ISOLATION, CHARACTERISATION AND BIOEFFICACY ANALYSIS OF *Bacillus thuringiensis* (BT) STRAINS AGAINST DENGUE VECTORS

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ISOLATION, CHARACTERISATION AND BIOEFFICACY ANALYSIS OF *Bacillus thuringiensis* (BT) STRAINS AGAINST DENGUE VECTORS

by

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LIST OF SYMBOLS

%	Percentage
µL	Microlitre
bp	Base Pair
g	Gravity
g	Gram
kDa	Kilodalton
M	Molar
m	Metre
mg	Milligram
mL	Millilitre
mm	Millimetre
nm	Nanometre
ºC	Degree Celsius
ºN	Degrees North
ºS	Degrees South
rpm	Revolutions Per Minute
V	Volt

LIST OF ABBREVIATIONS

A_{260}/A_{280}	Ratio of Absorbance at 260 nm and 280 nm
Ae.	*Aedes*
An.	*Anopheles*
Bt	*Bacillus thuringiensis*
Bti	*Bacillus thuringiensis* subsp. *israelensis*
Btk	*Bacillus thuringiensis* subsp. *kurstaki*
Cx.	*Culex*
DNA	Deoxyribonucleic Acid
LC_{50}	Lethal Concentration required to kill 50% of the population
LC_{95}	Lethal Concentration required to kill 95% of the population
NaCl	Sodium Chloride
OD	Optical Density
R^2	R-Squared Value
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
spp.	Species
UV	Ultraviolet

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ABSTRAK

Penyakit bawaan nyamuk semakin mengancam kesihatan orang awam dan menambahkan beban ekonomi sedunia. Tambahan itu, langkah-langkah kawalan nyamuk semakin kurang efektif akibat peningkatan ketahanan terhadap racun perosak. Salah satu agen kawalan nyamuk yang berjaya adalah bakteria entomopatogenik, *Bacillus thuringiensis*. Satu penyelidikan telah dijalankan untuk mengkaji profil ketoksikan isolat *B. thuringiensis* yang diasingkan dari satu kawasan bekas perlombongon bijih timah. Pengesahan isolat dilakukan melalui pengenalpastian mikroskopi dan pencirian biokimia. Selain itu, ciri morfologi protein kristal diperiksa menggunakan mikroskop elektron pengimbasan (SEM) dan penghantaran (TEM). Kehadiran gen insektisida dan protein kristal dikenalpasti melalui PCR dan analisis SDS-PAGE. Seterusnya, sifat larvisidal dan keberkesanan submaut isolat dikaji menggunakan strain makmal *Ae. albopictus* dan *Ae. aegypti*. Daripada tujuh tapak pensampelan, hanya 13 isolat *B. thuringiensis* yang diperoleh (A10C, D10D, N6Bss, 6a3s, 8a3, 8a3s, 8b3, R1, R2, R3, D1C, A2B dan C6D) dengan indeks *B. thuringiensis* 0.07. Protein kristal berbentuk sfera dijumpai pada semua isolat. Sebilangan kecil protein kristal berbentuk bujur dan kuboid juga dijumpai. Analisis profil protein mendapati kehadiran lapan kelas kristal protein (Cry2, Cry4C/4D, Cry6A, Cry10, Cry11, Cry34, Cry35, Cyt1/2). Empat dari isolat ini memiliki enam gen insektisida (*cry1*, *cry2*, *cry4*, *cry10*, *cry11* dan *cyt2*), yang menghasilkan delta-endotoksin yang spesifik terhadap Diptera. Walaupun begitu, hasil kajian menunjukkan terdapat

hubung kait yang minima antara profil protein dengan kehadiran gen insektisida dan morfologi protein kristal isolat *B. thuringiensis*. Analisis bioefikasi menunjukkan tahap ketoksikan yang berbeza, dengan tiga (A10C, R2 dan A2B) menunjukkan sifat larvisidal yang baik. Nilai LC_{50} dan LC_{95} dari rawatan sehingga 48 jam oleh ketiga-tiga isolat terhadap *Ae. albopictus* dan *Ae. aegypti* masing-masing menunjukkan nilai kurang dari 0.3 mg/mL dan 0.5 mg/mL. Selanjutnya, penilaian submaut terhadap ketiga-tiga isolat terbaik (A10C, R2 dan A2B) menunjukkan penjejasan ke atas perkembangan larva, pengurangan dalam nisbah betina ke jantan nyamuk dewasa, kesuburan serta ukuran sayap nyamuk dewasa. Antara ketiga-tiga isolat ini, R2 menunjukkan kesan submaut yang paling tinggi. Sebagai kesimpulannya, isolat *B. thuringiensis* dalam kajian ini berpotensi untuk digunakan sebagai agen biopestisida spektrum luas dalam program pengurusan kawalan vektor tempatan.

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ABSTRACT

A mounting public health concern over mosquito-borne diseases, a global economic burden, and failure of current control measures due to escalating development of insecticides resistance have intensified the search for alternative eco-friendlier vector control strategies. An investigation was conducted to explore the toxicity profiles of indigenous *B. thuringiensis* isolates from an ex-tin mining area. Verification was performed through microscopic identification and biochemical characterisations. Morphological features of the crystal proteins were assessed using scanning and transmission electron microscopy. Distribution of insecticidal genes and crystal protein classes were examined through PCR and SDS-PAGE analyses. Eventually, these isolates were screened for larvicidal and sublethal efficacies against laboratory strains of *Ae. albopictus* and *Ae. aegypti*. From the seven sampling sites, 13 *B. thuringiensis* isolates were obtained (A10C, D10D, N6Bss, 6a3s, 8a3, 8a3s, 8b3, R1, R2, R3, D1C, A2B and C6D) with an overall *B. thuringiensis* index of 0.07. Spherical-shaped crystal proteins were present in all isolates, while lesser numbers of ovoidal- and cuboidal-shaped crystal proteins were noted. Protein profiles revealed presence of eight classes of crystal proteins (Cry2, Cry4C/4D, Cry6A, Cry10, Cry11, Cry34, Cry35, Cyt1/2). Four of the isolates (N6Bss, 8a3, R1 and A2B) harboured all six of the examined insecticidal genes (*cry1*, *cry2*, *cry4*, *cry10*, *cry11* and *cyt2*), which are known to produce Dipteran-specific delta-endotoxins. Nevertheless, results suggested minimal correlations between the examined characteristics of the *B.*

thuringiensis isolates. Bioefficacy analysis showed varying degrees of toxicity, with three *B. thuringiensis* isolates (A10C, R2 and A2B) exhibited good larvicidal properties. Generated LC_{50} and LC_{95} values of all three isolates against *Ae. albopictus* and *Ae. aegypti* up to 48 hours' post-treatment were below 0.3 mg/mL and 0.5 mg/mL, respectively. Sublethal evaluations of the three best isolates saw inversely impacted life parameters on treated *Aedes* spp. larvae, such as decreases in adult emergence, gender ratio, fecundity, fertility, and adult wing sizes; with R2 giving the highest adverse sublethal effects. Findings in this study support the idea that distribution of *B. thuringiensis* with combinations of insecticidal *cry*/*cyt* genes is associated with presence of targeted insects and may serve as potential broad-spectrum biopesticides to be incorporated as part of local vector control management programmes.

