *D. melanogaster* (Min & Benzer, 1997) and *Ae. aegypti* infected with it (McMeniman et al., 2009). CI was also reported in *Ae. aegypti* when the *Wolbachia* *w*AlbB was transferred from *Ae. albopictus* into them via microinjection (Xi et al., 2005).

A recent study suggested that CI is only correlated with the density of *w*AlbA in *Ae. albopictus*. They crossed wild type superinfected males with female *Ae. albopictus* infected with *Wolbachia* from *Cx. pipiens*. When the wild type male have a high density of *w*AlbA, bidirectional CI was observed and when they had low density of *w*AlbA, partially viable eggs were produced. Another study has showed that the density of *w*AlbA decreases with aging of the male mosquito (Tortosa et al., 2010). Therefore, as the male ages, the chances for CI decrease (Calvitti et al., 2015). This concurs with a previous study carried out on *Ae. albopictus* from La Reunion and Madagascar (Tortosa et al., 2010). Both unidirectional and bidirectional CI was observed in a study done in 2010 which used wild *Ae. albopictus*, *Wolbachia* free *Ae. albopictus* and *w*Mel infected *Ae. albopictus* (Calvitti et al., 2010).

Taking into account the results obtained from all studies conducted so far on cytoplasmic incompatibility on various hosts, *Wolbachia* is capable of unidirectional CI in most cases when a *Wolbachia* infected male is mated with a *Wolbachia* uninfected females whereas bidirectional is only expressed when mating is done between host infected with different *Wolbachia* strains. Nevertheless, CI can also not be expressed in certain hosts. Expression of CI is not as predictable and may depend on geographical and environmental factors (Calvitti et al., 2015). Table 2.3 shows a list of common *Wolbachia* strains and their respective hosts.

Table 2.3: Strains and hosts (Werren et al., 2008).

Strain	Host
*w*AlbA	*Ae. albopictus*
*w*AlbB	*Ae. albopictus*
*w*Mel	*D. melanogaster*
*w*MelPoP	*D. melanogaster*
*w*Pip	*Cx. pipiens*
*w*Sim	*D. simulans*
*w*Ri	*D. simulans*

2.8 Effect of *Wolbachia* on host life characteristic and pathogen transmission

The most reported effects of *Wolbachia* on their host has been on the fecundity, longevity and egg viability. When a naturally *Wolbachia*-infected host is cleared of *Wolbachia* using antibiotic treatment, the *Wolbachia*-free strain tends to have decreased fecundity, longevity and egg viability (Dobson et al., 2002; Joanne et al., 2015). Whereas, when a *Wolbachia* uninfected host is infected by microinjecting *Wolbachia* from a different host, the new host experiences shorter lifespan, lower fecundity and decreased egg viability (McGraw et al., 2002; McMeniman et al., 2009; Moreira et al., 2009; Yeap et al., 2014). It seems to appear like considerable mutualistic relationship has occurred between *Wolbachia* and its natural host (Baton et al., 2013). However, infection removal was not observed to affect male fitness in terms of longevity, mating performances and sperm capacity (Calvitti et al., 2010).

As for host susceptibility towards pathogens, *Wolbachia* have shown to reduce or inhibit pathogen transmission when they are transferred into a new non-native host (Brownstein et al., 2003; Hancock et al., 2011). This could be either when they are transferred into a new *Wolbachia* uninfected host (Walker et al., 2011) or when a different strain of *Wolbachia* is transferred into an originally *Wolbachia* infected host (Suh et al., 2009).

*w*Mel infection artificially introduced into *Ae. albopictus* was reported to block dengue virus transmission (Blagrove et al., 2012). RNA viral inhibition by *w*Mel has been previously demonstrated in *Drosophila* as well (Osborne et al., 2009), and the same was shown when it was transferred into *Ae. albopictus*. Their results showed no major effect on fecundity, unlike the significant fecundity reduction previously observed with *w*Pip infection of *Ae. albopictus* (Calvitti et al., 2010). Besides that, a *w*Mel transinfected line

had a much higher egg viability than observed for a *w*MelPop strain transinfection in *Ae. albopictus* (Suh et al., 2009).

On the other hand, when native *Wolbachia* was examined for dengue virus and chikungunya virus inhibition properties within its natural host *Ae. albopictus*, for dengue virus, it did not affect the replication of dengue virus in *Ae. albopictus* but was able to reduce viral infection of salivary glands and limit transmission (Mousson et al., 2012) and for chikungunya virus, they found that *Wolbachia* led to optimization in chikungunya virus replication from day 4 post-infection onwards.

The first example of *Wolbachia* increasing host resistance against pathogens was observed in *D. melanogaster* (Hedges et al., 2008; Teixeira et al., 2008). Following that, when *Wolbachia* was transferred into *Ae. aegypti*, it made the mosquitoes resistant to dengue virus and chikungunya virus (Moreira et al., 2009; Walker et al., 2011).

Field trials have shown that releasing *Wolbachia* infected mosquitoes allows the bacterium to invade *Ae. aegypti* populations (Hoffmann et al., 2011) and reduces the susceptibility of the mosquitoes to dengue virus (Frentiu et al., 2014). Both *Anopheles* and *Ae. aegypti* are not naturally infected with *Wolbachia*, therefore *Wolbachia* transfer into these vector species are crucial to determine whether *Wolbachia* can limit pathogens transmitted by them. *w*MelPop-CLA strain transferred from *D. melanogaster* into *Ae. aegypti* reduces the ability of dengue, chikungunya virus and *Plasmodium* from establishing productive infections in the mosquito (Moreira et al., 2009). The *w*MelPop-CLA infection was demonstrated to reduce average mosquito lifespan by approximately 50% in the laboratory (McMeniman et al., 2009).

In another study, artificially *w*Pip infected *Ae. albopictus* was crossed with natural superinfected *Ae. albopictus* and *Wolbachia* cleared strain (Calvitti et al., 2012). Although no differences were observed in male longevity, the naturally superinfected females lived longer than the other two strains. Differences in female longevity did not appear to be because of the *w*Pip infection, but due to difference in features of the *Wolbachia* cleared strain compared with the superinfected strain, possibly determined by dissimilarities in genetic variability (Calvitti et al., 2012).

A very recent study analysed *Wolbachia*-host relationship using Semliki Forest virus and *D. melanogaster* cell line infected with *Wolbachia (Rainey et al., 2016).* Their data suggested that *Wolbachia* targets the virus after infection, and is likely blocking early replication of viral RNA within host cells. This could have been done by *Wolbachia* competing for source with the virus or by changing the intracellular environment to become not suitable for the virus to thrive (Rainey et al., 2016).

The effect of *Wolbachia* on the host life characteristics and on host pathogen transmission capabilities are not predictable and the mechanism underlying this effects are still not clear. *Wolbachia* tends to effect different host differently and inhibits pathogen in some and not in others. Therefore it is important to thoroughly study the effect of *Wolbachia* on every host separately and establish a clear understanding on these aspects.

2.9 Field studies involving *Wolbachia*

Field studies have been conducted on *Ae. aegypti* and *Ae. polynesiensis*. One field trial and one semi-field trial (field cage experiment) was conducted in Cairns, Australia. Both was done by releasing *Ae. aegypti* artificially infected with *Wolbachia* from *D. melanogaster* (Hoffmann et al., 2011; Walker et al., 2011). In the field trial, two locations

were chosen for the study which were Yorkeys Knob consisting of 614 houses and Gordonvale consisting of 668 houses. In the month before release, residential buildings within the release area were inspected and water was removed from breeding containers. *Wolbachia* infected *Ae. aegypti* were released weekly for ten weeks into those areas. They reported that *Wolbachia* infected *Ae. aegypti* managed to successfully invade both populations. Hence, they concluded that it is possible to introduce self-sustaining stable *Wolbachia* infected mosquitoes into a population with minimal fitness cost (Hoffmann et al., 2011).

The field trial was based on results obtained from a field cage experiment conducted earlier in the same year at Cairns, Australia as well. They established that rapid invasion of *Wolbachia* infected *Ae. aegypti* in their cages was possible and reported complete block of dengue virus transmission by *w*Mel in *Ae. aegypti* in their laboratory studies. Dengue virus titre in whole mosquito and dissemination rate into legs was also reduced compared to *Wolbachia* uninfected *Ae. aegypti* (Walker et al., 2011).

Semi-field experiments were carried out in South Pacific in 2011 using *Ae. polynesiensis*. They artificially introduced *Wolbachia* from *Aedes riversi* into *Ae. polynesiensis* and conducted a trial for 3 months. They reported bidirectional CI and reduced in brood hatch rate (Chambers et al., 2011). Ensuing that, a field study was conducted at the same area, using the same mosquitoes. They released incompatible male mosquitoes and performed a thirty week open field release trial. This study reported that male mosquitoes do not horizontally transfer *Wolbachia*. Despite the small amount of mosquitoes released, there was a significant drop in number of female able to produce viable embryos (O'Connor et al., 2012).

CHAPTER 3: NATURAL *WOLBACHIA* INFECTION STATUS OF MALAYSIAN *AEDES ALBOPICTUS* AND IN THEIR ORGANS

3.1 Introduction

Wolbachia is an intracellular endosymbiotic α-proteobacteria found in most arthropods and nematodes (Dobson et al., 2002; Zhou et al., 1998). This bacteria is usually vertically transmitted from a female host to their offspring and found at the highest density in their reproductive organs (Tsai et al., 2004). Very few examples of horizontal transmission have been reported. *Wolbachia* have been detected in about 40 arthropods. Most of these are in insects. Among the insects naturally infected with *Wolbachia* are *D. melanogaster*, *Cx. pipientis*, *Ae. albopictus* and *Lutzomyia* species. The *Wolbachia* was first identified from *Cx. pipientis* and named as *Wolbachia pipientis*. *Wolbachia* genome was then first determined from *Wolbachia* in *D. melanogaster*. Besides insects, *Wolbachia* have also been detected in two isopods and a mite (O'Neill et al., 1992). Filarial worms are the most common nematodes naturally infected with *Wolbachia*.

Wolbachia have developed a mutualistic relationship with their host (Dobson et al., 2004). When *Wolbachia* is removed from their natural host, the host reproductive capabilities tends to be effected such as the mosquito fecundity, longevity and egg viability (Das et al., 2014; O'Neill et al., 1997; Werren et al., 2008). In order to study *Wolbachia* effect on a particular host, the extent of *Wolbachia* infection on the host has to be first explored followed by its distribution range.

Aedes albopictus is native to Malaysia. It is the secondary vector for dengue (Chow et al., 1998) and primary vector for chikungunya virus (Tesh et al., 1976) and the newly emerged Zika virus (Wong et al., 2013). *Aedes albopictus* co-exist with *Ae. aegypti*

in most regions of Malaysia. Their population have been growing at an alarming rate over the past decade (Paupy et al., 2009; Rozilawati et al., 2007).

Aedes albopictus is a natural host of *Wolbachia* and commonly infected with two major supergroups of *Wolbachia*. *Wolbachia* supergroup A (*w*AlbA) and *Wolbachia* supergroup B (*w*AlbB) (Armbruster et al., 2003; Kittayapong et al., 2000). When *Ae. albopictus* is infected with both supergroups, they are known to be superinfected and when they are infected with only one of the supergroups, they are known to be singly infected (Zhou et al., 1998). Many studies have reported superinfected *Ae. albopictus* from North America, South America and Thailand (Armbruster et al., 2003; Kittayapong et al., 2000) and few singly infected *Ae. albopictus* (Sinkins et al., 1995). Specific primers of the *wsp* gene was designed by Zhou *et al*. for these two supergroups for quick identification of the *Wolbachia* infection type in their hosts (Wang et al., 2010; Zhou et al., 1998).

Although most *Ae. albopictus* worldwide are superinfected with *Wolbachia*, little is known about *Wolbachia* infection and their host in Malaysia. This is the first study conducted in Malaysia to study the distribution and relationship of *Wolbachia* in *Ae. albopictus*. The objective of this study was to determine the distribution, infection status and phylogenetic affiliation of *Wolbachia* in Malaysian *Ae. albopictus*. In addition to those, *Wolbachia* distribution in the mosquito organs was also studied.

3.2 Methodology

3.2.1 Sampling technique

Aedes albopictus larvae were collected from October 2012 to April 2013 using ovitraps. Ovitraps used were black plastic containers filled one third with overnight stored rain water (Figure 3.1). A strip of rough brown paper, 3 inch width lined the interior of the ovitraps for egg laying. A minimum of eight ovitrap and maximum of 20 ovitraps were set in each location. Ovitraps were equally placed at indoor and outdoor (under vegetation) locations. Outdoor location were under vegetation to prevent disturbance by animals. Each ovitrap was set approximately 200 metres apart to avoid obtaining all eggs from a single female mosquito. After one week, traps were collected. Larvae and eggs were transferred into rectangular trays with fitted white cloth as cover (Figure 3.2). Not more than 200 larvae were allowed to mature in each tray. Larvae were fed with tetramin fish food twice a day and allowed to develop into pupae. At the pupae stage they were transferred into respective cages and allowed to emerge as adults. Cages used were made of wooden frame with netting (Figure 3.3). Adults were maintained with 10% sucrose solution incorporated with B-Complex vitamin. All colonies were maintained at 27˚C and relative humidity of 85% with 12h: 12h light-dark photoperiod in insectarium of Department of Parasitology, University of Malaya.

Figure 3.1: Ovitrap with fitted brown paper.

Figure 3.2: Larvae rearing trays.

Figure 3.3: Cage used to rear adult mosquitoes.

3.2.2 Study site

Samples were collected from 21 sites; one site from Perak and Negeri Sembilan, two sites from Sabah, four sites from Federal Territory of Kuala Lumpur and 13 sites from Selangor (Table 3.1). DNA was extracted from individual mosquitoes between 4-6 days after emergence. Samples from Kudat Sabah, Banggi Sabah, Serendah, Sungai Tamu, Batang Kali, Kuala Kubu Baru, Kelumpang, Sungai Sendat, Pulau Indah and Sungai Merab were wild caught adults. These were obtained when other studies were conducted. Sample size varied between 4 to 20 adult mosquitoes per site. Among 21 sites, only eight locations had sample size below 10. Three sites using ovitrap method and the other five were wild caught mosquitoes.Once the mosquito was 4 to 6 day old, they were transferred into individual empty 1.5mL microcentrifuge tube and stored in -20˚C freezer prior to DNA extraction.

Table 3.1: Sample collection sites.

State	District	Sampling Site	Method	Coordinate	N
Perak	Kinta	Ipoh	Ovitrap	4.57 ˚ N, 101.08 ˚ E	20
Negeri Sembilan	Nilai	Nilai	Ovitrap	2.85 ˚ N, 101.81 ˚ E	5
Sabah (East Malaysia)	Kudat	Rural Kudat	Wild Caught	6.89 ˚ N, 116.83 ˚ E	9
Sabah (East Malaysia)	Kudat	Banggi Island	Wild Caught	7.27 ˚ N, 117.15 ˚ E	18
Kuala Lumpur	Titiwangsa	Ampang	Ovitrap	3.16 ˚ N, 101.75 ˚ E	16
Kuala Lumpur	Titiwangsa	Setapak	Ovitrap	3.19 ˚ N, 101.71 ˚ E	20
Kuala Lumpur	Titiwangsa	Keramat	Ovitrap	3.17 ˚ N, 101.73 ˚ E	20
Kuala Lumpur	Lembah Pantai	Bangsar	Ovitrap	3.11 ˚ N, 101.67 ˚ E	20
Selangor	Hulu Selangor	Serendah	Wild Caught	3.36 ˚ N, 101.60 ˚ E	19
Selangor	Hulu Selangor	Kampung Sungai Tamu	Wild Caught	3.37 ˚ N, 101.74 ˚ E	16
Selangor	Hulu Selangor	Batang Kali	Wild Caught	3.47 ˚ N, 101.64 ˚ E	10
Selangor	Hulu Selangor	Kuala Kubu Baru	Wild Caught	3.56 ˚ N, 101.66 ˚ E	8
Selangor	Hulu Selangor	Kelumpang	Wild Caught	4.90 ˚ N, 101.53 ˚ E	6
Selangor	Hulu Selangor	Sungai Sendat	Wild Caught	3.17 ˚ N, 101.72 ˚ E	8
Selangor	Petaling	Jalan Gasing	Ovitrap	3.10 ˚ N, 101.65 ˚ E	20
Selangor	Petaling	Petaling KTM Station	Ovitrap	3.46 ˚ N, 102.07 ˚ E	20
Selangor	Hulu Langat	Kajang	Ovitrap	2.99 ˚ N, 101.78 ˚ E	20
Selangor	Hulu Langat	Sungai Merab	Wild Caught	2.94 ˚ N, 101.75 ˚ E	5
Selangor	Klang	Pulau Indah	Wild Caught	2.95 ˚ N, 101.31 ˚ E	14
Selangor	Shah Alam	Section 2	Ovitrap	3.07 ˚ N, 101.52 ˚ E	4
Selangor	Gombak	Bukit Lagong	Ovitrap	3.26 ˚ N, 101.64 ˚ E	8

N: Number of samples

3.2.3 DNA extraction

A minimum of five and a maximum of 20 individual whole mosquitoes were processed from each collection site. A total 286 adult of *Ae. albopictus* consisting of 67 male and 219 female mosquitoes were processed. DNA was extracted using Dneasy Blood and Tissue extraction kit according to the protocol provided by the manufacturer (Qiagen, CA, USA). Individual whole mosquitoes were homogenized using hand held homogenizer (Kontes Thompson Scientific) with plastic pestle in 180 µL ATL buffer from the extraction kit in a 1.5 mL micro-centrifuge tube (Axygen, USA). After homogenizing for 30 seconds, 20 µL proteinase K was added to digest the protein and remove contamination from the nucleases. The mixture was then incubated at 56˚C, 300 rpm overnight in a thermomixer (Eppendorf, Germany).

The next day, tube was centrifuged to remove liquid from the lid. 200 µL of 97.0% ethanol and 200 µL buffer AL was added into the sample and mixed thoroughly by vortexing to yield a homogenous solution. The homogenized solution was then pipetted into a Dneasy Mini Spin column placed in a 2 mL collection tube. The sample was centrifuged at 8000 rpm for 1 minute. The flow-through and collection tube were discarded. The spin column was placed in a new clean 2 mL collection tube. A volume of 500 µL AW1 buffer was added into the spin column. The sample was centrifuged for 1 minute at 8000 rpm. Again, the flow-through and collection tube were discarded. The spin column was transferred into a fresh 2 mL collection tube and 500 µL of Buffer AW2 was added into it. The tube was centrifuged for 3 minutes at 14000 rpm. The flow through and collection tube was discarded. After transferring the spin column into a new collection tube, it was centrifuged again for 1 minute at 14000 rpm to remove any residue of the wash buffer. The spin column was then transferred into a new 1.5 mL micro-centrifuge tube and 100 µL of elution buffer was added. It was left for 1 minute at room

temperature and centrifuged for 1 minute at 8000 rpm. All 286 samples were extracted individually and stored in 1.5 mL autoclaved Eppendorf micro centrifuge tubes in -20˚C freezer.

3.2.4 PCR amplification and sequencing

All samples extracted were amplified using *wsp* primers from Zhou *et al*. (1998), targeting surface protein. Multiplex PCR were executed using Promega reagents (Promega, Madison, WI) and diagnostic primers from Genomics BioSci & Tech, China. The primer pair 328F and 691 R was used to amplify the 363 bp *w*AlbA DNA and primer pair 183F and 691R was used to amplify the 508 bp *w*AlbB DNA (*wsp* 183F: 5' - AAG GAA CCG AAG TTC ATG - 3'; *wsp* 328F: 5' - CCA GCA GAT ACT ATT GCG - 3'; *wsp* 691R: 5′ - AAA AAT TAA ACG CTA CTC CA - 3′) (Armbruster et al., 2003; Zhou et al., 1998)(Zhou 1998, Armbruster 2003). Multiplex PCR contained final volume of 20 µl containing 10 µl ddH_2O, 4 µl 1 X Green GoTaq Flexi Buffer, 1.6 µl 2mM $MgCl_2$, 0.4 µl 0.2mM dNTP, 0.2 µl Taq Polymerase, 0.6 µl of each 10 µM primers, and 2 µl template DNA. It was performed in T100 Thermal Cycler (Bio-Rad, Singapore) with the following conditions: initial denaturation at 94˚C for 5 minutes followed by 35 cycles of denaturation at 95˚C for 1 minute, amplification at 55˚C for 1 minute, extension at 72˚C for 1 minute, followed by final extension step of 10 minutes at 72˚C (Armbruster et al., 2003). Negative and positive controls were run alongside in each batch. Negative control was prepared by substituting template DNA with ddH_2O whereas positive controls for both *w*AlbA and *w*AlbB was obtained from Armbruster's laboratory, USA.

Amplified products were loaded along with 100bp ladder (Promega, Madison, WI) into freshly prepared 1% agarose gel using LE Grade Agarose powder and 0.5% 5X TBE buffer pH 8.3 (Vivantis, USA). The gel electrophoresis was run at 100V for 1 hour.

The gel was stained with SYBR® Green (Life Technologies, USA) and viewed under UV light.

PCR amplification and gel electrophoresis were repeated using CO1 gene primers for all samples with no *Wolbachia* infection to ensure they were truly negative. Three different amplified samples from each sampling site were sent for sequencing. The bands from the gel were cut and sent for sequencing to Genomics BioSci & Tech, China. Sanger method was used for sequencing.

3.2.5 Multiple alignments and phylogenetic analysis

Sequencing was done in both directions for three specimens from each sampling site. Sequences were aligned and cleaned using BioEdit software (version 7.1.11) (Applied Biosystem, UK). The quality of the sequences was ensured using Chromas Lite Version 2.1 software. The type of *Wolbachia* supergroup infection was confirmed by blasting each aligned sequences in Genbank BLAST. Since all samples had identical sequences within *w*AlbA and *w*AlbB, one representative sequence was taken for each sampling site for each supergroup. A total of 53 sequences consisting of 21 *w*AlbA sequences, 21 *w*AlbB sequences and 11 other *wsp* sequences retrieved from Genbank were aligned using ClustalW version 1.7. A phylogenetic tree was constructed using MEGA version 6.0 software. The phylogenetic relationship was inferred using Neighbour Joining method. Branches corresponding to partitions reproduced in less than 80% bootstrap replicates were collapsed. All sequences have been submitted to Genbank (Accession numbers: KF781993 to KF782108). *Wsp* gene sequences from *Cx. pipiens* and *D. melanogaster* were incorporated as outgroups to confirm outcome of the phylogenetic tree.

3.2.6 Detection of *Wolbachia* in different organs of *Ae. albopictus*

A total of 90 doubly infected female mosquitoes from Bukit Lagong, Gombak, Selangor colony were dissected at three different time points (6^{th}, 14^{th} and 30^{th} day) to isolate the salivary glands, ovaries and midguts. Care was taken to avoid contamination. DNA was extracted from the individual organs in individual 1.5 mL micro centrifuge tubes using Dneasy Blood and Tissue Extraction Kit as described above (Qiagen, CA, USA). DNA was stored at -20˚C freezer. Multiplex PCR were carried out for all extracted organ samples according to the protocol in Section 3.2.3. Amplified samples were confirmed for *w*AlbA and *w*AlbB infection using gel electrophoresis. Female mosquitoes used for dissection were defined as doubly infected when all ovaries had double infection of *w*AlbA and *w*AlbB.

3.2.7 Statistics

All statistical analysis was carried out using GraphPad Prism version 6.01 for Windows (GraphPad Software, Inc., 2012). Significant differences on distribution of *Wolbachia* in Malaysian *Ae. albopictus* and within *Ae. albopictus* organs were calculated using two-way ANOVA ($P<0.001$) and Tukey's multiple comparison test ($P<0.001$).

3.3 Results

3.3.1 *Wolbachia* distribution in Malaysian *Ae. albopictus*

The distribution of *Wolbachia* infection type represented by pie charts according to the districts is shown on map of Malaysia in Figure 3.4. Each colour represents one infection type; superinfected, singly infected with *w*AlbA only, singly infected with *w*AlbB only and uninfected. Only the districts of Titiwangsa and Kudat had all four infection types. Most pie charts had bigger blue section compared to the rest which indicates most samples were superinfected. Samples were from five states; Selangor, Kuala Lumpur, Perak, Negeri Sembilan and Sabah.

A detailed *Wolbachia* infection status of the mosquitoes according to the 11 districts are listed in Table 3.2 and a graphical representation in Figure 3.5. Overall infection status is represented in Figure 3.6. Out of the 286 samples, 74 of them were males and 212 females. Only nine of the males were not superinfected. Five of them were singly infected with *w*AlbB and 4 of them uninfected. None of the male samples had single infection of *w*AlbA only. As for the females, 197 were superinfected with both *w*AlbA and *w*AlbB. Three of them were singly infected with *w*AlbA and one of them with *w*AlbB. Eleven of the females were uninfected. Majority of the uninfected samples were from Kudat. All districts had superinfected exceeding 50%. Only samples from Shah Alam, Gombak and Hulu Selangor had 100.0% *w*AlbA and *w*AlbB superinfection. None of the districts had all samples negative for *Wolbachia* infection. The lowest was Nilai with only 60% superinfection. Overall, 91.6% (262/286) of the samples were superinfected, 5.2% (15/286) uninfected, 1.0% (3/286) singly infected with *w*AlbA and 2.2% (6/286) singly infected with *w*AlbB. Percentage of samples superinfected with *w*AlbA and *w*AlbB were statistically significant when compared to the rest.

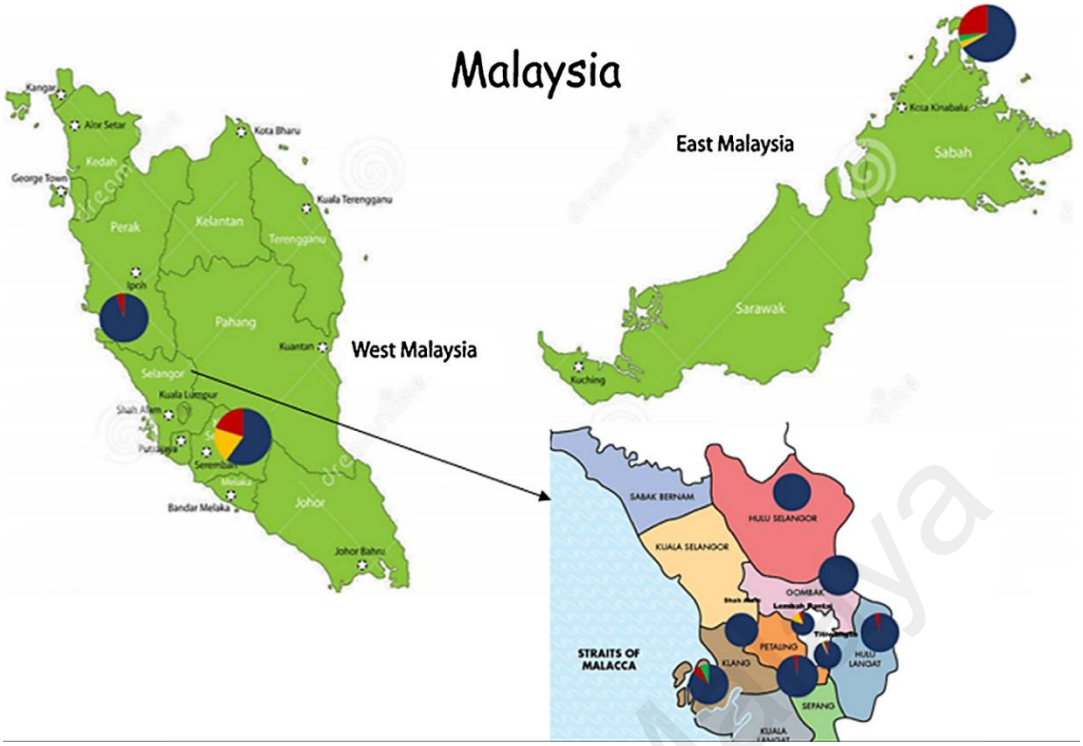

Figure 3.4: Map of Malaysia showing *Ae. albopictus* collection sites among 5 states of Malaysia. [Blue: Infected with both *w*AlbA and *w*AlbB; Red: Uninfected; Green: Singly infected with *w*AlbA; Yellow: Singly infected with *w*AlbB].

Table 3.2: Infection status of *Ae. albopictus* from 11 district in Malaysia.

District	Total samples		Both *w*AlbA and *w*AlbB		*w*AlbA only		*w*AlbB only		None	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Titiwangsa [a]	15	41	13	39	0	1	1	0	1	1
Lembah Pantai [b]	10	10	6	10	0	0	3	0	1	0
Hulu Selangor [c]	0	67	0	67	0	0	0	0	0	0
Petaling [d]	22	18	22	17	0	0	0	0	0	1
Hulu Langat [e]	8	17	7	17	0	0	0	0	1	0
Klang [f]	3	11	3	9	0	1	0	0	0	1
Shah Alam [g]	1	3	1	3	0	0	0	0	0	0
Gombak [h]	3	5	3	5	0	0	0	0	0	0
Kinta [i]	10	10	9	10	0	0	0	0	1	0
Nilai [j]	2	3	1	2	0	0	1	0	0	1
Kudat [k]	0	27	0	18	0	1	0	1	0	7
Total	286		262 (91.6%)		3 (1.0%)		6 (2.2%)		(5.2%)	

[a] Titiwangsa: Ampang, Setapak, Keramat
[b] Lembah Pantai: Bangsar
[c] Hulu Selangor: Sungai Sendat, Kuala Kubu Baru, Kelumpang, Batang Kali, Serendah, Kampung Sungai Tamu
[d] Petaling: Petaling KTM Station, Jalan Gasing
[e] Hulu Langat: Kajang, Sungai Merab
[f] Klang: Pulau Indah
[g] Shah Alam: Section 2
[h] Gombak: Bukit Lagong
[i] Kinta: Ipoh
[j] Nilai: Nilai 3
[k] Kudat: Rural Kudat and Banggi Island

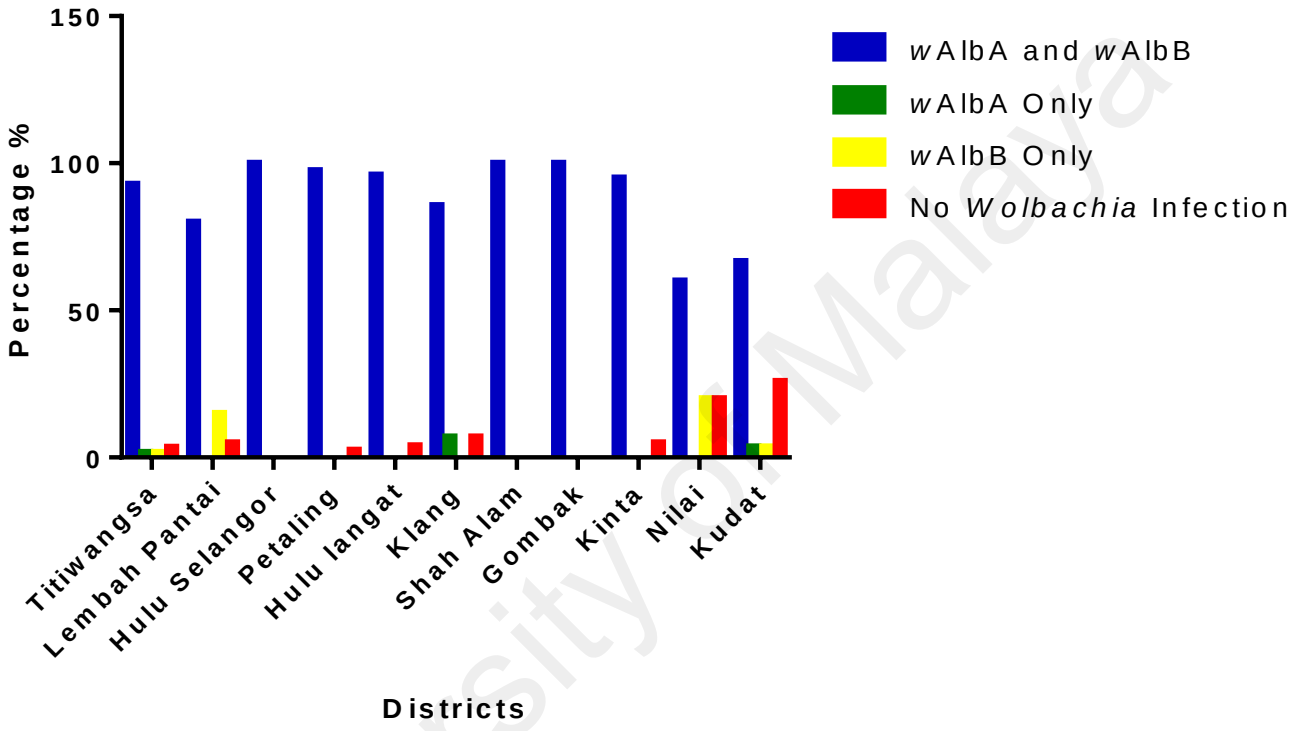

Figure 3.5: Total number of samples having different *Wolbachia* infection type grouped by districts.

