STUDIES ON HOST-SEEKING, RESTING BEHAVIOUR AND CONTROL OF THE DENGUE VECTOR *Aedes aegypti*

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Submitted by

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Studies on host-seeking, resting behaviour and control

of the dengue vector *Aedes aegypti*

Hadura Abu Hasan

Abstract

Aedes aegypti is the main vector of dengue worldwide. A highly anthropophilic and endophilic mosquito, its behaviour is a major influence on dengue epidemiology and a major challenge to vector control, which is the only dengue prevention method available. A series of studies into host-seeking and resting behaviour were carried out in the laboratory. In Penang, Malaysia, the efficacy of standard and a novel modified form of indoor residual spraying was evaluated in a field trial and the insecticide susceptibility of local vector populations was determined.

Arrival patterns of female *Ae. aegypti* were investigated at a seated human-bait protected by an adhesive-coated net. Mosquitoes preferentially landed on the top and nearest upper vertical surfaces of the net, clustering in a region above the volunteer's head. Although not previously reported in *Ae. aegypti*, this behaviour supported the proposition that a plume of potential host attractants rises from the human host.

Resting preferences of unfed female *Ae. aegypti* were investigated using simple two-dimensional panel targets and resting boxes. Exploring the influence of colour, texture, adhesive and target height, the highest resting rates were found on black targets in a vertical configuration at 90 cm above ground. Target texture and adhesive factor did not influence target attractiveness. Data also indicated that female *Ae. aegypti* were randomly distributed on the panels. In laboratory tests, significantly higher numbers of mosquitoes were captured in resting boxes by raising internal humidity to over 65%. However, a field test in Malaysia did not capture any *Aedes sp.*, although *Cx. quinquefasciatus* were caught.

A randomised-controlled trial was conducted in Penang, Malaysia to investigate the effect of indoor residual spraying (IRS) on *Ae. aegypti* and *Ae. albopictus* populations. Two insecticides (lambda-cyhalothrin and pirimiphos-methyl) were delivered either by standard (entire interior surface sprayed) or selective IRS (upper walls and ceilings sprayed) methods. Throughout the three-month study, entomological indices fluctuated considerably and, while there was some evidence of an overall effect throughout the study area, due to a number of confounders comparison between treatments was not possible and the outcome was ultimately inconclusive.

At the trial study site in Penang, the insecticide susceptibility status of local populations of *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* were investigated. All were found to be resistant to lambda-cyhalothrin. For pirimiphos-methyl, *Ae. aegypti* and *Ae. albopictus* remained susceptible but *Cx. quinquefasciatus* was classed as 'suspected resistance' and potential resistance management strategies are discussed.

The study has demonstrated the potential to improve traps or targets for *Ae. aegypti* by simple alterations to their design. The potential of IRS in the control of dengue vectors remains to be confirmed. The data on emerging insecticide resistance in the mosquito vector populations is timely and provides an evidence base for local authorities to reconsider management strategies that are currently in place for the control of dengue vectors in Malaysia.

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List of Abbreviations

AChE	Acetylcholinesterase
AeDNV	*Aedes densonucleosis* virus
Bs	*Bacillus sphaericus*
Bti	*Bacillus thuringiensis israelensis*
C	Carbamates
CDC	Centers for disease control and prevention
CO_2	Carbon dioxide
CS	Capsule suspension
CYD-TDV	CYD-tetravalent dengue vaccine
DALYs	Disability-adjusted life years
DDT	Dichlorodiphenyltrichloroethane
DEET	*N*,*N*-diethylmethyl-3-methylbenzamide
DEN	Dengue
DF	Dengue fever
DHF	Dengue haemorrhagic fever
DNVs	*Densonucleosis* viruses
DSS	Dengue shock syndrome
EC	Emulsifiable concentrate
FAO	Food and Agricultural Organization of the United Nations
GABA	Gamma-aminobutyric acid
GSTs	Glutathion-S-Transferases
HeDNV	*Hemagogus equines* densovirus
HIV/AIDS	Human immunodeficiency virus/ Acquired immunodeficiency syndrome
IGRs	Insect growth regulators
IPCS	International programme on chemical safety
IRS	Indoor residual spraying
ITMs	Insecticide-treated materials
ITNs	Insecticide-treated nets
kdr	Knock-down resistance
MFO	Mixed Function Oxidases

OC	Organochlorinated
OP	Organophosphates
PAHO	Pan American Health Organization
ppb	Part per billion
PY	Pyrethroides
RIDL	Release of insects carrying a dominant lethal gene
RT-PCR	Real-time reverse transcription -Polymerase chain reaction
SC	Suspension concentrates
SF	Surface film
SIT	Sterile Insect Technique
TropNetEurop	The European Network on Imported Infectious Disease
ULV	Ultra-low volume
VBDCP	Vector Borne Disease Control Programme
WG	Water dispersible granule
WHO	World Health Organization
WHOPES	WHO Pesticide Evaluation Scheme
WP	Wettable powder

CHAPTER 1

REVIEW OF DENGUE, DENGUE VECTORS AND ADULT MOSQUITO CONTROL MEASURES

1.1 Introduction to dengue

Dengue is the most significant mosquito-borne viral disease in the world. Recent reports estimate up to 400 million dengue infections occur in humans every year (Bhatt *et al.,* 2013), with nearly 3.6 billion more at risk of infection (Ferreira, 2012). Dengue viruses have widely spread across the globe and it is estimated that more than 100 tropical countries suffer the effects of the virus, and more than 60 of these countries have been documented with severe dengue (WHO, 2000). Severe dengue cases arise every year with at least 12,000 deaths, mostly in children (WHO, 2003a). Asia contributes 70% of this burden whereas the Americas contribute 14% of global apparent infections. The Africa dengue burden is nearly equivalent to that of the Americas, 16% of the global total, whereas the countries of Oceania recorded less than 0.2% of global apparent infections (Bhatt *et al.,* 2013).

Dengue is caused by four viral serotypes, DEN-1, DEN-2, DEN-3 and DEN-4, in the family of *Flaviviridae* (WHO, 2003a); however the first new serotype of dengue virus has been discovered and the emergence of this new virus has been reported in most recent years (Normile, 2013). The new dengue serotype 5 has been detected from blood and serum samples from a severe outbreak in Sarawak, Malaysia. The antibodies elicited in humans by this 5th serotype have been proven to be different from those elicited by the other previous four viral serotypes. This 5th serotype is believed not to have a sustained transmission cycle in humans but only circulates among non-human primates (Normile, 2013). These flaviviruses are mainly transmitted by *Aedes aegypti* and a secondary dengue vector, *Aedes albopictus*. *Aedes* mosquitoes can be infected by the virus during their feeding process and, once infected, the mosquito is capable of retaining the virus throughout its adult life. This factor causes the *Ae. aegypti* mosquitoes to be a

highly efficient vector, constantly transmitting the dengue virus to humans (WHO, 2009a). *Ae. aegypti* has also been determined to cause both dengue fever and severe dengue. Other than that, *Aedes polynesiensis* and several species of *Aedes scutellaris* complex may also be dengue vectors but these localised species are less efficient vectors and thus are of minor importance.

1.1.1 Dengue fever (DF) and severe dengue

Any of the four dengue virus serotypes can cause many clinical signs ranging from mild illness to severe and deadly disease. Both child and adult patients may have variable symptoms including nonspecific viral syndrome, especially in younger children (Gubler, 1997). Dengue fever (DF) is an acute febrile disease causing a fever lasting between 5 and 6 days with typically high temperature (38-40 ^{o}C) (Mairuhu *et al.,* 2004). During febrile periods, the patient may develop several symptoms such as headache, bone or joint and muscular pains, rash, leucopoenia, myalgia, arthralgia, nausea and vomiting (WHO, 1997; Gubler, 1998). The severity of dengue frequently depends on the age of the patients and increases with repeated infections (Cobra *et al.,* 1995; WHO, 1997). Most dengue virus infections among infants and young children are mildly symptomatic or asymptomatic (Rigau-Pérez *et al.,* 1998) whereas older children and adults may develop a mild febrile syndrome or a seriously incapacitating disease (WHO, 1997).

Dengue haemorrhagic fever (DHF) is characterised by high fever with other symptoms resembling DF (Rigau-Pérez *et al.,* 1998). During critical stages of DHF, thrombocytopenia ($\leq$ 100,000 cells/mm^3) and elevated haematocrit occur (Mairuhu *et al.,* 2004). Other bleeding complications such as epistaxis, gingival bleeding, gastrointestinal bleeding, haematuria and menorrhagia can also occur. DHF patients may also develop skin haemorrhages with hepatomegaly, and in severe cases it can cause capillary leak syndrome (WHO, 1997). If the plasma leakage is not detected and replaced with fluid therapy, this stage can be fatal (Gubler, 1997; WHO, 1997). The critical stages can lead to hypovolaemic shock, referred to as dengue shock syndrome (DSS) (WHO, 1997).

The classification of dengue was revised recently to recognise the differences between severe dengue and non-severe dengue, with non-severe dengue further divided into with and without warning signs groups (WHO, 2009a). Non-severe dengue with warning signs requires precise observation and medical care. The criteria for dengue with warning signs include abdominal pain, persistent vomiting, clinical fluid accumulation, mucosal bleed, lethargy, restlessness, liver enlargement and rapid decrease in platelet count (WHO, 2009a). For severe dengue, the criteria for diagnosing patients include plasma leakage, haemorrhage and organ impairment. The classification levels of severity are important especially for (1) practical use in clinical decisions, (2) reporting consistent medical results and (3) measuring the end point of dengue vaccines and drug trials (WHO, 2009a).

1.1.2 Treatment of dengue infection

An organised process is required for the early recognition of the disease to ensure that dengue mortality can be reduced in the future. The main component of the process is good clinical services with appropriate health care at all dengue stages. The effective supportive care management which has been applied for primary and secondary stages of dengue could help in identifying the risk of developing severe disease. Furthermore, efficiency of emergency assessment, treatment and hospital care is needed for the tertiary stage of dengue (WHO, 2009a). There is great variability of clinical symptoms; therefore early recognition and treatment is important for dengue virus infection. Patients with DF require appropriate fluid balance as fluids are lost during diarrhoea or vomiting. Resting is necessary whereas analgesic and antipyretics are used for high fever (Rigau-Pérez *et al.,* 1998). In addition, Harris *et al.* (2003) suggested that maintaining hydration is the key finding that could reduce the number of hospitalisations. They also indicated that dengue patients could be treated at home by additional fluid intake. However, if the symptoms and deterioration of health continue, hospital admission is highly necessary. Patients with a high risk of developing severe DHF also require hospitalisation so that they can be fully monitored by a physician (Malavige *et al.,* 2004).

1.1.3 Dengue vaccine development

Although the main treatments for dengue patients are based primarily on appropriate clinical services and case management, significant efforts have also been invested in the development of vaccines against dengue viruses. Dengue vaccine development has been in progress for more than 60 years but no effective and safe vaccine is yet available. Several factors such as the complexity of the disease agents (the vaccine must protect against all four virus serotypes), inadequate investment by dengue vaccine developers and insufficient animal models have delayed the progress (Hombach, 2007; Whitehead *et al.,* 2007). Since the disease is caused by four distinct serotypes (DEN-1 to 4), all four viruses may circulate in one endemic area at the same time. Infection by any of the four dengue serotypes has been shown to produce lasting protection against reinfection by the same serotype but only provisional protection against a secondary or tertiary infection of heterologous serotypes. Furthermore, higher risk of severe disease is associated with secondary infection (WHO, 2013a).

Despite these challenges, there has been considerable progress in dengue vaccine development in recent years. There are several vaccine candidates currently underway at different stages of development. The most advanced candidate vaccine is a live attenuated tetravalent dengue vaccine, which is under evaluation in phase II and phase III clinical trials in dengue endemic regions (Durbin & Whitehead, 2010; Guy *et al.,* 2011; WHO, 2012c). This vaccine candidate has been developed by Sanofi Pasteur (CYD-TDV) and has now completed phase IIb study in Thailand. Furthermore, the evaluation of this vaccine candidate has continued to phase III studies which are currently underway in 10 countries in Asia and Latin America (WHO, 2012c).

1.1.4 Geographical distribution and incidence of dengue

The geographical distribution and incidence of DF and DHF has continued to increase throughout the years as a result of the expansion of both dengue viruses and their mosquito vectors, *Ae. aegypti* and *Ae. albopictus*. Recently, it was estimated that over 40% of the world's population are now at risk, with the

disease being found mainly in urban and semi-urban areas in tropical and sub-tropical climates worldwide (Bhatt *et al.,* 2013; WHO, 2013a). The incidence of dengue has increased 30-fold during the past five decades. The annual average number of DF and DHF cases reported to the World Health Organization (WHO) has gradually increased, almost doubling between 2008 and 2010 (Figure 1.1). Currently, South East Asia and the Western Pacific have been identified as the most seriously affected regions (WHO, 2009a).

In Asia, India reported outbreaks of dengue in 1945 whereas Thailand recorded its first epidemic of DHF in the 1950s. DHF continue to occur in the 1990s, with the greatest incidence recorded from Thailand, Myanmar and Sri Lanka (Pinheiro & Corber, 1997). Several countries such as Bangladesh, Indonesia, the Maldives, Myanmar, Sri Lanka, Thailand and Timor-Leste reported dengue cases in 2003 and Bhutan in 2004. Thailand and Indonesia reported the highest number of dengue cases in the region in 2003 and 2004 respectively. Since then, many countries such as Bangladesh, Bhutan, Indonesia, Thailand, the Maldives and Sri Lanka have recorded increasing numbers of dengue cases, except in 2005 (WHO, 2009a). To date, WHO (2012a) showed that Indonesia reported the highest number of dengue cases, with more than 100,000 cases reported between 2004 and 2010 in this region (Figure 1.2).

In the Western Pacific region, the level of dengue activity is variable. During 1990-1991, a small dengue outbreak was recorded in Australia with a large outbreak being reported in 1992-1993. Several different Pacific islands such as Vanuatu, New Caledonia, Tahiti, Rarotonga, Fiji, American Samoa, Western Samoa, Yapa and Palau were also identified with sporadic cases of DHF (Pinheiro & Corber, 1997). Since the last major pandemic in 1998, more countries in the Western Pacific have been reported with dengue outbreaks. Several countries such as Cambodia, the Lao People's Democratic Republic, Malaysia, the Philippines, Singapore and Vietnam recorded an escalation in dengue cases in 2009. In 2010, over 100 cases were recorded from French Polynesia, New Caledonia, and Vanuatu, and more than 1,000 cases were reported by Australia, Cambodia, the

Lao, People's Democratic Republic, Malaysia, the Philippines, Singapore and Vietnam (Arima & Matsui, 2011).

In the Americas, dengue outbreaks have occurred since the 19th century. During 1941 to 1946, dengue epidemics were documented in other regions of the Americas including Mexico, Panama and Venezuela and several islands including Cuba, Puerto Rico and Bermuda (Halstead, 2006). Most recent data by WHO (2012a) illustrated that the highest numbers of dengue cases were recorded in Brazil with over 200,000 reported cases (Figure 1.2). In fact, dengue transmission has occurred in almost every country in this region except there has been no local transmission for Uruguay and continental Chile in Latin America (San Martín *et al.,* 2010).

In the European region, dengue fever was first detected in 1927 and 1928 in Greece and Turkey, when up to 1,500 people died (WHO Regional Office for Europe, 2012). The disease then disappeared until recently when dengue fever was found in residents returning from overseas travel, with approximately 8% of travellers returning to Australia and Germany being identified as dengue positive (Potasman *et al.,* 1999; Jelinek, 2000; O'Brien *et al.,* 2001). The European Network on Imported Infectious Disease Surveillance (TropNetEurop) suggested that the number of imported dengue cases was increasing in European travellers (Jelinek *et al.,* 2002). Recent significant outbreaks occurred in Madeira, Portugal in 2012 with over 2,100 cases confirmed (WHO Regional Office for Europe, 2012).

Epidemics of DF and DHF have significantly increased in the Eastern Mediterranean region. Reported cases have been documented since the mid-1990s particularly in the Arabian Peninsula (Zaki *et al.,* 2008). Djibouti, Pakistan, Saudi Arabia, Somalia, Sudan and Yemen have recently experienced outbreaks of dengue (WHO, 2014). An outbreak of DHF was first recorded in Karachi, Pakistan in 1994 (Chan *et al.,* 1995) in the same year that Saudi Arabia also reported its first dengue transmission, in Jeddah (Fakeeh & Zaki, 2001).

Subsequently, several dengue outbreaks have been documented and the expansion of dengue has occurred in the main cities of Pakistan (Jamil *et al.,* 2007). Three major outbreaks occurred in Saudi Arabia between 1993 and 2008 with over 2500 cases, 77 cases of DHF/DSS and 10 fatal cases (WHO, 2009a). Dengue was first identified in Makkah, Saudi Arabia during an outbreak in 2004 (Khan *et al.,* 2008). Ayyub *et al.* (2006) described how Jeddah could become a great potential for introduction and exchange of various infectious diseases including dengue, as this city is the main international transit in the country for large numbers of pilgrims from around the world who visit Saudi Arabia every year for the Haj. There were also more than 1,000 suspected dengue cases reported in Yemen between 2000 and 2005 and 84 febrile cases recorded in Sudan in 2005 (Ageep *et al.,* 2006; WHO, 2009a).

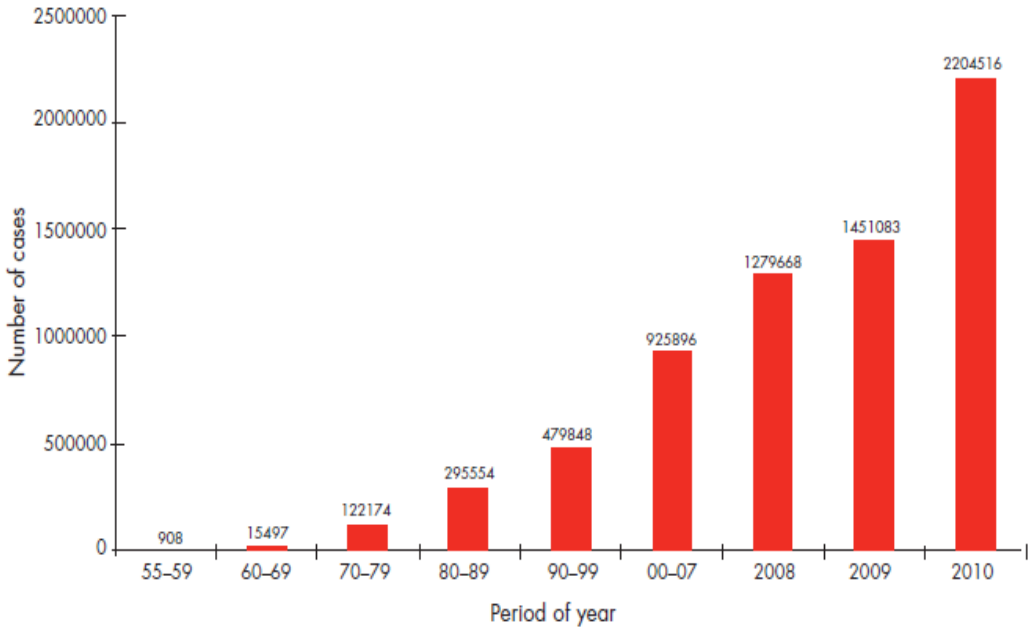


Figure 1.1 Average numbers of dengue and severe dengue cases reported to WHO annually over ten year periods from 1955-2007 and the annual number of cases reported from, 2008-2010 (WHO, 2012a).

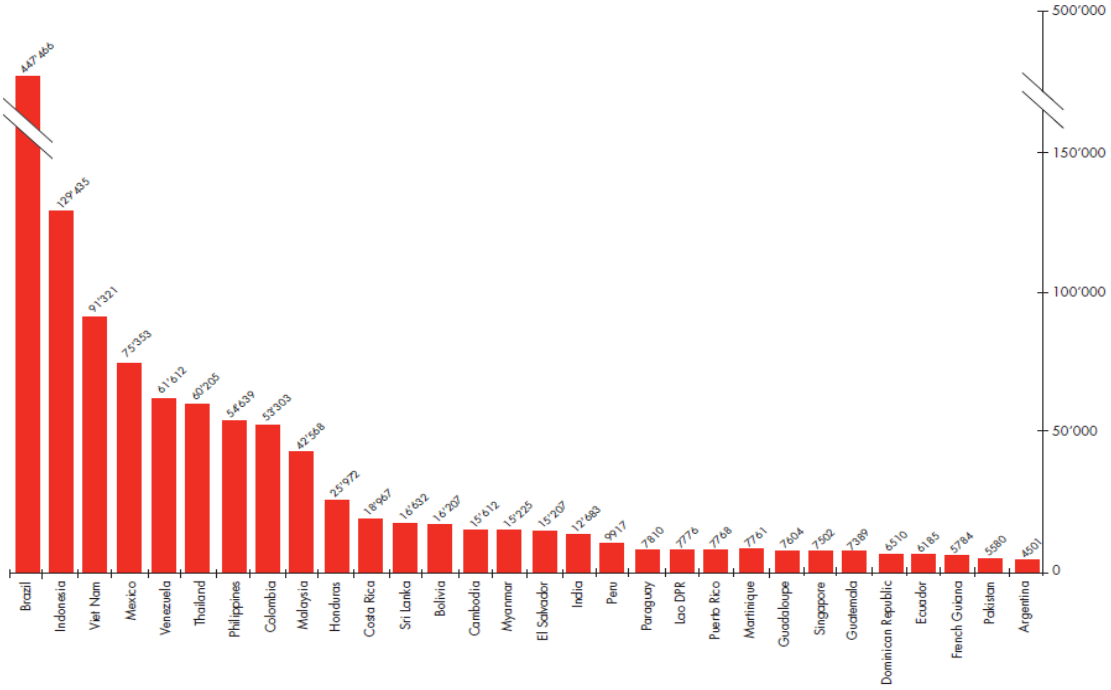


Figure 1.2 Average number of dengue cases in 30 most highly endemic countries /territories as reported to World Health Organization (WHO), 2004-2010 (WHO, 2012a).

The geographical areas in which all four dengue virus serotypes circulate have been illustrated by WHO (Figure 1.3). Dengue areas have been classified by WHO regions and dengue fever is currently endemic in more than 100 countries in Africa, the Americas, the Eastern Mediterranean, South East Asia and the Western Pacific (WHO, 2009a). For those countries, dengue epidemics can have major economic and health impacts. In dengue-endemic countries of Asia and the Americas, the burden of dengue is estimated at approximately 1,300 disability-adjusted life years (DALYs) per million people. In these regions, dengue exerts an economic impact comparable to malaria, tuberculosis, sexually transmitted diseases (excluding HIV/AIDS) and hepatitis, childhood diseases (polio, measles, pertussis, diphtheria) and other tropical diseases (*e.g.* schistosomiasis, filariasis, Chagas disease, leishmaniasis and onchocerciasis) (Gubler & Meltzer, 1999).

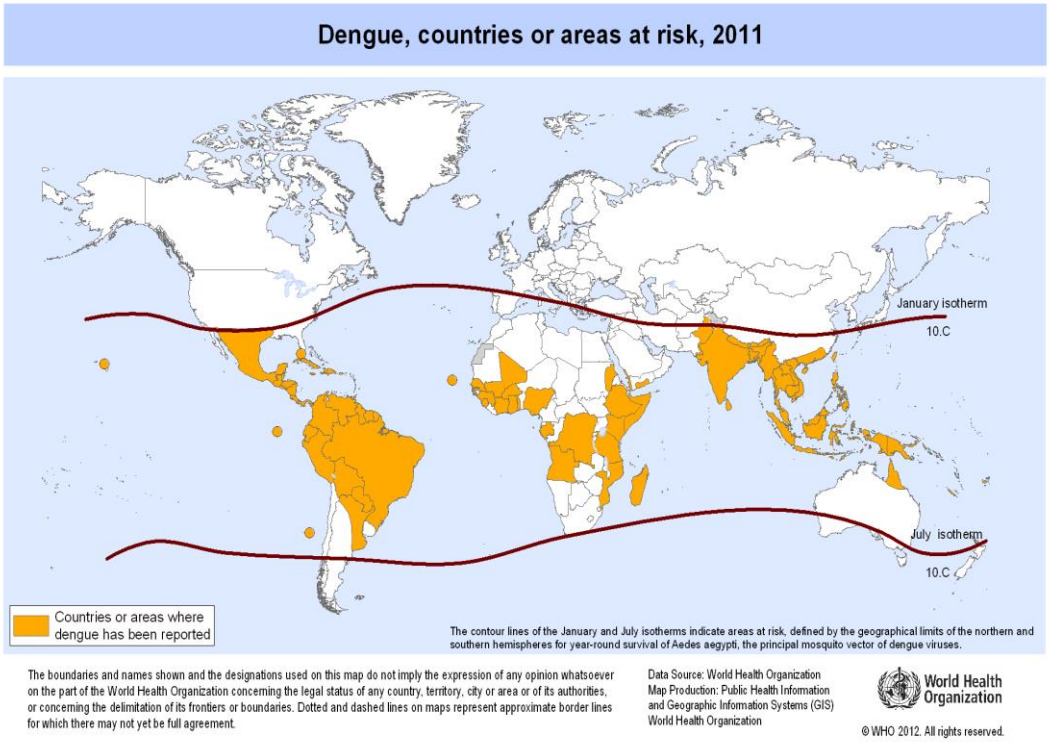


Figure 1.3 World map showing countries or areas at risk of dengue transmission in 2011 (Nathnac, 2011).

1.1.5 Transmission of dengue virus

Dengue viruses are transmitted to human hosts during feeding or probing of infective mosquito vectors of the genus *Aedes*. The *Ae. aegypti* mosquito has been recognised as the primary vector that is responsible for dengue transmission (arboviruses or arthropod-borne viruses) (WHO, 1997) whereas *Ae. albopictus* as secondary vector for transmission in urban and peri-urban settings (urban transmission cycle) (Durbin *et al.,* 2013). In contrast to most arboviruses, the dengue virus is limited to vertebrate hosts, which most likely includes only humans and non-human primates. The transmission of dengue virus in an enzootic cycle between non-human primates and arboreal *Aedes* species mosquitoes has been found in Southeast Asia and West Africa (Durbin *et al.,* 2013). Dengue virus transmission includes both intrinsic and extrinsic incubation periods (McBride & Bielefeldt-Ohmann, 2000). The intrinsic incubation period ranges from between 1 and 15 days before the infected individual human becomes viraemic. The female

Aedes mosquito obtains the virus from a blood meal taken from an infectious person and, once infected, the mosquito remains infected with the virus for life (WHO, 1997). During the extrinsic incubation period, the virus develops and replicates in the mosquito gut, brain, salivary glands and reproductive organs, typically taking 8 to 10 days before it can be transmitted to other individuals (McBride & Bielefeldt-Ohmann, 2000; WHO, 2009a). The period of extrinsic incubation depends on environmental conditions including ambient temperature and humidity (Kuno, 1995; WHO, 1997).

The dengue viruses could also be transferred to the next mosquito generation by vertical or transovarial transmission. The presence of the DEN virus has been experimentally demonstrated in *Ae. aegypti* that had been intrathoracically inoculated with DEN-3 (Joshi *et al.,* 2002) as well as larvae and progeny from *Ae. albopictus* that had been orally infected with DEN-2 (Castro *et al.,* 2004). Molecular tools such as immunofluorescence (Arunachalam *et al.,* 2008) and real-time reverse transcription PCR (RT-PCR) (Cecílio *et al.,* 2009) have been used for detecting vertical transmission. Previous studies indicated that transovarial transmission in *Ae. aegypti* occurred in the laboratory and only rarely in natural conditions (Joshi *et al*., 2002; Lee & Rohani, 2005; Gunther *et al*., 2007). This phenomenon is not well understood, occurs rarely and probably does not significantly contribute to the transmission of dengue (WHO, 1997). There are several other factors that contribute to the dynamics of dengue virus transmission and they include environmental and climate factors, population immunological factors and host-pathogen interactions (WHO, 2009a). The magnitude and duration of the viraemic phase of an infected person is also associated with dengue transmission. Patients with high viraemia provide a greater possibility for vector mosquitoes to become infected. However, persons with a low level of viraemia are also infectious, so may also cause some feeding vector mosquitoes to become infected with the virus (WHO, 1997). Other than that, transmission of dengue virus could also occur through demographic and societal changes such as population growth, uncontrolled rapid processes of urbanisation and the increase of global air and water transportation (Gubler, 2002).

1.2 Introduction to dengue vectors

The primary vector of dengue is *Ae. aegypti,* which is also called the 'yellow fever mosquito', first described by Linnaeus (1762). It is a member of the subgenus *Stegomyia* and the genus *Aedes* (Christophers, 1960). *Ae. aegypti* originated from Africa but is now found in tropical and subtropical regions throughout the world. The secondary vector of dengue is the 'Asian tiger mosquito', *Ae. albopictus.* It was first described by Skuse (1894) in Bombay, India as *Culex albopictus.* It is an invasive species originally from tropical and subtropical areas of South East Asia that has invaded many countries around the world (Watson, 1967). Apart from being responsible as dengue vectors, both *Ae. aegypti* and *Ae. albopictus* have also become efficient vectors of other human diseases including Chikungunya and yellow fever (Hochedez *et al.,* 2006; Phillips, 2008) as well as some encephalitis viruses (CDC, 2012) and filariasis parasites (Cancrini *et al.,* 2003).

1.2.1 Aedes aegypti (Linnaeus)

Adult *Ae. aegypti* is a small to medium-sized mosquito, approximately 3 to 6 millimetres in length, with two white stripes and a single curved line at each side forming a lyre shape on the dorsal thorax. The abdomen is generally dark brown to black, and is covered with white scales in the form of stripes and spots which create the unique distinguishing pattern. Each tarsal segment of the hind legs also possesses white stripes (Lee *et al.,* 2003b). The immature stages are mostly found in artificial containers including water tanks, flower vases, pot plant bases, discarded tyres, buckets, rain gutters or other manmade containers inside and outside the house (Christophers, 1960). Females also lay eggs in natural sites such as bromeliads, tree holes and discarded coconut shells (Gubler & Rosen, 1976). *Ae. aegypti* eggs can withstand desiccation for up to one year (Russell *et al.,* 2001a), enabling eggs to be spread to other new areas. The four larval stages usually take 5 - 10 days for development whereas the transformation from the pupal to the adult stage generally takes 2 - 3 days. Occasionally, under favourable climatic and environmental conditions, the life cycle of this species can occur in less than 10 days. The lifespan for adult mosquitoes typically ranges between 2 weeks to a month. Their daily flight distance is about 50 - 100 metres or a

maximum of 200 metres (Lee *et al.,* 2003b; Maricopa, 2006). *Ae. aegypti* is a day-biting mosquito that prefers to feed on humans even if other hosts are available (Scott *et al.,* 2000; Harrington *et al.,* 2001). This species breeds and rests close to human habitation. They are highly anthropophilic and often feed on multiple hosts during a single gonotrophic cycle (Huber *et al.,* 2008). The high human-biting rate and ability of eggs to survive periods of desiccation are important factors for the successful parasite and pathogen transmission in this species (Phillips, 2008).

1.2.2 Aedes albopictus (Skuse)

Adult *Ae. albopictus* is recognised by its bold black scales with a distinguishing white stripe down the central dorsum of the thorax. It is a medium-sized mosquito, 2 - 10 millimetres in length. The abdomen and legs are mostly covered in black scales and white stripes (Hawley, 1988). As with *Ae. aegypti*, the immature forms of *Ae. albopictus* can be found in artificial containers with stagnant water such as tyres, flower pots, plates under potted plants, vases, buckets, tins, cans, clogged rain gutters, ornamental ponds, drums, water bowls for pets, birdbaths and catch basins. They can also be found in natural habitats such as tree holes, rock holes, hollow bamboo stumps and leaf axils. This species was previously believed to be restricted to vegetated areas and forests but now has adapted to human environments in urban, suburban and rural areas (CDC, 2012). The entire aquatic cycle can occur between 7 and 9 days at optimum conditions, and the lifespan of an adult mosquito is around 3 weeks. Generally, this species has a less than 200-metre daily flight distance, and its breeding sites are likely to be in close proximity to its blood-feeding habitat (CDC, 2012). *Ae. albopictus* is a daytime feeder and normally found in shady areas where they prefer to rest in shrubs near the ground. Although similar in biology and ecology to *Ae. aegypti*, *Ae. albopictus* is less anthropophagic and has been described as an opportunistic biter with a wide host range including humans, mammals and birds (Hawley, 1988; Eastrada-Franco & Craig, 1995; Tandon & Ray, 2000). It is an aggressive biter which actively feeds early in the morning and late afternoon, indoors and outdoors, but mainly the latter (Ponlawat & Harrington, 2005; Chaves *et al.,* 2010; Valerio, 2010). It is well recognised that both *Ae. aegypti* and *Ae. albopictus* can

feed multiple times; therefore this behaviour may increase opportunities for the transfer of arboviruses to other vertebrate hosts (Harrington *et al.,* 2001; Delatte *et al.,* 2010). Although *Ae. albopictus* has been implicated in the transmission of dengue, it is in fact a far less efficient dengue vector than *Ae. aegypti* (Lambrechts *et al.,* 2010).

1.3 Behaviour of *Aedes sp.* mosquitoes

The most important behaviours of the mosquito life cycle in relation to human disease are those relating to reproduction and feeding. Reproduction in mosquitoes includes mating and oviposition whereas feeding consists of host-seeking and sugar-feeding behaviour. The behavioural responses depend on internal and external cues which are mostly genetically determined, or in certain circumstances governed by physiological state and external stimuli. These include age, size, nutrition, mating condition, gonotrophic status and circadian rhythms (Clements, 1999b; Takken & Knols, 1999). In addition, the behavioural response also depends on environmental factors such as temperature, humidity and light (Clements, 1999b).

1.3.1 Mating behaviour

Mating in mosquitoes generally involves swarming behaviour (Clements, 1999a) mate-finding and recognition of specific species (Takken & Knols, 1999). Swarming in mosquitoes can occur from only a few individuals up to groups of several thousand males. Swarming does not occur in all mosquito species (Becker *et al.,* 2010). *Ae. aegypti* males do not form large swarms (Clements, 1999a) but tend to aggregate and mate near their potential hosts in nature. When entering a swarm, the female mosquito will be grasped by a male but normally the mating process takes place outside the swarm (Clements, 1999a). Mating in female mosquitoes can begin soon after emergence, but are refractory to insemination for about 48 - 72 hours. Therefore, mating behaviour by newly emerged females does not result in insemination. On the other hand, the process of genitalia rotation through 180º must be completed before mating can occur in male mosquitoes. Therefore mating in male *Ae. aegypti* only occurs after complete rotation, which is

15 - 24 hours after emergence (Gwadz & Craig, 1968). The mating process normally completes in less than a minute (Clements, 1963; Spielman, 1964). In mosquitoes, sperm and seminal fluids are transferred from male to female's bursa copulatrix and finally remain in spermathecae (Clements, 1963). This sperm will be used by the female mosquito to fertilise several egg batches without further mating. Female *Ae. aegypti* are monogamous, mating only once in a lifetime and further mating is prevented by the secretion from the male accessory gland called the 'matron'. Male mosquitoes, in contrast, can mate several times during their lifetime.

Mating biology in mosquitoes is influenced by different physical factors. Age, body size and density affect mating success in *Ae. aegypti* (Ponlawat & Harrington, 2009). Previous studies have documented that environmental conditions affect the amount of sperm stored in the reproductive organs of older males. The study also indicated that males of *Ae. aegypti* more than 10 days old could produce and transfer a higher amount of sperm to females during mating, resulting in competitive advantage (Ponlawat & Harrington, 2007). It has been suggested that recognition of a female by male mosquitoes is dependent on visual cues, female flight tones and contact pheromones (Cabrera & Jaffe, 2007). Indeed, the existence of contact pheromones has been described in *Ae. aegypti* and *Ae. albopictus* (Nijhout & Craig, 1971).

1.3.2 Sugar-feeding behaviour

Both male and female mosquitoes start sugar-feeding within hours of emergence and continue for 1 - 2 days (Haramis & Foster, 1990). Most female mosquitoes take a sugar meal before a blood meal and some mosquitoes will not seek a blood meal until after a sugar meal (Hancock & Foster, 1993). In *Ae. aegypti*, higher survivorship has been recorded in females that fed on blood and sugar compared to females that fed on blood alone (Day *et al.,* 1994). In the absence of host stimuli, sugar-feeding is frequently observed in *Ae. aegypti* and *Ae. albopictus* under laboratory conditions. However, sugar-feeding normally ceases if these species are exposed to both sugar and host stimuli (Yee *et al.,* 1992).

The activity of sugar-feeding in mosquitoes can be diurnal, nocturnal or crepuscular, and often shows bimodal periodicity. Odours are important for locating nectar sources (Foster, 1995). In wind tunnels, *Ae. aegypti* responded to the odours of ox-eye daisy, *Leuchanthemum vulgare*, in a biphasic periodicity but not without visual or nutritional cues (Jepson & Healy, 1988). Numerous other nectar sources have been reported including extrafloral nectaries (Takken, 1999), honeydew (Yuval 1992; Smith & Gadawski; 1994; Foster, 1995; Takken & Knols, 1999), tree sap (Nasci, 1986), leaves damaged by phytophagous insects (Mogi & Miyagi, 1989), damaged fruits (Joseph, 1970), seed pods (Müller *et al.,* 2009) and several wild and ornamental flowers (Müller *et al.,* 2011).

Nectar is presumed to provide the energy sources for flight and host-seeking activity (Takken & Knols, 1999). It has been reported that females of many *Culex* and *Culiseta* species commonly take small sugar meals in between blood meals during gonotrophic development (Nasci & Edman, 1984; Andersson & Jaenson, 1987), while blood-fed females of *Anopheles freeborni* frequently take sugar meals at the end of gonotrophic development (Holliday-Hanson *et al.,* 1997). However, most female *Aedes* and *Anopheles* species only take sugar meals before blood-feeding or in the gravid stage (Edman *et al.,* 1992; Yee *et al.,* 1992) and the amount of the sugar meal required by female mosquitoes remains unclear (Müller & Schlein, 2006).

1.3.3 Host-seeking behaviour

Host-seeking behaviour is described as the orientation to a host for a blood meal from a distance (Takken, 1991). Female mosquitoes use a number of senses to locate a potential host, believed to be based on olfactory, visual and thermal cues. In general, there are three phases involved in host-seeking behaviour, namely long-range, middle-range and short-range orientation (Gibson & Torr, 1999). The host-seeking behaviour in most of the mosquito species is initiated with random dispersal flight. Dispersal flights enhance the chances of a mosquito coming into contact with potential host stimuli. Once a mosquito comes into contact with host stimuli, oriented flight towards the host stimuli increases and, therefore, they

become closer to each other. In the vicinity of the host, the mosquito identifies a suitable candidate and this ends up with the mosquito alighting on the host (Sutcliffe, 1987). The long-range orientation usually involves a combination of the reception and evaluation of olfactory and visual cues (Takken, 1991). The olfactory receptors, which are located on the antennae, maxillary palpi and labellum (Kwon *et al.,* 2006; Pitts & Zwiebel, 2006; Leal, 2013), are responsible for responding to specific host odour (Qiu *et al.,* 2006; Carey *et al.,* 2010). Olfactory cues also play an important role during middle-range orientation whereas visual cues are responsible for host identification and recognition in middle- and short-range orientation (Allan *et al.,* 1987). In short-range orientation, mosquitoes use thermal cues such as body heat and moisture to locate the host (Daykin *et al.,* 1965). The main olfactory cues are carbon dioxide (CO_2), lactic acid, octenol, acetone, butanone and phenolic compounds (Sutcliffe, 1987). It is widely acknowledged that host-seeking behaviour is elicited by a combination of odours rather than by a single compound. CO_2 is a general host odour detected by most of the haematophagous insect species (Clements, 1963; Gillies, 1980; Nicholas & Sillans, 1989) which triggers activation (Gillies, 1980) and attraction (Reeves, 1951; Russel, 2004; Smallegange *et al.,* 2010).

For blood-sucking insects, there are a series of physiological and behavioural steps which cause considerable variation among vector species. This series of steps has been described in mosquito vectors and it consists of (i) an appetitive search, (ii) activation and orientation and (iii) attraction (Lehane, 2005). Appetitive search involves the simplest form of behaviour which is likely to bring the hungry mosquitoes into contact with stimuli derived from a potential host. Activation occurs when the mosquitoes come into contact with a suitable signal from a potential host and orientation causes the mosquitoes to use the host-derived signal information to orientate towards the host. Attraction occurs when host stimuli such as the size, shape and colour of the target are used to bring the mosquito into the vicinity of the host and the final decision to contact or not to contact the host is made (Lehane, 2005).

In general, mosquitoes exhibit daily biting behaviour with one or several peak times of host-seeking activities. *Ae. aegypti* is a diurnal mosquito, typically with biting peaks close to dawn and dusk (Abu Hassan *et al.,* 1996), though other behavioural patterns differing between different localities and subspecies have been reported (Chadee, 1988). Diurnal behaviour has been reported in some studies in Asia, Africa and the Americas (Chadee, 1988). Nocturnal behaviour has been reported in *Ae. Aegypti*, possibly due to adaptation to artificial lighting in urban areas (Chadee & Martinez, 2000). Chadee (1988) reported diurnal activities of *Ae. aegypti* with bimodal peaks at 0600 - 0700 and 1700 - 1800 hours whereas several other studies reported trimodal peaks at varying hours in different locations during daytime (Chadee & Martinez, 2000). Beck (1968) suggested that an internal circadian rhythm controlled host-seeking behaviour. Nevertheless, the pattern of this behaviour can be altered by insemination, blood digestion (Rowland, 1989), and environmental abiotic factors (Klowden, 1994).

1.3.4 Blood-feeding behaviour

The male mosquito's mouthparts are well developed for sugar-feeding whereas female mosquitoes' mouthparts are better for piercing and blood-feeding (Magnarelli, 1979; Clements, 1992). Once landing on the host, the female starts searching for a blood capillary on the host's skin. The females might probe a few times using labellae before taking a blood meal. It has been suggested that thickness and temperature of the host skin are possibly important stimuli for probing since they are related to the number of blood vessels in the skin (Davis & Sokolove, 1975). There are receptors called sensilla, located on the ventral side of the pair of labellae and on the distal part of the labium, which may help to discover a suitable site for probing (Becker *et al.,* 2010). The ingestion of blood begins when the female successfully punctures the skin. The labium gradually bends backward when the mosquito penetrates deeper into the skin. The cibarial and pharyngeal pumps are the sucking organs which are responsible for pumping the blood or nectars into the mosquito's gut. To complete the blood meal, it is important for the female to prevent coagulation of the blood. The mosquito's saliva, which contains anticoagulants, will be transferred to the host's skin tissue

by the female during feeding. This process generally stimulates an immune response which causes an inflammatory reaction such as irritation at the site of the mosquito bites.

A previous study indicated that female mosquitoes can ingest more than three times their actual body weight (Nayar & Sauerman, 1975). In *Ae. aegypti*, approximately 5 µl blood can be ingested in a single feeding. The processes of blood ingestion and salivation continue until the mosquito is disturbed by the host or stretch receptors are triggered and the signal that the midgut is full of blood is attained. For young *Ae. aegypti,* the stretch receptors located at the anterior of the abdomen will be triggered when the total volume of blood exceeds 2.5 µl (Klowden, 1988; Klowden, 1990). At that time, the female removes its mouthparts so that it may prevent itself from bursting due to excessive blood intake. The study shows that the mosquito continuously feeds until it does burst if the nervous system pathways are cut surgically (Gwadz, 1969). The females will continue to exhibit active blood-feeding behaviour when the total volume of blood in the abdomen is below the required amount (Klowden, 1994). The blood could be from a single feeding or an accumulation of multiple feedings (Madhukar & Jones, 1974; Bowen, 1991). After feeding, the female mosquitoes prefer to rest for the process of blood digestion and egg maturation. Based on their physiological state, the behaviour of mosquitoes practically changes upon blood-feeding (Washino, 1977; Takken *et al.,* 2001). The female is inhibited from taking another blood meal until the first batch of eggs is laid. At first, the inhibition mechanism is activated by the dilation of the abdomen while the latter is the influenced by the development of oocyte (Klowden, 1994). This process is influenced by temperature and it may last 2 to 6 days (Klowden, 1995). The blood and its protein contents are highly important for the egg production. The production of eggs by anautogenous species can only be completed once the female takes a blood meal. However, there is an exception for some autogenous species such as *Culex pipiens* biotype *molestus*, which are capable of producing their first egg batch without a blood meal (Weitzel *et al.,* 2009).

1.3.5 Oviposition behaviour

The oocyte-induced behavioural inhibition mechanism is initiated from the start of the process of egg maturation inside the female mosquitoes until after oviposition. The development of eggs occurs between 25 and 30 hours after the digestion of a full blood meal (Klowden, 1988; Klowden, 1990). It has been suggested that ovaries release an initial factor 6 - 12 hours after a full blood meal. This initial factor is called an ecdysteroid, which stimulates the fat body to generate a haemolymph substance and therefore reduces the sensitivity of lactic acid receptors (Bowen, 1991). The reduction of this sensitivity inhibits host-seeking in female mosquitoes. The females undergo this inhibition process approximately 24 hours before the host-seeking behaviour starts again (Klowden, 1994). Pre-oviposition is described as attraction and orientation towards the oviposition site whereas the actual oviposition is a deposition of eggs on the substrate (Bentley & Day, 1989). Some mosquito species exhibit morning and evening oviposition flights. These species generally have two crepuscular biting peaks (Bidlingmayer *et al.,* 1974). In Trinidad, *Ae. aegypti* showed a diurnal pattern of oviposition activity which occurred 2 hours after sunrise and 2 hours before sunset (Chadee *et al.,* 1990; Corbet & Chadee, 1992; Corbet & Chadee, 1993) whereas in French Polynesia*, Ae. aegypti* oviposition peak occurred from noon to midnight (Russel & Ritchie, 2004).

Several ovipositional strategies have been found in nature. Mosquito genera such as *Anopheles, Sabethes, Toxorhynchites* and *Wyeomyia* lay their individual eggs on the surface of water. Other genus such as *Coquillettidia, Culex* and *Culiseta* directly lay egg rafts on the surface of water, whereas some *Aedes* species and *Psorophora* species lay their individual eggs on a substrate or above the water line. Some mosquitoes attach their egg rafts to vegetation below the water surface. This ovipositional strategy has been used by some subgenera, for example *Mansonioides* and species of *Aedeomyia, Culex* and *Anopheles* (Lounibos & Linley, 1987). Mosquito species that lay their eggs on a substrate above the water line are generally resistant to desiccation. The eggs could survive for many months or even years, especially when protected from direct sunlight. The

hatching process occurs when the source of water and suitable habitat for the development of immature stages are available (Knight & Baker, 1962). Oviposition in these species and other species that lay their eggs on permanent water can occur immediately after the development of the eggs is completed and the breeding site is located. In contrast, mosquito species that lay individual eggs or rafts on the surface of water or that attach the eggs to vegetation have to evaluate and select the specific plant and suitable breeding site for immediate habitation by immature stages. These species only deposit their eggs on water until suitable plants or aquatic habitats for the immature stages become available (Shroyer & Sanders, 1977; Day & Edman, 1988). For *Ae. aegypti,* it has been suggested that female mosquitoes visit a number of sites to lay eggs, and this behaviour is called 'skip oviposition'. This strategy is used to avoid intraspecific competition and to minimise the risks of temporary sites (Reiter, 2007).

Several factors are known to influence mosquito oviposition behaviour and oviposition site selection. A previous study by Canyon *et al.* (1999) documented that humidity and diet significantly influence *Ae. aegypti* oviposition behaviour. Their study indicated that low humidity and high sugar concentration (sugar-feeding has been shown to reduce subsequent blood-feeding frequency) significantly delayed oviposition. Tsunoda *et al.* (2010) also suggested that female body size and sugar availability possibly influence *Ae. aegypti* oviposition behaviour. Females with larger body size store more energy reserves; therefore they are able to disperse their eggs more widely. Factors such as salinity, pH and nutrient content have been identified as being involved in breeding-site selection by mosquitoes (Merrit *et al.,* 1992). Other than that, physical factors have also long been recognised as being important in site selection by mosquitoes. These factors have been identified as colour and optical density of the site, site surface, temperature and reflectance (Clements, 1963). Furthermore, water components or the presence of mosquito eggs and conspecific larvae or pupae may also influence the selection of oviposition sites by mosquitoes (Allan & Kline, 1998).

1.4 Dengue vector control

A variety of methods have been used for the control of dengue vectors in the past, including biological and chemical methods. No single approach has been proven to be effective and the integration of several control tools and strategies is considered a necessity in most vector control programmes to control dengue vector populations today (McCall & Kittayapong, 2007). Dengue vector control involves the suppression of dengue vector immature stages and/or their habitats, or control of adult mosquitoes. These habitats can be eliminated by frequently emptying, cleaning and disposing of containers that could be a potential breeding site for mosquitoes or by removing the immature stages using insecticides or biological control agents. The adult vector can be killed by using insecticides with several methods of application (WHO, 2009a).