CHAPTER 1

INTRODUCTION

Mosquitoes are the main cause of an extensive range of debilitating vector-borne diseases that are plaguing more than 50% of the world's population. These parasitic and viral diseases comprise 17% of total global infectious diseases, leading to an annually reported record of over 700,000 deaths worldwide (CDC, 2017; Lee *et al*., 2018). These vectors are on the top one ranking in human killing statistics, causing the death of almost 1 million people annually from their bites (Learish, 2016). No other animals can rival the kill counts by mosquitoes, not even humans (Gates, 2014).

Malaria alone causes the deaths of over 600,000 people annually while incapacitating another 2 million people, thus threatening the lives of half of the global population and causing the loss of billions of dollars in productivity each year. Apart from that, over 3 million people are at risk of contracting dengue. The World Health Organization (2019) ranked dengue fever as the most life-threatening vector-borne viral disease globally, reporting an estimate of 390 million dengue infections annually. Around 500,000 cases develop into severe dengue and dengue haemorrhagic fever which resulted up to 25,000 deaths per year (WHO, 2019).

Meanwhile, in Malaysia, dengue is known as the most prevalent mosquito-borne disease, followed by chikungunya and Zika infections (Institute for Medical Research, 2017). According to the statistics given by the National Crisis Preparedness Response (CPRC) team under the Ministry of Health (MOH) Malaysia, there are 130,101 dengue cases recorded in the year 2019, which was the highest ever reported figure in Malaysia. Additionally, there were new dengue cases which were reported from localities that used to be dengue-free as well. Besides that, a total of 990

Chikungunya cases and one Zika case were also reported within the same duration (Loh, 2020).

Apart from the development of vaccines and improvement in the treatments, vector control remains as one of the most imperative step in suppressing the spread of vector-borne human diseases. The classical method of vector control has always heavily relied on the usage of synthetic chemical adulticides and larvicides, which, to a certain extent, have managed to decrease the numbers of infections transmitted by mosquitoes. Chemical insecticides have demonstrated effectiveness in controlling the infectious rates in many endemic countries (Himeidan *et al*., 2012).

Although chemical controls are more economical and manageable in terms of accessibility, continuous deployment of such monotherapy treatments would ultimately lead to the development of resistance among the targeted mosquito population (Wilson *et al*., 2020). Widespread and extended usage of synthetic pesticides may inevitably cause long term adverse effects to the environment and its inhabitants. There have been countless reported cases of human morbidity and mortality caused by synthetic chemical pesticides either environmentally or occupationally. In addition, their non-selective toxicity has more often than not led to irreversible ecological damages (Hurtig *et al*., 2003; Özkara *et al*., 2016).

Despite the various studies that have been done to improve the efficacy of synthetic chemical insecticides and to minimise the hazardous impacts of its prolonged usage, there is still the unavoidable development of insecticides resistance among the mosquito populations. Insecticides resistance, a major challenge in mosquito control programmes, is an inheritable trait which typically results in a critical reduction in the insecticides susceptibility of the mosquito populations and subsequent ineffectiveness of the control strategies (CDC, 2017).

Biological control, which is one of the main components utilised in all integrated pest management strategies, can be defined as "the study and uses of parasites, predators, and pathogens for the regulation of host (pest) densities" (Kwenti, 2017). The main advantage of utilising biological control measures is that they would be able to reduce the targeted vectors while at the same time conserving the biodiversity and existing predators themselves. Additionally, non-targeted organisms are spared. Ever since the introduction and subsequent rediscovery of mosquitocidal agents, their advancements have been immense (Quesada-Moraga *et al*., 2004).

Usage of mosquitocidal agents which was once shelved following the introduction and efficacy of synthetic chemical insecticides was reintroduced two decades later as the more environmentally suitable choice of vector control (Niang *et al*., 2018). An interesting observation noted among the historical usages of biological agents is that although pests frequently develop resistance towards synthetic chemical pesticides, no records of host resistance towards an introduced biological agent have been documented thus far (Mills, 2017). Most of the naturally occurring entomo-pathogens employed as biological control agents are biodegradable, therefore, they generally do not persist in the environment (Gupta and Dikshit, 2010; Usta, 2013).

The interest on the usage of mosquitocidal bacteria for vector control was revived ever since the discovery of the larvicidal activities in *Bacillus sphaericus* and *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) (Usta, 2013). For centuries, humans have been well exposed to the ubiquitous Gram-positive, facultative anaerobic, and endospore-forming bacilli, *B. thuringiensis* (Berliner). Belonging to the *B. cereus* group, this soil bacterium has the unique feature of producing parasporal inclusion bodies categorically known as crystal (Cry) and cytotoxic (Cyt) proteins (Sanahuja *et al*., 2011). These crystalline inclusions possess host-specific delta-endotoxins that are

extremely potent, thus allowing them to affect only the target insects without harming non-target organisms. The high spectra of potency exerted by *B. thuringiensis* are due to the presence of these distinct-shaped crystalline inclusions that contribute to its well-known entomopathogenic properties (Chen *et al*., 2014; Monnerat *et al*., 2014).

Once ingested by unsuspecting host insects, these crystalline inclusions will initiate a cascade of events that eventually lead to their starvation and death within a few hours to days. The toxicity spectrum of *B. thuringiensis* is unique to the Cry and Cyt crystal proteins encoded by specific delta-endotoxin genes which are harboured by the different strains. The insecticidal spectra are typically limited to one or few species in a particular order of insects, ranging from the two-winged flies of Diptera, to the butterflies and moths of Lepidoptera, and the beetles of Coleoptera. Nonetheless, over the years, *B. thuringiensis* have been documented to exert their potencies against other insect orders such as Hymenoptera and Hemiptera, and even invertebrates such as nematodes (Palma *et al*., 2014; Fernández-Chapa *et al*., 2019).

Bacillus thuringiensis has been proven to be a valuable alternative to the traditional insecticides due to their endotoxins which remain innocuous to non-target organisms, completely biodegradable and environmentally friendly. Additionally, as the *B. thuringiensis* is not indigenous to aquatic environments, it will not be likely to proliferate in water. Hence, *B. thuringiensis* is considered a safer mosquitocidal bioagent alternative as it is applicable to both soil and aquatic environments, including drinking-water reservoirs (Fernández-Chapa *et al*., 2019).

Extensive research works have been done to assess the efficacy of *B. thuringiensis* as a potential microbial agent for various agricultural and medical pests. In spite of that, numerous aspects of the ecological distribution of this bacterium in areas with high burden of dengue cases remain unexplored (Quesada-Moraga *et al*.,

2004). Likewise, much of the distribution and toxicity profiles of the crystal proteins are unknown as most studies that have been done so far focused on mass isolation and characterisation of the different *B. thuringiensis* strains. Moreover, a large number of the bioefficacy studies that have been done were aimed at improving the already well-known and established *Bti* (Fernández-Chapa *et al*., 2019).

It is a given fact that the various *B. thuringiensis* strains that were isolated and characterised from around the world would exhibit varying degrees of genetic diversity and toxicity capacities within the countries and regions of their origins. Hence, with this in mind, an unexplored area within the town of Kampar was studied to search for indigenous *B. thuringiensis* isolates harbouring potential mosquitocidal crystal proteins to combat a primary health concern caused by the abundantly found vectors that transmit arboviral diseases in this locality.

