IMPACT OF INSECTICIDE TREATED-MATERIALS ON DENGUE VECTOR POPULATIONS IN VENEZUELA.

Thesis submitted in accordance with requirements of the University of Liverpool for degree of Doctor in Philosophy

by

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ABSTRACT

Currently, in terms of morbidity and mortality, dengue fever is considered the most important vector-borne viral disease in the world, potentially affecting 2.5 billion people in more than 100 tropical and sub-tropical countries worldwide. *Aedes aegypti,* a cosmotropical, day-biting mosquito, is the main vector. A highly synanthropic species, *Ae. aegypti* exploits a variety of man-made containers as breeding sites in and around the household and to date, prevention of dengue has been based on control of such breeding sites. This project evaluated the efficacy of two types of insecticide-treated materials (ITMs) that target adult *Aedes aegypti*, window curtains and domestic water container (known as drums) covers, in a cluster randomized controlled field trial in Trujillo, Venezuela.

As measured by the House, Breteau, Container and Pupae per Person (HI, BI, CI, PPI) indices, an immediate impact on vector populations was seen following introduction. In general, reductions in dengue vector populations were sustained throughout the study, particularly when both curtains and covers were used together. At baseline, efficacy of ITMs was highly heterogeneous as measured by bioassay, ranging from 45-100% mortality. Curtains remained intact for the duration and although became dirty, retained insecticidal efficacy (48-97% mortality). Water jar covers however, tore and became unusable (only 42% households still used them after 26 months). No bioassays were conducted in covers. Measurement of IgM seroconversion levels in the trial population were inconclusive regarding any impact on dengue virus transmission.

Using WHO criteria, *Ae. aegypti* populations from the external control site showed consistent susceptibility to deltamethrin, but populations from within the trial areas, including the internal control arm, were categorized as 'suspected resistant' by the end of the study.

In a series of studies to compare various parameters to measure evolutionary fitness, adult female *Ae. aegypti* emerging from pupae collected in drums lived longer, laid larger and more viable egg batches, retained fewer eggs and had higher insemination rates than those from small containers and tyres. Using geometric morphometric analysis, females from drums had significantly larger adult bodies than those from other sites, estimated by centroid size. Mosquitoes collected from drums were significantly smaller at baseline than those collected at the end of study. Since domestic water container covers can impact on the vector population and drums are known to be the most important sources of adult *Ae. aegypti* in this area, the implications of these findings are discussed.

The findings of this study are not yet sufficient to form a final conclusion on the efficacy of ITMs for dengue prevention globally, for which further trials are needed. However, they contribute to the growing data on that topic and provide an initial evidence base for local authorities to reconsider the direction of the control policies that are being applied routinely for the control of *Ae. aegypti* in Trujillo State.

DECLARATION

This work has not been previously accepted in substance for any other degree and is not being currently submitted in candidature for any other degree.

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Statement 1

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Statement 2

This thesis is the result of my own investigation, in which I carried out cluster selection and allocation, community sensitisation, ITM distribution, training and supervision of field entomology and serosurvey teams, processing of collected material and data collection, including cleaning and checking the dataset. I carried out all of the experiments investigating mosquito fitness and morphometry. I performed all the statistical analyses, following the advice of those acknowledged in the thesis, and I interpreted the results. I reviewed the literature and wrote the thesis.

Exceptions were:- the samples for dengue IgM capture ELISA were analysed in the Centre for Vaccine Development, Institute of Molecular Biosciences, Mahidol University, Bangkok, Thailand, and the insecticide bioassays were conducted in collaboration with L. Alvarez (NURR, ULA, Trujillo).

Signed.....(Candidate)

Date.....

CONTENTS

DEDICATION

This Thesis is dedicated, firstly to my parents, and secondly to my son Manuel Alejandro, who have always been the most important pillars supporting and encouraging me. With immeasurable gratitude and love I recognize this success was possible thanks to them.

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Many thanks to La Universidad de Los Andes and La Fundación Gran Mariscal de Ayacucho, whose financial support made this, the culmination this stage of my academic career, possible; European Union FP-6 for funding the DENCO consortium trial in Venezuela; Vestergaard-Frandsen for donating the insecticide-treated materials; Dr. Sutee Yoksan (Bangkok) for serology; the communities in Monay, Flor de Patria, Pampán, Pampanito and Motatán, whose collaborations were invaluable in development of this research. My thanks go to Abraham, Anthony and Carlos Villegas, especially for all the nights they spent with me in the lab, counting eggs and mounting wings of *Aedes aegypti* females. My sincere gratitude to Ing. Miguel Linares and Prof. Jose Maria Anderez, whose unconditional and timely support was decisive. My deepest gratitude to my brother Luis Manuel, I would not have made it without his technical support in data management. Special thanks to my dear Gastón for his love and support. Thanks to my friends and partners in: Laboratorio de Ecología de Parásitos (Dr. Eric and Humberto), CIDIS (Luis Miguel and Clemencia), Instituto "José Witremundo Torrealba" (especially Dr. Elci, Lic. Herminia, Dr. Milagros and Lic. Leslie Lavarez), Departamento de Biología y Química (especially to Drs. Carlos Navea and Delfin Viera). Huge and special thanks to the Gettings-Godfrey family and Gwen Finnegan who were invaluable pillars throughout this time.

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ABBREVIATIONS USED

AFS	Arbovirus Field Station
ANOVA	Analysis of variance
AUC	Area under Curve
BI	Breteau Index
Bti	*Bacillus thuringiensis israelensis*
C	Carbamates
CI	Container Index
CDC	Centers for Disease Control and Prevention
CDNA	Communicable Diseases Network Australia
CHIK	Chikungunya virus
DDT	Dichlorodiphenyltrichloroethane
DEN	Dengue
DNA	Deoxyribonucleic acid
DENCO	Dengue and Control study (multi-country study)
DF	Dengue fever
DHF	Dengue haemorrhagic fever
DSS	Dengue shock syndrome
ELISA	Enzyme-linked immunosorbent assay
EMRO	Regional Office for the Eastern Mediterranean
HCH	Hexachlorocyclohexane
HI	House Index
HIV/AIDS	Human immunodeficiency virus/acquired immunodeficiency
syndrome	
IgM	Immunoglobulin M
IGR	Insect growth regulator
ITM	Insecticide treated material
ITN	Insecticide treated nets
KD	Knock down

KDT	Knock down time
LLIN	Long lasting insecticide impregnated nets
LM	Land marks
MFO	Mixed function oxidase
OC	Organochlorinated compounds
OP	Organophosphorus insecticides
PAHO	Pan American Health Organization
PCR	Polymerase chain reaction
PPI	Pupae per person index
PY	Pyrethroides
RAPD	Random Amplification of Polymorphic DNA
RR	Resistance ratio
SPSS	Statistical Package for the Social Sciences
TB	Tuberculosis
TDR	Special Programme for Research and Training in Tropical Diseases
ULV	Ultra-low volume
WHO	World Health Organization
WHOPES	WHO Pesticide Evaluation Scheme

CHAPTER 1

INTRODUCTION: DENGUE HISTORY, VECTORS AND CONTROL

1.1 Overview

Dengue fever is globally the most widely distributed insect-borne human disease. The four dengue virus serotypes cause a variable spectrum of disease ranging from asymptomatic infections to an undifferentiated fever, the classical dengue fever (DF) and the severe disease, such as dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) (WHO, 1997, Ligon, 2005, Guzman *et al.*, 2004). Approximately two fifths of the world's population are living in areas where there is a risk of dengue infection and more than 50 million new infections occur annually. Of the reported cases, several hundred thousand cases of DHF are included, which is fatal in about 2.5% of cases (WHO, 2009b). Without proper treatment, DHF mortality rates can exceed 20%. However, through early recognition and treatment of haemorrhagic dengue, DHF fatalities can be reduced to less than 1% (WHO, 2009b).

Dengue infection is caused by one of four different dengue virus serotypes, called DEN-1, DEN-2, DEN-3, and DEN-4, which belong to the genus *Flavivirus* (Family: *Flaviviridae*) (Gubler, 1998). The viruses are transmitted by the bite of infected female *Aedes sp.* mosquitoes during blood-feeding on humans. As dengue infection provides lifelong immunity to only the infecting serotype and there is no cross-protective immunity between serotypes (Gubler, 1998, Guzman & Kouri, 2002), people can be infected up to four times during their lifetime. This has been one of the main obstacles to developing an effective vaccine for dengue as it requires the development of four immunogens inducing a protective immune response against all four serotypes (WHO, 2009a).

1.2 Dengue history

The first recorded epidemics of a disease resembling dengue date back to 1779–1780, when outbreaks occurred in Batavia (Jakarta) in Indonesia, Cairo in Egypt, and Philadelphia in the USA. The near simultaneous occurrence of dengue outbreaks on three continents indicates that dengue viruses and their mosquito vectors have had a worldwide distribution for more than 200 years (Mairuhu *et al.*, 2004). In the eighteenth and early nineteenth centuries, epidemics or regional pandemics of dengue fever occurred approximately every 10 to 40 years in tropical regions of the world. However, the pattern shifted such that during the late nineteenth and early twentieth centuries, the number of epidemics increased with epidemics of dengue cycling through countries in South-East Asia approximately every 3 to 5 years (Ligon, 2005).

1.3 Global distribution by WHO regions

The global incidence and distribution of dengue fever and dengue hemorrhagic fever (DHF) has increased dramatically in recent years (Figure 1.1 and Figure 1.2), partially as a result of the expanding geographical distribution of both of the viruses that cause dengue and of their mosquito vectors, *Aedes aegypti* and *Aedes albopictus.* According to the WHO (2009a), dengue fever is currently endemic in over 100 countries in Africa, the Americas, the Eastern Mediterranean, South-East Asia and the Western Pacific, with an estimated 2.5 billion people at risk of infection, with the exception of Europe, where locally reported cases originated in overseas territories or were imported from endemic countries (WHO, 2009a). South-East Asia and the Western Pacific are the most seriously affected regions. Prior to the 1970s, only nine countries had experienced DHF epidemics, but since then this number has increased more than four-fold.

Figure 1.1. World map showing countries / areas at risk of dengue transmission in 2009 (Source: http://www.who.int/csr/disease/dengue/impact/ en/index.html).

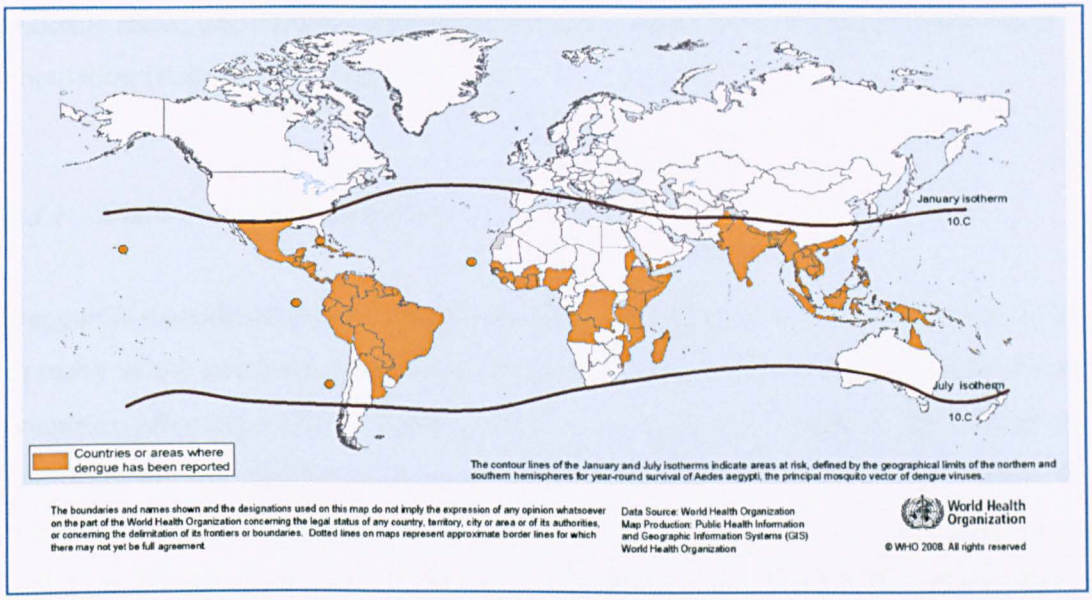
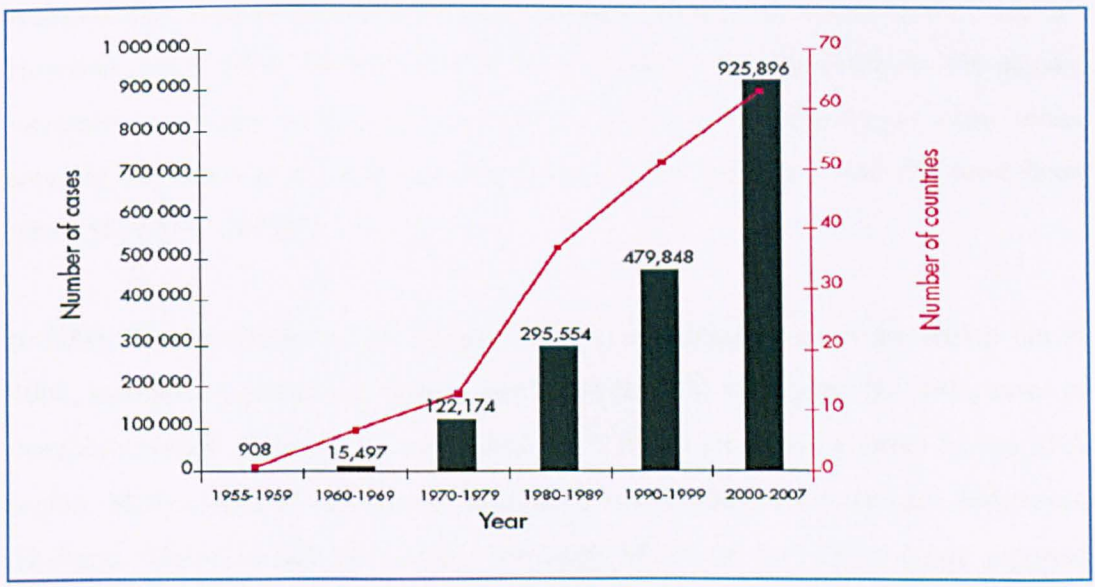


Figure 1.2. Average annual number of dengue fever (DF) and dengue haemorrhagic fever (DHF) cases reported to the WHO and the number of countries reporting dengue, 1955 -2007 (Source: http://www.who.int/csr/disease/dengue/impact/ en/index.html).



The main factors that have contributed to the rapid global spread of dengue include the failure to adequately control vector populations, increased air travel to dengue endemic areas, uncontrolled urbanization and an unprecedented growth in the world's population (Rigau-Perez *et al.*, 1998).

1.3.1 South-East Asian countries

Dengue is considered hyper-endemic throughout Asia as all 4 serotypes co-circulate in many of the countries. DHF is an important public health problem in many Asian countries, affecting mainly children and representing a major cause of hospitalization and death among children in many of these countries (Pinheiro & Corber, 1997). In the 1990s, DHF continued to occur, with a higher incidence in South-East Asia, mainly in Thailand, Myanmar and Sri Lanka (Pinheiro & Corber, 1997). Since 2000, dengue epidemics have spread to new areas and cases have increased in number in the zones already affected in this region. According to data collected by the WHO, several countries in the SEA region, (such as Bangladesh, Indonesia, Maldives, Myanmar, Sri Lanka, Thailand and Timor-Leste) reported dengue cases in 2003. Additionally, Bhutan reported its first outbreak dengue in 2004 (WHO, 2009a). However, since 2003, the case fatality rate has been maintained below 1% despite increases in dengue incidence. Nevertheless, in focal outbreaks away from urban areas in Myanmar and Indonesia case-fatality rates between 3 and 5% have been reported (WHO, 2009b).

In 2003, Thailand reported the highest number of dengue cases in the region but in 2004, Indonesia reported the highest number of cases in the region. By 2006, cases of dengue recorded in Indonesia accounted for 57% of all cases reported in the SEA region. Many countries reported an increase in cases (Bangladesh, Bhutan, Indonesia, Thailand, Maldives and Sri Lanka), although Myanmar and Timor-Leste reported slight decreases in cases from 2005 (WHO, 2009b). In Thailand by 2007, dengue was

reported in Northern, Central, North-Eastern and Southern regions. However, the case-fatality rate was reported to be below 0.2% (WHO, 2009b).

1.3.2 Western Pacific Region

Sporadic cases of DHF have been reported on different Pacific islands, including Vanuatu, New Caledonia, Tahiti, Rarotonga, Fiji, American Samoa, Western Samoa, Yapa and Palau, where all 4 serotypes have been detected (Pinheiro & Corber, 1997). Between 1990 and 1991 a small outbreak was reported in Australia and attributed to DEN-1. Also, a large outbreak due to DEN-2, affected Townsville during 1992-1993 (Pinheiro & Corber, 1997).

According to National Notifiable Diseases Surveillance System, imported dengue cases are reported every year in Australia, with occasional local transmission restricted to some places in northern Queensland. In 2007, a significant increase (68%) was observed compared to the number of cases reported in 2006. The locally-acquired cases represented 15% (46/314) of the total number of dengue cases recorded in 2007 (CDNA, 2010).

Although dengue is not considered endemic in north Queensland there are outbreaks annually, which coincide with the wet season from January to April (Hanna *et al.*, 2009, Ritchie *et al.*, 2009). Between January 2000 and November 2008, 16 outbreaks were recorded with 1142 confirmed dengue cases (Ritchie *et al.*, 2009), and two deaths (Mc Bride, 2005). These were the first deaths associated with dengue haemorrhagic occurring in Australia in 100 years (CDNA, 2009). The simultaneous circulation of all four serotypes of dengue virus, recorded in recent years, makes this region vulnerable to continuous outbreaks (Hanna *et al.*, 2009).

Since the last major pandemic in the Western Pacific in 1998, epidemics have been recorded in much of the region (WHO, 2009a). Between 2001 and 2008, a high number of dengue cases were reported in Cambodia, Malaysia, the Philippines and Vietnam, (1020333 cases). In addition, these countries reported the highest numbers of cases and deaths (WHO, 2009a). In this region, dengue has been reported predominantly in urban and peri-urban populations, where the main factor facilitating transmission is high population density. However, there is evidence to suggest that transmission is also occurring now in rural areas (WHO, 2009a).

1.3.3 Eastern Mediterranean Region

The Middle East has experienced significant increases in dengue outbreaks in recent years. An outbreak of dengue (DEN-2) was reported in Jeddah, Saudi Arabia in 1994, which resulted in at least two fatal cases (Fakeeh & Zaki, 2001). More recently (2005-2006), Pakistan, Saudi Arabia, Sudan and Yemen have reported outbreaks of dengue (WHO, 2009a).

In Pakistan, the first outbreak of DHF was reported in Karachi in 1994 (Chan *et al.*, 1995). Since then, a significant increase in dengue cases has been documented in several hospitals (Akram *et al.*, 1998, Paul *et al.*, 1998). Although previous publications report DEN-1 as the most commonly circulating serotype, detection of the DEN-3 serotype was reported recently (Wasay *et al.*, 2008). A dengue epidemic associated with DEN-3 was reported in 2005. Since then, the increased severity and expansion of dengue has been reported in the main cities of Pakistan (Jamil *et al.*, 2007).

In Sudan, in 2005, a hospital-based study recorded a small number (84) of febrile cases (Ageep *et al.*, 2006). In 88% of patient sera samples, diagnosis was confirmed by ELISA detection of dengue virus immunoglobulin M antibodies (Ageep *et al.*,

2006). In Yemen, more than 1,000 suspected dengue cases were reported between 2000 and 2005, with more than 50% of the reported cases, occurring in 2000. The two deaths recorded in this time period were associated to DEN-2 (WHO/EMRO, 2005).

Dengue transmission was first recorded in Jeddah, Saudi Arabia in 1994 from a fatal case of dengue haemorrhagic fever confirmed as DEN-2 (Fakeeh & Zaki, 2001). Since then, Saudi Arabia has reported several epidemics. The major one occurred in 2006 with 1269 cases of dengue reported, including 27 cases of DHF, 12 cases of DSS and 6 fatal cases. Jeddah is the main commercial port in the country and a transit place for large numbers of people from all over the world coming to visit Islamic Holy places. Thus, this city may potentially be of great epidemiological importance to dengue transmission (Ayyub *et al.*, 2006).

1.3.4 African countries

Due to the limited ability of dengue surveillance dengue in Africa, its epidemiology is poorly documented. It is mainly based on the particular interests of researchers, the surveillance of travellers returning from Africa and some publications on outbreaks (Sang, 2007, Franco *et al.*, 2010). Although the circulation of all four dengue serotypes has been recorded, dengue is not officially reported to the WHO by countries in this region. However, there is documented evidence that epidemic dengue fever has increased significantly since 1980 (WHO, 2009b). Unlike other regions, such as Asia, the Pacific and the Americas, where human-to-human transmission via *Ae. aegypti* and *Ae. albopictus* vectors is predominant, a sylvatic dengue transmission cycle have been also referred occurs in Africa (Gubler, 1997, Diallo *et al.*, 2003, 2005, 2008, WHO, 2009a).

Due to the threats posed by other major communicable diseases (such as malaria, TB and HIV/AIDS), dengue is not considered a major public health problem in Africa (WHO, 2009a). However, a lack of laboratory and diagnostic facilities may lead dengue infections to be misdiagnosed as other diseases (Rogers *et al.*, 2006). The first major outbreak of dengue caused by DEN-3 was documented in Pemba, Mozambique between 1984 and 1985 and resulted in two recorded deaths (WHO, 2009a). Dengue epidemics have been recorded in the Seychelles, Kenya, Mozambique and Somalia (Gubler & Clark, 1995), with DENV-2 and DENV-3 involved (WHO, 2009). More recently, in 2007, both dengue and chikungunya (CHIK) viruses were simultaneously reported in Gabon, resulting in more than 20,000 suspected cases (Leroy *et al.*, 2009). This coincided with the invasion of *Ae. albopictus* into Central Africa (WHO, 2009a).

The continuous increase in the number of dengue cases that has been recorded in the last two decades in Africa could be a warning about serious changes in the epidemiology of dengue in this region (Diallo *et al.*, 2005, Leroy *et al.*, 2009, WHO, 2009a, Franco *et al.*, 2010). Although, the viral isolation of all four dengue viruses has been documented (Diallo *et al.*, 2008), the transmission in urban areas is very limited according to Paupy and colleagues (2010). This may be due to low vector competence in local populations of *Ae. aegypti*, and may partially explain the absence of a major epidemic in this region (Paupy *et al.*, 2010). Likewise, Guzman and colleagues (1990) deemed it "likely that human genes regulating disease severity distributed unequally in blacks and whites" could be the most plausible explanation why DHF is not widely recorded during dengue outbreaks. More scientific research is needed to clarify the potential factors involved in this situation.

1.3.5 Dengue in the Americas

Dengue is believed to have occurred in the Caribbean since the first half of the seventeenth century (Oletta, 2006). The first recorded case of dengue in the Americas

dates from 1780, with the report of a case of a dengue-like illness in Philadelphia, USA (Halstead, 1992, Pinheiro & Corber, 1997). According to Halstead (2006), dengue outbreaks of unknown aetiology occurred frequently during the 19th century in port cities of the Caribbean and in North, Central and South America. Between 1941 and 1946, epidemics were documented which affected several countries, including Mexico, Panama and Venezuela, and several islands including Cuba, Puerto Rico and Bermuda (Ehrenkranz *et al.*, 1971, Halstead, 2006).

The Caribbean and Venezuela were affected by two pandemics of dengue in the 1960s. The first occurred in 1963 and was associated with DEN-3 and primarily affected Jamaica, Puerto Rico, islands of the Lesser Antilles and Venezuela (Pinheiro & Corber, 1997). The second occurred between 1968 and 1969. While DEN-3 was again the predominant serotype, DEN-2 was also isolated from several people (Ehrenkranz *et al.*, 1971).

Despite the success achieved in suppressing dengue vector populations through the *Ae. aegypti* eradication campaigns of the 1960s, the intense vector control efforts were not sustained. Between 1960 and 1970, vector re-infestation began to occur and was followed by the subsequent appearance of dengue outbreaks in the region (Guzman *et al.*, 2006, PAHO, 1997). By 1977, dengue outbreaks reported in the Caribbean and South America were associated with DEN-2 and DEN-3. After the introduction of DEN-1 into Jamaica in 1977, dengue spread widely in the Americas. For the first time since 1945, locally acquired cases were reported in the United States (PAHO, 1989).

Until 1981, only sporadic suspected cases of DHF had been reported in the Americas. However, in 1981, an outbreak of DHF/DSS occurred in Cuba that marked the start of epidemic DHF in the Americas. During this epidemic (attributed to DEN-2), a total of 344,203 classic dengue cases, 10,312 DHF cases and 158 deaths (of which 101

were children) were reported (Guzman *et al.*, 1984, Kouri *et al.*, 1989, Guzman *et al.*, 1990, PAHO, 1997, Arias, 2002).

In the following two decades, the region changed from an area of low dengue endemicity, with few countries registering few cases, to an area of hyper-endemicity, with many countries reporting multiple co-circulating serotypes, frequent epidemics and numerous cases of DHF (Guzman *et al.*, 2006). A characteristic of dengue transmission in the Americas is the cyclical occurrence of outbreaks every 3 – 5 years. Dengue incidence has typically increased with each outbreak (Guzman *et al.*, 2006), thus creating a situation that could worsen with the simultaneous circulation of the four serotypes in the region. This represents a high risk to DHF, as secondary infection by a different dengue serotype is considered be the most significant individual risk factor for DHF/DSS (Guzman & Kouri, 2002, Nimmannitya *et al.*, 1987).

1.3.6 Dengue in Venezuela

According to Coello (1992), the first mention of a dengue epidemic in Venezuela was described by Dr. Jose M. Vargas in 1828 and affected Caracas. The next publication on dengue was written by Rísquez (1890) and was followed by other sporadic reports [Salom, 1915, Razetti, 1919 (cited by Coello, 1992), Dominici, 1946, Echezuria, 1949, &Rossi, 1964 (cited by Coello, 1992)].

The first widespread outbreak of DHF/DSS in Venezuela occurred in October 1989 until April 1990. The epidemic reappeared in the second half of 1990 and in each of the subsequent years until 1993. During the period from 1989-1993, a total of 11,260 cases of DHF and 136 deaths were reported in Venezuela (WHO, 2001). Since then, Venezuela has reported large numbers of DHF cases every year. In 1995 the country recorded its largest outbreak up to that point, with over 30,000 cases of classic

dengue and over 5,000 cases of DHF (Torres & Castro, 2007). Between 1995 and 1997, in the Americas region was reported an annual increasing in dengue fever incidence rate of +12% and +35% respectively, and simultaneously an increasing in DHF incidence rate of +61.87%. During this period, Venezuela experienced a +61.29% annual increase in the DHF incidence rate (Guzman, 1999, Kouri *et al.*, 1998).

Between 1995 and 2002, Venezuela reported a total of 283,373 dengue cases with 33,477 cases of DHF and 169 deaths. Between 2003 and 2005, a total of 92,253 dengue cases were reported, including 6,913 cases of DHF and 16 deaths. From 2006 until 2008, Venezuela reported a total of 168,554 dengue cases with 12,586 cases of DHF (64,662 of the cases were laboratory-confirmed) (PAHO, 2005).

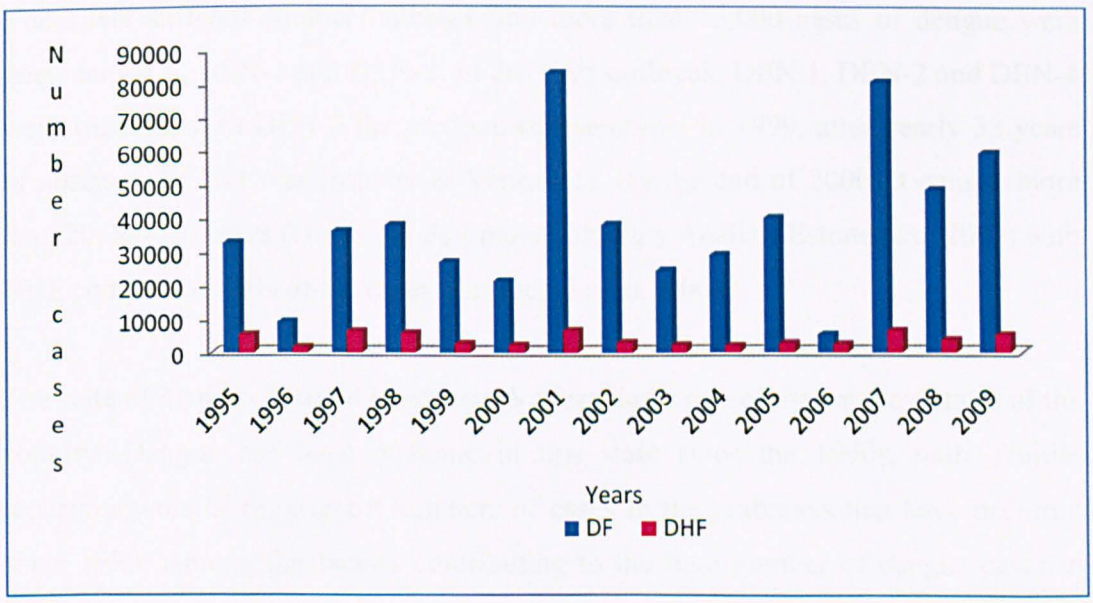
The highest number of reported cases in Venezuela were recorded in 2001 (83,180 DF cases, 6,541 DHF cases and an incidence rate of 337.69/100,000 people. In 1995 and 1997 when the number of DF cases was almost three times lower (32,280 and 35,654 respectively), there were similar numbers of DHF cases (5,380 and 6,300 respectively). However, mortality from DHF in 2001 was far lower (15 reported deaths) while in 1995 and 1997, 43 deaths were reported in each year (Dirección de Epidemiología y Estadística Vital, 2002). Epidemiologists in Trujillo state consider that one of the main factors contributing to the higher dengue fatality rate in 1995 and 1997 was the lack of clear guidance on how to manage dengue cases in the main hospitals (Director de Epidemiología Regional, *personal communication*).

Although multiple variables could explain the high incidence of dengue in 2001, several factors are of particular importance. It has been referred that the replacing of strains established previously by a more pathogenic DEN-2 strain (Rico-Hesse *et al.*, 1997), as well as, the appearance of DEN-3, which was absent from 1960 (Barrera *et al.*, 2002), could explain partially this situation. It is likely that climate played a role, as during 2000 and 2001 the *La Niña* phenomenon occurred, resulting in an increase

in rainfall particularly between June-August (Rifakis *et al.*, 2005). This contributed to a steady abundance of breeding sites, thereby leading to a high population density of *Ae. aegypti* in most of the country. Additionally, ongoing structural changes in the various institutions belonging to the Ministry of Health negatively impacted the policies established for the surveillance and control of *Ae. aegypti*. (Epidemiologists of Ministerio del Poder Popular para la Salud, *personal communication*, 2009).

According to statistics reported to the PAHO between 2003 and 2006, reported Venezuelan cases accounted for 35.95% of the total cases of DF and 33.64% of the total cases of DHF recorded in the Andean sub-region, as well as 9.71% of the fatalities (PAHO, 2005). In 2007 and 2008, Venezuela reported more than 50% of the total cases of DF in the region (54.23% and 53.30%, respectively), with a similar proportion of DHF cases (55.67% and 53.82% respectively). Although no deaths due to DHF were reported in Venezuela during these two years, these numbers show that dengue remains a significant public health problem in Venezuela.

Figure 1.3. Number of Reported Cases of Dengue and Dengue Hemorrhagic Fever in Venezuela to PAHO, from 1995 to 2009.



2009 Updated until epidemiological week 48

1.3.7 Dengue serotypes in Venezuela

Since 2001, more than 10 countries in the Americas have reported more than one dengue virus serotype circulating simultaneously, which in Venezuela, increasing the incidence of secondary infections and consequently increasing the risk of DHF/DSS (Oletta, 2006, Torres & Castro, 2007).

Schneider & Droll (2001) describe chronologically the introduction and circulation of dengue virus serotypes between 1963 and 2000. In 1963, DEN-3 was first documented in Venezuela. In 1978, it was re-introduced from Haiti causing an epidemic resulting in more than 100,000 DF cases. Simultaneously, DEN-1 was first reported (Pinherio & Corbet, 1997, Pinherio & Nelson, 1997, Schneider & Droll, 2001). In 1984, DEN-2 was first reported in Venezuela during a concurrent outbreak with DEN-1. In 1985, DEN-4 was first reported in Maracaibo and Caracas. During 1987, an outbreak occurred in Caracas, primarily due to DEN-2, although some DEN-4 cases were also reported. In 1989-90, the second major outbreak of DHF in the Americas occurred in Venezuela. During this time, DEN-2 was the predominant serotype but DEN-1 and DEN-4 were also circulating. Between 1991 and 1992, Venezuela suffered another outbreak and more than 12,000 cases of dengue were documented as DEN-1 and DEN-2. In the 1995 outbreak, DEN 1, DEN-2 and DEN-4 were isolated, with DEN-2 the predominant serotype. In 1999, after nearly 32 years of absence, DEN-3 was isolated in Venezuela. By the end of 2000, it caused more than 20,000 DF cases (Dirección de Epidemiología y Análisis Estratégico, 2000) with DHF comprising 10% of the cases (Uzcategui *et al.*, 2003).

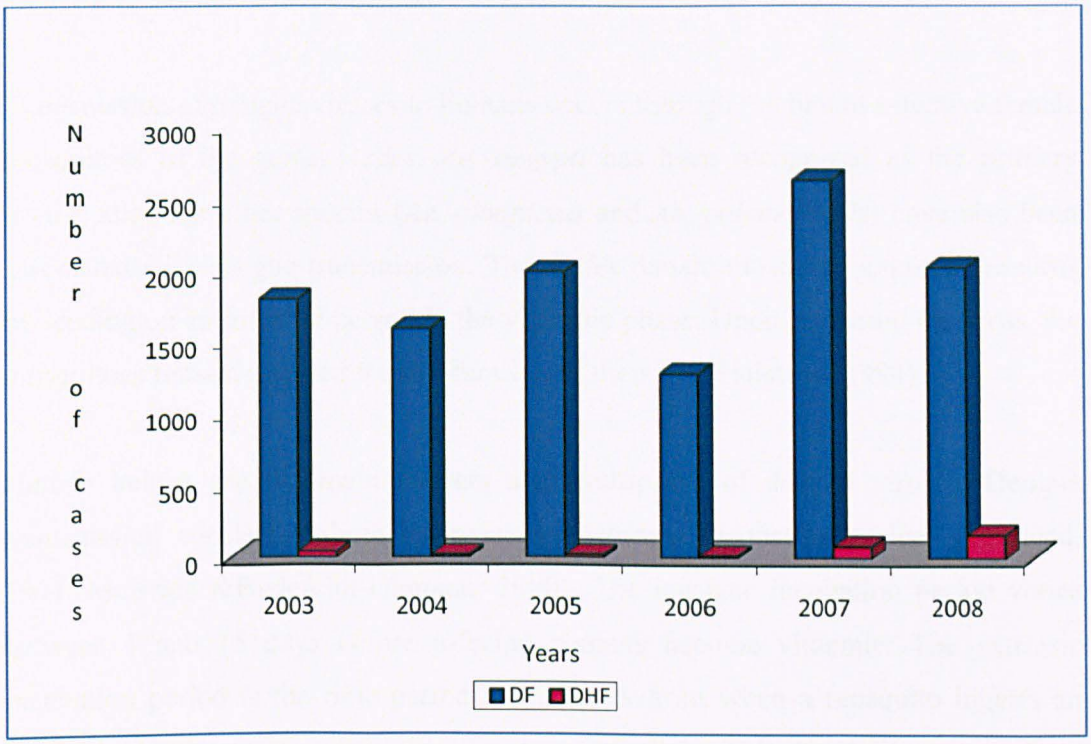
The state of Trujillo, located in western Venezuela, is one of the smallest states of the country. Dengue has been endemic in this state since the 1990s, with Trujillo reporting some of the highest numbers of cases in the outbreaks that have occurred since 1989. Among the factors contributing to the high number of dengue cases in this state is the lack of constant water supply service and a continued lack of

sanitation service in rural and urban areas of the state. Householders tend to maintain different types of containers for water storage, with drums (large containers of approximately 150 – 200 litres of capacity) representing the most important domestic water storage containers in these communities (Barrera *et al.*, 1995, Lenhart *et al.*, 2006, Nathan *et al.*, 2006). People do not take measures to cover these containers properly, so they become attractive breeding sites for *Ae. aegypti.* Likewise, the accumulation of discarded bottles, cans and car tyres in the vicinity of human dwellings also contributes significantly to increased infestation by *Ae. aegypti.*

Dengue control in Trujillo is the responsibility of the Vector Control Department, Bureau of Sanitation & Malariology of the Ministry of Popular Power for Health and Social Protection. The planning and design of control programmes are conducted at a central level and programmes are implemented vertically. In many cases, this process does not adequately take into account the importance of community participation, such that many communities have come to passively expect the health authorities to solve the problem of dengue transmission. However, effective dengue vector control requires the active participation of community inhabitants (Lloyd *et al.*, 1994, Chadee *et al.*, 2005, Troyo *et al.*, 2008), particularly when addressing the proliferation of multiple breeding sites due to household water storage practices.

Trujillo state reported a total of 11,679 dengue cases between 2003 and 2008, of which 2.95% (345 cases) were DHF. While overall dengue incidence is relatively stable (Figure 1.3), the number of DHF cases in 2008 was almost twice that observed in 2007 (Dirección Regional de Epidemiología y Estadística Vital, Trujillo 2009). There is no clear explanation for this increase in number of DHF cases. Furthermore, the lack of an active surveillance system, which includes the isolation and characterization of circulating serotypes, further hinders understanding of this epidemiological situation.

Figure 1.4. Number of dengue and DHF cases in Trujillo state from 2003-2008 (Source: Dirección Regional de Epidemiología y Estadística Vital, Trujillo-Venezuela, 2009).



1.4 Dengue epidemiology and disease

1.4.1 Transmission

Several key factors influence the complex dynamics of dengue transmission, primarily the virus, the vector and the presence of a susceptible host, as well as characteristics of the environment where they occur (Barrera *et al.*, 2000, Focks, 2003). Several researchers (Halstead, 1994, Rigau-Perez, 1998, Guzman & Kouri, 2002) have discussed how increases in density and geographic distribution of dengue vectors and viruses play an important role in disease transmission. This has been exacerbated by explosive human global population growth and uncontrolled urbanization observed in dengue endemic areas. In addition, the lack of regular piped

water supply, adequate waste removal, deteriorates health systems and ineffective *Ae. aegypti* control policies contribute to increases in dengue transmission (PAHO, 1996, Pinheiro & Corber, 1997, Guzman, 1999, Guzman & Kouri, 2002).

Transmission of dengue viruses to humans occurs through the bite of infective female mosquitoes of the genus *Aedes*. *Ae. aegypti* has been recognized as the primary vector, although other species (*Ae. albopictus* and *Ae. polynesiensis*) have also been associated with dengue transmission. The *Aedes* female mosquito acquires the virus by feeding on an infected person in the viraemic phase. Upon acquiring the virus, the mosquitoes remain infected for the duration of their life (Halstead, 1994).

Human beings are the main carriers and multipliers of dengue viruses. Dengue transmission consists of both intrinsic and extrinsic incubation periods (Halstead, 1994, McBride &Bielefeldt-Ohmann, 2000). The intrinsic incubation period varies between 1 and 15 days before infected humans become viraemic. The extrinsic incubation period is the time period that elapses from when a mosquito ingests an infected bloodmeal to when they become infective. This typically ranges from between 8 to 12 days (Halstead, 1994, McBride &Bielefeldt-Ohmann, 2000, WHO, 2009a), depending on the ambient temperature and humidity (Kuno, 1995). Virus replication occurs in the mosquito gut, brain, salivary glands and reproductive organs without any apparent harm to the infected mosquito (Halstead, 1994). An infective mosquito transmits dengue virus not only during blood feeding, but also during probing. Repeated interruption of blood feeding could be important to virus transmission (Putnam & Scott, 1995b), as this behaviour could increase significantly the efficiency of *Ae. aegypti* as a vector (Gubler, 1998).

