# Effects of Bed Nets on Host Seeking Behaviour of African Malaria Vectors

Thesis submitted in accordance with the requirements of the

University of Liverpool for the degree of Doctor in Philosophy by

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# Abstract

Long lasting insecticide treated bed nets (LLINs) are a key tool in malaria control in sub-Saharan Africa, and their widespread distribution has contributed significantly to recent reductions in malaria prevalence. Sustaining this impact will require thorough understanding of anopheline host seeking behaviour and LLIN mode of action. However, the behaviour of anopheline mosquitoes during interactions with LLINs, and how insecticides affect that behaviour, is poorly investigated. To pursue this, novel video systems, scaled to record and track nocturnally active free-flying mosquitoes at different levels of detail, were developed and evaluated in a series of behavioural studies, primarily with insecticide susceptible *Anopheles gambiae s.s.*.

The spatial repellent properties of deltamethrin and DDT were investigated using two small-scale cage assays that presented mosquitoes with a human thumb bait and LLIN with (larger choice test) or without (smaller single test) an untreated control alternative. Results from single tests indicated repellency (in deltamethrin only) but the larger choice tests, (and subsequent large-scale tracking), did not. The results highlighted the limitations of such assays, and the caution required when otherwise convenient laboratory behavioural assays are used.

The flight behaviour of host-seeking mosquitoes as they navigated through an open window was investigated in a laboratory environment using a novel 3D tracking system. The study proved the principle of this 3D tracking concept, which uses a retro-reflective material to identify a mosquito's position during flight using a single camera. Analyses of tracks showed that mosquitoes approached windows from higher flight elevations, consistently descending to low levels following passage from the window into the room.

Large scale tracking experiments used Fresnel lenses to illuminate a large field of view, and record activity of free flying *An. gambiae s.s*. at a human-baited bed net. These laboratory tests characterised mosquito flight into four behavioural modes, showing that insecticide treatment rapidly reduced mosquito activity around the net, and provoked a shift in flight behaviour resulting in less net contact. Highest levels of net contact were centred on the net roof above the volunteer's torso. Insecticide treatment reduced the time a mosquito spent in contact with the net, and an individual mosquito was estimated to accumulate less than 100 seconds of direct physical contact with the LLIN during a 60-minute test. Velocity measurements showed that mosquitoes detected nets, including unbaited untreated nets, prior to contact.

The large scale tracking system was transported to, and operated successfully at an experimental hut in Tanzania, to investigate the behaviour of a wild mosquito population consisting predominantly of *An. arabiensis*. Experimental outcomes were similar in both settings, though field tests did not show such pronounced activity decay as was observed in laboratory tests.

The large-scale system was used to explore the host seeking flight behaviour of *An. gambiae s.s.* in the absence of a bed net. Flight activity at a supine human host was separated into approaching or departing tracks. Flight elevation and speed were similar in both, but tortuosity was higher in tracks approaching the host. Mosquitoes showed no preferences for feeding on any part of the host's body and bites were distributed evenly across the volunteer's exposed skin.

This study delivers the most complete characterisations of mosquito-LLIN interactions to date. The tracking systems provide a new platform for a range of further studies and the findings contribute to evidence base required for vector control tool design, research on basic host seeking behaviour and the behavioural mechanisms of insecticide resistance.

## Declaration

This work has not been submitted previously for any other degree, and is not being currently submitted in candidature for another degree at this or any other university.

The work presented in this thesis is the result of my own investigations. The contributions of the collaborating partners involved in the work are detailed below:

All technological aspects of the insect tracking system, described in Chapter 2 and employed in Chapters 5-7, were designed and assembled by Dr Natalia Angarita-Jaimes, Dr Catherine Towers and Prof. David Towers. NAJ wrote the custom software used in Chapters 4-7, and undertook cleaning, tracking and post-processing of data used in Chapter 5.

The illumination method used for tracking in Chapter 4 was conceived by DT and realised by NAJ.

The “small scale assay A”, which was modified for use in the behavioural tests reported in Chapter 3, was designed by Mayumi Abe and Philip McCall.

I was assisted in carrying out the experiments in Chapter 5 (MA and NAJ), 6 and 7 (NAJ).

Signed:

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Date:

25th November 2015

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# Chapter 1 General Introduction

## 1.1 Malaria

Nearly half the world’s population is at risk from malaria, a disease which caused 198 million cases, and 584,000 deaths in 2013 (WHO, 2014). Over 75% of malaria deaths occur in children under 5 years old, and malaria is the fifth leading cause of death in this age group (WHO, 2015). Mortality data for infectious and parasitic diseases shows that malaria is one of the most significant causes of death in all age groups worldwide (table 1.1, Global Health Observatory, 2014), though an estimated 90% of malaria deaths occur in sub-Saharan Africa (WHO, 2014).

Malaria is caused by *Plasmodium spp.* parasites, which are transferred from human to human in the bite of an infected *Anopheles spp.* mosquito. Parasites infect liver cells and red blood cells, causing symptoms of severe anaemia, recurrent fever, and headache. In *Plasmodium falciparum*, the most deadly of the four species infecting humans, the disease may progress to the potentially disabling and life-threatening cerebral malaria.

**Table 1.1 Estimated death rates from infectious and parasitic diseases for all ages worldwide in 2012 (Global Health Observatory, 2014)**

| | Cause | Deaths |
|---|---|---|
| 1 | Respiratory infections | 3,060,166 |
| 2 | HIV/AIDS | 1,533,760 |
| 3 | Diarrhoeal diseases | 1,497,674 |
| 4 | Tuberculosis | 934,838 |
| 5 | Malaria | 618,248 |
| 6 | Meningitis | 395,225 |
| 7 | Childhood-cluster diseases | 266,267 |
| 8 | Acute hepatitis B | 149,162 |
| 9 | Measles | 130,461 |

Following a period of sustained motivation and investment in prevention and control since the year 2000, malaria prevalence in African children aged 2 to 10 fell by 48%, and prevalence in all age groups has decreased in most sub-Saharan African countries (Noor *et al.*, 2014; WHO, 2014). This unprecedented financial investment also enabled multifaceted improvements in diagnosis, drug treatment, and vector control (Bhatt *et al.*, 2015).

## 1.2 Prevention and treatment of malaria

Correct diagnosis is fundamental to disease control. For several decades light microscopy has been the most useful diagnostic technique, surpassing clinical diagnosis, which relies on non-specific symptoms such as fevers and breathing difficulty (Payne, 1988; Källander *et al.*, 2004). However this tool is not always available in health clinics as it requires a trained microscopist, and patients frequently rely instead on clinical diagnosis or self-treatment (Guerin *et al.*, 2002). This has brought about issues of misdiagnosis and over-diagnosis, problematic as where patients are not suffering from malaria, the real cause of their fever is not treated, and they are burdened with the expense of unnecessary drugs (Amexo *et al.*, 2004). New diagnostic tools have been developed using immunofluorescence methods, quantitative buffy coat centrifugal haematology systems, polymerase chain reaction (PCR) and enzyme linked immunosorbent assay (ELISA) but none are in wide scale use, as their cost or training requirements make them impractical for use in most field labs (Guerin *et al.*, 2002). Rapid diagnostic tests (RDTs) which analyse blood for presence of parasite antigens through immunochromatography can be used cheaply and with little training, with sensitivity and specificity that almost match diagnosis by light microscopy for *P. falciparum* and *P. vivax* malaria (Abba *et al.*, 2011, 2014). RDTs were used as a diagnostic tool in 52% of all suspected malaria cases in 2013, and have helped to reduce the misdiagnosis of malaria (WHO, 2014). At present, malaria control relies on drug therapies to target the *Plasmodium sp.* parasite stages in the human host, and vector control to prevent transmission by the mosquito vectors.

The earliest drug treatment to prevent or cure malaria was quinine, originally taken simply as an infusion of the bark of the Cinchona tree (Meshnick & Dobson, 2001). Chloroquine was the first synthetic drug to be used in malaria treatment, and by 1942, rapidly supplanted quinine as drug of choice (Coatney, 1963; White, 1996). However, resistance to chloroquine and other drugs emerged in the 1970s, (Payne, 1987) making these treatments virtually ineffective against the parasite by the 1990s (WHO, 2010a). The World Health Organisation (WHO) currently recommends that uncomplicated malaria is treated with artemisinin combination therapy (*i.e.* using two or more drugs simultaneously) (WHO, 2007a; Sinclair, 2009). This strategy of using combination therapy for the majority of malaria cases is designed to slow the spread of drug resistance (Hastings, 2011). Drug resistance to artemisinin has been found, but is currently confined to Southeast Asia (Ashley *et al.*, 2014). Containing resistance through early disease diagnosis and well-regulated treatment with high quality drugs will be key to maintaining recent gains in malaria control (Dondorp *et al.*, 2010).

Pregnant women living in endemic countries are particularly vulnerable to severe malaria that can be fatal or result in a range of adverse consequences, including birth complications (Steketee *et al*, 2001). As prophylaxis, pregnant women are advised to take intermittent preventive treatment with sulphadoxine-pyrimethamine (WHO, 2004a). This strategy is also applied to infants in high transmission areas, in a treatment schedule that coincides with childhood vaccinations, though fewer countries have formally adopted this policy (WHO, 2010b, 2014).

No vaccine is currently available for use against malaria, but a number of vaccines are in later stages of development including the RTS,S vaccine. This vaccine candidate is at the most advanced stage of development, and has shown 30-56% efficacy against clinical malaria over 12-14 months in Phase 3 trials in seven malaria endemic countries, though efficacy fell to 26-36% over the 3-4 year follow up period (RTS,S Clinical Trials Partnership, 2011, 2012, 2015).

Vector control is recommended by the WHO as an essential part of malaria control (WHO, 2006). Moreover, modelling studies suggest that even in the event that an effective vaccine becomes available, vector control will remain an important component of anti-malaria programmes in high transmission settings (Griffin *et al.*, 2010; Eckhoff, 2013; Artzy-Randrup *et al.*, 2015).

The main tools deployed in vector control are long lasting insecticide treated bed nets (LLINs) and indoor residual spraying (IRS), and to a lesser extent, control of immature stages using insecticides or other approaches (WHO, 2015). These tools have been improved in the last decades by development of new insecticide formulations, and better coordinated distribution and spray programs (Casida & Quistad, 1998; Hill *et al.*, 2006; Walker & Lynch, 2007). Vector control is discussed in detail later in this chapter.

### *1.2.1 Global Malaria Control Efforts*

Global malaria control efforts have been boosted in the last few decades by the formation of a number of partnerships which have in turn announced targets and funded the scale-up of control efforts. The most recent upsurge in control efforts began in 1997 and 1998 with the formation of the Multilateral Initiative on Malaria in Africa, and the Roll Back Malaria partnership, bringing together NGOs, international research institutes, the World Health Organisation, the UN, UNICEF and the World Bank. These bodies worked to strengthen healthcare systems, and build capacity in malaria endemic countries by investing in research and assisting countries in devising malaria control programs (Nabarro & Tayler, 1998; Miller, 2010). In 2000, the United Nations further committed to malaria control by including in its Millennium

Development Goals a target to “begin to reverse the incidence of malaria and other major diseases” by 2015 (United Nations, 2000). Progress towards these goals was greatly assisted by increases to funding, with the formation of the Global Fund to Fight AIDS, Tuberculosis and Malaria, the US President’s Malaria Initiative, and the Bill and Melinda Gates Foundation (Roberts & Enserink, 2007; Snow *et al.*, 2008). Working on the foundation of progress enabled by these groups, the Roll Back Malaria partnership set out updated targets in the Global Malaria Action Plan (GMAP), calling for universal coverage of malaria interventions to be achieved by 2015 (Roll Back Malaria, 2008). In July 2015, a further announcement by the Roll Back Malaria Partnership set out new strategies for the next 15 years as part of the UN sustainable development goals, calling for increased investment to assist in reaching the target of a 90% reduction of malaria mortality rates and case incidence from 2015 levels by 2030, to be achieved through improved prevention, diagnosis and treatment (Roll Back Malaria, 2015). Already, many countries are reducing disease incidence and mortality; four countries have been certified as malaria free, and a further nine are in the elimination phase of control (WHO, 2014). The GMAP proposes malaria elimination as a non-time-bound part of its global strategy, but funding constraints, and the need for new tools and approaches to control means that this remains a very distant goal for most endemic countries (Moonen *et al.*, 2008; Roll Back Malaria, 2008; Snow *et al.*, 2008). If gains made against malaria in the past decade are to be maintained, it will be important to employ synergistic use of drugs and vector control tools, making a sustained and long-term effort whilst practicing careful surveillance for and management of drug and insecticide resistance (Lines *et al.*, 2008). As such better knowledge of the mosquito vector’s biology, ecology and behaviour will be key to successful control efforts (Ferguson *et al.*, 2010).

## 1.3 Mosquito Biology

Mosquitoes are a large group of Culicidae in the order Diptera. There are two major subfamilies: the Anophelinae which comprises the *Anopheles* mosquitoes; and Culicinae, which includes *Aedes, Mansonia* and *Culex* genera, as well as the sugar feeding *Toxorhynochites* (Harbach *et al.*, 2007). Almost all mosquito genera require the female to take a blood meal as part of their life cycle (Service, 2008). Mosquitoes commonly feed on birds and mammals, but in some cases are able to take blood meals from reptiles and amphibians (Tempelis, 1975; Takken & Verhulst, 2013). While many haematophagous mosquitoes are important as vectors of animal and human infections, human malaria is transmitted only by female *Anopheles spp* mosquitoes. The mosquito becomes infected when taking a blood meal containing *Plasmodium spp.* gametocytes from an infected host. The gametocytes undergo fertilisation within the midgut but take a further 10 days (approximately, the rate of development is temperature-dependent [Beier, 1998]) to reach the mosquito's salivary glands as infective sporozoites; hence only older mosquitoes are capable of transmitting the disease.

After a blood meal, tropical mosquitoes have a 2-4 day maturation period during which the blood is digested prior to egg laying (Service, 2008). Following this the female lays her eggs in small bodies of standing water (Clements, 1999). Eggs hatch into aquatic larvae, which feed voraciously, undergoing three moults before pupation. The pupa is unusually mobile and like the larval stages, requires access to the surface film to breathe atmospheric oxygen. After metamorphosis the adult mosquitoes emerge at the water surface (Clements, 1999). In *Anopheles gambiae* mosquitoes the entire process from egg to adult can take between 10 and 23 days depending on environmental temperature (Bayoh & Lindsay, 2002). Only the adult female feeds on blood, using the nutrients of the blood meal to produce an egg batch. The blood meal, resting, oviposition cycle is termed the gonotrophic cycle and in African vectors, is repeated every two or three nights (Service, 2002).

## 1.4 Vectors of malaria

There are 537 members of the *Anopheles* genus, including subspecies (Harbach, 2013). Whilst 70 species are capable of acting as malaria vectors under natural conditions (Service, 2002), only 40 to 70 are considered to be of public health importance (Service, 2008; Hay *et al.*, 2010). A number of *Anopheles* species comprise morphologically indistinguishable complexes (Harbach, 2008). The *Anopheles gambiae sensu latu* complex consists of several sibling species: *Anopheles arabiensis*, *Anopheles gambiae sensu stricto*, *Anopheles coluzzi* (previously *An. gambiae* M form), *An. quadriannulatus*, *An. melas*, *An. merus*, *An. bwambe, An. amharicus* (Coluzzi *et al.*, 1979; Coetzee, 2004; Coetzee *et al.*, 2013). The *Anopheles funestus* group includes eleven species: *Anopheles funestus s.s., Anopheles rivulorum, An. funestus-like, An. fuscivenosus, An. vaneedeni, An. parensis, An. leesoni, An. confusus, An. brucei, An. rivulorum-like and An. aruni* (Coetzee & Koekemoer, 2013).

This review will focus on African malaria vectors, as this continent has the greatest malaria burden (WHO, 2014). The high malaria prevalence in this region results from environmental factors relating to climate, and the large populations of competent vector mosquitoes, as well as socio-economic factors such as land use, agricultural practices, and health sector infrastructure (Coluzzi, 1999; Stratton *et al.*, 2008). There has been a great body of research conducted on African malaria vectors and control efforts (Alilio *et al*, 2004), and trials of new interventions have been focussed here for many years (Snow *et al.*, 2012). With greater global attention towards shrinking the malaria map, countries in this region face the most challenging outlook as here the disease has high, stable transmission, and involves vector species that exhibit a wide variety of different behaviours and feeding habits and that, increasingly, are becoming insecticide resistant (malERA, 2011; Noor *et al.*, 2014).

In Sub-Saharan Africa, *An. gambiae s.s., An. coluzzi, An. arabiensis* and *An. funestus* are the main vectors of malaria (Coluzzi *et al.*, 1999). Though *An. gambiae s.s.* has often been viewed as the dominant vector on the continent, *An. funestus* and *An. arabiensis* populations are of growing importance in malaria transmission as control efforts impact *An. gambiae s.s.* populations (Russell *et al.*, 2011; Mwangangi *et al.*, 2013; Lwetoijera *et al.*, 2014a; McCann *et al.*, 2014). Additional vector species include *Anopheles nili* and *Anopheles moucheti*, though these are less important to disease transmission being less common, less widespread geographically, and less closely associated with human populations in their behaviour (Fontenille & Simard, 2004).

In addition to being highly susceptible to *Plasmodium falciparum* (Ndiath *et al.*, 2011) *An. gambiae s.s.* and *An. coluzzi* mosquitoes exhibit a number of behavioural characteristics which contribute to their high vectorial capacity for malaria. Firstly, these species are highly anthropophilic (Gillies & De Meillon, 1968; Takken & Verhulst, 2013), preferring to bite humans over cattle and other animals. Secondly they can be abundant, with a broad geographical distribution (Gillies & De Meillon, 1968; Sinka *et al.*, 2012).

*Anopheles funestus* is also generally anthropophilic but may opportunistically feed on cattle (Takken & Verhulst, 2013). *Anopheles arabiensis* is more zoophilic than *An. funestus* and *An. gambiae s.s.*, frequently feeding on cattle (Takken & Verhulst, 2013). Choice of host species is determined often by availability of humans and cattle: both *An. arabiensis* and *An. gambiae s.s.* may adjust their feeding strategies when their preferred host is not available (Mwangangi *et al.*, 2003; Lefèvre *et al.*, 2009a; Lyimo & Ferguson, 2009).

*Anopheles gambiae s.s.* and *An. funestus* are nocturnal and predominantly feed indoors (Gillies & De Meillon, 1968; Pates & Curtis, 2005). Such indoor feeding is termed endophagy, and contrasts with *An. arabiensis*, which though plastic in its behaviour, exhibits more exophagic (outdoor feeding) and exophilic (outdoor resting) behaviours (Sinka *et al.*, 2010). It has been suggested that endophagic behaviour could be an artefact of the fact that humans go indoors at night (Huho *et al.*, 2013). After feeding, indoor-resting (endophilic) mosquitoes will remain on the walls of the house for several hours, exiting after day break (Gillies, 1954). This behaviour means that most interventions targeting *An. gambiae s.s.* are designed to protect people from bites in the home.

These vector species also appear to have different preferences in larval habitats. *Anopheles funestus* and *An. coluzzi* lay eggs in permanent or semi-permanent large bodies of fresh-water, while *An. gambiae* oviposit in smaller, temporary puddles of water (Sinka *et al.*, 2010; Fillinger *et al.*, 2009). *Anopheles arabiensis* has been found to lay eggs in large and small water bodies (Sinka *et al.*, 2010; Fillinger *et al.*, 2009).

These differences contribute to differences in seasonal vector abundance: *An. funestus* and *An. arabiensis* may persist at low levels during the dry season, whereas *An. gambiae s.s.* and *An. coluzzi* proliferate during the rainy season and are less abundant at other times of year (Lindsay *et al.*, 1998; Minakawa *et al.*, 2002; Mzilahowa *et al.*, 2012). The result of this monthly variation in vector population is that malaria transmission is often seasonal, typically with the high transmission periods following rains (Mabaso *et al.*, 2007).

## 1.5 Control of malaria vectors

### *1.5.1 Bed Nets and Indoor Residual Spraying*

The history of malaria vector control dates back to the period immediately following the demonstration of the role of anopheline mosquitoes as vectors. Early approaches targeted breeding sites by draining or oiling water in suspected breeding sites (Ross, 1902). House screening was also a popular technique in the then malarious areas of Europe and America (Lindsay *et al.*, 2002). In the mid-20th century, DDT was used and the era of insecticides had begun (Casida & Quistad, 1998; Roberts *et al.*, 2010). This led to the global malaria elimination efforts of the 1960s, when WHO oversaw a global malaria eradication campaign using chloroquine drug treatment and house indoor residual spraying with DDT insecticide (Snow *et al.*, 2012). Malaria was eliminated from 30 countries, but ultimately the programme failed in its goal of eradication when widespread drug and insecticide resistance compromised the efficacy of the control tools: and as a result there was a resurgence of malaria cases in many countries that had been nearing control (Bruce-Chwatt, 1979).

The decrease in malaria cases resulting from the global malaria control efforts of the past decade can be attributed in a large part to vector control (Bhatt *et al.,* 2015). Of the methods available, the two most widely used are indoor residual spraying (IRS), and long lasting insecticide treated bed nets (LLINs), both of which have proven highly effective (Lengler *et al.*, 2004; Pluess *et al.*, 2010). Indoor residual spraying (IRS) was the first method used to deploy an insecticide, DDT, for control of adult female mosquito vectors of malaria (Casida & Quistad, 1998; Roberts *et al.*, 2010). IRS involves insecticide spraying of the walls and ceilings within homes. This technique exploits the propensity of many malaria vectors to rest indoors after feeding: mosquitoes are killed after making contact with the insecticide treated surface of the wall. Today, although IRS is used in 79 of the 97 malaria endemic countries, only 4% of the population at risk is protected by this control method (WHO, 2013a).

A Cochrane systematic review reported that evidence indicated that IRS has good protective efficacy against malaria in a range of transmission settings (Pluess *et al.*, 2010). However depending on formulation and type of insecticide used, the duration of protection may be as short as three months (Etang *et al.*, 2011; Tchicaya *et al.*, 2014), with a maximum of six months after a single treatment (WHOPES, 2015). As a result the sustainability and effectiveness of IRS hinges on appropriate timing of spray campaigns, which must be completed before the rainy season begins and transmission levels increase (Worrall *et al.*, 2007).

Sleeping within an LLIN at night provides both a physical and a chemical barrier to the endophagic nocturnal mosquito. LLINs all make use of pyrethroid insecticides, the only approved class for this use (Zaim *et al.*, 2000). The net blocks mosquitoes from reaching the host, and the insecticide is thought to repel or kill mosquitoes making contact with the LLIN surface (Strode *et al.*, 2014). Insecticide treated bed nets became a common part of malaria control following the initial trial where permethrin-treated nets were found to reduce malaria incidence amongst children in The Gambia (Snow *et al.*, 1988). In the first years of bed net use, new nets required manual application of the insecticide prior to use; this treatment would wear off relatively rapidly and nets required retreatment every six to twelve months (Curtis *et al.*, 1996). However achieving regular retreatment of all nets within a community proved challenging (Lines, 1996). Eventually these were replaced with long lasting insecticide treated nets in which insecticide is incorporated into the net fibres during the manufacturing process, and one net should retain efficacy for 3 to 5 years (WHO, 2001, 2003).

Today, programmes in many countries worldwide provide LLINs for free, or sell them at subsidised prices in mass distribution campaigns, and as a result it is estimated that 49% of people living in malaria-endemic countries have access to an insecticide treated net (WHO, 2014). There is good evidence for their efficacy: a systematic review of 20 net trials found use of insecticide treated nets reduced malaria incidence by 50%, and cut mortality in children under 5 years old by one fifth in areas of stable malaria transmission (Lengeler, 2004).

Initially, ITNs were intended to provide personal protection for the sleeper, but evidence indicates that when coverage in a population is high (50-75%) ITNs can provide a community wide protective effect (Hawley *et al.*, 2003). In this large-scale study in western Kenya, LLINs reduced the abundance of vectors, with an observable decrease in *An. gambiae* density within a radius of 600m from houses with nets (Gimnig *et al.*, 2003).

There is mixed evidence for advantages of using IRS and ITNs in combination compared to choosing one intervention alone (Kleinschmidt *et al.*, 2009; Corbel *et al.*, 2012; West *et al.*, 2014). The benefit of combining interventions is likely to depend on many factors, including the achievable level of coverage of the two interventions, the behaviour and the insecticide resistance status of the local mosquito species, and the malaria transmission intensity (Fullman *et al.*, 2013).

Both IRS and LLINs target endophagic and endophilic mosquitoes. Exophagic, exophilic mosquitoes present a major control challenge, maintaining residual malaria transmission after universal LLIN and IRS coverage has been achieved (Killeen, 2014). Other strategies will be necessary to tackle these vectors, if higher levels of malaria control or elimination are to be considered (Govella & Ferguson, 2012).

### *1.5.2 Other Methods of Vector Control: Larval Control*

Following the discovery that mosquitoes were the vectors of malaria, recommendations for mosquito control focussed on destruction of larval habitats (Ross, 1900; Grassi, 1901). Such larval source management (LSM) is still used today, and approaches include the use of chemical and microbial larvicides, or biological control agents such as larvivorous fish. Larviciding has been used to great effect in the past, most notably to halt the spread of accidentally introduced

*Anopheles gambiae* in Brazil (Killeen *et al.*, 2002). Following a resurgence of interest in this approach, recent trials in Tanzania and Kenya have shown reductions in prevalence of malaria infection using LSM, with further evidence showing it to cause reductions in mosquito density (Fillinger *et al.*, 2009; Geissbühler *et al.*, 2009; Fillinger & Lindsay, 2011). LSM may be limited in its potential by the need to identify mosquito breeding sites, a challenging and labour intensive task given the tendency of *An. gambiae s.s.* to breed successfully in sites ranging from large water bodies such as irrigated paddy fields to small temporary water bodies that proliferate in the wet seasons (Sinka *et al.*, 2010). However this has been achieved in the past, particularly in urban environments, and is made simpler by the limited host seeking range of mosquitoes: adult mosquitoes in urban settings tend to feed on humans living within 100m of their breeding sites, hence the search can be kept to a manageable area (Killeen *et al.*, 2002). One development that could eliminate this issue is the concept of auto-dissemination. In auto-dissemination adults are contaminated with a substance at resting sites and subsequently contaminate breeding sites with it when they oviposit (Devine *et al.*, 2009). Pyriproxyfen (PPF), a synthetic juvenile hormone analogue which kills larvae by halting their development, is a promising candidate for use in this dispersal method (Dhadialla *et al.*, 1998). Initial field trials in Peru, America, Italy and Brazil using PPF against container breeders *Aedes aegypti, Aedes albopictus* and *Culex sp.* have successfully shown auto-dissemination over distances of up to 400m from original contamination stations (Devine *et al.*, 2009; Caputo *et al.*, 2012; Suman *et al.*, 2014; Abad-Franch *et al.*, 2015). In translating this principle to malaria vectors, the different breeding sites must be considered; African *Anopheles sp.* breed in larger more exposed water bodies than *Ae. aegypti*, with rainfall regularly ‘flushing’ breeding sites (Devine & Killeen, 2010), but it is thought that this type of LSM could be an effective tool during the dry season. Results from early semi-field trials in Tanzania are promising (Lwetoijera *et al.*, 2014b).

This method has the significant advantage that it potentially minimises the need to search for every breeding site, in order to deliver effective control. It is hoped that in the future it could be used as a complementary tool to IRS and LLINs in control programmes.

### *1.5.3 Biological Control*

Other control techniques seek to reduce mosquito numbers through predation. The water dwelling larval stage of the mosquito is vulnerable to predation by larvivorous fish and copepods, and a number of studies have investigated the use of such predators in a method termed ‘biological control’. This method is only applicable to species breeding in permanent water bodies, but has been used successfully against *Ae. aegypti* in Vietnam (Nam *et al.*, 1998). There is no conclusive evidence for this intervention’s efficacy against *Anopheles* mosquitoes, but some trials showed that introduction of larvivorous fish reduced the number of breeding sites found to contain larvae and pupae (Walshe *et al.*, 2013). There are ecological constraints to the introduction of new fish species to an area, but biological control may yet prove a useful control tool, offering an opportunity for a community led intervention, in which the fish themselves can be used as food (Howard *et al.*, 2007).

### 1.5.4 House Screening

Studies in Uganda and Sri Lanka have found that malaria cases are associated with poor quality housing (Gamage-Mendis *et al.*, 1991; Wanzirah *et al.*, 2015). Controlling for the effect of socio-economic status on malaria risk, malaria infection was found to be higher in children living in traditional houses (Tusting *et al.*, 2013; Wanzirah *et al.*, 2015). Authors considered that this finding could relate to mosquito house entry: traditional homes in this study had thatched roofs and open eaves, whereas modern houses had closed eaves and tin roofs. Improvements to housing have the potential to limit mosquito entry and reduce disease exposure (Tusting *et al.*, 2015).

House screening is a simple and effective method of preventing entry into houses by endophagic or endophilic mosquitoes. Here, net screens or mesh are used as barriers to block mosquito entry through house doors, windows and eave gaps. This method can reduce the number of mosquitoes found in houses, and therefore minimise human contact with mosquitoes (Lindsay *et al.*, 2002; Kirby *et al.*, 2009). Tests with *Ae. aegypti* in Mexico used insecticide treated screens to reduce house entry (Che-Mendoza *et al.*, 2015), but other studies have found good effects using untreated netting against *Anopheles* and *Culex* vector species (Lindsay *et al.*, 2002, 2003; Kirby *et al.*, 2009). At present this method has relatively high cost and labour requirements, as screens need to be fitted to the individual dimensions of each house, however it is popular with householders, and has the advantage that it does not require use of insecticide so is likely to be robust to problems of insecticide resistance (Gimnig & Slutsker, 2009; Kirby *et al.*, 2009).

### 1.5.5 Repellents

Topical repellents, insecticide treated clothing and burnable repellent coils are useful in deterring mosquitoes (Goodyer *et al.*, 2010; Ogoma *et al.*, 2012a). Supplementing bed net use with topical repellents can reduce incidence of *P. falciparum* and *P. vivax* (Hill *et al.*, 2007; Deressa *et al.*, 2014), and use of DEET (N,N-Diethyl-meta-toluamide) by itself reduced *P. falciparum* incidence in an Afghan refugee camp in Pakistan where nets were not used (Rowland *et al.*, 2004). Use of repellents can protect people against bites in the early evening when mosquitoes are active but before people have entered their bed nets. However the method is likely to be expensive and, perhaps most importantly, requires frequent reapplication as repellent effects typically wear off after a few hours; hence, achieving sustained compliance at the levels required is challenging. This was the case in studies of repellents in Tanzania, Laos, Ecuador and Peru, that did not report reductions in malaria incidence (Curtis *et al.*, 1994; Kroeger *et al.*, 1997; Chen-Hussey *et al.*, 2013). Kroeger *et al.* suggested that the failure of the South American study was due to the low levels of consistent repellent use, and speculated that insects could have been diverted towards people not using repellents. Subsequent studies in Bolivia and Tanzania have provided evidence for such diversion when repellent coverage is patchy (Moore *et al.*, 2007; Maia *et al.*, 2013). An important consideration here is that repellent use is relatively expensive, and use of repellents could divert the malaria burden towards the poorer members of endemic countries who cannot afford to buy repellents.

### *1.5.6 Attractants*

Mosquitoes are attracted to chemicals such as $CO_2$, ammonia, and lactic acid, as these odours are associated with human and animal hosts (Takken & Knols, 1999). A number of traps use chemical attractants or live baits to attract and trap mosquitoes (Costantini *et al.*, 1993; Xue *et al.*, 2008; Okumu *et al.*, 2010a). Such traps are useful for the surveillance of exophilic mosquito populations (Mboera *et al.*, 2000). Some trials have found that placing attractant traps outside houses reduces the number of mosquitoes caught inside, but others failed to find an effect (Jawara *et al.*, 2009; Smallegange *et al.*, 2010). The complex suite of (largely unknown) cues that mosquitoes use in host location makes it difficult to design an artificial attractant trap that mosquitoes will prefer to a real human (Okumu *et al.*, 2010b). As such attractant traps may be more suited to monitoring insects than control, unless paired with other methods.

### *1.5.7 Zooprophylaxis*

Zooprophylaxis is a control measure which proposes the strategic use of cattle to divert host seeking mosquitoes from humans to cows, reducing the number of bites received by humans (WHO, 1982). This method is enacted by controlling availability of hosts, through placement of cattle in locations that would intercept the path of the host seeking mosquito. The term ‘zooprophylaxis’ may refer to the normal housing of animals near homes (passive zooprophylaxis), the transfer of animals to particular locations for the purposes of control (active zooprophylaxis; Bøgh *et al.,* 2001), or the insecticide treatment of cattle for vector control (insecticide prophylaxis; Donnelly *et al.,* 2015).

A recent review of studies covering all types of zooprophylaxis found presence of animals could decrease malaria risk to people when cattle were housed in shelters close to human homes, but that malaria risk was increased when humans shared a room with animals (Donnelly *et al.,* 2015). Some studies included in the review failed to find an impact on malaria risk, and in others the effect was modified by a number of other factors such as net use and wealth of study participants. Effectiveness of zooprophylaxis is predictably affected by mosquito species present in an area, with greater success in areas where more zoophilic vectors such as *An. arabiensis* and *An. pharoensis* were present (Donnelly *et al.,* 2015).

Zooprophylaxis has potential for use in disease control against these vectors, and may be particularly effective when paired with other protective measures such as IRS or LLINs that reduce availability of humans, producing a push-pull type control action (Iwashita *et al.,* 2014).

### *1.5.8 Push-Pull Strategies*

One practice that seeks to use the principles of attraction and diversion to its advantage is that of push-pull mosquito control, combining the use of mosquito repellents and attractants. In this method the ‘push’ aspect involves repelling or diverting mosquitoes away from humans, while in the simultaneous ‘pull’ element mosquitoes are caught in traps baited with attractive odours, or cattle. This concept is already used for control of agricultural pest insects, and is currently under development for use with mosquitoes (Cook *et al.*, 2007). A semi-field trial using four Mosquito Magnet X traps (MMX, baited with $CO_2$ and attractive odours) placed around a human-baited house, with repellents delivered using a modified MMX traps containing repellent treated nylon strips, hung at the four corners of the house, and found that this push-pull strategy significantly reduced numbers of mosquitoes found inside houses compared to repellents alone (Menger *et al.*, 2014). The MMX traps caught over half the mosquitoes released. An experimental hut trial in Kenya which used MMX attractant traps paired with repellent material partially blocking house eave gaps found that the reduction in house entry produced by ‘push-pull’ interventions (51.6%) was approximately equal to that achieved using ‘push’ (52.8%) or ‘pull’ (43.4%) alone (Menger *et al.*, 2015). At present this method is in its infancy, and implementation will require careful design of both push and pull tools to maximise its efficacy.

### *1.5.9 Attractive Toxic Sugar Baits*

Both male and female mosquitoes feed on plant sugars (Yuval, 1992; Foster, 1995). Attractive toxic sugar baits (ATSB) exploit this behaviour to kill mosquitoes. In general ATSBs spray a mixture of an attractive plant based scent, sugar solution and an oral toxin onto plants, poisoning mosquitoes as they ingest the toxin. This method has shown encouraging results against *Anopheles* mosquitoes, and has the benefit of affecting mosquitoes of both sexes, not just the blood-feeding female (Müller & Schlein, 2006; Müller *et al.*, 2010; Beier *et al.*, 2012). This method has also been adapted to work with endophilic mosquitoes, using treated cloths hung around a bedroom: experimental hut trials of this method in Tanzania found these baits could kill both *An. arabiensis* and *Cx. quinquefasciatus* mosquitoes (Stewart *et al.*, 2013). However the potential collateral damage ATSBs could do to other non-target insect populations has yet to be fully investigated in malaria endemic countries (Qualls *et al.*, 2013).

### *1.5.10 Sterile Insect Technique and novel mosquito pathogens*

In recent years two entirely novel approaches to vector control have emerged, with great potential: symbiont mediated control and sterile insect technique. Infection by commensal/symbiotic bacteria can affect insects’ susceptibility to other pathogens (Hedge *et al.*, 2008; Teixeira *et al.*, 2008). *Wolbachia,* the bacterium that is the main focus of current research, invades populations with great success as a result of cytoplasmic incompatibility, in which uninfected females do not produce offspring when mating with infected male insects (Yen & Barr, 1973). The bacterium has been shown to decrease *Ae. aegypti* susceptibility to viruses such as Chikungunya and dengue (Moreira *et al.*, 2009). *Wolbachia* has been used with demonstrable success in field trials targeting *Ae. aegypti* (Frentiu *et al.*, 2014). Natural *Wolbachia* infections have been reported in field populations of *An. gambiae* (Baldini *et al.*, 2014) suggesting this technique could be adapted for use against malaria, but at present this strategy requires several years of research and development before it can be used in public health campaigns.

The sterile insect technique was first proposed several decades ago when it was found that radiation could be used to sterilise male insects (Klassen & Curtis, 2005). This method has been used with great success to eradicate screwworm from North America and Mexico (Krafsur *et al.*, 1987). More recently this principle has been attempted using transgenic mosquito strains, modified for sterility or refractoriness to disease, though these are also at relatively early stages of development (Ito *et al.*, 2002; Benedict & Robinson, 2003; McGraw & O'Neill, 2013). Mosquitoes that are refractory to malaria could be used to flooding the mosquito population with insects that are less competent as vectors (Catteruccia *et al.*, 2000; Marshall & Taylor, 2009). Experiments with vectorial capacity have investigated several aspects of mosquito-parasite interactions, including the pathogen's invasion of the midgut or salivary glands, and the strength of the mosquito's immune response to the parasite (Wang & Jacobs-Lorena, 2013; Li *et al*., 2013).Use of sterile insects, or insects that are refractory to disease has most potential when populations are physically or ecologically isolated, and as such may be suitable for use against 'urban island' populations of *Anopheles stephensi* in India (Knols *et al.*, 2007).

Like house screening, these methods are not reliant on insecticides, but they are species specific, so would need to be adapted for use on each of the different malaria vector species.

### *1.5.11 Integrated Vector Management*

The WHO encourages the use of Integrated Vector Management (IVM) in malaria control (WHO, 2008a). This is the integration of two or more techniques, in a cost-effective, multi-sectorial approach to vector control. IVM can pair insecticide based interventions such as IRS or LLINs with LSM or house screening (Beier *et al.*, 2008; Chanda *et al.*, 2008). By avoiding reliance on one control tool, IVM is more robust to resistance to interventions through insecticide or behavioural resistance. IVM must be fitted to local conditions, and as such there is potential for community participation in programs: using clubs and community groups empowers a community to feel involved in control and can help disseminate public health messages about mosquito control (Mutero *et al.*, 2015).

## 1.6 Resistance to Interventions: Insecticide Resistance

There are four insecticide classes available for use in mosquito control: organophosphates, carbamates, chlorinated hydrocarbons and pyrethroids. Of these, only pyrethroids have been approved by the WHO for use with/ on insecticide treated bed nets (Zaim *et al*, 2000). Pyrethroids are suitable for this purpose because of their low mammalian toxicity, and rapid lethal action against mosquitoes (Chavasse & Yap, 1997).

IRS can use any of the four different insecticide classes. The flexibility of IRS is useful in managing insecticide resistance, as different insecticides can be rotated or combined to manage insecticide resistance (WHO, 2012). Pyrethroids are often cheaper than other insecticide classes, and as of 2012 the majority of countries using IRS employed pyrethroids as their primary insecticide (WHO, 2013a). However in the context of increasing insecticide resistance the WHO now advises the use of non-pyrethroids in IRS campaigns, particularly when LLINs are also in use (WHO, 2012).

Insecticides may be supplemented with the juvenile hormone mimic PPF or the synergist piperonyl butoxide (PBO) to increase or restore their toxic effects against mosquitoes (N'Guessan *et al.*, 2010; Ngufor *et al.*, 2014). Toxins from bacteria, such as *Bacillus thuringiensis israelensis* (Bti) can be used as biological insecticides/ biopesticides in larval control (Gill *et al.*, 1992; Lacey, 2007).

With this limited arsenal of chemicals suitable for use in vector control, insecticide resistance is a real challenge to malaria control. Insecticide resistance to DDT was noted in sub-Saharan Africa following large-scale IRS campaigns in the 1950s and 1960s (Kouznetsov, 1976). The first reported cases of pyrethroid resistance in *An. gambiae s.s.* were in Cote D'Ivoire in 1993 (Elissa *et al.*, 1993). Resistance of *An. gambiae* to organochlorines and pyrethroids has since become widespread across the continent, with resistance to carbamates and organophosphates emerging recently too (Knox *et al.*, 2014; irmapper.com, 2015).

Insecticide resistance can be caused by mutations to the voltage-gated sodium channel, which is the site of action of pyrethroids and DDT (Martinez-Torres *et al.*, 1998; Ranson *et al.*, 2000). Metabolic resistance is also widespread, occurring as a result of mutations to, or overexpression of, P450 or glutathione S-transferase enzymes involved in insecticide metabolism (Bergé *et al.*, 1998; Vulule *et al.*, 1998). Changes to and thickening of mosquito cuticle is a less common characteristic also suspected to contribute to insecticide resistance is some cases (Djouaka *et al.*, 2008). A single mosquito population may be resistant to several different insecticides, which creates a difficult control scenario (Martinez-Torres *et al.*, 1998; Ranson *et al.*, 2000; Edi *et al.*, 2014).

As insecticide resistance has only recently become so widespread, monitoring of its effects on control interventions is still at an early stage. A recent systematic review found no evidence to indicate that insecticide resistance was reducing the effectiveness (mosquito mortality) of insecticide-treated nets (Strode *et al.*, 2014). However the reviewers stated that available information was limited, as only four studies had been carried out since 2012.  Not only has insecticide resistance been spreading between countries in recent years, but it has increased in intensity, as resistant mosquitoes have become less sensitive to knockdown even after extended insecticide exposure times (Toé *et al.*, 2014). As such, the results of the review must be treated with caution as they may not reflect the impact of resistance on control in the current context of high intensity and geographically widespread insecticide resistance. Modelling results of mass LLIN distributions in different resistance scenarios present a more worrying picture. A mass distribution of LLINs in an area of highly insecticide resistant mosquitoes would avert roughly 40% fewer cases of malaria than if the insects were susceptible, according to recent modelling study by Briët *et al.* (2013). In the face of this threat to mosquito control it is more important than ever to get a clear picture of the full effects of how mosquitoes interact with LLINs, and how they are affected by insecticide exposure.

## 1.7 Behavioural Resistance

In Africa, the two most widely used control tools, LLINs and IRS, target mosquitoes that feed indoors at night such as *An. gambiae s.s.* and *An. funestus*. Fewer interventions are designed to counter exophagic and zoophagic mosquitoes such as *An. arabiensis*. High coverage with LLINs and IRS provides a strong selection pressure for behavioural changes which would reduce mosquito contact with insecticide: earlier biting times, outdoor biting, and zoophagy, which could undermine progress made in malaria control (Lockwood *et al.*, 1984; Govella & Ferguson, 2012; Gatton *et al.*, 2013).

### *1.7.1 Biting Times*

Use of LLINs protects the human population at night when they are sleeping. This intervention exploits the nocturnal biting behaviour of *Anopheles* mosquitoes (Clements, 1999). However some initial evidence has suggested that *An. gambiae s.l.* and *An. funestus* in south-eastern Tanzania may be adapting to high use of indoor control by biting earlier in the evening when nets will not be in use by most people (Russell *et al.*, 2011), presenting an obvious challenge for control.  That study did not clarify whether the shift in biting times observed was a result of changes in behaviour in *An. gambiae s.s.* or whether vector control has changed the proportions of different sibling species present, selecting for higher proportions of

*An. arabiensis*. Comparing recent population surveys against analyses of archived dried specimens and historical data favours the latter explanation as *An. gambiae s.s.* numbers appear to have decreased relative to *An. arabiensis* at a similar site in north-eastern Tanzania (Derua *et al.,* 2012). However there is also historic evidence for similar behavioural changes evolving in populations in response to insecticide selection pressure. On islands in the southwest Pacific, *Anopheles farauti* was found to change its biting times to attack earlier in the evening following widespread use of IRS (Russell *et al.*, 2013). This behaviour was maintained following cessation of IRS which suggests that, rather than responding flexibly to circumstances, the population had undergone genetic selection for earlier biting times. Changes in a species' behaviour, and/ or an increase in population of species with 'behaviourally resistant' characteristics have the potential to maintain residual malaria transmission (Killeen, 2014).

Shifts in biting times have thus far only been recorded in a few sites in sub-Saharan Africa (Russell *et al.*, 2011; Moiroux *et al.*, 2012). Recently some sites have noted *Anopheles* populations with early biting times that are not typical of the species, but lack historical data to document this as a change from past behaviour (Yohannes & Boelee, 2012; Ojuka *et al.*, 2015). Other studies have not found such changes. A recent survey of six sites in both West and East Africa where LLINs have been in use found that in the majority of cases mosquito activity still peaked late at night when most people are indoors and likely to be protected by LLINs (Huho *et al.*, 2013).

### 1.7.2 Exophagy and Exophily

One of the first reports of exophily following vector control found increased numbers of *An. gambiae* resting outdoors following IRS with an organochloride insecticide in Zimbabwe (Muirhead-Thomson, 1960). However this and other studies published after large scale IRS campaigns, lacked sufficient evidence to indicate change, did not distinguish between sibling species and, in some cases, could not exclude the possibility that 'adaptations' observed might have resulted from irritant or repellent properties of insecticide used in houses (Muirhead-Thomson, 1960).

Similar problems have also affected more recent data. Huho *et al.* (2013) found evidence for increased early evening and outdoor biting in *An. funestus s.l.* populations in Burkina Faso sites (though the same trend was not found in Tanzania, Kenya, or Zambia). Again, this change may result from behavioural adaptation or a change in sibling species composition.

In both Russell *et al.* (2011) and Huho *et al.* (2013), changes to earlier biting times and exophagic behaviour were found to occur together in the same mosquito population, but in other cases such changes were found to be independent. One recent study from western Kenya found that after ten years of LLIN use, there had been some subtle shifts towards outdoor biting in *An. arabiensis* and *An. funestus*, but the peak of activity was still late at night when most people would be indoors under insecticide-treated nets (Bayoh *et al.*, 2014).

### *1.7.3 Zoophagy*

Highly or entirely anthropophilic vectors can adapt their host preferences when humans are protected from bites (Bøgh *et al.*, 1998; Lefèvre *et al.*, 2009a; Lyimo & Ferguson, 2009). Such a switch could impact on mosquito fitness by reducing fecundity or longevity (Lyimo & Ferguson, 2009; Lyimo *et al.*, 2012, 2013). There is some evidence for host choice changing according to host abundance or following IRS in *An. funestus* and *An. arabiensis*, but *An. gambiae s.s.* appears less flexible in its preferences and therefore more vulnerable to existing control methods (Bruce-Chwatt *et al.*, 1966; Iwashita *et al.*, 2014). Simple models of other insect populations suggest that behavioural adaptations which avoid insecticide contact will slow the development of physiological resistance, and likewise that a population with physiological resistance will be less likely to develop behavioural resistance (Gould, 1984). However data collected on populations of various insect species has found that physiological and behavioural resistance may occur at the same time, and that traits are sometimes linked, making the relationship between the two adaptations harder to predict (Lockwood *et al.*, 1984).

Independently of behavioural changes, mosquito species such as *An. arabiensis* which exhibit exophagic and zoophagic tendencies already present a problem for control. Where large scale control programs predominantly use IRS and LLINs, residual malaria transmission may persist as a result of species which are less affected by indoor interventions (Killeen, 2014). In order to decrease malaria transmission to non-self-sustaining levels it will be necessary to use interventions capable of impacting these behaviourally resilient mosquitoes, such as larviciding and odour baited traps (Ferguson *et al.*, 2010; Govella & Ferguson, 2012). Any control strategy which fails to consider mosquito behaviour and behavioural change risks dulled impact, and will be vulnerable to disease rebound if control is not sustained (Ferguson *et al.*, 2010; Killeen, 2014).

## 1.8 Host location, selection and blood-feeding

Mosquitoes use a range of host-derived cues to locate and select their hosts for blood-feeding. Known and possible cues include individual or blends of chemicals (including carbon dioxide and water) in exhaled breath, in glandular secretions deposited onto the skin as well as the by-products of bacterial metabolism of those secretions (Takken & Knols, 1999). Although olfactory cues have received much attention in research, visual, thermal and other cues are also involved (Gibson & Torr, 1999; McMeniman *et al.*, 2014; van Breugel *et al.*, 2015).

As with other blood-feeding insects (Sutcliffe, 1987) different cues facilitate host location at different distances from the host (Takken, 1991). Different species rely on cues to different extents: day active mosquitoes are attracted to visual cues of an object, responding to colour and movement, whereas nocturnal mosquitoes may rely more on odour cues (Allan *et al.*, 1987).

A mosquito responds to a number of different cues when choosing to fly towards an attractant source. Several cues act synergistically to attract mosquitoes (Olanga *et al.*, 2010; Spitzen *et al.*, 2013; McMeniman *et al.*, 2014) and mosquitoes appear to respond to signals identifying the presence of a suitable host in a sequence of events (Takken *et al.*, 2001). Carbon dioxide in exhaled breath acts as a long-range cue, activating host seeking flight (Snow, 1970). Mosquitoes sense carbon dioxide via receptors on their maxillary palps (Kellog, 1970; Omer & Gillies, 1971). Background $CO_2$ stands at approximately 350 parts per million (Thoning *et al*, 1989), but electrophysiological tests of *Ae. aegypti* found $CO_2$ receptors on maxillary palps are capable of detecting concentrations as low as 150 parts per million, and are sensitive to changes in concentration as small as 50 parts per million (Grant & O'Connell, 1996, 2007). Other odour receptor genes are expressed on the antennae, palps and labellum (Hill *et al.*, 2002; Hallem *et al.*, 2006), and as with $CO_2$, responses to body odour are affected by concentration of cues (Gillies, 1980; Takken *et al.*, 1997a; Healy *et al.*, 2002).

Wind tunnel tests indicate that $CO_2$ activates flight upwind (Healy & Copland, 1995; Dekker *et al.*, 2005). Though a mosquito is sensitive to low concentrations of olfactory cues, due to the chaotic dispersion of odour in wind, they are thought to navigate towards a host over long ranges simply by following an odour plume upwind rather than navigating along a chemical gradient (Cardé & Willis, 2008). The chemical gradient leading to the host will likely only prove useful within centimetres' distance of the host (Lacey & Cardé, 2012).

Air currents play a key part in host location: mosquitoes fly upwind, moving anemotactically towards the source of attractive cues (Swellengrebel, 1929; Kennedy, 1939; Bertram & McGregor, 1956). The structure of an air current affects response. Mosquitoes respond best to carbon dioxide when it is presented in a turbulent plume of air, (Geier *et al.*, 1999; Dekker *et al.*, 2001).

Odours from human sweat act as both long and short range cues, both stimulating flight towards an attractant source, and as the mosquito approaches the host, eliciting landing and biting (De Jong & Knols, 1995; Healy & Copland, 2000; Verhulst *et al.*, 2009; Spitzen *et al.*, 2013). $CO_2$ is also important for short range host response, provoking landing in wind tunnel tests (Healy & Copland, 1995; Lacey *et al.*, 2014; McMeniman *et al.*, 2014; Webster *et al.*, 2015). Unlike carbon dioxide, body odours may be used by the mosquito in host choice, helping anthropophilic species to distinguish between humans and animals (Zweibel & Takken, 2004; McBride *et al.*, 2014). In dual choice olfactometers, *An. gambiae s.s.* will approach a source of human odour, but prefer clean air to a source of cow odour (Pates *et al.*, 2001). Mosquitoes are also sensitive to the concentration of odour cues and $CO_2$, and higher concentrations of these chemicals may deter mosquito approach (Takken *et al.*, 1997a; Dekker *et al.,* 2001; Mukabana *et al.*, 2004; Lefèvre *et al.*, 2009b; Webster *et al.*, 2015).

Wind tunnel flight tracking tests with *An. gambiae s.s.* have shown that heat can contribute to encouraging the mosquito to land at an attractant source, but is not used in initiating flight, suggesting that this acts as a short range attractive cue (Spitzen *et al.*, 2008; Spitzen *et al.*, 2013). Whilst moisture increases attraction of *Ae. aegypti* to heated baits over small scale distances, there have as yet been no equivalent investigations showing this effect in *Anopheles* species (van Breugel *et al.*, 2015).

Though *Anopheles* may use vision during host location and navigation, it is likely secondary to other cues such as olfaction and heat (Clements, 1999). The eyes of nocturnal mosquitoes are adapted to low levels of light, and it is thought that vision is used in gauging flight speed in the optomotor response (Gibson, 1995; Land *et al.*, 1999). Some nocturnal mosquito species show increased attraction to conspicuous dark objects, but as the dark objects caught both blood-fed and gravid mosquitoes this may not be related to host seeking alone (Bidlingmayer & Hem, 1979; Gillies & Wilkes, 1982). *Anopheles gambiae* can learn to associate visual cues with negative stimuli in host seeking (Chilaka *et al.*, 2012), and human landing catches of the nocturnal *Mansonia sp.* are slightly higher when the bait is surrounded by a dark canopy (Gillies & Wilkes, 1982).

Host seeking responses depend on appropriate environmental conditions. *Anopheles gambiae s.s.* will not respond to skin odour when humidity is too low, or feed following exposure to light (Takken *et al.*, 1997b; Das & Dimopoulos, 2008).

Cues act synergistically, and one cue can gate a response to another: attraction to human odour or a synthetic odour blend can be improved by addition of heat and moisture (Olanga *et al.*, 2010; Spitzen *et al.*, 2013). Tests with *Ae. aegypti* suggest that presentation of multiple cues may be necessary to engage host seeking*: e.g.* heat, lactic acid and human odour presented individually produced weak to no attraction, but when paired with $CO_2$, the two stimuli attracted many more mosquitoes, acting synergistically to release host seeking flight (McMeniman *et al.*, 2014).

### *1.8.1 Manipulation of mosquito behavioural responses by parasitic infections*

There is some evidence that mosquito biting behaviour may be influenced by malaria infection in both the host and the vectors. In some studies, infected mosquitoes have been shown to exhibit higher responses to host odour (Rossignol *et al.*, 1986; Smallegange *et al.*, 2013) Other studies have shown that the parasite may change an organism's odour profile, making the human hosts more attractive to mosquitoes (Lacroix *et al.*, 2005; De Moraes *et al.*, 2014). However, there is some debate as to whether behaviour of infected mosquitoes represents parasite manipulation, or a general response to immune challenge, since mosquitoes infected with heat killed *Escherichia coli* also show increased host seeking activity (Cator *et al.*, 2013).

