ESTABLISHMENT OF NOVEL *WOLBACHIA* SYMBIOSIS AND CHARACTERIZATION OF SEX DETERMINATION GENES IN DENGUE MOSQUITO VECTORS

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ABSTRACT

ESTABLISHMENT OF NOVEL *WOLBACHIA* SYMBIOSIS AND CHARACTERIZATION OF SEX DETERMINATION GENES IN DENGUE MOSQUITO VECTORS

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As one of the deadliest animals on earth, mosquitos transmit numerous diseases to humans, including dengue, Zika and malaria, which account for over one million human deaths every year. Due to insufficiency of traditional vector control strategies, significant efforts have recently been made to develop novel genetic approaches to either directly suppress mosquito populations or reduce mosquito's ability to transmit pathogens to humans. One of them is based on the maternally transmitted intracellular symbiotic bacterium *Wolbachia*. Estimated to infect more than 60% of arthropods in nature, *Wolbachia* can spread through host populations by means of a reproduction-interfering referred to as cytoplasmic incompatibility (CI). By altering the host's physiological environment, including immune priming or metabolic perturbation, *Wolbachia* can also confer antiviral resistance in mosquito vectors. Successful field trials have been conducted to release *Wolbachia*-infected mosquito males to induce incompatible matings for population suppression or spread *Wolbachia* into mosquito populations to reduce or block dengue transmission by population replacement. Both population suppression and replacement require for establishment of an artificial *Wolbachia* symbiosis in mosquito to make it incompatible with target populations. In order to develop a *Wolbachia*-based strategy for dengue/Zika control in Singapore and Mexico, I have established the transinfected line WB2. By comparing with another transinfected line WB1 which developed 15 years ago, I have demonstrated that *w*AlbB maintains a stable symbiosis with *Ae. aegypti*. Further assays show that *Wolbachia* induces strong resistance to dengue, Zika and Chikungunya viruses in WB2. WB2 line has now been

released for field trials in both Mexico and Singapore. In order to improve *Wolbachia*-based mosquito control, transinfected mosquitoes must be optimized to display maximum pathogen blocking, the desired CI pattern, and the lowest possible fitness cost. Achieving such optimization, however, requires a better understanding of the interactions between the host and various *Wolbabachia* strains. Thus, we transferred the *Wolbachia w*Mel strain into *Ae. albopictus*, resulting in a transinfected line, HM (*w*AlbA*w*AlbB*w*Mel), no CI was induced when the triply infected males were crossed with the wild-type GUA females or with another triply infected HC females carrying *w*Pip, *w*AlbA, and *w*AlbB, but removal of *w*AlbA from the HM line resulted in the expression of CI after crosses with lines infected by either one, two, or three strains of *Wolbachia*. These results show that introducing a novel strain of *Wolbachia* into a *Wolbachia*-infected host may result in complicated interactions between *Wolbachia* and the host and between the various *Wolbachia* strains, with competition likely to occur between strains in the same supergroup. In order to manage the potential risk of failure in population suppression in Singapore, I developed another *Ae. aegypti* carrying *w*Mal. The transinfected line showed 100% maternal transmission. To facilitate developing a perfect sex separation approach for *Wolbachia*-based population suppression, I established the CRISPR/Cas9 approach to characterize the function of sex determination pathway genes in *Ae. aegypti*. By individually knocking out *doublesex* (*dxl*) and *transformer-2* (*tra-2*), two essential genes in mosquito sex determination pathway, we show that *dxl* is not essential gene for female development while knockout of *tra-2* results in male-biased sex ratio and absence of female mosquito with homozygous *tra-2.* These results indicate that the *tra-2* is a potential sex determination target that can be explored to develop the female-specific lethality for mosquito sex separation.

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Chapter 1 Introduction

1.1 Aedes mosquito vectors and their transmitted arboviruses

Arboviruses are a group of viruses transmitted by arthropod vectors including mosquitoes and ticks. There are more than 534 viruses in the arbovirus group and, among them, at least 134 species are known to cause human disease[1]. Dengue, Chikungunya and Zika viruses are among the most commonly known arboviruses, with *Aedes aegypti* and *Aedes albopictus* as the primary mosquito vectors. These two mosquito species distribute worldwide and cause epidemic outbreaks of arbovirus diseases every year. According to the CDC, at least 25 US states are affected by these two mosquito vectors, with more areas at risk due to global warming[2].

1.2 Aedes aegypti

Ae. aegypti, first named in 1762[3], belongs to the order Diptera, family Culicidae, and genus *Aedes*. It is also named as yellow fever mosquito due to being identified as the vector of the yellow fever virus in 1900[4]. In 1906, Dengue virus (DENV), the cause of dengue fever, was also proved to be transmitted by *Ae. aegypti[5]*. In addition, *Ae. aegypti* is the primary vector of Chikungunya virus and Zika virus, the two arboviruses that cause significant public health problem in recent decades[6, 7].

The eggs of *Ae. aegypti* are the same as the other *Aedes* species, which are not fertilized until the moment of oviposition. The sperms are stored in the female mosquito's spermatheca after mating. During the oviposition, sperm enters the oocyte when it passes through the opening of the spermathecal duct. Once eggs leave the female, the embryonic development starts as well as a new mosquito life cycle[8]. Same as other *Aedes* in the subgenus, *Ae. aegypti* lay eggs singly, spreading around the location above the water surface[9]. Resistant to desiccation, *Ae. aegypti*

eggs can survive in a wide variety of environmental conditions. The desiccation resistance depends on the development stage of the egg. The early stage of the egg, the first four days after oviposition, is sensitive to dryness. If the eggs are allowed to developed in a humid condition during this period, then the eggs will develop to be highly resistant to desiccation[10]. The eggs can survive for more than 200 days under a favorable condition, such as at 25°C and 70% to 75% relative humidity (RH)[10].

After embryonic development is completed, or in other words, the eggs are mature, they will hatch when exposed to a specific stimulus, with the hatching time mainly dependent on water and food availability. Oxygen level in water is another factor that will influence eggs hatch and low oxygen levels in water usually indicate a high level of microbial activity and nutrients, and will trigger egg hatch[11].

Ae. aegypti larvae experience four instars, from L1 to L4, and can develop in water with PH ranging from 5.2 to 7.6 [12], and oxygen level from 0.6 – 6 ppm[12, 13]. The larval development takes 4 to 42 days, which is affected by the temperature, food supply, larval density and the sex of the larvae. Low temperature, low oxygen level, starvation, and high larval density not only extend the larval development time, but also increase the larval mortality rate[14].

Mosquitos develop into pupae after the larval stage L4. *Ae. aegypti* remains in the pupae stage for about two days under an ideal condition. Pupae do not feed at this stage, during which the water temperature is the main factor affecting pupal development. The pupae stage will take two days at 30°C, three days at 25°C or five days at 20°C[15]. *Ae. aegypti* males emerge before females, and, with enough food supply, the body size of female pupae is larger than males [15].

Mating can start shortly after adult emergence. In a common circumstance, the *Ae. aegypti* males swarm around human and females arrive later and mate as they approach the host to bite. Mating takes place in flight, with the entire process most lasting from 5 to 15 seconds[12]. One copulation is sufficient to fertilize all the eggs that a female will produce during its lifetime[12].

Females require blood-meal to develop their eggs, whereas males only feed on plants. As directly related to disease transmission, the blooding feeding behaviors of *Ae. aegypti* are well studied. It is generally accepted that females start to feed two days post-emergence. Females can take multiple blood meals during a single gonotrophic cycle[16, 17], a behavior that could increase their ability to acquire and deliver pathogens from and to different hosts [18]. The number of eggs laid by a female depends on the age and size of the mosquito and the amount of the blood meal.

As a disease vector, the longevity of mosquito is important due to the fact that greater longevity means a higher probability of transmitting diseases. In a lab condition, adult *Ae. aegypti* females can live up to 80 days. However, longevity is shorter in the field. Depending on the environmental factor and nutrition availability, longevity are varied from a few days up to 40 days[19, 20].

1.3 Aedes albopictus

Known as Asian tiger mosquito, *Ae. albopictus* is originally from tropical and subtropical areas of Asia, and has spread worldwide by international travel and goods. It was first reported in Europe in 1979 in Albania [21], then imported into Texas, USA in 1980 [22]. In 2015, at least 32 US states were reported to present *Ae. albopictus* [22, 23]. In 2017, this mosquito species was recorded in at least 1,568 counties in 40 U.S states[24].

Adapted to both tropical and temperate climates, *Ae. albopictus is* capable of using a wide range of habitats, including water-filled tires, cemetery vases, birdbaths, other artificial containers, and tree holes [13]. It is active all year long in the tropical regions, whereas it overwinters by diapausing in the egg stage in the temperate or subtropical regions[8, 25].

Ae. albopictus has a white/silver string on the back, a straightforward feature to distinguish *Ae. albopictus* from other *Aedes* mosquito species. In laboratory conditions with 26°C and 50% to 60% RH, the *Ae. albopictus* females can live up to 117 days [16]. However, in the field, its longevity is much shorter, varying from 15 to 40 days in 20°C [26]. The life cycle of *Ae. albopictus* is very similar to *Ae. aegypti.* However, the habitat of these two mosquito species is different. *Ae. aegypti* is more adapted to urban condition and prefers to lay its eggs in artificial, rather than natural containers, whereas *Ae. albopictus* prefer rural environment and can use the natural habitant to breed, such as a tree hole.

1.4 The arbovirus

1.4.1 Dengue virus

The dengue virus (DENV), belonging to the genus *Flavivirus* of the family Flaviviridae, is a positive-stranded RNA virus with four serotypes, DENV-1, DENV-2, DENV-3, and DENV-4. Structurally, DENV has about 11,000 bases of the positive-sense, single-stranded RNA genome, which codes three structural protein (capsid protein C, membrane protein M, envelope protein E), seven non-structural protein (NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5) and a short non-coding region on both 5' and 3' ends [27].

Formed as a dimer on the surface of the mature virus, E protein is a glycoprotein belonging to class II viral membrane fusion protein, around 400 amino acids long [28]. E protein monomer comprises one C-terminal transmembrane domain and three ectodomains (D-I, D-II and D-III).

The D-I protein resides at the center of the mature monomer. The D-II has a stable core structure with finger-like loops and is involved in the attachment of the virus to the host cell [29]. The D-III protein has an Ig-like structure, which may involve in the recognition of the host cell receptor [30]. Because the role of E protein during DENV infection is attachment and penetration into the host cell, it has been extensively studied for drug discovery and vaccine development [31].

The Precursor-membrane protein (prM) consists of seven antiparallel β-strands stabilized by three disulfide bonds called predomain, followed by an M-domain, stem region, and two transmembrane helices [32]. The transformation of the immature virus to a mature virus involves the rearrangement of prM and E-protein. A furin-mediated cleavage site at the interface of predomains and M-domains inside Golgi apparatus leads to the conversion of prM to M-protein and maturation of virus[33].

Essential for nucleocapsid formation, the Capsid protein (C protein) is involved in the very first step in viral assembly. Nucleocapsid comprises a single RNA strand and multiple capsid molecules arranged in an asymmetric manner. The aggregation of nucleocapsid is caused by the interaction of Capsid-RNA, due to the neutralization of positive charge by viral RNA [33].

The non-structural protein 1 (NS1) is a multifunctional dengue glycoprotein, and its functioning is still unknown. NS1 can be used as a diagnostic marker for early diagnosis of Dengue, because it is secreted into blood before the antibodies are produced [34]. The secretion of NS1 activates the immune response and results in the production of anti-NS1, which is positively related to severe dengue symptoms [33]. NS2A plays a role in viral replication, assembly, pathogenesis, and immune evasion [35]. NS2B protein majorly acts as a factor for NS3 proteolytic activity [36]. NS3 protein is a multifunctional protein that acts as a serine protease, adenosine tri-phosphatase (ATPase), RNA tri-phosphatase (RTPase), and RNA helicase[33]. NS3 has a crucial

role in viral replication, which is a target for antiviral drug development. NS4A is a scaffold for the virus replication complex, involving in altering cell membrane curvature and induction of autophagy [37, 38]. NS5 protein is the largest protein produced by DENV, and both NS3 and NS5 are the key catalytic enzyme in viral replication and capping. The N-terminal region of NS5 has a methyltransferase (MTase) domain, which is capable of catalyzing all four activities: guanylyl-transferase, guanine-N7-methyltransferase, nucleoside-2′O-methyltransferase, a prerequisite for 5′-RNA capping[33]. NS5 is also found to have a significant role in modulation host immune response[39].

The infection cycle of DENV starts when virus binding to the cell membrane by E protein, then receptor-mediated endocytosis occurs and virus enters the cell. In the endosome, the low pH leads to E protein trimerization and the structural alteration. Followed by the releasing of viral RNA into the cytoplasm. The host machinery is used by the virus to synthesis viral poly-protein. The poly-protein is processed and released by viral (NS2A, NS2B and NS3) and host proteases. The viral RNA replication occurs by viral NS1, NS3, NS4B, and NS5 protein. The viral assembly take place at host ER surface and NS1 and SN2A are involved in the process. Furin mediated cleavage of pre-domain of M protein at trans-Golgi network. The pre-domain continues to attach to the virus surface, which prevents the binding of the progeny virus to the host cell membrane. After the release of progeny virus, per-domain of M protein is then released from the virus[33].

Dengue fever is a severe flu-like infection affecting all age groups[40]. In 1997, dengue fever was divided into three groups: undifferentiated fever, classic Dengue fever (DF), Dengue hemorrhagic fever (DHF) [41]. With undifferentiated fever, it is difficult to differentiated from other viral diseases and usually remains undiagnosed, mostly seen in primary infection. Dengue

fever is a self-limiting fever, usually lasting 5 to 7 days. The clinical features of DF are varied according to the age of the patient. The infant and young children may have undifferentiated fever with rash, and the older children and adult may have mild fever or high febrile syndrome with severe headache, retroorbital pain, myalgia, arthralgia, nausea, vomiting, and petechiae [42]. The Dengue hemorrhagic fever is characterized by symptoms of DF with thrombocytopenia, hemorrhagic manifestations, and plasma leakage. Plasma leakage is the most important difference between DF and DHF, which is used to determine disease severity. High plasma leakage cases are marked by "frank shock with low pulse pressure, cyanosis, hepatomegaly, pleural and pericardial effusions, and ascites, which also known as dengue shock syndrome (DSS)" [40]. In severe cases, with critical plasma loss, DSS may be life threatening if not treated properly.

The primary infection is defined as the first-time exposure of an individual to any of the five dengue virus serotypes. The primary infection is less like to develop an asymptomatic infection. During the primary infection, IgM and IgG antibodies will reach high titers in 3-5 and 6-10 days, respectively. The IgG will persist for a lifetime [43]. A secondary infection with a previously unencountered DENV serotype increases the chance of developing DHF. This is caused by antibody-dependent enhancement (ADE). ADE occurs when preexisting antibodies present in the body (IgG) from a primary dengue infection bind to a different dengue serotype. The antibodies from the primary infection cannot neutralize the virus. Instead, the antibody–virus complex attaches to receptors called Fcγ receptors on monocytes, resulting in that the virus infects monocytes more efficiently[44].

1.4.2 Zika Virus

Zika virus (ZIKV) belongs to the Flaviviridae family consisting of the Flavivirus genus and Hepacivirus genus. It is a positive, single-stranded RNA virus with an envelope [45]. ZIKV was first isolated from a monkey in Uganda in 1947 [46], and the first case of human infection was reported in 1954 [47]. The first major zika fever outbreak took place in 2007 in the Western Pacific Island of Yap [48]. Since then, multiple outbreaks have been reported worldwide. The outbreak in 2013 and 2014 in French Polynesia affected over 30,000 people [49]. In March 2015, Brazil reported a severe outbreak of disease, soon be identified as Zika. Subsequently, WHO declared Zika virus epidemic as a Public Health Emergency of International Concern (PHEIC) in 2016. To date, a total of 86 countries and have reported mosquito-transmitted Zika infection[6]. Different from 4 serotypes presented in DENV, ZIKV has only 1 serotype.

The ZIKV RNA codes for a polyprotein that can be cleaved into 10 proteins, including the 3 structural proteins (C, prM/M, and E) and 7 non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The structure of the ZIKV is highly identical to DENV and other viral members in the Flaviviridae family.

The common symptoms caused by for ZIKV include fever, joint pain, red eyes, headache, and a maculopapular rash. Despite the similarity in the structure and the replication strategy of ZIKV with other flaviviruses,, the downstream diseases caused by ZIKV can be remarkably different. For example, Zika virus can traverse the placental barrier and causes robust infection in the fetal brains, resulting in teratogenicity or fetal demise[50].

1.4.3 Chikungunya Virus

In the early time, the Chikungunya fever was treated as Dengue fever. The first described case is in Tanzania in 1952 [51]. In 1953, the virus was isolated from patients as well as *Ae. aegypti* and

Culex spp. [52]. The virus belongs to the genus Alphavirus, a member of the Togaviridae family. After a large outbreak in Kenya in 2004, CHIKV spread to the Indian Ocean islands, India and Southeast Asia, with more than 6 million cases reported [53]. Subsequently, it caused 215,000 people infected in Comoros in 2005 [54], and 250,000 people infected in Reunion Island from March 2005 to April 2006 [55]. In 2007, the virus was introduced by a traveler from India to Italy [56]. In 2010, an autochthonous case was reported in France [57]. Until now, CHIKV infections have been reported in countries on all continents, except Antarctica, according to CDC[58].

CHIKV is an enveloped positive-strand RNA virus, and its genome is about 12 kb in length and encodes four non-structural proteins (NS1 to NS4) at 5' end, 5 structural protein (C, E1 to E3 and 6K) and poly(A) tail at 3' end. Four CHIKV lineages have been identified so far, including West African and East/Central/South African (ECSA), Asian lineage and Indian Ocean lineage. Each lineage has distinct genotypic and antigenic characteristics.

CHIKV enter the host cell by receptor-mediated endocytosis, and DC-SIGN, L-SIGN, heparin sulphate, laminin, integrins and prohibitin have been implicated in this process [59]. Following endocytosis, the acidic condition in the endosome triggers the formation changes in the viral envelope, the E1 peptide exposed and mediated virus-hose membrane fusion. The process allows cytoplasmic delivery of the viral core and releases the genome. Precursors of non-structural proteins are translated from the viral mRNA and cleaved into NS1–NS4 protein [60]. NS1 involves in negative-strand RNA synthesis, and NS2 is Helicases with proteases activity. NS3 functions in RNA synthesis and NS4 is RNA dependent RNA polymerase. These four proteins assemble to form the viral replication complex. The full length negative-strand viral RNA is synthesis intermediate by replication complex, and used as a template for both subgenomic and

genomic RNAs. The C–pE2–6K–E1 polyprotein precursor is then expressed from subgenomic RNA, and further processed PreE2 and E1 glycoproteins. In Golgi body, PerE2 is cleaved into E2 and E3. The viral RNA binding with nucleocapsid, and recruitment of the envelope glycoproteins promote viral assembly. The assembled alphavirus particle then buds at the cell membrane, and released from the cell [60].

The pathogenesis of CHIKV infection in humans is still poorly understood. Following the inoculation of the virus by infected mosquitos, the CHIKV enters the subcutaneous capillaries and infects macrophages, fibroblasts and endothelial cells in the skin, where viral replication is limited [61]. The virus that replicated in the skin then transports to secondary lymphoid organs and enters into the blood. Once in the blood, the virus has access to various organs and tissues, e.g. liver muscle, joints and even brain [62]. CHIKV infection induces strong host immune responses, including systemic innate response, inflammatory response and cellular immune response.

Chikungunya fever is characterized by an abrupt febrile illness polyarthralgia and maculopapular rash, although 5% to 15% infection is asymptomatic [63]. The acute symptoms usually last less than 2 weeks, and the arthralgia may last for weeks to years [64]. Generally, Chikungunya fever is not considered as life-threatening, nevertheless, severe forms also are reported. Patients with severe chikungunya fever tend to be older and with comorbidities such as cardiovascular, neurologic, and respiratory disorders or diabetes [65].

1.5 Interactions of arboviruses with mosquito hosts

In the natural course of virus infection in the mosquito, the virus is ingested with blood, and then the virus must overcome several barriers to establish infection in mosquito. The first barrier is in the midgut, where virus has to infect and replicate in the midgut epithelium, then escape from the midgut, and enter into the mosquito hemolymph [66]. The infection rate of the midgut is dose-dependent; the higher the virus titer will result in a higher infection rate[67]. After entering into the hemolymph, virus will cause systemic infection in the mosquito body. The final barrier is in the salivary glands, where the virus will infect the salivary gland and enter the saliva before transmitted to next host. The virus titers will reach a peak in the midgut between 7 to 10 days post-infection, in the salivary gland between 10 to 21 days post-infection[68]. The time from mosquito intake the virus to virus enter the saliva is called the extrinsic incubation period (EIP). The length of EIP is depended on the virus, the mosquito host, and the temperature. The EIP for Dengue and Zika viruses range from 10–15 days at 25 °C[69], and 5 days to 20 days[70-72], respectively.

In the mosquito host, the host innate immunity and microbiome functional together to against virus infection. The innate immunity includes three main pathways, Toll pathway, IMD pathway and JAK-STAT pathway. The immune pathway is activated by receptor recognizing virus, resulting in the expression of antiviral peptides and other antivirus effectors[73]. The microbiome in the mosquito gut, with more than 40 different of bacteria isolated from the gut of *Ae. aegypti*[74], forms a complex ecological environment and can play important role in the vector competence. Amon them, the maternal transmitted Wolbachia causes systematic infection in mosquitos, with impacts in different mosquito systems, e.g. immune system, metabolism

system. The detailed effect of *Wolbachia* on mosquito vector competence will be reviewed in the later sections.

1.6 Arboviral disease control

Vector control, drugs and vaccines are the primary methods used for vector-borne disease control. Similar to other infectious disease control, vaccines help individuals to reduce the infection risk. Currently there is no effective drug for most of arboviruses, including dengue, Chikungunya and Zika. Due to insufficiency of vaccines and absence of drugs, vector control becomes the primary intervention for vector-borne diseases.