Although it has long been recognized that humans are the only hosts capable of maintaining urban transmission of dengue, some authors have suggested transovarial transmission, whereby the infected female mosquito is able to transmit the virus to its progeny (Joshi *et al.*, 1996, Joshi *et al.*, 2002, Lee & Rohani, 2005, Gunther *et al.*,

2007). Transovarial transmission has been demonstrated in the laboratory in *Ae. aegypti*, but under natural conditions only a few cases have been documented (Hull *et al.*, 1984, Joshi *et al.*, 1996, Joshi *et al.*, 2002, Lee & Rohani, 2005, Gunther *et al.*, 2007). New and more detailed studies are needed to determine the epidemiological significance of this phenomenon in the transmission of dengue.

Ae. aegypti are highly anthropophilic, day-biting mosquitoes which live and breed in close proximity to human environments and human dwellings (Christophers, 1960, Scott *et al.*, 1993b, Scott *et al.*, 1997, Reyes-Villanueva & Rodriguez-Perez, 2004), and it is presumed that most dengue transmission occurs in and around homes. In South-East Asia, Halstead (1970) reported that the majority of dengue infections occurred in children who usually stayed indoors, where they were bitten by *Ae. aegypti*. Although several studies have been conducted regarding mosquito productivity in non-residential areas (Barrera *et al.*, 1979, Schultz, 1989, Vezzani & Schweigmann, 2002, Abe *et al.*, 2005, Wan Norafikah *et al.*, 2009), this has yet to be related to dengue transmission.

The typical flight range of *Ae. aegypti* is typically less than 300 meters (McDonald, 1977ab, Edman *et al.*, 1998, Scott *et al.*, 2000a, Ordonez-Gonzalez, *et al.*, 2001). However, this range can be longer, mainly influenced by the need for available oviposition sites (Reiter *et al.*, 1995). Although the movement of mosquitoes is important to dengue transmission, movement of people is also important as they can transport the viruses over greater distances in a shorter period of time (Getis *et al.*, 2003). Although typical control activities primarily address residential areas, public areas where large numbers of people frequently congregate, especially during peak *Ae. aegypti* biting times, should not be neglected by control programmes (Halstead, 1980, Kouri *et al.*, 1989, Scott *et al.*, 1993b, Morrison *et al.*, 1998).

1.4.2 Clinical features

Although millions of people annually acquire dengue virus infection, clinical disease develops only in a small percentage of them (Farrar, 2008). In many cases, the clinical differentiation of dengue from other viral diseases is not possible, particularly in the case of minor illness not requiring significant medical intervention (Kautner *et al.*, 1997). Dengue infections can cause a wide spectrum of symptoms ranging from mild to potentially deadly disease, with variable clinical presentations amongst endemic regions. The proportion of patients developing the more severe manifestations of illness may be associated with several factors, including the strain and serotype of dengue virus, the immunological and nutritional status of the host, the presence of secondary infections, chronic disease, ethnicity and the age of the human host (Kouri *et al.*, 1989, Kautner *et al.*, 1997).

1.4.2.1 Dengue fever

Classic dengue fever is a flu-like illness characterized by a rapid increase in temperature above 39°C, with fever lasting between 5 and 6 days (Mairuhu *et al.*, 2004). During the febrile period, the patient may experience severe headaches, retro-orbital pain, myalgia, arthralgia, nausea, and/or vomiting. More than half of infected patients report a rash during this period that is initially macular or maculopapular and becomes diffusely erythematous. Minor haemorrhagic manifestations such as petechiae, epistaxis and gingival bleeding can also occur (Gubler, 1998). While classic dengue fever may be temporarily incapacitating, its prognosis is favourable (Brigtner & Fantato, 1998) and complete recovery generally occurs after 7–10 days of illness (Mairuhu *et al.*, 2004).

1.4.2.2 Dengue Haemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS)

DHF is defined as an acute febrile illness with haemorrhagic manifestations, thrombocytopenia (≤100,000 cells/mm^3) and evidence of an increased vascular permeability resulting in loss of plasma (Nimmannitya, 1987, Kalayanarooj *et al.*, 1997, Mairuhu *et al.*, 2004). Hypoproteinaemia, an elevated haematocrit and evidence of serous effusion are distinctive indicators of plasma leakage (Nimmannitya, 1987). The severity of DHF is classified by the WHO (1997) according to the following clinical criteria:

Grade I: fever accompanied by nonspecific constitutional symptoms. The only haemorrhagic manifestation is a positive tourniquet test;

Grade II: spontaneous bleeding, usually skin, nose or gums, in addition to manifestations of Grade I;

Grade III *: circulatory failure manifested by rapid, weak pulse with narrowing of pulse pressure (< 20mm Hg) or hypotension;

Grade IV *: moribund patients with undetectable blood pressure and pulse.

* Grades III and IV are called DSS, while DHF encompasses all four grades (McBride & Bielefeldt-Ohmann, 2000).

When plasma loss becomes critical it may result in DSS, which is characterized by a complicated and deteriorating condition and is followed by circulatory failure (WHO, 2009). Generally, this condition presents with a rapid and weak pulse, narrowing of the pulse pressure (regardless of blood pressure levels) or hypotension with cold, clammy skin and restlessness (WHO, 1997). During the early phase of dengue infection, it is difficult to establish certain clinical criteria helping the physicians to predict whether or not a patient will evolve to severe stages of dengue infection (DHF/DSS). Therefore, it is extremely important to recognise quickly the appearance

of warning signs and manage appropriately to reduce the dengue fatality rate (WHO, 2009a).

1.4.3 Pathogenesis

The pathogenesis of dengue is considered very complex and difficult to understand (Stephenson, 2005). Although there is no clear agreement on the pathogenesis of dengue, available evidence suggests multiple factors such as virulence, the presence of cross-reactive and non-neutralizing antibodies, genetic predisposition, nutritional status, age and underlying chronic disease could play an important role (Halstead, 1988, Kautner *et al.*, 1997, Rothman, 2003, Chaundry, 2006).

Infection with one dengue serotype provides lifelong homologous immunity but only limited heterologous immunity (Kautner *et al.*, 1997). The immune enhancement hypothesis is currently favoured as an explanation of dengue pathogenesis and is based on the prior presence of heterotypic antibodies during a secondary infection (Halstead, 1988, Chaundry, 2006). According to this hypothesis, patients who experience a secondary viral dengue infection would be at greater risk of developing DHF or DSS.

1.4.4 Treatment

Despite recent significant efforts to develop an effective vaccine against all four serotypes of dengue virus, one is not yet available nor is there any specific treatment for dengue virus infection. The main treatment is based primarily on early and appropriate supportive care. Given its variable spectrum of unpredictable clinical presentations, it is difficult to know which dengue cases will progress in severity, but with early diagnosis and preventive care the fatality rate can be reduced to less than 1% (WHO, 2009b).

Some findings suggest that the maintenance of appropriate fluid balance could reduce the number of unnecessary hospital admissions (Harris *et al.*, 2003) because in its classic form dengue can often be successfully treated at home (Malavige, *et al.*, 2004). Early recognition of dengue symptoms is very important in dengue management and treatment, particularly taking into account the high number of dengue cases occurring in children (Burke *et al.*, 1988, Pinheiro & Corber, 1997, Guzman *et al.*, 2004, Halstead, 2006, Farrar, 2007). More time and greater effort should be invested in educating parents/carers, especially mothers, who can play an important role in avoiding dengue complications by knowing when to seek the support of health services (Roses & Guzman, 2007).

1.4.5 Epidemiology

The primitive or ancestral enzootic transmission cycle of dengue viruses is associated with canopy-dwelling *Aedes* mosquitoes and lower primates in the rain forests of Asia and Africa. Current evidence suggests that these viruses do not regularly move out of the forest to urban areas, such that sylvatic dengue is not considered a zoonotic infection (Rico-Hesse, 1990).

According to Gubler (1998), in some areas characterized by small, isolated human populations (such as rural villages or islands), an epidemic transmission cycle may occur whereby the majority of susceptible individuals in these areas become infected, thus increasing herd immunity and causing the virus to disappear from the population (Gubler, 1998). However, the most important transmission cycle from a public health standpoint is the urban endemic/epidemic cycle in large urban and peri-urban centres of the tropics and subtropics, in which the viruses are maintained in an *Ae. aegypti*-human-*Ae. aegypti* cycle alone, with epidemics occurring periodically (Gubler, 1998).

Adult *Ae. aegypti* prefer to rest indoors (Scott *et al.*, 1993b, Scott *et al.*, 2000b), and most blood-feeding activity on humans occurs during daylight hours (Chadee, 1988,

Chadee & Martinez, 2000). Although diurnal, typically there are two peaks of biting activity; early morning for 2 to 3 hours after daybreak and in the afternoon for several hours before dark (Chadee, 1988, Chadee & Martinez, 2000). However, these mosquitoes can feed all day indoors and outdoors on overcast days, when it is cooler and more humid. Not surprisingly, for a mosquito that does not feed on a sleeping host, *Ae. aegypti* are easily disturbed and the interruption of feeding with resumption on a different host is frequent (Edman & Scott, 1987, Chadee & Beier, 1997). Consequently, *Ae. aegypti* females will often feed on several persons to obtain a single complete blood meal and thus, if infected, may transmit dengue virus to multiple persons over a relatively short time period, even if they probe without taking blood (Putnam & Scott, 1995a, Platt *et al.*, 1997, Scott *et al.*, 1997).

1.5 Dengue vectors

1.5.1 Overview

The principal vector of dengue is *Ae. aegypti* (Linnaeus, 1762) (Family: Culicidae, Sub-family: Culicinae, Tribe: Aedini), which is also well-known as the "yellow fever mosquito" (Christophers, 1960). *Ae. aegypti* is a member of the subgenus *Stegomyia* (Theobald, 1901) and the genus *Aedes* (Meigen, 1818).

Ae. aegypti is thought to have originated in Africa and is presumed to have arrived in the Americas via water barrels on the ships of the early explorers and colonists. It is a cosmotropical species and is unique in its widespread global distribution (Christophers, 1960). However, its distribution (Figure 1.1) is limited to between latitudes of 45° N and 35° S and it dwells at altitudes below 2000m (Christophers, 1960).

A secondary dengue vector, *Ae. albopictus* (also known as the 'Asian tiger mosquito'), has historically been an important vector of dengue in South-East Asia.

Recently, it has been introduced into Europe, North and South America and Africa as a consequence of increased international commerce activities, primarily due to the transportation of dormant eggs in tyres (Hawley *et al.*, 1987, Reiter & Sprenger, 1987, Adhami & Reiter, 1998, Lounibos *et al.*, 2001, Gratz, 2004, Benedict, 2007) and the 'Lucky Bamboo' plant (*Dracaena* spp.) (Linthicum *et al.*, 2003). Other vectors of dengue viruses include *Ae. polynesiensis, Ae. scutellaris, Ae. pseudoscutellaris*, and *Ae. rotumae* in the Pacific region (Rosen *et al.*, 1954, Freier & Rosen, 1987, Gubler, 1988).

1.5.2 Life cycle of Ae. aegypti

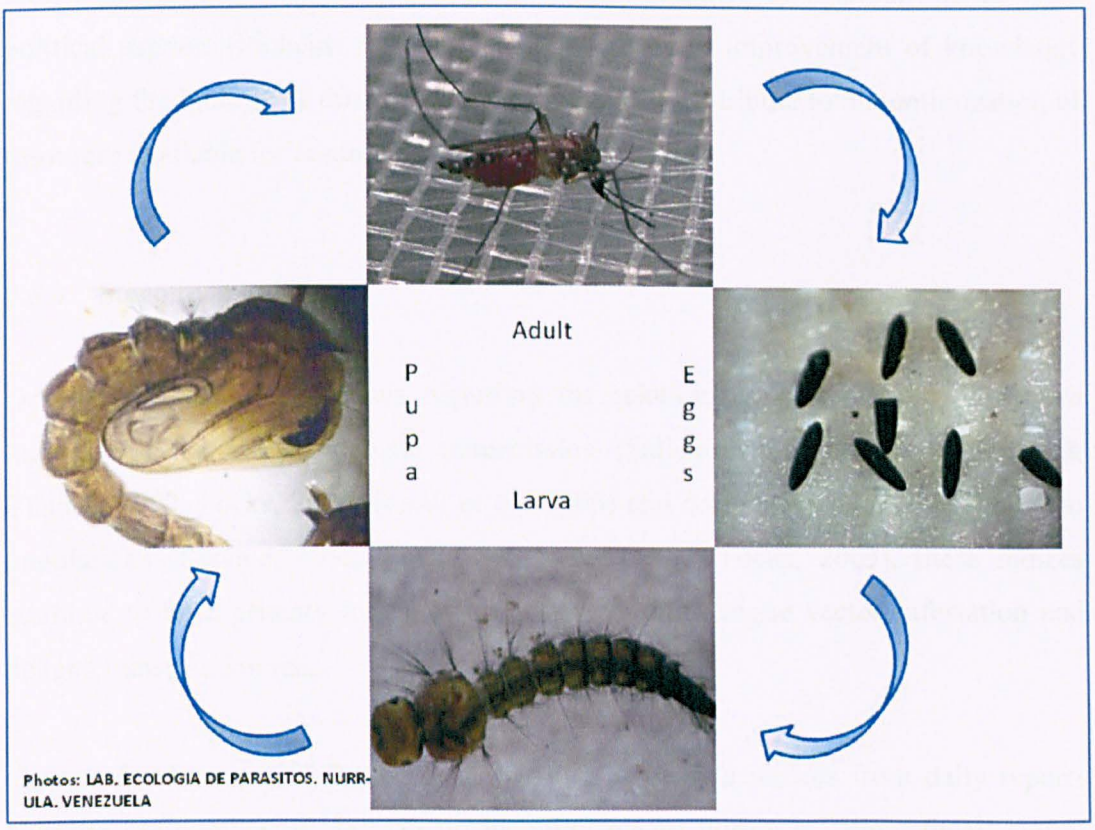
Ae. aegypti breed in close proximity to human settlements. The biotic (food, predation, competition) and abiotic (temperature, evaporation, rainfall) factors in these environments are determinants for hatching, larval performance and consequently for survival of progeny. Favourable breeding conditions result in larger adults with increased lifespan (Nasci, 1986, Tun-Lin *et al.*, 2000, Barrera *et al.*, 2006a, Jirakanjanakit *et al.*, 2007a).

The life cycle of *Ae. aegypti* is comprised of four phases, namely egg, larvae, pupae and adult stage (Figure1.4). The period from hatching to emergence lasts approximately one week in tropical areas (Christophers, 1960). With the exception of the adult stage, all other stages are aquatic. Before laying eggs, *Ae. aegypti* females require one (Christophers, 1960, Klowden & Briegel, 1994) or several blood feedings (Scott *et al.*, 1993a, Scott *et al.*, 1997, Reyes-Villanueva & Rodriguez-Perez, 2004) to produce mature eggs. Upon egg maturation, the female deposits eggs singly and intermittently on moist surfaces that are likely to become submerged (Christophers, 1960, McCall, 2002).

After submersion in water, the larvae hatch and pass through four larval instars, each ending in a moult, which are denoted as I, II, III or IV (Christophers, 1960). The

pupal stage corresponds to a transition period in which profound changes occur. *Ae. aegypti* pupae, unlike the pupae of other insects, are very active. Unlike larval stages, where males tend to develop faster than females, the pupal period has the same duration for both sexes. However, it has been observed under laboratory conditions that males emerge before females (Christophers, 1960). *Ae. aegypti* are not ready to reproduce immediately after emergence. Males require 24 hours for terminalia rotation and females require the same time to develop host-seeking behaviour (Christophers, 1960, Klowden, 1990, Briegel *et al.*, 2001).

Figure 1.5. The four developmental stages of Ae. aegypti.



1.5.3 Measures of dengue vector abundance

Knowledge of *Ae. aegypti* vector population abundance is of vital importance in the design and implementation of control policies directed against the mosquito in the prevention of dengue. In an absence of an effective vaccine or therapeutic chemotherapy, the main dengue control measure is focussed upon the reduction of vector mosquito populations (Guzman & Kouri, 2002). Understanding of temporal fluctuations in vector abundance and of the most important containers that lead to production of *Aedes* can enable campaigns to focus control efforts more effectively. The success of vector control campaigns against *Aedes* has been affected by several factors in the past, such as the availability or investment of economic resources / political support (Pinheiro & Corber 1997). However, improvement of knowledge regarding the biology of this mosquito could greatly contribute to the optimization of resources available for control purposes.

1.5.4 Stegomyia *indices*

Despite contradictory opinions regarding the relationship between the *Stegomyia* indices and the risk of dengue transmission (Sallehudim *et al.*, 1996, Focks & Chadee, 1997, Focks, 2003, Bisset *et al.*, 2006) and correlation with adult mosquito populations (Service, 1992, Focks & Chadee, 1997, Focks, 2003), these indices continue to be a primary indicator used in assessing dengue vector infestation and dengue transmission risk.

Connor & Monroe (1923) first described the *Stegomyia* indices from daily reports charting the number of *Ae. aegypti* breeding places during a yellow fever control campaign. The basic sampling unit is the house or premise, which is systematically searched for water-holding containers that are then carefully examined for mosquito

larvae and pupae. The following three *Stegomyia* indices are commonly used to record *Ae. aegypti* infestations (WHO, 2009a):

1. House Index (HI): Percentage of houses infested with larvae and/or pupae.

2. Container Index (CI): Percentage of water-holding containers infested with larvae and/or pupae.

3. Breteau Index (BI): Number of positive containers per 100 houses inspected.

The HI has been used for many years and was previously considered the most valuable (Tun-Lin *et al.*, 1996) record of *Ae. aegypti* infestation. Since 1954, the BI has come to be more widely used. From global surveys of *Aedes* mosquitoes and simultaneous determination and equating the three indices, the WHO (1972) developed a corresponding index, called the WHO Density Figure. This index attempted to relate these three indices to adult biting rates, commonly measured as the number of *Ae. aegypti* biting a human collector per hour (Tun-Lin *et al.*, 1996, Focks & Chadee, 1997). Although no specific values have been developed to determine thresholds for dengue transmission, the threshold values used to indicate the risk of transmission of yellow fever have been applied to dengue (Tun-Lin *et al.*, 1996). The Pan American Health Organization has recommended that both Breteau and house indices with values of <5 can be considered an indicator of low *Ae. aegypti* infestation and hence of low level risk for dengue transmission (PAHO, 1994). However, it is evident that more detailed scientific research is necessary to link these indices to dengue transmission risk.

Several researchers, including Connor & Monroe (1923), have noted that traditional *Stegomyia* indices have serious shortcomings. Tun-Lin (1995) reported that the indices had small correlations with adult mosquito density, while Focks & Chadee (1997) showed that the indices failed to account for the fact that containers vary in

their production of adult *Ae. aegypti*. In Cuba, Bisset & colleagues (2006) reported that the traditional indices failed to detect the risk of dengue transmission, because dengue transmission still occurred when indices were very low. Recently, WHO, (2009) advised that larval indices should be considered poor indicators of adult mosquito production, and indicated that in localities with similar larval indices but different container profiles, adult abundance and transmission potentials may vary considerably.

1.5.5 Pupal / demographic surveys

Given the shortcomings of the *Stegomyia* indices, the pupal survey method was proposed as a more appropriate means of identifying containers responsible for producing the majority of the local adult mosquito population (Focks, 2003). Mortality in the pupal stage of *Ae. aegypti* is typically low so the number of pupae recorded represents a reliable indicator of the number of adult mosquitoes that will emerge from a particular container (Focks & Chadee, 1997).

The pupal/demographic survey was developed to identify the most important types of containers in epidemiological terms and has been proposed as an operational tool. The pupal surveys take into account the total number of pupae in different container types in a particular location (WHO, 2009b). The pupae are counted and indices are calculated reflecting the number of pupae per container type, pupae per person, pupae per house and pupae per hectare (Focks, 2003). Current research is investigating how these indices correlate with other epidemiological parameters, such as serotype-specific dengue seroconversion rates and ambient temperatures. Also, models are being developed to estimate the degree of vector reduction required to impact on dengue transmission in a given location (Nathan *et al.*, 2006).

Results obtained from measuring *Ae. aegypti* productivity in multiple field studies for evaluation of the pupal survey technique suggest this technique is a potentially valuable tool (Focks & Chadee, 1997, Arredondo-Jimenez & Valdez-Delgado, 2006, Barrera *et al.*, 2006a, Lenhart *et al.*, 2006). In Puerto Rico, the number of *Ae. aegypti* larvae, the BI and the presence of any immature forms was not as efficient in identifying the most productive types of containers as direct pupal counts (Barrera *et al.*, 2006a). Data from Trujillo, Venezuela suggests that by counting the total number of pupae present in each container, *Ae. aegypti* control campaigns could focus their efforts on those that produce the greatest number of pupae to achieve the maximum impact (Lenhart *et al.*, 2006). Findings from México suggested that monitoring pupal productivity in different containers can be used to evaluate the impact of vector control strategies (Arredondo-Jimenez & Valdez-Delgado, 2006).

Although pupal/demographic surveys require higher labour than traditional *Stegomyia* indices, Focks & Alexander (2007) suggest that this tool should be used to identify and classify the most important containers as a function of their pupal productivity, as this provides important data for developing targeted control strategies.

1.5.6 Oviptraps

Although monitoring of dengue vectors has been based most commonly on the immature infestation indices (*Stegomyia* indices), important information can be obtained through the use of ovitraps (also known as oviposition traps), regarding the presence or absence of *Aedes* in a particular area. The original *Aedes* ovitrap design (Fay & Perry, 1965) has undergone a series of modifications in order to improve its ability in estimating the density of *Ae. aegypti* populations. Ovitraps are an inexpensive and sensitive tool and are especially useful in detecting the presence of *Aedes* when infestation levels are low. Ovitraps are also useful in assessing how

vector control programmes can impact mosquito populations, by monitoring dispersal and gravid female oviposition activity (Reiter & Nathan, 2001, Reiter, 2007). They can also be used for the early detection of re-infestation in areas where mosquitoes have been eliminated (WHO, 2009a). In addition, they are easy to implement and do not require specially trained personnel (Reiter & Nathan, 2001).

The "enhanced CDC ovitrap" employs two jars, one containing an olfactory attractant hay infusion and the other a 10% dilution of the same infusion. It is considered more attractive to ovipositing females than traditional ovitraps as it collects more *Aedes* eggs than conventional ovitraps (WHO, 2009b, Reiter & Gubler, 1997). A study conducted in Trinidad, West Indies, found significant differences comparing the number of eggs collected using different substrates. Wooden paddles yielded more eggs when compared with the water surface and inner surface of the ovitrap wall (Chadee *et al.*, 1995). However, a study conducted in México found a greater number of eggs were obtained when the inner surface of the ovitrap wall was lined with a cotton fabric substrate (Lenhart *et al.*, 2005).

Notably, several authors have stated the main disadvantage of standard ovitraps to be the requirement of eggs hatching in order to identify larvae or adults (Faccinelli *et al.*, 2007, Morrison *et al.*, 2008). However, this appears to have been overcome by sticky ovitraps, which allow quick quantification and identification of females laying eggs. Sticky ovitraps consist of small, dark-coloured plastic jars containing an infusion attractant which are supplied with an adhesive internal surface (Ordonez-Gonzalez *et al.*, 2001, Ritchie *et al.*, 2003, Faccinelli *et al.*, 2007, Morrison *et al.*, 2008). Different sticky ovitraps designs, such as mark-release-recapture experiments have been evaluated in the field to study different aspects of dengue vectors, (Ordonez-Gonzalez *et al.*, 2001, Russell *et al.*, 2005) including association between number of females captured and dengue risk transmission (Ritchie *et al.*, 2003, 2004), surveillance and oviposition behaviour and dispersal of *Aedes* (Russell & Ritchie

2004, Ritchie *et al.*, 2006) and sampling of container-breeding mosquitoes (Ritchie *et al.*, 2003).

It should be noted that the efficiency of sticky ovitraps and of standard ovitraps can be influenced by the availability and diversity of natural oviposition sites for gravid females in a given area (Focks, 2003, Faccinelli *et al.*, 2007). Sticky ovitraps target in collection of gravid rather than host-seeking females. Therefore, the results obtained with this tool cannot be extrapolated to estimate man-vector contact (Morrison *et al.*, 2007, Faccinelli *et al.*, 2007).

1.5.7 Surveillance of resting and host-seeking adult mosquitoes

Entomological surveillance data is crucial to evaluate the impact of vector control measures and to make proper and timely decisions regarding the most effective dengue control interventions. Surveillance based on adult mosquitoes tends to provide less reproducible results than those obtained by sampling *Ae. aegypti* immature stages (WHO, 2009a). Also, results are strongly dependent on the training and motivation of the collector and collaboration of the household, as this mosquito is typically found resting indoors. Although laborious and difficult to estimate accurately, adult vector abundance potentially provides the most useful information for assessing dengue transmission risk (Focks, 2003).

Several methods have been developed to capture adult mosquitoes, with one of the most common being the CDC backpack aspirator, which was constructed (Clark *et al.*, 1994), by improving of the AFS sweeper (Meyer *et al.*, 1983). The CDC back aspirator was constructed, especially for use in field studies on *Ae. aegypti*, and it was designed to provide the needed suction and increased mobility by a back pack design (Clark *et al.*, 1994). This device is considered the most effective method for indoor

collections of *Ae. aegypti* (Edman *et al.*, 1992, Clark *et al.*, 1994, Reiter & Gubler 1997, Scott *et al.*, 2000b, Scott & Morrison 2008).

A new trap developed to collect *Ae. aegypti* adults is the B-G Sentinel™ Mosquito Trap developed and manufactured by BioGents (Regensburg, Germany), which can be used with or without mosquito attractants. The trap is a collapsible pop-up container with white gauze covering its opening. In the middle of the gauze cover, air is sucked into the trap through a black catch pipe by an electrical fan, drawing approaching mosquitoes into a catch bag. Then, by generating ascending currents, the air exits the trap through the white gauze. Both of these surveillance tools have been compared to human-landing collections. Results obtained from a study in Peru revealed that there was no comparable alternative to backpack-aspirator or human-landing collections for monitoring adult *Ae. aegypti* populations (Schoeler *et al.*, 2004). However, two studies conducted in Brazil found the B-G Sentinel Trap to be a comparable alternative to human landing/biting collections and CDC backpack aspirator collections (Krockel *et al.*, 2006, Maciel-de-Freitas *et al.*, 2006).

1.5.8 Breeding sites

The intimate association of *Ae. aegypti* with humans contributes to its efficiency as a vector. Ancestrally, this species may have bred in small rock pools or tree holes, but it has adapted well to exploit the modern manmade environment. *Aedes* can be found breeding in an impressive variety of receptacles holding fresh water, including drums, large water tanks, buckets, flower vases, tyres, animal drinking pans and discarded bottles, to name a few (Service, 1992, Tun-Lin *et al.*, 1995, Focks & Chadee, 1997, Schneider *et al.*, 2004, Barrera *et al.*, 2006, Calderon-Arguedas, 2009).

The oviposition behaviour of female mosquitoes is separated into two distinct behavioural categories: preoviposition, which includes all acts related to attraction of

the fly to a potential oviposition site, and oviposition, during which eggs are deposited on the substrate (Bentley & Day 1989). *Ae. aegypti* follow both visual and olfactory cues to find suitable oviposition sites. They then use both physical and chemical factors of the water to discriminate between the most suitable places for oviposition (Clements, 1999).

Aedes deposit their eggs singly on moist surfaces that are likely to become submerged. This is vital for the normal process of hatching because if they are submerged prematurely, immersion can be fatal (Christophers, 1960). Under laboratory conditions, gravid female *Aedes* lay their eggs in any water source or wet surface available and in the absence of sources of moisture they will retain their eggs (Christophers, 1960).

Mogi & Morky (1980) first used the term "skip oviposition" to describe the behaviour of female *Wyeomyia smithii* that distributed the same batch of eggs among different plant pots. However, it had previously been reported by Christophers (1960) that *Ae. aegypti* deposit their eggs in several places. Subsequent laboratory studies confirmed this observation (Chadee & Corbet, 1987, Chadee & Corbet, 1990). Chadee & Corbet (1987) reported that female *Ae. aegypti* deposited only a fraction of their total egg batch in containers due to the small number of eggs found in those containers. Using RAPD-polymerase chain reaction (PCR) fingerprints, Apostol *et al.*, (1994) confirmed that eggs from multiple *Ae. aegypti* sibling families were laid in single oviposition containers. Likewise, Reiter *et al.*, (1995) used rubidium–marked eggs to demonstrate that females deposit eggs in a variety of places; while these studies demonstrate that "skip-oviposition" in *Ae. aegypti* appears to be a common behaviour, other research has not found enough evidence to support this hypothesis (Morrison *et al.*, 1999, Harrington & Edman, 2001).

Another widely discussed aspect of *Aedes* oviposition behaviour is whether gravid females are attracted to or repelled by water containing con-specific eggs or larvae.

Several authors have suggested that water containing con-specific larvae is preferential to tap water alone for oviposition (Soman & Reuben, 1970, Trimble & Wellington, 1980, Maire, 1984, Allan & Kline, 1998). There is evidence to suggest that female *Ae. aegypti* avoid laying eggs in containers in which they themselves have already laid eggs, and in which other females have laid eggs. However, when offered the choice of both, they tend to avoid the container bearing their own eggs (Chadee *et al.*, 1990).

Zahiri & Rau (1998) have reported that the attraction of *Ae. aegypti* to waters in which congeneric larvae lived was dependent on the biomass of larvae. Similar responses were obtained as the volume of water decreased or the number of larvae increased. These results could suggest the existence of a feedback mechanism to maintain larval populations at an optimal level i.e. in favourable environments, larvae may produce compounds to attract their con-specifics and under stressing situation may produce compounds repellent to oviposition (Zahiri & Rau, 1998). Additionally, in this study, it was reported that varying degrees of food deprivation may affect the chemical message for oviposition. Therefore, waters from fed larvae, which were initially attractive, progressively reduced in attractiveness as the duration of starvation increased (Zahiri, *et al.*, 1997). Different starvation periods (3 or 5 days and after 7 days) in different larval instars (2^{nd}, 3^{rd} and 4^{th} instars) appeared to render waters unattractive to gravid female *Ae. aegypti* (Zahiri, *et al.*, 1997).

Unlike *Culex*, which has been very well studied regarding the chemical nature and effect of pheromones affecting oviposition (Laurence *et al.*, 1985), *Aedes* has not been well-studied. Existing evidence suggests that certain substances act as chemical attractants for *Aedes*. However, it has not yet been determined whether they are mosquito-derived or are by-products of bacterial metabolism of mosquito faeces (McCall, 2002).

Gravid mosquito females presumably follow a variety of chemical cues when choosing oviposition places (Bentley & Day, 1989, Millar *et al.*, 1994, McCall & Cameron 1995, Torres-Estrada *et al.*, 2001). Amongst the variety of chemical cues to oviposition, olfactory cues received by contact chemoreception (Bentley & Day 1989) are considered to play a crucial role in both site selection and successful oviposition (Sharma *et al.*, 2008). The olfactory cues have been studied using odour-baited oviposition traps. Data suggests that odour can intensify sampling efficiency of mosquito ovitraps (Reiter *et al.*, 1991).

Avoidance of oviposition sites containing predators/competitors is an effective survival strategy for some insects (McCall, 2002). However, with *Ae. aegypti*, it appears to have an attractive effect. In particular, this is observed in water that previously at that point in time contained the predator copepod *Mesocyclops longisetus* (Torres-Estrada *et al.*, 2001). Similarly, Carrol (1979) reported that the presence of methoprene (a synthetic insect growth regulator used for larval control) increased by almost twofold the number of eggs laid by *Ae. aegypti* compared to untreated controls. These results have important implications for control strategies, since potential breeding sites treated for larval control could be more attractive to gravid females (Bentley & Day 1989).

Phytochemicals can also act as either attractants or deterrents to *Ae. aegypti* oviposition. Oviposition deterrents reported to date include derivatives from species of *Lemna minor* and *Hemizonia fitchii,* such as Eugenol, citronellal, thymol, pulegone, rosemary oil and cymene (Saxena & Sharma, 1972, Angerilli, 1980, Walitiya *et al.*, 2008), while compounds such as borneol, camphor and β-pinene act as attractants (Walitiya *et al.*, 2008).

While valuable studies have been conducted to evaluate the factors that modulate oviposition behaviour of *Ae. aegypti*, important gaps in knowledge remain, such as the nature of the substances referred to as potential attractants or repellents and their

mechanism(s) of action. Likewise, the relationship between results obtained in the laboratory with 'real life' field conditions must be further explored so information can be translated into possible control strategies based on the oviposition behaviour.

1.5.9 Blood feeding patterns

One of the most important parameters in vector borne pathogen transmission is feeding preference. *Ae. aegypti* generally do not disperse 300 meters beyond their breeding sites (Christophers, 1960). Females tend to rest inside houses, where they feed frequently on human blood (Scott *et al.*, 1993b). The highly anthropophagic behaviour exhibited by this species has important epidemiological implications regarding the transmission of viral pathogens (Edman *et al.*, 1992, Scott *et al.*, 1993b). Evidence suggests that the fitness of *Ae. aegypti* is increased when they feed on humans rather than other animals (Scott *et al.*, 1997, Scott *et al.*, 2000ab, Harrington *et al.*, 2001b, Styer *et al.*, 2007b). The highly anthropophilic nature of *Ae. aegypti* makes it highly efficient as a vector of dengue, which is a non-zoonotic human pathogen.

The majority of female hematophagous mosquitoes require both blood and a source of carbohydrates (Clements, 1992, Foster, 1995). However, this is not the case with *Ae. aegypti* (Edman *et al.*, 1992, Scott *et al.*, 1997, Costero *et al.*, 1998a). *Ae. aegypti* females are non-autogenous and thus require blood from a vertebrate host for egg production (Singh, 1957). It is widely accepted that most non-autogenous mosquitoes require a single blood meal for the development of each egg batch, known as 'gonotrophic concordance', with periodic meals of plant sugars to provide energy reserves used for survival and flight (Scott *et al.*, 1997, Edman *et al.*, 1992). However, *Ae. aegypti* females frequently imbibe multiple human blood meals during each egg-laying cycle (Scott *et al.*, 1993ab, 1997) and obtain all nutrients necessary for reproduction, flight and survival by feeding only on humans (Edman *et al.*, 1992, Van Handel *et al.*, 1994).

The exact neurologic and genetic mechanisms regulating mosquito feeding preferences are not well understood (Edman *et al.*, 1992). Amongst the factors affecting *Ae. aegypti,* feeding preference is host defensive behaviour (Edman & Scott, 1987). In environments where multiple alternative hosts are available for mosquitoes, as well as plant sources of nectar and fruit sugar, it would be expected that host preference would modify to include less defensive hosts than humans. However, a high percentage (over 90%) of *Ae. aegypti* was found to feed on human blood (Scott *et al.*, 1993b, Scott *et al.*, 2000a, Ponlawat & Harrington, 2005).

In a field study of female *Ae. aegypti* feeding preference in Thailand and Puerto Rico, Scott *et al.*, (2000a) found that 88 and 95%, respectively, were positive for human blood, with the remainder containing mixed meals between humans and other hosts. Likewise, Ponlawat & Harrington (2005) reported that *Ae. aegypti* from different locations in Thailand fed almost exclusively on humans (99%), with other hosts such as bovine, swine, cat, rat and chicken representing less than 1% of blood meals.

The reluctance or avoidance of plants as a source of sugar by *Ae. aegypti* (Edman *et al.*, 1992, Van Handel *et al.*, 1994, Martinez-Ibarra *et al.*, 1997, Costero *et al.*, 1999, Ponlawat & Harrington, 2005) appears to be balanced by frequent blood feedings to prevent starvation (Costero *et al.*, 1999). This pattern consequently increases the frequency of contacts between the mosquito and the host, which is interpreted as an increased likelihood that females acquire and transmit the dengue virus (Dye, 1992). Several authors suggest that this could explain how a mosquito that shows a low susceptibility to oral infection and a low rate of transovarial infection (Monath, 1994) can still efficiently transmit pathogens, even at a low population density (Gubler, 1992, Kuno, 1997).

Results obtained from both field and laboratory studies show that preferential and frequent human blood feeding behaviour confers a fitness advantage to *Ae. aegypti* (Scott *et al.*, 1997, Costero *et al.*, 1998b, Naksathit & Scott 1998, Harrington *et al.*,

2001) by increasing survival time, progeny size and the rate of reproduction, all of which increase the transmission potential of this vector. Additionally, Costero *et al,*. (1998) reported that this behaviour is not restricted geographically or temporally, such that *Ae. aegypti* from the Old and New World show similar behavioural patterns throughout different times of the year.

1.6 Dengue vector control

The history of mosquito control for disease prevention dates from the discovery that mosquitoes are the vectors of malaria by Ronald Ross in 1897. Although a considerable amount of control is possible without the use of insecticides, it is only since the discovery of synthetic organic insecticides in the 1940s that the modern era of insect control began (Becker *et al.*, 2003). Insecticides used in mosquito control belong to four major chemical groups, namely chlorinated hydrocarbons, organophosphates, carbamates and pyrethroids. An additional group includes the biological insecticides, such as Bti (the bacterial endotoxin from *Bacillus thuringiensis* var. *israelensis*) and the insect growth regulators (IGRs *e.g.* pyriproxyfen).

A variety of methods have been used for the control of *Aedes* spp., with varying degrees of success and failure (Curtis, 1990). These include chemical, biological and environmental approaches. Most vector control programmes integrate several of these control methods to suppress *Aedes* populations (Marquardt, 2005, McCall & Kittayapong, 2007). Control can be summarised as the elimination of *Ae. aegypti* habitats by prevention of access to potential breeding sites. This can be achieved either by covering the sites, by frequently empting and cleaning them, by disposing of unused or discarded containers or by killing developing mosquito stages within containers using insecticides or biological control agents. Adult mosquitoes can be killed using insecticides when they are in flight (space spraying) or in locations where they rest (residual spraying) (WHO, 2009a).

Given that, *Ae. aegypti* is a highly domesticated species (Christophers, 1960) with limited flight range (Muir & Kay, 1998, Harrington *et al.*, 2005), control or reduction of the peri-domestic vector population should be a feasible task. However, field based results have proven otherwise (McCall & Kittayapong, 2007). Despite the availability of powerful tools for *Ae. aegypti* control, their effectiveness has been affected by issues of delivery, coverage and acceptability (Farrar *et al.*, 2007). Regarding the latter issue, communities often reject the application of larvicides (such as temephos) in domestic water containers, citing unpleasant odour and water appearance. As such, low acceptance can minimize the effectiveness reported by laboratory studies (Lee *et al.*, 2005, Sihuincha *et al.*, 2005). For this reason, the selection of the most appropriate vector control method, or combination of methods, requires multiple factors to be taken into account, in particular, community participation. Likewise, the local ecology and behaviour of the target species, the resources available for implementation, the cultural context in which control interventions are undertaken , the feasibility of their application in a timely manner and the adequacy of coverage must be addressed (McCall & Kittayapong, 2007).

1.6.1 Environmental management

The early successes achieved in the control of *Ae. aegypti* before the advent of DDT were made by control measures aimed primarily at the destruction of breeding sites (Curtis, 1990, Reiter & Gubler, 1997). In recent years, increases in the abundance and diversity of man-made breeding sites suitable for *Ae. aegypti* has resulted in new challenges to this strategy. It is necessary to characterize the importance of these breeding sites as a function of abundance and productivity to streamline available resources and target control activities most efficiently. The WHO (1980) defined environmental management for vector control as "the planning, organization, carrying out and monitoring of activities for the modification and/or manipulation of

environmental factors or their interactions with humans, with a view to preventing or minimizing vector propagation and reducing human-vector-pathogen contact".