Insecticides and other chemicals were used to control the *Ae. aegypti* population during the first campaigns against yellow fever in Cuba and Panama in the early 20th century (Reiter & Gubler, 1997; Rogers *et al.*, 2006). During the clean-up campaigns, breeding sites were treated with oil whereas houses were treated with pyrethrins. After the discovery of DDT in the 1940s, this compound was widely used for the eradication of this species in the Americas before 1970. Initially, these programmes were largely successful in some countries but in many countries the success was only temporary (Rozendaal, 1997). The development of resistance to DDT by mosquito populations began in the early 1960s, after the extensive use of this chemical for malaria control (WHO, 2009a). Subsequently, alternative insecticides such as organophosphates, carbamates and pyrethroids were used for the control of mosquito populations but resistance continued to develop (Hemingway & Ranson, 2000), which created the ongoing need for new and more effective insecticides. Pyrethroids were developed in the 1960s-1970s and led to major improvements in the application of insecticides. Pyrethroids are a group of photostable insecticides with biodegradable compounds that can be used in the field at a rate of 10 to 100 times lower than other insecticides, which ultimately reduces the chemical burden on the environment (Becker *et al.*, 2010).

Biological control is defined as the reduction of the target species population by predators, parasites, pathogens, competitors or toxins from other organisms (Woodring & Davidson, 1996). These organisms include viruses, bacteria, protozoa, fungi, plants, parasitic worms, predatory mosquitoes and fish. Generally, biological agents are used to destroy mosquito larvae to avoid environmental pollution (Rozendaal, 1997). The use of beneficial organisms for the control of mosquitoes was no longer considered to be an important method after the discovery and the massive application of synthetic insecticides in the 1940s and 1950s. However, the excessive use of insecticides frequently causes environmental damage and the development of resistance in targeted mosquito species. As a result, interest in other alternatives to insecticides such as biological control and environmental management has been revived (Rozendaal, 1997; Becker *et al.,* 2010). In recent years, the use of several biological agents for the control of *Ae. aegypti* has been extensively reported; these agents have been largely successful in their control (Martinez-Ibarra *et al.,* 2002; Suarez-Rubio & Suarez, 2004; Kay & Nam, 2005; Seng *et al.,* 2008b; Iturbe-Ormaetxe *et al.,* 2011). Biological control can be an important component of integrated control strategies when applied with environmental management (Rozendaal, 1997).

Environmental management, which focuses on the destruction, alteration or elimination of natural and artificial breeding sites that produce the greatest number of *Ae. Aegypti*, is one of the most effective dengue control measures. This method is aimed at preventing and reducing vector breeding sites and minimising human-vector-virus interaction (WHO, 2009a). WHO (2009a) describes three types of environmental management: modification and/or manipulation of environmental factors and changes to human behaviour. Environmental modification involves physical transformation to reduce vector habitats, such as installation of a reliable water supply and water storage systems in communities, whereas environmental manipulation includes temporary changes to the vector habitats, such as emptying, cleaning, disposing of and removing discarded containers and pieces of household items together with the removal of plants that were frequently found to be breeding sites for the mosquitoes. Changes to human

behaviour include efforts by the communities to reduce human-vector contact, such installing mosquito screening on windows, doors and other entry points, using topical repellents as personal protection and bed nets while sleeping during the day.

Since *Ae. aegypti* is a domesticated species with short flight range and which often feeds on humans (Muir & Kay 1998; Harrington *et al.,* 2005; WHO, 2009a), the control or the elimination of this vector population would appear to be feasible. However, over the years, the results have proven otherwise (McCall & Kittayapong, 2007). Although many potential control measures for *Ae. aegypti* are available, their effectiveness has been affected by issues of delivery, coverage and acceptability (Farrar *et al.,* 2007). For this reason, several factors such as community participation, understanding of local ecology and behaviour of the target species, availability of resources for implementation, cultural context where the control intervention is undertaken, feasibility of the application in a timely manner and adequacy of coverage need to be considered in order to select the most appropriate vector control method or integration of control methods (WHO, 2009a).

1.4.1 Vector surveillance

The key to establishing the density, seasonal and geographical distribution of any vector population is through data from field-based entomological surveillance. These data are beneficial for evaluating control programmes and obtaining relative longitudinal measurements of vector populations, and they are also useful for facilitating interventions (WHO, 1997). Ovitrap and adult trap are widely used simple methods used in dengue vector surveillance, which also allow the detection and analysis of the virus. However, without knowing the immunological status of the population at risk, the significance of this method remains uncertain. Vector surveillance of adult mosquitoes tends to provide less reproducible results than that of immature mosquitoes (WHO, 2009a). Monitoring of late instar larvae and especially pupae can potentially be more informative (Focks, 2003). An invasive method such as serological tests, which involves the intake of periodic blood

samples from the representative participants including children in high-risk areas, is impractical in many communities. However, the development of non-intrusive (e.g. from saliva, tears and urine), serotype-specific, rapid sensitive and inexpensive tools for the detection of antibodies would be a great advance in entomological surveillance and assessment (Morrison *et al.,* 2008). It is important that surveillance data collected from the sampling of mosquito, virus and sera can be interpreted into meaningful information that relates to virus transmission risk. The complex epidemiology of virus transmission has resulted in difficulty in targeting and designing effective vector control (Lloyd-Smith *et al.,* 2005), and it is extremely important to focus on the distribution of confirmed cases and the most productive sources of adult mosquitoes so that the control programmes against *Aedes* mosquitoes can be carried out effectively.

1.4.2 Control of immature stages of Aedes aegypti

The most effective preventive measures for dengue transmission are reducing the population density of the vector *Ae. aegypti*. This control is achieved by sustained prevention of expansion of both artificial and natural breeding sites of this species. This method has been used widely in many dengue programmes in the past. McCall and Kittayapong (2007) reported that clean-up campaigns were widely conducted and promoted until recently and that many programmes were successful in many situations, despite limits on sustainability in affected or at-risk communities. The chance of success can be improved if control efforts are primarily focused on habitats that are most productive (containers which produce large numbers of adult mosquitoes rather than all types of containers), which is epidemiologically more important for the transmission of dengue viruses. Furthermore, if the elimination of larval habitats and other methods is not feasible or practical, control measures against this species can also be performed by the application of safe and effective larvicides using chemical or biological agents at breeding sites (WHO, 2009a).

1.4.2.1 Insecticides for larval control

Chemicals are extensively used in the control of immature stages of *Ae. aegypti.* The application of insecticides to larval habitats should be considered as complementary to environmental management except during an emergency situation, where it is mainly applied to the containers that cannot be eliminated or managed. Water storage containers, plant vases and flush tanks are some of the microhabitats found indoors for larval *Ae. aegypti* (WHO, 2009a; Dieng *et al.,* 2012). The difficulty of accessing indoor larval habitats in urban areas is one of the factors that limit the value of the application of insecticides onto breeding sites (WHO, 2009a).

Several insecticides have been listed and confirmed by the International Programme on Chemical Safety (IPCS) and WHO for use as larvicides (Table 1.1), the most common of which is the organophosphate Temephos (Abate). These insecticides need to demonstrate a low degree of acute and chronic toxicity before they can be considered safe for use in drinking water (Gratz & Halstead, 2008), but, although approved to be safe for use in drinking water, larviciding is often viewed with suspicion and less accepted by the communities than other methods (WHO, 2009a). Although many previous studies have demonstrated the effectiveness of this method (Ritchie *et al.,* 2001; Pinheiro & Tadei, 2002; Thavara *et al.,* 2004; Mulla *et al.,* 2004; Tawatsin *et al.,* 2007; Azirun, 2009) and it has been proven as a useful method in the control of dengue and dengue haemorrhagic fever vectors (WHO, 2009a), resistance to the insecticides used is widespread (Hemingway & Ranson, 2000).

Productive larval habitats can also be treated with chemicals by ‘perifocal treatment’. This method consists of hand-held or power-operated spraying of emulsifiable-concentrate formulations of insecticides on and around larval habitats and water surfaces. Generally, liquid insecticides can be applied with hand-operated compression sprayers whereas wettable powder can be applied with backpack sprayers. For indoor breeding sites, a syringe or pipette can be used to handle liquid insecticides whereas granules and other solid formulation can be

sprinkled using a spoon or protected hand. This type of treatment can be delivered into the non-potable containers that contain water or that are empty during the application time. This method must be treated according to the instruction given on the insecticide packaging and usually sufficient insecticide should be added for the volume of the container (e.g. 1 g of 1% temephos granules for 10 litres of container volume). The existing and subsequent larval infestation or adults found around the treatment area will be eliminated by using this method. This method also demonstrates both larviciding and residual adulticiding effects on mosquito populations. For the treatment cycle, several factors have to be considered in order to obtain the greatest impact on mosquito populations. These factors include mosquito species, seasonality of transmission, patterns of rainfall, duration of larvicide efficacy and types of larval habitat (WHO, 2009b; WHO, 2011).

Table 1.1 List of insecticides that may be used in mosquito control, WHO (2010)

Active ingredient	**Larvicides/ Adulticides**	**Class of insecticides**	**Toxicity class**
Allethrin	L/A	PY	III
Alpha-cypermethrin	A	PY	II
Bendiocarb	A	C	II
Binfenthrin	A	PY	II
Bioresmethrin	A	PY	III
Chlorpyrifos	L/A	OP	II
Chlorpyrifos-methyl	L	OP	II
Cypermethrin	A	PY	III U
Cyfluthrin	A	PY	II
DDT	A	OC	II
Deltamethrin	L/A	PY	II
Diazinon	L/A	OP	II
Dichlorvos	L/A[a]	OP	I B
Diflubenzuron	L	IGR	III U

Etofenprox	A	PY	III U
Fenitrothion	L/A	OP	II
Fenthion	L/A	OP	I B
Jodfenphos	L/A	OP	III U
Lambda-cyhalothrin	A	PY	II
Malathion	L/A	OP	III
Methoprene	L	IGR	III U
Methoxychlor	A	OC	III U
Naled	L/A	OP	II
Permethrin[b]	L/A	PY	II
Pirimiphos-methyl	L/A	OP	III
Propoxur	A	C	II
Pyrethrins	A	n.o.	II
Pyriproxyfen	L	IGR	III
Resmethrin	A	PY	III
Surface film	L	SF	-
Temephos	L	OP	III U

[a] Fumigant may be dangerous in oil solution
[b] May be dangerous in oil solution
Larvicides/Adulticides: *L* larvicide; *A* adulticide;
Class of Insecticides: *IGR* Insect growth regulator; *OC* Chlorinated hydrocarbons; *OP* Organophosphates; *C* carbamates; *PY* pyrethroids;
SF Surface film; *n.o*. natural organic
Toxicity Class: *IA* Extremely hazardous; *IB* Highly hazardous; *II* Moderately hazardous; *III* Slightly hazardous; *IIIU* a.i unlikely to present acute hazard in normal use

1.4.2.2 Bacterial endotoxins (Bti) and (Bs) for larval control

Bacillus thuringiensis israelensis (*Bti*) and *Bacillus sphaericus (Bs)* are two species of bacteria which produce an endotoxin that has high larvicidal activity in mosquitoes that is used as a control agent (Rodcharoen & Mulla, 1996; Glare & O'Callaghan, 1998). Both bacteria produce crystalline protein toxins (inactive protoxin) during sporulation, which, after ingestion by mosquito larvae, are solubilised and converted into biologically active toxins in the alkaline midgut, ultimately destroying the midgut cells (Zahiri & Mulla, 2006; Lacey, 2007;

Becker *et al.,* 2010). They are non-toxic to non-target organisms, have an extremely low mammalian toxicity, and have been approved for the control of mosquitoes in drinking water and water containers for household use (Rozendaal, 1997).

They are suitable for mass production, easy to handle, environmentally safe, stable under a range of storage conditions, can be applied using conventional equipment and are suitable for integrated control programmes (Becker *et al*., 2010, Becker & Margalit, 1993; Lacey, 2007). Factors that influence the effectiveness of *Bti* and *B. sphaericus* include mosquito species and their feeding behaviour, rate of ingestion, age and density of larvae, habitat conditions, formulation contents, storage conditions and duration of storage, medium for bacteria production, methods of application and frequency of treatment (Lacey, 2007). Various formulations have been commercialised for conventional use such as wettable powders, granules, briquettes, flowable concentrates and slow-release tablets (Lacey, 2007). For container breeders, such as *Ae. aegypti* and *Ae. albopictus*, slow-release or high-potency formulations are used to permit longer-term control and reduce the effect on taste and appearance of water (Mulla *et al.,* 2004; Vilarinhos & Monerat, 2004).

Previous studies have indicated that *Bti* is widely used as an insecticide and has shown its effectiveness against *Anopheles stephensi* and *Ae. aegypti* while *B. sphaericus* is most effective against the polluted-water breeder, *Cx. quinquefasciatus* (Fillinger *et al*., 2003, Monnerat *et al.,* 2004; Lee & Zairi, 2006; Zahiri & Mulla, 2006; WHO, 2011). A study in Malaysia showed that *Bti* rapidly killed the larvae but had short residual activity. This study also demonstrated that *Bti* performed better when used with insect growth regulators such as pyriproxyfen, which extended residual activity (Lee *et al.,* 2005). In Eastern Thailand, it has been demonstrated that the integration of copepods and *Bti* was successful in the control of dengue transmission and has a high potential to be used in other extended areas (Kittayapong *et al.,* 2006). Various degrees of larvicidal activity of *B. sphaericus* have been documented against *Aedes*

mosquitoes. Some of the species showed susceptibility to this bacterial agent whereas others, particularly *Ae. aegypti*, were largely unaffected by *B. sphaericus* (Monnerat *et al.,* 2004). Lacey (2007) recommended that bacterial control agents are combined with other types of biological control agents which could facilitate continuous and prolong suppression of immature mosquitoes.

1.4.2.3 Insect growth regulators (IGRs)

Insect growth regulators (IGRs) are effective tools for the control of a variety of disease vectors including mosquitoes. In general, IGRs have low mammalian toxicity and show extremely high levels of activity against mosquitoes (Mulla, 1991). IGRs that have been used for mosquito control include juvenile hormone mimics (e.g. pyriproxyfen, methoprene) and chitin synthesis inhibitors (e.g. diflubenzuron, triflumuron, novaluron) (Mulla *et al.,* 2003; Paul *et al.,* 2006). IGRs interrupt the development of mosquito immature stages by interfering with chitin synthesis during the moulting process in larvae or by disrupting the pupal and adult transformation processes (WHO, 2011). Pyriproxyfen is an insect-juvenile hormone analogue which is effective against mosquitoes, extremely effective at low concentration (≤ 1 ppb), and without any inhibition of oviposition at high concentration (Sihuincha *et al.,* 2005). At ppb quantities, pyriproxyfen sub-lethally decreases the fecundity and fertility of adult mosquitoes whereas contaminated adult females can transfer small quantities of pyriproxyfen into other breeding sites that are subsequently visited by the female (Dell Chism *et al.,* 2003). It has been documented that new pyriproxyfen formulations remain effective for 6 months (Seng *et al.,* 2008a). WHOPES recently recommended the use of pyriproxyfen for the control of some mosquito species (WHO, 2001). Also recently, WHO and FAO indicated that pyriproxyfen has low toxicity; therefore it is suggested that pyriproxyfen can be safely added to potable water at a rate of 0.01 mg AI/litre for mosquito control (FAO, 2001). Pyriproxyfen showed excellent activity as an IGR against *Ae. aegypti* (Nayar *et al.,* 2002; Paul *et al.,* 2006) whereas triflumuron, methoprene, diflubenzuron and novaluron showed considerable potential for the control of *Ae. aegypti* (Mulla *et al.,* 2003; Martins *et al.,* 2008; da Silva *et al.,* 2009) in a variety of laboratory and field studies.

1.4.2.4 Predatory copepods

A number of studies recently reported on the potential of various predatory *Mesocyclops spp.* to control mosquito larvae. Most of these species are omnivorous and offer the potential for control by consuming immature stages of mosquito populations (Marten & Reid, 2007). Several species have been reported as potential biological control agents of *Ae. aegypti* including *Mesocyclops aspercornis, Mesocyclops thermocyclopoides, Mesocyclops guangxiensis* and *Mesocyclops longisetus* (Rawlins *et al.,* 1997; Manrique-Saide *et al,* 1998; Schaper, 1999; Kay *et al.,* 2002). In Vietnam, dengue prevention includes the use of local predacious copepods, *Mesocyclops sp.*, together with community participation (Nam *et al.,* 2000). Many studies have documented that *M. thermocyclopoides* and *M. aspericornis* are two species that are exceptionally effective against dengue vectors (Mittal *et al.,* 1997; Schaper, 1999; Kay *et al.,* 2002; Nam *et al.,* 2005; Marten & Reid, 2007). Copepods can survive up to 6 months, and are most effective for use in large containers such as wells, concrete tanks and piles of tyres (Lardeux, 1992) which do not need to be cleaned regularly. Frequent cleaning of these containers may reduce the number of copepods and re-introduction of copepods will then be necessary for sustainable control (Chansang *et al.,* 2004). *Mesocyclops* is unlikely to be effective in household waste, discards and bases under potted plants but most effective in specified environments such as large-volume habitats (Kay & Nam, 2005). Field studies in Queensland, Australia and Thailand showed mixed results on the efficacy of *Mesocyclops* as a mosquito control agent. However, in Vietnam, the results were more robust, contributing to the successful eradication of *Ae. aegypti* in some provinces (Kay *et al.,* 2002). Furthermore, the efficacy of this method has been proven to be more successful with the integration of other bio-control tools such as predatory fish, *Bti*, and IGRs together with environmental management (Wang *et al.,* 2000; Russell *et al.,* 2001; Seleena *et al.,* 2001; Micieli *et al.,* 2001; Kay *et al.,* 2002).

1.4.2.5 Larvivorous fish

As early as the 1900s, macroorganisms such as larvivorous fish were used as a biological control agent to control mosquito populations (Bellini *et al.,* 1994; Legner, 1995). Several species of fish have been tested for their potential use against dengue vectors. However, only certain species of larvivorous fish have been proven as potential biological control agents for *Aedes* mosquitoes (Martinez-Ibarra *et al.,* 2002; Valero *et al.,* 2006). Larvivorous fish can only be used in specific aquatic habitats and ecological conditions. Furthermore, the target populations can only be effectively reduced if this control agent is well adapted to the target habitats (Becker *et al.,* 2010). Two species of larvivorous fish were successfully introduced in many countries to control mosquito larvae, namely *Gambusia affinis* and *Gambusia holbrooki* (Walton, 2007; Chandra *et al.,* 2008). Another larvivorous fish, *Poecilia reticulate*, is also extensively used for the control of *Ae. aegypti* in many countries in South East Asia (Seng *et al.,* 2008b). Walton (2007) suggested that sensible use of larvivorous fish is required to prevent detrimental effects on indigenous fish species. Moreover, under favourable conditions, larvivorous fish can be an important mosquito control, successful in eliminating immature mosquitoes, preventing environmental contamination and reducing the development of resistance in target populations.

1.4.2.6 Densonucleosis viruses (DNVs)

Densoviruses or densonucleosis viruses belong to the genus *Brevidensovirus* of the subfamily *Densovirinae* in the family *Parvoviridae* (Tijssen & Bergoin, 1995). The viruses that infect mosquitoes could be used as biological control agents to reduce mosquito populations or could be used to deliver and express genes to reduce the vectorial capacity of mosquitoes (Carlson *et al.,* 1995; Carlson *et al.,* 1996; Corsini *et al.,* 1996). Infected mosquito larvae experience only a slight response to stimulation, lose mobility, rise to the surface of water, the body becomes shortened and deformed and some have a semi-transparent body or whitish colouration, whereas infected pupae also reduce in mobility and remain at the bottom of the water (Buchatsky, 1989). *Aedes densonucleosis* virus (AeDNV) is one of the well-documented species that was originally isolated from *Ae.*

aegypti (Lebedeva *et al.,* 1972). This species was also shown to infect other *Aedes, Culex* and *Culiseta* mosquitoes (Buchatsky, 1989). A previous study examined the efficiency of three mosquito densoviruses including AeDNV, *Hemagogus equines* densovirus (HeDNV) and *Aedes Peruvian* densovirus (APeDNV) in *Ae. aegypti* mosquitoes. However, the results show that only larvae infected with AeDNV display a delayed development whereas less effect is exhibited by larvae infected with HeDNV and APeDNV (Ledermann *et al.,* 2004). Studies by Suchman *et al.* (2006) demonstrated that AeDNV potentially reduces the adult lifespan, daily survival and female fecundity. Recently, a study documented that the oviposition behaviour of female *Ae. aegypti* leads to successful distribution of densovirus from treated to untreated oviposition containers in large cages. However, the concentration of AeDNV was not maintained to reduce egg densities (Valdez *et al.,* 2010). Although mosquito densoviruses have a great potential as biological control agents for mosquitoes, further work needs to be carried out and various factors have to be considered in order to determine the efficiency of this control in the future.

1.4.3 Control of adult Ae. aegypti mosquitoes

Since a dengue vaccine is not expected to appear in the near future, dengue prevention and control strategies are primarily dependent on vector control, by decreasing mosquito abundance, reducing adult mosquito lifespan and preventing vector-human contact. Many control efforts have been implemented to reduce or eliminate dengue vector populations with various degrees of success in the past (Eisen *et al.,* 2009). The use of chemical control is the most practical method when the source reduction routines for a mosquito control programme have not been successfully achieved and the risks of dengue transmissions are currently high (Reiter & Nathan, 2001). Other methods such as mosquito lethal trap and the application of household insecticides also greatly contribute to the control of adult *Ae. aegypti* and provide personal protection against this species (WHO, 1997).

1.4.3.1 Insecticide space-spraying

Many control efforts directed at adult dengue vectors have been carried out using space-spraying. Space-spraying refers to the dispersion of small droplets of insecticides released into the air in the form of a vapour with the intention of killing adult mosquitoes on contact (WHO, 1999). For over 25 years, space-spraying has been used as the principal method for dengue control in many countries, particularly in the South East Asia region. In general, there are two forms of space-spraying used, namely thermal fogs and cold fogs. Both of these methods can be operated by vehicle-mounted or hand-held machines (WHO, 2011).

Thermal fogs involve the application of insecticide that normally condenses after being vaporized at a high temperature. Two forms of insecticides that are commonly used for thermal fogs are oil-based and water-based formulations. Oil-based formulations produce thick white smoke whereas water-based formulations produce a colourless fine mist (WHO, 2009a; WHO, 2011). Thermal fogs have been widely used for the control of *Ae. aegypti* both indoors and outdoors (Chung *et al.,* 2001; Perich *et al.,* 2001; Seleena *et al.,* 2001; Yap *et al.,* 2001; Perich *et al.,* 2003; Mani *et al.,* 2005).

The application of cold fogs (aerosol), which includes ultra-low volume (ULV) or mist, uses smaller quantities of insecticides. This method can be applied by vehicle-mounted, backpack ULV mist blower or hand-carried ULV aerosol generators. In general, portable sprays can be used to treat congested low-cost housing areas, multi-storey buildings, warehouses, covered drains, sewage tanks and residential or commercial premises. On the other hand, vehicle-mounted sprays can be used in the urban and suburban areas where a proper road system is available. This method can be used to cover up to 1,500 - 2,000 houses a day. In addition, aerial cold fogs are also used if the targeted areas exceed 1,000 ha or if there is no access to targeted areas, for example, due to density of vegetation. Aerial fogs delivered from aircraft are suited for rapid treatment but the accurate placement of insecticide application using this method is more difficult than with

ground application (WHO, 2009a; Becker *et al.,* 2010; WHO, 2011). Many studies have been conducted in the past to evaluate the efficacy of ULV for the control of *Ae. aegypti*. Although most of these studies investigate outdoor applications, indoor ULV is also conducted to control *Ae. aegypti* population inside the house (Perich *et al.,* 2000; Perich *et al.,* 2001; Perich *et al.,* 2003; Sulaiman *et al.,* 2002). Furthermore, Sulaiman *et al.* (2002) reported no significant difference in adult mortalities by both applications, inside and outside the house.

Insecticides used for space-spraying include organophosphates such as malathion, fenitrothion and pirimiphos-methyl, and alspyrethroids such as cyfluthrin, deltamethrin, lambda-cyhalothrin and permethrin. Various formulations of carbamates also can be used in such a method and, according to WHO (2006b), 15 active ingredients from the group of synthetic pyrethroids, organophosphates and carbamates are available for both thermal and cold fogs application (Table 1.5). Several factors have to be considered for the efficacy of these methods for controlling *Ae. aegypti*. These factors include targeted species, insecticides susceptibility, indoor penetration of the insecticides, and frequency and timing of applications (WHO, 2009a; WHO, 2011). Furthermore, the application of space-spraying also should be related to the behaviour of the targeted species (Bonds, 2012). Since *Ae. aegypti* and *Ae. albopictus* are active during the day, the control of these species is best conducted during their peak activities, which are early in the morning and late afternoon. Factors such as temperature, humidity and wind velocity also become a critical role in the efficacy of this method (WHO, 2009b; WHO, 2011).

Table 1.2 Insecticides suitable for cold aerosol sprays and thermal fogs in mosquito control (WHO, 2006b).

Insecticide	Chemical	Dosage [a] (grams of active ingredient per ha)	
		Cold	Thermal
Chlorpyrifos	OP	10 - 40	150 - 200
Cyfluthrin	PY	1 - 2	-
Cypermethrin	PY	1 - 3	-
Cyphenothrin	PY	2 - 5	-
Deltamethrin	PY	0.5 - 1.0	-
D-phenothrin	PY	5 - 10	-
Etofenprox	PY	10 - 20	10 - 20
Fenitrothion	OP	250 - 300	270 - 300
Fenthion	OP	150	-
Malathion	OP	112 - 693	500 - 600
Naled	OP	56 - 280	-
Permethrin[b]	PY	5 - 10	-
Primiphos-methyl	OP	230 - 330	180 - 200
Propoxur	C	100	-
Zeta-cypermethrin	PY	1 - 3	-

Class of Insecticides: *PY* Synthetic pyrethroid; *OP* organophosphorus; *C* Carbamate
a Because of their low dermal toxicity and on the basis of experience with their use, these products have been classified in the WHO Hazard Classification in Class III, Table 5 (WHO/PCS/94.2)
b Also used in mixtures with knock-down agents or synergists

Many studies have reported that space-spraying can rapidly reduce adult mosquito populations. However, the insecticides must be applied repeatedly to maintain the effectiveness of this method (Reiter & Gubler, 1997; WHO, 1997). The effectiveness of space-spraying could be reduced if the householders refuse to comply with the procedures required during insecticide spraying, such as requests to open the doors and windows, and not to cover any water sources inside the house except drinking water during the treatment (Renganathan *et al*., 2003; Mani *et al*., 2005). This is because the insecticides may not reach the resting or breeding sites of *Ae. aegypti* indoors (Reiter & Gubler, 1997; Perich *et al*., 2000).

The differences in insecticide dosage, type of equipment, resistance level of the vectors, structure of the houses and the direction of the spraying that is used during the ground-based vehicle treatment may contribute to the variable results of this method (Nelson, 1991; Mount, 1998). Furthermore, the seasonal factor, the frequency of the spraying time, spatial variability that exists among the buildings of the sprayed area, droplet size and type of insecticide used are also reported to give variation impacts on space-spraying (Perich *et al*., 2001; Mani *et al*., 2005; Koenraadt *et al*., 2007; Chadee, 2009).

It has been suggested that one of the main reasons for reduction in the effectiveness of space-spraying is the behaviour of *Ae. aegypti* (Perich *et al*., 1990). *Ae. aegypti* populations may be found resting on wardrobes, under beds, behind furniture and in closed rooms where it is difficult for aerosol droplets to reach (Perich *et al*., 2000). In Thailand, Pant and Yasuno (1970) demonstrated that 95% of the mosquitoes rest indoors, and over 90% were found to be resting on surfaces that could not be sprayed with insecticides, such as clothing, pictures, decorative objects, beddings and mosquito nets. Furthermore, it is believed that mosquitoes rest indoors most frequently in bedrooms, bathrooms and kitchens, where they prefer surfaces such as walls, ceilings, under furniture and hanging materials such as clothes, towels and curtains (Nelson, 1986). Moreover, Focks *et al*. (1987) hypothesised that gravid females remain sequestered during treatment period in places that are well protected from aerosols.

It has been documented that the effectiveness of space-spraying is still limited (Perich, 2000). Previous studies have shown that there was a relatively rapid recovery in mosquito populations after space-spraying (Koenraadt *et al*., 2007). The mosquito population recovered quickly, in some cases in more numbers than before spraying treatment in many studies (Esu *et al*., 2010). Koenraadt *et al*. (2007) also demonstrated that recovery of adult *Ae. aegypti* populations after insecticide spraying was rapid, consistent with other ULV application studies in Thailand. Within one week, the number of mosquitoes returned to approximately one-half of the numbers before spraying. Moreover, effectiveness of insecticides

could also be underestimated because part of the recovery is due to immigrating mosquitoes.

Esu *et al.* (2010) concluded that the effectiveness of space-spraying in reducing dengue transmission remains unclear. Due to the variety of entomological surveillance and sampling methodology used, it was difficult to directly compare the effectiveness of this method between the studies (Pilger *et al.,* 2010). Although the impact of space-spraying is only transient and unable to provide a long-term control, it is often used during emergency situations where massive and rapid destruction of adult vector populations can be attained. Esu *et al.* (2010) suggested that more research is needed so that a practical public health campaign can be drawn up either by recommending or rejecting the use of space-spraying for dengue vector control and to provide clear guidelines for appropriate implementation and monitoring of effects.

1.4.3.2 Indoor Residual Spraying

Indoor residual spraying is described as the application of insecticides on the surface of walls and roofs inside houses or domestic animal shelters. IRS operates by killing adult vector mosquitoes that land and rest on these surfaces before or after taking a blood meal (WHO, 2006a). Furthermore, IRS may also prevent the mosquitoes from entering the houses (Pluess *et al.,* 2010). However, IRS is not recommended for dengue vector control as it is believed that adult *Ae. aegypti* often rest on non-sprayable surfaces (Reiter & Gubler, 1997), although the application of IRS could reduce the lifespan of adult vector mosquitoes, prevent the transmission of pathogens and reduce the density of vector mosquitoes as well as decrease human-vector contact (WHO, 2006a). The application of IRS is often conducted on a large scale where the mass effect of insecticide could be maximised; therefore it increases the mortality of adult mosquitoes (Pluess *et al.,* 2010). In general, IRS is carried out between one and three times a year depending on the insecticides and season of transmission. A variety of insecticide products can be used for IRS, including wettable powder (WP), water dispersible granule (WG), emulsifiable concentrate (EC), suspension concentrate (SC) and

capsule suspension (CS) (WHOPES, 2007). Recently, 12 different insecticides within four different classes of insecticides have been recommended by WHO (2009) for IRS against malaria vectors (Table 1.3).

Table 1.3 WHO-recommended insecticides for indoor residual spraying against malaria vectors (WHO, 2009).

Insecticide compounds	**Insecticide formulations**	**Class group**	**Dosage (g A.I./m^2)**	**Mode of action**	**Duration of effective action (months)**
DDT	WP	OC	1 - 2	Contact	> 6
Malathion	WP	OP	2	Contact	2 - 3
Fenitrothion	WP	OP	2	Contact & airborne	3 - 6
Pirimiphos-methyl	WP & EC	OP	1 - 2	Contact & airborne	2 - 3
Bendiocarb	WP	C	0.1 - 0.4	Contact & airborne	2 - 6
Propoxur	WP	C	1 - 2	Contact & airborne	3 - 6
Alpha-cypermethrin	WP & SC	PY	0.02 - 0.03	Contact	4 - 6
Bifenthrin	WP	PY	0.025 - 0.05	Contact	3 - 6
Cyfluthrin	WP	PY	0.02 - 0.05	Contact	3 - 6
Deltamethrin	WP, WG	PY	0.02 - 0.025	Contact	3 - 6
Etofenprox	WP	PY	0.1 - 0.3	Contact	3 - 6
Lambda-cyhalothrin	WP, CS	PY	0.02 - 0.03	Contact	3 - 6

Insecticide formulations, *CS*: capsule suspension; *EC*: emulsifiable concentrate; *SC*: suspension concentrate; *WG*: water dispersible granule; *WP*: wettable powder.
Class group, *OC*: Organochlorines; *OP*: Organophosphates; *C*: Carbamates; *PY*: Pyrethroids.

The efficacy of indoor residual spraying is well documented for malaria control interventions and it has been widely used in many parts of the world especially in Asia, Latin America and Southern Africa since the 1950s (Lengeler, 2003; WHO, 2008). There is evidence that, during malaria control intervention, *Ae. aegypti* populations are dramatically reduced in the areas where IRS is implemented in some countries in the Americas (PAHO, 1994). In addition, the advantage of IRS is not only to reduce the densities and lifespan of dengue vectors but also to have an impact on other insect vectors such as *An. gambiae*, *An. funestus*, *Cx. quinquefasciatus*, sand flies and triatomine bugs. Moreover, pest insects such as bedbugs, cockroaches and houseflies inside the house may also be affected by this control strategy.

In more recent years, Chadee (2013) revealed that the behaviour and physiological features of *Ae. aegypti* may influence the resting behaviour of this species inside the house. This study was conducted to determine the primary resting places of *Ae. aegypti* in houses and the duration for female *Ae. aegypti* to have a blood meal after laying eggs. The results revealed that females rest for between 36 - 50 hours after laying eggs. Therefore, the re-introduction of IRS may provide an alternative control measure based on the evidence that the mosquitoes come into contact with the wall surfaces while undergoing physiological process such as diuresis (removing excess liquid from the blood meal) and vitellogenesis (the process of egg formation with nutrients being deposited in the oocyte) (Chadee, 2013).

1.4.3.3 Insecticide-Treated Materials

Insecticide-treated materials (ITMs), mainly bed nets, were originally effective in reducing diseases transmitted by nocturnally active vectors. The efficacy of ITMs on diurnally active vectors such as *Ae. aegypti* has been evaluated in recent years (Kroeger *et al.,* 2006). Many studies have demonstrated that dengue vector infestation could be reduced or controlled at household level by the implementation of ITMs (Kroeger *et al*., 2006; Lenhart *et al*., 2008; Seng *et al*., 2008c). It is hypothesised that adult vector mosquitoes come into contact with the ITMs during host-seeking. This could reduce the life expectancy of adult *Ae.*

aegypti and consequently reduce the transmission of dengue (Kroeger *et al*., 2006; Lenhart *et al*., 2008). Since ITMs are produced from long-lasting insecticide-treated materials, this method could maintain efficacy for a number of years (Vanlerberghe *et al.,* 2010). ITMs were also shown to impact on vector populations and have high acceptance levels by the householders (Kroeger *et al*., 2006; Seng *et al*., 2008c). Recent studies in Latin America and the Caribbean have demonstrated that insecticide-treated materials such as window curtains, container and jar covers and bed nets can reduce dengue vector densities to low levels and potentially have an impact on dengue transmission (Kroeger *et al*., 2006; Lenhart *et al*., 2008; Venlerberghe *et al.,* 2009). Such interventions essentially deliver a residual insecticide targeting adult mosquitoes inside the house and it is suggested that ITMs possibly become an effective alternative to IRS in targeting adult dengue vectors (Esu *et al.,* 2010). Recently, several studies have demonstrated that ITMs have potentially become an effective novel tool for controlling *Ae. aegypti*, with efficacy likely to be optimised when implemented in combination with other vector control tools, particularly when their use is integrated in a strategy that also involves the communities in given areas (Seng *et al.,* 2008c; Vanlerberghe *et al.,* 2011; Rizzo *et al.,* 2012; Lenhart *et al.,* 2013).

1.4.3.4 Lethal ovitraps

A lethal ovitrap is essentially a black bucket containing water with an attractant infusion (alfalfa pellet), a strip of cloth treated with a residual insecticide, and a plastic mesh cover (Williams *et al*., 2007). In Brazil, lethal ovitraps with deltamethrin-treated ovistrips killed 89% of *Ae. aegypti* adults and produced more than 99% larval mortality during first month field trials (Perich *et al*., 2003). The advantages of lethal ovitraps for controlling *Aedes* vectors include their simplicity, specificity and effectiveness against container breeders like *Ae. aegypti*. Ritchie *et al.* (2009) demonstrated that lethal ovitraps were well accepted by the communities in an efficacy trial in Australia. According to Morrison *et al*. (2008), the application of ovitraps for *Aedes* control is faster, less expensive, uses less pesticide and is less likely to affect non-target species than interior residual spraying.

1.4.3.5 Household insecticides

In many parts of the world, household insecticide products are used for personal protection against household insect nuisance pests and insect vectors including mosquitoes. Mosquito coils, fumigation mats, liquid vaporizers, aerosol and sticky baits are extensively used to combat mosquitoes. These products are also important against other household insects such as cockroaches, ants, fleas, flies, wasps, house flies, sand flies and bedbugs. Furthermore, in several parts of Latin America, these products are also widely used for triatomine bugs. Commonly, the active ingredients contained in household insecticide products are low in mammalian toxicity.

Mosquito coils are one of the most common products used in Asia, Africa and the Western Pacific. The active ingredients for these products are pyrethroids. A study on the bio-efficacy of mosquito coils found that the *d, d*-T-prallethrin and K-3050 coils with the synergist are highly effective against *Ae. aegypti* mosquitoes (Katsuda *et al.,* 2008). Although mosquito coils are inexpensive, they generate unpleasant smoke and pollutant from the burning particles (Liu *et al.,* 2003). Since the early 1980s, electric vaporizers and liquid vaporizers have been used and mostly marketed in urban areas (WHO, 2011). Adanan *et al.* (2005) demonstrated that the efficacy of vaporising mats depends on active ingredients and targeted species. In recent years, the development of non-heated formulations has increased the safety and easy use of insecticides. Most of these products are to prevent mosquitoes from entering the houses; they provide rapid exposure which subsequently results in death.

1.4.3.6 Spatial and topical body repellents

Repellents are mostly used for personal protection against mosquito vectors. Body repellents are available in a variety of formulations including liquid, lotion, waxes, creams, foams and soaps (Becker *et al.,* 2010). DEET formulations (*N*, *N*-diethylmethyl-3-methylbenzamide) have been widely used in repellents (WHO, 2011). For general protection against mosquitoes, body repellent should be applied to the exposed parts of the body. Impregnated clothes provide extra

protection with a longer-lasting effect whereas spray-on application only provides temporary protection. The current active ingredients available include DEET, botanicals, citronella and picaridin (Katz *et al.,* 2008). DEET has become the most efficacious and broadly used insect repellent with strong safety records and excellent protection against ticks, mosquitoes and other arthropods. Natural botanical products such as citronella and oil of lemon eucalyptus as well as newer agents such as picaridin are also widely used in insect repellent. These products are accepted by the public because of their low toxicity and comparable efficacy (Becker *et al.,* 2010).

1.4.3.7 Genetic manipulation

Genetic manipulation potentially provides new, species-specific and environmentally friendly novel traits for mosquito control strategies (Alphey, 2014). The potential of the symbiotic intracellular bacterium, *Wolbachia pipientis*, to reduce the lifespan of mosquito vectors, invade mosquito populations through the induction of cytoplasmic incompatibility and interfere with the replication of a variety of pathogens has placed this bacterium at the frontline of new approaches targeting mosquito-borne diseases in recent years (Iturbe-Ormaetxe *et al.,* 2011). *Wolbachia* is particularly a great agent for mosquito control because it is maternally transmitted and rapidly spread throughout the population (Hoffmann *et al.,* 2011). Cytoplasmic incompatibility causes unviable embryos when *Wolbachia*-infected males mate with uninfected females. On the other hand, when *Wolbachia*-infected females mate with uninfected males, the progeny will be produced but their offspring will be infected with *Wolbachia* (Hoffmann *et al.,* 2011; O'Connor *et al.,* 2012). The *w*Mel *Wolbachia* infection from Drosophila melanogaster was successfully introduced to two natural *Ae. aegypti* populations in Australia. A field trial was conducted in Queensland, Australia and *Wolbachia*-infected *Ae. aegypti* mosquitoes were released in the dengue fever outbreak area. Preliminary data showed promising results where 20% of the *Ae. aegypti* population was already infected with *Wolbachia* after three months of the trial (Iturbe-Ormaetxe *et al.,* 2011). This *Wolbachia*-based biocontrol showed no evidence for the transfer of *Wolbachia* to humans by mosquito bites (Popovici *et*

al., 2010) and the transmission of *Wolbachia* to non-target species is also unlikely to occur as *Wolbachia* are maternally inherited (Huigens *et al.,* 2004).

Another genetic control strategy is through the Sterile Insect Technique (SIT). The SIT involves rearing, sterilising and releasing large numbers of disabled insects (Dyck *et al.,* 2005). Mating of sterile insects with wild insects in the target population leads to a decrease of reproductive potential of the target, and, if sufficient numbers of insects can be released using SIT, control or elimination of the targeted population can be achieved (de Valdez *et al.,* 2011). Large-scale SIT programmes have successful suppressed or eliminated a number of agricultural pests (Dyck *et al.,* 2005) and one of the highly successful trials using SIT was for controlling screwworm fly *Cochliomyia hominivorax* Coquerel in the United States (Klassen & Curtis, 2005). For mosquito control, there are some factors that need to be considered for SIT programmes. These factors include the damaging effect of irradiated males (Helinski *et al.,* 2009; Alphey *et al.,* 2010); the need to release only male mosquitoes, as they do not take blood meals (Benedict & Robinson 2003); and the need to reduce larval mortality due to early-acting lethality (Phuc *et al.,* 2007).

An approach based on mosquitoes carrying a dominant lethal gene is an advancement to the SIT programmes. Release of insects carrying a dominant lethal gene RIDL has been developed to control the transmission of dengue viruses using a transgenic strain designated by a biotechnology company, Oxitec (de Valdez *et al.,* 2011). A sterile male *Ae. Aegypti*, known as OX513A, has been engineered to mate with females in a wild population in the Cayman Islands (Harris *et al.,* 2011). Other open field releases of RIDL mosquitoes were conducted in Malaysia (Lacroix *et al.,* 2012) and Brazil (Reis-Castro & Hendrickx, 2013) using the same transgenic strain. After a few years of the development of this transgenic strain, OX3604C was designed to have genetic features that produce a highly penetrant, dominant and late-acting, female-specific flightless phenotype (Fu *et al.,* 2010; de Valdez *et al.,* 2011). This phenotype is effectively lethal because flightless females are not able to mate, blood-feed or

avoid predators and, most importantly, they are unable to serve as vectors for dengue viruses. This study was conducted in laboratory-based, large-cage trials and showed success in competing against the wild-type mosquitoes, which supports further testing of this strain in confined field trials to evaluate its mating competitiveness in the future (de Valdez *et al.,* 2011).

1.5 Introduction to insecticides and insecticide resistance

During the late 1930s, the new synthetic insecticides were discovered and they were widely used in the control of insect pests and vectors. DDT was first introduced for mosquito control in 1949. Insecticides have become important tools in the control of major insect vectors such as mosquitoes, triatomine bugs, sand flies, house flies, lice and others (Hemingway & Ranson, 2000). Resistance to DDT was first recorded in *Aedes tritaeniorhynchus* and *Aedes solicitans* in the year after its introduction (Brown, 1986). Since then, the rapid development of insecticide resistance has been recorded in many mosquito species (Hemingway & Ranson, 2000).

1.5.1 Insecticides

Arsenic is one of the first generation of insecticides. The second generation insecticides are the one that used in mosquito controls. These insecticides belong to four major chemical groups, namely chlorinated hydrocarbon, organophosphates, carbamates and pyrethroids. The third generation of insecticides are the juvenile hormone analogues whereas the fourth generation of insecticides are used for insect-specific control agents which derived from the entomopathogenic bacteria (Becker *et al.,* 2010).

The chemical (second generation) insecticides are widely used for aerial spraying, ground fogging and indoor residual spraying, and for impregnated materials including bed nets, blankets and curtains to target mosquitoes. A study on global insecticide use in vector borne-disease control (Zaim & Jambulingam, 2007) demonstrated the use of four classes of insecticides according to the type of application in 2003 - 2005. More than 50% of the insecticides reported used

during the study were for IRS. Another 30.7% were used for space treatment whereas 7.6% were used for larviciding and 1.5% for insecticide-treated mosquito nets. In endemic areas of developing countries, the use of organochlorines and carbamates is limited for IRS treatment while pyrethroids are used for IRS, space-spraying and bed nets. OPs are used in all types of treatments except bed nets.

1.5.2 Insecticide resistance

Insecticide resistance occurs when a population of insects is exposed to insecticide for a period of time and at a frequent rate (Lee *et al.,* 2003b). The level or degree of insecticide resistance depends on the volume and frequency of insecticide application and other factors such as frequency of resistance gene(s) (genes with any mutation that modifies the behaviour or the physiology of the insect vectors in a way that impairs the function of molecular targets) in the population and type of gene(s) responsible for resistance (Nazni *et al.,* 1998; Pasteur & Raymond, 1996). Mosquitoes exhibit rapid insecticide resistance development because of their short life cycles and abundant number of progeny (Hemingway & Ranson, 2000). The first report of resistance to DDT occurred in 1947 (Brown, 1986). Since then more than 100 mosquito species have been reported to be resistant to one or more insecticides worldwide (WHO, 1992).

DDT resistance has been reported (Thanispong *et al.,* 2008; Fonseca-Gonzalez *et al.,* 2011) and pyrethroid resistance is widespread in *Ae. aegypti* (Bang *et al.,* 1969; Prasittisuk & Busvine, 1977; Chadwick *et al.,* 1977; Hemingway *et al.,* 1989) while the development of resistance to organophosphates and carbamates has also been recorded in this species (Canyon & Hii, 1999; Rodriguez *et al.,* 2001; Tikar *et al.,* 2009; Bisset *et al.,* 2011). Organophosphate resistance has also been shown in *Ae. albopictus* (Khong *et al.,* 1988). Permethrin resistance has been noted in both *Ae. aegypti* and *Ae. albopictus* (Ponlawat *et al.,* 2005; Chuaycharoensuk *et al.,* 2011) and resistance to pyrethroid also been reported in both species (Somboon *et al.,* 2003; Khan *et al.,* 2011).

1.5.3 Mechanisms of insecticide resistance

Insecticide-resistance mechanisms are based on alterations of target sites or detoxification mechanisms, and include either change in insecticide target sensitivity in the central nervous system such as sodium channels, GABA receptors and acetylcholinesterase or an increased rate of insecticide detoxification (Hemingway & Ranson, 2000). Metabolic resistance involves enhanced enzyme activity of non-specific esterases (α- and β-), Glutathion-S-Transferases (GSTs) and P450-mediated monooxygenases or Mixed Function Oxidases (MFO) (Hemingway & Ranson, 2000). GSTs have been associated with resistance to organophosphates, carbamates, pyrethroids and chlorinated hydrocarbons (Hemingway *et al.,* 2004) whereas non-specific esterases are mostly involved in resistance to organophosphates, carbamates and pyrethroids (Hemingway *et al.,* 2004). Target-site resistance such as knockdown resistance (*kdr*) is associated with pyrethroid and DDT cross-resistance while alterations in acetylcholinesterases (AChE) are accountable for organophosphate and carbamate resistance (Hemingway & Ranson, 2000; Soderlund & Knipple, 2003). Numerous mutations in the sodium channel gene are responsible for reducing channel sensitivity to target pyrethroids and DDT (Soderlund & Knipple, 2003) whilst a mutation in AChE results in a decreased sensitivity to inhibition by target insecticides (Weill *et al.,* 2003).

1.5.4 Dengue vector control programmes in Malaysia

The history of vector-borne disease control in Malaysia began in 1967 (Malaysia Ministry of Health, 2008). The first major outbreak of dengue haemorrhagic fever in West Malaysia occurred in 1973 (Wallace *et al.,* 1980). Temephos or Abate® was recommended by WHO (1985) in 1973 and has been widely used in the past 30 years for controlling *Ae. aegypti* and *Ae. albopictus* in Malaysia. After the introduction of temephos by WHO, malaria eradication and control programmes in Peninsular Malaysia, Sabah and Sarawak were conducted until 1980. The Vector Borne Disease Control Programme (VBDCP) established in 1983 is responsible for the control of seven vector-borne diseases in Malaysia: malaria, dengue, filariasis, Japanese encephalistis, plague, scrub typhus and yellow fever

(Malaysia Ministry of Health, 2008). However, there is no specific control programme for adult filariasis vectors in Malaysia but the vector is controlled through mass control programmes for dengue and agricultural pest control.

In Malaysia, dengue control mainly focuses on source reduction and adulticiding by fogging with chemical insecticides (Malaysia Ministry of Health, 2008). Normally, the operations against adult mosquitoes involve cold spraying or thermal fogging treatment with organophosphate and pyrethroid. The treatment is applied within 1 km of the suspected dengue case area with a repeat treatment 7 to 10 days after the first treatment (Tham, 1997). The used of malathion was stopped in 1996 based on observation and feedback by fogging teams which indicated lower acceptance rates by householders during the treatment. It has been replaced with water-based pyrethroid fogging formulations such as Resigen and Aqua Resigen. In addition, Abate® was also recommended for householders to use as a larvicide in potential larval habitats around the houses (Teng & Singh, 2001). Moreover, cold spray of *B. thuringiensis israelensis* combined with chemical insecticides has also been applied in the Vector Control Programme in Malaysia. The bacterial and chemical insecticides were proven to be compatible with each other and gave the maximum larval and adult mortalities in the field (Seleena & Lee, 1998; Seleena *et al.,* 1999).