Kampar, which is a small township within the Kampar district in the state of Perak, has its fair share of sporadic dengue and chikungunya cases. Between the years 2003 to 2018, there was an apparent increase in the number of dengue cases, with the highest peaks observed in the years 2014, 2015 and 2017, respectively (Majlis Daerah Kampar, 2020). During the first five months of the year 2020 alone, the state of Perak recorded a huge spike in the number of dengue cases (The Star, 2020). Additionally, Perak also saw a significant increase in the number of reported chikungunya cases, with Kampar being one of the major towns affected by this mosquito-borne viral disease (Marks *et al*., 2020). Therefore, this study aimed to investigate the toxicity profiles of indigenous *B. thuringiensis* isolates obtained from an ex-tin mining area with reported dengue and chikungunya cases, by conducting larvicidal bioefficacy assays and investigating their sublethal effects against laboratory strains of the dengue vectors, *Ae. albopictus* and *Ae. aegypti*.

1.1 Objectives of the Study

The objectives of this research project were as follow:

i. To identify indigenous *B. thuringiensis* isolates from an ex-tin mining land in Kampar, Perak through various morphological identifications, biochemical characterisations, and SEM and TEM analyses,

ii. To examine the protein profiles and delta-endotoxin genes of the *B. thuringiensis* isolates through SDS-PAGE and PCR analyses,

iii. To assess the potential larvicidal properties of the *B. thuringiensis* isolates against laboratory strains of *Ae. albopictus* and *Ae. aegypti* mosquitoes,

iv. To evaluate the sublethal effects of selected *B. thuringiensis* isolates based on generated LC_{50} values against the larval stages of *Ae. albopictus* and *Ae. aegypti* mosquitoes.

CHAPTER 2

LITERATURE REVIEW

2.1 *Aedes* Mosquitoes

Aedes spp. mosquitoes are small midge-like insects under the order of two-winged flies called Diptera in the family Culicidae. Up till now, about 3,500 species of Culicidae members have been identified (Kweka *et al*., 2018). Under the 110 mosquito genera from subfamily of Culicidae, the *Ae. aegypti* (Linnaeus) and *Ae. albopictus* (Skuse), which derive from the subgenus *Stegomyia*, are two important mosquito species responsible for several emerging pandemic-prone viral diseases (Kraemer *et al*., 2015).

Yellow fever mosquito, *Ae. aegypti*, is the primary vector transmitting major arboviral diseases such as dengue, chikungunya, Zika and yellow fever (ECDC, 2016a). On the other hand, the Asian tiger mosquitoes, *Ae. albopictus*, are not only secondary vectors of dengue and chikungunya viruses (Rezza, 2012), they are also known to transmit dirofilariasis, yellow fever, Rift Valley fever, Japanese encephalitis, and West Nile fever (ECDC, 2016b). In addition, infection studies on *Ae. albopictus* conducted by Wong *et al*. (2013) and Grard *et al*. (2014) collectively reported possible correlations between the mosquito population to Zika virus outbreaks.

Both of these *Aedes* spp. are widely dispersed around the world, being found within the coordinates of 35ºN and 35ºS. These latitudes relate to 10ºC winter isotherms, that are the limiting temperatures to which these mosquito species are able to tolerate while still overwintering (Fredericks and Fernandez-Sesma, 2014). Nonetheless, these mosquito species originated from different localities. The *Ae.*

aegypti was said to have been introduced to Asia from Africa some 500 years ago, whereas *Ae. albopictus* was a native tropical species, being endemic in Asia and the Pacific Islands (Paupy *et al*., 2009). Due to worldwide redistribution led by global trades, international shipping activities, rapid human population growth, movement, and massive urbanisation, they are now found throughout the tropical, subtropical and parts of the temperate regions (Kauffman and Kramer, 2017; Reinhold *et al*., 2018).

Aquatic habitats are where the three immature stages (eggs, larvae, and pupae) of *Aedes* spp. mosquitoes develop before metamorphosing into mature flying adult mosquitoes. The breeding sites of *Aedes* spp. mosquitoes are numerous, as larvae of *Aedes* spp. can be discovered in various natural sites such as rain-filled cavities in trees, bamboo internodes and leaf-axils of plants, to man-made containers such as used tyres, trash cans, buckets, tanks, plant pots and dishes, animal drinking pan, and unsealed septic tanks that collect rainwater or filled with water by people (Paupy *et al*., 2009; CDC, 2012a; CDC, 2012b). These water receptacles provide ideal aquatic habitats for *Aedes* spp. larvae, as they can develop without disturbances unless there are human interventions (CDC, 2017).

The adaptability of *Ae. aegypti* mosquitoes to inhabit a diversity of environments have made them a highly resilient species. Even after undergoing massive disturbances caused by natural phenomena (e.g., floods, droughts) or human interventions (e.g., larvicides, adulticides), this mosquito species can easily revert back to their initial population numbers (Kweka *et al*., 2018). One of the very important adaptations by the *Ae. aegypti* is the ability of their eggs to withstand long periods of desiccation by maintaining dormancy on the inner chambers of containers over extended periods of time.

Besides that, its counterpart the *Ae. albopictus*, which is naturally a tree-hole mosquito, can also establish themselves and survive in non-urbanised and rural localities that lack artificial containers, leading to increased concerns over public health in rural areas (Eritja *et al*., 2005). Additionally, as the *Aedes* spp. mosquitoes have accommodated to live in a wide range of temperatures, they can even thrive in temperate weathers successfully. This adaptability, including the fact that the *Aedes* spp. mosquitoes can easily breed in various natural and artificial sites, have made them to become the most successful species of all. Moreover, their anthropophilic (preference towards humans) behaviour, frequent biting, and aptitude to create habitats near human dwellings, have led them to be the most effective vectors for arboviruses (Reinhold *et al*., 2018).

Once the female mosquitoes become infected with the virus that they are transmitting, they will be able to transfer the virus to humans during blood meals. They may feed multiple times during one gonotrophic cycle, which has implications for disease transmissions (ECDC, 2016a; ECDC, 2016b). Besides, infected female *Aedes* spp. mosquitoes have the ability to transmit the virus to their offspring via a transovarial (vertical) transmission (Li *et al*., 2017). Previous studies confirmed that mosquitoes infected with flaviviruses, including dengue, Zika, yellow fever, West Nile and St. Louis encephalitis could undergo vertical transmissions (Ciota *et al*., 2017).

2.1.1 Life Cycle of *Aedes* Mosquitoes

Aedes spp. mosquitoes are holometabolous insects that undergo complete metamorphosis. Their life-cycle consists of four main stages; eggs, larvae and pupae which are immature stages living in aquatic environments, and the mature adults that

live as terrestrial beings (Figure 2.1). Process of maturation from egg to adult typically takes around 7 to 10 days at optimal conditions, being subjected to food availability and environmental conditions (Nature Education, 2011b; CDC, 2012a; CDC, 2012b).