1.6.1 Vaccines

The adaptive immune response to flaviviruses involves both humoral and cell-mediated components. Neutralizing antibody responses target the E protein and are cross-reactive with other flaviviruses. This cross-reactivity has been an issue for diagnostic tests and vaccine development. The presence of four DENV serotypes raises the main challenges for vaccine development because of the risk in developing Antibody-dependent enhancement (ADE) when the exposure of Dengue primed individuals to a different serotype. The vaccine must be tetravalent to balance the immunity against currently epidemic DENV. Another challenge is that Dengue does not induce long-lasting heterotypic immunity, and subsequent infection after a long-time interval may lead to severe dengue fever.

CYD-TDV, or Dengvaxia, is a vaccine commercially available in 2016, and was approved by FDA in the United States in 2019. Evidence indicates that Dengvaxia is partially effective in preventing infection. CYD-TDV is a live attenuated tetravalent chimeric vaccine, made by replacing the PrM and E structural gene of yellow fever 17D strain vaccine with genes from four dengue serotypes[75, 76]. However, CYD-TDV is suggested to be used only for those

individuals previously infected with Dengue. The uninfected individuals who receive CYD-TDV may develop severe disease. As such, the company made a statement that Dengvaxia poses higher risks to people without prior dengue infection.

Currently, no commercial Zika vaccine available in the market. As the end of 2019, four vaccines were entered phase-1 clinical trials, six vaccines were in the preclinical and animal studies and others still in early-stage research [77]. Similarly, no commercial vaccine against CHIKV is available at present. The outbreak of Chikungunya fever since 2004 have boosted CHIKV vaccine research and development. Some vaccine has entered the Phase-1 or Phase-2 stage of clinical trials, whereas others are still in early research [78].

1.6.2 Self-protection

For individual protection, especially during the outbreak, clothing that minimizes skin exposure during mosquito active time could reduce the risk of mosquito biting. Mosquito repellent may be applied to exposed skin and clothing. For those indoor-biting mosquitos, insecticide aerosol products, mosquito coils, insecticide-treated mosquito net may reduce the biting risk. Household fixtures such as door/window screens, air conditioning, mosquito traps can also reduce biting.

Planed travel and self-quarantine could reduce the infection risk as well as disease transmission. Avoiding travel to the area of the outbreak, following individual protection guidelines, choosing a hotel with air conditioning and windows/doors screen may reduce infection risk. After the return from the risk area, if a sign of infection is identified, seeking a doctor immediately. Even with no sign of infection, still prevent mosquito bit for 3 weeks to avoid potential spread infection by a mosquito to others. To prevent Zika transmission by sex, ensure using personal protection. If woman in the high risk area is pregnant or plans to be pregnant, seeking a doctor for specific guidelines always be the best way to prevent congenital Zika syndrome.

1.6.3 Vector control

The current mosquito control strategies falls into five categories: chemical control, physical control, environmental management, genetic control, and biological control. Each of the single control method/strategy has its advantage as well as disadvantage. Combining different methods can increase the effectiveness in mosquito control. For example, use of chemical Insecticide-treated bed net not only can physically prevent human-mosquito contact but also kill mosquito in contact and reduce environment damage. Modification of the water channel/river in the environmental management category which makes mosquito pupa and larva easier to be discovered by predator, combining with introducing a mosquito predator, will multiply the efficiency in vector control.

1.6.4 Chemical control

Chemical control involves insecticides and insect growth regulators. There are four main classes of insecticides: pyrethroids, carbamates, organophosphates, and organochlorines, which are all neurotoxic to insects. Nonetheless, indiscriminate use of those chemicals causes damages not only to non-target insects, but also to human health and the environment.

The organochloride group is the oldest group of insecticides used against insect pests. The most notorious one in this group is the DDT (Dichlorodiphenyltrichloroethane). Insecticides in this group affect the sodium channels, and cause these channels to remain open, resulting in hyperexcitation [79]. Organochlorides are lipids soluble, therefore, accumulate in fat bodies, which will affect the entire food chain. Organochlorides cause various damage and health risk in humans as problems are observed in the central nervous system, immune system, liver, kidney, heart and bone marrow [80]. Organochlorides also are suspected of causing hormone-dependent tissue cancer [81]. In addition, these insecticides disrupt the endocrine system and affect the

feeding behavior, immune system and reproduction of birds [82], resulting in decreases of the raptorial bird population. Consequently, the general use of DDT was ban on 1972.

Compared to organochloride, organophosphates have many advantages as they are biodegradable and do not accumulate in tissue. Its drawback is lack of stability, thus requiring for periodic applications [83]. In insects, organophosphates inhibit the cholinesterase, which is required for acetylcholine degradation, resulting in degradation of acetylcholine in nerve endings. The organophosphates are more toxic to vertebrates than organochlorides, they can circulate in the human body and reach adipose tissue, liver, kidneys, salivary glands, thyroid, pancreas, lungs, stomach, intestine, central nervous system, and muscles. Evidence shows that exposure to organophosphates increases the risk of Parkison Diseases [79]. Organophosphates were banned by EPA for most of the residential uses in 2001, although agricultural use is still permitted.

Carbamates are chemicals derived from carbamic acid. Similar to organophosphates, carbamates have rapid, lethal action against insects by inhibiting the acetylcholinesterase. The acetylcholinesterase is an enzyme catalyzing the hydrolysis of acetylcholine. Carbamates are potentially toxic to humans, have effects on the central and peripheral nervous systems, and are immune-suppressing or carcinogenic. Carbamates do not accumulate in the body; however, their effects are cumulative [84].

The last common insecticide category is pyrethroids, which are biodegradable, do not accumulate, and rarely cause acute poisoning in birds and mammals. The effect of pyrethroids on an insect is to paralyze the insect central and peripheral nervous system. However, pyrethroids are extremely toxic to aquatic animals[85]. It was also observed that the use of pyrethroids and DNA damage on sperms are correlated, which raises the concern about the indiscriminate use of pesticides[86].

Other than the environmental hazard and toxicity, a common issue for using insecticide to control mosquito is the development of insecticide resistance in the mosquito population. Until 2015, mosquito resistance to insecticides has been reported in more than 60 countries, affecting all of the major vector species and all classes of insecticides, according to CDC[87]. The mutation of sodium channel structure with either point mutations or substitutions, resulting in a reduction in or an elimination of the binding affinity of the DDT/pyrethroids to proteins. This heritable mutation results in the DDT/pyrethroids insensitivity of the mosquito population[88]. The AChE1 and AChE2 are acetylcholinesterase (target by insecticides carbamate and organophosphates) in mosquito, which is encoded by *ace-1* and *ace-2* genes. Two mutations from the active site of AChE1 in mosquitoes result in insensitivity or reduced sensitivity in response to organophosphates and carbamate insecticides[89]. Along with those described disadvantages, chemical control of mosquito needs to be combined with other methods like physical control method and environmental management to achieve effective disease prevention.

1.6.5 Physical control and environmental management

Many mosquito control methods hard to be classified into physical control or environmental management. For example, changing the water in the container, removing the possible mosquito larva habitat, placing the mosquito traps, screening of doors and windows. However, environmental management more focuses on large scale modification that preventing or minimizing vector propagation and reducing man-vector-pathogen contact. As the definition of environmental modification states: "A form of environmental management consisting in any physical transformation that is permanent or long-lasting of land, water and vegetation, aimed at preventing, eliminating or reducing the habitats of vectors without causing unduly adverse effects on the quality of the human environment[90]."

1.6.6 Genetic control

The strategies of genetic control of mosquito can be classified into suppression and replacement. The suppression strategy target to reduce the number of mosquito vectors, for example, sterile-male method, in which modified males are released to mate with wild females. The genetic modification will result in the death of the offspring after mating[91]. The female killing system is one of the suppression strategies. The modified/inserted female-specific lethal gene would inherit by the offspring and reduced female population numbers and finally lead to the collapse of the population[92]. A good example is that the genetically modified Ae. aegypti establish by Oxitec which use the approach called RIDL (release of insects carrying a dominant lethal gene). The RIDL Ae. aegypti have LA 513 transposon inserted into DNA. The insertion resulted the death of the mosquito larva unless feed on tetracycline[93]. The replacement strategy replaces the wild mosquito population with a genetically modified mosquito that carries a virus or parasite resistance gene[94].

Gene drive system is a key method to establish and spread the modified mosquito into the wild population. The gene drive is the genetic element that able to copy itself to another chromosome in order to have all offspring carrying the modified gene[95]. There are several candidate gene drive systems, one of the systems is transposable elements (TEs). The TEs are able to move to new locations in the host DNA, at the same time, increases the copy number of themselves. The increased copy number increased the frequency of inheritance from a heterozygous[96]. Another candidate system is homing endonuclease genes(HEGs). HEGs recognize and cut a specific sequence, then the cut chromosomes will be repaired use HEGs as the template[97]. CRISPR-Cas9 gene drive is one of the gene drive systems that have been applied in the mosquito, which has a similar function as HEGs[98]. The Cas9 protein uses a short RNA as a guide to recognize

and cut target, and then the cut gene is repaired by the host. During the reparation, a template also is required[98].

1.6.7 Biological control

The biological control strategy is the mosquito control through utilizing predatory species and pathogenic microorganisms or exploiting mosquito physiology, behavior and genetics to improve mosquito mortality and releasing mosquitoes that are either sterile or unable to transmit diseases [99-101].

Pathogenic microorganisms include entomopathogenic fungi and bacterial agents. Fungus species that are used for this purpose belong to the genera Beauveria, Coelomomyces, Metarhizium, Culicinomyces, Entomophthora and Lagenidium [102]. Bacillus thuringiensis var. israelensis (Bti) is a gram-positive, spore-forming bacterium that releases insecticidal toxins and virulence factors that selectively target the larval stages of insects[103, 104]. Plant-borne molecules are often effective at a few parts per million (ppm) against *Aedes*, *Anopheles* and *Culex* larva, more than 80 plant species have been employed for the successful synthesis of nanomosquitocides [105, 106].

Natural enemies (predatory species) feeding on mosquito larvae and pupae in aquatic environments can play an important role in reducing the mosquito population. A large number of aquatic organisms including fish, amphibians, copepods, odonate young instars, water bugs, and even larvae of other mosquito species, can feed on mosquito larva. The use of predator of mosquito larvae has been recorded in many habitats, from small containers to complex natural ecosystems, including coastal wetland environments, and has demonstrated to be very effective at reducing mosquito larval populations[107]. However, introducing predatory species may have

a negative impact on the local ecosystem, and the introducing species may not adapt to the new environment.

Another novel biology control strategy is using mosquitos to control mosquitos, which has recently received great attention. This strategy involved using radiation-based sterile insect technique (SIT), Wolbachia infected mosquito as well as the transgenic mosquito to either induce sterile mating or reduce mosquito's vector competence for arboviruses. Among them, *Wolbachia*-based mosquito control has shown great potential in disease control, which will be discussed in detail below.

1.7 Wolbachia and Wolbachia-based vector control

1.7.1 Wolbachia

Estimated to infect ~40% of all terrestrial insect species[98] and approximately 28% of the surveyed mosquito species [108, 109], Wolbachia is maternally transmitted, gram-negative intracellular α-proteobacterium, and belongs to the order of Rickettsidae and the genus of *Wolbachia pipientis*. It was first discovered by Hertig & Wolbach in the mosquito *Culex pipiens* in 1942 [110]. *Wolbachia* spp. can further be divided into six major clades/groups, from A to F. A, B, E and F have been reported from insects, arachnids, and crustaceans, whereas C and D groups are identified from filarial nematodes. Wolbachia carried by mosquito mainly falls into A and B groups [111].

In mosquito, *Ae. albopictus* is naturally infected by *Wolbachia* strains *w*AlbA and *w*AlbB, and most Culex mosquito species carry *Wolbachia* wPip strain. Wolbachia was considered absent from *Ae. aegypti* and *Anopheles* mosquitoes. However, recent studies show *Wolbachia* sequences were detected in those mosquito species[112]. These native infections present at low infection

prevalence and extremely low titer and do not induce cytoplasmic incompatibility. Further sequence-independent evidence is needed to understand these new symbiosis[112].

The first sequenced *Wolbachia* is *w*Mel, a strain from *Drosophila melanogaster* [113]. Until 2013, *w*AlbB, *w*Pip, *w*Au, *w*Ri and *w*Bm were sequenced[114] and more *Wolbachia* sequences were published and studied[115]. As an intracellular bacterium, *Wolbachia* has a smaller size of the genomes compare to those free-living bacteria. Evidence indicates that many of the recent gene losses are those involved in cell envelope biogenesis [113]. The size of the *Wolbachia* genome is around 0.9 Mb to 1.7 Mb [116]. In the *Wolbachia* genomes, large segments of mobile and repetitive DNA have been identified, the acquisition and preservation of these repeated and mobile elements are hypothesized to play crucial roles in *Wolbachia* evolution [113]. Another important mobile element found in the *Wolbachia* genome is the *Wolbachia* prophage/bacteriophages (WOs) sequence. It is reported that the WO phage in *Wolbachia* contains genes responses for regulating *Wolbachia* density and CI expression [117, 118], and the base composition of WO phage DNA is similar to *Wolbachia* chromosome, suggesting that WO phages have been associated with *Wolbachia* for a long time [119]. It is likely that the WO phage may provide a potential tool to study the function of *Wolbachia* genes, a field that has largely lag behind due to the lack of an effective tool in the genetic modification of *Wolbachia*.

1.7.2 Wolbachia-induced Cytoplasmic incompatibility (CI) and other reproduction alteration

An ability to alter host reproduction is the unique characteristic of *Wolbachia*. First noticed in *Culex pipiens,* CI is the most common host reproduction alteration induced by *Wolbachia* . It was initially discovered that certain intraspecific crosses within *Culex* mosquito were incompatible, and their offsprings had a very low hatch rate or even none were hatched [120-122]. In addition, this incompatibility factor had a cytoplasmic inheritance pattern – only passing

from mother to their offsprings - and thus the phenotype was named as cytoplasmic incompatibility [122]. In 1970, CI was associated with the presence of bacteria, based on the observation that males from an infected line were incompatible with antibiotically cured females derived from the same infected line, whereas the reciprocal cross was compatible[123].

CI is an early embryonic death which occurs when the *Wolbachia*-infected male mates with either an uninfected female or a female carrying a different strain of *Wolbachia*[124]. At least eight arthropod orders are documented with CI: Acari, Coleoptera, Diptera, Isopoda, Lepidoptera, Hymenoptera, Homoptera and Orthoptera. A mating incompatibility between uninfected female and infected male is called unidirectional CI, which can also happen when females carry the other *Wolbachia* strains in addition to the one carried by males. The Bi-directional CI occurs between female and male that are infected with different strains of *Wolbachia*[125]. CI can be explained using a modification-rescue (or poison-antidote) mode. The mosquito sperm is modified by *Wolbachia* in the male and the same *Wolbachia* strain has to present in the eggs to rescue the modification. Some Wolbachia strains have both modification (mod+) and rescue(resc+) function but other Wolbachia may have only one of those functions. Based on that, *Wolbachia* strains can be classified into four phenotypic categories: mod^{+} $resc^{+}$, mod^{-} $resc^{+}$, mod^{-} $resc^{-}$, and mod^{+} $resc^{-}$. The mod^{+} $resc^{-}$ strains are called suicide strains which have not been observed in nature[126]. It was observed that the modification induced by one *Wolbachia* strain was rescued by another *Wolbachia* strain, *e.g.* the modification induced by *w*Mel can be rescued by *w*Ri and *w*Tei can rescue the *w*Ri modification[127]. The modification and rescue phenotype may be affected by the host genetic background. For example, *w*Tei has both modification and rescue function in is natural host, *D. teissieri,* but the rescue function is lost when transinfected into *D. simulans*[126].

There is general consensus on the below three points when *Wolbachia*-induced CI is described. First, *Wolbachia* is not present in the matured sperm, as *Wolbachia* and cytoplasmic mass are eliminated into the waste bag during spermiogenesis [128]. Second, when sperm from infected male fertilize an uninfected egg, paternal chromosome segregation is observed to lag behind during the first mitotic division, resulting in early embryo death[129]. Third, the modified sperm can be rescued when the same *Wolbachia* present in the infected egg. Base on these observations, three different models, including ‘‘lock-and-key’’, ‘‘titration–restitution’’ and the ‘‘slow-motion’’ models [130], were proposed to explain the CI mechansim. All three models include the paternal chromosome modification. The differences are that Wolbachia is proposed to add a lock or slowdown factors into the chromosome in lock-and-key and slow-motion models, respectively, whereas Wolbachia removes host protein from the paternal chromosome in titration–restitution model. In the CI embryo, during the first mitoses, the added lock or removed protein prevent the chromosome from duplication, and the slow down factor results in asynchrony of male and female pronuclei. During the process of rescue, Wolbachia in the lock-and-key model functioned as a key to remove lock. In the titration–restitution model, the removed protein is given back by Wolbachia. In the slow-motion model, the slow down factors are added to the maternal chromosome and male and female pronuclei are once again synchronous. All these rescue processess resut in normal mitoses[130].

Based on mass spectrometry and SDS-page, Beckmann and Fallon identified *Wolbachia w*Pip prophage WO protein WPIP0282 in spermatheca, indicating that the prophage WO may contribute to CI[131]. A year later, genomic comparisons of CI inducing *Wolbachia w*Mel and no-CI inducing *Wolbachia w*Au identified nine genes that were absent in *w*Au, in which WD0631 is a *w*Mel homolog of wPip WPIP0282. WD0632 is adjacent to WD0631[132], and

both were identified as the CI factors, CifA and CifB, in *Wolbachia* *w*Mel and *w*Pip in 2017[118, 133]. It was observed that singly expression of CifA or CifB in males did not induce CI, but the dual expression of the genes caused a rescuable phenotype similar to CI. In addition, singly expression of CifA in females can rescue CI[134]. Comparative sequence analysis of Cif proteins suggests that at least five phylogenetic clades of CI gene, from type 1 to type 5, only type 1 cifs from *w*Mel and type 4 cifs from *w*Pip have been confirmed to cause and rescue CI experimentally. The structural study suggests that Type 1 CifA has three putative domains. One catalase-related domain is related to both CI inducing and rescue, and one unknown function domain with homology to a Puf-family RNA-binding domain is only related to CI inducing. The last domain is a sterile-like transcriptional regulator, which has no impact on CI. How CifA involved in both CI and rescue is still unknown. The Type 1 CifB encodes a single putative ubiquitin-like protease, which can cleave ubiquitin chains *in vitro*, and Type 4 CifB has a nuclease domain which can cause DNA breaks *in vitro*.

The discovery of CI factors uncovers bacterial factors involved in CI mechanisms. However, how they interact with the host factors to induce CI expression still remains unknown. In additon, how different *Wolbachia* strains interact each other during CI expression has yet to be understood.

1.7.3 Wolbachia-mediated pathogen interference

Wolbachia-mediated pathogen interference has been observed in *Drosophila*[135], mosquito[136] and planthopper[137], with both viral loads and prevalence (infection rate) significantly reduced. Mosquitoes transinfection with either of *w*AlbB, *w*Mel, *w*Melpop, or *w*Pip strains are resistant to flavivirus, including dengue virus (DENV), Zika virus (ZIKV) and West Nile Virus (WNV), Yellow fever virus (YFV)[136, 138-140], and alphavirus such as

Chikungunya virus (CHIKV)[140]. Both flaviviruses and alphaviruses are positive-strand RNA viruses. In *Drosophila*, *Wolbachia* also inhibits positive-strand RNA viruses, including Alphanodavirus (FHV), Cripavirus (DCV)[141]. *Wolbachia*-mediated inhibition of double-stranded RNA viruses is also observed in both planthopper and Drosophila [137, 142]. In addition to viral inhibition, Wolbachia also suppress gram-positive and gram-negative bacterial infection, filarial nematode, and malaria parasite in mosquitoes. However, evidence on inhibition of negative-sense RNA viruses is limited[143] and *Wolbachia* is even observed to enhance DNA viruses in Aedes cells[144].

Wolbachia inhibits virus at mutiple life stages. Evidence indicates that Wolbachia inhibits the binding of DENV and ZIKV to the mosquito cell[145], viral entrance into cells, and replication [143]. In additon, the viral RNA has a faster turnover in *Wolbachia* infected cells and the viruses released from Wolbachia infected cell are less infective [146].

The strength of *Wolbachia*-mediated viral inhibition depends on *Wolbachia* strains and host genetic background[147]. After being transferred into mosquitoes, *w*Melpop and *w*Au induced strong pathogen blocking but also resulted in high fitness costs to the host. *w*AlbB, *w*Pip and *w*Mel induced intermediate pathogen blocking, with fitness cost ranging from intermediate to low (Table 1). By contrast, some *Wolbachia* strain, such as *w*Ri and *w*AlbA indued low or no inhibition to viruses in the transinfected mosquitoes (Table 1).

Table 1.1 Pathogen blocking effect and fitness cost induced by different *Wolbachia* strains in transinfected mosquitoes[135, 136, 148-150].