Following arrival at the host, additional effects may also occur. Malaria infection can cause more frequent and persistent probing behaviour, a behavioural change believed to occur as a result of parasite-caused pathology to the salivary glands (Weseka *et al.*, 1992; Anderson *et al.*, 1999; Hurd, 2003).

## 1.9 Bite Site Selection

A number of studies (De Jong & Knols, 1995; Dekker *et al.*, 1998) reported that *An. gambiae s.s.* preferentially oriented to and landed on the feet and legs of a seated human. This response was partly attributed to human foot odour (De Jong & Knols, 1995), but subsequent work indicated that the body posture of the human volunteer significantly altered that response as mosquitoes showed no preference for any particular body part when the host lay horizontally on the floor (Dekker *et al.*, 1998). Moreover, when legs were raised in the air they were bitten comparatively less often, as mosquitoes attacked the (now lower) volunteer's arms and trunk instead, which suggested that selection of bite site was determined by which body part was closest to the ground. Qualitative observations in De Jong & Knols' paper found mosquitoes would often approach the volunteer at head height before descending towards the feet, and the authors speculated that mosquitoes were following convection currents towards the feet, though alternatively this descent could have been a consequence of the height at which mosquitoes were released in to the test arena. Convection currents around the human body extend a short distance around and above the human body and could, therefore, be used in short range host location (Lewis *et al.*, 1969). Body heat produces a thin homogenous air current around the feet and legs of a standing human, which is initially contained within a 3cm boundary layer, but forms a wider plume around the body as it rises, expanding to a 15-20cm thick air column around the head (Clark & Toy, 1975). The air plume becomes more turbulent as it reaches chest height, and is still detectable up to 50cm above the head of a standing human (Lewis *et al.*, 1969). Combining this physical evidence with subsequent observations of mosquito landing locations in bed net studies (Lynd & McCall, 2013; Sutcliffe & Yin, 2014), contributes to the hypothesis that mosquitoes follow convection currents carrying attractive cues to their source to locate a blood meal.

## 1.10 Effects of insecticides and other interventions on mosquito behaviour

### *1.10.1 Spatial Repellency and Contact Irritancy*

LLINs and IRS, the most commonly used vector control tools, use insecticide to reduce human exposure to (infective) bites and to suppress mosquito populations. The effectiveness of insecticide-based control tools on mosquito populations depends on how the insecticide alters or impacts the target mosquito's behaviour. For LLINs, of primary importance are the treated net's repellent properties, its contact irritancy, and toxicity to mosquitoes, and what duration of contact is required to lead to irritancy, mosquito knockdown or mortality. In this thesis, references to repellency and irritancy use the definitions of Grieco *et al.*, (2007): "contact irritant action stimulates directed movement away from the chemical source after the mosquito makes physical contact. A spatial repellent action stimulates directed movement away from the chemical source without the mosquito making physical contact with the treated surface". "Directed movement" is here taken as flight in any direction moving away from a treated surface, but in descriptions of repellency, it includes any reduced tendency to fly towards the host.

A bed net that has strong repellent properties will deter mosquitoes from approaching, but risks diverting them to unprotected individuals when not all members of a community are using nets (Killeen & Chitnis, 2014).

Irritant and repellent properties of insecticide may also affect mosquito exposure of the whole household. In tests of permethrin-treated nets, some studies have found the number of bites received by unprotected individuals sleeping in the same room decreased (Lines *et al.*, 1987). This effect was not observed in work with lambda-cyhalothrin treated nets, where experiments suggested that an unprotected individual will receive a greater burden of bites when sharing a room with a net user (Arredondo-Jiménez *et al.*, 1997). The different repellent and irritant properties of the insecticide used on bed net may be responsible for these contrasting findings, as permethrin-treated nets in this study prompted a much higher rate of house exit, thereby reducing the unprotected individual's exposure to mosquitoes. Differences in results may also relate to the different species of mosquitoes (*An. arabiensis* [Lines *et al.*, 1987], *An. albimanus* [Arredondo-Jiménez *et al.*, 1997]) used in the two studies.

Rapid-acting contact irritancy is undesirable in that it might cause mosquitoes to leave an insecticide treated surface before receiving a lethal dose of insecticide, though it could benefit the net user: for example, mosquitoes would not feed for a long time (perhaps not long enough to transmit infection) through a net if the sleeper's skin was in contact with the net surface (Hossain & Curtis, 1989). A modelling study compared the impact of different theoretical profiles of insecticides for use on bed nets, examining the relative effects of product toxicity and deterrency (a term that here encompasses both repellent and irritant effects) (Killeen *et al.*, 2011). Perhaps unsurprisingly, nets with high toxicity to mosquitoes and zero repellent effect were found to be most effective. Toxic but partially repellent products performed better at the community level than products offering users 100% repellent protection in this model, providing some protection to non-users in the community as mosquitoes would not survive exposure to feed on these people.

### *1.10.2 Action of Insecticide on Mosquitoes*

The four insecticide classes show different behavioural effects, though there is some similarity in properties of insecticides of the same class. Insecticide effects on behaviour vary according to dose used, but in general, higher doses of insecticide have more pronounced behavioural effects (Evans, 1993; Hougard *et al.*, 2003a).

Behavioural assays of *Ae. aegypti* which tested several members of the four classes found pyrethroids to be the most irritating class of insecticides (Achee *et al.*, 2009). Within the pyrethroid class, bifenthrin was found to be less irritating to *An. gambiae* and *Cx. quinquefasciatus* than other pyrethroids at WHO recommended doses (Hougard *et al.*, 2003a). Organophosphates had weak irritant effects on *Ae. aegypti* compared to other insecticide classes (Achee *et al.*, 2009). Within their classes, DDT was the most irritating organochlorine, and propoxur the most irritating carbamate. Other insecticides had moderate to weak irritating effects depending on concentration. Of all insecticides in all classes tested, only DDT was found to have significant repellent action against *Ae. aegypti* (Achee *et al.*, 2009). A laboratory study looking at escape responses of mosquitoes to insecticides found deltamethrin or permethrin were repellent to *Ae. albopictus* and *An. minimus*, but not to *Cx. quinquefasciatus* or *Ae. aegypti* (Sathantriphop *et al.*, 2014a). Field test results vary according to insecticide formulation, but many studies have failed to find a repellent effect of pyrethroids in field settings (Miller *et al.*, 1991; Tungu *et al.*, 2010), and as with laboratory tests some show differences in responses of *An. gambiae* and *Cx. quinquefasciatus* (N'Guessan *et al.*, 2010).

Further nuances have been found in investigations of the escape responses of three strains of *An. albimanus* (Chareonviriyaphap *et al.*, 1997), where DDT, deltamethrin and permethrin acted as contact irritants to all three strains tested, but only some strains found the chemicals repellent. There are also indications that behavioural responses can be affected by insecticide resistance status and the type of behavioural assay employed.

### 1.10.3 Insecticide Resistance and Mosquito Behaviour

There is some evidence that activity levels and behaviour of mosquitoes is genetically controlled. Selection experiments have been able to produce mosquitoes that are hyperirritable, or less responsive to repellents (Gerold & Laarman, 1964; Stanczyk *et al.*, 2010). Some mutations affecting insecticide susceptibility appear to be pleiotropic (impacting more than one unrelated phenotypic trait), affecting behavioural traits too. Resistant mosquitoes can be less responsive to behavioural effects of insecticide, as evidenced by reduced irritability responses (Rowland,1990; Chandre *et al.*, 2000; Hougard *et al.*, 2003a). This topic will be discussed in further detail in Chapter 3.

Insecticide resistance can also affect host seeking behaviours. Pyrethroid resistant mosquitoes with the kdr mutation were slower to reach a source of host odours

(Spitzen *et al.*, 2014). This suggests that insecticide resistance may affect other aspects of a mosquito's biology including host seeking abilities. A recent study examined mosquito success in reaching a guinea pig bait via a holed LLIN screen. Here kdr homozygotes were less successful than heterozygotes at navigating through the holes to blood-feed (Corbel *et al.*, 2004; Diop *et al.*, 2015), though this effect has not been found in all studies of kdr (Chandre *et al.*, 2000). More generally, homozygotic dieldrin resistant *An. gambiae* and *An. stephensi* have been found to be less active and less responsive to oviposition stimuli than homozygotic susceptible and heterozygote individuals (Rowland, 1991). If mutations contributing to physiological resistance carried a behavioural disadvantage this could slow the spread of the resistance mutation, prolonging the efficacy of insecticide based interventions. At present potentially detrimental effects have been observed predominantly in *An. gambiae* with kdr mutations, while other studies investigating the effect of metabolic resistance, or other insecticide resistance mechanisms in *Anopheles stephensi* found no evidence for behavioural changes resulting from differences in susceptibility (Hodjati & Curtis, 1997; Kolaczinski & Curtis, 2000; N'Guessan *et al.*, 2007).

### *1.10.4 Measuring effects of insecticides on behaviour*

Precisely determining the true nature of the effect of insecticide-treated surfaces on mosquitoes, and determining whether or not contact is required to achieve impact, is fundamental to ensuring optimal insecticide delivery in terms of highest impact and minimising resistance development. It is important to use appropriate behavioural assays to obtain representative behavioural data on insecticide effects, and impact of insecticide on host seeking. Until the recent availability of affordable camera systems, direct observation of mosquito host-seeking behaviour and the effects of insecticides were restricted to experimental hut studies or laboratory-based bioassays. Despite those limitations, many studies were undertaken and have formed the basis of how insecticide-altered behaviours are classified and for the development and evaluation of new chemicals. The behavioural assays used to examine insecticide impact on behaviour are reviewed here, focussing particularly on studies using pyrethroids, the insecticides used on LLINs.

One simple and rapid test for assaying contact irritant properties of an insecticide are 'time to first take-off assays', performed using plastic cone housings placed against insecticide treated material.  Here the outcome measured is simply the time elapsed between first landing and first take off (Hougard *et al.*, 2003a). This is a useful, high throughput test that allows basic comparison of irritant properties of several different insecticides. In these comparisons, WHO diagnostic doses of deltamethrin and permethrin cause the same levels of contact irritancy, but effects can vary with dose, mosquito species, and insecticide resistance status (Chandre *et al.*, 2000; Hougard *et al.*, 2003a). These assays are particularly useful in evaluating insecticides for use in IRS, indicating how long a mosquito will stay in contact with a treated wall before irritation leads to flight.

Small tube assays can be used in high throughput screening (Figure 1.1). Here mosquitoes are permitted to move towards or away from cylindrical chambers lined with insecticide treated papers, in contact and non-contact scenarios to distinguish repellent and irritant impacts of chemicals (Grieco *et al.*, 2005). Although the small scale of operation (tubes have an internal diameter of less than 10cm), and lack of either artificial or live bait could theoretically compromise result reliability, in practice experimental hut trials conducted by the same group found that field results compared well to laboratory findings, supporting the reliability of this testing method (Grieco *et al.*, 2007).

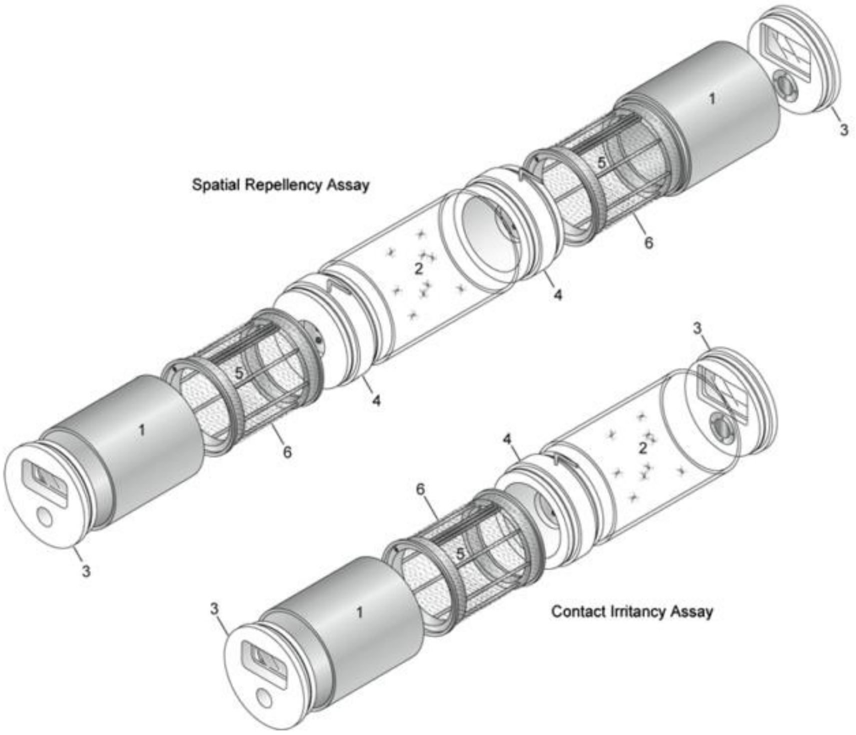

**Figure 1.1 Schematic showing the test arena used by Grieco *et al.* (2005).**

In contact irritancy assays, treated net is placed on an internal frame (5, 6) within a test cylinder (1). Mosquitoes are released in to the test cylinder (1) and permitted to pass through a funnel cap port (4) towards the clear cylinder (2). The apparatus is sealed with an end cap (3). In spatial repellent assays one test cylinder contains treated netting, the other is untreated, and mosquitoes are released in to the clear central cylinder (2). Figure from Grieco *et al.* (2007).

Cooperband & Allan (2009) extended this approach to examine landing frequency and duration, using infrared (IR) sensitive cameras to film behaviour around insecticide treated papers in an IR illuminated test chamber. As mosquitoes are unable to detect infrared wavelengths (Gibson, 1995) this ‘dark’ set-up mimicked their nocturnal feeding conditions. The system was used to examine landing preferences in *Ae. aegypti* and *Cx. quinquefasciatus* when given a choice of treated substrates or an untreated control. The study examined behavioural changes over time and found that both *Ae. aegypti* and *Cx. quinquefasciatus* made less contact with bifenthrin and deltamethrin as the test progressed, making fewer landings on this surface relative to the control paper, in response to irritant effects of the chemicals. Initial landing frequencies were the same on treated and untreated papers, indicating that the insecticide concentrations tested were not repellent.

In tests in which Permanet or Olyset LLINs were baited with a human hand, no repellency was found as insecticide treatment had no effect on latency until landing, but landing frequency was different over the full test period implying post-contact irritant effects (Siegert *et al.,* 2009). Though in this instance, the baited and unbaited test results agree, in other studies, landing duration and persistence can be affected by presence of bait, as mosquito behaviour can change when stimulated by attractive cues.

### *1.10.5 Importance of the host in behaviour tests*

Ideally a behavioural assay designed to demonstrate the efficacy of insecticide used in LLINs should include a bait to simulate the presence of a human. This can prove important, as responses may vary according to the context of odours experienced during exposure. Dogan *et al.* (1999) found that the mosquito repellent DEET was actually attractive to mosquitoes in the absence of accompanying human odours, highlighting the importance of testing chemicals in the context of their intended use.

The impact of a host’s presence on mosquito behaviour was investigated in Siegert *et al.* (2009) in experiments that alternatively presented mosquitoes with a gloved, and un-gloved hand covered with an LLIN. Results showed that the presence of an un-gloved hand had a significant effect on approaches to an LLIN, showing that stronger host cues elicited a more pronounced host seeking response. In the total absence of the hand (*i.e.* no attractant), mosquitoes did not contact the net sample.

Host effects have also been examined using contact irritant assays, in which mosquitoes are introduced to a test box lined with insecticide treated papers, and allowed to escape through a narrow exit slit which is monitored over time

(Chareonviriyaphap *et al.,* 2001, 2002). Responses to insecticide were assessed in the presence or absence of a guinea pig host in the test box (Figure 1.2). When the guinea pig was present, *Anopheles minimus* stayed longer in the box prior to escaping when exposed to the insecticides deltamethrin and bifenthrin (Kongmee *et al.*, 2012). Mated *Aedes aegypti* showed similar reduced escape responses to deltamethrin when a guinea pig was present (Boonyuan *et al.*, 2011). The effect was not seen in unmated *Anopheles harrisoni*, or unmated *Ae. aegypti*, and the authors suggested this was because unmated females were less likely to seek a blood meal.

Results of tests with baited exit response assays, which distinguish contact irritant and repellent insecticide properties suggest deltamethrin and bifenthrin had little repellent effect, and that escape responses to the chemicals were the result of contact irritancy: mosquitoes placed inside the baited test box but prevented from contacting insecticide treated paper by a net screen (Figure 1.2), did not escape from the box (Kongmee *et al.*, 2012).

Using a live bait, whether human or animal, in behavioural tests may present practical challenges in terms of recruiting volunteers or the need for additional ethical permits for research (Achee *et al.*, 2015). However artificial baits releasing odour blends or carbon dioxide do not always make adequate substitutes. For example  a choice test bioassay using synthetic odour baits made from blends of carboxylic acids, ammonia and $CO_2$ showed that mosquitoes were more attracted to the synthetic odour bait when it was paired with the repellent PMD (para-Menthane-3, 8-diol), than to the odour bait alone (Okumu *et al.*, 2009). PMD is repellent when used on human volunteers, so results show that tests with artificial baits may give unreliable impressions of how different chemicals affect mosquito behaviour.

It has also been suggested that the species of host used is important to mosquito behaviour. When *Ae. aegypti* are given the opportunity to feed through netting treated with permethrin on a mouse or, in separate tests, on a human, a low dose of insecticide was sufficient to completely deter feeding on mice, but over 50% of mosquitoes persisted in feeding through the net on humans even at the highest insecticide concentration (Hossain & Curtis, 1989). Given that guinea pigs are not the preferred host of the anthropophilic malaria vectors, it is possible that using them as baits in behavioural assays may deliver results that are not entirely representative of what occurs with human hosts.  Hossain and Curtis (1989) also showed that the presence of an accessible blood meal encouraged prolonged contact with the net, testing persistence of contact in a more realistic context than that used in unbaited cone tests. However as mosquitoes were enclosed in covered

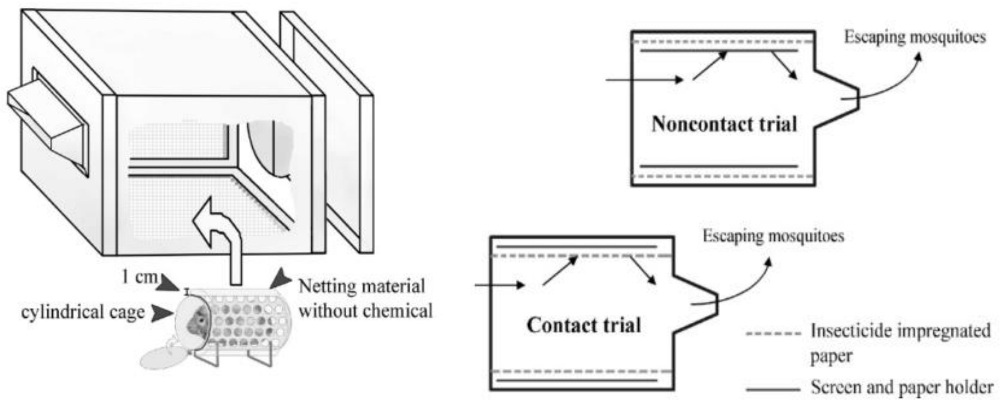

**Figure 1.2 Irritant and repellent assay used by Boonyuan *et al.* (2011).**

Mosquitoes are placed within the central test chamber, and allowed to escape through a vent. The test chamber is baited with a guinea pig, and lined with insecticide papers. In noncontact trials a screen excludes physical contact with insecticide. Chareonviriyaphap *et al.* (2001, 2002) employed this assay without the use of a guinea pig bait. Figure from Kongmee *et al.* (2012).

paper cups, the end of which was placed against the mouse's skin, little host seeking action was required on the part of the mosquito (Hossain & Curtis, 1989). The same study also performed room-scale free flight assays using a volunteer lying with their arm pressed against a bed net, in a test that required host seeking flight to locate a blood meal. They found that whilst over 70% of *An. gambiae* mosquitoes fed through an insecticide-treated net when placed in a paper cup against a mouse, none managed to successfully feed through the treated net in free flight assays. A similar effect of scale was found in host seeking tests with mice in *Ae. aegypti* which examined the behaviour of mosquitoes with mutations to their $CO_2$ receptors (McMeniman *et al.*, 2014): roughly 50% of mosquitoes successfully fed on the mouse in smaller 30x30x30cm cages, but less than 25% fed in a 61x61x91cm cage. Scale is therefore an important consideration in the design of behavioural assays.

A consideration when using real hosts as bait, is the likely increase in variability in test results. This variability has been well studied in humans. Mosquitoes are more attracted to some people than others due to differences in odour cues (Knols *et al.*, 1995; Logan *et al.*, 2008), which may in turn be influenced by factors including the individual's weight, age (Port *et al.*, 1980), and the composition of their skin microbiota (Verhulst *et al.*, 2011). Attractiveness of the same individual may fluctuate over time according to a person's alcohol intake (Lefèvre *et al.,* 2010), if they have washed recently (De Jong & Knols, 1995) or whether they are pregnant (Ansell *et al.*, 2000). However, humans remain the best host for tests that seek to elicit realistic behavioural responses from mosquitoes, and experiments can be designed to use multiple volunteers to avoid biasing results according to such variations in attractiveness.

### *1.10.6 Baited Tunnel Tests*

Baited tunnel tests allow the realisation of a more complete simulation of natural mosquito foraging around LLINs. Insects are released at the end of a tunnel screened with holed treated netting, which blocks the mosquitoes' path towards a host bait (generally a guinea pig) (WHO, 2005a). The outcomes measured describe blood-feeding inhibition and mortality. In this method, insects are tested in large numbers (100 per test) and confined within the tunnel for 15 hours. Whilst results can give an indication of a mosquito's ability to locate and move through holes, scores do not translate easily to the field for a number of reasons.  For instance, tunnel tests conducted by N'Guessan *et al.* (2010) reported that Permanet 3 nets inhibited blood-feeding in *An. gambiae* by almost 100%. However field experiments conducted in the same study reported blood-feeding inhibition of less than 27% when the same nets were tested with human hosts in experimental hut studies (see section 1.10.11). Similar discrepancies between laboratory and field experiments were reported by Malima *et al.* (2009), where tunnel tests indicated that carbosulfan treated netting inhibited blood-feeding in *An. gambiae* by almost 100%, but inhibition in experimental hut trials was negligible.

The disparity may result from a number of factors. Firstly the use of guinea pigs as bait may reduce blood-feeding rates in anthropophilic insects. Secondly, the tunnel uses 1cm holes which proportionally fit with the scale of the test: such small holes limit the number of mosquitoes that can successfully pass through the net, and increase the amount of net contact mosquitoes make during hole location and transit (Itoh *et al.*, 1986; Sutcliffe & Colborn, 2015). Experimental hut trials use larger 4x4cm holes in LLINs, which should be easier for mosquitoes to locate and enter with less physical contact with the net, therefore fewer mosquitoes will be knocked down prior to successful entry and host feeding. LLINs typically develop holes of up to 10cm diameter within their first year of use, and continue to accumulate greater damage over time (Morgan *et al.*, 2015). The larger holes are more representative of the damage sustained in normal net use. Tunnel tests therefore may give an exaggerated view of LLIN impact, and results could mislead as to a net's efficacy in the field.

### *1.10.7 Wind Tunnel Tests*

By introducing an air current to a baited tunnel, wind tunnels simulate the movement of mosquitoes upwind towards a host in response to attractant plumes. In such tests mosquitoes are released and observed as they approach a guinea pig host behind a barrier of insecticide treated netting that prevents feeding. Early work with this approach simply filmed and analysed recorded behaviour by observation and manual enumeration and reported that insecticide treatment of netting reduced time spent at rest on the net, provoking dose dependent irritancy and knockdown (Miller & Gibson, 1994). One of the most important results of this work was the demonstration that mosquitoes would persist in host location, spending long periods in contact with the insecticide-treated net when attempting to locate a host. The study design of Miller & Gibson (1994), where mosquitoes could not reach the bait, and persistence was induced simply by attraction to the host, contrasted with Hossain & Curtis' (1989) setup, in which mosquitoes contacting the net were allowed to feed through it. Miller & Gibson (1994) also showed differences in the responses of *An. gambiae* and *Culex quinquefasciatus* to insecticides, a point which has been noted in a number of other assays (Hossain & Curtis, 1989; Hougard *et al.*, 2003a). In addition the formulation of insecticide affected results, with an emulsifiable concentrate causing more irritation to mosquitoes than a wash resistant version (Miller & Gibson, 1994).

### *1.10.8 Tracking Mosquitoes in Flight*

More recent work using wind tunnels has used cameras to film and subsequently track mosquito flight towards attractive cues (Figure 1.3). Spitzen *et al.* (2014) extended this to tracking flight paths in 3D as mosquitoes moved towards a bait that was protected behind a barrier of deltamethrin treated netting. In tests of 5 minutes in duration, they found no differences in mosquito readiness to approach a treated net compared to untreated netting, concluding the insecticide had no repellent effect. 'Time to first take off' assays (Hougard *et al.*, 2003a) showed that deltamethrin took an average of 12 seconds to provoke take off (albeit at lower concentrations). This might explain why contact irritant effects were not found in Spitzen *et al.*'s (2014) tests, as mosquitoes accrued little physical contact with the nets in the filming period (Spitzen *et al.,* 2014). A longer test period may be required to detect an irritant effect as, even on untreated nets, mosquitoes accumulated only 14-20 seconds of net contact each in the 5 minute assay.

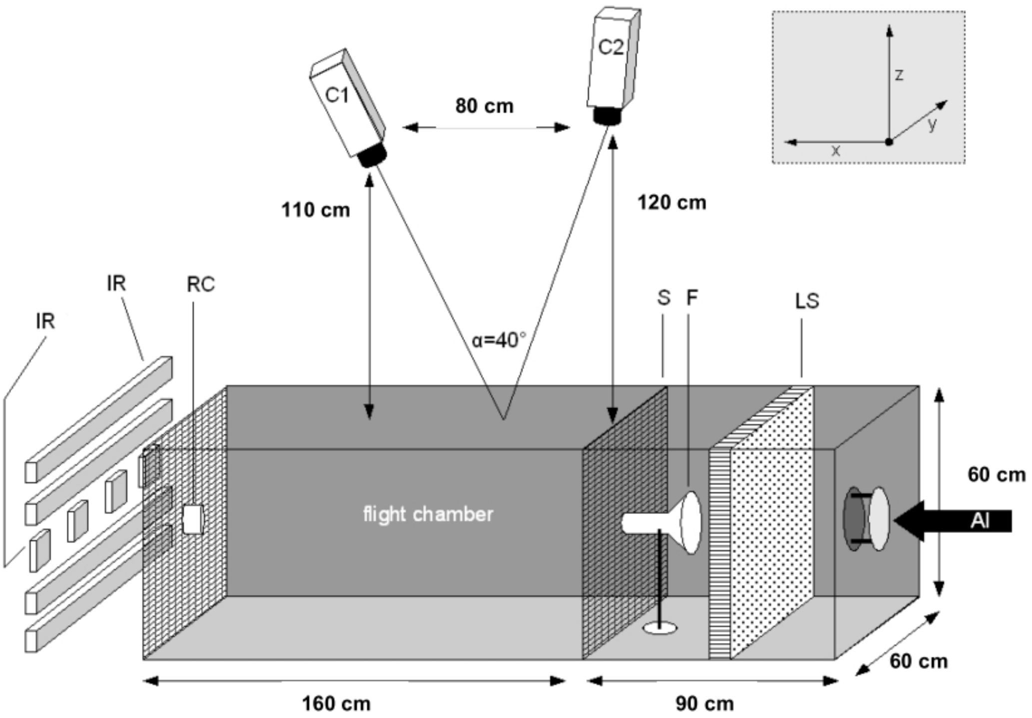

**Figure 1.3 Schematic of a wind tunnel used in 3D tracking of mosquitoes by Spitzen *et al.* (2008).** Mosquitoes are released from point RC, and flight is recorded by two cameras to produce a 3D track. The set-up is illuminated by infrared LEDs (IR). Air supplied via the air inlet (AI) passes through the lamination screen (LS). Mosquito tracks are recorded as they fly upwind towards the screen (S) covering a heating element that can also act as the release point for the odour attractant (F).

Cohnstaedt and Allan (2011) used 2D filming of a wind tunnel baited with attractive odours and $CO_2$ to assess the impact of sub-lethal insecticide exposure on mosquitoes' responses to a host. *Aedes aegypti* and *Cx. quinquefasciatus* treated with sub-lethal doses of deltamethrin and permethrin were less responsive to the odour bait. No such effect was seen in *Anopheles albimanus* but this might be attributed to the species' poor attraction to the bait under control settings. The finding that host seeking behaviour of mosquitoes can be affected over 24 hours after insecticide exposure, provides evidence for a heretofore little discussed aspect of insecticide action, showing that even if a mosquito survives its encounter with a bed net, its future blood-feeding activity may be compromised.

Wind tunnel assays of flight provide an exceptional level of detail on mosquito activity, though improvements could be made to the way tunnels are baited, as tests often use artificial odours, $CO_2$ with a heat stimulus, or worn socks. However the equipment required is expensive, and tests can be time consuming compared to short cone tests. Video recording of nocturnally-active species is also partly limited in scale by the need to illuminate the set-up with light outside of the visible spectrum. *Anopheles gambiae* is nocturnal, and exposing mosquitoes to light can inhibit blood-feeding behaviour (Das & Dimopoulos, 2008). Mosquitoes can detect visible light up to 600nm in wavelength (red light), but cannot detect infrared light (700-1000nm) (Gibson, 1995). Illuminating assays with infrared light will ensure insect behaviour is not affected but can prove technically challenging, requiring banks of hundreds of infrared LEDs (Spitzen *et al.*, 2014), or a compromise by using dusk level illumination with visible light (Miller & Gibson, 1994). This inability to use appropriate and realistic lighting has been a constraint on the scale of these behavioural tests, and flight tracking has so far been kept to boxed in tunnel tests less than 1m across.

Dimensions of the test arena are an important consideration in the design of behavioural assays. Wind tunnel experiments that provide track examples often show flights that expand to fill the limits of the test chamber (Figure 1.4), suggesting that mosquito movement may be restricted by the boundaries of the wind tunnel (Spitzen *et al.*, 2013; Lacey *et al.*, 2014; Spitzen *et al.*, 2014). With arena widths typically between 50 to 60cm (Lacey *et al.*, 2014; Spitzen *et al.*, 2014), wind tunnels used in mosquito studies offer limited space for movement, potentially affecting numerous behavioural parameters including time taken to locate an odour source, flight tortuosity and velocity.

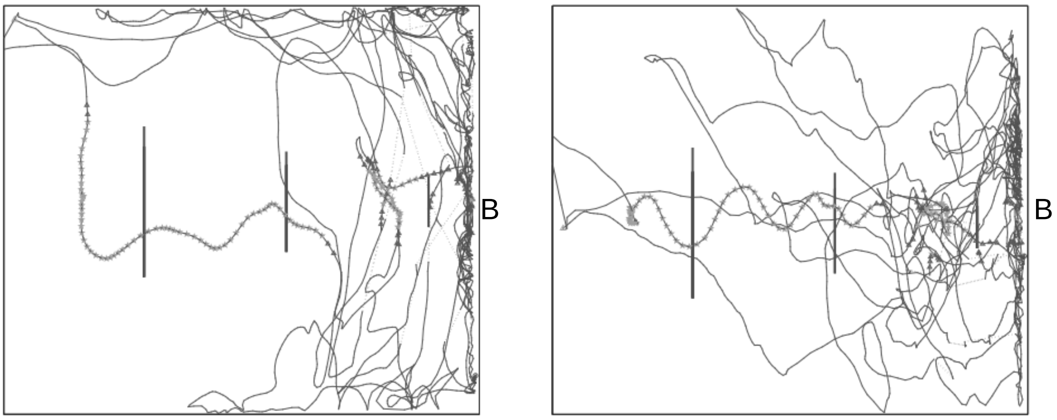

**Figure 1.4 Track example of a mosquito flying towards an odour bait**

These images show the same track viewed from above (left) and the side (right). The odour source is positioned behind the screen panel on the right of the arena (B) and the extent of the odour plume is denoted using vertical lines. The flight track approaches all walls of the test arena. Figure modified from Spitzen *et al* (2013).

This design of test may also rely on attractant plumes that do not realistically represent those a mosquito would encounter in the field. For example, many wind tunnel studies present mosquitoes with an odour emanating from a point source, or nylon sock, producing a regular conical odour plume (Dekker *et al.,* 2005; Beeuwkes *et al.*, 2008). In field settings, the odour plume of a human is likely to be highly heterogeneous in its shape and composition and subject to dispersal by air currents (Murlis *et al.*, 1992; Cardé & Willis, 2008). Mosquitoes have been shown to be sensitive to the turbulence of an odour plume (Dekker *et al.*, 2001; Dekker & Cardé, 2011), and may be less attracted to bait presented as a regular plume. Standard wind tunnel tests also restrict host seeking behaviour to a horizontal plane as mosquitoes fly upwind along the tunnel to the bait. As the selection of biting sites involves descending flight down the body (De Jong & Knols, 1995; Dekker *et al.*, 1998), by limiting the planes of host seeking one might affect mosquito behaviour around the upwind tunnel end wall.

### *1.10.9 Free-Flight Assays*

Other approaches allow mosquito interaction with hosts and insecticide treated materials on a larger scale, with movement in all planes around a bait. Free-flying mosquitoes released in to a room have been used to evaluate mosquito feeding success through insecticide treated materials. Here a known number of mosquitoes are released in to a sealed room containing a net. A volunteer sitting with an arm pressed against the net surface acts as bait, allowing recording of feeding success and knockdown of host seeking mosquitoes. Results have shown that *An. stephensi* feeding success was significantly reduced by pyrethroid treatment (Hodjati & Curtis, 1997; Kolaczinski & Curtis, 2000). Knockdown results suggest that host seeking mosquitoes contacted nets, and that blood-feeding inhibition was not therefore due to repellent properties of the insecticide but some post-contact mechanism (Hodjati & Curtis, 1997; Kolaczinski & Curtis, 2000).

Sticky trap tests, in which mosquitoes are released in a room and allowed to forage around a baited bed net covered in non-setting adhesive, have shown that *An. gambiae* and *An. albimanus* will preferentially attack the roof of the net rather than its sides (Lynd *et al.*, 2014; Sutcliffe & Yin, 2014). Such information is useful to the design of new nets, but this method is not viable for testing LLINs, as the net material must be coated with a non-setting adhesive, like Tangle trap. By its nature, this test can only indicate the point of first landing, and will not show how mosquito attack might change over time.

Domestic repellents may be evaluated in choice–test type assays which allow repelled mosquitoes to exit a test room and move in to a control room (Rapley *et al.*, 2009). This allows realistic assessment of a repellent’s knockdown effects and blood-feeding inhibition.

Such tests present useful results that are relevant and representative of an intervention’s real-world performance. The methods present practical issues though, not least of which is the large space required. Unless arenas are designed for the purposes of these tests, different labs will use rooms of different sizes, presenting challenges of standardisation.

### *1.10.10 Effects of Colonisation on Behaviour*

Large-scale laboratory tests offer an opportunity to study mosquito behaviour under controlled conditions, but in interpreting results based on laboratory mosquitoes, the possibility that behaviour has been altered by colonisation must be considered. There are a number of examples that appear to show behaviour being influenced by captive breeding.

Comparisons of three *Ae. aegypti* strains found that a colony that had been in captivity for 40 years showed different responses to the irritant effects of alpha-cypermethrin and DDT relative to more recently-caught strains (Thanispong *et al.*, 2009). Exit responses of the older strain to alphacypermethrin were  40% lower than the sensitive younger strain, but the older strain showed the strongest irritability response to DDT, and which of the two insecticides would have been classified as more irritating depended on the strain of mosquito being tested. The results could not be completely explained by differences in insecticide susceptibility, and suggested that colonisation may have affected mosquito behaviour. Experiments with several *Anopheles* species found that one *An. dirus* colony that had been kept in the laboratory for over 16 years was more likely to exit an escape box even when exposed to control untreated test papers (Chareonviriyaphap *et al.*, 2004). This study showed a lot of variation between species and between colonies of different ages. It is possible that this was partly due to variation within the wild mosquito population, as colonies were founded with insects caught from different locations.

Other work found that colonised *Ae. aegypti* were less responsive to odours from human hands, or from attractive chemical baits (Clark *et al.*, 2011). Field-caught mosquitoes adapted to exhibit the same behaviours as laboratory strains within 10 generations of colony rearing. Laarman (1958) showed that *Anopheles atroparvus* mosquitoes that had been fed for 20 generations on rabbits were more attracted to rabbit odours than field-caught mosquitoes.

Speed of blood-feeding may be affected by colony conditions, with some evidence that a colony kept *Ae. aegypti* strain lose their tendency for fast feeding within as few as three generations (Chadee & Beier, 1997; Chadee *et al.*, 2002). It has also been suggestions that colonisation may affect long range flight ability of mosquitoes: flight mill experiments with *Culex tarsalis* found that males that had been in colony for 2 years would fly for shorter distances, and for less time than field-caught individuals of the same age (Clarke *et al.*, 1983). This may be related to changes in muscle structure, something that has been observed in comparisons of colonised and wild caught *Ae. aegypti* (Beckett & Townson, 1982).

Biological differences between wild and colony mosquito strains have been observed in many other traits, including pathogen susceptibility and male swarming behaviour (O'Meara & Evans, 1974; Lorenz *et al.*, 1984; Scott *et al.*, 2006). As such, field tests may be the best way to ensure behaviours observed are genuine to a species and not an artefact of adaptation to colony conditions.

### *1.10.11 Testing Behaviour in the Field*

Semi-field testing offers a way to set experiments in a field context whilst controlling the mosquito population being tested using some form of enclosure. The term is used to encompass a broad range of methodologies, but generally encompasses methodologies that expose insects to local climatic conditions in netted screen-walled enclosures. Semi-field settings have been used for a range of tests of pyrethroid repellents. These use 'taxis boxes', which consist of a central test chamber with one-way movement ports at its front and back that can be used to study movement towards or away from a stimulus (Lorenz *et al.*, 2013). Taxis assays are set in an enclosed semi-field tunnel to determine the efficacy and range limits of the repellent's action (Ogoma *et al.*, 2014a). Such tests have the advantage that they allow mosquitoes to receive and respond to signals from the host, but do not expose volunteer hosts to the risk of bites from wild mosquitoes. Spatial repellents or attractants can be evaluated by placing the test material next to a volunteer, and examining taxis or movement towards (attraction) or away (repulsion) at different distances (Ogoma *et al.*, 2014a). The main limitation of this test method is the restricted size of the taxis box chambers: the central chamber measures 40x40x40cm, and mosquitoes move out of the chamber through small funnel entrances at each end. Such a scale has the potential to impede the broad turning flights mosquitoes make upon sensing a host, and compromise attractive flights towards the volunteer (Spitzen *et al.*, 2013).

Other semi-field enclosures operate on larger scales, consisting of closed houses, or screened structures that may contain a human volunteer, resting sites, vegetation and houses (Chandre *et al.*, 2000; Cilek & Hallmon, 2006). Indoor room-scale mesocosms may be used to similar effect in countries where the species being studied is not endemic (Jackson *et al.*, 2015). The enclosures play an important role in assessment of mating competitiveness of genetically modified or *Wolbachia* infected mosquitoes, as the large volume of the enclosures permits formation of normal mating swarms (Segoli *et al.*, 2014). Such enclosures offer a great opportunity for testing new vector control interventions under controlled conditions.

The most comprehensive approach to this style of testing, semi-field systems, include breeding pools to allow self-propagation of mosquito populations (Russell & Rao, 1942; Knols *et al.*, 2002; Ferguson *et al.*, 2008; Ng'habi *et al.*, 2010). Semi-field systems are often used for ecological studies, but have also been used to test new mosquito trapping methods, evaluate auto-dissemination of pyriproxyfen, and push-pull pairings of repellents and attractant traps (Mathenge *et al.,* 2002; Okumu *et al*., 2009, 2010a, 2010b; Lwetoijera *et al.,* 2014b; Meger *et al.*, 2014). Such enclosures offer scope to study behaviour under ambient climatic conditions, allowing for effects of air movement on insecticide volatiles and the bait's odour plume. As mosquitoes are not enclosed in small test boxes, it is assumed that their host seeking flights will not be impeded by the size limits of the behavioural arena. Volunteers can be safely exposed to laboratory-reared mosquitoes and, given appropriate ethical consideration, uninterrupted blood-feeding can be permitted without risk of disease transmission.

Semi-field studies and semi-field systems allow testing of a known mosquito population, with some experimental control of the species, age and insecticide resistance status of insects. This is particularly useful when working in an area with a mix of mosquito species, as the behaviour of non-vector species may be less relevant to the study. Open experimental hut trials by contrast may study the behaviour of multiple mosquito species varying in age and parity. However by removing all boundaries, one has the advantage that it is possible to study how mosquitoes respond to a host or insecticide when they have the option to exit and forage elsewhere.

The species being studied can be controlled to some extent by mark release tests. Achee *et al.* (2006) collected non-engorged wild *Anopheles dirus* females, which were marked, released and recaptured to study the host location and dispersion around a human baited experimental hut. Though this work has thus far only studied flight around unprotected human baits, it would be interesting to see how repellents affect dispersion, and how distance affects repellent efficacy.

Arredondo-Jiménez *et al.* (1997) collected field populations of *An. albimanus* for a different type of mark-release experiments. By coating insects with fluorescent powder, it was possible to use an ultraviolet lamp to directly observe mosquitoes flying around a lamdacyhalothrin treated net. The authors observed that use of the insecticide cut the time mosquitoes rested on the net to less than half the value for untreated nets. The number of mosquitoes that landed on the net decreased significantly when there was an alternative human host in the room who was not using a bed net. Whilst the powder coating and light could have affected mosquito behaviour, the quantitative field observations of this study are a step towards assessing mosquito behaviour in a realistic setting.

Experimental huts are more commonly used to look at entry, exit, and resting behaviour of mosquitoes (Muirhead-Thomson, 1945; Smith, 1965). They have been used extensively in experimental hut trials of bed nets to assess the impact of using a net on house entry, blood-feeding success and mosquito mortality (Lines *et al.*, 1987; Miller *et al.*, 1991; Graham *et al.*, 2005; N'Guessan *et al.*, 2010; Ngufor *et al.*, 2014). Experimental hut trials differ from semi-field trials in that they involve exclusively wild mosquitoes, which approach and depart the hut in response to stimuli contained inside the house. By using traps fitted to eaves, windows or doors, mosquitoes can be trapped as they enter or exit a hut. Further manual collection can catch mosquitoes knocked down or resting within a hut at the end of a test. By comparing catch results in a baited control hut, to a baited hut using an intervention (e.g. an LLIN) the effect of the intervention on mosquito behaviour within the home can be established (Silver, 2007; figure 1.5).

Data on number of mosquitoes caught in different scenarios reveals repellent effects of the intervention that may have deterred mosquitoes from approaching a hut (Silver, 2007). The number of mosquitoes exiting an intervention test hut can reveal whether contact irritant properties of an insecticide intervention led more mosquitoes to exit a hut, though this output is less clear due to potential for exit to be influenced by close-range repellent effects within the home. In some instances mosquitoes are permitted to bloodfeed on human volunteers within the hut, and this output is used to calculate bloodfeeding inhibition of an intervention. This term encompasses both mosquitoes deterred from feeding by behavioural properties of an insecticide, as well as mosquitoes that did not feed due to knock-down. Toxicity of an intervention is found by counting the immediate number of mosquitoes knocked down after a test, and after 24 hours.

Such tests have been used to stress the importance of insecticide treatment of nets in preventing blood meals, examine the wash-resistance of insecticide formulations, and determine the effect of insecticide exposure on mosquito mortality. Unlike laboratory behavioural tests which force mosquitoes to make contact with insecticide treated materials for set periods of time, experimental hut trials allow insects to approach and contact LLINs of their own volition, and to attempt to leave the house if they are repelled or irritated. Mortality rates can therefore be considered more representative of an intervention's true effect.

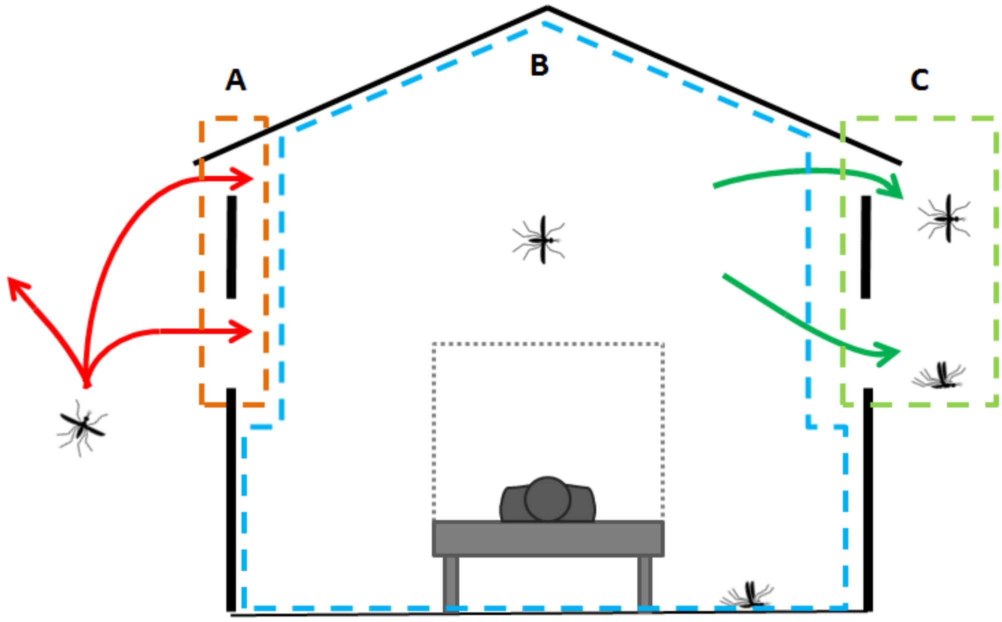

**Figure 1.5 Diagram of mosquito activity around experimental hut**

This diagram shows how catching mosquitoes at different points within the experimental hut can reveal behavioural impacts of an intervention (e.g. an LLIN). Entry traps fitted to windows and eaves (A) show the number of mosquitoes repelled prior to entering a hut (deterrency). Collection within the hut (B) shows the number of mosquitoes that have remained in the hut. If this number is equivalent to controls this demonstrates a lack of repellency or contact irritancy of the intervention. Exit traps fitted to windows and eaves (C) show the number of mosquitoes exiting the hut, potentially as a result of contact irritancy or repellency. Knockdown and bloodfeeding measured in mosquitoes collected in the hut (B) and exit traps (C) demonstrates insecticide toxicity and bloodfeeding inhibition.

The drawback of such tests is that they do not provide information about the extent of the mosquito's interactions with nets. Hence, they do not allow quantification of contact with the net prior to exit, nor indicate where contact might have occurred, or the mode by which an LLIN could have interfered with a mosquito's host seeking process (repellency or contact irritancy). Further to this, variation in hut design may influence mosquito behaviour: there is as yet no agreed upon standard for experimental hut design, and huts consequently vary in size and shape (Silver, 2007). This variation can influence catch results of experiments conducted in different locations (Massue *et al*., 2016). Though standardisation of hut design would remove this variation, experimental hut construction is often based on availability of local materials, and made to mimic architecture of houses in the area, thus whilst such differences can make data gained less directly comparable between field sites, they will reflect the impact an intervention would have in local domestic settings.

The development of better behavioural assays that allow observation of these behavioural interactions remains a major challenge in vector biology, and will be crucial to our understanding of mosquito control tools.

## 1.11 Summary

*Anopheles gambiae s.s.* is a very efficient malaria vector due to its highly anthropophilic and endophagic traits. The adult female mosquito locates blood meals using a suite of attractive cues emanating from the host. Blood-feeding can be prevented by use of interventions such as LLINs. The details of mosquito interactions with LLINs have yet to be fully elucidated. The various behavioural assays that exist at present have limitations to scale, bait or observable detail, and there is scope for the design of novel behavioural assays to fill this knowledge gap. Rigorous analysis of mosquito behaviour around humans and LLINs will be important as the vector control community seeks to maintain its progress against malaria in the face of increasing insecticide and behavioural resistance to existing intervention.

## 1.12 Aims and Objectives

### *1.12.1 Aim*

This project aims to develop new tools for the study of mosquito behaviour, and apply them in the laboratory and field to study the interactions of malaria vectors with insecticide treated materials. It aims to investigate the host seeking behaviour of malaria vectors, and how this is affected by insecticide.

### *1.12.2 Objectives*

The specific objectives of this thesis are:

1. To design behavioural assays to investigate the spatial repellent effects of insecticide-treated nets on *Anopheles gambiae* host seeking behaviour, determining whether mosquitoes repelled by insecticide divert their approach to an unprotected bait (Chapter 3).
2. To use novel flight tracking techniques to observe mosquito behaviour around LLINs, and quantify how malaria vector responses to the host are affected by contact irritant, repellent and toxic effects of the insecticide treatment (Chapter 5).
3. To transfer the technology developed in objective 2 to a field site in Tanzania, to study the behaviour of field populations of *Anopheles gambiae* s.l. at LLINs (Chapter 6).
4. To use novel 2D and 3D technologies to study the spatial aspects of the flight of host seeking malaria vectors at an unprotected supine human host, and during window passage en route to the host, investigating whether mosquitoes follow stereotypical paths towards the host (Chapters 4 and 7).

# Chapter 2 General Methods[1]

## 2.1 Introduction

Mosquito host seeking and behavioural interactions with insecticide treated surfaces can influence the success of vector control (Killeen *et al.*, 2011; Gatton *et al.*, 2013; Killeen *et al.*, 2014). Relatively little is known about these behaviours, primarily because unobtrusive observation of anopheline activity is difficult due to their nocturnal habits. Recent technological advances offer the opportunity to record mosquito activity without influencing their behaviour using infrared camera tracking systems. Two and three dimensional (2D and 3D) systems have been developed for this purpose, but many suffer constraints of scale, recording duration, or are unable to track multiple mosquitoes flying simultaneously. For this thesis a 2D tracking system was developed that can record mosquito host seeking activity on a significantly larger scale, for longer time periods and that can handle multiple flight trajectories.

A number of key technical challenges associated with recording nocturnal flight activity of organisms as small mosquitoes, had to be overcome to deliver a fully functional system. The challenges and their technical solutions are described and discussed here, together with materials and methods common to all experimental tests reported in the thesis.

**Illumination**

As *Anopheles sp.* mosquitoes seek hosts and blood-feed at night, this behaviour can be disturbed by exposure to visible light, and behavioural assays are best conducted under conditions of darkness (Gillies & De Meillon, 1968; Das & Dimopoulos, 2008). Arredondo-Jiménez *et al.*, (1997) attempted to observe flight behaviour under conditions of near darkness by dusting mosquitoes with fluorescent powder and using ultraviolet light to observe their movements in an otherwise darkened room. Though fluorescent dusting is not considered to adversely affect host seeking (Verhulst *et al.*, 2013), mosquitoes can perceive ultraviolet light, and

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[1] Some of the content of this chapter has been included in a published paper (N.C. Angarita-Jaimes, J.E.A. Parker, M. Abe, F. Mashauri, J. Martine, C.E. Towers, P.J. McCall, D.P. Towers, (2016) A novel video-tracking system to quantify the behaviour of nocturnal mosquitoes attacking human hosts in the field, *Journal of the Royal Society Interface, 13* (117), 20150974).

illumination could potentially influence behaviour (Costantini *et al.*, 1998).

The largest mosquito camera tracking system described to date uses natural dusk light to record activity of mating swarms with a field of view of over 1.5x1.5x1.5m (Butail *et al.*, 2012, 2013). However, mating occurs at dusk, hours before most anophelines begin host seeking, and the use of extraneous evening light violates our requirement for darkness. This system cannot be adapted for use inside a house to view host seeking behaviour.

*Anopheles sp.* mosquitoes can perceive visible light up to and including red wavelengths (600nm) but infrared light (>700nm) is invisible to them (Gibson, 1995). Illuminating a behavioural arena with infrared light enables recording with an infrared sensitive camera to record nocturnal activity.

Using diffuse, unfocussed illumination imposes scale constraints on filming: wind tunnels must use banks of 360 infrared LEDs to illuminate an area 0.6x0.6x0.6m (Spitzen *et al.*, 2014). Recording mosquito flight in a large volume is therefore problematic, and behavioural tests to date have been conducted in moderate to small scale arenas, which may constrain host seeking flights (see Chapter 1.10; table 2.1). With large Fresnel lenses it is possible to illuminate large recording volumes using a single point light source as the beam is collimated (refracted by the lens to form a parallel beam) across the gap between lenses, then focussed onto a camera by a second Fresnel lens (Figure 2.1). A Fresnel lens is structured to have one entirely flat side, and one side that consists of concentric circular grooves. As the refractory surface consists of a series of annular rings, lenses may be designed that have a short aperture, whilst being flat and thin. Were convex lenses to be used for the same purpose in this instance they would be very thick at their widest point, intruding on the filming volume and presenting practical challenges in support and alignment. By using paired Fresnel lenses to focus light, one infrared LED was capable of illuminating a recording space of 1.2x1.2m, in a uniquely optically efficient system.

### Tracking of Multiple Trajectories

Discriminating between the intersecting tracks of flying mosquitoes is a challenging component of track analysis, and it is common for laboratory tests to avoid the problems associated with this by testing individual insects separately (Lacey *et al.*, 2014; Spitzen *et al.*, 2014), or in groups of four (Dekker & Cardé, 2011). However, it is possible to track multiple mosquitoes, and use of multiple cameras to provide 3D coordinates of a mosquito can assist in this process. Field tests using two cameras in stereoscopic recording could track up to 25 individual males within a mating swarm for up to 90 seconds, (Butail *et al.*, 2013; Manoukis *et al.*, 2014). In the study by van Breugel *et al.,* (2015) five cameras recording at 100 frames per second (fps) were used to distinguish trajectories of 20 mosquitoes flying simultaneously.

To achieve the study objectives the camera tracking system eventually used in the present study, recorded movement in 2D, but was designed to be capable of distinguishing multiple mosquito tracks through use of a fast frame rate (50 frames per second), and track analysis that considered coherence of movement direction to distinguish multiple paths of numerous mosquitoes potentially adjacent in time and space.

### Recording Duration

Video recorded experiments are limited in their recording duration: this is principally constrained by a computer's video storage capacity, though larger files will also take longer to process (Manoukis *et al.*, 2014). Wilkinson *et al.* (2014) were able to record mosquitoes for a 30 hour period by recording directly on to an external hard drive, reducing file sizes through use of a lower frame rate and camera resolution. Other studies have recorded mosquito activity for 3 hour periods with 5 cameras recording at 100 frames per second, by employing a technically complex system with six linked computers to record the large amounts of data produced (Straw *et al.*, 2010; van Breugel *et al.*, 2015). Simpler laboratory systems recorded host seeking behaviour of mosquitoes in wind tunnels using two cameras to record activity for periods of 3 to 5 minutes (Dekker & Cardé, 2011; Spitzen *et al.*, 2014).