Aedes spp. are multivoltine species that are able to produce 5 to 17 generations per year depending on distribution of rainfall and temperature changes. Female mosquitoes lay between 50 to 500 eggs within 2 to 4 days after consuming a complete blood meal. Size of blood meals may affect the number of eggs produced. Female mosquitoes lay up to five batches of eggs during a lifetime, spreading them at varying distances from the water surface, rather than an entire batch in one area. The eggs are usually attached to the water's edge in the menisci, on inner damp surfaces of water receptacles or in moist soil (Muktar *et al*., 2016; CDC, 2017).

Singly laid eggs are initially white and soft, having a cigar-shaped appearance of approximately 1 mm in length and are without floats. Within minutes, these eggs will turn hard and shiny black. The anterior end of the eggs is blunt compared to the posterior side which is more tapered (Muktar *et al*., 2016). Developmental period of eggs can be as short as two days in warmer climates, and as long as one week in colder temperate regions (Gatt *et al*., 2009). Unlike *Ae. aegypti*, the eggs of *Ae. albopictus* undergo diapause during winter in temperate countries but are active throughout the year if they are in tropical and subtropical areas (Gatt *et al*., 2009). In a desiccated state, survivability of laid eggs is usually about one year. *Aedes* spp. eggs are not only desiccant-resistant, as they are also able to withstand months of dormancy, known as "extended quiescence", in unfavourable abiotic environments (Diniz *et al*., 2017).

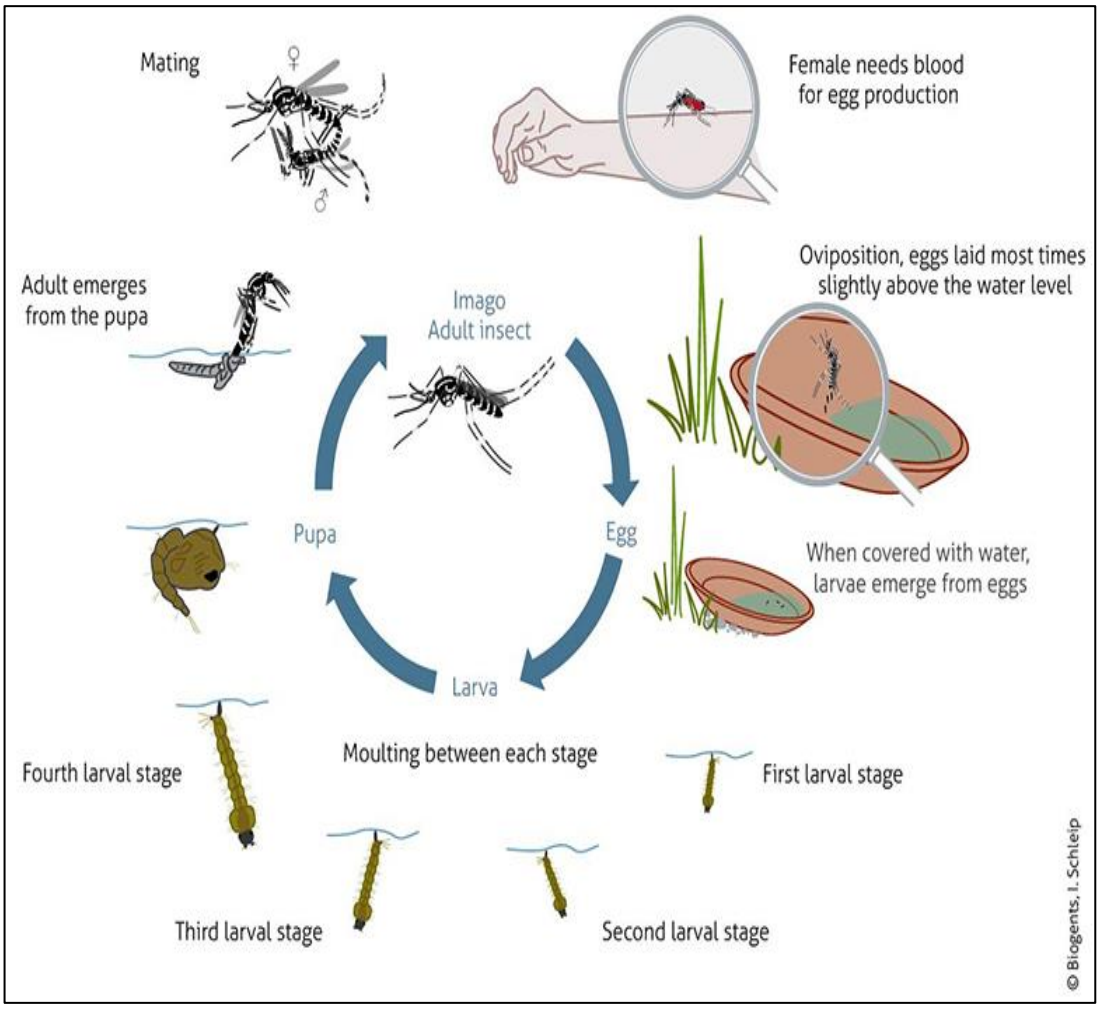

Figure 2.1: Life cycle of *Aedes* spp. mosquito which consists of four main stages (Biogents Mosquito Control, 2018).

Once immersed in water, they typically hatch within three days, emerging as first instar larvae. However, repeated inundations are usually required for eggs to hatch. Apart from food availability, water temperature and oxygen tension could greatly influence eggs hatching. Low oxygen tension, which is associated with high levels of microbial activity and nutrients in the water habitat, tends to stimulate egg hatching (Reinhold *et al*., 2018). The larvae of *Aedes* spp. are characterised by an ovoid head, thorax, and an eight-segmented abdomen. Four-lobed anal gills located at the posterior segment of the abdomen aid in osmotic regulation (Bar and Andrew, 2013). A siphon tube, which arises from the posterior region of the eighth abdominal segment at an angle of about 140º with the line of the body, aids in respiration at water surface while the rest of the body hangs vertically downwards (Bar and Andrew, 2013; Muktar *et al*., 2016).

The larval stage generally lasts between 6 to 21 days, depending on water conditions and food availability. Larvae feed on microorganisms and fine particulate matters in the water. *Aedes* spp. larvae are often called “wigglers”, “filter feeders” or “browsers” due to their active feeding behaviour in the water. A decrease in total growth and an extended developmental period occur when food supply becomes scarce (CDC, 2012c). The adaptability of *Aedes* spp. larvae to varying habitats have enabled them to breed successfully in a wide range of water-retaining containers. The larvae of *Ae. aegypti* can adapt and survive in either extremely acidic or basic conditions ranging from pH 4 to pH 11, whereas the larvae of *Ae. albopictus* can only adapt at a much lower range of between pH 6.8 to pH 7.6 (Clark *et al*., 2004). Occasionally, when both of these *Aedes* spp. larvae share similar habitats, they can be differentiated through their larval morphology (Reinhold *et al*., 2018; Das *et al*., 2019).

Only one branch is found on seta 7-C on the larval head of *Ae. aegypti*, but two branches are found on *Ae. albopictus*. Presence of long spines on the pleural hair groups is observed on the mesothorax and metathorax of *Ae. aegypti* larvae but are absent in the larvae of *Ae. albopictus*. Ventral brush of *Ae. aegypti* has four pairs of hairs, whereas *Ae. albopictus*' brush has five pairs. Further to that, comb scales of *Ae. aegypti* have a bare apical spine and a row of small spinules basally on each side, but the comb scales of *Ae. albopictus* are present with several shorter, stout, subapical spines (Figure 2.2) (Rueda, 2004).