***Wolbachia* variant**	**host**	**CI**	**Fitness cost**	**Pathogen blockage**
*w*AlbB	aegypti	Complete	Intermediate/low	Intermediate
*w*Mel	aegypti	Complete	Low	Low-Intermediate

Table 1.1 (cont'd)

*w*Ri	aegypti	Near-complete	Low	Low
*w*MelCS	aegypti	Near-complete	Intermediate	Intermediate
*w*Pip	aegypti	Complete	Low	Intermediate
*w*Au	aegypti	No	High	High
*w*AlbA	aegypti	Near-complete	High	None
*w*MelPop	aegypti	Complete	High	High
*w*AlbB/wMel	aegypti	Near-complete	Intermediate	Intermediate
*w*Au/wAlbB	aegypti	Complete		
*w*AlbA/wAlbB/wMel	aegypti	wMel no CI	High	
*w*Ri	albopictus	Near-complete	High	
*w*MelPop	albopictus	Intermediate	High	High
*w*Pip	albopictus	Complete	Intermediate	
*w*Mel	albopictus	Complete	None	High
*w*RivB	albopictus	Complete		
*w*AlbA /*w*AlbB/*w*Pip	albopictus	Complete	None	Intermediate
*w*AlbA/*w*AlbB/*w*MelPop	albopictus	High	Intermediate	
*w*Mel/*w*Pip	albopictus	Complete	None	Intermediate
*w*AlbA /*w*AlbB /*w*Mel	albopictus	Complete, but partially self-incompatible	High	

Although the mechansims underlying *Wolbachia*-mediated pathogen interference is not fully understood, evidence indicates that multiple pathways involve in this process. *Wolbachia* can prime mosquito immune system to induce virus resistance [151]. *Wolbachia* infection is known to activate Toll pathway in *Ae. aegypti*[152], which functions to defense against fungi, Gram-positive bacteria, and DENV[151]. This activation is induced by increased production of reactive oxygen species (ROS) due to *Wolbachia* infection[153]. The upregulation of Toll pathway leads to transcriptional upregulation of the antimicrobial peptides and other immune genes [154]. *Wolbachia* also induces the Imd pathway which may also contribute to an antiviral effect[155].

Small RNA pathways, RNA interference and miRNA are important for antiviral response in insects[156]. *Wolbachia* infection is also known to alter the expression of several miRNA in *Ae. aegypti* [157], which may contribute to *Wolbachia*-mediated viral interference.

Additional evidence also supports the competition between *Wolbachia* and pathogen for cellular resources as an alternative mechanisms. For example, *Wolbachia* is able to regulate host iron homeostasis which is critical for viral replication [158]. *Wolbachia* downregulates insulin receptor, and fatty acid synthase (FAS) - a key enzyme in the fatty acid biosynthesis pathway- and deletes certain lipid classes (eg., diacylglycerols) in mosquito hosts, resulting in a physiological environment hostile for pathogen growth [159-161]. Previous studies also indicate that depletion of cholesterol in the host cell by *Wolbachia* may contribute to viral blocking in mosquito[162].

1.7.4 Population replacement

Population replacement is a strategy to releases Wolbachia-infected mosquitoes, both male and female, to spread desired traits (such as an anti-pathogen phenotype) into the population. Because of the advantage of infected females in reproduction as compared to their uninfected counterparts, *Wolbachia* is able to invade a target population, even given the limited level of fitness cost it also confers. This invasion, together with *Wolbachia*-mediated pathogen interference, would eventually modify the population and reduce its ability to transmit diseases to humans. The successful field trials have been accomplished to reduce dengue transmission by replacing virus-susceptible populations with *Wolbachia*-infected, virus-resistant *Ae. aegypti* populations [163-165]. *Wolbachia*-based population replacement is advantageous in that it has the potential to provide a sustainable control effect at a relatively low cost, resulting in the permanent reduction of the high vectorial capacities of the dominant mosquito vectors.

1.7.5 Population suppression

In the population suppression strategy, releases of *Wolbachia*-infected males into the field is used to induce incompatible matings, resulting in a reduction in the target mosquito population.

As it is similar to the sterile insect technique (SIT), by analogy, this strategy is also called the "incompatible insect technique" (IIT)[166]. Since *Wolbachia* can not be transmitted from males to their offsprings, the released *Wolbachia* strain is not established in the field. One advantage of population suppression is that male mosquito neither bites humans nor transmits diseases and thus, their release causes less public concern than does the release of females. As the size of the wild population decreases due to the incompatible mattings, the repeated releases of incompatible males over time will lead to population suppression and even population elimination. In an early field test of this strategy, the release of bidirectionally incompatible males successfully eliminated a Culex mosquito vector population from a village in Burma (Myanmar) [122]. The recent success to develop population suppression for *Aedes* mosquito control includes > 90% reduction of *Ae. albopictus* population in China [167], and *Ae. aegypti* population in both US [168] and Thailand[169].

One of the bottlenecks of this strategy is the lack of a perfect sex separation approach to mass-producing the male mosquito. The common way to separate males from females using the mechanical method is based on the difference in the pupae sizes: generally, male pupae develops faster and have smaller size compared to female pupae. However, this method will result in about 0.5% residual female mixed with the male, raising the risk of population replacement which can result in failure in population suppression if the release of the males carrying the same Wolbachia strain continues. Different novel approaches have been explored to develop an efficient sex separation. For example, artificial intelligence is used to identify female and then remove them by laser[170]. Bacteria-expressing dsRNA is used to treat the mosquito larvae to selectively kill the females[171]. Drugs are used to kill females during blood feeding[172].

1.7.6 Wolbachia transinfection

Both population replacement and suppression strategies are based on the release of mosquitoes carrying a Wolbachia strain different from the target mosquito in the field. This requires establishing novel artificial Wolbachia infections. *Wolbachia* transinfection is the method that transfers a *Wolbachia* strain from a donor host to a recipient host, resulting in an artificial infection that can be stably inherited through maternal transmission, a hallmark of *Wolbachia* symbiosis. This is commonly accomplished through embryonic microinjection in the mosquito, which enables to directly introduce *Wolbachia* into the cytoplasm of early mosquito embryos, followed by intensive screening for transinfected females that can pass Wolbachia to their offsprings. Once transinfection is successfully established through embryonic microinjection, *Wolbachia* usually presents not only in the germline but also in the somatic tissues of mosquito. A high density of *Wolbachia* in somatic tissues, including the midgut and salivary gland, often provides a high level of resistance to arboviruses.

Rational selection of the donor species and *Wolbachia* strain is essential to success in establishing the novel transinfected mosquito line. In general, there is a higher success rate if the donor and recipient species are closely related. In order for a transinfected line to be used in vector control, it has to pass rigorous quality control in the lab before release in the field trial. This mainly includes *Wolbachia* maternal transmission efficiency, CI level, pathogen blocking effect and fitness. An ideal line will have perfect maternal transmission efficiency, complete CI, strong pathogen blocking and low fitness cost. However, there is a tradeoff between pathogen blocking effect and fitness cost and a high Wolbachia density (e.g., wMelPop) can induce both high viral blocking and fitness cost[173]. Certain Wolbachia strains (e.g., wAu) induce high viral

blocking but does not express CI[174]. Until now, a number of *Wolbachia* strains have been introduced into *Ae. aegypti* and *Ae. albopictus*, with the above traits well characterized (Table 1).

Several novel transinfected mosquitoes have already been used in the field trial. As the first *Wolbachia* strain released for population replacement in the field, wMel was introduced into Ae. aegypti and released in the field for dengue control in multiple countries including Australia, Indonesia, Vietnam, Brazil and Columbia by World Mosquito Program [175]. Recent cluster randomised trials show 77% reduction in dengue transmission in the release sites compared to control sites[176]. *Ae. albopictus* transinfected with a single *w*Pip infection has been released in US and Italy, resulting in a moderate suppression[177, 178]. In China, *Ae. albopictus* with a triple infection of *w*AlbA, *w*AlbB and *w*Pip was released for population suppression, resulting in near elimination of target mosquito population in the field[167]. Similar approaches were also used to suppress *Ae. aegypti* population by release of wAlbB-infected males in Thailand, Singapore, Mexico and US[178]. However, the above transinfected mosquito lines are not able to block virus transmission completely. Although Wolbachia strains wMelpop and wAu inducevery strong virus blocking effect but also impose high fitness costs to the hosts. In addition, using local *Wolbachia* strain for transinfection is preferred to minimize the risk of introducing the foreign organisms into the environment. Thus, a transinfected mosquito with optimal characteristics for release is still needed. As such, through this dissertation studies, we have made significant effort on identifying appropriate novel *Wolbachia* strain or strain combinations to develop transinfected line with a highest pathogen blocking effect and lowest fitness cost.

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Chapter 2 Establishment and characterization of a *Wolbachia* *w*AlbB transinfection in *Aedes aegypti* for Zika and dengue control in Mexico and Singapore

2.1 Introduction

Both the distribution range of mosquito vectors and the number of mosquito-borne arbovirus diseases have rapidly increased due to global tourism and global warming in the last decades [1-3]. An effective, sustainable and environment-friendly disease control strategy is urgently needed due to the insufficiency of traditional control approaches. The conventional chemical insecticides not only have negative environmental impacts but also face a significant problem with insecticide resistance in mosquito disease vectors[4]. Efforts have been made to develop novel genetic control approaches to either reduce mosquito's ability to transmit pathogens or suppress the mosquito density below the epidemic risk threshold, with the field trials completed for both transgenic mosquito and *Wolbachia*-based approaches[5, 6]. Among them, the *Wolbachia*-based mosquito suppression strategy offers an environmentally friendly species-specific suppression of the target population. Successful field trials show encouraging progress on suppressing or eliminating *Aedes* mosquito vector populations, through *Wolbachia*-induced incompatible mattings[5, 7, 8]. *Wolbachia* infected mosquito also can be released to spread *Wolbachia* into a population and make it become resistant to arbovirus infection, resulting in reducing or blocking disease transmission[9-11].

Wolbachia is a maternally transmitted endosymbiotic bacteria belong to the order of Rickettsiales, which estimated to infected more than 65% of insect species[12]. *Wolbachia* can be divided into eight supergroups based on their natural host species, with *Wolbachia* hosted by mosquito commonly falling into the groups A and B. *Wolbachia* is known to induce host reproduction alteration which includes feminization, parthenogenesis, male-killing and Cytoplasmic Incompatibility (CI). As the only phenotype induced by *Wolbachia* in the mosquito, CI is an early embryonic death when the *Wolbachia*-infected male mates with either an uninfected female or a female carrying a different strain of Wolbachia[9]. CI provide a reproductive advantage to those *Wolbachia*-infected females compared to uninfected females, as they can reproduce after mating both infected and uninfected males, and this advantage will facilitate invasion and spread of *Wolbachia* into the uninfected population, and eventually modify the entire population to be infected, referred to as population replacement.

Aedes aegypti is the primary vector of dengue, Zika and chikungunya viruses in Latin America and southern east Asia, including Mexico and Singapore. Although there is no native *Wolbachia* infection in *Ae. aegypti*, a variety of *Wolbachia* strains have been introduced into this mosquito species[13-15]. All those artificial *Wolbachia* infections can be maternally inherited in *Ae. aegypti* and maintain their ability to induce CI. Depending on the *Wolbachia* strain and host genetic background, these traninfected mosquitoes show either strong, moderate, or no resistance to arboviruses[13-15]. Evidence indicates that the strength of *Wolbachia*-mediated viral interference correlates with the density of *Wolbachia* in the somatic tissues, such as mid guts and salivary glands, of transinfected mosquitoes. Although not fully illustrated, immune priming and

altered metabolism are the two physiology changes that contribute to the underlying mechanism of *Wolbachia*-mediated viral blocking.

Aedes albopictus is an important arbovirus vector, and in some areas it is considered as a secondary or even primary arbovirus vector[16, 17]. In contrast to *Ae. aegypti*, *Ae. albopictus* is naturally superinfected with two *Wolbachia* strain *w*AlbA and *w*AlbB. These native *Wolbachia* infections in *Ae. albopictus* show little or no effect on mosquito vector competence for dengue virus replication[18]. Further studies show that *Wolbachia* density in *Ae. albopictus* is too low to induce any inhibitory effect on dengue virus[19]. However, after *w*AlbB was transferred from *Ae. albopictus* to *Ae. aegypti*, it induced a strong resistance to dengue viruses. In parallel, *w*AlbB developed very high density in the midguts and salivary glands of transinfected *Ae. aegypti*, dramatically activated both Toll and IMD pathways and boosted the production of ROS. It is unclear whether this increase in *Wolbachia* density and boosted immunity are caused by a recent association between *w*AlbB and *Ae. aegypti* or difference in genetic background between *Ae. aegypti* and *Ae. albopictus.* More importantly, it is unknown whether a long-term adaptation between *w*AlbB and *Ae. aegypti* will result in attenuation of *Wolbachia* density and its associated viral blocking effect.

We developed the first *w*AlbB-infected *Ae. aegypti line* WB1 fifteen years ago[11]. Here, in order to test the stability of *w*AlbB-*Ae. aegypti* association and examine the feasibility of developing *w*AlbB-infected *Ae. aegypti* as a tool for control of dengue, Zika and chikungunya, we repeated the previous transinfection assay to introduce *w*AlbB from *Ae. albopictus* into *Ae. aegypti*. By comparing the new established WB2 with WB1, we found no evidence of attenuation of *w*AlbB titer in WB1 line during the long-term maintenance.

We also showed that *w*AlbB induced strong inhibitory effect to dengue, Zika and chikungunya viruses in *Ae. aegypti* with both Mexico and Singapore background. In addition, *w*AlbB-infected *Ae. aegypti* males had mating competitiveness close to wild-type males. These results support the potential to release *w*AlbB-infected *Ae. aegypti* for disease control.

2.2 Result

2.2.1 Generation of the Aedes aegypti WB2 line with a wAlbB infection

The *Wolbachia w*AlbB strain induces resistance to flaviviruses in both mosquito cell line and mosquito [20, 21]. In order to examine the stability of *w*AlbB - mosquito interactions, we created a novel identical *w*AlbB infection in *Ae. aegypti* to compare with the WB1 line establish in 2005 [22]. The wild-type *Ae. albopictus* HOU line was used as the donor, and the cytoplasm of HOU embryo was transferred to wild-type *Ae. aegypti* by embryo microinjection. Experiments were repeated three times, each with approximately 200-400 embryos injected. Females (G0) developed from the embryos survived from microinjection were mated with wild-type males (Waco), followed by blood feeding. After their offspring (G1) were produced, the G0 females were sacrificed to screen for *Wolbachia* infection by PCR assay. In the experiment, 3 out of 5 survived females shows positive of *w*AlbB infection based on PCR diagnosis (Fig. 2.1a). Only the progenies from the positive female were selected to produce the next generation. Six out of twenty-one G1 isofemales (28.5%) were observed to carry *w*AlbB infection. At G2, there were 16 out of 45 females showing *w*AlbB infections in PCR assays. Among them, 7 females showing strong infections were selected to establish the next generation (G3). The

subsequent assay showed that all tested G3 individuals (n = 10) carried *w*AlbB infections in this transinfected line, hereafter referred to the line WB2. After G3, we randomly selected 20 or 10 individuals from WB2 line for PCR assay and observed 100% maternal transmission efficiency in all the tested generations (Fig. 2.1b). Egg hatch rates were low (29-50%) before G4, but recovered to a level close to 70% afterward (Fig. 2.1b), likely caused by removal of inbreeding effects through repeated crosses with wild-type male until G6.

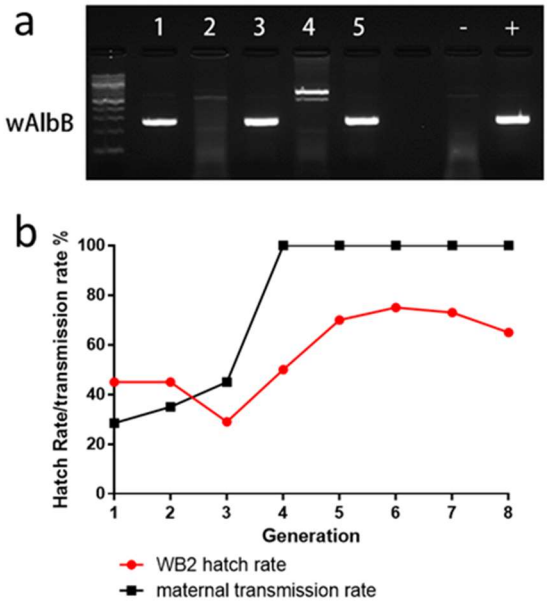
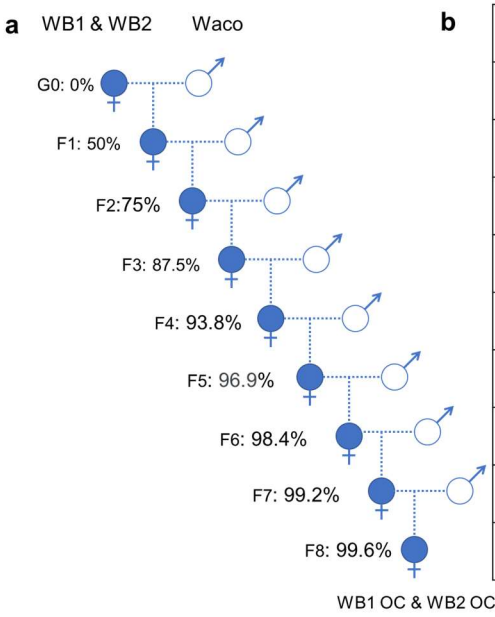


Figure 2.1 Establishment of the transinfected *Ae. aegypti* WB2 line with *w*AlbB infection.

a. Strain-specific amplification of the *w*AlbB infection in a PCR assay on five G0 females survived from injection in one of three experiments. +, WB1 (positive control), - wild-type *Ae. aegypti* (negative control)**. b.** Egg hatch rate and maternal transmission rate of WB2 line. Egg hatch was calculated as the percentage of eggs hatched divided by the total number of eggs. Transmission rate was calculated as the percentage of *w*AlbB-infected mosquito among all the tested mosquitoes.

2.2.2 Homogenization of the host genetic background in WB1 and WB2

In order to compare WB2 with WB1, we have crossed WB2 line and WB1 line with wild-type Waco line for 7 generation to homogenize their backgrounds. In each cross 50 infected females were crossed with 50 WT male. After each cross, the maternal transmission rates for the offspring were tested using PCR. The host genetic background was expected to be 99.6% identical after 7 outcrosses (Fig. 2.2a) and the maternal transmission rates were maintained at 100% after each cross (Fig. 2.2b).



b

maternal transmission		
generation	WB1	WB2
F1	100%	100%
F2	100%	100%
F3	100%	100%
F4	100%	100%
F5	100%	100%
F6	100%	100%
F7	100%	100%
F8	100%	100%

Figure 2.2 Introgression to establish WB1 and WB2 with homogeneous Waco genetic background.

a. WB1 line and WB2 line females were repeatedly outcrossed with wild-type *Ae. aegypti* Waco males for 7 consecutive generations. The number indicated the percentage of the Waco gene background. **b.** The maternal transmission rate of WB2 was assayed after each outcross. All the outcrossed generations had 100% maternal transmission rate.

2.2.3 CI induction by wAlbB in both WB1 and WB2 line when crossed with wild-type line and each other.

An ability to induce CI is a critical feature in order to develop *Wolbachia*-based strategies for mosquito-borne disease control. We set up the CI cross between WB1, WB2 and wild-type mosquito. The self-cross between WB1, WB2 and Waco yielded hatch rates of 44.9%, 51.5% and 53.6%. The WB1 males were compatible with WB2 females and their crosses had an average hatch rate of 52.4%. Similarly, WB2 males are also compatible with WB1 females with a hatch rate of 50.8%. Both WB1 and WB2 males induced a 100% CI when crossed with Waco females (Fig. 2.3). The result indicated that WB1 and WB2 were compatible to each other and both induced complete CI into wild-type *Ae. aegypti*, indicating stability of *w*AlbB in inducing CI after transfer into *Ae. aegypti*.

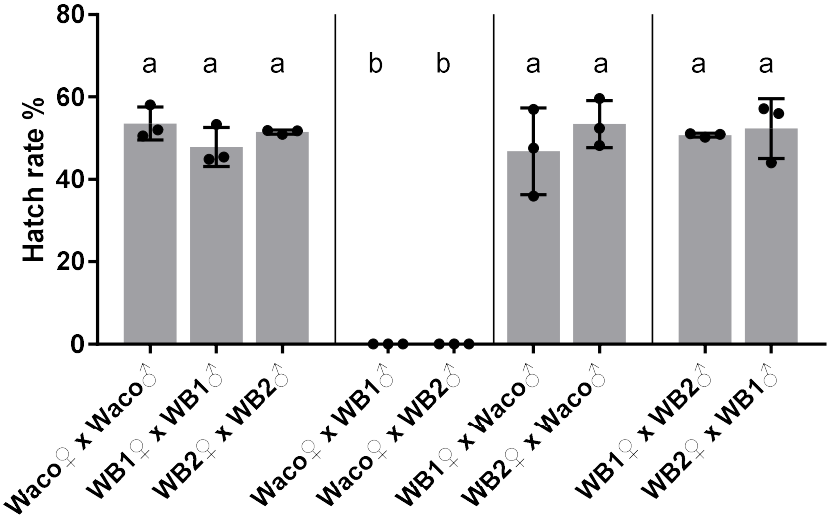


Figure 2.3 CI crosses between wild-type Waco, WB1 and WB2 mosquito.

The result expressed as mean for 3 replicates/cross, 10 female and 10 male per replicates. Dot indicated each sample value. Error bar indicated the standard deviation. Different letters above the column indicate significant difference ($P < 0.0001$) by ANOVA-Tukey's multiple comparison test.

2.2.4 Comparison of wAlbB distribution in both somatic and germline tissues between WB1 and WB2 lines.

In order to identify the impact of long-term adaptation on tissue distribution of *Wolbachia* in mosquito, we compared the densities of *w*AlbB in reproductive tissues, ovaries and testis, and the remaining carcass between WB1 and WB2 line. The results showed no difference in wAlbB density in testis and carcass tissues between WB1 and WB2. However, WB2 females shows higher *Wolbachia* density in ovaries compared to WB1 female (Fig. 2.4). The results indicated that *Wolbachia* density was stable in somatic tissues of WB1 line after maintained in laboratory conditions over 15 years.