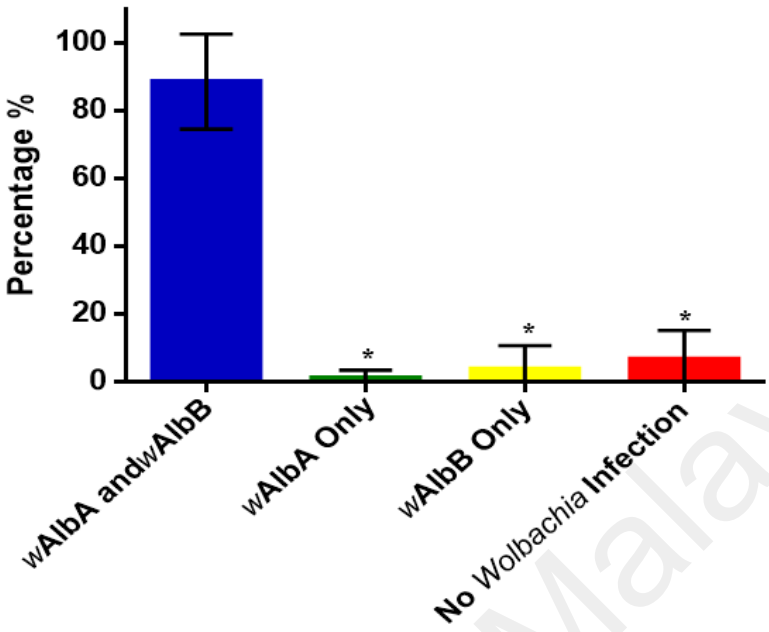

Figure 3.6: Overall distribution of *Wolbachia* supergroups (*w*AlbA and *w*AlbB) in 286 Malaysian *Ae. albopictus*. Each bar represent the mean of the infection type of the 11 districts. The vertical line represent the standard deviation. Asterisk indicates significant difference of the infection types compared to *w*AlbA and *w*AlbB infection type which is taken as the control ($P<0.001$) according to two-way ANOVA $F_{(3, 30)} = 183.8$ and Tukey's multiple comparison test ($P<0.001$).

3.3.2 Phylogenetic association

Phylogenetic tree resulted in two major clades with bootstrap values of 86 and 79 (Figure 3.7). All sequences that fell into the upper clade belonged to *w*AlbB supergroup and the lower clade belonged to *w*AlbA supergroup. Every sample was confirmed as *w*AlbA or *w*AlbB respectively by performing a BLAST in GenBank. No samples branched out of the major clades other than the out-group samples. All *w*AlbA sequences and *w*AlbB sequences were identical to each other, respectively.

Five ClustalW aligned sequences of *w*AlbA and *w*AlbB are shown in Table 3.3 and Table 3.4. Three of them were samples from this experiment whereas two of them were retrieved from GenBank. All five samples had identical sequences.

All 42 samples of *w*AlbA and *w*AlbB showed no differences within their own supergroups therefore further analysis of the phylogenetic tree was not necessary. The accession numbers (KF781993 to KF782108) for each sequence used in the phylogenetic tree are listed in Table 3.5 and Table 3.6. The districts Titiwangsa and Lembah Pantai are located in Kuala Lumpur; Hulu Selangor, Petaling, Hulu Langat, Klang, Shah Alam and Gombak are located in Selangor; Kinta in Perak; Nilai in Negeri Sembilan and Kudat in Sabah.

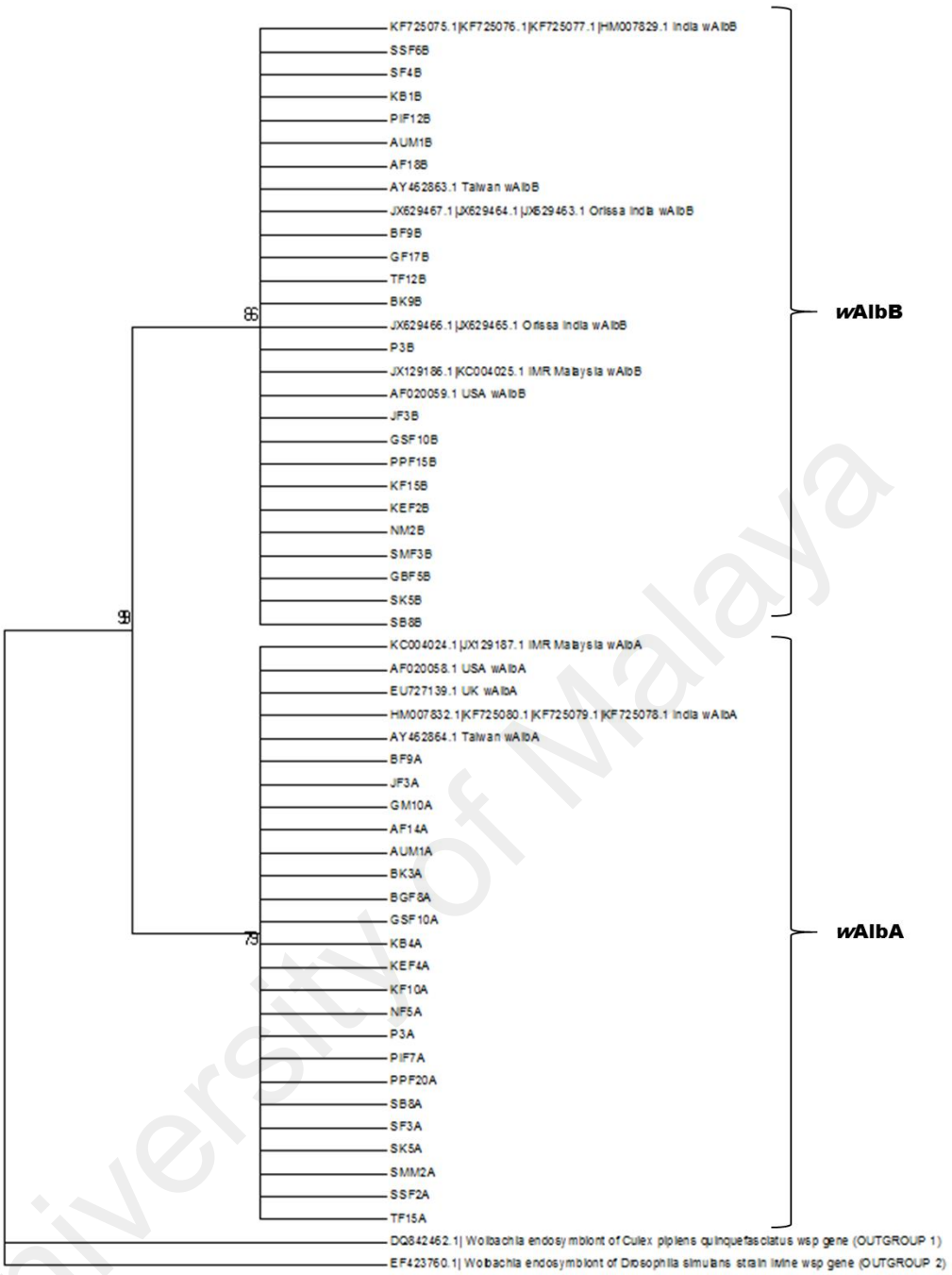

Figure 3.7: Phylogenetic tree inferred using neighbour joining method in Mega 6.0 software. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches.

Table 3.3: *w*AlbA sequences aligned in ClustalW.

Label	1-60 NUCLEOTIDE
SSF2A	AACAGTTTAACAGCAATTTCAGGACTAGTTAACGTTTATTACGATATAGCAATTGAAGAT
TF15A	AACAGTTTAACAGCAATTTCAGGACTAGTTAACGTTTATTACGATATAGCAATTGAAGAT
AF14	AACAGTTTAACAGCAATTTCAGGACTAGTTAACGTTTATTACGATATAGCAATTGAAGAT
KC004024.1(A)	AACAGTTTAACAGCAATTTCAGGACTAGTTAACGTTTATTACGATATAGCAATTGAAGAT
HM007832.1(A)	AACAGTTTAACAGCAATTTCAGGACTAGTTAACGTTTATTACGATATAGCAATTGAAGAT
Label	**61-120**
SSF2A	ATGCCTATCACTCCATATGTTGGTGTTGGTGTTGGTGCAGCGTATGTCAGCACTCCTTTG
TF15A	ATGCCTATCACTCCATATGTTGGTGTTGGTGTTGGTGCAGCGTATGTCAGCACTCCTTTG
AF14	ATGCCTATCACTCCATATGTTGGTGTTGGTGTTGGTGCAGCGTATGTCAGCACTCCTTTG
KC004024.1(A)	ATGCCTATCACTCCATATGTTGGTGTTGGTGTTGGTGCAGCGTATGTCAGCACTCCTTTG
HM007832.1(A)	ATGCCTATCACTCCATATGTTGGTGTTGGTGTTGGTGCAGCGTATGTCAGCACTCCTTTG
Label	**121-180**
SSF2A	AAAACCGCTATAAATAATCAAAACAGTAAATTTGGTTTTGCTGGTCAAGTAAAAGCTGGT
TF15A	AAAACCGCTATAAATAATCAAAACAGTAAATTTGGTTTTGCTGGTCAAGTAAAAGCTGGT
AF14	AAAACCGCTATAAATAATCAAAACAGTAAATTTGGTTTTGCTGGTCAAGTAAAAGCTGGT
KC004024.1(A)	AAAACCGCTATAAATAATCAAAACAGTAAATTTGGTTTTGCTGGTCAAGTAAAAGCTGGT
HM007832.1(A)	AAAACCGCTATAAATAATCAAAACAGTAAATTTGGTTTTGCTGGTCAAGTAAAAGCTGGT
Label	**181-240**
SSF2A	GTCAGCTATGATGTAACTCCAGAAGTCAAACTTTATGCTGGAGCTCGTTATTTCGGTTCT
TF15A	GTCAGCTATGATGTAACTCCAGAAGTCAAACTTTATGCTGGAGCTCGTTATTTCGGTTCT
AF14	GTCAGCTATGATGTAACTCCAGAAGTCAAACTTTATGCTGGAGCTCGTTATTTCGGTTCT
KC004024.1(A)	GTCAGCTATGATGTAACTCCAGAAGTCAAACTTTATGCTGGAGCTCGTTATTTCGGTTCT
HM007832.1(A)	GTCAGCTATGATGTAACTCCAGAAGTCAAACTTTATGCTGGAGCTCGTTATTTCGGTTCT
Label	**241-300**
SSF2A	TTTGGTGCTCACTTTGATAGCGAAACTACTGGTGCAGATAACAAAAAAGTAGTTACCAAA
TF15A	TTTGGTGCTCACTTTGATAGCGAAACTACTGGTGCAGATAACAAAAAAGTAGTTACCAAA
AF14	TTTGGTGCTCACTTTGATAGCGAAACTACTGGTGCAGATAACAAAAAAGTAGTTACCAAA
KC004024.1(A)	TTTGGTGCTCACTTTGATAGCGAAACTACTGGTGCAGATAACAAAAAAGTAGTTACCAAA
HM007832.1(A)	TTTGGTGCTCACTTTGATAGCGAAACTACTGGTGCAGATAACAAAAAAGTAGTTACCAAA
Label	**301-337**
SSF2A	GATGCATACAAAGTTCTTTACAGCACTGTTGGTGCAG
TF15A	GATGCATACAAAGTTCTTTACAGCACTGTTGGTGCAG
AF14	GATGCATACAAAGTTCTTTACAGCACTGTTGGTGCAG
KC004024.1(A)	GATGCATACAAAGTTCTTTACAGCACTGTTGGTGCAG
HM007832.1(A)	GATGCATACAAAGTTCTTTACAGCACTGTTGGTGCAG

Table 3.4: *w*AlbB sequences aligned in ClustalW.

Label	**1-60 NUCLEOTIDE**
JF3B	AACAGTGTGGCAGTATTTTCAGGATTGGTTAACGTTTATTACGATATAGCGATTGAAGAT
GF17B	AACAGTGTGGCAGTATTTTCAGGATTGGTTAACGTTTATTACGATATAGCGATTGAAGAT
GSF10B	AACAGTGTGGCAGTATTTTCAGGATTGGTTAACGTTTATTACGATATAGCGATTGAAGAT
KC004025.1(B)	AACAGTGTGGCAGTATTTTCAGGATTGGTTAACGTTTATTACGATATAGCGATTGAAGAT
AY462863.1(B)	AACAGTGTGGCAGTATTTTCAGGATTGGTTAACGTTTATTACGATATAGCGATTGAAGAT
Label	**61-120**
JF3B	ATGCCTATCACTCCATACGTTGGTGTTGGTGTTGGTGCAGCATATATCAGCAATCCTTCA
GF17B	ATGCCTATCACTCCATACGTTGGTGTTGGTGTTGGTGCAGCATATATCAGCAATCCTTCA
GSF10B	ATGCCTATCACTCCATACGTTGGTGTTGGTGTTGGTGCAGCATATATCAGCAATCCTTCA
KC004025.1(B)	ATGCCTATCACTCCATACGTTGGTGTTGGTGTTGGTGCAGCATATATCAGCAATCCTTCA
AY462863.1(B)	ATGCCTATCACTCCATACGTTGGTGTTGGTGTTGGTGCAGCATATATCAGCAATCCTTCA
Label	**121-180**
JF3B	GAAGCTAGTGCAGTTAAAGATCAAAAAGGATTTGGTTTTGCTTATCAAGCAAAAGCTGGT
GF17B	GAAGCTAGTGCAGTTAAAGATCAAAAAGGATTTGGTTTTGCTTATCAAGCAAAAGCTGGT
GSF10B	GAAGCTAGTGCAGTTAAAGATCAAAAAGGATTTGGTTTTGCTTATCAAGCAAAAGCTGGT
KC004025.1(B)	GAAGCTAGTGCAGTTAAAGATCAAAAAGGATTTGGTTTTGCTTATCAAGCAAAAGCTGGT
AY462863.1(B)	GAAGCTAGTGCAGTTAAAGATCAAAAAGGATTTGGTTTTGCTTATCAAGCAAAAGCTGGT
Label	**181-238**
JF3B	GTTAGTTATGATGTAACCCCAGAAATCAAGCTTTATGCTGGTGCTCGTTATTTTGGTT
GF17B	GTTAGTTATGATGTAACCCCAGAAATCAAGCTTTATGCTGGTGCTCGTTATTTTGGTT
GSF10B	GTTAGTTATGATGTAACCCCAGAAATCAAGCTTTATGCTGGTGCTCGTTATTTTGGTT
KC004025.1(B)	GTTAGTTATGATGTAACCCCAGAAATCAAGCTTTATGCTGGTGCTCGTTATTTTGGTT
AY462863.1(B)	GTTAGTTATGATGTAACCCCAGAAATCAAGCTTTATGCTGGTGCTCGTTATTTTGGTT

Table 3.5: Sample labels of *w*AlbA used in the phylogenetic analyses.

Label	Accession Number	Location	District	Supergroup
BF9A	KF782055	Bangsar	Lembah Pantai	A
JF3A	KF782058	Keramat	Titiwangsa	A
GM10A	KF782061	Jalan Gasing	Petaling	A
AF14A	KF782063	Ampang	Titiwangsa	A
AUM1A	KF782064	Setapak	Titiwangsa	A
BK3A	KF782066	Batang Kali	Hulu Selangor	A
GBF8A	KF782068	Bukit Lagong	Gombak	A
GSF10A	KF782070	Serendah	Hulu Selangor	A
KB4A	KF782073	KKB	Hulu Selangor	A
KEF4A	KF782077	Kelumpang	Hulu Selangor	A
KF10A	KF782079	Kajang	Hulu Langat	A
NF5A	KF782083	Nilai 3	Nilai	A
P3A	KF782085	Petaling KTM	Petaling	A
PIF7A	KF782088	Pulau Indah	Klang	A
PPF20A	KF782092	Ipoh	Kinta	A
SB8A	KF782093	Banggi Island	Kudat	A
SF3A	KF782096	Section 2	Shah Alam	A
SK5A	KF782099	Rural Kudat	Kudat	A
SMM2A	KF782102	Sungai Merab	Hulu Langat	A
SSF2A	KF782103	Sungai Sendat	Hulu Selangor	A
TF15A	KF782107	Sungai Tamu	Hulu Selangor	A
KC004024.1	KC004024.1	IMRKL	Gene Bank	A
HM007832.1	HM007832.1	Karnataka	Gene Bank	A
AY462864.1	AY462864.1	Taiwan	Gene Bank	A

Table 3.6: Sample labels of *w*AlbB used in the phylogenetic analyses.

Label	Accession Number	Location	District	Supergroup
BF9B	KF781993	Bangsar	Lembah Pantai	B
AF18B	KF781998	Ampang	Titiwangsa	B
JF3B	KF781999	Keramat	Titiwangsa	B
GF17B	KF782004	Jalan Gasing	Petaling	B
AUM1B	KF782005	Setapak	Titiwangsa	B
GSF10B	KF782008	Serendah	Hulu Selangor	B
TF12B	KF782011	Sungai Tamu	Hulu Selangor	B
PIF12B	KF782014	Pulau Indah	Klang	B
PPF15B	KF782017	Ipoh	Kinta	B
BK9B	KF782020	Batang Kali	Hulu Selangor	B
KB1B	KF782021	KKB	Hulu Selangor	B
KF15B	KF782026	Kajang	Hulu Langat	B
P3B	KF782027	Petaling KTM	Petaling	B
SF4B	KF782032	Section 2	Shah Alam	B
KEF2B	KF782033	Kelumpang	Hulu Selangor	B
SSF6B	KF782038	Sungai Sendat	Hulu Selangor	B
NM2B	KF782039	Nilai 3	Nilai	B
SMF3B	KF782043	Sungai Merab	Hulu Langat	B
GBF5B	KF782046	Bukit Lagong	Gombak	B
SK5B	KF782049	Rural Kudat	Kudat	B
SB8B	KF782052	Banggi Island	Kudat	B
KC004025.1	KC004025.1	IMRKL	Gene Bank	B
AY462863.1	AY462863.1	Taiwan	Gene Bank	B
AF020059.1	AF020059.1	New Haven USA	Gene Bank	B

3.3.3 Detection of *Wolbachia* in *Ae. albopictus* organs

All ovaries were positive at all time points (6^{th}, 14^{th}, and 30th). No midguts were positive on day 6, however, 50% of midguts were positive on day 14 and 93% on day 30. There was a significant difference in *Wolbachia* infection in midgut over the three time points ($P < 0.001$). It increased from 0% (0/30) to 50% (15/30) and to (28/30). Salivary glands were negative at all time points (Table 3.7).

Over the thirty days, 90 ovaries, 90 salivary glands and 90 midguts were tested. Overall, 100% of the ovaries were superinfected and 0% of the salivary glands were infected. A total of 47.8% of the midguts were infected.

Table 3.7: Distribution of *Wolbachia* in organs of *Ae. albopictus* at three different time points.

Day	Organ	N	Positive	Negative	Percentage infected with *Wolbachia* (%)
6th day post emergence	Salivary Glands	30	0	30	0.0
	Midgut	30	0	30	0.0*
	Ovaries	30	30	0	100.0
14th day post emergence	Salivary Glands	30	0	30	0.0
	Midgut	30	15	15	50.0*
	Ovaries	30	30	0	100.0
30th day post emergence	Salivary Glands	30	0	30	0.0
	Midgut	30	28	2	93.3*
	Ovaries	30	30	0	100.0

Asterisk indicates significant increase in the percentage of infected midguts between the three intervals according to two-way ANOVA ($P<0.001$); $F_{(2, 18)} = 45.31$ and Tukey's multiple comparison test ($P<0.001$). N: Total number of samples; Positive: number of samples which were superinfected with *w*AlbA and *w*AlbB; Negative: Samples uninfected with *Wolbachia*.

3.4 Discussion

3.4.1 *Wolbachia* infection status of Malaysian *Ae. albopictus*

Wolbachia is an abundant and wide spread endosymbiont parasitic bacteria (Chai et al., 2011; Hertig, 1936). Due to extensive *Wolbachia* infections in many arthropods and nematodes, it has become a subject of great interest to many researchers.

Hypothesis being studied in this chapter is that most *Ae. albopictus* are superinfected with *w*AlbA and *w*AlbB. The finding shows that 91.6% of Malaysian *Ae. albopictus* were superinfected with *w*AlbA and *w*AlbB. Hence, hypothesis is substantiated. It was similar to previous studies that reported 98.8% superinfection in Brazil (Albuquerque et al., 2011), 97.5% in Thailand (Kittayapong et al., 2002) and 100.0% superinfection in *Ae. albopictus* from several locations by Armbruster *et al*. in 2003 (Armbruster et al., 2003).

An older study conducted in 1995 reported that samples from Mauritius and Koh Samui, Thailand which were collected pre-1970 had only single infection (Sinkins et al., 1995). It was suggested by Armbruster *et al*. that it is possible that superinfection only occurred later in more recent years (Armbruster et al., 2003). Werren *et al*. suggested that superinfection became more common possibly due to infection of one supergroup facilitating infection by the other supergroup (Werren et al., 1995). Perhaps, cytoplasmic incompatibility aided the spread of *Wolbachia* superinfection in their hosts.

In this study, only 1.0% of females were singly infected with *w*AlbA, 2.2% were singly infected with *w*AlbB (mostly male) and 5.25% were free of *Wolbachia* infection. All samples that gave no band with *wsp* gene were reanalysed with CO1 gene primers to make sure they had DNA in them. Only samples that gave positive bands with CO1 gene primers and negative with *wsp* were taken as negative samples. The negative samples

were not excluded from the analysis as was done previously by Osei-Poku since the percentage was significantly higher than their estimation of 0.78% (Kittayapong et al., 2000; Osei-Poku et al., 2012). The 5.25% of the uninfected population may have been due to environmental factors. Several antibiotics have been reported to be able to cause bactericidal effect on *Wolbachia* (Dobson & Rattanadechakul, 2001; Otsuka & Takaoka, 1997). The uninfected samples may have been feeding from natural or synthetic bactericide containing food source causing the *Wolbachia* in them to perish in the wild. Besides, negative samples could indicate maternal transmission leakage of *Wolbachia*.

A study conducted in 2010 reported that all female *Ae. albopictus* were superinfected with *w*AlbA and *w*AlbB but *w*AlbA tend to deplete in male samples as the male ages. They proposed that it could have been due to evolutionary process selecting nuclear counter measures to *Wolbachia* manipulations (Tortosa et al., 2010). Another study conducted on cell line also reported depletion of *w*AlbA in the males with aging (Fallon, 2008).

A study by Ahantarig *et al*. from Thailand stated that density of *Wolbachia* passed on to offspring was always different. Some can be high and some can be low regardless of the offspring sex (Ahantarig et al., 2008). The singly infected samples in this study may have been due to varying low densities of *Wolbachia*. Perhaps one of the supergroup had density that was too low to be detected resulting in single infection. It was unlikely to be due to the aging of male mosquitoes as all the samples were below 6 days old. Wsp genes were used for *Wolbachia* supergroup detected since it's a fast evolving highly variable region (Zhou et al., 1998). It possesses 10 times higher variability compared to *fts*Z gene which makes it more suitable for evolutionary and phylogenetic studies.

The pattern of *Wolbachia* infections was not homogenous among the districts. Similar result were reported for *Wolbachia* infections in other countries (Doudoumis et al., 2012). Difference in sample size among the districts may have caused the heterogeneous *Wolbachia* infection pattern. Sample size analysed was considerably small for the establishment of the infection pattern because both empirical and theoretical data indicate that when a population gets infected with *Wolbachia*, it is likely to spread to fixation immediately (Armbruster et al., 2003). Additional studies on the diversified infection rate of *Wolbachia* with larger sample size may provide a better insight on the functional role of the bacteria in *Ae. albopictus* dynamics, evolution and ecology.

Samples from Sabah and Nilai resulted in more than 30.0% of *Wolbachia* free samples. More extensive sample collections and studies should be conducted in these regions to perhaps develop a naturally *Wolbachia* uninfected *Ae. albopictus* colony in Malaysia.

3.4.2 Phylogenetic studies

Two distinct clades of *w*AlbA and *w*AlbB were obtained. All *w*AlbA and all *w*AlbB were identical to each other respectively. This result concurs with previous studies conducted (Albuquerque et al., 2011; Armbruster et al., 2003). No further sequence analysis was carried out as the sequences were identical.

Lack of diversity in the *w*AlbA and *w*AlbB sequences may have been due to one strain being less dense compared to the other leading to decrease in likelihoods of homologous recombination. The other explanation could be these genes might have some role in cytoplasmic incompatibility, resulting in a high selective pressure that might hinder the occurrence of new strains (Albuquerque et al., 2011). Although the *wsp* gene is highly variable, it is perhaps more suitable to study variation in *Wolbachia* infection in

different species. Specific *wsp* genes are more applicable to identify infection status of a host.

3.4.3 *Wolbachia* distribution in *Ae. albopictus* organs

Although sufficient time was provided for the *Wolbachia* to infect different organs, only midguts and ovaries were found to be infected with *Wolbachia*. All salivary glands remained uninfected even after 45 days. Infection in ovaries and midguts concurs with previous studies but previous studies also reported infection in salivary glands of *Ae. albopictus* (Tsai et al., 2004; Zouache et al., 2009). Study conducted on La Reunion island, reported *Wolbachia* infections in their *Ae. albopictus* salivary glands, ovaries, guts and eggs (Zouache et al., 2009). Presence of *Wolbachia* in salivary gland may indicate inhibition of dengue virus dissemination whereas presence of *Wolbachia* in midgut may indicate inhibition of dengue virus replication. Absence of *Wolbachia* in salivary glands could not be explained. However, study conducted in Taiwan mentioned only moderate *Wolbachia* infection was found in their sample of salivary glands (Tsai et al., 2004). This probably could be due to difference in geographical origin.

Several studies have been reported high *Wolbachia* infection in the reproductive organs of their respective hosts (Werren, 1997). The presence of *Wolbachia* in ovaries may be the reason for *Wolbachia* often associated with abilities to alter reproductive phenotypes of their host (O'Neill et al., 1997). *Wolbachia* is a cytoplasmically inherited bacteria, therefore it has high possibility to promote mutualism and symbiotic relationships with their host (Werren, 1997). Since all ovaries in this study and previous studies have been superinfected with *Wolbachia*, it would be safe to hypothesize that *Wolbachia* may have an important impact on fecundity and egg viability of *Ae. albopictus*. Presence of *Wolbachia* in ovaries would also explain why *Wolbachia* is vertically transmitted to their offspring. *Ae. albopictus* being a vector for several

pathogens such as dengue and chikungunya virus, *Wolbachia* in the ovaries may effect transovarial transmission of these pathogens.

Wolbachia was found in both somatic and reproductive organs in this study. Detection of *Wolbachia* in midguts only on 14th day and in ovaries since the 6th day suggests that *Wolbachia* was first found in ovaries and then spread to the midguts. Infection of pathogens ingested by *Ae. albopictus* occurs from the midgut to other organs. When mosquitoes ingest dengue virus during blood meal, the virus penetrates into the midgut epithelial cells of mosquitoes. Secondary organs such as salivary glands and ovaries only gets infected after replication and release of virions (Mousson et al., 2012). *Ae. albopictus* is a less competent vector for dengue (Ahmad et al., 1997). Perhaps, presence of *Wolbachia* in midgut reduces the virus penetration into other organs such as salivary glands thus reducing virus dissemination.

3.5 Conclusion

A high proportion of Malaysian *Ae. albopictus* were superinfected with both *w*AlbA and *w*AlbB. *Wolbachia* was found in ovaries and midguts of the mosquitoes. Since majority were superinfected with *Wolbachia* and a high amount *Wolbachia* infection was found in their organs, these can make *Wolbachia* a valuable tool to influence host population as long as it can express inhibition of pathogen. The limitation of this study is that number of samples collected from each sampling site were not the same. All states in the country should have been sampled. It would be important to investigate the adult population in the wild. Currently samples was obtained only from a few places.

Future studies should be carried out with more extensive sample collection throughout Malaysia from different ecological settings to possibly obtain and develop colonies of *Ae. albopictus* singly or uninfected with *Wolbachia.*

CHAPTER 4: ESTABLISHING *AEDES ALBOPICTUS* COLONY WITH AND WITHOUT *WOLBACHIA* IN THE LABORATORY

4.1 Introduction

Aedes albopictus is naturally infected with the endosymbiont *Wolbachia* (Armbruster et al., 2003). In the previous chapter, it was established that 91.6% of Malaysian *Ae. albopictus* were superinfected with *w*AlbA and *w*AlbB (Joanne et al., 2015). Several studies have reported that *Wolbachia* have the ability to depress reproductive phenotypes in their hosts (O'Neill et al., 1997). It has also been reported that *Wolbachia* can cause cytoplasmic incompatibility in their hosts which may be used to reduce their host populations (Dobson et al., 2002; Dobson et al., 2004; O'Neill et al., 1997).

These traits have not been studied on Malaysian *Ae. albopictus* and only few other studies have been conducted to study these behaviours on naturally *Wolbachia* infected *Ae. albopictus* from other parts of the world (Dobson et al., 2002; Dobson et al., 2004).

In order to study cytoplasmic incompatibilities, the effect of *Wolbachia* on *Ae. albopictus* reproductive phenotypes and effect of *Wolbachia* on Malaysian *Ae. albopictus* susceptibility towards dengue virus, it is necessary to have both *Wolbachia* infected (WIS) and *Wolbachia* uninfected (WUS) stable colonies. Since none of the sample sites from the previous studies conducted on Malaysian *Ae. albopictus* had entirely *Wolbachia* free samples, artificial *Wolbachia* removal techniques had to be implemented (Afizah et al., 2015; Joanne et al., 2015).