For malaria, fogging activities are conducted periodically and householders provided with insecticide-treated bed nets (ITNs) (Ho & Zairi, 2013). Household insecticide products such as mosquito coils, liquid vaporizer and aerosol are also encouraged for personal protection. At present, residual sprays on wall surfaces is one of the strategies used on a larger scale for vector control of *Anopheles* mosquitoes in Malaysia (Lee & Yap, 2003). Indoor residual spraying (IRS) commonly uses deltamethrin, whereas temephos and permethrin are used as larvicide and for bed nets respectively. These methods are frequently used to control *Anopheles* mosquitoes such as *Anopheles maculatus*, *Anopheles donaldi* and *Anopheles balabaciensis* in Malaysia. Deltamethrin wettable powder (WP) has replaced DDT as the main insecticide used since 1988 in malaria-endemic

areas (Vector Borne Disease Control Programme, 1988). Recently, several control trials have been conducted using deltamethrin water-dispersible granule formulation which has a broad spectrum, and is fast acting and water dilutable for surface spraying (Rohani *et al.,* 2006).

1.5.5 Insecticide resistance status of dengue vectors in Malaysia

Permethrin has been widely used in the dengue control programme in Malaysia. Permethrin resistance had already developed in *Ae. albopictus* and *Ae. aegypti* in Kuala Lumpur (Rohani *et al.,* 2001; Wan-Norafikah *et al.,* 2008; Wan-Norafikah *et al.,* 2010). Furthermore, temephos resistance has been detected in *Ae. aegypti* from dengue-endemic areas in Selangor (Chen *et al.,* 2008). Selvi *et al.* (2010) reported that *Ae. albopictus* exhibited tolerance to malathion as observed from larval and adult bioassays. The development of resistance to temephos in dengue vectors has also been reported in Malaysia (Lee and Lime, 1989) and it has been suggested that resistance could possibly be attributed to continual selection pressure resulting from control programme by space-spraying (Lee *et al.,* 1996). The frequent use of temephos as a larvicide has also resulted in resistance in dengue vectors (Lee *et al.,* 1998). A recent study by Chen *et al.* (2005b) indicated that field strains of *Ae. aegypti* and *Ae. albopictus* in Kuala Lumpur city centre and Selangor state have developed some degree of resistance to temephos. Recently, a study conducted by Chan and Zairi (2013) demonstrated that a colony of permethrin-resistant *Ae. albopictus* originating from Penang Island was highly resistant to permethrin and cross-resistant to deltamethrin.

Studies on the susceptibility status of *Cx. quinquefasciatus* in Kuala Lumpur also demonstrated that this species is highly resistant to malathion and DDT. This species is believed to be under selection pressures of organophosphate groups from outdoor house spraying in dengue-endemic areas (Nazni *et al.,* 2005). Fenitrothion resistance has also been recorded in *Cx. quinquefasciatus* and it is suggested that this species is widely exposed to the fenitrothion used by the agricultural sector since this chemical is not being used in the Malaysia Vector Control Programme (Nazni *et al.,* 2005).

It is clear that insecticide resistance in mosquito vectors is currently emerging in Malaysia. The extensive use of chemical insecticides for vector control and also in agricultural pest control seems to have indirectly contributed to the resistance in dengue and other mosquito vectors. To date, rotation of the insecticides used in vector control activities has been suggested in Malaysia to delay and minimise the occurrence of insecticide resistance (Wan-Norafikah *et al.,* 2010). Since the mosquitoes may quickly develop a high level of resistance, it is essential to use advanced detection and monitoring tools so that a better management of insecticide resistance could be performed in the future.

CHAPTER 2

INTRODUCTION TO THE STUDY

2.1 Introduction

Today, dengue is the most important mosquito-borne viral disease in the world and improvements in vector control are urgently required. Some of the most successful control intervention methods have exploited vector behaviour (Manda *et al.,* 2011), and this study aimed to investigate a number of key aspects of *Ae. aegypti* behaviour in human habitation, where the female mosquito spends the majority of its life, and where it rests and blood feeds (Scott *et al.,* 2000).

A recent study by Lynd and McCall (2013) used a sticky net to trap *An. gambiae* mosquitoes as they approached a human host to feed, providing new insight into the movement of this important vector towards the host. This approach could also be utilised to explore orientation of *Ae. aegypti* to a seated or standing human bait, with a view to discovering how they move through three-dimensional space and where they arrive to feed on the human host.

Improved knowledge of the resting preferences of endophilic mosquitoes is important in order to target or deliver insecticidal residues more efficiently. *Ae. aegypti* has specific resting preferences based on trap or target visual cues, configurations and textures (Manda *et al.,* 2011). Early exploitation of *Ae. aegypti* preferences led to the development of oviposition, host-seeking and other adult traps such as the Fay Prince trap (Fay & Prince, 1970), counterflow geometry trap (Kline, 1999) and the BG Sentinel^{TM} trap (Geier *et al.,* 2006). Resting boxes for indoor sampling also exploit *Ae. aegypti* resting behaviour (Edman *et al.,* 1997; Kittayapong *et al.,* 1997). Some recent studies have been conducted to collect *Anopheles* mosquitoes using resting boxes (Kweka *et al.,* 2009; Sikulu *et al.,* 2009; Pombi *et al.,* 2014). However, there have been relatively few studies performed to explore these behavioural patterns further in *Aedes* mosquitoes (Edman *et al.,* 1997; Kittayapong *et al.,* 1997), particularly in recent years.

Although neither used nor recommended for control of *Ae. aegypti* today, indoor residual spraying (IRS) has the potential to target this vector effectively. However, in order to do so, all potential indoor resting surfaces should be sprayed – an extremely laborious, time-consuming and expensive exercise, particularly in high-density urban areas where dengue is common. In an earlier study of malaria vectors in Mexico (Arredondo-Jiménez *et al.,* 1995), selectively spraying of key wall surfaces proved to be as effective as spraying the entire house, at half the cost and taking half the time to complete. Combined with better knowledge of dengue vector resting behaviour (as described in the previous paragraph) this approach could make IRS feasible for dengue control.

In Malaysia today, insecticidal space-spraying such as fogging is extensively used for routine disease control (Yap *et al.,* 2000). Information is only available on vector susceptibility or resistance pattern to insecticides and resistance mechanisms for some areas of Malaysia (Nazni *et al.,* 2005; Wan-Norafikah *et al.,* 2008; Wan-Norafikah *et al.,* 2010; Rong *et al.,* 2012; Low *et al.,* 2013). Regular updates on insecticide susceptibility/resistance status are an essential component of any vector control programme, particularly when novel approaches, such as those described above, are being considered as future vector control tools.

Against this background, the objectives of the study were defined.

2.2 Objectives

The objectives of the study were:

1. To explore arrival patterns of host-seeking *Ae. aegypti* at a seated human bait using a sticky net barrier.

2. To investigate resting preferences of *Ae. aegypti* on simple two-dimensional panel targets and in resting boxes.

3. To compare the impact of two insecticides on peridomestic mosquitoes delivered either by standard (entire surface sprayed) or selective IRS (limited areas sprayed) method (ceiling and top 1 m of walls) in a field trial in Malaysia.

4. To characterise the insecticide susceptibility status of *Ae. aegypti, Ae. albopictus* and *Cx. quinquefasciatus* to lambda-cyhalothrin (pyrethroid) and pirimiphos-methyl (organophosphate) in Penang, Malaysia.

CHAPTER 3

INVESTIGATION OF ARRIVAL LOCATIONS OF HOST-SEEKING *Aedes aegypti* AT A HUMAN HOST

3.1 Introduction

Investigating the process and behaviour of how mosquitoes seek their host for feeding must face challenges presented by the space and distance mosquitoes travel during host-seeking, coupled with the fact that they are almost invisible to the naked eye, making it very difficult to accurately observe and monitor them, especially under natural conditions. Nonetheless, such problems can be resolved through controlled indoor and outdoor studies, e.g. observing how they locate their host by studying their responses to host cues within laboratory and field settings alike (Cardé & Gibson, 2010). We can attempt to overcome these issues but any experiment alters reality and the extent of which is remain unknown.

The mechanisms of host-finding can be characterised through observations of the responses of mosquitoes in the laboratory or field to host cues during the process of host location (Cardé & Gibson, 2010). These mechanisms are generally described as activation, orientation to wind direction and odour plume as well as settlement near the host (Cardé & Gibson, 2010). Once the airborne odour cues have been detected, a mosquito relies primarily on the olfactory and visual cues to move towards the odour source by optomotor-guided anemotaxis. A mosquito consequently comes into contact with the host odour plume and continues its orientation, until it finally arrives near the odour source when it responds to stimuli such as heat, moisture and visual features of the host (Cardé & Gibson, 2010). Evidence to date indicates that olfactory cues are the most important distance signals used, while visual and physical cues (e.g. heat and moisture) are used at close range, during the final stages of landing (Takken & Knols, 1999). The mosquito's physiological age (*e.g.* maturation of the host odour-sensitive neurons in a newly-emerged adult), stage in the gonotrophic cycle (*e.g.* abdominal distension following a blood meal, inhibition of host-finding following a blood

meal) also influence the host-finding behavioural response (Davis, 1995). In addition, the sensory and other physiological factors that modulate the host-finding process vary enormously between mosquito species (e.g. in the range of potential hosts, odour cues, structure of habitat and its influence on plume dispersion as well as environmental conditions such as wind speed, wind turbulence and light levels in nature) (Gibson & Torr, 1999). Therefore, studies on mosquito orientation to host odours and other cues are developed mainly to improve the effectiveness of odour-baited mosquito traps, and/or to identify the chemicals facilitating the mosquito's orientation towards the host (Cardé & Gibson, 2010).

Many methods have been used to evaluate mosquito host responses in the laboratory, which include direct observation by human landing catches (de Jong & Knols, 1995; Dekker *et al.,* 1998), video-recording behaviour (Dekker *et al.,* 2005; Cooperband & Carde, 2006; Beeuwkes *et al.,* 2008) and wind tunnel studies (Takken *et al.,* 1997; Costantini *et al.,* 2001; Dekker *et al.,* 2005). Mosquito responses to human hosts have been studied under laboratory conditions in large indoor cages such as bed nets or closed rooms. Experiments conducted using similar tools have also been used for studies on the selection of biting sites on human hosts (de Jong & Knols, 1995; Dekker *et al.,* 1998). The selection of biting sites by mosquitoes on the human body region appears to differ between mosquito species (de Jong & Knols, 1996). The head region of a sitting human was preferred by *Anopheles atroparvus* and *Anopheles albimanus* whereas the lower part of the human body, feet and ankles were more preferred by *Anopheles gambiae sensu stricto* (Knols *et al.,* 1994; de Jong & Knols, 1995). *Anopheles arabiensis* and *Culex quinquefasciatus* showed no pattern, with biting occurring over all parts of the body (de Jong & Knols, 1996). *Ae. aegypti* preferentially bit the head and upper part of the human chest (de Jong & Knols, 1996). This may be related to the odour cues emanating from specific regions of the host's body, or the interaction with the carbon dioxide plume and other compounds in the host's breath (de Jong & Knols, 1996). In other studies, mosquito orientation has been monitored by electrical nets and odour-baited entry trap (Torr, 1994; Knols *et al.,*

1998; Torr *et al.,* 2008). This approach identified responses to actual hosts, odour sources such as carbon dioxide or to synthetic chemical attractants.

In a later study, Lynd and McCall (2013) investigated host orientation by examining arrival locations of *An. gambiae* mosquitoes on a human-occupied bed net surface, using adhesive-coated bed nets. This method allowed investigation of mosquito behaviour without the need for a second person to observe and record data, potentially confounding the results. Evaluation of the method showed that the adhesive-coated net treatment was effective in capturing flying mosquitoes and there was no significant repellency effect of the adhesive. Hence, the method was used in the present study to investigate the preferred arrival location of host-seeking *Ae. aegypti* at the human host.

3.2 Methods

3.2.1 Mosquitoes used in the experiments

An *Ae. Aegypti* colony from a dengue susceptible strain originating from New Orleans, USA, was used in this study. The strain was provided by the Centers for Disease Control and Prevention (CDC), USA, and has been maintained in a continuous laboratory culture at Liverpool School of Tropical Medicine, UK, for many years. Mosquitoes were reared in a mean temperature-controlled insectary at 28^{o}C and 85% humidity with a photoperiod of light and dark (12:12). *Ae. aegypti* egg paper was placed into 2,000 ml of tap water in 30 x 20 cm trays. Five hundred ml of hay infusion were added to the tray to stimulate the eggs to hatch at the same time. Finely ground fish flakes and brewer's yeast (1:1) were given daily during the larval stages until pupation. Pupae in plastic cups were then transferred into 30 x 30 x 30 cm Bug Dorms cages to emerge. Adult mosquitoes were provided with constant access to 5% glucose solution and mates in the cages. Female mosquitoes were blood-fed through a Hemotek membrane feeding system twice per week and gravid females were provided with a wet filter paper to oviposit on. Mosquitoes aged between 3 and 12 days post-eclosion were used in all experiments.

3.2.2 Experimental room

In this experiment, mosquitoes orienting to the volunteer were trapped at an adhesive-coated net barrier placed around the volunteer, at the location of arrival at the host. All of the experiments were conducted in a room with dimensions of 510 cm length x 359 cm width x 222 cm heights, specifically modified for this study (Figure 3.1), and maintained at 24 to 28^{o}C and 60 to 80% relative humidity.

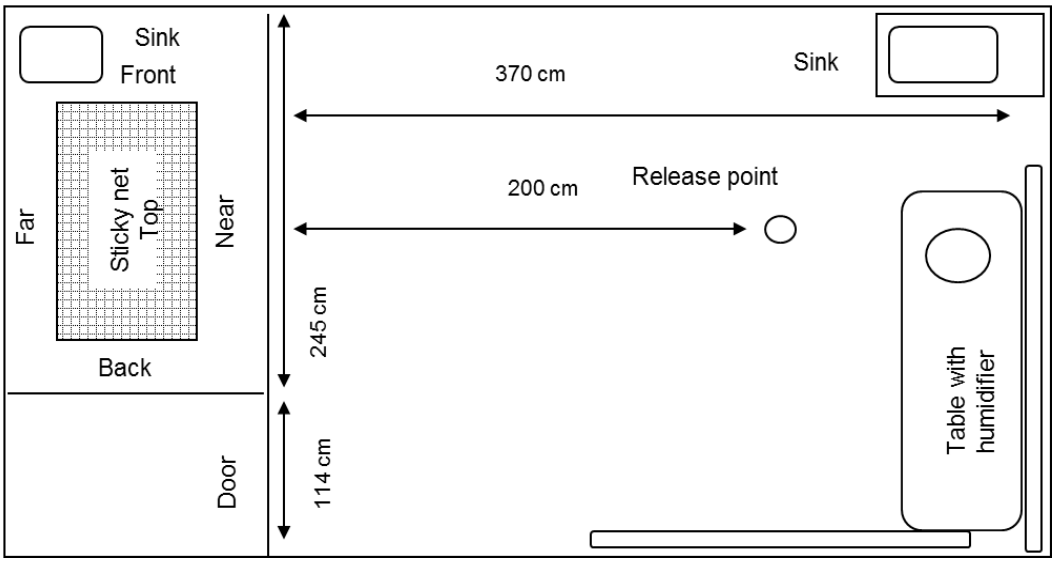


Figure 3.1 Diagram of the experimental room setup showing the contents of the room and the position of the human-baited sticky net and mosquito release point

3.2.3 Experimental setup and procedure

A metal frame measuring approximately 123.5 cm length x 62.5 cm width x 154 cm heights was covered with standard mosquito netting, fitted tightly. The netting was marked out into a 10 cm^2 grid with a permanent marker (Figure 3.2). The dimensions of each surface area are shown in Table 3.1. Each grid refers to 10 cm x 10 cm with a number of exceptions: column 1 was 5 cm x 5 cm and row 15 was 10 cm x 5 cm (Figure 3.2b). The head of the seated volunteer was always near the upper part of the cage whereas the volunteer's feet were always at the lower part of the cage. For each surface except for the top, the first row was at the upper part of the net whereas the final row is at the lower part of the net. The first column is read starting from the left to the right of the cage for each surface.

The net surfaces are designated based on the orientation of the volunteer within the sticky net, described as ‘front’ and ‘back’ (Figure 3.2 c & d). On the other hand, two net surfaces ‘near’ and ‘far’ are described based on the release point for the mosquitoes in flight (Figure 3.2 c & d).

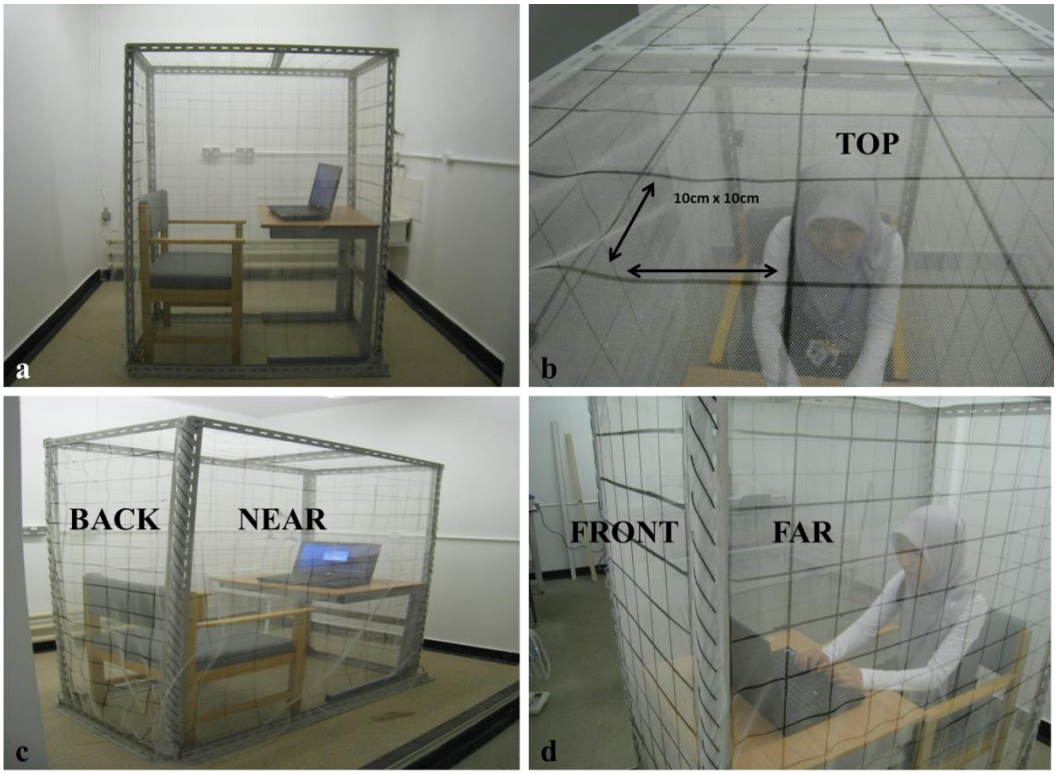


Figure 3.2 Experimental setup of the human-baited adhesive coated net a) Table and chair were set up to imitate a natural situation b) Top view illustrating 10 cm^2 sections c) ‘Near’ and ‘back’ view d) ‘Far’ and ‘front’ view

Table 3.1 Pattern of grid markings on each surface of the sticky net

Net surface	Dimensions
Near See Figure 3.2 (c)	**All 10 cm x 10 cm except;** **Column 1, Row 1 to 14 = 5 cm x 10 cm** **Column 2 to 12, Row 15 = 5 cm x 10 cm** **Column 1, Row 15 = 5 cm x 5 cm**
Far See Figure 3.2 (d)	**All 10 cm x 10 cm except;** **Column 1, Row 1 to 14 = 5 cm x 10 cm** **Column 2 to 12, Row 15 = 5 cm x 10 cm** **Column 1, Row 15 = 5 cm x 5 cm**
Front See Figure 3.2 (d)	**All 10 cm x 10 cm except;** **Column 1, Row 1 to 14 = 5 cm x 10 cm** **Column 2 to 6, Row 15 = 5 cm x 10 cm** **Column 1, Row 15 = 5 cm x 5 cm**
Back See Figure 3.2 (c)	**All 10 cm x 10 cm except;** **Column 1, Row 1 to 14 = 5 cm x 10cm** **Column 2 to 6, Row 15 = 5 cm x 10cm** **Column 1, Row 15 = 5 cm x 5 cm**
Top See Figure 3.2 (b)	**All 10 cm x 10 cm except;** **Column 1, Row 1 to 5 = 5 cm x 10 cm** **Column 2 to 12, Row 6 = 5 cm x 10 cm** **Column 1, Row 6 = 5 cm x 5 cm**

A water-based liquid formulation of Tangle-Trap glue (The Tanglefoot Company, MI, USA) was used as a non-setting adhesive. This glue has no human toxicity, is colourless and virtually odourless, and remains sticky for a period of time, and had been used previously for similar experiments with *An. gambiae* (Lynd & McCall, 2013). Three coats of glue were applied to the net using a paintbrush and it was allowed to set for one week before use.

In tests, a single volunteer sat at a small table and chair inside the sticky net. The volunteer was permitted to read books or magazines, and use any devices such as laptop and headphones during the experiments, which resembled a real daily routine indoors. Fifty unfed female mosquitoes were transferred to a paper bucket (10 cm diameter x 10 cm height) and placed on the floor at a distance of 2 metre from the host, for 24 hours to allow acclimatisation prior to release. These fifty mosquitoes were selected from those in the rearing cage on the basis that they were the most active in responding to the presence of the researcher and therefore most likely to respond in the test. After each experiment, all mosquitoes were discarded. All the trials were conducted in the morning and afternoon between 10:30 and 12:30 and 14:30 and 16:30 hours (colony mosquitoes were maintained under a 12:12 light: dark cycle with light period beginning at 07:00).

The 13 volunteers used in this experiment were aged between 23 and 50 years, both male and female (6:7) and from a range of ethnicities (Malaysian, Latin American, European and African). Control experiments (no human bait present) were also conducted in this study. Each experiment ran for one hour. The position of the table and chair inside the sticky net and release point remained unchanged in all experiments. At the end of the experiment, the number and position of each mosquito caught on the net was immediately recorded. All mosquitoes caught on the net were removed using forceps, and free-flying mosquitoes were collected and destroyed. The humidifier was turned off to ensure that any remaining mosquitoes died before the next test. The net was reused a maximum of 10 times or replaced if it became dirty, damaged or less sticky. A total of 30 replicates were carried out during the experiments with approximately two or three replicates for each volunteer. Following early observations that the hijab worn by some female volunteers might have influenced behaviour, two experimental groups were compared. In 15 tests, volunteers covered their heads by wearing a hat or hijab; 15 repeats were also done with volunteers who did not cover their heads. The data gathered on the recording grids were analysed in Microsoft Excel. To determine the differences mean numbers of mosquitoes caught on the net surfaces and to compare the mean numbers of mosquitoes caught on the different grid sections

and between both groups (covered head and uncovered head), the tests used were analysed using the Negative Binomial Distribution and Poisson Analysis in Stata software V.9.0. Goodness of fit tests was done with Stats Direct Software V.2.6.8.

3.3 Results

A total of 30 baited experiments involving 13 volunteers were conducted, amounting to a total of 1,500 *Ae. aegypti* released and tested in experiments with human bait. Six control experiments with 300 *Ae. aegypti* were also performed.

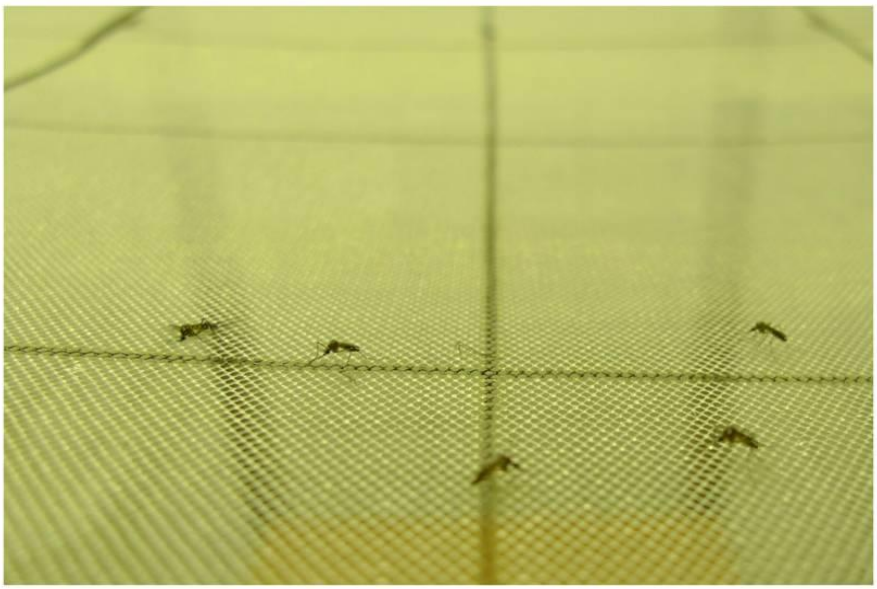


Figure 3.3 Photograph of mosquitoes caught on the top surface of the sticky net

The number and location of trapped mosquitoes are summarised in Table 3.2. The overall results show that mean caught for baited sticky nets was 53.8% of mosquitoes whereas this was only 0.9% mean caught for the control tests. Notably, while 24.6% of mean caught mosquitoes were on the top of the net in baited trials, none were caught in this position in controls. Also, the majority of the mosquitoes caught in the controls were caught on the 'near' side of the net, which was the closest location of arrival by mosquitoes. In baited trials, a mean of 11.9 ± 5.9 mosquitoes were caught on the release side (near). There were significantly higher numbers captured on the top and 'near' surfaces of baited trials compared to the control tests (Negative Binomial Distribution, $P < 0.001$).

Table 3.2 Arrival locations of *Ae. aegypti* at a human-baited sticky net and control test. The data are presented as mean ± SD caught and mean caught (%) on each of the net surfaces

	Human-baited test		Control test	
Net surface	**Mean ± SD Caught**	**Mean Caught (%)**	**Mean ± SD Caught**	**Mean Caught (%)**
Near	11.9 ± 5.9	23.8	2.0 ± 2.5	0.7
Far	0.7 ± 1.0	1.4	0.2 ± 0.4	0.07
Front	0.5 ± 0.9	1.0	0.3 ± 0.5	0.1
Back	1.5 ± 1.6	3.0	0.2 ± 0.4	0.07
Top	12.3 ± 7.7	24.6	0	0
Total	**26.9 ± 5.5**	**53.8**	**3.7 ± 0.2**	**1.23**

Results of experiments investigating the effect of head covering on location of mosquito arrival are shown in Figure 3.4. Number of mosquitoes captured on each surface was recorded as mean. The results showed that there were no significant differences in the mean of mosquitoes caught in human host with covered head and uncovered head (Poisson analysis, $P = 0.0578$).

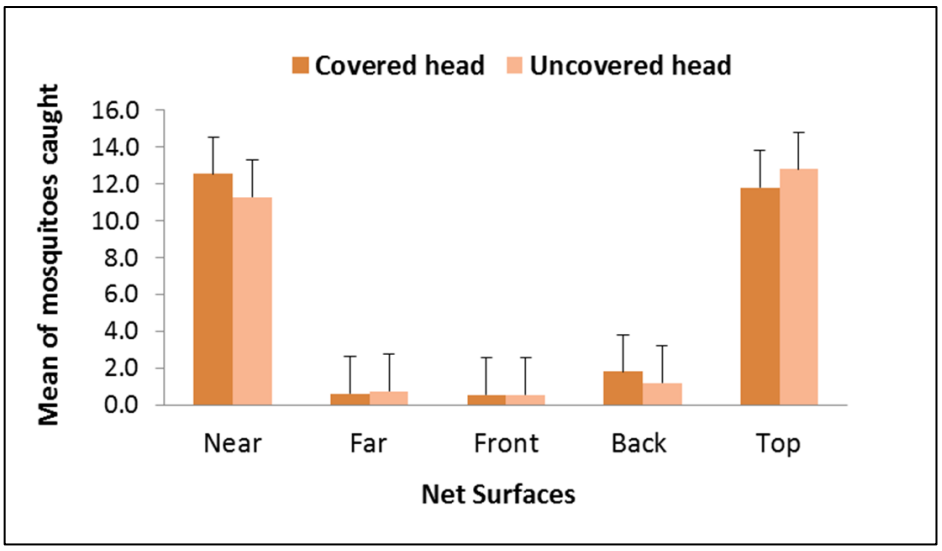


Figure 3.4 Comparison of number of mosquitoes caught on each surface of the sticky net when the volunteer's head was covered or uncovered

3.3.1 Overall distribution of mosquitoes on the sticky net

The results showed that there was no evidence of any differences between the human hosts who covered their heads and the human hosts who did not cover their heads (Negative Binomial Distribution, $P = 0.878$). Therefore, results obtained from both of the experiments were combined and the number of mosquitoes caught was presented as mean caught.

The results showed that the number of mosquitoes caught on each net surface was significantly higher on the 'near' surface compared to the 'far' surface (Poisson analysis, $P < 0.001$), 'front' surface (Poisson analysis, $P < 0.001$), and 'back' surface (Poisson analysis, $P < 0.001$). However, there was no significant difference in the mean number of mosquitoes caught between 'near' and top surface (Poisson analysis, $P > 0.05$). Furthermore, there was also significantly greater mean number of mosquitoes caught on the top surface compared to the 'far', 'front' and 'back' surface of the net (Poisson analysis, $P < 0.001$).

The results demonstrated that the mean number of mosquitoes caught on the 'near' and top surfaces was significantly greater than the 'back' surface (Poisson analysis, $P < 0.05$). However, the mean number of mosquitoes caught on the 'far' and 'front' surface was not significantly different compared to the 'back' surface (Poisson analysis, $P > 0.05$). A goodness of fit test was carried out to compare the number of mosquitoes caught on each net surface. Based on surface area, the higher number caught on top and 'near' surfaces of the net was clearly shown by the higher observed minus expected [O-E] values (Table 3.3). For control tests, all [O-E] values were negative except for the 'near' surface. The values showed that there were significantly more mosquitoes caught on the 'near' surface than on other net surfaces (Table 3.4).

Table 3.3 Comparison of arrival location in human-baited sticky net tests. The data show mean caught, the differences between the observed and expected values, and chi-squared values, $\chi^2 = 39.5$

Net surface	No. of 10 cm^2 cells	(Observed) Mean caught	Observed minus Expected	Chi-square statistic
Near	167.0	11.9	3.7	1.7
Far	167.0	0.7	-7.6	6.9
Front	74.5	0.5	-3.1	2.7
Back	74.5	1.5	-2.2	1.3
Top	63.5	12.3	9.2	26.9
χ^2 total				**39.5**

Table 3.4 Comparison of arrival location in control tests. The data show mean caught, the differences between the observed and expected values, and chi-squared values, $\chi^2 = 2.6$

Net surface	No. of 10 cm^2 cells	(Observed) Mean caught	Observed minus Expected	Chi-square statistic
Near	167.0	2.0	1.2	1.7
Far	167.0	0.2	-0.6	0.5
Front	74.5	0.3	-0.1	0.01
Back	74.5	0.2	-0.2	0.1
Top	63.5	0	-0.3	0.3
χ^2 total				**2.6**

3.3.2 Distribution of mosquitoes in 10 cm^2 sections on the top surface of the sticky net

Examination of the distribution of mosquitoes in 10 cm^2 sections on the top surface of the net revealed clustering over the centre of the net. The total number of mosquitoes landing in each 10 cm^2 section on the top surface of the net for all baited experiments was calculated and plotted by row and column (Figures 3.5 to 3.6). These data were then used to construct density plots of the number of mosquitoes caught in each section on the top surface of the net (Figure 3.7).

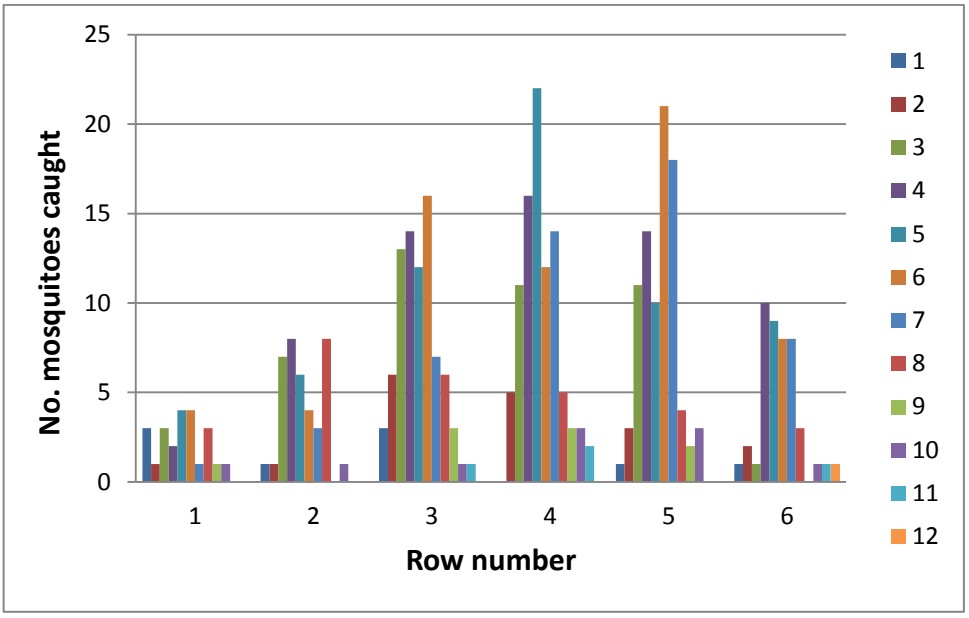


Figure 3.5 Number of mosquitoes in 10 cm^2 sections grouped by row on the top surface of the net. Colour bars on the right represent column numbers 1 to 12

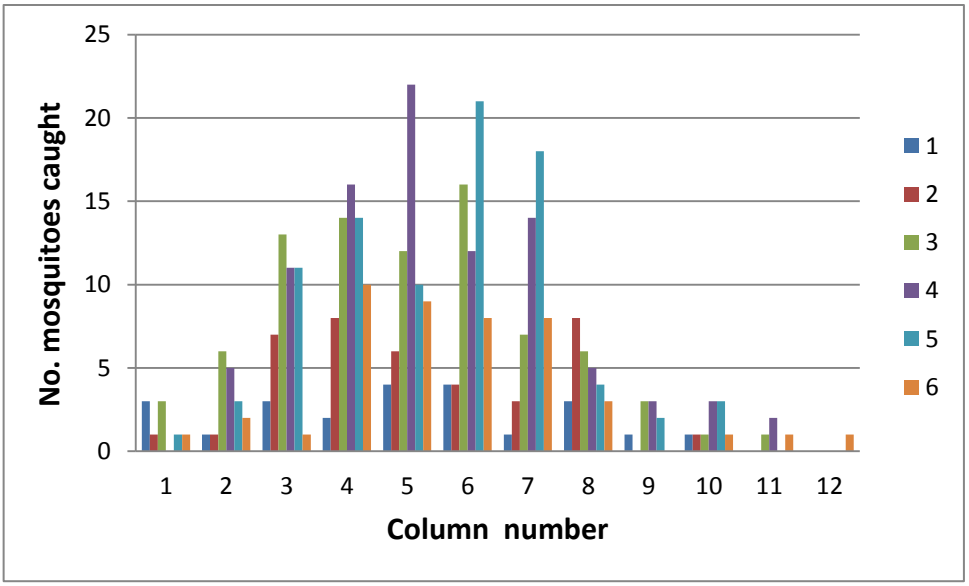


Figure 3.6 Number of mosquitoes in 10 cm^2 sections grouped by column on the top surfaces of the net. Colour bars on the right represent row numbers 1 to 6

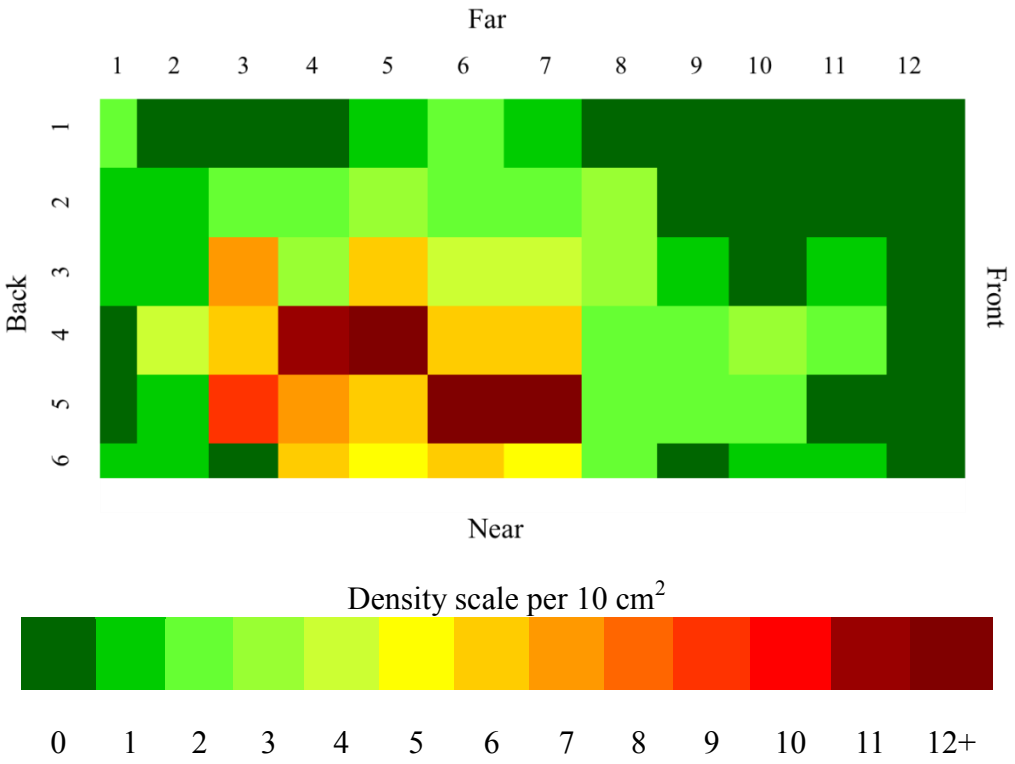


Figure 3.7 Density distribution plots of mosquitoes on the top surface of the sticky net. All sections were 10 cm^2 except those in column 1 and row 6, which were 5 cm x 5 cm and 10 cm x 5 cm

A goodness of fit analysis was also carried out to determine the differences in the number caught in each row and column of the top surface of the net. These tests could not be applied to individual experimental data since the expected values were below the recommended minimum of 5. Therefore, the total number caught in all experiments was used. Analysis of the distribution of mosquitoes on the top surface of the net clearly demonstrated clustering over the volunteer's head, rows 3 to 5 and columns 3 to 8 of the sticky net. However, there were fewer mosquitoes found on the area where the table was placed inside the sticky net. The statistical analysis was carried out on the number caught in each row and column on the top surface of the net. The total numbers caught in each row and column were significantly different (Chi-square, $P < 0.001$). In this test, it is assumed that mosquitoes had an equal chance to be caught on any parts on top of the net surface. The highest number of mosquitoes caught was on row 3 and column 4 with [O-E] values of 21.4 and 19.6 respectively (Tables 3.5 and 3.6).

Table 3.5 Distribution of mosquitoes caught within the top surface of the sticky net, distributed by row. Data presented as number caught, the differences between observed and expected values and chi-square values, $\chi^2 = 30.2$

Row number	No. of 10 cm^2 cells	(Observed) Number caught	Observed minus Expected	Chi-square statistic
1	11.5	17	-12.6	5.4
2	11.5	23	-6.6	1.5
3	11.5	51	21.4	15.5
4	11.5	39	9.4	3.0
5	11.5	30	0.4	0.0
6	11.25	17	-12.0	4.9
χ^2 total				**30.2**

Table 3.6 Distribution of mosquitoes caught within the top surface of the sticky net by column. Data presented as number caught, the differences between observed and expected values and chi-square values, $\chi^2 = 106.5$

Column number	No. of 10 cm^2 cells	(Observed) Number caught	Observed minus Expected	Chi-square statistic
1	2.75	4	-3.7	1.8
2	5.5	10	-5.4	1.9
3	5.5	22	6.6	2.8
4	5.5	35	19.6	25.0
5	5.5	30	14.6	13.9
6	5.5	30	14.6	13.9
7	5.5	20	4.6	1.4
8	5.5	17	1.6	0.2
9	5.5	4	-11.4	8.4
10	5.5	4	-11.4	8.4
11	5.5	0	-15.4	15.4
12	5.5	1	-14.4	13.5
χ^2 total				**106.5**

3.3.3 Distribution of mosquitoes within the 'near' surface of the sticky net

The total number of mosquitoes landing in each 10 cm^2 section on the 'near' surface of the net for all baited experiments was calculated and plotted by row and column (Figure 3.8 to 3.9). These data were then used to construct density plots of the number of mosquitoes caught in each section on the 'near' surface of the net (Figure 3.10).

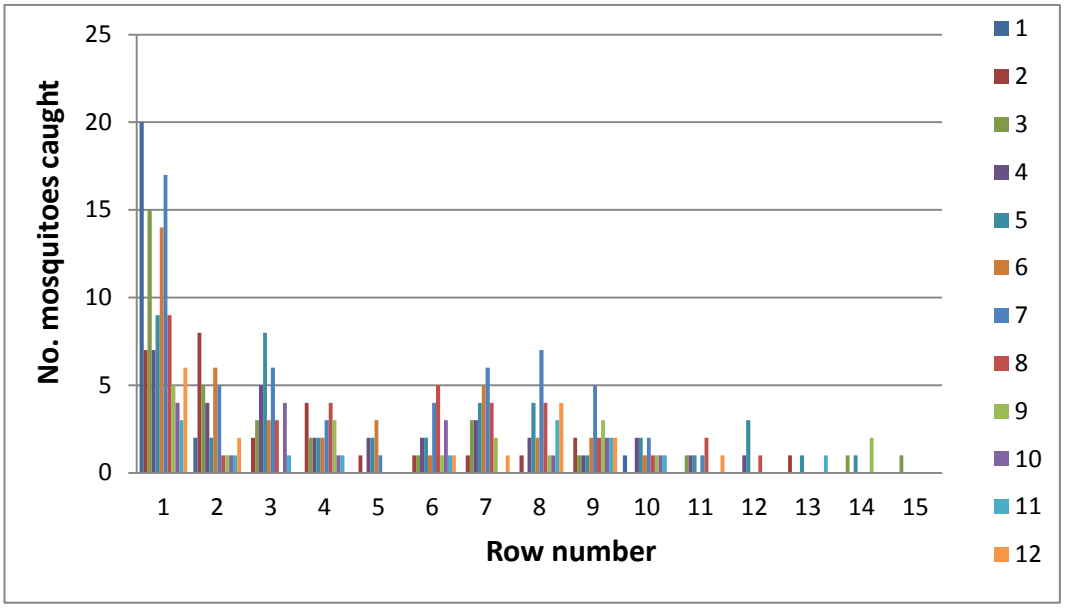


Figure 3.8 Number of mosquitoes in 10 cm^2 sections grouped by row of the 'near' surface of the net. Colour bars on the right represent column numbers 1 to 12

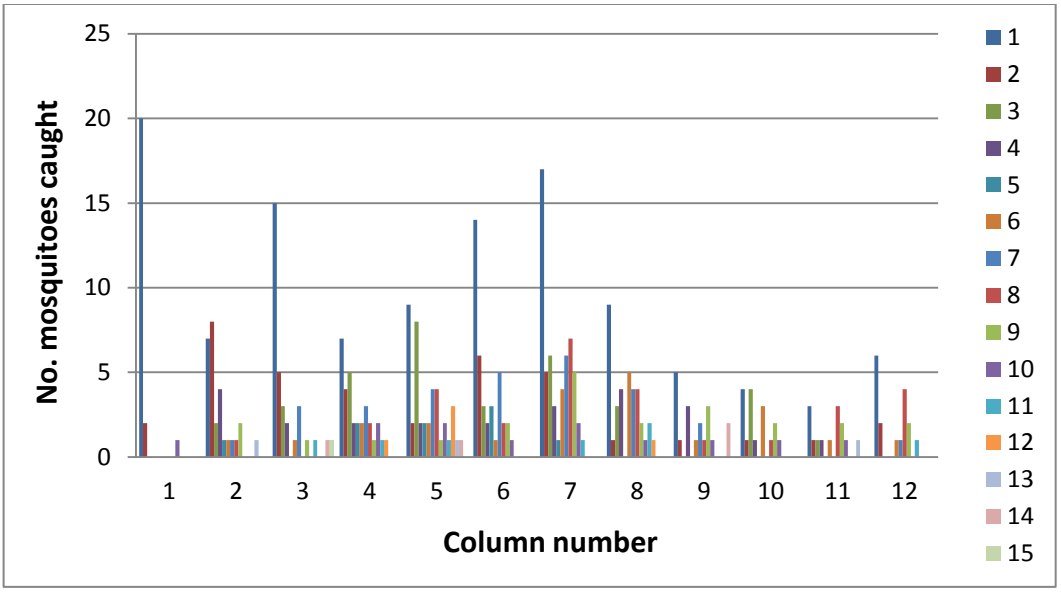


Figure 3.9 Number of mosquitoes in 10 cm^2 sections grouped by column of the 'near' surfaces of the net. Colour bars on the right represent row numbers 1 to 15

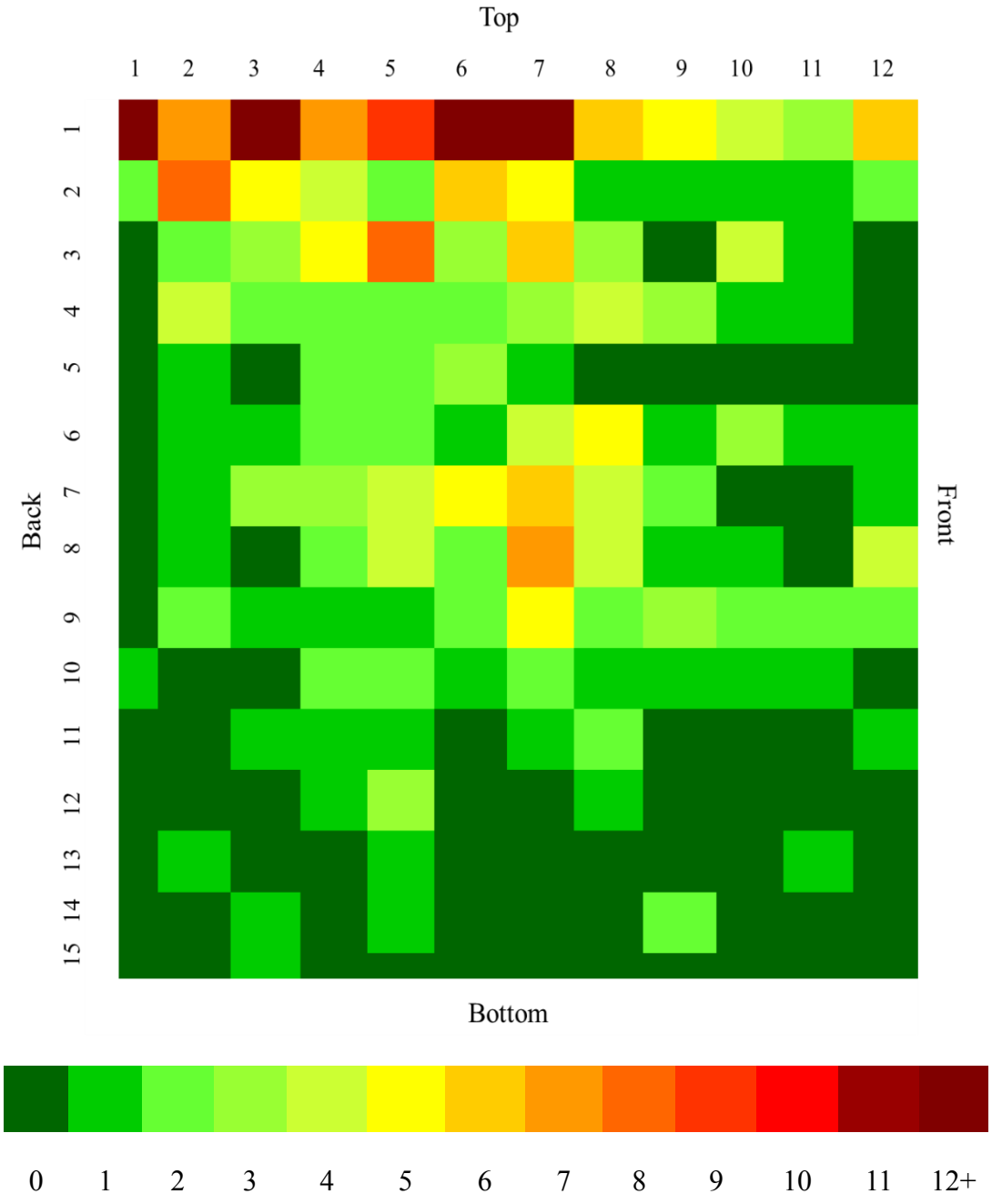


Figure 3.10 Density distribution plots of mosquitoes on the 'near' surface of the sticky net. All sections were 10 cm^2 except for those in column 1 and row 15, which were 5 cm x 5 cm and 10 cm x 5 cm

The total number of mosquitoes from all baited experiments was used to construct a colour density plot in each 10 cm^2 section on the 'near' surface of the net. The statistical analysis was also carried out on the number caught in each row and column on the 'near' surface of the net. The total numbers caught in each row and column were significantly different (Chi-square, $P < 0.001$). Examination of the distribution of the mosquitoes on the 'near' surface of the net revealed high clustering over row numbers 1 to 3 and 7 to 8. There was also clustering across column numbers 1 and 3 to 8, which were much closer to the volunteer's head (Table 3.7 and 3.8).