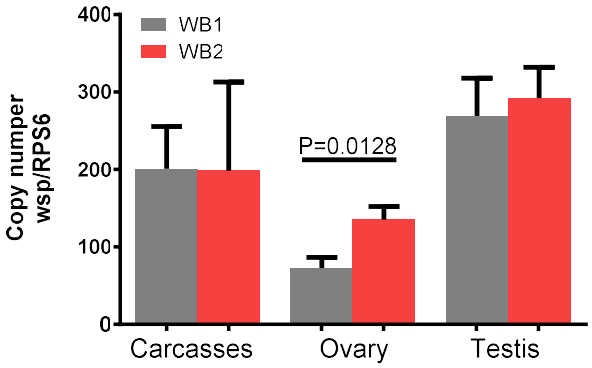


Figure 2.4 The density of *w*AlbB in various tissue.

After ovaries and testis were removed, the remaining carcass were collected. The copy number of the *Wolbachia wsp* gene was normalized by the mosquito *rps6* gene. The data are shown as the mean of eight replicates ± standard deviation.

2.2.5 Outcrosses to develop WB2 with Mexico and Singapore background

In order to introduce the local *Ae. aegypti* genetic background into WB2 line, we crossed WB2 line with wild-type mosquitoes collected from the field in Merida Mexico (AFM) for seven generations. During each cross, 100 virgin WB2 females and 100 AFM males

were randomly selected. The offspring from each cross were test for maternal transmission rate. The maternal transmission rates of the outcrossed line WBM were maintained at 100% during all the crosses (Fig. 2.5). The same procedure was performed to introduce the Singapore genetic background into WB2, with 100% maternal transmission rates of *w*AlbB maintained at each generation.

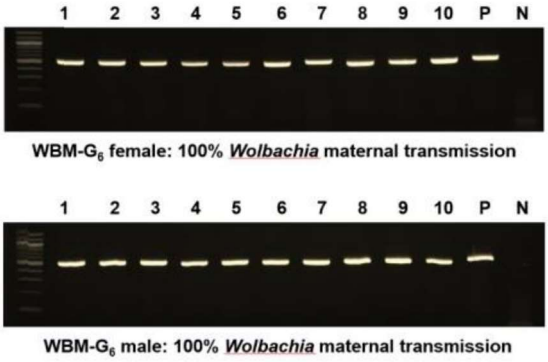


Figure 2.5 Maternal transmission of *w*AlbB in WBM line.

The WB2 outcrossed line WBM with Mexico genetic background maintained 100% maternal transmission rate of *w*AlbB. The results were showed by gel electrophoresis in PCR diagnosis of randomly selected 10 females and males at G6. P, positive control. N, negative control.

2.2.6 wAlbB-mediated viral inhibition in the outcrossed WB2 females with Mexico and Singapore background

Vector competence assays were performed to measure the ability of *w*AlbB to block Zika virus in the outcrossed *A. aegypti* strain with Mexico and Singapore background. Two Zika virus lineages, South American and Asian lineages, were used to infect both *w*AlbB-infected and wild type *A. aegypti*. Virus titers were measured in both midguts and salivary glands at Day 7 and 14 post infectious blood meal.

Under both Mexico and Singapore genetic backgrounds, *w*AlbB-infected *Ae. aegypti* showed strong inhibitions of both Zika lineages at 7 and 14 days post infections (DPI)

(Fig. 2.6). *w*AlbB showed nearly complete blocking of South American lineage in both Mexico and Singapore backgrounds. For the Zika virus Asian lineage, only *w*AlbB infected *Ae. aegypti* with Singapore genetic background showed complete blocking in salivary glands at 7, but not 14, days post infection. These results indicate the strength of *w*AlbB-mediated viral blocking differs between the two different Zika virus lineages.

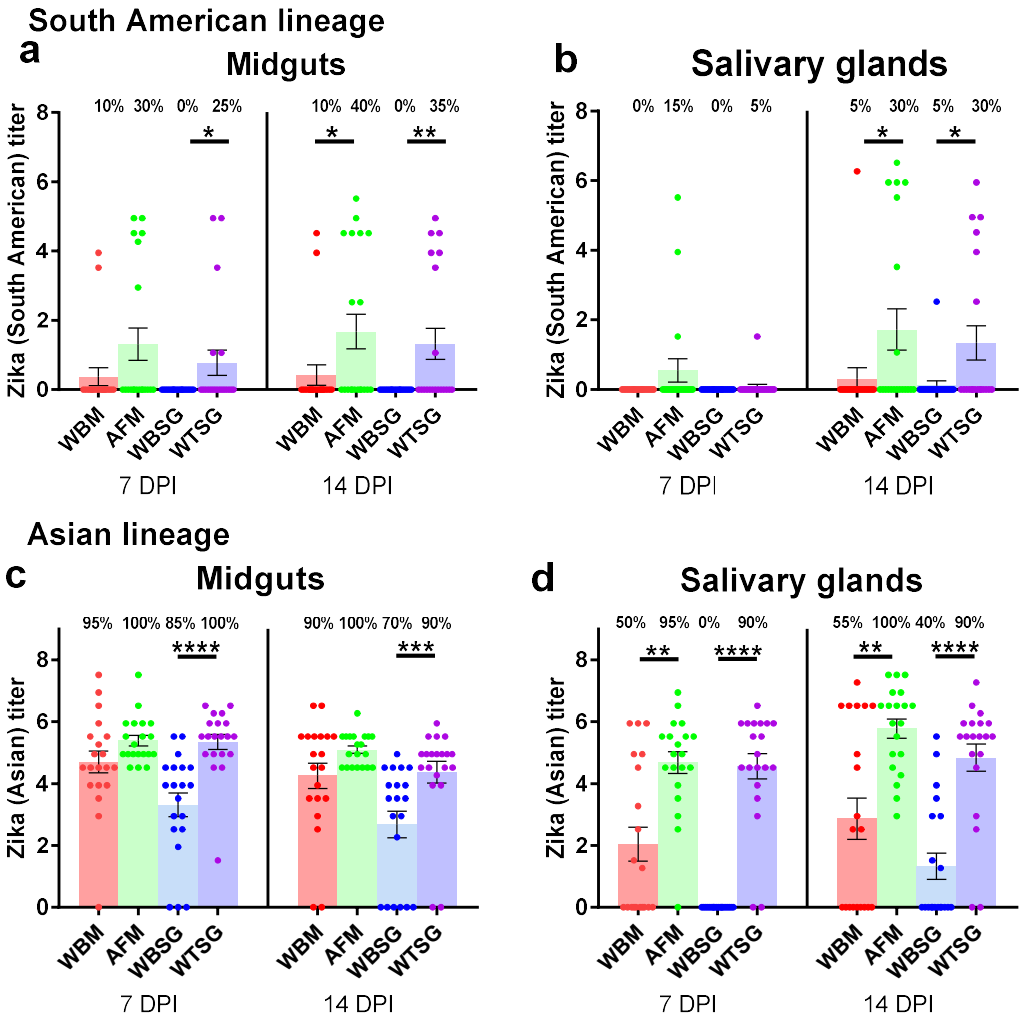


Figure 2.6 Vector competence of *w*AlbB-infected *Ae. aegypti* with either Mexico or Singapore genetic background for ZIKV.

a. The infection titer of Zika virus South American lineage in the midgut at 7 and 14 days post infection (DPI). **b.** The infection titer of Zika virus South American lineage in the salivary glands at 7 and 14 DPI. **c.** The infection titer of Zika virus Asian lineage in the midgut at 7 and 14 DPI. **d.** The infection titer of Zika virus Asian lineage in the salivary glands at 7 and 14 DPI. The infection titers were calculated as Log10TCID50/ml. WBM: outcrossed *w*AlbB-infected *A. aegypti* with Mexico genetic background; AFM: wild type *Ae. aegypti* (*Wolbachia*-free) with Mexico genetic background; WBSG, outcrossed *w*AlbB-infected *A. aegypti* with Singapore genetic background; WTSG, wild type *Ae. aegypti* with Singapore genetic background. Number above the bars indicated the infection rate. *, $P < 0.05$, **, P<0.01 ***, P<0.001, ****, P<0.0001 by Mann-Whitney test.

Further assay was performed to measure *w*AlbB-mediated inhibitory effects on DENV, CHIKV, and ZIKV in *Ae. aegypti* with Singapore genetic background. After mosquitoes were fed with virus-infected blood, midguts and salivary glands were collected at 5 and 13 DPI to measure the viral titers. In the assay with DENV serotype 2 (DENV-2), there was no significant difference in the infection rate and virus titer between *w*AlbB-infected and non-infected *Ae. aegypti*in midguts at 6 DPI. However, a significant reduction in DENV-2 titer was observed in both midguts and salivary glands at 13 DPI. Specifically, viral titers and infection rates were reduced by 80% and 30%, respectively, in salivary glands of *w*AlbB-infected mosquitoes compared to the wild-type mosquitoes (Fig. 2.7a). In ZIKV infection assay, there was significant reduction in viral titers in salivary glands of *w*AlbB-infected mosquitoes at both 6 and 13 DPI compared to those of wild-type

mosquitoes. But similar reduction was not observed in midguts (Fig. 2.7b). Remarkably, *w*AlbB induced much stronger resistance to CHIKV than DENV and ZIKV. In midguts at both time points, *w*AlbB reduced CHIKV infection rates and viral titers by 30% and 75%, respectively, as compared to the wild-type mosquitoes. In the salivary glands at both time points, 10% CHIKV infection rate was observed in *w*AlbB-infected mosquitoes compared to 100% in wild-type mosquitoes, and viral titers were reduced by 6-fold or 4-fold at 5 or 13 DPI, respectively (Fig. 7C). These results indicate that *w*AlbB in our WB2 line confers strong resistance against DENV, ZIKV and CHIKC, with near complete blocking effects on CHIKV transmission.

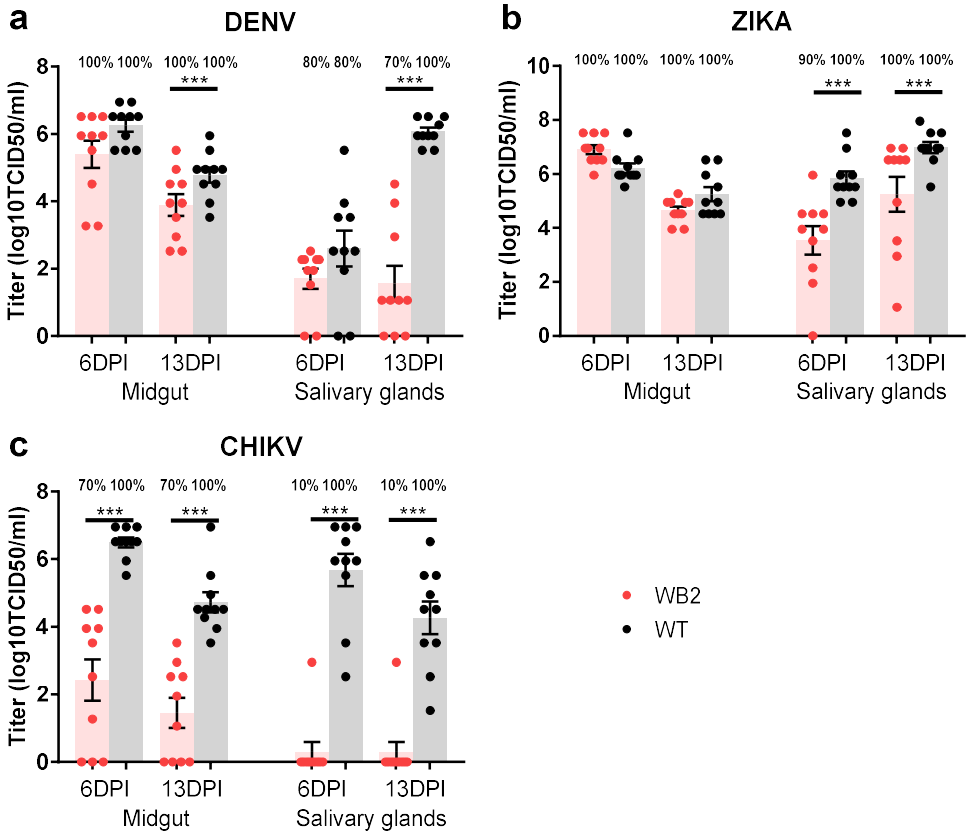


Figure 2.7 Vector competence of *w*AlbB-infected *Ae. aegypti* with Singapore genetic background for DENV, ZIKV, and CHIKV.

After mosquitoes were fed with blood infected with DENV-2 (**a**), ZIKV (**b**) or CHIKV (**c**), midguts and salivary glands were collected at 6 and 13 DPI and assayed for viral infection. Virus infection levels were determined using viral titration assay and expressed as Log10TCID50/ml. Bars indicate the average titer, and each point represents an individual midgut/salivary gland. *** denotes significant difference, $P < 0.001$ by Mann-Whitney test. The number above the bars indicated the infection rate.

2.2.7 WB2 males with mating competitiveness equal to wild-type males

As a key parameter for population suppression, male mating competitiveness was tested to measure the ability of the outcrossed *w*AlbB-infected *Ae. aegypti* males to compete with wild type males to mate with local wild type females. *w*AlbB-infected *Ae. aegypti* males with Mexico genetic background (WBM) was mixed with wild-type males (AFM) at three different ratios to compete for mating with wild-type females.

Although slightly reduced in the ratios 1:1 and 1:10, the mating competitiveness index reached 1.3 in the ratio of 5 to 1, which is known to be a critical over flooding ratio to reach population suppression for *Ae. albopictus* control based on both field trial and mathematical model[5] .The results indicated that *w*AlbB infected WBM had mating competitiveness equal to wild type males (Table 2.1). Similar results were observed in the test using *w*AlbB-infected *Ae. aegypti* in the Singapore genetic background.

Table 2.1 Induced sterility and male mating competitiveness index of WBM *Ae. Aegypti* males at different release ratios.

Male:Male	Ratio (Sterile: Fertile)	Hatching rate	IS(%)	C
WBM：AFM	0：1	0.772		
	1：0	0.000		
	1：1	0.419	45.7	0.842
	5：1	0.103	86.7	1.299
	10：1	0.106	86.3	0.628

WBM: outcrossed *w*AlbB-infected *A. aegypti* with Mexico genetic background; AFM: wild type *A. aegypti* (*Wolbachia*-free) with Mexico genetic background.
Induced sterility (IS) value: 100% minus the residual fertility value(Ho /Hn).Ho: the observed egg hatch rate from each experimental cage.
Hn: the hatch rate from eggs of females mated with fertile males.
Male mating competitiveness index (C):

$$C = [(Hn—Ho) / (Ho—Hs)] * (N / S).$$

Hs: hatch rate from eggs of females mated with sterile males.
N : the numbers of fertile males.
S : the numbers of sterile males.

2.3 Discussion

We have demonstrated the successful establishment of the *w*AlbB infected *Ae. aegypti* WB2 line with a stable 100% maternal transmission efficiency. Comparison of WB2 with WB1 shows a long-term stability of *w*AlbB-*Ae. aegypti* association, with neither alteration of CI expression nor attenuation of *Wolbachia* titer in mosquito somatic tissues. We also show that *w*AlbB confers a strong resistance to both South American and Asian lineages of ZIKV in WB2 line with either Mexico or Singapore genetic backgrounds. In

addition, it also inhibits DENV-2 and CHIKV in both midguts and salivary glands of WB2 mosquitoes. We also show WB2 males had mating competitiveness equal to wild-type males in the ratio of 5 to 1 (WB2 *vs* wild-type males). These results support the feasibility of developing *w*AlbB-based population replacement and suppression for controlling dengue, Zika and chikungunya.

Mosquito midgut is the first organ that arboviruses will replicate inside the mosquito body, and salivary gland is the last stop before viruses will be transmitted to human by mosquito biting. The higher resistance to viruses in the midgut means less likely that mosquito will acquire the viral infection. The lower number of virus load in salivary glands indicates less chance for the mosquito to transmit the virus to human. We focused on the susceptibility of the midgut and salivary glands to viral infections because they can represent the vector competence of WB2 line. The results indicated that WB2 line induced strong resistance against both Flavivirus and Alphavirus, including DENV, CHIKV and ZIKV, with strongest inhibition occurred in salivary glands. The significant reduction of virus loads and infection rates in salivary glands indicated a reduced potential of viral transmission. Interesting, WB2 line showed the strongest resistance against CHIKV , with infection rates reduced by 70% and 90% in midguts and salivary glands, respectively, and viral infection level reduced by 6-fold and 4-fold in salivary glands at 6 and 13 DPI. The results indicated that WB2 had a lower chance to be infected by CHIKV, and even lower possibility to transmit CHIKV to human compared to wild-type mosquitoes. The observed *w*AlbB-mediated viral blocking effects suggests to the

potential to develop WB2 for population replacement as a cost-effective method for disease control.

WB2 males had similar mating competitiveness compared to wild type males, which is essential when developing WB2 males to suppress the wild mosquito population. The sterile and insect technology (SIT) applies a similar concept as *Wolbachia*-based incompatible insect technologies (IIT) on control mosquito population[23]. However, a high dose of radiation requires for sterilizing males leads to high fitness costs, including reduction in the mating competitiveness. Consequently, the number of males to be released has to increase to offset these reduction in fitness, which prevents the successful development of SIT for mosquito control. The successful field trials in China, US and Thailand's suggest that great mating competitiveness of *Wolbachia*-infected males might be the key factor that determine the efficiency of population suppression[5]. In order to better assay the male mating performance, however, further studies should evaluate the male mating competitiveness in the field condition.

Although *w*AlbB induced resistance to both South American and Asian lineages of ZIKV in WB2 line with either Mexico or Singapore genetic backgrounds, the strength of blocking effects varied among different groups. WB2 in Singapore genetic background blocked the Asian lineage better than in Mexico genetic background. Such difference was not observed in the assay using South American lineage. We also saw a higher vector competence for ZIKV Asian lineage than South American lineage used in this experiment , resulting in a better blocking of South American lineage than Asian lineage in WB2 with both Mexico and Singapore genetic backgrounds. Our results suggest that the strength of *Wolbachia*-mediated viral interference is affected by virus genome type.

Wolbachia tissue tropism is an important factor determining its viral blocking effect and maternal transmission. The tropism could be affected by both *Wolbachia* factor and host genetic background. To examine the impact of long-term adaption on *Wolbachia*, we compared *w*AlbB density between WB1 and WB2 after outcrossed with Waco for 7 generations to remove any impact of host genetic backgrounds on *Wolbachia* infection. Although slightly decreased in ovaries, lack of changes in *w*AlbB densities in both somatic tissues and testis indicates that wAlbB maintain a stable association with Ae. aegypti, supporting robustness of WB2 when using in the field trial. In conclusion, we have established a recent *w*AlbB transinfection in *Ae. aegypti* and showed that *w*AlbB can reduce the potential of *Ae. aegypti* to transmit DENV, ZIKV and CHIKV. Together with perfect maternal transmission, complete CI and great male mating competitiveness, these results support the feasibility of developing field trials to release WB2 for controlling arboviruses in Singapore and Mexico. It is excited to see *w*AlbB-infected *Ae. aegypti* has been released in multiple countries to develop either population replacement or suppression strategies based on our work[11]. The long-term stability of *w*AlbB-*Ae. aegypti* association is expected to further boost cost-effectiveness and sustainability of these vector control strategies. Further studies will require for assaying these key characteristics in the field of disease endemic countries under tropical environments.

2.4 Material and method

2.4.1 Mosquito lines and maintenance

Wild-type *Aedes albopictus* lines, HOU carrying the native superinfection of *w*AlbA and *w*AlbB, was used as a donor to generate the WB1 line and WB2 line. Waco is a wild type

Aedes aegypti line which do not carriers *Wolbachia* and uses as the receiver of *Wolbachia*.

All the mosquito lines were maintained on 10% sugar solution at 27 ± 1 °C and 80 ± 10% relative humidity (RH), with a 12:12 h light:dark photoperiod, according to standard rearing procedures. For routine colony maintenance and experimental studies, female mosquitoes were provided with either human (MGYP2 line) or sheep (other lines) blood at day 7 post eclosion and eggs were collected two days post blood meal.

2.4.2 Transinfection to generate the WB2 line

The WB2 line was generated by the transfer of *w*AlbB from HOU to wild-type aedes aegypti using embryonic microinjection according to the approach described previously[11, 22]. In brief, cytoplasm from donor embryos was transferred into the posterior of 60-90 minutes old recipient embryos using an IM300 microinjector (Narishige Scientific). After injection, embryos were incubated at 85% RH and 27°C for one hour and transferred to wet filter paper. Embryos were allowed to mature for 5-7 days before being hatched. Females (G0) developed from the survived embryos were isolated and mated with HOU males. After blood-feeding and oviposition, G0 females were tested for wAlbB infection by PCR using strain-specific primers as described below. G1 females were again crossed with Waco male, blood-fed, isolated and allowed to oviposit. The offspring from *w*AlbB-positive G1 were selected for the next screen, and this process was repeated until *w*AlbB maternal transmission rate reach 100%. The wAlbA also been tested for those *w*AlbB positive females, none of the *w*AlbB positive females carry *w*AlbA infection.

2.4.3 PCR assay of Wolbachia infection

Primers were designed base on the sequence of Wolbachia surface protein *wsp* gene for strain-specific diagnosis of four different strains. The primers for The primers for *w*AlbA were: forward 5'-GTGTTGGTGCAGCGTATGTC-3'; reveres 5'-GCACCAGTAGTTTCGCTATC-3'. wAlbB were: forward 5'-ACGTTGGTGGTGCAACATTTG-3'; reveres 5'-TAACGAGCACCAGCATAAAGC-3'. The below primers for mosquito *rps6* were reported previously [30]: forward 5'-CGTCGTCAGGAACGTATTCG-3'; and reverse 5'-TCTTGGCAGCCTTGACAGC-3'. Standard curves were generated for each of the above genes to convert the Ct value of qPCR into the copy number of the target sequences.