Two promising techniques have been published previously in 1997 (Otsuka & Takaoka, 1997) and 2001 (Dobson & Rattanadechakul, 2001). Both involved treatment of larvae or adult mosquitoes with antibiotic tetracycline. The latter was published to

improve the first technique. However, when both the techniques were executed on separate occasions in this study, unsatisfactory outcomes were obtained. The objective of this study was to colonise *Ae. albopictus* with and without *Wolbachia* in the laboratory.

4.2 Methodology

4.2.1 Mosquito strain

Mosquito samples were collected using ovitraps from Bukit Lagong, Selayang, Kuala Lumpur. All samples were originally superinfected with *w*AlbA and *w*AlbB. Mosquito infection status was confirmed by randomly testing 30 adult mosquitoes monthly from each generation. Their DNA was extracted as mentioned in Section 3.2.3 followed by PCR amplification with *wsp* specific primers and gel electrophoresis (Figure 4.1) as mentioned in Section 3.2.

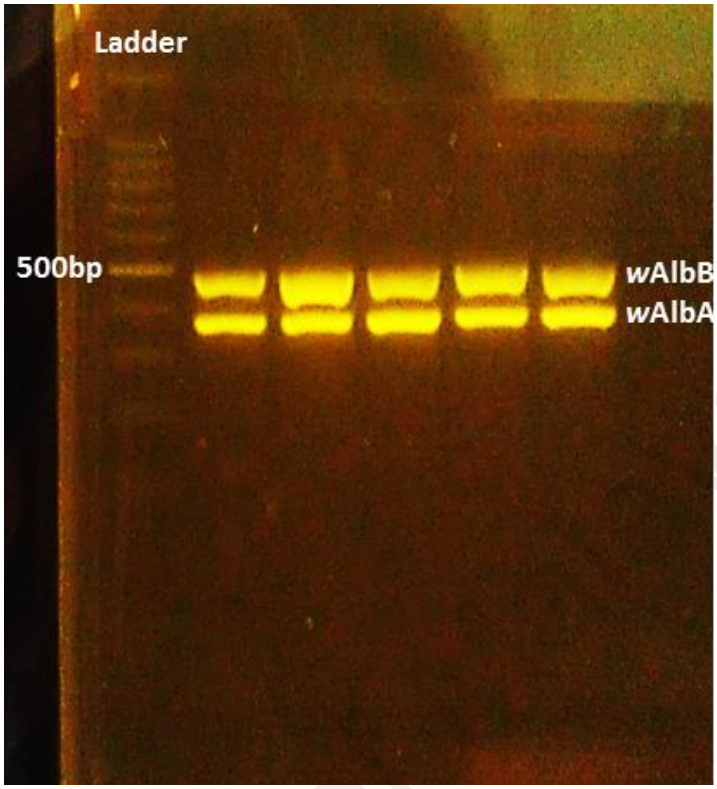

Figure 4.1: Gel electrophoresis result image. Lane 1 is the 100kB ladder. Lane 2-6 are the samples. The upper row are *w*AlbB amplified gene at 508bp and the lower row bands are *w*AlbA amplified gene at 363bp.

4.2.2 Colonization of WIS

4.2.2.1 Larvae rearing

Eggs obtained from the field were allowed to hatch in our insectarium in trays filled with overnight water as shown in Figure 3.2. About 200 eggs were hatched in each tray and fed with tetramin fish food twice daily. The overnight water was replaced every two days to prevent the growth of unwanted organisms.

4.2.2.2 Pupae collection

Once the larvae turned into pupae, they were transferred using Pasteur pipette into a clean container with unchlorinated water. No food was provided. They were placed in cages as shown in Figure 3.3. The container was removed once all pupae had become adult mosquitoes.

4.2.2.3 Adult rearing

Adult mosquitoes were supplied with 10.0% sucrose solution incorporated with 1.0% B-Complex soaked in cotton wool. Every two week, female mosquitoes were isolated into a separate cage and they were starved for 48 hours. They were then given a fresh blood meal using hemotek membrane feeder (Discovery Workshop, UK) in ACL-2 laboratory in Parasitology Department, University of Malaya with lights off. Fresh blood was voluntarily drawn from the author. After an hour, they were transferred back into the same cage.

Two days later, a petri dish with moist filter paper was placed into the cage for oviposition. Filter papers with eggs was taken out and replaced daily. The papers were dried in sterile environment for 24 hours and stored in a clean dry container to be hatched in future to obtain the next generations. Adult mosquitoes were randomly caught and

tested for *Wolbachia* infection at each generation to ensure *Wolbachia* superinfection were present. WIS colonies were maintained in Department of Parasitology insectary, University of Malaya at 27˚C and relative humidity of 85.0% with 12h: 12h light-dark photoperiod. This study obtained ethics approval from the UMMC Medical Ethics Committee (Ethics Committee /IRB Reference Number: 860.24 and 908.9).

4.2.3 Tetracycline treatment

All *Wolbachia* removal studies were conducted on the superinfected Bukit Lagong strain. Studies were conducted as stated in Table 4.1. Treatment 1 was conducted as previously described by Otsuka and Takaoka in 1997 on only the larvae (Otsuka & Takaoka, 1997). Treatment 5 and 6 was conducted as described previously by Dobson and Rattanadechakul on larvae and adult mosquitoes (Dobson & Rattanadechakul, 2001). Treatments 2, 3, 4 and 7 are the modified techniques. All generation subjected to treatments were considered F_0 generations.

Table 4.1: Tetracycline treatment design.

Treatment	Life cycle stage	Treatment period	Concentration of tetracycline
1[a]	24h – 48h larvae	24 hour	5.0 mg/mL in 2L overnight water
2[c]	24h – 72h larvae	72 hour	1.25 mg/mL in 2L overnight water
3[c]	48h – 120h larvae	72 hour	1.25 mg/mL in 2L overnight water
4[c]	48h – 72h larvae	24 hour	1.25 mg/mL in 2L overnight water
5[b]	48h – 72h larvae	24 hour	1.25 mg/mL in 2L overnight water
	Newly emerged adult mosquito	Continuous	0.50 mg/mL in 10% Sucrose solution with 100mg B-Complex
6[b]	Newly emerged adult mosquito	Continuous	1.00 mg/mL in 10% Sucrose solution with 100mg B-Complex
7[c]	Newly emerged adult mosquito	Continuous	1.25 mg/mL in 10% Sucrose solution with 100mg B-Complex

[a] Conducted as previously described by Otsuka and Takaoka (1997).

[b] Conducted as described previously by Dobson and Rattanadechakul (2001).

[c] Modified techniques.

Larvae after the treatment period in treatments 1, 2, 3 and 4 were transferred back into trays with overnight water without tetracycline and reared to adulthood. After each treatment, female mosquitoes were given a fresh blood meal and allowed to oviposit. Twenty-five female mosquitoes were randomly tested for *Wolbachia* infection after oviposition using PCR and gel electrophoresis method as described in Section 3.2.4. All PCR and gel electrophoresis were run alongside positive control. If the tested female was negative for *Wolbachia*, eggs laid by that female mosquito were hatched in a separate tray with overnight water and fed with tetramin fish food. They were reared to adulthood in the insectarium (F_1 generation). Meanwhile, larvae reared in tetracycline treated water in treatment 5 was transferred back into water without tetracycline after 24 hours and reared to adulthood.

Adult mosquitoes in treatment 5, 6 and 7 were blood fed bimonthly for egg collection and colony maintenance. Twenty five females were randomly tested for *Wolbachia* infection after oviposition for each generation as was done for treatment 1, 2, 3 and 4. Eggs collected from treatments 5, 6 and 7 were allowed to hatch in new trays with overnight water and also fed with tetramin fish food. Sucrose solution with tetracycline was continuously supplied for adult mosquitoes in treatment 6 and 7 throughout F_0, F_1 and F_2 generations. The tetracycline treated sucrose solution was replaced with tetracycline free sucrose solution from F_3 generation onwards.

4.2.4 Egg viability

Total number of eggs laid and number of larvae emerged by all female mosquitoes in treatment 6 and 7 was calculated and recorded. Egg viability of F_1 of treatment 6 and 7 as well as F_2 of treatment 7 was calculated using the formula below:

$$\text{Egg Viability (\%)} = \frac{\text{Total number of larvae emerged}}{\text{Total number of eggs laid}} \times 100$$

4.2.5 *Wolbachia* infection rate

Wolbachia infection rate was calculated using the formula below:

$$\text{WIR (\%)} = \frac{\text{Number of tested mosquito infected with } \textit{Wolbachia}}{\text{Twenty five randomly tested mosquito}} \times 100$$

4.3 Results

The WIS colony used in this study was previously established with superinfection of both *w*AlbA and *w*AlbB infection. The F_4 eggs were used in this *Wolbachia* removal study and treated as F_0 for WUS development study. Percentage of eggs hatched that survived to pupation, percentage of adult mosquitoes emerged, percentage of *Wolbachia* infection status of the F_0 and percentage of F_1 eggs hatched were calculated for all treatments 1-7, as shown in Table 4.2.

The results showed that treatments 1 and 2 yielded very low egg viability. In both treatments, less than 10.0% of the eggs produced hatched. Treatment 3 was slightly higher as the larvae subjected to tetracycline treatment were older (48-72 hr). When the treatment period was reduced to 24 hours in treatment 4, the egg viability improved but not all tested adult mosquitoes emerged from treatment 4 were free of *Wolbachia*. Treatment 5 was a combination of both larvae and adult treatment. Larvae were only treated for 24 hours as in treatment 4 but since complete removal of *Wolbachia* was not achieved in treatment 4, the adults were also treated with tetracycline incorporated into their sucrose solution. Egg viability was relatively good (56.6%) and all adult tested had no *Wolbachia* infection in them but F_1 eggs had only 17.9% of hatching rate. Therefore treatment 6 was designed by treating only the adult mosquitoes to reduce their exposure to tetracycline. Both F_0 and F_1 egg viability improved. Treatment 7 was an improvised technique with higher concentration of adult tetracycline treatment. Both F_0 and F_1 egg viability was high, which were 64.3% and 51.9% respectively.

Table 4.2: Percentage eggs hatch in F_0 and F_1 tetracycline treated strains.

Treatment	Number of eggs (F_0)	Pupae after 5 days (Egg viability)	Adult mosquito (F_0)	WIR	Number of eggs obtained (F_1)	Eggs hatched (Egg Viability)
1	132	4 (3.0%)	4 (100.0%)	All 4 *Wolbachia* free (100.0%)	0	NA
2	111	6 (5.4%)	5 (83.3%)	All 5 *Wolbachia* free (100.0%)	0	NA
3	122	42 (34.4%)	32 (76.2%)	All 25 tested *Wolbachia* free (100.0%)	5	0 (0.0%)
4	142	88 (62.0%)	83 (94.3%)	13 out of 25 *Wolbachia* free (52.0%)	29	0 (0.0%)
5[x]	145	82 (56.6%)	78 (95.1%)	All 25 tested *Wolbachia* free (100.0%)	230	41 (17.8%)
6[y]	153	107 (70.0%)	105 (98.1%)	All 25 tested *Wolbachia* free (100.0%)	249	142 (57.0%)
7[z]	143	92 (64.3%)	88 (95.7%)	All 25 tested *Wolbachia* free (100.0%)	189	98 (51.9%)

x Emerged mosquitoes were treated with 0.5 mg/mL tetracycline treated sucrose solution. F_0 adult mosquitoes were only *Wolbachia* free after 1 month of treatment.

y Emerged mosquitoes were treated with 1.0 mg/mL tetracycline treated sucrose solution. F_0 adult mosquitoes were only *Wolbachia* free after 1 month of treatment.

z Emerged mosquitoes were treated with 1.25 mg/mL tetracycline treated sucrose solution. F_0 adult mosquitoes were *Wolbachia* free after 2 weeks of treatment.

Treatment 5, 6 and 7 produced F_1 eggs. Therefore studies were continued to obtain the percentage of F_1 adult mosquitoes emerged from the eggs, *Wolbachia* infectivity status of F_1 colony and *Wolbachia* infectivity status of F_2 colony (only treatment 7). Results for this continuation studies are shown in Table 4.3.

Number of eggs produced in treatment 5 decreased after the first generation. No eggs were produced by F_1 adults. In treatment 6, the hatching rate for F_1 eggs was good (57.0%). However, when tested for *Wolbachia*, not all were free of *Wolbachia*. Only 72.0% was cleared of *Wolbachia*. Therefore eggs of F_1 adults were discarded. Treatment 7 had a high F_0 and F_1 egg hatching rate. In addition to that, all mosquitoes tested, F_0, F_1 and F_2 adults did not have *Wolbachia* infection in them. All were 100.0% free of *Wolbachia*.

Table 4.3: *Wolbachia* infection rate (WIR) of F_1 and F_2 tetracycline treated strains.

Treatment	No. of larvae	No. of adults (F_1)	Average WIR of F_1	Average WIR of F_2
5	41	32 (78.0%)	All 25 tested *Wolbachia* free (100.0%)	NA because no eggs were obtained
6	142	130 (91.6%)	18 out of 25 *Wolbachia* free (72.0%)	NA
7	98	92 (93.9%)	All 25 tested *Wolbachia* free (100.0%)	All 25 tested *Wolbachia* free (100.0%)

NA: Not applicable

4.4 Discussion

In order to study dynamics of *Wolbachia* on Malaysian *Ae. albopictus*, it was necessary to generate a stable WIS and WUS colonies. Initial protocols were carried out as mentioned by Dobson *et al* for the WUS colony development but not all *Ae. albopictus* was cleared from *Wolbachia*. Therefore this study was carried out by modifying previous methods to obtain a more viable method to completely remove *Wolbachia*.

Treatment 1 was conducted based on Otsuka method. Although all surviving adult were free of *Wolbachia*, it was ineffective as only 3.0% of the eggs obtained were viable and grew to become sterile adult mosquitoes (Otsuka & Takaoka, 1997). Similar findings were reported by Dobson (Dobson & Rattanadechakul, 2001). This may have been due to the very high concentration of tetracycline to treat the larvae.

Similar larval treatments were carried out with reduced concentration to 1.25mg/mL in treatment 2, 3 and 4 at different exposure periods. High larval mortality was observed when larvae were treated for more than 24 hours. However, improved larval mortality was observed when the larvae were treated after 48 hours instead of the 24 hour. This may be because 24 hour larvae are too young to withstand the tetracycline treatment.

Treatment 4 was designed to expose 48 hour larvae for 24 hours which gave lower larval mortality and a higher percentage of surviving adults. Although low larval mortality was observed, the treatment failed to remove *Wolbachia* completely from all surviving adults. Therefore it can be concluded that perhaps the period of treatment or tetracycline concentration was not sufficient.

Treatment 5 was conducted based on Dobson's study which subjects both larvae and adult mosquitoes to tetracycline (Dobson & Rattanadechakul, 2001). This method had low larval mortality and was able to completely remove *Wolbachia* from all surviving

F_0 adults. A good number of F_1 eggs were obtained but the hatching rate of the F_1 eggs was very low compared to untreated strains.

Treatment 6 was conducted based on the final method from Dobson's paper in 2001 which treats only the adult with 1.0 mg/mL tetracycline in sucrose solution (Dobson & Rattanadechakul, 2001). No alternative food source was provided for the mosquitoes. F_0 adult mosquitoes were tested for *Wolbachia* after 2 weeks exposure to tetracycline sucrose treatment. Mosquitoes were not found to be completely free of *Wolbachia*. F_0 adult mosquitoes were again tested for *Wolbachia* after 1 month tetracycline treatment and all were *Wolbachia* free. Eggs were collected and F_1 mosquitoes were obtained. Although the experiment was repeated three times, entirely *Wolbachia* free F_1 adult mosquitoes was not obtained. Therefore treatment 6 as proposed by Dobson & Rattanadechakul (2001) was not effective in this study.

Treatment 7 was designed exactly as treatment 6 with a slight increment of the concentration of tetracycline in the sucrose solution (Joanne et al., 2014). Complete *Wolbachia* removal from the F_0 adult mosquitoes was observed in two weeks tetracycline treated mosquitoes. This was confirmed with two replicates. Egg hatching rate was slightly lower than treatment 6 and 93.9% became F_1 adults. In contrast to treatment 6, F_1 adults were 100.0% *Wolbachia* free. Average was obtained from three replicates. All F_2 adults were also found to be *Wolbachia* free.

Tetracycline treatment of only adult mosquitoes simplifies the process, improves the egg hatchability, reduces larval mortality and increases adult fecundity. Tetracycline could be administered to adult mosquitoes either through sucrose solution or blood meal. Tetracycline was incorporated into sucrose solution so that both male and female would feed on it. The best concentration for the adult treatment was concluded to be 1.25mg/mL in sucrose solution with no alternative food source. This method was able to remove both

*w*AlbA and *w*AlbB completely in just two weeks and produces subsequent generations free of *Wolbachia*.

Tetracycline treatment was continued for three complete generations (F_0-F_2) to prevent the WUS from being re-infected with *Wolbachia*. The tetracycline treated sucrose was replaced with clean sucrose solution F_3 onwards. Several studies have mentioned possible degradation of host activity caused by loss of important microbiota during the tetracycline treatment (Bandi et al., 1999; Baton et al., 2013; Casiraghi et al., 2002). To prevent that, three generations were free of tetracycline treatment and only generations after that were used for experiments that followed to allow regrowth of any lost microbiota that may have been essential for *Ae. albopictus* functionality. Hypothesis of this chapter was to produce stable *Wolbachia* infected and *Wolbachia* free *Ae. albopictus* laboratory colonies. The difficulty faced in this chapter was to develop and sustain the *Wolbachia* free *Ae. albopictus* colony. Limitation of this study was not being able to develop a technique to produce singly infected *Ae. albopictus* colony with either *w*AlbA or *w*AlbB only.

4.5 Conclusion

In this chapter, it was found that the best method to remove *Wolbachia* from *Ae. albopictus* was by treating only the adult mosquitoes upon emergence with 1.25mg/mL tetracycline incorporated into their sucrose solution. Future research may be conducted to develop a singly infected *Ae. albopictus* strain with a modified antibiotic treatment as none has been established so far.

CHAPTER 5: DYNAMICS OF *WOLBACHIA* AND CYTOPLASMIC INCOMPATIBILITY IN MALAYSIAN *AEDES ALBOPICTUS*

5.1 Introduction

Wolbachia have been associated with tendency to develop a mutualistic relationship with their host (Werren et al., 2008). Mutualism is when both bacteria and their host exist in a relationship where each of them benefits from the other (Boucher, 1988).

Among areas that have been reported to be affected by *Wolbachia* are the host fecundity, longevity and egg viability (Fry et al., 2004; McMeniman et al., 2009; Walker et al., 2011). These characteristics were expressed differently in different hosts. In some of the natural *Wolbachia*-infected hosts such as *Ae. albopictus, D. simulans* and *D. melanogaster*, *Wolbachia* was reported to have a positive effect on their reproduction (Dobson et al., 2004). When *Wolbachia* was removed from them, their host fecundity, longevity and egg viability was reduced (Dobson et al., 2002), while, in other closely related species where this relationship is not found in nature, the presence of *Wolbachia* showed reduction of their host fecundity, egg viability and longevity (McMeniman et al., 2009; Yeap et al., 2011). *Wolbachia* appears to have developed a mutualistic relationship with their natural hosts and behaves as a parasite in new hosts. However, this is complex as studies report contradicting effects where *Wolbachia* have caused reduction in host fecundity and egg viability in their natural host such as *Culex quinquefasciatus* (de Almeida et al., 2011) and *Ae. polynesiensis* (Brelsfoard & Dobson, 2011).

Besides these properties, a very significant reproductive phenotype alteration caused by *Wolbachia* that has been of interest to many researchers from all over the world is their ability to cause cytoplasmic incompatibility (Dobson et al., 2004). Cytoplasmic

incompatibility (CI) enables *Wolbachia* to be transferred from one generation to the next in their host with minimal modification on their properties. This has been postulated to eradicate the necessity for horizontal transmission of *Wolbachia* (Turelli, 1994). There are two types of CI namely unidirectional and bidirectional. Unidirectional is when no viable offspring are obtained when infected males mate with uninfected females but viable offspring are obtained when uninfected males mate with infected females (Dobson et al., 2004; Laven, 1967; O'Neill et al., 1992). Bidirectional is when no viable offspring are obtained when either male or female are infected with different type of *Wolbachia* (Telschow et al., 2005).

Wolbachia is described as a saving mechanism that needs to be present in the female. *Wolbachia* modifies the sperm and when the sperm fertilizes an egg, the egg needs to be infected with the same strain of *Wolbachia* to save the modified sperm. If it doesn't, the mating becomes unsuccessful and results in unviable eggs (Telschow et al., 2005; Werren, 1997; Werren et al., 2008). In populations with both *Wolbachia* infected and uninfected hosts, CI reduces chances of obtaining *Wolbachia* uninfected offspring. This ensures their infection continuity in their host populations. Henceforth, this property can be manipulated to reduce their hosts' population (Werren et al., 2008).

Previous studies have reported unidirectional CI in both nematodes and arthropods (Dobson et al., 2002; Dobson et al., 2004; Kambhampati et al., 1993; Zabalou et al., 2004) and bidirectional CI in a few hosts (Blagrove et al., 2012; O'Neill & Karr, 1990; Telschow et al., 2005; Zabalou et al., 2004). There are studies where *Wolbachia* did not express CI in their natural host such as in *D. melanogaster* (Giordano et al., 1995; Hoffmann et al., 1996).

Populations of *Ae. albopictus* have been growing at an alarming rate in spite of all vector control methods currently carried out in Malaysia (Dieng et al., 2010; Rozilawati et al., 2007). If populations of *Ae. aegypti* are suppressed to very low levels, it is very likely that *Ae. albopictus* would emerge as the major vector. Therefore, it is important to study the dynamics of *Ae. albopictus* and possible measures to control its populations.

In order to develop a successful biological control measure, the sustainability of the method is crucial (Brownstein et al., 2003). If CI can be used as a tool to reduce *Ae. albopictus* population in Malaysia, other characteristics mentioned above specifically fecundity, egg viability and longevity of *Ae. albopictus* with and without *Wolbachia* needs to be considered to prognosticate the durability of the control measure. Malaysian *Ae. albopictus* are naturally infected with *Wolbachia*. If CI is expressed by Malaysian *Ae.albopictus*, field release of *Wolbachia* free *Ae. albopictus* should theoretically reduce number of female mosquitoes producing viable offspring which would eventually reduce population of *Ae. albopictus*.

In this chapter, the objective is to determine dynamics of *Wolbachia* and cytoplasmic incompatibility in Malaysian *Ae. albopictus*. *Aedes albopictus* fecundity, egg viability and longevity with and without *Wolbachia* were studied alongside cytoplasmic incompatibility.

To my knowledge, no prior study has been conducted to study these characteristics on naturally *Wolbachia*-infected Malaysian *Ae. albopictus*. These biological characteristics provided the initial data which were useful for the next chapter of this dissertation.

5.2 Methodology

5.2.1 Mosquito strain

Strains used in this study were from the colonies originated from Bukit Lagong, Gombak, Selangor (Chapter 4). Adult mosquitoes were supplied with 10.0% sucrose solution incorporated with 1.0% B-Complex soaked in cotton wool. In both dynamics and cytoplasmic incompatibility study, both *Wolbachia* infected strain (WIS) and *Wolbachia* uninfected strain (WUS) were used. Both colonies were maintained in insectary at the Department of Parasitology, University of Malaya at 27˚C and relative humidity of 85.0% with 12h: 12h light-dark photoperiod.

Samples were continuously confirmed to be *Wolbachia* superinfected or *Wolbachia* uninfected from each generation by randomly sampling adult mosquitoes with PCR amplifications and gel electrophoresis as mentioned in Section 3.2.4. The WIS strain used in this chapter was from F_7 onwards and the WUS was from F_5 onwards.

5.2.2 Dynamics of *Wolbachia*

5.2.2.1 Fecundity

Newly emerged male and female adult mosquitoes were placed in the same cage for 4 days with 10% sucrose solution incorporated with B-complex soaked in a cotton wool. On the fifth day, all females were isolated and transferred into a separate cage using mechanical suction tube. The sucrose solution was removed and the females were starved for 24 hours.

On the sixth day, the female mosquitoes were transferred into three polystyrene cups; 40 in each. They were provided a fresh human blood meal using hemotek membrane feeder (Discovery Workshop, UK) in ACL-2 laboratory in Parasitology Department,

University of Malaya with lights off for an hour. The fresh blood was voluntarily drawn from the author before each feeding session.

The mosquitoes were knocked down by placing the polystyrene cups in -20˚C freezer for 60 seconds. The cups were then taken out and placed on ice bath. Fully engorged female mosquitoes were isolated into a new cage and supplied with sucrose solution. On third day post feeding, 50 engorged mosquitoes were transferred into individual transparent 4 oz. containers lined with moist filter paper for oviposition. They were supplied with a small piece of cotton wool soaked in 10% sucrose solution and maintained in humidity chamber (27˚C, relative humidity of 85.0% and 12h: 12h light-dark photoperiod). Both sucrose soaked cotton wool and moist filter paper for oviposition were changed daily. For those females with no hatched eggs, the spermathecae were checked for the presence of spermatozoa to confirm the occurrence of mating and then transferred into 1.5 mL micro-centrifuge tubes. A total of 20 randomly selected ovaries were tested for *Wolbachia* infection using PCR amplification and gel electrophoresis as mentioned in Section 3.2.4. Only data obtained from female mosquitoes that had fertilized eggs were analysed. Number of eggs produced by individual female were recorded and analysed. This study was carried out three times.

5.2.2.2 Longevity

Eggs were hatched in trays with fitted cover as shown in Figure 3.2, half filled with overnight water. About 200 eggs were hatched in each tray and fed with tetramin fish food twice daily. Once pupae were formed, they were collected using a Pasteur pipette and transferred into a transparent container. They were placed into cages as shown in Figure 3.3.

Life span was calculated from the first day mosquito emerged from pupa. Adult mosquitoes were supplied with 10% sucrose solution incorporated with 1% B-Complex soaked in cotton wool. Mosquitoes were provided fresh blood meal using hemotek membrane feeder (Discovery Workshop, UK) in ACL-2 laboratory in Parasitology Department, University of Malaya every two weeks. Cages were lined with a white paper to ease collection of dead mosquitoes. Dead mosquitoes were collected daily and recorded according to their gender. The study was carried out two times. Both WIS and WUS colony longevity were studied simultaneously in each replicate.

5.2.2.3 Two weeks dried egg viability

Collected eggs were dried and stored for two weeks in a dry and clean container (27˚C, relative humidity of 85.0% and 12h: 12h light-dark photoperiod). Twenty five eggs on each filter paper were allowed to hatch in overnight water in four containers after examining them under a stereo microscope. Eggs with broken shells were not included in this study. Larvae were fed with tetramin fish food twice daily. The overnight water was changed every two days to prevent mould growth. The number of mosquitoes that emerged from the eggs was recorded. The study was repeated three times.

5.2.2.4 One day dried egg viability

Twenty five eggs on each filter paper were allowed to hatch in overnight water in four containers after drying them for one day after collection. Eggs with broken shell were not included in this study. Larvae were fed with tetramin fish food twice daily. The overnight water was changed every two days to prevent mould growth. The number of mosquitoes that emerged from the eggs was recorded. These steps were also carried out for both WIS and WUS in triplicates.

5.2.3 Cytoplasmic Incompatibility

5.2.3.1 Population cages

A total of four crosses were made as shown in Table 5.1. Fifty female mosquitoes were maintained in each cage with only sucrose that has been incorporated with B-complex. When the females were 8 days old, 20 male mosquitoes were introduced into their cages for mating. Three days later, a fresh blood meal was provided. Blood meal was drawn from the author each time and fed using hemotek membrane feeder (Discovery Workshop, UK) in ACL-2 laboratory. Fully engorged female mosquitoes were transferred into individual 4 oz. transparent containers layered with a filter paper for egg laying. Eggs were collected daily and recorded. Collected eggs were dried and hatched to measure the egg viability as previously described (Joanne et al., 2015).

Table 5.1: Crosses made for each cage.

	Cage 1	**Cage 2**	**Cage 3**	**Cage 4**
Crosses	IF × IM	UF × UM	UF × IM	IF × UM

IF: infected female; IM: infected male; UF: uninfected female; UM: uninfected male.

5.2.4 Statistics

All statistical analysis was carried out using GraphPad Prism version 6.01 for Windows (GraphPad Software, Inc., 2012). Significant difference in fecundity was analysed using unpaired two tailed t-test ($P<0.01$) while significance of longevity was analysed using Mantel-Cox and Gehan-Breslow-Wilcoxon tests ($P<0.01$) which are specific tests for survival data. Lastly, significant difference in egg viability was analysed using two-way ANOVA with Sidak test as post hoc ($P<0.01$).

In addition, one-way ANOVA ($P<0.01$) was used for all data obtained in cytoplasmic incompatibility studies and Sidak test as post hoc. Test for significant difference was analysed comparing each of the Cross 2, Cross 3 and Cross 4 with Cross 1 as control.

5.3 Results

5.3.1 Dynamics of *Wolbachia*

5.3.1.1 Fecundity

A total of 4620 eggs was laid by 150 *Wolbachia* infected mosquitoes and a total of 1080 eggs was laid by 150 *Wolbachia* uninfected mosquitoes. Figure 5.1 shows average number of eggs laid for both WIS and WUS colonies. The difference between the average number of eggs laid were statistically significant with p=0.0029.

When average number of eggs laid and highest number of eggs laid were compared statistically using unpaired two-tailed t-test between WIS and WUS, WIS showed to be significantly higher. An average of 1540 eggs were laid by 50 WIS female and this was significantly higher than WUS (360 eggs) with p=0.0029. Same goes to the highest number of eggs laid, WUS (102.7) was significantly higher than WIS (31.7) with p=0.011 (Table 5.2).

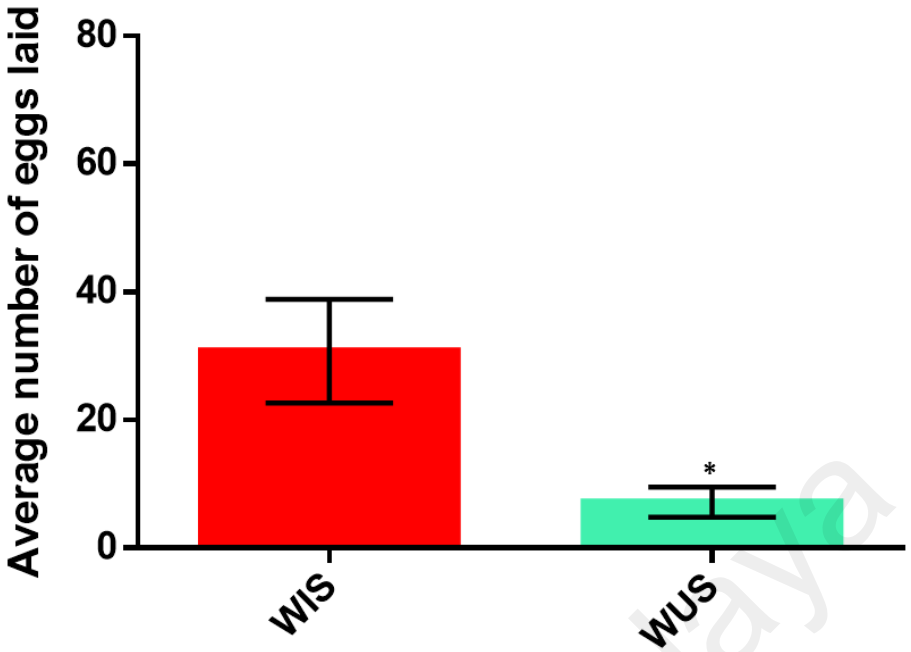

Figure 5.1: Average eggs laid by WIS and WUS. Each bar is the mean of three replicates and the vertical lines represent 95% confidence interval. Asterisk indicates significant difference ($p<0.01$). Significant difference between both WIS and WUS was calculated using unpaired two tailed t-test ($t = 5.613$ $df = 98$).