Table 3.7 Distribution of mosquitoes caught within the 'near' surface of the sticky net by row. Data presented as number caught, the differences between observed and expected values and chi-square values, $\chi^2 = 444.0$

Row number	**No. of 10 cm^2 cells**	**(Observed) Number caught**	**Observed minus Expected**	**Chi-square statistic**
1	11.5	116	91.4	339.2
2	11.5	38	13.4	7.3
3	11.5	35	10.4	4.4
4	11.5	24	-0.6	0.0
5	11.5	9	-15.6	9.9
6	11.5	22	-2.6	0.3
7	11.5	29	4.4	0.8
8	11.5	29	4.4	0.8
9	11.5	23	-1.6	0.1
10	11.5	12	-12.6	6.5
11	11.5	7	-17.6	12.6
12	11.5	5	-19.6	15.6
13	11.5	3	-21.6	19.0
14	11.5	4	-20.6	17.3
15	5.75	1	-11.3	10.4
χ^2 total				**444.0**

Table 3.8 Distribution of mosquitoes caught within the 'near' surface of the sticky net by column. Data presented as number caught, the differences between observed and expected values and chi-square values, $\chi^2 = 59.2$

Column number	No. of 10 cm^2 cells	(Observed) Number caught	Observed minus Expected	Chi-square statistic
1	7.25	23	7.5	3.6
2	14.5	28	-3.0	0.3
3	14.5	33	2.0	0.1
4	14.5	32	1.0	0.0
5	14.5	42	11.0	3.9
6	14.5	39	8.0	2.0
7	14.5	57	26.0	21.7
8	14.5	36	5.0	0.8
9	14.5	19	-12.0	4.7
10	14.5	17	-14.0	6.4
11	14.5	14	-17.0	9.4
12	14.5	17	-14.0	6.4
χ^2 total				**59.2**

The analysis of the distribution of mosquitoes caught on the top and 'near' surface of the net for control trials could not be carried out for each 10 cm^2 section as the expected values for number of mosquitoes caught in rows and columns were too low. The overall results from each 10 cm^2 section of the row and column data indicate clearly that distribution of mosquitoes over the top and 'near' surfaces of the net was clustered close to the volunteer's head inside the sticky net.

3.4 Discussion

This study is the first to evaluate the arrival location of *Ae. aegypti* using this 'sticky net' approach, unlike human-bait catches, which have been used for many years to collect anthropophagic species. The rationale for developing sticky nets was to determine the arrival location of female *Ae. aegypti* before landing and biting on human hosts indoors. A study using this simple technique which described the distribution of mosquitoes at a human-baited bed net was first described by Lynd and McCall (2013). The simple technique used in this current study is similar to those used in the previous study.

This sticky net is safe for the experimental test as it prevents human hosts from being bitten by mosquitoes. Therefore, it could be used with wild mosquitoes as it reduces the risk of disease transmission on volunteer hosts. Through this experiment, the sticky net was capable of capturing mosquitoes in the laboratory. Control experiments have proven that the sticky net caught more mosquitoes in the presence of the human host but fewer when the human host was absent. There were significantly lower numbers responding in the control trials, as measured by the number of mosquitoes caught on the sticky net in control trials when compared to the baited trials. The results obtained in this study greatly suggest that the human-baited sticky net captured many more mosquitoes than the non-baited sticky net because the mosquitoes showed a preference for the human host, rather than due to random or accidental flight.

The application of Tangle Trap glue on the net offers several advantages. It is relatively easy to apply on the net, transparent, has low human toxicity and remains effective for weeks. The effectiveness of the sticky net will decrease with the accumulation of dust or mosquitoes caught on it. However, it could be effectively used several times before a new layer of glue needs to be applied. The effectiveness of this sticky glue has been documented in a previous study which successfully caught mosquitoes on the net and there was also no repellent effect detected in the presence of a human host (Lynd & McCall, 2013).

In the control experiments, no mosquitoes were captured on the top surface of the net (Table 3.4) but in baited tests, the greatest numbers were caught on top (Table 3.3). This distribution is similar to Lynd and McCall (2013) and provides further evidence for the theory that rising plumes of attractants emanating from human bodies result in mosquitoes arriving above the host. On the other hand, the high numbers of mosquitoes caught on the ‘near’ surface, that closest to the release point, were likely due to the fact that the mosquitoes first reached this surface rather than the other surfaces. Interestingly, total numbers caught on the ‘near’ surface were significantly lower in control compared to baited experiments, suggesting that female mosquitoes being captured on the ‘near’ surface was also

due to the oriented flight towards the human host. Furthermore, mean numbers caught on the top surface of the net were not significantly different compared to the 'near' surface. Hence, in this observation, mosquitoes showed an equal preference for both the horizontal and vertical surfaces of the sticky net. Thus, as well as arriving from above, which indicates that they flew directly towards the host, they also arrived at the 'near' surface, which was the closest to the release site during the experiments.

Furthermore, the greater number of mosquitoes caught on the 'near' surface is also possibly due to the volunteer's position inside the sticky net. The volunteers tended to face the 'near' side of the sticky net to observe the mosquito's movement on the net immediately after release. This could lead to the exhaled breath being released on the 'near' side and funnelled up to the top of the net. It has been long established that carbon dioxide, sweat and exhaled breath increase the attractiveness of a site to anthropophilic mosquito species (Smallegange *et al.,* 2011). Movement of the volunteer inside the sticky net may also have contributed to the number of mosquitoes captured on the net. Visual cues are important in short-range orientation to a host, especially in *Ae. Aegypti*, which bites during daylight. *Ae. aegypti* greatly responds to host movement. In the experiment by Sippell and Brown (1953), they found that nearly double the amount of female *Ae. aegypti* approached the transparent airtight container containing an active rather than anaesthetised deer mouse (*Peromyscus*).

The evidence for host-biting site preference has been previously studied on mosquito species belonging to four different genera: *Aedes, Anopheles, Culex* and *Mansonia* (de Jong & Knols, 1995, 1996; Knols *et al.,* 1994), and later with three closely related *Anopheles* species under laboratory conditions (Dekker, 1998). In the studies on *Anopheles* species, *Anopheles gambiae s.s* preferred to bite feet and ankles whereas *Anopheles atroparvus* and *Anopheles albimanus* preferred to bite around the nose when humans were seated upright. *Ae. aegypti* showed a preference for biting the head and upper part of the chest of humans in a seated position (de Jong & Knols, 1996). In this current study, density distribution of

mosquitoes on caught on the top surface (Figure 3.7) clearly showed that there was a high clustering over the head of the human host, whereas density distribution of mosquitoes on the 'near' surface (Figure 3.10) also demonstrated high density on row number 1 of the net and around the upper part of the seated volunteers. Only mosquito arrival on the net could be measured in this current study; it was not able to detect biting sites because the sticky net prevents mosquitoes from flying to their final destination for blood-feeding. Conversely, previous experiments only measured the final biting site of the mosquitoes (de Jong & Knols, 1995) not arrival paths of mosquitoes before the mosquitoes landed. Nonetheless, results from this and previous studies (de Jong & Knols, 1996) suggest that *Ae. aegypti* approach the head region of a seated host, responding to breath and convection current from the human body, but eventually bite selected upper parts of the body region.

However, despite these results indicating the importance of the head in attraction, covering the head did not alter that effect. Covering the head did not affect the exhale breath but temperature and the odour of the skin might be the potential attractants to the mosquitoes. Previous studies demonstrated that removal of exhaled breath significantly reduced biting of *An. atroparvus* and *An. albimanus* on the head area (de Jong and Knols, 1995) while washing of the feet resulted in a significant change in the distribution of biting sites by *An. gambiae s.s* on human hosts (Knols et *al.,* 1994). However, a more recent study conducted in the laboratory suggested that human breath may have a repellent effect on *An. gambiae s.s.* (Mukabana *et al.,* 2004). These findings indicated that mosquitoes were guided by specific emanations when selecting a biting site on human hosts and this process differs between species. Results in this current study clearly indicated that no differences were found in the attraction of mosquitoes to the human host with covered head and with uncovered head. The exact process of how the mosquito differentiates between hosts still could not be identified. Olfactory cues such as carbon dioxide, organic compounds from expired breath and skin emanations together with physical cues are considered to be responsible for the attractiveness of the hosts (Takken, 1991).

In previous experiments, there was insufficiently evidence that gender and age of the host influence the host preferences of *An. gambiae s.s* (Qiu *et al.,* 2006). Therefore age and gender differences were not tested in this study. Other factors such as body weight and temperature, skin colour, blood type, pregnancy, disease status and skin flora have also been reported to influence attraction in mosquitoes but all these factors were not analysed due to the limited number of replicates and volunteers to be carried out. However, all the mentioned factors are believed not to greatly contribute to the female mosquito's attraction to a human host (de Jong & Knols, 1995; Lindsay *et al.*, 2000; Ansell *et al.*, 2002; Lacroix *et al.*, 2005).

In a study conducted in Haiti it was shown that ITNs may provide protection against dengue vectors (Lenhart *et al.,* 2008). They also presumed that the likelihood of endophilic mosquitoes contacting the insecticide-treated bed nets hung within sleeping areas in small houses would be high (Lenhart *et al.,* 2008). Since the bed net could provide a protective barrier against *Ae. aegypti*, the people on Samui Island use them to protect sleeping infants and children from mosquito bites during daytime (Thavara *et al.,* 2001). In Northern Thailand, the use of bed nets also appeared to protect against dengue whether the bed nets used were insecticide-treated or not (Vanwambeke *et al.,* 2007). A previous study by Lynd and McCall (2013) documented that the basis for developing sticky nets was to assess the potential effectiveness of a two-in-one insecticide-treated bed net which relies upon the mosquitoes' distribution on the net surface. Thus, the data obtained from this sticky net study may also contribute to essential knowledge of *Ae. aegypti* arrival locations, especially if the ITNs or ITMs and long-lasting insecticidal nets (LLINs) which available to combat malaria would be applied for the control of dengue transmission.

3.5 Limitations and further work

The implications and potential results from this current study might not immediately obvious but can be further explored in field by involving wild mosquitoes. It would also be interesting if the effect on an individual host could be pre-identified as attractive or unattractive individuals. Human host could also

be investigated by using local volunteers in certain study areas and compared to the foreign volunteers from other regions. The results might be different because the degree of attraction of mosquitoes to different humans might be varied due to differences in volatile emanations. This would be interesting too if *Ae. albopictus* species could be tested, as this species also contributes to the dengue transmission in many tropical regions. Further experiments also should be repeated with different mosquito release positions in order to prevent sampling bias. The experiment also should be repeated with the human host in different positions, such as upright standing, sitting or lying on the ground positions. To reduce the effect of the side nearest the release point accounting for a high proportion of captured mosquitoes, it is suggested that the distance from the net to the release point are same with other non-release sides within the experimental room. Furthermore, if the implication of this study is used for studying mosquito behaviour in wind tunnels, olfactometer or Y-tubes, a reliable result could be obtained. In some way, such techniques limit the mosquito ability to display a full range of mosquito behavioural responses. However, the previous laboratory studies using those techniques have demonstrated the role of human odour in the host-seeking behaviour mosquito and evidently revealed a mosquito's orientation to odour plumes even when presented only with extract or synthetic of human or animal odours (Pates *et al.,* 2001; Dekker *et al.,* 2005; Williams *et al.,* 2006; Lacey, & Cardé 2011). Moreover, the experiment could also involve direct observation using a non-lethal camera system and this will accurately demonstrate the behavioural events of the mosquitoes towards the human host. However, this current sticky net experiment certainly obtained satisfactory results even though it was originally anticipated that filming of mosquito movement on the net surfaces could also be carried out. Furthermore, the application of a camera system to track mosquitoes in the future would give promising and highly accurate data on the arrival location and final biting site of mosquitoes on the human host.

3.6 Conclusion

1) Sticky net effectively captured mosquitoes and is a useful tool for measuring mosquito behaviour in the laboratory.
2) There were significantly higher numbers of *Ae. aegypti* caught on the top and 'near' surfaces of the net.
3) Overall distribution of mosquitoes on the net was not random. The mosquito density distribution on the top and 'near' surfaces of the net revealed clustering on the volunteers' heads. However, random distribution was observed on the lower part of the human host.
4) The data obtained provide essential knowledge of the behavioural responses of female *Ae. aegypti* towards a human host which is useful for the improvements of ITMs or ITNs and the development of novel trap design in the future.

CHAPTER 4

STUDIES ON ADULT FEMALE *Aedes aegypti* RESTING BEHAVIOUR IN RESPONSE TO TWO DIMENSIONAL TARGETS AND RESTING BOXES

4.1 Introduction

Ae. aegypti are widely recognised in the tropics as day biting mosquitoes. This major urban vector of dengue viruses is endophilic, tending to feed and rest inside the house. When choosing resting sites, *Ae. aegypti* shows a marked preference for subjects with the least reflection, e.g. dark (especially black) coloured objects and preference for areas away from open spaces, with low light intensities or with higher degree of shadow as their resting habit (Sippell & Brown, 1953; Muir *et al.,* 1992). Attractiveness to different colour surfaces by the mosquitoes correlates with the percentage of the reflected light rather than any colour discrimination (Brown, 1954).

Brighenti (1930) investigated colour attraction in resting behaviour of *Anopheles maculipennis* by painting the ceilings of cattle sheds with different colours. It was shown that the colours that proved attractive by this species, in order, were carmine red, violet, chrome yellow, white, green and cobalt blue. On the other hand, Headlee (1937) demonstrated that light sources with different colours caught different numbers of mosquitoes per microwatt of light energy. He compared the attractive colour preferred by mosquitoes with a 25-watt white source frosted bulb. He found that blue was the most attractive colour with 21.5 times the attraction power of white, followed by green-yellow and red with 12.3 and 6.1 times the attraction power of white respectively.

Brett (1938) demonstrated that *Ae. aegypti* generally preferred darker-coloured clothing, showing that black is the most attractive colour for this species followed by red, which also has a similar low reflectance factor. White was avoided because of its high reflection factor. However, yellowish khaki tended to be more

repellent than white and yellow is also a repellent colour. Brett's (1938) test on colour preference was influenced by the presence of human hands, i.e. the cloth material in the experiment covered a volunteer's hand and Brett concluded that none of the three repellent colours was sufficient to prevent mosquitoes from feeding.

Brighenti's experiment tested colour preference in 'landing to rest' whereas Brett tested 'landing to feed', a considerably different reaction. Landing behaviour in host-seeking mosquitoes is influenced by several factors such as olfactory and visual cues, warmth, heat and movement by the host (Christophers, 1960). The combination of heat and odour is among the most prominent attractants for host-seeking mosquitoes, while resting is influenced more by visual cues or optical stimuli such as light intensity, luminous reflectance and contrast of the background. The responses of several species of mosquitoes to visual stimuli of various colours and shapes in the absence of hosts have been studied, e.g. Gjullin (1947), Brown (1954), Gilbert and Gouck (1957), Browne and Bennett (1981) and Muir *et al.* (1992). The behavioural experiments conducted by Muir *et al.* (1992) demonstrated that, although *Ae. aegypti* were able to discriminate between some wavelengths, luminous reflectance, vertical contrast and movement were likely to be more important than colours. Muir *et al.* (1992) concluded that stationary objects with low reflectance and solid colour were the most preferred by male and female *Ae. aegypti*.

Previous studies conducted in Africa, South East Asia, and Australia (Pant & Yasuno, 1973; Trpis & Hausermann, 1975; Muir & Kay, 1998) reported the resting habits of *Ae. aegypti*. This species is found beneath and inside the house and has also been reported to rest in other shelters. Adult *Ae. aegypti* are found particularly in furnished rooms where they can rest within clothes, furniture and other hiding places (Schoof, 1967). Other studies conducted in the Americas such as in Panama, Costa Rica, Dominican Republic, Puerto Rico and Mexico (Perich *et al.,* 1990; Clark *et al.,* 1994; Perich *et al.,* 2000; Perich *et al.,* 2003; Eisen & Beaty, 2008) also reported such secluded resting behaviour of *Ae. aegypti* within

homes. Therefore, it is proposed that visual targets be placed in shaded locations inside homes and other structures that *Ae. aegypti* mosquitoes might frequent while searching for blood meals, resting and oviposition sites.

Conventional methods of monitoring and controlling adult *Ae. aegypti* currently differ in efficiency, labour cost and technical requirements (De Santos *et al.,* 2012). Sampling mosquitoes by adult traps has been evaluated under different field conditions and found to be effective for monitoring *Aedes* population (Edman *et al.,* 1992; Scott *et al.,* 1993; Clark *et al.,* 1994; Hoel *et al.,* 2009). The collection of mosquitoes using CDC backpack aspirators (Clark *et al.,* 1994; Scott *et al.,* 2000; Maciel-de-Freitas & Lourenco-de-Oliveira, 2009) is considered as the most effective adult *Ae. aegypti* collection method because it collects males and all gonotrophic stages of female *Ae. aegypti*. However, this method is laborious and also requires diligence, skill, consistency of effort and free access to most of the house. Therefore, the BG-Sentinel trap was developed and used for evaluation in different field conditions (Krockel *et al.,* 2006; Gama *et al.,* 2007; Bhalala & Arias, 2009). Odour-baited traps such as CDC Wilton, Fay Prince (Williams *et al.,* 2006), Counter flow trap (Schmied *et al.,* 2008) and the MMX-trap (Njiru *et al.,* 2006) also have efficiency for mosquito sampling in different areas. Although the BG-Sentinel trap is claimed as the most efficient trap for sampling various adult stages of dengue vectors, this trap and other mentioned traps are generally not cost effective as they require a source of power and are highly labour intensive for daily mosquito collection (Krockel *et al.,* 2006; Maciel-de-Freitas *et al.,* 2006; Williams *et al.,* 2006; Facchinelli *et al.,* 2007; Facchinelli *et al.,* 2008).

Sticky ovitraps (Ritchie *et al.,* 2003; Facchinelli *et al.,* 2007) and Adultraps (Donatti and Gomes, 2007; Gomes *et al.,* 2007; Maciel-de-Freitas *et al.,* 2008) are alternative ovitraps that have been specifically developed to capture gravid *Aedes* mosquitoes. These ovitraps are based on the original ovitraps that are made from black containers filled with water and a paddle for egg-laying (Fay & Eliason, 1966). Ovitraps and sticky ovitraps have been widely used to obtain information on number of eggs laid and correlated to the spatial and temporal distribution of

mosquitoes (Facchinelli *et al.,* 2007; Reiter, 2007). In addition, the information from the collection of gravid females and eggs can be used to assess the efficacy of vector control, indices of dengue transmission risk and can be processed for arbovirus infection (Ritchie *et al.,* 2004).

Resting box techniques have shown to be an effective method in capturing several mosquito vector species in the field. There are several resting box methods developed for sampling mosquito vector populations as well as for surveillance and control programmes (Morris, 1981; Crans, 1989; Nasci *et la.,* 1993; Edman *et al.,* 1997; Kittayapong *et al.,* 1997; Harbison *et al.,* 2006; Burkett-Cabena *et al.,* 2008; Kweka *et al.,* 2009). These resting boxes have different shapes, colours and materials as mosquito attractants and manage to capture both male and female mosquitoes indoors (Yasuno *et al.,* 1976) and outdoors (Goodwin, 1942; Edman *et al.,* 1968; Morris 1981; Kay, 1983; Weathersbee & Meisch; 1988). Resting boxes designed by Edman *et al.* (1968) and Morris (1981) are examples of resting boxes that were specifically developed for collecting blood-fed adult *Culiseta melanura* as part of a surveillance programme for eastern equine encephalitis virus and for a study on mosquito fecundity, flight range and dispersal (Howard *et al.,* 1989, 1996; Oliver *et al.,* 1996).

The resting box method provides an effective alternative to human-bait sampling. The resting boxes developed in this current study were designed to capture resting mosquitoes with minimal labour requirements. The boxes are made from disposable hardboard and are fairly easy to produce using inexpensive materials and, more importantly, no power source is required to operate them. Furthermore, these boxes were provided with humidity, which was expected to attract more mosquitoes. If this cost-effective trapping method were shown to be an effective technique in collecting mosquitoes, it could therefore be used in dengue surveillance.

Thus, the first objective of this current study was to use a simple laboratory assay to investigate the responses of female *Ae. aegypti* to 2D panel targets as resting sites, at varying colour surfaces, material textures and orientations under controlled experimental conditions. Mosquito attractiveness was measured as a function of mosquito landing frequency and duration of resting time. Secondly, this study was also to investigate the use of novel resting boxes for *Ae. aegypti* under laboratory conditions. The abilities of resting boxes as outdoor devices for sampling resting mosquitoes were also assessed in the field. Research findings are discussed in the context of further resting trap development for this important urban vector.

4.2 Methods

4.2.1 Laboratory trials using two-dimensional panel targets as resting sites

The room measured 418 cm x 269 cm x 232 cm (length x width x height) (Figure 4.1). The panel target was set up 134.5 cm from the door, 134.5 cm from the window, 159 cm from the sink and 159 cm away from the wall (Figure 4.2). The distance between panel target and the camera was 85 cm (Figure 4.2).

All cameras, lighting wires and laptop cables were covered with white PVC insulated tape while the sink was covered with white cotton fabrics to provide the maximum contrast of the black target over the experimental room and all objects inside. A small hole was cut into the window so all the cables connected to the laptop and the computer were able to pass through it. One laptop and one computer were used to monitor the movement of the mosquitoes. They were placed inside a second insectary to ensure that mosquitoes were not influenced by the presence of a human observer inside the experimental room.

4.2.1.1 Experimental setup and procedure

The room remained at between 24°C to 28°C and relative humidity around 60% to 80%. The room temperature and humidity was recorded before and immediately after the experiment using a digital thermo-hygrometer placed inside the room.

4.2.1.2 Room preparation

The experimental room was prepared before each test (Table 4.1). Any previous mosquitoes that still alive were killed using a bug zapper and a new release container with a new batch of mosquitoes was placed within.

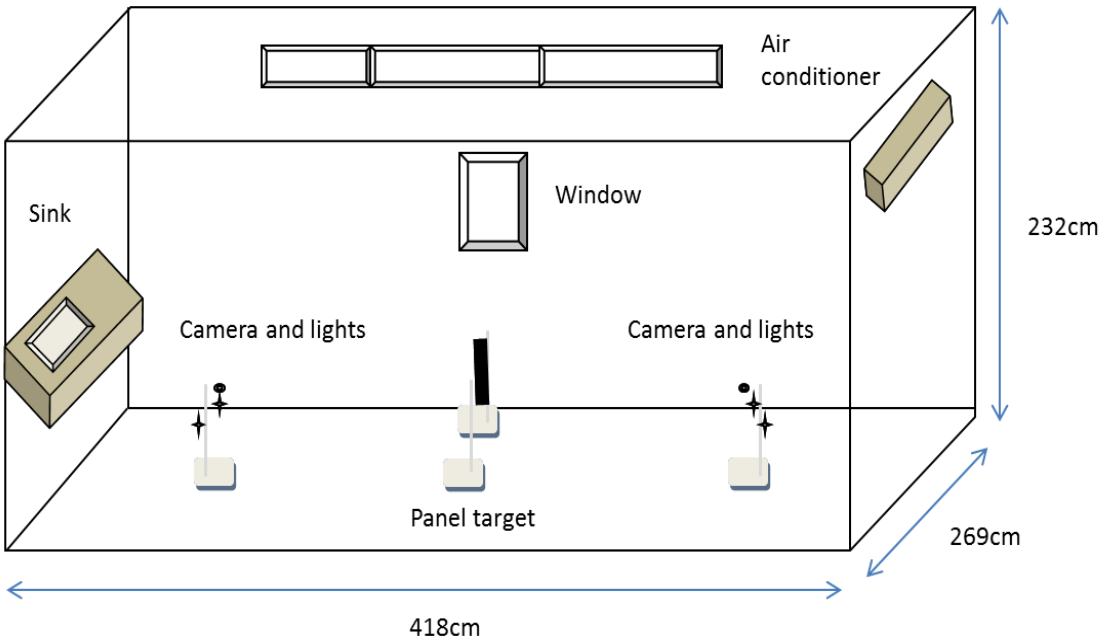


Figure 4.1 Experimental room dimension and setup to measure landing and resting behaviour of *Ae. aegypti*

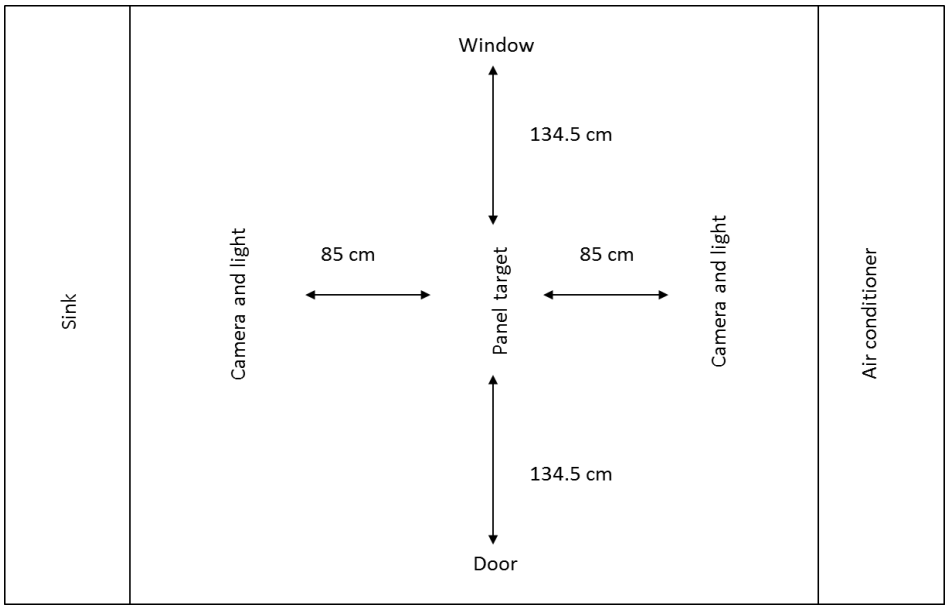


Figure 4.2 Arrangement of the usable areas inside the actual room

Table 4.1 Room preparation and experimental time

Method	Time (minutes)
Room preparation	30
Release mosquitoes and allow to rest	60
Transferring data	30
Ventilate the room after killing the remaining mosquitoes	30

4.2.1.3 Cameras and video recordings

Cameras were Basler acA2500 (Basler; Germany) with Tamron CCTV Lens 8mm (Tamron; Japan) and Logitech HD Pro Webcam C910 (Logitech; USA). For each piece of experiment, camera-monitoring properties were set to take an image with 0.2 frame rate for a period of 1 hour. Due to some variance in acquisition control (exposure time and frame rate) and lighting (aperture and power supply), the camera settings differed slightly between each experiment and between the two cameras. Camera specifications and setting properties that were used in this experiment are shown in Tables 4.2 and 4.3 for Basler camera, while Tables 4.4 and 4.5 are for Logitech HD webcam.

The operating cameras were monitored using the free software Pylon Viewer for capturing images and Virtual VCR for recording videos. All images and videos were directly saved onto the computer. All the recorded videos were immediately transferred to a secure hard drive for further analysis.

Table 4.2 Basler Ace series specifications

Camera specification	
Basler Ace	acA2500-14gm/gc
Resolution (h x w pixels)	2592 x 1944
Sensor Type	Aptina MT9P
Sensor Technology	CMOS, Rolling Shutter
Sensor Size (optical)	1/2.5″
Power Consumption (PoE/AUX)	2.5 W/2.2 W
Weight (typical)	<90 g
Maximum Frame Rate at full resolution	14
Pixel Size (µm)	2.2 x 2.2
Lense Mount	C-mount
Data Output Type (Interface)	Fast Ethernet (100 Mbit/s) or Gigabit Ethernet (100 Mbit/s)
Software Environment	
Software Driver	Basler Pylon SDK including filter and performance driver
Operating System	Windows, Linux -32 bit and 64 bit

Table 4.3 Optimum Basler Ace properties settings used for all experiments

	BLACK PANEL	**WHITE PANEL**
AOI		
Width	2592 (Maximum)	2592 (Maximum)
Length	1944 (Maximum)	1944 (Maximum)
Acquisition Control		
Exposure Time	52920-209930	40635-149975
Frame Rate	0.2	0.2
Lighting		
Aperture number	~4	~8
Power supply	0-2	0-2

Table 4.4 Logitech HD series specifications

Camera specification	
Logitech	HD Pro C910
Video recording	1080p Full HD
Photo resolution	15 MP
Focus type	20-step Auto Focus
Lens	Carl-Zeiss multi-element
Low light correction	Yes
Face tracking	Yes

Table 4.5 Optimum Logitech HD properties settings used for all experiments

Camera settings	
Brightness	100-200
Contrast	120-160
Saturation	20-25
Sharpness	100-150
White balance	2800
Backlight compensation	0
Gain	25-50
Zoom	1
Focus	30-70
Exposure	-7
Pan	0
Tilt	0
Power frequency (anti-flicker)	60 Hz

4.2.1.4 Investigation into the effect of surface texture

The purpose of this experiment was to evaluate the attractiveness to mosquitoes of black and white panels with two different material textures, in a vertical orientation. The black panels were placed in one side and the white panels were placed in the opposite-side. This experiment tested the hypothesis that female mosquitoes show a preference for black targets as resting surfaces. It was intended that this would demonstrate distribution patterns that are predictable and indicate the preference for material texture in definable location of the panels within the experimental room.

Foam boards (30 cm x 30 cm) were covered with black velvet, white velvet, black velvet with netting and white velvet with netting respectively (Figure 4.3). The black flock paper that was used to cover the foam boards was of absorbing light material, which has a texture similar to heavy construction paper with adhesive backing. Both white velvet and white velvet with netting operated as the control in this experiment whereas the black velvet and black velvet with netting were the targeted panels. Four foam boards were attached together using binder clips and was secured to a retort stand with two clamps at the base. The relative positions of each material were changed for each experiment. The first number chosen meant that the corresponding material was placed at the ‘upper black’. The second

number being assigned to the next place was ‘lower black’ while the next places were ‘upper white’ and ‘lower white’ respectively. Label ‘b’ refers to the black side whereas ‘w’ refers to the white side. Four different orientations were tested. The experiment was replicated 5 times (Figure 4.3).

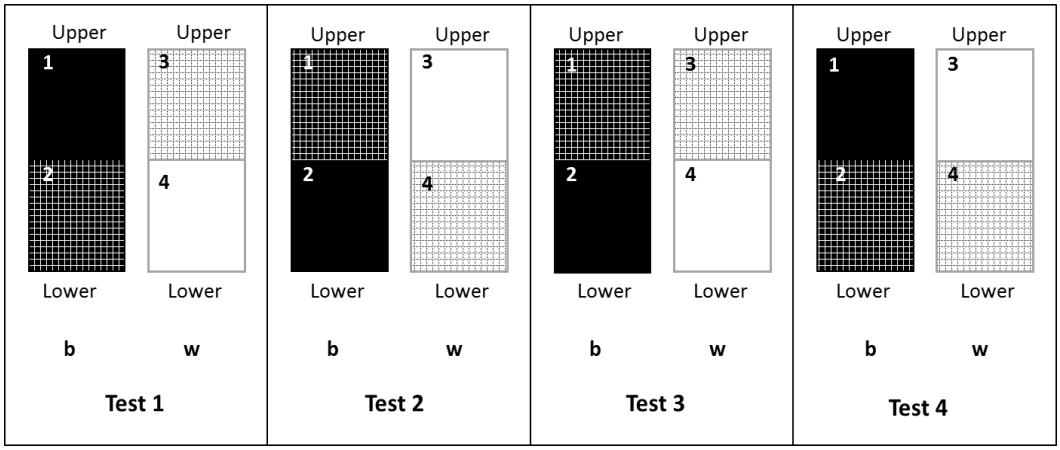


Figure 4.3 Test surfaces in four different arrangements (test 1 to test 4) as set up to observe *Ae. aegypti* resting preferences for colour and texture

In each experiment, black and white panels were placed together using clamps on retort stands suspended 90 cm above ground for the upper panel and 60 cm above ground for the lower panel (Figure 4.4). This height measurement was approximately the same as used by Brown and Sippell (1954) in their study on the role of visual factors in attraction of female *Aedes* mosquitoes. Two different views of the panels are shown in Figure 4.4.

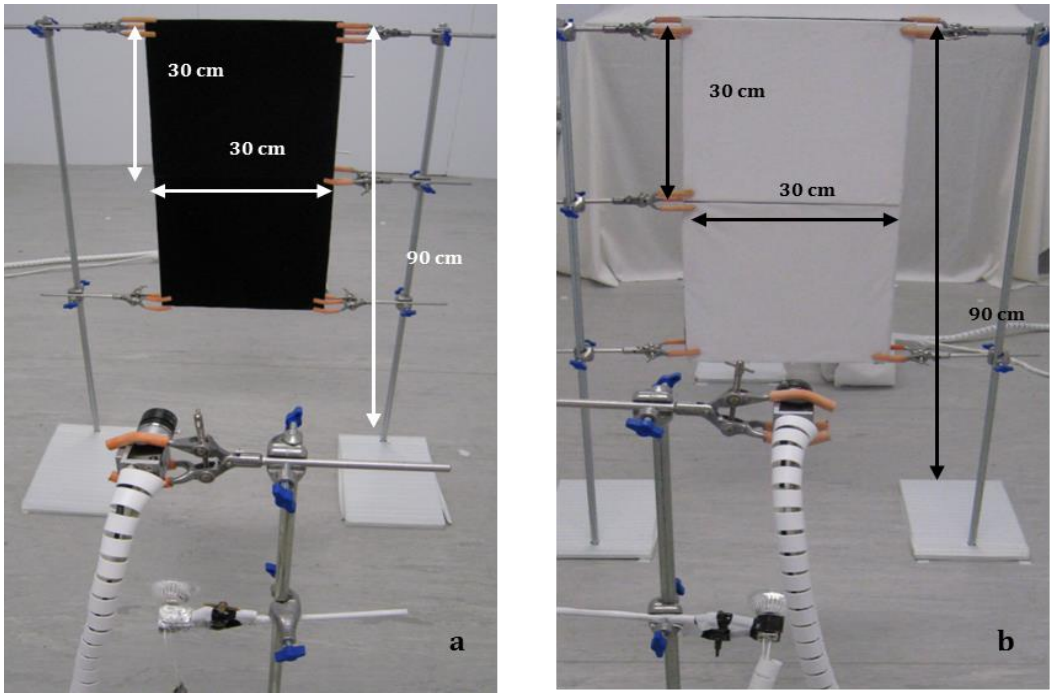


Figure 4.4 Photograph showing a) the front view camera with black panels, and b) the opposite-side camera with white panels' view

To observe the effect of panel size on landing, these panels were marked in a grid using permanent marker (Figure 4.5). The numbers of mosquitoes landing on each panel were counted based on location of the mosquitoes in the gridded area. The distribution was divided into three parts: the upper part was the top 10 cm of the panel, the middle part was the next 10 cm, and the lower part was the final 10 cms'.

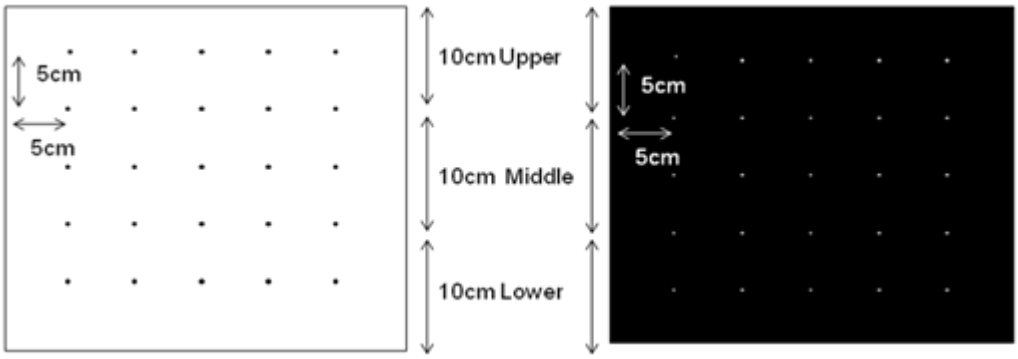


Figure 4.5 The 5 x 5 cm reference grids on black and white panels

4.2.1.5 Investigation into the effect of panel orientation and contrast

The experiments were conducted to observe the effect of contrast between black and white panels in two different orientations, vertical and horizontal: (a) comparing the contrast factor of black and white panels in vertical and horizontal orientations and (b) comparing the contrast of black and white panels in mosaic design in vertical orientation.

Experiment A - This experiment tested the hypothesis that mosquitoes prefer black panels and it was assumed that certain panel orientations are preferred by mosquitoes. It was also hypothesised that the orientation would have caused different landing durations or the amount of time spent on the preferred panel orientation.

A second experimental room setup was used for this experiment. The room measured at 230 cm x 180 cm x 200 cm (length x width x height). The temperature and humidity inside this room remained the same as in the previous experimental room. The panel board arrangement was set up at 115 cm from the camera and 90 cm from the side walls respectively. The distance between two stands was 115 cm for both sides. All other setup was the same as the previous experiment.

The black panel was placed on top of the white panel. This arrangement was then reversed and the white panel was placed on top of the black panel. Labels ‘a’ and ‘b’ refer to the two different sides of the panel (Figure 4.6). The fabric material was the same material as used in the previous experiment, except the netting material was not used. For both vertical and horizontal orientations, two different arrangements with five replicates for each arrangement were tested during the experiments.

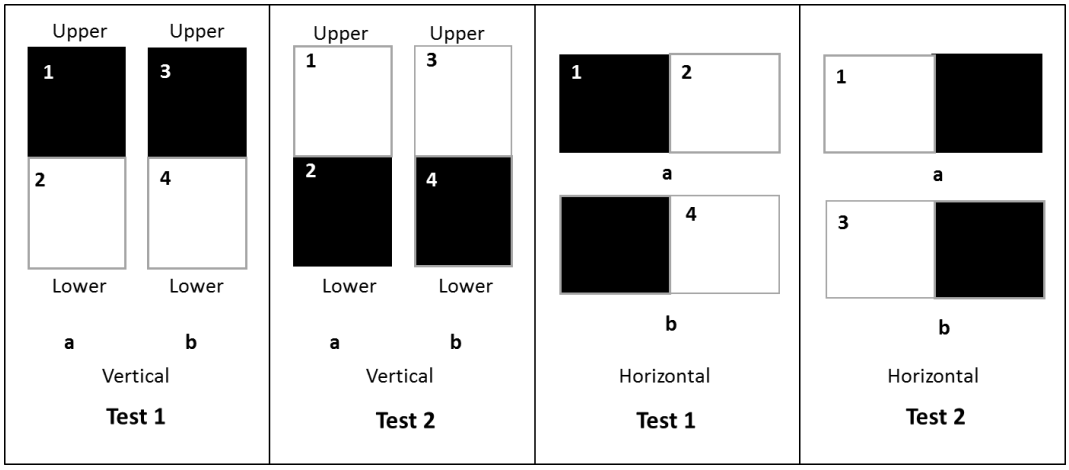


Figure 4.6 Panel boards with two different arrangements were set up to test *Ae. aegypti* on contrasting surfaces of two different orientations

The results from the first experiment indicated that further experiments were required to determine the mosquitoes' preference for black surface over white surface if both different surfaces were placed together on the same side. From the previous experiment, it was shown that the material textures did not contribute to significant differences in landing frequency and landing duration of the mosquitoes. Therefore, only plain velvet without netting was used in this experiment. Furthermore, the effect of surface orientation was also tested to determine the surface position preferred by *Ae. aegypti* during landing and resting indoors.

Experiment B - In order to further investigate the contrast factors that influence *Ae. aegypti* landing and resting behaviour, black and white panels of mosaic design were developed. It was hypothesised that black and white mosaic design would best attract mosquitoes. Since there were higher landing frequency and landing duration recorded on the vertical panel in the previous experiment, the mosaic design was only tested in the vertical orientation.

The experimental setup was the same as the previous experiment. Two different panel arrangements were set up in this experiment. In the first test (a), the black and white plain panels were placed on top of the black and white netting panels. In the second test (b), the black and white netting was placed on top of the black and white plain panels. Two different arrangements with five replicates for each arrangement were tested during the experiments (Figure 4.7).

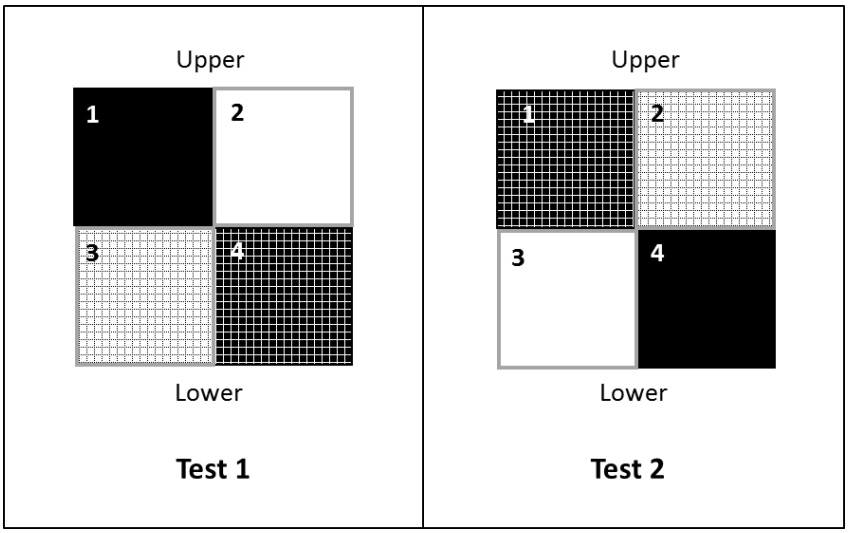


Figure 4.7 Mosaic design with two different arrangements (test 1 and test 2) was set up to test *Ae. aegypti* preference for colour and texture of resting surfaces using two different materials.

4.2.1.6 Investigation into the effect of resting panel height

Due to different mosquito responses between upper and lower arrangements in previous results, the experiment was repeated with the black panel placed at different heights. This experiment tested the hypothesis that, when flying indoors, mosquitoes prefer to fly at specific heights above ground and that they prefer certain heights of landing and resting sites. This experiment is only tested on black panel (Figure 4.8).

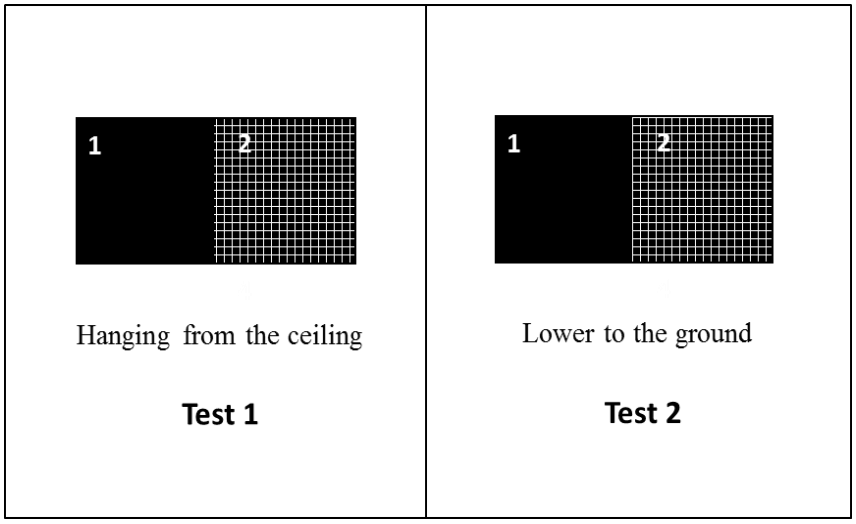


Figure 4.8 Two panel boards set at two different heights a) Test 1 - Hanging from the ceiling; 200 cm above ground and b) Test 2 - 90 cm above ground were set up to test *Ae. aegypti* preference for black colour and texture of resting surfaces using two different materials

4.2.1.7 Investigation into the effect of trap adhesive

A final experiment was conducted to determine the effects of the non-setting adhesive (Tangle-Trap Liquid Insect Trap Coating) on the responses of mosquitoes. It was theorised that *Ae. aegypti* attraction to panels coated with sticky glue was entirely visual, with no odour response involved. The experiment was carried out by recording the number of mosquitoes caught on the treated panels (Figure 4.9).

Both black plain and black netting panels were coated with Tangle-Trap Liquid Insect Trap coating. Three layers of sticky glue were applied to the surface of the panels. The panels were left for three days in an open area before the experiment, to ensure that volatile chemicals from the glue had disappeared from the treated panels. As in the previous experiment, five replicates for each position were carried out to determine the number of mosquitoes caught on the treated panels. However, landing frequency and landing duration in this experiment were not measured since this experiment only detected the final catch point of the mosquitoes.

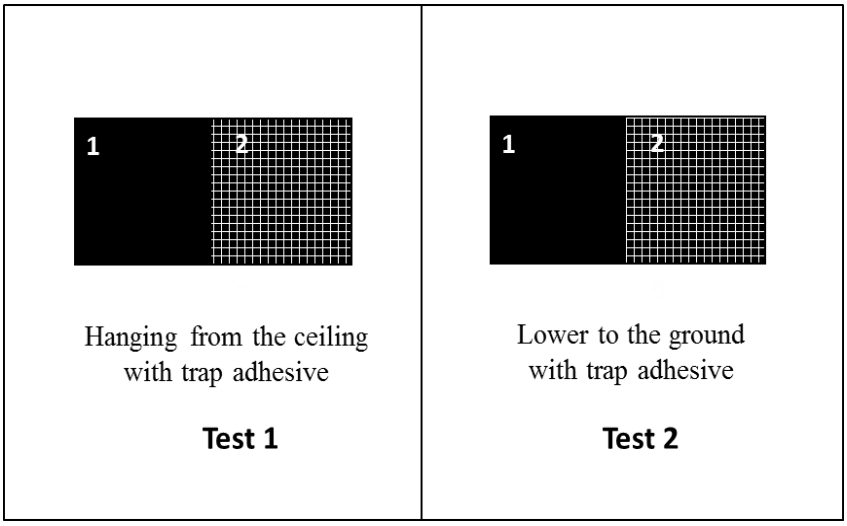


Figure 4.9 Two panel boards with triangle traps glue with two different heights a) Test 1 - Hanging from the ceiling; 200 cm above ground and b) Test 2 – Lower to the ground; 90 cm above ground were set up to test *Ae. aegypti* preference for black colour and texture of resting surfaces using two different materials

4.2.1.8 Analysis of images

The targeted and control panels were monitored directly using images captured by Pylon Viewer software and videos recorded by Virtual VCR software. All video recordings captured by Basler Ace were extracted to form a series of 60 images using free software Advanced X Video converter and JAI Tools. This series of images was then exported into Matlab software for further analysis. For images that were captured using Logitech webcam, the AutoClick 3.0 version software was used to set one-minute interval per image capture. Photographic images were recorded for 60 minutes at one-minute intervals. All of the mosquitoes' behavioural activities on targeted and control panels were analysed using Matlab R2012a software. A series of 60 images for each replicate was loaded into the program. The number of mosquitoes resting on the panels for a given frame and the duration of the mosquitoes' resting events were determined using 'records positions and tracks'. Beginning with frame one, an individual mosquito's position was visually identified. The mouse cursor was placed over the mosquito image and clicked over that particular position. A mosquito was assumed to have moved from a given position once its body was no longer within the specified

radius. Once all mosquitoes' positions were identified, the save 'positions' was selected in order to save the position of each mosquito at every frame. The save 'Tracks' was selected to record the frequency of one mosquito on the frame as well as the duration of one mosquito on the frame.

(a) Landing frequency

Every 60 minutes of the recording period, the number of landings at one-minute intervals was determined for both targeted panel and control panel. The total number of mosquitoes resting on each target panel and control panel for the entire 60 minutes of the recording period was taken, and then divided by 50 (total number of released mosquitoes) to determine the mean number of landings per mosquito for each panel.

(b) Duration of resting times

Resting time for each individual mosquito was totalled for the entire 60-minute test and divided by the number of landings to determine the mean resting duration per landing. A mosquito was assumed to have moved from a certain position when the mosquito was no longer observed to be within that specified radius.

(c) Landing frequency over a 60-minute period

Mean numbers of landings per mosquito for each orientation were compared at eight different time points: 1, 5, 10, 20, 30, 40 50 and 60 minutes.

(d) Immediate landing activities

Arrival activities of mosquitoes were recorded at first 60 seconds from the test start. This activity was considered as immediate landing on panel by mosquitoes after release into the experimental room.

(e) Distribution of resting mosquitoes on panels

The distributions of mosquitoes on a panel were constructed based on a 5 cm reference grid made on each panel. The upper part was the top 10 cm of the panel, the middle part was the next 10 cm, and the lower part was the final 10 cms'.

Mosquitoes landing distribution on the panel was evaluated using models of dispersion. There are three general patterns of dispersion found in nature: regular, random and contagious (clumped). Therefore, based on probability distributions using this model, sample data with small variance ($s^2/\chi < 1$) suggests regular dispersion, whereas sample data with intermediate ($s^2/\chi = 1$) and large variance ($s^2/\chi > 1$) suggests contagious and random dispersion respectively. The ratio of the variance to the mean (s^2/χ) was used as *Index of Dispersion*. The variance/ratio was standardised by multiplying by the number of observations in the sample minus one (n-1), that is the degrees of freedom (v). The mosquito landing distributions were recorded as an *Index of Dispersion* for which the value unit represented regular, random or contagious distribution.