In the tracking system used in the present study, limitations of software compatibility and write speed to external hard drives meant it was not possible to record to external HDDs. Instead, the high resolution, 50fps files were recorded on multiple internal hard drives in a RAID configuration. The maximum recording time of approximately 13 hours was set by PC memory capacity. Single tests were limited to 60 minutes or less, to balance the requirement to obtain meaningful behavioural information and processing time.

**Table 2.1 Key filming systems used in video tracking mosquito flight.**

Key tracking methods used to observe flight are detailed in this table, which compares scale of available tests, maximum number of mosquitoes that have been tracked simultaneously, and experimental details. Where field of view information is not specified assumptions have been drawn from diagrams (noted ‘approx.’ for ‘approximately’ in table).

| Method | Field of view (m) | No. mosquitoes tested at one time | Application | Reference |
|---|---|---|---|---|
| **Barber’s pole tunnel,**<br>**Single camera (2D)**<br>**Manual tracking** | Approx.<br>0.6 x0.18 | 10 | Assessing visual responses of mosquitoes to different light wavelengths | Gibson, 1995 |
| **Wind tunnel**<br>**Two cameras, 2D** | 2 x 0.6 | 1 | Activation of mosquitoes in response to attractive odours | Takken *et al.*, 1997a |
| **Wind tunnel**<br>**Two cameras (3D)**<br>**Automated tracking** | 0.6 x 0.6 x 0.6 | 1 - 4 | Mosquito responses to attractive odours | Dekker *et al.*, 2005;<br>Beeuwkes *et al*. 2008 |
| **Wind tunnel**<br>**Two cameras (3D)** | Approx.<br>1.5 x 0.7 x 0.7 | 4-6 | Responses of mosquitoes to $CO_2$ baited traps | Cooperband & Cardé, 2006a |
| **Field, wild mosquitoes**<br>**Two cameras (3D)** | Approx.<br>1.5 x 1.5 x 1.5 | 6 - 25 | Swarming activity of wild mosquitoes (90 second recording time) | Butail & Manoukis, 2012 |
| **Wind tunnel**<br>**Five cameras (3D)** | 1.2 x 0.33 x 0.33 | 20 | Interplay of attractive cues in host location, odour gated responses | Van Breugel *et al.* (2015) |
| **Room/ exp. hut**<br>**Two cameras (2D)** | 1.4 x 2.4 | 25 | Effects of insecticide treated materials on mosquito host seeking behaviour | Chapters 2, 5, 6, 7 |

## 2.2 Materials and methods

### *2.2.1 Recording System*

Mosquitoes were tracked using paired identical recording setups (*i.e.* to capture upper or lower body sections of the supine human host, Figure 2.1B-D), each comprising a single high power infrared LED (light emitting diodes; 850nm, 1000mA minimum; M850L2, Thorlabs, UK) and acrylic diffuser (Comar Optics, UK), aligned with a pair of Fresnel lenses (1400 x 1050mm and 3 mm thick; NTKJ Co., Japan) mounted either side of the bed (Figure 2.1A), and a camera. Each setup comprised a 12.5mm imaging lens (Kowa LM12HC 1”; Multipix Imaging, UK) mounted on a monochrome camera (Baumer HXC40NIR, Camera Link, 4Mpix; Lambda Photometrics, UK) (Figure 2.1C). Both cameras were operated by a single computer (Intel Core i7. 3.4 Ghz.8 Gigabytes RAM, Windows 7 Ultimate; 10 hard drives (2 Terabytes each), at 5 drives per camera). Total cost of the tracking system was approximately £80,000 at time of purchase (2011-2012).

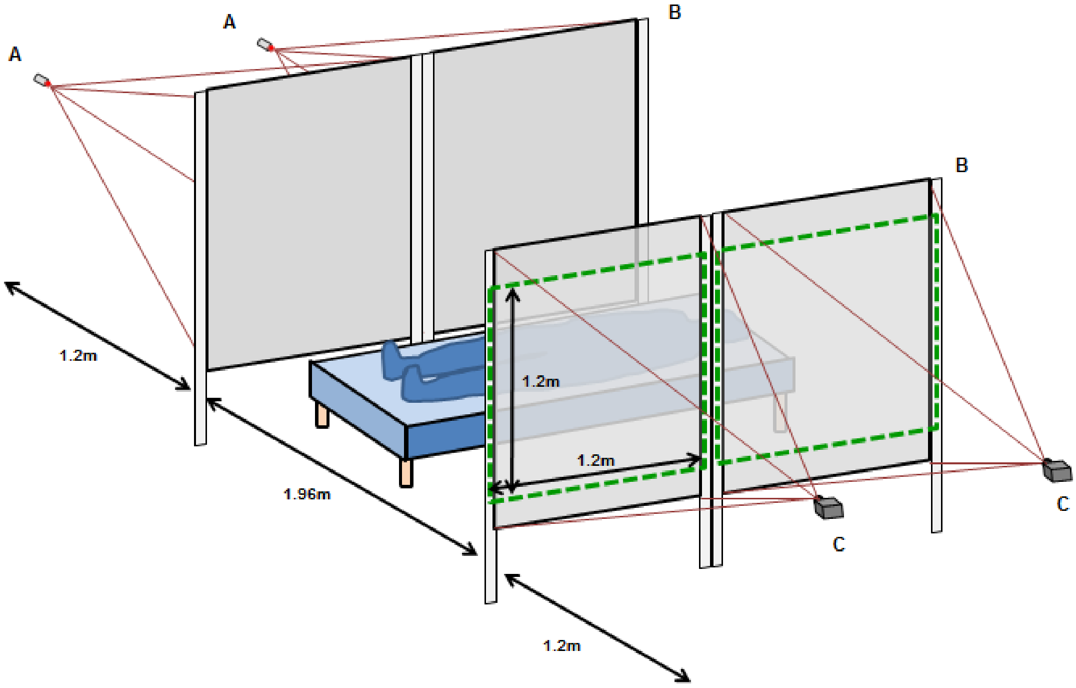


**Figure 2.1 Simplified diagram showing the complete recording system for tracking mosquitoes at a human host.**

LEDs (A) emit infrared light that is collimated by the paired Fresnel lenses (B; focal length 1.2m), and focussed on the monochrome camera (C). A mattress is positioned in between the lenses, and host seeking behaviour of mosquitoes in this volume is imaged. Red lines represent light rays on either side of the lenses. Dashed green lines show the field of view of each camera (1.2x1.2m, depth of field 1.96m).

In this back-lit set-up, the large aperture Fresnel lenses (Figure 2.1B) enabled the illumination source (a single infrared LED) to be formed into a large area approximately collimated beam. This allowed optically efficient illumination of a large volume from a single light source. The additional 3mm thick acrylic diffuser (Comar Optics, UK) placed between the light source and the Fresnel lens also helped homogenise the illumination across the entire field of view, while also ensuring that the LED source was not directly imaged.

Mosquitoes are imaged as dark shadows in the back-lit system. The efficiency of the illumination enabled the exposure time of each frame to be reduced to typically 3 milliseconds, thus ensuring that images were not overexposed.

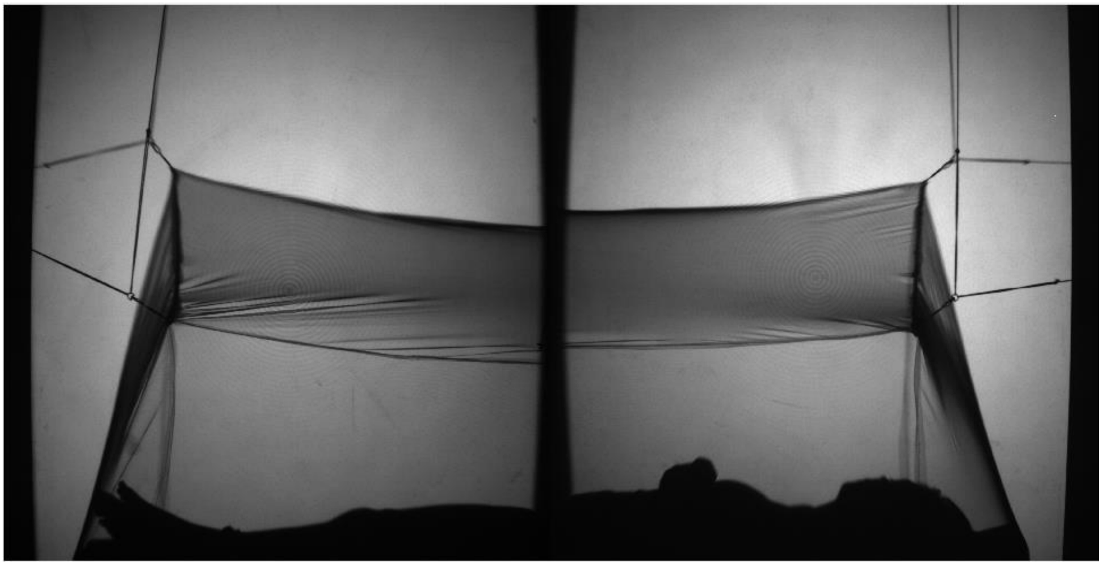


**Figure 2.2 Composite image showing the total field of view as observed by the two cameras.**

Visible beneath the bed net, suspended from the ceiling and walls with string, is the volunteer, positioned with their head at the lower edge of the field of view. The dark strips at the edges of the field of view and the dark vertical line in the centre are the aluminium frames supporting the Fresnel lenses.

Fresnel lenses had a focal length of 1.2m and were positioned with a gap of up to 1.96m between them to accommodate the width of the bed and mattress (Figure 2.1). Cameras were operated from a computer outside the insectary. Due to constraints of the aperture of the camera lens, this system did not capture the entire area of the Fresnel lens, instead the filmed area was 1.2 x 2.4m (Figure 2.1). Components were mounted on heavy tripods or aluminium frames to minimise sagging and movement. These covered the edges of the Fresnel lenses, producing blind zones of 0.1 x 1.2m in the centre and 0.05 x 1.2m on each side (Figure 2.2).

Minimal barrel type lens distortion was observed, as assessed in multiple planes along the optical axis between the Fresnel lenses (Angarita-Jaimes, *personal communication*).  Hence, any image distortions present would have affected the absolute positional accuracy across the entire field of view but have negligible effects on displacements when evaluated during tracking.

### *2.2.2 Recording*

Mosquito activity was recorded using StreamPix software (www.norpix.com) and data saved as .seq files.

Recordings with bed nets (Chapters 5 and 6) lasted for one hour and were recorded at 50 frames per second. Host seeking experiments (Chapter 7) used fewer mosquitoes and were recorded at 30 frames per second.

#### Post-Test Room Clearance

Following tests mosquitoes were collected using a prokopack aspirator (Vazquez-Prokopec *et al.*, 2009). Collection took 10-20 minutes, during which time mosquitoes may have been damaged by the strong vacuum effect of the prokopack aspirator. Therefore mosquito mortality was not followed up after tests, as it was considered that this may have been influenced by post-test collection methods.

#### Motion Detection

Recording a full hour of mosquito activity at an untreated bed net produced large files of over 700GB in size. To reduce storage requirements and to avoid recording periods of time when no mosquito activity occurred, motion detection was used. Here a processing algorithm ran simultaneously as a module within the StreamPix recording, and detected movement within the image by assessing maximum per pixel grey scale differences between consecutive image frames (Angarita-Jaimes *et al.*, 2016). When this difference exceeded an adjustable threshold, movement was inferred, and frames were recorded. When there was no activity, cameras did not record. Use of this algorithm was able to reduce the total file size of a video to less than 400GB, in tests with minimal mosquito activity. During recording, motion detection could be turned on or off by the user depending on observed activity levels.

### *2.2.3 Tracking System*

Segmentation and tracking algorithms were developed using bespoke software written in Matlab (Mathworks), to extract and interpret trajectory duration, time resolved velocity, distance travelled, tortuosity, and the number and duration of contacts made with a bed net or similar surface.

Video processing detected mosquito activity by image subtraction, in which consecutive frames were analysed and positions of moving points extracted (Figure 2.3, Angarita-Jaimes *et al.*, 2016).

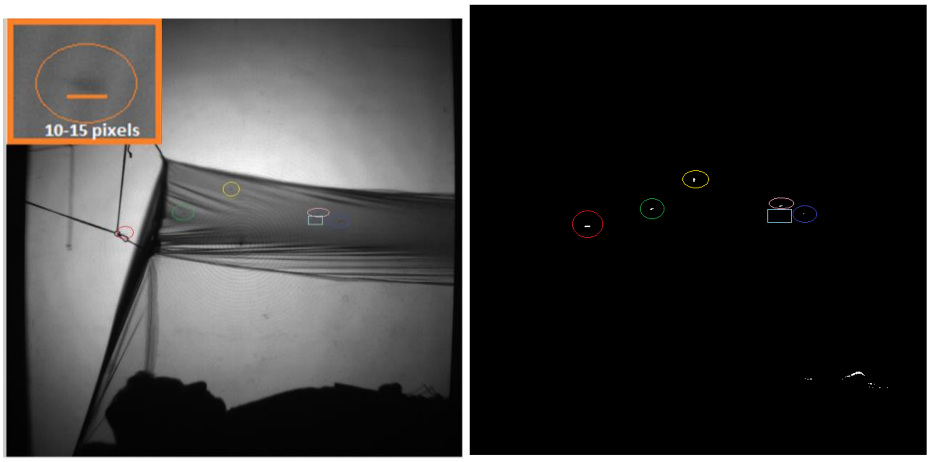


**Figure 2.3 Images of position analysis performed by the tracking software.**

A single camera frame showing the positions of six individual mosquitoes (left); inset shows a magnified image of a single mosquito on the bed net. Binarised version of the same image (right) shows the result of sequential frame subtraction and filtering. Moving objects are represented in white: the processing algorithm has detected five of the mosquitoes in the image, as well as some movement of the volunteer's hands. The sixth mosquito (within the rectangular box) is immobile so has not produced a movement trace. Figure from Angarita-Jaimes *et al* (2016).

Video processing generated a position set which included mosquito movement, and positions resulting from volunteer movement, bed net movement, and signal noise. Such noise was identifiable visually as differing from mosquito tracks (see Figure 2.4), and was removed manually by the system user in post-processing step prior to tracking.

Points were linked subsequently during tracking by referencing their spatial and temporal proximity. Tracking was based around search radius calculations and, in addition to basic tracking (single or unbroken tracks), included capacity to track mosquitoes that flew between the two camera views, mosquitoes that rested briefly on the net, and mosquitoes that flew out of the field of view and returned within a 10 second time period. Where two mosquito tracks crossed, ongoing paths were allocated based on coherence of movement direction based on the last 10 frames (0.2 seconds) of recorded movement of each individual.

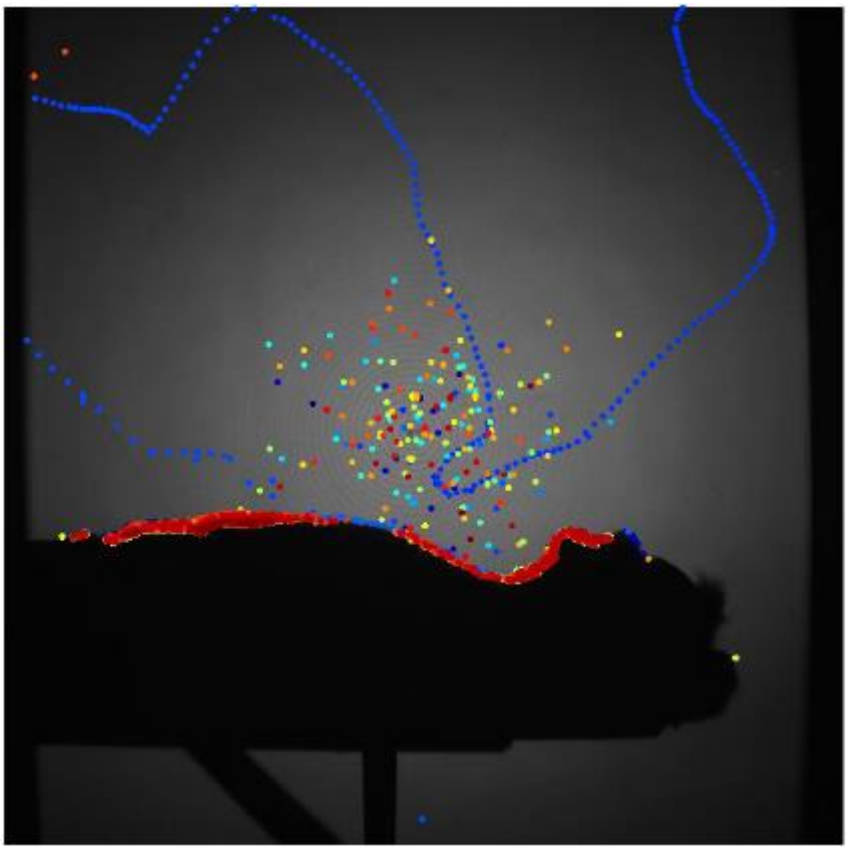


**Figure 2.4 Position image showing noise generated by volunteer movement, signal noise, and bed movement.**

This image shows positions from a ten minute recording in which mosquitoes were active around a volunteer not using a net (Chapter 7). Three types of noise are visible in this image. Red positions around the volunteer's body show noise created by volunteer movement. Multi-coloured positions close to the centre of the image are the result of small variations in LED intensity. A single yellow position at the right end of the bed is the result of the volunteer's movement shifting the bed. Mosquito movement appears as a set of consecutive positions moving coherently across the image. As noise is visibly different from the mosquito track, it could be removed by the user in post-processing steps prior to tracking.

### *2.2.4 Post Tracking Analysis*

Post-processing software enabled further manual track linking, and deletion of erroneous tracks created from noise. Though most noise was cleared in pre-tracking steps, some noise remained and was linked to form short tracks. These were identifiable as not representing genuine mosquito movement by their length, commonly, under 0.2 seconds long, and by their start and end points; noisy tracks commonly appeared to start in the airspace above the net, and finished abruptly in the same area. This contrasts to genuine mosquito tracks, which start and end at the edge of the field of view as mosquitoes fly in to the filmed area.

In post-processing, activity was categorised into different behavioural modes, and regional information regarding the mosquito's position on the net/ human assigned to track sections (Chapters 5 and 6).

### Mosquito Recording

Since multiple mosquitoes were present in all tests and the entire room was not visible, determining the total number of mosquitoes responding or tracking individual mosquitoes throughout the test was not possible.  Hence analyses were performed using individual flight track events, and as every track theoretically could have been a different mosquito, each track from entry and exit in the field of view was analysed independently.

### Quantifying velocity and tortuosity

Flight velocity values were calculated using whole swooping tracks, that is, tracks which did not make contact with the bed net or volunteer. Tortuosity values were calculated using whole swooping tracks, and track sections prior to first net contact for other flight types. To measure tortuosity, an index of the degree of flight meander, tracks were subdivided into sections comprising 40 sequential positions (average length 280mm), and tortuosity calculated as the ratio of actual distance travelled to the straight line distance between the two end points on the section; sub-section values were then averaged to provide track value. Though this method differs from standard tortuosity calculations, which work on entire path length from start to end, this alteration was used to compensate for the limits of the camera field of view,  removing bias resulting from extreme meandering tracks that started and ended in close proximity. Although speed and tortuosity data were not normally distributed, results from GLM analysis of square-root transformed data were unchanged, and the untransformed data are shown.

### Recording System Capacity

The recording system was theoretically capable of recording 5.5 hours of video from two cameras at 100fps. During position processing, mosquitoes were located to an average accuracy of 0.5mm.  On occasion, tracks could be lost at the point of contact with the bed net, when passing through creases on the net, or when moving in poorly lit regions of the net. Initial evaluation found that these track ‘breaks’ affected 12% of all trajectories derived from tests in the laboratory, and 17% in the field tests (Angarita-Jaimes *et al.*, 2016). Consequently, such ‘breaks’ may have led to the incorrect classification of some visiting tracks as swooping, and reduced the resting times recorded because when tracks were broken, the trajectories of mosquitoes arriving at and departing from the bed net would not have been connected.

### *2.2.5 Experimental conditions*

**Mosquitoes**

For experiments conducted at the LSTM (Chapters 3, 4, and 5), mated unfed 3-5 day old *An. gambiae s.s.* adult females from a long established colony of the Kisumu strain were used. This strain, originally collected from Kenya in 1953, is insecticide susceptible; the strain does not carry the kdr mutation, and has neither the gene mutations nor elevated expression of acetylcholinesterase that would confer insecticide resistance (Weill *et al.*, 2004; Constant *et al.*, 2014). Strain $LC_{50}$ against deltamethrin is 0.02µg/ml (Liverpool Insecticide Testing Establishment, 2016).

Mosquitoes were reared in insectaries maintained at 27±2˚C, 70±10% Relative Humidity, under a L12:D12 hour light: dark cycle. In routine colony maintenance, adult mosquitoes were fed on 10% sugar solution *ad libitum*, and blood-fed on human blood, and larvae were fed on ground fish food (Premium Tropical Flake, Aquarama).

During tests, adults were starved of sugar prior to testing (details as described in each experiment) and all tests started after the first hour of scotophase.

**Human volunteers**

All tests used human volunteers as bait to attract mosquitoes. Volunteers agreed to abstain from using perfumes and other scented cosmetics on the day of a test, and did not bathe for at least 4 hours prior to the start of testing. During tests volunteers were requested to remain as still as comfort permitted to avoid disturbing mosquitoes or producing erroneous tracks, though all video files were manually reviewed and cleaned to remove human movement ‘tracks’.

**Ethical Permission**

All methods were carried out in accordance with standard practices in the field. Informed consent was obtained from all participating human subjects.

The study was approved by Liverpool School of Tropical Medicine Research Ethics Committee (‘Behaviour of African malaria vectors’: Permit no. 12.13, issued 24th May 2012, Appendix C).

# Chapter 3 Spatial Repellency of Insecticide Treated Materials

## Abstract

The behavioural properties of an insecticide determine how much contact a mosquito will make with material treated with it. At an insecticide that acts as a repellent, or a contact irritant, mosquitoes will make less contact with the treated surface. Short exposure times may not kill mosquitoes, allowing them to survive and continue the cycle of disease transmission.

This chapter describes the use of two novel behavioural assays to test the spatial repellent properties of insecticides. In these assays mosquitoes were exposed to insecticide treated materials, but physical contact with the insecticide was prevented by the presence of an additional out untreated net barrier. Nets were baited with a human thumb, and mosquito responses filmed using infrared light to assess whether repellent properties of insecticides reduced host seeking activity, or prevented mosquitoes from approaching the bait. Behavioural assay A presented a single baited test panel. Behavioural assay B offered mosquitoes a choice between two baited panels, to test whether repellent materials diverted mosquito host seeking activity to an alternative untreated panel. These assays tested Permanet 2 nets and DDT treated nets, using insecticide susceptible *Anopheles gambiae s.s.* mosquitoes.

In behavioural assay A, Permanet 2 was found to be repellent, reducing the number of mosquitoes that contacted the panel ($X^2(1)=2.43$, $p=0.015$), and reducing probing activity at the panel ($p = 0.018$), resulting in mosquitoes spending less time in close proximity to the treated netting ($p=0.028$). DDT had no effect on mosquito activity, and was not found to be repellent. Non-contact exposure to insecticide in behavioural assay A reduced post-test longevity of mosquitoes in Permanet 2 tests ($p=0.010$), but not DDT tests ($p=0.213$). In behavioural assay B, neither insecticide was found to be repellent, and no evidence for a diversion effect was found.

The difference in results between the two behavioural assays provokes questions about the reliability of these small scale box tests. DDT had been included as a positive control for repellency, but was not repellent in either assay. It is concluded that such small scale bioassays may not yield accurate information on spatial repellent properties of insecticides, and alternative behavioural assays should be explored to ensure behavioural data are representative of interactions between mosquitoes and insecticides in field settings.

## 3.1 Introduction

As vector control faces growing problems of insecticide resistance, much research has focused on the toxic effects of insecticide (WHO, 2012). This work tends to emphasise the importance of mosquito mortality in results obtained from WHO bioassays and forced-contact cone tests, and the role that vector behaviour could play in mosquito interactions with insecticide has been largely overlooked. Toxic effects of insecticide depend on the time mosquitoes spend exposed to it, and if they disengage from a treated surface too soon, they will not be killed (Siegert *et al.,* 2009). Duration of contact will depend on whether a mosquito is repelled by insecticide prior to landing, or whether after contact, it is irritated by the chemical, leaving before receiving a lethal dose.

### *3.1.1 Definition of terms*

In early studies, mosquito behaviour was recorded in narrative terms, as they were noted to show ‘pleasure’, ‘indifference’ and ‘fear’ in response to stimuli (Rudolfs, 1922). Later, the behavioural effects of chemicals on mosquitoes were formalised by Dethier *et al.* (1960), who defined a repellent as “a chemical which causes insects to make oriented movements away from its source”. Davis (1985) applied stricter definitions of repellency that sought to account for the exact mechanism of action (inhibiting attraction to host, stimulating an inappropriate behavioural pattern, or activating noxious odour receptors). Identifying mode of action is useful for design of new repellents, but such definitions are beyond the scope of most behavioural studies, which investigate the behaviours chemicals elicit without reference to mechanism.

The definition of repellents was updated by Grieco *et al.*, (2007) to distinguish between pre and post contact effects: a spatial repellent “stimulates directed movement away from the chemical source without the mosquito making physical contact with the treated surface”, whereas a contact irritant stimulates the same response after a mosquito has made contact with the surface. The term ‘spatial’ repellent (Grieco *et al.*, 2007) distinguishes between personal or topical repellents, such as DEET which is applied topically to the skin or clothing to reduce mosquito landing on an individual, and spatial repellents, such as insecticide coils or volatile spatial repellents (Pates *et al.*, 2002; Kawada *et al.*, 2008; Ogoma *et al.*, 2014a) which can repel mosquitoes from a space around the host.

Miller *et al.* (2009) suggested that chemicals termed repellents may not act solely by provoking taxis away from the insecticide source, but may stimulate undirected ‘hyperactive’ movement or inhibit attraction towards a host. The authors raised the alternative, broader terms of “non-contact disengagent” and “contact disengagent” as alternatives to “spatial repellent” and “contact irritant” that explicitly include these effects.

This thesis will use the terms “contact irritant” and “repellent” to separate pre and post contact effects of insecticides respectively. “Repellent” will apply the same definition as Miller *et al*. (2009) for “non-contact disengagent”, *i.e.* a mechanistically neutral term describing a stimulus that diminishes interaction of a mosquito with its source, without direct physical contact. These terms have been chosen in order to maintain coherence with the majority of existing literature, and because they do not require the additional investigations that would be required to prove oriented movement away from a chemical source.

### 3.1.2 Behavioural Interactions with Insecticides

Contact irritancy can reduce the time a mosquito spends in contact with insecticide, limiting its efficacy. New technologies have attempted to reduce the problem of contact irritancy through use of higher concentrations of insecticide, synergists or other agents that can have toxic effects within a shorter contact time, or new powder-based delivery methods which immediately contaminate mosquito tarsi upon landing, with lethal effects (Raghavendra *et al.*, 2011; Ngufor *et al.,* 2014, Snetselaar *et al.*, 2014; Sternberg *et al.*, 2014; Andriessen *et al.*, 2015; Osinga *et al.*, 2015). However if a chemical with rapid toxic properties is repellent, a proportion of mosquitoes will not physically contact the treated surface and will survive (Achee *et al.*, 2012a).

Repellent chemicals also risk diverting mosquitoes to unprotected individuals within the same home, or to another house. This effect has been observed with topical repellents (Moore *et al.*, 2007; Maia *et al.*, 2013). In studies of bed nets the effect has either not been seen, or contact irritancy could not be excluded (Lines *et al.*, 1987; Arredondo-Jiménez *et al.*, 1997). Depending on the fitness cost of an extended host seeking period, use of repellent interventions could shift disease burden within a community to unprotected individuals. A modelling study investigating community protection in settings of partial intervention coverage found that highly toxic, non-repellent, non-irritant insecticides would give the best community protection while toxic products that were slightly repellent were better than an entirely repellent intervention (Killeen *et al.*, 2011).

Spatial repellents may be of some benefit to mosquito control however, and there are strategies that would exploit repellency in ‘push-pull’ control systems, in which diverted (pushed) mosquitoes are lured (pulled) to attractive traps (Cook *et al.*, 2007). Though this approach has been successful in some agricultural settings with crop pests, and in semi-field and small scale trails with mosquitoes (Menger *et al.*, 2014, 2015) its field efficacy in large scale control of malaria vectors has yet to be tested.

It is useful therefore to study the repellent properties of insecticides and the behavioural changes they elicit, as the details will aid in designing or improving the function of control tools using these chemicals. Different insecticides have different repellent properties, and behavioural effects vary according to concentrations used (Chareonviriyaphap *et al.*, 1997; Pothikasikorn *et al.*, 2007; Achee *et al.*, 2009). By using burnable coils or heated emanators, insecticide may be released into the air at faster rates than when sprayed on walls or applied to netting. Where insecticide is used in IRS or LLINs, the chemical’s volatility may influence its repellent properties, as this affects the rate at which a chemical is released into the air (Garson & Winnike, 1968). However this does not satisfactorily explain all differences in insecticide classes, as insecticides such as propoxur and fenitrothion have high vapour pressures but poor repellent properties (Sathantriphop *et al.*, 2006; Achee *et al.*, 2009; Sibanda *et al.*, 2011; Figure 3.1) while DDT has a lower vapour pressure, but shows some level of repellency (Taylor, 1975; Sibanda *et al.*, 2011; Achee *et al.*, 2012b; Figure 3.1).

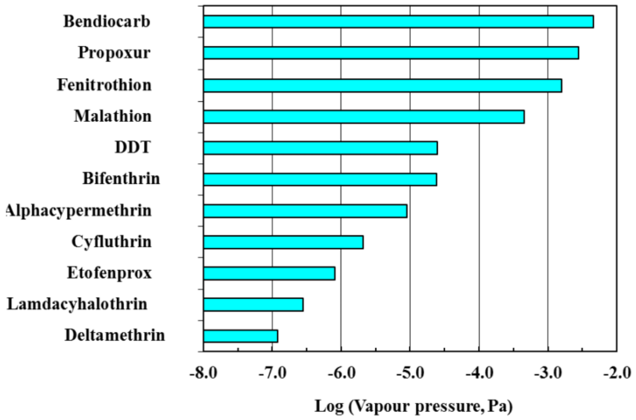


**Figure 3.1 Log vapour pressure plots of the insecticides at 25 °C**

Modified from Sibanda *et al* (2011)**.** References for individual insecticides as follows; Bendiocarb (WHO, 2009a); Propoxur (WHO, 2005b); Fenitrothion (FAO, 2010); Malathion (WHO, 2004b); DDT (Royal Society of Chemistry, 1991); Bifenthrin (WHO, 2010c); Alphacypermethrin (WHO, 2009b); Cyfluthrin (WHO, 2004c); Etofenprox (WHO, 2007b); Lambdacyhalothrin (WHO, 2007c); Deltamethrin (WHO, 2010d).

The rate at which an insecticide discharges into the air is a subject of interest as mosquitoes are thought to detect repellents via their olfactory system. This has yet to be investigated for the common insecticides, but there have been recent advances in understanding the mode of action of the repellent DEET. DEET functions both by inhibiting responses of mosquitoes to 1-octen-3-ol and other attractive cues released by animal hosts, and by acting as a repellent, detected by olfactory receptors in the antennae, ultimately reducing landings at the odour source (Syed & Leal, 2008). Antennae also respond to components of repellent essential oils, demonstrating the role of olfaction in behavioural responses to repellents (Deletre *et al.*, 2015). As such DEET may be considered to affect the mosquito both as a noxious odour, and an inhibitor of a normally attractive signal, thereby acting by two of the sensory mechanisms proposed by Davis (1985).

However, if repellents are detected by the olfactory system, insecticide resistance may not affect responses to repellents. DDT-resistant *Ae. aegypti* were still repelled by DDT (Polsomboon *et al.*, 2008), and variation in responsiveness to repellents in *An. albimanus* and *Cx. quinquefasciatus* is not explained entirely by insecticide susceptibility (Chareonviriyaphap *et al.*, 1997; Sathantriphop *et al.*, 2006). However, following selection for behavioural insensitivity to transfluthrin, insensitive *Ae. aegypti* populations show reduced knockdown susceptibility to transfluthrin, with results suggesting that the behavioural response is related to kdr alleles (Wagman *et al.*, 2015). Further investigation of this interaction will be important to understand how behavioural responses of mosquitoes influence or arise from the development of insecticide resistance.

### *3.1.3 Testing Behavioural Responses to Repellents*

There are important limitations to many of the common methods used to study mosquito behavioural interactions with insecticide. For example, behavioural assays such as baited tunnel tests, and those examining blood-feeding through nets in closed rooms do not separate repellency from contact irritancy (Chandre *et al.*, 2000; Kolaczinski & Curtis, 2000). Tests that are designed to assess the efficacy of topical repellents using a human arm with repellent applied, or an artificial odour-baited blood-feeding membrane (Deboun & Wagman, 2004; WHO, 2009c), typically measure landing and probing behaviour, both of which require contact with the test substance, and responses may be influenced by a chemical’s contact irritancy rather than by true repellency.

Other test designs, such as Y-tube olfactometers and laboratory assays that assess mosquito movement away from a repellent source, whilst able to isolate a chemical's spatial repellent properties, generally do so without employing a human bait (Grieco *et al.*, 2007; Kongmee *et al.*, 2012; WHO, 2013b).

Video recording mosquito behaviour offers an opportunity to observe mosquito behaviour in detail, and under appropriate lighting conditions. Using infrared (IR) lighting, a wavelength not visible to a mosquito (Gibson, 1991), but detectable with an IR-sensitive camera, it is possible to observe mosquito behaviour under conditions of complete 'darkness', appropriate to their natural nocturnal hunting activity. Potentially, different behavioural stages or sequences as the mosquito flies, lands, probes, and feeds, can be observed and defined and the precise character of and amount of time a mosquito spends on different surfaces can be accurately recorded during video review (Miller & Gibson, 1994; Healy & Copland, 1995). Short actions and rapid behavioural sequences which would be difficult to quantify by the human eye alone, can be recorded and accurately explored using video playback (Dickerson *et al.*, 2012).

This chapter reports on the use of a small portable and relatively simple video tracking system to investigate responses of *An. gambiae*, active around insecticide treated netting, in order to accurately assess the spatial repellent effects of insecticide treated netting.

This study aimed to evaluate novel behavioural assays to assess the spatial repellency of insecticides. The study hypothesis in the small scale behavioural bioassay A was mosquitoes repelled by insecticide would spend less time close to the thumb panel, and less time probing the net in front of the treated material. In small scale behavioural assay B the study hypothesis was that where mosquitoes were repelled by an insecticide they would be diverted to the untreated thumb panel in the same box, making more contacts with and spending more time at the untreated panel.

## 3.2 Methods

### *3.2.1 Mosquitoes*

Female *An. gambiae s.s.* of the insecticide susceptible Kisumu strain, were tested at 3-5 days post-eclosion.  Colonies were maintained as described in General Methods chapter (section 2.2.5). All tests were conducted between 0 and 5 hours after the start of scotophase (12:00-17:00). Prior to testing, mosquitoes were sugar starved for 8-12 hours. Mosquitoes were chosen for tests by placing an arm against the cage and selecting insects attempting to bite.

### *3.2.2 Small Scale Behavioural Assay A – no choice test*

The small scale behavioural assay A used a modified test box to assess repellent properties of insecticides. In brief, mosquitoes were presented with a human bait (a thumb) in combination with a piece of treated netting. In order to ensure only repellent effects were tested, treated netting was covered with an untreated net layer to block contact. As such, this test allowed examination of effects of test chemicals on mosquito movement within the box, and host seeking inhibition.

The assay made use of a new behavioural bioassay (Abe *et al.*, *unpublished*) in which mosquitoes are filmed in close focus as they respond to a human thumb behind netting. In this test box, mosquitoes can be presented with a piece of insecticide treated material placed a small distance behind an untreated net panel, exposing insects to any odours from the material but preventing tarsal contact with the insecticide on its surface (Figure 3.2A). A volunteer's thumb placed against the treated net was used to attract the hungry mosquito.

The test box was 10 x 10 x 10cm in size, with four black plastic sides, and two clear plastic sides through which mosquito activity could be filmed. The box had a 26mm diameter circular port, covered with untreated netting.

The insecticide treated material was presented 5mm behind the untreated net. This distance was chosen as it prevented physical contact with the net, but still allowed the mosquito to come into close proximity with the insecticide. This ensured only the spatial repellent properties of the net were being tested.

A volunteer put their thumb behind the insecticide treated material to act as a bait to encourage mosquitoes to approach the netting. As a mosquito's proboscis is between 1.2-1.6mm long (Adeleke *et al.*, 2008) mosquitoes could probe towards the thumb but could not make contact and were unable to feed.

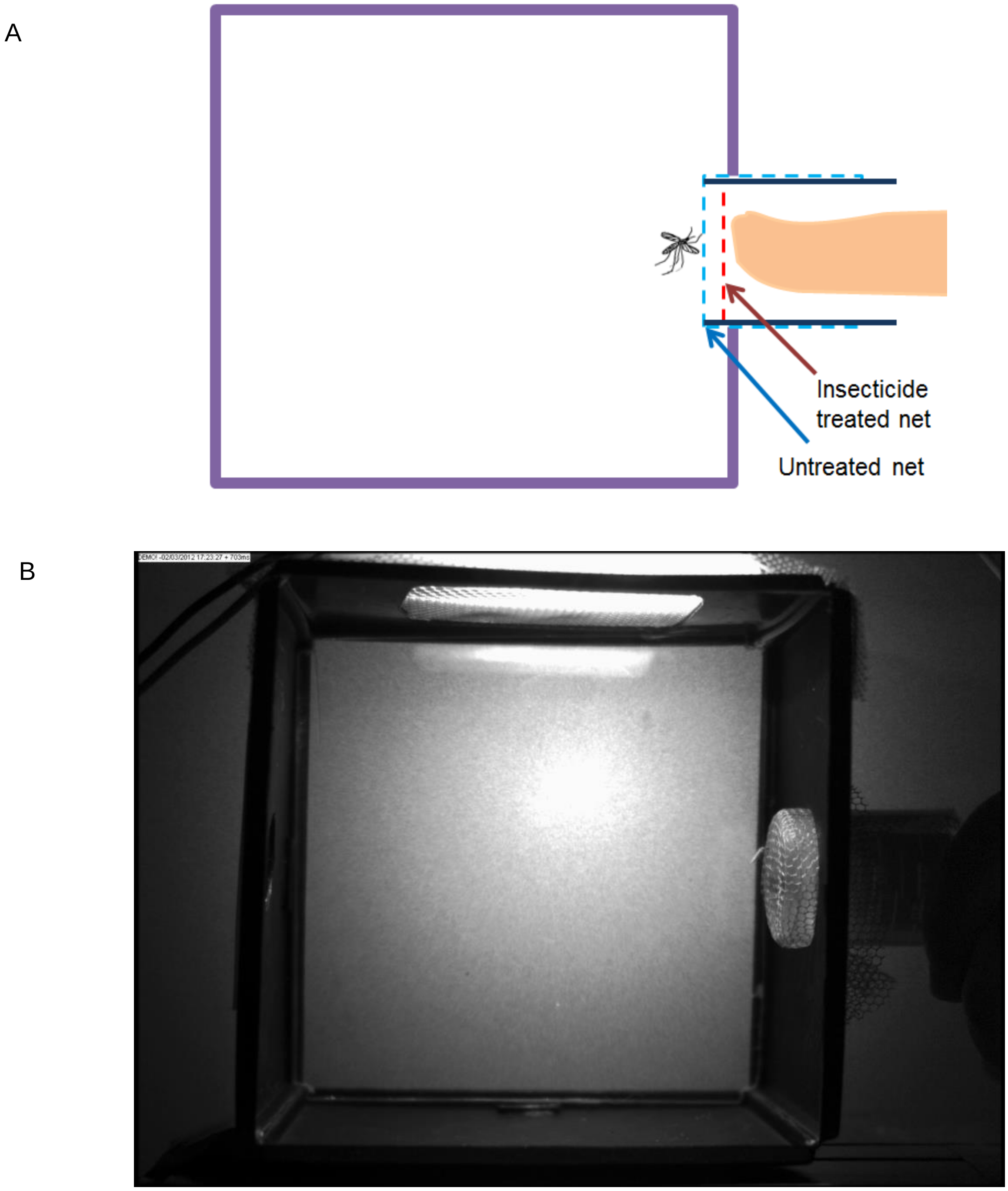


**Figure 3.2 Small Scale Assay A.** (A) Diagram of the small-scale (10x10x10cm) behavioural assay device showing the arrangement of the netting layers within the test box as used to investigate repellency. Contact with the insecticide treated net is prevented by the untreated (blue) net layer. The net panel is baited with a volunteer's thumb. (B) Video screenshot of the behavioural assay, as seen during operation. A mosquito is visible on the netting of the port on the right side of the box, which is baited with the volunteer's thumb. The apparatus is illuminated in infrared light by LEDs placed above the test box, and behind (directly facing the camera lens).

During the test the volunteer kept their hand as still as possible to avoid disturbing the mosquito. The same volunteer was used for all tests. To avoid unnecessary variation in odour, the volunteer did not use scented toiletries on test days. In the three hours prior to testing, the volunteer did not eat with the hand being presented as bait, nor wash the hand using anything other than water.

The test box roof had a 6cm x 6cm net covered hole in it to allow illumination of the interior, with four infrared LEDs providing 860nm wavelength light (RS Components, UK). A fifth LED was placed behind the box with light directed at the camera lens, dispersed using a 25cm x 25cm, 3mm acrylic diffuser (Comar Optics, UK) (Figure 3.2B).

Recordings (as avi files) were made at 20fps using a DALSA Falcon 1.4M100 Camera (Teledyne DALSA, Canada), with a Nikon 24mm/f2.8 lens and CVB Movie Interactive software as configured using CamExpert (both from Stemmer Imaging, UK). Unlike methods described in chapters 2 and 4, mosquito activity was not tracked, but instead reviewed and scored by eye.

Separate boxes were used for each net type to avoid insecticide contamination, and at the end of each test day equipment was washed in Virkon solution. All tests took place in an insectary under the environmental conditions described for colony maintenance.

### *3.2.3 Small Scale Assay A: Test Protocol*

A single mosquito was introduced to the test box and allowed to acclimatise for one hour. One minute prior to the start, the test netting was fitted into position 5mm behind the barrier netting and the volunteer's thumb placed behind it (Figure 3.2B). Activity was filmed for 20 minutes. At the end of the test the mosquito was aspirated out of the box and placed in a paper cup with 10% sugar solution on a cotton wool pad. Mortality/ survival was recorded 24 hours after the test, and mosquitoes were held and inspected daily until death, to measure total longevity after testing. Mortality results at 24 hours were adjusted using Abbott's formula to account for the mortality in controls (WHO, 2013c).

Recordings were processed manually, and the observer was blinded to the net treatment. Behavioural states and mosquito location within the box were logged using The Observer 5.0 software (Noldus Information Technologies, The Netherlands), using a Speedlink Strike PC Gamepad (Jöllenbeck GmbH, Germany) to input multiple key codes for both activity classes simultaneously. Recordings were reviewed in real time playback (not frame-by-frame) and changes to behavioural modes logged using key strokes.

Location of the mosquito within the box was allocated to one of seven categories; four pertaining to each of the dark plastic box walls; a fifth describing contact with the clear plastic sides of the box; a sixth category encompassed the air space, when the mosquito was in flight; and the seventh location category was used for the net panel which covered the thumb port. Behaviour was allocated to one of four categories: flying, probing, walking, or resting. Resting and walking could occur on any solid box surfaces (including the thumb port). Probing could only occur on the thumb port, and all flying was classed as occurring in the air space of the box.

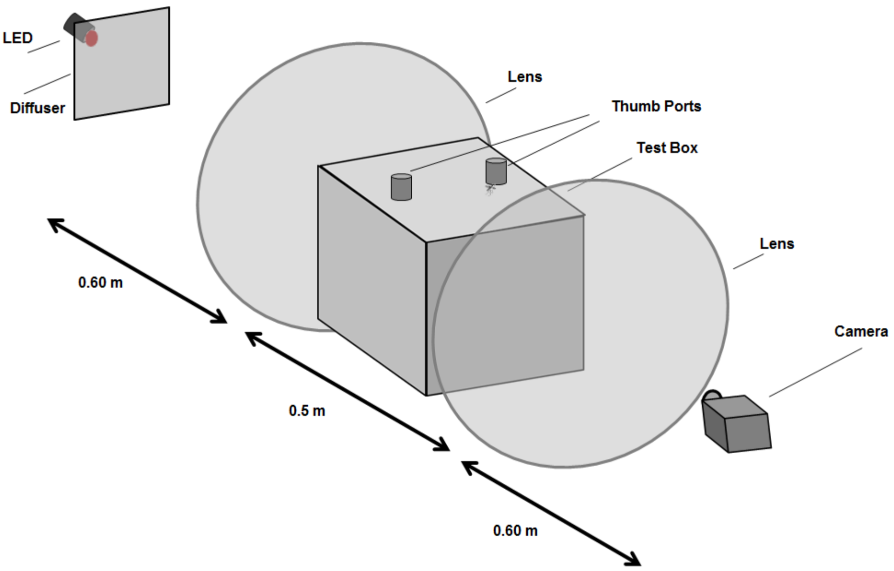
No response threshold was set to include or discard mosquitoes from the study, as it was considered that failure to approach the panel could be a result of insecticide net treatment, and that discarding inactive mosquitoes could mask such effects.

Tests were conducted between the 27/2/12 and 22/4/12. A total of 73 mosquitoes were tested in this bioassay; 25 against untreated netting (controls), 24 with Permanet 2, and 24 with DDT-treated netting. During testing, one net type was tested each day, and order of testing was assigned by block randomisation, in which the three net types were presented in each repeat block, and the order in which net types were used was randomly generated.

### *3.2.4 Small Scale Behavioural Assay B – choice tests*

This assay used a larger choice test arena, 30cm x 30cm x 30cm in size, in which mosquitoes were presented with a choice between two thumb ports of 2.6cm in diameter, set 15cm apart on the roof of the test box (Figure 3.2A and B). As before, each thumb port was covered with a surface of untreated netting with the test material placed 5mm behind. The volunteer acting as bait placed one thumb from each hand on the test material to attract the mosquito.

A



B

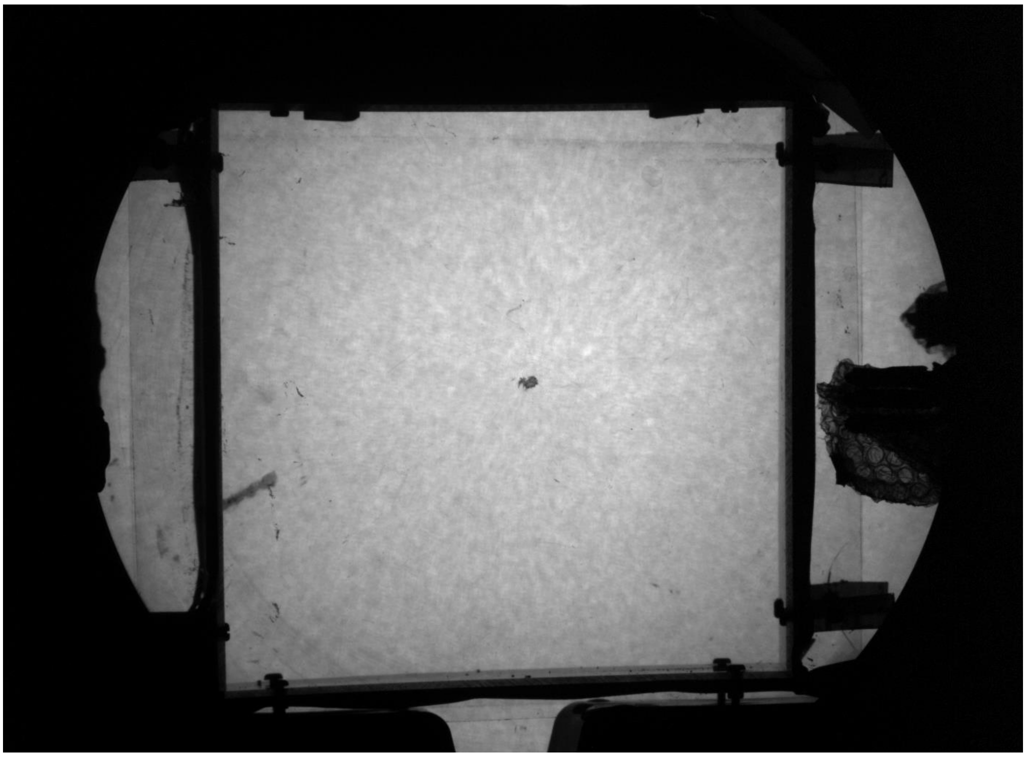


**Figure 3.3 Small Scale Assay B.** (A) Diagram of small scale behavioural B: choice test. The test box (30x30x30cm), with two thumb ports on its roof, is placed between two Fresnel lenses. The setup is illuminated with a single LED through a diffuser, positioned behind the test box. The camera is situated 0.60m from front the Fresnel lens. The mosquito is presented with two thumbs covered with netting layers. As in assay A, contact with the insecticide treated net is prevented by the untreated net layer. (B) Video screenshot of the behavioural assay, as seen during operation. A mosquito is visible resting on the netting of the right thumb port. The edges of the two circular Fresnel lenses produce the curved edges of the field of view in this image.

The box was illuminated using a single infrared LED (wavelength 860nm, RS Components, UK) and a pair of Fresnel lenses (46 cm diameter). The test box was placed in the space between the Fresnel lenses. The camera and LED were placed 60cm from the Fresnel lenses, on either side of the test box (Figure 3.3A). Light from the LED was dispersed using a 3mm acrylic diffuser (25 x 25cm, Comar Optics, UK). Videos were recorded as .bmp images at 20 frames per second using a DALSA Falcon 1.4M100 Camera (Teledyne DALSA, Canada), and 12.5mm lens using CVB Movie Interactive software, and configured using CamExpert (both from Stemmer Imaging, UK).

### *3.2.5 Small Scale Assay B: Choice Test Protocol*

Prior to the test a single mosquito was placed in the test arena and allowed to acclimatise for one hour. At the start of the test, the treated nets were placed on the ports and the volunteer’s thumbs were placed on top of the test nets. Mosquito activity was recorded for 10 minutes.

At the end of each test day equipment was soaked in virkon for 24 hours, then washed thoroughly with water before reuse to avoid any possible insecticide contamination.

The position of the thumb on the left and right ports and pairing with the treated netting was ordered in a Latin square design to guard against arbitrary side or thumb preferences in mosquitoes.

The volunteer remained as still as possible during tests to avoid disturbing the mosquito. On test days the volunteer did not use scented toiletries, and in the four hour period prior to starting tests did not eat, or use soap.

Recordings were processed manually, with an observer noting the time the mosquito spent in contact with thumb ports, the box walls, or in flight. The observer was blinded as to which thumb port contained the treated netting.

Choice tests were conducted between 4/9/12 and 7/10/12. In total, 86 tests were completed with the choice test; 28 with DDT-treated nets tests, 29 with Permanet 2 netting (deltamethrin) and 29 using untreated (control) netting.

### *3.2.6 Net Treatments*

Mosquito behaviour was studied in response to Permanet 2.0 netting (55mg/m$^2$ deltamethrin; Vestergaard, Lausanne, Switzerland) DDT-treated netting (2g/m$^2$) and an untreated net (Abakhan Fabrics, UK). The Permanet 2 net was aired for three weeks prior to starting tests, to allow for evaporation of volatile solvents. Nets were treated with DDT in the laboratory by applying DDT (Sigma Aldrich) dissolved in acetone solvent and silicone oil (556, Dow Corning) to untreated netting using a hand spray bottle. The net was weighed before and after treatment to verify the dose retained. To avoid contamination of small test boxes, only one net type was tested each day. For choice tests, the three net types were tested on the same day and the volunteer washed their hands between tests to avoid residual effects of insecticide contaminating subsequent results. The order of presentation, and the pairing of the volunteer's right or left thumb with the test netting types was determined by Latin square test design.

### *3.2.7 Data Analysis*

*Small Scale Assay A*

The number of mosquitoes that contacted the thumb panel at least once in tests that used insecticide was compared to values of the untreated test using Pearson Chi Squared tests. Effect of net treatment on the amount of time the mosquito spent probing, the time spent on the net panel, and the time spent on the back wall of the test box were analysed using a Kruskal-Wallis H test, using SPSS version 21 (IBM), as data were not normally distributed. Pairwise comparisons were performed using Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons. Adjusted p-values are presented in results.

Longevity after the test was analysed using a Cox proportional hazards model, in the R software package (Fox & Weisberg, 2011). This test was selected as it demonstrates the effect of treatment on time to an event (in this case, death of the mosquito). In this model, longevity in days after the test was the outcome variable, and net treatment type and the week in which the test was conducted were fit as factors.

*Small Scale Assay B*

The number of mosquitoes landing on either thumb (treatment or control) in Permanet 2 and DDT tests was compared to the number landing on either thumb in control tests using Pearson Chi Square tests. The number mosquitoes that landed on the treatment thumb first in Permanet 2 and DDT tests was compared with the average number of landings made at one port on the control box using a Pearson Chi Squared test. Due to low expected probabilities, a Fisher's exact test was used to assess whether insecticide treatment influenced the number of mosquitoes switching thumbs in tests (landing on the treatment thumb, then moving to the control thumb). In analysis of switching in control boxes  (in which both thumb ports used untreated netting), one thumb port was randomly allocated as the ‘treatment’ thumb port, in order to obtain a figure for movement between this and the ‘control’ port, also untreated. As time data were not normally distributed, two Kruskal Wallis H tests were performed to assess effect of treatment on (1) the time mosquitoes spent on the ‘treated thumb panel’ and (2) the time mosquitoes spent on the ‘untreated thumb panel’. As distributions of data were not similar, as assessed by visual inspection of box-plots, the test was used to assess differences in mean rank of data.

In both Small Scale Assay A and B, data are reported as means with 95% confidence intervals. Confidence intervals around mean outcome times were calculated using the t probability distribution, to account for small sample sizes. Where lower bounds of confidence intervals reached negative values as a result of large standard errors in data, the lower bound was truncated at zero.