Table 5.2: Fecundity parameters of WIS and WUS.

	WIS				**WUS**			
Replicates	1st	2nd	3rd	Average	1st	2nd	3rd	Average
Mean number of eggs laid	30.0	24.9	37.4	30.8*	6.8	7.6	7.2	7.2
Total number of eggs laid by 50 female	1501	1247	1872	1540*	341.0	379.0	360.0	360
Highest number of eggs laid by individual female	76.0	130.0	102.0	102.7*	28.0	34.0	33.0	31.7

Asterisk indicates significant difference ($p<0.01$) using two tailed unpaired t-test ($t = 5.613$, $df = 98$).

5.3.1.2 Longevity

Each colony consisted of more males than females. There was no significant difference in sex ratio between *Wolbachia* infected and uninfected colony.

WIS males died by the sixth week whereas (38 days), WUS males died by the 18th week (121 days). WIS females survived for about 19 weeks (131 days) whereas WUS females survived for about 18 weeks (125 days). Lifespan of WUS male was significantly higher than WIS male when tested with Mantel-Cox and Gehan-Breslow-Wilcoxon tests ($p=0.0001$ and $p=0.0019$). Longevity of WUS female was slightly lower than WIS female but the difference was not significant in both tests ($p=0.7936$ and $p=0.5064$). (Figure 5.2).

In both strains, most death occurred within the first 6 weeks. The graph had higher gradient in the first six weeks especially for the males. Although WUS males lived longer than 6 weeks, less than 20.0% of the initial male population survived after the seventh week in both WIS and WUS.

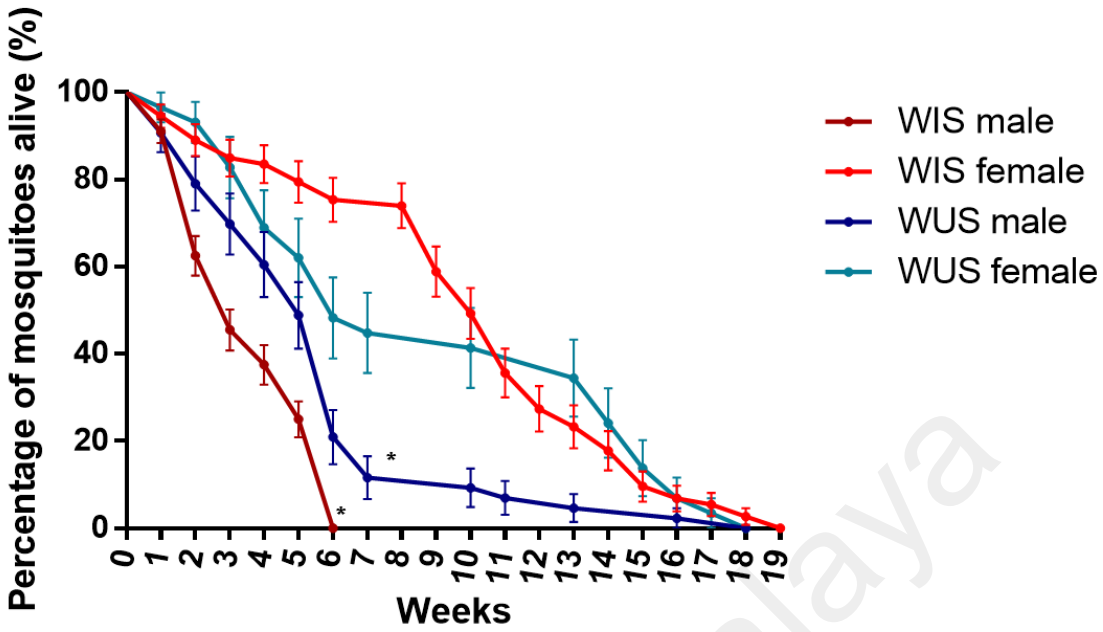

Figure 5.2: Lifespan of WIS and WUS mosquitoes. Each point represents percentage of mosquitoes alive at each respective week. Vertical lines show standard error. Asterisk indicates significant difference ($p<0.01$), ($x^2=105.5$, df= 3).

5.3.1.3 Egg viability

Egg viability is equivalent to the percentage of eggs hatched to become adults. Larvae and pupae emergence was faster in WIS eggs. Pupation started on day six for WIS eggs while on day eight day for WUS eggs.

One day dried WIS eggs had the highest percentage of eggs hatched (43.5 ± 7.0%) while two weeks dried WIS eggs had the lowest (27.3 ± 1.8%). The difference between WIS one day dried eggs and two weeks dried eggs were statistically significant with p=0.0005 when tested with two-way ANOVA and Sidak multiple comparison.

Two weeks dried WUS eggs (41.0 ± 1.3%) had significantly higher egg viability compared to two weeks dried WIS eggs (27.3 ± 1.8%) with p=0.0009. When the one day dried eggs were compared, WUS (36.5 ± 3.0%) had a lesser egg viability compared to WIS (43.5 ± 7.6%) but the difference was not significant (p=0.371) (Figure 5.3).

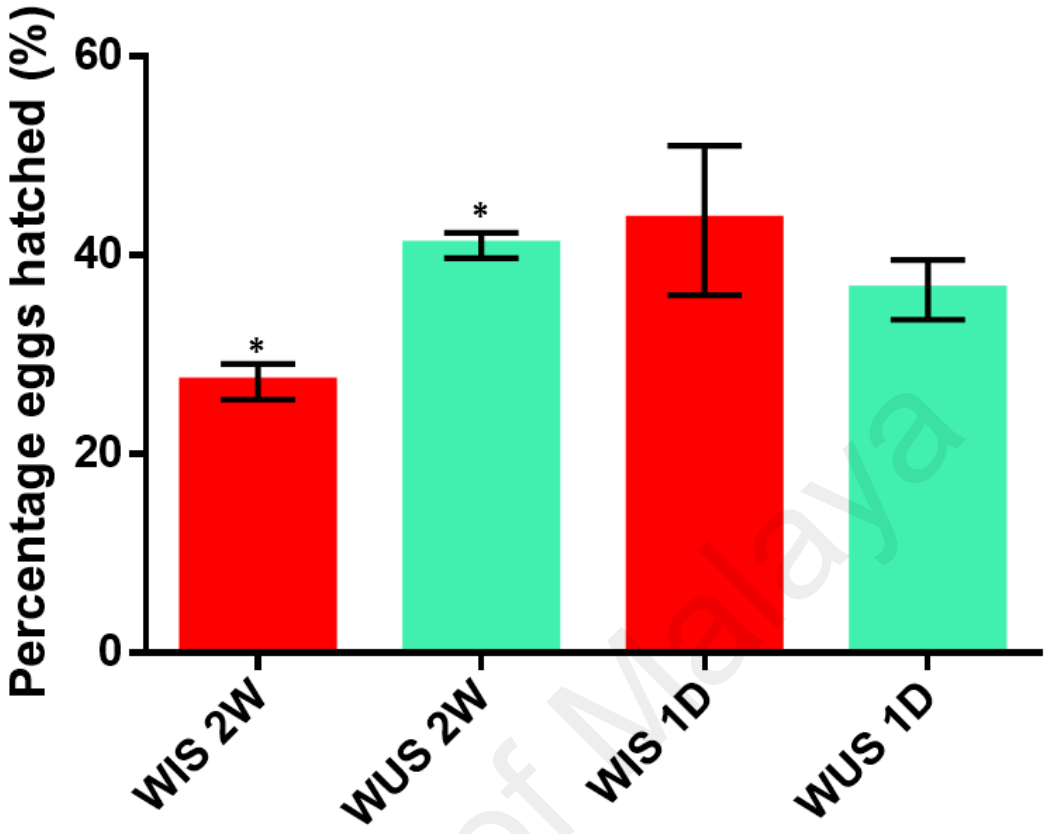

Figure 5.3: Egg viability of WIS and WUS. Each bar represents the mean percentage eggs hatched for the triplicates. Vertical line represents the standard deviation. [2W: Eggs left to hatch after drying for two weeks and 1D: Eggs left to hatch after drying for one day]. Asterisk indicates significant difference ($p<0.01$) between each bar marked with (*) according to two-way ANOVA $F_{(3, 24)} = 9.831$ and Sidak test for multiple comparison.

5.3.2 Cytoplasmic Incompatibility

Infection status of cytoplasmic incompatibility was determined using PCR amplification and gel electrophoresis as mentioned in Section 3.2.4.

5.3.2.1 Fecundity

In crosses that were superinfected with *w*AlbA and *w*AlbB, the larger number of eggs (2799) were laid by 81 females with mean of 35.90 compared to only 621 eggs by 82 females in the uninfected cross with mean of 7.86. While when the infected male was crossed with the uninfected female, 491 eggs were produced by 66 females with mean of 7.38. However, in the reverse cross 2147 eggs were produced by 72 females with mean of 29.80 (Figure 5.4). Total number of eggs laid in cross 1(IF X IM) was significantly higher compared to cross 2 (UF X UM) ($p<0.0001$) and cross 3 (UF X IM) ($p<0.0001$).

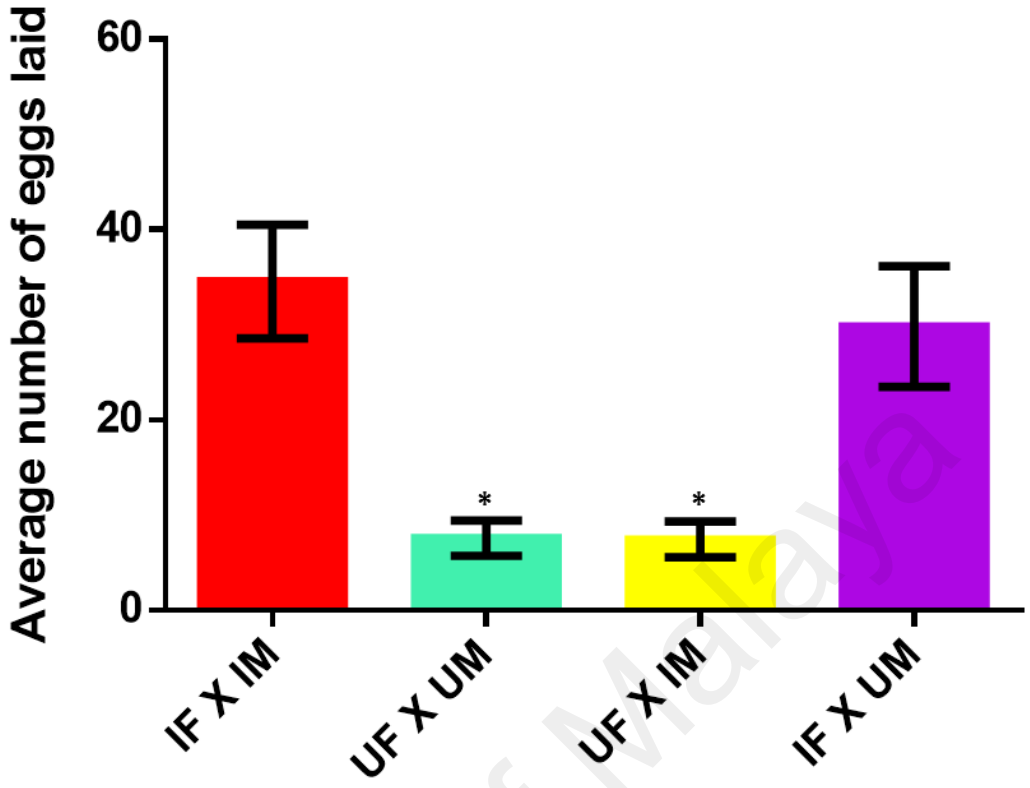

Figure 5.4: Mean number of eggs laid by each female mosquito of each cross. Each bar represent the overall mean value of the triplicates for each cross. The vertical lines represent 95% confidence interval. Asterisk indicates significant difference of the cross compared to cross 1 which is taken as the control ($P<0.01$) according to one-way ANOVA $F_{(3, 297)} = 38.86$ and Sidak test for multiple comparison ($P<0.001$). IF: Infected female; IM: Infected male; UF: Uninfected female; UML Uninfected male.

5.3.2.2 Egg viability

Overall egg viability was highest when *Wolbachia* was present in both sexes (93.8% with mean of 50.4%). However, when both were not infected, the total egg viability was only 50.0% with mean of 14.7%. In the cross between uninfected female and infected male, the total egg viability was 8.0% with mean 0.7% while for the reverse it was 39.0% with mean 5.4% as shown in Figure 5.5. Cross 1(IF X IM) egg viability was significantly higher compared to Cross 2 (UF X UM) ($p<0.0001$), Cross 3 (UF X IM) ($p<0.0001$) and Cross 4 (IF X UM) ($p<0.0001$).

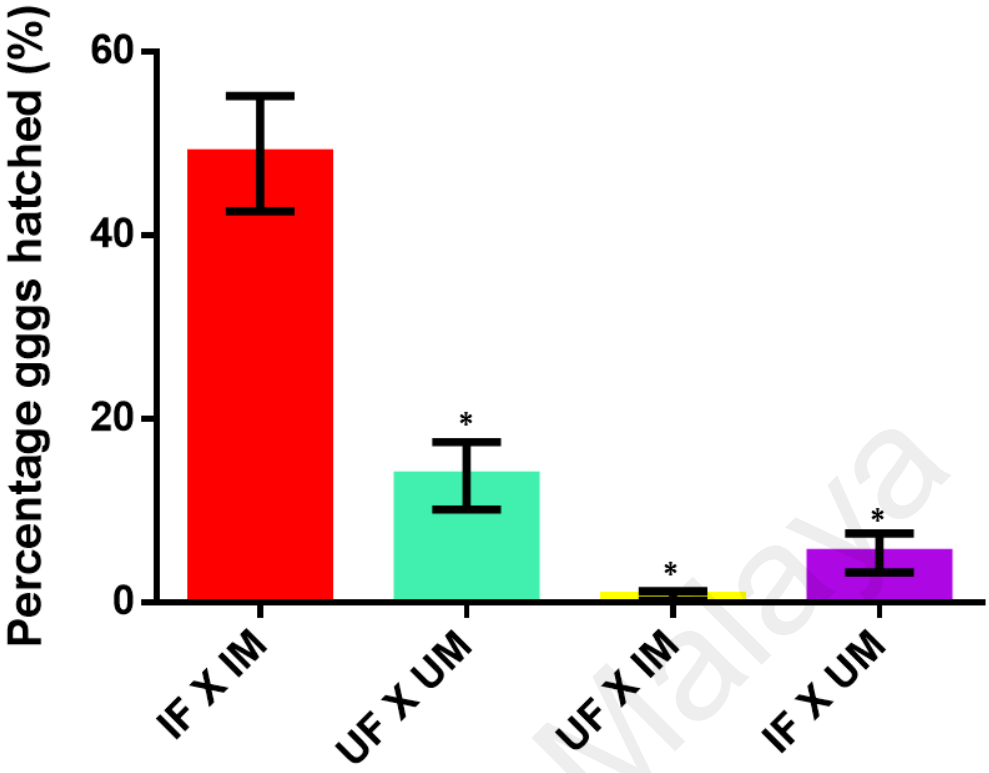

Figure 5.5 Mean of percentage of eggs by each female mosquito hatched for each cross. Each bar represent the overall mean value of the triplicates for each cross. The vertical line represent 95% confidence interval. Asterisk indicates significant difference of the cross compared to cross 1 which is taken as the control ($P<0.01$) according to one-way ANOVA $F_{(3, 297)} = 117.0$ and Sidak test for multiple comparison ($P<0.001$). IF: Infected female; IM: Infected male; UF: Uninfected female; UML Uninfected male.

5.3.2.3 Infectivity of offspring

All offspring tested in Cross 1 (IF X IM) were superinfected with *w*AlbA and *w*AlbB while all offspring tested from Cross 2 (UF X UM) and Cross 3 (UF X IM) were clear from *Wolbachia* infection. Among offspring tested in Cross 4 (IF X UM), 82.0% were superinfected with *w*AlbA and *w*AlbB and the rest were clear.

5.4 Discussion

This chapter of the dissertation discusses two main behaviours of *Wolbachia*. Firstly, how *Wolbachia* affects the reproductive output of Malaysian *Ae. albopictus* specifically the fecundity, longevity and egg viability. This was done by comparing these characteristics between WIS and WUS mosquitoes. Secondly, the ability of *Wolbachia* to exhibit cytoplasmic incompatibility on Malaysian *Ae. albopictus*. This was done by carrying out four different crosses and comparing them in terms of the female fecundity and their eggs viability.

All studies were conducted in replicate population cages. In order to prevent cross contaminations, trays and cages used in experiments involving WIS was not used for WUS. Separate trays, containers and cages were assigned and used for each strain.

The results showed that removal of *Wolbachia* from their native host Malaysian *Ae. albopictus* affected their female fecundity, egg viability, female longevity and their male lifespan. The results also show that *Wolbachia* expressed unidirectional cytoplasmic incompatibility in Malaysian *Ae. albopictus*.

Wolbachia have been previously associated with forming mutualistic relationship with their hosts (Werren et al., 2008). The results suggest that this could be true since when *Wolbachia* was cleared from *Ae. albopictus* using tetracycline treatment, their

females fecundity was noticeably reduced. This decline was statistically significant. Malaysian *Ae. albopictus* is a natural host for *Wolbachia*. Highest density of *Wolbachia* is usually found in the gonads of their host (Tsai et al., 2004). It would be reasonable to assume that *Wolbachia* plays a major role in the activities that occurs in their gonads such as their eggs development, fecundity and hatching capabilities. Hence, removal of *Wolbachia* would affect their host negatively. Previous studies on naturally infected *Ae. albopictus*, *D. simulans* and *D. melanogaster* have reported similar findings when *Wolbachia* was removed from them (Dobson et al., 2002; Dobson et al., 2004; Fry et al., 2004; Poinsot & Mercot, 1997; Weeks et al., 2007). However, in a study conducted with naturally *Wolbachia* infected *Cx. quinquefasciatus*, fecundity and their eggs viability improved when *Wolbachia* was removed from them (de Almeida et al., 2011). This could have been due to host and geographical differences. Previous study also reported that infected southern Californian *D. simulans* females to have 10-20% lower fecundity compared to their *Wolbachia* cleared strain (Hoffmann et al., 1990). However, when *D. simulans* from the same region was analysed 20 years later, they found that fecundity of their infected strain was 10% higher compared to uninfected strain (Weeks et al., 2007). In my opinion, *Wolbachia* role in them might have evolved from parasitism to mutualism over the years. Taking this into account, Malaysian *Ae. albopictus* might have been infected with *Wolbachia* for more than two decades since *Wolbachia* do seem to have developed mutualistic relationship with them.

WUS females had higher mortality rate compared to the WIS in between the third week to the eleventh week, then improved and survived as long as the WIS females did. Longevity experiments were carried out on three different generations and similar outcomes were observed. The first generation studied was three generations after discontinuing tetracycline treatment. This could have been due to the females needing time to adapt the elimination of *Wolbachia* although *Wolbachia* was removed from them

generations earlier. Overall, WUS female had similar lifespan as WIS females but with lower survival rate though the difference was not statistically significant. Similar reduced longevity in uninfected strain were observed in *Cx. quinquefasciatus* (de Almeida et al., 2011), *Ae. albopictus* (Calvitti et al., 2009; Dobson et al., 2002; Dobson et al., 2004), *Ae. polynesiensis* (Brelsfoard & Dobson, 2011), *D. simulans* (Weeks et al., 2007) and *D. melanogaster* (Fry et al., 2004). Probably the living capacity of female insects without *Wolbachia* needs a longer time to adjust and adapt compared to natural host colonies. As for the male mosquito longevity, WUS males had a statistically significantly longer lifespan and survival rate compared to WIS males. Among reproductive phenotype alteration induced by *Wolbachia* is male killing. Male killing is when the male eggs do not hatch to become an adult. Male eggs do not pass the embryogenesis or first instar larvae stage (Hurst et al., 2003; Hurst et al., 1999). Perhaps, infection of *Wolbachia* in Malaysian *Ae. albopictus* induces a delayed male killing. Instead of male eggs not making it through the embryogenesis, *Wolbachia* could be killing them in their adult stage. That would be able to explain WIS males having one third of the lifespan of WUS males. Nevertheless, this is just a postulation and further studies need to be carried out to explain this observation. A study done in 2009 on naturally infected *Ae. albopictus* reported that no difference in male reproduction phenotypes was observed when *Wolbachia* was removed but their female had a shorter lifespan, fecundity and egg viability (Calvitti et al., 2009). Both their male strains had lifespan approximately 65 days circa the time the WUS males survived in this study.

It is a common practice in most studies to dry the eggs before hatching them (Bellini et al., 2007; Gerberg et al., 1994). When that was done, WUS eggs had a higher viability compared to WIS eggs. Studies which reported similar findings were not conducted on native host of *Wolbachia*. When non-native *Wolbachia* strain was embryonically microinjected into *Ae. aegypti* or *Wolbachia* cured *Ae. albopictus*, egg

viability of the artificially *Wolbachia* infected hosts were reduced (Blagrove et al., 2012; Suh et al., 2009; Turley et al., 2013; Yeap et al., 2011). In studies conducted with natural host of *Wolbachia*, *Ae. albopictus* and *D. simulans*, the opposite was observed. Removal of *Wolbachia* reduced the egg viability of their host (Dobson et al., 2002; Poinsot & Mercot, 1997).

However, when the eggs were dried only for a day before hatching, WIS had higher egg viability than WUS eggs.

It is possible that *Wolbachia* in Malaysian *Ae. albopictus* might be somehow causing the eggs to be more readily hatched after a short drying period to benefit their survival in nature during rainy seasons. Storing it for longer time might be reducing the eggs health by decreasing moisture, hence decrease viability. When *Wolbachia* is removed, this property is no longer expressed, thus WUS eggs are more viable after two weeks. Instead of carrying out the common practice of dying the eggs for a period of time before hatching them, perhaps hatching them immediately after a day of collection might help their colony development since one day dried WIS eggs had significantly higher hatching rate compared to two weeks dried WIS eggs.

In order to make sure that the only factor contributing to the observations and results is absence of *Wolbachia*, all WUS samples used for these studies were free from tetracycline treatment for at least generations before starting these studies. This is to allow re-establishment of any other microbiota that may have lost during the antibiotic treatment (Baton et al., 2013). Mosquitoes were continuously tested at each generation to ensure absence of *Wolbachia* in them.

Cytoplasmic incompatibility is an effect caused by *Wolbachia* to their host species. This modification is carried out to sustain *Wolbachia* infection in every generation since it reduces the chance of developing *Wolbachia* free offspring (Dobson et al., 2004). The *Wolbachia* infection in Malaysian *Ae. albopictus* appears to cause unidirectional CI under laboratory conditions.

Study conducted using *Ae. albopictus* from Houston, Thailand and Mauritius in 2001 reported similar unidirectional cytoplasmic incompatibility (Dobson et al., 2002). Other than *Ae. albopictus*, *Wolbachia* have been reported to cause unidirectional cytoplasmic in most of their natural hosts such as *Cx. quinquefasciatus*, *D. simulans*, *D. melanogaster* and spider mites *Tetranychus urticae* and *Tetranychus turkestani* (Bourtzis et al., 1996; Breeuwer, 1997; de Almeida et al., 2011; Sinkins et al., 1995). Study done on *D. mauritiana* and *D. sechellia* reported that *Wolbachia* was not able to express CI in *D. mauritiana* and expressed partial CI in *D. sechellia* (Giordano et al., 1995). Similar inability to exhibit CI was also observed in *D. melanogaster* studied in 1997 (Hoffmann et al., 1996). *Drosophila melanogaster* generally experiences a weak CI compared to the other hosts (Bourtzis et al., 1996; Hoffmann et al., 1996; Hoffmann et al., 1998).

The first inter population CI was reported in a study conducted using Mauritius *Ae. albopictus.* Complete embryonic death was observed when females from Mauritius island were mated with males from five different geographical locations. All matings carried out within the same geographical location were compatible (Kambhampati et al., 1993). Studies were also conducted to observe CI in *Ae. albopictus* artificially infected with *Wolbachia* from different hosts such as *w*Mel from *D. melanogaster* and *w*Pip from *Cx. pipientis*. These studies mostly showed bidirectional CI (Blagrove et al., 2012; Calvitti et al., 2015). These show that the same species of host may have different types of CI caused by *Wolbachia* and CI expression in each of their host is unpredictable.

In this study, embryonic death with very high mortality was observed in Cross 3 (UF X IM) and embryonic death with lower mortality in Cross 2 (UF X UM) and Cross 4 (IF X UM). The reduced fecundity and egg viability in Cross 2 (UF X UM) was due to removal of *Wolbachia* in them as *Wolbachia* exerts a major impact on the reproductive properties of *Ae. albopictus*.

The lowest egg viability was from the cross between uninfected females and infected males which was significantly lower compared to all other crosses. Cross 3 (UF X IM) fecundity was also significantly lower compared to cross between infected females and males (control). This shows a very strong incompatibility with 99. 3% embryonic death in Cross 3.

A recent study reported crosses between *Ae. albopictus* males with low density of *w*AlbA and *w*Pip infected *Ae. albopictus* females resulted in complete CI whereas crosses between males with high densities of *w*AlbA and *w*Pip female did not. Analysis of *Wolbachia* density by quantitative PCR of the *wsp* gene showed that *w*AlbA densities were generally lower than *w*AlbB titre in their naturally-infected mosquitoes (Calvitti et al., 2015). In this study, the *Wolbachia* titre was not quantified but none of the infected samples from field collections were singly infected with *w*AlbA. The reason 100.0% embryonic death was not achieved in Cross 3 (UF X IM) could have been due to varying density of *w*AlbA in the samples which can be tested in future studies by quantifying density of each *Wolbachia* supergroup in them.

Unlike other studies, even mating between infected females and uninfected males (Cross 4) resulted in 94.7% unviable eggs. Given these results, in my opinion, if a field release of *Wolbachia* uninfected males is carried out, decrease in *Ae. albopictus* population may occur although resulting offspring have 82.0% superinfection.

Hypothetically, if *Wolbachia* free *Ae. albopictus* males are released, eggs produced by every female mosquito they mate with becomes almost unviable.

Study has shown that non-virgin female mosquito mated less readily than virgin females and indicated that female *Ae. aegypti* in nature may normally utilize sperm from only a single male (Spielman et al., 1967). Assuming *Ae. albopictus* has a similar mating behaviour, if an uninfected male have mated with infected female from the field, all eggs that will be produced by this female mosquito become permanently unviable. This would theoretically reduce the *Ae. albopictus* population.

Since only female mosquitoes are capable of transmitting pathogens, it would be irrational to release both male and females. Hence only uninfected males should be released but by executing this, sustainability of the release would never be achieved. In order to sustain the field study, a continuous release of uninfected male mosquitoes would be necessary and since it does not transmit any disease, it should not be an issue. A semi-field release done using *Ae. polynesiensis* reported that a mass release of the modified males could supress their natural population (Chambers et al., 2011). Another study describes how release of artificially *Wolbachia*-infected *Ae. aegypti* successfully invaded *Ae. aegypti* populations in Australia (Hoffmann et al., 2011). These finding demonstrates that *Wolbachia* based strategies can be deployed as a practical approach to vector population suppression. Hypothesis of this chapter was that *Wolbachia* reduces *Ae. albopictus* fecundity, longevity and egg viability. Instead, it was found that removal of *Wolbachia* reduces *Ae. albopictus* fecundity, longevity and egg viability.

5.5 Conclusion

Wolbachia causes unidirectional cytoplasmic incompatibility in Malaysian *Ae. albopictus*. Removal of *Wolbachia* reduces *Ae. albopictus* fecundity, female longevity and one day dried egg viability. Removal also increases male life span and two weeks old dried egg viability. Malaysian *Ae. albopictus* eggs had a better hatching rate when the eggs were dried only for a day instead of for a longer period of time. In my opinion, these findings could be used as a possible control measure to reduce *Ae. albopictus* population in Malaysia by conducting a field release of *Wolbachia* free *Ae. albopictus*.

CHAPTER 6: EFFECT OF *WOLBACHIA* ON MALAYSIAN *AEDES ALBOPICTUS* SUSCEPTIBILITY TOWARDS DENGUE VIRUS

6.1 Introduction

Dengue is the most important global arboviral disease. Its threat is most imminent in tropical and subtropical countries (Guzman et al., 2010). Malaysia has an equatorial climate, which means it is hot and humid all through the year and suited for mosquito breeding. Only *Aedes*, *Anopheles*, *Mansonia*, *Armigeres* and *Culex* genera are active for the entire year in warm and humid tropical countries. Each of these mosquito genus is capable of transmitting deadly pathogens and circa 700 million people are infected by mosquito borne diseases yearly according to CDC (Centre for Disease Control, 2015).

Aedes mosquitoes are the vectors of the dengue virus. Dengue virus is a single stranded RNA virus which maintains its life cycle on successful replication within both the human host and mosquito vector (Yang et al., 2014). Dengue can be caused by the infection of four closely related antigenic serotypes recognised as DENV-1, DENV-2, DENV-3, DENV-4 (Shekhar & Huat, 1992; Yang et al., 2014). All four serotypes have been circulating in Malaysia (Mudin, 2015). DENV-1 predominated in 2005, DENV-1 and DENV-3 in 2006, DENV-1 and DENV-2 in 2007, and DENV-3 in 2008 and 2009 (Mohd-Zaki et al., 2014; Mudin, 2015). Developing insecticide resistance by the mosquitoes and absence of available clinical cures for dengue has stimulated interest in using biological control measures such as copepods (Kay et al., 2002), larvivorous fish (Nam et al., 2000) and *Wolbachia* (Werren et al., 2008).

Recently, *Wolbachia* based incompatible insect technique has been acknowledged to be an effective technique to reduce pathogen transmission rate. The combination of several pathogen replication suppression ability and rapid spread of its own population

has led researchers to propose that *Wolbachia* can be used to develop public health strategies against dengue vector (Dobson et al., 2002; O'Neill et al., 1997). Several studies have been conducted to study the effect of *Wolbachia* in disease transmission on various vectors such as *Ae. aegypti*, *D. melanogaster*, *Cx. quinquefasciatus* and *Anopheles gambiae* (Bian et al., 2010; Glaser & Meola, 2010; Hughes et al., 2011; Moreira et al., 2009; Van den Hurk et al., 2012; Walker et al., 2011).