The *Aedes* spp. larvae moult three times at intervals, growing through four larval instar stages, before reaching pupal stage. Larval development involves the changes in chaetotaxy and other external features, for example, number and branching of spines, branching of hairs, and the complexity of larval structures such as the mouthparts, anal segment, and siphon tube (Sivanathan, 2006). The larvae which appear transparent immediately after every moulting, will be darkened right before the next moult occurs (Bar and Andrew, 2013). The presence of dark bands across the thoracic and abdominal segments due to the circularly-wrapped lateral hairs of the next instar seen through the cuticle, are the typical indications of pre-moulting stage (Sivanathan, 2006).

Emerged *Aedes* spp. pupae are typically found living within aquatic environments as well. The pupal body consists of a large globular anterior portion, followed by the cephalothorax, and the posterior is made up of a narrower articulated abdomen. The nine-segmented, dorsoventrally-flattened pupal abdomen is usually kept flexed under the cephalothorax and used to propel while swimming. The last abdominal segment is characteristically small and carries terminal paddles (Becker *et al*., 2010).

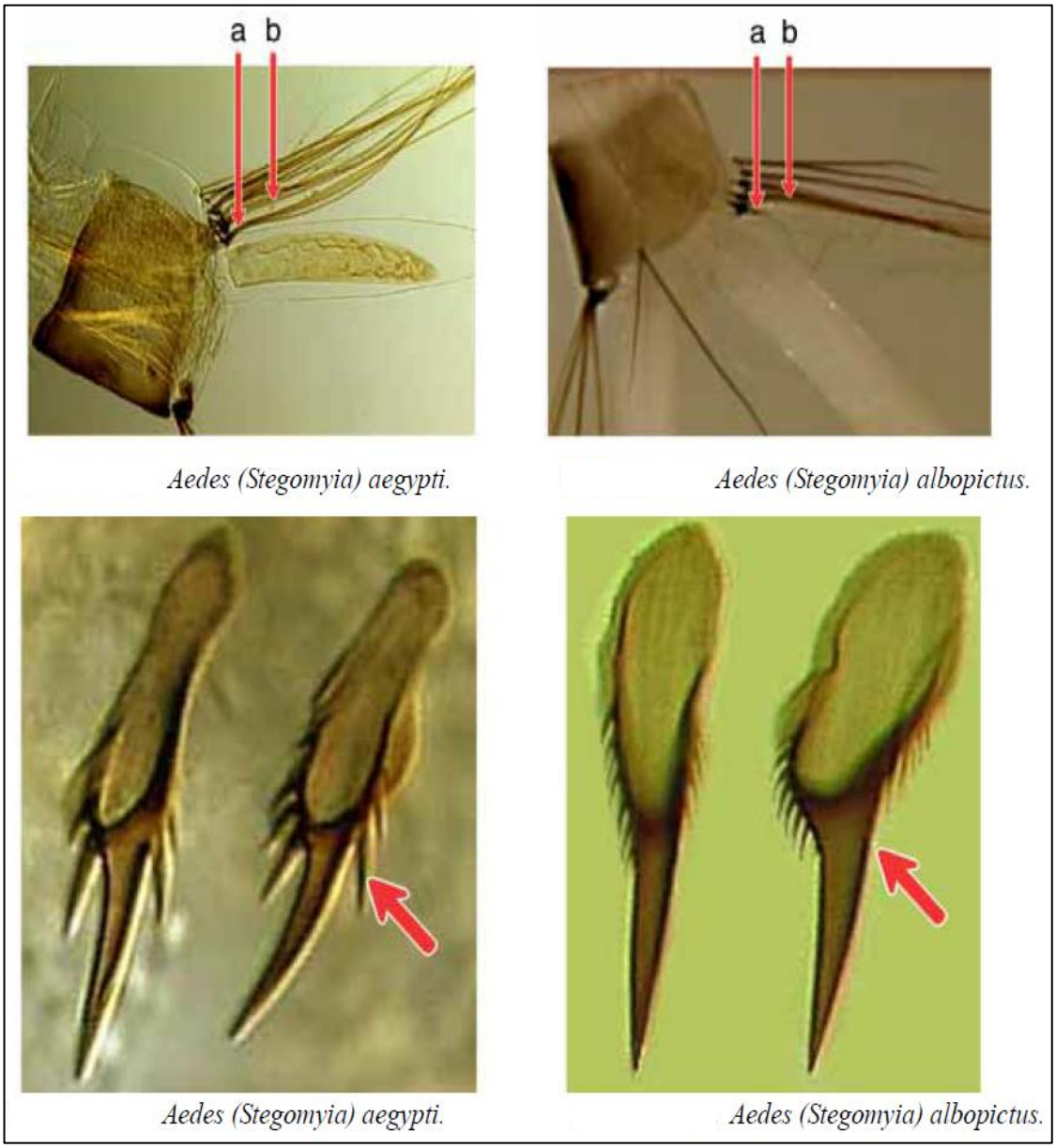

Figure 2.2: Differences in the ventral brush (above) and comb scales (below) between the *Ae. aegypti* and *Ae. albopictus* larvae (Rueda, 2004). In *Ae. aegypti*, ventral brush (4-X) is with 5 pairs of setae, seta 4-a, b X is branched; in *Ae. albopictus*, ventral brush (4-X) is with 4 pairs of setae, seta 4-a, b X is single.

Pupal stage of *Aedes* spp. typically lasts about two days, but the duration may be reduced or extended depending on temperature changes. These comma-shaped non-feeders are usually found floating near water surfaces to acquire oxygen through two breathing tubes known as "trumpets" (CDC, 2012c). The pupae are commonly known as "tumblers" since they tend to dive deeper under water in a tumbling motion when they detect threatening stimuli (Rivera-Pérez *et al*., 2017). As pupal stage is generally short-lived, the time will be spent to metamorphose, after which adult mosquitoes will begin to emerge from the pupal skin from the third day onward (CDC, 2012c).

Paddles of the *Ae. albopictus* pupae are hairier than those of *Ae. aegypti*, which appear as stubbles (Carvalho *et al*., 2014). Unlike larval or adult stages, the pupal stage provides less distinct external morphology and chaetotaxy characteristics for mosquito species identification. The male *Aedes* spp. pupae are comparatively smaller in size than the female pupae. Moreover, the shape of the genital lobe at the last abdominal segment just below the terminal paddles can reliably determine the gender of the pupae (Figure 2.3) (CDC, 2012a; CDC, 2012b).

Emerging adult mosquitoes break through the pupal skin to first release their head, subsequently ingesting air to expand the abdomen. The newly emerged adult mosquito rests on the water surface until its body becomes fully dried and hardened (CDC, 2012c; Muktar *et al*., 2016). Pupal development in male mosquitoes would generally be shorter than females. Due to the delayed sexual maturity of adult male mosquitoes at the time of emergence, males would usually emerge a few days earlier in order to reach sexual maturity at the same time as the later emerging adult females. Corresponding to pupal size differences between genders, the adult males in a typical mosquito population are also comparatively 20% smaller in size than the females (Becker *et al*., 2010).