## 3.3 Results

### *3.3.1 Small Scale Behavioural Assay A – no choice tests*

In total, 25 mosquitoes were tested using control treated netting, 24 with Permanet 2, and 24 with DDT-treated netting. The number of mosquitoes making at least one landing on the baited net panel was similar in control and DDT-treated net tests, where 72% (55-89%), and 63% (44-81%) respectively contacted the thumb panel at least once ($X^2$ (1)=0.71, *p*=0.48). However, Permanet 2 netting significantly reduced the proportion contacting the net panel, with only 38% (19-56%) of mosquitoes approaching the thumb panel ($X^2$(1)=2.43, *p*=0.015).

Distribution of activity between different behavioural categories is shown in figure 3.4. A broad range of probing times was recorded in all tests, with some mosquitoes probing the net for up to 15 minutes, and others not probing at all. To investigate insecticide impact on host seeking, a Kruskal-Wallis H test was conducted to determine if there were differences in probing times between different insecticide treatment groups. Distributions of probing times were not similar for all groups, as assessed by visual inspection of a boxplot. The mean ranks of probing times were statistically significantly different between groups, $X^2$(2) = 8.962, *p* = 0.011.

Mean probing times were 49s [11-87] in assays with Permanet 2 (mean [95% CI]), 215s [111-318] in DDT assays, and 226s [113-338] in control assays. Post hoc pairwise comparisons revealed statistically significant differences in mean ranks of probing times between the Permanet 2 group (rank 26.9) and control (rank 42.9) (*p* = 0.018) but not between the control and DDT group (rank 41.0) (*p* = 1.000). This indicates that the presence of Permanet 2 decreased probing.

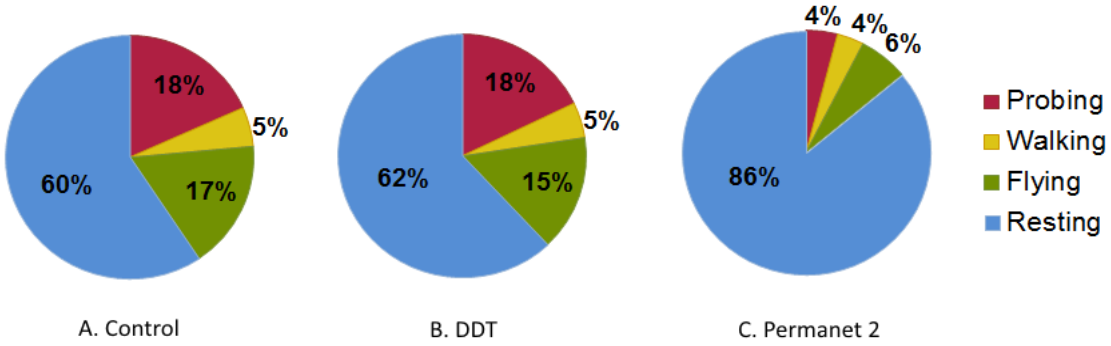


**Figure 3.4 Pie chart showing proportion of time spent by An. gambiae s.s., in each behavioural activity at the thumb panel in the small-scale behavioural bioassay A (no choice test).** Pie charts show data for control (A), DDT (B) and Permanet 2 (C) assays.

The time mosquitoes spent in different parts of the test box is shown in figure 3.5. Net treatment also had a significant impact on where the mosquito spent its time within the test arena (Figure 3.5). Time spent on the thumb panel (i.e. in close proximity to the treated material) was influenced by net treatment. This was tested using a Kruskal-Wallis H test. Time spent on the panel showed different distributions between groups, as assessed by visual inspection of the box plot. The mean ranks of time spent on the panel were significantly different between groups, $X^2(2) = 7.63$, $p = 0.022$. Pairwise comparisons with Bonferroni correction for multiple comparisons (Dunn, 1964) showed that mosquitoes spent less time in contact with the panel in Permanet 2 tests (mean rank 27.7) than in control tests (mean rank 42.9, $p=0.028$). DDT panel contact time did not differ significantly from control tests (DDT mean rank 40.2, $p=0.100$).

Insecticide treatment had a significant effect on time spent on the back wall of the box (away from the treated material). A Kruskal-Wallis H test was conducted to investigate impact of insecticide treatment on time spent on the back of the box. Distributions of time spent on the back wall were different between different groups, as assessed by visual inspection of a boxplot. The mean ranks of time spent on the back wall were statistically significantly different between groups, $X^2(2) = 6.933$, $p = 0.031$. Mean ranks were higher in DDT (42.1) and Permanet 2 (40.9) than in controls (28.3); pairwise comparisons, adjusted for multiple comparisons, found no significant differences between control and DDT tests ($p = 0.092$). The pairwise comparison between Permanet 2 and control tests bordered on statistical significance ($p = 0.053$), after correction for multiple comparisons.

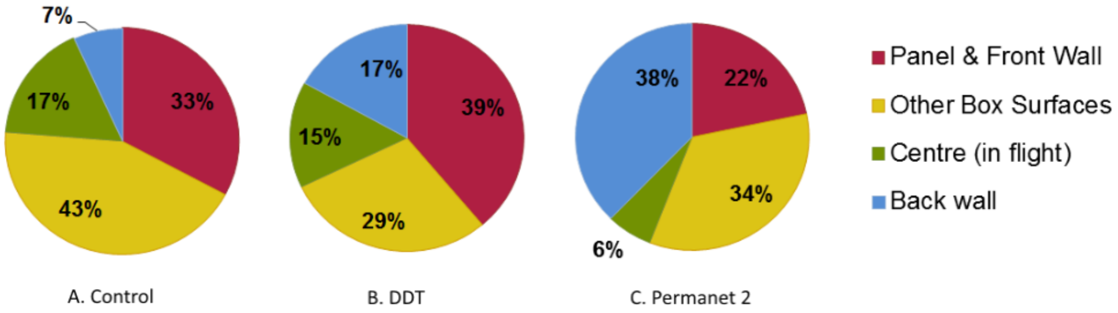


**Figure 3.5 Pie chart showing proportion of time spent by An. gambiae s.s. in different locations on the test box in the small-scale behavioural bioassay A (no choice test).** Pie charts show data for control (A), DDT (B) and Permanet 2 (C) assays.

### *3.3.2 Survival rates following tests*

Mosquitoes in the control group lived for a median of 6 days (95% CI 5.3 to 6.7) after the test, compared with 5 days (95% CI 3.0 to 7.0) for the DDT group and 4 days (95% CI 3.5 to 4.5) for the Permanet 2. Using a cox proportional hazards analysis to compare longevity across all three groups, mosquitoes exposed to Permanet 2 were found to have significantly reduced longevity relative to mosquitoes in control tests (OR= 1.97, 95% CI 1.04 to 3.73, z=2.09, *p*=0.036). DDT exposure did not significantly impact on longevity (OR= 1.69, 95% CI 0.92 to 3.10, z=1.68, *p*=0.093). The week in which the test was conducted did not significantly affect longevity results (OR= 0.91, 95% CI 0.80 to 1.02, z=-1.66, *p*=0.098). No mosquitoes were knocked down during the 20 minute test period, or 1 hour after the test. At 24 hours, corrected mortality was 18.5% and 4.9% with DDT and Permanet 2 nets, respectively.

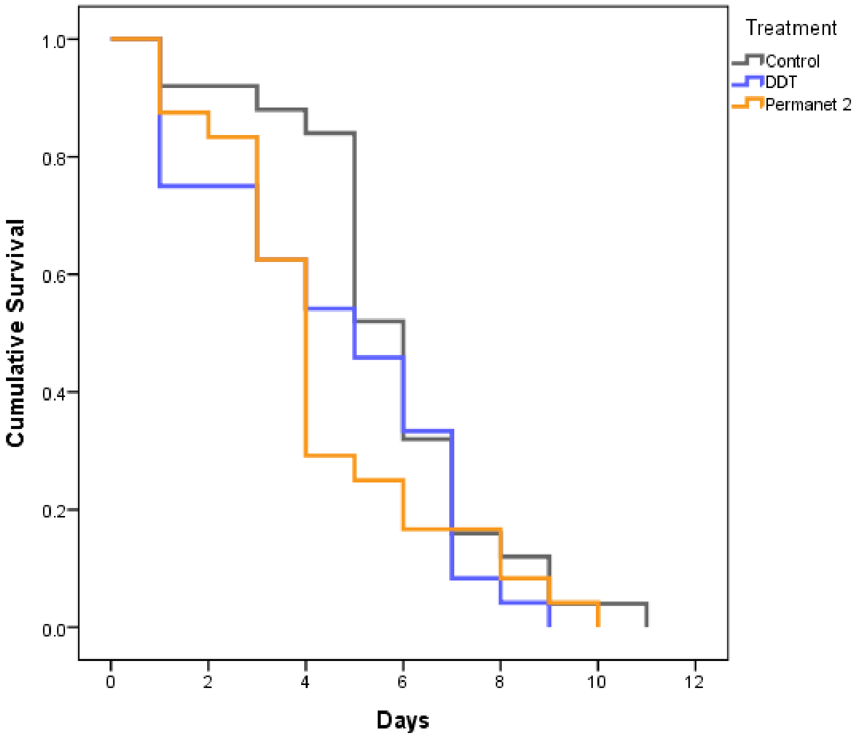


**Figure 3.6 Pie chart showing proportion of time spent by An. gambiae s.s. in different locations on the test box in the small-scale behavioural bioassay A (no choice test).** Graph shows the proportion of insects surviving in the days after the test.

### *3.3.3 Small scale Behavioural Assay B – choice tests*

To measure responses to treated nets in the presence of an untreated alternative, 28 mosquitoes were tested individually with DDT-treated nets tests, 29 with Permanet 2 netting (deltamethrin) and 29 with untreated control netting. Net treatment did not affect the number of mosquitoes that contacted the thumb ports; 41% (23-60%) of mosquitoes contacted a thumb port in control tests, compared to 39% (20-58%) in DDT tests and 55% (36-74%) in Permanet 2 tests ($X^2(2)=1.049$, $p=0.421$). Moreover, the treatment did not affect the choice of which thumb port to approach first (Figure 3.7). The number of mosquitoes that landed on the treatment thumb port were not significantly different between the three tests ($X^2(2)=0.608$, $p=0.826$).

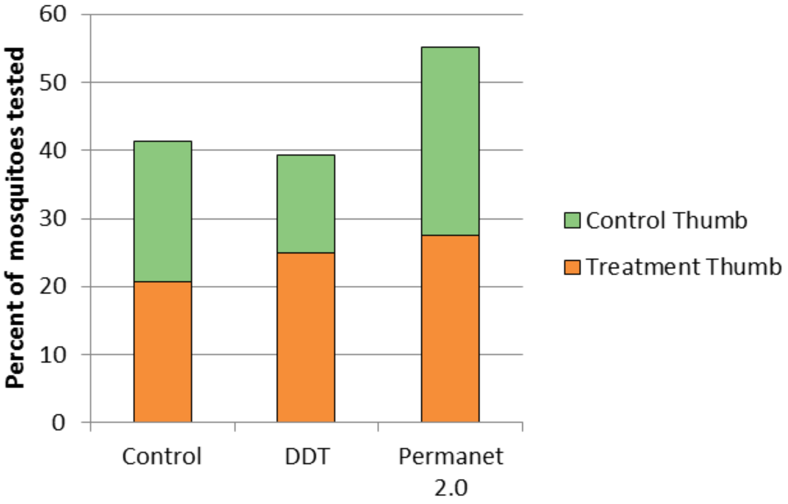


**Figure 3.7 The percentage of mosquitoes approaching thumb ports in small scale behavioural assay B (choice test).** The full extent of the bar shows the percentage of all mosquitoes tested that approached either thumb port. Colours show the proportion of mosquitoes that made their first contact with the treatment thumb port (in which the test material was DDT, Permanet 2, or untreated material, respectively) or the control port (in which the test material was untreated netting). Net treatment had no significant effect on the proportion of mosquitoes contacting the test material, or their first choice of thumb port (see text).

Following first approach, the presence of insecticide did not increase the likelihood of 'switching thumbs' *i.e*. moving to the untreated netting thumb port after landing on the treatment thumb (df=2, $p=0.211$). In controls, switching between ports occurred in 3% (0-10%) of replicates, in DDT tests, the occurrence was 7% (0-17%), and in Permanet 2 tests, where switching thumbs was observed in 17% (3-32%) of cases.

Insecticide treatment did not affect location of activity as mosquitoes spent approximately the same amount of time on the 'treatment thumb' port in all tests (Figure 3.8). Mosquitoes spent an average of 1.0 minute (0.3-1.8 min) and 1.2 minutes (0.3-2.1 min) on the treated thumb panel in DDT and Permanet 2 tests respectively, and 1.4 minutes (0.3-2.5 min) on an untreated thumb panel in control box tests. Results of a Kruskal Wallis H test found mean ranks of time spent in contact on the 'treated thumb' port was not affected by treatment type ($X^2(2)=0.105$, $p=0.949$). There was no evidence for a diversion effect: a Kruskal Wallis H test found use of insecticide did not result in any increase to the amount of time mosquitoes spent on the untreated control thumb port within the same box (DDT control, 0.5 minutes [0.1-1.0min]; Permanet 2 control, 1.6 minutes [0.6-2.6min]; Control test, alternative untreated port, 1.1 minutes [0.2-2.0min]; $X^2(2)=4.025$, $p=0.134$).

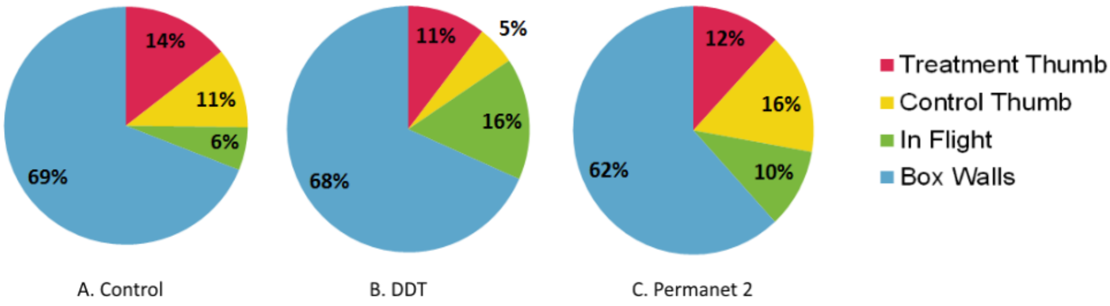


**Figure 3.8 Pie chart showing proportion of time spent by An. gambiae s.s. resting in different locations or in flight, within the small scale behavioural assay B (choice test) arena.** Net treatment had no effect on mosquito activity within the box.

## 3.4 Discussion

These tests were conducted to assess the spatial repellent properties of insecticide, and the impact of close exposure to insecticide without contact, on mosquito longevity. Results from the two different bioassay setups delivered different results regarding the non-contact repellency of the insecticides tested. Small scale behavioural assay A provided evidence for repellency of Permanet 2, as mosquitoes made fewer contacts with the treated panel, spending less time in contact with the panel, and less time probing towards the thumb. Mosquitoes in tests with Permanet 2 were found to spend more time on the back wall of the test box (i.e. the furthest point from the treated panel). This suite of behavioural effects were considered evidence of repellency. In assay B (choice assay) there was no indication of repellency as insecticide treatment did not affect the number of contacts on the thumb ports, the time mosquitoes spent at the ports, or their choice of which thumb to approach. DDT treatment was not found to exert a repellent effect in either assay. Non-contact exposure to Permanet 2 resulted in a small but significant reduction in longevity in small scale assay A.

The discrepancy in bioassay results may have been a product of the confining conditions of the smaller bioassay (assay A) which, at 10x10x10cm, may have constrained mosquito flight and host seeking behaviour. Test assay scale has been suggested to affect behavioural assay results, as in the instance of *Ae. aegypti* responses to 10% carbon dioxide, which was found to be repellent in a very small olfactometer, but attractant in a large olfactometer (Willis & Roth, 1952). Test chamber size and mosquito density were found to affect repellent efficacy of DEET to *Ae. aegypti* and *Anopheles quadrimaculatus* (Barnard *et al.*, 1998). Presenting mosquitoes in different sized cages with an arm covered in DEET, the repellent's protective time against biting was longer when tested in a medium size box (46 x 38 x 37cm), than for mosquitoes in smaller (30cm cubed) and larger cages (50cm cubed), after controlling for the effect of density. By varying the number of mosquitoes in the cage, it was found that testing insects at higher density reduced the repellent's apparent protective time. Intuitively one would expect that a test arena allowing more natural host-seeking flight would produce more realistic, biologically relevant data. If so, then this would be an argument to reconsider the reliability of studies on insecticides and repellents reported using bioassay methods based on smaller scale behavioural assays (Achee *et al.* 2009; Chareonviriyaphap *et al.*, 1997).

The size of a test arena may also affect the level of insecticide exposure, as smaller test box volumes are more likely to hold a higher concentration of volatiles from a standard surface area or section of netting. Mosquitoes detect repellents such as DEET using olfactory receptors on their antennae and palps (Davis, 1985; Syed & Leal, 2008; Stanczyk *et al*, 2010). Assuming they respond to volatiles of pyrethroids and DDT using the same mechanism, the volatile concentration they are exposed to has the potential to influence behavioural responses observed, and it is therefore important to try and simulate realistic exposure levels in behavioural assays. A test of DDT treated material found the concentration of insecticide in the air within small scale behavioural assays was greater than concentrations present in the air of room scale semi-field experiments (Martin *et al.*, 2013). This presents the danger that small chamber behavioural assays conducted in the laboratory may inadvertently exaggerate an insecticide's spatial repellency by exposing mosquitoes to higher volatile doses than are likely to be experienced in regular use.

Small enclosed behavioural assays risk air becoming saturated with insecticide, losing the odour gradient that would be necessary for escape responses away from the odour source. Longer term exposure to saturated air could risk habituation or adaptation of mosquito response to a chemical. Using an assay ventilated with artificial airflow, Martin *et al.* (2013) found that an odour gradient could exist between adjoining untreated and insecticide treated test compartments (each of which was a 30cm sided cube).

In the smaller behavioural assay (assay A), the ceiling panel comprised a large net-covered window to allow LED illumination from above, which would have allowed some ventilation and air movement and helped reduce volatile saturation. As choice tests were wholly back-lit, boxes contained no open panels other than the thumb ports. This may have affected the concentration of insecticide in the air within the test boxes, and been a factor in differences in the results of the two tests.

It is also possible that the position of the thumb ports played a role in the different responses from the two test setups being reported here. Comparing untreated control results, 72% of mosquitoes contacted the thumb panel in small box tests (port on side wall of cage; Figure 3.2A), compared to 41% in the choice test arena (ports on the roof; Figure 3.3A). The placement of choice test ports on the arena ceiling in the latter may have influenced the landing rates. Lyski *et al.* (2011) tested responses of *Ae. aegypti* and *Ae. albopictus* to blood-feeding targets in different positions, and found that mosquitoes were more successful in feeding on targets placed against the side of the cage than on the floor.

To evaluate which testing method gave the most accurate representation of spatial repellent effects it is helpful to consider existing data on the properties of these insecticides. A number of field and semi-field tests have found 2g/m$^2$ concentrations of DDT to be repellent when used in IRS or as net treatment (Taylor, 1975; Achee *et al.*, 2012b; Tangena *et al.*, 2013). Hence it was included in tests in the present study as an intended positive control for repellency.  However neither behavioural assay found evidence for any repellent effect of DDT and there was no evidence that presence of DDT reduced approaches to the thumb bait (assay A), or that in a choice test (assay B) that the insecticide diverted more mosquito activity towards the control thumb.

In this study, DDT was prepared using an acetone solvent, and silicone oil as carrier, a different preparation process to that used in IRS where a wetting agent and dispersing agent are used (WHO, 2013d), and which could have repellent properties of their own. This issue was reported in early studies of bed nets, in which the emulsifiable concentrate carrier chemicals used to apply insecticide to the bed net were found to be repellent (Lindsay *et al.*, 1991). However DDT netting prepared with acetone solvent was found repellent in field trials against *Ae. aegypti* (Achee *et al.*, 2012b), which supports the hypothesis that DDT is itself repellent, and would indicate that the failure to find a repellent effect in the present study may be indicative of flaws in the assays used.

Laboratory tests also found evidence for spatial repellency of DDT: trials in a well-controlled experiment using *Ae. aegypti* and *Anopheles albimanus* reported that non-contact exposure to DDT at the same concentration used in the present study resulted in 28% of mosquitoes exiting test boxes in a repellent-induced escape response (Chareonviriyaphap *et al.*, 1997; Thanispong *et al.*, 2009).  These tests found variations in responses between mosquito species and some strains did not respond to DDT.  Later work using this bioassay found that *Anopheles minimus* could be repelled by DDT but that mosquito responses were dependent on nutritional status: unfed insects were not repelled, whereas sugar-fed and blood-fed insects were (Sungvornyothin *et al.*, 2001).

The lack of repellency found in the behavioural assays in the present study may be a result of the smaller surface area presented to the insects. In the escape bioassay used by Chareonviriyaphap and colleagues, mosquitoes were exposed to an area of over 3250 cm$^2$ of treated material (Chareonviriyaphap *et al.*, 1997; Thanispong *et al.*, 2009), whereas in both assays A and B used in the present study, the area used was 7.1 cm$^2$. A larger surface area would allow for greater volatilisation of DDT, increasing the levels in the air within the test chamber and explain why the same chemical concentration induced different responses in these assays.

Differences in results from different test types and mosquito species highlight the importance of conducting tests in realistic bioassays, ideally using more than one test, and using the insect strain that an intervention is intended for. Smaller scale bioassays can be valuable in describing relative contact irritant or repellent properties of insecticide types (Achee *et al.*, 2009), but care must be taken in extrapolating these results to predict the efficacy of insecticide-based interventions in the field, and ultimately field tests will provide the most reliable.

Results from these behavioural assays indicated that deltamethrin-treated LLINs are repellent in small single port test chambers, but not in the choice test. Escape response experiments measuring mosquito exit rate from guinea pig baited test boxes found no repellent effect of deltamethrin to *Ae. aegypti, Anopheles harrisoni* or *An. minimus* (Boonyuan *et al.*, 2011; Kongmee *et al.*, 2012), using test chambers that were approximately the same size as those used here in choice tests. Other small scale tests reported findings similar to those reported in the present study. A high throughput screening system measuring movement away from insecticide treated netting showed DDT to be repellent to *Ae. aegypti* at doses as low as 9mg/m$^2$, but found no repellent effect of deltamethrin at doses up to 1.2g/m$^2$ (Grieco *et al.*, 2007; Achee *et al.*, 2009). However, as no attractant or host bait was used, the results may not translate to responses to a human baited LLIN. The technique also risks problems arising from crowding and constrained flight; in each test 10 mosquitoes are released in to a chamber cylinder measuring only 14cm in length and 10.2cm in diameter.

Using a simple small scale camera system to measure landing of *An. arabiensis*, *Cx. quinquefasciatus*, and *Ae. aegypti* on insecticide treated paper, Cooperband & Allan (2009) found no evidence for repellency by deltamethrin (20mg/m$^2$), instead mosquitoes only showed behavioural effects after landings had been made.

Experimental hut trials offer a way to address the question in a more realistic setting, using human bait and free flying wild mosquitoes. However occasionally studies contain methodological flaws that could compromise results. For instance a number of studies have been published showing a repellent effect of deltamethrin using entry traps fitted to experimental huts, in which huts were not stated to be protected from ant attack (Darriet *et al.*, 2000; Asidi *et al.*, 2004). Failure to properly ant-proof a set-up can seriously compromise results, as ants can enter a trap and remove knocked-down or otherwise immobilised mosquitoes, whether the effect is fatal or temporary. Such results would imply repellency by reducing catch within a trap. Experimental huts should be protected by stilts set in water buckets, or by a small moat to ensure results are unaffected by predation.

Darriet *et al*. (2004) employed unwashed treated nets, which may allow for carrier chemical effects in the initial weeks of testing. In tests of permethrin-treated nets in The Gambia, solvents used to apply insecticide have been found to be responsible for the deterrent effects on house entry (Lindsay *et al.*, 1991). The same effect has been found using topically applied repellents, where solvents have been found capable of reducing as well as enhancing a treatment's repellency (Dethier, 1947). Volatile solvents on bed nets are thought to evaporate over a period of weeks (Lindsay *et al.*, 1991) and thus to avoid misattribution of repellent effects, test nets should either be aired for a period of time or washed to remove these chemicals prior to testing. LLINs incorporate insecticide directly into net fibres, or apply it to the net using a resin, and do not require use of solvents. Residues remaining from the manufacturing process have not been studied for repellency, though washing has been shown to influence repellency, hence a similar airing time may be warranted when testing LLINs as in tests of nets treated by immersion.

A large number of experimental hut trials using deltamethrin treated nets (applied at between 25-55mg/m$^2$) found no evidence for insecticide repellency on *An. gambiae* (*e.g.* Miller *et al.*, 1991; Mosha *et al.*, 2008; Tungu *et al.*, 2010). One additional trial has reported Permanet 2.0 to be repellent, but the effect was lost upon washing, suggesting that it may have been caused by carrier chemicals (N'Guessan, 2010).

Field and laboratory tests suggest that DDT is a spatial repellent whilst deltamethrin is not. Results from small-scale behavioural assays conducted in the present study indicated that deltamethrin had repellent properties but DDT did not. When mosquitoes had a choice between the insecticide or a control, neither insecticide was repellent. The small scale of the behavioural tests was designed to afford detailed observation of mosquito flight, landing and probing behaviour, and permit the easy use of a human thumb as attractant bait. However the constraints of such a small arena may have elicited aberrant responses from mosquitoes in atypical conditions, as discussed earlier. These tests could be improved by increasing the test box volume to allow more room for flight, by using a larger attractant bait (*i.e.* a volunteer's arm) placed on the side or base of the test arena. The test might produce better quality data if the sides of the arena were not solid, to allow better air movement and avoid saturation of air with test volatiles.

Currently repellent tests expose mosquitoes in behavioural assays to material treated with the same insecticide concentration (g/m$^2$) as is intended for use in the field. However, the concentration of insecticide in the air within the test arena and experienced by mosquitoes, will be higher in enclosed laboratory assays (Martin *et al.*, 2013). Future work might consider the relationship between air concentration in laboratory tests and field settings, and attempt to scale test concentrations in laboratory tests, to ensure mosquitoes are exposed to appropriate levels of airborne insecticide.

Different repellent properties of insecticides may be related to their chemical properties. It has been proposed that a high vapour pressure allows a chemical to vaporise easily thereby making it more repellent, though practical evidence for this is mixed (Garson & Winnike, 1968). In addition to vaporising directly, it has been suggested that an insecticide could become airborne through contamination of dust particles, in a process referred to as ‘flaking’ (Smith & Webley, 1969; Somboon, 1993). There is limited evidence to support this mechanism though, and flaking is unlikely to play a major role in short laboratory tests. Deltamethrin has a lower vapour pressure than DDT (figure 3,1), which would make it less likely to act as a repellent at the same concentration, as the chemical is emitted from the net into the air at a lower rate (Site, 1997; WHO, 2010d). Clear repellent effects have been shown for other members of the pyrethroid insecticide class that have much higher vapour pressures, such as transfluthrin and metofluthrin, and that are intended as spatial insecticides (Achee *et al.*, 2012a), often dispersed from paper emanators or on hessian fabric strips (Lucas *et al.*, 2005; Ogoma *et al.*, 2012b). One may conclude that whilst the pyrethroid chemical class can induce directed movement away from the chemical source in mosquitoes, this depends on the mosquito encountering a detectable concentration of the chemical during its flight. Analysis of air samples in experimental huts lined with 2g/m$^2$ DDT net panels found appreciable concentrations of the insecticide in the air (0.7-1.4 µg/m$^3$), which were noted to have a deterrent effect on *Ae. aegypti* approaching the hut (Achee *et al.*, 2012b). Work has yet to be conducted to establish the insecticide concentration found in the air around LLINs treated with pyrethroids.

At present it is uncertain whether responses to repellent insecticides occur via aversive physical effects, whereby a mosquito experiences neurotoxic effects on approach, or through detection by odorant receptors. Pyrethroid insecticides and DDT both act by the same physical mechanism to cause mortality, disrupting a mosquito’s voltage gated sodium channels (Zlotkin, 1999). Exposure can bring about excitation, impairment of movement, paralysis and death (Kennedy, 1947; Davies *et al.*, 2007). It is unknown whether a mosquito approaching these repellents can detect insecticide in air before physical effects occur, as no electroantennography or similar studies have been carried out with mosquitoes. The few available studies of other insects show poor to no antennal response to pyrethroid insecticides in mole crickets and moths (Sower & Shorb, 1985; Kostromytska, 2010). However, both of these insect groups occupy very different ecological niches to mosquitoes restricting any comparisons with mosquitoes.

If sensation of insecticide by odorant reception is discounted, a mosquito approaching an insecticide must at some point experience aversive sensations on approach, provoking movement away from the insecticide source. In the results reported in this chapter, the slightly reduced lifespan of mosquitoes following exposure to insecticide without contact (see Figure 3.6) indicates that direct contact with the insecticide may not be necessary to achieve toxic effects.

This study found some evidence for a small reduction in mosquito longevity following non-contact exposure to insecticide (from 6 days in control tests to 4 days after tests with Permanet 2). A similar study by Kongmee *et al.*, (2012) exposed *An. harrisoni* and *An. minimus* mosquitoes without contact to 20mg/m$^2$ deltamethrin for 30 minutes, but found no increased mortality 24 hours later. Using a similar set-up, Boonyuan *et al*. (2011) found no increase in 24 hour mortality in *Aedes aegypti* following 60 minutes of non-contact exposure. *Ae. aegypti* housed in experimental huts in which metofluthrin coils were burnt, or where DDT netting panels were applied to the walls, showed no significant knockdown or increase in 24 hour mortality (Achee *et al.*, 2012b). A study of non-contact mortality using paint containing pyriproxyfen, chlorpyriphos and diazinon (Mosqueira *et al.*, 2013) reported up to 100% mortality in *An. gambiae* and *Cx. quinquefasciatus* at 24 hours following exposure for 12 hours in an experimental hut at 1m distance from the painted walls.

The absence of mortality effects in non-contact exposure assays (Boonyuan *et al.*, 2011; Achee *et al.*, 2012b; Kongmee *et al.*, 2012) might be related to the lower insecticide concentration used, but may also be a consequence of the shorter follow up time (24 hours). In this assay, mortality results 24 hours after the test did not show any significant impact of treatment on death rate, and it was only in following mosquitoes up to the day they died that differences became apparent. Interventions that reduce longevity are of particular importance to disease control, because malaria is usually transmitted by mosquitoes that are more than ten days old. In

Ross-MacDonald style models of mosquito borne disease transmission, reduction in longevity can reduce disease transmission, and plays a major role in controlling malaria (Smith *et al.,* 2012).

### *3.4.1 Summary*

Two small scale bioassays were designed to investigate the repellent effects of insecticide on mosquitoes. Small scale behavioural assay A examined the close range effect of insecticide on mosquitoes' probing activity at a human bait, and movement within a test box. Small scale behavioural assay B assessed whether mosquitoes could be diverted from a source of insecticide towards a control thumb. These behavioural bioassays provided ambiguous data on the repellent properties of deltamethrin and DDT, as small scale behavioural assay A found the Permanet 2 to be repellent whilst small scale behavioural assay B showed no such effect. DDT, an insecticide that other assays have shown to be repellent, was not shown to be repellent in the present study.  The results may have been compromised by a small sample size, or the design of the test in which flight was constrained and insecticide exposure was not typical of field conditions. A small reduction in longevity was observed following non-contact exposure to deltamethrin treated LLINs, although further research would be necessary to establish whether such an effect would occur in a full scale test in a natural setting.

# *Chapter 4* Flight Patterns of Host-Seeking *Anopheles gambiae s.s.* During Movement Through a Window

## Abstract

Host seeking *An. gambiae* enter houses through eave gaps and windows to feed on humans. This stage in the host seeking pathway may be exploited for vector control, either through the use of screens or barriers to block entry in to the home, or by intercepting mosquito flight with insecticide treated materials. This chapter evaluated a novel 3D tracking method for use in observing house entry flight of mosquitoes. Flight tracks were examined for evidence of stereotypical entry behaviours that may behave the potential to guide design of new vector control tools.

Flight of *An. gambiae s.s.* through a window towards a bait was observed in 3D using a novel single-camera tracking technique. In this method, video recordings could be analysed with custom-written tracking algorithms to identify a mosquito's position in 3D, using calculations based on the relative position of a mosquito and its shadow on a retro-reflective screen. This tracking principle has been applied to studies of diurnal insects, but the work presented here represents the first use of such methods with nocturnal insects using artificial lighting and a retro-reflective screen.

Tests found that the novel tracking method could successfully locate a mosquito's 3D coordinates in space, and link flight tracks of mosquitoes entering a window during host seeking. Track analyses provided some evidence for non-random, stereotyped pathways of room entry. Mosquitoes decreased their flight elevation as they moved through a window, and after entry, flew downwards out of the camera's field of view.

This work has demonstrated successful proof of principle of this tracking method, which has potential to be applied to a variety of studies of mosquito behaviour. Further work will need to investigate the limits of system resolution, and the extent to which flight entry tracks are influenced by context of house design and environmental conditions.

## 4.1 Introduction

The preference of *An. gambiae s.s.* for blood-feeding indoors is thought to be a consequence of its high anthropophilic tendencies (Costantini *et al.*, 1999), facilitated by its host's ancestral societal transition from a nomadic hunter-gatherer culture to settled agricultural communities (Coluzzi, 1999). Such communities show tendencies to manipulate land in a way that generates habitats for mosquito larval development, fostering a strong association between mosquitoes and humans (Coluzzi, 1999; Costantini *et al.*, 1999). This in turn may have enabled selection for endophagy, as houses provide protective micro-environments for resting mosquitoes, and are where humans sleep at night, thereby offering potential blood meals for mosquitoes (Costantini *et al.*, 1999; Carter & Mendis, 2002).

Several important anopheline disease vectors show endophagic tendencies, in Africa (*An. gambiae s.s.*, *An. funestus*), Asia (*Anopheles minimus*, *Anopheles culicifacies*) and Latin America (*An. darlingi*; Pates & Curtis, 2005). Typically mosquitoes fly indoors to blood-feed, resting within the house after the blood meal until they are gravid, when they exit to lay eggs in outdoor breeding sites (Gillies, 1954; Smith, 1965). Houses also may act as important refugia for mosquitoes during the dry season (Charlwood *et al.*, 2000; Lehmann *et al.*, 2010), and the favourable microclimate within houses can permit endophilic populations to exist at altitudes or persist during seasonal periods when conditions would not permit completion of the mosquito life cycle (Tchuinkam *et al.*, 2010; Paaijmans & Thomas, 2011). As such, detection of and navigation through house entry points demands behavioural capabilities that are important at many stages of the mosquito's life cycle.

Mosquitoes enter houses primarily through eave gaps, windows and doors. Entry routes may be opportunistic, and vary according to house design, and which routes are accessible. Eaves are important routes of entry for anophelines, and house surveys of indoor resting or using light trap catches report fewer mosquitoes in houses with closed eaves (White, 1969; Kirby *et al.*, 2008; Lwetoijera *et al.*, 2013; Wanzirah *et al.*, 2015). Screening eave gaps can reduce the numbers of *An. gambiae s.l.* and *An. funestus* caught inside houses by more than 66% (Lindsay *et al.*, 2002; Lindsay *et al.*, 2003; Atieli *et al.*, 2009; Njie *et al.*, 2009; Ogoma *et al.*, 2010). The fact that eave screening does not completely eliminate ingress indicates that doors and windows are also important points of house entry for *An. gambiae s.l.*. Routes of entry may vary according to what areas of the house are vulnerable to mosquitoes: closing the doors or windows may not significantly reduce the number of *An. gambiae s.l.* entering a house (Ogoma *et al.*, 2010), as mosquitoes may be diverted to enter through alternative routes (Diabaté *et al.*, 2013). Screening is a popular intervention and typically well accepted by target communities, and has the additional benefit of impacting on more than one mosquito vector or nuisance species (Atieli *et al.*, 2009; Ogoma *et al.*, 2010; Manrique-Saide *et al.*, 2015).Effective screens and ceilings also can reduce the incidence of mild malaria or anaemia among householders (Lindsay *et al.*, 2002; Kirby *et al.*, 2009; Tusting *et al.*, 2015).

Screening windows can reduce mosquito numbers indoors even when screens are damaged, with partial cover providing better protection than an open window (Lwetoijera *et al.*, 2013). This suggests that the size of the opening may be important in facilitating or permitting entry.  This is supported by studies where partial covering of the window space with curtains (without insecticide) reduced but did not eliminate mosquito house entry (Majori *et al.*, 1987; Fanello *et al.*, 2003), and by studies of indoor resting anophelines which reported that mosquito density was directly proportional to eave width (White, 1969; Kirby *et al.*, 2008).  A recent laboratory study examining mosquito passage through holes in LLINs, found that a higher proportion of mosquitoes successfully passed through larger holes (Sutcliffe & Colborn, 2015). It may be that smaller openings limit the release of attractive odours in to the air surrounding a building, thereby providing weaker stimulus for long range host location. Alternatively, smaller gaps may be more difficult to locate and/ or eventually fly through. Video observations of flight through small holes (9-13mm) showed that mosquitoes often collided with net edges during their attempt to pass through the gap (Sutcliffe & Colborn, 2015).

The importance of hole or gap size appears to differ between species. To enter houses in a field study, *An. gambiae s.l.* were reported to be capable of passing through small eave gaps, whereas *An. funestus* and culicine species such as *Cx. thalassius* and *Cx. quinquefasciatus* did not use these openings as often, and will instead enter houses through larger gable end openings or doorways (Njie *et al.*, 2009; Kampango *et al.*, 2013). However, studies have not investigated whether these preferences were determined or influenced by hole or gap size, or whether the height, shape and position of the openings also played a role in route choices.

Arriving at a house and choosing whether to enter or not may be a stepped process, as the mosquito interprets signals from the potential host within. Torr *et al.* (2008) found that *An. arabiensis* would approach an experimental hut or odour trap when it was baited with either human or cattle odours, but would only enter it if the odours were of human origin. In the same study, the zoophagic and exophilic *An.*

*quadriannulatus* was attracted to both human and ox odours, but rarely entered traps or huts. Precisely what comprised or was perceived as ‘indoor’ and ‘outdoor’ in that situation remains unknown. In fact, knowledge of spatial aspects of mosquito movement in general is quite limited.

### *4.1.1 Height of Flight, Navigating Barriers*

The majority of *Anopheles gambiae* caught in open, un-vegetated land will fly at less than 1m above the ground (Snow, 1979). Vertical barriers of up to 1.72m in height placed around a volunteer reduced the number of mosquitoes attacking a human host to less than 60% of that approaching an unprotected host (Snow, 1987).

Another study found that *An. gambiae* and *An. funestus* were capable of flying over a 6m tall fence when responding to a human or cow bait, and that such a barrier did not reduce the number of mosquitoes caught within a circular fenced enclosure (Gillies & Wilkes, 1978). In that study, analysis of the flight elevation of *Mansonia sp.* mosquitoes, suggested that a mosquito’s movement upwards must occur very close to the barrier: even within 25cm of the fence there was no detectable increase in mosquito elevation. Catches inside a smaller (2.9m high, 3m radius) fenced enclosure found that whilst some mosquitoes reaching the centre of the ringed circle had returned to ground level (less than 1m), the number still flying at elevations of 1-3m had proportionally increased.

It is not known whether mosquitoes navigate up a barrier by contacting its surface during flight. Results of a study of passage over short insecticide treated fences around cattle enclosures suggested that culicine mosquitoes were contacting the fence during navigation over it (Maia *et al.*, 2012). However little other information is available on how mosquitoes navigate barriers in flight.

Detailed knowledge of how the main mosquito vectors and nuisance species enter houses could be useful to guide house design or modification to reduce exposure to mosquitoes inside the home without, or at least reducing the reliance on, insecticides (Lindsay *et al.*, 2002; Ogoma *et al.*, 2009). There is potential to exploit the house entry behaviour of mosquitoes for distribution of insecticide or bio-control agents such as fungi using treated curtains on eaves or windows (Sexton *et al.*, 1990; Fanello *et al.*, 2003; Farenhorst *et al.*, 2011; Mnyone *et al.*, 2012). These methods place treated materials across house openings, on the assumption that insects will contact them as they enter the house. Little is currently known about how mosquitoes move through such openings/ apertures/ spaces: whether they exhibit spatial patterns or preferences, *e.g.* preferring the boundary or the centre, or move randomly, when entering via a window.

### *4.1.2 3D Tracking Methods in Entomology*

Single camera 3D imaging has yet to be fully explored in mosquito tracking. The majority of systems that track insects in three dimensions use stereoscopy, in which insect activity is viewed from two perspectives using two cameras, and the data from each camera are coordinated to generate a 3D track (Reynolds & Riley, 2002; Lacey & Cardé, 2011). Using retroreflective screening (RRS) it is possible to generate a 3D track using a single camera. This is achieved by placing the RRS at the rear of the field of view; using light from an infrared LED positioned adjacent to the camera lens, an image is obtained showing stereo positions of the insect and its shadow on the RRS. With calibration, the distance between the insect and its shadow is used to estimate the mosquito’s proximity to the RRS. Using the sun as a light source, this approach has been applied to obtain 3D flight data on diurnal insects including bees, wasps and midges, in studies examining a variety of insect behaviours including nest approach, swarming and landing (Okubo *et al.*, 1981; Zeil *et al.,* 1993; Srinivasan *et al.*, 2000). Though the majority of those studies tracked flight of single insects, this method has also been used successfully to study interactions of multiple insects in swarms (Okubo & Chiang, 1974).

Single viewpoint imaging has many advantages in that it requires only one camera – hence it is cheaper, easier to transport and considerably simpler to calibrate, and has a faster tracking procedure following recording, as only one video file requires processing. Set-ups must be recalibrated according to the moving position of the sun (Zeil *et al.,* 1993; Srinivasan *et al.*, 2000), although this does not affect tracking of nocturnal insects where an infrared light can be installed in a fixed position to illuminate the entire test set-up. Clearly, single viewpoint 3D tracking offers many advantages for studying flight patterns of nocturnal insects such as mosquitoes in the laboratory and in the field.

This chapter reports on a set of studies utilising a newly developed single camera 3D tracking system, which explored the movement of *An. gambiae s.s.* during passage through an aperture or ‘window’ fitted between two experimental rooms. This study aimed to evaluate the capabilities of this novel tracking system for use in studies of mosquito behaviour, through experimental proof-of-principle of the use of retro-reflective screens in 3D flight tracking. The objective of this study was to test the viability of this tracking method for use in research into house entry behaviour of mosquitoes. The secondary research objective of the study was to characterise flight patterns of mosquitoes entering a room, and to determine whether insects exhibited spatial preferences or patterns in their flight paths. The study held the null hypothesis that mosquitoes would enter houses through random paths, and that no trends in spatial activity would be observed.

## 4.2 Methods

### *4.2.1 Mosquitoes*

All tests were carried out using 3-5 day old female *An. gambiae s.s.* Kisumu strain, reared in the LSTM insectaries (conditions described in chapter 2, section 2.2.5). Behavioural recordings were made in the initial 1-6 hours of the scotophase (13:00-18:00). On the morning of the test day, individual mosquitoes were selected based on their attraction to an arm placed against the side of their cage. The selected mosquitoes were sugar starved for 4-6 hours before testing.

### *4.2.2 Insectary conditions and equipment*

All tests were carried out in a purpose-built insectary at the Liverpool School of Tropical Medicine. Two adjoining rooms were linked by an open window (width 40cm, height 45cm; depth of recess 12cm; bottom edge of window 120cm above floor level; figure 4.1). Mosquitoes were released in one ‘release’ room (3.95x2.70, 2.33m high) and allowed to fly freely towards a human host in the second ‘bait’ room (4.77x2.70, 2.33m high). In the release room, a 1.22x1.05m retro-reflective screen (ritrama.com) supported by solid plastic backing was mounted 0.9m above floor level on a metal stand, 0.94m from the window frame. The release room was empty otherwise. In the bait room, a human volunteer sat on a stool 1.5m from the window frame, next to the table holding the camera, LED and PC. The wall in which the window was set was covered in retro-reflective screening on the bait room side (Figure 4.1[4]). The retro-reflective screen acted to reflect light back towards the LED, and consequently the adjacent camera. This returns more light to the camera, and provides a sharper shadow than the alternative filming background of an unmodified wall, which would scatter light in diffuse reflection. Both rooms were maintained at 27 ± 1.5$^0$C and 80 ± 8% RH. The humidifiers and air conditioners were turned off in both rooms during tests to avoid creating uneven gradients or air currents that potentially could have affected flight behaviour. Since visible light was minimised (see next section), the operator/human bait was sitting in a room in near total darkness.

### *4.2.3 Video tracking equipment and software*

The camera used was a DALSA Falcon 1.4M100 (1400x1024 pixel resolution, Stemmer Imaging, UK), with a 12.5mm lens (f1.4 aperture imaging lens (Kowa LM12HC 1”; Multipix UK). Recordings were made at 20 frames per second, using CVB Movie Interactive 2 (Common Vision Blox, Stemmer Imaging, UK). The set-up was illuminated using an infrared LED (850nm, RS components, UK), which was positioned directly above the camera lens. Light emitted from the PC was restricted by using the lowest brightness level, a black desktop background, and a purpose-built pyramidal screen cover that allowed the screen to be viewed through a 7cm by 15cm slot. The total area captured by the camera and lens was 0.93 x 0.53m.

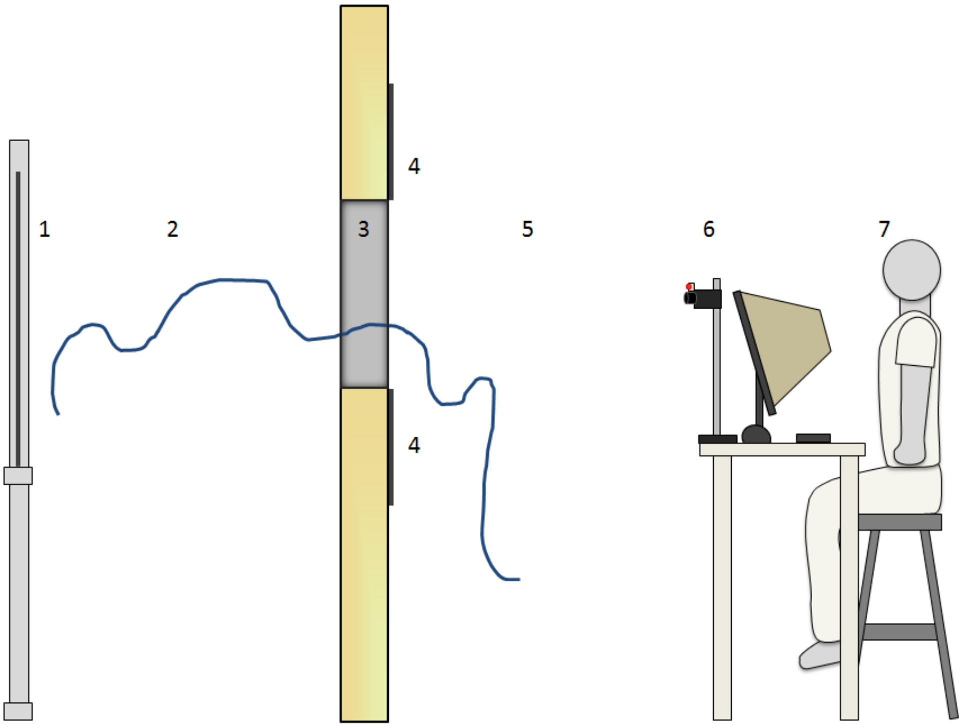


**Figure 4.1 Simplified diagram of experimental set-up.**
The retro-reflective screen (1) was placed 0.94m from the window in the release room (2). Mosquitoes released in this room were permitted to pass through the window frame (3) into the bait room (5). The front wall of the bait room was covered with retro-reflective screen (4). The camera, illuminated by an infrared LED (6) recorded flight, and the system was operated by the volunteer acting as bait (7).

At one hour prior to each test, the window was closed and 10 mosquitoes were freed into the 'release room', and allowed to acclimatise. After acclimatisation, recording was initiated, and the window was opened by the volunteer who sat in the bait room. Mosquito activity was recorded for 30 minutes. Mosquitoes were permitted to feed undisturbed on the volunteer. At the end of this period, mosquitoes were caught using a prokopack aspirator (Vazquez-Prokopec *et al.*, 2009), and the number that had blood-fed was recorded. The test was repeated 18 times, using a total of 180 mosquitoes. Tests took place within a 4 week period, in August to September 2013.

### *4.2.5 Calibrating the tracking system*

Calibration images were used to produce reference points that could be used by the tracking algorithm in calculating 3D positions of mosquitoes. To calibrate the recording volume, cameras recorded images of an A3 sheet of transparent acetate, marked with a grid of black dots of 7mm in diameter, spaced 24mm apart across the sheet (Figure 4.3). A single image of the sheet was recorded when it was attached to the release room side of the window frame recess, and a second image taken when it was attached at the bait room side (*i.e.* 12cm closer to the camera). These images were then analysed using a custom written Matlab application (Angarita-Jaimes *et al.*, 2016), that analysed the object-shadow distance of dots on the acetate sheet and the spacing of dots across the page, to calibrate the 3D volume of the window frame and the surrounding area.

The position of the LED next to the camera necessitated some correction to account for variation in the appearance of the mosquito's shadow when flying at different heights in the field of view. Such variation would not occur if the camera and LED were directly superimposed, however since the LED was positioned above the camera lens, the object-shadow distance changed according to object height: the object-shadow distance appeared greater at the base of the window frame than at the top (see Figure 4.3A). This introduced errors into calibration, and calculation of a mosquito's z coordinates. A number of corrections were considered to address this issue. The initial solution proposed was to employ an angular selective light filter that would have allowed the light source to be optically superimposed on the centre of the camera (D. Towers, *personal communication*). To implement this a dichroic beamsplitter was tested to improve alignment, superimposing the centres of the light and the camera, but as available devices were too small for the equipment used in this set-up, and obstructed the camera lens, this option was not considered feasible here. Instead this difference in shadow appearance across the image was corrected for at the calibration stage. Calibration images of a calibration sheet were used to identify the extent of variation in shadow-object distances across the image's y axis, and this data was then used to calculate accurate z coordinate outputs that accounted for variation in the conditions across the field of view.

Calibration images were taken each test day to ensure accuracy was not compromised by small movements of the camera's position.

### *4.2.6 Tracking mosquito positions and other data*

After the tests, videos were reviewed visually by the operator to identify frames in which mosquitoes were seen passing through the window aperture, and the x, y coordinates of entry position were recorded through tracking.  This was a two-stage process. Firstly mosquito positions were identified using software custom written in Matlab (Angarita-Jaimes *et al.*, 2016), which enabled identification of moving objects using the same image subtraction principles as those applied in the large scale tracking system (Chapter 2, section 2.2.3). Tracking was based on proximity of consecutive positions, using a 100 pixel search radius to link between points, and a minimum track length of 0.25 seconds. Following calibration in a separate application, the second stage of the tracking application used the pixel distance between a mosquito and its shadow (the object-shadow distance) to determine distance of the mosquito from the retro-reflective screen (RRS) in the release room, and assign 3D coordinates. Thus, when a mosquito flew close to the screen, its image was close to, or indistinguishable from, its shadow; conversely, when further from the screen, the distance between the mosquito and shadow was greater (Figure 4.1, and 4.2).

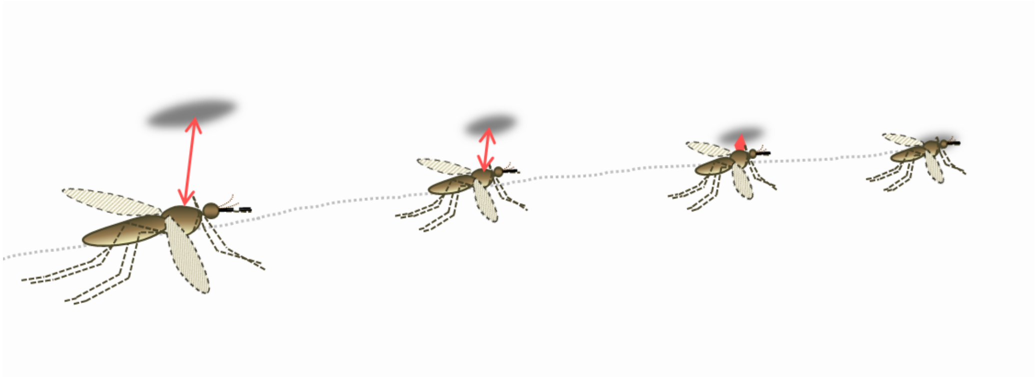


**Figure 4.2 Illustration of mosquito flying towards a retro-reflective screen, shown from the perspective of the front-facing camera**

In this image, the distance between a mosquito and its shadow (indicated by the red arrow) becomes shorter as the mosquito moves towards the screen. As the mosquito comes in to close proximity with the screen, its position becomes indistinguishable from its shadow. The 3D tracking process identifies the position of a mosquito and its shadow, in a single recorded frame, and uses the pixel distance between them to identify the mosquito’s z coordinates.