Genomic DNA was extracted from samples using Thermo Scientific Phire Animal Tissue Direct PCR Kit (F-140WH). Samples were pre-treated in 20 µl dilution buffer with 0.5 µl DNARelease Additive. The reaction mixture contained 10 µl 2X Phire Animal Tissue PCR Buffer, 0.4 µl Phire Hot Start II DNA Polymerase, 0.2 µl for both forward and reverses primer and 7.2 µl dsH2O. The regular PCR condition comprised of initial denaturation at 98 °C for 6 min, followed by 40 cycles of 5 s at 98 °C, 5 s at 56 °C, and 45 s at 72 °C. The quantitative PCR (qPCR) was performed using the QuantiTect SYBR Green PCR Kit (Qiagen) and ABI Detection System ABI Prism 7000 (Applied Biosystems, Foster City, California, USA). Samples were homogenized in 100 µl 1 x STE buffer and incubated with 4 µl Proteinase K at 55°C for 1 h and followed by 97°C for 5 min.

2.4.4 Experimental crosses to determine cytoplasmic incompatibility

Cytoplasmic incompatibility assays were conducted as previously described[11, 22]. Ten virgin males were mated with ten virgin females with five replicated cages for each cross. Blood meal was provided to females at day 7 post eclosion. Two days after blood meal, eggs were collected using oviposition cups containing wet filter paper, which was subsequently desiccated for 7 d at 27 °C and 80% relative humidity. Eggs were counted and then hatched in water containing 6% m/v bovine liver powder. Larvae were counted at the L2-L3 stage to record the hatch rate.

2.4.5 Vector competence assay

The viruses used were DENV2: Clade1B Cosmopolitan Genotype Y13, ZIKV: MR766 strain and CHIKV: A226V Y08. The 7-days old mosquito was feed on virus-spiked bloodmeal for 45 min. At 6 days and 13 days post-infection, the midgut and salivary glands were sampled for virus titer measurement. The virus levels were determined using viral titration assay and expressed as Log10TCID50/ml.

2.4.6 Mating competitiveness

Adult cages were contained with fifty AFM female and male. Varying numbers of WBM males (0, 50, 250 or 500). Another cage with fifty AFM female and fifty WBM males also was prepared. Mosquitoes were allowed to mate for two days before blood-fed for 20 min. Two days after blood-feeding, egg cups were placed the cages for eggs collection. The eggs was then hatched and the hatch rates were calculated. The egg hatch rate was compared to the expected hatch rate assuming: (i) random mating and equal mating

competitiveness between WBM and AFM males, and (ii) complete unidirectional cytoplasmic incompatibility between WBM males and AFM females[5].

2.4.7 Statistical analysis

Differences between Wolbachia density and virus titer were analyzed using t-test (between two group, and normal distribution was tested before t-test) and one-way ANOVA with values <0.05 considered significant. All analyses were performed in GraphPad Prism v. 6 (GraphPad Software, San Diego, California USA).

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Chapter 3 Characterization of symbiont-host interactions in *Aedes albopictus* mosquito with a *Wolbachia* triple-strain infection

3.1 Introduction

A rapid increase in the number of arbovirus diseases transmitted by mosquitoes, such as dengue and Zika, in recent decades has underscored the urgency in developing effective intervention strategies [1, 2]. The insufficiency of traditional control approaches, including vaccines, drugs, and chemical insecticides, has led to significant efforts to develop novel vector control methods to combat disease transmission. Rather than using chemical insecticides to directly kill the vector, an approach that is being challenged by the rapid development of insecticide resistance and the negative impacts on both the environment and non-target insect species, these new tools have focused on modifying the mosquito population in a species-specific manner, with the goal of either reducing the mosquito's ability to host a pathogen or of suppressing (or even eliminating) the mosquito population to break the viral transmission between vector and host [3]. Among these strategies, *Wolbachia*-based interventions have recently shown encouraging results in field trials, successfully demonstrating either reduced dengue transmission through *Wobachia*-induced viral inhibition in the mosquitoes or the elimination of the *Aedes* vector populations through *Wobachia*-induced incompatible mating [4-8].

Estimated to infect >65% of all insect species, *Wolbachia* are maternally transmitted endosymbiotic bacteria belong to the order Rickettsiales and family Anaplasmataceae [9]. Designated based on their naturally associated host species and divided into eight

supergroups, different *Wolbachia* strains can interact with their hosts in their own manner, with phenotypes determined by the genetic background of both *Wolbachia* and the host, as well as the environment [9-11]. In the mosquito and many other insects, *Wolbachia* causes a reproduction alteration known as cytoplasmic incompatibility (CI), in which early embryonic death occurs when the *Wolbachia*-infected male mates with an uninfected female or a female carrying a different strain of *Wolbachia*. The CI can be rescued, resulting in compatible mating, if the *Wolbachia* strain carried by the male is also present in the female. Recent studies have shown that two CI determination genes, *cifA* and *cifB,* in *Wolbachia* modify the sperm development to induce CI, but only *cifA* mediates CI rescue in females (or eggs) [12-15]. However, it is still unknown how these CI factors interact with their host targets and how the CI determination factors of different *Wolbachia* strains interact with each other to induce CI expression in a host with a *Wolbachia* superinfection.

Since the ability to generate novel *Wolbachia* symbiosis (transinfection) in mosquitoes was first developed through embryonic microinjection [16-18], a number of transinfected mosquito lines carrying different *Wolbachia* strains have been established and characterized, with the goal of using them for disease control [17-24]. Many of these transinfected mosquito lines show different levels of resistance to dengue, Zika, and Chikungunya viruses, with the strength of the viral inhibition being associated with the density of *Wolbachia* in somatic tissues such as the midgut and salivary glands, where the viruses reside, migrate, and replicate. Whereas transinfected lines with three *Wolbachia* strains (*w*Mel, *w*AlbB, and *w*Pip) have been well characterized and successfully tested in field trials [4-6], significant interest remains in developing improved transinfected lines

with maximal viral blocking and optimal fitness under field conditions in order to reach the highest efficiency in disease control or to be able to replace the released lines if viruses develop resistance to the released strains in the future [24].

Naturally carrying two Wolbachia strains, *w*AlbA and *w*AlbB, *Aedes albopictis* is the world's most invasive mosquito vector. As the density of these two native *Wolbachia* is too low to induce viral inhibiton in *Ae. albopictus* [25], efforts have been made to introduce novel strains into this mosquito species to develop transinfected lines that are both incompatible with the wild-type line and resistant to viruses [6, 26]. Experiments are often designed by either directly adding a novel strain to *Ae. albopictus* to generate a superinfection [6, 27-29] or replacing the native *Wolbachia* with a novel strain, by removing the native *Wolbachia* with an antibiotic and then introducing the novel strain [17, 22]. The first approach results in a triple infection that can induce a unidirectional CI in wild-type mosquitoes [6, 27], with the advantage that it increases the invasion and spread of *Wolbachia* into the population more effectively than does the second (replacement) approach, which often induces a bi-directional CI [17, 22]. However, for a host with a triple-strain infection, the outcome of the transinfection is difficult to predict, given the complicated interactions between the various *Wolbachia* strains and between *Wolbachia* and the host [28, 29].

We previously developed the transinfected *Ae. albopictus* line HC, featuring a triple infection with *w*Pip, *w*AlbA, and *w*AlbB [6]. The HC line induces high uni-directional CI in crosses with the wild-type line and shows a strong resistance to both dengue and Zika viruses [6]. In the present study, we have introduced *w*Mel into *Ae. albopictus* and generated another transinfected line, HM, infected with *w*Mel, *w*AlbA, and *w*AlbB. The

HM line show complete efficiency in maternal transmission of the triple infection, with *w*Mel showing the highest density in ovaries. Multiple crosses showed that the ability of *w*Mel to induce CI was blocked by *w*AlbA in the HM line and that double infection with *w*Mel and *w*AlbB induced a high level of CI in crosses with the lines having a single, double, or triple infection.

3.2 Results

3.2.1 Generation of the Ae. albopictus HM line with a triple Wolbachia infection: wMel, wAlbA, and wAlbB

The ability of a single *w*Mel infection to inhibit arbovirus transmission in both *Ae. aegypti* and *Ae. albopictus* [19, 22] motivated us to test whether a triple infection with *w*Mel, *w*AlbA, and *w*AlbB could be established in *Ae. albopictus* to produce enhanced viral blocking effects for disease control, and whether there was competition among the various *Wolbachia* strains that might affect the nature of the symbiosis between *Wolbachia* and its mosquito host. The cytoplasm of *w*Mel-infected *Ae. aegypti* (MGYP2) embryos [19] was transferred by microinjection into embryos of the *Ae. albopictus* HOU line with a native superinfection of *w*AlbA and *w*AlbB (Fig. 3.1a). The virgin females (G0) developed from embryos surviving the microinjection were outcrossed with HOU males to produce offspring (G1). Eighteen G1 isofemales were outcrossed with HOU males. After their eggs (G2) were collected, PCR assay was used to diagnosis the *Wolbachia* strain profile in these females, with 15 of 18 isofemales (83%) being seen to carry the triple *Wolbachia* infection (Fig. 3.1b, c); the offspring of the females without a triple infection were discarded. Among the G2 offspring of these triply infected mothers,

18 of 20 (90%) males and 15 of 20 (75%) females maintained a triple *Wolbachia* infection. Without further screening, the offspring from the triply infected G2 females were then pooled together to establish a new transinfected line, hereafter referred to as HM. At G3 and G4, we randomly selected 20 and 10 individuals, respectively, for PCR assay. All of the tested mosquitoes carried a triple infection, indicating a 100% maternal transmission efficiency. Subsequently, the infection status of the HM line was monitored every other generation from G8 to G24, and all the tested samples (n = 126) were positve, confirming the stability of the triple infection in the HM line (Fig. 3.1c). These results suggest that *w*Mel can coexist with *w*AlbA and *w*AlbB to exhibit symbiosis within *Ae. albopictus*.

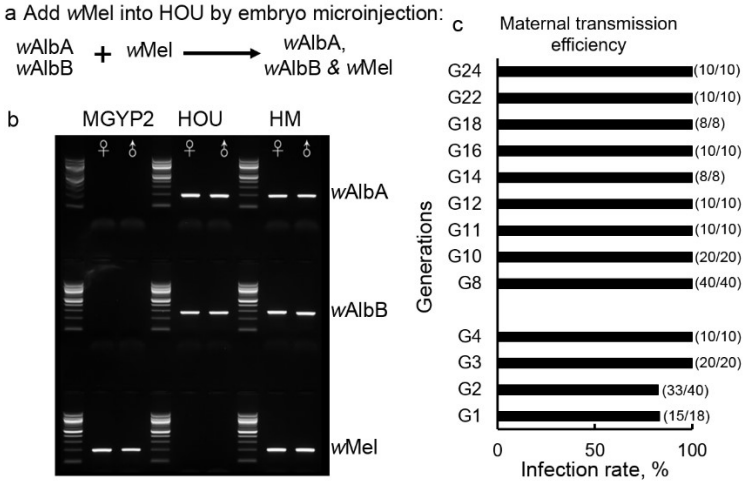

Figure 3.1Establishment of the transinfected *Ae. albopictus* HM line with the triple infection (*w*Mel, *w*AlbA, and *w*AlbB).

a. Schematic diagram of the experimental design to establish the HC line with a *Wolbachia* triple-strain infection. b. Representative results of the strain-specific amplification of the three *Wolbachia* strains, *w*Mel, *w*AlbA, and *w*AlbB, in a PCR assay. MGYP2, the transinfected *Aedes aegypti* line carrying *w*Mel, serving as a donor during

the embryonic microinjection experiment in this study. HOU, Ae. albopictus HOU line carrying *w*AlbA and *w*AlbB, serving as the recipient. HM, transinfected Ae. albopictus line carrying the triple infection *w*Mel, *w*AlbA, and *w*AlbB. c. Maternal transmission efficiency was monitored by randomly selecting individuals from each generation, as indicated, and diagnosis of Wolbachia infection by PCR using strain-specific primers. The infection rate was calculated as the percentage of positive individuals in the tested samples at the designated generation.

3.2.2 Introduction of a new host genetic background into the HM line to increase its fitness

The newly established HM line suffered from a strong fitness cost associated with the triple-strain infection, with an extremely low egg hatch rate ranging from 1 to 12% between G2 to G5 (Fig. 3.2). Therefore, we outcrossed HM females with HOU males to remove the potential inbreeding effect, which has been observed to cause a low egg hatch rate in previous transinfected lines [16, 17]. The egg hatch rate increased to 60% at G6, then dropped to 12% and 6% at G10 and G12, respectively (Fig. 3.2). From G13 to G27, the egg hatch rate continued fluctuating and varied from 8% to 65%, indicating that the low egg hatch rate may not be only caused by inbreeding; the maladaptation of the novel triple-strain infection to the HOU genetic background may also have contributed to this fitness cost. Thus, at G16, we started to outcross the HM females with males of the GUA strain, a wild-type *Ae. albopictus* recently collected from the field in Guangzhou, China [6]. A steady increase in the egg hatch rate of the outcrossed HM line was then observed, from 21% at G19 to 84% at G27 (Fig. 3.2), the higher level being similar to that in the

GUA strain. Thus, it appears that the GUA genetic background is able to overcome the triple infection-associated decrease in egg hatch rate in the HM line.

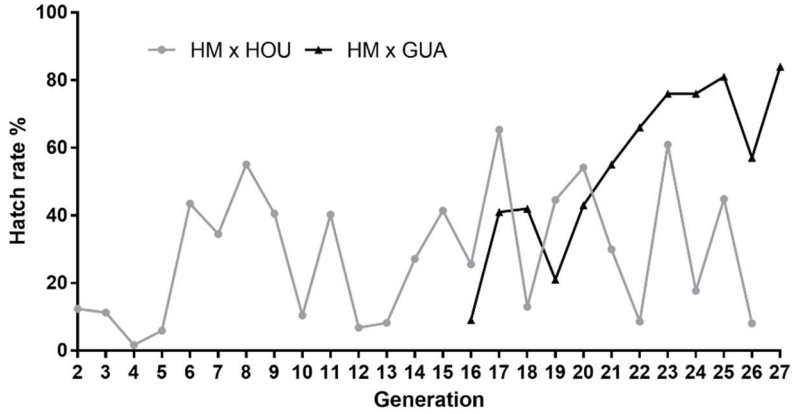

Figure 3.2 Egg hatch rate of HM females outcrossed with wild-type males from G2 to G27.

Egg hatch was calculated as the percentage of eggs hatched divided by the total number of eggs. Outcrosses are indicated as female x male. HM, the transinfected *Ae. albopictus* line carrying the triple infection with *w*Mel, *w*AlbA, and *w*AlbB. HOU and GUA, two wild-type *Ae. albopictus* lines carrying *w*AlbA and *w*AlbB.

3.2.3 wMel distribution in both the somatic and germline tissues in the HM line

Wolbachia tissue tropism is an important determining factor underlying its viral blocking effect and maternal transmission. We first compared the densities of the three *Wolbachia* strains, *w*Mel, *w*AlbA, and *w*AlbB, in somatic tissues (salivary glands and midgut) and germline tissues (ovaries) of HM mosquitoes by quantitative PCR (qPCR). In the salivary glands at G6, the density of *w*AlbB was significantly higher than that of *w*AlbA, but there was no significant difference in density between *w*Mel and *w*AlbA or between *w*Mel and *w*AlbB (Fig. 3a). In the midgut, a higher density of *w*AlbB than either *w*Mel or *w*AlbA was observed, whereas the the densities of *w*Mel and *w*AlbA did not differ significantly

(Fig. 3b). These results indicates that *w*AlbB is dominant in the somatic tissue of HM mosquitoes. By contrast, a higher density of *w*Mel than of *w*AlbA or *w*AlbB is apparent in HM ovaries (Fig. 3c). This distribution pattern was consistently maintained at G6 and G18 despite some degree of fluctuation.

To better understand the strain-specifc interactions in transinfected mosquitoes with triple infections, we also compared the densities of *w*Pip, *w*AlbA, and *w*AlbB in HC ovaries and observed a different order of *Wolbachia* density: *w*Pip > *w*AlbA > *w*AlbB (Fig. 3c). Consistent with previous observations [25], *w*AlbB was present at a higher level than was *w*AlbA in the ovaries of HOU mosquitoes, from which both the HC and HM lines were originally derived (Fig. 3c). We further compared the density of the same *Wolbachia* strain in ovaries across various mosquito lines to examine the impact of the host's genetic background on infection levels. *w*AlbA showed its highest level of infection in HM ovaries at G6 but decreased by 7.8-fold at G18, when it reached a level closer to that in HOU ovaries. The density of *w*AlbB was stable in HM ovaries from G6 to G18 and was consistently maintained at a level significantly higher than that in both the HOU and HC lines (Fig. 3d). The density of *w*Mel decreased by 47% in HM ovaries from G6 to G18 (Fig. 3d) but was still much higher than that of the other *Wolbachia* strains. Interestingly, as compared to HOU ovaries, *w*AlbA was 1,022-fold higher-fold in HM ovaries at G6, and *w*AlbB was 1,411-fold lower in HC ovaries(Fig. 3d). Taken together, these results indicate that *Wolbachia* density is regulated in triply infected *Ae. albopictus* in a strain-, host-, and temporally specific manner.

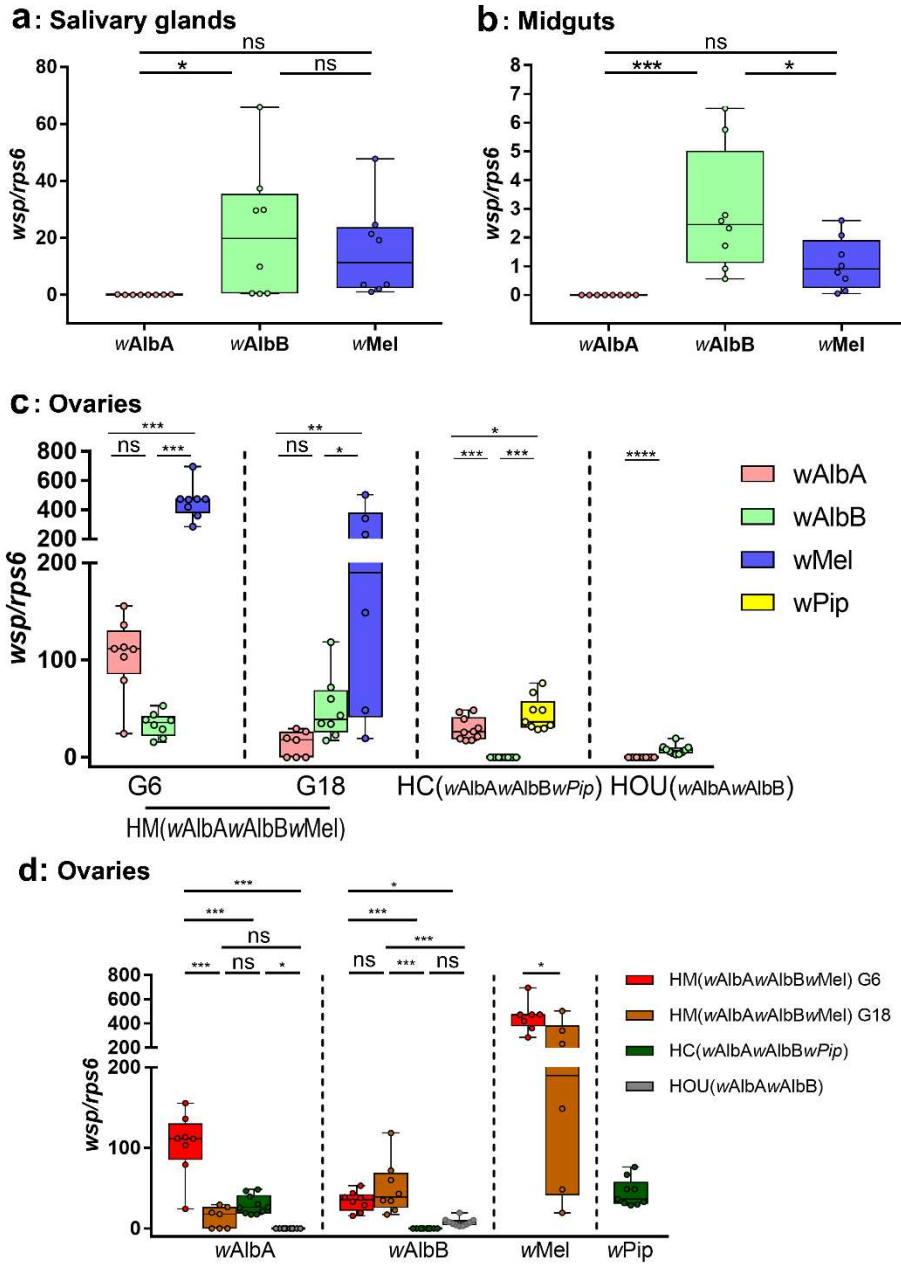

Figure 3.3The densities of various *Wolbachia* strains in the salivary glands, midguts, and ovaries of HM mosquitoes.

The densities of *w*Mel, *w*AlbA, and *w*AlbB in salivary glands (**a)** and midguts (**b**) of HM mosquito were measured by quantitative PCR (qPCR). The densities of the three *Wolbachia* strains in the ovaries of HM mosquitoes from two generations, G6 and G18, were compared within (**c**) and across (**d**) mosquito lines. The copy number of the *Wolbachia wsp* gene was normalized by the mosquito *rps6* gene. The data are shown as

the mean of nine replicates ± standard deviation. ****, P <0.0001; ***, P <0.001; **, P <0.01; *, P <0.05; ns, not significant; ANOVA and Tukey's multiple comparisons test.

3.2.4 Failure of the HM line to induce CI when crossed with wild-type or transinfected lines

The ability to induce CI is a key feature that is required in order to develop *Wolbachia*-based strategies for mosquito-borne disease control. We therefore set up a series of reciprocal crosses among the HM, GUA, and HC lines to measure the relative strength of their *w*Mel-mediated CI expression. All compatible crosses yielded egg hatch rates ranging from 51% to 56% (Table 3.1). Unexpectedly, two presumably incompatible crosses, matings between HM males and either GUA or HC females, resulted in high egg hatch rates (46.7% and 43.2%, respectively), indicating compatible mating between them. By contrast, consistent with the ability of HC males to induce a strong CI when crossed with GUA females [6], near-complete CI was observed in the crosses between HC males and HM females (Table 3.1). These results indicate that the ability of *w*Mel to induce CI, as observed previously [19, 22], is blocked in the HM line when it co-exists with *w*AlbA and *w*AlbB.