4.2.1.9 Statistical Analysis

An assessment of the normality distribution of data was determined using Shapiro-Wilk test. The significant value of the Shapiro-Wilk test that is greater than 0.05 indicated that the data was normally distributed. The differences in mean numbers of mosquito landing frequency on the black target were compared to the white control. The mean numbers of mosquito landing frequency on the top black target were also compared to the bottom black target. Data that were normally distributed were compared using Paired T-test whereas data that were not normally distributed were compared using Wilcoxon Sign Rank test. The mean mosquito landing duration on the black target was compared to the white control using Paired T-test. The difference in mosquito landing frequency over 60 minutes was compared using Wilcoxon Sign Rank test.

4.2.2 Preliminary field trials and laboratory trials using resting boxes

Two resting boxes were developed based on three-dimensional cuboids. The top surface of each box had an opening, and the whole box was covered with black binding tape over the entire outer surface. The black binding tape was used to ensure that the boxes remained stable in hot and wet weather during the experiment. The dimensions of box type 1 were 30 cm length x 14 cm width x 12 cm height, while the dimensions of box type 2 were 28 cm length x 14 cm width x

9 cm height. The interior of each box was covered with black velvet measuring 21 cm length x 12 cm height (two sides of the box) and 21 cm length x 14 cm width (base of the box) for box type 1 and box type 2 respectively. This flock paper has a texture similar to heavy construction paper which available with adhesive backing. This velvet like material is also able to absorb light. Tap water was used to wet the interiors of the boxes and the black velvet material in order to maintain the humidity of the boxes throughout the day. The black surfaces and the humidity inside the boxes were believed to be the main attraction for the mosquitoes. Two different openings were designed to compare how attracted mosquitoes were to these two boxes: box type 1 with two openings and one 3 cm gaps in the middle and box type 2 with one opening and one 3 cm gaps in one end (Figures 4.10 & 4.11).

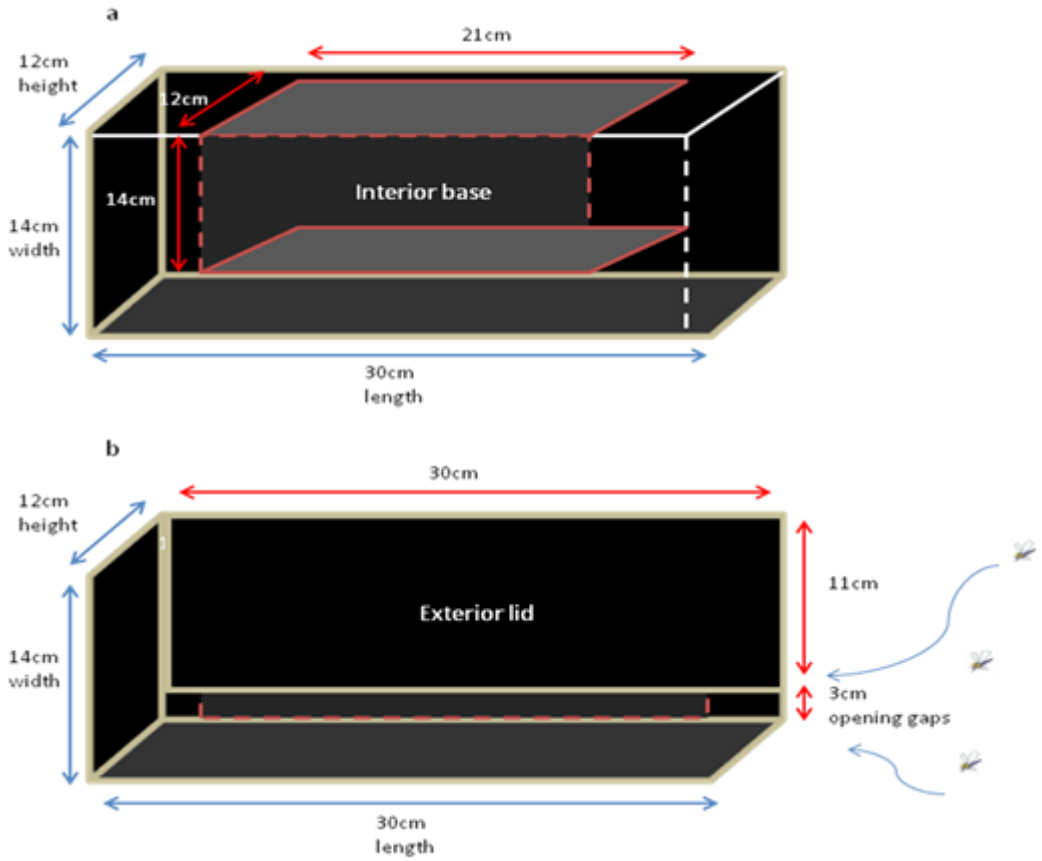


Figure 4.10 Dimensions of the three-dimensional resting box type 1 a) Interior image with black velvet inside the resting box b) One opening gaps at one end was designed for mosquitoes to enter the resting box during landing and resting behaviour

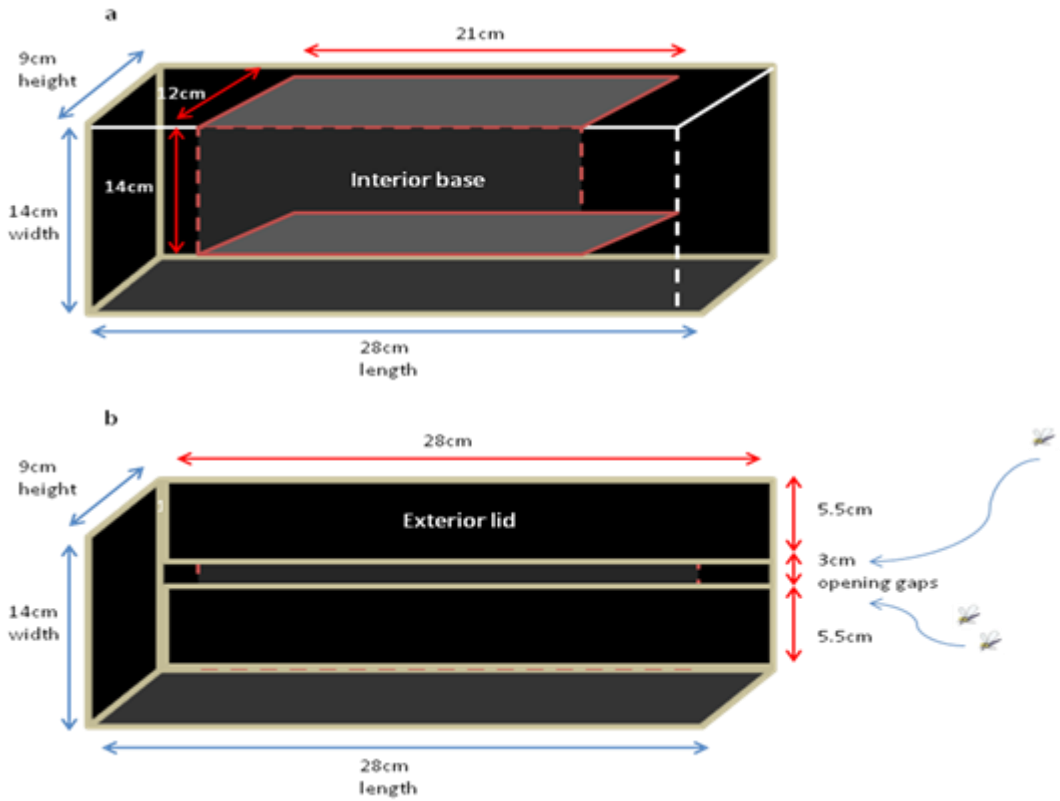


Figure 4.11 Dimensions of three-dimensional resting box type 2 a) Interior image with black velvet inside the resting box b) Two opening gaps were designed for mosquitoes to enter the resting box during landing and resting behaviour

4.2.2.1 Study area

This study was conducted inside a house at a small village in the township area of Penang Island during the month of July 2012. The study area was located in Bayan Baru (5°19'53.85"N 100°17'19.4"E; altitude 6 m) and consisted of approximately 250 houses. The typical design of the houses in this area is single storey with open eaves and metal roofs. The houses here are built close to one another creating a large, close-knit housing community, consisting of a mixed Malay and Chinese population.

4.2.2.2 Experimental setup and procedure

Two resting boxes were placed in two different locations inside and outside of the selected house. The first rectangular box with one opening and one 3 cm gaps in one end was placed under a small parking area with corrugated roof where the owner of the house parked motorcycles and hung clothes during the day (Figure 4.12). The second rectangular box with two opening and 3 cm gaps in the middle was placed inside the house, in the open wooden rack which was used by the owner to place the used and unwanted plastic bottles, tins, plastic sheets and fabric sheets (Figure 4.13).

Mosquitoes were captured in the experimental resting boxes to determine the species and number of resting mosquitoes. Two resting boxes were placed in the selected location one hour before the first data recording was taken. The first trial of mosquito collection was conducted at a) four different times: 07:00, 11:00, 15:00 and 19:00 (every four hours) and second trial at; b) three different times: 07:00, 13:00 and 19:00 (every six hours). The internal sides of the resting boxes were sprayed with tap water every time before they were placed in both selected areas in order to keep them moist for mosquitoes to rest inside. The collection of mosquitoes was carried out using a hand aspirator, after which the samples were brought to the laboratory for identification. The experiment was conducted in 10 replicates for 10 consecutive days. The temperature and humidity were recorded using devices called TinyTag. The temperature ranged from 28°C to 31°C while the humidity ranged from 71% to 72% inside and outside the house.

Figure 4.12 a) Three-dimensional resting box type 1 in black colour with one opening and one 3 cm gaps in one end b) The rectangular box placed outside the house, at the bottom of a pile of wood c) Black rectangular box as a resting box to trap mosquitoes d) The resting box placed outside the house, under a small shaded area where the owner of the house used to park motorcycles and hang their clothes during the day

Figure 4.13 a) Three-dimensional resting box type 2 in black colour with two openings and one 3 cm gaps in the middle b) The rectangular box placed inside the house, in the bottom of a wooden cupboard c) Black rectangular box as a resting box to trap mosquitoes d) The resting box placed inside the house; the opening door is towards the kitchen area of the house

4.2.2.3 Evaluation of the resting box in the laboratory

To confirm the results observed in the field trial in Penang and to control temperature and humidity inside and outside resting boxes, a further series of experiments was carried out in a climate-controlled experimental room in Liverpool School of Tropical Medicine, using laboratory-reared *Ae. aegypti* (New Orleans strain). Since the numbers of mosquitoes caught did not differ between the two designs of resting box in the previous field trial, the resting box type 2 was used in this trial. The resting box was placed in the middle of the experimental room. Dimensions and setup of the experimental room were the same as described in previous landing and resting behaviour experiments using panels. The internal sides of the resting box were sprayed with tap water each day of the trials. A TinyTag data logger was placed inside the resting box to monitor the humidity and temperature, which were kept between 25^{o}C and 26^{o}C and 70% to 80% inside the resting box. Fifty mosquitoes were placed into a holding container, provided with 5% sucrose solution and kept in the experimental room for one-day to acclimatise. The mosquitoes used in this experiment were aged between 3 and 12 days post-eclosion. The mosquitoes that rested inside the box were collected using a hand aspirator while the remaining mosquitoes in the experimental room were killed using an electrocution zapper.

Experiment A measured the effect of temperature and humidity, to determine the optimum conditions for resting mosquitoes. A resting box was placed on a retort stand suspended 90 cm above the floor of the experimental room. This experiment tested the hypothesis that mosquitoes preferred lower temperature and high humidity area for the resting site. The numbers of mosquitoes captured were compared to the control resting box, which had the same background temperature and humidity as the experimental room. The trial was conducted by controlling both the room temperature and humidity of the experimental room and the inside of the resting box as follows:

Table 4.6 Room temperature and humidity controlled in the experimental room.

Treatment	Experimental room		Resting box	
	Temperature	Humidity	Temperature	Humidity
Control	27-32^{o}C	50-64%	27-32^{o}C	50-64%
1	27-32^{o}C	50-64%	25-30^{o}C	65-80%
2	25-30^{o}C	65-80%	27-32^{o}C	50-64%

Experiment B examined the effect of opening surface orientation, to determine if flight orientation influenced entry to a resting box. A resting box was arranged in either a vertical or a horizontal position, and placed approximately at the same height above ground as described in experiment A.

4.3 Results

4.3.1 Laboratory trials using two-dimensional panel targets as resting sites

In this experiment, mosquito attraction was measured as a function of mosquito landing frequency and duration of resting times. These results were recorded as mean number with standard error. Landing frequency over 60-minute periods, immediate landing activities and landing distribution on the panels were also recorded.

4.3.1.1 Investigation into the effect of surface texture

(a) Landing frequency

The overall results showed that landing frequency of unfed female *Ae. aegypti* ranged from a low of 0.03 ± 0.03 mosquito per minute for the white panel (control), to a high of 11.9 ± 1.5 mosquitoes per minute for the black panel (target) (Table 4.7). The black panel was significantly more attractive than the white panel. Therefore the upper black was highly attractive than upper white (Paired T-Test, $P < 0.05$). The upper panels were significantly more attractive than the lower panels (Paired T-Test, $P < 0.05$). However, there were no significant differences between upper white and lower white (Paired T-Test, $P > 0.05$).

The second, third and fourth tests showed similar patterns to the first test and all the black panels were significantly more attractive (Paired T-Test, $P < 0.05$) than the white panels. Furthermore, regardless of colour, the upper panels were significantly more attractive (Paired T-Test, $P < 0.05$) than the lower panels. The results showed that, when the black panel was placed at the lower position, (60 cm above ground), the mosquitoes were not attracted to it. Thus, in this experiment, the results suggested that the height of the panels was a more important factor than surface texture in influencing mosquito landing behaviour.

Table 4.7 Mean ± SE of mosquitoes' landing frequency on investigation into the effect of surface texture. Mean number of mosquitoes per minute test on four different orientations with each test n=5.

(See **section *4.2.1.4*** and refer to **Figure 4.3**, page 86)

Test 1 (n=5)

Mean ± SE Number of mosquitoes/minute	
Black Plain (Upper)	White Net (Upper)
11.0 ± 1.0 **	1.8 ± 0.3
Black Net (Lower)	White Plain (Lower)
0.8 ± 0.1	0.1 ± 0.05

Test 2 (n=5)

Mean ± SE Number of mosquitoes/minute	
Black Net (Upper) **	White Plain (Upper)
11.9 ± 1.5	0.8 ± 0.5
Black Plain (Lower)	White Net (Lower)
1.2 ± 0.3	0.6 ± 0.2

Test 3 (n=5)

Mean ± SE Number of mosquitoes/minute	
Black Net (Upper)**	White Net (Upper)
9.3 ± 1.2	1.1 ± 0.2
Black Plain (Lower)	White Plain (Lower)
1.2 ± 0.1	0.6 ± 0.1

Test 4 (n=5)

Mean ± SE Number of mosquitoes/minute	
Black Plain (Upper)**	White Plain (Upper)
11.2 ± 1.6	0.2 ± 0.1
Black Net (Lower)	White Net (Lower)
1.2 ± 0.6	0.03 ± 0.03

*Black panel is significantly different from white panel (Paired T-Test, $P < 0.05$)

*Upper panel is significantly different from lower panel (Paired T-Test, $P < 0.05$)

(b) Duration of resting times

In the first test, mean resting time of unfed female *Ae. aegypti* per landing ranged from a low of 1.6 ± 0.6 minutes per landing on white plain to a high of 20.9 ± 1.8 minutes per landing on black plain (Table 4.8). There was significantly longer time recorded for those mosquitoes landing on the black panels compared to those landing on the white panels (Paired T-Test, $P < 0.05$). In contrast, the second and third tests showed no significant differences in resting time of mosquitoes between black panels and white panels and furthermore there were no differences in the resting time of mosquitoes on upper black compared to lower black (Paired T-Test, $P > 0.05$). On the other hand, in test four, there were significantly longer times recorded in both black plain and black net compared to both white plain and white net (Paired T-Test, $P < 0.05$). However, the resting time of those mosquitoes landing on the upper black were not different compared to those mosquitoes landing on the lower black (Paired T-Test, $P > 0.05$). Overall results showed that mean resting time per landing was found to vary from a minimum of 0.3 ± 0.3 minutes for white panels to a maximum of 20.9 ±1.8 minutes for black panels (Table 4.8).

Table 4.8 Mean ± SE of duration of resting times on investigation into the effect of surface texture. Mean resting time (min) per landing test on four different orientations with each test (n=5).

(See **section *4.2.1.4*** and refer to **Figure 4.3**, page 86)

Test 1 (n=5)

Mean ± SE Resting time (min)/landing	
Black Plain (Upper)**	White Net (Upper)
20.9 ± 1.8	7.6 ± 1.1
Black Net (Lower)	White Plain (Lower)
8.3 ± 2.6	1.6 ± 0.6

Test 2 (n=5)

Mean ± SE Resting time (min)/landing	
Black Net (Upper)	White Plain (Upper)
12.6 ± 5.4	5.4 ± 2.2
Black Plain (Lower)	White Net (Lower)
12.2 ± 2.9	6.0 ± 3.7

Test 3 (n=5)

Mean ± SE Resting time (min)/landing	
Black Net (Upper)	White Net (Upper)
9.0 ± 2.7	9.2 ± 4.2
Black Plain (Lower)	White Plain (Lower)
12.1 ± 3.0	6.6 ± 2.8

Test 4 (n=5)

Mean ± SE Resting time (min)/landing	
Black Plain (Upper)*	White Plain (Upper)
18.7 ± 2.9	0.3 ± 0.3
Black Net (Lower)*	White Net (Lower)
17.2 ± 3.9	2.7 ± 1.3

*Black panel is significantly different from white panel (Paired T-Test, $P < 0.05$)

*Upper panel is significantly different from lower panel (Paired T-Test, $P < 0.05$)

(c) Landing frequency over a 60-minute period

There were no significant differences in mean landing frequency at different time intervals over the 60 minutes for each test (Kruskal-Wallis Test, $P > 0.05$) (Figures 4.14 to 4.17). There were also no changes in the numbers of mosquitoes resting at each interval time. The mean number of mosquitoes at each interval was compared between 1, 5, 10, 20, 30, 40, 50 minutes to 60 minute and there were no significant differences at each interval time (Wilcoxon Signed Rank Test, $P > 0.05$). There was no significant difference between the textures of the black panel. The upper panel was more attractive than the lower panel to this species and it was proved that any black texture which was on the upper position was the most preferable to these mosquitoes.

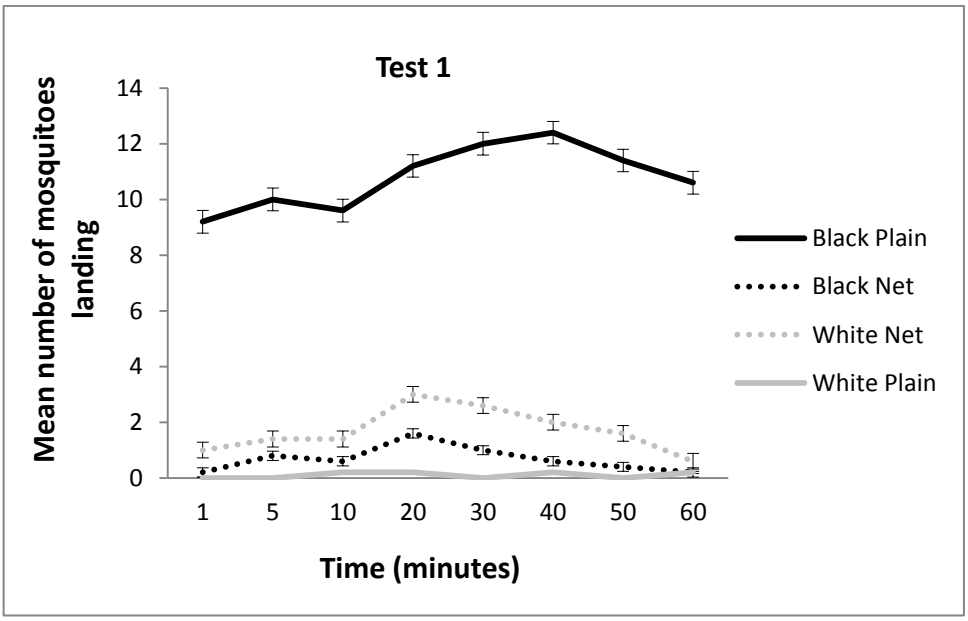


Figure 4.14 Comparison of mean number of female *Ae. aegypti* mosquitoes resting on each panel at eight different interval times. Means are displayed with ± standard errors; n=5 for Test 1

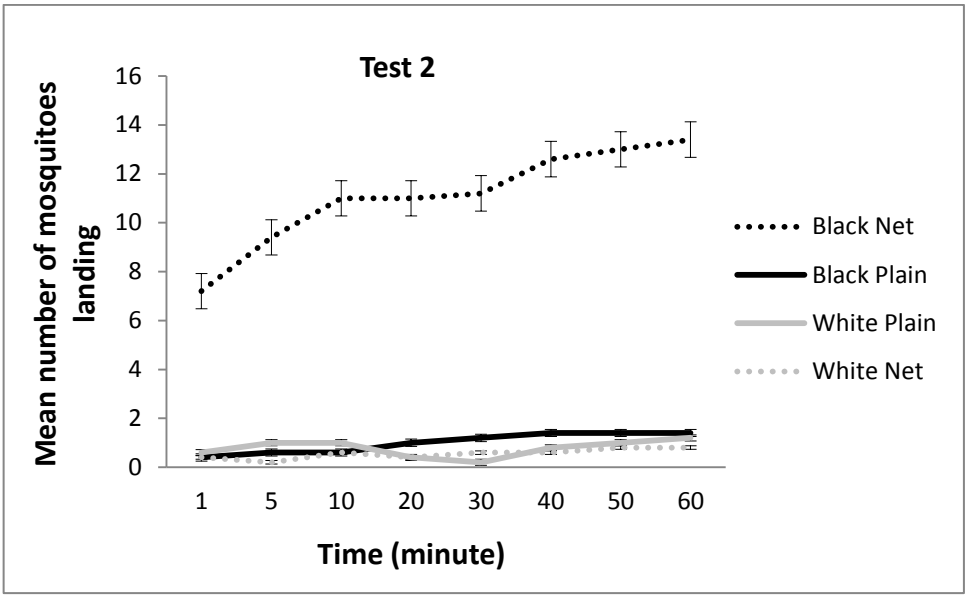


Figure 4.15 Comparison of mean number of female *Ae. aegypti* mosquitoes resting on each panel at eight different interval times. Means are displayed with ± standard errors; n=5 for Test 2

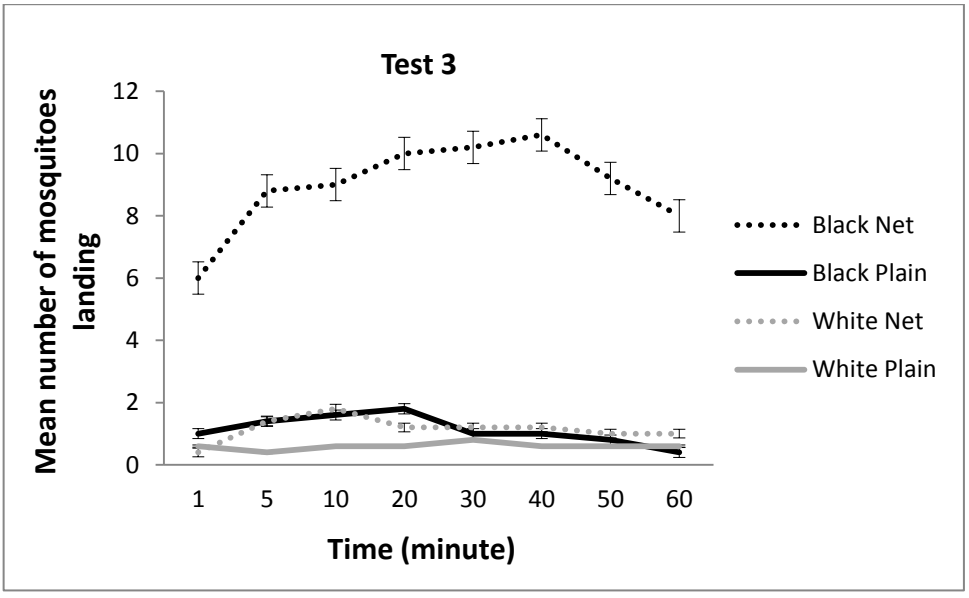


Figure 4.16 Comparison of mean number of female *Ae. aegypti* mosquitoes resting on each panel at eight different interval times. Means are displayed with ± standard errors; n=5 for Test 3

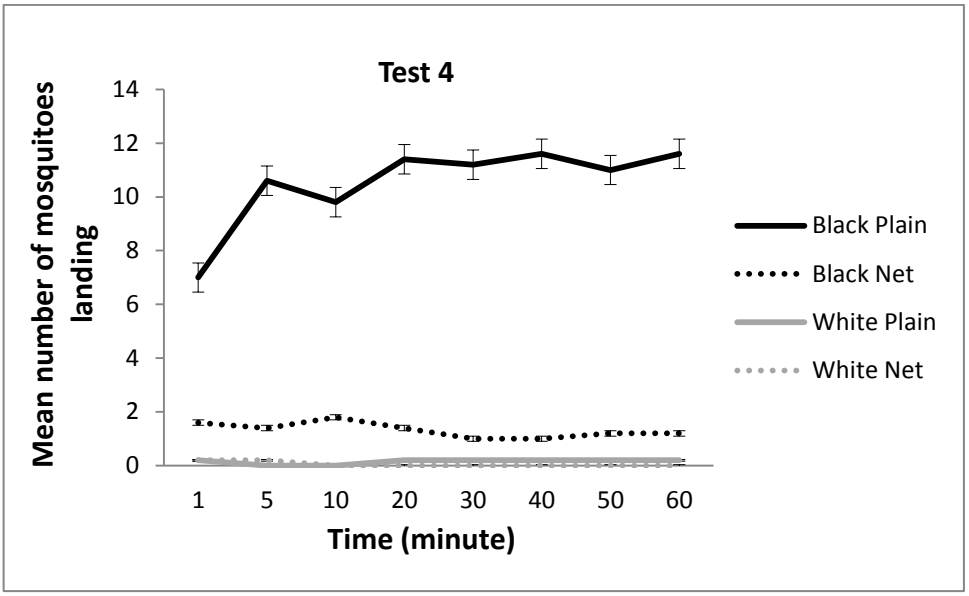


Figure 4.17 Comparison of mean number of female *Ae. aegypti* mosquitoes resting on each panel at eight different interval times. Means are displayed with ± standard errors; n=5 for Test 4

(c) Immediate landing activities

Mean number of mosquitoes landing in the first 60 seconds after release into the experimental room was considered as immediate landing. Immediate landing activities for four different tests are shown in Figure 4.18. The mean numbers of mosquitoes immediately landing during the first 60 seconds on the overall panels in four different tests were recorded from 8 to 10.4 mosquitoes. This result exhibited that an average of 16% to 20% unfed female *Ae. aegypti* immediately responded to the panels after being released into the experimental room.

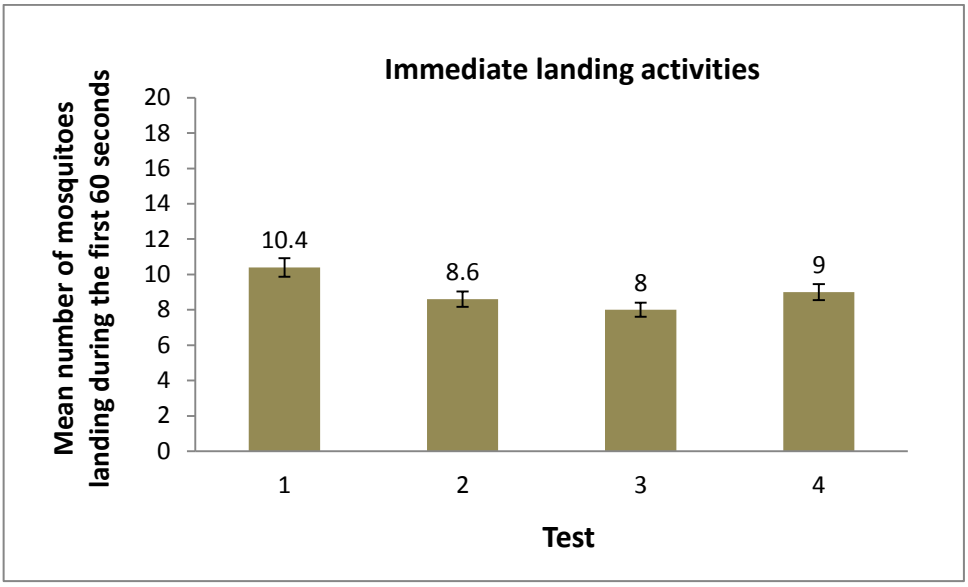


Figure 4.18 Mean numbers of mosquitoes landing during the first 60 seconds in four different tests

(d) Distribution of resting mosquitoes on panels

The ratio of the variance to the mean (χ^2, v) was calculated for mosquito distribution on resting panel. Dispersion indexes recorded in test 1 were (0.07, 4) for black plain, (0.37, 4) for black net, (0.22, 4) for white net and (1.66, 4) for white plain. In test 2, the results showed (0.08, 4) for black net, (0.4, 4) for black plain, (0.69, 4) for white plain and (0.57, 4) for white net. In test 3, there were (0.09, 4) for black net, (0.17, 4) for black plain, (0.3, 4) for white net and (0.5, 4) for white plain. In test 4, the dispersion indexes were (0.09, 4) for black plain, (0.47, 4) for black net, (1.5, 4) for white plain and (4.7, 4) for white net. Thus, it was concluded that resting position was random.

4.3.1.2 Investigation into the effect of surface orientation and contrast

(a) Landing frequency

For test 1 (vertical), the mean number ± SE of mosquito landings per minute recorded for black plain was 9.34 ± 0.5 mosquitoes per minute whereas it was only 1.89 ± 0.4 mosquitoes per minute for white plain. Significantly more landing per minute occurred on upper black compared to lower white (Paired T-Test, P =

0.001) (Table 4.9). For test 2 (vertical), the mean number ± SE of mosquito landings per minute recorded for white plain (upper) was 4.59 ± 0.9 mosquitoes per minute whereas 14.71 ± 0.8 mosquitoes per minute was recorded for black plain (lower). There was a significantly higher number recorded on black panel compared to white panel (Paired T-Test, $P = 0.001$) (Table 4.9).

In horizontal test 1, the mean mosquito landing per minute was 4.10 ± 0.9 for black plain and 1.84 ± 0.6 for white plain. The mean number landing on the black plain was not significantly different compared to the white plain (Paired T-Test, $P > 0.05$) (Table 4.9).

Furthermore, the data were compared between vertical and horizontal panels. For black panel, the results showed that significantly higher landings per minute occurred on vertical panel compared to horizontal panel (Paired T-Test, $P = 0.029$). For white panel, there was no significant difference recorded for both vertical and horizontal panels (Paired T-Test, $P = 0.142$). Therefore, this result revealed that more female mosquitoes rested on the vertical compared to the horizontal panel.

Table 4.9 Mean ± SE of mosquitoes' landing frequency for the investigation into the effect of surface orientation, vertical and horizontal. Mean number of mosquitoes per minute test on four different orientations with each test n=5
(See **section *4.2.1.5***, **Figure 4.6**, **page** 89)

Vertical Test 1 (n=5)

Mean ± SE Number of mosquitoes/minute	
Black Plain (Upper)**	White Plain (Lower)
9.34 ± 0.5	1.89 ± 0.4

Vertical Test 2 (n=5)

Mean ± SE Number of mosquitoes/minute	
White Plain (Upper)**	Black Plain (Lower)
4.59 ± 0.9	14.71 ± 0.8

*Black panel is significantly different from white panel (Paired T-Test, $P < 0.05$)
*Upper panel is significantly different from lower panel (Paired T-Test, $P < 0.05$)

Horizontal Test 1 (n=5)

Mean ± SE Number of mosquitoes/minute	
Black Plain	White Plain
4.10 ± 0.9	1.84 ± 0.6

Horizontal Test 2 (n=5)

Mean ± SE Number of mosquitoes/minute	
White Plain*	Black Plain
2.40 ± 0.004	14.02 ± 0.52

*Black panel is significantly different from white panel (Paired T-Test, $P < 0.05$)

The mosaic tests were conducted to investigate the effect of contrast on mosquitoes' landing behaviour on panels. There were significant differences in mean numbers of mosquitoes landing per minute for mosaic test 1 (Paired T-Test, $P < 0.05$). The mean landing frequency was significantly different among test

pairs except for [white plain (upper) compared to black net (lower)] and [white plain (upper) compared to white net (lower)] with $P = 0.60$ and $P = 0.270$ respectively. On the other hand, mosaic test 2 showed significant difference in overall test pairs except for [black net (upper) compared to black plain (lower)] and [white net (upper)] compared to white plain (lower)] with $P = 0.116$ and $P = 0.308$ respectively (Table 4.10). Moreover, the mean landing frequency was compared between mosaic test 1 and mosaic test 2. There were no differences recorded for both tests (Paired T-Test, $P > 0.05$). Nevertheless, the number of mosquitoes landing on the black panel was significantly greater than on the white panel, as recorded in both mosaic tests.

Table 4.10 Mean ± SE of mosquitoes' landing frequency for the investigation into the effect of contrast (mosaic test). Mean number of mosquitoes per minute test on two different orientations with each test n=5

(See **section *4.2.1.5***, **Figure 4.7**, page 90)

Mosaic Test 1 (n=5)

Mean ± SE Number of mosquitoes/minute	
Black plain (Upper)**	White plain (Upper)
14.26 ± 1.5	1.64 ± 0.4
White net (Lower)*	Black net (Lower)
3.37 ± 1.2	6.86 ± 1.8

Mosaic Test 2 (n=5)

Mean ± SE Number of mosquitoes/minute	
Black net (Upper)**	White net (Upper)
10.77 ± 2.7	2.65 ± 1.1
White plain (Lower)*	Black plain (Lower)
0.95 ± 0.3	4.86 ± 1.4

*Black panel is significantly different from white panel (Paired T-Test, $P < 0.05$)

*Upper panel is significantly different from lower panel (Paired T-Test, $P < 0.05$)

(b) Duration of resting times

In vertical tests, the mean resting time was 3.6 ± 0.5 minutes per landing for black plain and 5.5 ± 1.0 minutes per landing for white plain test (Table 4.11). Both vertical tests showed no significant differences in resting time of mosquitoes between black plain and white plain (Paired T-Test, $P > 0.05$). On the other hand, in the horizontal test, there was greatly longer mean resting time on the white panel compared to the black panel with average time 24.3 ± 10.0 to 55.9 ± 4.1 minutes per landing (Paired T-Test, $P = 0.001$) (Table 4.11).

Furthermore, analysis for both vertical and horizontal tests was carried out to examine if there were any differences in mosquitoes' resting time. For the black panel, the resting times were significantly longer on the horizontal test compared to the vertical test (Paired T-Test, $P = 0.028$) while for the white panel, the resting times were also significantly greater on the horizontal panel (Paired T-Test, $P = 0.048$). Thus, it is shown that longer resting times occurred in the horizontal test for both black and white panels.

Table 4.11 Mean ± SE of mosquitoes' duration of resting times. Mean resting time (min) per landing test on four different orientations with each test n=5
(See **section *4.2.1.5***, **Figure 4.6**, page 89)

Vertical Test 1 (n=5)

Mean ± SE Resting time (min)/landing	
Black Plain (Upper)	White Plain (Lower)
3.6 ± 0.5	5.5 ± 1.0

Vertical Test 2 (n=5)

Mean ± SE Resting time (min)/landing	
White Plain (Upper)	Black Plain (Lower)
5.5 ± 0.5	4.0 ± 0.8

Horizontal Test 1 (n=5)

Mean ± SE Resting time (min)/landing	
Black Plain*	White Plain
3.0 ± 0.4	24.3 ± 10.0

Horizontal Test 2 (n=5)

Mean ± SE Resting time (min)/landing	
White Plain*	Black Plain
55.9 ± 4.1	12.1 ± 2.0

*Black panel is significantly different from white panel (Paired T-Test, $P < 0.05$)

There were significant differences in mean resting time for test 1 (mosaic) (Paired T-test, $P > 0.05$) whereas in test 2 (mosaic), there were no significant differences in mean resting time (Paired T-test, $P > 0.05$). In addition, there were no differences recorded in mosquito duration of landing when compared between mosaic test 1 and mosaic test 2 (Paired T-test, $P > 0.05$).

Table 4.12 Mean ± SE of mosquitoes' duration of resting times for the investigation into the effect of contrast (mosaic test). Mean resting time (min) per landing test on two different orientations with each test n=5
(See **section *4.2.1.5***, **Figure 4.7**, page 90)

Mosaic Test 1 (n=5)

Mean ± SE Resting time (min)/landing	
Black plain (Upper)	White plain (Upper)
9.16 ± 2.0	18.6 ± 9.7
White net (Lower)	Black net (Lower)
31.32 ± 12.0	16.82 ± 5.7

Mosaic Test 2 (n=5)

Mean ± SE Resting time (min)/landing	
Black net (Upper)	White net (Upper)
8.09 ± 1.2	11.44 ± 2.5
White plain (Lower)	Black plain (Lower)
28.6 ± 13.0	10.51 ± 1.0

(c) Landing frequency over a 60-minute period

The combined mean numbers of landing frequency over 60 minutes were recorded in (Figures 4.19 to 4.21) for vertical, horizontal and mosaic tests respectively. There were no significant differences in mean landing frequency for both tests at eight time points of 60 minutes for each test (Kruskal Wallis Test, $P > 0.05$). There were also no changes in the numbers of mosquitoes resting between time points. The mean numbers of mosquitoes at each point were compared to 60 minutes and there were no significant differences at each time interval (Wilcoxon Sign Rank Test, $P > 0.05$).

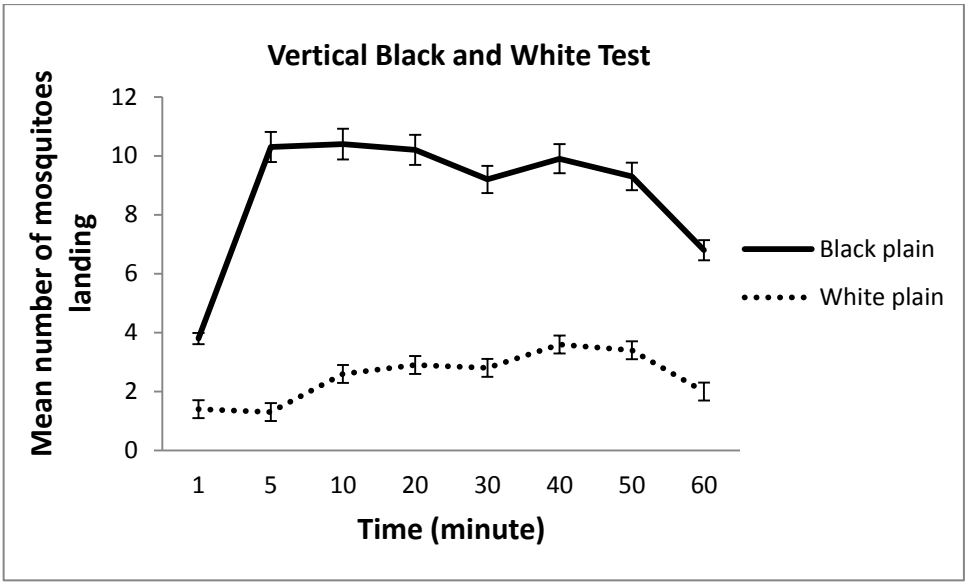


Figure 4.19 Comparison of combined mean number of female *Ae. aegypti* mosquitoes resting on vertical black and white panels at eight different time interval. Combined means are displayed with ± standard errors; n=10

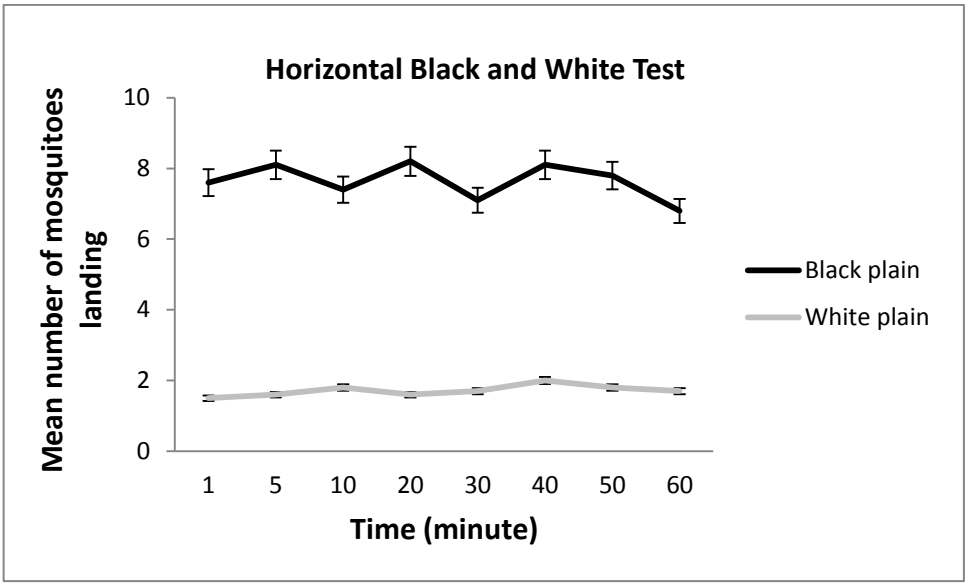


Figure 4.20 Comparison of combined mean number of female *Ae. aegypti* mosquitoes resting on horizontal black and white panels at eight different time interval. Combined means are displayed with ± standard errors; n=10

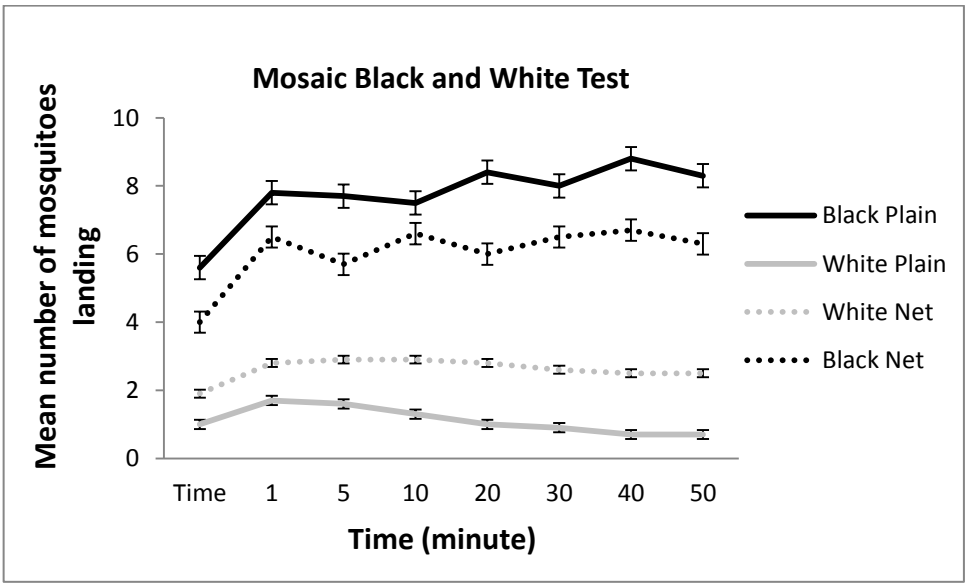


Figure 4.21 Comparison of combined mean number of female *Ae. aegypti* mosquitoes resting on mosaic black and white panels at eight different time intervals. Combined means are displayed with ± standard errors; n=10

(d) Landing distribution

Indexes of dispersion recorded from vertical and horizontal tests were less than one. For vertical tests, the ratio of the variance to the mean (χ^2, v) was (0.07, 4) for the black panel and (0.3, 4) for the white panel. In horizontal tests, the values were recorded as (0.15, 4) for the black panel and (0.26, 4) for the white panel. Thus, it was concluded that resting position was random.

The combined ratios showed that values recorded both in mosaic test 1 and 2 were (0.14, 4) black plain, (0.4, 4) white plain, (0.27, 4) white net and (0.15, 4) black net. Therefore, there was no evidence for over dispersal of the resting positions and it was concluded that resting position was random.

4.3.1.3 Investigation into the effect of height on landing preference

(a) Landing frequency

The mean number ± SE of mosquitoes landing per minute recorded in the black test at 200 cm height was 5.4 ± 2.7 mosquitoes per minute for black plain whereas it was 6.18 ± 3.8 mosquitoes per minute for black net. The experiment at 90 cm height showed that black plain was 12.1 ± 1.3 whereas black net was 12.5 ± 2.2 mosquitoes per minute. There were no significant differences in mean mosquitoes landing per minute for both black plain and black net panels (Paired T-Test, $P > 0.05$) (Table 4.13). Further analysis was carried out to compare mosquito landing frequencies between black test at 200 cm and black test at 90 cm. The results showed that there were differences recorded between black plain (200 cm) and black net (90 cm) (Paired T-Test, $P = 0.001$). Again, there were significantly greater numbers of mosquitoes landing per minute recorded in the black test at 90 cm compared to the black test at 200 cm.

Table 4.13 Mean SE of mosquitoes' landing frequency in the investigation into the effect of height on landing preference. Mean number of mosquitoes per minute on two different heights with each test n=10

(See **section *4.2.1.6***, **Figure 4.8**, page 91)

Black Test at 200 cm height (n=10)

Mean ± SE Number of mosquitoes/minute	
Black Plain*	Black Net*
5.4 ± 2.7	6.18 ± 3.8

Black Test at 90 cm height (n=10)

Mean ± SE Number of mosquitoes/minute	
Black Plain	Black Net
12.1 ± 1.3	12.5 ± 2.2

*Black test at 90 cm height is significantly higher than black test at 200 cm height (Paired T-Test, $P < 0.05$)

(b) Duration of resting times

For the test of black coloured material, hanging from the ceiling, 200 cm height, mean resting time per landing was recorded as 7.9 ± 4.0 minutes per landing for black plain and 7.8 ± 4.3 minutes per landing for black net. For the black test placed at 90 cm height, mean resting time was recorded as 10.9 ± 4.7 for black plain and 10.8 ± 3.5 for black net (Table 4.14).

Table 4.14 Mean ± SE of mosquitoes' duration of resting times in the investigation into the effect of height on landing preference. Mean resting time (min) per landing test on black surfaces at two different heights (n=10)
(See **section *4.2.1.6***, **Figure 4.8**, page 91)

Black Test at 200 cm height (n=10)

Mean ± SE Resting time (min)/landing	
Black Plain	Black Net
7.9 ± 4.0	7.8 ± 4.3

Black Test at 90 cm height (n=10)

Mean ± SE Resting time (min)/landing	
Black Plain	Black Net
10.9 ± 4.7	10.8 ± 3.5

(c) Landing frequency over a 60-minute period

In black test at 200 cm height and black test at 90 cm height, the combined mean numbers of landing frequency over 60 minutes were recorded in (Figures 4.22 to 4.23). There were no significant differences in mean landing frequency for both tests at eight time points of 60 minutes for each test (Kruskal Wallis Test, $P > 0.05$). There were also no changes in the number of mosquitoes resting at eight time points (Wilcoxon Sign Rank Test, $P > 0.05$).

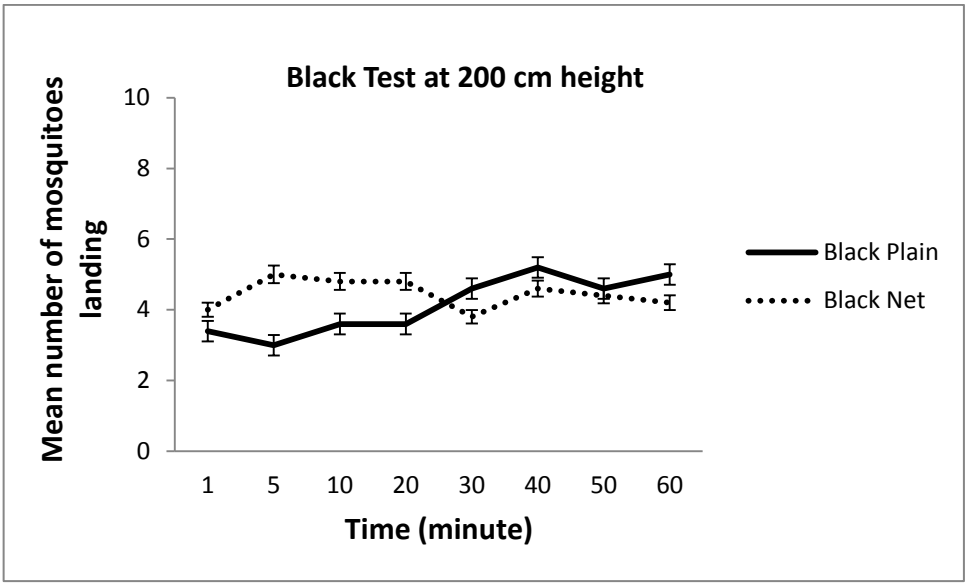


Figure 4.22 Comparison of combined mean number of female *Ae. aegypti* mosquitoes resting on high black plain and black net panels at eight different time points. Combined means are displayed with ± standard errors; n=10

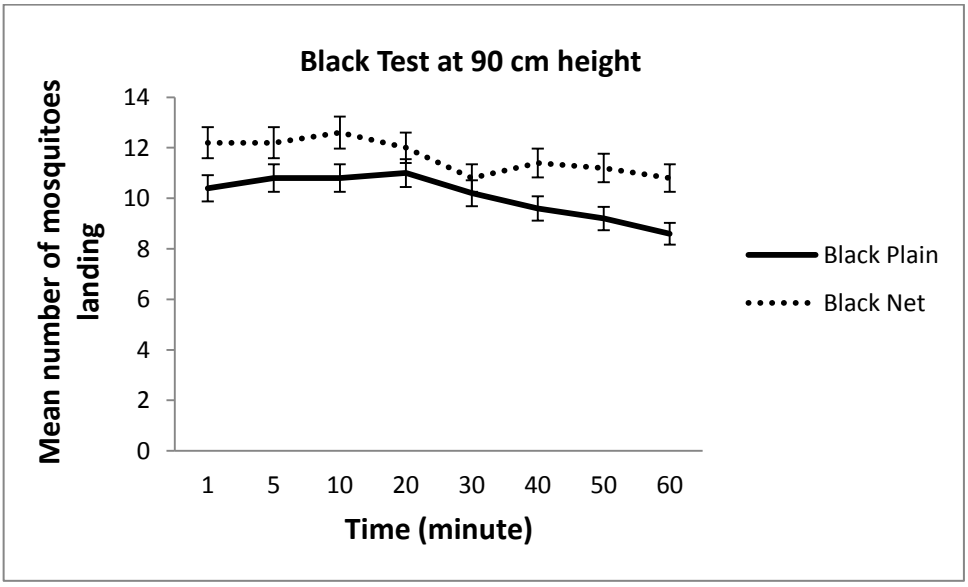


Figure 4.23 Comparison of combined mean number of female *Ae. aegypti* mosquitoes resting on low black plain and black net panels at eight different time interval. Combined means are displayed with ± standard errors; n=10

(d) Landing distribution

For the high black tests, the ratio of the variance to the mean (χ^2, v) was (0.2, 4) for black plain and black net. In the low black tests, the values were recorded as (0.04, 4) for black plain and (0.05, 4) for black net. These results concluded that the mosquitoes were also randomly distributed on the panels.