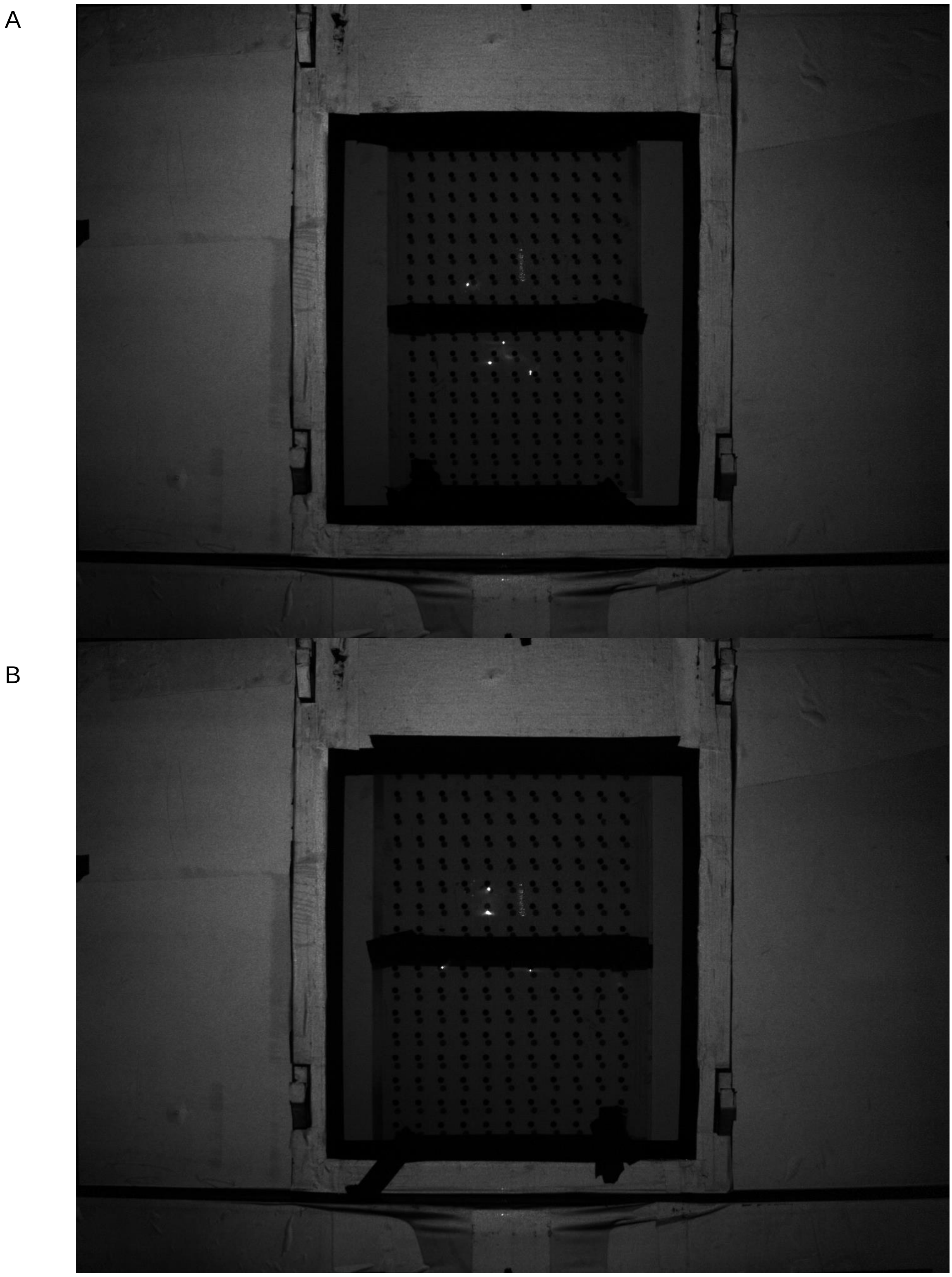


**Figure 4.4 Example calibration images from window recordings.**
The A3 acetate calibration sheet was positioned in the window frame in line with the release room wall (A) and then in line with the bait room wall (B). The window opening was 12cm deep and object-shadow distance was less when the object was closer to the retro-reflective screen in the release room (A). The dot grid pattern on the A3 calibration sheets was used to establish the object-shadow distance for objects at the back (A) and front (B) of the window frame. Using the pixel distance between an object and its shadow it was possible to determine whether that object was behind, within, or in front of the window. Due to placement of the LED above the camera, shadow-object distances varied across the y-axis of the image. This source of error was particularly visible when comparing dot-shadow spots at the top and bottom of the window in (A).

### *4.2.7 Data Analysis*

The study hypothesised that the flight paths of mosquitoes during approach, passage through and exit from the window (to the bait room) were randomly distributed in space. Quadrat and point dispersion analyses were used to assess distribution of activity during flight through the window, and investigate data for clustering.

Only tracks of mosquitoes that passed through the window and entered the bait room were included in analyses. Tracks that were seen to approach but failed to enter the window recess, or that performed a U-turn and exited the window from the bait room, were discarded as the objectives of the study were to use 3D tracking methods to observe house entry. Tracks used in analyses were grouped into three regional categories based on their z coordinates in space: activity occurring in the release room prior to window entry (*i.e.* during window approach), activity within the 12cm depth of the window recess itself, and final track point recorded in the bait room (*i.e.* following entry).

All images and representations of the window are shown from the viewpoint of the bait room, and all descriptions refer to the window from that position.

Activity prior to entry, and activity within the window recess were analysed to assess spatial homogeneity of flight activity. In order to do this, the area of the window frame was subdivided into nine quadrats. The outcome variables tested were time observed in flight prior to entry, and time observed in flight within the window frame space. The quadrant number activity occurred in was used as an explanatory factor in the model. No random effects were applied to the model as the nine quadrants were applied as categorical variables, and to add to additional random effects would risk overfitting the model. These data were analysed by unit of the 30 minute filmed test, rather than by individual mosquito, as it was not possible to distinguish whether tracks flying in and out of the field of view behind the window frame were generated by several mosquitoes, or one highly active individual. Analyses were conducted in SPSS Statistics version 21 (IBM) and values expressed as mean activity with 95% confidence intervals.

The final point coordinates of the mosquito tracks as exiting the field of view were analysed for clustering using Ripley's K function (Ripley, 1976; Baddeley & Turner, 2005), with edge correction to remove positions that were closer to the window boundary than to other positions (R Core Team, 2015; RStudio, 2015). This test, conducted using R statistical software (version 3.2.0) assesses distribution of points in space, and provides an indication of whether points are randomly distributed, or clustered. Since a negligible number of mosquitoes were observed returning from the bait room to the release room (on average 0.5 ± 0.9 tracks per test; see Results 4.3.1) and since each mosquito track entering the bait room was clearly identifiable as that of a distinct individual mosquito, these data were analysed as individual tracks. As clustering was found, a further quadrat analysis was conducted on this data. Here the filmed space was subdivided into three equally sized quadrats, and Pearson Chi-squared tests were used to assess the number of points found in different quadrats, using RStudio (R Core Team, 2015; RStudio, 2015; R Statistical Software version 3.2.0). Two such analyses were conducted, one dividing the space into vertical bars (figure 4.8C), and one into horizontal bars (figure 4.8C) to determine whether clustering was occurring in the x axis, y axis, or both.

## 4.3 Results

### *4.3.1 Performance of the simulated window entry insectary setup*

A total of 180 mosquitoes were released in 18 tests, from which 114 entry tracks were identified; equivalent to 6.3 tracks ± 2.8 (mean ± SD) per test. An average of 46.4% of the mosquitoes entering the bait room blood-fed on the volunteer (SD=39.4). After entering the bait room, mosquitoes rarely returned through the window to the release room; 0.5 ± 0.9 return tracks recorded per test).

### *4.3.2 Performance of the retro-reflective screen tracking system*

The retro-reflective tracking system was capable of detecting moving mosquitoes and tracking their flight, as well as generating the 3D coordinates of mosquitoes passing through the window. However, although this tracking worked well in the region of the window frame, in spatial regions closer to the RRS, the coordinates were less accurate. In the air space directly in front of the screen (0-45cm from screen surface in z plane), the z coordinates of mosquito tracks could not be distinguished because the object and shadow images were viewed by the camera as being a single point (Figure 4.2). The limits of z resolution were not tested, but it can be assumed that the z coordinates of mosquitoes with shadow-object distances of a few pixels may also be difficult to resolve, because the tracking software may not be capable of identifying overlapping shadow-object points. Considering the pixel size of a mosquito, a conservative estimate is that this limitation would be likely to have affected mosquitoes flying within 45-55cm of the screen. However, as the RRS was positioned 94cm from the window, movement through the window frame could be tracked in 3D successfully.

Shadows within the field of view imposed a further limitation on tracking. Due to poor reflection of light at the edges of the 12cm deep window recess, mosquitoes could not be detected as they traversed the inner window recess (Figure 4.4). Although this resulted in the loss of some information, errors in tracking events were partly avoided by selecting tracking parameters capable of bridging this distance, as demonstrated in figure 4.5A. Despite this, a portion (27%) of tracks were broken (Figure 4.5B).

Erroneous linking of shadow and object tracks sometimes occurred as tracks exited the window into the bait room (Figure 4.5). However this did not affect the data and, so was not corrected for. Nonetheless, all tracks were verified manually in order to identify the position of the final flight point of the mosquito entering the bait room.

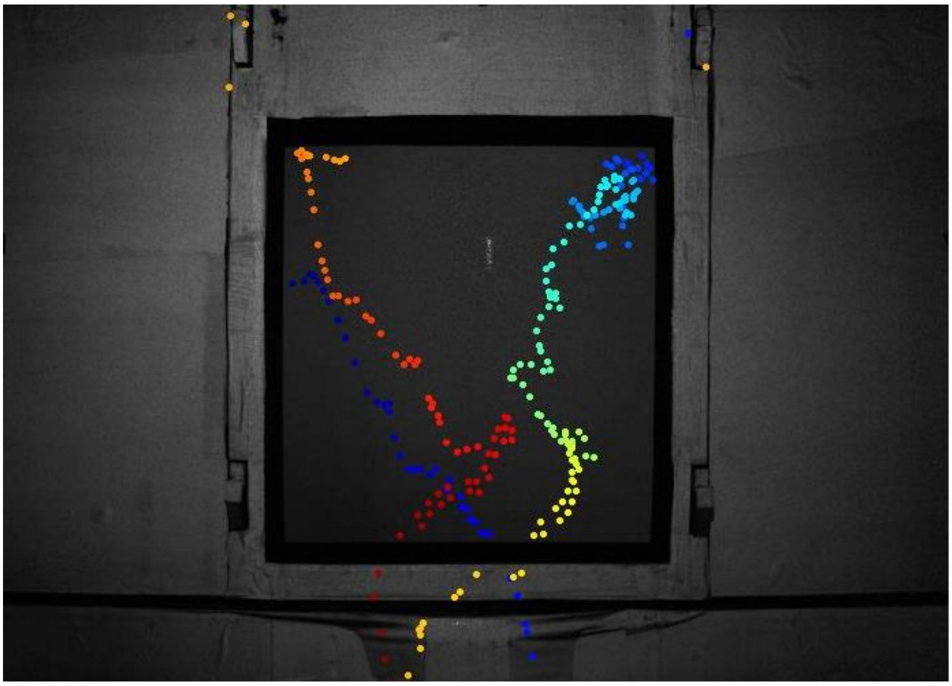


**Figure 4.5 Tracks of *An. gambiae s.s*. during passage through a window from a release room into a room with human bait.**

Recordings were made at 20 fps, and consecutive points in each track show the mosquito's movement at 0.05 second intervals. Position colour indicates time recorded; points of similar colour occurred simultaneously. Colour scale is blue-red (blue points occurring first, going to red at end of recording).

As mosquitoes moved closer towards the camera, the distance between the mosquito and shadow increased and allows resolution of both object and shadow separately. In this image, single position tracks descending through the window split in to paired position points (object and shadow) as mosquitoes approach the window and enter the bait room.In this track image, this forwards movement coincides with descent of tracks.

Mosquitoes were not visible when crossing the darkest space within the window recess, resulting in the gaps in the tracks seen in this image.

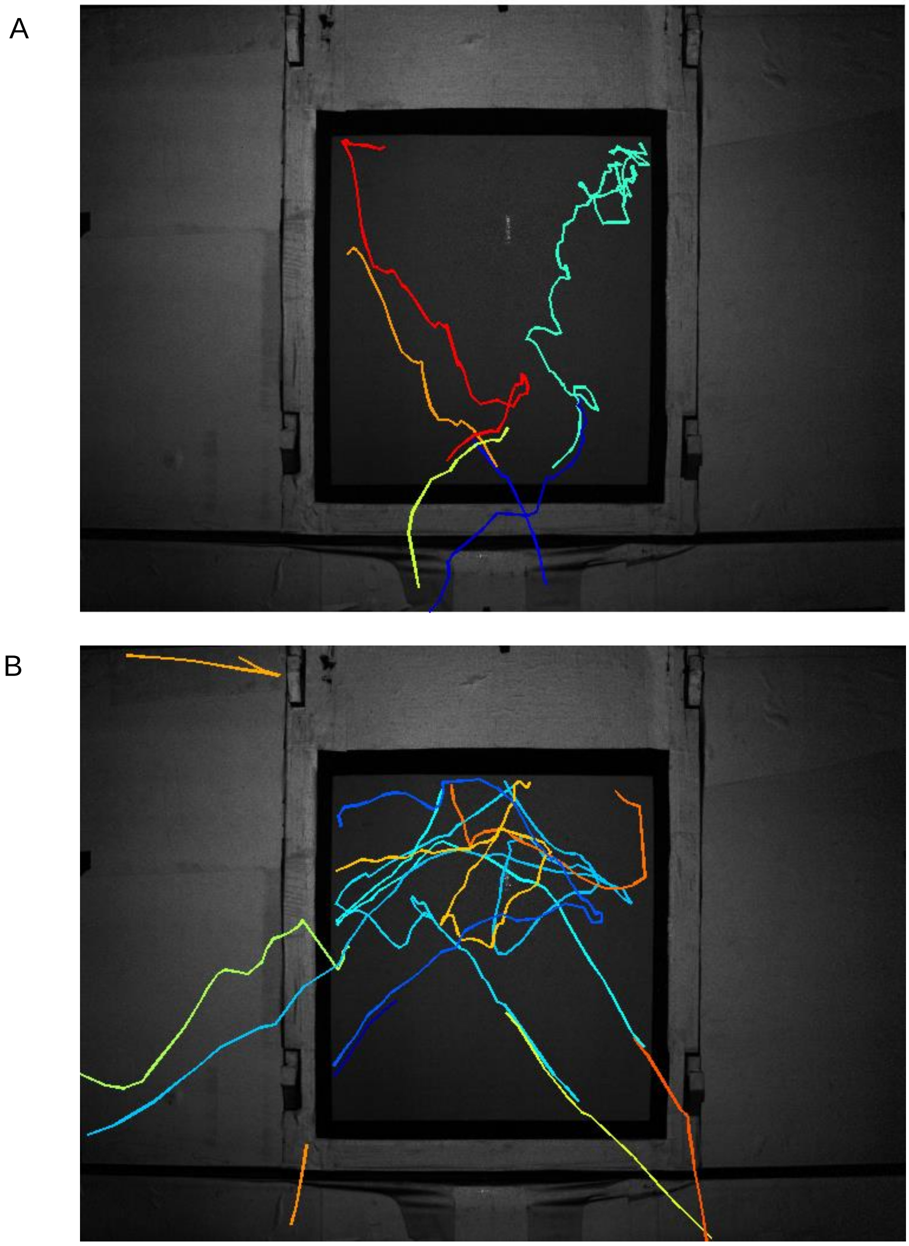


**Figure 4.6 Tracks of *An. gambiae s.s*. during passage through a window from a release room into a room with human bait.**

Each coloured line shows the track of a mosquito, or of its shadow, generated by the retro-reflective screen (RRS). Tracks are created by linking sequential positions, identified by segmentation in an automatic tracking process. Mosquitoes appear as single tracks when close to the RRS (located in the mosquito release room on the wall facing the window and camera, or on the wall of the bait room, facing the camera). When the mosquito is further from the RRS, the shadow is detected separately, and mosquitoes appear as two adjacent tracks of different colours.

In figure (A) all flight trajectories are successfully tracked from start to finish. In figure (B) the shadowed region within the window recess has led to a ‘false’ track break in one trajectory entering the bait room (dark blue and orange tracks, bottom left of window frame). Tracking also could erroneously link the shadow track within the window recess to the object (mosquito) track entering the room. This is visible in tracks here: *e.g.* (figure A) red-yellow track and (figure B) blue-yellow tracks.

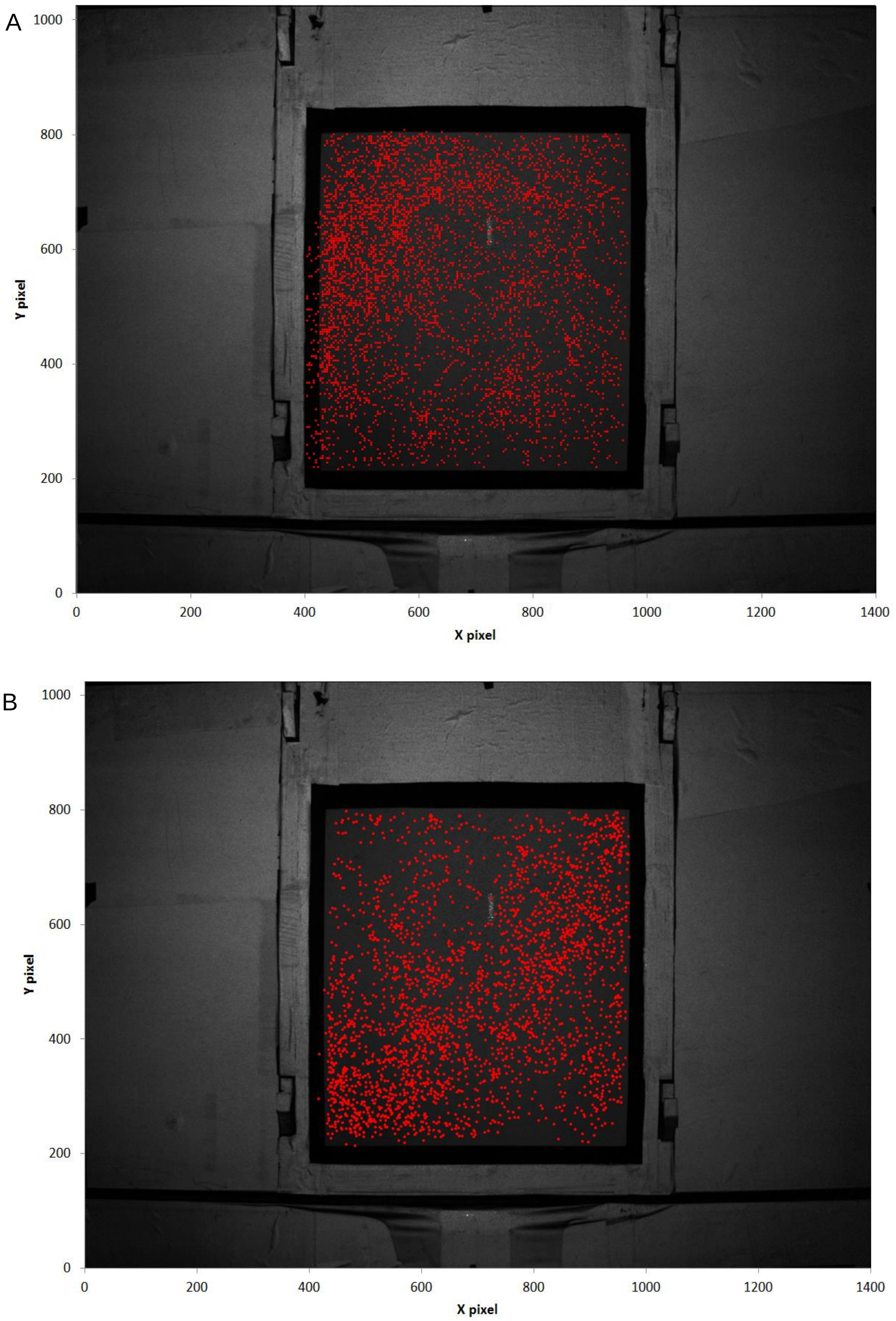


**Figure 4.7 Spatial aspects of window entry by host seeking *An. gambiae s.s.***

A) Track positions of *An. gambiae s.s.* immediately prior to entering the window frame (*i.e.* arrival points at the window); includes all points on all tracks that were recorded in the release room in all 18 tests. B) Track positions of *An. gambiae s.s*. during activity within the window frame (*i.e.* flight within the 12cm depth of the window recess); includes all positions recorded in this location in all 18 tests.

### *4.3.4 Flight behaviour of Anopheles gambiae s.s. during window entry*

#### Approaching the window prior to entry

Positions of flight tracks within the release room prior to entering the window recess are shown in figure 4.6A. These positions represent all recorded movement of tracks that were classified by the 3D tracking software as having occurred in the release room, prior to reaching the window. This movement is referred to as approach activity.

Analysis of approach activity by quadrat showed that positions were not randomly distributed throughout the available area. In fact, significantly more mosquitoes approached the window from the upper left hand side of the release room ($X^2$=21.2, d.f.=8, *p*=0.007) and activity was highest in regions 1, 2 and 4, where mean (SD) flight times per test of 1.9s (± 0.7), 1.5s (± 0.6) and 2.6s (±1.4) respectively were recorded in each test (Figure 4.7A).

#### Passage Through the Window Recess

Positions of flight tracks within the space contained within 12cm deep window recess are shown in figure 4.6B. These positions represent all movement of tracks within the window recess, as identified by 3D tracking software, and are referred to as window entry activity.  Analysis of activity by quadrat showed a heterogeneous distribution of movement as mosquitoes crossed the window recess ($X^2$=49.8, d.f.=8, *p*<0.001). Highest levels of activity were recorded in regions 5, 6 and 7 (Figure 4.7B), where individual mosquitoes spent an average of 1.2s (± 0.5), 1.2s (± 0.4) and 1.4s (± 0.6) of time respectively (means ± SD).

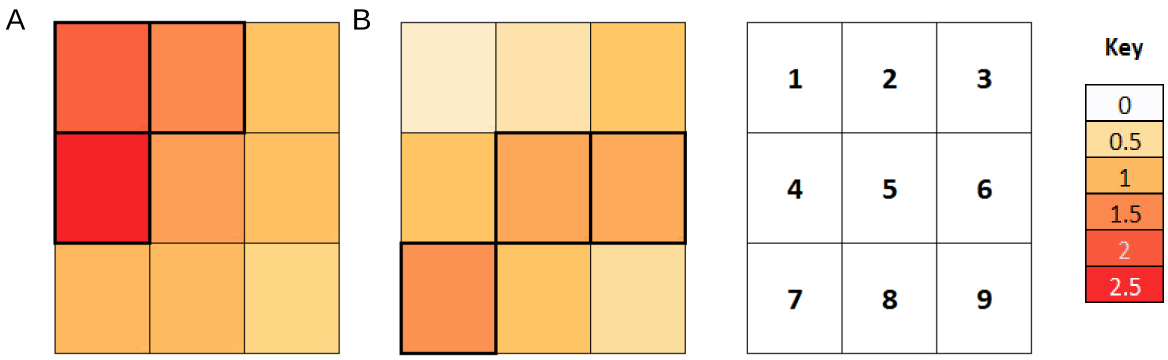


**Figure 4.8 Heat maps of flight activity in and behind test room window.**
Heat maps show the mean levels of flight activity in each of 9 quadrats of the window as viewed from the camera: A) flight activity in the release room prior to arrival at the window; B) flight activity within the window recess. The colour scale represents the mean duration of individual mosquito flight tracks in each quadrat section per test (seconds). Double thickness lines denote quadrats where activity was significantly higher than others (*p*<0.05).

### Exiting the window

The direction of mosquito flight following passage through the window and entering the bait room was investigated by quantification of the level of dispersal or scatter into the space within the bait room. The position of the final point on each flight track (as recorded in the camera's field of view) of every mosquito that entered the bait room was test for aggregation using Ripley's K function analysis (Figure 4.8A). The analysis indicated high levels of aggregation (Figure 4.8B). In this plot the green envelope indicates the simulated line of spatially random positions *i.e.* the expected distribution if the exit points showed no spatial patterns or preferences. Clearly, the plotted line of L(t) against distance falls outside of this envelope, running above the boundaries of this random simulation, suggesting strong clustering of points. The quadrat count analysis showed that points were distributed unevenly in both the vertical and horizontal axes, as the majority of mosquitoes exited the filmed area through the lower left of the field of view ($X^2$=180.35, d.f.=2, *p*<0.001, $X^2$=14.42, d.f.=2, *p*=0.001 respectively, Figures 4.8C, D).

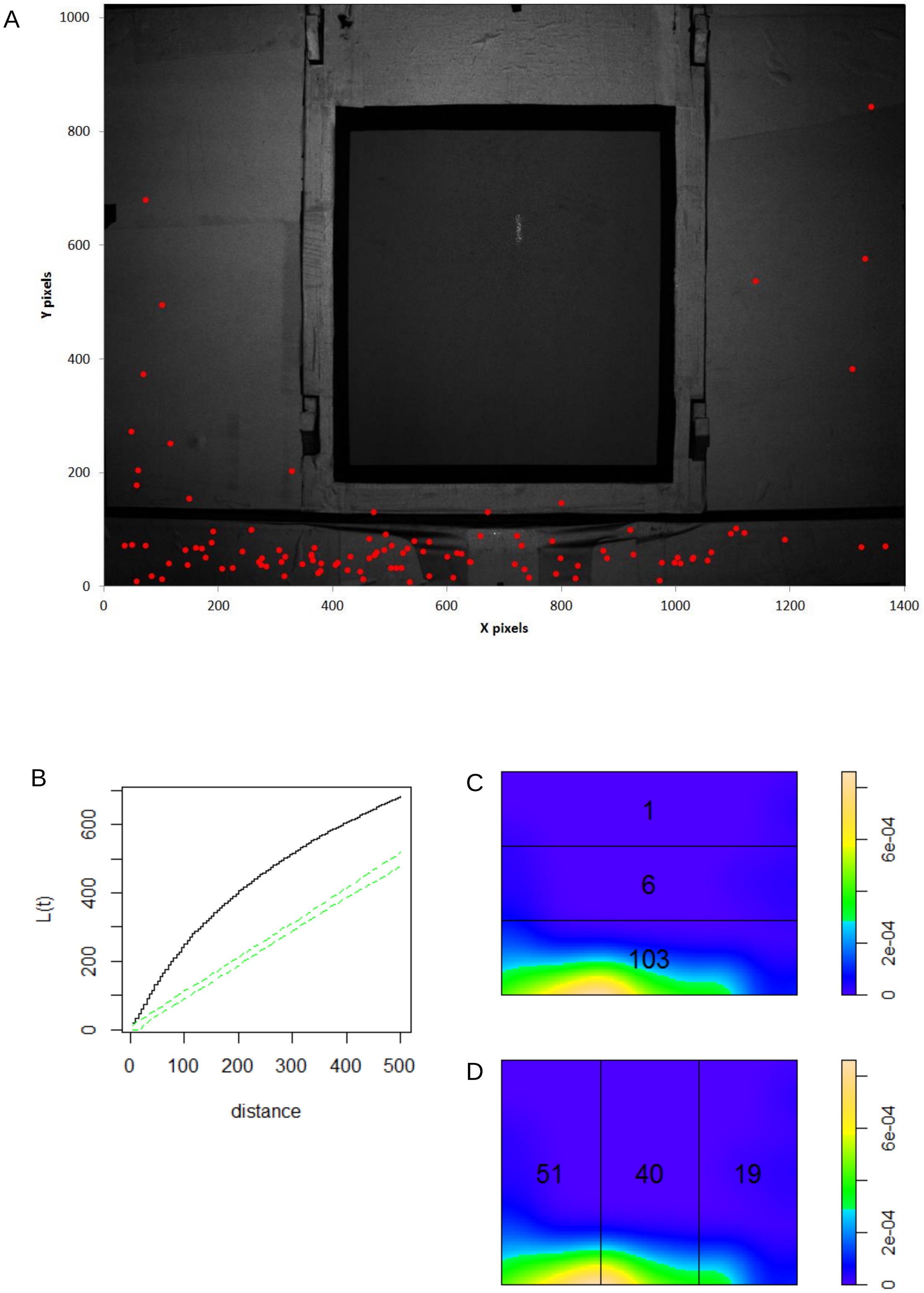


**Figure 4.9 Dispersal of *An. gambiae s.s* from a window on entry into a room with human bait**
A) The position of the final point (as recorded by the camera) on each flight track of every mosquito that entered the bait room. B) Ripley's K function plot of points: green lines show the envelope of a simulated line of complete spatial randomness. C-D) Density plot of the final exit points across the entire camera field of view, with quadrat count numbers for vertical (C) and horizontal (D) point distribution. Yellow-white shows highest density, and lowest density is shown in violet.

## 4.4 Discussion

House entering and exit behaviour is a key activity of endophagic and endophilic mosquitoes that has potential to be exploited for vector control. First however, a better understanding of all elements of passage into a human habitation is required. This set of experiments set out to characterise the spatial flight paths of *An. gambiae s.s.* during orientation to a host through an open window.

Results indicated that flight trajectories of mosquitoes approaching a window were biased towards particular areas during approach, passage through the window, and exit. Approaching the window, mosquitoes entered through the upper region of the window area. They appeared to descend as the flight paths continued through the window at lower levels, closer to the base of the frame, before exiting by flying downwards out of the camera's field of view.

Previous studies in the field reported that *An. gambiae* flew at heights of under 1m (Snow, 1979), when outdoors. In the present study, the window opening was positioned at 1.2-1.65m above ground level. If the tested mosquitoes began flying from a similarly low elevation, they would have had to rise to pass through the window, although potentially flight could have started from higher locations on the walls in the release room. Regardless of entry considerations, the results indicated that after passing through the window, mosquitoes descended to lower flight elevations.

Low flight elevation might be attributed to the mosquito's use of optomotor flight control, whereby insects use visual information on the rate of movement of their surroundings to control their trajectory and speed (Kennedy, 1939, 1951; Gibson, 1995). Several diurnal and nocturnal insect species, including *Anopheles gambiae*, are thought to use optomotor flight control in navigation (Kennedy, 1939; Gibson, 1995; Warrant & Dacke, 2011). It has been suggested that an insect's flight speed and elevation are coordinated to keep their surroundings moving at their 'preferred' retinal velocity, which for mosquitoes entails flight close to the ground (Kennedy, 1939; Kuenen & Baker, 1982; Franceshini *et al.*, 2007). Preferred or optimal flight height is also likely to be influenced by air currents: ultimately, low flight heights may be preferred as air at this elevation moves more slowly and mosquitoes would be less likely to be affected by wind interference. Insects tend also to fly within the boundary layer (*i.e.* the air space close to the ground in which wind speed is slower), at heights at which their mean flight velocity exceeds air speed (Kennedy, 1951; Taylor, 1974). At lower flight elevations odour plumes may also be more easily tracked, as odour plumes are less disrupted by wind (Cooperband & Cardé, 2006b). In the present study, the air conditioning was inactivated during the test period to avoid unequal air movements in the different rooms and within rooms. As the air would have been relatively static throughout the airspace of the tests, it can be assumed that mosquito flight elevation was likely determined by optomotor flight control with no correction for wind.

### *4.4.1 Navigation Through the Window*

Activity within the window frame was approximately evenly dispersed across the x-axis. If mosquitoes were showing a preference for the centre of the window a strong bias in activity towards the central quadrat would have been expected, but this was not observed. Neither was there an 'edge-clinging' effect, which would have seen higher activity in the quadrats on the sides of the window.

In other insects, flight routes and navigation through or around obstacles is often visually controlled. Tests with bees have used flight tunnels with moving walls to investigate the impact of changing visual cues on flight paths. Results of those studies showed that bees navigated corridors using 'optic flow balance', *i.e.* centring their flight path so that the walls of the corridor appear to have equal angular speeds (Kirchner & Srinivasan, 1989; Srinivasan *et al.*, 1991). Tests with stationary tunnels ranging in width from 12-95cm found that *Apis mellifera* honey bees centred their flight down the midline of a corridor when flying towards a reward (Kirchner & Srinivasan, 1989; Serres *et al.*, 2008). In the present study, the 'corridor' of the window frame, at 12cm long, may have been too short for the mosquitoes to exhibit such a response. Nonetheless, at subsequent sections of flight paths through the window, mosquitoes showed some evidence of central-biased flight. Movement both before and after entry was biased towards the left side of the field of view. If flights through the window had conformed to this bias, one would have expected to see trajectories predominantly at the left side of the window frame. However activity was evenly spread in the x-axis, indicating that mosquitoes corrected this skew whilst moving through the frame. Potentially, the 'corridor' of the window frame may have been too short for full centring behaviour to occur, but as data suggest, mosquitoes may have adjusted their flight paths to move away from the frame edge when passing through the opening. As air movements within the rooms could not be measured, there is no way to distinguish whether this behaviour was a response to visual cues from the window frame, or a plume-following behaviour as the mosquito followed air currents or airborne attractive cues towards the host. Moreover, though the dimensions of the window are not dissimilar from domestic windows found worldwide, the artificial nature of the experimental setup, and its possible influence on air currents, temperature and humidity gradients, might also account for observed flight behaviour.

Eyes of the nocturnal *An. gambiae* are adapted to low lighting, with conical rhabdomeres that allow a wider acceptance angle for incident light (Land *et al.*, 1997). Eyes comprise wide fused rhabdoms, and larger interommatidial angles, with poor visual resolution but much greater sensitivity than day adapted eyes of other mosquito species (Land *et al.*, 1999). Since *An. gambiae* responds to dim levels of visible light ($1x10^{-5}$ W/$m^2$, slightly more than starlight [Gibson, 1995]), it is possible that the dim level of light emitted by the shielded computer screen in the bait room provided sufficient illumination for the mosquitoes approaching the window to detect it visually.

However, this low level of background or ambient lighting is not likely to have been a serious confounder, or to have compromised the validity, of the test system used here. In the tests being reported, infrared light was used to illuminate the room as it is invisible to mosquitoes (Gibson, 1995), whilst visible light was avoided as it can inhibit host-seeking behaviour (Jones *et al.*, 1972; Sheppard, 2014), typically with bright light (70 lux - equivalent to an artificially illuminated room in a house). In natural or field settings, mosquitoes will have to fly under illumination of the stars and moon, which can reach up to 0.2 lux in brightness (Bowden, 1973), or in human settlements where fires or artificial lighting can often be widely used. Investigations into effects of moonlight mosquito activity have given conflicting results, with some studies finding higher numbers of anophelines when the moon is full, and others catching more during the new moon (Bidlingmayer, 1985; Guimarães *et al.*, 2000; Kampango *et al.*, 2011). Hence, it might be argued that low levels of light as emitted by the dimmed covered PC screen in an otherwise unlit room would not be expected to compromise natural host seeking behaviour of the mosquitoes.

Failure to centre when passing through a window does not exclude the option that *An. gambiae* navigate entrances using visual cues. Visual cues are important to the navigation of nocturnal moths along flight tunnels towards pheromone sources, although the symmetry of such cues may have little role in navigation. Vickers & Baker (1994) suggested that *Heliothis virescens* moths navigated primarily using cues related to pheromone plume concentration rather than visual cues, reasoning that in the field, insects must navigate through a highly asymmetric environment of irregularly spaced vegetation to locate a pheromone source, and that centring flight would be less helpful to navigation than directly following the plume. For insects that locate targets using odour plumes, such as moths and mosquitoes, visual cues may play a secondary role in determining flight paths.

In-room air movement and the odour plume of the volunteer may also have played a part in guiding mosquito movement. *Culex quinquefasciatus* are capable of rapidly locating a source of host odour over distances of 1.2m in still air (Lacey & Cardé, 2012), thus in the present study it is possible that mosquito flight was guided by odour cues. Dispersal of the volunteer's odour plume across the two test rooms could also have influenced the spatial patterns of activity observed here.

### *4.4.2 Implications for vector control*

Results indicated non-random patterns of flight within the window; flight tracks were not equally distributed across the wind space, as some quadrats of the filming area contained 4-5 times the flight activity of less preferred quadrats. However only one navigation scenario was tested (a single open window between two similarly sized rooms). Field evidence suggests that mosquito house entry paths are flexible, and can change according to local conditions, house construction, and accessibility of the window (Njie *et al.*, 2009; Diabaté *et al.*, 2013; Wanzirah & Tusting *et al.*, 2015). As such it may be impossible to discover 'true' routes of mosquito entry, as these will vary in different circumstances. It might be more useful to focus on how mosquitoes locate an opening in a house, both for house entrance and exit, and how they respond to barriers and baffles obstructing their path.

House screening is a useful method for mosquito control: the risk of malaria infection remains highest indoors at night, and screens can prevent mosquitoes from entering the home (Lindsay *et al.*, 2002; Bayoh *et al.*, 2014). Tests with *Aedes aegypti* using the same experimental rooms, setup and recording system as those used in the present study, found that 75% coverage of a window opening with a screen barrier could reduce room entry rates by over 20%, and increasing screen coverage to 90% reduced entry rates by over 50% (Riesen, 2014). Riesen's analysis of *Ae. aegypti* flight paths showed that the majority approached the window frame through the upper regions, as seen with *An. gambiae* in the present study, though activity following entry did not show a descent in elevation after entering the bait room like that seen here with *An. gambiae*. Though specific tests with *An. gambiae* will be needed, the similarities in routes during window passage, together with results from field collection studies suggest that even partial screening of windows with damaged netting will impact on entry rates (Lwetoijera *et al.*, 2013).

Riesen (2014) noted that in tests of net screens across windows, mosquitoes made contact with the screen barrier as they attempted to navigate through it, implying a trial-and-error method in locating the gap. This type of 'pinball' entry through holes was also noted in Sutcliffe & Colborn (2015) and it could be suggested that this behaviour resulted from responses to the odour plume permeating or leaking through the entire net screen barriers, and that behaviour around a hole in a solid wall would differ. However observations of *Anopheles vestipennis* house entry reported that mosquitoes searching for openings in walls made a number of short flights interspersed with brief wall contacts prior to successfully locating and entering a house opening (Grieco *et al.*, 2000). Insecticide treatment of fence material has been shown to reduce the number of culicine mosquitoes successfully passing through or over net barriers, likely as a result of toxic or contact irritant properties of insecticide experienced during such brief exploratory contacts (Maia *et al.*, 2012). Clearly, further research of how mosquitoes locate window openings, and holes in screens and bed nets, is potentially very useful, providing information about the function of existing interventions, and how these vulnerabilities might be made less attractive to mosquitoes.

### *4.4.3 Study Limitations*

An important limitation of the study is the artificial nature of the laboratory setup where the lack of air movement in the artificial release or 'outdoors' room, and the similarity in conditions between both 'indoors' and 'outdoors' would have differed from a natural situation. In the field, mosquitoes move upwind towards a bait, tending to enter houses from the downwind side (Bertram & McGregor, 1956), but in the present study, mosquitoes were released in to a closed room with no air currents, preventing normal upwind navigation. Despite its convenience and advantages, using two 'indoor' settings may not be a good simulation for mosquito flight into a house from outdoors. Moreover, results from a study on *Ae. aegypti* carried out in the same two-room setup indicated that window entry positions could differ depending on which room was used as the release room, an effect that was caused by unknown variations in conditions within each room (Riesen, 2014)

No tests were performed in the absence of a volunteer, and therefore room entrance may have been motivated by something other than the attraction to host cues, such as taxis towards the light (albeit very low levels), an escape response from unfavourable or sub-optimal conditions. However, the fact that 46.4% of insects entering the bait room proceeded to blood-feed on the volunteer, indicates that approximately half of the mosquitoes were actively host seeking. House entry behaviour can also be exhibited by non-host seeking insects; *e.g.* approximately 5%

of *An. gambiae s.l.* caught in window entry traps in Burkina Faso were gravid (Diabaté *et al.*, 2013). Further experiments without a volunteer would be important to explore this.

### 4.4.4 Notes on the performance of 3D tracking system

To our knowledge, this is the first time the retro-reflective screen (RRS) method for single camera 3D imaging has been used in the tracking of nocturnal insects, using artificial light sources. In general terms, this RRS technique worked satisfactorily in this context, and proved a useful non-invasive method with sufficiently high resolution for observing mosquito house entry, with the potential to deliver valuable results.

This system was calibrated, and tracking software written such that the z-axis 3D coordinates were only pertinent to the movement of the insect through the window and did not apply to mosquitoes flying within the bait room; therefore, the volume of the field of view used for 3D tracking was approximately 0.4 x 0.45 x 2.2m (width x height x depth). The depth of field in which accurate z-coordinates could be obtained would be less than 2.2m however, because at distances very close to the RRS (*i.e.* within 45cm of the screen), the mosquito and its shadow could not be distinguished as separate points. Even with this caveat however, the volume is close to dimensions that can be imaged in wind tunnels using stereoscopic 3D tracking in infrared, and that operate currently with a filming volume of 0.6x0.6x0.6m (Spitzen *et al.*, 2013). Stereoscopic systems tracking swarming insects in the ambient light of dusk have reached volumes of over 1 x 1 x 1m (Butail *et al.*, 2012), but can only record for periods of 90 seconds. The use of a single camera in the RRS adds value to the system used here in that equipment may be cheaper, the need to synchronise capture in two cameras is eliminated, video storage requirements are halved and processing times are expected to be substantially lower than a double camera system (Manoukis *et al.*, 2014).

The RRS system has not been validated yet for tracking error, or assessed for spatial resolution, which is expected to vary with z-axis distance from the RRS (D. Towers, *personal communication*). However these experiments provide a proof of principle for the tracking system’s capabilities. There is potential to modify the test setup so that the entire field of view can be tracked in three dimensions. This could be achieved using a test arena with a plain flat background covered with a retro-reflective screen, rather than more complex scenarios using windows or eave gaps.

In this respect, some improvements to the optical set-up of the tracking system are needed. The dark edges of the window frame presented a tracking problem, as mosquito positions could not be observed in this area. As a result, some insect tracks were broken or potentially would be assigned erroneous 3D coordinates, as their position or the position of their paired shadow was lost as they crossed the dark edge of the window frame (Figure 4.5B). Using Fresnel lenses to collimate the LED light would be expected to narrow the appearance of the dark edges of the window frame, and reduce these errors (see Chapters 5 and 6). The paired Fresnel lens system was deliberately not used in the window tests to avoid placing an additional barrier in between the bait and the entering mosquito. Although one potential barrier existed, *i.e.* the large RRS covered board positioned less than 1m from the window, a previous study with *Ae. aegypti* experimented with the placement of the board and found no evidence of it exerting any influence on mosquito flight paths (Riesen, 2014).

Reducing the field of view by using a camera lens with a longer focal length, or moving the camera and LED closer to the RRS would improve the resolution of the tracks’ z-coordinates; by placing the same camera closer to the RRS, shadows and objects would occupy more pixels, increasing the shadow-object distance for objects near the camera. This would enable more accurate measurement of a mosquito’s coordinates. As mentioned previously (section 4.2.5), the variable shadow displacement caused by the position of the LED above the camera lens could be addressed either using a dichroic beamsplitter or fibre optic cable, both devices which would bring the position of the light source closer to the centre of the camera lens. An improved RRS system using Fresnel lenses to achieve an estimated spatial resolution of 20mm is currently under development by the same team (D. Towers, *personal communication*). The camera configuration used in this set-up was chosen to allow recording of mosquito movement within the bait room, but for a project with different recording goals, the 3D resolution of the system could be improved. Single camera 3D tracking is a promising technique that offers a faster, less expensive method of recording flight of nocturnal insects (Manoukis *et al.*, 2014), and will be a useful new tool in behavioural studies.

### *4.4.5 Summary*

Results from an experimental system indicated that host seeking *An. gambiae s.s.* fly through window openings via non-random paths, typically approaching from higher elevations before descending in height as they pass through the window into the room. How fixed these preferences are has yet to be tested under more natural conditions, where house entry may be more likely to be influenced by house structure than by innate behaviours (Njie *et al.*, 2009; Diabaté *et al.*, 2013; Wanzirah & Tusting *et al.*, 2015). The 3D tracking system developed for this type of investigation and tested in the present study, performed satisfactorily and demonstrated its considerable potential to investigate these entry paths further, under natural conditions in the field.

# Chapter 5 Characterising Flight Behaviour of *Anopheles gambiae s.s.* around Bed Nets in Laboratory Settings

## Abstract

Long-lasting insecticidal bed nets (LLINs) protect humans from malaria transmission and are fundamental to malaria control worldwide, but little is known of how mosquitoes interact with nets. Elucidating LLIN mode of action is essential to maintain or improve efficacy, an urgent need as emerging insecticide resistance threatens their future. Tracking multiple free-flying *Anopheles gambiae* responding to human-occupied bed nets in a novel large-scale system, and key behaviours and events were characterised. Four behavioural modes with different levels of net contact were defined: swooping, visiting, bouncing and resting. Approximately 75% of all activity occurred at the bed net roof where multiple brief contacts were focussed above the occupant's torso. Total flight and net contact times were lower at LLINs than untreated nets but the essential character of the response was unaltered. LLINs did not repel mosquitoes but impacted rapidly: LLIN contact of less than 1 minute per mosquito during the first ten minutes reduced subsequent activity; after thirty minutes, activity at LLINs was negligible. Velocity measurements showed that mosquitoes detected nets, including unbaited untreated nets, prior to contact. This is the most complete characterisation of mosquito-LLIN interactions to date, and reveals many aspects of LLIN mode of action, important for developing the next generation of LLINs.

## 5.1 Introduction

Many of the important mosquito vectors of malaria feed indoors at night, where and when most human malaria is transmitted in Africa (Huho *et al.*, 2013; Bayoh *et al.*, 2014). Long-lasting insecticidal bed nets (LLINs) exploit this behaviour and are one of the most effective methods for reducing malaria transmission, fundamental to malaria control (spending on malaria control amounting to $2.5bn in 2012) and to ambitious plans for its elimination (WHO, 2014; The Roll Back Malaria Partnership, 2008). Recent analysis suggests that mass distribution of LLINs is the principle factor driving decreases in *P. falciparum* cases in Africa since 2000 (Bhatt *et al.*, 2015). However, the future of LLINs is seriously threatened by emerging resistance in vector populations to pyrethroids, the only insecticide class that can be used with LLINs (Strode *et al.*, 2014; Toé *et al.*, 2014) and the need for novel LLIN designs that enable safe use of other insecticides or entirely new control devices or strategies is a global health priority (The Roll Back Malaria Partnership, 2008).

Delivering the 'next generation' of LLINs or similar tools will require a thorough understanding of how LLINs function, yet remarkably little is known of the mode of action or of precisely how mosquitoes behave at the LLIN interface. Recent studies using 'sticky-nets' reported that host-seeking female *Anopheles spp.* landed preferentially on the top surface of bed nets (Lynd & McCall, 2013; Sutcliffe & Yin, 2014) but that lethal capture method recorded only a single landing event and no other behaviours before or after. Although clustering at the net roof is thought to be a response to an attractant convective 'plume' rising from the human beneath (Sutcliffe & Yin, 2014), this too remains speculative because knowledge of mosquito flight behaviour prior to blood-feeding and of the identity and location of the key attractants that mediate the host-seeking response is limited (Cardé *et al.*, 2010; Okumu *et al.*, 2010b; Spitzen *et al.*, 2013; McMeniman *et al.*, 2014). Host seeking behaviour appears to rely on a range of attractive cues acting over different distances, which induce tortuous and persistent flights towards their source (Siegert *et al.*, 2009; Lacey & Cardé, 2011; Spitzen *et al.*, 2013; Webster *et al.*, 2015). Importantly, how insecticide treatments influence that response is unclear.

Some studies reported that insecticide residues repelled mosquitoes prior to contact (Achee *et al.*, 2009; Chareonviriyaphap *et al.*, 2013), which would reduce or eliminate the chance of mosquitoes contacting an LLIN and receiving an effective dose, and potentially divert them to unprotected hosts (Killeen *et al.*, 2011). Others found no evidence for such repellency (Lindsay *et al.*, 1991; Mathenge *et al.*, 2001; Kirby *et al.*, 2008; Cooperband *et al.*, 2009; Spitzen *et al.*, 2014) indicating that LLINs attract and impact on mosquitoes by direct contact.

A further complication is the existence of what is termed 'contact-irritancy' or 'excito-repellency', whereby brief exposure to an insecticide can result in mosquitoes exhibiting avoidance behaviour, potentially before a lethal dose has been delivered (Kennedy, 1947; Achee *et al.*, 2009). Remarkably, some basic details are missing: *e.g.* the minimum duration of LLIN contact necessary to deliver an effective dosage is not known. Despite these phenomena being recognised for decades (Kennedy, 1947; Muirhead-Thomson, 1960; Roberts & Andre, 1994), when and how they occur and their relative importance in selecting for insecticide resistance have never been fully elucidated. Beyond this, basic details of host-seeking behaviour of mosquitoes remain unknown. How mosquitoes detect net surfaces, and persistence of attack at a barrier have not been investigated, yet play an important role in net function.

Consequently, behavioural resistance to insecticides remains poorly understood and rarely reported in mosquitoes, though the risk of vector populations switching blood-feeding times, locations or host preferences in order to avoid LLINs is recognized and closely monitored today (Russell *et al.*, 2011; Briët *et al.*, 2013; Govella *et al.*, 2013). Additional but less apparent or detectable behavioural changes might also exist, conferring partial or complete insecticide resistance (*e.g.* changes in sensitivity to repellents, attractants, or modified flight or resting behaviours). In the absence of definitions or quantifications of the basic behavioural events likely to be affected (Rivero *et al.*, 2010; Gatton *et al.*, 2013), these changes cannot be investigated, let alone monitored.

Ideally, characterisation of mosquito behaviour requires direct observation under conditions that are as ‘natural’ as possible. Informative studies to date have been limited to wind-tunnel or small-scale laboratory tests, potentially restricting mosquito flight. Frequently, tests use artificial or incomplete attractants such as human breath or limited body parts, carbon dioxide, single attractant chemicals or simple odour blends (Dekker *et al.*, 2011; Spitzen *et al.*, 2013; Spitzen *et al.*, 2014), rather than an entire human host. Experimental huts (Ferguson *et al.*, 2008; Okumu *et al.*, 2010b; Ogoma *et al.*, 2014b), electrocution grids (Torr *et al.*, 2008; Majambere *et al.*, 2013), taxis boxes (Lorenz *et al.*, 2013) and other methods overcome some of these obstacles but are unsuitable for detailed exploration of behavioural sequences.

Addressing many of the technical challenges that hindered progress to date, I have developed and constructed a novel system that enables tracking, recording and analysis of the flight paths of multiple individual mosquitoes over long periods in the dark at large volumes around the entire human host.

The aim of this study was to use a novel large scale tracking system to observe mosquito behaviour around bednets. The study objectives were to use flight parameters of mosquitoes to assess effects of insecticide on mosquito approach to, contact with, and host seeking behaviour at an LLIN. The study hypothesised that the presence of a human bait sleeping under the bednet would increase mosquito activity at the bed net. This study further hypothesised that behavioural impacts of insecticide would reduce mosquito host seeking at LLINs, leading to measurable differences in flight activity around nets.

## 5.2 Methods

### 5.2.1 Mosquitoes & Insectary Environment

Tests used three to five day old unfed adult female (25 per experiment) *An. gambiae s.s.* “Kisumu” strain, reared at the Liverpool School of Tropical Medicine (LSTM). Mosquitoes were starved of sugar and water for 4-6 hours, and introduced into the experimental room at least 1 hour before testing. Mosquitoes were selected for testing by placing an arm against the cage and using an aspirator to collect insects that attempted to feed. All tests were conducted within 1-5 hours of the end of scotophase.

### 5.2.2 Room Set-Up

Tests were conducted in a dedicated insectary at the LSTM (5.6m x 3.6m in area 2.3m high; climate controlled at 27±2˚C, 70±10% Relative Humidity). The room contained a bed, covered by a bed net, surrounded on its long axis by pairs of Fresnel lenses (see Figure 5.1). The complete filming system captured an area of 1.2x2.4m, and was illuminated using infrared light.

The mosquito release point was located 1.4m from the end of the net, at a height of 2m (chosen to simulate entry at eave height). To avoid any influence of air movements or climate gradients, humidification and air conditioning were switched off during tests.

### 5.2.3 Bed Nets

The LLINs used were Permanet® 2.0 (75 denier polyester net with deltamethrin at 55mg/m$^2$; Vestergaard, Lausanne, Switzerland), a WHOPES approved product (WHO, 2008b). Untreated nets were assembled from untreated polyester net of similar mesh. LLINs were removed from packaging and hung (in a separate room) for four weeks prior to tests. To facilitate image capture, bed nets were altered and sewn to fit the mattress tautly to eliminate wrinkling or folding, and the top surface of the net was tilted on its long axis (measuring 750mm and 450mm high on opposite sides; Figure 5.1). Human volunteers lay on a fresh sheet on a 2m x 0.88m mattress (180mm thick, 480mm above the floor at the top) on timber slats mounted on bricks to ensure rigidity and reduce vibration.

**Figure 5.1 Photograph of filming set-up.**

Seen here is the experimental insectary, showing the bed and fitted bed net, with two pairs of Fresnel lenses visible on the left and right. Mosquitoes were released on the wall behind the photographer at a height of 2m.

### *5.2.4 Volunteers*

Ten human volunteers were used, a sample size exceeding that used in previous studies investigating similar behaviours (Lynd & McCall, 2013; Sutcliffe & Yin, 2014; Dekker *et al.*, 1998). Volunteers (5 males and 5 females of different ages and a range of ethnicities) were recruited from staff and students at the Liverpool School of Tropical Medicine. Volunteers were clothed but barefoot and lay on their backs, as immobile as comfort permitted. To control for any influence of body orientation, half the participants were randomly assigned to one position (*i.e.* 50% with head and 50% with feet towards the mosquito release point), which they retained for both tests. Volunteers were each tested with LLIN and untreated net, with tests held on different days, with an average interval of 13 days between them.

### *5.2.5 Experimental Procedure*

One hour before tests, the volunteer entered the bed net, the mosquitoes were placed in a paper cup connected to an external release cord, and the room was closed. After one hour’s acclimatisation, the release cord was pulled, removing the cup’s net cover, inverting it, and releasing mosquitoes. Activity of mosquitoes around the bed was recorded for 60 minutes.

At the end of the 60-minute test period, mosquitoes in the room were collected with aspirators. Between tests with treated and untreated nets, surfaces in the insectary were washed (5% Decon 90) and rinsed and air vented with a fan in the doorway.

Data were recorded and analysed from 23 laboratory tests (25 mosquitoes/ test): 10 with an untreated net and 10 with an LLIN; 3 tests used an unbaited (*i.e.* no human bait) untreated net. Tests were conducted in a ten week period in April to June 2013.

### 5.2.6 Mosquito tracking

Mosquitoes were tracked using the systems described in the General Methods chapter (Chapter 2). Flight track segments were categorised in behavioural modes using existing quantification algorithms (Angarita-Jaimes *et al.*, 2016). A track could comprise up to three different behavioural modes (all except swooping, where no net contact occurred) and where more than one mode occurred, the times spent in each mode were recorded separately.

### 5.2.7 Quantifying net activity

Track duration was analysed using a linear generalised linear model with normal probability distribution. Track numbers were analysed using a generalised linear model with Poisson distribution.  The time lag between the first mosquito's first appearance in the field of view (using the natural log to correct for skew) and its first contact with the net, and the effect of net type were assessed with Kaplan Meier Survival Analysis.

### 5.2.8 Quantifying velocity and tortuosity

Tortuosity and velocity values were calculated using whole swooping tracks, as described in the Chapter 2. Although speed and tortuosity data were not normally distributed, results from GLM analysis of transformed data were unchanged, and the untransformed data are reported here.

### 5.2.9 Quantifying net approach

Analyses were applied only to activity recorded in the first ten minutes.  The point where a track first appeared in the field of view was classed as either high (*i.e.* over the net: regions 12 and 13 Figure 5.4A) or low (all other positions). Tracks starting on the net were considered likely to be fragments of incomplete tracks (*e.g.* track continuity was lost during movement between lenses or darker net regions or tracks could not be linked with confidence) and discarded; rigorously applying this rule eliminated 24% of tracks from analysis.