Table 3.1 Results of CI crosses among the HM, GUA, and HC lines.

Expected CI type	Cross (♀ × ♂)	Infection type		Percent egg hatch*
		female	male	
Incompatible	HC X HM	*w*AlbA,*w*AlbB, *w*Pip	*w*AlbA,*w*AlbB, *w*Mel	43.2 ± 11.6 a
	HM X HC	*w*AlbA,*w*AlbB, *w*Mel	*w*AlbA,*w*AlbB, *w*Pip	0.01 ± 0.04 b
	GUA X HM	*w*AlbA,*w*AlbB	*w*AlbA,*w*AlbB, *w*Mel	46.7 ± 7.8 c
Compatible	HM X GUA	*w*AlbA,*w*AlbB, *w*Mel	*w*AlbA,*w*AlbB	55.5 ± 17.8 c
	HM X HM	*w*AlbA,*w*AlbB, *w*Mel	*w*AlbA,*w*AlbB, *w*Mel	55.1 ± 9.0 c
	HC X HC	*w*AlbA,*w*AlbB, *w*Pip	*w*AlbA,*w*AlbB, *w*Pip	51.3 ± 11.7 c

* Expressed as the mean for 15 replicates/cross type ± standard deviation. Different letters following the data indicate significant differences ($P < 0.001$) by ANOVA-Tukey's multiple comparison test.

3.2.5 CI induction by wMel after removal of wAlbA from the HM line in Ae. albopictus

In order to understand whether the ability of *w*Mel to induce CI in the HM line is being blocked by the other two native *Wolbachia* strains, we treated the HM line with a subdose of tetracycline for four generations and monitored the infection profile by strain-specific PCR from G3 to G5 after tetracycline treatment (Fig. 3.4a-c). This treatment resulted in

the specific removal of *w*AlbA from the HM line and establishment of the HM2 line, with a double infection with *w*Mel and *w*AlbB (Fig. 3.4c). CI crosses were were then performed using HM2, GUA, HC, and an *Ae. albopictus* HB line with a single *w*AlbB infection. Strikingly, we observed a strong, although not complete, CI when HM2 males were crossed with GUA, HC, or HB females (Table 3.2). As expected, HM2 induced bi-directional CI when crossed with the GUA and HC lines, but uni-directional CI when crossed with the HB line. Among all of these incompatible crosses, HC males induced the highest level of CI, with 100% embryonic death. These results indicate that *w*AlbA may block the expression of CI by *w*Mel in the HM line.

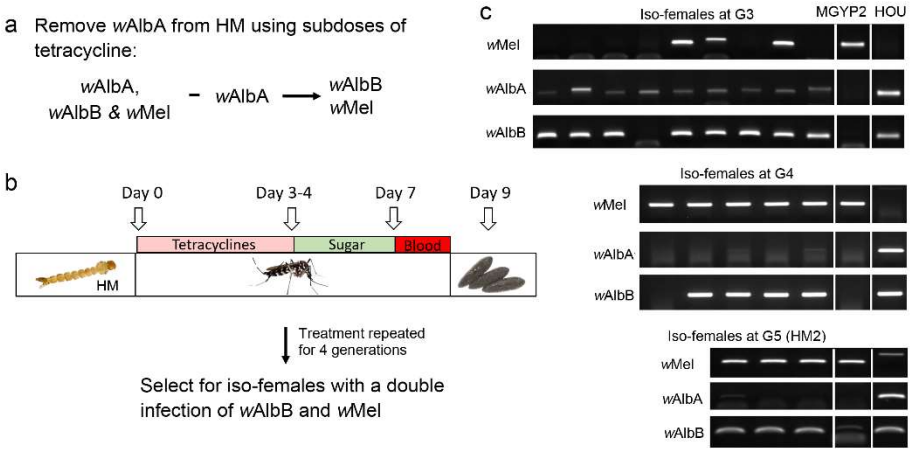

Figure 3.4 Establishment of the HM2 line carrying the double infection with *w*AlbB and *w*Mel.

a, b. Schematic diagram of the experimental approach to remove *w*AlbA from the triply infected HM line using subdoses of tetracycline. **c.** Representative results from the PCR screening for isofemales carrying a double infection with *w*AlbB and *w*Mel. *w*AlbA was specifically removed after treatment for four consecutive generations.

Table 3.2 Results of CI crosses among the HM2, GUA, HB, and HC lines.

Expected CI type	Cross (♀ × ♂)	Infection types		Percent egg hatch*
		Female	Male	
Incompatible	HM2 × HC	*w*AlbB, *w*Mel	*w*AlbA, *w*AlbB, *w*Pip	0 ± 0 a
	HC × HM2	*w*AlbA,*w*AlbB *w*Pip	*w*AlbB, *w*Mel	9.0 ± 7.8 b
	GUA × HM2	*w*AlbA, *w*AlbB	*w*AlbB, *w*Mel	14.6 ± 8.3 b
	HM2 × GUA	*w*AlbB, *w*Mel	*w*AlbA, *w*AlbB	9.3 ± 4.2 b
	HB × HM2	*w*AlbB	*w*AlbB, *w*Mel	14.6 ± 6.1 b
Compatible	HM2 × HB	*w*AlbB, *w*Mel	*w*AlbB	79.6 ± 8.2 c
	HM2 × HM2	*w*AlbB, *w*Mel	*w*AlbB, *w*Mel	64.8 ± 17.7 d
	HB × HB	*w*AlbB	*w*AlbB	83.7 ± 6.9 c
	HC × HC	*w*AlbA, *w*AlbB, *w*Pip	*w*AlbA,*w*AlbB *w*Pip	80.8 ± 7.8 c
	GUA × GUA	*w*AlbA,*w*AlbB	*w*AlbA,*w*AlbB	85.6± 4.8 c

* Expressed as the mean for 15 replicates/cross type ± standard deviation. Different letters following the data indicate significant differences ($P < 0.001$) by ANOVA-Tukey's multiple comparison test.

3.3 Discussion

We have demonstrated the successful establishment of a novel triple *Wolbachia* infection with *w*Mel, *w*AlbA, and *w*AlbB in the *Ae. albopictus* HM line, with 100% maternal transmission efficiency. Despite a severe reduction in the egg hatch rate associated with

the triple infection, the rate was returned to normal levels by outcrossing with the wild-type GUA line, but not the HOU line. Among three different *Wolbachia* strains, *w*Mel and *w*AlbB were highest in expression in the ovaries and midguts, respectively, of HM mosquitoes, whereas *w*Pip and *w*AlbB were present in the highest and lowest levels, respectively, in HC ovaries. The densities of *w*AlbA and *w*Mel, but not *w*AlbB, were reduced from G6 to G18 in HM ovaries. Experimental crosses showed that CI is not induced when HM males mate with either GUA or HC females, but removal of *w*AlbA from the HM mosquitoes results in CI when these mosquitoes are crossed with three *Ae. albopictus* lines carrying either a single- (HB), double- (GUA), or triple- (HC) strain infection. These results indicate the existence of complicated interactions in term of both tissue tropism and CI expression when various *Wolbachia* strains co-exist in a host, providing important information to guide the design and establishment of transinfections in mosquito with optimal *Wolbachia* strains or their combination for disease control.

Our results indciate that competion for tissue tropism may occur between *Wolbachia* strains in the same supergroup. In the phylogeny of *Wolbachia*, both *w*Mel and *w*AlbA belong to supergroup A, whereas *w*AlbB and *w*Pip belong to supergroup B [9]. With the introduction of *w*Mel into *Ae. albopictus* HOU mosquitoes carrying *w*AlbA and *w*AlbB, we observed that the density of *w*AlbA decreased by 7.8-fold in HM ovaries from G5 to G18, but *w*AlbB density remained stable. The level of *w*Mel infection also decreased from G5 to G18, but this decrease could have been caused by either the adaption of *w*Mel to a novel host background or competition from *w*AlbA, or both. Consistent with a previous report [25], the *w*AlbB density was higher than that of *w*AlbA in HOU ovaries. In the triply infected HC mosquitoes, generated by transfer of *w*Pip to HOU mosquitoes

[6], *w*AlbB was suppressed to a minimal level in the ovaries. Specifically, the density of *w*AlbB (5.6 x 10^{-3} *wsp*/*rps6*) was 7,934- and 5,226-fold lower than that of *w*Pip (44.6 *wsp*/*rps6*) or *w*AlbA (29.4 wsp/rps6), respectively. It is worth noting that this low number of *w*AlbB was still sufficient to induce CI, given that unidirectional CI has been observed in crosses of HC and GUA mosquitoes [6]. Thus, when *Wolbachia* is being introduced into an infected host, choosing a novel strain belonging to a supergroup different from that of the orginal infection may prove useful for avoiding competition. Caution should be used if the native strain provides an essential benefit to the host, since the novel strain will likely outcompete the native strain in the transfected line, based on our observations from the HM and HC lines.

Competition for CI induction can also occur among different strains within the same supergroup. Although a single *w*Mel infection is able to induce CI in both *Ae. aegypti* and *Ae. albopictus* [19, 22], HM males did not induce CI when crossed with either GUA or HC females. After removal of *w*AlbA from the HM line, however, we observe a strong CI expression in crosses of HM2 with either GUA, HC or HB. These results indicate that the ability of *w*Mel to modify the HM sperm may be blocked by the presence of *w*AlbA, instead of *w*Mel-modified sperm being rescued by *w*AlbA or *w*AlbB in HC or GUA mosquitoes. Consistent with our observations iconcerning HM2 crosses, double infection of *w*AlbB and *w*Mel in transinfected *Ae. aegypti* was able to induce CI in the crosses with either non-infected, *w*AlbA-, *w*AlbB-, or *w*Mel-infected lines [21]. A similar effort to develop a triple infection (*w*AlbA, *w*AlbB, and *w*Mel) in *Ae. Albopictus* has been reported, but it resulted in self-incompatibility [29], which was not observed in our studies. One possible explaination for the difference from our study is that the *w*AlbA

density in the embryos of their triply infected line was inhibited to such an extent that it was impossible for *w*AlbA to rescue the CI modification in the males; by contrast, in our case the infection level of *w*AlbA was not significantly reduced in the HM ovaries when compared to wild-type.

Blocking by *w*AlbA of the *w*Mel-induced modification of sperm in the HM line suggests a potential competition for host targets of CI factors between *w*Mel and *w*AlbA. Recent studies have suggested a "two-by-one" model underlying the CI mechanism in which *Wolbachia*-induced sperm modification is determined by two CI factors, *cifA* and *cifB*, whereas CI rescue is determined only by *cifA* [12-15]. Further evidence has suggested that *cifB* targets nuclear protein import and protamine-histone exchange and that *cifA* rescues embryos by restricting the access of *cifB* to its targets [15]. We hypothesize that the *cifB* genes of *w*Mel and *w*AlbA are very similar, so that they bind to the same sites that affect the host's nuclear protein import and then are translocated to the nucleus, where their substrates for sperm modification reside. The affinity of native *w*AlbA for host targets may be higher than that of *w*Mel, thus preventing the *w*Mel from entering the nucleus to induce CI expression.

Very low rates of egg hatching were observed in the HM line before G6. Suprisingly, the outcross with wild-type HOU only increased egg hatch rates temporarily in some generations (e.g., G6, G17, and G23); in these cases, there was an immediate decline afterwards, resulting in a fluctuation wave across 26 generations. After the HC line was established, low hatch rates were also observed for almost a year. The situation was different for the other transfected lines that we established, in that egg hatching quickly returned to a normal level after the outcrosses with wild-type for several consecutive

generations [16-18]. It would presumably be more challenging for the host to establish a symbiotic relationship with a *Wolbachia* triple strain than with a single or double strain because of the overload of symbionts and the complicated interactions between various strains and the host. Interestingly, outcrosses of HM with another wild-type line, GUA, effectively recovered normal egg hatch rates, indicating that the GUA genetic background can facilitate the host's adaptation to the novel triple infection. Because HM was derived from HOU, which had been maintained for a long time in the laboratory, outcrosses with HOU may not be able to introduce as much genetic heterogeneity to foster a novel symbiosis as can outcrosses with GUA, which was recently established from field samples [6].

Here, we have demonstrated the successful extablishment of a transinfected *Ae. albopictus* HM line carrying a *Wolbachia* triple-strain infection. Unfortunately, the newly introduced *w*Mel strain failed to induce CI, and our experimental evidence indicates that its ability to modify the sperm was blocked by the native strain, *w*AlbA. Further studies are needed to compare the CI determination factors associated with *w*Mel and *w*AlbA and to understand the molecular mechanism undergirding their potential competition in utilizing host targets for CI expression. The tissue tropism of the three *Wolbachia* strains in the HM line indicates their complicated interactions, with competition likely to happen between *Wolbachia* strains in the same supergroup. The differences in both CI expression and *Wolbachia* tissue tropism between the two triply transinfected lines HM and HC also indicate that caution is necessary when predicting the outcome of transinfected lines with multiple infections. These results provide important information to guide the future selection of *Wolbachia* strains for the development of transinfected lines in order to

obtain the maximum pathogen-blocking efficiency, the lowest fitness cost, and ideal CI patterns.

3.4 Materials and Methods

3.4.1 Mosquito lines and maintenance

Two wild-type *Aedes albopictus* lines, HOU [16] and GUA [6], carrying a native superinfection with *w*AlbA and *w*AlbB were used in this study. Two transinfected *Aedes albopictus* lines, HB and HC, carrying a single *w*AlbB infection and a triple infection with *w*Mel, *w*AlbA, and *w*AlbB, respectively, had been generated previously [6, 16] and were used in the CI crosses. The transinfected *Aedes aegypti* MGYP2 line [19], carrying *w*Mel, was used as a donor to generate the HM line.

All the mosquito lines were maintained on a 10% sugar solution at 27± 1°C and 80±10% relative humidity, with a 12:12 h light:dark photoperiod, according to standard rearing procedures. For routine colony maintenance and experimental studies, female mosquitoes were provided with either human (for the MGYP2 line) or sheep (for the other lines) blood at day 7 post-eclosion, and eggs were collected 2 days post-blood meal.

3.4.2 Transinfection to generate the HM line

The HM line was generated by transfer of *w*Mel from *Ae. aegypti* MGYP2 to *Ae. albopictus* HOU using embryonic microinjection according to the approach described previously [16, 18]. In brief, cytoplasm from donor embryos was transferred into the posterior of 60- to 90-min-old recipient embryos using an IM300 microinjector (Narishige Scientific). After injection, the embryos were incubated at 85% relative

humidity and 27°C for 1 h, then transferred to wet filter paper. Embryos were allowed to mature for 5-7 days before hatching. Females (G0) developing from the surviving embryos were isolated and mated with HOU males. After blood-feeding and oviposition, G0 females were tested for *w*Mel infection by PCR using strain-specific primers as described below. G1 females were again crossed with HOU males, blood-fed, isolated, and allowed to oviposit. The offspring from *w*Mel-positive G1 were selected for the next screen, and this process was repeated until the wMel maternal transmission rate reached 100%. Diagnosis of *Wolbachia* *w*AlbA and *w*AlbB was also performed to ensure that the transinfected line carried the triple infection.

3.4.3 PCR assays of Wolbachia infection

Primers were designed for strain-specific diagnosis of four different strains on the basis of the sequence of the gene encoding the *Wolbachia* surface protein *wsp* . The primers for *w*AlbA were: forward 5'-GTGTTGGTGCAGCGTATGTC-3'; reverse 5'-GCACCAGTAGTTTCGCTATC-3'. The primers for *w*AlbB were: forward 5'-ACGTTGGTGGTGCAACATTTG-3'; reverse 5'-TAACGAGCACCAGCATAAAGC-3'. The primers for *w*Mel were: forward 5'-CCTTTGGAACCCGCTGTGAATG-3'; reverse 5'-GCCTGCATCAGCAGCCTGTC-3'. The primers for *w*Pip were: forward 5'-TATTTCCCACTATATCCCTTC-3'; reverse 5'- GGATTTGACCTTTCCGGC-3'. The primers given below for mosquito rps6 have been reported previously [30]: forward 5'-CGTCGTCAGGAACGTATTCG-3'; and reverse 5'-TCTTGGCAGCCTTGACAGC-3'. Standard curves were generated for each of the genes listed above to convert the Ct value from qPCR to the copy number of target sequences.

Genomic DNA was extracted from the samples using a Thermo Scientific Phire Animal Tissue Direct PCR Kit (F-140WH). Samples were pre-treated in 20 µl of dilution buffer with 0.5 µl DNARelease Additive. The reaction mixture contained 10 µl 2X Phire Animal Tissue PCR Buffer, 0.4 µl Phire Hot Start II DNA Polymerase, 0.2 µl of both the forward and reverses primer, and 7.2 µl dsH_2O. The regular PCR conditions were: initial denaturation at 98°C for 6 min, followed by 40 cycles of 5 s at 98°C, 5 s at 56°C, and 45 s at 72°C. Quantitative PCR (qPCR) was performed using a QuantiTect SYBR Green PCR Kit (Qiagen) and ABI Detection System ABI Prism 7000 (Applied Biosystems, Foster City, California, USA). Samples were homogenized in 100 µl 1 x STE buffer and incubated with 4 µl of roteinase K at 55°C for 1 h, followed by 97°C for 5 min.

3.4.4 Tetracycline treatment of the HM line to generate the HM2 line with a double infection with wMel and wAlbB

Once the HM mosquitoes had emerged as adults (day 0), they were provided with 0.5 mg/ml tetracycline HCl in a 10% sugar solution. This solution was replaced with a 10% sugar solution from day 3 or 4, and a blood meal was provided on day 7. Two days after the blood feeding, the mosquitoes were provided with oviposition cups containing wet filter paper. These treatments were repeated for four generations. At G3, after blood-feeding, the females were isolated for oviposition. After their eggs collected, individual isofemales were sacrificed to extract genomic DNA, and a PCR assay was used to identify each of the three *Wolbachia* strains. Only the eggs from females showing a double infection with *w*Mel and *w*AlbB were allowed to hatch to establish the line. The isofemale selection described above was repeated at G5 to ensure the removal of *w*AlbA, and the resulting HM2 line carried only the double infection with *w*Mel and *w*AlbB.

3.4.5 Experimental crosses to determine CI

CI assays were conducted as previously described [16, 18]. Ten virgin males were mated with ten virgin females in five replicate cages for each cross. A blood meal was provided to the females at day 7 post-eclosion. Two days after the blood meal, eggs were collected using oviposition cups containing wet filter paper, which was subsequently desiccated for 7 days at 27°C and 80% relative humidity. The eggs were counted and then hatched in water containing 6% (m/v) bovine liver powder. Larvae were counted at the L2-L3 stage to record the hatch rate.

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Chapter 4 Establishment of transinfection with the local *Wolbachia* strain *w*Mal in the local *Aedes aegypti* line for risk management in Singapore

4.1 Introduction

Dengue fever and dengue hemorrhagic fever were first reported in Singapore in the 1960s, and, since then, they have been a significant health issue threatening the local population. The number of dengue virus infection cases has increased in recent years. As reported by Singapore National Environment Agency (NEA), during the dengue outbreak, there were 22,170 cases and 7 deaths in 2013, the peak number of cases reached 660 patients per week in 2019, and the number reached 1,800 cases in the episode in 2020[1]. During the Zika outbreak in 2016, 455 cases were confirmed in Singapore[2]. Both dengue and Zika are primarily transmitted by *Aedes aegypti* in Singapore, which presents in built up areas, with the ubiquitous species *Aedes albopictus* as the secondary vector. Due to insufficiency of traditional vector control approaches in dengue control, Singapore NEA started to explore the use of *Wolbachioa*-based population suppression after comparing different novel vector control tools[3]. Currently *w*AlbB-infected *Ae. aegypty* developed through this dissertation study has been released for field trial in Singapore, resulting in over 90% reduction in mosquito population and 65-80% fewer dengue case compared to the control sites without release[3].

Encouraging progress in field trial, however, is recently challenged by emerging risks that may compromise the sustainable vector control for the long-term. Absence of perfect

sex separation technique to produce incompatible males results in releasing small appropriation (~0.5%) of residual females into the field. This could cause invasion of *w*AlbB into the population and failure of population suppression if *w*AlbB-infected males continue to be released as the matings between males and females carrying the same type of *Wolbachia* are compatible. The situation becomes even more complicated when Malaysia, the neighboring country of Singapore with causeway directly connected each other, started the population replacement trial by releasing *w*AlbB-infected mosquito[4]. The increasing possibility of establishment of *w*AlbB in Singapore by both unintentionally release of *w*AlbB-infected females and spreading from Malaysia to Singapore requires for a solution to mitigate the risk. In addition, as the *w*AlbB infection in *Ae. aegypti* used in Singapore field trial is derived from *Ae. albopictus* Waco strain originally collected from Texas, US, there is a new need by Singapore government to release only local *Wolbachia* strain into Singapore's environment to remove any potential risk associated with a foreign organism.

Aedes malayensis is another local *Aedes* mosquito species found in Singapore. [5] This mosquito species is infected with the *Wolbachia* strain *w*Mal, belonging to the supergroup A, but its associated phenotypes, including host reproduction alteration, are undetermined. Previous studies show that *Ae. malayensis* may be a potential vector for yellow fever virus[5], indicating the native *w*Mal infection may be similar to the superinfection of *w*AlbA and *w*AlbB in *Ae. albopictus* and may confer either very minor or no resistance to arboviruses in *Ae. malayensis*. This new strain of *Wolbachia* motivated us to generate a novel *w*Mal infected *Ae. aegypti* line and to test whether it has

desired characteristics in *Ae. aegypti* for use in disease control and risk management if it becomes necessary in the future.