Actions required for the sustainable environmental management of potential breeding sites for *Ae. aegypti* include improved joint participation of the health authorities with the education, public service and environmental sanitation sectors to build infrastructure and community participation. Public information programmes, including legislation regarding the management and maintenance of properties to prevent them from harbouring suitable *Ae. aegypti* breeding sites, should form a part of any environmental management strategy. Public places, such as cemeteries, can become significant breeding sites for *Ae. aegypti* and other species of medically important mosquitoes. Studies conducted in cemeteries in Trujillo (Abe *et al.*, 2005) and in Caracas (Barrera *et al.*, 1979, 1982, Navarro, 2009) showed high infestation levels of *Ae. aegypti* and other mosquito species. While the initial costs of managing and maintaining environmental control programmes may seem expensive, the long-term benefits can justify the necessary investment (Becker *et al.*, 2003). Additionally, environmental control methods have the benefit of not requiring the application of expensive chemical or biological agents.

The most notorious and successful campaign in the fight against dengue vectors was achieved by development of the *Ae. aegypti* eradication programme, initially in Brazil and later in the Americas (Soper, 1967). Unfortunately, the sustainability of any control effort aiming to obtain long-term effective prevention of dengue transmission would be difficult to maintain without continued economic support from government, and active involvement of communities in design and implementation.

1.6.2 Biological control

The definition of biological control has been modified by several authors trying to unify terminology (Eilengber *et al.*, 2001) However, the definition most often cited was proposed by Debach in1974, who defined biological control as "the utilization of natural enemies to reduce the damage caused by noxious organisms to tolerable levels".

With the discovery and large-scale use of synthetic insecticides between the 1940s and 1950s, biological control of mosquitoes was no longer considered to be important (Becker *et al.*, 2003). However, excessive use and abuse of chemicals has resulted in both environmental pollution and the development of resistance in target mosquitoes. As a result, in the search for new strategies to combat the mosquito vectors of human pathogens, biological control strategies again have come to be regarded as useful and valuable control tools (Becker *et al.*, 2003).

Several organisms have been tested in the laboratory and field to explore their potential use against dengue vectors. Only certain species of larvivorous fish (Mulla, 1971, Martinez-Ibarra *et al.*, 2002, Valero *et al.*, 2006, Senng *et al.*, 2008) and predatory copepods (Copepoda: Cyclopoidea) have proven effective in operational contexts in specific container habitats (Brown, *et al.*, 1991, Marten, 1994, Kay *et al.*, 2002, Nam *et al.*, 2005). While biological control avoids many of the complications chemical contamination of the environment may pose, there may be operational limitations such as expense, difficulty of large-scale rearing of the organisms, difficulty of distribution to sites and their limited utility in aquatic sites where temperature, pH and organic pollution may exceed the narrow requirements of the organism (WHO, 2009a).

The best known fish predator of mosquitoes is the so-called 'mosquito fish' *Gambusia affinis*, which is native to the south-eastern United States, eastern Mexico

and the Caribbean, and the common guppy *Poecilia reticulata*, which is native to tropical South America. Both types of fish are effective predators of mosquito larvae, but they also feed on the eggs and offspring of indigenous fish. The introduction of *G. affinis* can also lead to the destruction of extensive aquatic predatory invertebrate populations (Becker *et al.*, 2003, Service, 1995). The WHO recommends that the use of indigenous fish is preferred over exotic species, due the former are well adapted to local environmental conditions, and the biological control based on larvivorous fish is best achieved, if it is conducted as a part of an integrated control programme (WHO/EMRO, 2003).

The most promising new form of biological control for container-breeding *Aedes* larvae is the cyclopoid copepods (Crustacea). Numerous larvivorous species of these microscopic crustaceans occur naturally worldwide. They have shown significant potential for the control of dengue vector populations (Marten, 1990), particularly in Vietnam. Although not evaluated in a controlled trial, *Mesocyclops* spp. used in tandem with normal clean-up and education campaigns, successfully controlled *Ae. aegypti* populations in water storage containers, in north and central Vietnam (Kay & Nam, 2005, Nam *et al.*, 2005). In some areas, the *Ae. aegypti* population disappeared entirely for a number of years and no dengue cases occurred across large geographical areas for a number of years following initiation of the scheme.

1.6.3 Chemical insecticides

In 1947, the Pan American Health Organization began the Western Hemisphere campaign to eradicate *Ae. aegypti* through the use of DDT (Schliesman & Calheiros 1974). Thus, by 1962, *Ae. aegypti* was eradicated from 22 countries in the Americas and from all countries bordering the Mediterranean in 1972 (PAHO, 1994). Unfortunately, early DDT resistance represented a serious problem (Brown & Pal 1971) and in combination with political and logistical factors contributed to

interruption of the campaign before eradication was achieved in all of the Western Hemisphere (Soper, 1965). However, Soper (1967) pointed out that although initially, "the objective of the Brazilian campaign was not to eradicate *Ae. aegypti*, but to eradicate yellow fever through the reduction of *Ae. aegypti* breeding and the maintenance of low *Ae. aegypti* indices in the principal cities". Thus, Brazil eradicated *Ae. aegypti* by the destruction of breeding sites during the 1940s. In order to maintain the country free of reinfection, they started to export their programmes to neighbouring countries (Soper, 1967).

Insecticides have played an important role in the control of major insect vectors of human pathogens, such as mosquitoes, triatomine bugs, sandflies, flies, lice and others (Hemingway & Ranson, 2000). In the history of insecticides, dichlorodiphenyltrichloroethane (DDT) occupies a special place. DDT was first introduced for mosquito control in 1946. The first cases of DDT resistance in mosquitoes were reported the following year in *Ae. tritaeniorhynchus* and *Ae. sollicitans* (Brown, 1986). Since then, resistance to one or more insecticide has been reported in more than 100 mosquito species. The rapid development of resistance in mosquitoes led to the use of alternative insecticides. However, this did not solve the problem of resistance as it continued to develop to the newer insecticides such as organophosphates, carbamates and pyrethroids (Hemingway & Ranson, 2000).

After appearance of DDT resistance, the strategy changed to the use of organophosphate insecticides, such as larvicides (temephos) and adulticidal (fenthion, fenitrothion and malathion), the latter mainly recommended to be applied as ultra-low-volume (ULV) concentrates in emergency control measures during epidemics. However, in spite of this recommendation its use was adopted as a routine control measure (Reiter & Gubler, 1997). Some studies conducted in several countries to evaluate in the field the efficacy of ULV applications by ground or aerial equipment have demonstrated that little or no lasting impact is obtained on *Ae. aegypti* populations (Chadee, 1985, Reiter & Gubler, 1997, Perich *et al.*, 2000, 2001).

In the 1960s-1970s, the discovery and development of photostable pyrethroids, mainly in Japan and the UK, led to major improvements in the practice of insecticide utilisation. Pyrethroids are exceptionally potent, biodegradable compounds that may be used in the field at rates as low as 20g/ha, (10-100 times lower than other insecticides), eventually leading to a lower burden of chemicals in the environment. However, pyrethroids are toxic to many aquatic vertebrate and invertebrate species, including fish, and many beneficial insect species (Becker *et al.*, 2003).

At present, the main classes of insecticides used for vector control are: organochlorines (OCs), of which DDT and HCH are the only products still used in vector control; organophosphates (OPs), such as fenitrothion, malathion and temephos; carbamate (C) such as bendiocarb and propoxur and pyrethroids (PYs), such as cyfluthirn, cypermethrin, deltamethrin, etofenprox, lambdacyalthrin and permetrhin (Zaim, 2002). However, microbial insecticides, such as *Bacillus thuringiensis* H-14 and *Bacillus sphaericus,* and the Insect Growth Regulators (IGRs), such as methophrene and piryproxifen, are being used in large-scale control programmes (Bisset, 2002).

Zaim (2002), in a review of global insecticide use for vector-borne disease control, informs that between 1995 and 2000 in the Americas region most countries have reported use of insecticides for *Ae. aegypti* control . Additionally, about 60% of the organophosphate and 94% of pyrethroids insecticides used for dengue control are applied by space spraying. Organophosphates, such as fenitrothion and malathion were the insecticides used more frequently, whilst pyrethroids, such as cypermethrin and permethrin were the most commonly insecticides used for dengue vector control during the mentioned period. As use of organophosphates for dengue control has shown a significantly decrease, use of pyrethroids has increased progressively in recent years (Zaim, 2002).

Unfortunately, *Ae. aegypti* has demonstrated the ability to develop resistance to organochlorine, organophosphate, carbamate and pyrethroid insecticides (Brengues *et al.*, 2003, Lima *et al.*, 2003, da-Cunha *et al.*, 2003, Rodriguez *et al.*, 2005).

1.6.4 Dengue vector control-immature stages

In most countries, where *Ae. aegypti* and dengue represent a public health problem, the main mosquito control interventions are focused on the immature *Ae. aegypti* stages. Control measures against immature stages most commonly consist of the application of larvicides to water containers. Until now, only four products have met the criteria required by the International Programme for Chemical Safety and the WHO: the organophosphate temephos (Abate), two insect growth regulators (methoprene and pyriproxifen) and Bacillus thuringiensis var. israelensis (WHO, 1991, 1993). To be considered safe for use in drinking water, a larvicide must demonstrate a low degree of acute and chronic toxicity, and ideally be tasteless, odourless and colourless (Gratz & Halstead, 2008). For the treatment of drinking water, temephos and methoprene can be applied at dosages of up to 1 mg of active ingredient (a.i.) per litre (1 ppm); pyriproxyfen can be applied at dosages up to 0.01 mg a.i. per litre (0.01 ppm), and Bti at 1-5 mg per litre (WHO, 2006).

1.6.4.1 Larval control with biological insecticides

Biological agents for mosquito control include the mosquitocidal bacteria *Bacillus thuringiensis* var. *israelensis* (Bti) and *Bacillus sphaericus*. Both bacilli produce crystalline proteinaceous toxins during sporulation (parasporal bodies), which when ingested by mosquito larvae are activated by enzymatic action in the alkaline medium of the midgut, ultimately destroying the gut lining (Becker *et al.*, 2003, Zahiri & Mulla, 2006). After their discovery (Golberg & Margalit, 1977), these products have been widely used as insecticides and have shown effectiveness against *Aedes* and

other mosquitoes such as *Culex* and *Anopheles* (Mulla *et al.*, 1988, Becker *et al.*, 1998, Fillinger *et al.*, 2003, Zahiri & Mulla, 2006).

In addition to the relative ease with which they can be mass-produced, bacterial control agents are highly efficient, environmentally safe, easy to handle, stable when stored and suitable for integrated control programmes based on community participation (Becker *et al.*, 1992, Becker & Margalit, 1993). A basic requirement for the successful use of bacterial control agents is the development of effective formulations suited to the biology and habitats of the target organisms (Becker *et al.*, 2003).

Apart from differences in susceptibility of mosquitoes to bacterial toxins, several factors have been identified that may interfere with the efficacy of these products in the field. These include the feeding behaviour of the target species as well as the environmental conditions in which they are applied (Becker *et al.*, 1993, Mulla, 1990). In developing countries, the most important factor limiting their use has been the relatively short period of action of the toxins because sunlight quickly renders the parasporal body inactive. Mosquito populations can recover within two weeks of treatment if breeding sites are exposed to direct sunlight (Mulla, 1990, Castillo & Scorza, 1997).

1.6.5 Dengue vector control- adult mosquitoes

The main goal of control methods focused on adult mosquitoes is the reduction of populations to levels that can impact upon the transmission of pathogens. This may be achieved either by killing the mosquitoes or interrupting the contact between humans and infective insects. Traditionally, control directed at adult dengue vector mosquitoes has been attained through use of ULV application of insecticides, generally by vehicle-mounted equipment. Despite controversial opinions regarding

the efficacy of this control (Perich *et al.*, 2000, 2003, Reiter & Gubler, 1997, Esu *et al.*, 2010) these interventions tend to be reactive, particularly to combat epidemic outbreaks (Rawlins *et al.*, 1995, McCall & Kittayapong, 2007). Malathion is the adulticide most widely applied by thermal fogging or ULV (Georghiou *et al.*, 1987, Rawlins *et al.*, 1995), although from the 1990s many Latin American countries have also applied the pyrethroids deltamethrin, permethrin, cyhalothrin, cypermethrin and cyfluthrin against adult mosquitoes (Bisset *et al.*, 2009).

1.6.6 Lethal ovitraps

Although ovitraps were originally designed as a tool for detecting and monitoring *Ae. aegypti* populations in United States (Fay & Perry, 1965), they can also be modified into lethal traps to kill *Ae. aegypti* adults by incorporating insecticides (primarily pyrethroids such as delthamethrin (Perich *et al.*, 2003, Sithiprasasna *et al.*, 2003) and bifenthrin (Williams *et al.*, 2007) onto the oviposition substrate. In Brazil, lethal ovitraps were tested in urban areas, resulting in a significant decrease in *Ae. aegypti* populations at the test sites (Perich *et al.*, 2003). Likewise, a significant impact on natural *Ae. aegypti* populations was recorded after deploying lethal ovitraps in three villages in western Thailand (Sithisprasasna *et al.*, 2003). (Williams *et al.*, 2007) Laboratory and field studies from North Queensland, Australia suggest that lethal ovitraps could be used for regular *Aedes* control, having demonstrated efficacy for at least 4 weeks (Williams *et al.*, 2007, Perich *et al.*, 2009). Although further studies are required to determine the impact of lethal ovitraps on a larger scale (Williams *et al.*, 2007), they can be considered potentially important tools for vector control programmes (MacCall & Kittayanpong, 2007).

1.6.7 Insecticide treated materials (ITMs)

The concept of insecticide treated materials (ITMs) was originally introduced to prevent diseases transmitted by nocturnally-active vectors in Asia, Latin America, and Africa (WHO, 1996). Recently, ITMs have been evaluated in control of the diurnally-active mosquito *Ae. aegypti* (Kroeger *et al.*, 2006, Lenhart *et al.*, 2008). Among these materials, insecticide treated bednets are the most widely used, primarily to prevent malaria transmission. ITMs in the form of curtains have been implemented to control several medically important insects (*Anopheles gambiae, Anopheles funestus, Rhodnius prolixus, Rhodnius robustus, Culex quinquefasciatus, Phlebotomus argentipes, Lutzomyia youngi* and *Lutzomyia ovallesi*) with significant results upon vector populations observed in different regions (Lines, 1987, Majori *et al.*, 1989, Sexton *et al.*, 1990, Herber & Kroeger, 2003, Kroeger, 2002, Kroeger *et al.*, 2006, Joshi *et al.*, 2009, Kasili *et al.*, 2010).

Pyrethroid insecticides are most commonly used to treat ITMs due to their low mammalian toxicity and rapid insecticidal action (WHO, 1991). Several pyrethroids have been approved by the WHO Pesticide Evaluation Scheme (WHOPES) or are in the field trial phase. Amongst them, deltamethrin is one of the pyrethroids typically used to treat insecticide-treated bednets (ITNs). Several studies have been conducted to evaluate the efficacy of pyrethroids used on ITMs, either in the laboratory or in the field, (Cheng *et al.*, 1995, Hii *et al.*, 1995, Sharma & Yadav, 1995, Curtis *et al.*, 1996, Carnevale *et al.*, 1988, Yadav *et al.*, 1998, 2001).

In a relatively short period (one decade), work on insecticide impregnated bednets has grown significantly from small-scale trials to the widespread operational use of more than 10 million treated bednets (WHO, 1997). In the broadest sense, the treatment of bednets may be considered as the most rational form of selective insecticidal treatment because the treated surfaces are those which blood-seeking mosquitoes are

highly likely to encounter during their efforts to approach human hosts (Mouchet, 1987).

Traditionally, effectiveness of ITNs for malaria control required the re-impregnation of bednets after one year of use (Lengeler, 2004b). This proved a major obstacle, particularly in developing countries (Yates *et al.*, 2005). The incorporation of new techniques to improve the diffusion of the insecticide in the material during manufacture, creating long-lasting insecticidal nets (LLIN), is providing a potential solution to this problem (Itoh & Okuno, 1996, Graham *et al.*, 2005).

Although initially ITMs were developed and introduced to evaluate the protective effect against malaria, there is accumulating evidence that insecticide-treated window curtains and long-lasting insecticidal fabric covers for domestic water-storage containers can reduce dengue vector densities to low levels in some communities (Kroeger *et al.*, 2006, Lenhart *et al.*, 2008). Although further studies are needed to confirm that transmission can be reduced by this type of intervention, ITMs appear to hold promise for dengue vector control (WHO, 2009a).

1.6.8 Protective efficacy of ITMs

ITMs, primary deployed as bednets, have show significant impact upon malaria transmission in different Africa regions, especially in preventing malaria mortality in children (Lengeler, 2004a).The application of insecticide to bednets dates from Second World War when they were used by Russian troops (Blagoveschensky *et al.*, 1945, Nevill *et al.*, 1996). However, it was in the 1980s that controlled trials demonstrated a significant impact upon the incidence of febrile cases due to *Plasmodium falciparum* infection through the use of bednets impregnated with pyrethroid insecticides (Graves *et al.*, 1988, Snow *et al.*, 1988, Sexton *et al.*, 1990, Nevill *et al.*, 1996).

In several African, Asian and Latin American countries, the WHO and other agencies have encouraged ITNs trials to evaluate the effects on malaria mortality and morbidity. Choi *et al.*, (1995) reported that studies in Asia and Africa in different epidemiological situations demonstrated that insecticide-impregnated bednets reduced the incidence of clinical attacks of malaria by 50%. In the same way, a trial in the Gambia (Alonso *et al.*, 1991) found that permethrin-impregnated bednets reduced overall deaths by >50%. Although trials carried out later in other countries did not report the same effect on mortality rates, there was a reduction in overall child mortality of 17 to 33% (Mutambu & Shiff, 1997, Snow *et al.*, 1996). The high acceptance and high usage by the population, even without expensive promotional programmes, makes ITNs promising and beneficial malaria control tools (Phillips, 2001).

Research conducted between 1997 and 2000 in Tanzania to evaluate the impact of ITNs reported a reduction of 27% in mortality risk, decreased anaemia and parasitaemia in children and pregnant women and demonstrated the cost-effectiveness of ITNs (Armstrong *et al.*, 2001, Abdulla *et al.*, 2001, Marchant *et al.*, 2002, Hanson *et al.*, 2003).

A Cochrane review of ITNs in the scientific literature concluded that the beneficial effect of ITNs in reducing overall mortality in Africa has largely been demonstrated under trial conditions (Lengeler, 2004a). There is evidence to suggest that the widespread use of ITNs with full coverage could prevent 370,000 child deaths per year (Lengeler, 2004a).

In South-East Asia, where drug resistance in *Plasmodium falciparum* is a problem, several studies investigating bednets with or without impregnation reported a significant reduction in *P. falciparum* infections after 6 months of using impregnated bednets (Luxemburger *et al.*, 1994, Binka *et al.*, 1998).

ITMs have also been used for malaria control in Latin America. A study undertaken in Guatemala compared the protective efficacy of ITNs (impregnated with a solution 10% permethrin formulation, diluted to deliver 500mg/m^2 of insecticide) with untreated bednets. After 13 months, a significant reduction in the density of malaria incidence among people using both treated and untreated bednets was observed. It was concluded that the principal protective effect was conferred by the physical barrier between hosts and mosquitoes. An additional benefit reported by people was a reduction in nuisance insect biting thus permitting better sleep, which may have resulted in the high acceptance of bednets observed in this study (Richards *et al.*, 1993). In a multi-country intervention programme to control malaria carried out in Ecuador, Peru and Colombia, communities using bednets impregnated with lambdacyhalothrin and permethrin reported a reduction of 40% in malaria cases, as well as a repellent effect that reduced mosquito-host contact (Kroeger *et al.*, 1991, 1995).

In Latin America, ITMs have recently been proposed as a potential dengue control tool. A cluster-randomized trial conducted in Mexico and Venezuela demonstrated that insecticide-treated window curtains and/or insecticide-treated domestic water container covers significantly reduced dengue vector densities (Kroeger *et al.*, 2006). Likewise, a preliminary study conducted in Haiti with insecticide-treated bednets demonstrated an immediate decrease in *Ae. aegypti* populations (Lenhart *et al.*, 2008). Larger scale trials of ITMs for dengue control are being conducted to further evaluate their impact and potential for widespread application in dengue vector control programmes.

1.7 Dengue control in Venezuela and in Trujillo state

The *Ae. aegypti* eradication campaign in Venezuela began in 1947 and continued until 1977, resulting in extremely low infestation levels at the house level (HI ranging

from 0.2-2%). When the eradication campaign concluded, Venezuela transformed the eradication campaign into a control programme, which was limited to 9 states. The resulting control programme was not allocated sufficient resources to maintain the low levels of infestation achieved during the eradication campaign. Consequently the infestation rates began to increase, with HIs ranging between 24% and 39.5% in 1989, when the epidemic of dengue haemorrhagic fever occurred (Coello, 1992).

It is assumed that *Ae. aegypti* are present in all urban places below 2,000 meters above sea level in Venezuela. In a study on the abundance and characteristics of breeding places of *Ae. aegypti* in 30 Venezuelan coastal localities, it was found that all places were infested and water storage containers represented the most common breeding sites (Barrera, 2000).

In Venezuela, the organophosphate temephos (applied as 1% granules) is the only chemical larvicide widely used by dengue control programmes. Despite its continuous use over the past 40 years, *Ae. aegypti* fortunately remain susceptible to this insecticide (Perez & Molina, 2009). Malathion is most commonly used to control adult populations but, in contrast, its prolonged use has created problems of resistance in this vector (Perez, 2006).

Currently, in Trujillo state, *Ae. aegypti* control is based primarily on the use of organophosphate insecticides. Ultra-low-volume formulations of malathion and fenthion are used to control adult mosquitoes and larval control consists of applying 1% temephos granules. To date, only containers that store water for human consumption are treated. Applications in other types of breeding sites has been suspended (Vector Control Department, Trujillo state, personal communication), presumably, due to the lack of resources allocated to vector control activities. Consequently, treatment of storage water containers for human consumption may have been prioritised as they may consider these are the most productive breeding sites.

Clearly, dengue is a growing public health problem in Venezuela. The vertical control programmes implemented by health authorities have not obtained the desired effect in controlling populations of dengue vectors. In the absence of an effective vaccine against the four serotypes of dengue virus, the control of *Aedes* populations remains the most effective strategy for dengue control. The series of studies reported in this thesis deal with this challenge, in an effort to deliver an effective and acceptable new strategy for dengue prevention.

CHAPTER 2

INTRODUCTION TO THE STUDY

2.1. Introduction

Dengue is the most common and fastest spreading human arboviral disease worldwide. Control of the vector mosquito, *Aedes aegypti*, is the only effective preventive measure. Reduction of mosquito breeding in household water vessels through larvicides, predatory crustaceans, or elimination of discarded containers, and control of adult mosquitoes by spraying with insecticide, require a continuous effort by the community, and can be difficult to sustain and expensive.

Insecticide-treated materials (ITMs) have been shown to prevent transmission of a range of vector-borne diseases by preventing vector bloodfeeding, thus protecting the individual, and by reducing both vector population and lifespan, thus protecting the community. While great success has been achieved by targeting with insecticide-treated bednets, the nocturnal endophilic vectors of malaria (Nahlen *et al*, 2003), Chagas disease (Kroeger *et al.*, 2003), leishmaniasis (Kroeger *et al.*, 2002) and lymphatic filariasis (Pedersen & Mukoko, 2002), control of the diurnal endo/exophilic vectors of dengue/DHF had not been evaluated until recently.

In preliminary small-scale studies carried out in Latin America, Kroeger *et al.*, (2006) showed that ITMs can significantly reduce dengue vector populations. In 2003 in Venezuela, the combined effect of insecticide-treatment of both window curtains and water storage container covers on the local *Ae. aegypti* population was assessed in a cluster-randomised trial. Data indicated a marked drop in vector infestation indices in houses that received both curtains and water storage container covers as compared to control houses, indicating a clear effect on individual households. Vector infestation indices also dropped to a certain degree in control group houses. This indicated a

community effect of the interventions, whereby the entire dengue vector population was suppressed such that the intervention also benefited houses that did not have any protection. A study in Mexico investigating the effect of insecticide treated window curtains alone also showed significant reductions in household vector infestations (Kroeger *et al.*, 2006). This exciting breakthrough had to be repeated in other locations and contexts to confirm the potential of these novel dengue vector control tools.

The studies reported in this thesis were undertaken as part of the EU FP-6 funded DENCO consortium trial in Venezuela which evaluated insecticide-treated curtains and water jar covers in a cluster randomised trial to determine their efficacy in reducing dengue vector populations and dengue transmission, as assessed by indices of vector abundance. A related trial was undertaken in Thailand, but is not reported here.

The target population were communities in urban or peri-urban areas in a series of adjoining villages and small towns in Trujillo state, Venezuela, an endemic dengue transmission area where all households were at risk of dengue infection. Housing structure typified those in Venezuelan that are considered at risk for dengue infection - urban, 'open' (no glass in windows, large spaces between timbers that comprise the walls), with little or no access to sanitation and refuse disposal. Although the *Ae. aegypti* vector population breeds in a wide variety of containers, it was known that the most important, *i.e.* those container types from which the majority of the adult vector population emerged, were the large metal drums, that serve as domestic water storage containers in these communities. The intention therefore was to target these breeding sites only with a cover, simplifying both the intervention and the educational messages encouraging its adoption.

The insecticide-treated materials (ITM) to be used were made from long-lasting, insecticide-treated (the pyrethroid deltamethrin is applied during manufacture)

polyester netting that requires no re-impregnation (PermaNet®, Vestergaard-Frandsen, Switzerland). PermaNet materials are designed to retain their insecticidal properties and efficacy for the lifespan of the net (several years in the case of bednets) and have been approved by WHOPES for use as bednets. Safety aspects for use of the product as covers for domestic water storage containers have been assessed by WHO.

If effective, it is inevitable that this intervention would profoundly affect *Ae. aegypti* populations, with a number of possible outcomes. First, elimination of the vector population must be considered the ultimate outcome of a 100% effective intervention tool and strategy, though such a dramatic result was not expected here, based on knowledge of ITM impact on a range of vectors. Secondly, materials that targeted the vectors efficiently and that retained their insecticidal efficacy but that fell short of vector elimination, would be expected to exert powerful evolutionary selection pressure on the *Aedes aegypti* population, eventually leading to increases in detectable levels of insecticide resistance.

A third potential outcome was also possible with one of the interventions deployed in this study. If water container covers did not effectively deliver insecticide but instead formed only a physical barrier that prevented vector access to the preferred breeding sites (a real possibility where water jar cover) then the *Ae. aegypti* population would comprise only those that emerged from smaller less favoured containers (such as bottles, cans, car tyres, etc.) in the household or from untreated public areas. Assuming that adult mosquitoes emerging from these high-density highly-competitive environments would be likely to be less fit in evolutionary ecology terms than those emerging from the large domestic water drums, it was hypothesised that while the local vector population would be likely to survive, albeit at a much reduced level, its fitness and vectorial capacity could be significantly compromised.

These basic premises formed the basis on which the thesis' aims were formulated.

2.2 Objectives

The aims of the study were:

1. To evaluate in controlled trials, the efficacy of two appropriate interventions, insecticide-treated window curtains and insecticide-treated domestic water container covers, by entomological indices and seroconversion rates

2. Determine susceptibility to deltamethrin of *Ae. aegypti* natural populations assayed at regular intervals throughout the study to confirm any change in susceptibility status as a consequence of the intervention.

3. Investigate the association between fitness and body size of adult female mosquitoes and the type, of containers (drums, small containers and tyres) in which they developed before adulthood.

Intervention efficacy would be evaluated by a range of standard entomological indicators and by IgM seroconversion rates in the study population. Fitness would be evaluated by standard measures of reproductive output and a novel method estimation of body size.

CHAPTER 3

INSECTICIDE TREATED MATERIALS FOR THE CONTROL OF THE DENGUE VECTOR *AEDES AEGYPTI*: A CLUSTER-RANDOMISED TRIAL IN TRUJILLO STATE, VENEZUELA

3.1 Introduction

Prevention and control of dengue and dengue haemorrhagic fever (DHF) are best achieved by vector control, primarily by the reduction of mosquito numbers in their domestic breeding sites. However, these control methods target only the immature stages of the vector. An effective method for either reducing adult *Aedes aegypti* populations or impacting upon adult mosquito life expectancy (thus reducing the transmission rate) has long been hoped for. In the past, vector control directed at adult mosquitoes has been limited to the use of ultra-low-volume (ULV) application of insecticides, usually by vehicle-mounted apparatus. However, this method is unlikely to have any lasting effect upon the majority of adult mosquito vectors (McCall & Kittayapong, 2007, Esu *et al.*, 2010).

Currently, insecticide treated materials (ITMs) are essential tools employed in many vector borne disease control programmes worldwide. Notably they are routinely used in the form of insecticide treated bednets (ITNs) for malaria prevention and control. ITNs affect vector populations by either killing adult mosquitoes when they come into physical contact with the insecticide treated net or by restricting access to hosts and thus preventing mosquitoes from feeding (Gimnig *et al.*, 2003). There is substantial evidence demonstrating that ITNs are a cost-effective intervention against malaria; significantly reducing the density of malaria vectors (Lindblade *et al.*, 2005, 2006), sporozoite rates in *Anopheles* (Gimnig *et al.*, 2003) and malaria transmission (Eisele *et al.*, 2005). In addition, they significantly reduce overall childhood mortality and morbidity rates (Lengeler *et al.*, 2004). There is also evidence that suggests ITNs

have a significantly beneficial effect during pregnancy, in areas with moderate to intense malaria transmission in sub-Saharan Africa (Hawley *et al.*, 2003b, Gamble *et al.*, 2007). As a result, the scaling up of ITNs for significant malaria control across Africa is now being widely promoted (Magesa *et al.*, 2005, Hanson *et al.*, 2008, Abdisalan *et al.*, 2009).

Due to their success in malaria prevention, research into the efficacy of ITMs for the control of other vector borne diseases has also been undertaken. In Colombia, Kroeger (1999) first demonstrated the effect of ITNs upon triatomine bug population numbers (the vector of Chagas disease). Subsequently, a community study undertaken in Venezuela (Kroeger *et al.*, 2003) demonstrated that deltamethrin-impregnated bed nets successfully protected against *R. prolixus* bites. Furthermore, Herber & Kroeger (2003) showed that pyrethroid-impregnated curtains were highly efficient in protecting against *Rhodnius prolixus* and *R. robustus.*

The impact of using ITMs upon filarial worm transmission, particularly in Africa, has also been demonstrated. In Kenya, Bogh *et al.*, (1998) observed the protective effect of permethrin-impregnated bednets against the transmission of *Wuchereria bancrofti* by *Anopheles gambiae, An. funestus and Culex quinquefasciatus.* Pedersen & Mukoko (2002) later showed that ITNs reduced mosquito density and were effective in lowering the spread of lymphatic filarial parasites. Similar effects of ITNs use have been shown in Cambodia (Odermatt *et al.*, 2008).

There is growing evidence to support the effectiveness of ITMs in preventing the transmission of both visceral and cutaneous leishmaniasis (Joshi *et al.*, 2009, Emami *et al.*, 2009, Kroeger *et al.*, 2002), although conflicting evidence exists (Dinesh *et al.*, 2008).

However, it is important to note that all of the vector species mentioned above are nocturnally active vectors. While, ITNs were shown to impact upon *Ae. aegypti* in a

small trial in Haiti (Lenhart *et al.*, 2008), other more appropriate and effective methods to deploy the insecticide to target the diurnally active dengue vectors had not been considered until recently. In two cluster-randomised trials in Venezuela and in Mexico, Kroeger and his colleagues used insecticide-treated curtains (to target vectors during entry and exit into houses) or insecticide-treated water jar covers (targeting gravid female mosquitoes during oviposition or males and females emerging from the water beneath) to reduce dengue vector densities to low levels with some evidence showing a slight impact on dengue transmission (Kroeger *et al.*, 2006). These ITMs, particularly the curtains, were well accepted by the communities studied. This was attributable in part to the daily sight of dead insects like cockroaches, houseflies and other pests, as well as mosquitoes, beneath the treated curtains. The authors suggest that, as in the case of other disease vectors, the risk of a host-seeking mosquito contacting an ITM at some point during its frequent visits to houses to blood-feed meant that its life expectancy was reduced (*i.e.* the age structure of the vector population was altered and few individuals were likely to live long enough to become infective with dengue). The study also demonstrated a spill-over effect, whereby the intervention reduced vector populations in neighbouring control clusters. As such, houses without ITMs that were located close to treated houses were less likely to have high mosquito infestations than those further away from treated houses. This is also referred to as a "mass effect" and will reduce vectors throughout a community, as was previously shown with insecticide-treated bednets and malaria vectors in Africa (Gimnig *et al.*, 2003, Hawley *et al.*, 2003a). This is a highly beneficial outcome given that the coverage of any intervention tool is typically less than 100% in any community worldwide.

The Kroeger *et al.*, a Venezuelan trial (2006) was undertaken in Trujillo State, where, despite its low fatality rate, dengue represents a major public health problem. Although its control has been considered a main priority by health authorities, dengue cases have increased considerably. In 2008, the number of DHF cases almost doubled

compared to the number reported in 2007 (Dirección Regional de Epidemiología y Estadística Vital, 2009; see chapter 1, section 1.4.3, Figure 1.3).

The objective of this study was to investigate whether insecticide-treated window curtains and insecticide-treated covers for domestic water storage containers, both made from long-lasting insecticide-treated netting and deployed either separately or in combination, could lower populations of *Ae. aegypti* to levels at which reduced dengue virus transmission occurred in treated communities.

3.2 Methods

3.2.1 Study area

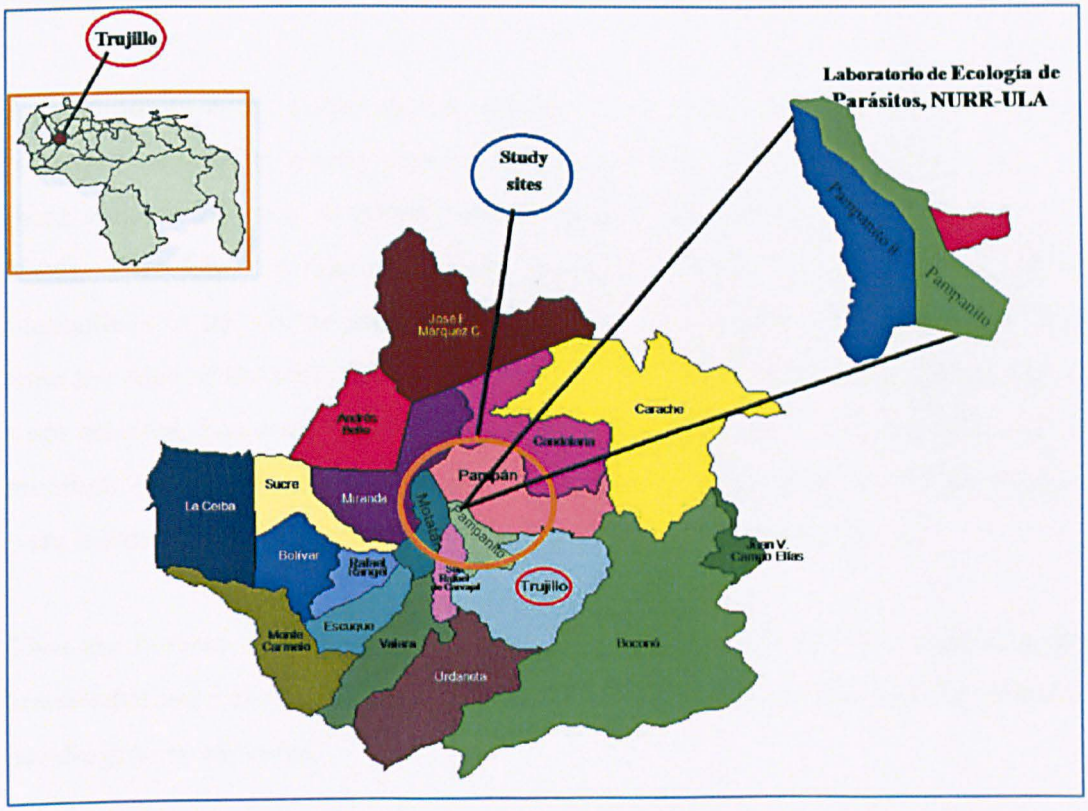
This study site was located in the western region of the Venezuelan Andes, in Trujillo State. The territory of Trujillo State occupies an area of 7,400 km^2, which represents 0.81% of the national territory, extending from 9° 51′ 00″ to 8° 58′ 20″ latitude North and from 70° 01′ 00″ to 71° 06′ 00″ longitude West. Trujillo is divided into 22 municipalities and 93 parishes and has a population of over 600,000 (Oficina Central de Estadística e Información, 2001). The state capital is Trujillo City and the research laboratory at which this study was based, Laboratorio de Ecología de Parásitos, NURR-ULA, is in the Pampanito municipality in Trujillo State (Figure 3.1).

Trujillo State has a mean annual rainfall of 750 mm, falling mainly in one season (March to October), while the driest months are January and July. Temperatures range between 16-37°C, with the hottest period occurring between July and September and the coldest period between December and February (Ministerio del Poder Popular para el Ambiente, 2009).

Within this study, 5 parishes were selected located in 3 municipalities based on dengue incidence rates in previous years. In these parishes, the total number of

reported dengue cases rose from 42 cases in 2001 to 194, 410 and 480 cases in 2002, 2003 and 2005 respectively (Dirección Regional de Epidemiología y Estadística Vital, 2005). The parishes selected were Monay, Flor de Patria, Pampán, Pampanito and Motatán, which represented a total human population of 91,500 inhabitants.

Figure 3.1. Map of Trujillo State illustrating the 3 municipalities (Pampan, Pampanito and Motatan) where are located the 5 parishes under study and the research laboratory at which this study was based
(Source: http://www.a-venezuela.com/mapas/map/imag/1estados/trujillo.jpg).



3.2.2 Study design

The study was undertaken as a cluster-randomised controlled trial, across 60 clusters (each cluster occupying an area greater than 500 x 500 m and comprising between 50 and 100 houses per cluster. Sample size calculations were made according to Hayes & Bennett, 1999) and based on the data of a previous pilot study. Allocation to treatment arm was assigned randomly: (1) 15 clusters received insecticide-treated curtains, (2) 15 clusters received insecticide-treated covers, (3) 15 received both curtains and covers and (4) a control group of 15 clusters received no intervention.

A community-wide effect (when effective treatments in intervention clusters 'overspill' and affect nearby control clusters, resulting in reduced vector activity in those clusters too) was considered likely based on an earlier study (Kroeger *et al.*, 2006). Therefore, to function as an 'external control' to monitor any natural fluctuations in the vector population, an additional 15 clusters located at least 5km from the edge of the study sites (and therefore unaffected by any of the interventions) were selected, based on similarities in dengue incidence (from the preceding 12-24 months), household density and other geographical characteristics. These clusters were monitored in exactly the same way as the study clusters (see below).

Thus the number of households required for the trial totalled 7,500. All occupied households were eligible for inclusion while business-only premises and multi-storey buildings were excluded.

3.2.2.1 Baseline surveys

After permission from health authorities and informed consent from community populations (June-July 2006) were obtained in all localities under study, entomological surveys to determine the standard indices for the abundance of

immature stages of *Ae. aegypti* were conducted at the household level in all houses: Breteau index (BI), house index (HI), pupae per person index (PPI) and container index (CI). Potential and actual breeding sites where immature stages were found were categorised as: drums, tanks, tyres, small (such as vases, [discarded] bottles, cans, and plastic cup) medium and big containers and others. A 'drum' is a plastic or metallic container with a capacity of 150 – 200 litres, typically used in these localities for storage, particularly drinking water. 'Tanks' are underground or elevated containers that usually have a capacity of over 1,000 litres. 'Car or motorcycle tyres' are typically cut in the middle to build water troughs for domestic animals or to prevent plant roots from drying out. 'Vases' are small containers used to maintain natural flowers. Medium and big containers were defined as those containers whose shape and size did not fall within one of the other categories and were categorised according to their volume -between 3 and 20 and more than 20 litres respectively. They are often used on a more temporary basis (i.e. than a drum) to store water inside houses for use in household chores. 'Other' was a category used to incorporate containers of indeterminate shape and size, for example, discarded pieces of household items like toys, furniture, electrical appliances, that were infrequently found as breeding sites.