There is a paucity of data on the effect of *Wolbachia* on all four dengue virus serotypes interaction with naturally *Wolbachia* infected *Ae. albopictus*. This could be due to fact that *Ae. albopictus* is considered as the secondary vector for dengue virus transmission in most countries where *Ae. aegypti* co-exist (Paupy et al., 2009). Both species are known to transmit dengue virus in Malaysia (Dieng et al., 2010). *Aedes albopictus* is probably generally considered as the secondary vector primarily due to their opportunistic and zoophilic feeding behaviour (Richards et al., 2006). However, there have been studies reporting that *Ae. albopictus* also feeds on humans (Delatte et al., 2010; Gratz, 2004; Kek et al., 2014; Richards et al., 2006).

Due to large populations of *Ae. albopictus*, expansion and more anthropophagic feeding habits (Kek et al., 2014) , this study is important especially since dengue is still a major public health problem in Malaysia.

6.2 Methods

6.2 1 Mosquitoes

Ae. albopictus was obtained from the laboratory colony maintained as described in Chapter 4. The two strains of mosquitoes used were WIS (with *Wolbachia*) and WUS (without *Wolbachia*). The WUS strain used was free from antibacterial treatment for four generations before being used in this study to allow recovery from any potential side

effects from the prior tetracycline treatment. WIS colony was confirmed to be superinfected and the WUS was confirmed to be free of *Wolbachia* by randomly testing 30 mosquitoes from each generation for infection using PCR and gel electrophoresis. Both strains were maintained with 10% sucrose solution incorporated with B-Complex vitamins. Colonies were maintained at 27˚C and relative humidity of 85% with 12h: 12h light-dark photoperiod.

6.2.2 Dengue virus

All four dengue virus serotypes used were provided by Professor Sazaly Abu Bakar, WHO Collaborating Centres (WHOCC), The Tropical Infectious Diseases Research & Education Centre (TIDREC), University of Malaya. Virus stocks were obtained by inoculating monolayers of C6/36 cells at 80% confluency with initial virus inoculum diluted in foetal bovine serum (FBS) free medium. After 1 hour of adsorption at room temperature with gentle rocking, FBS-free medium was replaced with minimum essential medium (MEM) supplemented with 2% FBS. The cells were then cultured for up to 7 days at 28°C in 3% CO_2. Culture medium containing the viruses were collected and centrifuged at 1000 × *g* for 10 minutes to remove cell debris. Supernatant having the viruses were sterile-filtered using a 0.2 µm syringe filter (Sartorius Stedim Biotech, Germany), aliquoted and stored at -80°C. Final virus titre of each virus strain used were standardized to be above 1.0 x 10^6 CFU. The details of dengue virus serotypes are shown in Table 6.1.

Table 6.1: Dengue virus serotype details.

Dengue Virus	Isolate Number	Initial concentration (FFU/mL)	Final concentration (FFU/mL)
DENV-1	10245	8.5×10^6	4.0×10^6
DENV-2	83995	3.0×10^7	6.0×10^6
DENV-3	310	3.0×10^8	8.1×10^7
DENV-4	1659	2.5×10^{10}	6.8×10^9

6.2.3 Oral infection of mosquitoes

Five to seven days old female mosquitoes were used in the experiments. Female mosquitoes were starved for 48 hours prior to blood feeding. Fifty female mosquitoes were transferred into paper cups. The paper cups were 13 cm in height and 7 cm in diameter and the top covered with netting. Five cups were filled with 50 mosquitoes in each. A total of 250 mosquitoes of each strain was used for each serotype infection. Four cups were fed with dengue virus infected blood whereas one cup was fed with clean blood and served as control. Fresh blood was drawn from the author ten minutes before beginning the procedures. Each virus infected blood meal contained a 2:8 ratio of virus suspension in Minimum Essential Medium (MEM) to fresh blood. Feeding was done using Hemotek membrane feeder (Discovery Workshop, UK) in ACL-2 laboratory in the Parasitology Department, University of Malaya (Figure 6.1). The Hemotek feeder maintained the blood at 37.0^{o}C throughout the feeding. Each feeder were filled with 2mL of blood. Feeding was carried out for an hour with all lights off. After feeding was completed, cups containing mosquitoes were cold anaesthetised by placing in the freezer at -20˚C for 30 seconds. The cups were then transferred into an ice box inside the glove box. Then, fully engorged female mosquitoes were transferred to a new cup using sterile forceps. Each cup contained 10 mosquitoes. On day zero the mosquitoes were provided with plain water by placing cotton soaked in water on the top of the netting in each cup. From day one onwards 10% sucrose solution with Vitamin B complex was provided instead of water. The cotton with sucrose solution was changed every two days to avoid fungal growth. The cups were placed in lock and lock type containers and maintained in a humidified chamber at 27˚C and relative humidity of 85% with 12h: 12h light-dark photoperiod. This study obtained ethics approval from the UMMC Medical Ethics Committee (Ethics Committee /IRB Reference Number: 860.24 and 908.9).

Figure 6.1: Hemotek membrane feeder.

6.2.4 Dissection

Ten mosquitoes each from the WIS and WUS strain were dissected at 4, 8, 12 and 16 days post-feeding (pi). Mosquitoes were dissected to obtain the salivary glands, midguts and ovaries. Dissecting needles were thoroughly washed in alcohol between each dissection to prevent cross contaminations. Prior to dissection, two drops of saline were transferred on glass slides and 1.4 mm of Zirconium Beads tubes (OPS Diagnostics, Lebanon) were filled with 500 µL of MEM solution. They were all labelled to prevent mix up. Mosquitoes were anesthetized by placing the cup in -20˚C freezer for 30 seconds and transferred into a polystyrene box filled with ice. The wings and legs were removed and the mosquitoes placed on the glass slide. The abdomen was separated from the thorax and each placed next to the drop of saline. Dissected organs were placed inside the tubes containing Zirconium beads filled with 500 µL of MEM solution. They were homogenized using rapid homogenizer (Bertin Technologies, Lebanon) at 8000 rpm and stored in -80˚C freezer until needed for nucleic acid extraction.

6.2.5 Nucleic acid extraction

Both DNA and RNA were co-purified and extracted using Qiagen Cador Pathogen Extraction Kit according to the protocol provided by the manufacturer (Qiagen, CA, USA). Firstly, 100 µL of VXL buffer, 20 µL Proteinase K and 1 µL of carrier RNA was transferred into a clean 1.5 mL micro-centrifuge tube. Then, 200 µL of the thawed MEM solution containing homogenized mosquito organs were transferred into the micro-centrifuge tubes containing the VXL buffer and carrier RNA. The contents were mixed thoroughly by vortexing to yield a homogenous solution and incubated to 15 minutes at room temperature. After incubation, tube was centrifuged for 30 seconds at 8000 rpm to remove liquid from the lid. Next, 350 µL of ACB buffer was added into the tubes and vortexed. The homogenized solution was then pipetted into QIAamp Mini column placed

in a 2 mL collection tube. The sample was centrifuged at 8000 rpm for 1 minute. The flow-through and the collection tube were discarded. The spin column was placed in a new clean 2 mL collection tube. A volume of 600 µL of buffer AW1 was added into the spin column. The sample was centrifuged for 1 minute at 8000 rpm. Again, the flow-through and the collection tube were discarded. The spin column was transferred into a fresh 2 mL collection tube and 600 µL of Buffer AW2 was added into it. The tube was centrifuged for 1 minute at 8000 rpm. The flow through and collection tube was discarded. After transferring the spin column into a new collection tube, it was centrifuged again for 2 minute at 14000 rpm to remove any residue of the wash buffer. The spin column was then transferred into a new 1.5 mL micro-centrifuge tube and 60 µL of elution buffer was added. It was incubated for 1 minute at room temperature and then centrifuged for 1 minute at 14000 rpm. Extracted nucleic acids were stored in two aliquots. One was used in qPCR to quantify dengue virus genome copy number and the other for PCR to detect the presence of *Wolbachia*. Both aliquots were stored in -80˚C freezer.

6.2.6 Polymerase chain reaction (PCR) amplification

PCR amplification with specific primers *w*AlbA (primer 328F and 691 R) and *w*AlbB (primer 183F and 691R) was conducted on each organ of both mosquito strains to determine the presence of *Wolbachia* as previously described in Section 3.2.4. PCR products were analysed by agarose gel electrophoresis stained with Syber green (Life Technologies, USA). Negative samples were tested with CO1 mitochondrial primers. Only samples which gave positive results for CO1 but negative for *wsp* were taken as truly negative samples.

6.2.7 Real time quantitative PCR amplification of the dengue RNA

The virus genomic RNA copy number was quantified using the genesig Real-Time PCR DENV Detection Kit (PrimerDesign Ltd., UK) (Lau et al., 2015; Teoh et al., 2013). In each run, triplicates of qRT-PCR assay standard plot were included. It ranged from 10 to 10^6 RNA copies, made by preparing a 10-fold serial dilution of the genesig DENV RNA standard. The genesig DENV RNA standard is a synthetic RNA template with known copy number. The PCR mix was performed in a final volume of 20 µL containing 10 µL of real time master mix, 1µL of probe-primer mix, 4 µL of DNase free water and 5 µL of diluted RNA. Amplification profile was performed according to the genesig manufacturer's protocol: 10 min at 55°C, 8 min at 95°C followed by 50 cycles of denaturation for 10 s at 95°C, annealing for 60 s at 60°C. Raw data were analysed with StepOne Software v2.2.1 to determine a copy number based on the threshold cycles (Ct). The efficiency of the qRT-PCR was measured from the slope of the standard curve. The threshold level of fluorescence for Ct determination was optimized manually so that the slope of the standard curve was as close to the theoretical value of -3.32. Every run had a standard curve R^2 value more than 0.99 and the detection limit was 10^1.

6.2.8 Infection and dissemination rate

Presence of virus antigen in the midgut, salivary gland and ovaries indicated infection of the organs. The Midgut Infection Rate (MIR), Virus Dissemination Rate (VDR) and Ovary Infection Rate (OIR) were calculated as shown below.

Midgut Infection Rate (MIR)

$$\frac{Number\ of\ Positive\ Midguts}{Total\ Number\ of\ Mosquitoes}\ X\ 100\%$$

Virus Dissemination Rate (VDR)

$$\frac{Number\ of\ Positive\ Salivary\ Glands}{Number\ of\ Positive\ Midguts}\ X\ 100\%$$

Ovary Infection Rate (OIR)

$$\frac{Number\ of\ Positive\ Ovaries}{Number\ of\ Positive\ Midguts}\ X\ 100\%$$

6.2.9 Statistics

All statistical tests were conducted using GraphPad Prism 6.1 (GraphPad Software, USA). Two way ANOVA test was used for all multiple comparisons of replication kinetics between WIS and WUS and also between serotypes. Tukey and Bonferroni tests were used as post-hoc. Significant differences in all MIR, VDR and OIR were determined using Fisher's exact test. P-values >0.05 were considered non-significant.

6.3 Results

6.3.1 MIR and VDR comparison between WIS and WUS in Malaysian *Ae. albopictus*

On day four, no mosquitoes dissected had blood in their midguts. There were positive midguts and salivary glands from day four onwards but at different infection rates as shown in Figure 6.2. DENV-1 infected WUS *Ae. albopictus* midguts at a higher overall rate (31/40 = 77.5%) compared to WIS (24/40=70.0%; p>0.05). However, overall DENV-1 VDR was higher for WIS (20/28=71.4%) compared to WUS (19/31=61.29; P>0.05).

DENV-2 and DENV-3 had a higher overall MIR for WIS (20/40=50%;17/40=42.5%) in relative to WUS (18/40=45%; 9/40=22.5%; P>0.05). Overall VDR of DENV-2 for WIS (16/20=80%) was higher than WUS (10/18=55.6%; P>0.05) whereas it was the reverse for DENV-3 (WUS:8/9=88.9%;WIS:12/17=70.6% P>0.05). Overall MIR and VDR of DENV-4 were similar for both WIS and WUS. No statistically significant differences were observed in both parameters for all four dengue virus serotype between WIS and WUS.

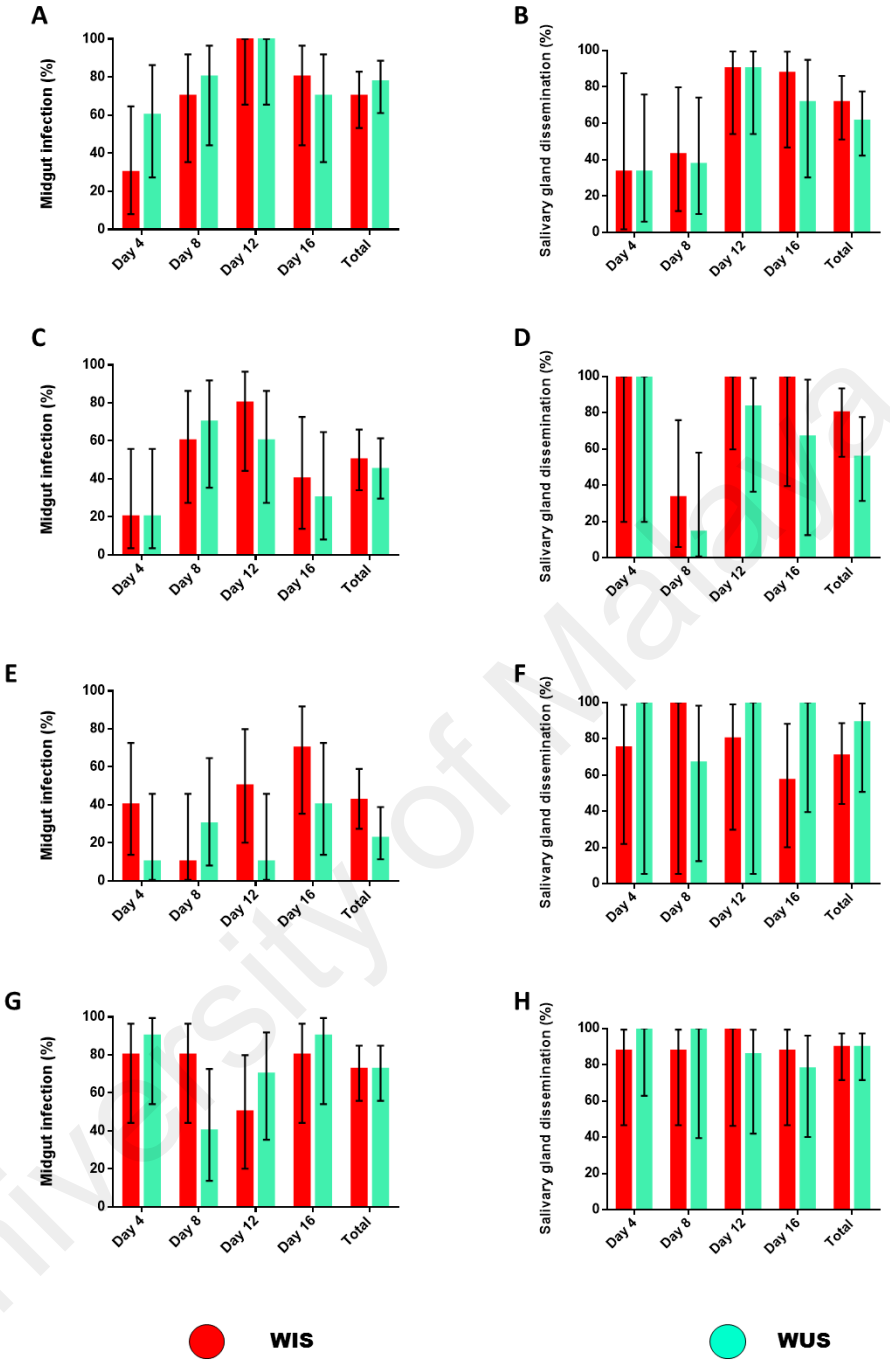

Figure 6.2: Comparative midgut infection rate and viral dissemination rate for each dengue serotype between WIS and WUS. Vertical lines represent upper and lower limits with 95% confidence interval. Multiple comparisons were conducted using two-way ANOVA ($p<0.05$). A and B: DENV-1; C and D: DENV-2; E and F: DENV-3; G and H: DENV-4.

6.3.2 Comparative dengue virus replication kinetics between WIS and WUS in Malaysian *Ae. albopictus*

Virus loads for salivary glands and midguts from day 4, 8, 12 and 16 pi for both *Wolbachia* infected (WIS) and *Wolbachia* uninfected (WUS) mosquitoes are presented in Figure 6.3 for all four dengue virus serotypes. Samples that had virus load less than 10 FFU/mL were not included and taken as negative for dengue virus in all analysis performed. No significant difference between WIS and WUS was found for all four DENV serotypes.

The highest average replication of DENV-1 in salivary gland and midgut was for WIS on day 12 (2.93 $\log_{10}$ copies/mL and 4.51 $\log_{10}$ copies/mL) respectively. As for DENV-2, the highest average replication for midgut was on day 8 (2.03 $\log_{10}$ copies/mL) and for salivary gland on day 16 (2.01 $\log_{10}$ copies/mL). However, for DENV-3, the highest average replication kinetics in salivary gland and midgut were for WUS on day 12 (2.05 $\log_{10}$ copies/mL) and day 4 (1.79 $\log_{10}$ copies/mL) respectively. Same was observed for DENV-4, the highest average replication in salivary gland and midgut was for WUS as well but both on day 12 (2.12 $\log_{10}$ copies/mL and 2.46 $\log_{10}$ copies/mL) respectively. No significant difference between WIS and WUS was found for all four dengue virus serotypes.

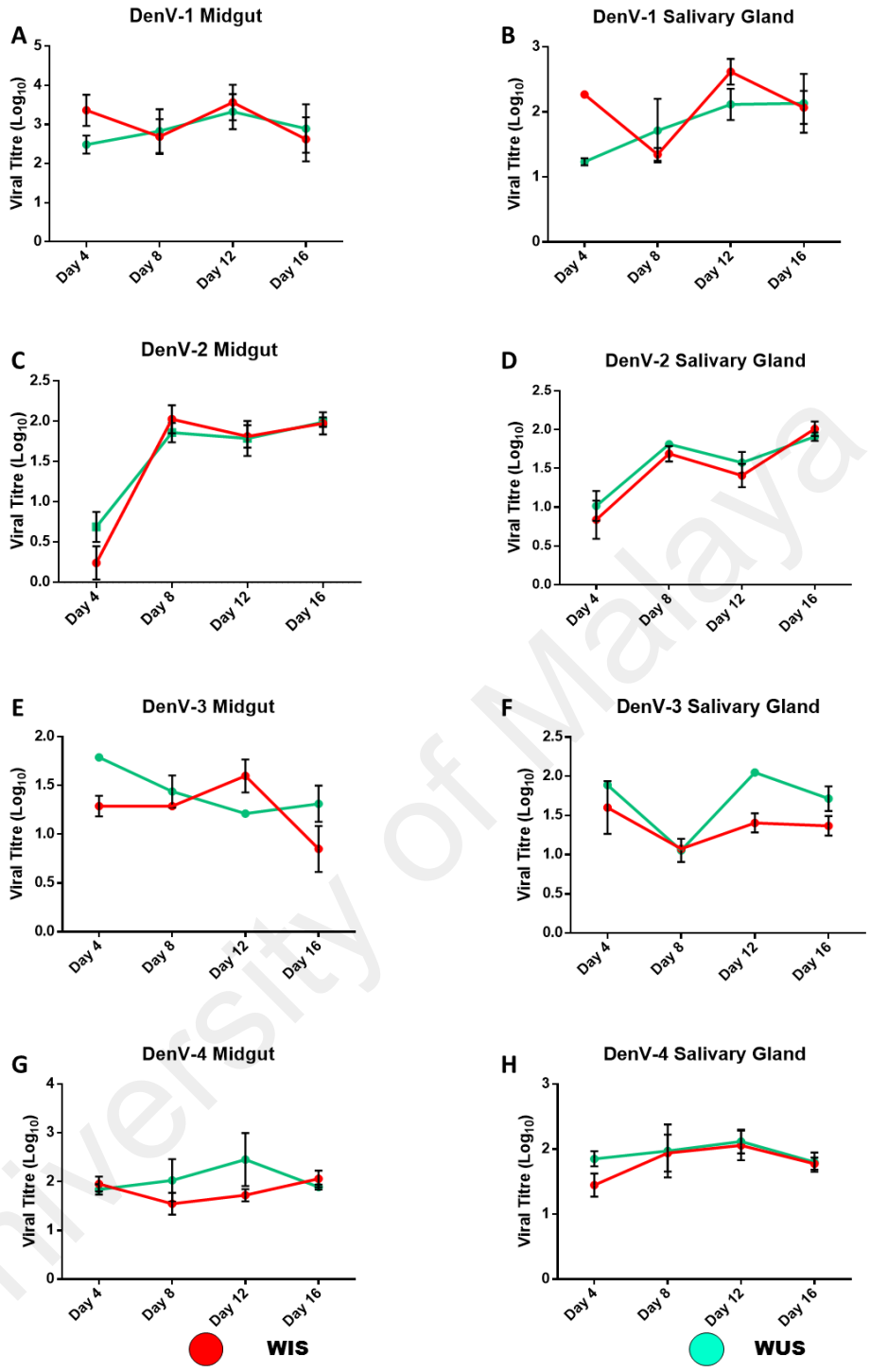

Figure 6.3: Comparative replication kinetics for each dengue virus serotypes between WIS and WUS. Viral titres were quantified using qPCR and their means ± SEM for each interval were plotted. (A, B) are titres for DENV-1; (C, D) are titres for DENV-2; (E, F) are titres for DENV-3; (G, H) are titres for DENV-4.

Table 6.2: Highest dengue virus replication kinetics for each serotype.

	Salivary glands			**Midguts**		
	Strain	Day	Titre (log_{10} copies/mL)	Strain	Day	Titre (log_{10} copies/mL)
DENV-1	WIS	12	2.93	WIS	12	4.51
DENV-2	WIS	16	2.01	WIS	8	2.03
DENV-3	WUS	12	2.05	WUS	4	1.79
DENV-4	WUS	12	2.12	WUS	12	2.46

6.3.3 MIR and VDR between the four dengue serotypes in Malaysian *Ae. albopictus*

The MIR and VDR were compared between the four serotypes and results was presented in Figure 6.4. MIR of WIS had significant difference on day 8 between DENV-4 (8/10=80%) and DENV-3 (1/10= 10%; P=0.035), $F_{(3, 12)} = 2.512$ whereas for WUS, significant differences were observed on day 4 [DENV-2 (2/10 = 20%) and DENV-4 (9/10= 90% p= 0.024); DENV-3 (1/10 = 10%) and DENV-4 (9/10 = 90% p=0.012)], day 12 [DENV-1 (10/10=100%) and DENV-3 (1/10=10% p=0.006); DENV 3 (1/10=10%) - DENV-4 (7/10=70% p=0.047)] and day 16 [DENV-2(3/10=30%) and DENV-4 (9/10 =90% p= 0.047)], $F_{(3, 12)} = 8.929$.

VDR of WIS had significant difference on day 4 [DENV-1 (1/3=33.33%) and DENV-2(2/2=100%; p=0.048)] and day 8 [DENV-2 (2/6 = 33.33%) and DENV-3 (1/1 =100%; p=0.048)], $F_{(3, 12)} = 1.250$ whereas in WUS, significant difference was only observed on day 8 between DENV-2 (1/7=14.3%) and DENV-4 (4/4=100% p= 0.011), $F_{(3, 12)} = 3.683$.

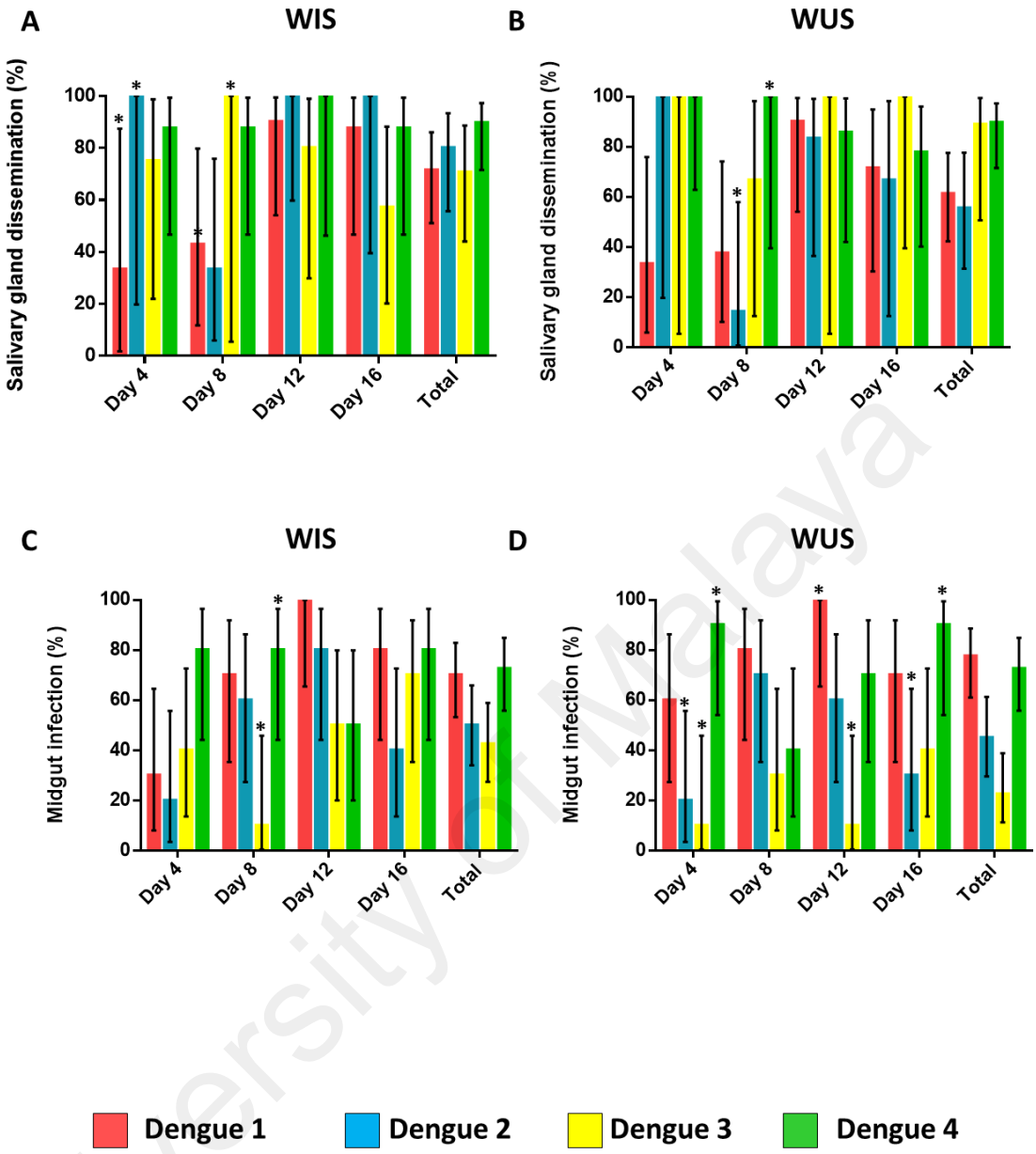

Figure 6.4: Comparative viral infection rate and viral dissemination rate between the four dengue serotypes for both WIS and WUS. Multiple comparisons were conducted using two-way ANOVA ($p<0.05$) and Tukey test as post hoc. Significances were reconfirmed using Fisher's exact test. Vertical lines represent upper and lower limits with 95% confidence interval. Asterisks indicate significant differences at the same time interval between the serotypes marked.

6.3.4 Comparative dengue virus replication kinetics between the four dengue serotypes

Results comparing the replication kinetics of the four viruses are shown in Figure 6.5 for both WIS and WUS. Significant differences are shown in Table 6.3.

In salivary glands of WIS, significant differences were observed between DENV-1 and DENV-3 on day 12 ($p= 0.0002$) and day 16 ($P=0.0304$), between DENV-1 and DENV-4 on day 16 ($P<0.0001$), $F_{(3, 54)} = 3.525$. Salivary glands of WUS had no significant difference between serotypes at any time points.

While in midguts of WIS, significant difference were observed in between DENV-1 and DENV-3 on day 4, 12 and 16 ($p=0.021$;$p<0.0001$;$p=0.0001$), DENV-1 and DENV-2 on day 4 ($p=0.0001$) and 12 ($p<0.0001$), between DENV-1 and DENV-4 on day 4, 8 and 12 ($p=0.0171$;$p=0.0116$;0.0002) between DENV-2 and DENV 4 ($p=0.0131$) on day 4, between DENV 2 and DENV-3 ($p=0.039$) on day 16 and between DENV-3 and DENV-4 ($p=0.0076$) on day 16, $F_{(3, 78)} = 16.21$. In midguts of WUS, significant differences were observed in between DENV-1 and DENV-3 on day 8, 12 and 16 ($p=0.044$; $p=0.048$; $p=0.0141$), between DENV-1 and DENV-2 on day 4 ($p=0.0314$) and day 12 ($p=0.004$), $F_{(3, 71)} = 7.757$.

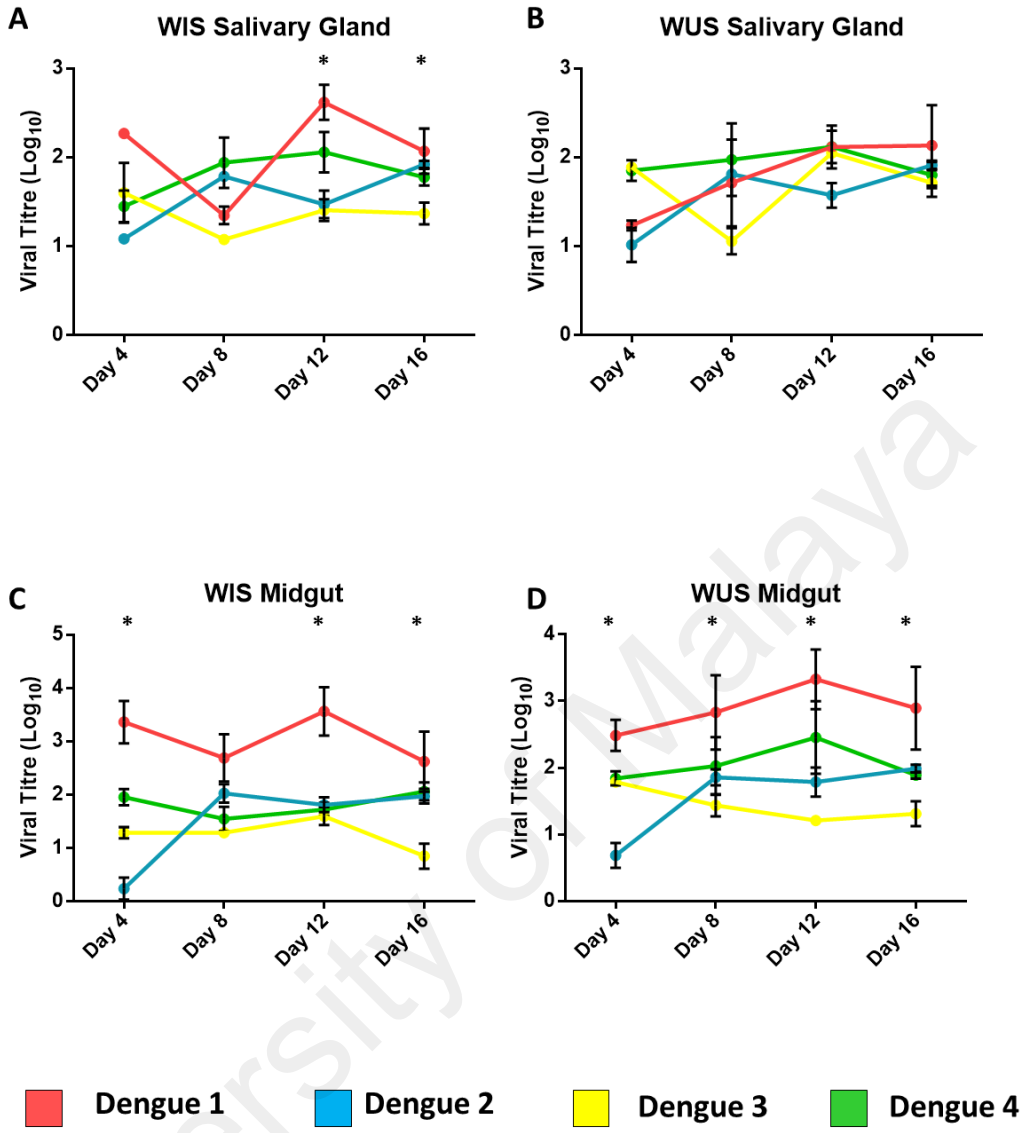

Figure 6.5: Comparative replication kinetics between the four dengue serotypes in Malaysian *Ae. albopictus*. Viral titres were obtained using qPCR and mean ± SEM were plotted. Asterisks indicates significant difference ($p<0.05$) at each time interval when performed multiple comparison with two-way ANOVA and Bonferroni as post hoc test.