In this work, we have transferred *w*Mal from *Ae. malayensis* into *Ae. aegypti* using embryonic microinjection. In order to develop a "pure" local line, these two mosquito species collected in Singapore were used as both donor and recipient of *w*Mal during transinfection. This results in successful establishment of *w*Mal-infected *Ae. aegypti* with a perfect maternal transmission. Additional experiments are ongoing to examine *w*Mal-mediated CI expression and viral interference.

4.2 Results

4.2.1 Establishment of wMal transinfection in Ae. aegypti Singapore line

The *w*Mal from *Ae. malayensis* was transferred into embryos of wild-type (WT) *Ae. aegypti* Singapore line to establish a novel transinfected line. The virgin females (G0) developed from embryos survived from microinjection were outcrossed with WT males to produce offsprings (G1). Eight G1 isofemales were outcrossed with WT males. After their eggs (G2) were collected, PCR assay was used to diagnose the *Wolbachia* strain profile in those females, with 5 out of 8 isofemales (62.5%) observed to carry the *w*Mal *Wolbachia* infection. We also observed that 6 out of 7 (85%) G1 male were infected. The average maternal transmission rate at G1 is 73.3%. The offspring of those females without a *Wolbachia* infection were discarded. Among the G2 offsprings, 13 out of 18 individuals carried *Wolbachia* infections, with the infection rate 72.2%. Without further screening, G3 females were pooled together to establish the transinfected line, referred to as WSM hereinafter. At G5 and G6, we randomly selected 20 and 10 individuals,

respectively, for PCR assay, and all mosquitoes carried a *w*Mal infection, indicating a 100% maternal transmission efficiency (Fig. 1). Although low at G1, the egg hatch rate gradually increased from G2 to G5 (Fig. 1).

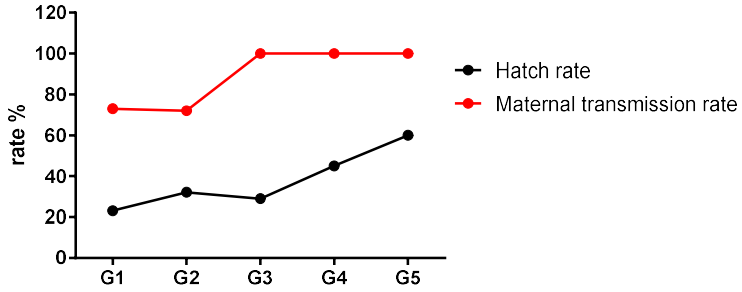

Figure 4.1 The hatch rate and maternal transmission rates of *w*Mal-infected *Ae. aegypti*.

The hatch rate was calculated as the number of hatched larvae divide by the total number of eggs. The maternal transmission rate was determined by the percentage of infected individuals among the total number of mosquitoes assayed by PCR at each generation.

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4.3 Discussion

In order to minimize the impacts of field release on environment, release of local mosquito species with local *Wolbachia* strain has been proposed to be an optimal solution. In this work, we have generated *w*Mal-infected *Ae. aegypti* by transferring *Wolbachia* from local donor species into local recipient species in Singapore. The transfected line WSM showed a perfect maternal transmission. A low egg hatch rate was observed in the WSM line, but was gradually recovered each generation after outcrosses. Further studies will characterize *w*Mal-induced CI expression, blocking effects on dengue virus strains circulating locally in Singapore and the fitness costs associated with *w*Mal.

Biosafety concerns are the major hurdle to prevent a novel mosquito control approach from deploying in an endemic country. These concerns commonly include its non-target effects and impacts on environment. Among different novel techniques, *Wolbahcia*-based population suppression has advantage in that it involves release of males that do not bite human and will disappear from environment quickly (i.e., about 10 days) after release. However, due to the current limitation in sex separation, unintentional release of infected females into environment can not be completely avoided. Thus, there is a need to release mosquito with local genetic background and local *Wolbachia* strain, such that no alien organism is introduced into the environment. Our studies provide the first example how to build a mosquito with "purely local" materials for release.

Although to be validated by the experiment, *w*Mal-infected *Ae. aegypti* may provide a practical solution for risk management in Singapore if it can induce bio-directional CI with *w*AlbB-infected *Ae. aegypti*, which is currently released in both Singapore and Malaysia. This will provide a weapon to eliminate *w*AlbB infection if it is established in the field and further suppression is needed for disease control, or an unexpected consequence associated with *w*AlbB release is noticed in the future.

As very little knowledge on *w*Mal is available, it is also likely that *w*Mal may not induce CI and /or viral interference. This would mainly be determined by the genetics of *w*Mal, e,g., presence of CI determination factors in its genome. Due to lack of essential CI determination factors, *Wolbachia w*Au strain did not induce CI after transferred from *Drosophila melanogaster* into *Ae. aegypti* [6]. However, *w*Au developed at a high density in *Ae. aegypti* and induced very strong resistance to arboviruses, indicating independent expression of CI and viral interference. If *w*Mal does not induce CI but has strong virus

blocking effects, we will combine it with the other CI-inducing strains to develop a superinfection for use in population replacement. Future studies will explore additional novel strains to develop a perfect transfection system, with maximum viral blocking effect, minimum fitness cost and ideal CI patten, for disease control.

4.4 Material and method

4.4.1 Mosquito lines and maintenance

Wild-type *Aedes malayensis* lines, carrying the native *Wolbachia w*Mal, was used as a donor to generate the WSM line. A Singapore wild-type *Aedes aegypti* (Ae.sin) line which does not carry *Wolbachia* was used as a recipient during the transinfection.

All the mosquito lines were maintained on 10% sugar solution at 27 ± 1 °C and 80 ± 10% relative humidity (RH), with a 12:12 h light:dark photoperiod, according to standard rearing procedures. For routine colony maintenance and experimental studies, female mosquitoes were provided with either human (MGYP2 line) or sheep (other lines) blood at day 7 post eclosion and eggs were collected two days post blood meal.

4.4.2 Transinfection to generate the WB2 line

The WSM line was generated by the transfer of wMal from Singapore wild-type Aedes malayensis to Singapore wild-type aedes aegypti using embryonic microinjection according to the approach described previously[8, 19]. In brief, cytoplasm from donor embryos was transferred into the posterior of 60-90 minutes old recipient embryos using an IM300 microinjector (Narishige Scientific). After injection, embryos were incubated at 85% RH and 27°C for one hour and transferred to wet filter paper. Embryos were allowed to mature for 5-7 days before being hatched. Females (G0) developed from the

survived embryos were isolated and mated with HOU males. After blood-feeding and oviposition, G0 females were tested for wMal infection by PCR using strain-specific primers as described below. G1 females were again crossed with Ae.sin male, blood-fed, isolated and allowed to oviposit. The offsprings from wMAl-positive G1 were selected for the next screen, and this process was repeated until wMal maternal transmission rate reach 100%.

4.4.3 PCR assay of Wolbachia infection

Primers were designed base on the sequence of Wolbachia surface protein wsp gene for strain-specific diagnosis of four different strains. The primers for The primers for wMal were: forward 5'-GTGTTGGTGCAGCGTATGTC-3'; reveres 5'-GCACCAGTAGTTTCGCTATC-3'. The below primers for mosquito rps6 were reported previously [30]: forward 5'-CGTCGTCAGGAACGTATTCG-3'; and reverse 5'-TCTTGGCAGCCTTGACAGC-3'. Standard curves were generated for each of the above genes to convert the Ct value of qPCR into the copy number of the target sequences.

Genomic DNA was extracted from samples using Thermo Scientific Phire Animal Tissue Direct PCR Kit (F-140WH). Samples were pre-treated in 20 µl dilution buffer with 0.5 µl DNARelease Additive. The reaction mixture contained 10 µl 2X Phire Animal Tissue PCR Buffer, 0.4 µl Phire Hot Start II DNA Polymerase, 0.2 µl for both forward and reverses primer and 7.2 µl dsH2O. The regular PCR condition comprised of initial denaturation at 98 °C for 6 min, followed by 40 cycles of 5 s at 98 °C, 5 s at 56 °C, and 45 s at 72 °C. The quantitative PCR (qPCR) was performed using the QuantiTect SYBR Green PCR Kit (Qiagen) and ABI Detection System ABI Prism 7000 (Applied Biosystems, Foster City, California, USA). Samples were homogenized in 100 µl 1 x

STE buffer and incubated with 4 µl Proteinase K at 55°C for 1 h and followed by 97°C for 5 min.

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Chapter 5 Functional characterization of sex determination genes in *Aedes aegypti* mosquito.

5.1 Introduction

Significant progress has been made to reduce reproduction of insect pests for population suppression by release of artificially reared insects to induce sterile matings in the field. Three different genetic approaches are currently under development for birth control of mosquito vectors: radiation-based sterile insect technique (SIT), *Wolbachia*-based incompatible mating, and the use of transgenesis to induce sterility[1]. While SIT has been successfully used to control several agriculturally important insect pests, both *Wolbachia* and transgenic technologies have shown great potential in suppressing / eliminating mosquito population in the field trials. All these approaches require for releasing males to mate with wild females and thus effective sex separation is essential in the application for mosquito control. Although male and female mosquito can be separated manually based on their morphological differences in laboratory experiments, it is not feasible when millions of males to be released in order for them to surpass the critical over-flooding ratio required for suppression to be attained in the field. To meet this challenge, a mechanical sorter has been developed for sex separation based on the size difference between male and female pupae. However, not only it has low efficiency and high costs, but also its accuracy is subject to rearing condition of mosquito larvae. For example, a low nutrition or larvae reared in crowds can reduce the size of female pupae, resulting in lack of sufficient difference between male and female pupae. In

addition, the mechanical sorter for *Aedes* mosquitoes is difficult to be used for *Anopheles* mosquitoes because of lack of synchronism in larval development. Additional efforts have been made to use a toxic chemical contained in the blood to kill the females (as only females take blood), or develop genetic sex strains for sex separation, but have resulted in either limited progress or failure when tested in mass production condition. Although transgenic technique and artificial intelligence have been recently developed to address the need for sex separation, their appropriateness has not been fully determined.

One novel approach has been recently proposed to induce the female-specific lethality during the larvae development by feeding with *E. coli* expressing double strand RNA (dsRNA) to silence the mosquito sex determination genes. However, controversial observations have been reported from different experiments. One study showed that nearly 100% adults were developed into males after *Ae. aegypti* larvae were treated with dsRNA of the female specific sex determining gene *doublesex* (*dxl*). Other studies, including our work, showed no male-biased sex ratio after *dxl* silencing using dsRNA. Thus, identifying the key genes involved in mosquito sex determination and validating their function are essential to develop such a novel genetic approach for mosquito sex separation.

The sexes of mosquitoes are controlled by the sex determination pathway (Fig. 5.1). In *Ae. aegypti* male, the male -determining factor Nix prevents the expression of *transformer* (*tra*), resulting in default male splicing of *dsx* during the early embryonic development. The male specific *dsx*-M leads to the expression of other male specific genes, and the embryo follows the development path to male mosquito[2, 3]. In the

female embryo, the absence of *Nix* gene enables the expression of *tra*, which, together with *transformer-2* (*tra-2*), activates the female-specific splicing of *dsx*, by interacting with a splice acceptor upstream of exon 4[3, 4]. In other insects, silencing or knocking out *tra* results in eliminating female[5]. However, *tra* has not been identified in mosquito. In contrast, both *dsx* and *tra-2* have been identified in *Ae. aegypti*, although their roles in sex determination have not been fully understood.

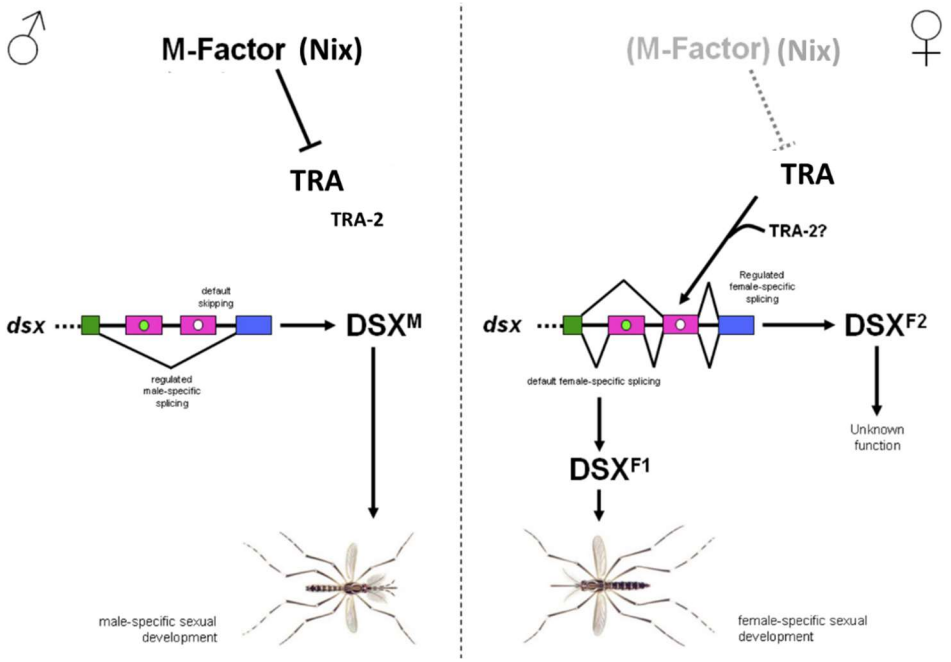

Figure 5.1 The sex determination pathway in *Ae. aegypti*.

In male mosquito, the male-determining factor Nix blocks the expression of *tra* expression. The *dsx* gene undergoes default splicing, and the male specific dsx^{M} mRNA is expressed, resulting in development into male characteristics. In female mosquito, absence of Nix leads to expression of *tra*. The tra and tra-2 proteins together activate female specific splicing, resulting in female sexual development[2, 3].

Since first discovered, clustered regularly interspaced short palindromic repeats (CRISPR)/ Cas9 genome editing system has become the most versatile and precise method of genetic manipulation to characterize the gene function[4]. The cas9 protein uses CRISPR sequences as a guide to recognize and cleave sections of the DNA sequence at particular locations in the genome. In mosquito, success has been made to generate high mutation rate by directly injecting Cas9 protein and single guide RNA (sgRNA) into mosquito embryos[6].

In this work, we have used CRISPR/Cas9 as a tool to screen and identify potential gene targets that can result in female-specific lethality after their knockout. To establish this approach, we first knocked out *Ae. aegypti* white gene, which encodes the black eye pigment, using CRISPR/Cas9 system, to optimize the experimental procedure[7]. Following the successful establishment of CRISPR/Cas9 approach, we further individually knocked out *dsx* and *tra-2* in *Ae. aegypti*. Although female-specific killing or altered sex ratio was not observed in the *dsx* mutant line, knockout of *tra-2* resulted in male-biased sex ratio and absence of female mosquito with homozygous *tra-2* mutant. These results indicate that *tra-2* is a potential sex determination target that can be explored to induce the female-specific lethality for mosquito sex separation tool.

5.2 Result

5.2.1 Measure the CRISPR/Cas9 knock-out efficiency by targeting the White gene in Ae. aegypti

Figure 5.2 Experimental design to knock out white gene by CRISPR/Cas9.
Two guide RNAs were designed to target white gene, with the sgRNA 1 located on exon 4 and the sgRNA2 on exon 6.

In order to develop the CRISPR/Cas9 approach and examine its knockout efficiency in *Ae. aegypti*, White gene (AAEL016999) was selected as a target for mutation to characterize its phenotypes. Two independent sgRNA were designed using Chopchop.com to target white gene exon 4 and exon 6. Cas9 protein and two sgRNA mixture was injected into fresh laid eggs (approximately 90 min after egg laying). After injection, the eggs were incubated for 5 days before hatching. After hatching the mosquito were examined to determine their phenotypes at both pupa and adult stage. Among 250 injected embryos, 49 individuals were developed into adult stage, with the survival rate 19.6% (Table 5.1). Furthermore, 38 out of 49 survived mosquitoes showed completed or partial white eye mutation, with the mutation rate 77.6%. Amon them, 6 individuals showed complete white eyes (Table 5.1).There were three different mutation phenotypes in G0: (1) loss of pigment in partial eye (Fig. 5.3a), (2) eye with reduced pigment (Fig. 5.3b), (3) complete loss of pigment in eye (Fig. 5.3c). By contrast, the eye was completely black in wild-type mosquito (Fig. 1d). The same phenotypes were also

observed in adult stage (Fig. 5.4). These results indicate the success in establishment of CRISPR/Cas9 approach to knock out the target gene in *Ae. aegypti*.

Table 5.1 Survival and mutation rates in *Ae. aegypti* with White gene knocked out using CRISPR/Cas9.

	No.	**Survival/mutation rates**
Total embryos	250	
Survival mosquito	49	19.6% (49/250)
Mutated mosquito	38	77.6% (38/49)
Complete white eye	6	12.2% (6/49)

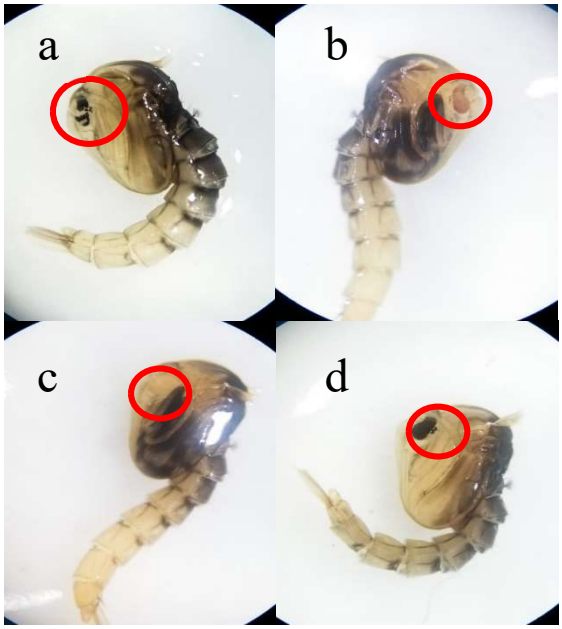

Figure 5.3 The eye phenotypes in *A. aegypti* pupae (G0) with white gene knock-out using the CRISPR/Cas9 approach.

a. Loss of pigment in partial eye. **b.** Eye with reduced pigment. **c.** Complete loss of pigment in the eye. **d.** Control, wild-type mosquito, with eye showing dark black pigment.

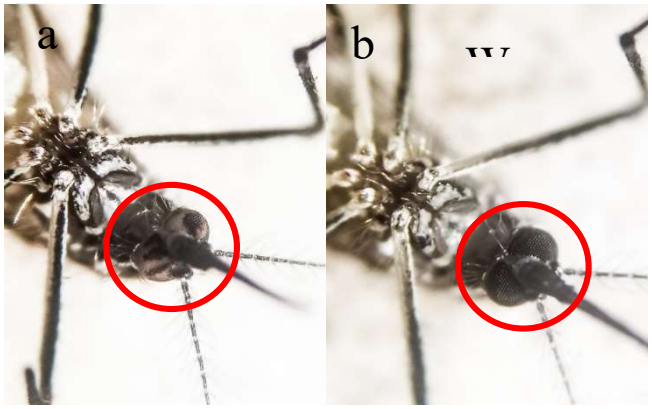

Figure 5.4 The eye phenotypes in *A. aegypti* adults (G0) with white gene knock-out using the CRISPR/Cas9 approach.

a. Loss of pigment in the partial eye. b. Control, wild-type mosquito, with eye showing dark black pigment.

5.2.2 Selecting the target site on dsx gene for the CRISPR/Cas9 knock-out.

We then used the CRISPR/Cas9 approach to knock out *dsx* to characterize its function in sex determination in *Ae. aegypti*. Among 3 transcripts of *dsx* gene, two are female-specific, the other is male-specific, and exon 5b are present in both female-specific transcripts (Fig. 5.5a). Two Cas9 targets were selected within exon 5b, with 155 bps between two targets (Fig 5.5b). In order to increase mutation efficiency and survival rate[6], Cas9 protein and *in vitro* transcribed sgRNA were used in this study. The mutants were screened based on deletion of the region targeted by guide RNA using PCR assay (Fig 5.5b).

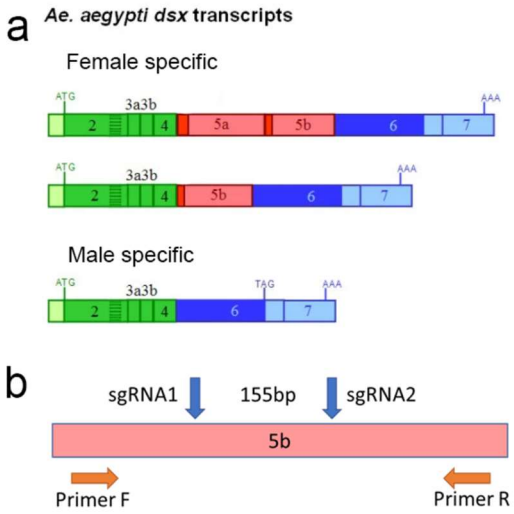

Figure 5.5 Genomic splicing variants of *dsx* in *Ae. aegypti* and sgRNA design.

a. Two female-specific and one male-specific transcripts, with exon 5b presented in both female-specific transcripts. **b.** Two sgRNA were designed to target exon 5b, with 155bps between them. Orange arrows indicate the locations of primers for PCR screen.