As the BG-Trap has been reported to be an effective method for collecting domestic populations of *Ae. aegypti* (Kröckel *et al.*, 2006, Maciel-de-Freitas, *et al.*, 2006, Williams *et al.*, 2006), five units were used in an attempt to sample *Ae. aegypti* adults. During each entomological survey (which lasted between 3 to 4 weeks) one BG-Trap was deployed in a randomly selected house within each study arm per day.

3.2.2.2 Interventions

The interventions being tested were insecticide-treated curtains or water storage container covers. Both were made from deltamethrin-coated polyester netting (long

lasting impregnated netting; PermaNet® 2.0, Vestergaard-Frandsen, Lausanne, Switzerland) which has been approved for indoor use by WHOPES (World Health Organization Pesticide Evaluation Scheme). Curtains were hung in all windows regardless of the presence or absence of other window coverings (Figure 3.2).

Figure 3.2. Hanging insecticide-impregnated curtains on the windows of treated households. In houses where the homeowner preferred to maintain their own curtains, rather than replace them, the impregnated ones were hung on top of the existing curtains.

Insecticide-treated covers for domestic water storage containers (referred to here as covers) were provided as ready-to-use products (a pre-packaged standard size with an elasticated border to close around the water container rim). Treated households were provided with enough covers for all big household receptacles that held water for longer than 1 week (Figure 3.3), as it is from these containers that most *Ae. aegypti* were known to emerge in Trujillo (Kroeger *et al.*, 2006, Lenhart *et al.*, 2006).

The ITMs were deployed immediately after the baseline surveys were completed.

Figure 3.3. Members of the field team demonstrating to householders how to fit and remove the water jar covers.

3.2.2.3 Post-intervention entomological surveys

In all localities under study, entomological surveys were carried out in all selected households at 4 weeks (August 2006), 8 months (April 2007), 14 months (October 2007), 20 months (April 2008) and 26 months (October 2008) after the interventions were introduced. April and October were chosen as these are the months when highest rainfall typically occurs, and therefore when numbers of *Ae. aegypti* are at their highest.

3.2.2.4 Serological surveys

Two serological surveys for dengue IgM ELISA screening were undertaken. The first was conducted nine months after the ITMs were delivered into the localities under study (April 2007; n=1039) and the second, 22 months after the intervention began (May 2008; n=783). After explaining the objective of the sampling, a written informed consent was obtained from the head of each household. A blood sample was obtained from one child under 8 years of age in each house on an individual piece of (Whatman) filter paper following finger capillary puncture. All samples were analysed by the dengue IgM capture ELISA in the Centre for Vaccine Development, Institute of Molecular Biosciences, Mahidol University, Bangkok, Thailand.

Members of each household under fifteen years of age were specifically selected for the study. With 1500 households in each treatment group and assuming a seroprevalence rate of 50% (realistic for high transmission areas), the study had ample power to detect differences of 5-8% between treatment groups.

3.2.2.5 Insecticide susceptibility bioassays

To evaluate insecticide treated window curtains, bioassays were conducted according to the WHOPES cone bioassay protocol, as described in Chapter 4.

3.2.2.6 Ethical approval

Ethical permission for the study was granted by the Research Ethics Committee of the Liverpool School of Tropical Medicine (Ref. 06.12) on 02/02/2006, and from the bio-ethical committee of the Jose Witremundo Torrealba Research Institute, Trujillo, Venezuela (18/06/2006).

Community representatives of each participating cluster approved the intervention and written informed consent was obtained from each individual household surveyed.

3.2.2.7 Statistical analyses

Data files were prepared in Microsoft Access, transferred to Excel and analysed using SPSS statistical analysis software version 17.0. The Mann-Whitney U test was used to compare entomological indices between intervention arms at each time point. To summarise the means of four entomological indices after intervention to the end of study, the areas under the curve (AUC) were calculated (Matthews *et al.*, 1990), values transformed into logarithm and one-way ANOVA used to compare each index in the five intervention arms. The effect of ITMs on *Ae. aegypti* populations were determined by decreasing entomological indices throughout the study compared with internal and external control arms. A one-way ANOVA was also used to compare the number of houses through the study. Statistical significance was accepted when p values were less than 0.05.

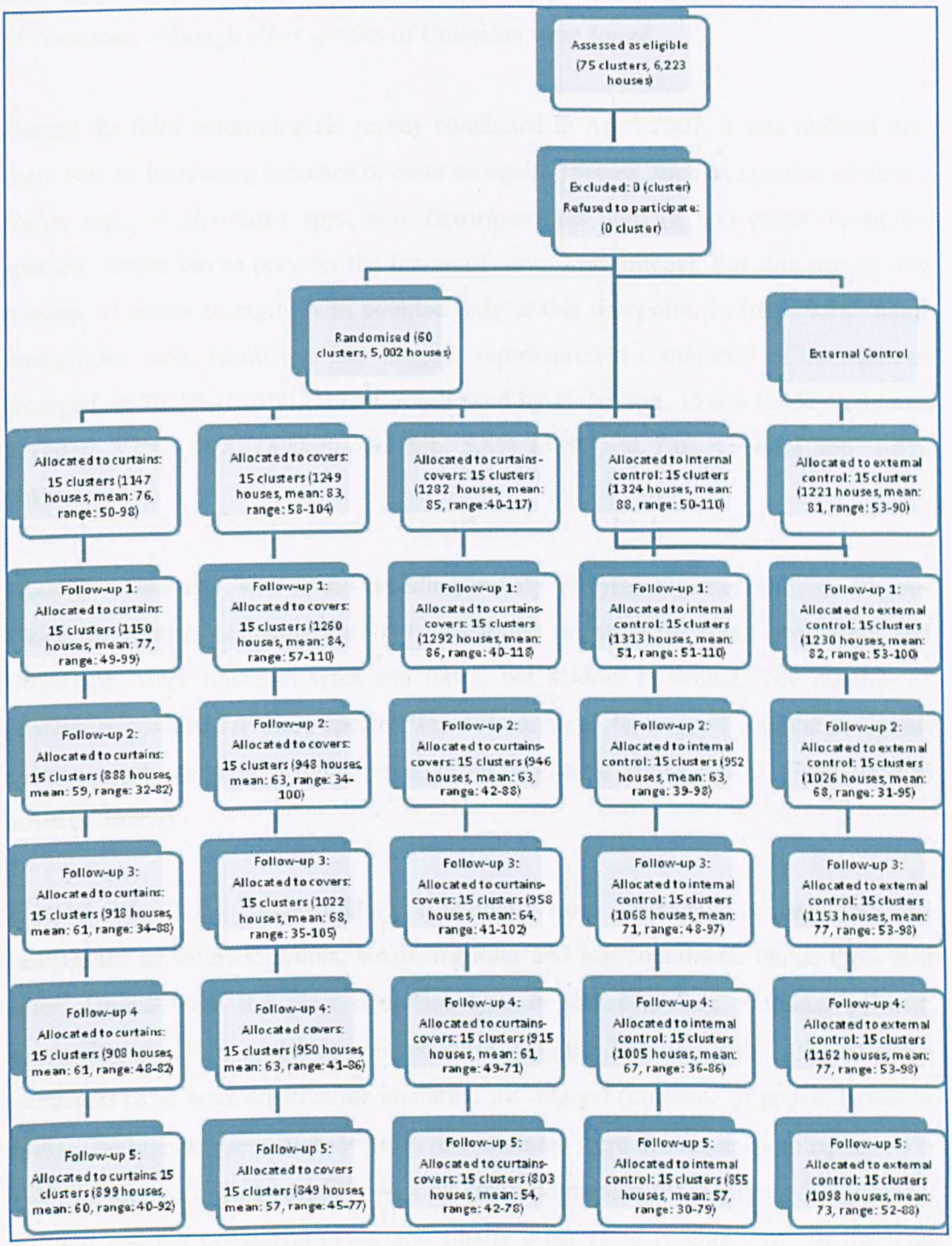
3.3 Results

Of the estimated total of 7,500 houses surveyed within the study site, 6,223 were determined to be eligible for inclusion and recruited to the study. This was equivalent to a mean of 1247 houses per treatment arm, with a minimum of 1147 (in the curtains arm) and maximum of 1324 (in the internal control arm).

Figure 3.4 summarises the number of household units recruited and maintained throughout the study, their distribution per cluster and by intervention arm and the changes that occurred throughout the trial from baseline until the end of the trial. At baseline and throughout the study no statistically significant differences were observed in the number of houses per arm. No clusters were lost during the study, but

at the end of the study, a total of 4940 houses remained from those recruited at baseline. This is equivalent to a loss of 11% from baseline and of 34% from the 7,500 stated in the original protocol (see Section 3.2.2).

Figure 3.4. Trial flowchart showing the total, the mean and the range of the number of houses in all intervention arms and the external control, throughout the entire study period.



3.3.1 Baseline data

Ae. aegypti was the most common immature mosquito species found within all types of container, although other species of Culicidae were found.

During the third entomological survey conducted in April 2007, it was realised that there was an increasing presence of other mosquito species, such as *Limatus durhami, Culex* spp., *Ochlerotatus* spp., and *Toxorhynchites* spp. (a non-vector mosquito species, whose larvae prey on the larvae of other mosquitoes). For this reason, the number of adults emerging was counted only at this timepoint. In total, 9,685 adult mosquitoes were identified. *Ae. aegypti* represented the majority of mosquitoes emerged, at 70.2% (6,799). This was followed by *Culex* spp. 15.0% (1452), *Limatus durhami* 7.7% (746), *Ochlerotatus* spp. 5.6% (539) and *Toxorynchites* spp. 1.5% (149).

Toxorhynchites spp. was found breeding mainly in tyres together with *Aedes* and *Culex*. In contrast, *Limatus durhami* was found primarily in small containers and *Culex* spp. were found in tyres and tanks, but seldom in drums. The number of containers positive for these species was not counted. *Ae. aegypti* was the mosquito species most commonly found breeding in domestic water drums (73% of 896 positive drums).

A total of 12,564 water-holding containers were inspected at baseline, and categorised as follows: drums, small, medium and big containers, tanks, tyres and other. Drums were the most abundant type of water-holding container present, accounting for 52% of all containers found (Figure 3.5). In total, 1,023 of these containers (8%) were positive for immature *Ae. aegypti* (presence of pupae, larvae or both). The highest proportion of positive containers were drums, accounting for 66% (672) of the total. This was followed by small containers (15%), tyres (6%), tanks, medium and big containers (4%), and finally other (1%) (Figure 3.6). Of the total

positive containers, 53% were positive for the presence of pupae, the highest proportion again being drums (62%), followed by small, tyres, tanks and medium and big containers, and the lowest, other (1%) (Table 3.1, Figure 3.7).

Figure 3.5. Relative proportions of water - holding containers in the entire study site at Trujillo, recorded at baseline, in July 2006.

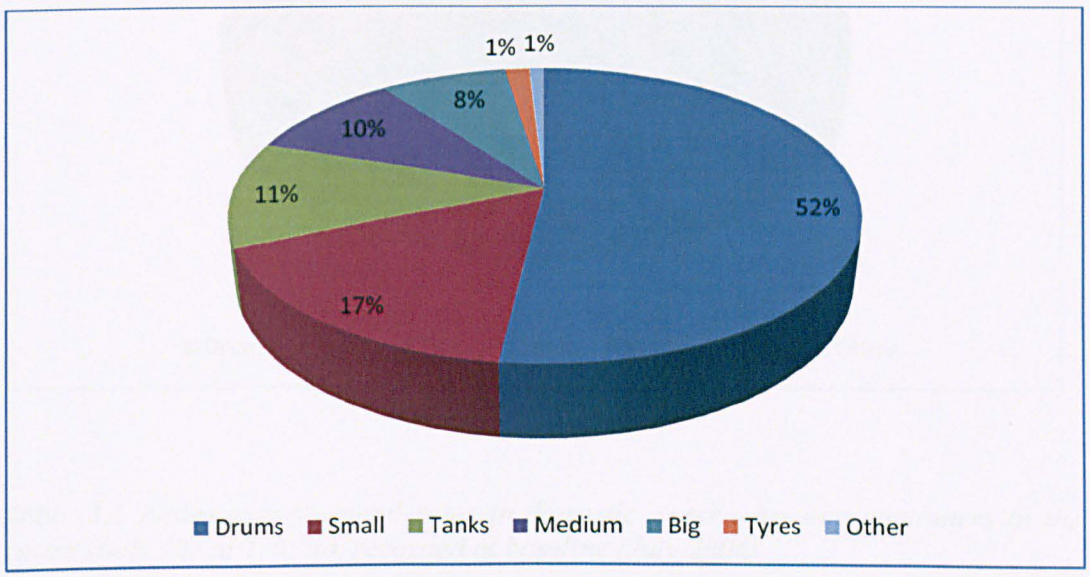


Figure 3.6. Relative proportions of water-holding containers with immature stages (larvae, pupae or both) of Aedes aegypti, *recorded in the entire study site at Trujillo, at baseline, in July 2006.*

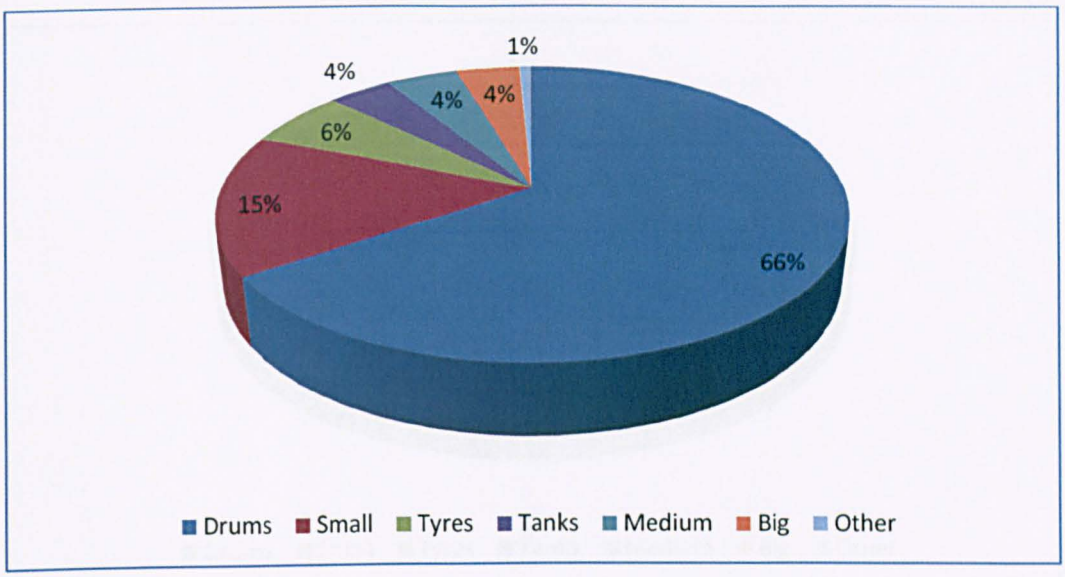
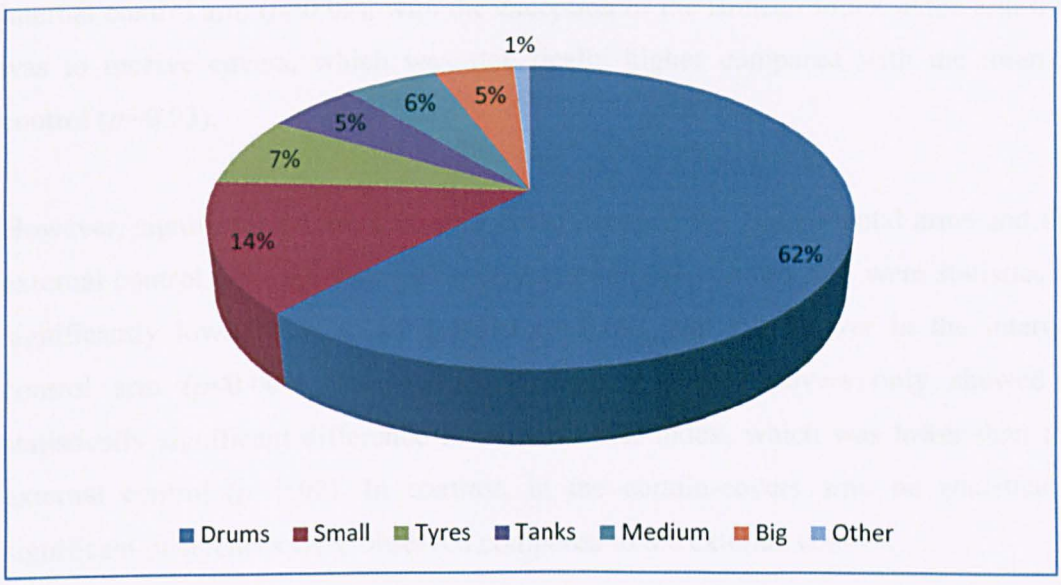


Table 3.1 Aedes aegypti *pupal rates in domestic water - holding containers in the entire study site at Trujillo, recorded at baseline (July 2006).*

Categories Containers	Total Containers (%/n)	Positive containers (%/n)	Positive for pupae (%/n)
Drums	52 (6521)	66 (672)	62 (338)
Small	17 (2127)	15 (158)	14 (77)
Tanks	12 (1447)	4 (40)	6 (30)
Medium	10 (1207)	4 (44)	6 (30)
Big	8 (962)	4 (40)	5 (26)
Tyres	2 (193)	6 (62)	7 (39)
Other	1 (107)	1 (7)	1 (5)

Figure 3.7. Relative proportions of water-holding containers positive for the presence of pupae of Ae. aegypti, *recorded in the entire study site at Trujillo, at baseline, in July 2006.*



3.3.1.1 Adult sampling

Data on adult mosquito abundance were not obtained because BG-traps did not function efficiently during the early stages of the trial. The primary reason was the persistent problem of night-time power outages, when traps stopped running. Catches were low in those few cases where traps were apparently functioning and it was suspected that, sometimes at least, householders may have disconnected the traps either because of the noise they made or worries about their electrical consumption.

3.3.1.2 Entomological indices

Table 3.2 shows the values of the *Stegomyia* and pupal indices as measured in the study site at baseline and classified according to the groups to which the clusters were

allocated. At baseline, there were no statistically significant differences in any of the entomological indices between the three arms that later received ITMs ($p>0.05$). Similarly, there were no such differences in the indices between these groups and the internal control arm ($p>0.05$), with the exception of the Breteau Index in the arm that was to receive covers, which was statistically higher compared with the internal control ($p=0.03$).

However, significant differences were noted between the experimental arms and the external control arm. All four indices recorded in the curtains arm were statistically significantly lower with a low p-value ($p<0.05$), and even lower in the internal control arm ($p<0.001$). Meanwhile, the arm receiving covers only showed a statistically significant difference in the container index, which was lower than the external control ($p=0.02$). In contrast, in the curtain-covers arm, no statistically significant differences were observed compared to the external control.

The highest entomological indices were recorded in the sectors selected to receive the combination of ITMs (curtains plus covers). The lowest values were recorded in the sectors selected as internal control (Table 3.2).

Table 3.2. Mean Breteau, Pupae per Person (PPI), House and Container Indices recorded at baseline in the study arms and external control arm, in Trujillo, Venezuela.

BASELINE				
	Breteau	**PPI**	**House**	**Container**
Curtains	14.57*	0.38*	6.61*	8.68*
	(174/1147)	(2084/5020)	(80/1147)	(174/2030)
Covers	15.51	0.38	8.46	6.37
	(200/1249)	(2181/5557)	(107/1249)	(200/3202)
Curt-Cov	25.18	0.88	11.42	9.97
	(326/1282)	(5189/5671)	(152/1282)	(326/2805)
Int-Ctrl	6.31**	0.22**	5.08**	4.28**
	(87/1324)	(1426/5706)	(69/1324)	(87/2230)
Ext-Ctrl	19.02	0.61	13.22	10.49
	(236/1221)	(3171/5261)	(162/1221)	(236/2297)

* $p<0.05$ ** $p<0.001$

3.3.2 Results of the intervention

Following the introduction of the insecticide-treated materials in July 2006, follow up surveys were made at 1 month and at approximately 6-month intervals for 26 months (1 month-August 2006, 8 months-April 2007, 14 months-October 2007, 20 months-April-2008, and 26 months-October 2008). The findings are summarised in Figures 3.8a and 3.8b.

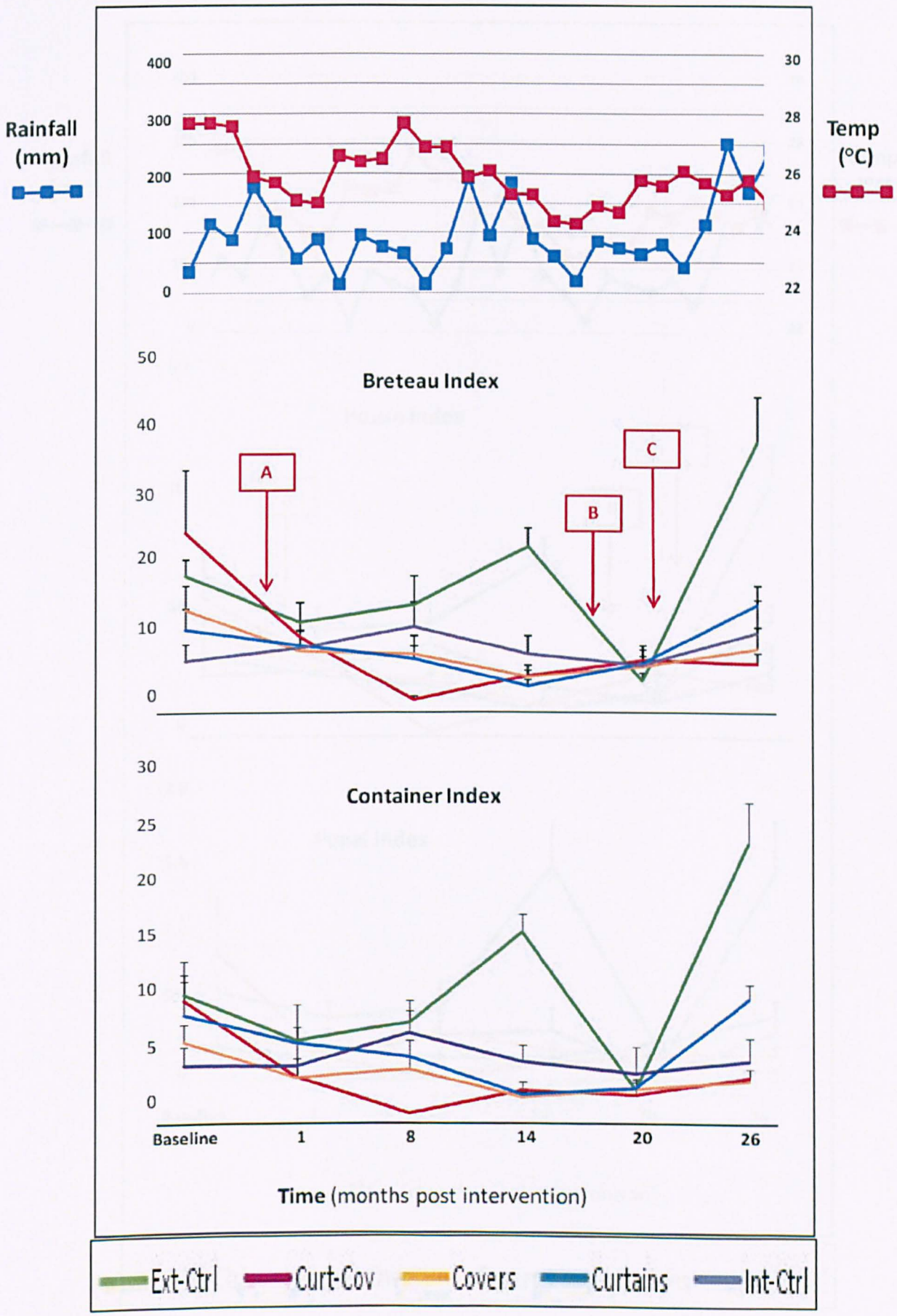
[*Following pages*]

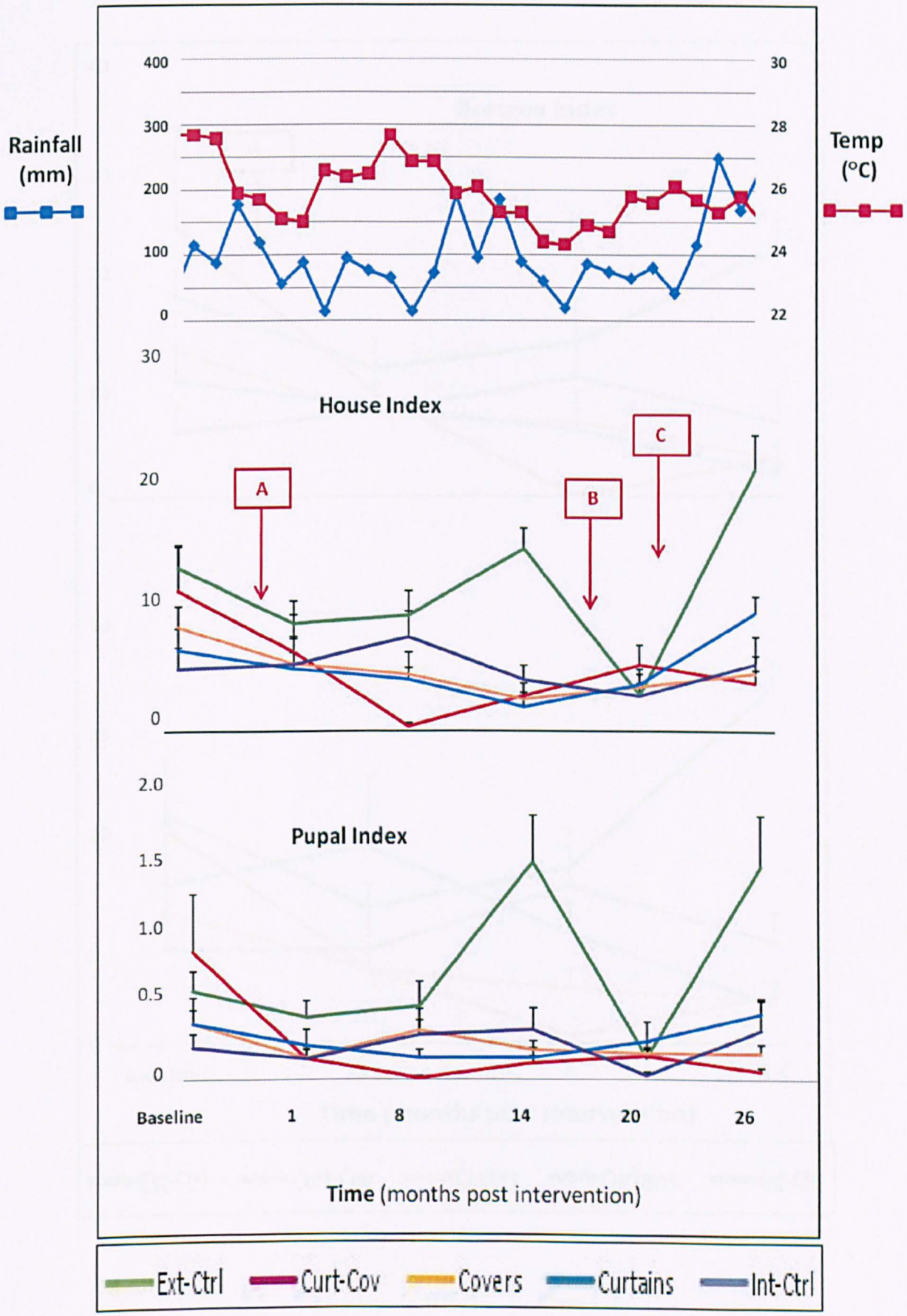
Figure 3.8. Summary of (a) Breteau and pupal entomological indices and (b) house and container entomological indices measured during the intervention in all arms, including the external control, with rainfall and temperature data (from Ministerio

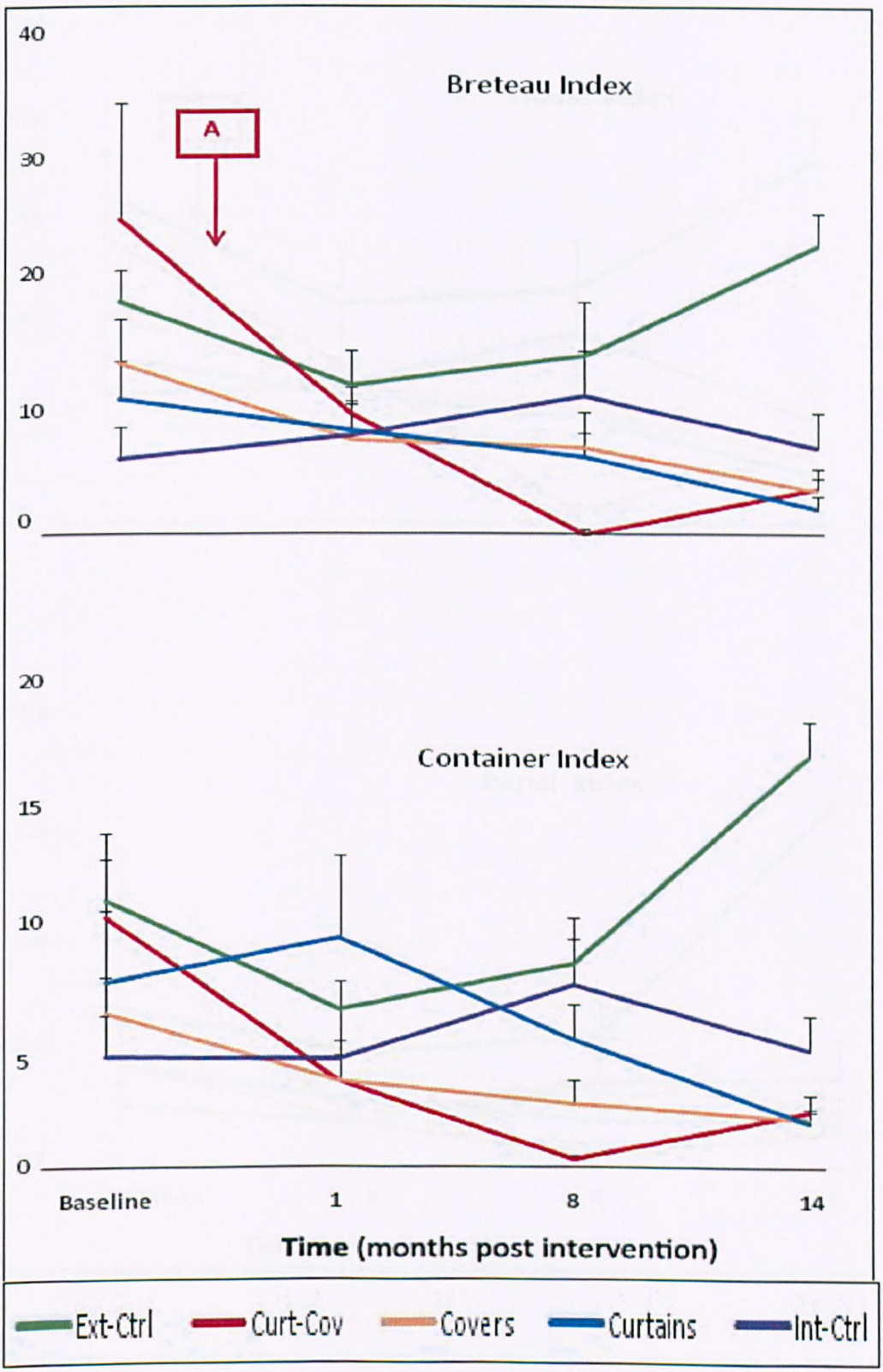
del Poder Popular para el Ambiente, Trujillo)(mean and standard errors for each index). The baseline survey was undertaken in July 2006 and the final survey 26 months later, in October 2008.

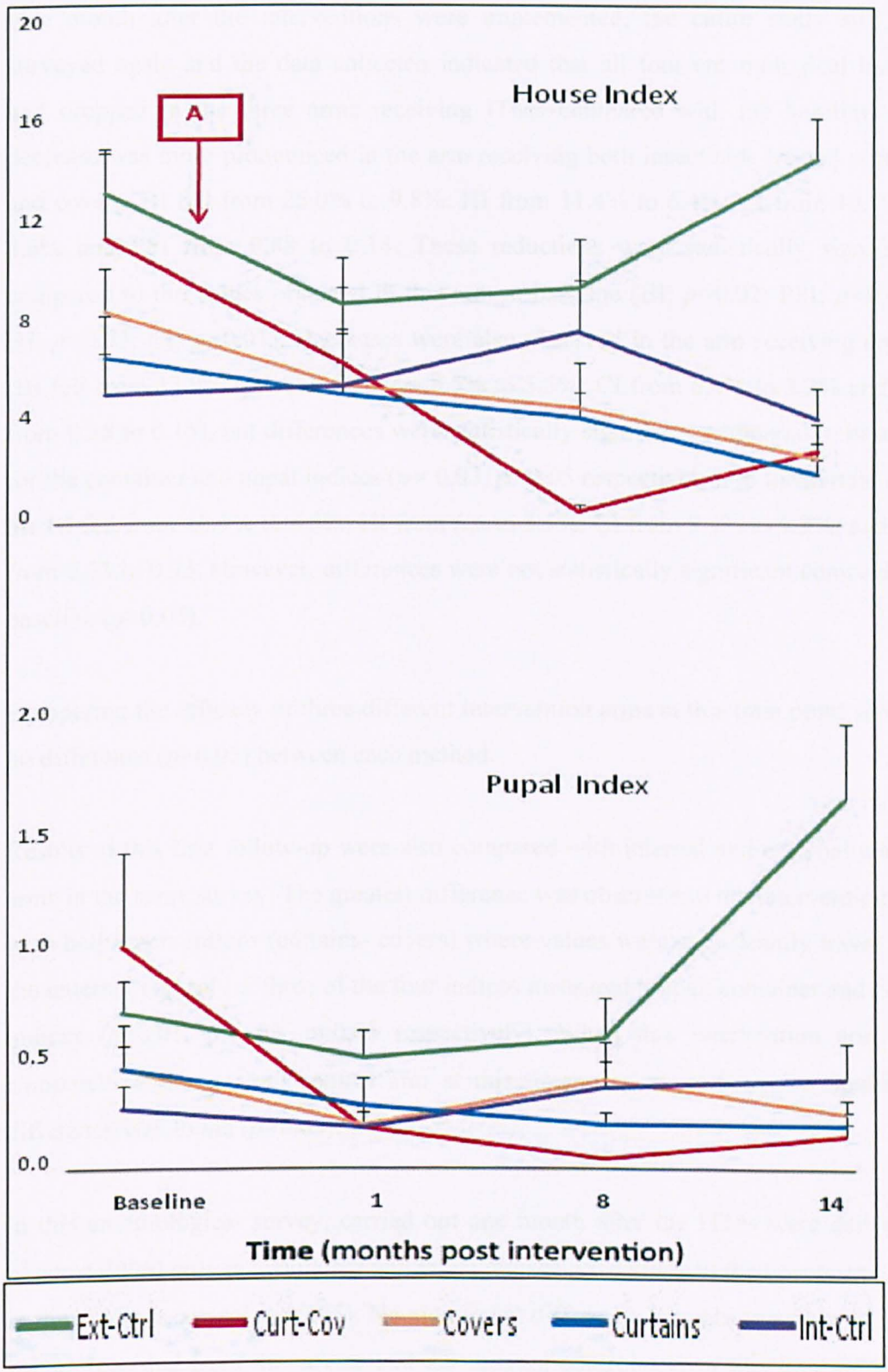
Labelled dates indicate (A) when the intervention began (July 2006); (B) when the local health authority intensified widespread dengue vector control measures in response to a dengue outbreak (the campaign to fight the outbreak started in June 2007, but due to the continuing increase in dengue cases, it was intensified from October 2007);(C) when ITMs in the trial were replaced (April 2008).

Figure 3.9. Summary of the first 14 months of the trial (the period prior to the dengue outbreak), showing (a) Breteau and pupal entomological indices and (b) house and container entomological indices in all arms, including the external control (mean and standard errors for each index), measured from the baseline in July 2006 until October 2007,when the coverage was more than 50%. Label 'A' indicates when the intervention began.


Rainfall (mm)
Temp (°C)
400
300
200
100
0
30
28
26
24
22
Breteau Index
50
40
30
20
10
0
A
B
C
Container Index
30
25
20
15
10
5
0
Baseline
1
8
14
20
26
Time (months post intervention)
Ext-Ctrl
Curt-Cov
Covers
Curtains
Int-Ctrl


Rainfall (mm)
Temp (°C)
400
300
200
100
0
30
28
26
24
22
House Index
20
10
0
A
B
C
Pupal Index
2.0
1.5
1.0
0.5
0
Baseline
1
8
14
20
26
Time (months post intervention)
Ext-Ctrl
Curt-Cov
Covers
Curtains
Int-Ctrl


40
30
20
10
0
Breteau Index
A
20
15
10
5
0
Container Index
Baseline
1
8
14
Time (months post intervention)
Ext-Ctrl
Curt-Cov
Covers
Curtains
Int-Ctrl


House Index
A
20
16
12
8
4
0
Pupal Index
2.0
1.5
1.0
0.5
0.0
Baseline
1
8
14
Time (months post intervention)
Ext-Ctrl
Curt-Cov
Covers
Curtains
Int-Ctrl

One month after the interventions were implemented, the entire study site was surveyed again and the data collected indicated that all four entomological indices had dropped in the three arms receiving ITMs compared with the baseline. The decrease was more pronounced in the arm receiving both insecticide-treated curtains and covers: BI fell from 25.0% to 9.8%; HI from 11.4% to 6.4%; CI from 10.7% to 3.6% and PPI from 0.88 to 0.14. These reductions were statistically significant compared to the values obtained in this arm at baseline (BI: p=0.02; PPI: p=0.005; HI: p=0.03; CI: p=0.01). Decreases were also observed in the arm receiving covers (BI fell from 13.9% to 7.8%; HI from 8.5% to 5.5%; CI from 6.4% to 3.2% and PPI from 0.38 to 0.15), but differences were statistically significant compared to baseline for the container and pupal indices (p= 0.03, p=0.05 respectively). In the curtain arm, the BI fell from 10.9% to 8.5%; HI from 6.6 to 5.1%; CI from 8.4% to 6.3%; and PPI from 0.38 to 0.23. However, differences were not statistically significant compared to baseline (p>0.05).

Comparing the efficacy of three different intervention arms at this time point showed no difference (p>0.05) between each method.

Results at this first follow-up were also compared with internal and external control arms in the same survey. The greatest difference was observed in the intervention arm with both interventions (curtains- covers) where values were significantly lower than the external control for three of the four indices measured: pupal, container and house indices (p=0.01, p=0.02, p=0.05 respectively). When this intervention arm was compared to the internal control arm at this timepoint, no statistically significant difference was found (p>0.05).

In this entomological survey, carried out one month after the ITMs were delivered, (August 2006) only the container and pupal indices were lower in the covers arm than in the external control (p=0.05). No significant difference was observed between the values recorded from the covers and the internal control arm at this time (p>0.05).

Furthermore, in the curtains arm at this timepoint, only the container index was lower than the internal control (p=0.04). No significant difference was observed between the values recorded in the curtains arm and the external control arm in this survey (p>0.05).

The values of four entomological indices recorded one month post intervention in the internal control compared to the values recorded at baseline in this arm, showed no significant difference. In contrast, comparing the values recorded in the external control arm versus the values recorded in this arm at baseline, container and Breteau indices were lower at this time than at baseline (p=0.02 and p=0.03 respectively). The values of the internal control arm, although lower than the external control at this time, were statistically significant in both pupal and container indices (p=0.02 and p=0.04 respectively).

At the next two follow-up surveys, at 8 and 14 months (April and October 2007), the pattern in all three intervention arms was similar, with indices continuing to fall. Within each treatment arm, this decrease was far more pronounced, particularly in the combined curtains and covers arm.