Table 6.3: Replication kinetics between serotypes which were significantly different.

WIS Salivary Glands $F_{(3, 54)} = 3.525$					
Day	Serotype	Viral titer (log_{10} copies/mL)	Serotype	Viral titer (log_{10} copies/mL)	p-value
12	DENV-1	2.619	DENV-3	1.406	0.0002
16	DENV-1	2.070	DENV-3	1.369	0.0203
16	DENV-1	2.070	DENV-4	1.770	<0.0001
WIS Midgut $F_{(3, 78)} = 16.21$					
Day	Serotype	Viral titer (log_{10} copies/mL)	Serotype	Viral titer (log_{10} copies/mL)	p-value
4	DENV-1	3.360	DENV-3	1.290	0.021
12	DENV-1	3.563	DENV-3	1.599	<0.0001
16	DENV-1	2.623	DENV-3	0.850	0.0001
4	DENV-1	3.360	DENV-2	0.240	0.0001
12	DENV-1	3.563	DENV-2	1.811	<0.0001
4	DENV-1	3.360	DENV-4	1.955	0.0171
8	DENV-1	2.691	DENV-4	1.549	0.0116
12	DENV-1	3.563	DENV-4	1.723	0.0002
4	DENV-4	1,955	DENV-2	0.2402	0.0131
16	DENV-2	1.974	DENV-3	0.850	0.039
16	DENV-4	2.060	DENV-3	0.850	0.0076
WUS Midgut $F_{(3, 71)} = 7.757$					
Day	Serotype	Viral titer (log_{10} copies/mL)	Serotype	Viral titer (log_{10} copies/mL)	p-value
8	DENV-1	2.831	DENV-3	1.439	0.044
12	DENV-1	3.328	DENV-3	1.213	0.048
16	DENV-1	2.895	DENV-3	1.989	0.0141
4	DENV-1	2.486	DENV-2	0.688	0.0314
12	DENV-1	3.326	DENV-2	1.787	0.004

6.3.5 Comparative replication kinetic and infection rate of the ovaries between the four dengue serotypes in Malaysian *Ae. albopictus*

Infection in ovaries may indicate possibility of transovarial transmission. To compare the difference in dengue virus genome copy number in the ovaries, we quantified the virus copy number and compared between the serotypes. Figure 6.6 represents comparison of OIR and replication kinetics in ovaries between dengue serotypes for both WIS and WUS. OIR of WIS had significant difference between DENV-2 (2/2=100%; 4/4=100%) and DENV-3 (0/4=0%; 1/7=14.3%) on day 4 (p=0.0103) and day 16 (p=0.0231) respectively, $F_{(3, 12)} = 7.546$. OIR of WUS also had significant difference between DENV-2 (2/2=100%) and DENV-3 (0/1=0%) on day 4 (0.0099), $F_{(3, 12)} = 3.876$. DENV-2 had the highest overall OIR (WIS 15/20=75%; WUS 16/18=88.9%) compared to the other serotypes but not significant (p>0.05).

As for the replication kinetic in ovary, WIS had significant differences between DENV-1 (2.823 $\log_{10}$ copies/mL; 2.798 $\log_{10}$ copies/mL) and DENV-2 (1.846 $\log_{10}$ copies/mL; 1.435 $\log_{10}$ copies/mL) on day 12 (p=0.0215) and day 16 (p=0.0079) respectively, between DENV-1 (2.727 $\log_{10}$ copies/mL; 1.726 $\log_{10}$ copies/mL; 2.823 $\log_{10}$ copies/mL) and DENV-3 (0 $\log_{10}$ copies/mL; 0 $\log_{10}$ copies/mL; 1.330 $\log_{10}$ copies/mL) on day 4 (0.0079) , day 8 (p=0.0278) and day 12 (p=0.0079) respectively, between DENV 1 (2.823 $\log_{10}$ copies/mL) and DENV 4 (1.802 $\log_{10}$ copies/mL) on day 12, between DENV-2 (2.689 $\log_{10}$copies/mL; 1.974 $\log_{10}$ copies/mL) and DENV-3 (0 $\log_{10}$ copies/mL; 0 $\log_{10}$ copies/mL) on day 4 (p=0.0028) and day 8 (0.0126) respectively, and between DENV-2 (2.689 $\log_{10}$ copies/mL) and DENV-4 (1.437 $\log_{10}$ copies/mL) on day 4 (p=0.0361), $F_{(3, 42)} = 9.954$.

In WUS ovaries, significant differences were observed between DENV-1 (1.895 $\log_{10}$ copies/mL) and DENV-3 (0 $\log_{10}$copies/mL; p=0.025) in addition to DENV-2 (1.783 $\log_{10}$ copies/mL) and DENV-3 (0 $\log_{10}$ copies/mL; p=0.0158) on day 4 respectively, between DENV-1 (2.315 $\log_{10}$ copies/mL) and DENV-2 (1.484 $\log_{10}$ copies/mL; p=0.0158) on day 12 and lastly between DENV-1 (2.752 $\log_{10}$ copies/mL) and DENV-4 (1.774 $\log_{10}$ copies/mL; p=0.0497) on day 16, $F_{(3, 39)}$ = 3.218.

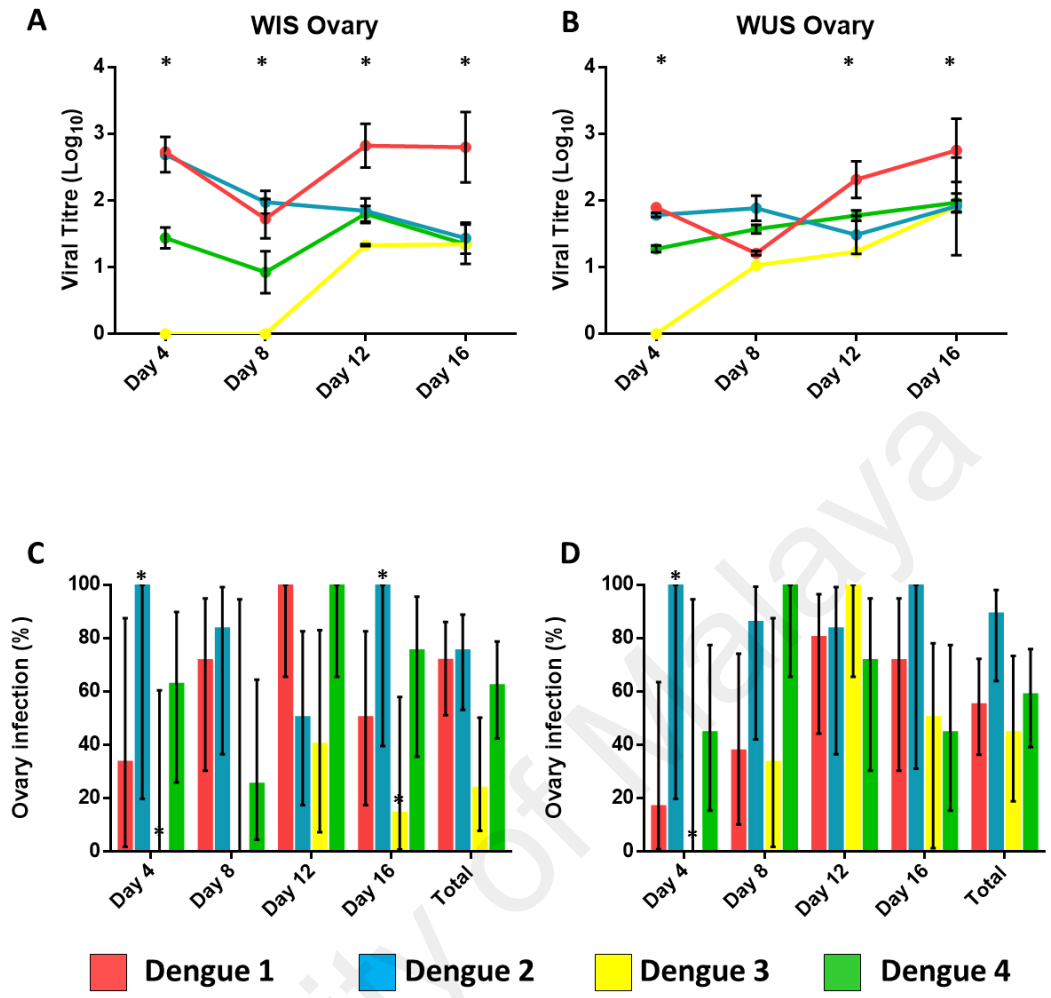

Figure 6.6: Comparative replication kinetics in the ovaries and OIR between the four dengue serotypes for both WIS and WUS. (A) and (B) are the replication kinetics in ovaries of WIS and WUS (mean ± SEM). They were analysed using two way ANOVA with Tukey test as post hoc. (C) and (D) are the ovary infection rate (OIR) of WIS and WUS. Vertical lines represent upper and lower limits with 95% confidence interval. They were analysed using Fisher's Exact test. Asterisks (*) indicate significant difference ($p<0.05$) at that time point between the serotypes.

6.4 Discussion

In Malaysia, most dengue epidemics are caused by *Ae. aegypti*. The exact role of *Ae. albopictus* in the transmission of dengue virus remains unclear (Dieng et al., 2010). . Even though both species exist alongside, *Ae. aegypti* persisted as the primary vector in Malaysia. *Ae. albopictus* is a competent vector for dengue virus transmission and can cause epidemics (Benedict et al., 2007; Gratz, 2004). Even if *Ae. albopictus* may not be the major vector in Malaysia at the moment, there are possibilities for them to displace the population of *Ae. aegypti* in near future as was reported in Brazil, Hawaii, Guam and Taiwan previously (Braks et al., 2004; Lambrechts et al., 2010). Limited research has been conducted to study effect of native *Wolbachia* on their host vectorial capacity.

Taking previous studies into account, it is clear that *Wolbachia* do not exhibit a predictable effect on their host infectivity. This study, to my knowledge, is the first study reporting the effects of *Wolbachia* on Malaysian *Ae. albopictus* susceptibility towards all four dengue virus serotypes co-circulating in Malaysia. Previous studies were conducted on only one dengue virus serotype in *Ae. albopictus* or effect of *Wolbachia* on dengue virus transmission in *Ae. aegypti* (Moreira et al., 2009).

Organs were scored as being infected with DENV when genome copies were detected above 10 FFU/mL using quantitative RT-PCR. Although viral genome copies detected using quantitative RT-PCR may not reflect presence of infectious and replicating virus, in this study, it was considered to represent infection rate in the particular organ (Mousson et al., 2012; Whitehorn et al., 2015) since plaque assay nor cell culture were not carried out. Future studies should be conducted by incorporating either plaque assay or cell culture in addition to quantitative RT-PCR for a more accurate results (Wong et al., 2016). In order to obtain the WUS colony, adult mosquitoes were subjected to tetracycline treatment. Strains used in these studies were 4 to 6 generations free from

tetracycline. Enough time was given to reobtain any lost microbiota within *Ae. albopictus* (Baton et al., 2013).

When comparison was made between WIS and WUS strain for DENV-1 infection, *Wolbachia* did not show any apparent difference in any of the parameters which includes the MIR, VDR and viral loads in both midgut and salivary glands. WIS had higher DENV-2 infected midguts and salivary glands compared to WUS. In contrary to this observation, study done on La Reunion *Ae. albopictus* reported that native *Wolbachia* in them reduced DENV-2 viral infection rate of salivary glands (Mousson et al., 2012). Their study suggested that a high density of *Wolbachia* was necessary to induce pathogen inhibition in their host. Malaysian *Ae. albopictus* could be having a lower *Wolbachia* density compared to La Reunion samples which may have contributed to the differences in the results. However, this can only be confirmed by quantifying *Wolbachia* infection. Perhaps future study can be conducted with quantification of *Wolbachia* to understand the differences.

Mosquitoes infected with DENV-4 had identical MIR and VDR in both WIS and WUS. However, removal of *Wolbachia* yielded slightly higher genome copy detection in salivary glands and midguts. *Wolbachia* may be enhancing DENV-4 viral replication in these organs as well. As for DENV-3 infection, removal of *Wolbachia* increased VDR and viral load in salivary glands. To sum up, *Wolbachia* could be stimulating DENV-2 infection rate but lessening DENV-3 VDR and DENV-4 viral load in Malaysian *Ae. albopictus*. However, these observations were not statistically significant.

In the second part of this study, Malaysian *Ae. albopictus* susceptibility towards the four dengue serotypes were compared. Malaysian *Ae. albopictus* are naturally infected with *Wolbachia*. Result shows that native WIS had the highest viral replication kinetics for DENV-1 followed by DENV-4 and lowest for DENV-3. Mosquitoes infected with

DENV-3 had significantly low MIR, OIR and viral load in all three organs. Malaysian *Ae. albopictus* may not be contributing much during DENV-3 epidemics.

Briefly, native WIS had the highest MIR and replication kinetics for DENV-1. Infection with DENV-2 yielded high number of infected ovaries but lesser infected midguts while DENV-3 infected mosquitoes had the lowest replication kinetics, OIR, MIR and VDR. Lastly, DENV-4 infection had the highest VDR and MIR but average replication kinetics and OIR.

These findings may give some insight as to why *Ae. albopictus* is not a good vector for dengue virus in Malaysia. Based on the results obtained, when dengue DENV-1 is circulating Malaysia, *Ae. albopictus* will be able to replicate the virus efficiently but not disseminate as efficiently whereas when dengue DENV-2 or DENV-4 becomes epidemic, the replication of virus in *Ae. albopictus* would not be great to make it very infectious although VDR for DENV-4 was very high.

Detection of dengue virus genome in ovaries suggests that Malaysian *Ae. albopictus* may vertically transmit dengue virus to their offspring. Previous studies have reported that *Ae. albopictus* exhibits high potential for vertical transmission which makes them a suitable candidate to maintain dengue virus infection in a population (Lourenço-de-Oliveira et al., 2003; Mitchell & Miller, 1990). Since infection with DENV-2 exhibits high OIR, *Ae. albopictus* could be playing a role in maintaining the infection during DENV-2 epidemics.

A recent study reported that *Ae. albopictus* had similar susceptibility towards dengue virus as *Ae. aegypti*. However, they had significantly lower chance to become infectious with DENV-2 and DENV-4 due to lower VDR observed for these serotypes compared to DENV-1 and DENV-3 (Whitehorn et al., 2015). In contrary to their study,

Ae. albopictus in this study had the lowest susceptibility for DENV-3 and highest for DENV-1 and DENV-4. Difference could have been due to difference in mosquito's geographical origin.

Aedes albopictus may not be such a competent vector as *Ae. aegypti* to start an epidemic in Malaysia yet due to the lower virus load in their organs, but according to this results on the possible high vertical transmission rate, it might be playing a major role in maintaining the dengue infection throughout and between the epidemics depending on the serotype circulating. Yet again, data were all based on qPCR analysis. Although virus was detected, the virus may not have been infectious. This has to be further confirmed using plaque assays. Two major limitations of this chapter are lacking of plaque assay to quantitate dengue virus and having small sample size at each time point and the inability to repeat experiments due to financial constraint.

6.5 Conclusion

In summary, *Wolbachia* which naturally exist in Malaysian *Ae. albopictus* does not significantly affect dengue virus replication in *Ae. albopictus*. Malaysian *Ae. albopictus* are susceptible to dengue virus infections and capable of transmitting dengue virus especially DENV-1. The removal of *Wolbachia* from Malaysian *Ae. albopictus* would not reduce their susceptibility.

CHAPTER 7: CONCLUSION

Aedes albopictus is the secondary vector of dengue virus globally and in Malaysia (Dieng et al., 2010). The benefits of studying *Ae. albopictus* vectorial capacity and possible population control methods are numerous. Populations of *Ae. albopictus* have been growing at an alarming rate (Paupy et al., 2009). There are countries where *Ae. albopictus* have displaced *Ae. aegypti*, the primary vector of dengue (Lambrechts et al., 2010). Studies have reported that *Ae. albopictus* have higher survival chance if both species are to compete for existence (Paupy et al., 2009). Although most epidemics in Malaysia occurred in regions with high density of *Ae. aegypti*, the role of *Ae. albopictus* in future outbreaks should not be ignored. A recent study in Vietnam stated that *Ae. albopictus* have similar susceptibility rate towards dengue virus infection as *Ae. aegypti* (Whitehorn et al., 2015). If *Ae. aegypti* populations in Malaysia are displaced by *Ae. albopictus*, this mosquito can become a big threat due to their widespread nature. On the other hand, although they are an effective vector for dengue virus, questions to why *Ae. albopictus* has a lower susceptibility towards the virus transmission remains unclear. In contrary to *Ae. aegypti*, *Ae. albopictus* is naturally infected with *Wolbachia*. In a study done in La Reunion, it was reported that *Ae. albopictus* with *Wolbachia* infection in them decreased DENV-2 dissemination rate (Mousson et al., 2012). Could the same be applied for *Ae. albopictus* from all other regions of the world to explain their susceptibility status?

This research was designed to study the effect of *Wolbachia* on Malaysian *Ae. albopictus* reproductive phenotypes and its susceptibility status towards the four dengue virus serotypes circulating in Malaysia.

It has been found that among the nine supergroups of *Wolbachia* that have been reported, most arthropods were found to be infected with supergroup A and supergroup B (Armbruster et al., 2003; O'Neill et al., 1997). As high as 91.6% of Malaysian *Ae. albopictus* were superinfected with both *w*AlbA and *w*AlbB. *Wolbachia* superinfection was found in mosquitoes from both Peninsular Malaysia and East Malaysia.

The highest density of *Wolbachia* were usually found in the host reproductive organs. Concurring to that, all *Wolbachia* infected *Ae. albopictus* had *Wolbachia* infection in the ovaries. In addition to the ovaries, most of their midguts were also found positive but at a later time point. None of their salivary glands at any time point had *Wolbachia* infection.

The removal of *Wolbachia* from native Malaysian *Ae. albopictus* reduced the mosquito's fecundity, female longevity and one day stored eggs viability. Removal also caused an increase in male lifespan and longer stored eggs viability. Based on the results obtained, *Wolbachia* infected *Ae. albopictus* would yield better egg hatching rate if the eggs were dried for only a day before hatching instead of drying them for a longer period of time. This may aid colonization of *Ae. albopictus* in laboratories.

In addition, *Wolbachia* exhibits unidirectional CI in Malaysian *Ae. albopictus*. Release of *Wolbachia* uninfected male mosquitoes may reduce *Ae. albopictus* population in Malaysia.

Finally, the removal of *Wolbachia* does not significantly reduce nor inhibit dengue virus infection and transmission by Malaysian *Ae. albopictus*. However, it was observed that Malaysian *Ae. albopictus* had the highest viral replication kinetics for DENV-1 but highest viral infection and dissemination rate for DENV-4. It had the lowest replication

kinetics, infection rate and dissemination rate for DENV-3. Results show that they may not be playing much role in DENV-3 epidemics.

Since dengue is a serious public health problem, more measures need to be instituted so as to reduce the incidence of dengue in the country. As a start, semi-field trials followed by proper field trial can be conducted using *Wolbachia.* A semi-field trial can be carried out by weekly releasing a known number of *Wolbachia* uninfected male mosquitoes into an enclosed space with a controlled environment that would resemble actual field ambience which would contain a known number of naturally *Wolbachia* infected female and male mosquitoes. Theoretically, using findings of this dissertation, number of mosquitoes in the proposed study should decrease over time as the number of females that would produce unviable eggs would increase. This will provide some information and confirm our laboratory findings.

It is also timely that *Wolbachia* from a different host should be injected into Malaysian *Ae. albopictus* to study the ability of the foreign *Wolbachia* to reduce *Ae. albopictus* susceptibility towards pathogens. In this study, native *Wolbachia* was not able to inhibit dengue virus transmission in *Ae. albopictus*. It has been mentioned in other studies that a high density of *Wolbachia* might be necessary to reduce transmission of pathogens in their respective hosts. Probably native *Wolbachia* density in Malaysian *Ae. albopictus* is low. Introduction of foreign *Wolbachia* into native *Ae. albopictus* may increase density of *Wolbachia* in them, especially by a fast replicating strain such as *w*MelPOP. *w*MelPOP have successfully reduced pathogen infection and dissemination rate in various hosts. Theoretically, embryonically injecting *w*MelPOP into Malaysian *Ae. albopictus* should cause bidirectional CI which would aid in population control and decrease the mosquito's susceptibility towards dengue virus.

Future viral studies conducted should include plaque assay in addition to quantitative RT-PCR to make sure detected genomes are infectious. Quantification of *Wolbachia* should also be carried out alongside.

Currently most measures and studies are targeted at *Ae. aegypti*. This would in future increase *Ae. albopictus* population which has already been growing momentously. Since studies have shown that *Ae. albopictus* is equally susceptible to dengue virus (Whitehorn et al., 2015), proactive measures need to be instituted to reduce the population of *Ae. albopictus*. It is also known that *Ae. albopictus* is also a vector for Zika virus, chikungunya virus and other arboviruses. Thus future research should be conducted to study the susceptibility status of Malaysian *Ae. albopictus* towards these viruses.

Finally, since the dengue control programme in Malaysia has been ongoing for decades, integration of *Wolbachia* as a tool for dengue vector population control and to reduce their susceptibility towards dengue virus should be given more thought.

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Modified technique of *Wolbachia* removal from Malaysian *Aedes albopictus*

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PEER REVIEW

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Comments

The study has provided a viable method to produce *Wolbachia* free *Ae. albopictus* which is increasingly implicated as a vector of dengue transmission in many endemic countries. Susceptibility studies with dengue serotypes in *Wolbachia* positive or negative mosquitoes will be easily available now.

Details on Page 560

ABSTRACT

Objective: To develop an artificial and modified *Wolbachia* removal technique using tetracycline from naturally *Wolbachia* infected *Aedes albopictus* (*Ae. albopictus*) so as to be able to produce generations of *Wolbachia* free offsprings.

Methods: In this study, seven different tetracycline treatment methods were conducted to obtain the best removal method. Four methods focused on larvae tetracycline treatment, one method on both larvae and adult tetracycline treatment and the last two methods on adult mosquito sucrose treatment.

Results: All larval tetracycline treatments resulted in either high larvae mortality, sterile F_0 adult mosquitoes or unsuccessful *Wolbachia* removal. Treatment of both larvae and adults resulted in reduced larvae mortality, successful *Wolbachia* removal but slow mosquito fecundity. As for the adult treatment, 1.0 mg/mL as previously published was not able to completely remove *Wolbachia* in F_1 generation whereas 1.25 mg/mL successfully removed *Wolbachia* from F_1 and F_2 mosquitoes in 2 weeks.

Conclusions: This method is different from the previously published methods as it provides an improved *Wolbachia* removal technique from *Ae. albopictus* with high egg hatchability, low larvae mortality, increased fecundity and better *Wolbachia* removal rate.

KEYWORDS

Wolbachia, tetracycline, *Aedes albopictus*

1. Introduction

Wolbachia pipientis is an intracellular bacteria found in most of the arthropods, nematodes and isopods[1,2]. They are vertically transmitted rickettsia endosymbiont bacteria[3]. In order to ensure the parasite being successfully transmitted maternally, *Wolbachia* tend to alter reproduction properties of their host[4]. Common alteration that have been reported are male killing, feminization, parthenogenesis and cytoplasmic incompatibility (CI)[5,6]. *Wolbachia* modifies the spermatogenesis causing no viable offspring to be produced when infected male mates with uninfected female or female infected differently from the male[7]. Well understood CI can be used to reduce population of the host.

Aedes albopictus (*Ae. albopictus*) is an arthropod known to be naturally infected with *Wolbachia pipientis* bacteria. Most strains of *Ae. albopictus* screened in Malaysia were superinfected with both two *Wolbachia* strains (wAlbA and wAlbB). *Aedes aegypti* and *Ae. albopictus* are the major vectors for dengue in Malaysia. They are lethal vectors which transmit many deadly pathogens including dengue fever virus, chikungunya fever virus and West Nile Virus[8]. The combination of dengue blocking activity and rapid spread due to CI has led researchers to suggest that

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Wolbachia can be used to develop public health strategies to reduce dengue incidence in humans[9,10].

In order to study the CI and effect of *Wolbachia* on Malaysian *Ae. albopictus*, it is necessary to have both *Wolbachia* infected and uninfected strains.

Ae. albopictus is a species naturally infected with *Wolbachia* therefore obtaining a natural strain without *Wolbachia* would be very rare[11]. Therefore an artificial *Wolbachia* removal technique is needed.

Previous studies have suggested treatment of larvae with tetracycline antibiotic to remove *Wolbachia* from *Ae. albopictus*[3,11]. However, reduced fecundity and egg viability was observed when the above mentioned method was implemented. Another study proposed treatment of only the adult mosquito with tetracycline antibiotic[11]. This managed to overcome the reduced fecundity and egg viability issue. However, when the method was implemented, the resulting offsprings were found not to be totally free from *Wolbachia*.

In this study, a modified *Wolbachia* removal method from *Ae. albopictus* is reported. It has minimal effect on the mosquito fecundity and egg viability and was able to produce generations of *Wolbachia* free offsprings.

2. Materials and methods

2.1. Mosquito strain

A strain of *Ae. albopictus* obtained from Bukit Lagong, Selayang, Kuala Lumpur, Malaysia in August 2013 was used in this study. Mosquitoes were maintained in cages with 10% sucrose with 100 mg B-Complex solution. They were blood fed and eggs were collected weekly. Mosquito infection status was confirmed using polymerase chain reaction (PCR) amplification and sequencing.

2.2. Infection status

A minimum of 30 blood fed mosquitoes were randomly caught for each new generation, blood fed, allowed to lay eggs and then extracted using Dneasy Blood and Tissue Extraction Kit according to the protocol provided by the manufacturer (Qiagen, CA, USA). Extracted DNA were stored at −20 °C until needed. All samples were screened for the presence of *Wolbachia* using multiplex PCR with Promega (Promega, Madison, WI) reagents for amplification of the wsp gene with diagnostic primers (Genomics BioSci & Tech, China).

The *w*AlbA strain gene was amplified with the wsp 328F and 691R primer pair whereas *w*AlbB strain gene was amplified with the wsp 183F and 691R primer pair. PCR was conducted in a 20 μL reaction per individual. This consisted of 10 μL ddH_2O, 4 μL 5X Green GoTaq® Flexi Buffer, 1.6 μL magnesium chloride, 0.4 μL dNTPS, 0.6 μL of each primer (183F, 328F and 691R), 0.2 μL of GoTaq® Flexi DNA polymerase and 2 μL template. Samples were denatured for 5 min at 94 °C, followed by 35 cycles of 1 minute at 94 °C, 1 min at 55 °C and 1 min at 72 °C. A negative control was run along with each batch of PCR amplification by substituting 2 μL of sample with 2 μL of ddH_2O[12].

A total of 8 μL of each sample was run in 1% agarose gel to detect the presence of amplified DNA fragments. One hundred kilobyte ladder (Promega, Madison, WI) was used to confirm presence of *w*AlbA (363 bp) and *w*AlbB (508 bp) genes[12](Figure 1).

Figure 1. Gel electrophoresis result image. Lane 1 is the 100 kB ladder. Lane 2–6 are my samples.
The upper row are *w*AlbB amplified gene at 508 bp and the lower row bands are *w*AlbA amplified gene at 363 bp.

2.3. Tetracycline treatment

All *Wolbachia* removal studies were conducted on strains with confirmed *w*AlbA and *w*AlbB superinfection. Studies were conducted as stated in Table 1. Treatment 1 was conducted as previously described by Otsuka and Takaoka in 1997[3]. Treatment 5 and 6 were conducted as described previously by Dobson and Rattanadechakul in 2001[11]. Treatments 2, 3, 4 and 7 consisted of a modified technique were conducted by this group. Larvae after the treatment period in treatment 1, 2, 3 and 4 were transferred back into water without tetracycline and reared to adulthood. In each treatment, randomly caught 25 blood fed adult mosquitoes were allowed to lay eggs first and then tested for presence of *Wolbachia* using PCR method as mentioned above. If no *Wolbachia* infection was found in all tested mosquitoes, the eggs obtained were hatched. Larvae after 24 h treatment in treatment 5 was transferred back into water without tetracycline and reared to adulthood.

Table 1
Tetracycline treatment design.

treatment	Life cycle stage	treatment period/h	Concentration of tetracycline
1[a]	24th–96th hour larvae	72	1.00 mg/mL in 1 L overnight water
2[b]	24th–72nd hour larvae	72	1.25 mg/mL in 1 L overnight water
3[b]	48th–120th hour larvae	72	1.25 mg/mL in 1 L overnight water
4[b]	48th–72th hour larvae	24	1.25 mg/mL in 1 L overnight water
5[a]	48th–72th hour larvae	24	1.25 mg/mL in 1 L overnight water
	newly emerged adult mosquito	continuous	1.00 mg/mL in 10% sucrose solution with 10 mg/L B-complex
6[a]	newly emerged adult mosquito	continuous	1.00 mg/mL in 10% sucrose solution with 10 mg/L B-complex
7[b]	newly emerged adult mosquito	continuous	1.25 mg/mL in 10% sucrose solution with 10 mg/L B-complex

[a]: Conducted as previously described by Otsuka and Takaoka in 1997[3].
[b]: Conducted as described previously by Dobson and Rattanadechakul in 2001[9].
[c]: Modified techniques conducted by this group.

Adult mosquitoes in treatment 5, 6 and 7 were blood fed

after two weeks and one month for egg collection. Twenty five mosquitoes from which eggs were collected were tested for presence of *Wolbachia* using PCR method as mentioned above. Eggs collected from the treatment 5, 6 and 7 were allowed to hatch in 2 L overnight water. Egg hatching rate were calculated to determine egg viability for each treatment. Once the F_1 generation mosquitoes were obtained, 25 blood fed adult mosquitoes were randomly caught from each colony, allowed to lay egg first and then tested for presence of *Wolbachia* using PCR method as mentioned above. Average *Wolbachia* infectivity of F_1 for treatment 6 and 7 was obtained by calculating the mean infected mosquito numbers for three replicates of *Wolbachia* testing. The same calculation was done for average *Wolbachia* infectivity of F_2 for treatment 7.