(e) Immediate landing activities

Immediate landing activities for overall tests are shown in Figure 4.24. The combined mean numbers of mosquitoes that landed during the first 60 seconds on the target panel are illustrated for each test. The result showed that an average of 10.4% to 25.0% of the unfed female *Ae. aegypti* responded to the target panels after being released into the experimental room.

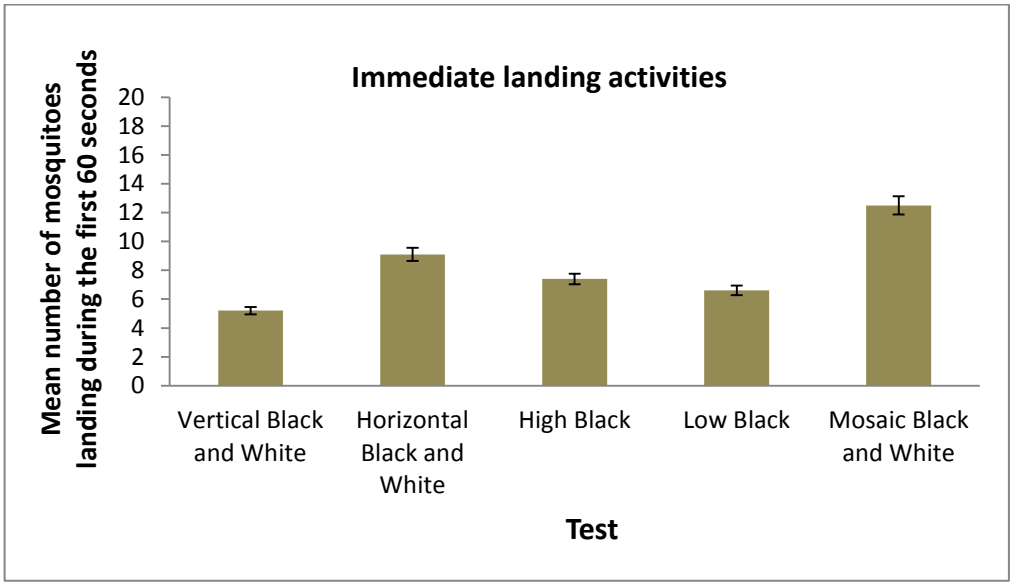


Figure 4.24 Combined mean number of mosquitoes landing during the first 60 seconds on overall panels in each different test

4.3.1.4 Investigation into the effect of adhesive

Two different tests were performed to test for any effect of the glue. There were black test at 200 cm height and black tests at 90 cm height. Black plain and black net panels were both coated with sticky glue. The combined mean number of mosquitoes caught in black test at 200 cm height and the black test at 90 cm height were 1.4 ± 2.0 and 2.2 ± 1.5 respectively. Analysis for landing frequency and landing distribution of mosquitoes caught on the panels could not be carried out since the numbers of mosquitoes caught in these tests were too low.

4.3.2 Preliminary trials and laboratory trials using resting boxes

In order to test the resting box as a potential tool for measuring mosquito landing and resting behaviour, two different types of resting boxes were developed and used in the field to determine if the resting boxes were capable of capturing mosquitoes (see **section *4.22***, **Figure 4.10** to **Figure 4.11**, page 96-97).

The only mosquito species collected during field trials was *Culex quinquefasciatus* and no *Aedes* mosquitoes caught inside these two resting boxes. Total numbers of mosquitoes captured daily in one house was recorded between 13th to 17th July for every four hours' collection time and 18th to 22nd July 2012 for every six hours' collection time. The results of this preliminary trial are shown in Tables 4.15 and 4.16 below.

Table 4.15 Number of mosquitoes (*Culex quinquefasciatus*) collected inside resting boxes over four hours

Day	Resting box Type 1	Resting box Type 2
1	11	8
2	1	0
3	3	8
4	5	3
5	3	6
Total of mosquitoes caught	23	25
Mean ± SE per day	4.6 ± 3.8	5 ± 3.5

*resting box type 1(outside the house); resting box type 2 (inside the house)

Table 4.16 Number of mosquitoes (*Culex quinquefasciatus*) collected inside resting boxes over six hours

Day	Resting box Type 1	Resting box Type 2
1	3	4
2	1	4
3	2	2
4	4	7
5	6	6
Total of mosquitoes caught	16	23
Mean ± SE per day	3.2 ± 1.9	4.6 ± 2.0

*resting box type 1 (outside the house); resting box type 2 (inside the house)

In resting box type 1, a total of 39 mosquitoes were collected, consisting of 33 (84.6%) fed and unfed female mosquitoes and 6 (15.4%) male mosquitoes. In resting box type 2, a total of 48 mosquitoes were captured consisting 44 (91.7%) fed and unfed female mosquitoes and 4 (8.3%) male mosquitoes. The data showed that resting boxes collected more female than male mosquitoes. No significant difference was observed between the mean numbers of mosquitoes collected in resting box type 1 and 2 (Wilcoxon Signed Rank Test, $P = 0.786$) for every four-hour sampling time and (Wilcoxon Signed Rank Test, $P = 0.102$) for every six-hour sampling time. The number of mosquitoes collected at four hours was not significantly different compared to the number of mosquitoes collected at six hours (Wilcoxon Signed Rank Test, $P > 0.05$).

4.3.2.1 Evaluation of the resting box in the laboratory

(a) Effect of temperature and humidity

The resting box trials carried out in the field conditions were replicated in the laboratory to determine if mosquitoes were affected by the temperature and humidity inside the resting box. The room temperature and humidity for the experimental room as well as the temperature and humidity inside the resting boxes were recorded using TinyTag and fully described in Table 4.6 (page 86). A total of 10 replicates with a total of 1,500 mosquitoes were released during the experiment. For the control experiment, 25.2% (n = 500) were found resting

inside the box. There were 61.4% (n = 500) and 21.6% (n = 500) mosquitoes recorded resting inside the box for test 1 and test 2 respectively. Mean ± SE of mosquitoes collected from resting boxes for control, test 1 and test 2 were 12.6 ± 3.3, 30.7 ± 5.1 and 10.8 ± 2.3 respectively.

Pairwise comparisons between each of the experiments found that there were significantly higher numbers of mosquitoes collected from the resting box in test 1 compared to the control (Wilcoxon Sign Rank Test, $P = 0.005$). There were also significantly higher numbers of mosquitoes collected from the resting box at test 1 compared to test 2 (Wilcoxon Sign Rank Test, $P = 0.005$). However, there was no difference found in the numbers of mosquitoes resting inside the box between the control and test 2 (Wilcoxon Sign Rank Test, $P = 0.172$).

(b) Effect of orientations of resting box entry

Two different tests were conducted to determine if the mosquitoes had any preferences regarding entry-point orientation. When the opening of the resting box was placed in an upward-facing position, it was described as a vertical entry whereas if the opening of the resting box was placed in the side position, it was described as a horizontal entry. Ten replicates were conducted for each test, with a total of 1,000 mosquitoes released during the experiments. For both experiments, 57% (n = 500) mosquitoes were found resting inside the boxes with vertical entry whereas 57.4% (n = 500) mosquitoes were found resting inside the boxes with horizontal entry. Mean ± SE numbers of mosquitoes resting inside the boxes with vertical and horizontal entry were 28.5 ± 3.1 and 28.7 ± 2.6 respectively. Therefore, the numbers of mosquitoes resting inside each of the resting boxes were not significantly different (Wilcoxon Signed Rank Test, $P > 0.05$)

4.4 Discussion

Female *Ae. aegypti* mosquitoes exhibited a marked preference for the black panel placed at 90 cm height compared to the black panel placed at 200 cm in the experimental room. There was higher landing frequency recorded on the vertical orientation. Although many landing activities occurred on the vertical orientation,

the longer resting times were, in contrast, recorded on the horizontal orientation. On the other hand, there was no evidence that texture of the panel (plain or net) or the adhesive-coated panel were attractive to the mosquitoes. Overall, experiments revealed that female *Ae. aegypti* were randomly distributed on the panels based on models of dispersion.

In this study, it was found that female *Ae. aegypti* clearly preferred the black panel compared to the white panel. Mean landing frequency on the white panel was significantly lower compared to the black panel (Table 4.7). This evidently shows that this species prefers to be in dark areas rather than bright areas during resting. This result has a similar finding to Manda *et al.* (2011) and Thainchum *et al.* (2013), which showed that the landing rate of the female mosquito was higher on dark materials rather than on lighter ones. This mirrors natural settings, as this species shows a preference for shade and dark corners and generally avoids bright light and open spaces (Sippell & Brown, 1953; Muir *et al.,* 1992). The studies by Gjullin (1947), Brown (1954) and Gilbert and Gouck (1957) pointed out that the landing rate of different species of *Aedes* was influenced by surface colour. Black colour was recognised to have the lowest reflectance factor whereas white colour has the highest reflectance factor. It is also previously recorded that dark colours including black are highly attractive as a resting surface to female *Ae. aegypti* (Sippell & Brown, 1953; Schoof, 1967; Muir *et al.,* 1992).

In the investigation into the effect of texture, the plain or net surfaces did not affect landing and resting behaviour of this species. The flock papers or velvet material with and without netting were used to investigate the effect of texture on mosquito landing rate. There were no differences between black or white velvet material and black or white velvet with net. This finding suggested that the reflectivity from this material is the same as if it is used with netting. However, if this material is compared to other materials such as cotton, polyester, nylon, and satin, it is expected that there would be differences in their reflectivity and texture profiles. A recent study by Manda *et al.* (2011) demonstrated that the landing rates on cotton texture are much greater than on polyester texture both under insecticide

and insecticide-free conditions. On the other hand, Thainchum *et al.* (2013) reported that a higher number of mosquitoes rested on cotton fabric compared to polyester. Different fabric materials or textures have been proven to have different humidity and reflectance properties. For instance, cotton exhibits greater moisture absorption than polyester (Su *et al.,* 2007). Earlier studies by Brown (1951) demonstrated that the number of mosquitoes attracted to black crepe is significantly higher than to black satin. According to Merton and Merton (1956), certain textures of surfaces were preferred for settling and resting by other insect pests. This includes wool jersey, wood and Styrofoam. However, smooth surfaces such as cellulose, acetate sheet, glass and surfaces coated with polytetrafluorethylene were less favourable for settling behaviour. The information on texture profiles and preferences could be valuable in optimising various vector control tools and products designed for resting target attraction.

The results reported in this study indicate that female *Ae. aegypti* prefer black vertical surfaces compared to horizontal surfaces (Table 4.9). The reason for this preference is unclear and it might be due to some innate behaviour of *Ae. aegypti*. The greater numbers observed resting on vertical surfaces in this current study are possibly due to the landing position and the number of legs that were in contact with the surface areas. Generally, in the landing position, support is mainly given by the fore-legs and mid-legs whereas the hind-legs are raised up in the air (Christophers, 1960). Studies reported by Hansel (1970) discovered that *An. gambiae* mosquitoes resting on a vertical surface were often in contact with the surface by only two pairs of front legs. However, he also observed that the number of legs in contact with the surface differs depending on the activities of the mosquitoes. In contrast, a previous study on mosquito resting position in *Anopheles maculipennis atroparvus* by Ungureanu *et al.* (1961) demonstrated that each pair of legs has a different amount of contact when on a vertical surface. The fore-legs were found to have minor contact whereas the hind-legs have major contact when resting on a vertical surface.

Duration of resting times recorded here demonstrated that mean resting time is significantly higher on the horizontal surface compared to the vertical surface (Table 4.11). This finding is contrary with the results of landing frequency. This result is also contrary to the finding by Manda *et al*. (2011) where they found that number of resting mosquitoes increased when the resting material was in horizontal orientation. Although many landing activities were recorded on the vertical surface, the mean resting time on this surface is lower compared to the horizontal surface. This result showed that, in the horizontal position, mosquitoes tend to rest longer than in the vertical position but fewer landing activities occurred in this position.

In the experimental tests on the effect of contrast, the results showed that there were significant differences in mean mosquito landings per minute in most black and white test pairs (Table 4.10). In addition, the second contrast test also showed significant differences in most test pairs (Table 4.10). These results explain that the principal contrast of black and white significantly attracted mosquitoes to land and rest on the dark areas compared to the lighter one. Therefore, it is evidently shown that the contrast factor affects the visual stimuli of mosquitoes in their landing behaviour. In Zimbabwe, a similar contrast principle has been used in sampling tsetse flies, *Glossina morsitans morsitans* Westwood and *Glossina pallidipes* Austen in the field. The biconical trap used the same contrast factor (dark and light surfaces) in attracting tsetse flies (Flint, 1985). The performance of this trap has been improved with various colour and shade combinations, both inside and outside the trap. The comparison was made between outside lower cone with white, black or blue and also the same range of colours inside of the lower cone. Furthermore, black on the upper cone and white on the lower cone was also compared with white on the upper cone and black on the lower cone. The biconical trap was used for sampling tsetse flies at dense population level. Initially, this trap efficiently captured tsetse flies without any additional odour attractant. However, the performance of this trap has been increased by the presence of chemical attractant, particularly at low population densities (Flint, 1985). Further experiments using screens or panels to capture tsetse flies have

been conducted using a two-coloured screen. The studies by Green (1989) demonstrated that the diagonal bi-coloured screen was highly attractive to the tsetse flies. This combination of blue and white screen caught 2.4 times more tsetse flies than the full blue screen.

In the height preference test, landing frequency on the black test at 90 cm height was significantly higher compared to the black test at 200 cm height (Table 4.13). Similar finding have been obtained from experimental hut studies where greater proportions of female *Ae. aegypti* population were observed resting on the lower part of the wall (Thainchum *et al.,* 2013). Other laboratory studies have also reported similar observations on *Anopheles* and *Culex* mosquitoes, where these species preferred to rest on the lower part than the upper part of the test box (Gjullin *et al.,* 1963). On the other hand, the duration of resting times reported here was not significantly different.

The rationale for developing the adhesive panel was to assess its potential to trap mosquitoes. The idea of coating attractive panels with adhesive is to convert them into traps. However, the numbers of mosquitoes caught in this test was too low. Low numbers of mosquito caught in this test possibly due to the fact that the mosquitoes could simply escape from the coated glue when they first contact the panel. This result supported the finding reported by Browne and Bennett (1981) in their field experiments. They found that coating a trap with 'Tanglefoot' was ineffective in catching *Coquillettidia perturbans*, *Ochlerotatus cantator* and *Ochlerotatus punctor*. These species of mosquito tended to hover around and 'feel' the surface by extending a leg before landing. Their experience of the sticky surface caused these mosquitoes to reverse flight and escape. In other situations, the disadvantage of the sticky surface was that the panels could be covered by extraneous materials such as dust, sand, seeds and any unwanted trapped insects.
The results of this study have demonstrated that there were low numbers of mosquitoes captured inside the current resting boxes during preliminary field trials. The low capture rate may be due to a number of reasons, which include: 1) the placement of the resting boxes, 2) difference in temperature and humidity

inside and outside the resting boxes and 3) numbers of resting boxes installed per house. Other explanations could also be due to the numbers of houses and sampling periods involved for this preliminary trial, i.e. only one house was used to sample resting mosquitoes in 10 days, arguably capturing low numbers of mosquitoes.

There was no *Aedes* species collected from this trial although it was originally expected to be present inside the current resting box. *Aedes* species possibly experienced less competition for other nearby resting sites and therefore only *Cx. quinquefasciatus* were captured inside the resting boxes. Although the resting boxes have been designed to collect *Ae. aegypti*, they successfully collected *Cx. quinquefasciatus*. The data demonstrated here may serve as a starting point for further investigations on the use of resting boxes to capture *Cx. quinquefasciatus*. The numbers of mosquitoes captured were not significantly different between resting boxes placed indoors or outdoors. The four-hour and six-hour sampling times also had no effect on the catch. Similar results were obtained by Kittayapong *et al.* (1997) where 3 - 4 hour sampling interval in the morning had no effect on the number of mosquitoes collected. Although there were no differences between the numbers caught inside the current resting boxes, the number of female mosquitoes (84.6%) was higher than male mosquitoes (15.4%). Although the differences were not significant, the study by Kittayapong *et al.* (1997) also demonstrated that more resting females were captured from the boxes placed in the dark corners of the house.

In the laboratory trials, the study concentrated on the effect of temperature and humidity that might affect resting box performance and opening surface orientation (vertical or horizontal). The total number of mosquitoes caught during the control test (50% - 64% humidity) was significantly lower compared to test 1 (65% - 80% humidity). The humidity inside the resting box in test 1 was considerably higher than the experimental room. This result indicated that female *Ae. aegypti* preferred to rest in the area where the humidity is high. This finding is similar to the results obtained by Yasuno *et al.* (1976) where their traps were

effective when positioned in low humidity areas. The boxes contained a sponge saturated with water that certainly increased internal humidity, which was significantly different compared to the surrounding areas. Field trials conducted in Trinidad by Nathan (1981) used similar resting boxes to those used by Yasuno e*t al.* (1976). A total of 1,720 female *Cx. quinquefasciatus* and substantial numbers of *Ae. aegypti* were collected from resting boxes placed inside houses. These resting boxes comprised open-ended 30 cm^2 plywood painted white on the outside and black inside. The resting boxes were also provided with a screened jar of water to increase the internal humidity and therefore improve their attractiveness.

In addition, the opening surface orientation of the resting box in the current laboratory trials did not make a difference to the number of mosquitoes collected. The opening surface orientation also did not affect its attractiveness to the mosquitoes. The results obtained in this trial indicated that the temperature and humidity inside and outside the resting box was the most important factor in improving the efficacy of this sampling tool. Other studies have used a variety of resting box models, therefore revealing considerably varying results with regard to their performance in the field. A great variety of simple resting boxes (Morris, 1981; Crans, 1989; Nasci *et al.,* 1993; Edman *et al.,* 1997; Kittayapong *et al.,* 1997; Harbison *et al.,* 2006; Burkett-Cabena *et al.,* 2008; Kweka *et al.,* 2009) has been used to collect a variety of mosquito species. Nevertheless, it was relatively impossible to compare their efficiencies with the present resting box because each of them have operated in different areas to collect different species and have varied in material, colour and location (Silver, 2008). Although only *Cx. quinquefasciatus* mosquitoes (87 mosquitoes in 5 days) were caught inside the resting boxes during the field experiments, this encouraging result may lead to the development of an efficient resting box for sampling adult *Cx. quinquefasciatus* mosquito populations in the future.

4.5 Limitations and future work

The important information obtained from these laboratory trials could be used to improve the effectiveness of mosquito visual targets and traps in the future.

Laboratory trials on wild mosquito strains should be conducted with males as well as females. Responses to insecticide-treated surfaces would also be required to ensure there was no additional repellent effect. Improved behavioural information on mosquito landing frequency and duration of resting times could be beneficial in the implementation of surveillance and vector control programmes in the future. Little experimental data exist for the evaluation and performance of resting boxes for dengue vectors. Further work is critically needed to explore the potential of this method before this could be used as one of the sampling tools in dengue surveillance and control programmes in the future.

4.6 Conclusions

Laboratory trials using two-dimensional panel targets as resting sites

1) The best combination of parameters on a resting panel for attracting *Ae. aegypti* was black colour, vertical orientation and at 90 cm height. The contrast of the panels also plays an important role in the attractiveness for mosquito landing behaviour.
2) There was no evidence that texture of the panel (plain or net) and adhesive factor had any significant effect on the attractiveness of the resting panel.
3) Overall experiments demonstrated that females *Ae. aegypti* were randomly distributed on target panels and therefore demonstrated no clear preferences for the upper, middle or lower part of each target panel.

Preliminary field trials and laboratory trials using resting boxes

1) Two resting box designs were ineffective in capturing *Ae. aegypti* and *Ae. albopictus* in field trials, but successfully trapped *Cx. quinquefasciatus* both inside and outside the house, when deployed for four or six hours.
2) In laboratory trials, significantly greater numbers of *Ae. aegypti* were captured in resting box type 1, with higher numbers captured in the box with raised humidity (65-80% humidity).
3) The orientation of the entry to the resting box did not affect capture efficacy.

CHAPTER 5

EXPLOITING VECTOR BEHAVIOUR FOR DENGUE CONTROL BY INDOOR RESIDUAL SPRAYING (IRS): A FIELD EXPERIMENT IN PENANG, MALAYSIA

5.1 Introduction

Dengue was first recorded in Penang over a century ago by Skae in 1902. In Penang, *Ae. aegypti* mainly occurs in the urban areas whereas *Ae. albopictus* is present in high numbers mostly in rural areas (Saifur *et al.,* 2012a). The study by Saifur *et al.* (2012a) also indicated that these mosquito species have spread to the northeast district of Penang Island, with moderate to high population densities. Dengue cases were reported from both urban and rural areas in Penang dominated by *Ae. aegypti* and *Ae. albopictus*. The numbers of dengue patients recorded in Penang were higher in *Ae. aegypti*-infested areas than those in *Ae. albopictus*-infested areas (Saifur *et al.,* 2012a). The dominant indoor breeder is *Ae. aegypti*, though both *Ae. aegypti* and *Ae. albopictus* show an equal preference for outdoor containers. In 2009, a survey conducted in Northern Peninsular Malaysia revealed that more than half of the immature stages of *Ae. aegypti* were found in outdoor containers (Saifur *et al.,* 2012b). The adaptation of this species to outdoor or peridomestic breeding together with indoor breeding behaviour potentially increases the biting activity of this vector species both indoors and outdoors, which may contribute to disease transmission (Saifur *et al.,* 2012b). However, Dieng *et al.* (2010) observed that *Ae. albopictus* also breeds indoors in many part of Penang Island.

Vector control programmes can involve the spraying of insecticide inside and outside houses of positive dengue cases. Space-spraying is commonly used during an outbreak of dengue fever, when regular application is required to maintain control of adult mosquitoes (WHO, 1997). Although Indoor Residual Spraying (IRS) is not recommended for dengue control, IRS is likely to impact on dengue vectors, as shown by the fact that *Ae. aegypti* was eliminated from some areas

where IRS had been used for malaria control in the past (WHO, 2006b). WHO (2013d) consider IRS to be highly effective in the areas where the vectors preferentially feed and rest indoors, with the potential to rapidly reduce adult mosquito vector density and longevity, and therefore reduce disease transmission.
A previous study in Mexico by Arredondo-Jiménez *et al.* (1995), demonstrated that control of *An. albimanus* mosquitoes could be achieved by spraying only the preferred indoor resting sites and that this required less time and was more cost-effective than conventional IRS. In a preliminary study of *Ae. aegypti* resting behaviour in Yucatan, Mexico, it was found that resting was highest on the ceiling and upper walls inside houses (Bowman & McCall, *unpublished data*). The study demonstrated that *Ae. aegypti* preferentially rested on the upper half of the wall or the ceiling when room temperatures were less than 30^{o}C inside the house. Therefore, it was hypothesised that treatment of the upper parts of the walls and the ceilings inside a house would provide as much protection as treatment of the entire house. Moreover, this 'strip IRS' treatment potentially would cost less and be carried out more rapidly than standard IRS and therefore represent a more satisfactory and effective approach for use in urban areas of high human population density. This was tested in a small field trial in Penang in Malaysia.

The aim of the present study was to compare the impact of two insecticides delivered either by standard IRS (entire surface sprayed) or the 'strip IRS' method (ceiling and top 1m of walls) on *Aedes spp.* and *Cx. quinquefasciatus*. Thus, this study was also conducted to determine whether treating only the preferred indoor resting sites of *Ae. aegypti* could target and reduce populations of this vector.

5.2 Methods

5.2.1 Study area

The area of the study covered the unregulated 'squatter' housing area of Ujong Batu in Bagan Dalam, Butterworth, located on the mainland of Penang state (05°23.289" N 100°22.397" E; altitude 11 m) (Figure 5.1). This site is approximately 8.5 km from Universiti Sains Malaysia (USM), which is located in the island part of *Penang*. The site was chosen as it was close to USM, and the

housing provides a micro-representation of the larger housing communities elsewhere within the region (i.e. a dense housing area with small individual houses) and, by extension, housing communities in many urban areas worldwide. The area experiences a typical tropical climate of a hot season from April to July, followed by heavy rains from August to November, with the dry season normally beginning in December and ending in March. The average annual rainfall is 267 mm with a consistent temperature ranging from 23°C to 32°C.

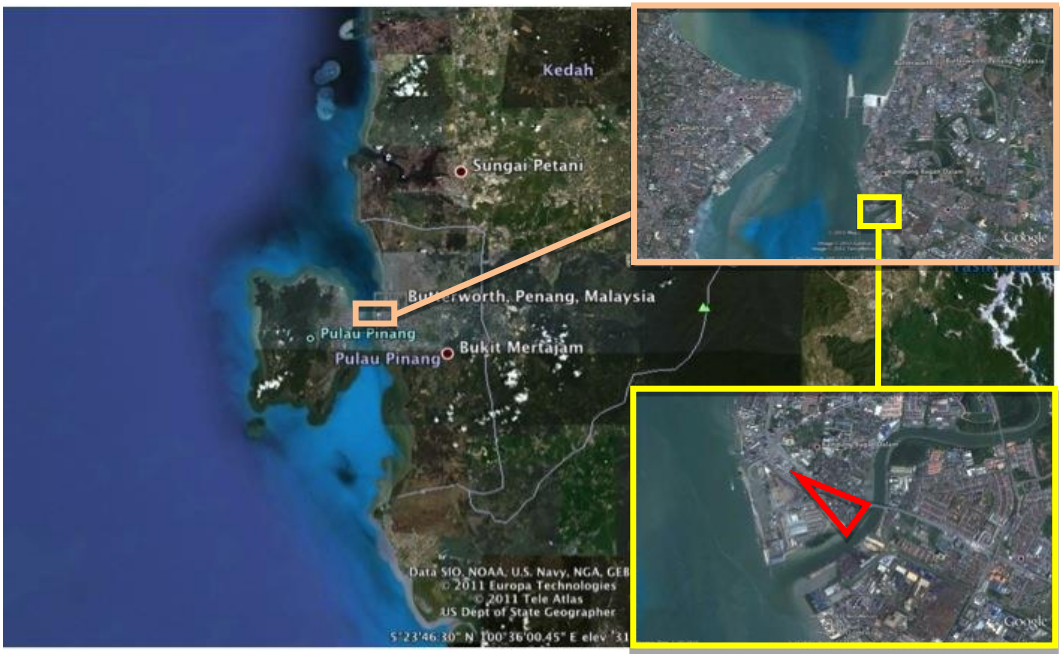


Figure 5.1 Location of Bagan Dalam, Butterworth (yellow square) and the study area of Ujong Batu (red triangle) in Penang, Malaysia

5.2.2 Study design

The study area was Ujong Batu, a village containing 123 houses. The village was divided into five clusters, with each cluster containing an approximate equal number of houses. With a cluster randomised trial design, allocation to treatment arm was assigned randomly: (1) Cluster 1 received Actellic strips, (2) Cluster 2 received no intervention, (3) Cluster 3 received Actellic 100%, (4) Cluster 4 received Icon 100% and (5) Cluster 5 received Icon strips. An image showing this classification is provided in Figure 5.2 and photographs showing the baseline activities are provided in Figure 5.4.

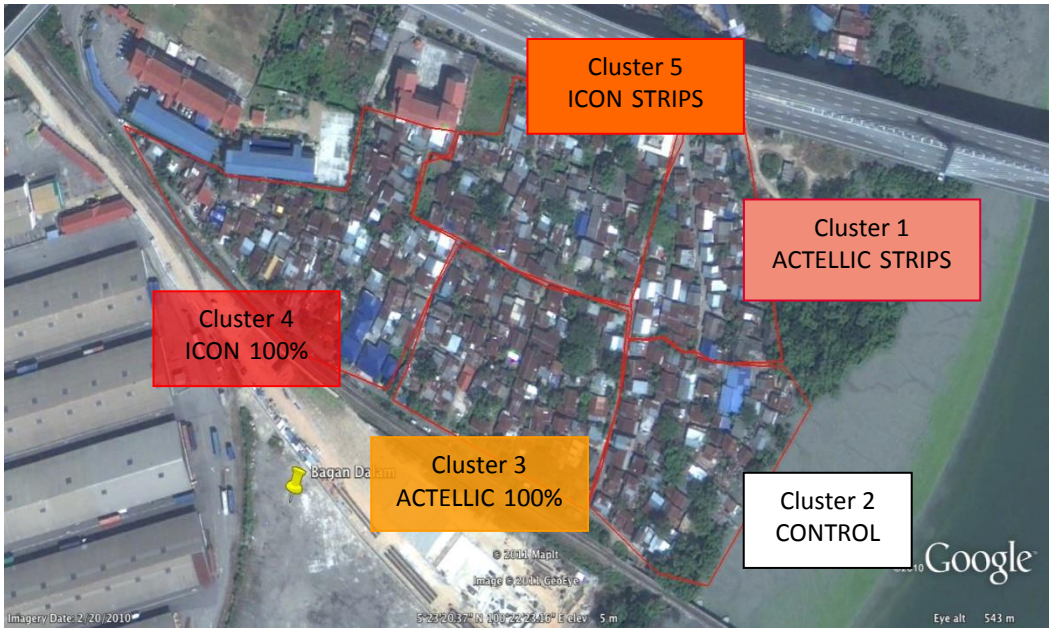


Figure 5.2 Treatment arms, area was divided into five clusters (approximately 25 houses per cluster), and each was randomly allocated a treatment

Bagan Dalam is a low-lying suburban village community which is situated close to the sea and the Kuala Prai River. There is little vegetation, and the railway track passes along one side of the village the village before it crosses a bridge on the Kuala Prai River. The area is regularly flooded at high tide and during the rainy seasons. Drainage is poor and contaminated wastewater often collects on the site. There are also a number of industrial dumping sites, which are always surrounded by domestic waste.

A typical type of house in this area is a single-storey house, built with low-grade timber walls and corrugated metal roofs (some with full or partial ceilings). The houses also normally have open eaves or numerous gaps in walls to the outside or to the neighbouring rooms. The houses usually consist of one or two sleeping areas with a small kitchen and living space (Figure 5.3).

Figure 5.3 The study site at Ujong Batu, showing typical houses and streets in the area

5.2.2.1 Ethical approval

The ethical approval for this study was granted by Liverpool School of Tropical Medicine Ethics Committee (Ref 11.82) on 17 October 2011.

5.2.2.2 Baseline surveys

Baseline surveys were conducted in November and December 2011. The first step was to recruit five clusters for an intervention study. All houses at the site were eligible for inclusion and every participating house was georeferenced using a handheld GPS unit (Garmin Oregon® 550 and Garmin eTrex Legend® HCx). The data obtained from the GPS units were transferred into Mapsource database, and all participating houses were labelled with a house ID number. A representative from each house was given a brief introduction to the study and a questionnaire to complete in the process of obtaining informed consent. Participants who did not understand the questionnaire or were unable to read and write were orally interviewed with questions based on the questionnaire paper (Figure 5.4). Subsequent to the process of obtaining informed consent, baseline data (pre-

intervention) on vector population were collected using ovitraps for *Aedes* mosquitoes and CDC Miniature Light Traps for *Culex* mosquitoes.

Figure 5.4 Images show the baseline activities, including obtaining informed consent and subsequent IRS treatment

5.2.2.3 Entomological surveys

The percentage of houses infested with larvae and pupae of *Aedes* and *Culex* (House Index) and the percentage of water-holding containers infested with larvae and pupae of *Aedes* and *Culex* (Container Index) were calculated from the inspection data. The overall density of mosquitoes was calculated as the number of positive containers per 100 houses inspected (Breteau Index). As the pupae per person index (PPI) is considered to be a reasonable proxy estimation of adult

mosquito density (since mortality is normally observed to be low among pupae and emerging adults of *Ae. aegypti* (Focks & Chadee, 1997; Chadee, 2004), this was also calculated. This index indicates the association between positive containers and houses; and is considered to be the most informative measure of household-level mosquito density (PAHO, 1994).

Surveys were conducted as follows: (1) Baseline studies in November 2011, (2) Treatment by indoor residual spraying in January 2011 (3) one-month follow-up in January 2012, (4) three-month follow-up in April 2012 and (5) six-month follow-up in July 2012 (Figure 5.5).

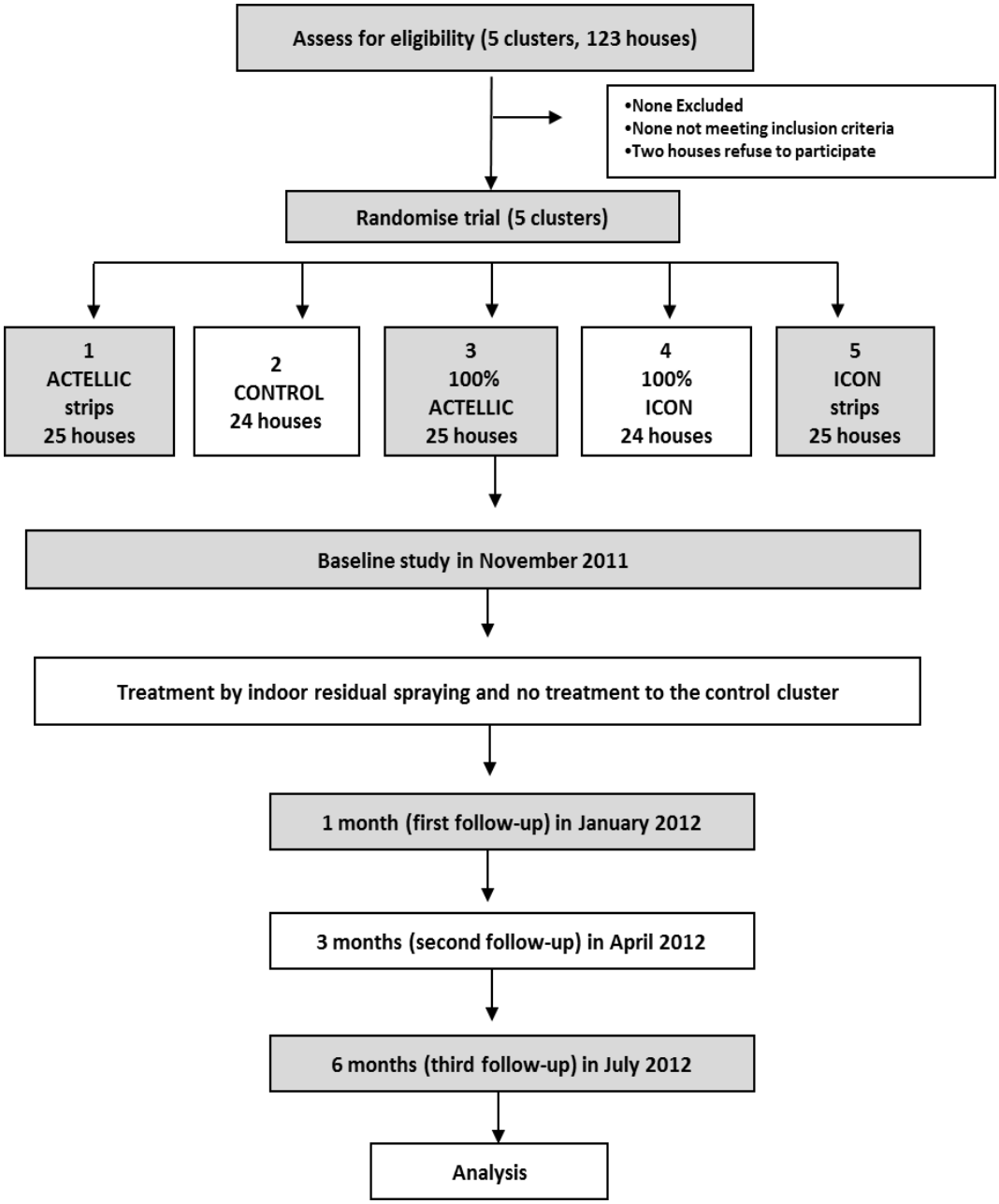


Figure 5.5 Diagram of the trial in Bagan Dalam

5.2.2.4 Ovitrap surveys and CDC Miniature Light Traps

The ovitrap surveys were conducted during the study. Each ovitrap consisted of a cylindrical black tin (13 cm height x 6 cm diameter) containing approximately 200 ml of tap water and a removable paddle (tongue depressor, 17 cm x 2.5 cm) on which the mosquitoes could lay their eggs just above the water level. There

were no overflow holes on the walls of the tins. Two ovitraps were placed in each of the 123 participating houses. Both ovitraps were placed outdoors at about 1.2 metres above ground level, in open areas near trees and/or shrubs. All ovitraps were collected after six days of the placement for laboratory examination. The numbers of eggs laid on the paddles were counted, and the numbers of larvae and pupae were recorded. Larvae and pupae were reared to adults for identification. *Ae. aegypti* were separated from *Ae. albopictus* using a hand aspirator. Both of these species were maintained in the laboratory for insecticide susceptibility tests (see Chapter 6).

Culex sp. mosquito samples were collected using CDC Miniature Light Traps. Two houses were chosen from each cluster and traps were hung indoors on the ceiling at about 2 metres above ground level. The addition of dry ice provided a source of carbon dioxide (CO_2) to increase the attractiveness of the light traps. A plastic container with a small tube at the bottom (designed to allow CO_2 gas to escape) was filled with small blocks of dry ice and placed above the top covers of the light traps. The traps were used three nights per week during the hours of just before dark to slightly after midnight. Traps were collected in the morning and mosquitoes placed in a labelled container and taken back to the laboratory for identification. Since there were only small numbers of adult mosquitoes collected from the light traps, the samples of immature stages of *Culex* mosquitoes were also taken back and maintained in the laboratory for insecticide susceptibility tests (see Chapter 6).

5.2.2.5 Interventions

A week before the intervention began, notices of spraying were distributed to all participating houses to ensure the residents were prepared for the spraying treatment day. The dimensions of the inner walls were measured and recorded in order to ensure the right amount of chemical concentration needing to be sprayed. Spraying was conducted in the living room and bedrooms, but not in the kitchen and dining room, of each house. The types of walls and ceilings were also recorded. The intervention activities were carried out in January 2012 by a team

of eight people from the Vector Control Research Unit (VCRU), with technical supervision provided by one representative from Syngenta. The 123 houses under study were allocated to five treatment groups: one untreated control group, and four other groups that received lambda-cyhalothrin (Icon 10CS; 25mg A.I./m^2) and pirimiphos-methyl (Actellic; 1000mg A.I./m^2). The treated groups were divided further into two groups: one group with 100% complete spray of Icon 10CS or Actellic on the wall, and the other group with only 1 metre (upper walls) strip spray of Icon 10CS or Actellic on the wall.

5.2.2.6 IQK assay and HPLC analysis

Small felt pads (1 cm diameter) were attached to the wall surfaces of each treated house. They were applied one day before spraying and removed the day after spraying. The samples were labelled with house ID, cluster number, date of spraying and type of treatment received. Samples were placed in labelled, sealed plastic bags and taken back to the laboratory in the Liverpool School of Tropical Medicine (LSTM). The purpose of this test was to measure the efficiency and quality of the spraying operation, as well as the efficacy of the insecticide residues over time after spraying. Furthermore, at second and third follow-up, some of the houses were sampled with two 5 cm x 20 cm strips of Sellotape. A pair of Sellotape samples was attached at three heights: high, middle and low in all houses for each cluster. By using gloves, the treated surfaces were rubbed using fingers to ensure that the chemical would be attached to the adhesive tapes. The Sellotape strips were stuck to pieces of Whatman 1 filter paper (100 cm^2). The samples were handled with care to ensure that the adhesive tapes did not fold in on themselves or overlap each other. The samples taken from each houses were tested using insecticide quantification kits (IQK). The lambda-cyhalothrin content was quantified using colorimetric assay, using the protocol described by Russell *et al.* (2014). The remaining samples were taken for HPLC analysis for determination of insecticide content over time.

5.2.2.7 Climate and hydrology data

Temperature and humidity data inside the houses were collected using TinyTags data loggers. These devices were randomly distributed to five houses (one for each cluster) and the data were downloaded at each follow-up survey. The rainfall and humidity data from November 2011 until July 2012 for the city of Butterworth were obtained from the Climatology and Hydrological Section, Malaysian Meteorological Department.

5.2.2.8 Acceptability and response of communities

During the intervention, at least one person in every household was verbally given an explanation of the possible side effects of IRS. The characteristics and response of communities towards IRS were also assessed using KAPB (Knowledge, Attitudes, Practices and Beliefs) survey by informal interviews during every follow-up.

5.2.2.9 Bioassays of insecticide-susceptible status of mosquitoes

The tube bioassays for susceptible status of mosquitoes in study areas were conducted during the baseline, one month, three months and six months post-intervention. The bioassays were conducted according to the WHOPES tube bioassays protocol as described in Chapter 6.

5.2.2.10 Statistical analyses

All data were prepared in Microsoft Excel and all these data were analysed using Stata 9.0 and SPSS 20.0 statistical analysis software. From the entomological surveys, the number of immature stages found in positive containers was tested using Negative Binomial Distribution analysis and Poisson analysis. The effects of IRS treatment on mosquito populations were determined by comparing the entomological indices between intervention and control clusters. Furthermore, the Friedman Test was used to compare entomological indices at baseline and entomological indices at each follow-up. Statistical significance was accepted when P values were less than 0.05.

5.3 Results

5.3.1 Entomological surveys

During the nine-month study period, *Ae. aegypti, Ae. albopictus* and *Cx. quinquefasciatus* were the only mosquito species found within all types of containers. Breeding containers were categorised as small containers (< 3 L) such as vases, discarded bottles, cans and plastic cups, medium containers (3 L - 20 L) such as plastic tanks, barrels and buckets, and others referring to undetermined containers such as discarded pieces of household items.

A total of 85 water-holding containers were inspected at baseline, of which 18 were positive for immature stages of *Ae. aegypti, Ae. albopictus* or *Cx. quinquefasciatus* (Table 5.1). The greatest number of positive containers was small containers (13) containers followed by four medium containers and only one 'other' container (Table 5.2). One month after intervention, 13 out of 101 containers inspected were positive with immature stages.

Table 5.1 Numbers of containers inspected and positive water-holding containers with immature stages (larvae, pupae or both) of the three species recorded in the entire study area at Bagan Dalam at baseline and each follow-up

Test	Baseline	1 month	3 month	6 month
No. of containers inspected	85	101	60	51
No. of positive containers	18	13	8	12

Table 5.2 Summary of types of containers found during entomological surveys at baseline, one-month, three-month and six-month follow-up

Time	Small container % (n)	Medium container % (n)	Others % (n)
Baseline	72.2 (13)	22.2 (4)	5.6 (1)
First follow-up	69.2 (9)	30.8 (4)	-
Second follow-up	100 (8)	-	-
Third follow-up	83.3 (10)	-	16.7 (2)

The total number of positive containers found throughout the study was 51 out of 297 containers inspected, from which 372 mosquitoes were collected and identified. *Ae. albopictus* represented the majority of mosquitoes found in all areas of study, accounting for 38.4% (143), whereas *Ae. aegypti* represented 34.4% (128), followed by *Cx. quinquefasciatus* with 27.2% (101) (Table 5.3).

Table 5.3 Numbers of *Ae. aegypti, Ae. albopictus* and *Cx. quinquefasciatus* at baseline and follow-up

Time	***Ae. aegypti***	***Ae. albopictus***	***Cx. quinquefasciatus***
Baseline	53	33	39
First follow-up	40	30	31
Second follow-up	20	28	17
Third follow-up	15	52	14
Total % (n)	**38.4 (128)**	**34.4 (143)**	**27.2 (101)**

The number of *Ae. aegypti* and *Cx. quinquefasciatus* gradually decreased from baseline to six months follow up. The number of *Ae. albopictus* also decreased from baseline to three months after intervention but increased dramatically at six months post-intervention. Although the amount of rainfall had drastically dropped at one-month follow-up, the numbers of mosquitoes found did not change markedly throughout the study (Figure 5.6).

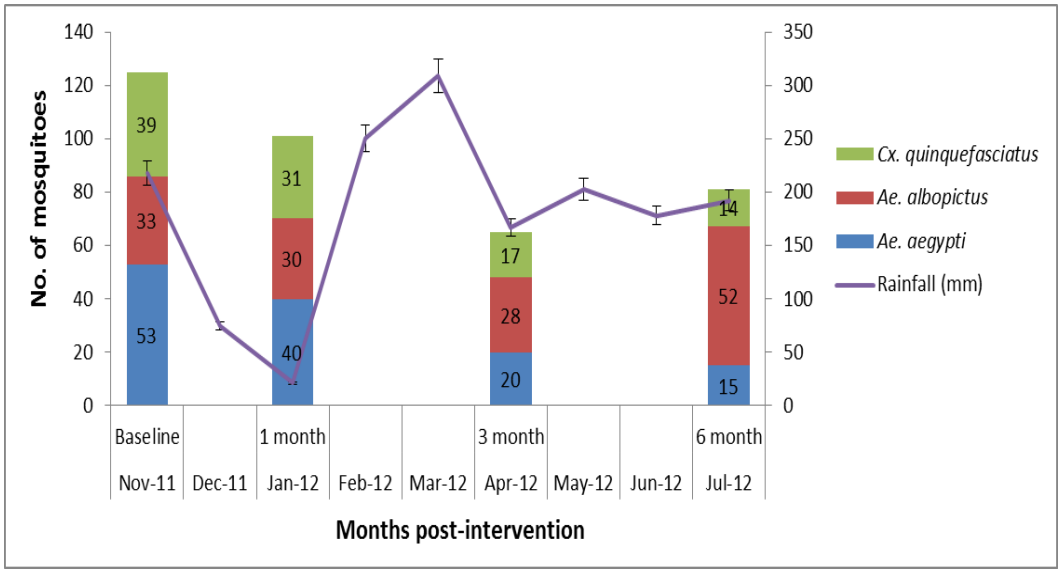


Figure 5.6 Summary of the mosquito species' samples per positive containers and correlated rainfall data at baseline and follow-up

5.3.2 Entomological indices

Table 5.4 shows the value of entomological indices as measured in the study area of Bagan Dalam before and after treatment. The House index and Breteau index values gradually decreased from baseline to three-month follow up but slightly increased at six months' post-intervention. In contrast, reductions were observed in Container index at one month after intervention but gradually increased later. The Pupal index fluctuated throughout the study. For all treatments, values of all entomological indices were not statistically different between four time points (Friedman Test, $P > 0.05$).

Table 5.4 Summary of entomological indices in all clusters (combined data) at baseline and follow-up; total number of houses investigated = 123

Summary of study area	**Baseline**	**1 month**	**3 months**	**6 months**
No of houses infested with immature stages	12	10	3	11
No of houses inspected	123	123	123	123
No of positive containers	18	13	8	12
No of containers inspected	85	101	60	51
No of pupae found in house	32	11	20	20
No of people occupying the house	12	24	13	41
Entomological indices				
House index (HI)	9.8	8.1	2.4	8.9
Container index (CI)	21.2	12.9	13.3	23.5
Breteau index (BI)	14.6	10.6	6.5	9.8
Pupae per person index (PPI)	2.7	0.5	1.5	0.5

House index = % of houses infested with larvae or pupae of Aedes mosquitoes. Container index = % of water holding containers infested with larvae or pupae. Breteau index = number of positive containers per 100 houses inspected. Pupae per person index = number of pupae per number of occupants of a house.