At 8 months after the ITMs delivery, all four entomological indices recorded in curtains-covers were significantly lower than at baseline (p<0.001). Likewise, the values recorded in the covers in this survey were lower than the values recorded in this arm at baseline (house and container: p=0.01 and Breteau and pupal indices: p=0.03). Even though, in the curtains arm the recorded values were lower than at baseline, the difference did not reach statistical significance.

At this time (8 months post intervention, April 2007), all four entomological indices recorded in curtains-covers were lower than both the internal and external controls (p<0.001). However, in this survey, in the covers arm, only the values of container and house indices were lower than the external control (p=0.001 and p= 0.003

respectively) and internal control (p=0.03 and p=0.05 respectively). In contrast, the values of all four entomological indices recorded in curtains were not different from internal and external control arms at this time (p>0.05).

To explore the impact of the three intervention measures on entomological indices, a Kruskal-Wallis test was conducted, which revealed statistically significant differences in the values of almost all the entomological indices (p<0.05). For this reason, it was further explored using the Mann-Whitney test. The results of this test revealed that the values recorded in the arm receiving both curtains and covers were significantly lower than the values recorded in the separate covers and curtains arms (p<0.05), compared at this time. On the other hand, when comparing curtains and covers no significant difference was observed between the indices values recorded from both intervention arms (p>0.05).

At fourteen months post intervention (October 2007), as the rainfall increased with the arrival of the wet season, *Ae. aegypti* populations increased in the external control, and this was reflected in the significant increase in all four indices recorded at this time (p<0.05), compared to the values recorded in this arm in the entomological survey conducted 6 months before, in April 2007. Interestingly, in the sectors receiving ITMs, none of the values of any index were seen to increase at this time compared to the values recorded in April 2007, i.e. six months before. Even in the internal control arm, no significant increase was recorded in the entomological indices (p>0.05).

At this time, (October, 2007) the values recorded in the curtains-covers arm were lower than those recorded in this arm at baseline in July 2006 (p=0.02), with the exception of the house index (p=0.06). A similar pattern was observed at this timepoint in the cover arm values compared to baseline, with 3 entomological indices significantly lower (Breteau: p=0.007; container: p=0.006 and house index: p= 0.009) yet the pupal index was not significantly different (p=0.06). Also, at this time

the values of three entomological indices recorded in the curtains arm were significantly lower than at baseline (Breteau: p=0.01; container: p=0.02, and house index: p=0.03).

At the same time (October 2007) the values of all four entomological indices recorded in the three arms receiving ITMs (curtains-covers, covers and curtains) were statistically significantly lower than the values recorded at the same time in the external control arm (p<0.001 in each index). In contrast, when comparing the values from these three intervention arms to the internal control at this timepoint no difference was observed (p>0.05). Again, the impact of the three intervention arms on entomological indices was compared, but no significant difference was found (p>0.05).

However, by this time, it was apparent that the covers had deteriorated to extent that they were no longer providing any form of protection (Figure 3.9). For this reason, in April 2008, the damaged covers were replaced, immediately before the entomological survey at 20 months post intervention was carried out.

Following the dengue outbreak, which started in June 2007, the municipal authorities, through the vector control department of Ministerio del Poder Popular para la Salud in Trujillo State, responded by increasing *Ae. aegypti* control measures (for details, see Section 3.3.3). Thus, the results obtained (in April 2008), in the survey at 20 months after the interventions were deployed, revealed a statistically significant decrease in indices in the external control arm, compared at baseline (p<0.001). Although all areas in this study were simultaneously treated, the impact of these actions on the treatment groups was less obvious.

Figure 3.10. Physical deterioration in covers in the study site, after fourteen months of use (photographs from January 2008).

At this time (April 2008), the values of all four entomological indices were significantly lower in the arm receiving covers than the values recorded in this arm at baseline (Breteau: p=0.02; house: p=0.03; container: p=0.01 and pupal index: p= 0.02). In contrast, while the values recorded in curtains-covers at this time compared to baseline were lower, this difference was not statistically significant (p>0.05), with the exception of the container index, which was lower than at baseline (p=0.04). Likewise, although lower, none of the values recorded in the curtains arm at this timepoint reached statistical significance compared to baseline (p>0.05).

When comparing the values of the four entomological indices recorded at this time (April 2008) in each arm receiving ITMs, they did not reveal any significant differences compared to the internal and external control arms (p>0.05). Surprisingly, only the pupal index recorded in the arm receiving the combination of ITMs,

curtains-covers, was higher than the value recorded at this time in the internal control arm (p=0.01). When contrasting the impact of ITMs on entomological indices the results revealed no significant difference (Breteau: p=0.93; house: p=0.74; container: p=0.98, and pupal index: p=0.96).

The values of the *Stegomyia* and pupal indices measured at the end of study, (26 months post intervention, in October 2008) are showed in the Table 3.3. With the covers replenished and 12 months after the vector control activities of the local authorities, the means of all four indices were statistically significantly lower in all of the three arms with interventions (curtains, covers and curtains-covers) compared to the external control (p<0.001).

The values of all four indices recorded in covers at the end of study were lower than the values recorded in this arm at baseline (house: p=0.04; container: p=0.03, and pupal index, Breteau: p=0.05). In contrast, although values were recorded to be lower in the arm which received curtains-covers than at baseline, only the pupal index reached statistical significance (p=0.003). Interestingly, the opposite was observed in the curtains arm, which exhibited higher values than at baseline. However, only the container index value reached statistical significance (p=0.01).

By the end of the study, whilst the values recorded in curtains-covers and covers were not significantly different (p>0.05) compared to the internal control arm, the curtains arm showed higher values than internal control arm, at this timepoint (house: p=0.03; container: p=0.01; pupal index: p=0.03, and Breteau: p=0.05). On the other hand, the values recorded in this survey in October 2008 in the arms which received curtains-covers and covers, were lower than curtains (p<0.001). No statistically significant difference was found between the curtains-covers arm and the covers arm at this timepoint (p<0.05).

Table 3.3. Mean Breteau, Pupae per person (PPI), House and Container Indices recorded in all arms under intervention in Trujillo, Venezuela at the end of the study, in October 2008.

26 MONTHS (OCT-08)				
	Breteau	**PPI**	**House**	**Container**
Curtains	14.50**	0.45**	9.63**	10.02**
	(129/899)	(1799/4095)	(86/899)	(129/1458)
Covers	8.02**	0.18**	4.70**	2.94**
	(70/849)	(729/3796)	(41/849)	(70/1882)
Curt-Cov	5.88**	0.06**	3.97**	3.27**
	(52/803)	(224/3669)	(35/803)	(52/1666)
Int-Ctrl	10.28	0.34	5.47	4.69
	(106/855)	(1893/3777)	(56/855)	(106/1436)
Ext-Ctrl	36.33	1.46	21.16	23.55
	(391/1098)	(6595/4869)	(229/1098)	(391/1743)

** p <0.001

The differences in means of entomological indices recorded in the internal control were not substantially different throughout the study compared to the values recorded at baseline. Notably, the values of entomological indices recorded in the external control arm post intervention were more fluctuating. One month post intervention (August 2006), in the external control were recorded values of Breteau and container indices lower than at baseline (p= 0.03 and p=0.02 respectively). At eight months (April 2007), only the Breteau Index was lower than at baseline (p=0.04). In the survey conducted at fourteen months after the study began, in the external control were recorded values higher than at baseline, although, only the p value of the container and the pupal indices reached statistical significance (0.01 and 0.02 respectively). However, by the end of the study, the *Ae. aegypti* population had recovered and all four indices registered values significantly higher than at baseline (p<0.05).

Calculating the AUC to summarise the means of the entomological indices throughout the study in the entire site, the comparison revealed that curtains-covers had the greatest impact on these indices (Table 3.4). The mean of the Breteau index was significantly lower than the mean recorded in the curtains, the internal and external control arms ($p<0.001$), but not when compared to covers alone ($p=0.06$). Likewise, the container index mean recorded in curtain-covers was significantly lower than the mean obtained in the curtains, the internal and external control arms ($p<0.001$), as well as the cover arm ($p=0.04$). The mean of the PPI obtained from the curtain-cover arm was significantly lower than the other arms (compared to covers: $p=0.01$; curtains: $p=0.01$; internal control: $p=0.04$), especially when compared with the external control: $p<0.0001$). An exception was the house index mean obtained from curtains-covers, which differed significantly only when compared to the external control ($p<0.0001$).

Otherwise, the Breteau and container mean AUC obtained from covers were significantly lower than the curtains, the internal and external control arms ($p<0.05$), and with regard to house and pupal indices, the values of AUC were lower than the external control ($p=0.0004$ and $p=0.003$). In contrast, the values obtained from the curtains arm, were only lower than external control (Breteau: $p=0.0008$; house: $p=0.006$ and container and pupal indices: $p=0.002$).

The values of AUC obtained from the internal control regarding all four entomological indices were lower than the external control (Breteau: $p=0.0003$; house and container indices: $p=0.001$ and pupal index: $p=0.009$).

Table 3.4. Mean of Area under the Curve of Breteau, Pupae per person (PPI), House and Container Indices recorded in all study arms in Trujillo, Venezuela.

	Breteau	Container	House	PPI
Curt-Cov	4.8	3.39	4.02	0.03
Covers	5.33	3.97	4.38	1.31
Curtains	5.98	4.6	4.43	1.28
Int-Ctrl	5.89	4.55	4.53	1.05
Ext-Ctrl	6.92	5.5	5.47	2.76

Thus, overall the greatest impact appeared to result from covers, either alone or in combination with curtains. From the second entomological survey post intervention, which was carried out in April 2007, all four entomological indices obtained from covers showed a sustained decrease up until the end of the study with statistically significant differences compared to the values obtained at baseline. In the curtains-covers arm, here, the pupal index decreased steadily until the end of the study with statistically significant differences compared to baseline. The container index showed the same trend, but only up to 20 months post intervention. Breteau and house indices were statistically significantly lower until 14 months compared to the baseline, and although at the end of the trial, they were again lower than the values recorded at baseline, these differences were not statistically significant.

In each survey, the use of curtains alone did not indicate any effect on the entomological indices measured during the study, when considered alongside the values recorded at baseline. Only the Breteau, container and house indices were significantly lower at 14 months, compared at baseline ($p<0.05$). In addition, container index, at the end of study, was statistically significantly higher than at baseline ($p=0.012$).

The differences in the means of entomological indices recorded in the internal control were not statistically significant throughout the study when viewed against the values recorded at baseline. In the external control arm, a statistically significant decrease was observed, in contrast to the baseline, in the results obtained from the survey conducted at 20 months, but by the end of study, the *Ae. aegypti* population had recovered and all four indices registered values significantly higher than at baseline.

3.3.2.1 Coverage

Table 3.5 shows the coverage of ITMs throughout the study in the three arms receiving curtains, covers and the combination of curtains-covers. In curtains, the coverage was defined as the proportion of houses with 1 or more curtains, and in covers as the proportion of drums with covers in use. In the arm receiving both ITMs together (curtains-covers) the coverage was defined as the proportion of houses with 1 or more curtains or drums with covers, while the coverage in curtains-covers was defined as the proportion of house with 1 or more curtains and 1 or more drums with covers.

During the three follow-up entomological surveys conducted at one month (August 2006), eight months (April 2007) and fourteen months post intervention (October 2007), the coverage recorded was similar between curtains and covers intervention arms (80% - 75%; 79% - 76%; 46% - 57% respectively). At the end of the study the coverage was the same in covers and curtains intervention arms (42%). Likewise in the arm receiving curtains-covers together, until 14 months more the 50% of the houses had one or another tool in use, but in the next surveys the coverage decreased significantly and at the end, only 20% of the houses were using curtains and 40% of drums was covered. People, who had removed the curtains for washing, were encouraged to hang up again, so some houses recorded without curtains in April 2008, were recorded with curtains in using in the last survey in October 2008.

Table 3.5. Percentage of houses using ITMs through the study per intervention arm at each timepoint. The row A shows the proportion of houses in the curtains arms using curtains throughout the study. The row B shows the proportion of drums using covers in the covers arm. The row C shows the proportion of houses in the curtains-cover arms using curtains throughout the study. The row D shows the proportion of drums using covers in the curtains-covers arm throughout the study.

		1 month %	8 months %	14 months %	20 months %	26months %
A	Curtains	80 (924/1150)	79 (698/888)	46 (422/918)	21 (193/908)	42 (376/899)
B	Covers	75 (1509/2014)	76 (1014/1337)	57 (733/1284)	31 (436/1404)	42 (499/1199)
				Curtains - Covers		
C	Curtains	76 (981/1292)	84 (797/946)	50 (481/958)	12 (108/915)	20 (161/803)
D	Covers	63 (1327/2091)	75 (1124/1501)	59 (680/1147)	10 (132/1299)	40 (439/1908)

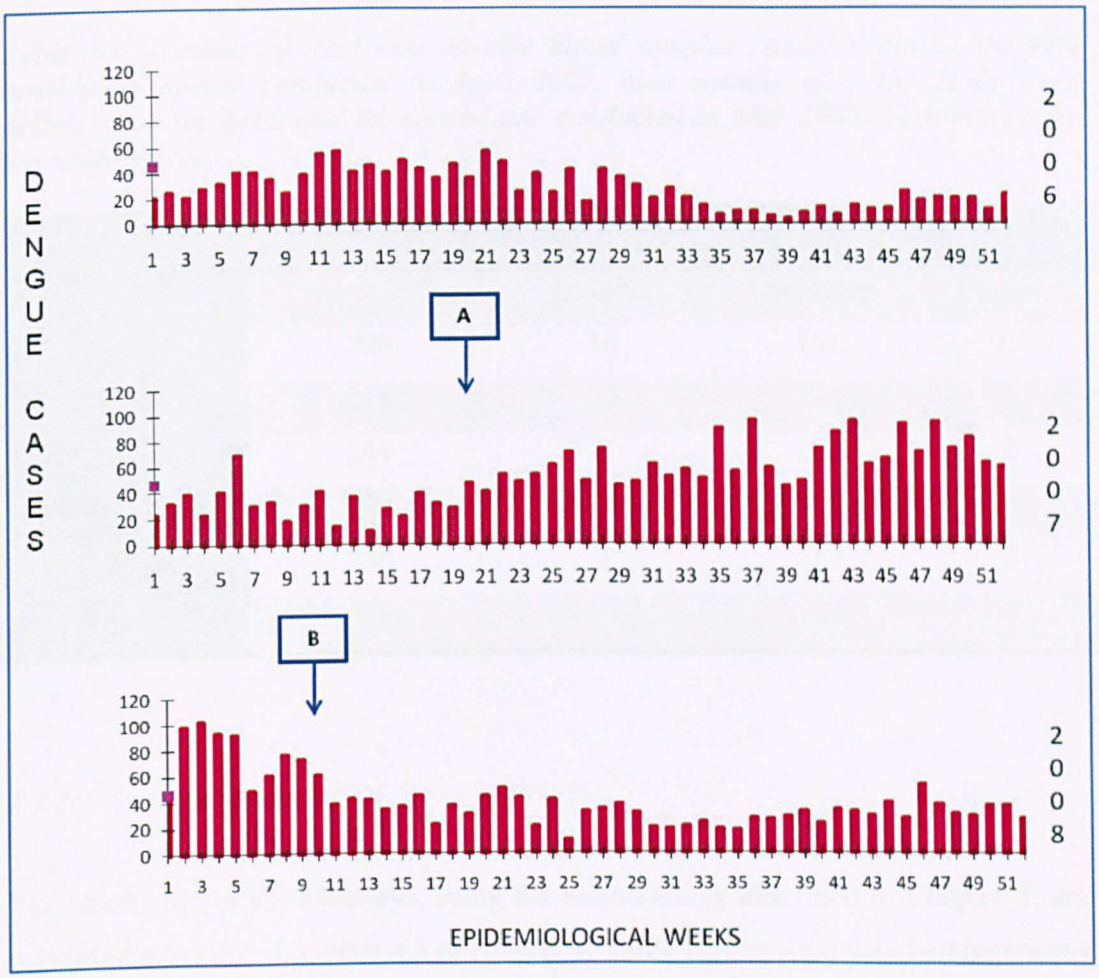
3.3.3 Dengue in the study site

During the first year of the study, the incidence of dengue in Trujillo State appeared to be stable throughout the year (Figure 3.10), with cases increasing only in March and June, as expected. This situation changed completely after May 2007 [epidemiological week 20, in Figure 3.10 (A)], when increases in reported cases were sustained during the rest of the year, including the first ten epidemiological weeks in 2008. This situation in Trujillo State reflected what was occurring throughout Venezuela, which recorded an increase in dengue cases of over 40% (Dirección Regional de Epidemiología y Estadística Vital, Trujillo-Venezuela, 2008).

In response to this, an intensive national campaign was deployed by the Ministerio del Poder Popular para la Salud, to face the dengue outbreak. In Trujillo State, the municipal authorities, through the vector control department of Ministerio del Poder

Popular para la Salud in Trujillo State, responded by a campaign which involved mainly an increase in of the use of ultra-low-volume adulticides malathion (94%) and fenitrotion (50%) applied by vehicle-mounted apparatus. Given that the results were not as successful as expected by the authorities and the number of dengue cases continued to increase, from October 2007, control activities were intensified, including educational campaigns, breeding sites reduction (cleaning campaigns), and the treatment of domestic water storage containers and public sites, such as cemeteries with temephos [granule (1%) and emulsifiable concentrations (4%) respectively). Thus, the results obtained (in April 2008), in the survey at 20 months after the interventions were deployed, revealed a statistically significant decrease in indices in the external control arm, compared at baseline ($p<0.001$). Although all areas in this study were simultaneously treated, the impact of these actions on the treatment groups was less obvious.

Figure 3.11. Dengue cases in Trujillo State between 2006 -2009 distributed by epidemiological weeks. (A) when dengue outbreak began and (B) when this finished (Source: Dirección Regional de Epidemiología y Estadística Vital, Trujillo-Venezuela, 2009).



3.3.4 Serological results

Table 3.6 shows the results of serological surveys. A total of 85 filter paper blood samples were positive for IgM dengue in the first serological survey. Overall, the prevalence of dengue IgM was higher in the sectors receiving jar covers (31%; 26/218), followed by curtains-covers (25%; 21/244), curtains (19%; 16/200), internal control (18%; 15/147) and the lowest value was recorded in the external control area

(8%; 7/230). In the second serological survey, only 6 blood samples were found positive for IgM dengue, 3 in external control area, two samples in curtains-covers, and one in covers sectors.

Table 3.6. Number of total and positive blood samples collected during the first serological survey conducted in April 2007, nine months after the ITMs were delivered on the field, and the second one conducted in May 2008, 22 months after the study began.

	1st serosurvey		2nd serosurvey	
	Samples	Positive	Samples	Positive
Curtains	200	16	161	0
Covers	218	26	174	1
Curtain-Covers	244	21	183	2
Internal Control	147	15	108	0
External Control	230	7	157	3
Total	1039	85	783	6

3.3.5 Curtain bioassays

The results of curtain bioassays, using the methodology described in Chapter 4, are presented in section 4.3, table 4.5 of chapter 4. Those results were very heterogeneous at baseline and throughout the study. The mortality rates of the susceptible *Ae. aegypti* mosquitoes exposed to new curtains at baseline varied between 45 and 100%. Six months after the curtains were hung, the results of mortality rates ranged between 56 -100%. One year post-intervention it ranged between 60 – 97% and two years after the study began the results established a mortality rate of between 48 and 97 %. No assays were conducted in covers.

3.4 Discussion

The results from this cluster randomized controlled trial of insecticide treated window curtains and water container covers for the control of dengue vector *Ae. aegypti*, demonstrated an immediate reduction in entomological indices in the communities under study, following deployment of the intervention.

Thus one month after the ITMs were delivered, all four entomological indices recorded in sectors receiving the combination of curtains-covers (the most complete protection possible) showed a statistically significant reduction compared with baseline values, with the external control, but not with the internal control, at this time point. In fact, statistically significant differences were only observed between this intervention arm and the internal control at the 8 month timepoint. However, when compared with the external control, the values of all entomological indices were statistically significantly lower at all time points, except at 20 months, when only the container index value was lower in the intervention. At the end of the study, even though the values of all four entomological indices recorded in this arm were more than three times lower than at baseline, only the PPI was statistically significantly different.

Looking at water container covers alone, at one month post intervention, covers had a major impact upon pupal and container indices only, reducing both significantly compared with baseline values. However, from this timepoint onwards, a statistically significant sustained decrease in all *Ae. aegypti* entomological indices, in comparison with baseline, was seen; the only difference with the internal control was in HI and CI at 8 months. However, when compared to the external control, a significant difference was observed between some entomological indices. Furthermore, at 14 months post intervention and at the end of the study, all entomological indices were significantly lower in covers than at the external control.

Curtains appeared to have the least impact upon *Ae. aegypti* entomological indices, with significant differences to baseline levels occurring only occasionally (HI, CI and BI at 14 months only). Importantly, at the end of study, only the container index was significantly lower than at baseline. Comparing the values with the internal control at the same timepoint, indices were significantly higher than the internal control arm at the final timepoint only. However, all four entomological indices were significantly lower at two time points, i. e. at 8 and 14 months after the study began.

Notably, at all timepoints throughout the study, the variations in entomological indices in the internal control arm without intervention (the internal control) were not statistically significant, with one exception (pupal index at 20 months). Not surprisingly however, significant fluctuations in the external control values (which measure the seasonal variation in local *Ae. aegypti*) populations varied considerably. At the end of study, all four entomological indices recorded in the external control arm were significantly higher compared with those recorded at baseline in this arm.

During the undertaking of this study, a serious epidemiological dengue situation started to be recorded in several states of the country. From October 2007, this increase in reported cases was sustained up until the beginning of 2008 (*Dirección Regional de Epidemiología y Estadística Vital*, Trujillo-Venezuela, 2009). In response to this situation, the local Health Authority intensified its dengue vector control measures around Trujillo State. The intervention activities planned included the training of a youth group called "*Defensores de la Salud*". The small pilot project was conducted in Monay parish which was the external control arm selected in this study. These activities were similar to those applied by field technicians when a case of haemorrhagic dengue is reported. The activities included mainly the application of temephos and cleaning campaigns. Also, the programme included training the community to prevent dengue by the reduction of breeding sites in and around houses. The impact of this small but intense programme was effective in the short term. However, it was not sustainable over time and mosquito populations recovered

rapidly, rising to levels comparable with those when the government intervention activities started. This is the most plausible explanation for the statistically significant reduction of entomological indices recorded in every arm, including the external control arm, during the survey conducted at 20 months post intervention in April 2008. Interestingly, the recovery of the vector population in the external control group by the time of the final survey at 26 months, indicates the failure of these interventions to achieve sustainable reductions in vector populations, in contrast to the ongoing effect of the intervention groups (and allied overspill in the internal control group) which maintained significantly lower levels at the same timepoint.

This indicates strongly, that the effectiveness of control programmes cannot depend solely on 'traditional' intensive measures alone. These have to be planned in order to obtain a significant and sustained impact on *Ae. aegypti* populations in the short, medium and long term. For this reason, with control measures or campaigns undertaken on an ad hoc basis, and without regard for local environmental factors and sufficient knowledge to guide and evaluate their activities, it is impossible for them to achieve sustainable results in the long term. Knowledge of the most prevalent as well as productive mosquito larval breeding sites and seasonal changes in mosquito populations is crucial to address mosquito control efforts, especially in those areas where dengue is endemic.

Acceptance of ITMs was high in the three intervention sectors in the early stages, but was not sustained. Eight months after the ITMs were delivered, they were still in use in more than 70% of the study houses. This observation was comparable with previous reports concerning acceptance of these tools in Trujillo State and in other places (Socheat *et al.*, 2004, Kroeger *et al*, 2006, Seng *et al.*, 2008). However, deterioration of the condition and performance of ITMs led to reduced usage over time. Thus, despite replacement of damaged materials at 20 months post intervention, by the end of the study fewer than 50% of houses had ITMs in use. By this time, the

lowest coverage value (20% of houses using curtains and 40% of drums was covered) was recorded in the sectors receiving the curtains and jar covers.

Overall the reduction in immature stage densities was similar to figures previously reported in Trujillo State (Kroeger, *et al.*, 2006) and Asia (Soachet *et al.*, 2004, Seng *et al.*, 2008). The significant differences between intervention arms and the external control, and the absence of such differences between intervention groups and neighbouring internal control groups suggest that as previously recorded here and in Mexico (Kroeger *et al.*, 2006) an overspill effect occurred, impacting on the adjacent untreated houses. Given that covered with any ITM did not exceed 42% during the second year of the study, this is a very encouraging result.

There is little evidence to suggest that peridomestic space spraying impacts on mosquito populations (McCall & Kittayapong, 2007, Esu *et al.*, 2010); indeed, there is evidence to the contrary (Hudson, 1986, Castle *et al.*, 1999, Koenraadt *et al.*, 2007). This must be taken into consideration to redirect *Aedes aegypti* control policies, which in the case of Venezuela and in particular in Trujillo State have proved to be ineffective in controlling dengue. The introduction of new tools such as ITMs could represent an alternative approach within an integrated management program.

Although the experience with ITNs has indicated that it is often human behaviour that limits the potential of the tool rather than the technical or entomological challenges, the latter issues have to be considered in this study. The quality of materials was quite sub-standard, in terms of durability of the actual netting, such that the reductions observed in the first months of the study were not sustained. A 'knock-on' effect of this was the fall in acceptance of the intervention by the treated communities, which could explain the low coverage registered in the later surveys.

This was an unexpected and unfortunate event and reduced our ability to evaluate completely, the impact of these ITMs on dengue vector populations.

It is essential that manufacturers produce better quality insecticide-treated curtains and covers that can resist the harsh environmental conditions in tropical countries and that have an extended duration of insecticidal activity to ensure efficacy and maximise community participation in the use of these new tools.

Results from the serosurveys did not indicate any change in infection rates between the two survey dates. A fundamental problem here was that the surveys failed to collect sufficient numbers of samples of both occasion: the original power calculation indicated the need to test 6,000 blood samples, far more than the number collected in the study (1039 and 789). The main reason for this was low compliance by the human population. Consequently, it was not possible to determine the impact of the interventions on dengue transmission in any way.

CHAPTER 4

IMPACT OF ITMS ON PYRETHROID SUSCEPTIBILITY IN *AEDES AEGYPTI* IN TRUJILLO, VENEZUELA.

4.1 Introduction

A variety of methods have been used for the control of *Aedes* spp. around the world (Curtis, 1990), and while the reduction of breeding sites and the implementation of environmental sanitation programmes are important components of vector control strategies, these alone are insufficient in reducing *Ae. aegypti* populations below dengue transmission thresholds. For this reason, most *Aedes* control programmes rely heavily on chemical control methods (WHO, 2009a). The most significant consequence of the widespread use of insecticides to control *Ae. aegypti* is that populations have developed resistance to organophosphates, pyrethroids, carbamates, and/or organochlorines in several countries (da-Cunha *et al.*, 2005, Rodriguez *et al.*, 2007, WHO, 2009a).

The history of insecticide use for vector control in Venezuela began with the introduction of DDT in 1945 (Gabaldon, 1949). DDT was first used in Trujillo State in 1954 as part of a widespread indoor house spraying campaign against malaria. After the World Health Organization certified that malaria had been eradicated in Venezuela in 1961 (Ache & Matos, 2001), DDT spraying continued to be used for Chagas disease control until 1968 against the main vectors *Rhodnius prolixus*, *Triatoma maculata* and *Panstrongylus geniculatus* (Cova-Garcia & Suarez, 1959). The implementation was in both rural and urban areas, but in 1967, when DDT resistance was detected in Rhodnius prolixus, and incidentally the first resistance reports were from Pampanito municipality of Trujillo State (Mogollon, 1997), the use of DDT was suspended and replaced with Baygon (a carbamate insecticide, 2-(1-

Methylethoxy-phenol methylcarbamate), applied as a 70% wettable powder (Dirección de Endemias Rurales, 1989, 1997, 1998).

In 1990, routine control of *Ae. aegypti* began in Trujillo State, employing malathion and fenitrothion sprays for adults and temephos as a larvicide. In 1994, lambdacyhalothrin was introduced as part of a research project for leishmaniasis control, conducted by the Research Centre "J.W. Torrealba" at the University of Los Andes in Trujillo. At the same time, the local vector control department (La Dirección de Endemias Rurales en el Estado Trujillo) incorporated the use of the pyrethroid deltamethrin into its control programmes for both *Ae. aegypti* and R. prolixus, in addition to the organophosphates already in use.

In 1999, the use of pyrethroids against *Ae. aegypti* was officially suspended in Trujillo, by central order from the División Central de Malariología, in Aragua State. It was not as result from local pyrethroids resistance evaluation in Trujillo State. This was due to reports of pyrethroid resistance in other regions of the country, mainly as consequence of intensive pyrethroid use to control the moth Hylesia metabus (Lepidoptera: Hemileucidae), which is a serious public health problem in the east region of the country. Despite this recommendation to suspend pyrethroids and continue using the organophosphates malathion and fenitrothion, during outbreaks of dengue and in the absence of availability of other insecticides, deltamethrin remained in use for control by local authorities in Trujillo State (Dirección de Endemias Rurales, 1989, 1997, 1998). This illustrates how the use of insecticides for *Ae. aegypti* control has been quite irregular in Trujillo State, because it has not been governed by a policy of local susceptibility / resistance status of this species, but rather by external factors.

Although the monitoring of the susceptibility of *Ae. aegypti* populations to insecticides commonly used for control is important, there is a no organised system of resistance surveillance in Trujillo State. Studies have been conducted focally, often

influenced more by the interest of researchers than by an organised policy at a national or regional level. Therefore, it is currently not possible to obtain timely information or early warnings of the development of resistance. For this reason, it is difficult to plan, design and implement strategies to delay or manage insecticide resistance in this mosquito in Trujillo. Moreover, traditionally the purchase of insecticides has not followed an overall policy of evaluating their efficacy. Instead, decisions have been made centrally, and then insecticides distributed to the various states, ignoring the geographical variability in the degree of susceptibility / resistance of *Ae. aegypti* throughout the country (Villegas C., 2009, personal communication).

The first report of resistance to DDT in *Ae. aegypti* from Venezuela was described by Quaterman & Schoof in 1958. Mouchet reported resistance to dieldrin / BHC in 1967, and Georghiou and colleagues reported resistance to organophosphates and carbamates in 1987. There are several more recent reports of organophosphate resistance in *Ae. aegypti* in Venezuela. Temephos resistance was detected in Trujillo State in 2006, (Alvarez *et al.*, 2006), where a strain of *Ae. aegypti* was shown to have a resistance ratio (RR) of 6.3, which according to WHO criteria suggests resistance to this larvicide. Municipalities in Aragua State and Falcón State, have also previously reported *Ae. aegypti* populations resistant to both temephos and malathion (Mazzarri & Georghiou, 1995). Later, Bisset and colleagues (2001) investigated the mechanisms of resistance to organophosphate insecticides in strains of *Ae. aegypti* from four Venezuelan states (Aragua, Apure, Táchira and Miranda), and they recorded high levels of resistance to temephos in a strain from Apure, whereas the strains from the other states were susceptible to temephos. However, recent data from municipalities in Aragua state show only resistance to malathion, and susceptibility to temephos (Perez & Molina, 2009). Only the last report stated the name of municipalities where the mosquitoes were collected, making it impossible to compare whether the mosquitoes from Aragua state evaluated in all three studies were from the same places. These differences could be attributable to focalized resistance in the places where the mosquitoes were caught, as perhaps the mosquito populations were

not under similar insecticidal pressures. The difference in results could also be explained by the times of the year in which the specimens were collected for bioassays, as the reproduction rate can affect resistance by diluting (or further concentrating) the genes that can confer resistance (Gazave *et al.*, 2001, Urdaneta-Marquez *et al.*, 2008).

The earlier reports regarding *Ae. aegypti* pyrethroids resistance (Chadwick *et al.*, 1977, Prasittisuk & Busvine, 1977) suggested that pyrethroid resistance could be associated partially with resistance to DDT, as consequence of earlier exposure to DDT. Pyrethroids and DDT share a common target site in insects, the voltage-gated sodium channel, and therefore specific mutations in the sodium channel gene sequence confer insensitivity to both types of insecticides (Martinez-Torres *et al.*, 1998, Ranson *et al.*, 2000, Brengues *et al.*, 2003, Saavedra-Rodriguez *et al.*, 2007). This type of resistance, called 'knockdown resistance' (kdr), describes the phenomenon that occurs when insects do not lose the capacity of coordination in their movements immediately after they are exposed to insecticides, and it was characterised by the first time in the housefly, *M. domestica* (Farnham, 1977). Since then, several researchers have reported on the phenomenon of cross-resistance in *Ae. aegypti*, particularly between pyrethroids and DDT, implicating a kdr mechanism (Hemingway *et al.*, 1989, Brengues *et al.*, 2003). Although, against *Ae. aegypti*, the widespread use of DDT was suspended in 1960 (Mazzarri & Georghiou, 1995), the legacy of its use remains in the form of DDT resistant vector populations (Brengues *et al.*, 2003).

In Venezuela, in the early 1990s moderate resistance to the pyrethroids permethrin and lambdacyhalothrin was detected in *Ae. aegypti* strains from Aragua and Falcon states (Mazzarri & Georghiou, 1995). Resistance to pyrethroids was not affected by the synergists S,S,S-tributyl phosphorothioate or piperonyl butoxide, further suggesting the presence of altered target site sensitivity (Mazzarri & Georghiou, 1995). Perez & Molina (2001) also reported resistance in *Ae. aegypti* from Aragua

State to the pyrethroids lambdacyhalothrin, cyfluthrin and deltamethrin. However, their results showed elevated mixed-function oxidases (MFO) to be the primary resistance mechanism. Rodriguez and colleagues (2007), reported that several strains of *Ae. aegypti* from Venezuela showed high resistance to DDT and deltamethrin, while remaining susceptible to other pyrethroids such as lambdacyalothrin, betacypermethrin and cyfluthrin. In Trujillo State, Alvarez and colleagues (2008) reported diminished mortality after exposure to deltamethrin in seven strains of *Ae. aegypti*. However, the mechanisms have not yet been classified.

Due to the reports of resistance to pyrethroids in *Ae. aegypti* in Venezuela as described above, it was important to assess the susceptibility status of this species prior to and as a consequnce of the use pyrethroids in the trial that formed the basis of this thesis. The target vector populations were surveyed at baseline to determine susceptibility to deltamethrin, and were assayed at regular intervals throughout the study to confirm any change in susceptibility status.

4.2 Materials and Methods

4.2.1 Study area

A complete description of the study site has been given in Chapter 3. Briefly, the study was carried out between July 2006 and October 2008 in Trujillo State, located in the Andean region of western Venezuela. Four parishes (Flor de Patria, Pampán, Pampanito and Motatán) spanning 3 municipalities (Pampán, Pampanito and Motatán) were selected (Table 4.1). The study area was divided into 60 clusters of approximately 100 houses each. Clusters were randomized to either receive one of 3 possible combinations of ITMs or act as untreated controls. The ITMs were deployed as window/door curtains, water jar covers or combination of both curtains and jar covers. An additional site, Monay (located in the municipality of Pampán), did not

receive ITMs and acted as an external control area, geographically isolated from the study area. Table 4.2 shows the allocation of clusters to each of the study arms.

Table 4.1 Geographical characteristics of the study site and external control site.

Place	Coordinates	Altitude (m above sea level)	Municipality
Monay	9°32'51" N/70°27"06W	451	Pampán
Flor de Patria	9°24'61" N/70°30"05W	451	Pampán
Pampán	9°26'47" N/70°28"26W	497	Pampán
Pampanito	9°25'03" N/70°31"39W	380	Pampanito
Motatan	9°23'45" N/70°35"34W	340	Motatán

Table 4.2. Distribution of clusters intervention and control arms throughout the study site.

Locality	External Control	Internal Control	Curtains	Jar-Covers	Curtains plus Covers	Total
Monay	15	0	0	0	0	15
Flor de Patria	0	2	2	4	7	15
Pampán	0	6	1	2	2	11
Pampanito	0	5	9	7	4	25
Motatan	0	2	3	2	2	9
Total	15	15	15	15	15	75

4.2.2 Evaluation of mosquito populations

Ae. aegypti eggs were collected in ovitraps (Lenhart *et al.*, 2005) deployed throughout the study area at baseline and at 8, 14, 20 and 26 months after ITMs distribution. These eggs were pooled based on study arm and reared to adults under insectary conditions (23 +/- 2°C and 70% relative humidity). *Ae. aegypti* mosquitoes from a known susceptible laboratory strain (Rockefeller strain, donated by the Centre for Disease Control and Prevention in San Juan, Puerto Rico) were used as control in the bioassays.

4.2.3 Bioassay procedure

Adult mosquito insecticide susceptibility bioassays were conducted according to the standard World Health Organization methodology (WHO, 1981 and 1998), using WHO bioassay tubes and impregnated papers. Five groups of 15 to 20 unfed females (aged from 1 to 3 days old) were introduced into the control (insecticide-free) tubes and kept for 20 minutes to check their status. After this pre-test period, they were transferred into the tubes lined with deltamethrin impregnated papers at the diagnostic dose suggested by the WHO of 0.05%. The control groups were exposed to papers impregnated with silicone oil alone. The mosquitoes were exposed for 60 min., and knockdown (KD) was recorded at 10, 15, 20, 30, 40, 50 and 60 min. The tubes were maintained in the vertical position throughout the tests. After completing the exposure period, the mosquitoes were transferred to recovery chambers and provided with sugar solution-soaked cotton. They were maintained for 24 hours under insectary conditions and mortality/recovery at 24 h was recorded.

Each bioassay was repeated 3 times (on different days), with 5 replicates for each population under evaluation.

4.2.4 Evaluation of insecticide-treated materials

There are currently no specific guidelines for evaluating long-lasting ITMs as curtains against *Ae. aegypti*, so therefore assays were conducted following the WHOPES cone bioassay protocol (WHO, 1998), substituting *Anopheles* with *Ae. aegypti* from the susceptible Rockefeller strain insectary colony maintained at the Research Centre "J. W. Torrealba" were used for the cone bioassays.

Bioassays were performed on curtains collected at 6 months, 12 months and 24 months after they were delivered in the field. Curtains collected at 6 months, as well as, curtains without use were bioassayed at 9 months, when the cones were available (see table 4.5). From the database, houses where curtains were in use were selected by using random numbers generated by a computer to collect curtains for bioassays. The collections were made in the early morning or during the early evening to be sure of finding the owners of selected houses, and were offered a new curtain to replace the used one, and thus in this way, they did not refuse to participate in the study. Information was also collected regarding whether they were exposed to sunlight and how often they had been washed. Unused curtains, stored in plastic bags at the laboratory were used as positive controls throughout the study.

The following protocol was devised to evaluate curtains. Three cones were placed on separate areas of the curtain, and ten mated but non-blood fed, 2-5 day old *Ae. aegypti* females were introduced into each cone, and exposed for three minutes. Mosquitoes were then removed and placed into holding cups, and "knockdown" effects recorded. They were provided sugar solution-soaked cotton and maintained under insectary conditions for 24 hours, when mortality / recovery was recorded.

The covers were not evaluated as it was difficult to produce enough mosquitoes from the Rockfeller susceptible laboratory strain.

4.2.5 Data analysis

The results of the bioassays were analyzed using probit analysis (SPSS for Windows version 17, SPSS Inc., Chicago, IL) to obtain KDT_{50} and KDT_{95} values, which represent the time required to knockdown 50% and 95% of the mosquitoes exposed to the insecticide. The mortality results were categorised based on the criteria suggested by WHO (1998): 98-100% mortality = susceptible; 80-97% mortality = suspected resistance and <80% mortality = resistant. When control mortality was between 5 – 20%, the average mortality was corrected using Abbott's formula [(% test mortality - % control mortality)/ (100 - % control mortality) x 100]. The resistance ratios were calculated from KDT_{50} and KDT_{95} values for the tested population divided by the KDT_{50} and KDT_{95} values for the reference susceptible Rockefeller strain. If data (even after log transformation), did not meet the assumptions of normality and homogeneity of variances, the non-parametric Kruskal-Wallis and Mann-Whitney U tests were conducted to explore differences in mortality rates. Any p value less than 0.05 was considered statistically significant.