3. Results

The strain of *Ae. albopictus* used in this study had 100% both *w*AlbA and *w*AlbB infection. Figure 1 shows the result of PCR amplification when both *w*AlbA and *w*AlbB is present. The F4 eggs were used in this *Wolbachia* removal study.

Percentage of eggs hatched that survived to pupation, percentage of adult mosquitoes emerged, percentage of *Wolbachia* infection status of the F_2 and percentage of F_1 eggs hatched were calculated for all treatments 1-7. Results are shown in Table 2.

Table 2

Percentage eggs hatch in F_2 and F_1 tetracycline treated strains.

Treatment	No. of eggs (F_2)	Pupa after 5 d	Adult mosquito (F_2)	*Wolbachia* infection status	No. of eggs obtained (F_1)	Eggs hatch
1	131	4 (3.05%)	2 (100.00%)	All 2 *Wolbachia* free (100.0%)	0	NA
2	111	9 (8.11%)	3 (33.33%)	All 3 *Wolbachia* free (100.0%)	0	NA
3	122	92 (75.41%)	92 (76.72%)	All 25 tested *Wolbachia* free (100.0%)	0	0 (0.00%)
4	143	89 (62.24%)	83 (93.25%)	11 out of 25 *Wolbachia* free (0.0%)	29	0 (0.00%)
5[a]	145	82 (56.55%)	78 (95.12%)	All 25 tested *Wolbachia* free (100.0%)	230	41 (17.83%)
6[b]	191	157 (76.96%)	150 (95.54%)	All 25 tested *Wolbachia* free (100.0%)	249	142 (57.03%)
7[c]	145	97 (66.90%)	96 (98.97%)	All 25 tested *Wolbachia* free (100.0%)	102	98 (96.08%)

[a]: Emerged mosquitoes were treated with 0.5 mg/mL tetracycline treated sucrose solution. F_2 adult mosquitoes were only *Wolbachia* free after 1 month of treatment. [b]: Emerged mosquitoes were treated with 1.0 mg/mL tetracycline treated sucrose solution. F_2 adult mosquitoes were only *Wolbachia* free after 1 month of treatment. [c]: Emerged mosquitoes were treated with 1.25 mg/mL tetracycline treated sucrose solution. F_2 adult mosquitoes were *Wolbachia* free after 2 weeks of treatment. NA: Not applicable.

Treatment 5, 6 and 7 had F_1 eggs therefore studies were continued to obtain the percentage of F_1 adult mosquitoes, *Wolbachia* infectivity status of F_1 colony and *Wolbachia* infectivity status of F_2 colony (only treatment 7). Results for this continuation studies are shown in Table 3.

4. Discussion

Tetracycline is a group of broad-spectrum antibiotics. Its overall usage has been reduced with the increasing bacterial resistance[13]. Since *Wolbachia* is an endosymbiotic bacteria, tetracycline at the right concentration and delivery method should be able to remove them from their respective hosts. This concurs with previous studies conducted[11].

Treatment 1 which was conducted based on Otsuka method was not effective in this study as it caused low egg viability, high larval mortality and sterile adult mosquitoes[3]. Same issue have been reported by Dobson and Rattanadechakul in 2001[11]. This may have been due to the high concentration of the tetracycline used to treat the larvae.

Similar larval treatments were carried out with reduced concentration to 1.25 mg/mL in treatment 2, 3 and 4 at different exposure periods.

High larval mortality was observed when larvae were treated for more than 24 h. However, improved larval mortality was observed when the 48 h larvae were treated instead of the 24 h larvae. This may be because 24 h larvae are too young to withstand the tetracycline treatment.

Treatment 4 was designed to expose 48 h larvae for 24 h which gave lower larval mortality and a higher percentage of adults.

Although low larval mortality was observed, the treatment failed to remove *Wolbachia* completely from all surviving adults. Therefore it can be concluded that perhaps the period of treatment or tetracycline concentration was not sufficient.

Treatment 5 was conducted based on Dobson report in 2001 which subjects both larvae and adult mosquitoes tetracycline[11]. This method had low larval mortality and was able to completely remove *Wolbachia* from all surviving F_2 adults. A good number of F_1 eggs were obtained but the hatching rate of the F_1 eggs was very low compared to untreated strains.

Treatment 6 was conducted based on the final method from Dobson paper in 2001 which treats only the adult with 1.0 mg/mL[11]. No alternative food source was provided for the mosquitoes. F_2 Adult mosquitoes were tested for *Wolbachia* after 2 weeks exposure to tetracycline sucrose treatment. Mosquitoes were not found to be completely free of *Wolbachia*. F_2 adult mosquitoes were again tested for *Wolbachia* after 1 month tetracycline treatment and all were *Wolbachia* free. Eggs were collected and F_1 mosquitoes were obtained. Although the experiment was repeated three times, we failed to obtain entirely *Wolbachia* free F_1 adult mosquitoes. Therefore treatment 6 as proposed by Dobson was not effective in this study.

Treatment 7 was designed exactly as treatment 6 with a

Table 3

Wolbachia infectivity status of F_1 and F_2 tetracycline treated strains.

Treatment	No. of larvae	No. of adults (F_1)	Average *Wolbachia* infectivity of F_1	Average *Wolbachia* infectivity of F_2
5	41	32 (78.05%)	All 25 tested *Wolbachia* free (100.0%)	Not applicable because no eggs was obtained
6	142	130 (91.55%)	18 out of 25 *Wolbachia* free (72.0%)	Not applicable
7	98	92 (93.88%)	All 25 tested *Wolbachia* free (100.0%)	All 25 tested *Wolbachia* free (100.0%)

slight increment of the concentration of tetracycline in the sucrose solution. Complete *Wolbachia* removal from the F_0 adult mosquitoes was observed in two weeks tetracycline treated mosquitoes. This was confirmed with two replicates. Egg hatching rate was slightly lower than treatment 6 and 93.88% became F_1 adults. In contrast to treatment 6, F_1 adults were 100% *Wolbachia* free. Average was obtained from three replicates. All F_2 adults was also found to be *Wolbachia* free.

Tetracycline treatment of only adult mosquitoes simplifies the process, improves the egg hatchability, reduces larval mortality and increases adult fecundity. The best concentration for the adult treatment is concluded to be 1.25 mg/mL in sucrose solution with no alternative food source. This method is able to remove both *w*AlbA and *w*AlbB completely in just two weeks and gives subsequent generations free of *Wolbachia*.

This self-sustaining *Wolbachia* free *Ae. albopictus* colony developed can be used to study the effect of *Wolbachia* on Malaysian *Ae. albopictus*. Future research may be conducted to develop a singly infected *Ae. albopictus* strain with a modified antibiotic treatment as none has been established so far.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

There is increasing interest on *Wolbachia* endosymbionts in *Aedes* vectors as they are related to fecundity and to dengue transmission. There is a need to obtain strains of *Ae. albopictus* free from endosymbionts but existing methods through treatment of larvae with tetracycline have not been satisfactory.

Research frontiers

An improved method to obtained *Wolbachia* free and viable *Ae. albopictus* mosquitoes for further studies.

Related reports

Although the use of tetracycline to obtain *Wolbachia* free *Aedes* has been previously studied, the dosage and methods used did not produce satisfactory results.

Innovations and breakthroughs

An improved method of using tetracycline in obtaining subsequent generations of *Wolbachia* free *Ae. albopictus*.

Applications

This study is important for research on *Wolbachia* and dengue susceptibility.

Peer review

The study has provided a viable method to produce *Wolbachia* free *Ae. albopictus* which is increasingly implicated as a vector of dengue transmission in many endemic countries. Susceptibility studies with dengue serotypes in *Wolbachia* positive or negative mosquitoes will be easily available now.

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Distribution and dynamics of *Wolbachia* infection in Malaysian *Aedes albopictus*

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ABSTRACT

Wolbachia are maternally transmitted bacteria found in most arthropods and nematodes, but little is known about their distribution and reproductive dynamics in the Malaysian dengue vector *Aedes albopictus*. In this study, polymerase chain reaction (PCR) was used to determine the presence of *Wolbachia* from field collected *Ae. albopictus* from various parts of the country using wsp specific primers. *Ae. albopictus* had *Wolbachia* infection ranging from 60 to 100%. No sequence diversity of *wsp* gene was found within all wAlbA and wAlbB sequences. Our findings suggest that *Wolbachia* infection amongst the Malaysian *Ae. albopictus* were not homogenously distributed in all districts in Malaysia. The presence of *Wolbachia* in different organs of *Ae. albopictus* was also determined. *Wolbachia* were only found in the ovaries and midguts of the mosquitoes, while absent in the salivary glands. The effects of *Wolbachia* on *Ae. albopictus* fecundity, longevity and egg viability were studied using infected and uninfected colonies. The removal of *Wolbachia* from *Ae. albopictus* resulted in reduced fecundity, longevity and egg viability, thus. *Wolbachia* seem to play a vital role in *Ae. albopictus* reproductive system.

1. Introduction

Dengue is the most important arthropod borne viral disease affecting humans in tropics and subtropics countries (Lambrechts et al., 2010). In Malaysia dengue is on the increase. There were 39,222 dengue cases and 83 deaths in 2013 compared to 103,610 dengue cases and 199 deaths in 2014. As of March 2015, there were 23,966 cases and 62 deaths in just three months (MOH, Malaysia; unpublished record from www.moh.gov.my). Number of cases and deaths are increasing in an alarming manner yearly. In the absence of a vaccine and suitable drugs, new paradigms for vector control approaches are needed to replace archaic ones. One of the novel methods proposed is bio control approach where *Wolbachia* from naturally infected arthropods are introduced into *Aedes aegypti* to reduce the transmission of dengue (Moreira et al., 2009; Bian et al., 2010; Walker et al., 2011). *Wolbachia* are a group of intracellular bacteria which are maternally transmitted and found in most arthropods and nematodes (Werren, 1997; Stouthamer et al., 1999). The capability of *Wolbachia* to alter their host reproductive properties allow them to increase their frequency in host population without necessity of horizontal transmission (Werren et al., 2008; Das et al., 2014). In many occasions, *Wolbachia* have been transferred from natural hosts like *Ae. albopictus* and *Drosophila melanogaster* into other vector mosquitoes to supress diseases such as dengue (Moreira et al., 2009; Bian et al., 2010) and malaria (Hughes et al., 2011; Baton et al., 2013).

Wolbachia play a crucial role in dynamics, evolution and reproductive system of their host. Several studies have indicated that *Wolbachia* play a mutualistic role in reproduction (O'Neill et al., 1997; Werren et al., 2008). To ensure continuous successful maternal transmission, studies have shown that the bacteria tend to alter reproductive properties of their hosts (O'Neill et al., 1997; Stouthamer et al., 1999). Common alterations observed in their hosts are male killing, parthenogenesis, feminization and cytoplasmic incompatibility (CI) (O'Neill et al., 1997; Werren, 1997).

Wolbachia consist of eight super groups (Werren et al., 1995). Supergroup wAlbA and wAlbB are found in arthropods and studies have revealed that *Ae. albopictus* is superinfected with both while *Ae. aegypti* is not infected (Kittayapong et al., 2000; Armbruster et al., 2003; Tsai et al., 2004). *Aedes albopictus* is native to Malaysia (Rudnick, 1965) and is a secondary vector of dengue (Knudsen et al., 1996; Chow et al., 1998) while *Ae. aegypti* is the primary vector (Dieng et al., 2010). In Malaysia, the *Ae. albopictus* population is on the increase (Rozilawati et al., 2007). It is a competent laboratory

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vector of more than 20 arboviruses including chikungunya and dengue virus (Hawley, 1988). *Aedes albopictus* is naturally infected singly and doubly with *Wolbachia pipientis* strains namely wAlbA and wAlbB. However, the singly or doubly infected mosquito's distribution in Malaysia has not been studied extensively. There have been no previous studies on the phylogenetic relationships of *Wolbachia* in Malaysia.

Field trials were conducted in Australia in 2011 releasing *Wolbachia* infected *Ae. aegypti* which had a reduced ability to transmit the virus (Hoffmann et al., 2011). The combination of dengue blocking activity and rapid spread due to CI has led researchers to suggest that *Wolbachia* can be used to develop public health strategies to reduce dengue incidence in humans (Moreira et al., 2009; Bian et al., 2010).

Our studies were conducted to elucidate the dynamics of *Ae. albopictus* with and without *Wolbachia*. These include the egg viability, fecundity and longevity. Previous studies have been conducted vastly to observe these characteristics on artificially infected *Ae. albopictus* (Dobson et al., 2004; Blagrove et al., 2012) and *Ae. aegypti* (Xi et al., 2005; McMeniman et al., 2009; Bian et al., 2010) but very limited on native *Ae. albopictus*. These biological characteristics will provide the initial data which will be useful for the next phase of the study of viral infection on *Ae. albopictus* with and without *Wolbachia*.

Increased risk of any arbovirus transmission over the past few decades may have been due to the current global expansion of *Ae. albopictus* (Benedict et al., 2007; Lambrechts et al., 2010). In Asian countries, *Ae. albopictus* has been incriminated in dengue epidemics (Chow et al., 1998; Ali et al., 2003; Almeida et al., 2005; Thenmozhi et al., 2007). However, information on the role of *Ae. albopictus* in the transmission of dengue in Malaysia is limited (Ahmad et al., 1997). Thus, it is important to carry out these studies since dengue is now a major problem in Malaysia.

2. Materials and methods

2.1. Sample collection

Aedes albopictus were collected from October 2012 to April 2013 using ovitraps and larval surveys. Minimum of eight ovitraps were set in each location and were at least 200 m apart so that all progeny would not be from the same parent. After a period of one week, traps were collected and larvae were allowed to mature into adults in the laboratory. Larvae were fed with fish food twice a day. At the pupae stage they were transferred into respective cages and allowed to emerge as adults. Adults were fed with 10% sucrose solution incorporated with B-Complex. *Aedes albopictus* were collected from 21 locations for the distribution study; one from Perak (Ipoh) and Negeri Sembilan (Nilai), two from Sabah (Banggi Island and rural Kudat), four from Wilayah Persekutuan Kuala Lumpur (Ampang, Setapak, Bangsar and Keramat) and 13 from Klang Valley (Serendah, Sungai Tamu, Batang Kali, Kuala Kubu Baru, Kelumpang, Sungai Sendat, Gasing, Petaling, Kajang, Sungai Merab, Pulau Indah, Shah Alam and Bukit Lagong) (Fig. 1). DNA was extracted from individual mosquitoes between 4 and 6 days after emergence. Samples from Kudat Sabah, Banggi Sabah, Serendah, Sungai Tamu, Batang Kali, Kuala Kubu Baru, Kelumpang, Sungai Sendat and Sungai Merab were wild caught adults.

2.2. DNA extraction

A minimum of five and maximum of twenty individual mosquitoes from each location were processed for DNA extraction. Samples consisted of both male and female mosquitoes. Two hundred and eighty six *Ae. albopictus* consisting of 67 male and 219 female mosquitoes from 21 locations were studied. DNA was extracted from individual mosquitoes using Dneasy Blood and Tissue Extraction Kit according to the protocol provided by the manufacturer (Qiagen, CA, USA). Extracted DNA was stored at −20 °C until needed.

2.3. DNA amplification and sequencing

Minimum of five and maximum of 20 samples from each location were first screened for the presence of *Wolbachia* by PCR amplification. Multiplex PCR was conducted using Promega (Promega, Madison, WI) reagents for amplification of the *wsp* gene with diagnostic primers (Genomics BioSci & Tech, China). wAlbA strain was amplified with the *wsp* 328F and 691R primer pair whereas wAlbB strain was amplified with the *wsp* 183F and 691R primer pair (Zhou et al., 1998; Armbruster et al., 2003). A total of 286 samples from 21 locations were amplified. PCR was conducted in a 20 μL reaction volume using 1× Green GoTaq Flexi Buffer, 2 mM $MgCl_2$, 0.2 mM dNTP, 1 unit of Taq polymerase, 0.3 μM of each primers and 2 μL of DNA template.

PCR was carried out using a T100 thermal cycler (Bio-Rad, Singapore). The PCR conditions were as follows: initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 1 min, amplification at 55 °C for 1 min, extension at 72 °C for 1 min followed by a final extension step of 10 min at 72 °C. (Armbruster et al., 2003). A negative and a positive control were run along with each batch of PCR amplification. Negative control was prepared by substituting 2 μL of sample with 2 μL of ddH_2O. Positive controls for wAlbA and wAlbB were included for all multiplex PCR assays (provided from Armbruster's lab, USA). PCR products were analyzed by agarose gel electrophoresis stained with Syber green (Life Technologies, USA).

PCR was conducted on three different occasions for all singly infected and negative samples to ensure they were truly singly infected and truly negative, respectively.

The 363 bp of the wAlbA strain and 508 bp of wAlbB strain of each sample amplified and separated on gel were sent for sequencing at Genomics BioSci & Tech, China. Sanger method was used for sequencing.

2.4. Multiple alignments and phylogenetic analysis

Sequences were aligned and checked manually using BioEdit (version 7.1.11) (Applied Biosystem, UK) (Hall, 1999). The sequencing was done in both directions for at least three specimens from each sampling site. Since all samples were identical, one representative sequence was taken for each location. A total of 42 sequences consisting of wAlbA, wAlbB and 11 other representative *wsp* sequences obtained from the Genbank were aligned using ClustalW version 1.7. Phylogenetic tree was constructed using MEGA version 6.0 software. The phylogenetic relationships were inferred using Neighbour Joining method. Branches corresponding to partitions reproduced in less than 80% bootstrap replicates were collapsed. All sequences have been submitted to Gene Bank (Accession numbers KF781993 to KF782108). *Wsp* gene sequence from *Culex pipiens* and *D. melanogaster* was used as out groups to confirm findings of the phylogenetic tree.

2.5. Colonisation of Ae. albopictus free of Wolbachia

Aedes albopictus with and without *Wolbachia* were maintained in the insectary of the Parasitology Department of University of Malaya. *Aedes albopictus* obtained from Bukit Lagong, were colonised since the infection frequency was 100% double *Wolbachia* infection in all tested samples. Mosquitoes were constantly tested from each generation for *Wolbachia* infection at different time

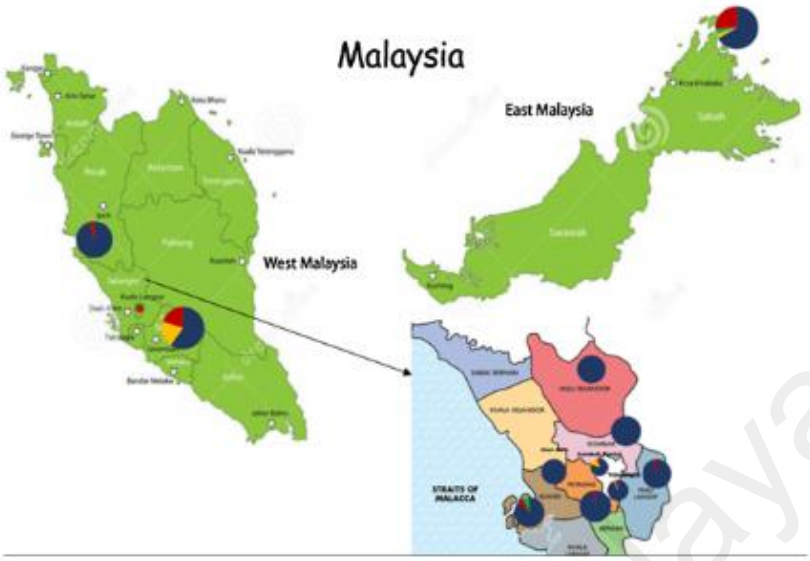

Fig. 1. *Map of Malaysia showing Aedes albopictus collection sites.* Samples were collected from 5 states of Malaysia. Single site from Perak and Negeri Sembilan. Two sites from Sabah. (One from rural Kudat and another from Banggi Island). Thirteen sites from Selangor and four sites from Kuala Lumpur. [Blue: Infected with both wAlbA and wAlbB; Red: Uninfected; Green: Singly infected with wAlbA; Yellow: Singly infected with wAlbB]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

points. All tested samples were doubly infected with wAlbA and wAlbB. *Wolbachia* free *Ae. albopictus* was maintained as described in Joanne et al. (2014). Briefly, to remove *Wolbachia*, newly emerged adult mosquitoes were fed with 1.25 mg/mL tetracycline treated sucrose solution with B-Complex for the first two generations. PCR confirmation that mosquitoes are free of *Wolbachia* was continuously carried out for each generation by randomly screening individual mosquitoes. Colonies of *Wolbachia* free *Ae. albopictus* were used for experiments after two generations free of tetracycline to allow growth of other necessary microbiota loss due to the initial high concentration tetracycline treatment. These colonies were used for the dynamics study (fecundity, longevity and egg hatchability). All studies were conducted in triplicates.

2.6. Detection of Wolbachia in different organs of Ae. albopictus

A total of 90 doubly infected female mosquitoes from Bukit Lagong colony were collected at three different time points (6th, 14th, and 30th day) and were dissected to isolate the salivary gland, ovaries and midgut. Care was taken to avoid contamination. DNA was extracted from the individual organs using Dneasy Blood and Tissue Extraction Kit as described above (Qiagen, CA, USA). DNA was stored at −20 °C freezer until needed for PCR. Multiplex PCR was performed as described above.

2.7. Dynamics of Wolbachia

2.7.1. Fecundity

Six day old female mosquitoes were provided a blood meal. All unfed mosquitoes were removed from the cage. On third day post feeding, fifty mosquitoes were transferred into individual transparent 4oz containers lined with moist filter paper for oviposition. They were provided with 10% sucrose solution and maintained in humidity chamber (85% humidity and 26 °C). Resulting eggs were counted and recorded.

2.7.2. Longevity

One hundred pupae were collected and left in a new cage. Life span was calculated from day one mosquitoes emerged from pupae. Adult mosquitoes were fed with 10% sucrose with B-complex. Mosquitoes were given blood meal once every two weeks. Cages were lined with white paper to ease collection of dead mosquitoes. Dead mosquitoes were collected daily and recorded.

2.7.3. Egg viability

Collected eggs were dried and stored for two weeks. Twenty five eggs on each filter paper were allowed to hatch in overnight water in four containers. Larvae were fed with fish food daily. The number of mosquitoes that emerged from the eggs was recorded. The larval and pupae development times were also recorded. This experiment was also conducted on one day old eggs.

2.8. Statistical analysis

SPSS version18 and GraphPad Prism version 6.01 for Windows (GraphPad Software, 1992–2007, San Diego, CA, www.graphpad.com) were used for all statistical analysis performed. McNemar and Fisher's exact test in SPSS was used to test for significant difference of *Wolbachia* in the different organs. The normality of the life history traits (dynamic study) was determined using Shaphiro Wilk test in SPSS.

3. Results

3.1. Wolbachia distribution in Malaysian Ae. albopictus

Two hundred and eighty six *Ae. albopictus* were collected from 21 sites in Malaysia and screened for *Wolbachia* infection using wAlbA and wAlbB supergroup specific *wsp* gene primers. The length of wAlbA strain was 363 bp and wAlbB strain was 508 bp. The infection frequency of the samples according to districts is listed on Table 1. Samples consisted of 67 male and 219 female *Ae. albopictus*.

It was found that 91.60% of samples screened were superinfected with both wAlbA and wAlbB supergroups and 3.15% of samples were singly infected with either wAlbA (1.05%) or wAlbB (2.05%) supergroup. However, 5.25% were uninfected. Samples from three districts; Hulu Selangor, Shah Alam and Gombak (mixed urban and rural) were 100% superinfected. Nilai (urban) had the lowest infection rate which was 60%. Only Titiwangsa, Lembah Pantai, Nilai (urban) and Kudat (rural Kudat and Banggi Island) had mosquitoes singly infected with either wAlbA or wAlbB supergroup only. Only infection frequency of samples from Nilai and Kudat showed significant difference compared to the other sites.

3.2. Phylogenetic association

The tree resulted in two major clades with bootstrap values of 71 and 80. The first clade was wAlbA and the second one was wAlbB supergroup (Fig. S1). Samples were confirmed as wAlbA and wAlbB, respectively, by performing a BLAST in the GenBank. No samples branched out from the two major clades. All wAlbA amplified sequences and wAlbB amplified sequences were identical to each other, respectively. Few sequences of wAlbA and wAlbB aligned samples are shown in Tables S2 and S3. The sequence obtained showed no deviation have occurred within the *wsp* genes of wAlbA and wAlbB samples. Two out-group samples were included in the phylogeny tree to ensure credibility. All 42 samples of wAlbA and wAlbB showed no difference between them therefore, no further analysis of the tree were required (Fig. S1). The accession numbers (KF781993 to KF782108) for each sequence used in the phylogenetic tree are shown in Table S1.

3.3. Detection of Wolbachia in organs of Ae. albopictus

All ovaries were positive on all days (6, 14 and 30th). No midguts were positive on day 6, however, 50% of midguts were positive on day 14 and 93% on day 30. All salivary glands were negative on all days tested (Table 2). There was a significant difference in *Wolbachia* infection in midgut over the three time points ($P < 0.05$) by Mc Nemar tests.

3.4. Dynamics of Wolbachia

3.4.1. Fecundity of Wolbachia infected and uninfected Ae. albopictus

A total of 4620 eggs were laid by 150 *Wolbachia* infected mosquitoes and a total of 795 eggs were laid by 124 *Wolbachia* uninfected mosquitoes (Fig. 2). Highest number of eggs laid and mean of total eggs for *Wolbachia* infected colony were 130 and 37.58, respectively, whereas for *Wolbachia* uninfected colony were 33 and 9.58, respectively. Both values were four times higher for *Wolbachia* infected colony.

There was a significant difference between *Wolbachia* infected and *Wolbachia* uninfected *Ae. albopictus* using two-tailed *t*-test ($P < 0.005$). Normality of the values obtained was confirmed using the Shaphiro–Wilk test. Thus, the assumption that the difference scores were normally distributed was met.

$n_{mosquito}$	150	124
n_{eggs}	4620	795

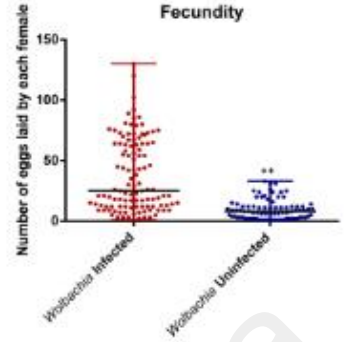

Fig. 2. *Eggs laid by individual female mosquitoes.* Highest number of eggs laid by a female for *Wolbachia* infected colony is 130 eggs and for *Wolbachia* uninfected colony is 33 eggs. Mean eggs laid for *Wolbachia* infected colony was 37.58 and mean eggs laid for *Wolbachia* uninfected colony was 9.578. Comparison marked with (**) showed there was a significant difference using two tailed *t*-test ($P < 0.005$). Results are from three different experiments from different generations have been combined.

3.4.2. Longevity of Wolbachia infected and uninfected Ae. albopictus

The average female and male lifespan for *Wolbachia* infected and uninfected study is shown in Fig. 3. Each colony consisted of more males than females. There were no significant difference in sex ratio between *Wolbachia* infected and uninfected colony. Male lifespan was three fold higher in the *Wolbachia* uninfected colonies. Female lifespan was almost similar for both colonies.

In the test for male lifespan, the 95% confidence interval for mean difference does not have the tested value of 0 ($P < 0.005$). Thus, there was a significant difference in male lifespan between *Wolbachia* infected and uninfected colonies. Normality of the values obtained was confirmed using the Shaphiro–Wilk test. Thus, the assumption that the difference scores were normally distributed was met.

In the test for female lifespan, the 95% confidence interval for mean difference does not have the tested value of 0 ($P > 0.05$). This concludes that the difference in female lifespan between *Wolbachia* infected and uninfected colonies were not significant thus, test of normality was not conducted.

3.4.3. Egg viability of Wolbachia infected and uninfected Ae. albopictus

Egg viability of *Wolbachia* uninfected colonies were higher for two weeks old eggs whereas egg viability of *Wolbachia* infected colonies were higher for the one day old eggs as shown in Fig. 4. Larvae and pupae emergence were shorter in *Wolbachia* infected eggs. Pupation started on day six for *Wolbachia* infected eggs with mean of 5.67 and on day eight for *Wolbachia* uninfected eggs with mean of 8.33.

One way ANOVA performed showed there was a significant difference in egg viability ($P < 0.005$) between *Wolbachia* infected and uninfected *Ae. albopictus* (two weeks and one day old eggs) Fig. 4.

4. Discussion

Wolbachia are known to be most abundant and wide spread endosymbiont parasitic bacteria ever since found in

Table 1
Infected status of *Aedes albopictus* from 11 district in Malaysia.

District	Total samples		Both A and B		A only		B only		None	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Titiwangsa[a]	15	41	13(23.2%)	39(69.6%)	0	1	1	0	1	1
Lembah Pantai[b]	10	10	6(30.0%)	10(50.0%)	0	0	3	0	1	0
Hulu Selangor[c]	0	67	0(0%)	67(100%)	0	0	0	0	0	0
Petaling[d]	22	18	22(55.0%)	17(42.5%)	0	0	0	0	0	1
Hulu Langat[e]	8	17	7(28.0%)	17 68.0%	0	0	0	0	1	0
Klang[f]	3	11	3(21.4%)	9(64.3)	0	1	0	0	0	1
Shah Alam[g]	1	3	1(25.0%)	3(75.0%)	0	0	0	0	0	0
Gombak[h]	3	5	3(37.5%)	5(62.5%)	0	0	0	0	0	0
Kinta[i]	10	10	9(45.0%)	10(50.0%)	0	0	0	0	1	0
Nilai[j]	2	3	1(20.0%)	2(40.0%)	0	0	1	0	0	1
Kudat[k]	0	27	0(0%)	18(66.7%)	0	1	0	1	0	7
Total	74(25.9%)	212(74.1%)	65(22.7%)	197(68.9%)	0(0%)	3(1.0%)	5(1.8%)	1(0.35%)	4(1.4%)	11(3.8%)
Total	286		262(91.6%)		3(1.0%)		6(2.2%)		(5.2%)	

[a] Titiwangsa: Ampang, Setapak, Keramat.
[b] Lembah Pantai: Bangsar.
[c] Hulu Selangor: Serendah, Kampung Sungai Tamu, Batang Kali, Kuala Kubu Baru, Kelumpang, Sungai Sendat.
[d] Petaling: Jalan Gasing, Petaling KTM Station.
[e] Hulu Langat: Kajang, Sungai Merah.
[f] Klang: Pulau Indah.
[g] Shah Alam: Shah Alam.
[h] Gombak: Bukit Lagong.
[i] Kinta: Ipoh.
[j] Nilai: Nilai 3.
[k] Kudat: Kudat and Banggi Island.

Table 2
Distribution of *Wolbachia* in organs of *Ae. albopictus* on day 0, 14 and 30.