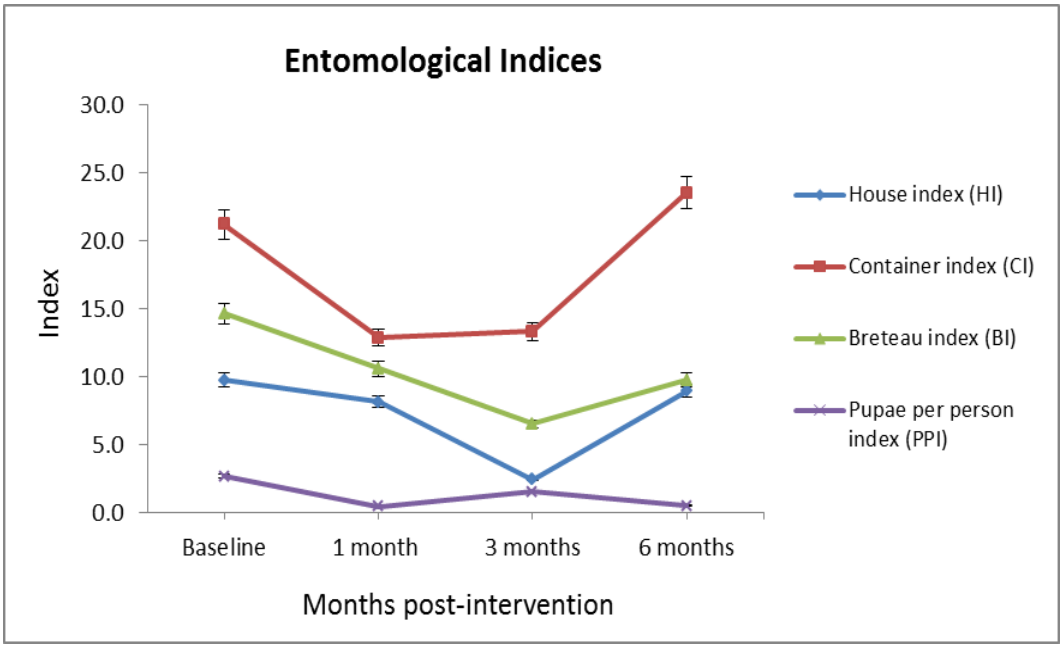


Figure 5.7 Summary of entomological indices in all treatments at study area during baseline (November 2011), one month (January 2012), three months (April 2012) and six months post-intervention (July 2012)

5.3.3 Comparison of entomological indices by treatment

The entomological indices were classified according to the treatment group. At baseline, there were no statistical differences in any of the entomological indices between all treatments (Friedman Test, $P > 0.05$). The highest entomological indices were recorded in the control cluster (cluster 2) whereas the lowest values were recorded in the cluster with Actellic 100% treatment (cluster 3). However, there were no positive containers found in cluster 3 during entomological surveys before and after the intervention. The overall data indicated that there was no significant reduction in entomological indices within treatments at the same time point (Kruskal-Wallis Test, $P > 0.05$). Furthermore, the entomological indices were also not significantly different within treatments at different time points (Friedman Test, $P > 0.05$). At one month and three months post-intervention, not only cluster 3 but also cluster 5 (Icon strip treatment) were found to be negative in all entomological indices. The findings are summarised in Table 5.5.

Table 5.5 Mean Breteau, Pupae per Person, House and Container Indices measured at baseline study and follow-up in all clusters

BASELINE				
	Breteau	**PPI**	**House**	**Container**
Cluster 1 (Actellic strips)	4	1	4	5.56
Cluster 2 (Control)	33.33	0.75	25	36.4
Cluster 3 (Actellic 100%)	0	0	0	0
Cluster 4 (Icon 100%)	25	3.63	16.67	31.58
Cluster 5 (Icon strips)	12	0	4	17.65

1 MONTH				
	Breteau	**PPI**	**House**	**Container**
Cluster 1 (Actellic strips)	4	0.4	4	4.76
Cluster 2 (Control)	25	0.5	25	27.3
Cluster 3 (Actellic 100%)	0	0	0	0
Cluster 4 (Icon 100%)	25	0	12.5	31.58
Cluster 5 (Icon strips)	0	0	0	0

3 MONTHS				
	Breteau	**PPI**	**House**	**Container**
Cluster 1 (Actellic strips)	4	1.17	4	12.5
Cluster 2 (Control)	16.67	2	4	33.3
Cluster 3 (Actellic 100%)	0	0	0	0
Cluster 4 (Icon 100%)	12.5	1.67	4.17	18.75
Cluster 5 (Icon strips)	0	0	0	0

6 MONTHS				
	Breteau	**PPI**	**House**	**Container**
Cluster 1 (Actellic strips)	8	0.33	8	14.29
Cluster 2 (Control)	20.83	0.57	17	41.7
Cluster 3 (Actellic 100%)	0	0	0	0
Cluster 4 (Icon 100%)	12.5	0.45	12.5	25
Cluster 5 (Icon strips)	8	0.46	8	29

The impacts of four treatment methods on entomological indices were compared during the intervention. Again, there were no significant differences found between these four treatments at the same time point (Kruskal-Wallis Test, $P > 0.05$). Furthermore, the impacts of two methods, full standard IRS sprayed (cluster 1 and 5) and strips sprayed (cluster 3 and 4) on entomological indices were also compared, but no significant difference was found (Kruskal-Wallis Test, $P > 0.05$). The efficacy of four treatments was again compared but no significant difference was recorded at different time points (Friedman Test, $P > 0.05$).

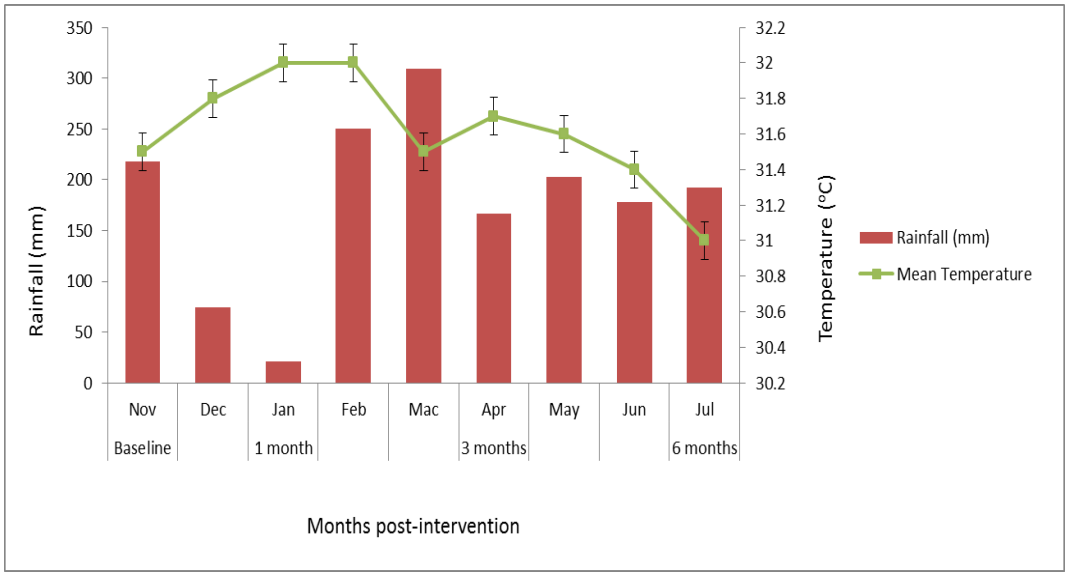
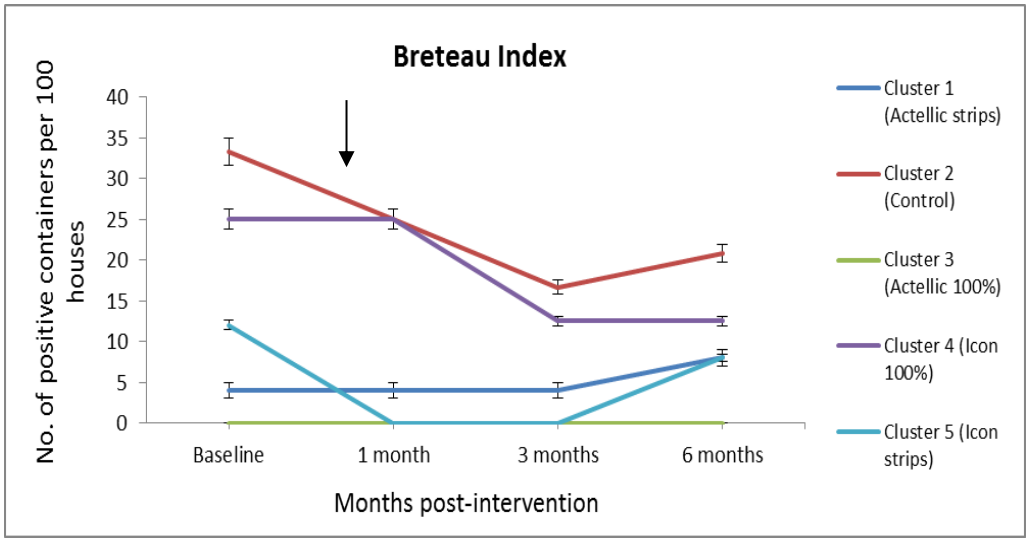
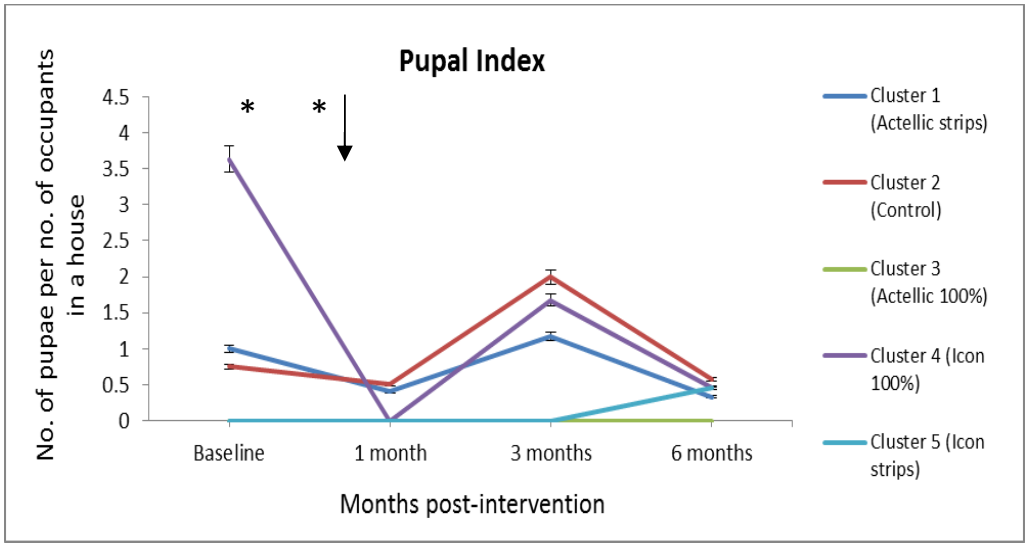
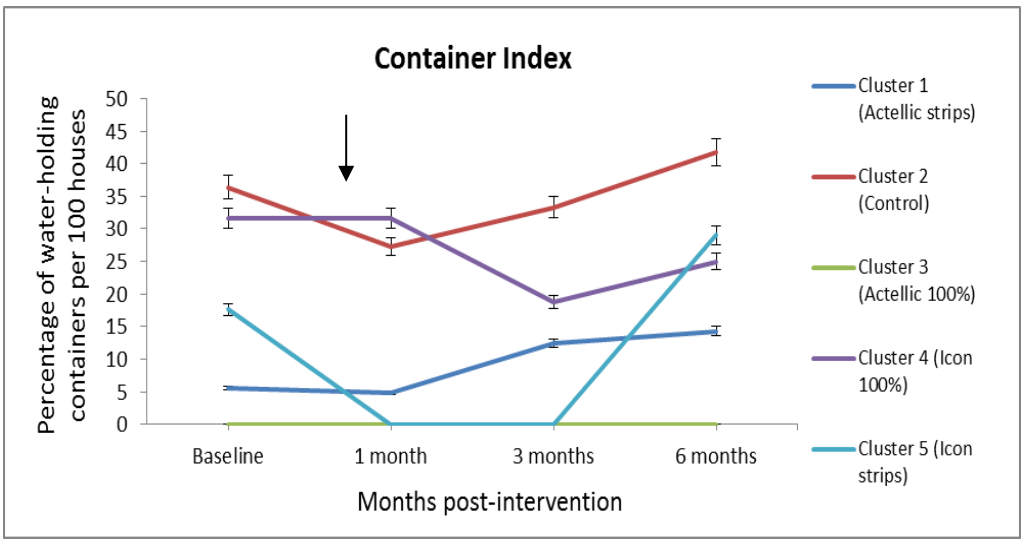


Figure 5.8 Rainfall and mean temperature data for nine-month study period from November 2011 until July 2012 for the city of Butterworth obtained from the Climatology and Hydrological Section, Malaysian Meteorological Department


Breteau Index
No. of positive containers per 100 houses
40
35
30
25
20
15
10
5
0
Baseline
1 month
3 months
6 months
Months post-intervention
Cluster 1 (Actellic strips)
Cluster 2 (Control)
Cluster 3 (Actellic 100%)
Cluster 4 (Icon 100%)
Cluster 5 (Icon strips)


Pupal Index
No. of pupae per no. of occupants in a house
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
Baseline
1 month
3 months
6 months
Months post-intervention
Cluster 1 (Actellic strips)
Cluster 2 (Control)
Cluster 3 (Actellic 100%)
Cluster 4 (Icon 100%)
Cluster 5 (Icon strips)


Container Index
Percentage of water-holding containers per 100 houses
50
45
40
35
30
25
20
15
10
5
0
Baseline
1 month
3 months
6 months
Months post-intervention
Cluster 1 (Actellic strips)
Cluster 2 (Control)
Cluster 3 (Actellic 100%)
Cluster 4 (Icon 100%)
Cluster 5 (Icon strips)

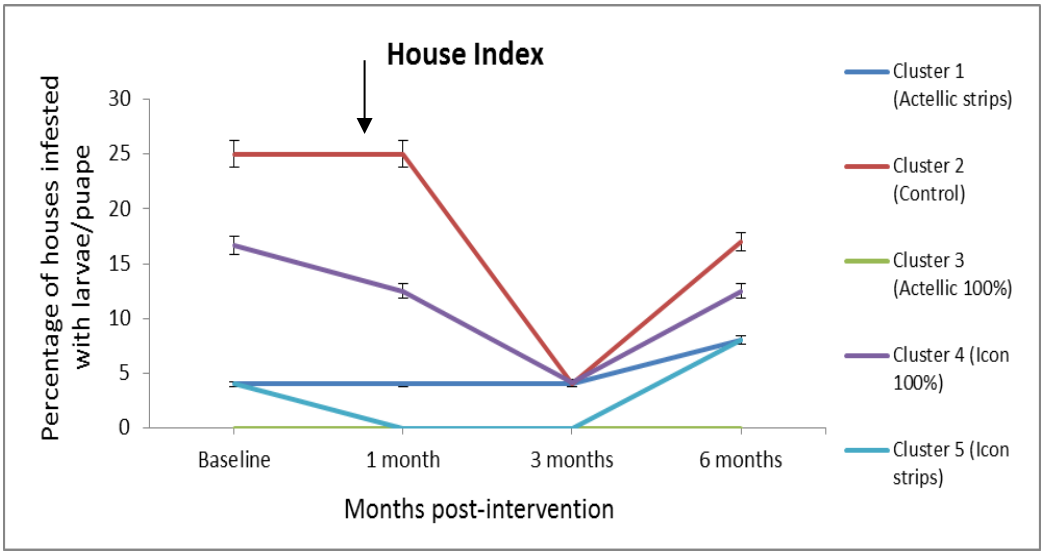


Figure 5.9 Summary of (a) Breteau Index, (b) Pupal Index, (c) House Index and (d) Container Index measured during the intervention in all clusters (mean and standard error for each index). Labelled arrows indicate when the intervention began (January 2012) and the asterisk (*) indicates when the local Ministry of Health in Butterworth conducted vector control responses to dengue cases (2 November 2011 and 7 December 2011)

5.3.4 Ovitrap Index

A total of 984 ovitraps were placed during the nine-month study period with 246 ovitraps placed at each sampling date. Results are summarised in Figure 5.10. The total ovitrap index did not change significantly throughout the study from 66.7% at baseline to 91.9% at six-month follow-up (Chi-Square Test, $P > 0.05$). There were also no significant differences between each follow-up at different time points (Chi-Square Test, $P > 0.05$).

To evaluate the relationship between the eggs collected from ovitraps and climate (rainfall, mean temperature and relative humidity), the correlation between variables was evaluated using the Pearson's correlation coefficient (r) and its significance was determined. The ovitrap index showed no significant correlation with rainfall (r = -0.327) ($P > 0.05$), mean temperature (r = 0.003) or relative humidity (r = 0.205) ($P > 0.05$).

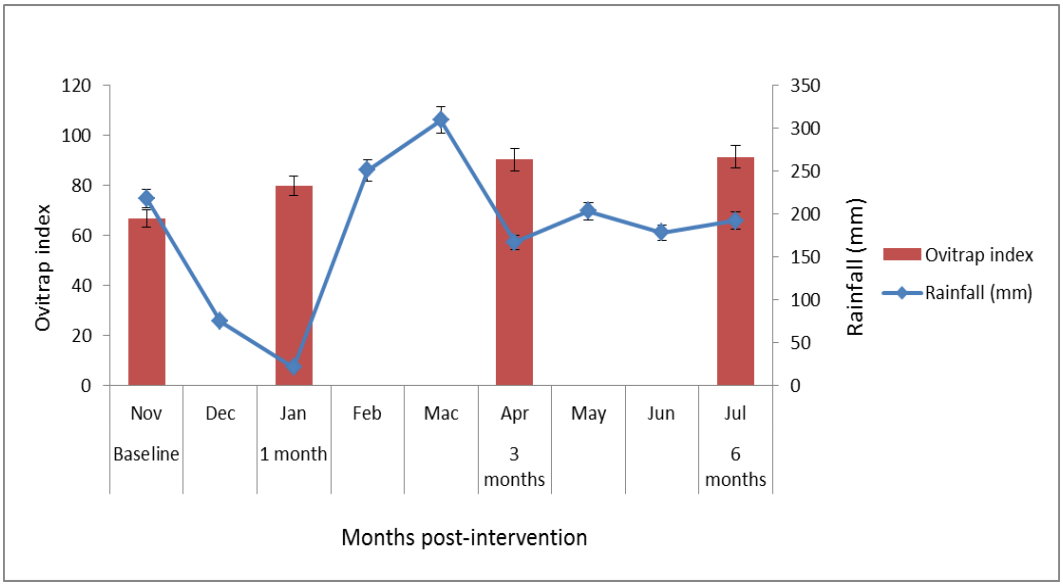


Figure 5.10 Summary of ovitrap index and rainfall in Bagan Dalam

Comparison of means of eggs, larvae and pupae found in all five clusters (Wilcoxon Signed Rank Test with Bonferroni correction; $P > 0.025$) revealed that there were no significant differences when compared between mean eggs and larvae at different sampling times. Means of eggs, larvae and pupae of *Aedes spp.* across four time points are summarised in Tables 5.6 to 5.9.

Table 5.6 Summary (Mean ± SD) calculated from eggs, pupae and larvae at baseline study per ovitrap observed

Treatment	Eggs (Mean ± SD)	Larvae (Mean ± SD)	Pupae (Mean ± SD)
Cluster 1 (Actellic strips)	6.8 ± (14.3)	10 ± (19.7)	6.2 ± (10.8)
Cluster 2 (Control)	7.4 ± (12.8)	11.3 ± (20.3)	5.3 ± (8.2)
Cluster 3 (Actellic 100%)	11 ± (16.5)	28.5 ± (46.8)	9 ± (11.5)
Cluster 4 (Icon 100%)	12.2 ± (22)	31.1 ± (48)	8 ± (11.2)
Cluster 5 (Icon strips)	8.6 ± (10.9)	8.7 ± (19)	7.3 ± (12)

Table 5.7 Summary (Mean ± SD) calculated from eggs, pupae and larvae at one month post-intervention

Treatment	Eggs (Mean ± SD)	Larvae (Mean ± SD)	Pupae (Mean ± SD)
Cluster 1 (Actellic strips)	8.9 ± (19)	18 ± (25.8)	1.5 ± (3.5)
Cluster 2 (Control)	3 ± (6.1)	11.1 ± (19.8)	0.8 ± (2)
Cluster 3 (Actellic 100%)	7 ± (19.9)	19.8 ± (26.3)	0.8 ± (2.1)
Cluster 4 (Icon 100%)	4.7 ± (11.2)	15.8 ± (15)	0.4 ± (1.1)
Cluster 5 (Icon strips)	7.9 ± (13)	16.7 ± (22.6)	1 ± (1.7)

Table 5.8 Summary (Mean ± SD) calculated from eggs, pupae and larvae at three months post-intervention

Treatment	Eggs (Mean ± SD)	Larvae (Mean ± SD)	Pupae (Mean ± SD)
Cluster 1 (Actellic strips)	44.8 ± (33.7)	27.3 ± (30.4)	0.1 ± (0.3)
Cluster 2 (Control)	28.8 ± (25.5)	25.8 ± (27.6)	0.2 ± (0.6)
Cluster 3 (Actellic 100%)	19.6 ± (23.3)	23.6 ± (30.5)	0.8 ± (3.3)
Cluster 4 (Icon 100%)	19.3 ± (30.1)	12.9 ± (18.9)	0.1 ± (0.2)
Cluster 5 (Icon strips)	16.8 ± (22.2)	10.5 ± (15.6)	0.7 ± (4)

Table 5.9 Summary (Mean ± SD) calculated from eggs, pupae and larvae at six months post-intervention

Treatment	Eggs (Mean ± SD)	Larvae (Mean ± SD)	Pupae (Mean ± SD)
Cluster 1 (Actellic strips)	18.4 ± (17)	18.9 ± (27.9)	0.2 ± (0.5)
Cluster 2 (Control)	3 ± (8.2)	7.2 ± (10)	0.5 ± (1.6)
Cluster 3 (Actellic 100%)	19.6 ± (21.4)	30.6 ± (34.2)	0.2 ± (0.9)
Cluster 4 (Icon 100%)	16.5 ± (18.8)	14.5 ± (21.4)	0.2 ± (0.7)
Cluster 5 (Icon strips)	14 ± (20.6)	16.8 ± (22.3)	0.1 ± (0.7)

Totals of adult *Ae. aegypti* and *Ae. albopictus* emerged from ovitraps were recorded at four sampling times (Table 5.10 to Table 5.13). *Ae. albopictus* populations were not significantly higher than *Ae. aegypti* (Kruskal-Wallis Test, $P > 0.05$). Total adult numbers for both species were also not significantly different at four sampling times (Friedman Test, $P > 0.05$).

Table 5.10 Summary (Mean ± SD) adults *Ae. albopictus* and *Ae. aegypti* emerged from ovitraps in study areas at baseline

Treatment	***Ae. albopictus*** **(Mean ± SD)**	***Ae. aegypti*** **(Mean ± SD)**
Cluster 1 (Actellic strips)	14.4 ± (16.8)	8.7 ± (9.2)
Cluster 2 (Control)	15.8 ± (21.8)	10.9 ± (12.4)
Cluster 3 (Actellic 100%)	33.6 ± (34.1)	19.9 ± (18.7)
Cluster 4 (Icon 100%)	36.4 ± (29.2)	12.8 ± (10.2)
Cluster 5 (Icon strips)	27.3 ± (25.4)	18.8 ± (19.3)

Table 5.11 Summary (Mean ± SD) adults *Ae. albopictus* and *Ae. aegypti* emerged from ovitraps in study areas at one month post-intervention

Treatment	***Ae. albopictus*** **(Mean ± SD)**	***Ae. aegypti*** **(Mean ± SD)**
Cluster 1 (Actellic strips)	12.6 ± (9.8)	4.8 ± (7.7)
Cluster 2 (Control)	8.2 ± (8.6)	1.8 ± (3.2)
Cluster 3 (Actellic 100%)	10.7 ± (9.3)	3.2 ± (3.7)
Cluster 4 (Icon 100%)	9.8 ± (10.9)	6 ± (7.4)
Cluster 5 (Icon strips)	8.7 ± (6.4)	5.4 ± (7.9)

Table 5.12 Summary (Mean ± SD) adults *Ae. albopictus* and *Ae. aegypti* emerged from ovitraps in study areas at three months post-intervention

Treatment	***Ae. albopictus*** **(Mean ± SD)**	***Ae. aegypti*** **(Mean ± SD)**
Cluster 1 (Actellic strips)	29 ± (18.3)	14.4 ± (13.9)
Cluster 2 (Control)	23.2 ± (18.1)	15.2 ± (17.9)
Cluster 3 (Actellic 100%)	25.9 ± (21.6)	16.9 ± (17)
Cluster 4 (Icon 100%)	11.2 ± (11.1)	6.5 ± (11)
Cluster 5 (Icon strips)	14.6 ± (15.2)	5.9 ± (9.7)

Table 5.13 Summary (Mean ± SD) adults *Ae. albopictus* and *Ae. aegypti* emerged from ovitraps in study areas at six months post-intervention

Treatment	***Ae. albopictus*** **(Mean ± SD)**	***Ae. aegypti*** **(Mean ± SD)**
Cluster 1 (Actellic strips)	15.1 ± (15.7)	4.7 ± (9.6)
Cluster 2 (Control)	9.5 ± (9.9)	5.2 ± (20.3)
Cluster 3 (Actellic 100%)	10.5 ± (10.7)	4.4 ± (8.1)
Cluster 4 (Icon 100%)	6.7 ± (5.1)	3 ± (4.2)
Cluster 5 (Icon strips)	17.6 ± (21.7)	3.5 ± (5.1)

5.3.5 Light Trap results

A total of 10 CDC light traps were used to collect adult *Cx. quinquefasciatus* in all clusters. The total number of adults *Cx. quinquefasciatus* caught is presented in Table 5.14. The number of adult *Cx. quinquefasciatus* was significantly different between four time points (Friedman Test, $P < 0.05$). Further *post hoc* analysis demonstrated that there was a significant difference between one month and three months post-intervention (Kruskal-Wallis Test, $P < 0.05$). The number of adults *Cx. quinquefasciatus* detected was related to the levels of rainfall (Figure 5.11).

Table 5.14 Total number of adult *Cx. quinquefasciatus* collected from two houses in each cluster at baseline study and one month (1st follow-up).

Treatment	Baseline	1 month	3 months	6 months
Cluster 1 (Actellic strips)	2	2	2	9
Cluster 2 (Control)	5	10	5	8
Cluster 3 (Actellic 100%)	15	17	4	4
Cluster 4 (Icon 100%)	2	5	12	2
Cluster 5 (Icon strip)	18	43	6	13
Total	**42**	**77**	**29**	**36**

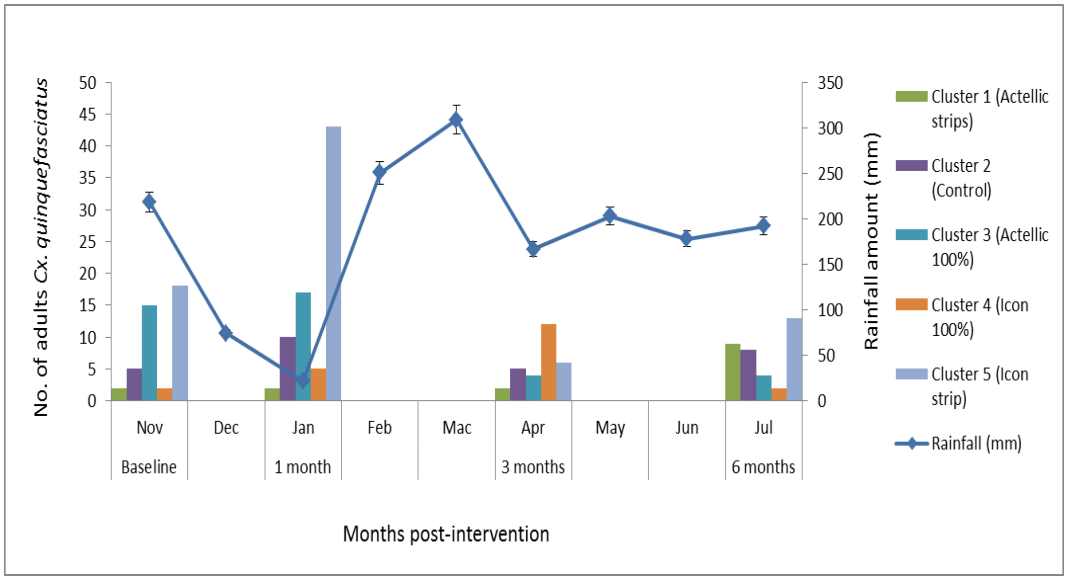


Figure 5.11 Summary of total number of adult mosquitoes caught by CDC light traps placed inside two houses in each cluster at baseline, one month, three months and six months post-intervention with rainfall (mm) data

5.3.6 Potential breeding sites

During the baseline study conducted in November 2011 and intervention in January 2012, most potential breeding sites found were artificial containers. From the water-holding containers inspected from the 123 houses in the study area, it was categorised as tanks (> 1000 litres), drums (150 - 200 litres), tyres (≤ 1 litre), small containers (< 3 litres), medium containers (3 - 20 litres), large containers (> 20 litres) and others (with intermediate shape and size). The potential breeding sites in the study area for each cluster were grouped as in Table 5.15.

5.3.7 KAPB survey results

5.3.7.1 Baseline study

At baseline, 93.5% of households stated that mosquitoes were a problem in the house; 43.5% mentioned that mosquitoes bite during the day and night, whereas 39.4% stated that mosquitoes bite at any time during the whole day. Only 7.8% referred to night biting. Over 45% were bitten mostly on hands and legs, whereas 52.2% were bitten throughout the body.

To prevent being bitten, 59.4% of the householders used mosquito coils at home, 13.8% used aerosol can, electric mat and liquid vaporizer, and 26.8% used fan, blanket and bed nets. The best preventive method was believed to be insecticide usage (75.6%) (Table 5.16).

Table 5.15 Type of potential breeding sites inspected by cluster

Cluster	Tanks	Drums	Tyres	Small containers	Medium containers	Large containers	Others
Cluster 1 (Actellic strips)	No	No	No	Flower vase Oil bottle Plastic containers	Paint barrel Oil barrel	Rubbish bin	Drawer Pot of water inside small temple Pile of garbage Chicken coop
Cluster 2 (Control)	No	Yes	Yes	Detergent bottle Plastic bottles Flower vase Cans, Paint cans Paint containers Polystyrene saucer	Aquarium Paper buckets Paint barrel	Rubbish bin	Push chair Plastic basket Husk
Cluster 3 (Actellic 100%)	No	No	No	Tins Cans Plastic containers Flower vase	Plastic barrel	Rubbish bin	Slippers and old shoes Drawer Plastic basket Pile of old fans

							Chicken coop Pile of garbage Stack of rusty iron roof
Cluster 4 (Icon 100%)	No	No	Yes	Flower vase Small bucket Plastic bottles Plastic cups Plastic containers Paint cans	Plastic barrel Paint barrel Paint container	No	Small basket Pot cover Pile of garbage Drawer Barrel filled with rubbish, slippers, rice pot and plastic cups
Cluster 5 (Icon strips)	Yes	No	No	Cans Drink bottles Paint cans Plastic bottles Flower vase Plastic containers	Plastic barrel	No	Chicken coop Pile of garbage Pile of boards and irons

Table 5.16 Pre-intervention questionnaire results (baseline study)

Question	**Answer**			
Question 1 Are mosquitoes a problem in your house?	Yes **93.5%**	No **6.5%**		
Question 2 If 'yes', *when* do they bite?	Daytime and night **43.5%**	Night only **7.8%**	All the time **39.4%**	Others (raining season) **9.3%**
Question 3 If 'yes', *where* do they bite?	Hands and legs **45.2 %**	Throughout the body **52.2 %**	Others (face, forehead, head) **2.6 %**	
Question 4 What measures do you carry out to reduce or prevent mosquito bites at present?	Mosquito coils **59.4 %**	Aerosol, electric mat and liquid vaporizer **13.8 %**	Others (fan, blanket, bed net) **26.8 %**	
Question 5 Where do you think the mosquitoes come from or where do they live?	Natural condition (sea, river, puddle, forest, tree) **26.8 %**	Artificial manmade container (drain, abandoned house, containers, rubbish) **69.1 %**	Others (do not know the answer) **4.1 %**	
Question 6 What do you think is the best method to prevent mosquito in the house?	Clean up the house and surrounding area **5.7 %**	Insecticide usage **75.6 %**	Bed net and fan **13.0 %**	Others (do not know the answer) **5.7 %**

5.3.7.2 First follow-up

At one month post-intervention, 50.4% agreed that the number of mosquitoes was reduced after treatment, but 42.3% believed that the number of mosquitoes was the same as before treatment, and 2.4% thought that the number of mosquitoes was greater after the treatment. A total of 22.6% believed that the number of mosquitoes was reduced only for one day after treatment. Only 4.9% believed that other insects such as cockroaches, ants and flies had been killed after spraying. Furthermore, 100% of participating householders agreed that no medical problems or any symptoms such as headache, nausea or chest pain had occurred after the implementation of the treatment (Table 5.17).

5.3.7.3 Second follow-up

Three months after the IRS implementation in the study areas, 96.7% believed that the intervention had reduced the number of mosquitoes in that area. Of these, 17.1% mentioned that the reduction lasted for only a day and nobody agreed that the lower numbers of mosquitoes lasted for more than 7 days. The result also indicated that participated householders agreed that no medical problems or any symptoms such as headache, nausea or chest pain had occurred after the treatment (Table 5.18).

Table 5.17 Post-intervention questionnaire results (first follow-up)

Question	Answer			
Question 1 Did the insecticide treatment reduce the number of mosquitoes inside the house?	Yes **50.4 %**	No, same as before treatment **42.3 %**	No, the numbers are greater than before treatment **2.4 %**	Others (raining season, water tide) **4.9 %**
Question 2 If 'Yes', *how long* since you were last bitten by mosquitoes?	1 day **22.6 %**	2-3 days **67.7 %**	7 days **6.5 %**	Others **3.2 %**
Question 3 Have you seen any dead mosquitoes inside the house after spraying?	Yes **0 %**	No **97.6 %**	Do not know **2.4 %**	
Question 4 Did the insecticide treatment reduce the number of other insects (cockroaches, ants, flies) inside the house?	Yes **4.9 %**	No, same as before treatment **95.1 %**	No, the numbers are greater than before treatment **0 %**	
Question 5 Have you or your family experienced any problems following the treatment?	Yes **0 %**	No **100 %**		

Table 5.18 Post-intervention questionnaire results (second follow-up)

Question	**Answer**		
Question 1	Yes	No, same as before treatment	No, the numbers are greater than before treatment
Did the insecticide treatment 3 month ago reduce the number of mosquitoes inside the house?	**96.7 %**	**3.3 %**	**0 %**
Question 2	1 day	2-3 days	7 days
How long since you were last bitten by mosquitoes?	**17.1 %**	**82.9 %**	**0 %**
Question 3	Yes	No	Do not know
Did the insecticide treatment 3 month ago reduce the number of other insects (cockroaches, ants, flies) inside the house?	**0 %**	**100 %**	**0 %**
Question 4	Yes	No	
Have you or your family experienced any problems following the treatment?	**0 %**	**100 %**	

5.3.8 IQK assay and HPLC analysis

The IQK results from 10 treated houses showed that the chemical content remained within the target dose (20 - 30 mg AI/m^2) throughout the trial, indicating that the treated houses were correctly sprayed.

For HPLC analysis, only five samples of treated houses were used to determine the level of insecticide residue over time. The result showed a gradual reduction of chemical residue on the walls at the three-month follow-up. However, due to some unclear reasons, the resurgence of chemical content was noticed from two samples of Icon 100% treatment at the six-month follow-up. The level of insecticide residue over time is illustrated in Figure 5.12.

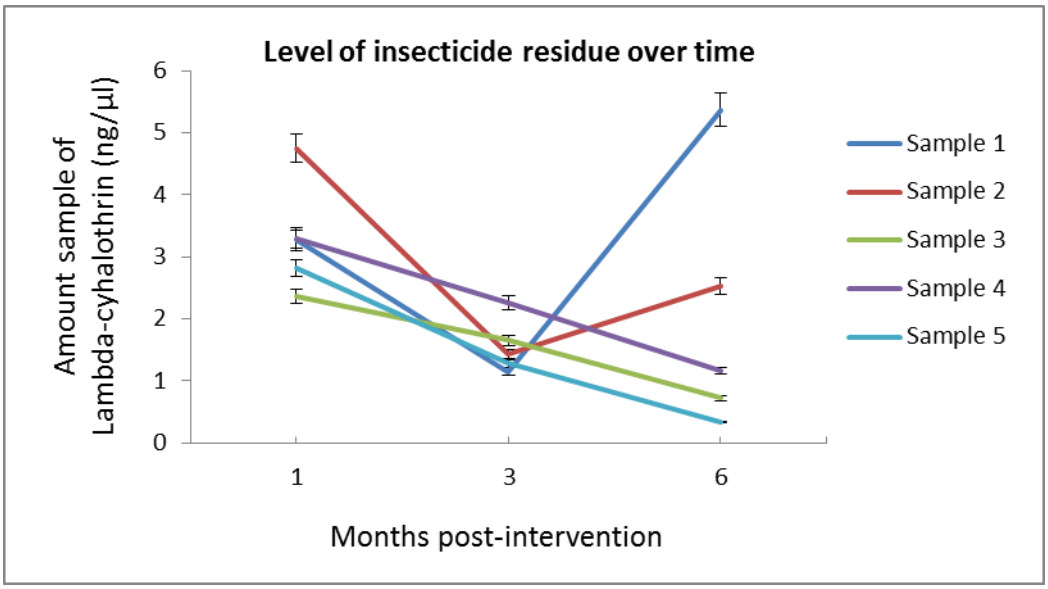


Figure 5.12 Determination of insecticide residue over time using HPLC analysis. The level of insecticide residue (lambda-cyhalothrin concentration ng/µl) over the six-month period

5.3.9 Cost-effectiveness analysis

Insecticide treatment was applied at a rate of 24 houses per day by two spray-men for standard IRS sprayed areas. In the area with selective IRS treatment, 36 houses were sprayed per day by spray-men. It is shown that there is about a 50% net saving for insecticide used in the selected IRS areas compared to standard IRS. For operational time, the standard IRS method was more than 50% longer than selective IRS. The mean time of 10 minutes spraying time per house for selective IRS was recorded whereas the mean time of 22 minutes per house spraying time for standard IRS. In terms of labour costs, the standard IRS was approximately 25% more expensive than selective IRS (Table 5.19).

Table 5.19 Comparison of the costs of the selective and full IRS spraying with Icon 10CS during intervention

	Selective spraying	**Full spraying**
Labour costs		
Total houses treated	50	49
Personnel per day (USD)	15	20
Insecticide		
Insecticide used per house (g)*	2.5	5.1
Total insecticide used (g)	125	250
Operational time		
Mean time spend per house (min)	10	22
Mean total time spend (min)	500	1078

* 62.5 g in 10 L tank capacity or 62.5 g per sprayer

5.4 Discussion

Major improvements in the control of dengue vectors are urgently needed worldwide. IRS, though it has never been used in dengue control, offers one method of meeting this challenge, but the existing approach – to treat every interior surface of all houses at risk – is time consuming and expensive. The trial reported here was the first attempt to determine whether targeted IRS could be a solution and improve the cost-effectiveness of IRS.

In the trial, the effectiveness of two residual sprayed insecticides, Icon 10CS and Actellic CS, was evaluated in a small community of traditional housing in a single location in Bagan Dalam, Penang. Icon 10CS is an advanced capsule suspension (CS) formulation of second-generation pyrethroid, lambda-cyhalothrin whereas Actellic CS is a long-lasting, microencapsulated formulation of the organophosphate insecticide pirimiphos-methyl. Icon 10CS has a contact mode of action whereas Actellic CS has both contact and airborne modes of actions. Both of these formulations have been specifically designed for use in IRS programmes. According to WHO (2009), the duration of effectiveness action is 2-3 months for pirimiphos-methyl and 3-6 months for lambda-cyhalothrin, as evaluated against malaria vectors. Previous study demonstrated that Icon 10CS provided long-lasting residual control in field trials in Tanzania (Mashauri *et al.,* 2013). Oxborough *et al.* (2014) reported that Actellic CS effectively controlled pyrethroid-resistant *Cx. quinquefasciatus* also in Tanzania. Rowland *et al.* (2013) and Tchicaya *et al.* (2014) reported effective control of pyrethroid-resistant *An. gambiae* in hut trials in Benin and pyrethroid-susceptible *An. arabiensis* in hut trials in Cote d'Ivoire, respectively. The study reported here was the first study to test indoor residues of both insecticides against dengue vectors.

The results showed that insecticide treatment by IRS has only a small reduction in and slight impact on mosquito populations. The effect of both IRS methods was low. It is highly probable that this is due to the fact that the clusters in this study consisted of small areas with approximately 150 houses surrounded by urban areas. The dispersal of mosquitoes within the clusters or migration of mosquitoes from adjacent areas is likely to occur to a higher extent. It is suggested that IRS treatment is applied in larger areas so that the effect of the intervention is more pronounced. This small-scale study could be the major reason for insignificant results between control and intervention clusters. However, there were no differences between treatment methods and this showed that there was no evidence that the strip IRS were effective as standard IRS. The effectiveness of IRS strip is not prominent in this study as there was no evidence for less time-consuming and more cost-effective than conventional spraying.

The overall results for all clusters showed that HI, CI, BI and PPI fluctuated throughout the study. There was a small reduction in all indices from baseline to one-month follow up (Table 5.4) but this reduction was not statistically significant. However, there were no positive containers found in cluster 3 constantly at four time points, whereas in cluster 5 there were no positive containers found at one- and three-month follow-up. Although the positive containers were not found, the ovitrap data showed that the highest mean pupae was recorded in cluster 3 during baseline study and immature stages of mosquitoes were found during each ovitrap survey. This suggested that, although there were no positive breeding sites found in that area, the mosquito populations remained high but possibly dispersed to other clusters. In other studies, ovitrap surveys have provided valuable data for detecting the presence of mosquitoes and seasonal abundance of *Ae. aegypti* and other container-breeding mosquitoes (Ritchie, 1984; Iriarte *et al.,* 1991). It has been reported that ovitrap index could be more sensitive than the traditional Stegomyia indices in detecting low populations (Micks & Moon, 1980; Marques *et al.,* 1993). Ritchie *et al.* (2004) also documented that ovitraps are inexpensive to produce in large numbers and easy to setup and monitor in the field.

One month after the IRS treatment, there were no significant reductions in entomological indices when compared to baseline study. Similarly, after six months, there were also no significant differences found between four different treatments compared to baseline. Furthermore, the impact of two delivering methods (selective and standard IRS) was compared, but no significant difference was detected.

It is likely that the small area and high density of housing in the study site compromised the study, with movement of mosquitoes between the treatment clusters, leading to the masking of any impact. This was unfortunate but unavoidable as Bagam Dalam was the only location available for the trial at that time.

Clearly, the strip IRS method is faster and cheaper than standard IRS. The usage of chemical amounts and spraying time was half of the usage for standard IRS (Table 5.19). However, there was no evidence that selectively targeting sites for insecticide application in this study provides a more cost-effective means of controlling mosquito vectors since the number of mosquitoes were not significantly different to before the treatment. Nevertheless, previous studies by Arredondo-Jiménez *et al.* (1995) have demonstrated that selective spraying was as effective as full spraying in controlling *Anopheles* species for malaria control. Only one-third of the insecticide amount was required and half of the manpower was essential for spraying application compared to full treatment (Bangs *et al.,* 1981; Gandahusada *et al.,* 1984; Arredondo-Jiménez *et al.,* 1995).

Based on the ovitrap surveys, the percentage of *Ae. albopictus* was higher compared to *Ae. aegypti*. One possible reason for this is that the ovitrap surveillance was only conducted outdoors where *Ae. albopictus* are mostly outdoors (Chen *et al.,* 2006). Although *Ae. aegypti* are primarily found indoors, the results showed that *Ae. aegypti* are also active outdoors in Penang. It has been hypothesised that, in some parts of Southeast Asia, *Ae. aegypti* have completely replaced the indigenous *Ae. albopictus* in urban areas (Pants *et al.,* 1973), and the preferred breeding habitats of these two species slightly overlap (Gould *et al.,* 1970; Thavara *et al.,* 2001). In Malaysia, Yap and Thiruvengadam (1979) found an extensive sharing of 55.4% of total positive ovitraps by *Ae. aegypti* and *Ae. albopictus* in Georgetown, Penang. The mixed breeding of *Ae. aegypti* and *Ae. albopictus* have also been reported by Chen *et al.* (2006), which showed that co-breeding occurred in these two species.

For adult *Cx. quinquefasciatus*, the total number collected during one-month follow-up was higher compared to baseline, 3 months and 6 months post-intervention. This result indicated that the number of *Cx. quinquefasciatus* was low when the rainfall increased. The water flow after heavy rainfall possibly flushed out the immature stages of this species. However, there was recovery of the mosquitoes once the rainfall decreased.

5.4.1 Notes on dengue vector breeding sites in Penang

Several categories of potential breeding sites were found in the study, including tanks, drums, tyres, small containers, medium containers, large containers and 'others' (including pieces of household items). The most productive containers, as measured by the numbers of pupae, were small containers. The classification was different to Nyamah *et al.* (2010) where breeding sites were classified based on the intended used of containers such as garden accoutrements (flower pots, flower pot plates, vases and watering cans); water storage containers (earthenware jars, plastic containers, metal drums, assorted tin cans and water compartments); discarded items (electrical goods and old furniture); kitchen utensils (pots, pans, plates, cups, and saucers); and other habitats (animal drinking pans and aquariums). A study by Danis-Lozano *et al.* (2002) classified *Ae. albopictus* habitats in Southern Mexico as controllable containers and disposable containers. The former category includes pails, drums, plastic containers, tubs, small bottles, flower pots, flower pot plates, wells, water tanks, and pots, whereas the latter category includes water-storage containers, tyres, tin cans, broken flower pots and others. The classification of potential breeding sites for *Aedes* mosquitoes is important in promoting appropriate long-term control and preventive measures to be taken by the householders to reduce the number of mosquito infestations.

5.4.2 Community response to indoor residual spraying

Mosquitoes were described as a problem in all clusters. Most of the participants were aware of the health problems and diseases related to mosquitoes. Some of the participants expressed their concern about acquiring infection as someone in their neighbourhood or someone that they knew had been diagnosed with dengue. They reported that biting occurred anytime during the day and night, and anywhere around the uncovered body. The most useful control as perceived by this community was insecticides such as aerosols, mosquito coils and electric vaporizer.

The results indicated that IRS was highly accepted in all clusters of the study area. There were no rejections from participants, while many asked for more spraying

to be conducted around the houses. There were opinions also regarding the fogging or space-spraying interventions carried out by the Ministry of Health prior to this study, which were considered to be effective for only a few days after the treatment. Some participants felt that the fogging caused "more mosquitoes" than before as this treatment left the mosquitoes able to fly away from their hiding places rather than being knocked down or being killed. Moreover, the smell of the insecticides was strong and many people were uncomfortable during the treatment. In contrast, regarding the IRS used in this study, the participants never complained as both of the chemical compounds used were considered to be odourless. However, the persistence of effectiveness of IRS was seen as being no different to fogging treatments, only lasting for a few days.

5.4.3 Quantification of insecticide treatment and quality control of IRS

One of the important factors required for the success of vector control trials is ensuring high-quality control of procedures. In this current study, pyrethroid quality assurance was conducted using simple colorimetric assays, developed recently by Dr Mark Paine and colleagues at the Innovative Vector Control Consortium (IVCC) (Russell *et al.,* 2014). This colorimetric assay relies on the chemical detection of cyanide released by alkaline hydrolysis (Dowd *et al.,* 2009; Green *et al.,* 2009). This method is preferably used in the field as it only requires simple equipment without a need for highly skilled staff (Russell *et al.,* 2014).

However, despite their ease of use and suitability for field studies, in the current study, many of the test pads were lost before they could be collected for analysis, most likely because they were removed by children or because some fell off the wall at high humidity. Therefore, only 10 samples were tested using colorimetric assay and five samples were used for HPLC analysis. Since the insecticide samples were limited, the samples from adhesive tapes were not used for colorimetric assay and HPLC analysis; only felt pads were used to compare these data. The limited results showed that the treated houses had been adequately sprayed. Since the individuals in a spray team were the same, it is probable that other houses were also treated the same.

For HPLC analysis, three out of five samples of treated houses showed that the level of insecticide declined over time, while in the other two samples, a reduction of insecticide residue was seen at three months but this had reverted six months after spraying (Figure 5.12) The causes of such reversal are not known but might be associated with environmental factors such as temperature and humidity in combination with the type of wall surface. However, the results are similar to Ansari *et al.* (1997), where the insecticide residue was tested to determine the mortality of *Anopheles culicifacies*. The mortality of mosquitoes declined to 80% on the 10^{th} week but then returned to 100% on the 11^{th} week after spraying of 25mg AI/m^2 deltamethrin WP. Another study, by Rohani *et al.* (2007), also showed that residual activity declined at first and then increased.