4.3 Results

4.3.1 Evaluation of mosquito populations

The KDT_{50}, KDT_{95}, and RR values for *Ae. aegypti* populations from the external control site, internal control arm and the susceptible Rockefeller strain from baseline through the end of the study are shown in Table 4.3. The laboratory Rockefeller strain was evaluated at each bioassay time point, and remained completely susceptible throughout the study, showing mortality levels of 99.7- 100%.

According to the WHO criteria (1998), the *Ae. aegypti* from the external control site were classified as susceptible throughout, given that their mortality at 24 h ranged

between 98.5- 99%. Knock down after one hour of deltamethrin exposure was 100% in this strain throughout the study. The values of KDT_{50} and KDT_{95} ranged from 14.8 -18.3 and 25.2 – 38.4 minutes respectively, and no statistically significant differences were observed in KDT values (Table 4.3). The high values of slopes of regression lines suggest a homogeneous response by mosquitoes to the insecticide evaluated.

The mosquito population from the internal control arm showed diminishing mortality rates from the beginning of study (95.8%) to the end (90.3%). The values recorded at 14, as well as, at 26 months after the study began, were statistically significantly lower than at baseline (U=54,000, z=-2.44, p=0.015, and U=56,000, z=-2.36, p=0.018). KDT_{50} and KDT_{95} values ranged between 16.7 - 22.9 and 31.1 – 41.1 minutes respectively, but no significant differences between post-intervention and baseline values were observed. Knockdown after one hour of exposure to the insecticide evaluated was 100% throughout the study. As in the external control strain, high slope values for regression lines were observed (6.4 – 9.3), suggesting homogeneity in the response of mosquito populations.

At baseline, the *Ae. aegypti* populations from the three arms under intervention with ITMs were categorised as 'suspected resistant' per WHO guidelines (1998), and their status remained consistent throughout the course of the study (Table 4.3).

Throughout the course of the study, mosquitoes from the curtain intervention arm showed mortality ranging between 90 – 96%. The values recorded at 8, 14 and 20 months post intervention were statistically significantly lower than at baseline, (U=24,000, z=-3.69, p<0.0001; U=46,000, z=-2.78, p=0.005 and U=46,500, z=-2.77, p=0.005, respectively) but the the value recorded at the end of study was not. KDT_{50} and KDT_{95} values ranged between 18.3 – 24.3 and 34.2 – 45.6 minutes, respectively. RR_{50} and RR_{95} values ranged from 1.5 to 2.0 and 1.2 to 2.0, respectively. The values of KDT_{50} recorded at 8 and 14 months were significantly higher compared to baseline [95% CI (21.4 - 25.2; 22.4 - 26.3 and 16.6 - 20.1, respectively)], but no significant

difference was observed in the evaluations at 20 and 26 months, compared to baseline. In contrast, the recorded values of KDT_{95} were not statistically significant different throughout the study, as this is shown by its confidence intervals overlapped (Table 4.4).

Mortality results recorded from mosquitoes from the arm that received jar covers dropped steadily from 93.6% at baseline to 86.7% at the end of the study. The value recorded at 8 months was not statistically different from the value recorded at baseline. However, steadily statistically significant differences were observed from this time point until the end of study, (U=65,000, z=-1.98, p=0.047; U=44,500, z=-2.84, p=0.005 and U=55,500, z=-2.34, p=0.017, 14, 20 and 26 months respectively). KDT_{50} and KDT_{95} oscillated between 16.4 – 26.1 and 32.0 – 50.6 minutes, respectively. The values of RR_{50} and RR_{95} ranged from 1.4 - 2.2 and 1.1 – 2.4 fold, respectively. The lowest values of KDT_{50} and KDT_{95} were recorded at baseline and the highest ones at 8 months after the study started. When comparing the values of KDT_{50} recorded at 8 months post intervention vs. baseline, the former was statistically significant higher [95% CI (15.8 - 17.1 and 24.5 - 27.92 respectively)]. After this time point, the values of KDT50 fell, but were statistically significantly higher than the value recorded at baseline [95% CI (15.8 - 17.1; 21.2 - 25.3; 19.2 - 20.8, and 18.0 - 19.2 respectively)]. A similar pattern was observed with regard to KDT_{95}, but the final value was not statistically different from baseline (Table 4.4).

In the study arm which received both curtains and jar covers, the mosquitoes showed values of mortality between 83.3 – 91.2 %. Although, a decreasing was observed after the materials were deployed, only at 20 months the mortality percentage was statistically significant lower than at baseline (U=44,000, z=-2.86, p=0.004). At the end of study, the mortality percentage was higher than at baseline, but this difference did not reach statistical significance. The values of KDT_{50} and KDT_{95} ranged from 16.7 - 23.2 and 33.4 – 46.2 minutes, respectively. RR_{50} and RR_{95} values varied from 1.4 – 2.0 and 1.2 – 2.1 fold, respectively. In this intervention arm the values of KDT_{50}

recorded at the end of study (26 months) were not significantly different from the values recorded at baseline; whereas, the values obtained at 8, 14 and 20 months were statistically significant higher compared to baseline [95% CI (16.1 - 17.3; 20.9 - 25.4; 19.8 - 26.2, and 18.5 - 20.1, respectively)]. In contrast, with regard to the values of KDT_{95} recorded throughout the study no statistically significant difference was observed (Table 4.4). The relationship registered between knock-down rate and log-transformed exposure time was linear in all mosquito populations. The high values obtained for slopes of regression lines (>5) suggest a homogeneous response by mosquitoes to deltamethrin from baseline to the end of the study.

Table 4.3. KDT_{50}, KDT_{95} and RR values for Ae. aegypti *populations from external and internal control arms and the Rockefeller (pyrethroid susceptible) strain after one hour of exposure to 0.05 % deltamethrin.*

Time	24 h Mortality %	% KD after 1 h exposure	KDT50 in min (95% CI)	RR50	KDT95 (95% CI)	RR95	Slope ± SE
External Control							
Baseline	99.0[(1)]	100	14.8 (12.2 - 17.2)	1.2	31.2 (25.5-44.9)	1.5	5.96±0.32
8 months	98.8[(1)]	100	15.9 (15.4 - 16.3)	1.4	25.2 (24.1-26.7)	1.2	9.80±0.50
14 months	98.8[(1)]	100	18.3 (15.9 - 20.7)	1.4	34.7 (29.1-46.6)	1.3	7.48±0.35
20 months	98.7[(1)]	100	17.8 (15.5 - 20.2)	1.4	34.2 (28.8-45.9)	1.3	7.26±0.34
26 months	98.5[(1)]	100	17.9 (14.3 - 21.3)	1.7	38.4 (30.3-60.7)	1.3	6.20±0.28
Internal Control							
Baseline	95.8[(2)]	100	19.5 (17.6 - 21.6)	1.6	41.9 (35.5-54.1)	2.0	6.40±0.32
8 months	95.7[(2)]	100	22.9 (21.0 - 25.1)	2.0	39.8 (35.2-47.7)	1.9	9.38±0.38
14 months	90.1[(2)]	100	19.4 (17.9 - 20.9)	1.4	37.2 (33.2-43.3)	1.4	7.46±0.32
20 months	92.2[(2)]	100	19.2 (17.5 - 21.0)	1.6	34.9 (30.7-42.2)	1.3	8.14±0.37
26 months	90.3[(2)]	100	16.7 (16.1 - 17.3)	1.6	31.1 (29.3-33.3)	1.1	7.49±0.37
Rockefeller strain							
Baseline	99.7[(1)]	100	12.1 (11.1 - 12.9)	-	20.7 (18.7-24.4)	-	7.60 ±1.01
8 months	100[(1)]	100	11.6 (10.3 - 12.7)	-	21.1 (18.6-25.9)	-	6.78±1.08
14 months	100[(1)]	100	13.4 (11.8 - 14.8)	-	26.4 (22.7-33.5)	-	6.31±0.96
20 months	100[(1)]	100	12.4 (10.9 - 13.7)	-	26.5 (22.9-33.3)	-	5.49±0.80
26 months	100[(1)]	100	10.5 (9.4 - 12.2)	-	28.7 (21.8-62.7)	-	3.80±1.12

[(1)]: Susceptible; [(2)]: Resistance suspected (WHO, 1998)
Slope ± SE: Slope of regression line ± standard error
RR: Resistance ratio KDT field strain/ KDT ROCKEFELLER strain

Table 4.4. KDT_{50}, KDT_{95} and RR values for Ae. aegypti *populations from ITMs intervention arms after one hour of exposure to 0.05 % deltamethrin.*

Time	24 h Mortality %	% KD after 1h exposure	KDT_{50} in min (95% CI)	RR_{50}	KDT_{95} (95% CI)	RR_{95}	Slope ± SE
Curtains							
Baseline	96.2[(2)]	100	18.3 (16.6 - 20.1)	1.5	38.8 (33.9-46.7)	1.9	6.38±0.34
8 months	90.6[(2)]	100	23.3 (21.4 - 25.2)	2.0	43.0 (38.2-50.5)	2.0	8.43±0.35
14 months	90.9[(2)]	100	24.3 (22.4 - 26.3)	1.8	45.6 (40.6-53.3)	1.7	8.34±0.35
20 months	90.3[(2)]	100	20.9 (19.2 - 22.7)	1.7	42.9 (37.9-50.6)	1.6	6.98±0.29
26 months	95.6[(2)]	100	20.6 (19.5 - 21.7)	2.0	34.2 (31.5-38.1)	1.2	9.83±0.46
Jar Covers							
Baseline	93.6[(2)]	100	16.4 (15.8 - 17.1)	1.4	34.1 (32.1-36.5)	1.6	6.33±0.29
8 months	92.2[(2)]	100	26.1 (24.5 - 27.9)	2.2	50.6 (45.9-57.2)	2.4	8.13±0.33
14 months	90.2[(2)]	100	23.2 (21.2 - 25.3)	1.7	46.7 (41.0-55.7)	1.8	7.41±0.25
20 months	87.4[(2)]	100	20.0 (19.2 - 20.8)	1.6	40.3 (37.1-43.5)	1.5	7.02±0.34
26 months	86.7[(2)]	100	18.6 (18.0 - 19.2)	1.8	32.0 (30.3-34.1)	1.1	6.96±0.32
Curtains-Covers							
Baseline	91.2[(2)]	100	16.7 (16.1 - 17.3)	1.4	36.7 (34.5-39.2)	1.8	5.89±0.25
8 months	89.8[(2)]	100	23.2 (20.9 - 25.4)	2.0	45.4 (39.6-55.0)	2.1	7.68±0.31
14 months	88.0[(2)]	100	22.9 (19.8 - 26.2)	1.7	46.2 (38.5-62.5)	1.8	7.35±0.30
20 months	86.6[(2)]	100	19.3 (18.5 - 20.1)	1.6	40.8 (38.3- 43.7)	1.5	4.17±0.43
26 months	93.3[(2)]	100	17.1 (16.4 - 17.7)	1.6	33.4 (31.5-35.7)	1.2	8.85±0.43

[(2)] Resistance suspected (WHO, 1998)
Slope ± SE: Slope of regression line ± standard error
RR: Resistance ratio KDT field strain/ KDT ROCKEFELLER strain

4.3.2 Evaluation of insecticide-treated materials

Overall the results obtained from curtain bioassays were very heterogeneous, even in curtains without use, with mortality values ranging between 45-100%. After six months of use, the percentage mortality varied from 52 -100% (mean=79%). Mortality from curtains that had been hanging for one year ranged between 60 – 97% (mean=80%), and after two years in use, mortality ranged between 48 - 97 % (mean=74%). No mortality was observed in the control groups.

Table 4.5. Number of curtains assayed at baseline and post intervention. Mean mortality (%) for Ae. aegypti *Rockefeller strain after three minutes of exposure to ITMs, recorded at 24 hours. In each curtains three cones were tested at each assay.*

Curtains assayed (n)	Period of use (months)	Age of net at bioassay (months)	No. Mosquitoes/ cone	24 h Mean Mortality % (± SD)	Min (%)	Max (%)
26	0	9	10	83 ± 17	45	100
16	6	9	10	79 ± 14	52	100
19	12	12	10	80 ± 9	60	97
23	24	24	10	74 ± 13	48	97

4.4 Discussion

Ae. aegypti has shown great ability to develop different levels of resistance to various classes of chemicals used for its control in countries where its presence represents a public health problem. Although the incorporation of pyrethroid insecticides into dengue vector control programmes has been relatively recent, pyrethroid resistance is widespread in *Ae. aegypti* (Chadwick *et al.*, 1977, Prasittisuk & Busvine, 1977, Hemingway *et al.*, 1989, Mazzarri & Georghiou, 1995, Rawlins, 1998, Rodriguez *et al.*, 1999, Brengues *et al.*, 2003, Ponlawat *et al.*, 2005, Jirakanjanakit *et al.*, 2007b, Rodriguez *et al.*, 2007, Saavedra-Rodriguez *et al.*, 2007, Bisset *et al.*, 2009,

Marcombe *et al.*, 2009). In some places, *e.g.* Brazil, resistance has developed remarkably quickly, where after only three years since the introduction of the pyrethroid cypermethrin, decreased susceptibility to this insecticide was observed in the city of Rio de Janeiro (da-Cunha *et al.*, 2005).

As described earlier, reports of pyrethroid resistance in Venezuela are highly variable, even in mosquitoes from the same state (Mazzarri & Georghiou, 1995, Perez & Molina, 2001, Rodriguez *et al.*, 2007). The mortality results obtained in this study indicate that *Ae. aegypti* populations in Trujillo state present differing levels of susceptibility to deltamethrin according to WHO criteria (1998). *Ae. aegypti* populations from the external control site showed consistent susceptibility to deltamethrin, but populations from the other study areas, including the internal control arm, should be categorised as 'suspected resistant'.

Although the mortality steadily dropped over time in clusters receiving jar covers, the mosquito populations remained within the same WHO resistance category at the end of study. Likewise, in the other intervention sectors, as well as in the internal control area, a similar pattern was observed with some variation in the mortality rates during the study, but within the same category of 'suspected resistant'.

Pyrethroid resistance sometimes leads to a significant increase in "knockdown" time before death, so knockdown time can serve as a good indicator for early detection of resistance to pyrethroids (WHO, 1992). As such, this parameter can be included in resistance monitoring programmes to provide initial information about the potential presence of kdr-type resistance (Chandre *et al.*, 1999, Corbel *et al.*, 2004).

In this study, KDT values were significantly higher in the three populations under intervention with ITMs and the internal control arm, compared to the susceptible Rockefeller strain. In the external control area KDT values were slightly higher, but not statistically significant to the susceptible Rockefeller strain.

Although the resistance ratio values in the external control were lower than the values from intervention areas, the difference was not significant. In no case, did the RR values exceed 2.2 fold, so they remained below the value established by WHO to estimate resistance to insecticides in a mosquito population. This could indicate that the decreased susceptibility to deltamethrin observed in the *Ae. aegypti* populations evaluated in this study is still a manageable phenomenon. In this context, Hemingway and colleagues (1997) emphasize the need to implement strategies to manage resistance before it becomes a limiting factor for the use of a particular insecticide. It could recommend evaluating the susceptibility status of *Ae. aegypti* local population to different pyrethroids than deltamethrin in order to determine the introduction of other insecticides, particularly with different modes of action, as well as, design a integrate programme including different alternative measures such as biological control, either Bti or IGRs, well-designed clean-campaign, in order to diminish the pressure exerted to *Ae. aegypti* natural populations by insecticides.

In addition, the low KDT values found in this study could suggest the absence of the kdr mutation. The two main mechanisms responsible of pyrethroids resistance in insects are increased detoxification of the insecticide and changes in target site insensitivity, thus the former might be implied in the developing resistance of these *Ae. aegypti* populations. To date in *Ae. aegypti* populations from Trujillo state, mechanisms of resistance are unknown. To clarify the actual mechanisms involved, detailed studies are needed, but would be important to manage *Ae. aegypti* control strategies in Trujillo state.

The high level of susceptibility detected throughout the current study in the external control area suggests that different insecticidal pressures may exist. Historically, malaria, Chagas disease and dengue transmission have been lower here than elsewhere in the region, and insecticide-based vector control interventions were not as aggressively implemented here. It was only after the introduction of DEN-3 in

2003 when a dengue epidemic occurred in Monay that insecticides started to be widely used (Control Vector Department, Trujillo, personal communication).

The results reported here correspond with the only other previous report on insecticide resistance in Trujillo, which found recently that out of nine *Ae. aegypti* populations evaluated with deltamethrin, only two were susceptible, while seven others were categorised as 'suspected resistant' (Alvarez *et al.*, 2008). These results suggest altered susceptibility to deltamethrin, indicating the need to implement a monitoring programme and plan for management of *Ae. aegypti* resistance as a key step in dengue control. Likewise, health authorities should consider a comprehensive plan that further includes the study of mechanisms that may be involved in places reporting insecticide resistance, as this would allow for better planning of both routine control strategies and outbreak responses. As dengue is an increasing public health in Trujillo, as well as, in Venezuela, there is an urgent need to introduce alternative control measures, to preserve the use of pyrethroids as valuable tool in *Ae. aegypti* control programmes. Health authorities must redirect control policies, by using different or rotating insecticides, previously resistance become a more concern issue into dengue control programmes.

A large-scale trial conducted in Mexico in a malaria endemic area (Penilla *et al.*, 2006, 2007) demonstrated the ability of resistance management strategies to reduce or delay the effect of resistance, either by rotation of insecticides or the implementation of insecticide mosaics to control Anopheles albimanus. Garcia and colleagues (2009) suggest that similar patterns could be adapted as a strategy in *Aedes* control, particularly to manage the pyrethroid resistance, which has increased dramatically in México in recent years (Garcia *et al.*, 2009).

Assessing the performance of these ITMs in this study, a definitive conclusion cannot be drawn about the residual effect of deltamethrin in these materials used under field conditions, given the high variability in the mortality recorded at each time point.

Although the sample examined was not very large, no clear trend was observed regardless of whether the curtains had been exposed to direct or partial sunlight. Likewise, no differences were observed regarding the frequency of washing. The heterogeneity of the results may have been due to differences in the distribution and/or the content of insecticide on the curtains, resulting from inconsistencies in the production process.

Although factors such as sunlight exposure and dust could affect the efficacy of ITMs in the field, the dominant belief is that the main factor causing loss of insecticide from bednets is repeated washing (Gimnig *et al.*, 2005, Graham *et al.*, 2005, Kayedi *et al.*, 2007ab). To overcome this obstacle, manufacturers developed the technology of 'long-lasting insecticidal nets' (LLIN), which have increased wash resistance, allowing products to retain insecticidal efficacy without the need for frequent re-treatment (Kayedi *et al.*, 2007ab).

One of the first types of LLIN recognized by the World Health Organization Pesticide Evaluation Scheme (WHOPES) was the Olyset Net®, which contains permethrin 2% w/w incorporated during manufacture (Itoh & Okuno, 1996, WHO, 2001). Vestergaard-Frandsen subsequently developed PermaNet (deltamethrin 55mg ai/m^2) (Graham *et al.*, 2005), which was the material used in this study. Given that PermaNet should retain its efficacy (according to the manufacturers' information), it was surprising that the results presented here reported such inconsistencies. To estimate whether it was a problem in the chemical content in the materials received in Venezuela for this study or incorrect storage after arrival, a more detailed and larger analysis regarding the chemical content distribution and bioefficacy have to be conducted.

Some variability has been encountered with regard the persistence of efficacy after washing LLINs as compared to conventionally treated materials. Ordonez-Gonzalez and colleagues (2002) evaluated washing regimes on PermaNet and conventionally

treated ITNs and found that after three gentle washes, no difference in insecticidal efficacy was observed. However, after more vigorous washing, PermaNet performed better than conventionally treated ITNs. On the other hand, Asidi and colleagues (2004) reported that, although Permanet retained a significant protective power after five washes and eight months of using, it was not significantly better than conventionally nets treated with alphacypermethrin, lambdacyhalothrin or permethrin, under the same testing conditions.

More recently, other researchers (Gimning *et al.*, 2005, Kayedi *et al.*, 2007ab, 2009b) evaluated PermaNet nets, using similar laboratory washing protocols. They found PermaNet, when compared to other LLINs and conventionally treated nets, retained significant efficacy after several washes. However, as washing removes insecticide from the fibres, insecticidal efficacy declines with increasing numbers of washes (Kayedi *et al.*, 2009b). In addition to wash resistance, the physical durability of the materials, particularly under tropical conditions, could also affect the long-term efficacy of LLINs.

As dengue continues to increase as a public health problem in Venezuela, the use of pyrethroid insecticides must be handled carefully to ensure their longevity as a valuable tool in the control of *Ae. aegypti*. Consequently, non-chemical methods should be considered for use together with ITMs and other insecticide-based approaches to impact on vector populations. Typically, while non-chemical interventions, such as the reduction of breeding sites, environmental sanitation programmes, and the use of biological control tools remain important elements of effective *Ae. aegypti* control programmes, they are most effective when applied together with insecticidal interventions. ITMs offer a novel delivery system of residual insecticides within the home. Indeed, ITMs employing pyrethroid insecticides have been shown to lower domestic populations of *Ae. aegypti* (Nam *et al.*, 1993, Nguyen *et al.*, 1996, Madarieta *et al.*, 1999, Kroeger *et al.*, 2006, Lenhart *et al.*, 2008), highlighting the importance of preserving the susceptibility of target

mosquito populations to pyrethroids. In managing and preventing resistance, it is also important to explore the possibility of applying other classes of insecticides to ITMs.

Due to the development and spread of pyrethroid resistant mosquitoes in Africa, investigations on alternative insecticides on nets to replace or complement the existing pyrethroid-based LLINs have been carried out (Gulliet *et al.*, 2001b, Hougard *et al.*, 2003a, Asidi *et al.*, 2005, Oxborough *et al.*, 2008, Adeniran *et al.*, 2009, N' Guessan *et al.*, 2010).

The concept of a '2-in-1' bednet has been developed as strategy to manage pyrethroid resistance in *Anopheles* mosquitoes. Bednets of this design are treated with pyrethroids on the lower part and with non-pyrethroid insecticides (such as organophosphates or carbamates) on the upper part. Theoretically, host-seeking mosquitoes would make consecutive contact with both insecticides (Guillet, 2001b). Also, as organophosphates and carbamates are less irritating to mosquitoes than pyrethroids (Hougard *et al.*, 2003a, Oxborough *et al.*, 2008), the mosquitoes would maintain contact with them for longer, ultimately resulting in greater mosquito mortality (Hougard *et al.*, 2003a). Among the potential benefits of a '2-in-1' net is the fact that the more toxic insecticide can be applied to the roof of the net to minimize potential human contact (Guillet *et al.*, 2001b, Oxborough *et al.*, 2008).

Insecticide-treated curtains potentially have an advantage in this respect as they are contacted far less frequently than bednets and might therefore, be acceptable vehicles for delivery of other insecticide classes.

The results obtained in this study indicated that changes had occurred in the susceptibility of *Ae. aegypti* populations, potentially in response to the delivery of the pyrethroid deltamethrin via impregnated curtains and water jar covers in three municipalities in Trujillo state. These findings suggest that use of ITMs or pyrethroids delivered by any other method in control programmes in the area should

include a strategy for the monitoring of *Ae. aegypti* susceptibility to those insecticides being used by the vector control authorities. It is also important to determine the mechanism(s) of resistance operating here, to further inform which resistance management strategies would be optimal. The evaluation of the susceptibility of *Ae. aegypti* populations to the insecticides used on ITMs should be a prerequisite to their widespread introduction in the field, to ensure their impact on mosquito populations reaches its maximum potential.

CHAPTER 5

EFFECT OF BREEDING SITE TYPE ON FITNESS OF ADULT FEMALE *Aedes aegypti.*

5.1 Introduction

The environment in which the immature stages of *Aedes aegypti* develop can have an important influence on the condition of the adult mosquitoes that emerge from it. Simple parameters such as container size, food availability, and population density of the invertebrate fauna (both *Ae. aegypti* and other species) can alter the quality of the emerging mosquitoes, ultimately influencing important components of vectorial capacity such as body size, frequency of blood-feeding and longevity, and components of an individual's fitness, such as body size, reproductive success, fecundity and longevity (Nasci, 1991, Chadee & Beier, 1997, Scott *et al.*, 2000, Schneider *et al.*, 2004, Jirakanjankit *et al.*, 2007).

This chapter reports on a study that was conducted to explore whether *Ae. aegypti* fitness might be associated with the type of containers (drums, small containers and tyres) where the mosquitoes grew as immature stages. Most importantly here were possible effects on adult body size (Christophers, 1960, Carpenter, 1983, Nasci, 1988, Tun-Lin *et al.*, 2000, Lounibos *et al.*, 2002, Juliano *et al.*, 2004, Arrivillaga & Barrera, 2004, Schneider *et al.*, 2004, Barrera *et al.*, 2006, Harrington *et al.*, 2008, Jirakanjanakit *et al.*, 2007a) as measured by wing length, which is considered a good estimator of global body size in mosquitoes (Lounibos, 1994, Lehman *et al.*, 2006).

The information obtained here, together with the information from Chapter 3 about the impact of ITMs on *Ae. aegypti* entomological indices, and the results from Chapter 4 with regard to insecticide susceptibility of *Ae. aegypti* to deltamethrin, will be analysed in combination to provide useful insight regarding the impact of ITMs on

entomological indices of *Ae. aegypti*, as well as, the epidemiological significance of the main breeding container categories taken into account in this study.

5.1.1 Short review on the concept of 'fitness'

The idea of fitness has been one of the most frequently discussed topics in evolutionary biology and an ideal definition, satisfactory to all in the field has yet to be accepted; in fact most work carried out in recent years deal with the quantification or measurement of fitness with biological parameters rather than the concept itself (Dawkins, 1982, Beatty, 1992, Iseda, 1994, Michod, 1999, Scott, 2005, Mills & Beatty, 1979). The first use of the word fitness in evolutionary biology is credited to Herbert Spencer (1864) who introduced the phrase "survival of the fittest" as an interchangeable phrase with Darwin's "natural selection or preservation of favoured races in the struggle for life". Darwin used the phrase "the continuous preservation of the individual best fitted" in almost the same sense as Spencer's "survival of the fittest", but the terms fitness and fittest were not central to his theory of natural selection (Iseda, 1994).

The concept of fitness was interpreted freely until the 1930s (Iseda, 1994), when population geneticists attempted exact measurements of fitness through mathematical models: *e.g.* Fisher's fundamental theorem of natural selection (Kimbrough, 1980). Fisher (1930) introduced the word fitness in terms of m = *the relative rate of increase (or decrease) of a population*; in a case where two populations have sets of different genes and consequential different relative rates of increase, the population which has the large rate of increase has the greater fitness. Fisher applied the fitness concept to the population and not the individual organism; an individual's fitness was represented by the expectation of offspring derived from the population average of offspring (Fisher, 1930). Haldane used the word fitness to refer to individuals of such a constitution that they are most likely to spread by themselves in larger number than

their partners, as a result of being better adapted to their environment, to be more fertile or both (Haldane, 1937, 1938). Dobzhanky (1955) introduced the term fitness in the Darwinian sense, saying that the viability and the reproductive success were what determined the contribution that a carrier of a genotype mad to the gene pool of the next generation of a population, the first reference to the notion of a gene pool (Iseda, 1994).

Thus fitness came to be used as a definition of reproductive success: Waddington (1939) believed to fitness of an organism could be measured as the number of offspring left by that individual and that the "fittest" was not necessarily the strongest or healthiest, but simply the one leaving more offspring. Mills & Beatty (1979), suggested that fitness could be described as the propensity of an individual to leave offspring. Iseda (1994) pointed out that care was needed here, because if it was said that those organisms with the higher propensity to survive, will survive, this was not universally certain, because the possibility also existed that those with the highest propensity to survive, do not always have the luck to survive. Thus, probability was introduced such that it could now be said that "the probability that those who have the largest propensity to survive will survive, is high" (Iseda, 1994).

During this period, Hamilton (1964) introduced two notions of fitness: *neighbour modulated* fitness and *inclusive* fitness, because he believed that the orthodox measure of fitness by reproduction was not enough. To explain *neighbour modulated fitness*, he defined the reproductive success of an organism as the sum of the basic unit (the fitness grade), the effect of the individual genotype and the total effect provided by relatives (which will depend on their genotype). Thus one genotype influences the reproduction of the organism that possesses it and also contributes to the reproductive success of the nearest relatives. To incorporate the latter, he introduced the phrase *inclusive* fitness, which is defined in terms of the expectation of an individual organism's survival and reproductive success, plus the expectation of their relatives' survival and reproductive success. The base of this definition is the

genotype of the organism which is applicable to individuals and to particular genes. An important factor influencing this is the social behaviour associated with that genotype (Hamilton, 1964). Subsequently, Dawkins (1982), who was heavily influenced by Hamilton, rejected the use of the word 'individual'. Instead he considered that the unit of natural selection was not the individual organism, but the individual genes.

With the development of population genetics between 1920 and 1930, the word 'fitness', in relation to evolutionary biology, evolved to its present form, which is interpreted as the success in the average number of offspring left, independently of the causes that originate it (Paul, 1992). The works developed by Fisher (1930) and Haldane (1938), contributed to identify the gene as the target of selection, and to redefine selection as a change in gene frequencies (Paul, 1992). Futuyma (1998) considers that a general term for fitness is reproductive success, including the average number of offspring that survive from birth to reproductive age, since survival is prerequisite to reproduction. Scott (2005) pointed out that it was also necessary to take into account environmental effects, and that fitness should be expressed as the average contribution of individuals, genotypes or alleles to the next or successive generations. Futuyma (1998) stated that fitness must be evaluated by the number of descendants after two generations.

A century and half after the introduction of the term fitness into biology, the term remains without universal agreement as to its definition, such that today, most research studies aim to measure fitness, however unclear its definition might be, rather than to continue to try to define it (Beatty, 1992).

5.1.2 Measuring fitness in mosquitoes

In studies of population ecology, fitness is typically measured by two key factors: the net replacement rate and instantaneous growth rate (Scott *et al.*, 2005). However, the population's fitness is dynamic, strongly influenced by the environment, since an organism may reveal different degrees of fitness in particular places and at specific times (e.g. different seasons), with no variation in its genotype (Beatty, 1992, Futuyma, 1998, Tabachnick, 2003, Scott *et al.*, 2005). Clearly, as Tabachnick (2003) warned, extrapolation from one situation to another, particularly from the laboratory to the field, should be avoided. Similarly, laboratory colonies should be avoided because the colonization process selects genotypes that are not representative of the population target (Scott *et al.*, 2005). Day *et al.*, (1994) suggested that the best, possibly the only way to reduce experimental error associated with measuring mosquito reproductive success, would be to measure mosquitoes individually. As yet, however, as with other organisms, there is no consensus on a single best general method to measure fitness in mosquitoes (Scott *et al.*, 1997, Harrington *et al.*, 2001, Irvin *et al.*, 2004).

There has been a resurgence in interest in this topic as the result of genetic manipulation of species (Scott *et al.*, 2005), as the success of a control strategy based on genetically modified mosquitoes would depend strongly on the effect of the transgene on the fitness of transformed mosquitoes (Catteruccia *et al.*, 2003, Lambrechts *et al.*, 2007). Studies have been carried out to assess survival, fecundity and fertility of transformed mosquitoes in population cage experiments, with contradictory results (Catteruccia *et al.*, 2003, Moreira *et al.*, 2004). Lambrechts *et al.*, (2008) remarked that studies have evaluated the fitness costs more than possible fitness advantages of genetically modified mosquitoes.

On the other hand, according to Roush & Mckenzie (1987), the evolution of resistance against a pesticide, is determined, in a natural population of arthropods, by

allelic substitution at a single locus. Thus, the introduction of a resistant allele (*R*) into the genome implies a deleterious effect of the *R* allele on the physiological processes associated with development (Brown & Pal, 1971, Clarke & Mackenzie, 1987, Mckenzie & Clarke, 1988). Studies conducted on fitness components, suggest that fitness of susceptible individuals is relatively greater than the fitness of either resistant heterozygote or homozygote organisms (Brown & Pal, 1971, Clarke & Mackenzie, 1987). In this context, Bourguet and colleagues (2004), studying the fitness cost of insecticide resistant *Culex pipiens*, found that the effect of resistant genes was an increasing of developmental time as well as a decreasing of wing length in mosquitoes resistant compared to susceptible individuals. Otherwise, Roush & McKenzie (1987) have referred that the presence of the pesticide may alter in great extent the relative fitness for individuals carrying the *R* allele to be favoured by selection.

Fitness also has been evaluated in relation to biological control agents. *Bacillus sphaericus* susceptible and resistant strains of *Culex quinquefasciatus* from both the laboratory and field were compared in absence of *Bacillus sphaericus* (Rodcharoen & Mulla, 1997). Resistant strains had significantly lower fertility and fecundity and were disadvantaged in the absence of *B. sphaericus*.

Ae. aegypti are able to use the bloodmeal for flight energy and survival. Thus, in mark-release-recapture studies in Thailand, Day *et al.*, (1994) found that sugar-starved *Ae. aegypti* females could survive without significant reduction of reproductive fitness, because they can use blood for eggs production and alternatively for other no reproductive energy activities (Day, *et al.*, 1994) Moreover, this observation has important implications for virus transmission, because without sugar, females increase their blood-feeding frequency (Scott *et al.*, 1993, 1997, Harrington *et al.*, 2001) and consequently virus transmission. In the same context, Scott & colleagues (1997) reported that *Ae. aegypti* fed only human blood had a greater net

replacement rate and intrinsic growth average, during all the phases of their reproductive life, in comparison with females fed on human blood and sucrose.

One very important factor to consider here is the effect or impact of competition, where the ultimate effect on individuals is a decreasing in contribution to the next generation compared to what would be without any competitors (Begon, 1996). Such competition can be intra or interspecific. Expressed simply: in intraspecific competition, only some individuals become deprived, but in interspecific competition all individuals of one species suffer a reduction in fecundity, survival or growth as a result of resource exploitation or interference by individuals of another species (Begon, 1996).

Studies on the competition in container-breeding mosquitoes such as *Ae. aegypti* (which is most typically intraspecific), have documented negative density-dependent effects on population growth, individual growth, individual fecundity, developmental time and adult longevity (Ho *et al.*, 1989, Walker *et al.*, 1991, Juliano, 1998, Lounibos *et al.*, 2001, Alto *et al.*, 2005, Reiskind & Lounibos, 2009).

Alto and colleagues (2005), evaluated the effects of intra and interspecific larval competition, and whether competitive effects carried over into the adult stage may influence the competence for arbovirus infection (Sindbis virus) in both *Ae. aegypti* and *Ae. albopictus* females. They found, poor performance under crowded larval conditions compared to consistently shorter time to emergence, greater survivorship, and greater adult size under uncrowded larval conditions. However, regarding to arbovirus infection, for *Ae. albopictus*, but not for *Ae. aegypti*, the competition resulted in greater infection, body titter, and dissemination rates compared to low-competition conditions (Alto *et al.*, 2005).

Similarly, contrasting results have been obtained in *Ochlerotatus triseriatus*, whose smaller adults obtained from larvae exposed to low food availability, were more

likely to transmit La Crosse encephalitis virus than their larger counterparts (Grimstad & Haramis 1984, Grimstad & Walker 1991), whilst adults obtained from pupae collected from field, when orally infected with the same virus, transmission rates were negatively correlated with adult size (Paulson & Hawley 1991). On the other hand, Sumanochitrapon and colleagues (1998), reported that large *Ae. aegypti* females, obtained under varying conditions of larval crowding and food availability disseminated dengue-2 virus more efficiently than did smaller females (Sumanochitrapon *et al.*, 1998).

Apparently, this seems to indicate that different ecological conditions encountered by larvae in breeding sites, may exert variable effects on the interaction of mosquitoes with arboviruses that they transmit (Alto *et al.*, 2005).

Reiskind & Lounibos (2009), evaluated in the laboratory how intraspecific larval densities affect the longevity of mosquitoes adults maintained at high (85%) or low (35%) of relative humidity. They found a significant negative effect of competition on adult longevity in *Ae. aegypti*, but not in *Ae. albopictus*. Thus, their results suggest that longevity of adults in desiccating conditions is affected by the degree of larval competition in *Ae. aegypti* (Reiskind & Lounibos, 2009).

In these environments breeding mosquitoes, biotic and abiotic factors can influence larval development either positively or negatively. It has been reported that shaded habitats, food availability, container size, and temperature play an important role on development rates and survival of mosquitoes (Christophers, 1960, Nasci, 1988, Tun-Lin *et al.*, 2000, Alto & Juliano, 2001, Barrera *et al.*, 2006, Harrington *et al.*, 2008). These factors are not the only important ones to normal development of immature stages, but they are directly associated with the size and vectorial capacity of mosquitoes emerging from such containers (Nasci, 1988, 1991, 1994, Klowden *et al.*, 1988, Mahmood *et al.*, 1997, Schneider *et al.*, 2004, Reiskind *et al.*, 2009).

In high-density environments, intraspecific competition may be high, and a major limiting factor on the size and fitness of those mosquitoes that emerge successfully (Broadie, 1991, Mahmood, 1997), with important consequences for vectorial capacity of the mosquito population and disease transmission locally. Smaller female mosquitoes live shorter lives (Packer & Corbet, 1989), and have reduced reproductive success (Nasci, 1987); importantly, they can be poorer dengue vectors (Sumanochitrapon *et al.*, 1998).

Several researchers have shown that larval competition could affect susceptibility of adult *Aedes* to virus infection. In addition adult size of vectors also may affect their ability to transmit pathogens (Nasci, 1994, Alto, 2008).

5.1.3 Measuring mosquito size by morphometry

Given the important influence of larval environment on adult mosquito body size and the consequential impact potentially that this can have on vectorial capacity, measuring body size accurately is paramount. In the past, the commonly used proxy measurement for adult mosquito size was wing length, measured simply as the length from the wing tip to the arculus (reviewed in Spiers, 2003). This simplistic method measures only wing length and does not take into consideration any variation in shape that might also occur in response to changes in overall body shape, asymmetry, or body mass. A more thorough method for this purpose is the use of morphometrics.

Morphometrics is the measurement of shape, which following the modern computer breakthroughs of the 1980s, which permitted the invention of coordinate-based analytical methods, the discovery of the statistical theory of shape, and the computational realization of multidimensional grids evolved into 'Geometric morphometrics' or 'geomorphometrics' (Mitteroecker & Gunz, 2009). The name derives from its ability to preserve the geometry of the coordinate or landmark configurations throughout the analysis, thus permitting the representation as statistical

results of the actual shapes or forms of the organisms. This permits the separation of metric variation into size and shape, and the visualisation of the changes occurring in the shape (Jirakanjanakit *et al.*, 2007a).

In geometric morphometrics, size and shape are very closely related concepts. Kendall (1984), defined shape as all of the geometric information remaining after the effects of location, scale and rotation are removed from an object. The scale with which the coordinate data is captured provides a definition of size which is independent of the definition of shape (Zelditch *et al.*, 2004). For this reason, before computing geometric scale, it is necessary to establish the location of the centre of the form (termed the "centroid") and calculates the distance between each landmark and the centroid (Zelditch, *et al.*, 2004). After this procedure, the geometric scale is computed by calculating the square of each of those distances, summing all the squared distances, and then taking the square root of that sum (Zelditch *et al.*, 2004). In geometric morphometrics, this quantity is called the "centroid size" (Zelditch *et al.*, 2004).

Dujardin & Slice (2007), describe geometric morphometrics as a powerful and cheap tool, which can be applied to characterize different organisms, particularly in groups of medically important insects. It has been successfully applied to different studies on natural populations of Chagas disease vectors (Jaramillo, *et al.*, 2002, Villegas *et al.*, 2002, Feliciangeli *et al.*, 2007, Dujardin *et al.*, 2007); leishmaniasis vectors (Dujardin *et al.*, 2002) and tsetse flies, the vectors of sleeping disease (Camara *et al.*, 2006).