Day	Organ	*N*	Positive	Negative	*Wolbachia* infectivity (%)
0th day post emergence	Ovaries	30	30	0	100
	Midgut	30	0	30	0
	Salivary glands	30	0	30	0
14th day post emergence	Ovaries	30	30	0	100
	Midgut	30	15	15	50
	Salivary glands	30	0	30	0
30th day post emergence	Ovaries	30	30	0	100
	Midgut	30	28	2	93
	Salivary glands	30	0	30	0

[*] The *P* value for Fisher's exact test obtained was 0.033 (significant, $P < 0.05$) and for Mc Nemar test was 0.004 (significant, $P < 0.005$).

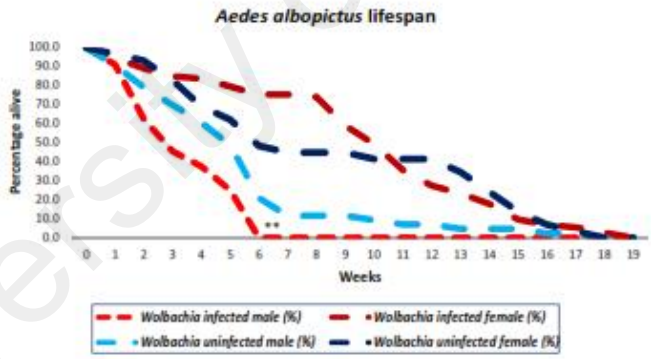

Fig. 3. *The lifespan of Wolbachia infected and uninfected Ae. albopictus male and female.* Percentage was calculated by dividing number of mosquitoes alive weekly over total initial number of mosquitoes. Comparison marked with (**) showed there was a significant difference using paired sample *t*-test ($P < 0.005$). Shaphiro–Wilk *P*-value 0.200; Test of normality: Shaphiro–Wilk ($P > 0.05$); Thus there is significant difference between *Wolbachia* infected and uninfected male *Ae. albopictus*. Results are from three different experiments from different generations have been combined.

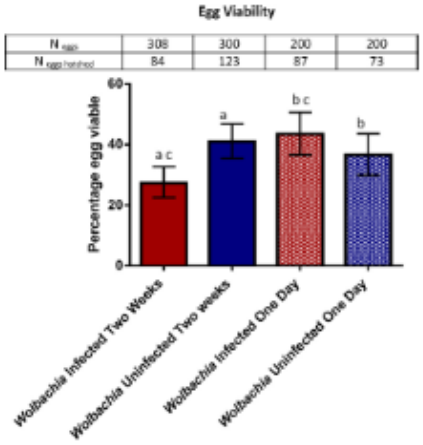

Fig. 4. *Percentage egg viability for Wolbachia infected and Wolbachia uninfected* colonies. Percentage was calculated by dividing total eggs became adult over number of eggs subjected to hatch. The egg viability with similar alphabets shown above the bar are significantly different using one-way ANOVA ($P < 0.005$).

Culex pipientis in 1936 (Hertig, 1936). Among many arthropods to be naturally infected with *Wolbachia* are *D. melanogaster*, *Cnaphalocrocis medinalis* (Asiatic leafroller), *Lutzomyia* sp. (sand fly) and *Ae. albopictus* (Tsai et al., 2004; Chai et al., 2011; Serpa et al., 2013; Das et al., 2014). Several studies have been conducted to determine the average infected rate of *Wolbachia* in their respective hosts.

Our findings concur with other published results showing *Ae. albopictus* to be naturally infected with *Wolbachia* (Kittayapong et al., 2000; Armbruster et al., 2003). The negative samples confirmed to be truly negative. The negative samples were not excluded from the analysis as was done previously by Osei-Poku since the percentage was significantly higher than their estimation of 0.78% (Kittayapong et al., 2000; Osei-Poku et al., 2012). The 5.25% of the uninfected population may have been due to environmental factors. Several antibiotics have been reported to be able to cause bactericidal effect on *Wolbachia* (Otsuka and Takaoka, 1997; Dobson and Rattanadechakul, 2001). The uninfected samples may have been feeding from natural or synthetic bactericide containing food source causing the *Wolbachia* in them to perish in the wild. Besides, negative samples may indicate maternal transmission leakage of *Wolbachia*. Future studies should take this into consideration. As for the singly infected samples, it could mean that cytoplasmic incompatibility was incomplete. In a study by Armbruster of 18 populations, all were positive (Armbruster et al., 2003). This clearly shows that more extensive sampling has to be conducted before concrete conclusions can be deciphered.

Since Malaysian *Ae. albopictus* are superinfected with *Wolbachia* and their infection is able to be sustained due to CI for generations (Kambhampati et al., 1993), it is possible to introduce disease transmission blocking genes into these species and expect them to be passed on within their host for generations (Kitrayapong et al., 2002).

The pattern of *Wolbachia* infection frequency was not homogenous among the districts. Similar patterns have been reported on *Wolbachia* infection in different host from other countries (Doudoumis et al., 2012). It is difficult to explain why some singly infected samples were found in urban areas (e.g. Titiwangsa, Lembah Pantai, and Nilai) and also in isolated locations like rural Kudat and Banggi Island in Sabah. Single infections could not be related to ageing of mosquitoes in this study, as all mosquitoes were not older than 6 days during sampling. Phylogenetic study of *Wolbachia* on *Ae. albopictus* in India and spiders in China indicates that geographical factors would impact *Wolbachia* infectivity rate and pattern (Wang et al., 2010; Das et al., 2014).

Besides, the sample size difference for each district may have caused the un-uniformed result. Sample size analysed was considerably small for the establishment of the infection pattern because both empirical and theoretical data indicate that when a population gets infected with *Wolbachia*, it is anticipated to spread to fixation immediately (Armbruster et al., 2003). Additional studies on the diversified infected rate of *Wolbachia* may provide a better insight on the functional role of the bacteria in *Ae. albopictus* dynamics, evolution and ecology.

Lack of diversity in the *w*AlbA and *w*AlbB sequences may have been due to one strain being less dense compared to the other leading to decrease in likelihoods of homologous recombination. The other explanation could be these genes might have some role in CI, resulting in a high selective pressure that might hinder the occurrence of new strains (Albuquerque et al., 2011).

Although sufficient time was provided for the *Wolbachia* to infect different organs, only midgut and ovaries were found to be infected and salivary glands were not infected even after 30 days. This contradicts with other studies where *Wolbachia* were found in salivary glands, ovaries and midguts of *Ae. albopictus* (Tsai et al., 2004; Zouache et al., 2009). This perhaps could be due to different strains of *Ae. albopictus* and that may be the reason why *Ae. albopictus* is a vector of dengue in some countries (Lambrechts et al., 2010) and only support a minor role in other countries (Gratz, 2004).

Presence of *Wolbachia* in salivary glands may indicate inhibition of dengue virus dissemination whereas presence of *Wolbachia* in midgut may indicate inhibition of dengue virus replication. When mosquitoes ingest dengue virus during blood meal, the virus penetrates into the midgut epithelial cells of mosquitoes. Secondary organs such as salivary glands only gets infected after replication and release of virions (Mousson et al., 2012).

Midgut being infected with *Wolbachia* may be the reason for reduced transmission of dengue virus by *Ae. albopictus*. In a laboratory study with the La Reunion Island strain of *Ae. albopictus* it was found that *Wolbachia* limits DENV-2 viral density and dissemination rate (Mousson et al., 2012). However, further studies will be required to determine if this is true for all *Ae. albopictus* strains.

According to Baton et al. (2013), no effect on host fitness was observed in the mosquito during continuous treatment of mosquito with tetracycline or in the period immediately following its withdrawal. The fecundity study and egg viability study was conducted for three generations without ongoing tetracycline treatment in the *Wolbachia* free colony and no significant difference was found compared to the colony maintained with 0.1 mg/mL (result not shown). Thus we conclude that all difference in reproductive dynamics reported in this study was due to the absence of the *Wolbachia* from *Ae. albopictus*.

Dynamics of *Ae. albopictus* with and without *Wolbachia* showed that fecundity, longevity and egg viability were affected. Previous study conducted on effect of *Wolbachia* on *Ae. aegypti* found that presence of *Wolbachia* in *Ae. aegypti* were able to reduce the mosquito fecundity, longevity and egg viability (Xi et al., 2005). However, *Ae. albopictus* is a natural host for *Wolbachia*. *Wolbachia* have been residing in the testes and ovaries of mosquitoes for a very long time. It has been suggested in previous studies that the *Wolbachia* have formed a symbiotic relationship with the mosquito (Dobson et al., 2002). This, suggest that removal of *Wolbachia* from *Ae. albopictus* would affect the normal reproductive behaviour of the mosquito.

The dynamics of *Ae. albopictus* with and without *Wolbachia* has been little explored. Dobson had studied effect of *Wolbachia* on CI and fecundity of native *Ae. albopictus* in 2001 (Dobson et al., 2002). He reported that *Wolbachia* induces CI and increases host fecundity.

Many have studied effect of *Wolbachia* on artificially infected *Ae. aegypti* (Xi et al., 2005; Ruang-Areerate and Kittayapong, 2006; Bian et al., 2010) and some on artificially infected *Ae. albopictus* with *wMel* strain (Blagrove et al., 2012; Calvitti et al., 2012).

Fecundity is the ability to reproduce an organism or population, measured by the number of successful progeny. Our *Wolbachia* infected colony had significantly higher fecundity compared to *Wolbachia* uninfected colony. This concurs with study done by Dobson et al. (2002). This clearly displays that removal of *Wolbachia* reduces the mosquito's natural ability to oviposit. Perhaps the removal of symbiotic *Wolbachia* from its natural host *Ae. albopictus* retards the reproductive system of the host.

Longevity is the total lifespan of the mosquitoes. A study by Gavotte have shown that *Wolbachia* infected *Ae. albopictus* females are less competitive relative to uninfected females when competing under highly competitive conditions (Gavotte et al., 2010). Therefore under low competitive environment as in the laboratory, *Wolbachia* uninfected females should live longer compared to *Wolbachia* infected females. However, in this study infected females had longer lifespan compared to uninfected females. Mousson et al. (2012) reported that removing *Wolbachia* did not affect the longevity of *Ae. albopictus* even with or without dengue virus infection.

Male lifespan in the *Wolbachia* uninfected colony were 3.18 times higher than *Wolbachia* infected colony. Males in the uninfected colony tend to live as long as the female mosquitoes. These indicate presence of *Wolbachia* causes reduction in male lifespan in *Ae. albopictus*.

Wolbachia have been associated to alter their host reproductive phenotype such as male killing (Kamgang et al., 2011). This causes their host to kill the male progeny that inherited *Wolbachia* (Kamgang et al., 2013). This would explain why the males die earlier in the *Wolbachia* infected colonies and live longer in the *Wolbachia* uninfected colonies.

Egg viability is considered as the number of adult mosquitoes that successfully merged from the hatched eggs. It has an important role in the lifecycle of the mosquito. Egg viability was higher for *Wolbachia* uninfected colony compared to *Wolbachia* infected colony. This was reverse from the results of fecundity experiments. However, when repeated using one day dried eggs, average egg viability for *Wolbachia* uninfected colony remained almost the same but the average egg viability for *Wolbachia* infected colony was significantly higher by 16.23% compared to the two weeks old eggs.

This difference could indicate that eggs of *Wolbachia* uninfected mosquitoes remains more viable after two weeks of storage whereas eggs obtained from *Wolbachia* infected colony becomes less viable upon storage. This perhaps could be one of the reasons why in the laboratory colonisation of *Ae. aegypti* and *Ae. albopictus* using two weeks egg drying method, more progeny is obtained from *Ae. aegypti* compared to *Ae. albopictus*. Perhaps, in order to obtain good egg viability for *Ae. albopictus*, the eggs need to be hatched after 24 h of drying.

5. Conclusion

Information on natural infection rate is important in order to assess the prospective of *Wolbachia* as a vehicle to modify *Ae. albopictus* populations. We conclude that 91.60% of Malaysian *Ae. albopictus* studied were superinfected with *Wolbachia* making them suitable vehicle for genetic control studies. Supporting the suggestion of *Wolbachia* to alter and affect the reproduction system of the host, all samples were found to be infected with *Wolbachia* in the ovaries. In this study, significant *Wolbachia* infection was found only in the midgut but not in salivary glands. As for the dynamics, removal of *Wolbachia* causes reduced fecundity, longevity and egg viability in *Ae. albopictus*. These findings would form the basis for further studies to be conducted on the biological roles of *Wolbachia* on Malaysian *Ae. albopictus* in relation to dengue virus transmission.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actatropica.2015.04.003

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Unidirectional Cytoplasmic Incompatibility in Malaysian *Aedes albopictus* (Diptera: Culicidae)

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Abstract

Aedes albopictus (Skuse) is a secondary vector of dengue and has naturally occurring *Wolbachia*, which is an intracellular bacteria found in most nematodes and arthropods. Previous findings demonstrated that 91.60% of Malaysian *Ae. albopictus* were superinfected with two major *Wolbachia* supergroups, namely, wAlbA and wAlbB. *Wolbachia* has been associated with manipulation of reproductive phenotypes of their host such as male killing, parthenogenesis, feminization, and cytoplasmic incompatibility (CI). Cytoplasmic incompatibility is when no viable offspring are produced between *Wolbachia*-infected male and *Wolbachia* uninfected or differently infected female host. To evaluate CI, we performed a study in the laboratory using *Wolbachia*-free and *Wolbachia*-infected *Ae. albopictus*. Four different crosses were made, and viable eggs produced from each cross were recorded. The percentage of viable offspring produced were compared and analyzed. Results obtained suggest that native *Wolbachia* causes strong unidirectional CI in Malaysian *Ae. albopictus*. Perhaps this can be used as a possible vector control or suppression tool.

Key words: cytoplasmic incompatibility, *Aedes albopictus*, dengue, unidirectional

Aedes albopictus (Skuse) is a vector for several pathogens including chikungunya virus (Tesh et al. 1976), yellow fever virus (Mitchell et al. 1987), Zika virus (Wong et al. 2013), and dengue virus (Mitchell et al. 1987). They originated from Asia and have spread across the globe (Gratz 2004). They were first introduced to Malaysia in 1894 from India (Skuse 1894). Since then, it has become a major public health threat to the nations, causing outbreaks of dengue (Gubler 1998) and chikungunya (Charrel et al. 2007). In Malaysia, *Ae. albopictus* is the secondary vector for dengue virus while *Aedes aegypti* (L.) is the primary vector (Sulaiman et al. 1996; Chow et al. 1998).

Wolbachia are vertically transmitted intracellular bacteria found in most insects—nematodes and arthropods (Werren 1997). *Aedes albopictus* is among the vectors naturally infected with *Wolbachia*. Several studies have transinfected *Wolbachia* into *Ae. aegypti* to supress its population (McMeniman et al. 2009). *Wolbachia* have been associated with ability to alter host reproductive phenotype (O'Neill et al. 1992, Dobson et al. 2002). They have been implicated to induce cytoplasmic incompatibility (CI) in most of their hosts (O'Neill et al. 1992, Breeuwer 1997, Dobson et al. 2002), parthenogenesis in wasps (Stouthamer et al. 1993), and feminization in isopods (Rigaud et al. 1991). Mechanism that causes this modification to reproductive phenotypes remains unknown (Breeuwer 1997, Dobson et al. 2002).

However, importance of CI for *Wolbachia* has been hypothesized in many cases (Hoffmann et al. 1990). Cytoplasmic incompatibility caused by *Wolbachia* helps to spread or maintain their infection in natural host populations by reducing or preventing *Wolbachia*-uninfected offspring to be produced (Hoffmann et al. 1990, Dobson et al. 2002).

There are two types of CI, namely, unidirectional and bidirectional. Unidirectional is when no viable offspring are obtained when infected males mate with uninfected females but viable offspring obtained when uninfected males mate with infected females (Laven 1967, O'Neill et al. 1992, Dobson et al. 2004). Bidirectional is when no viable offspring are obtained when either male or female are infected with different type of *Wolbachia* (Telschow et al. 2005).

Wolbachia is described as a saving mechanism that needs to be present in the female. *Wolbachia* modifies the sperm and when the sperm fertilizes an egg, the egg needs to be infected with the same strain of *Wolbachia* to save the modified sperm. If it doesn't, the mating becomes unsuccessful and results in unviable eggs (Werren 1997, Telschow et al. 2005). Bidirectional incompatibility seemingly occurs when different strains of *Wolbachia* have different saving mechanisms, for instance, when both male and female have *Wolbachia* that originated from different hosts (Telschow et al. 2005). In populations with both *Wolbachia*-infected and uninfected hosts, CI reduces

chances of obtaining *Wolbachia*-uninfected offspring. This ensures their infection continuity in their host populations.

Previous studies have reported unidirectional CI in both nematodes and arthropods (Kambhampati et al. 1993; Dobson et al. 2002, 2004; Zabalou et al. 2004) and bidirectional CI in limited hosts (O'Neill and Karr 1990, Telschow et al. 2005). In few cases, *Wolbachia* did not have an effect on host reproduction (Giordano et al. 1995, Hoffmann et al. 1996).

Currently various control methods like fogging, larviciding, source reduction, and biological control are being instituted to reduce the populations of the major vector *Ae. aegypti*. In spite of all control methods, populations of *Ae. albopictus* have been growing at an alarming rate (Rozilawati et al. 2007). If populations of *Ae. aegypti* are suppressed to very low levels, it is very likely that *Ae. albopictus* would emerge as the major vector. Therefore, it is important to study the dynamics of *Ae. albopictus* and possible measures to control its populations. Cytoplasmic incompatibility caused by *Wolbachia* on their host has been suggested to be a possible biological control mechanism (Zabalou et al. 2004, Moreira et al. 2009, Bian et al. 2010). Therefore, it is crucial to study CI status on Malaysian *Ae. albopictus*, as it has not been established to date. *Aedes albopictus* would be a suitable host to study CI, as they are naturally infected with *Wolbachia*. *Wolbachia*-free *Ae. albopictus* colony has been generated in the laboratory using tetracycline antibiotic treatment (Joanne et al. 2014).

To demonstrate the ability of *Wolbachia* to cause CI in Malaysian *Ae. albopictus*, four crosses were made between *Wolbachia*-infected and *Wolbachia*-uninfected colonies. In this study, we have examined fecundity and egg viability for each cross. We established that the Malaysian *Ae. albopictus* exhibits unidirectional cytoplasmic incompatibility. We also discuss how these findings could be used to reduce the size of *Ae. albopictus* populations in Malaysia.

Materials and Methods

Mosquito Strains

Two *Ae. albopictus* strains were used in this study: *Wolbachia*-infected strain—infected female (IF) and infected male (IM)—and *Wolbachia*-uninfected strain—uninfected female (UF) and uninfected male (UM). From each strain, the males and females were maintained in separate cages till crossed for mating. The *Wolbachia*-infected strain was from Bukit Lagong, Gombak, that has been established in insectarium of Parasitology Department of University of Malaya (Joanne et al. 2015). Maximum of 200 larvae were allowed to grow in each tray. They were fed with tetramin fish food. Adults were maintained with 10% sucrose solution incorporated with B-Complex vitamin. All colonies were maintained at 27°C and 85% relative humidity, with a photoperiod of 12:12 (L:D) h in an insectarium of Department of Parasitology, University Malaya. Fifth-generation offspring were used in this study. The *Wolbachia*-uninfected strain was generated via tetracycline treatment as mentioned in Joanne et al. (2014) also originated from Bukit Lagong, Gombak. Uninfected strain used was free from antibacterial treatment for complete two generations before being used in this study to allow recovery from any potential side effects from the prior tetracycline treatment. Infected colony was confirmed to be superinfected and the uninfected was confirmed to be free of *Wolbachia* by randomly testing 30 mosquitoes from each generation for infection.

Table 1. Crosses made for each cage

	Cage 1	Cage 2	Cage 3	Cage 4
Crosses	IF × IM	UF × UM	UF × IM	IF × UM

IF, infected female; IM, infected male; UF, uninfected female; UM, uninfected male.

Population Cages

Total of four crosses were made as shown in Table 1. Fifty female mosquitoes were maintained in each cage with only sucrose. When the females were 8 d old, 20 male mosquitoes were introduced into their cages for mating. Three days later, a fresh bloodmeal was provided. Bloodmeal was drawn from volunteer (the first author) each time and fed using hemotek membrane feeder (Discovery Workshop, United Kingdom) in ACL-2 laboratory. Fully engorged female mosquitoes were transferred into individual 4-oz. transparent containers layered with filter paper for egg laying. Eggs were collected daily and recorded. Collected eggs were dried and hatched to measure egg viability as previously described (Joanne et al. 2015).

PCR Amplification

PCR amplification with *wsp*-specific primers *w*AlbA (primer 328F and 691R) and *w*AlbB (primer 183F and 691R) was conducted on offspring to determine the presence of *Wolbachia*. PCR was conducted in a 20-µl reaction volume using 1× Green GoTaq Flexi Buffer, 2 mM $MgCl_2$, 0.2 mM dNTP, 1 unit of Taq polymerase, 0.3 M of each primer, and 2 µl of DNA template. PCR was carried out using a T100 thermal cycler (Bio-Rad, Singapore). The PCR conditions were as follows: initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, amplification at 55°C for 1 min, extension at 72°C for 1 min followed by a final extension step of 10 min at 72°C (Armbruster et al. 2003).

PCR products were analyzed by agarose gel electrophoresis stained with Syber green (Life Technologies, USA). PCR was repeated using CO1 gene primers for the negative samples to ensure they were truly negative and not due to extraction failure. Only samples that gave bands for CO1 gene primer and no bands for *wsp* primers were considered negative samples.

Statistical Analysis

All statistical analysis conducted in this study was carried out using GraphPad Prism 6.01 (GraphPad Software, Inc., 2012). One-way ANOVA ($P < 0.001$) was conducted for both data in Figs. 1 and 2. Test for significant difference was analyzed comparing each of the Cross 2, Cross 3, and Cross 4 with Cross 1 (control).

Results

Infection status was determined using PCR amplification and gel electrophoresis.

Fecundity

In crosses that were superinfected with *w*AlbA and *w*AlbB, the larger number of eggs (2,799) were laid by 81 females, with mean of 35.90, compared with only 621 eggs by 82 females in the uninfected cross, with mean of 7.86 (Fig. 1), and when the infected male was crossed with the uninfected female, 491 eggs were produced by 66 females, with mean of 7.38. However, in the reverse cross, 2,147 eggs were produced by 72 females, with mean of 29.80 (Fig. 1).

Egg Viability

Overall, egg viability was highest when *Wolbachia* was present in both sexes (93.80%, with mean of 50.40%). However, when both

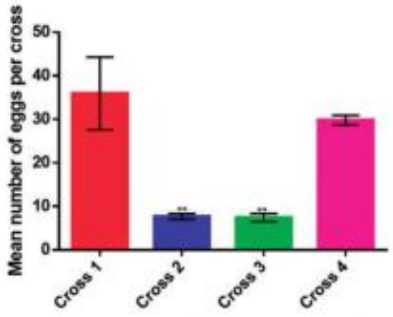

Fig. 1. Mean number of eggs laid by each female mosquito of each cross. Each bar represents the overall mean value of the triplicates for each cross. The vertical line represents the standard deviation between the triplicates. Cross 1: IF×IM; Cross 2: UF×UM; Cross 3: UF×IM; Cross 4: IF×UM. Asterisk (**) indicates significant difference of the cross compared with cross 1, which is taken as the control ($P < 0.001$) according to one-way ANOVA $F_{(3, 297)} = 38.66$ and Sidak test for multiple comparison.

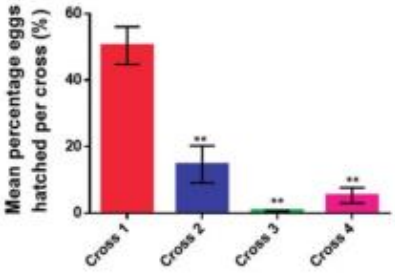

Fig. 2. Mean of percentage of eggs by each female mosquito hatched for each cross. Each bar represents the overall mean value of the triplicates for each cross. The vertical line represents the standard deviation between the triplicates. Cross 1: IF×IM; Cross 2: UF×UM; Cross 3: UF×IM; Cross 4: IF×UM. Asterisk (**) indicates significant difference of the cross compared with cross 1, which is taken as the control ($P < 0.001$) according to one-way ANOVA $F_{(3, 297)} = 117.0$ and Sidak test for multiple comparison.

were not infected, the total egg viability was only 50.00%, with mean of 14.70%. In the cross between uninfected female and infected male, the total egg viability was 8.00%, with mean 0.74%, while for the reverse it was 39.00%, with mean 5.35%, as shown in Fig. 2.

Infectivity of Offspring

All offspring tested in Cross 1 were superinfected with *w*AlbA and *w*AlbB, while all offspring tested from Cross 2 and Cross 3 were clear from *Wolbachia* infection. Among offspring tested in Cross 4, 82.00% were superinfected with *w*AlbA and *w*AlbB and the rest were clear.

Discussion

Cytoplasmic incompatibility is an important modification caused by *Wolbachia* to their host species. This alteration is carried out in order to sustain *Wolbachia* infection for generations. It reduces chances for developing *Wolbachia*-free offspring. As mentioned earlier, there are two kinds of CI that have been reported previously. In both types of CI, either complete or incomplete embryonic death is possible. The *Wolbachia* infection in Malaysian *Ae. albopictus* appears to cause unidirectional CI under laboratory conditions.

Study conducted on Australian *Drosophila simulans* that were naturally infected with *Wolbachia* (as is *Ae. albopictus*) reported that *Wolbachia* did not show CI (Hoffmann et al. 1996). Prior to 1990, *Wolbachia* was reported to cause bidirectional CI in *D. simulans* (O'Neill and Karr 1990). These show that the same species of host may have different types of CI caused by *Wolbachia*.

Currently, many strategies such as fogging, use of repellents, reducing human–vector contact, insecticide-treated netting are being used to reduce *Aedes* populations (Paupy et al. 2009). Recent approaches such as Release of Insects with Dominant Lethality (RIDL) and *Wolbachia* are being considered to supplement these control measures (McDonald et al. 1977, Beech et al. 2009, Lacroix et al. 2012). However, all these methods are targeted mainly toward *Ae. aegypti*. Since *Ae. albopictus* has a role in transmission of dengue, Zika, and chikungunya viruses, the results of this CI study is important. We may be able to use the findings of this study to develop measures to reduce population of *Ae. albopictus* in Malaysia. Cytoplasmic incompatibility caused by *Wolbachia* can be used to produce nonfertile males that can be released to the field as a suppression tool against this mosquito (Calvitti et al. 2015).

An older study from India reported unidirectional CI caused by *Wolbachia* in *Ae. albopictus* with complete embryonic death when female from Mauritius island were mated with males from five different geographical locations. All mating carried out within the same geographical location were compatible. Despite the usual CI caused due to infected male and uninfected female, this study reported CI between interpopulation mating (Kambhampati et al. 1993).

A more recent study conducted in USA portrayed *Wolbachia* infection causing unidirectional CI in *Ae. albopictus* with complete embryonic death, suggesting that combination of CI and increased host fitness caused by *Wolbachia* can accelerate the rate of *Wolbachia* invasion in a new population (Dobson et al. 2004).

Studies were also conducted to observe CI in *Ae. albopictus* artificially infected with *Wolbachia* from different hosts such as *w*Mel from *D. melanogaster* and *w*Pip from *Cx. pipiens*. These studies mostly showed unidirectional CI (Blagrove et al. 2012, Calvitti et al. 2015).

In our study, embryonic death with very high mortality was observed in Cross 3 and embryonic death with lower mortality in Cross 2 and Cross 4. The embryonic death and the apparent depression of egg production in Cross 2 between *Wolbachia* uninfected males and females have been discussed previously in an earlier research conducted in 2015. We reported that the reduced fecundity and egg viability in Cross 2 was due to absence of *Wolbachia* in them and that *Wolbachia* had a major impact on the reproductive properties of *Ae. albopictus* (Joanne et al. 2015). In order to make sure that the only factor contributing to the observations and results is absence of *Wolbachia*, we discontinued tetracycline treatment of the adult mosquitoes after the third generation and allowed continuity of two complete generations free from tetracycline treatment. This is to allow reestablishment of any other microbiota that may have lost during the antibiotic treatment (Baton et al. 2013, Joanne et al. 2015).

The lowest mean percentage eggs hatched obtained was 0.74% in the cross between uninfected females and infected males, which was significantly lower compared with all other crosses. Cross 3 fecundity was also significantly lower compared with cross between

infected females and males (control). This shows a very strong incompatibility, with 99. 26% embryonic death in cross 3.

In our previous study, we showed that 91.60% of Malaysian *Ae. albopictus* were superinfected with *w*AlbA and *w*AlbB (Joanne et al. 2015). A recent study reported only crosses between *Ae. albopictus* male with low density of *w*AlbA and *w*Pip-infected *Ae. albopictus* female resulted in complete CI whereas crosses between males with high densities of *w*AlbA and *w*Pip female did not. Analysis of *Wolbachia* density by quantitative PCR of the *wsp* gene showed that *w*AlbA densities were generally lower than *w*AlbB titer in their naturally infected mosquitoes (Calvitti et al. 2015). In our study, *Wolbachia* titer was not quantified but none of the infected samples from field collections were singly infected with *w*AlbA only (Joanne et al. 2015). The 99.26% embryonic death from the cross between infected males and uninfected females in our study could have been due varying density of *w*AlbA in the samples.

Mean percentage eggs hatched for cross between uninfected male and infected female was only 5.35%, and this is significant when compared with control Cross 1. A total of 94.65% of them had complete embryonic death. Although fecundity of the mosquitoes in this cross was not significantly different from control, we would conclude that cross between infected female and uninfected male can also play an important role in population control.

In other studies investigating cytoplasmic incompatibility, only the mating between uninfected females and infected males resulted in unviable eggs. In our study, even the mating between infected females and uninfected males resulted in 94.65% unviable eggs.

In our opinion, if a field release of *Wolbachia* uninfected male and female mosquitoes is carried out, decrease in *Ae. albopictus* population is very likely. However, since mating between these uninfected adults would result in highly unviable eggs and the adults having reduced longevity (Joanne et al. 2015), the sustainability of this release might be at risk. In order for the field trial to work, a continuous release might be necessary. Field release is suggested to target reduction of *Ae. albopictus* population and not the reduction of dengue cases. Currently studies on effect of *Wolbachia* on *Ae. albopictus* susceptibility toward dengue virus is being carried out.

Since only female mosquito bites and has the potential to transmit possible diseases, it would be desirable if a smaller number of female mosquitoes are released compared with the male mosquitoes. Naturally occurring *Ae. albopictus* in nature is superinfected with *Wolbachia*. Even if fewer uninfected female and more uninfected males are released, whenever the uninfected male mates with the naturally *Wolbachia*-infected female, the eggs produced becomes unviable, which would still decrease the mosquito population. However, the next step would be to carry out controlled field releases in a contained environment to observe the potential results of CI. Similar studies should also be carried out with *Ae. albopictus* from different countries crossed with Malaysian *Ae. albopictus* to observe for possible differences. In addition, future studies can be conducted by introducing other *Wolbachia* strains from other host into Malaysian *Ae. albopictus* to study bidirectional cytoplasmic incompatibilities.

In summary, Malaysian *Ae. albopictus* do exhibit unidirectional cytoplasmic incompatibility, and these findings could be used as a possible control measure to reduce *Ae. albopictus* population in Malaysia.

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