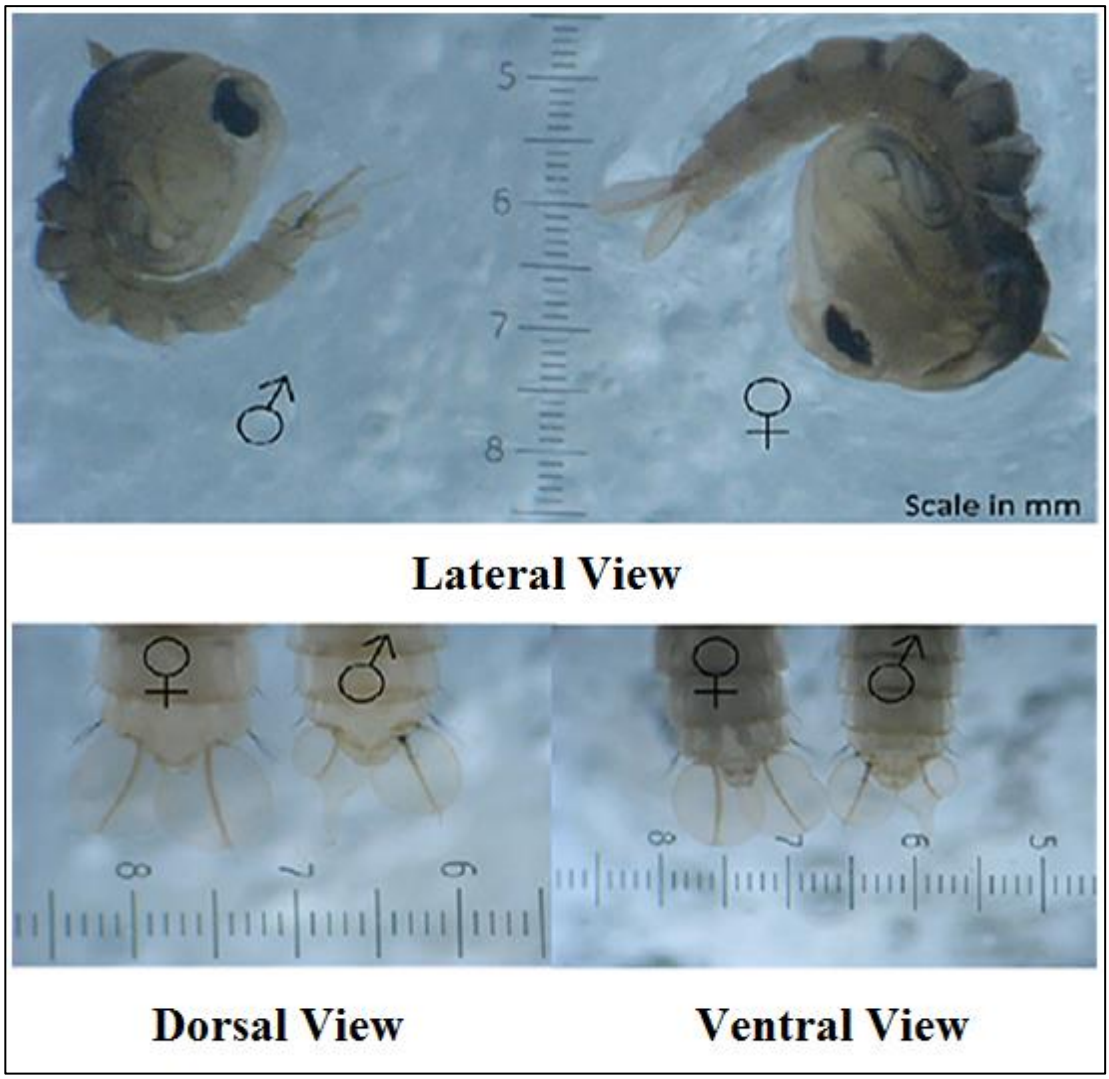

Figure 2.3: Distinguishing features of the male and female *Ae. aegypti* pupae based on the shape of the genital lobe (Carvalho *et al*., 2014).

Following emergence, the adult mosquitoes will be ready to embark on their life cycle of mating, feeding and oviposition. The female mosquitoes aggressively seek blood meals as protein sources right after mating. Studies on mosquitoes are naturally focused on the adult females due to their primarily haematophagous (blood feeding) behaviour and the ability to produce offspring. Nutrition greatly affects the physiology and behaviour of mosquitoes. A sugar meal typically sustains the active requirements of a female mosquito, but blood meals are necessary in order to produce eggs (Rivera-Pérez *et al*., 2017). Plant juices such as floral nectar provide the primary food source for the male mosquitoes, and as energy meals for the females. The female adult mosquitoes generally have a longer lifespan, being able to live up to two to three weeks, but the male adult mosquitoes can only survive for about one week (Becker *et al*., 2010).

An adult *Aedes* spp. mosquito is characterised by its small-sized, dark-coloured body of approximately 4 to 10 mm in length with the typical, white-banded markings on the legs (CDC, 2012a; CDC, 2012b). The main morphological feature that differentiates *Ae. aegypti* from *Ae. albopictus* is the conspicuous lyre-shaped silver patterns on the scutum of the former, compared to the distinct longitudinal silver line found on the latter. In addition, white scales cover the clypeus of female adult *Ae. aegypti* mosquitoes, unlike the black scales that are present in the female adult mosquitoes of *Ae. albopictus* (Figure 2.4).

Besides that, adult *Ae. aegypti* and *Ae. albopictus* can also be differentiated by observing their mesepimeron and anterior portion of the mid-femur regions. White scale patches on the mesepimeron of *Ae. aegypti* are well separated, but these patches are fused on the mesepimeron of *Ae. albopictus*, giving off a V-shaped white patch

design. Moreover, a longitudinal white stripe that is present on the anterior mid-femur region of the *Ae. aegypti* is non-existent in *Ae. albopictus* (Figure 2.5) (Rueda, 2004).

2.1.2 Bionomics of *Aedes* Mosquitoes

As flooding is important for their eggs to hatch, *Aedes* spp. mosquitoes are also known as "floodwater" mosquitoes. They have significant adaptational mechanisms, which aid them in surviving different environments. Any aquatic habitats or water bodies can become their breeding habitats. In addition to that, they can acclimatise to various climates as well as changing environmental conditions (Becker *et al*., 2010). These peridomestic mosquitoes mainly breed in clean waters and being anthropophilic, they prefer to feed on human blood (anthropophagic) rather than animals (zoophagic) (Akiner *et al*., 2016; Das *et al*., 2019).

Aedes aegypti prefers biting indoors and is usually called the "sneaky biter". The female mosquitoes are most active in blood-feeding throughout the day, with peaks of activity several hours after sunrise and a few hours before sunset (Kweka *et al*., 2018). After the third day of blood-feeding, the female mosquitoes lay their eggs on top of waters containing organic materials. Also known as an urban mosquito, *Ae. aegypti* prefers dark places with moisture for blood feeding and resting (Zahouli *et al*., 2016). The breeding sites of this container-breeding mosquito may vary from natural to artificial habitats, ranging from dense vegetation, discarded tyres and water storage containers, flowerpots and vases, to metal oil drums, open or unsealed septic tanks, water metres and even uncovered wells (Mathias *et al*., 2017).