To test whether mosquitoes made more approaches from above or below the net, A chi-squared test was conducted on data, pooled by treatment, to assess if the observed number of mosquito tracks approaching ‘from above’ differed from that which would be expected if height of approach was evenly distributed across all elevations. Separate tests were conducted for each net type. The expected value was calculated based on the relative sizes of regions 11 and 12 ‘above the net’, and regions 10 and 11 ‘below or level with the net’. Location of first net contact was assessed using the definitions of contact stated in relation to activity modes *i.e.* a sharp change in track direction, or frequent semi-periodic change.

### 5.2.10 Localisation of activity at the bed net interface

The field of view was divided into 16 regions, ten on the net surface and six in the surrounding space (Figure 5.4A). A mosquito track was assigned to regions 1-10 when contact with that region was detected.  Swooping tracks in regions 1-10 were assigned to region 15 or 16 (left or right camera fields, respectively). Mosquito activity showed no bias towards either the right or left camera field (t-test, t=0.65, df=21, *p*=0.523). Total activity, swooping, visiting, bouncing and resting were scaled by region area, giving values of seconds/mm$^2$, to compensate for size differences between regions. Point of first net contact, and duration of net contact were analysed without scaling for area. Larger combined regions were used for analysis of point of first contact (Figure 5.4B) as low numbers of data points occurred in the first ten minutes of some tests.

### 5.2.11 Determination of velocity/ deceleration prior to contact

To explore mosquito velocity during approach and landing at bed net surfaces, trajectories in which mosquitoes flew for at least one second prior to contacting the net were selected, and a 65-point section of each trajectory, from 1 second before contact to 0.3 seconds after contact was selected. Velocity of these tracks was calculated at each of the points along its length using the equation $v_i = \frac{\|\overrightarrow{r_{i+1}} - \overrightarrow{r_{i-1}}\|}{t_{i+1} - t_{i-1}}$ where $v_i$ is the velocity at point *i*, $r_i$ is the position vector at point *i*, and $t_i$ is time stamp at point *i*. In plain terms, an individual velocity value was calculated for each of the 65 points on the track section. The velocity calculation for any point “$r_i$” was based on the speed at which the mosquito flew from the preceding point ($r_{i-1}$) to the point after this ($r_{i+1}$). Velocities for each track were filtered with a low pass 3rd order Butterworth filter, with a cut-off frequency of 11.25Hz and a sampling frequency of

50Hz. This removes small sharp points of variation in data, averaging against neighbouring points to smooth the line of velocity during approach. Acceleration at each point was calculated using $a_i = \frac{v_{i+1} - v_{i-1}}{t_{i+1} - t_{i-1}}$ where $a_i$ is acceleration at point *i*.

The track point at which the mosquito began decelerating prior to contacting the net was calculated using methods modified from Cooperband *et al.* (2006a). An algorithm starting at the point of contact worked backwards along the track to identify the first incidence acceleration in the track (defined as two consecutive points with positive acceleration values). The point following this (*i.e*. closer to the net) was classified as the start of the mosquito's deceleration prior to contact. Using this algorithm, the closest point that could identified as the start of deceleration was that immediately prior to net contact. Track length between the deceleration start point and net contact point was calculated to find the mosquito's distance from the net when it commenced deceleration.

With front legs extended during flight (Baird *et al.*, 2013), mosquitoes potentially could have contacted the bed net with their tarsi before the tracking algorithm that detected the mosquito's body, could detect the change of direction indicating 'collision'.  On this basis, the numbers of tracks where deceleration began within 3mm of the net surface (*i.e.* when leg contact could not be excluded) or that accelerated on their last two points of flight towards the net, were quantified for each repeat test.  As the study interest was in determining whether mosquitoes decelerated prior to contact, these events were classed as "contacts without deceleration" and were excluded from further analysis. Remaining tracks were used to calculate a mean track distance between the point at which deceleration occurred and the net, for each of the 23 test replicates. Average instantaneous velocity at the deceleration was calculated for each test.

### *5.2.12 Defining and quantifying contact with a bed net surface*

Bed net contacts were identified as resting tracks, or by sharp changes in mosquito flight direction at the net surface, defined as minimum angle changes of 80° in visiting mode (Figure 5.3B). In bouncing mode (Figure 5.3C), angle changes during repetitive contacts were often lower, and repetitive oscillations in '*x'* and/or '*y'* co-ordinates were detected from zero crossings of a bandwidth-filtered position vs. time history (Angarita-Jaimes *et al.*, 2016). To avoid spurious connections between unrelated net-arrival and net-departure tracks, resting periods were limited to a maximum of 300 seconds per event.

Time spent in contact with the net was calculated from the sum of all contacts accrued through single visits or rests, and multiple bounces. Since tracking behaviour of individual mosquitoes over the course of any test was not possible, maximum and minimum values of net contact time per mosquito were estimated as follows: the maximum value was total contact divided by the maximum number of mosquitoes observed attacking the net simultaneously in each test; the minimum value assumed that all 25 mosquitoes responded simultaneously, and calculated each mosquito's activity as $\frac{total\ contact}{25}$. If the total number of trajectories recorded was fewer than 25 (only found in estimates for the first 10 minutes) the actual value was used as the total number of recorded trajectories.

### 5.2.13 Rates of mosquito activity throughout the 60 minute test period

Mosquito activity over the hour's test was grouped in to 12 five-minute intervals. Using Prism 6, these were fitted to an exponential decay equation to find the value of the decay constant (*k*) in the equation $Activity_t = Activity_{t=0}e^{-kt}$ (where *t*=time in minutes).

### 5.2.14 Statistical Analyses

Statistical analyses used SPSS Statistics 21 (IBM) and Prism 6 (GraphPad). Multiple generalized linear models with normal probability distribution were used to analyse the effect of the explanatory variable of net type (unbaited, untreated, LLIN) on the dependent variables of the total duration of time for which mosquitoes were active. Generalized linear models with Poisson distribution were used to assess the effect of net on numbers of tracks counted in different tests.

Qualitative graphical summaries are given of the proportion of time mosquitoes spent in different behavioural modes. However to avoid issues of non-independence in testing multiple related outcomes in analysis of behavioural budgets, only behavioural categories of 'swooping' and 'bouncing' were used in statistical analyses. In these analyses, a generalized linear model was used to assess the effect of the explanatory variable net type on time spent swooping, and time spent bouncing.

Generalised linear models were used to assess the effect of net treatment on track tortuosity and track velocity. To investigate whether mosquitoes approached the net preferentially from above, or level with/below the net, the number of tracks recorded approaching the net from above was counted and pooled for each of the three treatments. Three chi squared tests were used to compare these values to the expected value that would be observed if activity was evenly distributed across all flight elevations, for each of the net treatment types (unbaited, untreated, LLIN).

The net was split into 16 different regions (section 5.2; figure 5.4), and spatial distribution of mosquito activity around the net was compared using generalised linear models. These investigated the effects of the explanatory variables of net type, region, and an interaction term between net type and region on the dependent variables of activity density, and activity density in the four different behavioural modes.

Statistical analysis of the percentage of tracks contacting the net without deceleration, and the distance from the net and velocity of track at the deceleration point used generalized linear models to assess the effect of net treatment on these outcomes. This method was also used to test the explanatory effect of net treatment on the dependent variable of time spent in direct physical contact with the net. A spatial analysis which used net treatment, net region, and an interaction term between the two was used to investigate how these variables influenced the time mosquitoes spent in physical contact with the net, testing whether contact was evenly distributed across all net areas, and the extent to which net treatment influenced distribution of contact.

Kaplan Meier survival tests were used to assess differences in lag between appearance and net contact. These were chosen as they allow investigation of the effect of net treatment on a time to an event (lag time between appearance and contact). For analyses of rates of activity decay over time, *k*-values were tested for significant differences between untreated nets and LLINs using generalized linear models, and 95% confidence intervals used to determine time intervals with significant differences in activity (Figure 5.6A). For all tests, the α threshold used was 0.05. Where values were not normally distributed according to calculations of skewness and kurtosis, averages were calculated as geometric means. In all cases except for ‘all treatment’ data 95% confidence intervals were calculated using the *t* distribution to account for small replicate numbers. Unless stated otherwise, outcomes are reported as arithmetic means with 95% confidence intervals.

### *5.2.15 Ethical Permission*

Methods were carried out in accordance with the approved guidelines. Informed consent was obtained from all participating human subjects. The study was approved by Liverpool School of Tropical Medicine Research Ethics Committee (‘Behaviour of African malaria vectors’: Permit no. 12.13, issued 24th May 2012).

## 5.3 Results

### *5.3.1 Classification of mosquito behavioural modes*

In all treatments, mosquito activity was classified into four quantifiably distinct types of behaviours, termed ‘modes’ (Figure 5.3A-D; Supplementary Video 5.1 [available in enclosed CD, and online), defined as follows:

1. Swooping: Tracks that did not contact the bed net (Figure 5.3A).

2. Visiting: Tracks where relatively long periods of flight were interspersed with infrequent contacts with the bed net (Figure 5.3B). Contacts were characterized by sharp turns of 80° or more in the trajectory and when multiple contacts occurred, the minimum interval between them was 0.4 seconds (*i.e.* an interval of at least 20 frames, at 50 frames per second).

3. Bouncing: Tracks where the mosquito made multiple rapid contacts with the bed net surface, at intervals of less than 0.4 seconds; includes events where the mosquito executed short flights between contacts, or maintained contact with the bed net surface without being static (Figure 5.3C). The latter were brief pauses in movement lasting less than 0.75 seconds and included ‘walking’ on and ‘probing’ the bed net.

4. Resting: Mosquito tracks where insects were either completely static for at least 0.75 seconds, or where the velocity of mosquito movement was less than 1.33 mm/s (equivalent to movement of up to one mm in the minimum resting time); assumed constant contact with the bed net surface (Figure 5.3D). Dead mosquitoes were excluded by limiting resting events to a maximum of 300 seconds. Notably, no dead mosquitoes were found on nets at the end of tests.

### *5.3.2 Responses at unbaited, baited and insecticide-treated nets*

Figures 5.2A-C show representative examples of recorded flight tracks at unbaited untreated (henceforth termed ‘unbaited’), baited untreated (‘untreated’) and baited LLIN (‘LLIN’) nets during 1 hour of recording. Across all treatments, individual flight track durations ranged from 0.22 to 445.1 seconds, with a geometric mean track length of 4.2 seconds (4.0 - 4.3; n = 7729 tracks).

Activity at an untreated bed net was significantly lower in the absence of human bait, as measured by the geometric mean number of flight tracks (182 [30-1114] at unbaited and 517 [404-661] at untreated nets; generalized linear model, $X^2$ (1)= 17.9, $p < 0.001$) and the total duration of activity recorded for each test (*i.e.* 25 mosquitoes for 1 hour, maximum of 25 hours: geometric means of 19.0 [1.6-223.5] and 124.6 mins [101.5-153.0] at unbaited and untreated respectively; $X^2$ (1)=55.3, $p < 0.001$).

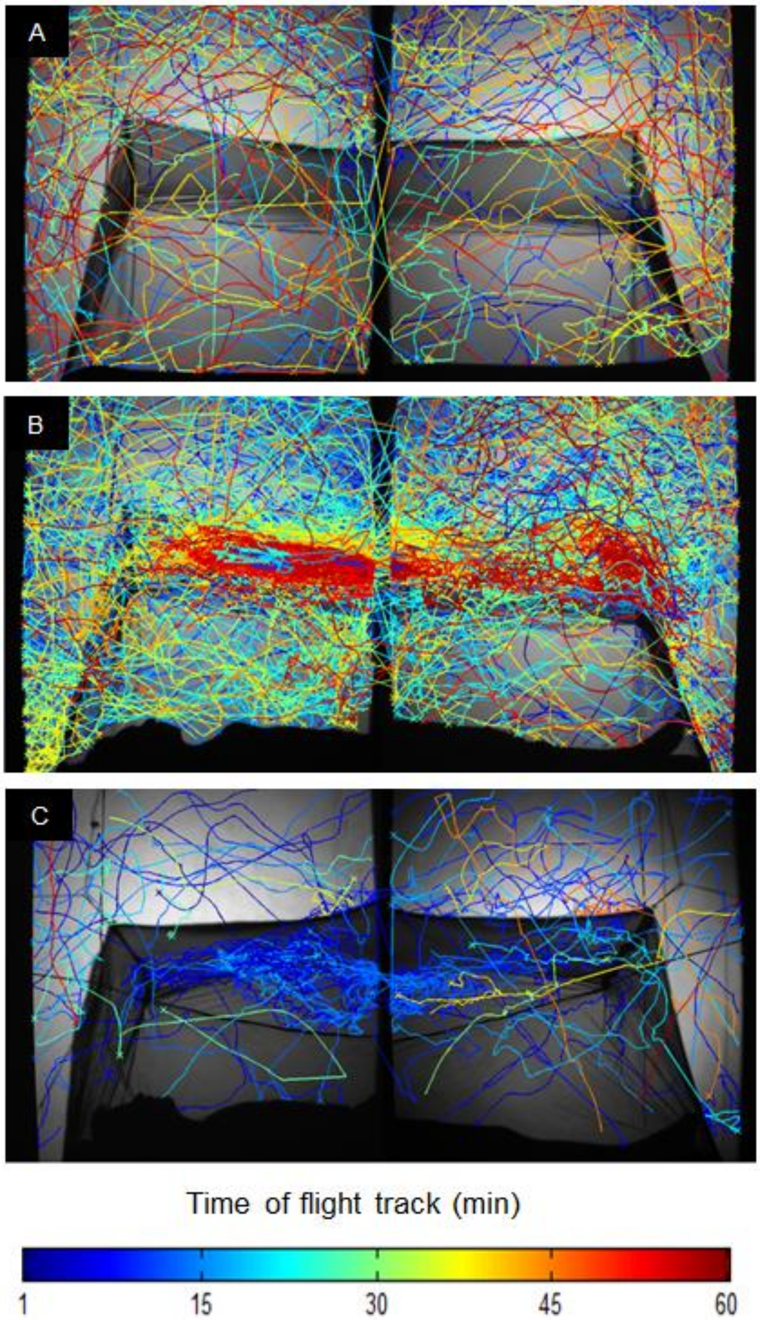


**Figure 5.2 Flight activity of *Anopheles gambiae* at unbaited, baited and insecticide-treated bed nets.**

A-C Track images showing 60 minutes' of mosquito activity at: (A) an unbaited untreated bed net; (B) a human-baited untreated bed net; (C) a human-baited insecticide-treated bed net (LLIN; Permanet 2®; Vestergaard-Frandsen, Lausanne, Switzerland). Each coloured track is the path of a single mosquito flight event. Tracks are colour-coded according to time they first appeared in the field of view as shown in the key: blue tracks at the start through to red at the end of the 60-minute test.

**Table 5.1 Total activity time of *Anopheles gambiae* recorded in each behaviour mode.**

Total duration of all tracks classed in each behaviour mode over 60 minute tests (geometric mean and 95% confidence interval, minutes). Since multiple mosquitoes were often active simultaneously in the field of view, the total activity times could exceed 60 minutes. Values for each mode followed by the same letter are not significantly different at $p<0.05$ (Generalized Linear Models), between different net types. Statistical tests were only conducted for swooping and bouncing activity modes.

| | N | Swooping | Visiting | Bouncing | Resting |
|---|---|---|---|---|---|
| **Unbaited** | 3 | 7.5 (0.5-116.1) | 10.6 (1.2-96.2) | 0.5 (0-22.1) | 0.1 (0-20.0) |
| | | ab | | a | |
| **Untreated** | 10 | 7.7 (6.1-9.8) | 33.2 (24.0-46.1) | 70.1 (57.7-85.1) | 10.3 (7.0-15.3) |
| | | a | | b | |
| **LLIN** | 10 | 3.4 (1.9-6.2) | 6.9 (3.5-13.6) | 7.7 (3.1-18.7) | 2.0 (0.8-5.0) |

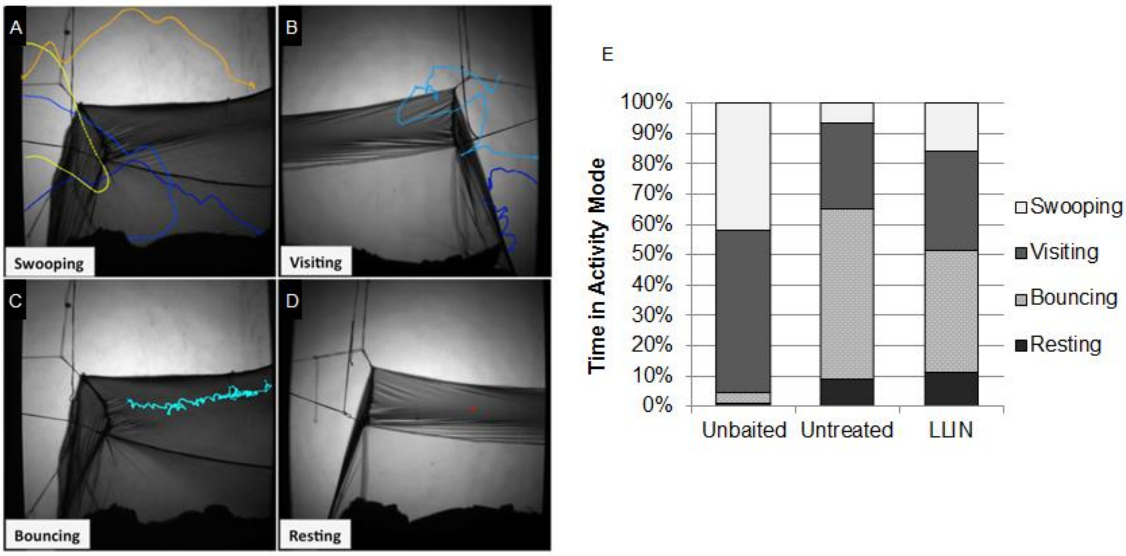


**Figure 5.3 Behavioural modes of *Anopheles gambiae* at unbaited, baited and insecticide-treated bed nets.**

(A-D) Images showing representative tracks for *Anopheles gambiae* flight in each of the four behaviour modes as defined in the text. (E) The proportion of time spent in each behaviour mode for each bed net type: Unbaited = unbaited untreated bed net; Untreated = human-baited untreated bed net; LLIN = human-baited insecticide-treated bed net (LLIN).

Track numbers recorded at LLINs (geometric mean 131 tracks [75-232]; 21.2 mins, [10.6-42.7]) were significantly lower than at untreated nets ($X^2$ (1)=34.9, $p < 0.001$) but both track number and duration were similar to unbaited nets ($X^2$ (1)=0.6, $p = 0.456$; $X^2$ (1)=0.2, $p = 0.649$ for track number and duration, respectively).

Activity was investigated qualitatively win all behavioural modes. However to avoid analysing multiple related variables, only swooping and bouncing behavioural modes were tested quantitatively for effect of net treatment using generalized linear models. Exploring activity by behavioural mode (Figure 5.3E) shows that 93.7% of activity on a baited (untreated) net involved net contact (*i.e.* visiting, bouncing or resting modes) compared to 58.1% on an unbaited net. In fact, 65.3% of the activity at a baited net involved frequent (bouncing) or continuous (resting) net contact, in contrast to 4.7% on the unbaited net.

The mean times spent in each mode involving contact were higher at baited nets (Table 5.1), as visible in the large statistically significant increase in bouncing type flight ($X^2$ (1)=46.5, $p < 0.001$) in the presence of human bait, while swooping was not significantly different ($X^2$ (1)=0.003, $p = 0.953$). At LLINs, activity in all four behaviour modes was lower than at untreated nets, particularly in the three modes with net contact where treated net activity fell to 27% or less than the untreated net values (Table 5.1). Statistical tests found significant decreases in seconds mosquitoes spent swooping, and bouncing at LLINs compared to untreated nets ($X^2$ (1)=9.4, $p = 0.002$; bouncing, $X^2$ (1)=33.2, $p < 0.001$). However, a response to the host persisted despite the insecticide presence, and the time spent attacking the LLIN in bouncing mode was significantly higher than at unbaited nets ($X^2$ (1)=11.4, $p = 0.001$).

### 5.3.3 Flight speed, tortuosity and height during net approach

The instantaneous velocity of individual swooping flight tracks ranged from 84 to 986 mm/s across all tests, with a mean velocity of 346mm/s [342-351] (n = 3234 tracks). Mosquitoes flew slightly faster at baited untreated nets (356mm/s [340-372]) than at unbaited nets (321mm/s [266-376]; $X^2$ (1)=7.8, $p = 0.005$) and LLINs (323mm/s [293-353]; $X^2$ (1)=5.4, $p = 0.020$), which were not significantly different from each other ($X^2$ (1)=0.009, $p = 0.923$).

Track tortuosity was higher in both baited net groups than in the unbaited nets (1.31 [1.16-1.47] unbaited, 1.66 [1.52-1.79] untreated, 1.63 [1.43-1.83] LLIN; $X^2$ (1)=15.0, $p < 0.001$), but not different between LLINs and baited untreated nets ($X^2$ (1)=0.1, $p = 0.783$).

Simple analysis of the spatial location of flight path prior to arrival did not indicate any notable bias for low (below top net surface level) or high (above the net) spatial preferences. Adjusting for differences in the visible field of view between high and low areas, equal distribution would result in 36% of tracks starting in the high region. In unbaited tests, there was no preference (38.0% [34.7-41.2] of tracks starting above the net; $X^2$ (1)=0.77, $p$ = 0.380). However in both baited untreated and LLIN tests, a slightly higher proportion of mosquitoes approached from above the net (40.3% [32.8-47.9] in untreated nets, $X^2$ (1)=10.05, $p$ = 0.002; 41.5% [37.0-46.1] in LLINs $X^2$ (1)=5.76, $p$ = 0.016).

### 5.3.4 Location of activity at the bed net interface

After accounting for the effect of net type on activity levels, the distribution of total activity (seconds/m$^2$) around the bed net was significantly different at each net type ($X^2$ (2)=115.927, $p$<0.001; Figures 5.4D and 5.2A-C). Without human bait, 49.9% of flights occurred in the spatial regions around the net (regions 11-16 in Figure 5.4A), compared with 5.5% at untreated nets and 10.5% at LLINs (Figure 5.4D). In contrast, activity in baited tests was located primarily on the net roof directly above the human body and to a lesser extent, near the feet: 74.7% and 78.3% of activity occurred on the roof (regions 1-6) and 10.9% and 8.8% at the feet (region 10) in untreated nets and LLINs respectively (Figure 5.4D).

Comparing nets by behaviour mode, swooping (Figure 5.4E) was distributed unevenly between different net regions in all treatments ($X^2$ (5)=102.208, $p$ < 0.001), with less activity occurring in regions 15 and 16 in front of the vertical net sides. In visiting mode, there was a significant interaction between net type and activity distribution ($X^2$ (26)=40.532, $p$ < 0.001; Figure 5.4F): higher visiting rates were recorded in regions 3 and 4 (17% and 16% of activity) on untreated nets, but at LLINs, visiting was higher in regions 3, 7 and 10 (12%, 11% and 10%, respectively). Treatment also affected activity distribution in bouncing mode ($X^2$ (2)=43.322, $p$ < 0.001): bouncing flight was higher in regions 2, 3 and 4 (21%, 35% and 17% respectively) in untreated nets, whereas most bouncing activity at LLINs occurred in regions 1, 2, and 3 (18%, 30%, 24%; Figure 5.4G). Finally, net type also significantly affected resting activity ($X^2$ (18)=99.714, $p$ < 0.001; Figure 5.4H): on untreated nets, more resting occurred in regions 2, 3 and 10 (26%, 26%, 17%) but on LLINs, resting was higher in regions 1, 2 and 3 (17%, 20%, 33%), with 11% of resting recorded at the feet (region 10).

Hence, although there were marked significant differences between baited and unbaited nets for bouncing and resting modes (Figures 5.4G, 5.4H), there was no evidence that insecticide treatment significantly altered the preference for the roof of the bed net as the focus of activity.

### *5.3.5 Velocity of mosquitoes during landing on bed nets*

The mean velocities of mosquitoes during final approach to the net surface, immediately prior to net contact, were determined for each test and compared between bed net types. In total, 896 tracks fitted the conditions for contact analysis. Of this subset, the geometric mean percentages of net contacts classed as ‘contacts without deceleration’ (*i.e.* tracks that accelerated on their last two points of flight before contact or where deceleration did not start until within 3mm of the net, and leg contact could not be excluded) were calculated as 3.3% [1.4-15.0] at unbaited nets, 9.1% [7.4-11.3] at untreated nets and 5.1% [2.6-11.7] at LLINs, and were not significantly different between treatments ($X^2$ (1)=1.872, $p = 0.392$). Hence, over 90% of mosquitoes decelerated prior to net contact, with deceleration starting at approximately 0.12 seconds prior to landing, at a distance of 26-41mm from the net.

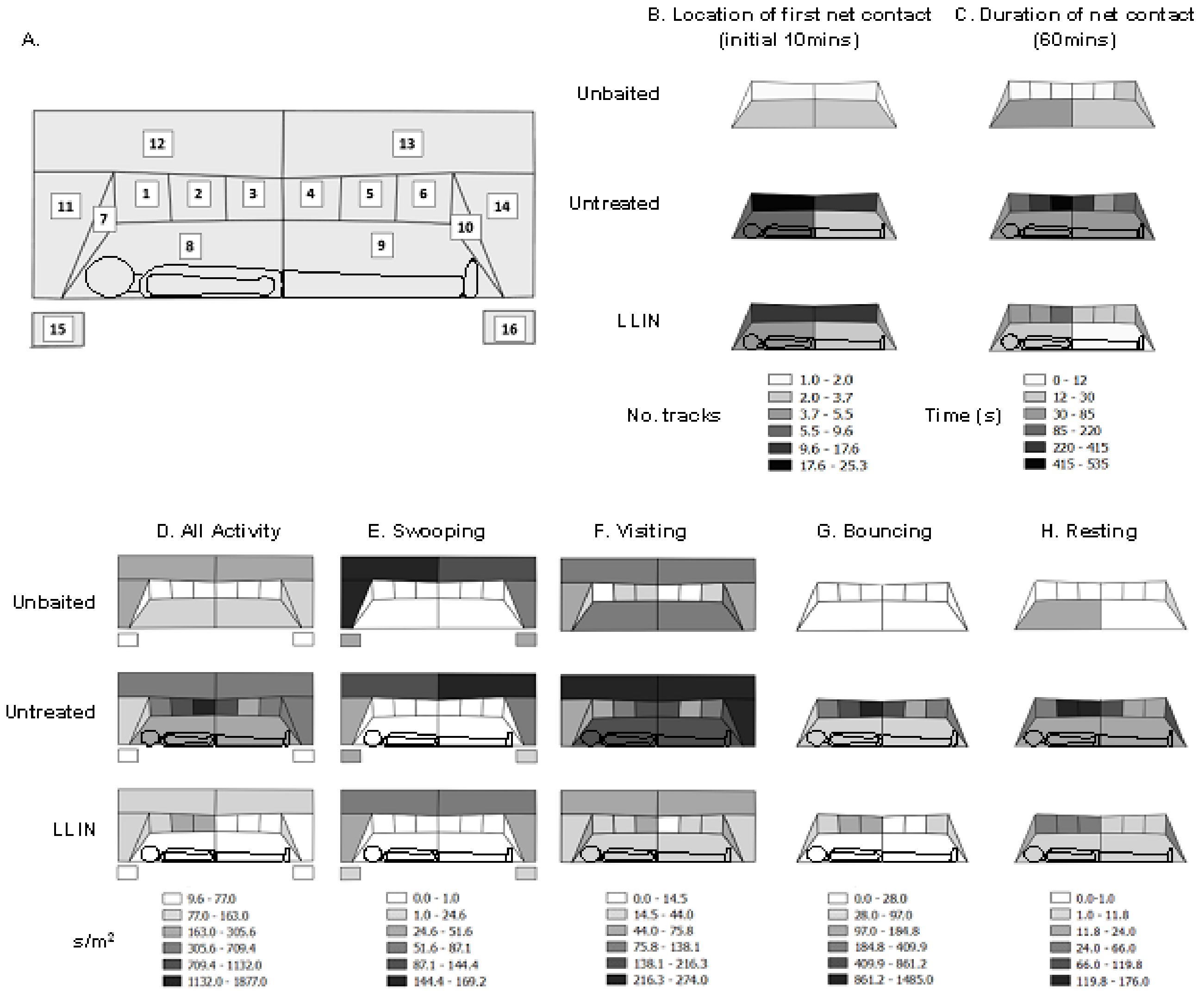


**Figure 5.4. Distribution of *Anopheles gambiae* flight activity, behaviour modes and net contact at different regions on and around a bed net**

(A) Distribution map key showing region codes to which each mosquito track was assigned. Bed net surfaces 1-6 were on the horizontal roof, 7 and 10 the vertical head and foot end walls, respectively, 8 and 9 the vertical side walls. Portions of flight tracks visible beyond the net surfaces were assigned to the spatial regions 11-14 as shown. Regions 15 and 16 record flight activity without net contact (i.e. swooping) that occurred in front of net, on the left (15) and right (16) portions of the field of view, respectively. (B) Distribution of initial net contacts by region, showing the first point of net contact for those tracks occurring in the first ten minutes of testing. (C) The total duration (seconds) of all contacts (includes mid-flight brief contacts made during visiting and bouncing, and resting behaviour) by all mosquitoes on each region of the bed net surface over the 60 minute test (means of 3,10 and 10 replicate tests for unbaited, baited and LLIN, respectively). Charts D-H show the density of activity (s/m2) around and on the bed net surface: (D) All activity combined; (E) Swooping; (F) Visiting; (G) Bouncing; (H) Resting. Although tests controlled for the orientation of the human bait in relation to the mosquito release point, all figures show the volunteer (when present) with the head on the left.

However, the point at which mosquitoes started to decelerate (Figure 5.5) was significantly closer to the net at unbaited nets (distance from the net 26.3mm [18.5-34.1]) than at baited nets, both untreated (41.5mm [36.8-46.2], $X^2$ (1)=6.7, $p$ = 0.010) and LLINs (40.0mm [31.0-49.0], $X^2$ (1)=5.5, $p$ = 0.019), which were not significantly different from each other ($X^2$ (1)=0.14, $p$ = 0.708). In addition, unbaited arrival flight velocities (277mm/s [212.6-340.7]) were significantly slower than those at untreated (384mm/s [365-404], $X^2$ (1)=13.0, $p$ < 0.001) and LLINs (357mm/s[310-341], $X^2$ (1)=7.2, $p$ = 0.007), which were not significantly different from each other ($X^2$ (1)=1.8, $p$ = 0.175).

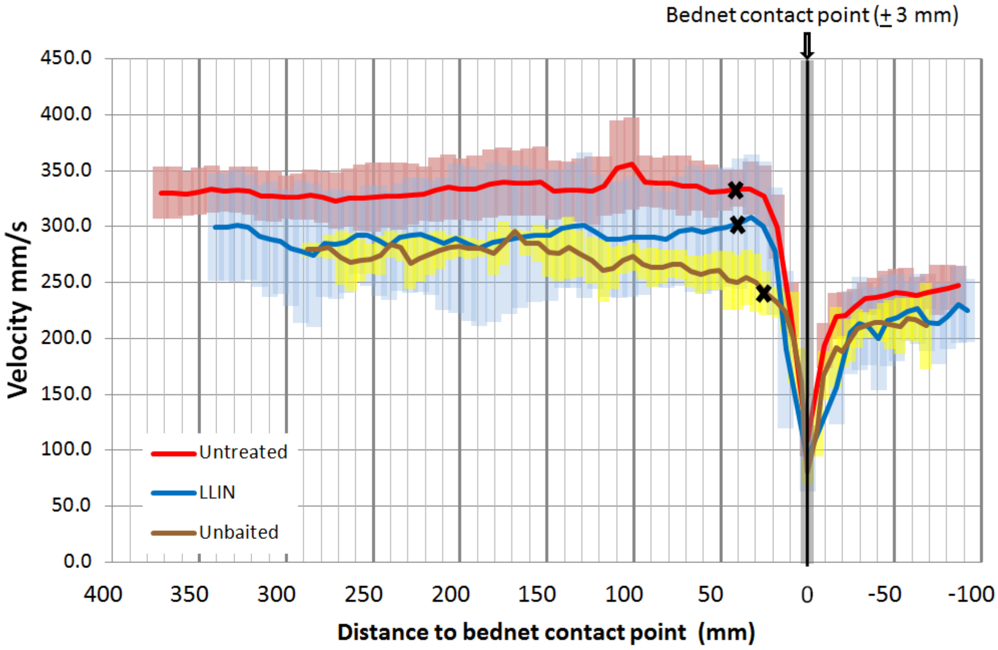


**Figure 5.5 Velocity of *Anopheles gambiae* during landing at bed nets.**

Mean velocity of mosquitoes during final approach, contact and departure from the bed net surface. The figure represents a 1.3 s track segment, with the bed net contact point at 0 mm; positive x-axis values indicate position before contact, negative values represent track distance after contact. The grey region either side of the contact point represents the ± 3mm region where tarsal contact with the bed net was possible. The average points at which deceleration started for each net type are marked with 'X'. Note that the graph presents the averages of multiple repeat test values and hence the position of the point of deceleration does not correspond perfectly with the average approach track as illustrated. Coloured bars show standard deviation at each track point.

### *5.3.6 Quantifying duration of net contact*

The geometric mean total time per test where mosquitoes were in physical contact with nets (Table 5.2) was significantly higher on the untreated baited net (31.3 minutes [31.1-31.4]) than on both the LLIN (5.1minutes [2.4-10.9]; $X^2$ (1)=42.2, $p <$ 0.001) and unbaited nets (1.7 minutes [1.0-28.7]; $X^2$ (1)=65.3, $p < 0.001$; generalized linear model). However, contact time was significantly higher also on LLINs than on unbaited nets ($X^2$ (1)=8.8, $p = 0.003$). The longest contact time recorded for a single mosquito track was 37.4 seconds on an unbaited net, 160.4 seconds on an untreated net and 110.5 seconds on an LLIN. Since it was not possible to measure the actual total contact time for individual mosquitoes, a plausible mean minimum and maximum contact time values were determined (as defined in Table 5.2) for a single mosquito of 75.0 to 321.9 seconds at an untreated baited net and 12.3 and 78.0 seconds at an LLIN over 60 minutes of a test.

Total contact time was significantly affected by net type, region, and interactions of net type and region ($X^2$ (2)=126.951, $p < 0.001$, $X^2$ (9)=70.511, $p<0.001$, $X^2$ (18)=81.054, $p<0.001$; Figure 5.4C). Highest contact times (extracted contact data, of all types, from all tracks) were recorded on the roof in untreated nets (regions 2, 3, 4: 410s, 531s, 306s, respectively), and in the centre of the roof in LLINs (region 3: 126s).

**Table 5.2 Duration of *Anopheles gambiae* contact with bed nets.**

Geometric mean duration of contact with the bed net surface for 60 minutes tests, as calculated for: [1] geometric mean total of all contacts observed; [2] geometric mean contact time per mosquito assuming all 25 mosquitoes responded; [3] geometric mean contact time per mosquito based on the maximum number of individual mosquitoes observed simultaneously (mean (95% CI), values for each mean followed by the same letter are not significantly different between net types at $p<0.05$).

**Duration of physical contact with the bed net surface (60 min test)**

| | N | Geometric mean total time (all contacts)[1] (min) | Geometric mean time/mosquito (25 mosquitoes) [2] (s) | Geometric mean time/mosquito (observed max number)[3] (s) |
|---|---|---|---|---|
| **Unbaited** | 3 | 1.7 (1.0-28.7) $^a$ | 4.1 (0.2-69.0) $^a$ | 35.1 (4.8-259.3) $^a$ |
| **Untreated** | 10 | 31.3 (31.1-31.4) $^b$ | 75.0 (74.7-75.3) $^b$ | 321.9 (320.8-323.0) $^b$ |
| **Treated** | 10 | 5.1 (2.4-10.9) $^c$ | 12.3 (5.8-26.2) $^c$ | 78.0 (45.3-134.3) $^c$ |

### *5.3.7 Interactions with the bed net over time*

Over 60 minutes, total mosquito activity decayed significantly more rapidly at LLINs compared to untreated nets ($X^2$ (1)=8.4, *p*=0.004, Generalized Linear Model; Figure 5.6A). Reduced activity at the LLIN was indicated after 5-10 minutes, becoming significantly lower from the 10-15 minute period onwards. By 30 minutes, activity at LLINs was negligible and did not recover, while sustained levels of host seeking were recorded at untreated nets for the entire 60 minutes. This rapid fall was apparent in the three behavioural modes involving flight, swooping, visiting, and bouncing ($X^2$ (1)=5.8, *p* = 0.016; $X^2$ (1)=4.7, *p* = 0.031; $X^2$ (1)=9.6, *p* = 0.002, respectively, Generalized Linear Model; Figure 5.6B, C).

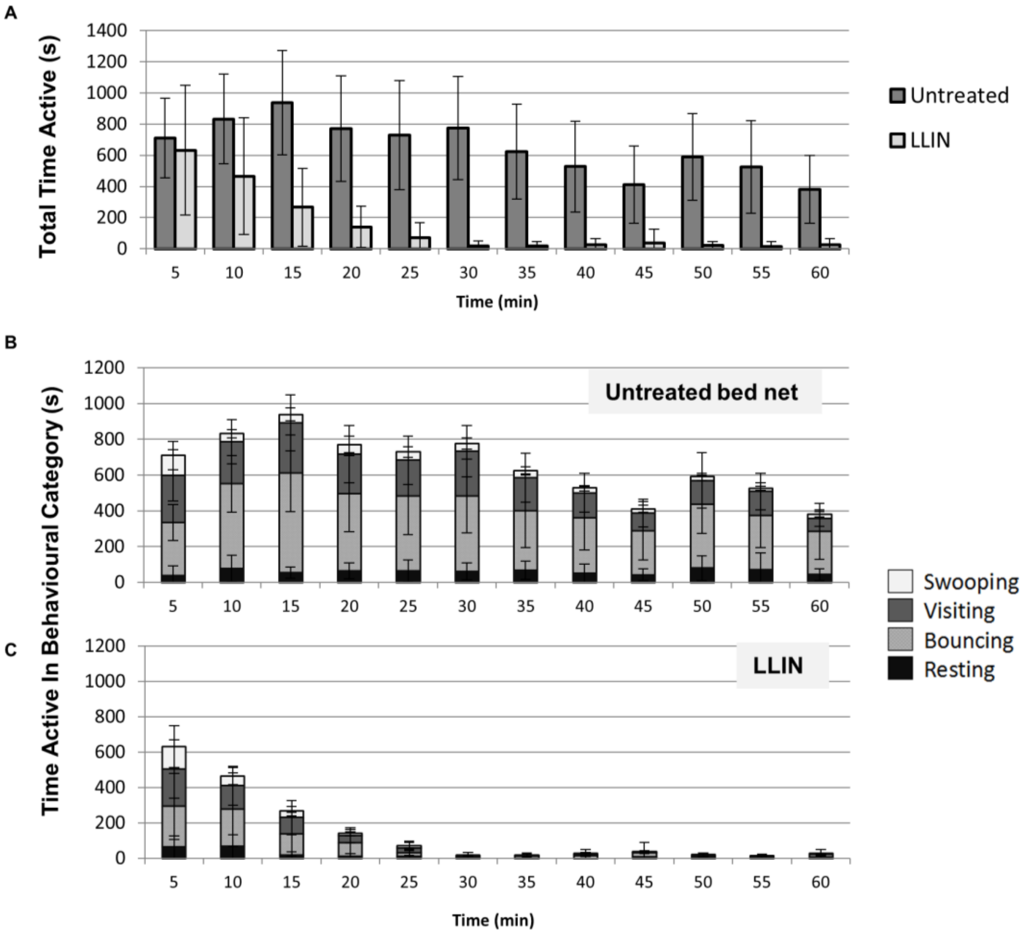


**Figure 5.6 Rates of *Anopheles gambiae* activity throughout the 60 minute test period.**

(A) Total activity at untreated baited nets and LLINs. (B, C) Mosquito activity as in figure A separated by behavioural mode, at untreated baited nets (B) and LLINs (C). X-axis units are mean (± SD) activity per 5-minute inclusive interval, *i.e.* 5 (0 – 4 min 59 s), 10 (5 min – 9 min 59 s), 15 (10 – 14 min 59 s), etc.

Mosquito activity during this key initial 10-minute period was explored further. The time lag between appearance of the first mosquito and first net contact was unaffected by the presence of the insecticide or human bait: geometric means unbaited = 18s (0-994); baited = 6s (1-33) ($X^2$ (1)=0.6, $p = 0.438$); LLINs = 17s (6-43) ($X^2$ (1)=0.6, $p = 0.432$). Comparing untreated baited nets with LLINs, there were no differences in the number (72.8 [53.1-92.5] at untreated nets, 50.3 [28.6-72.0] at LLINs; $X^2$ (1)=2.891, $p = 0.084$) or the distribution of contacts on different net regions ($X^2$ (5)=8.2, $p = 0.145$), with a significant majority ($X^2$ (5)=70.92, $p < 0.001$) of first contacts on the net roof in both (60.6% and 56.5% in untreated and LLINs, respectively; Figure 5.4B). At unbaited nets in contrast, significantly fewer contacts (12.3 [2.3-22.4]; $X^2$ (1)=85.731, $p < 0.001$) occurred in a significantly different pattern (*i.e.* near uniform distribution) on the net ($X^2$ (10)=21.5, $p = 0.018$; Figure 5.4B).

These results indicate that LLINs did not repel mosquitoes to any significant level prior to net contact. Yet, while contact with LLINs was significantly lower than untreated nets over 60 minutes (Figure 5.3E, Table 5.1), the majority of LLIN contacts occurred during the first ten minutes: 62.2% on LLINs (4.6 minutes [2.2-6.9]), 17.9% on untreated nets (5.9 minutes [4.0-7.8]). Moreover, this impact was preceded by surprisingly brief time in contact with the LLIN. It was calculated that during the initial ten minute period, one mosquito made between 14.3 and 70.3 seconds of contact with an untreated net or 11.0 and 57.1 seconds with an LLIN (minima and maxima calculated as described in the previous section).

**See enclosed CD for: Supplementary Video 5.1: Mosquito flight at a human-occupied bed net in swooping, visiting, bouncing and resting behavioural modes**

The video demonstrates the characteristic movement patterns within the different behavioural modes: in swooping, mosquitoes fly without contacting the net; visiting flights make infrequent net contacts; bouncing mosquitoes make frequent short persistent attacks on the net surface. In resting the mosquito is stationary, or slow moving. During the resting video, the marker disappears when movement ceases (start of resting) and reappears at the same point when movement restarts (resting mode ends). As markers are attached to moving objects, the mosquito is not highlighted when it stops moving, though the tracking algorithm continues to follow its position. Video also accessible online: https://www.dropbox.com/s/h1rbwc0rnkoaxds/SupplementaryVideo_Chapter5_1.wmv?dl=0

## 5.4 Discussion

These results provide detailed insight into the behaviour of *An. gambiae* at an LLIN. On detection of a human host within a bed net, with or without insecticide, mosquitoes responded immediately in four distinct behaviour modes, with persistent attempts to reach the host resulting in multiple brief net contacts focussed on the net roof above the human torso. Behaviour at an LLIN retained the essential character of the response to untreated nets for the first ten minutes, during which time less than one minute of total contact was made with the LLIN. In LLIN tests a rapid decay in all modes of activity resulted and after thirty minutes, mosquito activity was negligible and did not recover. Lag times to response, and velocities and deceleration rates prior to net contact were similar in LLINs and untreated nets, providing no evidence for a repellent effect of the LLIN. The results demonstrate that an LLIN is a highly efficient fast-acting baited insecticide trap.

These results were obtained with an optical imaging and flight-tracking system allows remote tracking, recording and quantitative analysis of multiple mosquitoes simultaneously flying without restriction in large fields of view over long periods while they respond to a complete human host in complete darkness. For studies at this scale, the system offers a number of advantages over other approaches. Despite their undoubted value, existing tracking systems, including some three-dimensional (3D) systems, are restricted, in relation to this study's goals, in terms of temporal resolution and test arena size constraints, short recording durations (up to 15 minutes), the low numbers of mosquitoes that can be observed simultaneously (1-4 mosquitoes per experiment) or the need to use isolated host cues such as heat or odour rather than complete human baits (Spitzen *et al.*, 2013; Dekker *et al.*, 2011; Lacey *et al.*, 2011; Dekker *et al.*, 2005). Studies that track multiple mosquitoes have been restricted by short recording periods of less than 3 minutes or the ability to track only initial and final behavioural events (Lacey *et al.*, 2011; Dekker *et al.*, 2005; Butail *et al.*, 2011). An effective stereo video system tracked up to 25 mosquitoes in wild mating swarms (Butail *et al.*, 2011; Butail *et al.*, 2012; Manoukis *et al.*, 2014) but required sunlight to generate the images.

The findings are novel and a significant contribution to our understanding of mosquito behaviour generally, and specifically how it is targeted by LLINs. Although the LLIN tested is only one of many types commercially available today, the Permanet® 2.0 is one of the most purchased and widely used LLINs in sub-Saharan Africa (Bahl *et al.*, 2012). Clearly further studies must investigate other LLINs.

The immediate and rapid effect of the LLIN and the low level of net contact required to achieve that has never been reported. Less than one minute of contact within the first 10 minutes of recording reduced subsequent foraging such that all flight and host location activity was virtually eliminated by 30 minutes. This may be considered an accurate measurement of LLIN contact duration as whilst it is acknowledged that a limited number of net contacts may not have been captured by the tracking system (*e.g.* potentially obscured by the host, net seams or wrinkles, or during processing) the majority of net contact occurred on the net roof in areas clearly visible to the camera, hence losses to recording are expected to be minimal.

Activity at baited nets, both untreated and LLINs, was higher than at unbaited nets, particularly in the bouncing and resting behaviour modes (where the highest levels of net contact occur). While activity over 60 minutes was lower at LLINs than at untreated nets, there was no difference in the number, distribution or duration of net contacts in the first 10 minutes. Furthermore, velocities measured immediately prior to net contact were virtually identical in both untreated nets and LLINs, with no indication that mosquitoes were repelled or deterred by the insecticide at close range. Finally, there were no significant differences in the time lag prior to the initial mosquito's response, confirming a previous report (Spitzen *et al.*, 2014), and indicating there was no distant or spatial insecticidal effect on behaviour.

This finding partially allays fears that LLINs might divert unfed but still hungry mosquitoes to non-users of nets without any LLIN contact (Quiñones *et al.*, 2000; Grieco *et al.*, 2007; Killeen *et al.*, 2007; Briët *et al.*, 2012). However, it remains to be determined whether the observed elimination of activity at the LLIN (Figure 5.6) resulted from insecticide-induced knockdown or death, an irreversible sub-lethal flight or sensory impairment, or some other reversible condition. Contact irritancy and impairment of host seeking responses by deltamethrin have been described (Hougard *et al.*, 2003a; Cohnstaedt *et al.*, 2011) but it was not possible to recapture sufficient mosquitoes to determine mortality rates or sub-lethal effects post-exposure. Calculations showed that an individual mosquito made on average between 12-78 seconds of contact with the LLIN (Table 5.2; although the true value depended on the proportion of released mosquitoes responding). Earlier tests with *An. gambiae* and deltamethrin-treated nets reported knockdown and death of some individuals following net contact times of only 0.4 seconds while others survived after 40 seconds of contact (Spitzen *et al*, 2014). These results suggest that, in reality, the effects of LLINs on individual mosquitoes may be wide ranging in severity. Hence, although our results demonstrate clearly that host seeking ceases rapidly when an LLIN is used, determining the proportion of mosquitoes that survive and remain capable of locating and feeding successfully on a different host following contact with an LLIN, is an important next step.

Even if insecticide contact is insufficient to induce mortality, it may impair subsequent host seeking behaviour of mosquitoes. Sub-lethal insecticide exposure can affect an insect's ability to locate a pheromone source, impair locomotion, and reduce feeding activity (Haynes, 1988). In wind tunnel studies, topical sub-lethal doses of deltamethrin have been shown to reduce flight towards odour attractants in *Cx. quinquefasciatus*, *An. albimanus* and *Ae. aegypti* (Cohnstaedt *et al.*, 2011). Topical sub-lethal exposure to pyrethroids can inhibit blood-feeding in *Ae. aegypti* (Liu *et al.*, 1986). However these effects may be short-lived, as normal *An. gambiae s.s.* behaviours can be restored within 24 hours of a short deltamethrin exposure (Siegert *et al.*, 2009). The behavioural impact and recovery time of sub-lethal insecticide exposure will partly define an LLIN's mode of action, and is a topic meriting further research.

An important additional point is that these (Table 5.2 and Spitzen *et al.*, 2014) LLIN contact values derive from observed behaviour and are considerably lower than the WHO standard method used for LLIN evaluation (WHO, 2013e), where mosquitoes are forced into contact with treated surfaces for 3 minutes. Although further accurate data are essential to confirm this, the duration of exposure used in standard evaluation of LLINs may need to be re-examined to avoid any possibility of overestimating the effectiveness of any material being tested.

Though mosquito attack at untreated nets did not decay as rapidly as when attacking LLINs, there was a small decrease in activity over the course of the 60 minute test. This differs from experiments that recorded *Ae. aegypti* activity as they responded to $CO_2$ and visual stimuli in wind tunnels, and found that host seeking activity persisted undiminished over the course of a 3 hour test (van Breugel *et al.*, 2015). In the present study, mosquitoes were able to fly away from the net and rest on the walls, contrasting to wind tunnel methods in which insects are confined in the presence of host stimuli, which may explain the differences observed in study results.

Our results emphasise the importance of the bed net roof (Lynd & McCall, 2013; Sutcliffe & Yin, 2014) by showing that it is the predominant first point of contact (Figure 5.4B), the most commonly visited surface (Figure 5.4C), and that most flight activity is also focussed at or around the roof, regardless of the flight path of the arriving mosquito (Figures 5.4D-F, 5.2A-C; Supplementary Video 5.1). Though there was some additional activity near the feet, activity at the net sides was very low (Figure 5.4C, G) indicating that mosquitoes oriented primarily to putative olfactory and thermal attractants rising from the prone host (Dekker *et al.*, 1998; Cardé *et al.*, 2010; Smallegange & Takken, 2010). Within hypothesised models of vector host location (Dekker *et al.*, 1998; Cardé *et al.*, 2010; Smallegange & Takken, 2010; Dekker *et al.*, 2011), the mosquitoes tracked in this study were relatively close to the host throughout, and therefore likely to have been flying in response to 'broad plumes' of host cues that would ultimately lure them to the net. Without knowing the actual location of those 'plumes' or their boundaries, it is not possible at this stage to interpret the observed flight trajectories or assign them to recognised behaviours such as 'casting' (Dekker *et al.*, 2011), where mosquitoes exhibit counterturning on leaving the plume in order to relocate it, or where increased tortuosity and decreased velocity occur as mosquitoes attempt to locate the source of the attractant (Cooperband *et al.*, 2006a, 2006b; Beeuwkes *et al.*, 2008; van Breugel *et al.*, 2015). This study tested responses of mosquitoes to a rectangular box shaped net, the most common net shape sold worldwide (Bahl & Shaw, 2012), but several LLINs are also available as circular cone shapes, and there is some question as to how mosquito interactions with an LLIN will be impacted by net shape. Lynd & McCall (2013) tested responses of *An. gambiae s.s.* to rectangular LLINs, and 'tent' nets with pitched sides, and found in both instances that mosquitoes made most contact with the net surfaces that were approximately directly above the volunteer's torso. It is thus suggested that net contact is determined by attraction to the volunteer rather than preferences for flat or vertical net surfaces, and it is therefore hypothesised that on cone-shaped nets, most contact would be made with the analogous area of the LLIN above the volunteer's torso.

Different manufacturers use different net mesh densities, which can affect air flow speed across net surfaces (von Seidlein *et al.*, 2012). Nets are shared between an average of 2.19 occupants (Kilian *et al.*, 2010). The number of people under a net, as well as the net's design could affect the distribution of attractive cues on the net surfaces, which in turn could influence and potentially alter the areas mosquitoes preferentially attack.

The four newly described behaviour modes provide a means to measure and compare the effectiveness of different treatments, including repellents or attractants. It is also hoped that the results will contribute towards the identification of possible new approaches to target anophelines. To maximise LLIN performance, new designs should ensure that novel chemistries or other treatments do not impair the essential attractiveness of a human-baited LLIN; indeed, efforts to enhance or exploit it should be pursued.  The results might also lead to improved vector sampling, *e.g.* CDC light traps or other devices placed directly above bed nets might yield better samples (Mboera *et al.*, 1998).

Mosquito velocities, measured here in free-flying anophelines responding to complete human hosts, were variously equal to or higher than those recorded in previous tracking studies with the same mosquito species (Beeuwkes *et al.*, 2008; Takken *et al.*, 1997a; Spitzen *et al.*, 2013). This is in spite of the consideration that 2D tracking will underestimate velocity, as movement in the z axis cannot be recorded. Hence the high velocities of the present study may be explained by: (1) the larger test setting used in the present study, as without the confinement of a wind tunnel, mosquitoes were free to move faster; (2) the method by which velocity was calculated in the present study: – using only track sections prior to first net contact, and not including post contact activity. The latter point would produce a higher average flight speed than methods which follow short contacting flights as short bouncing or visiting mode attack flights on the net will be slower than activity in free flight (Angarita-Jaimes *et al.*, 2016).

Velocities were faster in the presence of the host (previously reported with wind tunnels [Spitzen *et al.*, 2013]) but significantly slower (approximately 10%) when the host was protected by an LLIN. However, the velocities measured close to the bed net surface, reported here for the first time, were similar at LLINs and untreated nets.  The results are significant, first, because there were no significant differences in the proportions of mosquitoes that decelerated prior to contact and the distance from the net where deceleration occurred, further evidence for the inability of *An. gambiae* to detect the LLIN, and the absence of significant repellent properties, even at close range.  Secondly, they indicate that prior to contact, mosquitoes detected the presence of net barriers, including the unbaited untreated net. Landing behaviour in insects is strongly linked to visual interpretation of proximity to a surface (Goodman, 1960; Wagner, 1982; Baird *et al.*, 2013) and the eyes of nocturnally active mosquitoes like *An. gambiae* are sensitive to conditions of low visible light (Land *et al.*, 1999).  Tests were carried out using LEDs with a peak wavelength of 850nm, beyond the visual perception range of *An. gambiae* (Gibson, 1995).  Despite our efforts, it cannot be guaranteed that the test insectary was totally dark and it is possible that a light leak from visible LEDs on various devices within the test room might have allowed *An. gambiae* to navigate visually.

Alternatively, mosquitoes may have detected changes in air movement or the odour plume on coming in to proximity with the net surface (Gillett, 1979; Belanger & Willis, 1996), using the Johnston's organ or halteres, which are involved in the detection of mechanosensory cues (Dickinson, 1999; Gewecke *et al.*, 1974; Yorozu *et al.*, 2009). Notably, *An. gambiae* showed similar responses by avoiding 'invisible' clear plastic obstacles when orienting to host cues in a wind tunnel study (Hawkes, 2013), which gives evidence that response is not entirely visually controlled.