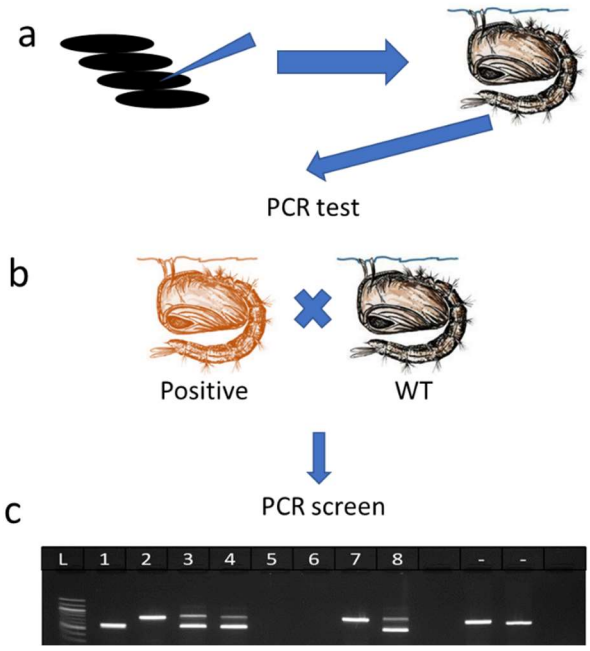

Figure 5.6 Experiment procedures.

a. After embryonic microinjection, genomic DNA were extracted from pupa shell of survived individuals for PCR diagnosis for mutants. **b.** The positive sample was outcrossed with wild-type (WT) and the offspring pupa shell was extracted for PCR test. **c.** PCR screen result. Sample 3, 4 and 8 indicate heterozygote with one copy of deletion and another copy of wild-type gene. Sample 1 indicates homozygote with two copies of gene deleted. Sample 2 indicates absence of deletion. –,WT.

5.2.3 Mutation screening

After injection, all survival pupae were individually placed in isolated tubes until eclosed. The pupa shells were collected to extract genomic DNA for PCR screening. The results showed three different genotypes: (1) homozygous mutants, with both copies of *dsx* deleted, (2) heterozygous mutation, containing one copy of gene with deletion and another copy of wild-type gene, and (3) absence of deletion, with PCR products in the same size as the wild-type control (Fig. 5.6). The heterozygous and homozygous mutants

were selected and crossed with wild-type mosquitoes, and all offspring were screened for mutation. All mutant offspring were crossed with WT type again for another round screen. The mutation was further confirmed by sequencing to locate the 100 bps deletion in the *dxl* gene (Fig. 5.6).

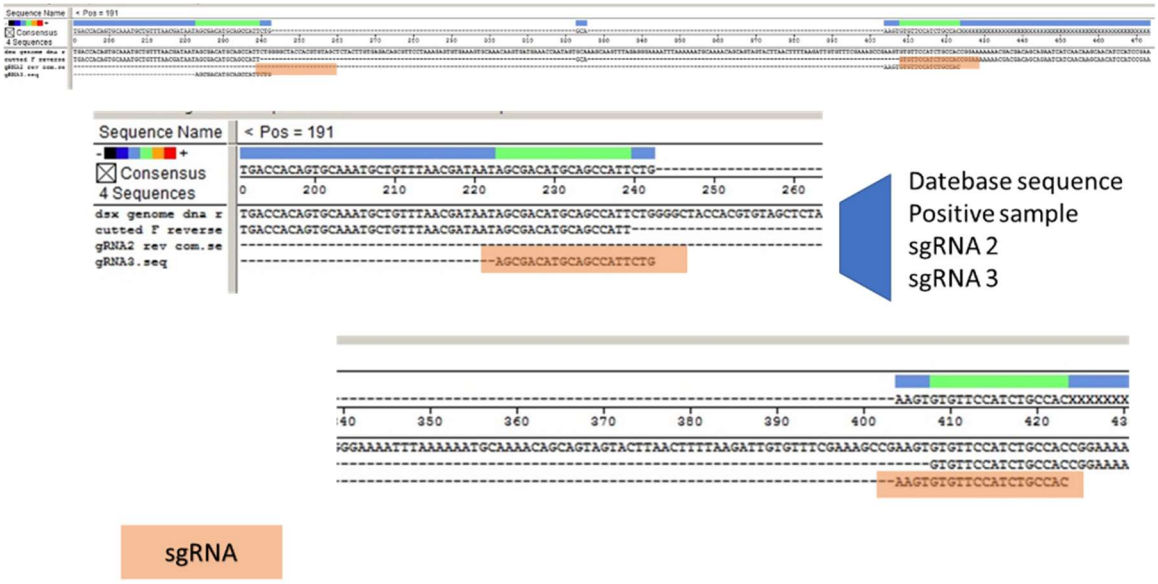

Figure 5.7 The sequence of *dxl* mutant mosquito.

A 100 bps was deleted in *dxl* of the mutant mosquito (the second line) as compared to the database sequence (the first line). The two sgRNA sequences and their locations were also shown (the third and fourth lines).A total of 13 female homozygote and 1 male homozygote were identified from G6, G7 and G8. These results indicate that *dxl* female-specific deletion does not cause female-specific killing lethality. Thus, *dxl* is not essential for female sex determination and development.

Table 5.2 The sexes of dxl homozygous Ae. aegypti mutants.

Generation	Female	Male
G6	7	0
G7	5	1
G8	1	0
Sum	13	1

5.2.4 Selecting the target site on tra-2 gene for the CRISPR/Cas9 knock-out

We then wanted to test whether *tra-2*, the upstream gene of *dsx*, play a role in sex determination in *Ae. aegypti*. To knock out *tra-2* and determine its function in mosquito sex determination, we designed three sgRNA targeting its exon 2. The distances between the two targets next each other were about 100 bps (Fig. 5.8b).

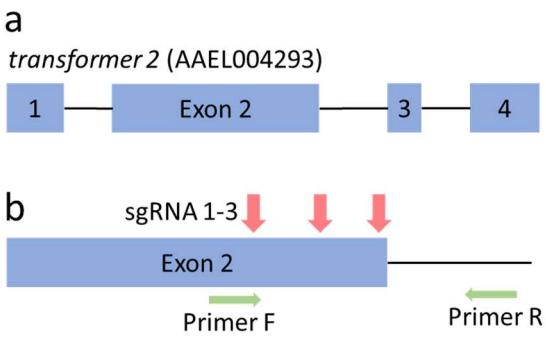

Figure 5.8 The locations of *tra-2* sgRNA and primers for PCR screening for mutants.

a. There is total of 4 exons in *tra-2* gene. **b.** Three gRNA were designed to target for exon 2 and three sets of primer were used for PCR screening.

5.2.5 Screening for tra-2 mutant and characterization of their sex ratios.

After embryo microinjection, the surviving G0 mosquitoes were sacrificed to screen for mutants. In order to determine the effect of *tra-2* mutation on mosquito sex development, the number of male and female mutants were counted and compared. As described in the above *dxl* knockout experiment, there were three different genotypes in the samples collected from *tra-2* knockout assay, including homozygous mutants, heterozygous mutation, and absence of deletion mutation (Fig. 5.9). Among the 28 survived mosquitoes (14 females and 14 males), 19 individuals had deletion in *tra-2*, including two homozygous deletion (Fig. 5.9). Males showed a higher mutation rate than females (Fig. 5.10a) ($P < 0.05$). Furthermore, 12 mutants were males and 7 mutants were females. Although not statistically different, there was a trend of male-biased sex ratio (63.1%) (Fig. 5.10b).

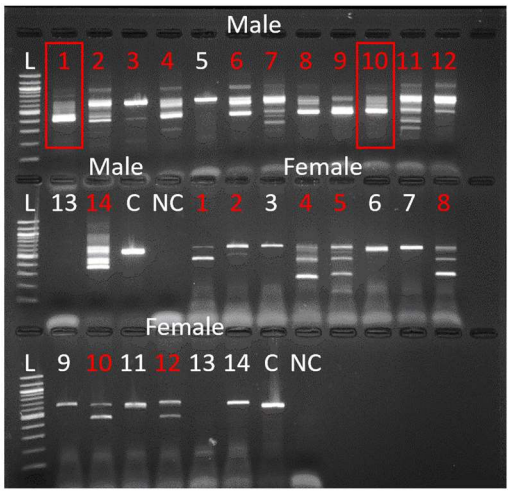

Figure 5.9 PCR screening for *tra-2* mutants.

A successful deletion resulted in band size smaller than the wild-type. Numbers in red indicate positive in screening with at least of one copy of deletion in *tra-2*. Among them,

samples 1 and 10 are homozygous mutants. C, the control group (wild-type mosquito); NC, negative control.

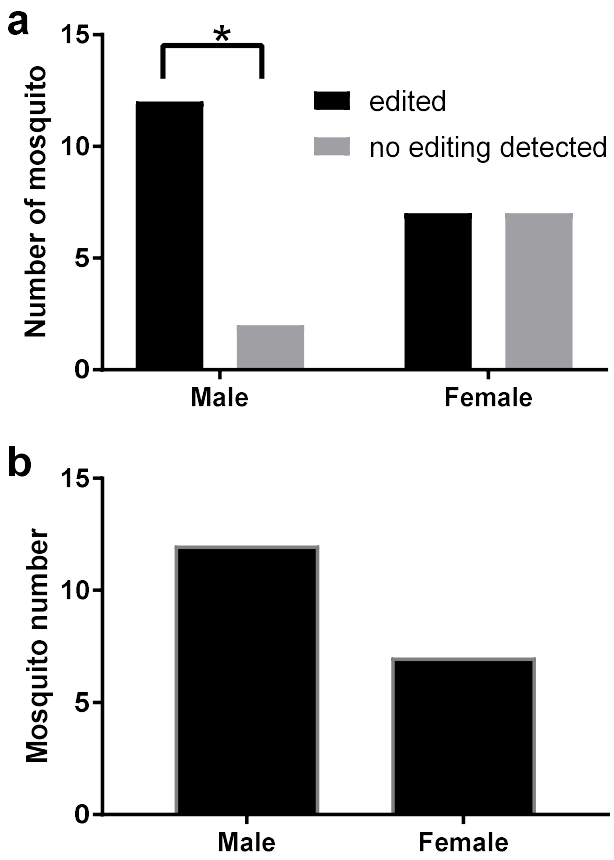

Figure 5.10 Mutation rate and sex ratio.

a. Mutated and unmutated number of males and females (G0). **b.** Sex ratio in individuals with deletion mutation in Tra-2. $P < 0.05$, Fisher's exact test.

5.3 Discussion

Here, we have demonstrated successful establishment of CRISPR/Cas9 approach to knock out target genes in *Ae. aegypti*, with both genotype and phenotype of mutants characterized. We began with knocking out *White* gene to test the efficacy of this system

and obtained the mutation rate 77.6% at G0, indicating robustness of CRISPR/Cas9-mediated gene knock-out. To characterize the function of two sex determining genes, *dxl* and *tra-2*, and evaluate their potential in use for sex separation, we individually knocked out them and examined their effects on female-specific killing and mosquito sex ratio. The results indicated that knock out of female-specific *dxl* did not cause more detrimental effects on survival and development of females than those of males. In the *tra-2* knockout experiment, we observed that males showed a higher mutation rate than females, and there was a trend of male-biased sex ratio in mosquito with *tra-2* mutants.

Although a previous study reports that dsRNA-mediated knockdown of female-specific *dxl* causes the female-specific lethality[8], these results are controversial. In order to examine whether different results are caused by variation in knockdown efficiency, we designed guide RNA targeting the female-specific *dxl* to generate its deletion mutants using CRISPR/Cas9. We assumed that no females could survive to adults in homozygous mutants if the target female-specific *dxl* was essential for female determination and differentiation. However, we observed 13 out of 14 homozygous mutants were females, indicating that there was no female-specific lethality associated with this mutation during development. This is consistent with the previous study showing no obvious impacts on mosquito survival after disruption of female-specific *dxl* by knock-in of eGFP in *Anopheles gambiae* [9]. To our surprise, we saw more homozygous mutants were females than males. This may be related to the position of the deletion in exon 5b, located just next to the male specific exon 6. When the exon 5b of female-specific *dxl* was disrupted in *An. gambiae*, half of male had developmental anomalies[9]. Thus, locating in the

downstream of sex determination pathway, *dxl* is not essential gene that determines the sexes of *Ae. aegypti* although it may facilitate sex differentiation during development.

As shown in other insects[10], *tra* is a key sex-determining gene at upstream of *dxl*. However, the *tra* gene has not been identified in *Ae. aegypti*. As the cofactor of tra, tra-2 is is more conserved than *tra* and has been identified in *Ae. aegypti*. Thus, we knocked out *tra-2* to test whether it could result in sex-specific lethality. We observed two homozygous males in G0, indicating that *tra-2* is not essential for male determination and development. Lack of homozygous females indicate the potential that *tra-2* homozygous mutation might be lethal to females. Consistent with this predication, we saw a higher mutation rate in males than females, probably caused by mutation-induced female-specific lethality. Although not statistically different, there was a trend of male-biased sex ratio in mutants. It is likely to see significant effects when the sample size is increased.

To establish CRISPR/Cas9 approach for gene editing in *Ae. aegypti* system, we performed *white* gene knock-out deletion with Cas9 protein plus two sgRNA experiments. The results show a survival rate of 19.6% and a mutation rate of 77.6%, with efficiency comparable to the other studies[6]. In other words, 100 injected embryos will result in about 15 mutants, and approximately 2% of mosquitoes will be homozygous mutants. Varied between 5% and 15%, the mutation rate can be gene-dependent, with some genes knocked out more efficiently than others. Together with *dxl* and *tra-2* results, we show that gene knock-out using CRISPR/Cas9 is a practicable method to characterize the function of mosquito genes in sex determination pathway. In order to validate the phenotype associated with mutation, further screening for mutants and out-crosses with WT are necessary, especially for those haploid-sufficient genes.

In summary, our experiments indicate that CRISPR/Cas9 is an effective tool to dissect sex determination pathway in *Ae. aegypti*. By targeting the key gene in the pathway, a novel mosquito sex separation can be developed by interfering with female sex determination and differentiation. Lack of female-specific lethality in *dxl* knock out mutants suggests future studies should focus on the gene in the upstream of the pathway. Although its role in sex determination needs to be validated, the results from our preliminary studies on *tra-2* knock-out warrant further characterization of this gene and explore its potential to produce mosquito with male-biased sex ratio for both SIT and *Wolbachia*-based population suppression.

5.4 Method

5.4.1 The sgRNA design and synthesis

The tool used to design is https://chopchop.rc.fas.harvard.edu/. In order to obtain large section deletion, 2 to 3 set of sgRNA is required. After obtaining sgRNA sequence from Chopchop, T7 promoter T7 sequence GAAATTAATACGACTCACTATA is added into upstream of the sgRNA, If gRNA design does not have GG at 5' end, GG will be added right after T7 promoter. A sgRNA backbone sequence GTTTTAGAGCTAGAAATAGC was added into downstream of sgRNA, which will allow PCR reaction to finish sgRNA. A Cas9 reverse primer: 5'–AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTT GCTATTTCTAGCTCTAAAAC–3' was also ordered from IDT.

5.4.2 PCR amplification of completing sgRNA template

The sgRNA with T7 promoter and backbone was mixed with Cas9 reverse primer, also A high fidelity mix was used for the reaction. The detail mix and thermocycler settings were listed below

Table 5.3 PCR mix

PCR reaction mix	1 reaction(μL)	2 reaction(μL)
10x PCR buffer	10	20
dNTPs (10mM each)	3	6
25mM MgSO4	2	4
CRISPR F primer (50 μM)	1	2
CRISPR R primer (50 μM)	1	2
Pfx DNA Polymerase	0.8	1.6
nuclease free water	82.2	164.4
TOTAL	100	200

Table 5.4 thermocycler settings

Steps	Temperature (°C)	Time	cycles
Initial denaturation	94	5 min	1
Denaturation	94	20 s	35
Annealing	58	30 s	
Extension	68	20 s	
Final Extension	68	2 min	1

The PCR products were purified using OMEGA E.Z.N.A Gel Extraction kit fowling the manufacturer protocol. After purification, Invitrogen 5X MEGAscript T7 kit was used for vitro transcription of sgRNAs. The detail mixture is list below:

Table 5.5 *Invitro* transcript mix

IVT reaction mix	1 reaction	2 reaction(µL)
nuclease free water	To 20	To 40
free ribonucleotides (ATP,CTP,GTP,UTP)	2 µl each (8 µl total)	4 µl each (16 µl total)
10x reaction buffer	2 µl	4 µl
sgRNA PCR template	500 ng	1000 ng (1µg)
T7 enzyme mix	2 µl	4 µl
TOTAL	20	40

The mixture was incubated at 24 to 48 hours at 40°C. At end of incubation, 1µl of Turbo DNAse was added per 20µl reaction to degrade DNA template.

5.4.3 Embryo microinjection:

The sgRNA and Cas9 protein was diluted to the appropriate concentration and mixed together to reach a final concentration of 400ng/ul Cas9 protein and 80ng/ul of each sgRNA. After 5 minutes of room temperature incubation, the mixture was loaded into the microinjector. The mixture was injected into the posterior of 60-90 minutes old recipient embryos using an IM300 microinjector (Narishige Scientific). After injection, embryos were incubated at 85% RH and 27°C for one hour and transferred to wet filter paper. Embryos were allowed to mature for 5-7 days before being hatched.

5.4.4 Mosquito lines maintain

All the mosquito lines were maintained on 10% sugar solution at 27 ± 1 °C and 80 ± 10% relative humidity (RH), with a 12:12 h light:dark photoperiod, according to standard rearing procedures. For routine colony maintenance and experimental studies, female mosquitoes were provided with human blood at day 7 post eclosion and eggs were collected two days post blood meal.

5.4.5 PCR screening

Mosquito was isolated during pupa stage. After eclosion, the pupa shell was sampled for PCR test. Genomic DNA was extracted from samples using Thermo Scientific Phire Animal Tissue Direct PCR Kit (F-140WH). Samples were pre-treated in 20 µl dilution buffer with 0.5 µl DNARelease Additive. The reaction mixture contained 10 µl 2X Phire Animal Tissue PCR Buffer, 0.4 µl Phire Hot Start II DNA Polymerase, 0.2 µl for both forward and reverses primer and 7.2 µl dsH2O. The regular PCR condition comprised of initial denaturation at 98 °C for 6 min, followed by 40 cycles of 5 s at 98 °C, 5 s at 56 °C, and 45 s at 72 °C. The mosquitoes with WT band only were identified as WT or no

mutation. Mosquitoes with both WT band and the mutated band were considered as heterozygote or chimeric, and the mosquitoes with the only mutated band were identified as a homozygote.

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Chapter 6 General conclusion

In Chapter 2, *w*AlbB was transferred from *Ae. albopictus* into *Ae. aegypti*, resulting in establishment of the transinfected line WB2. Comparison of WB2 with WB1 line established 15 years ago showed a stable association between *w*AlbB and *Ae. aegypti*, with no difference in both *Wolbachia* density in somatic tissues and CI expression. The *w*AlbB infection induced resistance to DENV, ZIKV and CHIKV in the transinfected WB2 line under both Singapore and Mexico genetic backgrounds, with the strength of viral inhibition subjected to a particular virus and genome type. In addition, WB2 mosquitos had comparable mating ability as the wild mosquito. These characteristics support the feasibility to release WB2 for field trial. In chapter 3, a transinfected *Ae. albopictus* HM line carrying a trip strain infection of *w*Mel, *w*AlbA and *w*AlbB was established. No CI was induced when the triply infected males were crossed with the wild-type GUA females or with another triply infected HC females carrying *w*Pip, *w*AlbA, and *w*AlbB, but specific removal of *w*AlbA from the HM (*w*AlbA*w*AlbB*w*Mel) line resulted in the expression of CI after crosses with lines infected by either one, two, or three strains of *Wolbachia*. The transinfected line showed perfect maternal transmission of the triple infection, with fluctuating egg hatch rates that improved to normal levels after repeated outcrosses with GUA line. Strain-specific qPCR assays showed that *w*Mel and *w*AlbB were present at the highest densities in the ovaries and midguts, respectively, of the HM (*w*AlbA*w*AlbB*w*Mel) mosquitoes. In chapter 4, a transinfected *Ae. aegypti* carrying *w*Mal was established to manage the potential risk associated with release of WB2 in Singapore, with further assays to test the CI and vector competence. In chapter 5, three genes were knocked out in *Ae. aegypti* using CRISPR/Cas9 to characterize their

functions. The *white* gene knockout resulted in the loss of pigment in the eye. The *doublesex (dxl)* knockout did not increase the male mosquito ratio, and female mosquito with homozygote *dxl* mutant still survived. The *transformer-2 (tra-2)* knockout increased male-biased sex ratio, with homozygous mutant potentially lethal to female. These results support the potential to develop a novel sex separation approach by silencing the expression of *tra-2* in *Ae. aegypti*.

My future research will focus on several directions. The first direction is mechanisms underlying *Wolbachia* inter-strain interaction. As discovered in the chapter 3, the *Wolbachia* *w*AlbA affects the sperm modification of *w*Mel. The recently discovered CI genes provide a good foundation for in-depth study of the similarities and differences between *w*AlbA and *w*Mel in term of *cifa* and *cifb* gene. The study of two CI genes of *w*AlbA and *w*Mel may provide not only the answer to CI modification interference but also the clue of the interaction between *Wolbachia* CI factors and host chromosome. The second direction is *Wolbachia* - host interaction. This research will include using transgenic mosquito to express a *Wolbachia* gene to study it*s* function as well as its interactions with mosquito host. The study may involve to establish a easy gene knock-in system in mosquito. *Wolbachia* transformation still is a unsolved challenge in the field, mainly due to lack of approaches for extracellular growth and screening for mutant. I plan to use mosquito embryos as an incubator for the growth of *Wolbachia* and screening. Chemical or radiation-induced mutation also would be a useful method to study the function of *Wolbachia* genes. The third direction is to identify better *Wolbachia* strains for disease control. This includes selection of different *Wolbachia* strain, and different phenotypes within a strain. It has been noticed that two transinfection studies involved

the same *Wolbachia* strain and same mosquito species can result in different phenotypes, which raises a question on the intra-strain variation of *Wolbachia*. The future study will focus on examining the phenotype difference among transinfected mosquitoes derived from the same *Wolbachia* strain but from different embryo microinjection experiments. Suceessful completion of the above work is anticipated to identify t a *Wolbachia* strain or variantwith “better” virus blocking effect in mosquito