The method has been used numerous times to investigate different aspect of insect biology, particularly inter and intraspecific differences in insects, including vectors (Dujardin *et al.*, 1997, 1998, 2003, Aytekin, *et al.*, 2007, Rodriguez *et al.*, 2007, Dujardin, 2008, Schachter-broide, *et al.*, 2009). Thus, studies on the morphological differences between a *Pastrongylus geniculatus* wild population and its laboratory descendants, suggested that by morphometrics analyses it is possible to decipher the

effect of synantrophic behaviour upon the metric properties of insects (Dujardin *et al.*, 1997, Schofield *et al.*, 1999, Jaramillo *et al.*, 2002). Similarly, Soto-Vivas and colleagues (2007) reported that it was possible to identify various samples of *Triatoma maculata* captured in and around dwellings as belonging to the same population, ruling out the possibility that sub-populations of this species were developing adaptive mechanisms to human dwellings.

Feliciangeli *et al.*, (2007) used the technique to show that sylvatic populations could re-infest human dwellings after insecticide applications (Feliciangeli *et al.*, 2007). Morphological studies also have demonstrated their usefulness for identifying geographical and seasonal variations in medically important insects (Casini *et al*, 1995, Monroy *et al.*, 2003, Schachter-Broide *et al.*, 2004, 2009, Dujardin & Le Pont, 2004, Dujardin *et al.*, 2005).

Morphometric geometry has been successfully applied to differentiate *Ae. aegypti* and *Ae. albopictus* natural populations. Henry and colleagues (2009) compared *Aedes sp.* populations from Pacific Islands, North and South America and South East Asia. They found that *Aedes* wing geometry was able to accurately distinguish between both *Aedes* species studied, and also significant intraspecific geographic differentiation was encountered for *Ae. aegypti* from Colombia and for *Ae. albopictus* from Thailand (Henry, *et al.*, 2009). Likewise, Morales and colleagues (2010) studied the relation of metric properties of *Ae. aegypti* natural populations collected in a dengue hyperendemic area of Thailand, in relation to temperature and relative humidity. Although they did not find any significant correlation between temperature and wing geometry, a significant correlation with relative humidity was found (Morales, *et al.*, 2010).

Jirakanjanakit and colleagues (2005, 2008) showed the power of this tool to discriminate the geographic origin of *Ae. aegypti* laboratory lines through successive generations based on wing geometry. These authors also studied how larval density

and food availability can affect adult metric properties based on *Ae. aegypti* wing geometry (Jirakanjanakit *et al.*, 2007a). They found a positive correlation with increasing food concentration and a negative correlation with larval overcrowding.

5.1.4 Long-term effects of ITMs on mosquito fitness

In Trujillo state, although *Ae. aegypti* can be found breeding in many kinds of domestic water containers, data obtained from the control vector department indicated that the main breeding sites for immature *Ae. aegypti,* are the large domestic water storage drums used to store water (see Figure 3.3, Chapter 3). Other containers such as tyres and numerous different kinds of discarded small bottles and cans, are also important for *Ae. aegypti* (Figure. 5.1).

It was on this basis that the use of the insecticide-treated water jar covers was implemented (Chapter 3). If effective, it is inevitable that this intervention would profoundly affect *Ae. aegypti* populations in the long term: elimination of the population is one possibility; if covers retained insecticidal efficacy but did not have such a complete effect, then insecticide resistance might arise (see Chapter 4); if water container covers did not effectively deliver insecticide but formed an effective physical barrier that prevented vector access to the preferred breeding sites, then the *Ae. aegypti* population would comprise only those that emerged from smaller containers (such as bottles, cans, car tyres) in the household or from untreated public areas. In these high-density environments, intraspecific competition would be expected to be high, with fewer and smaller mosquitoes emerging (Broadie & Bradshaw, 1991, Mahmood *et al.*, 1997). Moreover, the smaller volumes will be more susceptible to wider diurnal fluctuations in water temperature and may reach higher daytime maxima than larger domestic water barrels (although this may not be an entirely simple relationship; smaller vessels might contain more organic matter and result in large adults – see Tun-Lin *et al.*, 2000).

The consequences could be significant. Smaller female mosquitoes live shorter lives (Packer & Corbet, 1989, Takken *et al.*, 1998), and have reduced reproductive success (*e.g.* Nasci, 1987, Briegel, 1990) and importantly might be poorer dengue vectors (Sumanochitrapon *et al.*, 1998). Shorter-lived females are less likely to transmit infections, as they may not live long enough to become infective (Watts *et al*, 1987). Reduced fecundity might also negate vertical transmission of dengue in *Ae. aegypti*, a species with already low rates of filial transmission. Finally, poorly-fed *Ae. aegypti* larvae often fail to seek hosts as adults (Klowden *et al.*, 1988). However, whereas some studies have failed to demonstrate interaction between body size and dengue viruses dissemination (Schneider *et al.*, 2007), other have reported that smaller *Ae. aegypti* females showed greater probability of a dengue virus infection or disseminated infection than larger females (Alto *et al.*, 2008). Thus, while the local vector population would be likely to survive, albeit at a much reduced level, its fitness and vectorial capacity could be significantly compromised. The trial reported in chapter 3 provided a unique opportunity to pursue this investigation, the results of which are dealt with here.

5.2 Materials and methods

5.2.1 Sampling Method

This study was carried out between July 2006 and April 2008, in Trujillo state, Venezuela, in the same locations (Flor de Patria, Pampán, Pampanito, Motatán and Monay) and coinciding with the cluster randomised trial of ITMs for dengue control reported in chapter 3.

Pupae for the study were collected during four different time periods: June – July 2006, April 2007, October 2007 and April 2008, when all internal and external containers holding water in households were inspected for immature stages of *Ae.*

aegypti. Using fine mesh colanders with a long handle and pipettes, all pupae were collected (Figure 5.1), counted and transported live to the laboratory, for completion of development to adulthood. Containers were categorized as either drums, small containers (including [discarded] bottles, cans, plastic cups) or tyres. Only pupae were collected for use in these experiments to ensure the entire larval developmental period had been spent under the influence of that site (Scott *et al.*, 1997).

Figure 5.1. Different types of holding-water containers as Ae. aegypti *breeding sites.*

5.2.2 *Rearing in the laboratory*

On confirmation of identity in the laboratory, pupae were maintained (Figure 5.2) separately (according to the type of container and locality that the females originated from) at density of 100 pupae distributed into 4 small polystyrene trays (12 x12 x 4

cm), with 200ml of water and then maintained into 30x30x30 cm screened entomological cages, or 20 pupae per polystyrene cup of 700 ml of capacity, filled with 400 ml of water and then covered with a nylon mesh top. Both cages and cups were provided with water-soaked cotton pledgets. They were reared at room temperature. After emergence mosquitoes adults identified as *Ae. aegypti* were kept for the experiments and the species of mosquitoes as *Limatus*, *Ochlerotatus*, *Toxorrynchites* and *Culex* were excluded.

Figure 5.2. Maintainance of Ae. aegypti *from pupa to adult*

5.2.3 *Adult emergence*

Females and males were caged together for at least 3 days to allow mating to occur. From each rearing batch, one group of mosquitoes were bloodfed and used in subsequent fitness experiments (see below) and a second group was provided with water only in order to be used for morphometric analyses (see section 5.2.7).

5.2.4 *Blood-feeding and maintenance of mosquitoes*

At 3 days post emergence, females were fed with fresh human blood, using an artificial membrane feeder with newborn chicken skin membrane (Figure 5.3). All females fed at the same time were maintained together until the next day, when they were separated and stored individually if they fed to repletion (verified by visual inspection). From the group of fed females, subgroups of 15 mosquitoes from each container from each intervention sector were individualised at each time point (July-2006, April-2007, October-2007 and April-2008).

Figure 5.3. Artificial membrane feeding system for Aedes aegypti, *with fresh human blood and newborn chick skin membranes.*

Individual females were kept in white 350ml polystyrene cups, with a damp cotton pad on the bottom and lined with filter paper for oviposition. Cups were stored in white polystyrene containers and protected from ants with Vaseline. Humidity and temperature were recorded every day. Each female was offered one opportunity to feed every 48 hours for 5 minutes, using the methodology described above.

5.2.5 Oviposition

Every day between 07.00 and 11.00, every female was checked and papers with eggs were removed for counting and replaced with a new paper. After 3 or 4 days if some females did not lay eggs, oviposition papers and sometimes the containers, were changed to avoid fungal attack.

Mosquitoes that were damaged or killed accidentally were excluded from the study.

5.2.6 Protocol for comparison of fitness

The different parameters that were measured in each mosquito were defined as follows:

1. *Longevity:* adult mosquito lifespan, measured in days from day of emergence until death.

2. *Insemination rate*: In the three groups of females (from drums, tyres and small containers) the insemination rate was recorded as the percentage of spermathecae positive for sperm presence, by dissecting *Ae. aegypti* females after death. (Figure 5.4).

3. *Number of Bloodmeals*: The number of blood meals taken by each female from 3 days post emergence until death, and the number of bloodmeals required before the first egg-oviposition.

4. *Total number of eggs laid*: The total number of eggs laid by each female during its lifetime, providing an estimate of reproductive performance (*mx*: half of eggs laid, assuming a 1:1 sex ratio).

5. *Number of days for eggs laid*: the number of days before the first eggs batch was laid laid by each *Ae. aegypti* female.

6. *Retained eggs*: The numbers of eggs retained by each female, which were counted on dissection of females after death.

7. *Hatching rate*: The percentage of eggs that hatched from the total number of eggs that each female laid in its lifetime.

Life tables were constructed to compare the fitness parameters of females from the three categories of container (drums, small containers and tyres) from where they were collected as pupae. With the data on survival and reproduction the following were calculated for mosquitoes coming from each container (Price, 1984):

- reproductive expectation (*lxmx*)
- net replacement rate ($R_0 = \sum l_x m_x$)
- intrinsic rate of growth ($r = \log_e R_o/T$)

Where *lx* = age-specific survival

mx = reproduction (half the eggs laid, assuming a 1:1 sex ratio)

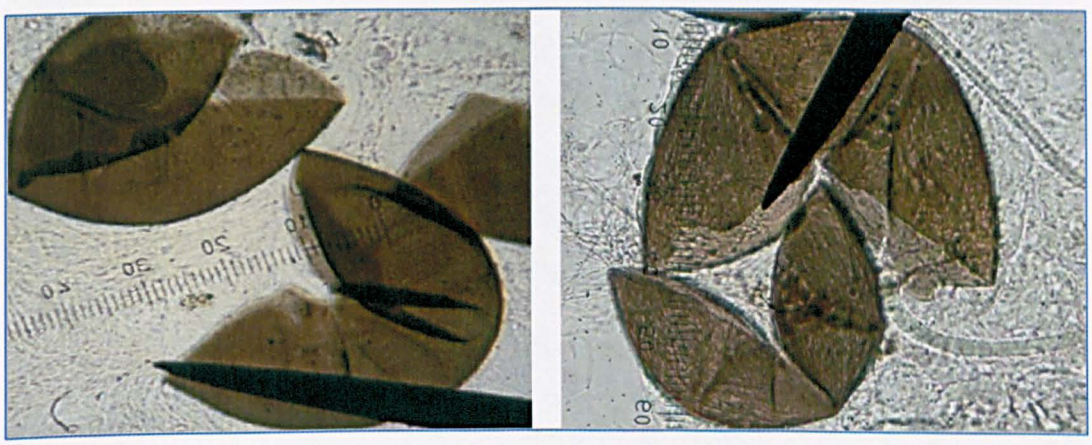
R_0 = *net replacement rate*

r = *intrinsic rate of growth*

T = the mean generation time

The net replacement rate is defined as "the number of daughters that replace an average female in the course of generation" (Price, 1984). The intrinsic rate of growth is defined as "the number of (female) progeny produced per unit time" (Price, 1984). Thus it is considered that females exhibiting a high net replacement rate and a high intrinsic rate of growth are fitter, in evolutionary terms (Scott *et al.*, 1997).

Figure 5.4. Dissection of Ae. aegypti *female following death, to examine for the presence of sperm in the spermatheca; the individual on the left was not inseminated, while the figure on the right shows the presence of numerous sperm.*



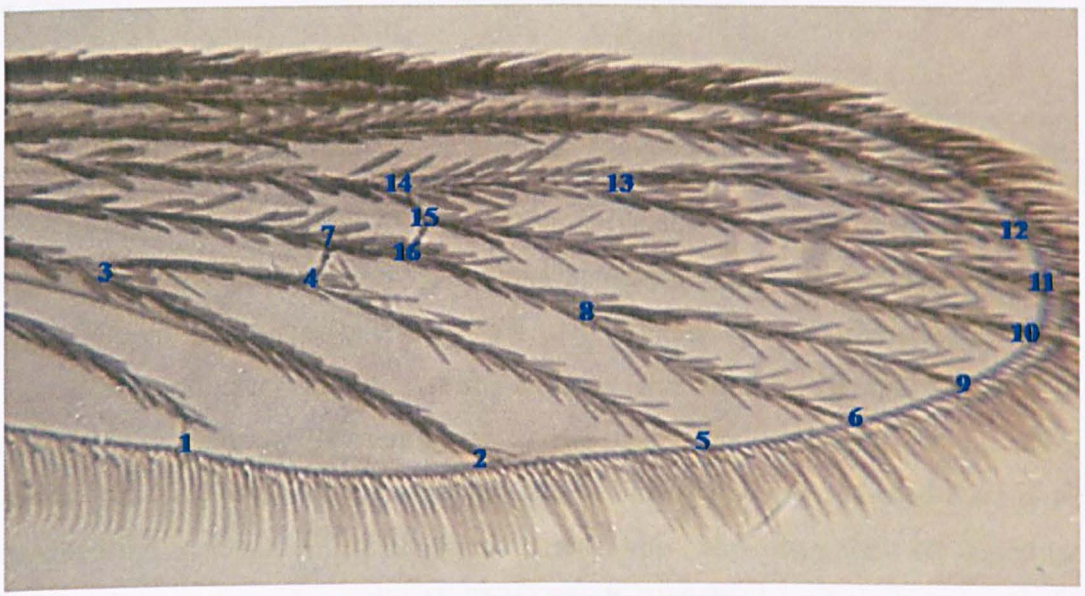
5.2.7 *Sample preparation and data collection for estimation of body size by the centroid size*

Three days after emergence (to allow their wings to attain full size), females were removed from their cups by mouth aspirator and killed by freezing. The right wings were detached from the thorax, placed on a new clean microscopic slide, and then secured with Euparal under the cover slip. Removing wing and positioning the cover

slip were done under a stereo-microscope Leica (EZ4). After slides were dried, using a phase contrast microscope with a 6.54X lens and a digital camera (Nikon Coolpix P5100, 12.1 mega pixels), wing images were captured, by placing each wing at the centre of the visual field, to avoid any possible distortion at the optical lens periphery. Only the right wing was measured, as it has referred that both right and left wigs have the same size (van den Heubel, 1963, Gleisser *et al.*, 2002).

A set of 16 landmarks (LM) covering most of the wing surface was selected and digitized on the computer screen. All of LMs were "type I" (venation intersections, see Bookstein, 1991) (Figure 5.5).

Figure 5.5 Landmarks on the Aedes aegypti *female wing used for morphometrics analysis, with numbers referring the order of landmarks collection.*



5.2.8 Statistical analysis

Data were introduced in Excel and analysed using SPSS (Statistical Package Social Sciences software, SPSS Inc 17.0) by Kruskal-Wallis and Mann-Whitney *U* non-

parametric tests. A Kaplan-Meier procedure was conducted to compare longevity among all three groups of mosquitoes coming from drums, tyres and small containers. The comparison of the frequency of positive inseminated females was analysed by a Chi-square test procedure for independence among groups. To obtain the centroid size values, collections of LMs, as well as, all other following analysis were done by using the modules (COO, TET, MOG, VAR, PAD and COV) developed by Dujardin, J.P. to performance morphometrics analysis, which are available on http://www.mpl.ird.fr/morphometrics/ provided under GPL license (General Public License). Coordinates data taken in pixels was later transformed into mms, to conducted non-parametric test for comparisons among the groups of females from different containers (drums, small containers and tyres).

5.3 Results

A total of 1020 mosquitoes from each type of breeding sites were processed from pupae collected in July 2006, April 2007, October 2007, April 2008. The results obtained in the first analysis (Kaplan-Meier Survival Time) revealed no statistically significant differences between the groups of mosquitoes from the same container types (drums) that were collected at the four different times or from the different localities under intervention where the study was undertaken. Similar results were recorded analysing the mosquitoes from small containers comparing all four times and by intervention arms. Likewise the analysis of mosquitoes collected from tyres revealed the same pattern, at all four different times, and when were compared by interventions sectors (Appendix 1). These results permitted pooling the mosquitoes for comparison of fitness according to container type. From each intervention arm and from the internal and external controls 15 mosquitoes were analysed at each timepoint.

5.3.1 Longevity

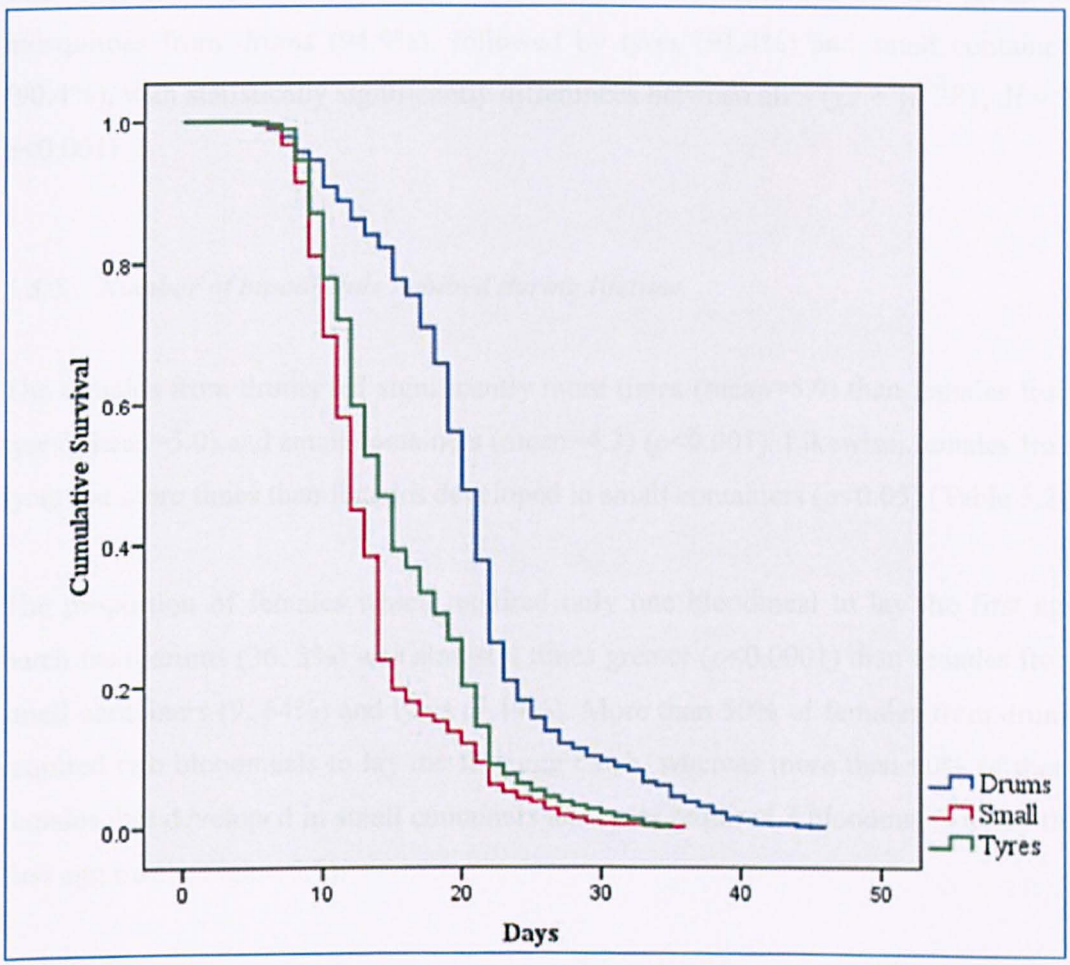
Mosquitoes that developed in drums had a significantly longer lifespan ($p<0.001$) than those originating in small containers and tyres. Likewise females developed in tyres had a significantly longer lifespan than females developed in small containers ($p<0.001$). These results are showed in the table 5.1 and Figure 5.6.

Table 5.1 Mean lifespan in days (SE and 95% CI) of Ae. aegypti *from drums, small containers and tyres.*

			95% Confident Interval		
Container	**No. Mosquitoes**	**Mean lifespan (days ± SE)**	**Lower Bound**	**Upper Bound**	**Max lifespan (days)**
Drums	**1020**	20.31 ± 0.22^{a}	19.88	20.75	46
Tyres	**1020**	15.35 ± 0.18^{b}	15.01	15.70	35
Small	**1020**	13.34 ± 0.15^{c}	13.04	13.64	36

Means followed by different letter were statistically significant from each other ($p<0.001$)

Figure 5.6 Cumulative survival of Ae. aegypti *female from different container type. Each line represents the data from mosquitoes pooled according to container type. From each container typer, int toal 1020 mosquitoes were evaluated during the study.*



Initially, survival of the three groups of females was similar, however by day ten, this trend changed significantly, and females from drums had a survival advantage compared to females collected from small containers and tyres. Likewise, by day twelve, females from tyres demonstrated longer survival than those from small containers (Wilcoxon (Gehan) Statistic=602,130, df=2, p<0.001).

5.3.2 Insemination rate

The highest proportion of females inseminated was recorded in the group of mosquitoes from drums (94.9%), followed by tyres (91.4%) and small containers (90.4%), with statistically significantly differences between all 3 ($\chi 2 = 15\ 381$, df = 2, $p<0.001$).

5.3.3 Number of bloodmeals imbibed during lifetime

The females from drums fed significantly more times (mean=5.9) than females from tyres (mean=5.0) and small containers (mean=4.3) ($p<0.001$). Likewise, females from tyres fed more times than females developed in small containers ($p<0.05$) (Table 5.2).

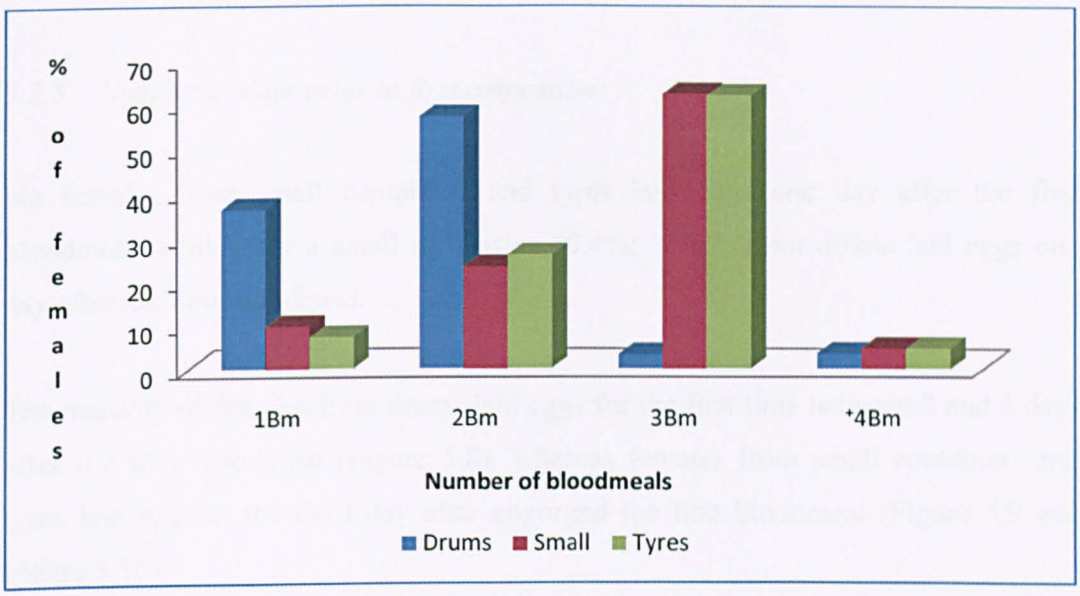
The proportion of females which required only one bloodmeal to lay the first egg batch from drums (36. 3%) was almost 4 times greater ($p<0.0001$) than females from small containers (9. 64%) and tyres (7.19%). More than 50% of females from drums required two bloodmeals to lay the first egg batch, whereas more than 60% of those females that developed in small containers and tyres required 3 bloodmeals to lay the first egg batch (Figure 5.7).

Table 5.2 Mean and standard deviation of the number of bloodmeals, total numbers of eggs, retained eggs and hatched eggs recorded from groups of Ae. aegypti *females collected as pupae from drums, small containers and tyres.*

No. Blood meals			
Containers	**No. mosquitoes**	Mean	(±) Sdev
Drums	**1020**	5.9^{a}	2.7
Small	**1020**	4.3^{b}	2.2
Tyres	**1020**	5.0^{c}	2.5
	Total no. Eggs		
Drums	**1020**	75.9^{a}	59.2
Small	**1020**	44.3^{b}	37.0
Tyres	**1020**	45.2^{b}	39.4
	Retained Eggs		
Drums	**1020**	6.2^{a}	12.8
Small	**1020**	7.9^{b}	15.0
Tyres	**1020**	7.7^{c}	11.9
	Hatched Eggs		
Drums	**1020**	39.9^{a}	25.9
Small	**1020**	19.3^{b}	24.4
Tyres	**1020**	20.8^{b}	27.4

Means followed by different letter were statistically significant from each other ($p<0.05$)

Figure 5.7 Proportion of Ae. aegypti *females from drums, small containers and tyres that laid their first egg batch after one to four bloodmeals (Bm).*



5.3.4 Total number of eggs laid

From the total of females evaluated from each container, 26% (263/1020), 25% (252/1020) and 29% (297/1020) (drum, small and tyres respectively) never laid eggs during their life. The mean total number of eggs laid by females from drums was significantly ($p<0.001$) higher than means of females from tyres and small containers (drums=75.9; tyres=45.2 and small=44.3). The difference between females developed in tyres and small containers was not statistically significantly ($p=0.83$) (Table 5.2).

Of those females from drums which did not lay eggs (263/1020), 80% (211/263) were inseminated but only 9% (19/211) had retained eggs. On the other hand, from the proportion of females from small containers which did not lay eggs in their lifetime (252/1020), 62% (157/252) were inseminated and 27% (43/157) retained eggs, whereas, in the group from tyres, from the proportion which did not lay eggs

(297/1020), 68% (201/297) were inseminated, and of these, 38% (77/201) retained eggs.

5.3.5 Number of days prior to first oviposition

No females from small containers and tyres laid eggs one day after the first bloodmeal, while only a small proportion (0.4%; 3/757) from drums laid eggs one day after the first bloodmeal.

The majority of females from drums laid eggs for the first time between 2 and 3 days after the first bloodmeal (Figure 5.8), whereas females from small containers and tyres laid eggs at the third day after engorged the first bloodmeal (Figure 5.9 and Figure 5.10).

Figure 5.8 Proportion of Ae. aegypti *females from drums which laid the first eggs batch, one, two, three, four and five days after their first bloodmeal.*

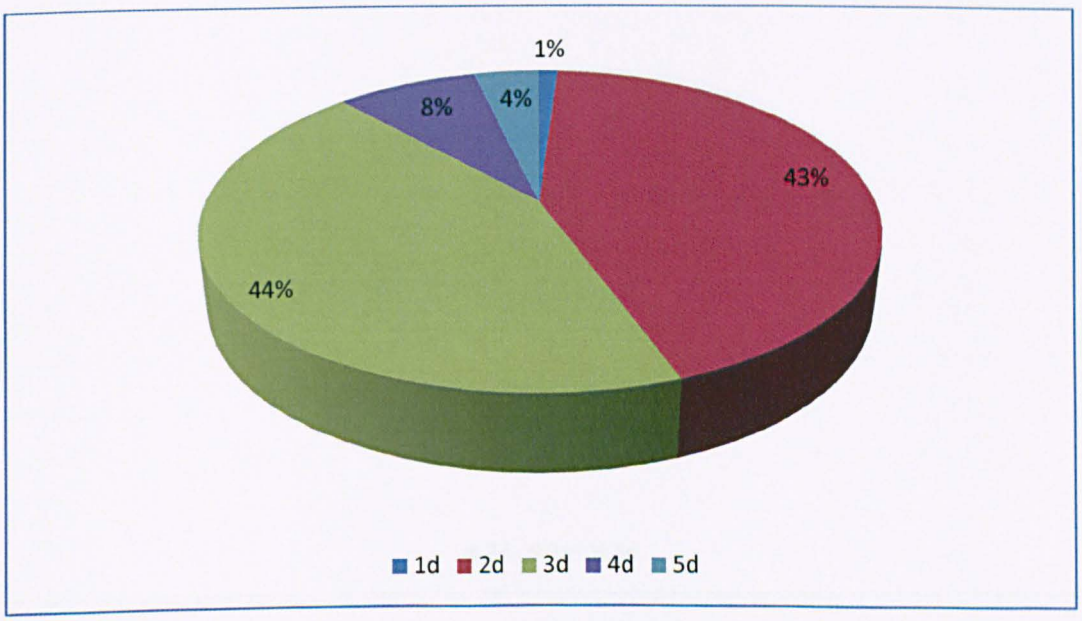


Figure 5.9 Proportion of Ae. aegypti *females from small containers, which laid the first eggs batch two, three and four days after their first bloodmeal.*

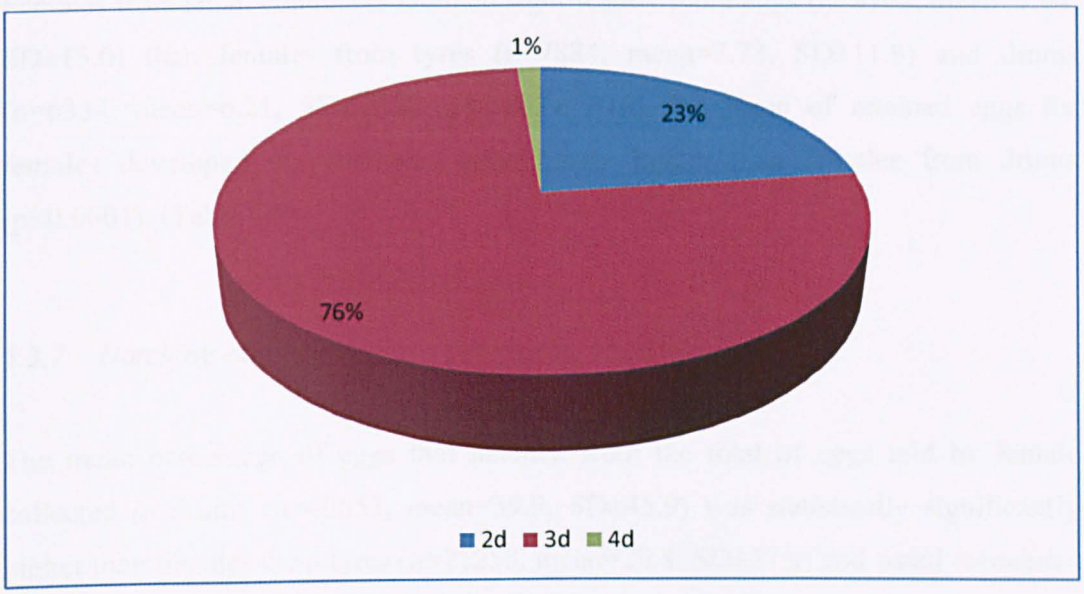
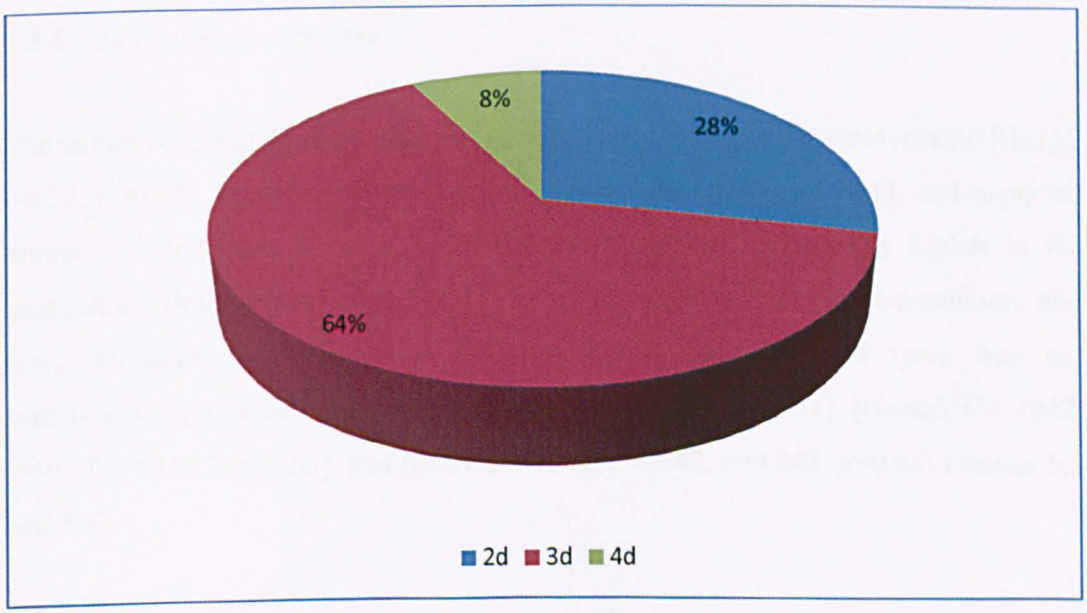


Figure 5.10 Proportion of Ae. aegypti *females from tyres, which laid the first eggs batch two, three and four days after their first bloodmeal.*



5.3.6 *Retained eggs*

Females from small containers retained significantly more eggs (n=8102, mean=7.94, SD±15.0) than females from tyres (n=7884, mean=7.73, SD±11.9) and drums (n=6334, mean=6.21, SD±12,8) ($p<0.001$). Also, the mean of retained eggs for females developed in tyres was significantly higher than females from drums ($p<0.0001$). (Table 5.2).

5.3.7 *Hatching rates*

The mean percentage of eggs that hatched from the total of eggs laid by female collected in drums (n=40653, mean=39.9, SD±45.9) was statistically significantly higher than females from tyres (n=21258, mean=20.8, SD±27.4) and small containers (n=19647, mean=19.3, SD±24,4) ($p=0.001$). However the difference between the last two groups was not significant ($p=0.81$).

5.3.8 *Life tables parameters*

The values obtained from the analysis of parameters age-specific survivorship [(lx) $\chi 2 = 6.27$, $p=0.04$], reproductive expectation [(lxmx) $\chi 2=18.79$, $p<0.001$], and expected number of daughters [(mx) $\chi 2=26.19$, $p<0.0001$], were statistically higher in the mosquitoes from drums compared to those recorded in both small containers and tyres. However the comparison between small containers and tyres was not statistically significant [(lx) $U= 1038$, $z=-0.51$, $r=0.052$, $p=0.61$], [(lxmx) $U= 1042$, $z=-0.48$, $r=0.047$, $p=0.63$], and [(mx) $U= 1044$, $z=-0.47$, $r=0.048$, $p=0.64$] (Tables 5.3 and 5.4).

The net replacement rate from drums (Ro= 460) was three times greater than that of mosquitoes from small containers (Ro=151) and 2.7 times greater than that

mosquitoes from tyres (Ro=171). Similarly the results obtained for the intrinsic rate of growth (r=0.7) indicated that mosquitoes from drums had a higher potential for an increase in population size, followed by the group from small containers (r=0.5) and tyres recorded the lowest value (r=0.4) (Table 5.3).

Table 5.3 Demographic parameters measured in Ae. aegypti *females originating in drums, small containers and tyres [lx = age-specific survival; mx = reproduction (half the eggs laid, assuming a 1:1 sex ratio); lxmx = reproductive expectation; Ro = net replacement rate; r = intrinsic rate of growth].*

	lx	*mx*	*lxmx*	*Ro*	*r*
Drums	1.8	35.8	9.8	460	0.7
Small containers	1.6	16.9	3.2	151	0.5
Tyres	1.1	15.1	3.6	171	0.4

Table 5.4 Chi-square and statistical significance of demographic parameters of Ae. aegypti *females originating in drums, small containers and tyres [lx = age-specific survival; mx = reproduction (half the eggs laid, assuming a 1:1 sex ratio); lxmx = reproductive expectation].*

	lx	*mx*	*lxmx*
$\chi 2$	6.27	26.19	18.79
U	1038	1044	1042
z	-0.51	-0.47	0.48
r	0.052	0.048	0.047
p	0.61	0.64	0.63

r = z/square root of *N*

5.3.9 Adult body size estimated by centroid size

In total 5,103 *Ae. aegypti* female wings from drums, small containers and tyres were digitalised. The numbers of wings from each container, as well as the number digitalised at pre and post intervention are shown in the Table 6.2. Overall, *Ae. aegypti* female size, as estimated by centroid size, of mosquitoes from drums was significant greater than those from both small containers and tyres [X^2 (2, n=5,103) = 3440, p<0.001].

Comparisons of females collected at baseline with those at the end of study, also revealed some differences in size between individuals within the same breeding site type. Mosquitoes from drums and small containers collected at baseline were significantly smaller than collected at the end of study from the same site types (U=246258, z=-8.44, p<0.001 and U=227272, z=-14.812, p<0.001 respectively). In contrast, the mosquitoes from tyres were of similar size at both time points (U=366028, z=-1.004, p=0.316).

Table 5.5 Number of Ae. aegypti *female wings from drums, small containers and tyres digitalised in both time points, before and after the insecticide treated materials were deployed in the localities under study. Mean, Std. Deviation, lower and upper limits at 95% CI and ranges are shown for groups of mosquitoes from each breeding site.*

	Drums		Small		Tyres	
	pre	post	pre	post	pre	post
Mean (mm)	2.28	2.32	1.82	1.89	1.80	1.80
Std Dev	0.11	0.07	0.10	0.08	0.10	0.05
Lower Bound	2.27	2.31	1.82	1.89	1.78	1.80
Upper Bound	2.29	2.32	1.83	1.90	1.81	1.81
Min	2.05	2.11	1.63	1.73	1.62	1.70
Max	2.58	2.45	2	2	2	1.94
Number of wings	776	838	884	869	847	889

Table 5.6 Chi-square and statistical significance of Ae. aegypti *female wing size from drums, small containers and tyres digitalised in both time points, before and after the insecticide treated materials were deployed in the localities under study.*

	Drums (pre vs. post)	Small (pre vs. post)	Tyres (pre vs. post)
$\chi 2$	**	74.28	101.9
U	246258	227272	366028
z	-8.44	-14.812	-1.004
p	<0.001	<0.001	0.316

* This value was not possible to calculate, due to the presence of several identical values in the data.

5.4 Discussion

The conditions in a particular container have a great influence on the development and survival of mosquitoes breeding there. Therefore the choice of environment, made by *Ae. aegypti* gravid females laying eggs is crucial as it will have a profound

impact on the condition and chance of reproductive success of her offspring, especially in a species which does not invest in parental care of its progeny, (Christopher, 1960, McCall & Cameron, 1995, Torres-Estrada *et al.*, 2001).

The main goal of this study was to evaluate whether the type of container in which the immature stages developed could affect fitness of *Ae. aegypti* females, hypothesising that mosquitoes emerging from bigger containers such as drums would exhibit a greater level of fitness than those from small containers or tyres, because of the lower rates of intraspecific competition in the larger sites. In these low volume/ surface area and highly competitive environments, one of the most important influences on larval development, and subsequent adult fitness, is the availability of food. Adult mosquito fitness depends on reserves accumulated during larval development and adult life (Briegel, 1990), which is supported by the observations in the study reported here.

The results obtained clearly demonstrated that this hypothesis seems to be correct. A statistically significant difference was recorded with regard to all measures of adult fitness in *Ae. aegypti* from drums as compared to other container types. *Ae. aegypti* females from drums lived significantly longer than conspecifics from small containers and tyres. Moreover, females from drums were able to lay a significantly higher number of eggs than the others, and were bigger, as estimated by centroid size. These results are consistent with previous reports showing that smaller female mosquitoes live shorter lives (Packer & Corbet, 1989, Takken *et al.*, 1998), and have reduced reproductive success (Nasci, 1987, Briegel, 1990).