Unlike coordinated landing on the tarsi, uncontrolled collision could potentially influence the quantity of insecticide deposited onto a mosquito, and it was questioned whether mosquitoes were responding sufficiently far in advance to avoid 'crashing' into the LLIN. Deceleration started at only 0.12 seconds prior to net contact (26-41mm from the net). There are no appropriate studies available on mosquitoes for comparison, but *Drosophila melanogaster* flying at 300 mm/s began deceleration when 27 mm away from the landing point (van Breugel & Dickinson, 2012), values that are remarkably similar to those measured in our study. That study also showed that the deceleration point varied with flight velocity: slower-flying *Drosophila* began deceleration closer to the landing point, also seen in our data. This provides further evidence that mosquitoes detect the presence of net barriers prior to contact.

Ongoing work will explore flight trajectories further and investigate responses in resistant malaria vector populations, other LLINs and other mosquito species. The tracking system has been deployed in rural locations in Africa where preliminary results indicate that these laboratory findings are representative of wild populations (Angarita-Jaimes *et al.*, 2016; Chapter 6). Though still at an early stage, already these findings significantly contribute to the evidence base required for improved vector control tools by identifying previously unrecognised vector behaviours that may be vulnerable to targeting via simple interventions, and mechanisms that identify potential routes for reducing quantities of insecticide used or for the use of previously unavailable insecticide classes. They also provide a base for further research on basic behaviour and much-needed studies into behavioural mechanisms of insecticide resistance. Not least, the study provides a new platform for elucidation of LLIN function and evaluation of new LLINs (Malima *et al.*, 2009; Farehnorst *et al.*, 2011; Ngufor *et al.*, 2014) and other vector control tools such as spatial repellents (Achee *et al.*, 2012a), at a rapid and cost-effective screening stage prior to larger scale testing in the field (WHO, 2008a).

### *5.4.1 Summary*

This study made first use of a newly developed flight tracking system to observe mosquito flight around bed nets in a laboratory setting. Results were used to characterise movement of host seeking mosquitoes in to four behavioural modes (swooping, visiting, bouncing and resting). Around untreated baited nets, mosquitoes engaged in persistent bouncing flight, concentrating activity on the net roof above the volunteer’s torso. Insecticide treatment of the net decreased the proportion of activity spent in behavioural modes with high bed net contact, and reduced persistence of attack, but did not change the areas of the net mosquitoes made most contact with. No evidence was found for repellency, with the reduction in activity indicating either contact irritant or toxic insecticide effects. Velocity measurements indicated that mosquitoes are able to detect the bed net surface (treated or untreated) prior to making contact. Results reveal that LLINs have rapid impact, causing a decrease in mosquito attack following brief contact with the net.

# Chapter 6 Behaviour of a Wild *Anopheles arabiensis* Population Host Seeking at an LLIN in a Semi-Field Trial in Tanzania

## Abstract

Determining the behavioural impacts of insecticide treatments is fundamental in understanding the mode of action of long lasting insecticide treated nets (LLINs). Laboratory-based behavioural tests often suffer constraints of scale or are forced to operate under conditions that are far from representative of field settings. The present study used a large scale semi-field behavioural assay to gather data on the impact of LLINs on a wild population of *Anopheles gambiae s.l.* mosquitoes, at a field site in Mwanza, Tanzania. Here, the host seeking behaviour of mosquitoes around human-baited bednets was observed in a closed experimental hut using the infrared camera tracking system described in Chapters 2 and 5 to record flight in 2D. The mosquitoes tested were collected as larvae from local breeding sites, and were identified as predominantly *Anopheles arabiensis*. Insecticide treated nets reduced mosquito activity to 32% of the levels recorded at untreated nets. This reduction principally impacted on resting and ‘bouncing’ behaviour (flights making brief repetitive contacts interspersed with short flights across the net surface; see Chapter 5). As in previous reports, the majority of activity occurred on the net roof (85.0% of activity on untreated nets, 56.8% on LLINs). The total time that a mosquito spent in contact with the net was estimated at 204-290s on an untreated net and 46-82 seconds on an LLIN. Latency to net contact was not significantly affected by treatment, implying that insecticide-treated nets were not repellent, but instead impacted mosquitoes only post-contact with the net. This study successfully demonstrated the feasibility of using a complex 2D tracking system to observe flight of wild host seeking mosquitoes in a semi-field setting in Tanzania. The tracking results obtained will be useful to our understanding of net function, and design and testing of new interventions.

## 6.1 Introduction

Investigating vector behaviour in the laboratory has an advantage in that many experimental conditions, including time of testing, light cycles and light intensities, temperature and humidity, can be controlled. However, to complete a series of tests, mosquitoes must be reared in large numbers in colonies, usually maintained for many generations in an insectary, leading to concerns that the inevitable inbreeding that occurs during colonisation could alter behaviour to an extent where results obtained in laboratory studies may not be entirely representative of the true behaviour of insects in the field.

A number of studies have directly compared behavioural traits of mosquitoes in field and laboratory settings. Experiments in Benin compared the effects of holed Permanet 3 LLINs on *Cx. quinquefasciatus* in tunnel tests and experimental hut trials. The authors reported that blood-feeding inhibition appeared higher in tunnel tests than in huts, though this could have been in part due to effects of scale (see below) and accessibility of the host, or to the use of a guinea-pig as bait, a less favoured host for this species (N'Guessan *et al.*, 2010).

Tests of *Ae. aegypti* behaviour in high throughput screening in labs and experimental hut work in Thailand examined the impact of insecticides on mosquito movement (Grieco *et al.*, 2007). Contact irritant effects of alphacypermethrin observed in the laboratory appeared to be faster and stronger than when examined in the field, but behavioural results with other chemicals were consistent between the two studies, *e.g.* dieldrin elicited no behavioural response from mosquitoes in either setting.  The differences in response to alphacypermethrin might be attributable to the differences in scale, inclusion of a human bait in field work, or the relative accessibility of exit routes in smaller vs. larger tests.

Field testing commonly entails an increase of scale from small assay tests to experimental huts. As discussed in chapters 1 and 3, the size of a test chamber may affect mosquito behaviour (Hossain & Curtis, 1989; Barnard *et al.*, 1998; McMeniman *et al.*, 2014). However with the camera system developed and used throughout the studies in this thesis, there was no such change in test scale, which consistently filmed an volume within a room of 1.2 x 2.4 x 2.0m (height x width x depth).

There is little evidence that mosquito attraction to humans is affected by ethnicity of the human host (Schreck *et al.*, 1990). However the nature of mosquitoes used in experiments can affect experimental results. As discussed in the general introduction, colonisation effects may result in reduced attraction of mosquitoes to host odours, or adaptation of olfactory preferences (Laarman, 1958; Clark *et al.*, 2011).

The length of time a colony has been kept in captivity may affect responses to insecticide. Several studies have compared the behaviour of different mosquito strains when responding to chemicals in escape box tests, where rates of mosquito escape from boxes lined with treated papers are recorded over time. Tests found that recently colonised *Aedes aegypti* showed stronger reactions to DEET repellent and alphacypermethrin treatment than strains reared in colonies for over 10 years (Thanispong *et al.*, 2009; Sathantriphop *et al.*, 2014b). Conversely, newer strains were less responsive to DDT, while responses to other repellents and control treated papers were not affected by times kept in colony (Thanispong *et al.*, 2009; Sathantriphop *et al.*, 2014b). In a different study, recently colonised *An. minimus* showed lower responses to alpha-cypermethrin than a 15 year old colony strain (Malaithong *et al.*, 2011). However, in these studies, the more recently colonised strains had higher levels of insecticide resistance than older strains, which may have influenced results. Chareonviriyaphap *et al.* (1997) removed this variable in a study of two insecticide susceptible strains of *An. albimanus*, and still found differences in behaviour: the more recently colonised population showed stronger escape responses to permethrin, deltamethrin and DDT than the matched strain that had been in colony for 20 years. However in tests with deltamethrin resistant *Culex quinquefasciatus* populations, the recently colonised strain showed the weaker escape response to deltamethrin and fenitrothion, while no there was no difference seen in response to propoxur (Sathantriphop *et al.*, 2006). This shows that the relationship between insecticide responses and duration of colonisation is unpredictable. Furthermore, the available evidence does not clarify whether behavioural changes might have occurred over generations, or resulted from differences in the ancestral wild population from which the colony was established.

Air movement has been shown to affect host seeking activation rates, and the dispersal of host generated odour plumes (Murlis *et al.*, 1992; Geier *et al.*, 1999; Lacey & Cardé, 2012). In laboratory tests, such as those reported in Chapter 5, experiments are conducted typically in closed, draught-proof or sealed rooms, in order to maintain stable conditions of high humidity and temperature and to avoid any uncontrolled or unpredictable air movements. This could result in artificially regular odour plumes emanating from the host, unlike in field settings in which air movements are possible through windows and eave gaps in housing, and where plumes are likely to be more turbulent and complex (von Seidlein *et al.*, 2012). In addition, tests conducted in field settings will more closely match conditions of temperature and humidity mosquitoes experience during host seeking in the wild (Ferguson *et al.*, 2008).

Malaria is transmitted by several mosquito species and sub-species. To provide a comprehensive picture of LLIN function in different settings, it will be important to investigate the behaviour of a range of vector species. Though *An. gambiae s.s.* is the most anthropophagic vector, with the strongest endophagic tendencies (Gillies & De Meillon, 1968; Pates & Curtis, 2005; Takken & Verhulst, 2013), *An. funestus* and *An. arabiensis* are also significant vectors in sub-Saharan Africa.

*An. arabiensis* may display different behavioural responses to insecticide than *An. gambiae s.s.*, for example, showing slightly less blood-feeding inhibition in tunnel tests with carbosulfan insecticide (Malima *et al.*, 2009). Experimental hut tests conducted in Tanzania further suggested that *An. gambiae s.s.* was less susceptible than *An. funestus* and *An. arabiensis* to blood-feeding inhibition by various insecticides (Malima *et al.*, 2009). Controlling for insecticide resistance, *An. funestus* showed higher responsiveness to irritant effects of deltamethrin than *An. gambiae s.s.* in high throughput screening assays (Kawada *et al.*, 2014). However in the same study, *An. gambiae s.s.* and *An. arabiensis* exhibited similar behavioural responses to permethrin in contact irritancy assays.

Species of the *An. gambiae* complex may prefer to bite different parts of the human body: *An. arabiensis* has a stronger tendency to bite the feet and legs of a seated human than *An. gambiae s.s.*, which prefers the legs but will also bite other body parts (Dekker *et al.*, 1998; Braack *et al.*, 2015). This could influence the surface of a bed net preferentially attacked by different species. Testing of new interventions should therefore consider various vector species, in order to ensure effectiveness in the event of such potential behavioural differences.

The use of camera tracking systems in field and semi-field situations presents some practical difficulties, including challenges in organising an uninterrupted power supply for long filming periods in remote areas, the potential for ‘invasion’ or confounding of a test by non-target insect species, and difficulties in achieving the laboratory performance of sensitive equipment under field conditions .

These challenges are not insurmountable, and other studies have tracked flight of swarming mosquitoes in their natural habitats. In Japan, swarming male *Culex sp.* were visualised in 3D using a three camera system, which captured a filming volume greater than 1.2x1.2x1.2m, illuminated with a flashlight (Ikawa *et al.*, 1994). This method captured three single photographs of a mosquito swarm, which were used to generate 3D coordinates of all individuals in the swarm. Due to technical limitations of camera resolution (the study was carried out over 20 years ago), this system procured single snapshots of mosquito position in time, rather than video sequences of trajectories.

More recently, advances in digital recording and tracking methods allowed extension of this principle to track swarming male *Anopheles gambiae s.s.* in flight in 3D, using two cameras to observe a 1.5x1.5x1.5m filming volume (Butail *et al.*, 2012). Swarming male mosquitoes were tracked at natural field sites in Mali. Power supply in this instance was limited to 30 minutes, but due to constraints in track handling capabilities of the analysis software, the periods recorded did not exceed a maximum of 90 seconds. The use of visible light by both tracking systems means that they cannot be directly applied to nocturnal host seeking flight by females: tracking flight in conditions of darkness requires development of novel techniques to overcome illumination challenges of filming using other light wavelengths that cannot be perceived by mosquitoes.

In the study described here, the large scale filming system used previously in the UK (Chapters 2 and 5) was deployed at a rural field site in Mwanza, Tanzania. Using 2-dimensional tracking the effects of an LLIN on host seeking flight by mosquitoes reared from local wild populations were quantified.  This experimental setup offered the opportunity to investigate the responses of wild vector populations, of additional species, and to compare with or validate the results from laboratory-based behavioural studies with colonies in UK, reported in Chapter 5.

This study aimed to apply large scale 2D tracking methods to a semi-field setting in Tanzania, recording effects on insecticide on host seeking behaviour of *An. gambiae s.l.* mosquitoes. Building on the work of laboratory results the experiment hypothesis was that mosquitoes would be irritated but not repelled by insecticide, spending less time host seeking at insecticide treated than untreated nets. Insecticide treatment is not hypothesised to influence the net surfaces mosquitoes approached, but is expected to reduce the persistence of mosquitoes attacking the net.

## 6.2 Methods

### *6.2.1 Mosquitoes*

Larvae were collected from a rice paddy area in Magu, Tanzania (-2°33'41"S 33°18'11"E). These were returned to the insectaries at the National Institute of Medical Research, Mwanza, for rearing. Larvae were fed on a diet of fish food, and adults were allowed access to 10% sugar solution *ad libitum*. Adults were maintained on a 12:12 light: dark cycle which approximately matched the hours of sunrise and sunset in Mwanza. Upon eclosion, mosquitoes were sorted to select members of the *An. gambiae* species complex using morphological keys of Gillies and Coetzee (1987) and Gillies and de Meillon (1968). Several larval collections were made across the duration of the experiments, and only the F0 insects were used in tests. No data is available on the biting times of mosquitoes in Mwanza district. Species used in this test are generally found to be most active in host seeking between 22:00 and 24:00pm, but the peak of this activity varies by location (Mathenge *et al*, 2001; Fornadel *et al*, 2010; Russell *et al*, 2011; Yohannes & Boelle, 2012; Bayoh *et al*, 2014).

**Species Identification and Insecticide Susceptibility**

*An. gambiae* species complex PCR ID was conducted periodically throughout the testing period using adult mosquitoes that had been classified using morphological characters as members of the *An. gambiae* species complex (Scott *et al.*, 1993). A WHO insecticide susceptibility bioassay was performed on 3-5 day old mosquitoes (n=22) using 0.05% deltamethrin treated papers, with one hour exposure as per standard methodology (WHO, 1998). Mortality outcomes were corrected according to Abbott’s formula.

### *6.2.2 Study Site*

The study was conducted in Kayenze, Mwanza, Tanzania ($2^{0}$23’43”S, $33^{0}$0’5”E), in a rice growing area roughly 13km north-east of Mwanza city. Mwanza region has the one of the highest values for malaria prevalence of Tanzania’s 30 regions; rapid diagnostic tests of children under 5 find 18.6% disease prevalence in this area (Demographic and Health Surveys, 2012). Survey data found approximately 95% of households had access to at least one insecticide treated net, and indoor residual spray programs using pyrethroids cover 40% of households (Demographic and Health Surveys, 2012; President’s Malaria Initiative, 2014).

The experimental hut was situated in a savannah area, with scattered trees, and a small stream to the south. The surrounding land was predominantly used in rice growing, and for grazing cattle and goats, with some additional farming of mango trees and tomato plants. The experimental site was situated 70m from the nearest house, and 240m from a cattle enclosure. Experiments were conducted in July and August 2014.

### *6.2.3 Experimental Hut*

Tests were conducted in a 5x5x2.5m wooden experimental hut (Figure 6.1A). The hut was built using locally purchased plywood, which had been treated with anti-termite paint over a year prior to the start of these tests. The roof was of gable design, and the height from its apex to the floor 3.5m. The roof had interior wooden panels, and the exterior was covered by thatch made from local grass, which was itself covered with tarpaulin to ensure the structure was weather-proof. A 10cm eave gap ran around all four sides of the house, which was covered with plastic mesh netting to prevent mosquito entry or exit, when reared mosquitoes were being released inside the hut.

**Figure 6.1 Photograph of experimental hut in Mwanza, Tanzania**

Image A shows the large plywood experimental hut, and the adjacent plastic control shed from which recordings were operated. Camera cables ran through the wall of the experimental hut to the computer in the control shed. Image B shows experimental hut interior, with paired Fresnel lenses surrounding the bed. LEDs (out of view on left of image) illuminate the field of view, and images are recorded by two cameras (image right).

The floor of the experimental hut was raised off the ground by 50cm on wooden stilts which were placed in buckets of water to prevent entry of ants. The door measured 0.6m by 2m, and was closed during tests so that no insects could enter or exit the room.

Cables from the two cameras ran out of the experimental hut through a port in the wall, to the computer housed in an external control shed, 1.3m away from the experimental hut. The observer operated the recording system from the external shed. The equipment was powered using a Honda EU20i generator, with a rated output of 1600W (Seddon Direct, UK). The complete tracking recording system was estimated to draw approximately 600W, and could be powered by the generator without refuelling for over 6 hours.

### *6.2.4 Test Procedure*

Tests were performed using ten female *An. gambiae* complex mosquitoes, 3-5 days post eclosion. Mosquitoes were selected for the experiment 2 hours prior to testing by placing an arm against a cage and aspirating mosquitoes that attempted to feed. Mosquitoes were placed in a paper cup and sugar starved for 1-2 hours prior to the start of the experiment. One hour prior to recording, the volunteer entered the bed net, and the paper cup of mosquitoes was hung at eave height on the wall of the experimental hut. At the start of tests a cord was pulled from outside the experimental hut to remove the net cover from the paper cup, inverting it and releasing mosquitoes in to the room. Activity was recorded for 60 minutes.

At the end of the 60 minutes, mosquitoes were collected using a prokopack aspirator (Vazquez-Prokopec *et al.*, 2009), but post-test mortality was not recorded as it was considered that collection process was highly likely to have caused sufficient damage and stress to the mosquitoes that it would influence results.

Tests used Permanet 2 LLINs (deltamethrin concentration 55mg/m$^2$) and untreated bed nets (assembled from untreated polyester net of similar mesh), both of which had been tailored to fit the field of view. Ten tests were conducted with each net (*i.e.* 20 tests in total, using a total of 200 mosquitoes), and test order was randomly allocated. Up to two tests could be conducted per night, though on days when two tests were conducted the same net was used for both tests.

Members of the local community volunteered to act as bait. 4 female and 6 males participated in the ten tests. Half of the volunteers lay with their heads on the left hand side of the field of view, and half with their heads on the right, to control for possible effects of the position of the mosquito release point on net approach.

Tests were conducted between the hours of 21:00 and 00:30, to coincide with peak biting periods for *An. gambiae* complex mosquitoes.

### *6.2.5 Tracking Wild Populations*

Further to the test described a number of tests were conducted in which the door of the experimental hut was left open, and the mosquitoes from the surrounding area permitted to approach. However initial trials found that a mixed population of numerous mosquito species within 3 genera, *Anopheles*, *Culex*, and *Mansonia*, entered the hut, along with insects from other families, including Lepidoptera and Chironomidae.  Since it was not possible to identify the mosquitoes flying around the bed net, this approach was not taken forwards for tracking and analysis.

### *6.2.6 Tracking*

Mosquito activity was recorded using the tracking system described in the General Methods of this thesis (Chapter 2.2). In brief, two cameras running at 50fps recorded a 1.2x2.4m field of view illuminated with two high powered infrared LEDs. Four Fresnel lenses were used to collimate and focus the light between the LEDs and the cameras (Figure 6.1B). The lens pairs were spaced 1.96m apart, producing a filming volume of 1.2x2.4x1.96m, which contained the bed and human baited bed net. Videos were recorded to .seq files using StreamPix software. Mosquito tracks were identified and analysed using custom written Matlab applications (Angarita-Jaimes *et al.*, 2016).

### *6.2.7 Mosquito Activity and Behavioural Modes*

Data and observations on flight activity and behavioural modes were obtained and extracted as described in chapters 2 and 5. Effects of net treatment on activity were analysed using cluster adjusted regression analysis (StataCorp, 2013), adjusting for matched identity of volunteers (STATA). Almost all volunteers participated in two tests (one with an untreated net, one with an LLIN). Using cluster adjustment in the model ensured standard errors incorporated the intragroup variation of 'volunteer identity'. By specifying cluster, the model accounted for correlation in mosquito activity that would be attributable to variation in attractiveness of the individual volunteers to mosquitoes.

The effects of net treatment were only investigated statistically for swooping and bouncing behavioural modes, to avoid analysing multiple non-independent variables. For these, impact of net treatment on time spent in the behavioural mode (swooping or bouncing) was assessed using cluster adjusted regression analysis (StataCorp, 2013), as described above (STATA). Activity times spent in all behavioural modes are presented in qualitative summaries in graphs, tables and text.

### *6.2.8 Flight Speed and Tortuosity*

Flight speed and tortuosity were calculated as described in the General Methods chapter of this thesis. As described, speed values only describe swooping tracks, as net contacting tracks were presumed to be slower, and not equally represented in the two net treatment types. The explanatory variable of net treatment on the dependent variables speed and tortuosity were analysed using cluster adjusted regression analysis (StataCorp, 2013), adjusting for repeated tests by the same volunteers (STATA).

### *6.2.9 Distribution of Activity on the Bed net*

As in chapter 5, the field of view was sub-divided in to 16 regions (Figure 6.4), activity was scaled by region size (s/m$^2$) in analysis of total activity, and activity within the behavioural modes and physical contact time were analysed by unscaled time (seconds). Spatial preferences were assessed using a generalised linear model that included terms for net treatment, region, and an interaction term between net treatment and region to assess whether activity was evenly distributed across the field of view (SPSS Statistics, version 21, IBM).

### *6.2.10 Physical Contact with Net*

Total duration of physical contact made with the net incorporated resting and walking on the net, as well as shorter contacts made during bouncing and visiting. The effect of net treatment on time spent in physical contact with the net was assessed by cluster adjusted regression analysis (StataCorp, 2013), adjusting for possible variations resulting from different levels of attraction with different volunteers (STATA). The range of times an individual mosquito may have spent in contact with the LLIN were calculated as described in results chapter 5.

### *6.2.11 Activity Over Time*

Repellency was investigated using measures of time lag to first appearance of a mosquito in field of view and time to the first mosquito’s net contact. These values were evaluated using a Log Rank Mantel-Cox survival analysis in SPSS version 21 (IBM). Two tests were conducted to assess the effect of the explanatory variable of net treatment against the outcome variables of time lag to first appearance, and the time between mosquito release and net contact. If nets were repellent, mosquitoes in treated tests would be expected to take longer to appear in the camera field of view, and take longer to make first contact with the net.

Mosquito activity over the 60 minute recording period was assessed for suitability for exponential decay modelling, but many of the tests violated equation constraints. Instead, tests assessed the difference between activity in the first five minute interval (0-5 minutes) and the final interval (55-60 minutes). To do this, total activity recorded in the first interval was subtracted from total activity recorded in the final time interval to produce a value which if negative indicated activity decay, and if positive indicated that activity had increased during between mosquito release and test end. These values were compared using cluster adjusted regression analysis (StataCorp, 2013), adjusting for volunteer effects (STATA) to investigate effect of net treatment on attack persistence.

## 6.3 Results

A total of 20 tests (200 mosquitoes) were completed using mosquitoes reared from local populations at Kayenze, Mwanza, between the 14th July and 23rd August 2014.

### *6.3.1 Identification of mosquito species*

The identity of 142 mosquitoes that had been identified morphologically as members of the *Anopheles gambiae* complex were investigated using the standard PCR method for this species complex (Scott *et al.*, 1993). A majority of 86.6% (123/142) were identified as *An. arabiensis*; 4.2% (n=6) were *An. gambiae s.s.*, but in 9.2% (13), the PCR failed to produce a result.

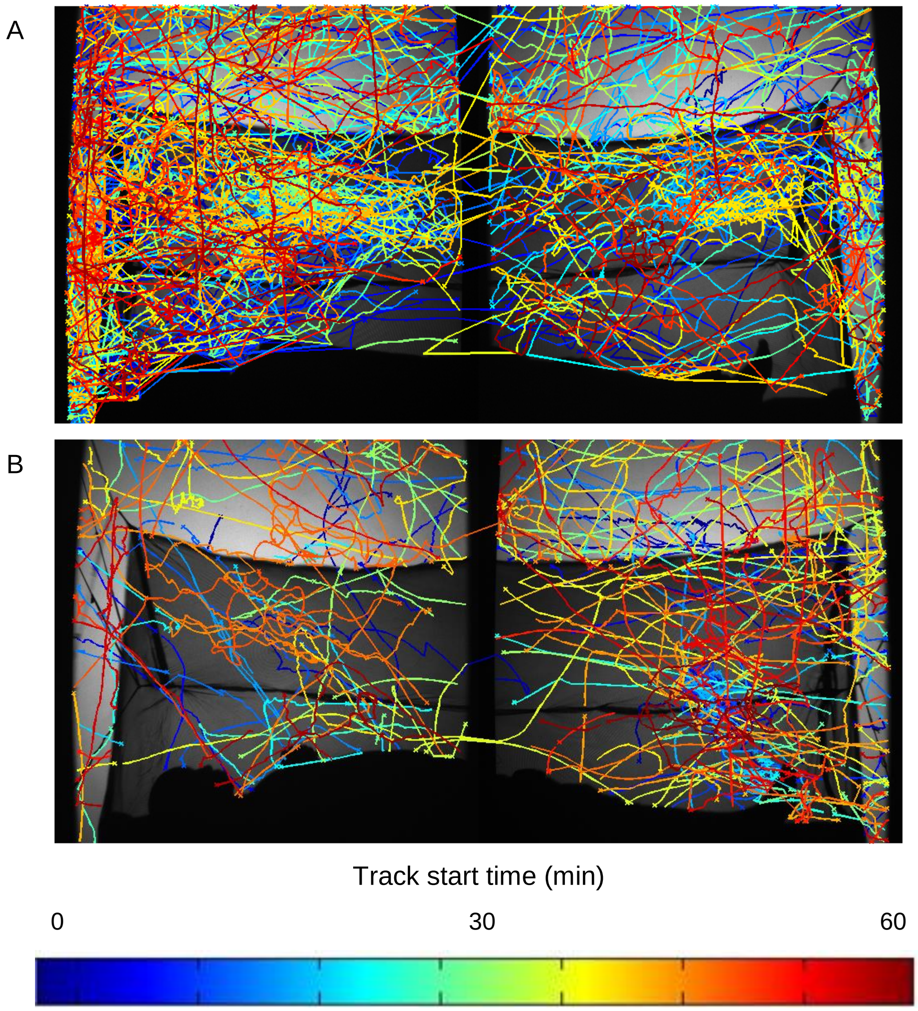


**Figure 6.2 Flight activity of field-caught mosquitoes at untreated nets and LLINs.**

Track images show activity of 10 female mosquitoes recorded over 60 minutes in response to a human volunteer protected within (A) an untreated and (B) an insecticide-treated bed net (Permanet 2, Vestergaard Frandsen, Switzerland). Each track shows an individual flight path. Tracks are colour coded by time of occurrence (blue at start of hour, red at end, see colour bar).

### *6.3.2 Insecticide Susceptibility*

Mosquitoes were classified as insecticide susceptible. Using WHO insecticide bioassays, in which mosquitoes were exposed to 0.05% deltamethrin treated papers, knockdown after 1 hour was 95%, and mortality after 24 hours was 100%.

### *6.3.3 Responses of Mosquitoes to LLINs and Untreated Nets*

In total, ten untreated and ten LLIN tests were completed, each using 10 mosquitoes. Across both net treatments, track durations ranged from 0.79 seconds to 13.6 minutes. The geometric mean of track duration was 1.53s (1.47-1.59, n=7631 tracks). As shown in Figure 6.2, total activity per test was higher in tests with untreated nets (73.5 minutes [42.6-126.8]) than with LLINs (23.8 minutes [14.7-38.5])(F(1, 10)=9.26, *p*=0.012; difference estimate 62 minutes, 95% CI 17-109 minutes). The Supplementary Video 6.1 (available online and in Supplementary CD) provides a representative sequence of *An. arabiensis* flight around an LLIN. Tracks in this clip demonstrate visiting and bouncing activity.

Comparing time spent in swooping and bouncing modes (Table 6.1), differences in activity between nets were more pronounced in some behavioural modes than others. Net treatment did not significantly affect mean times spent swooping (F(1, 10)=1.04, *p*=0.332,), but the presence of insecticide significantly reduced activity in in bouncing (F(1, 10)=18.48, *p*=0.002,). The proportion of total activity time spent in either bouncing or resting activity was 77% at untreated nets and 37% at LLINs (Figure 6.3). As mean time spent swooping was not reduced by insecticide treatment, the reduction in activity in other modes meant that the proportion of time spent in swooping flight rose from 6% at untreated nets to 23% at LLINs (Figure 6.3).

**Table 6.1 Mean total activity time (minutes) female mosquitoes spent in different behavioural modes over 60 minute tests in the field hut.**

Geometric mean with 95% confidence intervals, 10 repeat tests per treatment; 10 mosquitoes per test. As multiple mosquitoes were active simultaneously, the total activity time may exceed 60 minutes. Asterisks indicate results where activity for a given behavioural mode was significantly different between net treatments (*p*<0.05). Statistical tests were only conducted on total activity, and activity in swooping and bouncing behavioural modes.

| | Swooping | Visiting | Bouncing | Resting | Total |
|---|---|---|---|---|---|
| Untreated Net | 4.0<br>(2.8-5.7) | 11.7<br>(6.9-19.8) | 38.6<br>(19.5-76.4) | 13.4<br>(7.6-23.5) | 73.5<br>(42.6-126.8) |
| LLIN | 4.9<br>(3.2-7.5) | 8.7<br>(5.0-15.2) | 5.3<br>(2.5-11.5)* | 2.8<br>(1.7-4.8) | 23.8<br>(14.7-38.5)* |

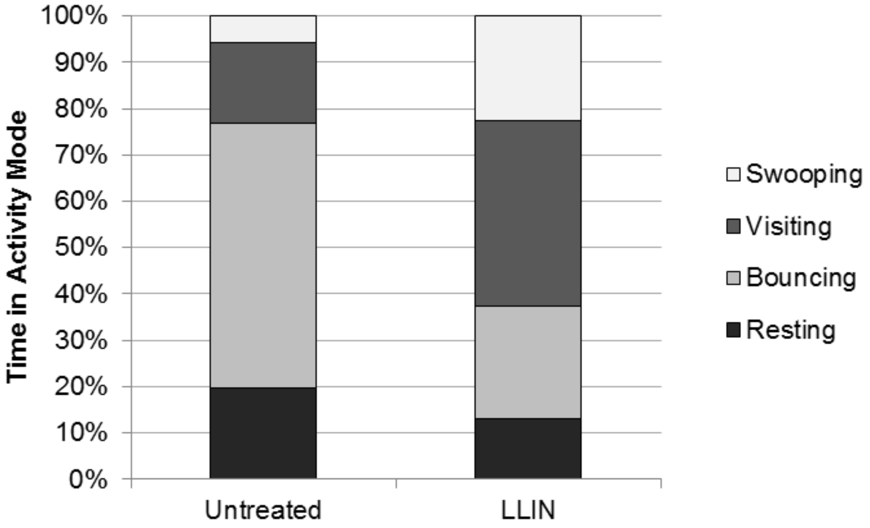


**Figure 6.3 The proportion of time spent by female mosquitoes in each behavioural mode, for the two net types (untreated and LLIN), during tests conducted in the experimental field hut.**

### *6.3.4 Flight Speed and Tortuosity*

Swooping mosquitoes in untreated tests flew at a mean instantaneous velocity of 327 mm/s (306-348). In LLIN tests mean swooping velocity was not significantly different (353 mm/s [318-388] LLIN, F(1, 10)=3.09, *p*=0.109).

Track tortuosity was not significantly affected by net treatment (F(1, 10)=0.22, *p*=0.650); mean tortuosity of tracks at an untreated net was 1.35 (1.20-1.50), and at LLINs mean tortuosity was 1.40 (1.27-1.53).

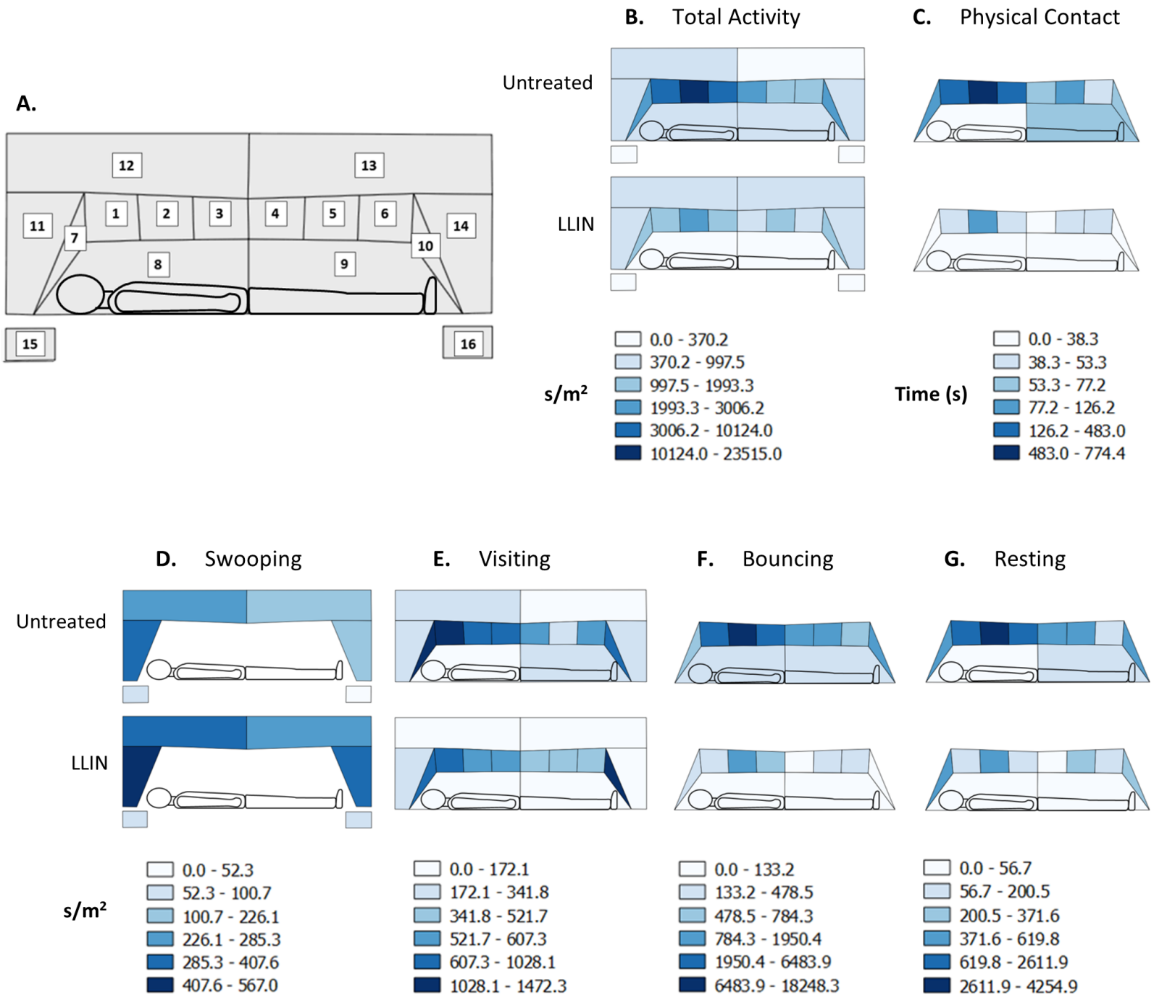


**Figure 6.4 Distribution maps of mosquito flight activity on untreated and treated nets showing activity in different behavioural modes and net contact at different regions.**

(A) Distribution map key showing region codes for different areas of the field of view. Regions 1-6 represent the bed net roof; 7 and 10 are the vertical surfaces at the head and foot ends; 8 and 9 are vertical side surfaces. Activity in the space around the net was assigned to regions 11-14. Regions 15 and 16 contain swooping activity occurring in front of the net, on the left (15) and right (16) side of the field of view.

(B) Density of total activity (all behavioural modes) s/m$^2$ (C) Distribution of physical contact with the net (in seconds). Includes resting, and brief mid-flight contact made during visiting and bouncing.

(D-G) Distribution of activity for each behavioural mode: D. Swooping, E., Visiting, F. Bouncing, G. Resting. Values are expressed as activity density (s/m$^2$). Colour coding is specific to each image, as shown in the legend beneath each chart. Charts only include regions relevant to each behavioural category, hence swooping chart (D) does not use net regions 1-10 , and resting chart (G) does not include the space around the net (regions 11-16). Although the volunteer's position was rotated in experiments, all charts are presented with the volunteer's head shown on the left.

### 6.3.5 Location of Activity at the Bed Net Interface

Insecticide treatment significantly reduced total activity levels ($X^2$ (1)=17.81, *p*<0.001). This decrease was seen for bouncing ($X^2$ (1)=16.01, *p*<0.001) and resting ($X^2$ (1)=21.96, *p*<0.001), but not swooping ($X^2$ (1)=3.77, *p*=0.052) or visiting ($X^2$ (1)=0.92, *p*=0.337). Accounting for these effects, generalised linear models also indicated spatial differences in activity distribution.

Total activity density was unevenly distributed across the entire field of view ($X^2$ (15)=234.69, *p*<0.001), with most activity occurring on the net surfaces (regions 1-10). Only 3.8% and 15.9% of total activity occurred in the spatial regions around the bed net in untreated net and LLIN tests respectively. The majority of activity occurred on the net roof (regions 1-6: 85.0% on untreated nets, 56.8% on LLINs; Figure 6.4B), with a small proportion occurring on the net end next to the feet (region 10, 4.6% untreated, 2.0% LLIN). There was a significant interaction between net treatment and total activity distribution ($X^2$ (15)=33.54, *p*=0.004): the proportion of activity occurring in the important regions of 1-3 (*i.e.* over the host torso) was significantly higher for untreated nets (74.2%) than for LLINs (38.4%).

Regions 15 and 16 (the spaces in front of the bed net, Figure 6.4A) were the sites of least swooping activity ($X^2$ (5)=66.77, *p*<0.001); 10.4% of untreated net swooping, and 10.9% of LLIN swooping density occurred here. Net treatment did not affect distribution of swooping flights ($X^2$ (5)=3.71, *p*=0.592; Figure 6.4D).

Most visiting activity occurred on the roof of the net above the volunteer’s torso regions 1-3 (39.9% on untreated nets; 29.9% on LLINs; $X^2$ (13)=89.91, *p*<0.001). Regions 7 and 10 respectively accounted for 16.1% and 10.2% of visiting activity on untreated nets, and 13.7% and 20.8% of visiting at LLINs. Net treatment did not significantly affect the distribution of visiting activity ($X^2$ (13)=10.42, *p*=0.659; Figure 6.4E).

The majority of bouncing activity occurred in region 2 on both untreated nets (50.4%) and LLINs (42.4%; $X^2$ (9)=45.73, *p*<0.001; Figure 6.4F). In contrast, very low levels of bouncing occurred at the lower body portion of the net regions 4-10 and 13-16 (13.8% of untreated bouncing, 22.8% of LLIN bouncing occurred in these regions). Net treatment affected distribution of bouncing ($X^2$ (9)=28.14, *p*=0.001), as mosquitoes on untreated nets showed a stronger preference for region 2 of the roof (Figure 6.4F).

Activity in resting mode was unevenly distributed between net regions ($X^2$ (9)=63.12, *p*<0.001). High levels of resting were observed on region 2 above the volunteer's chest in both untreated nets (38.6%) and LLINs (21.0%; Figure 6.4G). However, there were significant differences ($X^2$ (9)=27.59, *p*=0.001) in distribution of resting events according to net treatment: in LLINs, the highest density of resting (21.8%) was recorded on the vertical surface of the net adjacent to the head (region 7), where only 5.6% of resting occurred on untreated nets. Thus, the majority of resting events occurred on regions 1-3 at untreated nets, but were distributed across regions 2, 5, 7 and 9 on LLINs (Figure 6.4G).

### *6.3.6 Quantifying Duration of Net Contact*

Levels of physical contact with the net are derived from a combination of visiting, bouncing and resting activity, and hence the distribution of contact mirrors that of these behavioural modes (Figure 6.4C). On both net types, the highest level of physical contact occurred in region 2, where the ten mosquitoes collectively made a mean duration of 774s of contact in untreated nets and 126 seconds in LLINs (equivalent to 37.7% and 26.6% of total contact time; $X^2$ (9)=30.09, *p*<0.001). Regions of net contact were influenced by net treatment ($X^2$ (1)=20.00, *p*=0.011): in untreated nets, the majority of net contact (76.7%) occurred at roof regions 1-3, whereas in LLINs, net contact occurred here (47.0%), and at roof regions 5 and 6, above the volunteer's feet (21.2%).

**Table 6.2 Duration of physical contact made with the net during the 60-minute test.**

Duration of physical contact made with the net during the 60-minute test (seconds). Table shows mean total contact time observed (all mosquitoes); the minimum mean contact time per mosquito (assuming all 10 mosquitoes responded), and the calculated maximum mean contact time per mosquito (based on the maximum number of individual mosquitoes observed simultaneously in each test). Values shown are mean (seconds) with 95% CI. Mean total contact time was significantly higher at untreated nets than LLINs (*p*=0.010).

| | Mean total contact time (s; all mosquitoes) | Minimum contact time per mosquito (s; 10 mosquitoes responding) | Maximum contact time per mosquito (s; max observed no. responding) |
|---|---|---|---|
| Untreated Net | 2036 (947-3126) | 204 | 290 |
| LLIN | 465 (266-663) | 46 | 82 |

Total net contact duration was significantly higher in untreated nets than LLINs (Table 6.2; F(1, 10)=10.07, *p*=0.010 [mean difference = 1572 seconds; 95% CI=468-2675). The longest contact time recorded for a single track was 285 seconds on an untreated net, and 155 seconds on an LLIN. Tracking limitations meant it was not possible to measure actual total contact time for individual mosquitoes over the 60 minutes, so information on the maximum number of mosquitoes observed simultaneously active in the field of view was used to calculate a range of plausible estimates of contact time for single mosquitoes. This range was estimated at 82-290 seconds at an untreated net, 46-204 seconds at an LLIN over the 60 minute test (Table 6.2).

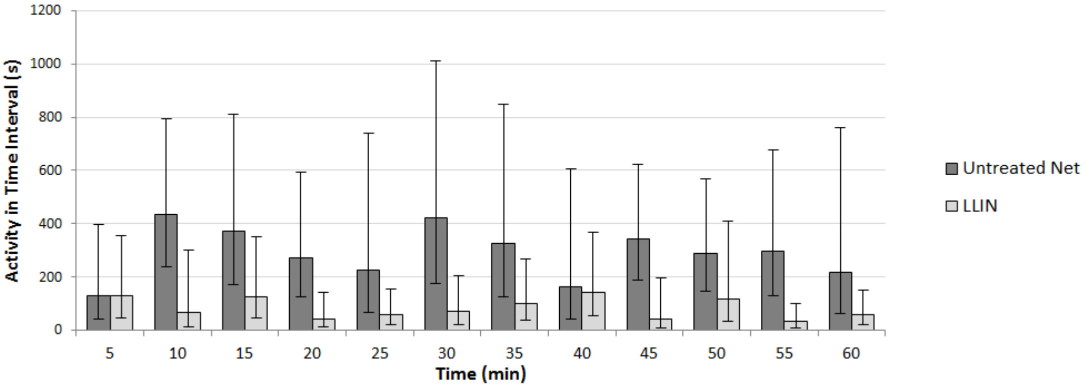


**Figure 6.5 Activity decay over time in untreated nets and LLINs across the 60 minute test.** Values show geometric mean (±95% CI) per 5-minute interval of the 60 minute test. *i.e.* 5 (0 – 4 min 59 s), 10 (5 min – 9 min 59 s) etc.

### *6.3.7 Interactions with the bed net over time*

Results did not provide any evidence for repellent effects of the LLIN on net approach. The delay prior to the first mosquito's appearance in the field of view was not significantly affected by net treatment ($X^2$ (1)=0.60, *p*=0.438), with a geometric mean delay from release to appearance of 8 seconds (95% CI = 4-14) in untreated nets, and 16 seconds (95% CI = 1-39) in LLINs.

In untreated nets, mosquitoes first contacted the net at a geometric mean of 36 seconds (95% CI 7-89) after release, whereas in LLINs first contact occurred at a geometric mean of 46 seconds (95% CI 9-119) after release, times that were not significantly different ($X^2$ (1)=0.89, *p*=0.766). Individual test results could not be modelled for exponential decay over the 60 minute test, as only 5 of 10 untreated net tests and 8 of 10 LLIN tests fit model assumptions of decreasing activity over time. As shown in figure 6.5 activity in untreated net and LLIN tests commenced at similar levels but showed different trends over time, with a greater decrease in activity when nets were insecticide treated (F(1, 10)=6.81, *p*=0.026).

**See enclosed CD for: Supplementary Video 6.1: Mosquito flight at a human-occupied LLIN in an experimental hut in Mwanza, Tanzania** The video is a 45 second clip of mosquito activity at an LLIN in the experimental hut. Tracks on the roof of the net engage in bouncing and visiting behaviour, before one disengages with the net and exits the field of view. Video also accessible at:

https://www.dropbox.com/s/n2l4d8eqd92pcyv/SupplementaryVideo_Chapter6_1.avi?dl=0

## 6.4 Discussion

The results presented here are the first flight trajectories recorded from a wild or natural population of mosquitoes using this tracking system and as such, may represent the most detailed flight data of any malaria vector recorded under such natural conditions. The mosquitoes used in the tests were predominantly pyrethroid susceptible *An. arabiensis.*

Comparing responses at LLINs and untreated nets, insecticide treatment reduced net attack, as measured by the number or frequency of flights, chiefly affecting behavioural modes involving higher levels of net contact, and activity in the behavioural modes of bouncing flight, and resting. The majority of flight activity occurred on the roof of the bed net in the area above the volunteer's chest (regions 1-3; Figure 6.4B), though the preference for these three regions was more pronounced in untreated nets than LLINs. Net contact estimates suggested that an individual mosquito made at least 46 seconds of physical contact with the LLIN. No evidence was found for repellency, hence the insecticide treatment would likely have exerted post-contact effects on host seeking behaviour, reducing activity at the net following physical contact.

Findings provide an important comparison for laboratory test results reported in Chapter 5, which were conducted using Kisumu strain *An. gambiae s.s.*. Results of the two test settings were broadly similar: insecticide reduced activity after net contact, and most attack occurred on the roof surface above the volunteer’s chest. As tests conducted in chapter 5 and the present chapter examined similar outcomes using the same recording methods it is interesting to compare the results. However such comparisons must be viewed in light of the fact that not only were tests in the present chapter conducted in Tanzania, but they used a different mosquito species, released lower numbers (10 in Tanzania, 25 in the laboratory), and worked in a larger test room. While recognising that these differences are important, for convenience, the two sets of tests will be referred to simply as laboratory or field within this discussion.

### *6.4.1 Effects of Insecticide on Activity*

Insecticide treatment of the net caused a reduction in mosquito activity around the net compared to untreated nets, and this reduction affected behavioural modes which have most contact with the net (bouncing and resting). These results are in agreement with a number of behavioural studies that have found insecticide treatment of nets to reduce host seeking activity (Siegert *et al.*, 2009; Strode *et al.*, 2014). However the extent of this reduction will vary according to net treatment and test methods.

Due to lower availability of mosquitoes in the field, field experiments used 10 mosquitoes in each test, whereas laboratory work used 25. Mosquitoes showed higher activity in field trials: though fewer mosquitoes were released, total activity time was 91.9 minutes at untreated tests, compared to 124.6 minutes at untreated net tests in the laboratory. Insecticide treatment reduced activity to 32% of untreated net values in the field, whereas laboratory results had found a reduction to 17% of untreated net activity.

Previous comparative studies have suggested that some species and genera are more active than others during host seeking and during behavioural bioassays (Chareonviriyaphap *et al.*, 2004; Cooperband & Allan, 2009; Sutcliffe & Yin, 2014; Angarita-Jaimes *et al.*, 2016). As Tanzanian tests used *An. arabiensis* rather than *An. gambiae s.s.*, the higher levels of activity observed may result from innate differences between species, rather than effects of the field setting, or impact of colonisation on the laboratory strain. Data from tests with the Kisumu strain of *An. gambiae s.s.* in the experimental hut in Tanzania have been collected but have not yet been analysed. Future analysis of these data will help elucidate the relative importance of these different variables in host seeking behaviour at bed nets.

### *6.4.2 Velocity and Tortuosity*

Neither laboratory nor field tests detected any effect of net treatment on track velocity and tortuosity. Velocities of *An. arabiensis* in all baited tests were between 322-355mm/s, and tortuosity ranged from 1.36-1.66. In the laboratory, *An. gambiae s.s.* speeds were comparable (Chapter 5): mean velocities ranged from 321-327 mm/s, and mean tortuosity values fell between 1.63-1.66. Velocities recorded in the present study with *An. arabiensis* were higher than those observed in 3D tracked wind-tunnel host seeking experiments, where upwind velocities ranged from 50-260 mm/s, and the highest average downwind flight velocity recorded was 272mm/s (Takken *et al.*, 1997a; Beeuwkes *et al.*, 2008; Spitzen *et al.*, 2013). Yet the Fresnel lens system is likely to underestimate true velocity and tortuosity since recording tracks in 2D does not capture information on movement in the z-axis. Values will therefore be underestimated for tracks flying in the z direction (*i.e.* towards the camera or LED), and true values of flight velocity and tortuosity probably lie slightly above the range reported in these tests. However even with this recording constraint, values exceeded those recorded in smaller bioassays, suggesting that mosquito flight is constrained by the dimensions of wind tunnels in smaller scale laboratory tests (Takken *et al.*, 1997a; Beeuwkes *et al.*, 2008; Spitzen *et al.*, 2013).

### *6.4.3 Activity Distribution on the Bed net*

Total activity, bouncing, and resting flight densities were highest in the region of the roof above the volunteer's chest. This agrees with previous results reported by other authors (Lynd *et al.*, 2013; Sutcliffe & Yin, 2014) and with laboratory tests on *An. gambiae s.s.* (Chapter 5) which also noted high levels of activity at this part of the net. As discussed in detail elsewhere (Chapters 1, 5 and 7) this behaviour suggests attraction to odours of the volunteer's breath and body which may be carried up towards the roof of the net by convection currents created by heat, in what has been termed the 'chimney effect' (Guillet *et al.*, 2001; Sutcliffe & Yin, 2014).

Though preference for the region of the net directly above the volunteer's chest was seen in LLINs and untreated nets, insecticide treatment significantly dampened this preference relative to other areas of the net. Sutcliffe & Yin (2014) found a similar effect when examining flight of *An. gambiae s.s.* and *An. albimanus* around a Permanet 2; though the insecticide did not alter the focus of activity at the roof of the net, there was some variation in the clustering of net contact at different surfaces. As data on individual mosquitoes' physical contact with the net show that mosquitoes at LLINs cease attacking the net sooner, it might be speculated that net exploration patterns change with time spent on the net, ultimately focussing on the area presenting strongest host cues. This could account for some of the differences in behaviour observed. Alternatively small differences in net material and mesh size, which were closely but not exactly matched might influence airflow and host cue concentrations at different areas of the net.

It is notable that for both laboratory (Chapter 5) and field tests, untreated nets recorded comparable proportions of activity on the net roof and foot end of the net (laboratory test activity, 74.7% and 10.9%; field test activity 85.0%, 4.6%; regions 1-6, and 10 respectively). Values for LLINs were markedly different as activity on the roof and foot end of the net respectively accounted for 78.3% and 8.8% in laboratory, and 56.8% and 2.0% in the field. There were also some minor differences in activity distribution at LLINs; in laboratory tests the highest levels of activity occurred at region 3 of the net roof (above the volunteer's stomach) whereas in field tests, region 2 (above the volunteer's chest) was the focus of activity on the roof. Ultimately attraction to the net surface may depend on the specific conditions in the room, varying according to the size and number of net users. WHO LLIN distribution goals aim to provide one net for every two household occupants (WHO, 2014). Further tests will be necessary to examine whether spatial preferences of mosquitoes are affected by the number of people sleeping under the net.

Lowest levels of swooping flight were recorded in regions 15 and 16, which represent the space between the bed net and the Fresnel lenses. This difference may be related to experimental design, as the volume of these regions was lower than areas 11-14; the 3D volumes of these regions were smaller than the 3D volumes of regions above and around the bed net, as in regions 15 and 16 the bed net occupied almost half of the space in the z axis, a point that was not accounted for in analyses of the 2D flight tracks. Alternatively, the low activity observed in regions 8 and 9 (the sides of the net) could have been artificially reduced by the placement of the Fresnel lenses, which may have blocked approach to these surfaces, thus forcing or channelling flight towards the higher regions around the roof. This potential source of bias was investigated by repeating these tests with the bed net and lenses in a different configuration (*i.e.* with the bed and bed net rotated through $90^o$, such that the head and feet ends of the bed now faced the Fresnel lens). It was not possible to complete analysis of this data in time to include it in this thesis, but early qualitative observation has suggested that net orientation did not significantly alter mosquito activity around the net, and that the placement of the Fresnel lenses is unlikely to be the cause of the preferences for different regions of the net observed in this thesis.

The 2D nature of our tracking system may have underestimated contact with the bed net in regions 8 and 9, as the sharp angle movement towards and away from the net that constitutes a visit would not be visible to the cameras when occurring in the z axis on these surfaces. As such some tracks contacting the sides of the net would have been wrongly classified as swooping. However, since total activity (Figure 6.4B), which includes all four behavioural modes, upholds the mosquito preference for the net roof observed when measuring total physical contact times (Figure 6.4C), any bias that may have occurred, is unlikely to have been significant. Data from earlier sticky net studies, which are not susceptible to skew by lens placement and tracking bias, also showed similar patterns of attack, (Lynd *et al.*, 2013; Sutcliffe & Yin, 2014) increasing the credibility of the data recorded here. Work of this thesis has built on previous observations of sticky net studies to characterise the type and duration of contacts mosquitoes make with the net during host seeking, providing new insights in to mosquito activity around bed nets.

The relative proportions of activity in the different regions were largely similar to those observed in the laboratory, as most physical contact with the net occurred on the net roof above the volunteer's torso, though high levels of activity on the end walls of LLINs in field tests (regions 7 and 10) were not seen in laboratory tests.