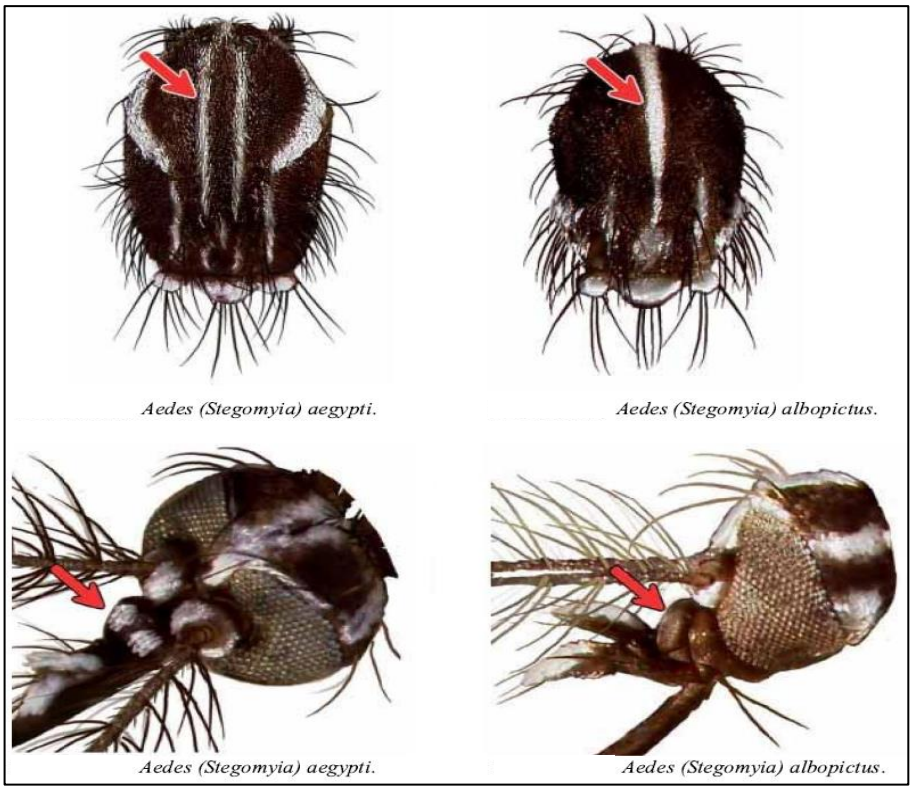

Figure 2.4: Morphological differences in the scutum (above) and clypeus (below) of the *Ae. aegypti* and *Ae. albopictus* adult mosquitoes (Rueda, 2004).

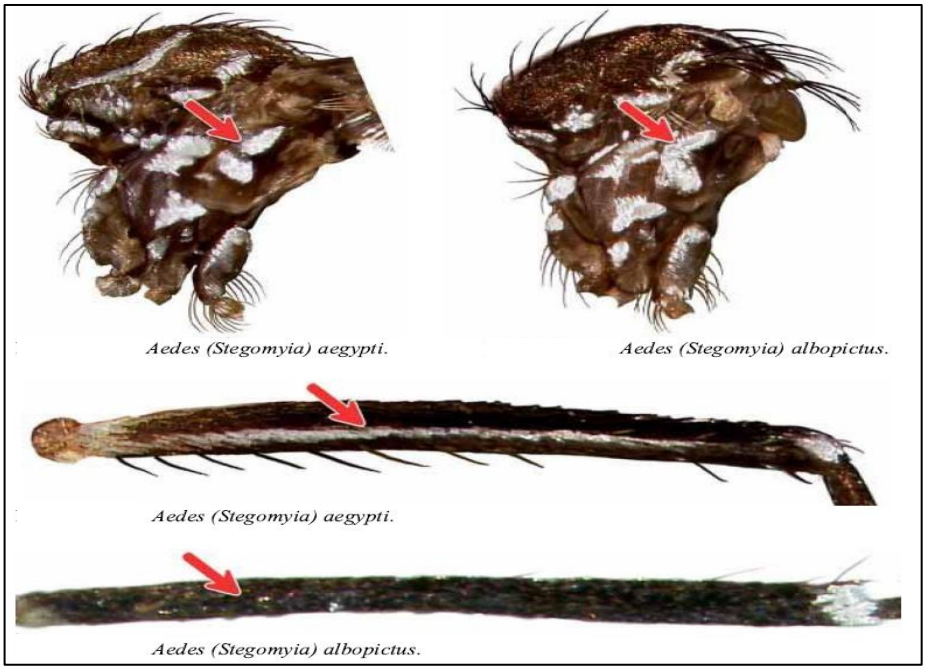

Figure 2.5: Morphological differences in the mesepimeron (above) and anterior portion of the mid-femur (below) between the *Ae. aegypti* and *Ae. albopictus* adult mosquitoes (Rueda, 2004).

It is not an overstatement to claim that the overall life cycle of the *Ae. aegypti* mosquito is fully dependent on environments which are created by humans. Indoors and urban areas provide vast compositions of favourable artificial breeding habitats which the *Ae. aegypti* prefers over outdoors and suburban or rural areas. Furthermore, as this species is commonly found within 100 m of residential locations, its close association with humans is the main factor for its effectiveness in the transmission of various arboviral diseases in urban areas (Higa, 2011).

On the other hand, the biting rates of female adult *Ae. albopictus* mosquitoes can be as high as 30 to 45 bites per hour, giving them the name "aggressive daytime biters". They are usually found biting during the early mornings and late afternoons, preferring to bite outdoors rather than indoors (Kweka *et al*., 2018). Although they may feed on a variety of hosts, including humans as well as mammals, reptilians, amphibians, and birds, they prefer mammalian blood (anthropophilic). As they are semidomestic breeders and are also known as "tree-hole mosquitoes", the breeding places of *Ae. albopictus* are generally within confined spaces in shaded bodies of water surrounded by vegetation. Although they inhabit densely vegetated rural areas, their ecological flexibility also allows them to colonise a wide range of artificial water containments and also in urban localities (Gatt *et al*., 2009).

There are reports of *Ae. albopictus* eggs being detected in birdbaths, empty cans, flowerpots, water containers and receptacles found in cemeteries (Kweka *et al*., 2018). Presence of decaying leaves which provides similar environmental conditions to tree-holes further attracts the oviposition of female adult *Ae. albopictus* mosquitoes. Despite their preference to breed in habitats of lower temperatures, *Ae. albopictus* mosquitoes are able to survive a wide range of temperature. Due to their short flight

range, the eggs are most likely to be laid closer to where the female mosquitoes have previously had their blood meals (Day, 2016).

2.1.3 Epidemiology of *Aedes*-Borne Diseases

The complex interactions between demographical, environmental and social factors proportionately affect the overall distributions of vector-borne diseases. The growth of urban slums, which lacked the basic facilities of reliable piped water or adequate solid waste management would lead to the increased risk of arboviral and parasitic diseases transmitted by mosquitoes (Kweka *et al*., 2018). The burden of these diseases is typically highest in tropical and subtropical areas, affecting the most impoverished populations. For the past decade, massive epidemics involving major vector-borne diseases have plagued populations, claimed lives, and overburdened the region's health systems (WHO, 2018).