5.4.4 The effectiveness of IRS control

The impact of IRS on adult dengue vectors in this study was unclear because adult mosquitoes were not sampled. Based on questionnaire data alone, the effects were short-lived and the mosquito population recovered rapidly two to three days after the treatments. Reductions in vector populations not only depend on the chemical control but also on other important factors such as the behaviour of the mosquitoes, the availability of breeding sites and resting sites for adult mosquitoes. For IRS, the last point is especially important. Some of the homeowners mentioned that mosquitoes were found in dark areas or rooms, under beds and furniture, in closets, on doors and windows. They also stated that mosquitoes were frequently found in humid locations such as bathrooms and washing areas in the kitchen. People also pointed out that biting by mosquitoes frequently occurred while watching television, hanging clothes in backyards or washing motorcycles in front of the house. Such information on landing and resting preferences of this species as perceived by the homeowners is highly valuable, together with the spraying efficiency, is important especially when planning for the control trials in the future.

5.5 Recommendations for future trials and additional research

1) While the susceptibility of the targeted vectors to the chemical compounds used is the major factor in ensuring IRS, the degree of indoor resting of the mosquitoes is also critical. It is hypothesised that the behaviour of landing and resting of *Ae. aegypti* might be different in different localities and, prior to any study, this should be confirmed in each new locality.
2) The frequency of treatment is important: clearly, cost will increase with more frequent treatment and an optimal lag time between treatments must be determined.
3) The trial should be repeated in a larger-scale study area, e.g. large treatment clusters/arms with $\geq$ 500 participant houses and with a 'buffer-zone' between clusters/treatments, to avoid spill-over effects - e.g. 200 m distance is beyond the flight range of *Aedes* mosquitoes (Getis *et al.,* 2003).
4) How IRS can be integrated with other approaches, including larval source reduction or educational campaign must be considered.
5) Adult vector populations must be sampled in any future trials, in order to accurately measure impact (Bowman *et al.,* 2014).

5.6 Conclusion

The study has presented results of a cluster-randomised trial of indoor residual spraying (IRS) of a pyrethroid and an organophosphate insecticide, delivered by standard (full indoor surface spraying) and a novel 'strip' treatment approach (treatment of only preferred vector resting surfaces) in a small study site in Penang. Results were inconclusive as to which was the better approach, primarily because significant overspill between clusters may have confounded the results. Results of a KAPB survey indicated that the population was receptive to IRS for dengue vector control, although they did not perceive it to have any persistent impact on vector biting.

CHAPTER 6

SUSCEPTIBILITY STATUS OF *Aedes aegypti*, *Aedes albopictus* AND *Culex quinquefasciatus* IN BAGAN DALAM, PENANG, MALAYSIA

6.1 Introduction

Since mosquitoes transmit parasites and viruses that cause disease worldwide, effective methods are essential to control the mosquito populations. Although dengue mortality and morbidity can be reduced by implementing early case detection, and improving management of severe cases, health services and surveillance systems, vector control is the most effective preventive measure against dengue fever and dengue haemorrhagic fever (WHO, 2012a). Currently, the application of insecticides is one of the most important methods of controlling major disease vectors including dengue, malaria and filariasis vectors (Chen *et al.,* 2005a; Nazni *et al.,* 2005; WHO, 2006b). However, extensive use of various chemicals has enabled the development of insecticide resistance, which has led to failures in vector-borne disease control (Selvi *et al.,* 2006). The wide-scale use of insecticide-based malaria control strategies such as indoor residual spraying (IRS) and for treating bed nets and other materials (WHO, 2006b) has contributed to the development of resistance to multiple classes of insecticide in some vectors (WHO, 2012b). High prevalences of resistance to organophosphates, pyrethroids, carbamates and organochlorines have also been documented in dengue vectors in many parts of the world (Vontas *et al.,* 2012).

The Health Department of Seberang Perai Utara reports that Resigen (permethrin, pyrethroid adulticide) and Abate 1% (temephos, organophosphate larvicide) have been extensively used in the study location of Bagan Dalam, Penang for the past 10 years or more (Table 6.1).

Table 6.1 Insecticide used in Bagan Dalam and the duration of its application

Location	Type	Chemicals	Duration
Bagan Dalam	Adulticide	Resigen (Pyrethroids)	More than 10 years
	Larvicide	Abate 1% (Organophosphates)	More than 10 years

Dengue fever and dengue haemorrhagic fever outbreaks are still being reported in Malaysia and control programmes rely heavily on chemical controls such as fogging and larvicides. Resistance to insecticides in dengue vectors and *Cx. quinquefasciatus* have been reported in many parts of Malaysia (Chen *et al.,* 2005; Nazni *et al.,* 2005; Wan-Norafikah *et al.,* 2008; Wan-Norafikah *et al.,* 2010; Rong *et al.,* 2012; Low *et al.,* 2013) but there is little data on insecticide susceptibility status of mosquitoes in Penang.

Therefore, the target vector populations were monitored to determine the susceptibility status of three mosquito species, *Ae. aegypti, Ae. albopictus* and *Cx. quinquefasciatus*, against lambda-cyhalothrin (pyrethroid) and pirimiphos-methyl (organophosphate) in study area of Bagan Dalam. These species were assessed at each follow-up throughout the study to confirm their susceptibility status.

6.2 Methods

6.2.1 Study area

The study was conducted in the area of *Ujong Batu* in *Bagan Dalam*, *Butterworth* located on the mainland of *Penang* state (05°23.289”N 100°22.397”E; altitude 11 m). A complete description of the study area has been given in Chapter 5.

6.2.2 Mosquito strains

The collection of *Aedes* mosquito samples from ovitraps and immature stages of *Cx. quinquefasciatus* from drainage system was carried out during baseline, 1 month, 3 months and 6 months after the implementation of IRS in the study area.

All these immature stages were reared to adult stage under insectary conditions maintained at 25°C ± 2°C and 80% ± 10% relative humidity. Susceptible laboratory strain of *Ae. aegypti, Ae. albopictus* and *Cx. quinquefasciatus* were used as control in the bioassay tests. These susceptible laboratory strains have been maintained in the Vector Control Research Unit (VCRU), Universiti Sains Malaysia for more than 30 years without exposure to insecticides.

(a) Aedes aegypti and *Ae. albopictus*

Standard ovitraps containing 200 ml of tap water were used to collect field strains of *Ae. aegypti* and *Ae. albopictus*. Two ovitraps were placed randomly in each of the 123 participating houses in the study area. Both ovitraps were placed outdoors, at about 1.2 metres above ground. All ovitraps were collected after 6 days of the placement and transported back to the laboratory. The paddles with mosquito eggs were transferred into plastic containers and topped up with fresh water and a small piece of finely ground fish flakes and brewers' yeast (1:1) as larval food. The mosquito species were identified morphologically when they reached the adult stage. Adult *Ae. aegypti* were separated from adult *Ae. albopictus* using a mouth aspirator and transferred to two different cages. Both species were supplied with white mice for blood-feeding to obtain eggs of the F1 progeny. The mosquito larvae were further reared until they pupated and all pupae were transferred to emergence cages. These F1 adults were provided with 10% sucrose solution and female mosquitoes 3 to 5 days old from the F1 or F2 generation were used in all bioassay tests.

(b) Cx. quinquefasciatus

The immature stages of *Cx. quinquefasciatus* collected from the study area were brought back to the laboratory and were fed with larval food. Mosquito larvae were further reared to adult stages for adult bioassay. The adult mosquitoes were provided with 10% sucrose solution and all susceptibility tests were conducted using F1 of F2 progeny.

6.2.3 Insecticides

The insecticides used in the adult susceptibility test were diagnostic dosages of WHO-impregnated papers obtained from the Vector Control Research Unit, Universiti Sains Malaysia, Penang. According to WHO diagnostic dosages of insecticides, the adults of *Ae. aegypti* and *Ae. albopictus* were tested against (0.03% lambda-cyhalothrin) whereas *Cx. quinquefasciatus* were tested against (0.025% lambda-cyhalothrin). Furthermore, all these three species were tested against (0.25% pirimiphos-methyl) to monitor the susceptibility of adult mosquitoes towards these insecticides (WHOPES, 2007).

6.2.4 WHO adult bioassays

The tube bioassays for susceptible status of mosquitoes in the study areas were conducted during the baseline, 1 month, 3 months and 6 months post-intervention. The bioassays were conducted according to the WHOPES tube bioassays protocol (WHO, 2013c). For these analyses, five groups of 20 unfed female mosquitoes aged 3 - 5 days were introduced into the control (insecticide-free) tubes and held for 20 minutes. After this pre-test period, they were transferred into the test tubes lined with a piece of WHO test paper (12 cm x 15 cm). Mosquitoes were introduced from the holding chamber and exposed to the insecticide for 60 minutes before being blown back into the holding chamber. After exposure to diagnostic dose of insecticides, female mosquitoes were left inside the holding chamber with sucrose solution provided, and maintained in a climatic room for 24 hours at 25°C ± 2°C and 80% ± 10% RH. For pyrethroid insecticide, observation of the number of knock-down (KD) mosquitoes was recorded at regular intervals of 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 minutes. After completing the exposure period, the mortality rate after 24 hours was also recorded for each test.

Female mosquitoes of F1 progeny were used in bioassay tests. However, for most field strains there was an insufficient number of F1 progeny produced and therefore F2 progeny were used to complete the tests. For all bioassay control tests, susceptible VCRU strains were used to compare with field strains. Each bioassay test with five replicates was conducted on the same day. Two additional

tests were repeated for *Cx. quinquefasciatus* Bagan Dalam to confirm the resistance status of this strain since their mortality after 24 hours' exposure was below 98%.

6.2.5 Data analysis

Bioassay data were analysed using a regression log time-probit statistical model (IBM SPSS Statistic 20.0 version). For pyrethroid insecticide, the results obtained were presented as knock-down time KT_{50} for adult bioassay. Based on KT_{50} values, resistance ratio (RR) was determined by the ratio of field strain (Bagan Dalam) to the ratio of susceptible strain (VCRU) as calculated below:

$$\text{Resistance ratio (RR)} = \frac{KT_{50} \text{ of field strain}}{KT_{50} \text{ of laboratory strain}}$$

The presence of resistance was indicated when the value of RR > 1 whereas the value of RR ≤ 1 indicated the susceptibility of the mosquitoes. The following revised criteria were also used for interpretation of adult susceptibility test, as recommended by WHO (2013c):

1. Mortality in the range 98-100% indicates susceptibility.
2. Mortality of less than 98% suggests the possibility of resistance, which needs to be further confirmed. Mortality between 90% and 97% suggests at least two additional tests need to be repeated and, if they consistently show mortality below 98%, then resistance is confirmed.
3. Mortality less than 90% confirms resistance.

If the control mortality was between 5% and 20%, the percentage mortalities should be corrected using Abbott's formula [(% test mortality - % control mortality)/ (100 - % control mortality) x 100]. To compare mortality rates, any P value less than 0.05 was considered statistically significant.

6.3 Results

The susceptibility tests of adult mosquitoes to diagnostic concentration were investigated against *Ae. aegypti, Ae. albopictus* and *Cx. quinquefasciatus* of both

field and laboratory strains. For pyrethroid, the KT_{50} and RR values for all these three species from the study site and susceptible VCRU strains from baseline through the end of the study are illustrated in Table 6.2 to Table 6.4. The control tests without insecticides were conducted at the same time as those for the field strain with impregnated paper. The control test results showed less than 5% mortality of the mosquitoes after 24 hours and were therefore not corrected with Abbott's formula. The laboratory strain of *Ae. aegypti, Ae. albopictus* and *Cx. quinquefasciatus* remained completely susceptible throughout the study, with mortality range between 98% and 100% after 24 hours exposed to diagnostic dosage of lambda-cyhalothrin and pirimiphos-methyl.

Ae. aegypti from Bagan Dalam showed resistance to lambda-cyhalothrin with 0% mortality 24 hours after exposure to the diagnostic concentration. This indicated that *Ae. aegypti* in Bagan Dalam is highly resistant to lambda-cyhalothrin. Knock-down rate after one hour of lambda-cyhalothrin exposure was 100% throughout the study. The values of KT_{50} ranged from 22.11 to 36.64 in this species and the mean percentage of knock-down after one hour exposure to lambda-cyhalothrin were 100% across time point. The laboratory VCRU strains remained susceptible to lambda-cyhalothrin, showing a mean percentage of 100% knock-down after one hour and a mean percentage of 100% mortality after 24 hours exposed to this insecticide (Table 6.2).

Results obtained for *Ae. albopictus* Bagan Dalam strain showed that this species was also resistance to lambda-cyhalothrin with 0% mortality after 24 hours' exposure to the diagnostic dose. The mean percentage of knock-down after one hour exposed to lambda-cyhalothrin was 100% throughout the study. The KT_{50} values ranged from 31.88 to 36.62 throughout the study. The mean percentage of knock-down was 100% at any time point during the study. For VCRU strain, there was 100% mortality after 24 hours and KT_{50} values ranged from 32.10 to 36.11. The mean percentage of knock-down remained 100% across time point in this strain (Table 6.3).

The diagnostic dose of 0.025% lambda-cyhalothrin for *Cx. quinquefasciatus* resulted in 4 - 10% mortality and therefore this species is resistant to this insecticide. Mean percentage of knock-down ranges from 19 - 38% for Bagan Dalam strain and this dose did not confer full mortality against this strain. On the other hand, mean percentage of knock-down recorded for the VCRU-susceptible strain was from 96 - 98%. A high level of RR value (3.66) was observed in *Cx. quinquefasciatus* Bagan Dalam strain at baseline study. The results showed that resistance ratio was decreased after 6 months' study (1.86). This field strain showed fluctuation and was inconsistent in the percentage of 24 hours' mortality across time point, which ranges between 4% and 10%, whereas KT_{50} values were decreased throughout the study. Although the RR values recorded decreased at the end of the study, the resistance to lambda-cyhalothrin remained in this species. The laboratory VCRU strain remained susceptible throughout the study as the values recorded were between 98% and 100% mortality after 24 hours exposed to lambda-cyhalothrin.

In *Ae. aegypti* and *Ae. albopictus* populations under intervention with pyrethroid, the KT_{50} values were not significantly different compared to the susceptible VCRU strain (Kruskal-Wallis Test, $P > 0.05$). There was also no change in KT_{50} values over time in both *Ae. aegypti* and *Ae. albopictus* (Friedman Test, $P > 0.05$). For *Cx. quinquefaciatus*, the KT_{50} values were significantly higher in the Bagan Dalam strain compared to the VCRU strain (Kruskal-Wallis Test, $P < 0.05$). The KT_{50} values were also significantly different across time point in this species (Friedman Test, $P < 0.05$).

For organophosphate insecticide, 0.25% pirimiphhos-methyl was evaluated against *Ae. aegypti, Ae. albopictus* and *Cx. quinquefasciatus.* Bioassay results indicated that both *Ae. aegypti* and *Ae. albopictus* Bagan Dalam were susceptible to pirimiphos-methyl (Table 6.5). Both of these species showed 100% mortality after 24 hours' exposure to this insecticide.

Cx. quinquefasciatus of the Bagan Dalam strain showed 94 - 96% mortality after 24 hours exposed to diagnostic dose. This result suggested that there was suspected resistance in this strain. Two additional tests were conducted to confirm the resistance status of this strain. The percentage mortality obtained from these two additional tests was consistent and therefore suspected resistance was suggested in this strain (Table 6.5).

Table 6.2 Susceptibility status of *Ae. aegypti* towards diagnostic dose of 0.03% lambda-cyhalothrin

Ae. aegypti						
Strain	**Time**	**Mean % ± SD 24 h Mortality**	**Mean % ± SD KD after 1 h exposure**	**KT_{50} in min (95% Cl)**	**RR50**	**Slope ± SE**
Bagan Dalam	Baseline	0[(3)]	100 ± 0.00	22.11 (21.38 - 22.77)	2.37	13.79 ± 1.27
	1 month	0[(3)]	100 ± 0.00	23.51 (22.72 - 24.25)	1.90	11.69 ± 0.96
	3 months	0[(3)]	100 ± 0.00	26.47 (24.42 - 28.37)	1.26	10.06 ± 0.76
	6 months	0[(3)]	100 ± 0.00	30.49 (29.67 - 31.31)	1.34	12.08 ± 0.92
VCRU	Baseline	100 ± 0.00 [(1)]	100 ± 0.00	34.32 (32.34 - 36.21)	-	13.86 ± 0.92
	1 month	100 ± 0.00 [(1)]	100 ± 0.00	36.64 (34.98 - 38.28)	-	14.09 ± 0.95
	3 months	100 ± 0.00 [(1)]	100 ± 0.00	33.36 (31.64 - 35.00)	-	12.56 ± 0.84
	6 months	100 ± 0.00 [(1)]	100 ± 0.00	33.43 (32.43 - 34.40)	-	9.73 ± 0.69

[(1)]: Susceptible; [(2)]: Resistance suspected; [(3)]: Resistant
Slope ± SE: Slope of regression line ± standard error
RR: Resistance ratio KT field strain/KT laboratory strain

Table 6.3 Susceptibility status of *Ae. albopictus* towards diagnostic dose of 0.03% lambda-cyhalothrin

Ae. albopictus						
Strain	**Time**	**Mean % ± SD 24 h Mortality**	**Mean % ± SD KD after 1 h exposure**	**KT_{50} in min (95% Cl)**	**RR50**	**Slope ± SE**
Bagan Dalam	Baseline	0[(3)]	100 ± 0.00	36.62 (35.39 - 37.85)	1.06	6.82 ± 0.39
	1 month	0[(3)]	100 ± 0.00	32.92 (31.79 - 34.03)	0.91	7.16 ± 0.41
	3 months	0[(3)]	100 ± 0.00	32.64 (31.57 - 33.68)	0.93	7.87 ± 0.44
	6 months	0[(3)]	100 ± 0.00	31.88 (30.79 - 32.94)	0.99	7.52 ± 0.43
VCRU	Baseline	100 ± 0.00 [(1)]	100 ± 0.00	34.68 (31.54 - 37.68)	-	9.22 ± 0.66
	1 month	100 ± 0.00 [(1)]	100 ± 0.00	36.11 (33.76 - 38.52)	-	9.27 ± 0.67
	3 months	100 ± 0.00 [(1)]	100 ± 0.00	35.25 (32.63 - 37.84)	-	10.0 ± 0.70
	6 months	100 ± 0.00 [(1)]	100 ± 0.00	32.10 (31.12 - 33.02)	-	10.14 ± 0.72

[(1)]: Susceptible; [(2)]: Resistance suspected; [(3)]: Resistant
Slope ± SE: Slope of regression line ± standard error
RR: Resistance ratio KT field strain/KT laboratory strain

Table 6.4 Susceptibility status of *Cx. quinquefasciatus* towards diagnostic dose of 0.025% lambda-cyhalothrin

Cx. quinquefasciatus						
Strain	**Time**	**Mean % ± SD 24 h Mortality**	**Mean % ± SD KD after 1 h exposure**	**KT_{50} in min (95% Cl)**	**RR50**	**Slope ± SE**
Bagan Dalam	Baseline	4 ± 0.98 (3)	19 ± 0.75	149.04 **	3.66	2.21 ± 0.83
	1 month	10 ± 1.67 (3)	21 ± 1.17	106.63 **	2.79	3.21 ± 0.86
	3 months	4 ± 1.17 (3)	22 ± 1.62	110.6 **	2.94	3.12 ± 0.87
	6 months	7 ± 1.02 (3)	38 ± 0.80	69.71 **	1.86	3.96 ± 0.74
VCRU	Baseline	98 ± 2.45 (1)	96 ± 0.75	40.68 (37.82 – 43.64)	-	7.88 ± 0.49
	1 month	99 ± 2.00 (1)	98 ± 0.49	38.10 (35.53 – 40.57)	-	8.01 ± 0.49
	3 months	100 ± 0.00 (1)	97 ± 0.49	37.66 (35.15 – 40.10)	-	7.27 ± 0.47
	6 months	100 ± 0.00 (1)	96 ± 0.75	37.55 (34.53 – 40.42)	-	7.00 ± 0.46

(1): Susceptible; (2): Resistance suspected; (3): Resistant
Slope ± SE: Slope of regression line ± standard error
RR: Resistance ratio KT field strain/KT laboratory strain
** cannot be computed by probit

Table 6.5 Susceptibility status of *Aedes species and Cx. quinquefasciatus* towards diagnostic dose of 0.25% pirimiphos-methyl

Strain	Time	*Aedes aegypti*	*Aedes albopictus*	*Culex quinquefasciatus*
		Mean % ± SD 24 h Mortality	**Mean % ± SD 24 h Mortality**	**Mean % ± SD 24 h Mortality**
Bagan Dalam	Baseline	100 ± 0.00 [(1)]	100 ± 0.00 [(1)]	96 ± 3.74 [(2)]
	1 month	100 ± 0.00 [(1)]	100 ± 0.00 [(1)]	94 ± 4.00 [(2)]
	3 months	100 ± 0.00 [(1)]	100 ± 0.00 [(1)]	95 ± 4.00 [(2)]
	6 months	100 ± 0.00 [(1)]	100 ± 0.00 [(1)]	96 ± 4.00 [(2)]
VCRU	Baseline	100 ± 0.00 [(1)]	100 ± 0.00 [(1)]	99 ± 2.00 [(1)]
	1 month	100 ± 0.00 [(1)]	100 ± 0.00 [(1)]	98 ± 2.45 [(1)]
	3 months	100 ± 0.00 [(1)]	100 ± 0.00 [(1)]	98 ± 2.45 [(1)]
	6 months	100 ± 0.00 [(1)]	100 ± 0.00 [(1)]	99 ± 2.00 [(1)]

[(1)]: Susceptible; [(2)]: Resistance suspected; [(3)]: Resistant

6.4 Discussion

The susceptibility status of *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* from the study area was investigated. Bioassay results indicated widespread resistance to pyrethroids with 0% mortality recorded for both *Aedes* species in Bagan Dalam. *Cx. quinquefasciatus* mosquitoes showed only 4 - 10% mortality after 24 hours. This pyrethroid insecticide has been widely used as an adulticide for more than 10 years in this study area. A study conducted by Chan *et al.* (2011) revealed that *Ae. albopictus* have developed resistance to 0.2% deltamethrin and 0.7% permethrin in two dengue hotspots on Penang Island. Pyrethroid resistance is also known to be widely developed in both *Aedes* species and *Cx. quinquefasciatus* in other dengue areas in Peninsular and East Malaysia (Nazni *et al.,* 2005; Wan-Norafikah *et al.,* 2008; Wan-Norafikah *et al.,* 2010; Rong *et al.,* 2012; Low *et al.,* 2013).

Pyrethroids' mode of action can be described as neurotoxic to insects. Pyrethroids can cause agitation, hyperactivity, lack of coordination and paralysis to insects. For flying insects such as mosquitoes, the knock-down effect is rapid but the symptoms vary depending on the type and dosage of pyrethroids used. The lethal effect of pyrethroids includes the action on both peripheral and central neurons, while the knock-down effect possibly involves peripheral intoxication (Becker *et al.,* 2010). The knock-down rate in this study was observed to be contradictory to mortality results. In order to discriminate between mortality and knock-down effect, after exposure to a diagnostic dosage of pyrethroids, these mosquitoes were transferred to holding chambers (insecticide-free tubes). The effects of pyrethroids on both *Ae. aegypti* and *Ae. albopictus* were rapid, with 100% knock-down rate, but, in this case, 100% recovery was observed after 24 hours in the absence of insecticide. Therefore, the results showed that *Aedes* mosquitoes in this study area were extremely resistance to pyrethroids. On the other hand, the knock-down rate is lower in *Cx. quinquefasciatus* compared to *Aedes* mosquitoes. This species is also resistant to pyrethroids as their recovery rate was up to 90% after 24 hours observed in the absence of insecticide.

The rapid knock-down induced by pyrethroids demonstrated that the rapid action is on the nervous system, suggesting the main penetration route of pyrethroids was in insect spiracle (Sugiura *et al.,* 2008).

Pyrethroid resistance was observed in both *Aedes* species, raising the question of the selection source. In this study, both *Ae. aegypti* and *Ae. albopictus* were found in the same breeding containers. The pyrethroid resistance in *Aedes* species is possibly due to the exposure to insecticide, particularly during the control intervention, which targeted the adult stage. *Ae. aegypti* prefer resting indoors and are likely to be exposed to household insecticides inside the house whereas *Ae. albopictus* prefer resting outdoors and may be more exposed to insecticides during fogging or space-spraying. According to Chan *et al.* (2011), low level of pyrethroid resistance was detected in *Ae. albopictus* strain from two dengue hot spots on Penang Island.

The extensive usage of pyrethroid insecticides has demonstrated the development of mosquito resistance in the Bagan Dalam strain. Pyrethroids are widely used in many household insecticide products and have now become one of the most important classes of insecticide with major usage in dengue control and public health (Yap *et al.,* 2000). Various mosquito controls such as ultra-low volume (ULV) fogging, thermal fogging, surface residual spray and household insecticide products are widely used for the control of adult mosquitoes in Malaysia (Yap *et al.,* 2000). The introduction of fogging activities using pyrethroid insecticide has contributed to pyrethroid resistance in *Cx. quinquefasciatus* (Nazni *et al.,* 1998) and *Aedes* species in this country (Rohani *et al.,* 2001). Many household insecticide products such as mosquito coils and mats, liquid vaporizer and aerosol which contain pyrethroids have been widely commercialised in Malaysian markets. All the active ingredients in these products are pyrethroids, including d-allethrin, d-*trans* allethrin, transfluthrin, prallethrin, s-bioallethrin, deltamethrin, d-phenothrin, permethrin, and tetramethrin (Yap *et al.,* 2000). Over-reliance on these products may also contribute to pyrethroid resistance development in these species.

Pirimiphos-methyl is an important member of the organophosphate group, which is derived from phosphoric acid. Organophosphates are chemically unstable but are more toxic to vertebrates (Becker *et al.,* 2010). In insects, pirimiphos-methyl produces toxic action at synapses by binding to and inhibiting acetylcholinesterase (AChE), an important enzyme in the nervous system. This inhibition causes the accumulation of acetylcholine, which interferes with the neuromuscular junction, producing rapid twitching of muscles and finally killing the insects (Becker *et al.,* 2010). This killing effect has been observed in *Aedes* mosquitoes in Bagan Dalam as the mortality rate after 24 hours was 100%. Therefore, full susceptibility to pirimiphos-methyl was detected in both *Aedes* species. In contrast, *Cx. quinquefasciatus* from the Bagan Dalam strain showed a low prevalence of resistance to pirimiphos-methyl (94 - 96% mortality after 24 hours). Two additional tests were conducted to confirm this resistance status and the percentage mortality remained consistent in both tests. Therefore, this species was categorised into 'suspected resistance' as suggested by WHO (2013c).The results reported here suggest that the application of organophosphate as adulticide in this area should be rotated with pyrethroid during control programmes as this class of insecticide still remains largely effective against both *Aedes* species and *Culex*.

Furthermore, it is essential to implement a resistance management strategy as these mosquitoes could rapidly develop high levels of resistance (WHO, 2013c). This strategy should include frequent resistance monitoring and using an accurate detection tool such as topical application to confirm the susceptibility status of these mosquito species. Based on their results, Chan *et al.* (2011) concluded that topical application is a more sensitive and indicative bioassay than the standard WHO kit. This method would be more effective against mosquitoes, especially those with a low level of resistance. Subsequently, it is also important to determine and characterise the mechanism(s) of resistance involved in mosquito populations by biochemical and molecular studies to further inform which management strategies would be the most relevant and reach their maximum impact (Selvi *et al.,* 2007).

The data obtained from this current study suggest that the Ministry of Health or the health authority should consider a comprehensive plan for further routine control strategies and outbreak responses. Bacteria such as *B. sphaericus* and *B. thuringiensis* are currently used in vector control programmes and these microbial agents produce a crystal toxin which is beneficial to their performance as biological insecticides (Brogdon & McAllister, 1998). Therefore, a possible alternative is the use of these biological insecticides (Loke *et al.,* 2010) or other non-chemical methods for use together in the control of dengue vector and *Cx. quinquefasciatus*. Generally, non-chemical methods involve destroying mosquito breeding sites, clean-up campaigns and the use of biological controls. To complete this, these methods could be combined with health education and public health communication. There are no easy solutions to dengue problems in Malaysia. Public participation in dengue control would be the biggest challenge in this situation. The effectiveness of any control programmes is associated with the public's attitude, knowledge and practice. Although most of the people are aware of the campaign, they are not willing to practice the suggestions in their real surrounding. Thus, to overcome this, the public should be made aware of their responsibilities to control dengue and encouraged to develop positive attitude to keep their houses free from mosquito breeding sites.

6.5 Limitations and future works

As dengue remains unremitting in Malaysia, especially in Penang, the use of pyrethroid and organophosphate insecticides must be organised appropriately to ensure their protracted durability as an effective tool in the control of *Aedes* species and *Cx. quinquefasciatus.* Insecticide resistance was detected in this study, therefore biochemical and molecular studies should be carried out in the future to characterise the mechanism for resistance involved in these mosquito species. The best strategy for controlling these vectors is the rotation of different types of insecticide. This strategy is important to extend the longevity of current available insecticides and consequently lower the risk of re-emergence of vector-borne diseases in the future.

6.6 Conclusion

1) *Ae. aegypti, Ae. albopictus* and *Cx quinquefasciatus* were highly resistant to lambda-cyhalothrin throughout the study, with less than 10% mortality after 24 hours.
2) *Ae. aegypti* and *Ae. albopictus* remained susceptible to pirimiphos-methyl, with 100% mortality recorded after 24 hours. However, *Cx. quinquefasciatus* has been detected as 'suspected resistance' to pirimiphos-methyl as the mortality recorded after 24 hours was between 94 and 96%.

CHAPTER 7

GENERAL DISCUSSION

At present, dengue infection continues to be a serious public health concern in Malaysia while Chikungunya infection is now classified as an emerging disease (Lam *et al.,* 2001). Malaysia has continuously recorded rising cases of dengue infection in recent years with an incidence of 167.76 per 100, 000 population with 0.02 mortality rate recorded in 2008 (Ministry of Health Malaysia, 2008). As development advances in Malaysia, the rapid massive infrastructure expansion in urban areas has contributed to this high incidence because *Ae. aegypti* proliferates in urban areas. Control of this difficult vector remains a major public health challenge for the population at risk of infection in Malaysia and in all tropical countries worldwide (Farrar *et al.,* 2007; Morens & Fauci, 2008; Gómez-Dantés & Willoquet, 2009; WHO, 2009), with an urgent need to improve dengue prevention and control efforts. Thus the fundamental purpose of this research was to consider new perspectives and approaches for the management of *Aedes aegypti* control by incorporation of behavioural factors in evaluating potential new tools.

It is well understood that host-seeking in mosquitoes is mediated primarily by chemical cues and many previous studies focused on host odours and identification of specific compounds or odour blends that mediate the attraction of host-seeking mosquitoes (Smallegange & Takken, 2010; Bernier *et al.,* 2007; Spitzen *et al.,* 2008). In chapter 3, results showed that female *Ae. aegypti* were attracted to the top of the human body, particularly around the head. This suggested that a plume of host odour and other cues emanating from the host and rising up, possibly carried by thermal currents from the torso, provided a powerful host cue for host-seeking females. Previous studies provided a similar proposition for *Anopheles gambiae*, showing that rising emanations attracted that species to the host (Dekker *et al.,* 1998; Guillet *et al.,* 2001; Lynd & McCall, 2013). This activity has not been reported previously for *Ae. aegypti* and therefore, enhances our understanding of this species' host-orientation

behaviour. Further studies should investigate the routes of travel before mosquitoes arrive and land on the host. The results obtained in Chapter 3 demonstrated that adhesive-coated nets can be a useful tool for measuring landing behaviour of female *Ae. aegypti* in the laboratory. The results from this chapter also provided new knowledge on the behavioural responses of female *Ae. aegypti* to the human host. As the result mentioned above demonstrates, the arrival location of female *Ae. aegypti* on a human host were recorded clustering on the top surface of the net and the limited areas of the side surface of the net (the nearest surface to the release side of the mosquitoes). This begged the question as to why the mosquitoes did not significantly land on the remaining sides (i.e 'front', 'back' and 'far' of the net surface). In a laboratory study on *An. gambiae* landing behaviour, Lynd (2008) has shown that multiple release points did not influence the landing outcome in her study. A conclusion can be made that release points were not determining factors of landing behaviour. Adopting this finding, multiple released points were not tested for this study as well. To that end, the release point for this study (i.e. 'near' side) was considered as a valid test point of release. In retrospect this issue should be given more attention. The landing preference to the right and top might just have been due to the closeness between the cage and the release point. In other words, the mosquitoes might have been caught or stuck at the closest point before they were able to go to the other sides of the cage. This is recognized as a limitation of this study, and thus, better method for release point has to be investigated in future studies. In addition to further investigations of the arrival locations of host-seeking female mosquitoes, studies must be undertaken in human habitation (field evaluation). The significance of this finding is principally for improving the effectiveness of ITMs or ITNs and for developing novel trap for mosquito control. For this reason, various kind of odour-released equipment which imitate the natural plume pattern released by a host could be tested and thus improve the trapping effect of the artificial baits used. It is also believe that mosquito surveys could be conducted with odour-baited traps and these traps also could be used to interrupt the host-seeking behaviour of female *Aedes* mosquitoes in the future.

Chapter 4 provided a further investigation of female *Ae. aegypti* behavioural response but it predominantly focused on the landing responses of female *Ae. aegypti* on panel targets in the absence of a human host. The laboratory trials presented in Chapter 4 explored *Ae. aegypti* resting behaviour by studying preferences for different target panels. The long-term objective here was to identify visual and tactile cues that might be employed to design insecticide-treated resting targets or traps. Results indicated that landing responses were significantly altered by contrast, orientation and height of the target panels, but not by surface texture. The combination of parameters that delivered the most attractive, and therefore the most effective, target were black colour and in a vertical configuration at 90 cm above ground. The distribution of female *Ae. aegypti* on the targets indicated that landing patterns were random, with no evidence for any preference for the edges or for other, already-trapped mosquitoes.

Trials of resting box parameters were also undertaken and showed that, in the laboratory, the best combination of parameters was placed at 90 cm height with over 65% internal humidity. The purpose of assessing this method is to develop the simplest and most inexpensive method of collecting *Aedes* mosquitoes in the field. However, resting boxes developed in this current study demonstrated that collection of resting mosquitoes only comprises *Cx. quinquefasciatus*. According to Bentley *et al.* (2009), if the resting boxes were used in the field, this method also could be incorporated into surveillance studies for identifying the status or stages of infectivity as well as to prevent humans from being bitten by infected mosquitoes. Furthermore, Bentley *et al.* (2009) documented that when he compared with HLC, resting boxes are cheap, as well as easy to transport, deploy and collect, unlike baited traps or those requiring CO_2 or other chemical attractants. In recent years, some studies have also evaluated the resting box performance with different forms but similar aims, primarily to target resting mosquitoes. These studies include one by Kweka *et al.* (2009), which evaluated resting boxes for anopheline sampling in the field, and they considered that this method is potentially useful in replacing human landing catches (HLC). Furthermore, recently, Pombi *et al.* (2014) found that the sticky resting box

(demountable wooden box with an opening at the upper front side, internal walls covered by black cotton cloth and coated with glue) also showed promise as a tool for monitoring anopheline resting population in the field.

Although the results obtained in Chapter 4 suggested that a height of 90 cm in the laboratory was preferential for resting by female *Ae. aegypti*, this result was not tested in the field trials conducted in Chapter 5. Since the field trials were conducted before the results from Chapter 4 were analysed, therefore this logistic problem was inevitable and the results from the preliminary experiments conducted in Mexico, instead, were adopted to use for the protocol in Chapter 5. The same situation occurred for the adult mosquito sampling techniques. Resting boxes that were developed in Chapter 4 were not used during the field trials in Chapter 5 because the adult mosquito sampling (specifically for *Cx. quinquefasciatus* mosquitoes) was conducted before those resting boxes were examined both in the laboratory and field. As a result, CDC light traps were used to collect adult *Cx. quinquefasciatus* during field trials in Chapter 5. To this end, it is recognize that the preferred high of landing for female *Ae. aegypti* found most effective in the lab analysis (i.e. 90 cm above ground level) was not tested in the fieldwork for this study. This is acknowledged as one of the limitation of this study, and that the preferential height found from the lab analysis should be redone in future fieldwork studies.

The control trial using IRS described in Chapter 5 set out to explore the effectiveness of IRS against *Ae. aegypti*. The area where the trial was conducted revealed an abundance of potential breeding sites for *Ae. aegypti*. The improper management of discarded domestic items means that these items, as well as more natural or permanent manmade locations, may potentially become breeding places for this species. As shown in this study, small containers were the most common breeding site in all cluster areas. Elsewhere, other studies (Nathan & Knudsen, 1991, Tun-Lin *et al*., 1995, Chadee, 2004, Barrera *et al*., 2006, Burkot *et al*., 2007, Maciel-de-Freitas *et al*., 2007) reported that large containers such as drums were the main breeding sites

for *Ae, aegypti*. However, in areas with a reliable water supply, such large containers are not common and dengue vectors breed elsewhere (McCall & Kittayapong, 2007, Troyo *et al*., 2008). Further surveys would be required to confirm the importance of small containers as dengue vector breeding sites, but, if true, then local public health efforts might focus on education and clean-up campaigns to eliminate such sites from Penang.

For IRS control to be effective, several factors are important. Firstly, *Ae. aegypti* resting behaviour must occur primarily within houses (Chadee, 2013). Secondly, IRS requires effective leadership and management for planning, organisation and implementation, with a good knowledge base on local epidemiological data, transmission patterns and insecticide resistance status, all of which require skilled professional teams (WHO, 2006). Thirdly, IRS is expensive and sufficient and sustained funding is necessary to achieve success. Finally, acceptance and cooperation by the communities regarding spraying such as allowing teams to enter their premises and tolerating the disruption are critical for the success of IRS.

Ensuring sustained acceptance by the communities is crucial because it has been observed that if home access is rejected, it can result in IRS failure anywhere in the world (WHO, 2006). Throughout the study, based on KAPB surveys, the IRS control trial appeared to be well accepted by the communities in the Bagan Dalam study site. During follow-up, the majority of the householders also gave their full support for the trial. To ensure the participation of communities, ongoing education may be necessary to maintain the awareness and often the behavioural changes required for sustaining efforts to reduce mosquito populations. Minor practical changes in daily routines by individuals, families and communities can be critical to ensure that they live in a dengue-free area. Thus, communities should be encouraged to actively participate in the implementation of control programmes, with education about dengue and its prevention fundamental in implementing new strategies to control dengue.

Public health education must be designed to target different groups of people, taking into account their different cultural backgrounds, education and socio-economic levels. Ideally, this education system might be introduced to students at schools, colleges and universities. It is also necessary to ensure the control strategy is adaptable to different communities because it will need to fit within routine household activities as much as possible. Furthermore, community health education could help behavioural changes which in future could improve both physical health and human well-being in all aspects of health.

Back in 1990, 27.5 cases per 100,000 populations were reported in Malaysia and drastically increased to 123.4 cases per 100,000 populations in 1998 during the global pendemic (Teng & Singh, 2001). In 2010, the number of cases increased to 46,171 cases which are 1648.96 per 100,000 populations. However the dengue cases reported in 2011 decreased to 19,884 cases with 36 deaths. During 2013, the number of dengue cases reported in Malaysia increased by 98% over the previous year, with a total of 43,346 cases reported compared to 21,900 cases in 2012. Deaths from dengue infections also increased, with 92 deaths in 2013 compared to 35 in 2012 (Ministry of Health, 2014). These figures reveal that Malaysian dengue vector control programmes are still unable to impact the increasing challenge of dengue, despite considerable efforts in recent years. Clearly, better methods are needed. However, if a single control method is unable to achieve the required impact on the vector population, integrated control must be used. We should therefore remain optimistic and use all the useful information and available control measures to achieve the greatest impact on dengue control.

It is well established that mosquito control measures used in Penang primarily rely on chemical control. Since pyrethroids and organophosphates were used for an extensive periods in mosquito control in this study area, therefore it is important to update the susceptibility status of this mosquito population. Chapter 6 reported the insecticide susceptibility status of *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* in the

study area, demonstrating the development of insecticide resistance in these three species. One of the most important parts of any integrated vector control programme is the monitoring of vector resistance status, as the early detection of insecticide resistance is a critical factor for insecticide resistance management. The high level of resistance to lambda-cyhalothrin reported in this study is worrying, because if this class of insecticide is used continuously, it may confer resistance at a rapid rate in the province and subsequently in the region. Therefore, alternative strategies (*e.g.* non-insecticide-based, such as biological control using predators, bacteria and viruses) are required to resolve this problem. Since IRS has the advantage of using a wide variety of insecticide classes (WHOPES, 2007), it extends the range of insecticides available for the management of insecticide resistance in this situation.

In 2013, the Malaysian Ministry of Health implemented several new action points and initiatives in preparation for dengue threats in 2014. 'Program Ayuh Gempur *Aedes* Perdana' aiming to eliminate *Aedes* breeding sites was launched by the Deputy Minister of Health Malaysia in January 2014 and has operated on a monthly cycle. A COMBI (Communication for Behaviour Impact) programme was conducted to provide incentives, especially for private companies or entrepreneurs, to help and promote social responsibility for controlling mosquitoes and preventing dengue. There are other ongoing research and control strategies conducted in Malaysia. The 'OPS Gempur *Aedes*', the enforcement activities have been conducted since July 2013. This programme was also carried out to eliminate breeding places of *Aedes* and actions are taken against householders if positive breeding sites are found in their premises. Recently, some studies have been carried out by the Institute for Medical Research (IMR) in Kuala Lumpur. These studies include (a) the use of 'autocidal trap' to kill immature stages and adult mosquitoes, (b) the use of 'insect chemical control agent' to ensure that the mosquitoes spread the chemicals to the potential breeding sites and (c) the use of 'outdoor residual spraying'. The insecticides used are 'rain-resistance' formulation and have been applied on the exterior walls of the building and trees around the premises (Ministry of Health, 2014).

As a final observation, no control programme will be able to eliminate dengue as long as *Aedes* are able to breed and new mosquitoes can emerge and spread the disease. Therefore, the incessant research and control strategies as mentioned above should be continued. The public and health authorities involved should remain optimistic in order to ensure that the efforts to prevent and control mosquito disease are successful. This also could be achieved through adequate knowledge on *Aedes* species as dengue vectors and hence the development of positive attitude and practice to retain the suppression of dengue and dengue vectors. Thus, with regard to providing a tailored solution, the existing information on dengue vectors and control tools should be utilised according to the needs of the particular community and its environment.

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Appendices

Appendix 1

Liverpool School of Tropical Medicine

Universiti Sains Malaysia

Informed Consent to Participate In the Research Study Entitled

Exploiting vector behaviour for dengue control by indoor residual spraying

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Village Executive Officer _____Signature_____GPSNumber_____

Name of household head _____Signature _____Household ID _____

This letter is to explain the purpose of the study and to ask for your permission to include you in the study.

Our project aims to test new methods for killing mosquitoes inside houses, to reduce the number of mosquito bites and to prevent disease. The method we will use is to spray the insecticide onto the walls inside the house; by doing this, we put a residue onto the wall that kills mosquitoes (and other pest insects like flies and cockroaches) when they land on the wall. Although this is a very old method and has been used worldwide in the past, we will test new formulations of two insecticides, called ICON and ACTELLIC. Both have been approved by the World Health Organization for this purpose and both are used safely worldwide for pest control, and we will find out if the new versions can kill mosquitoes for linger than previously.

Each insecticide will be tested in two ways: (1) by spraying the entire walls and ceilings (if in the house) in the living and sleeping areas, or (2) by spraying only the top 1m section of each wall and the ceiling. So we also want to know if spraying only the tops of the walls as effective as spraying the entire room - if it is, then it will be faster, cheaper and less trouble for everyone, than spraying the whole house.

To do this work, we need to test this in a real community. So we are asking if you will participate in our study. We have divided all the houses in Bagan Dalam into 5 groups: 4 of these will receive a treatment and the fifth group will receive no treatment. At the end of the study (about 10 months after spraying), every house will be treated with whichever treatment we discover is the most effective.

All the work will take place during short visits to each house, in the daytime. At the beginning, we will come to your house to examine for mosquitoes in and around the house, and about 1 week later, we will return to spray the walls and ceilings. After then, we will visit your home on 4 different occasions, to look for mosquitoes.

At the beginning of the study, we will also ask you some questions about mosquitoes in Bagan Dalam, and in your house. We may return to ask you more questions later in the study.

All of the results and all of the personal information about you and your home that we collect will be kept entirely confidential and will not be given to anybody outside this project. We will report to you what the study discovers and how best to protect your family from mosquitoes, and to provide a final best treatment for your house at the study's end. We hope the study will help us understand more about controlling mosquito-borne disease, not just here in Malaysia, but throughout SE Asia and elsewhere in the world.

Thank you very much.

If you do not wish to participate, you are free to tell us so. We will accept your decision immediately.

Also if you do let us begin, you may tell us freely at any time if you change your mind and want to withdraw.

Refusing to participate will not affect your rights to receive treatment for any health problems or to participate in any other projects by this or any other institutions at any time or in any way, in the future.

Thank you very much for your cooperation.

Ms Hadura Abu Hasan (USM) Signature_____ Date_____

Prof Zairi Bin Jaal (USM) Signature_____Date_____

Dr Philip J McCall (LSTM) Signature_____Date_____

Willing to take part_____Date_____

Not willing to take part _____Date_____

If you experience any ill-effects after the study begins, please contact Ms Hadura Abu Hasan on 04-6533053 or Prof Zairi Jaal on 04-6574776.

Appendix 2

Indoor Residual Spraying for control of *Aedes aegypti* in Bagan Dalam

2011/2012

An experimental trial by Universiti Sains Malaysia and

Liverpool School of Tropical Medicine

Questionnaire 1

We would like to ask you some questions about mosquitoes and how to control them. Your answers will be kept confidential and will be used only for the purposes of this study. The information you give will not be passed on to anybody else. If you do not want to answer these questions, or if you wish to stop at any time, we will be happy to do so. This will not affect your participation in this trial or any other programs in the future.

Date_____ **House ID no**_____

1. Are mosquitoes a problem in your house? *Yes*____ *No*____ *Don't know* ____

2. If 'yes', *when* do they bite? _____

3. If 'yes', *where* do they bite? _____

4. What measures do you carry out to reduce or prevent mosquito bites at present?

_____

5. Where do you think the mosquitoes come from or where do they live?

_____

6. What do you think is the best method to prevent mosquito in the house?

_____

Appendix 3

Indoor Residual Spraying for control of *Aedes aegypti* in Bagan Dalam

2011/2012

An experimental trial by Universiti Sains Malaysia and

Liverpool School of Tropical Medicine

Questionnaire at follow-up immediately after insecticide treatment

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We would like to ask you some questions about the treatments just carried out. Your answers will be kept confidential and will be used only for the purposes of this study. The information you give will not be passed on to anybody else. If you do not want to answer these questions, or if you wish to stop at any time, we will accept your decision. This will not affect your participation in this trial or any other programs in the future.

Date_____ **House ID number**_____

1. Did the insecticide treatment reduce the number of mosquitoes inside the house?

Yes____ ***No, same as before***____ ***No, the numbers are greater than before treatment*** _____

2. If 'Yes', *how long* since you were last bitten by mosquitoes?

1 day____ ***2 -3days***_____ ***7 days***_____

3. Have you seen any dead mosquitoes inside the house after spraying?

Yes ____ ***No*** _____ ***Don't know*** _____

4. Did the insecticide treatment reduce the number of other insects (cockroaches, ants, flies) inside the house?

Yes____ ***No, same as before***____ ***No, the numbers are greater than before treatment*** _____

5. Have you or your family experienced any problems following the treatment?

Yes ____ ***No*** _____

6. If 'yes': what were the symptoms, how long did they last and what did you do about it?

Appendix 4

Indoor Residual Spraying for control of *Aedes aegypti* in Bagan Dalam

2011/2012

An experimental trial by Universiti Sains Malaysia and

Liverpool School of Tropical Medicine

Questionnaire at second follow-up after insecticide treatment

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We would like to ask you some questions about the treatments just carried out. Your answers will be kept confidential and will be used only for the purposes of this study. The information you give will not be passed on to anybody else. If you do not want to answer these questions, or if you wish to stop at any time, we will accept your decision. This will not affect your participation in this trial or any other programs in the future.

Date_____ **House ID number**_____

1. Did the insecticide treatment 3 month ago reduce the number of mosquitoes inside the house?

Yes____ ***No, same as before***____ ***No, the numbers are greater than before treatment*** _____

2. How long since you were last bitten by mosquitoes?

1 day____ ***2 -3days***_____ ***7 days***_____

3. Did the insecticide treatment 3 month ago reduce the number of other insects (cockroaches, ants, flies) inside the house?

Yes____ ***No, same as before***____ ***No, the numbers are greater than before treatment*** _____

4. Have you or your family experienced any problems following the treatment?

Yes ____ ***No*** _____

5. If 'yes': what were the symptoms, how long did they last and what did you do about it?

_____

ENTOMOLOGICAL SURVEY FORM

Exploiting vector behaviour for dengue control by indoor residual spraying

Start time: ..... No. of people in the house: ..... adult..... child Observer: .....

Date: ..... House ID: ..... Finish time: .....

No	Type of container with water	Location		Under the sun	Under the shade	Volume of water	Presence of organic material	Diameter	Height	No. of larvae	No. of pupae	Other organisms
		Inside	Outside									
1												
2												
3												
4												
5												
6												
7												
8												
9												
10												

Containers key: Tanks >1000 litres; Drums 150-200 litres; Tyres ≤1 litres; Small containers <3 litres ; Medium containers 3-20 litres; Large containers >20 litres; Others