Schneider and colleagues (2004) earlier reported a positive correlation between *Ae. aegypti* adult female mosquito size (wing length) and the size (diameter) of containers they emerged from. Elsewhere it was found that smaller females emerging from breeding sites with reduced food availability require more blood meals before start reproduction than larger females (Takken *et al.*, 1998). Scott and colleagues (2000),

studying natural populations of *Ae. aegypti* from Thailand and Puerto Rico, also reported that smaller *Ae. aegypti* females took more blood meals per gonotrophic cycle than larger females, which increases the number of hosts visited and the probability of acquiring and transmitting dengue virus. Results from the present study showing that mosquitoes from small containers and tyres required more blood meals before laying their first egg batch, support this finding. Conversely, the data showing that the smaller *Ae. aegypti* females from tyres and small containers lived shorter lives, would counteract that.

Although females from drums engorged a significantly higher number of bloodmeals, these results may well be a consequence of the higher longevity in the same group. Females from small containers and tyres took longer to lay their first eggs batch and fewer laid eggs after only one bloodmeal, compared to females from drums.

Importantly, drums represent the most important containers (in terms of numbers of pupae found) utilised by *Ae. aegypti* as breeding sites in most of municipalities in Trujillo State. Without a reliable water supply, the habit of storing water in inappropriate ways will continue to be the most significant obstacle to achieving sustainable dengue vector control. However, the impact of insecticide-treated water jar covers on *Ae. aegypti* as shown in this study (Chapter 3), suggests that an effective method for reducing the importance of these sites in producing large numbers of potentially highly competent vectors, may be available.

The results obtained in this study clearly confirm previous observations regarding the variable productivity of different container types (Focks & Chadee, 1997, Tun-Lin *et al.*, 2009), and emphasize, particularly in these localities, the epidemiological importance of drums. Targeting only the most productive water storage container type (drums) can be an alternative option, especially when the control resource budget is one of the main constraints faced by the control vector departments in developing countries. In this case, covers represent a useful and simple control

measure, which can have an immediate impact (which would be sustained, if the quality of the covers was better) in reducing entomological indices as shown in this study.

Moreover, the results suggest that clean-up campaigns, where householders are encouraged or instructed to remove discarded tyres, bottles, cans, etc. from their private properties, are unlikely to impact dramatically on dengue transmission, since not only do they waste time on less productive containers, but also because the evidence here suggests that the mosquitoes that emerge from them are far less likely to be important dengue vectors.

CHAPTER 6

GENERAL DISCUSSION

Dengue is a growing worldwide public health problem and it is generally accepted that current control measures available for use by health authorities worldwide have been and will be unable to greatly change this situation (McCall & Kittayapong, 2007). In Venezuela, reports by the Pan American Health Organisation (PAHO), show that here too, the incidence of dengue has been growing significantly in recent years (Figure 1.3, Chapter1). In Trujillo State, where important vector-borne diseases such as Chagas disease and leishmaniasis have been endemic for centuries, dengue has become another problem which further complicates the situation. The high prevalence of dengue, the presence of all four dengue virus serotypes and the high levels of infestation of *Ae. aegypti* all highlight the urgent need for Venezuela to improve dengue prevention and control efforts. In the absence of a vaccine for dengue in the short term, control of natural populations of *Ae. aegypti* remains the main weapon to achieve this. It is therefore essential that the current strategies implemented by the control vector departments should be subject to a detailed review in order to better redirect their activities. A new perspective for the management of the programmes must be adopted in order to address this serious and growing public health problem. Fundamental to these reviews is the incorporation of new effective vector control tools, such as ITMs.

The research carried out in this thesis puts forward evidence regarding the effectiveness of ITMs against *Ae. aegypti*. The results of the trial of ITMs in Chapter 3 indicates that while they may not be able to achieve the reductions in malaria vector abundance (and consequent reduction in malaria transmission) that insecticide-treated bednets can do, they clearly impact on domestic infestations of dengue vectors in treated and adjacent untreated households, and may be capable of achieving such reductions even at coverage levels of substantially less than 100%. The reduction in

entomological indices recorded during the study correlates with results obtained in previous trials carried out in Trujillo - Venezuela (Ogar, 2002, Kroeger *et al.*, 2006). Of the two types of ITMs used here, evidence suggests that insecticide-treated water jar covers in particular, may have the greatest potential in achieving the desired impact, all the more so as they also appear to target those individuals that potentially may have the highest vectorial capacity.

The assessment presented in Chapter 4 highlighted the importance and the need to study the insecticide susceptibility of wild populations prior to the introduction of control measures, especially if they involve the use of insecticides not previously used here. The systematic monitoring of *Ae. aegypti* populations would allow for timely detection of changes in susceptibility in advance of problems; permitting altering the control measures before resistance becomes a problem. The rapid appearance of reduced susceptibility to deltamethrin during the study is worrying and a reminder of the need for alternatives, or strategies that do not depend on a single family of insecticides.

On the other hand, Chapter 5 shows the results of the study on the fitness and size of mosquitoes breeding in the three most common types of water container in the studied areas, highlighting that the environmental conditions encountered by immature stages in these habitats, can prolong their effects well into the lifetime of the adult stages. Of importance here was the evidence that adult females that spent their immature period of development in domestic water containers were potentially the fittest individuals in the local population, since it was those breeding sites that were targeted by one of the ITMs used in the study.

Ae. aegypti is a highly domesticated mosquito species. Human habitations provide the conditions necessary for its complete development from immature to adult stages. It is therefore clear that the dengue health problem is highly linked to human behaviour, whether at the individual, community or institutional level (Rodriguez, 2002). The

implementation of ITMs, as used in this study, demonstrates that it is possible to have a significant impact on natural populations of *Ae. aegypti*. This was clearly verified by the immediate reduction recorded in entomological indices just one month after ITMs were delivered in this study. However, it has been suggested that sometimes the control of mosquito populations has little effect in preventing dengue outbreaks (Chadee *et al.*, 2005), and that such a reduction in mosquito populations by current approaches is unable to attain the threshold at which dengue transmission can be prevented (Troyo *et al.*, 2008).

Ironically, we do not know the level at which mosquito densities must be reduced in order to avoid dengue transmission, but it is clear that while immediate reductions in vector populations can be achieved by many methods, sustainability of control remains a challenge. ITMs, presented as covers and curtains, might be better accepted by communities than many other methods but their use will still require a permanent behavioural change by people living in areas under dengue risk, to achieve effectiveness. Acceptance, trust and sustained use might be accepted if these new ITMs can be shown to protect against the other vector-borne diseases such as leishmaniasis and Chagas disease, which are co-endemic alongside dengue in some places in Venezuela, and particularly, in Trujillo State (Kroeger *et al.*, 1999, 2003, Emami *et al.*, 2009, Joshi *et al.*, 2009).

This study revealed that in some areas of Trujillo State, such as those in which this research was conducted, there is an abundance of containers of varying shape and size that are potential breeding sites for *Ae. aegypti*. However, as has been shown previously (Kroeger *et al.*, 2006, Lenhart *et al.*, 2006) the preferred breeding containers for this particular mosquito are the large drums used widely for domestic water storage. More than 50% of containers holding water recorded in this study were drums, and of the total percentage of positive containers for *Ae. aegypti*, either pupae or larvae, more than 60% were drums. Taken together with the new data here showing the superior fitness and size of mosquitoes developing there, drums clearly

are the most epidemiologically significant breeding sites in Trujillo State. Although drums are also the main breeding sites in many areas (Nathan and Knudsen, 1991, Tun-Lin *et al.*, 1995, Chadee, 2004a, Barrera *et al.*, 2006, Burkot *et al.*, 2007, Maciel-de-Freitas *et al.*, 2007), this is not always true and in some places people rarely use them, particularly in those places where a regular water supply service exists (McCall & Kittayapong, 2007, Troyo *et al.*, 2008). In some areas, small containers have been identified as the most important target for source reduction (Focks & Chadee, 1997). What types of breeding container should be given priority in order to ensure the success of control programmes based on source reduction? The answer can be difficult, firstly, because one size or type does not fit all, and secondly, because the spatial distribution pattern of *Ae. aegypti* is very heterogeneous and is influenced by many different human and environmental factors which vary considerably from place to place, as well as the climatic conditions.

In addition, the effective targeting of vectors is only one of the factors that determine dynamic dengue transmission. The failure of dengue vector control programmes (reflected by figures of dengue cases), implemented in Venezuela up until now, and specifically in Trujillo State, has resulted more from a failure to take into consideration the factors determining the presence of high densities of mosquitoes leading to active transmission of dengue, than from dengue vector measures being inefficient by themselves. Dengue as a public health problem is exacerbated by numerous factors including: deficient public services; a lack of educational programmes to encourage community participation in removing potential breeding sites; economic troubles that have seen a decrease in the budgets of regional health authorities; insufficient training for the staff responsible for implementing control activities; the absence of leadership in both communities and in institutions and repeated bureaucratic changes in the organisational structure of the Ministry of Health. Two additional points can be identified, firstly the weakness in available information on the biology of the mosquito and the dynamics of disease transmission,

and secondly, a failure to integrate both research institutions and the institutions that design and implement control strategies at national and regional levels.

It is against this pessimistic outlook on the status of dengue transmission, that the arrival of ITMs as an affordable and potentially effective alternative must be considered. Obviously, for these new control tools to be sustainable over time, it is necessary that health authorities include new strategies to encourage and promote the participation of local communities, which play a very important role in an integrated control programme. This would be a more viable alternative and would achieve better success in controlling *Ae. aegypti*, which in turn should reduce the transmission of the dengue virus. To ensure the success of any control strategy on *Ae. aegypti*, an investigation is needed at a national, regional and local level to outline the major factors causing the high density of mosquito populations that contribute to the endemicity of dengue.

Throughout the investigation conducted in this thesis it became absolutely evident that at the beginning of control programmes people were very interested in participating (this was also demonstrated by the high number of households willing to participate in the trial and the low drop-out rate). Considerable evidence points to the conclusion that the key factor for dengue control is first and foremost, the participation of the community. Unfortunately, this initial positive attitude does not last long and must be dealt with to ensure sustainability in any new programme. Often, it appears that people are waiting for a magic solution to their mosquito/dengue problem. A detailed and extensive awareness-raising programme is needed as communities do not comprehend the need for a sustained effort to reduce mosquito populations. The requirements of a community led programme are not hugely demanding and only require individuals and families to make minor, but sustained practical changes to their every day routines. Sadly, it seems that to some extent, communities have adapted to accept or just live with *Ae. aegypti*, and consequently with dengue.

Communities should be encouraged to actively participate, not only in the implementation of control measures, but also in their design. The idiosyncrasies and individuality of people must be considered when introducing new schemes, even those that are for the benefit of the whole community. The education of communities is a prime factor to be considered in planning new strategies to control dengue, regardless of the tool used. However, this has to be analysed further than has been the case up until now. As such, policies must be designed at the national and regional level aimed at achieving results in the short, medium and long term.

For public health education to produce the desired effect in controlling dengue, it is necessary to design educational programmes targeted to different groups of people, taking into account different socio-economic and cultural backgrounds. This must be considered in order to give each group the individual involvement required and therefore the "ownership" for an educational programme intended to have the desired impact.

A programme designed by experts in the education system might be introduced in the curriculum area at kindergarten, primary, secondary, technology colleges, and universities. This should be neither an isolated activity nor optional, but a compulsory subject, expecting the transference of practical knowledge from educational institutions into homes. In addition, it is necessary to design a flexible and adaptable educational health programme addressed to the communities, because control programmes based on source reduction depend on household activities. This type of community education would help foster permanent behavioural changes. This kind programme has to be systematically evaluated to achieve the objective planed. It is understood that to achieve permanent behavioural change via community education represents a long and arduous task, but the rewards will be exciting, and it will build cohesion in local and larger communities. Above all, this will be effective in terms of improving both physical health and people's well-being.

Although *Ae. aegypti* is a mosquito with a high preference for containers close to human dwellings, public places such as cemeteries and tyre dumps can also be of great significance as potential breeding sites, as occurs in Trujillo (Abe *et al.*, 2005). This is not just the case for this type of mosquito but also for other species as well. It is necessary that public health legislation be implemented to limit or ban the use of water in cemeteries. The promotion of wet sand in which to place flowers and the use of plastic flowers as viable alternatives would have a significant impact. Local governments must also be vigilant when enforcing sanitary legislation against companies dealing with tyres, in order to prevent their activities exacerbating the dengue situation. There is no doubt that these places represent a continuous source of re-infestation to the containers of neighbouring houses.

There is also a requirement that government agencies engaged in environmental management prioritise attention to basic services. These include water supply, wastewater disposal, and solid waste management. Those with experience and influence in these areas, for example, environmental groups and ecological research centres in different national universities, could provide advice on how to design policies to achieve a greater environmental impact and improved quality of life.

Ae. aegypti, besides being a highly domestic mosquito breeding very close to human settlements, has demonstrated remarkable ability to develop resistance to insecticide. This includes resistance to pyrethroid insecticides which have only relatively recently been incorporated into dengue vector control programmes (Hemingway *et al.*, 1989, Mazarri & Georgiou, 1995, Rawlins, 1998, Brengues *et al.*, 2003, Rodriguez *et al.*, 2007, Marcombe *et al.*, 2009).

Although the monitoring of vector resistance ought to be a key part of any integrated mosquito control programme, a surveillance programme is not currently in place in Trujillo State. During this study, mosquito populations showed the early signs of a

decreasing susceptibility to deltamethrin. This indicates the need to implement a monitoring programme and plan for the management of *Ae. aegypti* resistance as a key step in dengue control. The early detection of insecticide resistance is a vital element of resistance management. In the absence of a vaccine to protect people from the dengue virus, the control has to be focussed on dengue vectors. Pyrethroids are the only class of insecticides that have been recommended by the WHO for impregnating materials for vector-borne disease control, which highlights the urgent need to preserve their use for the control of *Ae. aegypti*. In this context ITMs can be a potential tool for managing and preventing resistance, and could be evaluated to determine the feasibility of applying other classes of insecticide to them.

The results presented in this thesis evidence the immediate impact of ITMs in reducing *Ae. aegypti* populations. They highlight the importance of establishing a baseline of susceptibility of natural populations of this mosquito to insecticides prior to its introduction in different localities. Furthermore, they show that the conditions of larval habitats can influence the performance of the adult females. Together, this information could serve as an incentive for the local health authorities responsible for the control of *Ae. aegypti,* to reconsider the direction of the control policies that are being applied routinely in Trujillo State.

Despite the many failures to control *Ae. aegypti*, we should remain optimistic. We should not continue in our search for a standard solution which just does not exist, but instead, search for an integrated solution, whereby all available information and tools are used to provide an efficient and tailored solution to meet the requirements of each different community and its unique environment.

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APPENDICES

Appendix 1. Entomological survey forms

ENCUESTA ENTOMOLOGICA WP3 (CORTINAS)

DATOS:

Hora de Inicio:_____hh mm Fecha:_____ Encuestador: _____

Localidad: _____ Intervención: _____

Identificación de la Vivienda:_____ Nombre del Jefe de Familia: _____

No. de Personas que viven en la casa: _____ No. de niños entre 2 y 8 anos: _____

No. de cortinas en uso: _____ Recogidas: Si ☐ NO ☐

	Tipo de Envase con agua	Locali-zación		Bajo Sol	Bajo sombra	Tapado	Volumen de Agua	Materia Organica	Diámetro	Altura	No. Pupas	Larvas	Otros Organismos
		Int	Ext										
1													
2													
3													
4													
5													
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9													
10													
11													
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13													
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19													
20													
Observaciones													

Envases: Pipas; Tanque; Tanque elevado; Cauchos, Botellas, Floreros, Envases pequeños < 3Lts, Envases medianos 3-20 Lts, Envases grandes >20 Lts,: hh/mm: Hora/minutos.

Hora finalizada: _____ hh/mm.

ENCUESTA ENTOMOLOGICA WP3 (TAPAPIPAS)

DATOS:

Hora de Inicio:_____hh mm Fecha:_____ Encuestador: _____

Localidad: _____ Intervención: _____

Identificación de la Vivienda:_____ Nombre del Jefe de Familia: _____

No. de Personas que viven en la casa: _____ No. de niños entre 2 y 8 anos: _____

No. de tapapipas en uso: _____

Cuanto tiempo mantiene usted el envase cubierto con el tapapipa: _____

	Tipo de Envase con agua	Locali-zación		Bajo Sol	Bajo sombra	Tapado	Volumen de Agua	Materia Organica	Diámetro	Altura	No. Pupas	Larvas	Otros Organismos
		Int	Ext										
1													
2													
3													
4													
5													
6													
7													
8													
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10													
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12													
13													
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15													
16													
17													
18													
19													
20													

Observaciones

Envases: Pipas; Tanque; Tanque elevado; Cauchos, Botellas, Floreros, Envases pequeños < 3Lts, Envases medianos 3-20 Lts, Envases grandes >20 Lts,: hh/mm: Hora/minutos.

Hora finalizada: _____ hh/mm.

ENCUESTA ENTOMOLOGICA WP3 (CORTINAS + TAPAPIPAS)

DATOS:

Hora de Inicio:_____hh mm Fecha:_____ Encuestador: _____

Localidad: _____ Intervención: _____

Identificación de la Vivienda:_____ Nombre del Jefe de Familia: _____

No. de Personas que viven en la casa: _____ No. de niños entre 2 y 8 anos: _____

No. de cortinas en uso: _____ Recogidas: Si ☐ NO ☐

Cuanto tiempo mantiene usted el envase cubierto con el tapapipa: _____

	Tipo de Envase con agua	Locali-zación		Bajo Sol	Bajo sombra	Tapado	Volumen de Agua	Materia Organica	Diámetro	Altura	No. Pupas	Larvas	Otros Organismos
		Int	Ext										
1													
2													
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Observaciones													

Envases: Pipas; Tanque; Tanque elevado; Cauchos, Botellas, Floreros, Envases pequeños < 3Lts, Envases medianos 3-20 Lts, Envases grandes >20 Lts,: hh/mm: Hora/minutos.

Hora finalizada: _____ hh/mm.

ENCUESTA ENTOMOLOGICA WP3 (CONTROL INTERNO)

DATOS:

Hora de Inicio:_____hh mm Fecha:_____ Encuestador: _____

Localidad: _____ Intervención: _____

Identificación de la Vivienda:_____ Nombre del Jefe de Familia: _____

No. de Personas que viven en la casa: _____ No. de niños entre 2 y 8 anos: _____

	Tipo de Envase con agua	Localización		Bajo Sol	Bajo sombra	Tapado	Volumen de Agua	Materia Organica	Diámetro	Altura	No. Pupas	Larvas	Otros Organismos
		Int	Ext										
1													
2													
3													
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20													

Observaciones

Envases: Pipas; Tanque; Tanque elevado; Cauchos, Botellas, Floreros, Envases pequeños < 3Lts, Envases medianos 3-20 Lts, Envases grandes >20 Lts,: hh/mm: Hora/minutos.

Hora finalizada: _____ hh/mm.

ENCUESTA ENTOMOLOGICA WP3 (CONTROL EXTERNO)

DATOS:

Hora de Inicio:_____hh mm Fecha:_____ Encuestador: _____

Localidad: _____ Intervención: _____

Identificación de la Vivienda:_____ Nombre del Jefe de Familia: _____

No. de Personas que viven en la casa: _____ No. de niños entre 2 y 8 anos: _____

	Tipo de Envase con agua	Localización		Bajo Sol	Bajo sombra	Tapado	Volumen de Agua	Materia Organica	Diámetro	Altura	No. Pupas	Larvas	Otros Organismos
		Int	Ext										
1													
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20													
Observaciones													

Envases: Pipas; Tanque; Tanque elevado; Cauchos, Botellas, Floreros, Envases pequeños < 3Lts, Envases medianos 3-20 Lts, Envases grandes >20 Lts,: hh/mm: Hora/minutos.

Hora finalizada: _____ hh/mm.

Appendix 2. Informed consented serological survey form

Formulario de Consentimiento informado Versión 2, 11/12/06
"Nuevas Herramientas para el Control del Dengue"
Instituto de Investigaciones "José W. Torrealba" NURR-ULA,
Trujillo - Venezuela.

Formulario de Consentimiento informado para participantes del estudio.

Introducción

El Dengue es una enfermedad viral, transmitida por un zancudo, que causa un espectro de enfermedades clínicas que van desde fiebre del dengue (DF) a fiebre de dengue hemorrágico (DHF) y síndrome de shock por dengue (DSS). Los síntomas clínicos incluyen fiebre alta, de 5 a 7 días de duración con 2 o más de las siguientes manifestaciones: dolor de cabeza, dolor muscular, dolor de los ojos, dolor en los huesos, malestar general y sangramientos en piel, encías o nariz.

Personal del Instituto de Investigaciones "José W. Torrealba" conjuntamente con investigadores en Brasil, Nicaragua, Tailandia, Vietnam, Filipinas y Malasia estamos realizando un estudio para evaluar Nuevas Herramientas para el Control del Dengue, y así poder generalizar la aplicación a todas las regiones geográficas.

Nosotros sospechamos que usted o su representado podría tener dengue. Nosotros queremos preguntarle si usted y/o su representado, le gustaría tomar parte en este estudio.

Procedimientos del Estudio

Su participación en este estudio es completamente voluntaria. Si usted decide participar o que su representado participe, se le pide que nos de su permiso por escrito y se le harán los siguientes exámenes: Toma de Muestra de Sangre pon Punción Capilar.

Riesgos

Los riesgos asociados a la toma de la muestra de sangre podrían incluir: dolor al momento de la extracción, le puede aparecer un pequeño morado en la zona de punción. Para minimizar los riesgos, la muestra será tomada por personal experimentados (Licenciados en Bioanálisis y Auxiliares).

Responsabilidad:

Al formar parte de este estudio, el participante se compromete a cumplir con las visitas que el estudio requiere.

Confidencialidad

Toda la información del participante obtenida durante el estudio será confidencial. En todos los formularios del estudio se utilizará solamente un número e iniciales para guardar su información. El nombre de los participantes no aparecerán en ningún reporte o publicación de este estudio. Solamente los investigadores del estudio y el personal clave tendrán acceso a su información. Los colaboradores de este proyecto que procesen sus muestras las recibirán codificadas y no tendrán acceso a la información personal de cada muestra.

Compensación

A usted no se le pagará ni se le cobrará por el procesamiento de las muestras del estudio ni por los exámenes que el estudio requiera de sus muestras.

Personas que puede contactar

Si usted o su representado tiene alguna pregunta acerca de este estudio, puede llamar a la Dra. Elci Villegas Ávila al teléfono 0416 - 7718172 o a la Lic. Carmen Castillo al teléfono 0424 - 7048771.

Si usted o su representado tienen alguna pregunta acerca de sus derechos como participante de este estudio, puede Hamar al 0416 -9766121 (Dra. Carmen Morales, miembro del Comité de Bioética).

Retirarse del estudio

Si usted o su representado deciden participar en el estudio, usted o su representado están en todo su derecho de retirar su participación en el estudio en cualquier momento. Si usted elige dejar el estudio, no será castigado de ninguna manera. Cualquier información que se haya recolectado sobre usted o su representado hasta ese momento podrá ser utilizada por los investigadores del proyecto.

Formulario de Consentimiento informado Versión 2, 11/12/06

Yo, _____, de _____ años de edad, voluntariamente quiero o acepto que mi representado, _____, de _____ años de edad, participe en el estudio "Nuevas Herramientas para el Control del Dengue", estudio bajo la supervisión de la Dra Elci Villegas Avila y conducido por el Instituto de Investigaciones "José Witremundo Torrealba" del NURR-ULA.

EI propósito de este estudio, la forma en que será realizada, que tengo que hacer, y las ventajas y los riesgos que puedo experimentar, todos me han sido explicados por: _____.

Entiendo que mi participación en este estudio es voluntaria. Si elijo participar en el estudio, y después cambio de idea, sé que no me castigarán de ninguna manera. He leído esta información. La persona encargada me permitió hacer todas las preguntas que quise acerca del estudio y todas me fueron contestadas. Sé que puedo hacer preguntas más tarde si así lo deseo. Estoy de acuerdo con ser parte de este estudio y permito que los investigadores tomen un poquito de mi sangre para que sea usada en el estudio.

_____ _____

(Nombre del Participante/representante) (Firma de Participante/ representante)

He sido testigo de la lectura precisa del formulario consentimiento al participante potencial (su representado) y él /ella ha tenido la oportunidad de hacer todas las preguntas que quiso. Confirmo que el individuo ha dado libremente su consentimiento para participar en este estudio.

Appendix 3. Ethical approval

UNIVERSIDAD DE LOS ANDES

jwT

Comité de Bioética
Instituto de Investigaciones "José W. Torrealba
NURR-ULA

Trujillo, 2 de Junio, 2006

Ciudadana:
Dra. Carmen Morales
Presidente del Comité de Bioética
Instituto Experimental de Investigaciones "José W. Torrealba"
NURR – ULA. Trujillo
Presente.

Muy respetuosamente me dirijo a usted para solicitarle formalmente la aprobación del Protocolo para el subproyecto titulado: "DENCO WP3 (Dengue vector control", que estará bajo la responsibalidad de esta institución.

Se adjunta para su consideración los siguientes documentos:

1. Protocolo del estudio
2. Formulario para el Consentimiento informado
3. Cuestionario a aplicar
4. Curriculum Vitae del Investigador Principal
5. Resumen del protocolo en español

En espera de una respuesta favorable,

Atentamente

Dra. Elci Villegas
Investigador Principal del Proyecto DENCO

UNIVERSIDAD DE LOS ANDES

jwT

Comité de Bioética
Instituto de Investigaciones "José W. Torrealba
NURR-ULA

Trujillo, 18 de Junio de 2006

Ciudadana:
Dra. Elci Villegas
Investigador Principal del Estudio
Instituto de Investigaciones "Josè W. Torrealba"
NURR – ULA, Trujillo
Presente.

Por este medio me dirijo a usted para informarle que el Comité que presido, ha revisado y aprobado, sin modificaciones, el protocolo de investigación titulado:" DENCO WP3 (Dengue vector control)", que será coordinado por usted en el estado Trujillo Venezuela. Así mismo le informamos que se revisó la documentación y los mismos se ajustan a los requisitos exigidos por este Comité:

1. Protocolo del estudio
2. Formulario para el Consentimiento informado
3. Cuestionario a aplicar
4. Curriculum Vital del Investigador Principal
5. Resumen del protocolo en español

Atentamente.

Dra. Carmen Morales
Presidente del Comité de Ética del Instituto

Dra. Carmen Morales Pelle
CI: 5753251
M.S.A.S. 42596

UNIVERSIDAD DE LOS ANDES

jwT

Comité de Bioética
Instituto de Investigaciones "José W. Torrealba
NURR-ULA

CONSTANCIA

Yo, Carmen Morales, C.I. N° 5.759.251, de Profesión Médico Cirujano Msc. en Protozoología y en mi condición de Presidente del Comité de Bioética del Instituto Experimental de Investigaciones "José Witremundo Torrealba del NURR-ULA, por medio de la presente hago constar la composición del mencionado comité de la siguiente manera:

- Dra. Carmen Morales Peña
Médico Investigador del Instituto Experimental de Investigaciones "José W. Torrealba"
Médico Adscrito al Ministerio de Salud.

- Dra. Laura Vasquez
Médico Coordinador Docente-Investigador de la Extensión Valera de la Escuela de Medicina de la ULA

- Dr. Nelson Vicuña
Médico Docente-Investigador de la Cátedra de Toxicología de la Facultad de Medicina de la ULA

- Dr. Iván Lobo Quintero
Médico Docente de la Cátedra de Medicina interna de la Facultad de Medicina de la ULA

- Dr. Marcos Barreto
Abogado. Edo. Trujillo

- Sr. Armando Torres
Miembro de Comité de Salud de la Comunidad "La Chapa" – Pampanito. Edo. Trujillo.

En Trujillo a los 18 días del mes de Junio de 2006

REPUBLICA BOLIVARIANA DE VENEZUELA
UNIVERSIDAD DE LOS ANDES
NUCLEO UNIVERSITARIO "RAFAEL RANGEL"
Instituto Experimental
"José Witremundo Torrealba"
DIVISIÓN DE SERVICIOS DE SALUD
TRUJILLO - VENEZUELA

Dra. Carmen Morales
Presidente del Comité de Bioética del Instituto "J.W.T."
0058416-9766121

Dra. Carmen Morales Peña
CI: 5759251
M.S.A.S. 42598
Médico Cirujano

Appendix 4. Entomological indices recorded throughout the study in all intervention arms, including internal and external control.

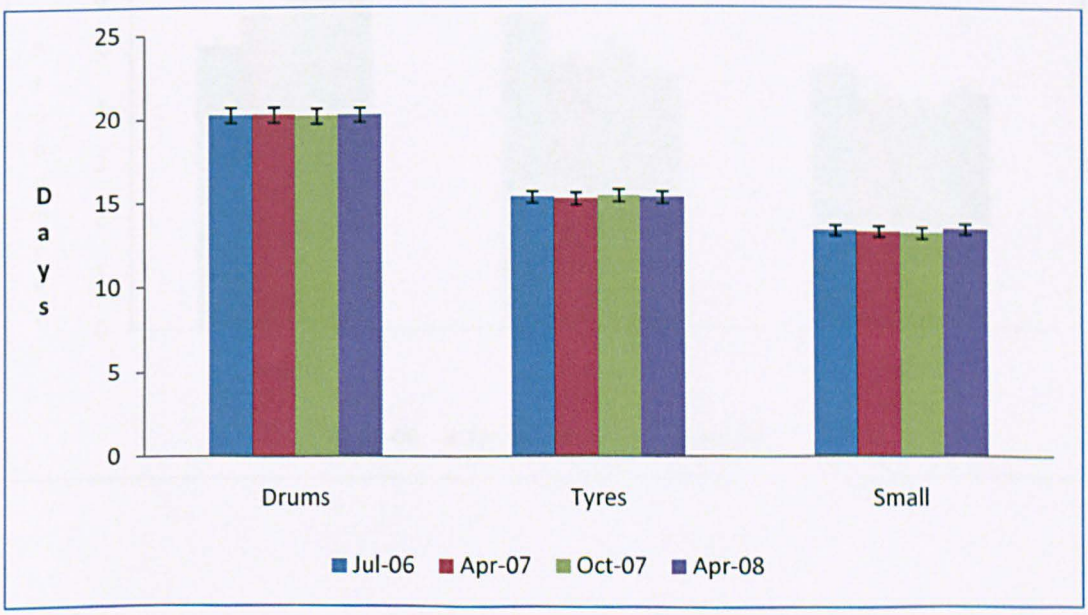
BRETEAU						
	Baseline	**1 m**	**8 m**	**14 m**	**20 m**	**26 m**
Curtains	14,57	8,47	6,48	2,45	5,88	5,88
Covers	15,51	7,75	13,60	3,31	5,42	14,50
Curt-Cov	25,18	9,87	0,50	3,99	6,43	8,02
Int-Ctrl	6,31	8,36	10,91	7,20	5,70	10,28
Ext-Ctrl	19,02	12,49	14,40	22,04	3,34	36,33
HOUSE						
	Baseline	**1 m**	**8 m**	**14 m**	**20 m**	**26 m**
Curtains	6,61	5,08	4,14	1,93	3,82	9,63
Covers	8,46	5,48	4,55	2,56	3,60	4,70
Curt-Cov	11,42	6,44	0,38	2,84	5,41	3,97
Int-Ctrl	5,08	5,44	7,67	4,15	2,85	5,47
Ext-Ctrl	13,22	8,77	9,41	14,72	2,89	21,16
CONTAINER						
	Baseline	**1 m**	**8 m**	**14 m**	**20 m**	**26 m**
Curtains	8,68	6,23	5,01	1,74	2,32	10,02
Covers	6,37	3,22	3,97	1,57	2,24	2,94
Curt-Cov	9,97	3,29	0,18	2,08	1,74	3,27
Int-Ctrl	4,28	4,25	7,04	4,56	3,60	4,69
Ext-Ctrl	10,49	6,40	7,97	15,79	2,20	23,55
PPI						
	Baseline	**1 m**	**8 m**	**14 m**	**20 m**	**26 m**
Curtains	0,38	0,23	0,15	0,15	0,26	0,45
Covers	0,38	0,15	0,33	0,20	0,18	0,18
Curt-Cov	0,88	0,14	0,01	0,11	0,16	0,06
Int-Ctrl	0,22	0,14	0,30	0,34	0,03	0,34
Ext-Ctrl	0,61	0,42	0,50	1,49	0,15	1,46

Appendix 5. Kuskal-Wallis Test compared all parameters measured in *Aedes aegypti* from drums, small containers and tyres at different times in each locality studied.

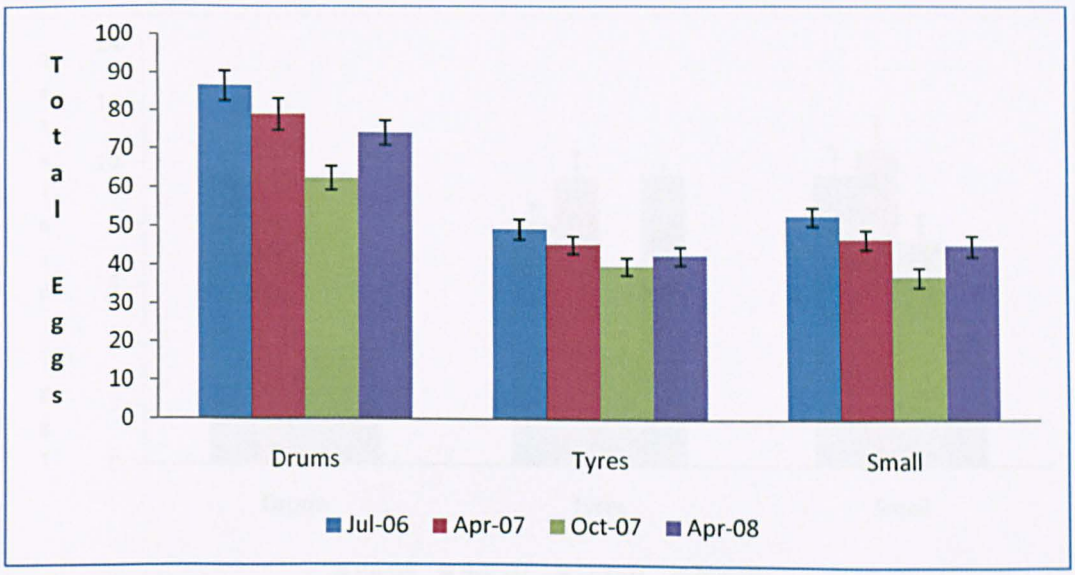
Chi-Square values (χ2), the degree of freedom (fd) and the significance level (p) obtained from Kuskal-Wallis Test conducted to compare the mean values of longevity, number of total eggs, bloodmeals, reatained eggs and hatched eggs from Ae. aegypti *females developed in drums, small containers and tyres. The test was conducted to explore any difference regarding the time when data was collected and according to the intervention site where pupae were collected, before to pool all data for following statistical comparisons.*

	Drums		**Small Containers**		**Tyres**	
	Times	Intervention	Times	Intervention	Times	Intervention
χ2	0.19	2.66	0.64	3.69	0.57	4.92
fd	3	4	3	4	3	4
p	0.98	0.62	0.89	0.45	0.90	0.30

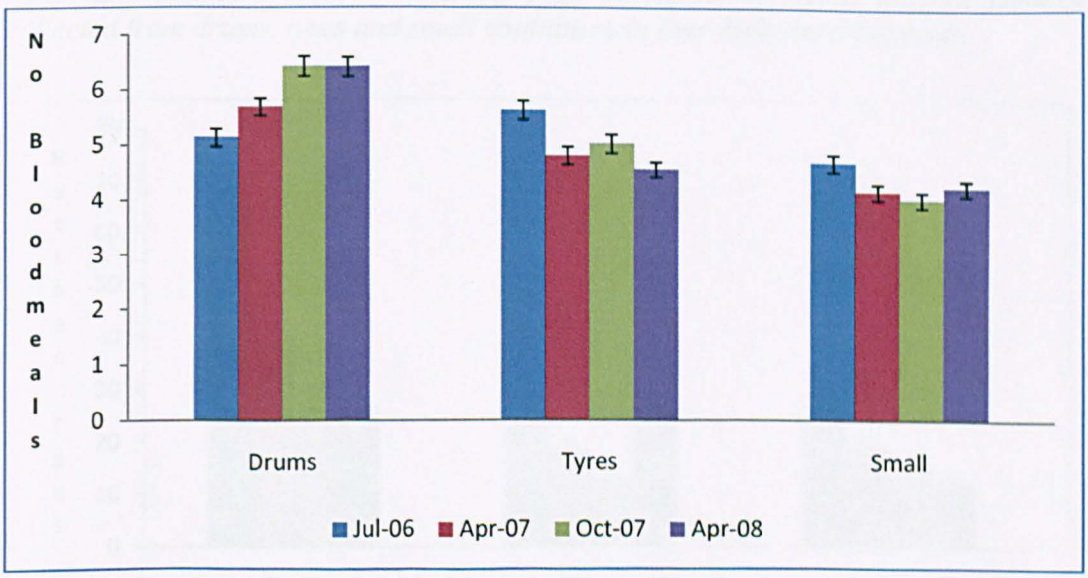
Mean and standard error of longevity measured in Aedes aegypti *females collected from drums, tyres and small containers in four different timepoints.*



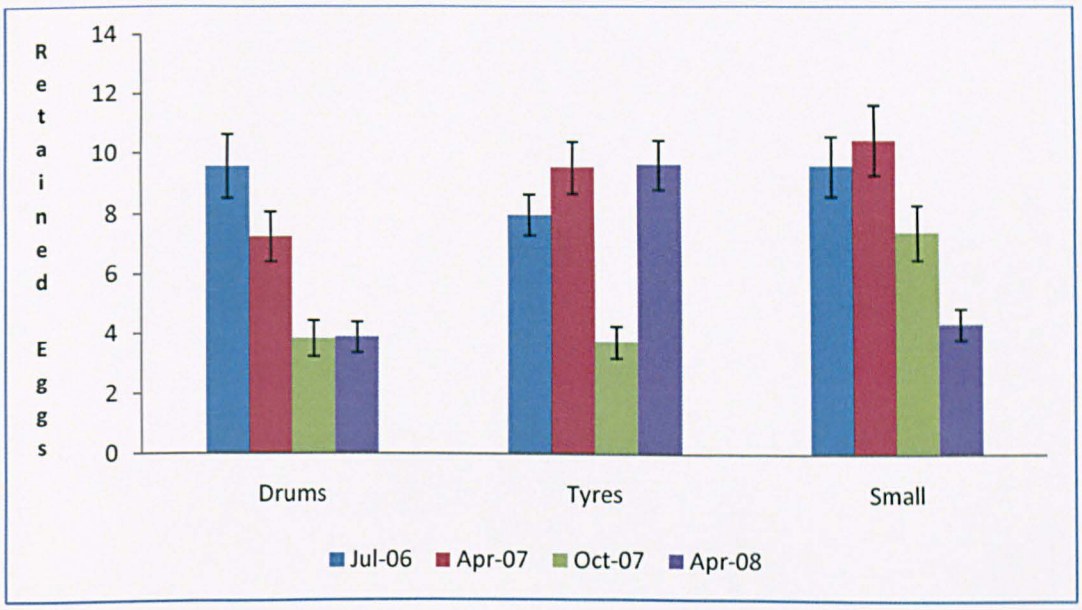
Mean and standard error of the total eggs laid by Aedes aegypti *females collected from drums, tyres and small containers in four different timepoints.*



Mean and standard error of bloodmeals engorged by Aedes aegypti *females collected from drums, tyres and small containers in four different timepoints.*



Mean and standard error of retained eggs measured in Aedes aegypti *females collected from drums, tyres and small containers in four different timepoints.*



Mean and standard error of hatched eggs measured in Aedes aegypti *females collected from drums, tyres and small containers in four different timepoints.*