Moreover, they hamper economic developments directly through medical costs, and indirectly through the loss of productivity and tourism (Kweka *et al*., 2018). The *Ae. aegypti* and *Ae. albopictus* mosquitoes are major contributors to the spread of various vector-borne diseases. Due to its anthropophilic behaviour and adaptability to domesticated environments and different geographical regions, *Ae. aegypti* is generally recognised as the species with the higher vectorial capacities. Nonetheless, both *Aedes* spp. are responsible for vectoring many arboviral infections and transmissions caused by dengue, chikungunya, Zika, and yellow fever viruses (WHO, 2018).

Dengue virus (DENV), which is the cause of dengue fever, is mainly transmitted by the female mosquitoes of *Ae. aegypti* and, to a lesser extent, *Ae. albopictus*. This virus is a single positive-stranded RNA virus from the family Flaviviridae under the genus Flavivirus. Five closely-related but antigenically different serotypes of the virus have been found (DENV 1 to DENV 5), all of which can cause the full spectrum of disease (Dwivedi *et al*., 2017). Nevertheless, it remains unclear whether the fifth serotype DENV 5 is capable of sustained transmission among humans (Normile, 2013). The lifelong immunity protection acquired upon recovery from infection by one of the serotypes is only effective against that particular serotype, and subsequent or secondary infections by other serotypes will increase the risk of developing the severe form of dengue (WHO, 2006).

Although dengue fever (DF) and dengue haemorrhagic fever (DHF) are major concerns in tropical countries, their incidence rates have increased dramatically worldwide. More than 3.9 billion people in over 128 countries are at risk of contracting dengue (WHO, 2006). Uncontrolled urbanisation, overexpansion of the urban population, poverty, ineffective health infrastructure, indiscriminate disposal of water, globalisation of trade, and increased international travels have all contributed to the dissemination of dengue around the world (Gill, 2017).

Despite a drop in the number of cases between the years 2017 and 2018, there was an increase of dengue incidences reported in 2019. In the report by the CPRC team, in 2019 Malaysia recorded 130,101 dengue cases (increase of 61.4%) compared with 80,615 cases during the same period the year before. There were 182 deaths in 2019 compared to 147 deaths in 2018. There were also reports of spike in dengue cases in the neighbouring countries. A report from the World Health Organization's Dengue Situation Update showed an escalation of dengue cases in Singapore, Taiwan, Laos,

Vietnam, Cambodia, the Philippines, and China by between one and eight times higher compared to the previous year (Loh, 2020). Environmental factors such as rainfall, maximum relative humidity, and temperature were the common factors implicated for the increase (Ahmad *et al*., 2018; WHO, 2019).

Chikungunya is a mosquito-borne viral disease first described during an outbreak in southern Tanzania in 1952. It is an RNA virus (CHIKV) that belongs to the alphavirus genus of the family Togaviridae (WHO, 2017). Both *Ae. aegypti* and *Ae. albopictus* have also been incriminated in the large outbreaks of chikungunya. Transmission by *Ae. aegypti* is generally confined within the tropics and subtropics, whereas *Ae. albopictus* is more widespread, being prominent even in temperate regions (WHO, 2017).

Chikungunya is characterised by an abrupt onset of fever frequently accompanied by joint pain, which is often debilitating and usually lasts for a few days or may prolong to weeks. Hence the virus can cause acute, subacute, or chronic diseases. Most patients recover fully, but in some cases, joint pain may persist for several months or even years (WHO, 2017). Malaysia experienced its first chikungunya outbreak in the late 1990s, followed by a reemergence in 2006 (Chua, 2010). Last year, the country experienced another outbreak of the virus with 37 confirmed cases in the Kedah state (The Sun Daily, 2019). In general, human globalisation and expansion of the mosquitoes' area of activity are creating new opportunities for viruses such as dengue and chikungunya to emerge into new territories (Rezza, 2012).

Zika virus (ZIKV) belongs to the family Flaviviridae and genus Flavivirus. This mosquito-borne flavivirus was first identified in humans in 1952 in Uganda and the United Republic of Tanzania and is primarily transmitted by infected *Ae. aegypti*.

It has also been known to be transmissible from mother to foetus during pregnancy, through sexual contact, transfusion of blood and blood products, and organ transplantation (Kaufmann and Kramer, 2017). Most people infected with Zika virus are either asymptomatic or may display symptoms which are similar to other diseases. Hence, many Zika cases were not identified and reported (CDC, 2019).

The first Zika outbreak was reported in 2007 in the Federated States of Micronesia, followed by a larger outbreak in French Polynesia and the Pacific in 2013 (WHO, 2018). Few years later, Brazil reported an association between Zika virus infection and microcephaly (de Araújo *et al*., 2016). Outbreaks and evidence of transmissions through reports of emergence of various Zika virus-related complications soon appeared throughout the Americas, Africa, and other regions of the world. To date, a total of 86 countries and territories have reported the occurrence of mosquito-transmitted Zika infections. Despite having a history of Zika virus transmission in Malaysia, there is currently no evidence of any ongoing outbreaks in the country (WHO, 2018; IAMAT, 2019).

2.2 Vector Control Programmes

Although having been granted a license for the Sanofi Pasteur vaccine (CYD-TDV – Dengvaxia) to control dengue outbreaks, the high cost of this vaccine prompted a doubt on whether it can be used in low-income countries. Moreover, as these live-attenuated dengue vaccines are only effective and safe for people with evidence of a previous dengue infection (seropositive individuals), it may increase the risk of development of severe dengue and hospitalisation for those who experience their first natural dengue infection after being vaccinated (seronegative individuals) (Aguiar *et*

al., 2016; WHO, 2019). Treatment is directed primarily at relieving the symptoms, including joint pain using antipyretics, optimal analgesics and fluids. Besides, there are no specific antiviral drugs, vaccines or treatments for other arboviral diseases such as chikungunya and Zika (WHO, 2017).

As such, reduction of the mosquito populations remains as the primary prevention method for vector-borne arboviral diseases. Additionally, there were suggestions of combining the various vector control strategies with dengue vaccines once they become globally available. Vector control is believed to be able to help in achieving the vaccination objective, which is to either reduce to its most minimal or to totally eradicate vector-borne disease transmissions. As a result, information on the worldwide distribution, and the ecological and geographical limitations of these vectors are necessary for the effective implementation of suitable control measures. Inevitably, knowledge on the common trends of distribution of these vectors would be the most important in order to identify the areas which are at risk of succumbing to vector-borne diseases. Generally, vector control strategies with the aim of reducing the size of the targeted vector populations, can be grouped into three main categories which are chemical control, environmental management, and biological control (WHO, 2019).

2.2.1 Chemical Control

The usage of chemical insecticides is usually recommended during outbreaks of any vector-borne diseases in order to reduce the vector population. Chemical control, which is a process of applying insecticides to kill mosquitoes, can be broadly divided into larvicides and adulticides. Larvicides are chemicals applied directly to