At the time this study was undertaken, it was not possible to obtain sufficient numbers of *An. gambiae s.s.* mosquitoes in this field location, or a 'pure' population of *An. arabiensis* and all tests used a population consisting predominantly of *An. arabiensis*. *An. arabiensis* mosquitoes have been found to show a slightly stronger preference for biting the feet and legs of seated humans than *An. gambiae* and *An. quadriannulatus* (De Jong & Knols, 1995; Dekker *et al.*, 1998; Braack *et al.*, 2015). Laboratory and field based comparisons of bites received by volunteers seated on chairs and lying on the ground suggest that biting preferences of *An. gambiae s.s.*, *An. funestus* and *An. arabiensis* are determined more by proximity of body to the ground than innate attraction to the odours or other attractive cues emanating from the feet or lower body (Dekker *et al.*, 1998; Braack *et al.*, 2015). How these preferences affect mosquito flight paths during host approach, which will in turn affect net surfaces contacted, is as yet unstudied, but differences in the behaviour of these closely-related sibling species could be responsible for the increased activity on the ends of the net observed in experimental huts here.

### 6.4.4 Persistence of Net Attack, and Impact of Insecticide

In these field tests with wild *An. arabiensis*, activity started at relatively low levels and remained low at the LLIN with little fluctuation over the entire test period. Though activity across the 60-minute test was lower at treated than untreated nets, there was no evidence for the decay in activity at the LLIN as seen in laboratory tests in chapter 5.

In the laboratory tests of Chapter 5 it was possible to analyse activity over the hour's test period using models of exponential decay, but the data of the present study showed an increase in activity on the untreated net after the initial 0-5 minute time interval and remained at high levels for the duration of the test. LLIN activity declined over the test period, but though laboratory tests found activity was reduced to near negligible levels within 30 minutes of release, field tests recorded low levels of net attack continuing until the end of the test. No repellent effects of the Permanet 2 LLIN have been observed in any of the large scale filming trials, as assessed by the time mosquitoes take to approach and contact the net. The finding that activity levels were lower around LLINs than untreated nets could therefore be the result of knockdown, or toxic impact of insecticide impairing host seeking, either in addition to or instead of contact irritant insecticide effects. Further detailed data on the lethal effects of short contact periods with insecticide will be crucial to determining the action of LLINs.

Since only 10 mosquitoes were released at one time it was possible to obtain a more precise estimate of the range of time a single mosquito may have spent in contact with the net, than in previous laboratory tests where 25 mosquitoes were released. In laboratory tests, a single mosquito was estimated to have made 18-96 seconds of physical contact with the bed net, whereas in these field tests with *An. arabiensis,* the range was 46-82 seconds. Although this range is within that of the laboratory test results, the significant decay in activity seen in the laboratory tests did not occur in the field. Mortality of mosquitoes following the test was not recorded as it was considered that damage sustained in collection methods could affect results, therefore consequences of this insecticide exposure to *An. arabiensis* must instead be inferred from bioassay results. Few data are available evaluating the effects of brief insecticide contact periods on mortality and knock-down, but it is possible to extrapolate trends from flight tracking tests and cone bioassays. Median knock-down times (KD50) demonstrate duration of insecticide contact required to induce immediate knock-down. *Anopheles gambiae s.s.* exposed to 50mg/m$^2$ deltamethrin have KD50 times between 5-8 minutes depending on insecticide formulation in forced contact bioassays (Skovmand *et al.*, 2008). This dosage is roughly equivalent to the 55mg/m$^2$ concentration applied to Permanet 2, the LLIN used in field tests. Using the WHO diagnostic dose of 20 mg/m$^2$ gives KD50 times of 10-17 minutes in *An. gambiae s.s.*, and 25 minutes in *An. arabiensis* (Hougard *et al.*, 2003a; Kawada *et al.*, 2014).

In flight tracking experiments that precisely quantified individual *An. gambiae s.s.* contact with treated materials, any mosquito contacting a net treated with a deltamethrin for over 40 seconds was knocked down at 1 hour post-testing, and dead after 24 hours (Spitzen *et al.*, 2014). The insecticide concentration used was approximately equivalent to that employed in LLIN tests in the present study. As *An. arabiensis* and *An. gambiae s.s.* display similar knock-down times (Kawada *et al.*, 2014), it is reasonable to assume that mosquitoes attacking the LLIN in field tests reported here would have been knocked down by the insecticide treatments used. However, Spitzen's results must be interpreted with some caution due to the low number of mosquitoes tested (35 in total, only 6 of which were knocked down), tests of more mosquitoes and different sub-species would be useful to further investigate knock-down effects.

It is crucial to note that bioassays assessing KD50 times (WHO, 2013c) show the constant contact period required to cause knock-down in real-time observation, whereas Spitzen *et al.* (2014) recorded knock-down 60 minutes after testing. Employing a 24 hour follow up period, 3 minutes of exposure to deltamethrin in a WHO cone test is sufficient to cause 100% mortality in insecticide susceptible *An. gambiae s.s.* one day after exposure (Hougard *et al.*, 2003a). The difference between KD50 tests and tracking studies suggests that the 46-204 seconds of contact accrued in LLIN testing (Table 6.2) may not induce immediate knock-down of mosquitoes, but would affect mosquito survival 1-24 hours after net attack. One may speculate therefore that mosquitoes attacking an LLIN may survive long enough to attempt feeding on close neighbours in the period following insecticide exposure, but mortality effects will be visible within one day of net contact.

It would be useful to assess effects of different insecticide exposure periods on mosquito mortality and host seeking abilities, using methods similar to those employed by Spitzen *et al.* (2014). In Spitzen's method insecticide contact was not forced by use of small enclosure bioassays, but instead encouraged by placement of attractive cues behind the treated net. In the large-scale tracking system tested in this chapter, data on lethal effects of insecticide could be gathered by recapturing mosquitoes after tests using mouth aspirators, which would be less damaging than the vacuum prokopack aspirator. However since filming can only track mosquito activity in the space of the camera field of view, unless insects were released individually the time an individual mosquito had spent in contact with insecticide would not be known. This would be a very time consuming series of tests but would generate a substantial quantity of valuable and reliable basic data on the performance of the insecticides used widely to prevent transmission of this serious and often fatal human infection.

As discussed, the transition from laboratory to field setting, and use of *An. arabiensis* may explain some of the differences between results reported in this chapter and chapter 5. A number of conditions changed between tests, which may also have impacted on results: field tests were conducted in a slightly larger room, with a higher ceiling, and the potential for movement of air currents through the screened eaves. Test chamber size has been shown to affect behaviour in small-scale laboratory assays, but less is known about impact of room size on behaviour (Hossain & Curtis, 1989; Barnard *et al.*, 1998; McMeniman *et al.*, 2014). Mosquitoes were released in smaller groups (10 rather than 25), and were sugar starved for a shorter time prior to experiment start. There is some evidence that mosquito biting behaviour may be influenced by the number of individuals attacking, with mosquitoes showing increased likelihood to feed on a host when others have already bitten (Alekseev *et al.*, 1977; Charlwood *et al.*, 1995). However, this is thought to relate to odours released upon biting the skin (Ahmadi & McClelland, 1985), and there is no evidence that responses differ when numbers exceed 10 mosquitoes.  Hence there is thus far no evidence for interactive effects of mosquito group size on host location and bed net approach.

### *6.4.5 Summary*

A key outcome of this work is the demonstration that this novel camera tracking system can be applied to semi-field settings, the first time host seeking flight has been recorded at this scale, using exclusively infrared light, outside of the laboratory. The development of a technically complex system than can be applied in this setting provides opportunities for further detailed field observation of mosquito behaviour. In this work, mosquitoes of known species were released in to a closed 'semi-field' room, as the insect population in the area comprised a mix of *Culex*, *Anopheles*, and *Mansonia* mosquitoes and the objectives of the study encompassed only malaria vectors. If a field site comprised a single dominant species however, experiments could be conducted in true field conditions, allowing host seeking mosquitoes to locate, enter, and depart from the room independently. This would avoid influencing attack persistence by blocking mosquito exit from a sealed semi-field room. The potential for identification of individuals to genus or species level by trajectory differences remains to be investigated, but development of such algorithms would require an extensive period of data gathering and evaluation.

Different levels of activity were observed with *An. arabiensis* in the field and *An. gambiae s.s.* in the laboratory, with higher and more persistent activity levels of *An. arabiensis* affecting the amount of time mosquitoes spent in physical contact with the bed net. Though general trends of attack were similar, the proportional reduction in activity resulting from insecticide treatment of the net, and details of activity distribution showed some differences between the laboratory and field tests. *An. arabiensis* showed a less marked preference for the roof and foot ends of the net, and the focus of the field mosquitoes' activity was directed towards the volunteer's mid-torso, rather than the lower torso, as observed in laboratory tests with *An. gambiae s.s.*. As field tests also used different mosquito species, smaller group sizes, and a larger test room, it is not possible to conclude which factors might have caused these changes. However field results provide encouraging evidence that rearing mosquito populations in colonies has not fundamentally affected host seeking behaviour of mosquitoes within the home, and that broadly similar trends in attack and deterrence are maintained in different settings.

Further work undertaken for this project has attempted to elucidate the relative importance of mosquito species on behaviour through tests using Kisumu strain *An. gambiae s.s.* in the experimental hut in Tanzania. Results of these tests are not yet available, but will help establish whether results observed are the consequence of differences of experimental setting between the laboratory and field, or biological differences between mosquito sub-species.

# Chapter 7 Host Seeking of *An. gambiae s.s.* in the Absence of a Bed Net

## Abstract

The host seeking activity of mosquitoes is often investigated in response to individual attractant cues, but due to technological limitations, few studies have been able to unobtrusively record host seeking behaviour of mosquitoes in response to a human bait in a domestic setting. Information on how mosquitoes approach unprotected humans is useful in evaluating how vector control tools disrupt the normal host seeking flight path. This study uses infrared camera tracking methods to observe flight of *An. gambiae s.s.* around an unprotected human. Numbers and locations of bites were recorded to investigate biting preferences of mosquitoes at the supine host.

Flight tracks of mosquitoes approaching, and departing the host following potential bloodfeeding, were recorded and track attributes compared to assess if there were differences between flight tracks of active host seeking and (probable) bloodfed female mosquitoes.

Results indicated that mosquitoes entered the field of view at flight elevation of 1m (95% CI: 0.95 to 1.06), and moved towards the bait in tortuous ‘visiting’ flight modes, making only 1.8 (95% CI: 1.6 to 2.0) contacts with the human prior to settling on the volunteer’s body. After bite numbers were scaled by the area of the volunteer’s exposed skin, there was no preference for any part of the body, and bites were evenly distributed across available skin. Mosquitoes leaving the host flew in less tortuous flight paths, though flight velocity was unaffected, and they exited the field of view at a similar flight elevation of 1.0m (95% CI: 0.95 to 1.05). The high tortuosity of flights approaching the host is suggestive of casting behaviour, and the descent of mosquitoes from above onto the host may be speculated to suggest the insects are responding to convective currents around the host. However, as used for this purpose, the 2D back-lit tracking method had limitations, as mosquito tracks were not visible when moving on or adjacent to the volunteer’s body. This issue could be overcome by release of individual mosquitoes rather than groups to account for track breaks resulting from this visual block, or by the introduction a 3D filming element to partially recover the lost section of the field of view.

## 7.1 Introduction

LLINs have been widely distributed for malaria control and are thought to be responsible for a significant fraction of the recent decrease in cases (WHO, 2014; Bhatt *et al.*, 2015). However it is difficult to achieve full compliance with nightly bed net use, with some people choosing not to use nets due to discomfort experienced whilst sleeping under them (Pulford *et al.*, 2011; WHO, 2014). LLINs are further restricted by the emergence of insecticide resistance (Mnzava *et al.*, 2015; discussed in Chapter 1). Additional vector control tools that might be used to augment or replace LLINs could potentially be designed rationally to target specific behaviours of host seeking mosquitoes (malERA Consultative Group on Vector Control, 2011). The design of alternative vector control interventions would benefit from information on the routes mosquitoes use in approaching a host. Earlier studies tracked mosquitoes attacking human hosts at a bed net (Arredondo-Jiménez *et al.*, 1997; see Chapters 5 and 6). The presence of a bed net will alter how mosquitoes approach a sleeping human, possibly by changing air currents in the space around the sleeper (von Seidlein *et al.*, 2012), or by physically blocking preferred routes. Hence it is essential to study mosquitoes at unprotected hosts to gain a fuller understanding of their flight patterns in the absence of an intervention.

*Anopheles* mosquitoes use odours from breath and body, heat and moisture to locate a host (Takken & Knols, 1999; Cardé & Gibson, 2010), and have been shown capable of detecting a bait from distances of over 30m away (Gillies & Wilkes, 1970; Lorenz *et al.*, 2013). Volatile attractive cues are thought to form odour plumes which are dispersed by air movement, expanding outwards from the host, and these may be used by insects moving upwind to locate a human over long ranges (Bowen, 1991; Murlis, 1992; Takken *et al.*, 1997a). *Anopheles gambiae* move upwind in visually guided flight termed optomotor anemotaxis (Gibson, 1995), integrating chemical cues from the host in tortuous flight up a chemical gradient, termed chemotaxis (Kennedy, 1983; Cummins *et al.*, 2012; see literature review in chapter 1).

A number of tracking studies reported that mosquitoes fly in more tortuous paths when approaching host cues (Beeuwkes *et al.*, 2008; Spitzen *et al.*, 2013). Turns appear to relate to passage through the odour plume, as mosquitoes moving out of the plume rapidly turn to re-enter the plume when flying upwind towards a host (Spitzen *et al.*, 2013), producing tracks that resemble the casting flight of moths following pheromone plumes upwind (Cardé & Willis, 2008).

Combinations of attractive cues release stronger behaviours than when presented individually, and this has been shown to impact short-range host location. For example, likelihood of successful location of and landing on an odour source in a wind tunnel is greatly improved when the stimulus is heated, or supplemented with $CO_2$ (Pates *et al.*, 2001; Healy & Copland, 2002; Spitzen *et al.*, 2013; Webster *et al.*, 2015), although when presented alone, heat or odour stimulate poor landing responses (Kröber *et al.*, 2010; Webster *et al.*, 2015). Further to this, odour cues have been found to be more attractive when presented as blends than when offered to a mosquito individually (Smallegange & Takken, 2010). Though $CO_2$ has often been described as a long distance cue (Gibson & Torr, 1999), mosquitoes also use it during close range navigation: $CO_2$ must be presented simultaneously with odour cues to evoke increased landing responses, as a prior encounter with the cue is not sufficient to cause heightened odour responses (Webster *et al.*, 2015). This is yet another reason (see Chapter 1) why it is important to test host location behaviours using a live human as bait.

Many tests to date have employed wind tunnels to simulate upwind flight of mosquitoes approaching a host from a distance. However within the home, wind movement is unlikely to guide a mosquito to the host. It is thought instead that convection currents play a role in the final discovery of the host, as evidenced by observations around human volunteers, activity around bed nets, and movements in vertical tunnel tests (Khan & Maibach, 1966; Khan, 1968; Dekker *et al.*, 1998; Sutcliffe & Yin, 2014; Chapters 5 and 6).

Upon reaching the body of seated hosts, *An. gambiae s.s.* and *An. arabiensis* preferentially bite the feet (De Jong & Knols, 1995; Dekker *et al.*, 1998; Braack *et al.*, 2015). However volunteer posture is important and a volunteer lying on the ground will be bitten evenly across their body (Dekker *et al.*, 1998; Braack *et al.*, 2015). Mosquitoes fly close to the ground (Snow, 1979), and it has been suggested that bites concentrate on low parts of the body either because these are located easily or as a result of innate biting preferences (Dekker *et al.*, 1998; Braack *et al.*, 2015).

Much less is known about the mosquito's behaviour as it departs from the host following blood-feeding. During blood-feeding a mosquito may imbibe a blood weight greater than 60% of its own body mass (Reid *et al.*, 2014), which has concomitant impact on flight ability. A blood-fed mosquito may fly at a lower velocity, and make more tortuous tracks (Roitberg *et al.*, 2003). The effects this has on vulnerability to predation are unclear: there is some evidence that blood-fed mosquitoes are less able to evade spider predators during flight (Roitberg *et al.*, 2003). Other work on mosquito predation by geckos found that blood-fed mosquitoes suffered less predation (Canyon & Hii, 1997). Those authors suggested that following feeding, mosquitoes spend more time resting and less time in flight, a behavioural change that reduces their exposure to the geckos and conceals their decreased mobility. After or during engorgement, mosquitoes may commence prediuresis (the excretion of water from ingested blood plasma), excreting urine and concentrating the blood meal. This involves a substantial loss of mass, roughly 50% of the blood meal (Mahmood & Nayar, 1989, Gray & Bradley, 2005). The majority of the excess fluid is excreted within 20-30 minutes, but it can take a full 90 minutes before the prediuresis is complete (Williams *et al.*, 1983, Mahmood & Nayar, 1989). Critically however, when the blood-fed mosquito completes her blood meal and flies away from the host, she must carry the additional weight of the largely unconcentrated blood meal in her abdomen.

In the hours and days following blood-feeding, mosquito circadian flight activity is reduced (Jones & Gubbins, 1978, Rowland, 1989, Lima-Camara *et al.*, 2014), and host seeking behaviour is inhibited (Takken *et al.*, 2001). However this effect is not due to the additional load of the blood meal as the change is contingent on insemination: virgin females show little if any reduction in locomotor activity following blood-feeding (Rowland, 1989, Lima-Camara *et al.*, 2014). Work to date has focussed on a longer time scale, and little is known about the immediate impact of blood-feeding on mosquito flight.

The flight paths and responses of mosquitoes during close range approach have yet to be studied in detail. Most large scale tests investigating host seeking behaviour have tended to report quantities of bites received rather than quantifying characteristics of approaching flight (Lines *et al.*, 1987; Arredondo-Jiménez *et al.*, 1997; Dekker *et al.*, 1998; Braack *et al.*, 2015). In such studies volunteers are required to monitor mosquitoes and in Braack *et al,* 2015, aspirate insects from the body themselves, introducing potential for host movement to disturb mosquito activity. Further to this little is known of the impedance caused to flight by feeding, as studies investigating host seeking behaviour commonly remove mosquitoes before biting is completed (De Jong & Knols, 1995; Dekker *et al.*, 1998; Braack *et al*, 2015). As such there is much still to learn about the close-range host-seeking behaviour of mosquitoes around humans. This study will examine the flight of mosquitoes immediately prior to, and after landing on the human bait, observing activity within 1 metre of the host. Though this range of host seeking has been examined in wind-tunnels (Lacey *et al.*, 2014; Spitzen *et al.*, 2014), such tests take place under influence of moderate air movements, and commonly use artificial baits to attract mosquitoes. By contrast, this study will follow mosquito activity through approach, host attack and departure, in a semi-field setting, in order to provide new insight into host seeking within the home.

This study aimed to investigate the routes of approach chosen by host seeking mosquitoes flying towards a supine human. The tracking system described in the General Methods (Chapter 2), and installed in the semi-field system in Tanzania, was used to observe flight during host approach and departure, investigating how mosquitoes chose a site for blood-feeding, and how the flight behaviour changed after ingestion of a blood meal.

The objective of this study was to investigate flight of mosquitoes approaching a host to blood-feed, and compare tracks to those of mosquitoes that were leaving the host, and therefore no longer considered to be responding to host cues. It was expected that a high proportion of mosquitoes departing the host would have bloodfed, and it thus hypothesised that this would impede flight ability of mosquitoes, which might be observed through slower flight speeds and shorter, less tortuous flights  A secondary objective of this study was to record bite sites of mosquitoes at the supine volunteer, testing the hypothesis that insects host seeking at a human lying down will not show preferences for feet, instead biting opportunistically at exposed skin.

## 7.2 Materials and Methods

### *7.2.1 Mosquitoes*

Mosquitoes were 3-5 day old female Kisumu strain *An. gambiae s.s.* reared in NIMR Mwanza insectaries at 27 ± 1.5$^0$C and 80 ± 8% relative humidity (RH). Larvae were fed ground fish food, and adults were maintained on 10% glucose solution. Adults were blood-fed on rabbits. The insectary ran a 12:12 light:dark cycle coinciding with the hours of sunrise and sunset in Tanzania, and tests were performed between 9pm and 1am at night, within a one week period in June 2014.

### *7.2.2 Volunteers*

Three volunteers were used for this test (one male, two female) age range 26-54. Volunteers all went barefoot, with their arms either fully bare or with a thin cardigan over upper arms, and legs uncovered from the knee down. Volunteers wore no perfumed cosmetics, and had not washed for at least ten hours prior to testing. The volunteer alternated their orientation between tests, lying with their head in the left or right side of the field of view on alternate tests. The three volunteers alternated their participation in the test, but fully random allocation order was constrained by volunteer availability. The three volunteers participated in 12, 7 and 3 tests respectively.

### *7.2.3 Experimental Setting*

Tests were conducted in the sealed experimental hut described in chapter 6. The bed used in this study slightly higher than in the bed net tests; the horizontal surface of the bed here sat 62cm off the ground, to ensure that mosquito flight beneath the level of the bed was contained within the field of view and could be recorded. The flat surface of the bed measured 67x168cm.

### *7.2.4 Recording*

Mosquito activity was recorded using the equipment and experimental hut described in chapters 2 and 6. Unlike other tests conducted with this system, recordings in these experiments were made at 30 frames per second. This frame rate was considered sufficient to distinguish between individual flight paths as in these tests, only 10 mosquitoes were released, and activity was lower with fewer intersecting paths than in chapter 6.

### *7.2.5 Test Procedure*

Mosquitoes were sugar starved for 2 to 4 hours prior to release in tests. Immediately prior to testing, mosquitoes were presented with a human arm against their cage and those attempting to feed were aspirated out. Each test used 10 mosquitoes, which were placed in the test room in a paper cup at eave height on the hut wall, in a position 0.68m from the short end of the bed. Following cup placement, the volunteer lay prostrate and motionless on the bed. Mosquitoes were released from the cup by means of a string pulled from outside of the experimental hut, which removed the net cover and inverted the cup. An observer viewed activity on StreamPix in real time. Recordings began 30 seconds prior to mosquito release and continued until the observer had seen no activity for 5 minutes, or after 30 minutes of filming, whichever occurred soonest. At the end of the test, volunteers reported where on their body they had been bitten. It was not possible to collect mosquitoes to count number bloodfeeding due to the size and height of the test room, mosquitoes could not be collected when resting of the roof interior (height at apex 3.5m). Bites were categorised as being sited on the head & neck, torso, arms, hands, legs, ankles, or feet. 25 test repeats were conducted, in which a total of 250 mosquitoes were released.

### *7.2.6 Tracking Procedure*

Mosquito flight was tracked using methods described in chapter 2. Specifically in this study, a minimum track length of 0.1 seconds was applied (tracks shorter than this were deleted). A higher level of manual tracking was also applied to link tracks that rested on the volunteer’s body beyond the automatic joining time of 2000 frames (67 seconds).

### *7.2.7 Classification of Track Types*

Uncertainties when track linking in recordings where multiple mosquitoes were active at the same time and when activity on the body was obscured by the silhouette of the volunteer, meant that it was not possible to link approaching and departing tracks of mosquitoes that rested for a long time on the volunteer’s body, or to match tracks with reported bite sites. Hence tracks were categorised as approaching and departing flight types, in order to isolate tracks that were approaching to blood-feed and distinguish from those departing after a probable blood-feed.

This was done by manual track analysis. This process selected “approaching tracks” (tracks in which mosquitoes were considered to be responding to host cues), and “departing tracks” (tracks of mosquitoes that had rested on the volunteer’s body, some of which were likely to have blood-fed).

Approaching tracks were defined as incoming tracks that contacted the volunteer's body for more than 1 second. Approaching tracks could consist of visiting, bouncing and resting. Due to breaks in tracks, some visiting tracks were erroneously classified as swooping tracks.

Departing tracks were defined as exiting tracks that were stationary on the volunteer's body for more than 30 seconds prior to flying away. In order to isolate the effects of blood-feeding on flight, trajectories were analysed from the point of departure from the body, excluding small bounces prior to departure.

All other tracks that did not fall in to these categories were discarded. Modes of activity (swooping, visiting, bouncing, resting) were classified as defined in chapter 5 (section 5.3.1).

### *7.2.8 Data Analysis*

Bite numbers per test were assessed for normal distribution using an ungrouped Shapiro-Wilk test. In order to provide an output of mosquito activity that would be comparable to previous studies, which were conducted using different numbers of mosquitoes, for different lengths of time, a value of "activity per mosquito per minute" was calculated. This was done by dividing total tracked activity (in seconds) of all mosquitoes by test duration (in minutes), and further scaling this to the number of mosquitoes released (10 in the present study, 25 in net tests; Chapter 5). This provided an output value for the number of seconds that one mosquito was active during a single recorded minute of testing. The data used for 'total tracked activity' used all tracks *i.e.* not solely those classed as approaching and departing tracks. Comparative values have been calculated using data used from experiments with *An. gambiae s.s.* and bed nets in chapter 5. Differences in the experimental designs of these studies prevented full statistical comparisons.

Data on approaching and departing tracks in the present study was analysed to compare time spent in different modes of activity were compared using generalised linear models (SPSS, version 21, IBM). This used the outcome variable of seconds spent active, and employed explanatory variables of direction of track (approaching or departing), behavioural mode, and an interaction term between behavioural mode and direction of track. Though summary data is provided for all behavioural modes, to avoid issues of non-independence of behavioural modes statistical outcomes will be reported for visiting and resting only.

Track tortuosity and velocity were compared between approaching and departing tracks using a paired t-test that. These tested the effect of the explanatory variable ‘direction of track’ (approaching or departing) on the dependent variables of ‘average value of track tortuosity’ and ‘average velocity’ (SPSS, version 21, IBM).

To determine whether track direction influenced flight elevation, the Y-coordinates of track start and end were recorded in pixels and converted to height (mm) above ground to calculate average height of entry and exit to the field of view per test. This calculation discounted unlinked approaching tracks that started within, and unlinked departing tracks that ended within the boundary of the human body, as these represent broken tracks, the flight elevation of which does not contribute to the research question. The Y-coordinate values were compared between approach and departure using paired t-tests (SPSS, version 21, IBM). Height band settings were based on the position of the bed (low-mid tier) and mid-point of the field of view (mid-high tier; see figure 7.3). All tracks were summed to obtain the percentages entering at each height tier, and the percentage of tracks that ascended or descended between tiers following entry.

To determine whether mosquitoes preferred to make their approach or departure from the volunteer’s head or feet, tracks were classed as being on the ‘head’ or the ‘foot’ side of the human host according to whichever camera had recorded their entry into or exit from the field of view. The effect of track direction (approaching, departing) on the percentage of tracks initiated or ending on the ‘head’ camera view was assessed using paired t-tests (SPSS, version 21, IBM).

Preferred regions of mosquito activity were investigated by sub-dividing the video space in to different regions, and assessing whether activity was evenly distributed amongst them. Filming space was split into 12 individual regions (Figure 7.4), which encompassed different areas around the human host. Vertical divisions between regions were at the volunteer’s neck, hips and ankles.  Horizontal divisions were those described above for height bands. The visible area of each region was calculated  as flight could not be observed when mosquitoes were adjacent to the bed net volunteer: hence, in regions 3, 4, 6, 9, 10 and 11 the visible area value was adjusted to subtract those areas blocked by the silhouette of the human body and/or the bed. The duration of activity occurring in each region was scaled by the area of that region to obtain values of activity density expressed in seconds/m$^2$. Generalized linear models investigated the dependent variable of total activity, visiting,  and resting activity to establish effects of the explanatory variables trajectory direction (approaching or departing), region, and the interaction between the two explanatory variables (SPSS, version 21, IBM).

Track contacts with the body were defined by sharp angle track turns of over 80°, or high frequency oscillations in bouncing mode (Chapter 5, section 5.2.11). Contact positions within different regions were analysed using generalized linear models to examine the effects of region, trajectory direction, and interaction between region and trajectory direction (SPSS, version 21, IBM).

Biting location preference was analysed using generalised linear models, using the explanatory variables of trajectory direction, region, and an interaction term (SPSS, version 21, IBM). The dependent variable of ‘number of bites’ was analysed unscaled for size of different body parts. Data were then transformed to calculate numbers of bites per $cm^2$ of exposed skin, using information on relative sizes of the exposed skin areas on the volunteer’s body, to assess whether biting rates were influenced by preference for particular body parts, or the relative availability of uncovered skin. Pearson’s correlation tests were performed to investigate the relationship between the number of mosquitoes observed simultaneously active (log transformed to achieve normal distribution), and the number of bites received. This output was used to assess whether the presence of multiple mosquitoes at a site disturbed or enhanced biting tendencies of the group. Unless stated otherwise, data are reported as means with 95% confidence intervals.

## 7.3 Results

### 7.3.1 Overview

A total of 25 tests with 10 mosquitoes per test were completed, and a total of 119 bites were reported by the volunteers and logged, equating to 0.48 bites per mosquito. One test was discarded because the mosquitoes made no contact with the volunteer, and therefore 24 tests were used in subsequent analysis. In these 24 tests, the volunteers reported an average of 5.0 (3.6-6.3) bites per test, though room dimensions meant that this could not be verified by assessing numbers of bloodfed insects. Bite numbers per test were normally distributed (W=0.924, df=24, *p*=0.073).

Analysis of total activity (before isolating approach and departure tracks) found that one mosquito was on average active for 1.8 seconds [1.3-2.2] for every minute of footage recorded. Tests were of variable lengths, running up to 30 minutes according to persistence of mosquito activity. For comparison, the equivalent values for activity at bed net protected hosts in laboratory tests were 5.2 s [4.1-6.3] (untreated nets) and 1.2s [0.7-1.7] (LLINs) for every minute of footage recorded.

### 7.3.2 Comparison of flight during host approach and departure

Flights were classed as approaching tracks or departing tracks (Figure 7.1). Incoming tracks were classed as approaching if they contacted the human for at least 1 second: thus, whilst the mosquito may not have blood-fed, it was assumed to have been responding to cues from the host. Outgoing tracks were classed as departing if they had rested on the body for more than 30 seconds. Outgoing tracks departing the human host were considered to be non-host seeking.

In total, 582 tracks were classed as approaching and 262 as departing tracks. An estimated 1252 tracks were not included in this analysis simply because they did not fit the conservative classification definition (section 7.2.7). Analyses were therefore based on the 40% of track activity recorded that were determined reliably to have had direct interactions with the host. As volunteers did not move, in an effort not to disturb mosquitoes during feeding, it was assumed that individual mosquitoes did not bite any volunteer more than once, and that all volunteers felt and reported all bites that they received.  On this basis, 45% (262 flights after 119 bites) of departing tracks were from mosquitoes that had blood-fed.

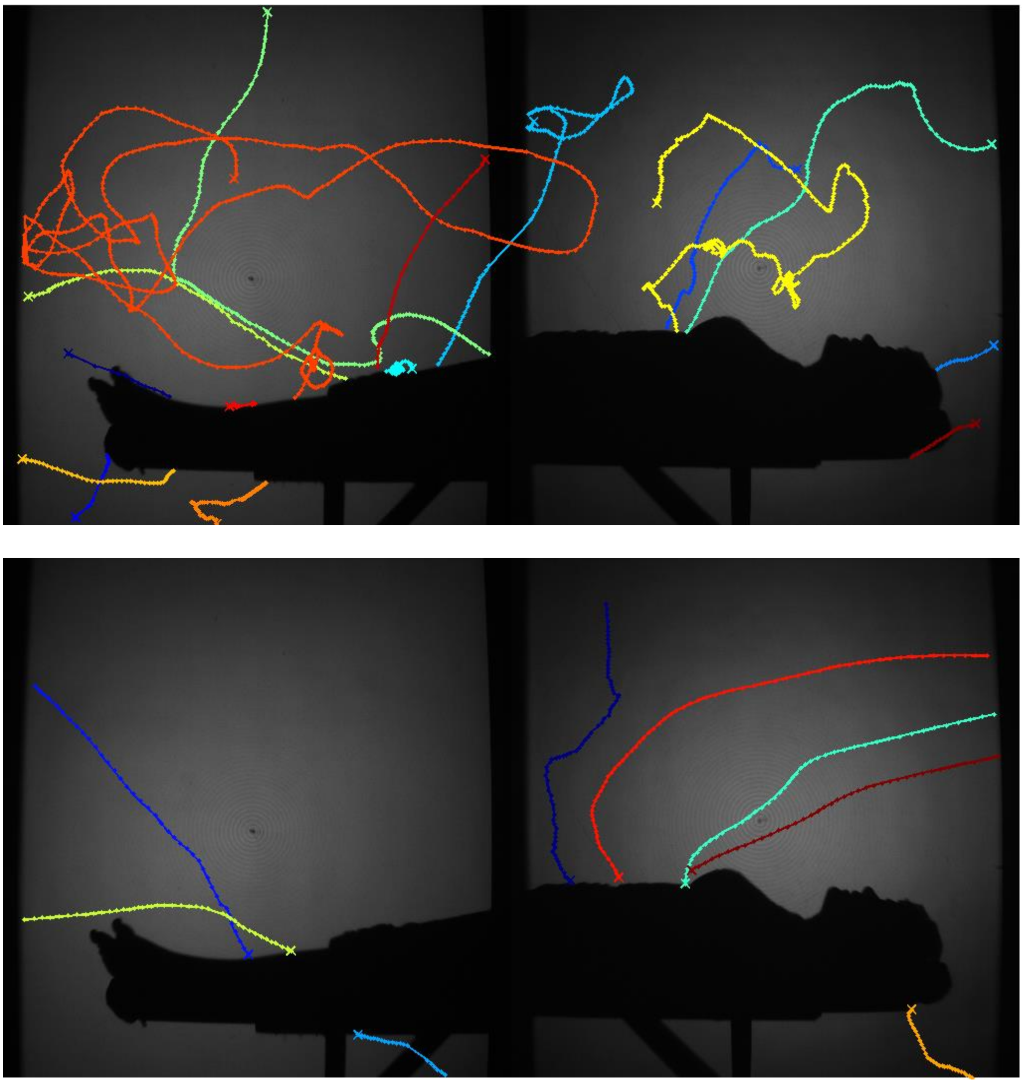


**Figure 7.1 Images from a single test (20 mins) showing all tracks classed as 'Approaching' (top; n = 17) and 'Departing' (below; n = 8) the volunteer.**

In this test, the volunteer received three bites to the arms, and one on their legs (not illustrated in images). Tortuosity of approaching tracks was significantly higher than departing tracks (t=2.51, df=23, *p*=0.020).

### *7.3.3. Modes of Activity*

The average total duration of all tracks classed as approaching was longer than in tracks classed as departing the body (geometric mean approaching activity 95s [71-128] per test; geometric mean departing activity; 30s [19-46] per test, $X^2$ (1)=21.44, *p*<0.001). A significant interaction was noted between track direction (approaching, departing) and the proportion of time spent in each behavioural ($X^2$ (3)=38.507, *p*<0.001, Figure 7.2).

As mentioned earlier (section 7.2.7), very low levels of swooping activity were included in the subset of tracks used in approach and departure analysis (geometric mean 0.5s [0.2-1.1] and geometric mean 0.1 [0.0-0.4] respectively). In both approach and departure the majority of flight activity was classified as visiting ( geometric mean 50.1s [38.9-64.4], approaching tracks; geometric mean 22.3s [15.1-32.8], departing tracks; $X^2$ (3)=157.671, *p*<0.001).

Resting behaviour (which in these tests included those periods when mosquitoes were blood-feeding at the skin) was the second most common activity recorded in both approaching and departing tracks (geometric mean 26.1s [15.0-44.9], approaching tracks; geometric mean 3.7s [1.6-7.3] departing tracks; $X^2$ (1)=8.509, *p*=0.004), followed by bouncing (geometric mean 6.4s [3.8-10.5], approaching tracks; geometric mean 0.2s [0.0-0.3] departing tracks). Category definitions and track sections used in analysis meant that approaching tracks comprised higher levels of bouncing than departing tracks.

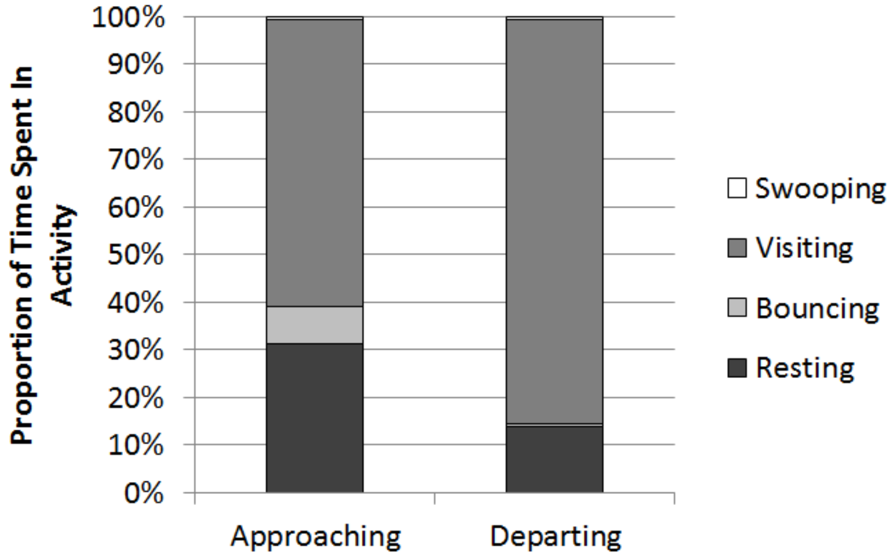


**Figure 7.2 Activity distribution by behavioural mode for approaching and departing tracks.**

Proportions shown are averages for the 24 test repeats, representing behavioural modes of approaching tracks (geometric mean 95 seconds [71-128] per test) and departing tracks (geometric mean 30 seconds [19-46] per test).

### *7.3.4 Velocity and Tortuosity*

Comparison of the velocity of tracks approaching and departing from the human did not indicate any significant differences. Average track velocity was 285 mm/s (264-306) during approach, and 293mm/s (271-314) during departure (Paired t-test, t=-0.567, df=23, *p*=0.576). However, tortuosity was significantly different (t=2.51, df=23, *p*=0.020), as approaching tracks were more tortuous (1.88 [1.65-2.11]) than departing tracks (1.47 [1.25-1.69], Figure 7.1). Supplementary video 7.1 (available in enclosed CD, or online) shows an example of a highly tortuous flight track approaching a volunteer’s feet.

### *7.3.5 Paths taken during approach to and departure from the host*

The height at which mosquitoes entered and exited the field of view was not affected by track direction: approaching mosquitoes entered at 1.0m (0.95-1.06), and departed at 1.0m (0.95-1.05) above the ground (t=-0.166, df=23, *p*=0.870, Figure 7.3). At this approach height, approximately 50% of all tracks first appeared in the ‘high’ tier (H, Figure 7.3). These tracks therefore had to descend in flight elevation to contact the volunteer: only 3% of all host seeking tracks ascended between height tiers during approach (from low to middle, or middle to high, Figure 7.3).

Analyses indicated a slight but significant preference for approaching the body from the ‘head’ side rather than the ‘foot’ side of the filming area, as 59% [54-64%] of approach tracks first appeared in ‘head’ field of view, t=3.677, df=23, *p*=0.001). When leaving the body, tracks departed in equal proportions from the head and feet sides of the recording area (50% [40-60%] exited from head camera, t=0.042, df=23, *p*=0.967).

### *7.3.6 Distribution of Activity at the supine host*

The distribution of visiting flight activity was significantly different between different regions ($X^2$ (11)=106.305, *p*<0.001, Figure 7.4B) with the majority of flights occurring in the regions closest to the volunteer’s body: regions 3, 4, 9 and 10. There were also differences between the flight paths of approaching and departing tracks with a small increase in the proportion of departure activity occurring in regions 1 and 8, (the upper left and right corners of the field of view; $X^2$ (1)=31.096, *p*<0.001; Figure 7.4B).

Time spent in bouncing activity did not appear evenly distributed across the body surface, with highest levels of this activity recorded in regions 4 and 9 (around torso and upper legs, Figure 7.4C). Departing tracks showed very little bouncing, and the activity appeared more evenly distributed between camera regions. Duration of resting activity varied significantly between different regions of the volunteer’s body (Figure 7.4D; $X^2$ (7)=37.196, *p*<0.001). Approaching and departing tracks showed significantly different patterns in resting distribution ($X^2$ (7)=26.181, *p*<0.001); in approaching tracks most resting was observed in regions 4 and 9 (the torso and upper legs), whereas departing tracks accumulated high resting times in regions 3, 6 and 9 (head, back and upper legs).

During approach, mosquitoes settled on the body after making a geometric mean of 1.8 (1.6-2.0) contacts with the volunteer (visits or bounces).

Contacts were evenly distributed across the body surface ($X^2$ (3)=6.242, *p*=0.100, table 7.1). Departing tracks made fewer contacts than approaching tracks ($X^2$ (1)=173.397, *p*<0.001), but sites at which contact occurred were not significantly affected by trajectory direction ($X^2$ (3)=2.057, *p*=0.561).

In keeping with patterns of flight activity observed around the volunteer, bite numbers were not evenly distributed across the 4 regions, with most bites received within region 4 of the field of view, around the volunteer’s torso ($X^2$ (3)=24.295, *p*<0.001, Table 7.1). However, when scaling bites received to area of exposed skin (bites/$cm^2$, Table 7.1), there was no significant preference for one body part over another ($X^2$ (6)=9.925, *p*=0.128).

### 7.3.7 Variation in biting rates at different human hosts

Amongst the volunteers tested, there was no indication that any individual was more or less acceptable to mosquitoes ($X^2$ (2)=0.057, *p*=0.972), as no volunteer received significantly more bites than the others.

There was no evidence of correlation between the number of mosquitoes observed active, and the number of bites received by the volunteer (Pearson Correlation Coefficient r=0.38, N=24, *p*=0.859).

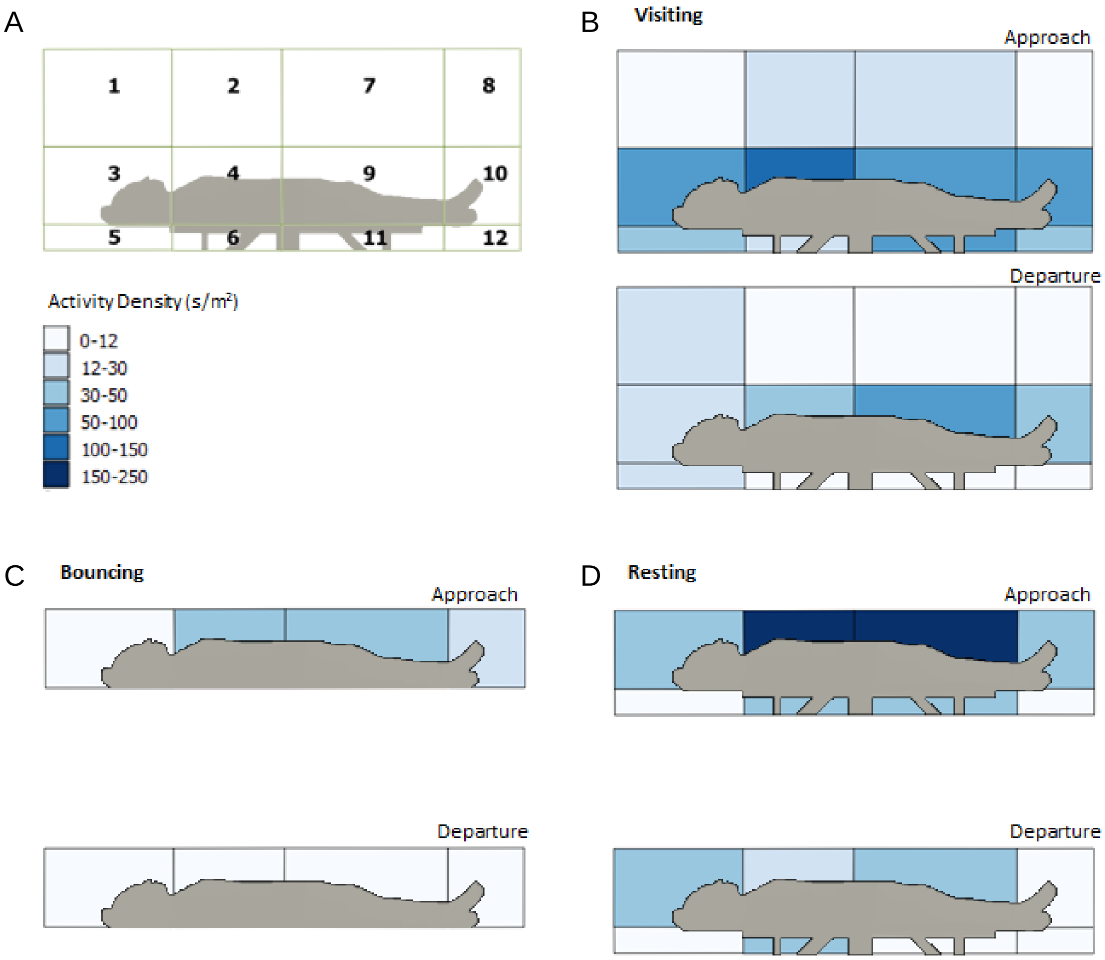


**Figure 7.3 Activity expressed as density (s/m$^2$) for different behavioural modes during flight approaching and departing the volunteer.**

The division of the filming area is shown in A. Other images respectively show activity during visiting (B), bouncing (C) and resting (D) activity. The same colour key of activity density applies to all charts.

**See enclosed CD for: Supplementary Video 7.1: Approach track landing on a volunteer**

The video demonstrates a highly tortuous approach track landing on the volunteer's feet. Approach tracks were significantly more tortuous than departing tracks (t=2.51, df=23, *p*= 0.020). Video also accessible online:

https://www.dropbox.com/s/hq1w1lompozxxv5/SupplementaryVideo_Chapter7_1.wmv?dl=0

**Table 7.1. Distribution of body contacts made and bites received at the four different body regions** (see figure 7.4A for key) expressed as the mean number (with 95% CI), and percentage of total per column. The columns on the right show the biting rates at the corresponding body parts, expressed as the geometric mean number of bites per $cm^2$ skin exposed for each region (with 95% CI) and the same figure as a percentage of the total. Asterisks (*) indicate values that were significantly higher in a region than in other regions in the same column ($p<0.05$).

| | Contacts/ Region | | | | Bites/ Region | | Body Part | Bites/ $cm^2$ skin exposed | |
|---|---|---|---|---|---|---|---|---|---|
| | Approach | | Departure | | | | | | |
| Region | No | % | No | % | No | % | | No | % |
| 3 | 9.4<br>[3.0-15.8] | 13.7 | 0.8<br>[0.1-1.4] | 22.1 | 0.9<br>[0.4-1.5] | 19.8 | Head & Neck | 0.10<br>[0.04-0.16] | 17.9 |
| 4 | 23.3<br>[12.0-32.5] | 34.1 | 1.1<br>[0.4-1.8] | 30.2 | 2.3*<br>[1.4-3.1] | 49.5 | Torso | 0.06 [0.00-0.15] | 10.6 |
| | | | | | | | Arms | 0.07 [0.04-0.10] | 12.6 |
| | | | | | | | Hands | 0.11 [0.04-0.18] | 20.2 |
| 9 | 24.3<br>[9.9-38.8] | 35.7 | 0.9<br>[0.3-1.5] | 25.6 | 0.9<br>[0.6-1.3] | 19.8 | Legs | 0.04 [0.03-0.06] | 7.9 |
| 10 | 11.3<br>[5.4-17.1] | 16.5 | 0.8<br>[0.1-1.4] | 22.1 | 0.5<br>[0.1-0.9] | 10.8 | Ankles | 0.11 [0.00-0.25] | 20.9 |
| | | | | | | | Feet | 0.05 [0.01-0.10] | 9.8 |

## 7.4 Discussion

A series of tests investigating the host seeking behaviour of *An. gambiae s.s.* at hosts not protected by bed nets found that the mosquitoes approached the host from flight elevations level with or above the volunteer, descending to land and made, on average, fewer than two contacts with the host's body before settling. Tracks of these approaching flights exhibited high tortuosity and most approaches occurred in the area around the volunteer's torso and legs. Adjusting for skin area available for feeding, bites were evenly distributed across all of the exposed skin of the volunteer, and mosquitoes showed no biting preference for any particular body part over another. Although nearly half of the departing tracks were estimated to have blood-fed height and speed were not different to approaching tracks, but tortuosity was significantly lower in departing flights.

### *7.4.1 Mosquito Host Seeking*

Mosquitoes respond to cues from the volunteer's body, including body odour, chemicals in breath, heat and humidity (Brown, 1951; Clements, 1999; Takken *et al.*, 2009; McMeniman *et al.*, 2014; Webster *et al.*, 2015). Attractive cues operate over different ranges, with carbon dioxide attracting insects from a greater distance than heat and moisture (Snow, 1970; Cardé & Willis, 2008; Cardé *et al.*, 2015). Individual attractive cues act synergistically to augment attraction to the host (McMeniman *et al.*, 2014; Cardé *et al.*, 2015; Webster *et al.*, 2015).

It has been suggested that in host location, mosquitoes follow convection currents carrying attractive odours towards the human body to locate a blood meal (Dekker *et al.*, 1998; Lynd *et al.*, 2013; Sutcliffe & Yin, 2014). *Ae. aegypti* have been shown to follow rising air currents moving at 12 cm/s, by flying down to their source (Daykin, 1967). Mosquitoes in Daykin's study (1967) had been activated by human breath, but the air current was clean and contained no bait chemicals. Artificial air currents employed by Daykin fell within the range of those that would be found around a host's body, as convection currents created by heat from the human body move at between 5 to 30 cm/s, depending on body position (Lewis *et al.*, 1969; Clark & Toy, 1975). Further experiments using a real human bait have shown that mosquitoes will descend a down a vertical chamber to locate a bait beneath, and that damping convection currents by raising the chamber temperature to the same temperature as the body may impair the ability of *Ae. aegypti* to locate and land on a human hand (Khan *et al.*, 1966; Khan *et al.*, 1968).

A superficial comparison of activity per mosquito was made between results of the present study (all activity, not limited to tracks classed as approach and departure, was included), and tracks attacking bed nets (obtained in experiments in Chapter 5). As the data was gathered using different methods, and in different test settings, a quantitative statistical comparison was not performed. However, data suggested that mosquitoes were more active when attacking untreated nets than when feeding on an unprotected volunteer. It could be speculated that as blood-feeding lasts for just a few minutes (Roitberg *et al.*, 2003), when able to feed a mosquito may approach, feed on, and leave a host within a short period of time. Presence of a net prolongs time spent in proximity to the host as mosquitoes persistently attack the net surface but are unable to feed, so do not depart from the host as rapidly. One might extrapolate that a mosquito that was able to successfully feed through a net would depart sooner, and spend less time in contact with a net than that that recorded in chapters 5 and 6.

### 7.4.2 Flight Elevation

In these recordings, mosquitoes entered the field of view at heights of approximately 1m above the experimental hut floor (approximately 20cm above the human volunteer, Figure 7.3). In outdoor field settings, *An. gambiae* is reported to fly at heights of 0-1m (Snow, 1979; Gillies & Wilkes, 1976). Flight below 0.4m was not visible to cameras, giving a slightly higher value for average flight elevation around the human, hence flight elevation of mosquitoes entering and exiting camera view in the present study fell approximately within this expected 0-1m range. The majority of mosquitoes flew in tortuous descending flights after entry in to the field of view. In fewer than 5% of cases did mosquitoes initiating flight at 1m ascend to the “high” elevation band. These results support the theory that mosquitoes descend, following attractive cues to locate a host. Though convective currents could not be imaged, it seems plausible that these air currents were used in guiding downward navigation.

Curtis *et al.* (1992) attempted to block host seeking flights of mosquitoes using bed curtains (a roofless bed net made from permethrin-treated polypropylene sacking). The formulation proved highly repellent, but those mosquitoes that were able to enter the experimental room were almost all able to locate the host and feed, demonstrating the ability to ascend over barriers in host location (a behaviour also observed in chapter 4). As host seeking flights in the present study predominantly took paths descending from above the body, it seems that this strategy might have been more successful had it used a roof-only ‘bed net’ (a horizontal material panel placed above the human), as this would intersect with the majority of mosquito flights, provided it did not compromise the volunteer’s odour plume. This design would likely be impractical in other ways however, since to be effective the panel would need to be placed within 15-20cm of the body (Figure 7.3), since otherwise, mosquitoes would pass underneath.

The finding that departing mosquitoes exited the field of view at similar height elevations to approaching flights was surprising given the expectation that the additional mass of the blood meal would compromise the flight abilities of fed mosquitoes (Reid *et al.*, 2014). However blood-fed *An. gambiae s.l.* resting indoors are commonly found on the ceiling and walls of houses (Haddow, 1942; Smith *et al.*, 1966), and they are capable of exiting through eaves after feeding (Smith & Webley, 1969; Port & Boreham, 1982). The departing flight patterns recorded in the present study may therefore represent these ascending flights from the host towards these resting and exit points.

Unlike Mboera *et al.* (1998), results did not indicate a strong preference for flight in the air space high above the volunteer’s feet: activity was approximately evenly split between the head and foot region. However, Mboera’s study used a CDC trap and bed net, one or both of which might have influenced mosquito movements enough to drive flight paths to different areas.

### *7.4.3 Tortuosity and Velocity of Approach*

Mosquitoes approached the host in highly tortuous paths. Tortuosity during approach was significantly higher (1.88) than in departing tracks (1.47). This is likely to be related to host seeking; in laboratory tests reported in chapter 5, tortuosity of all flights of Kisumu strain *An. gambiae s.s.* around untreated and treated bed nets was significantly higher (1.66 and 1.63 respectively) than at unbaited nets (1.31).

The tortuosity of host seeking flights is suggestive of casting behaviour. Wind tunnel tests of host seeking mosquitoes find that tracks become more tortuous as mosquitoes follow host cues (Beeuwkes *et al.*, 2008; Dekker & Cardé, 2011; Spitzen *et al.*, 2013), as upon flying out of an odour plume, mosquitoes make crosswind flights to relocate the plume (van Breugel *et al.*, 2015). The high tortuosity of the approaching tracks relative to the departing tracks suggests plume-tracking behaviour of approaching mosquitoes. The odour plume of a supine human has not been precisely mapped, but Schlieren thermal imaging, used to visualise air movement, has been applied to basic studies of airflow around the human body, showing that the air rising from a human body is turbulent, influenced by exhaled breath, with different air speeds over different body parts according to heat release, and skin exposure (Clark & Edholm, 1985; Tang *et al.*, 2009). Considering also that the sweat glands and bacteria responsible for production of some attractive odours are unevenly distributed across the body (Schreck *et al.,* 1990; Verhulst *et al.*, 2010; Grice & Segre, 2011), the approaching mosquito will experience an uneven plume of host cues, prompting a tortuous flight track as the mosquito navigates towards the most attractant areas of the host. Roitberg *et al.* (2003) found that blood-feeding increased flight tortuosity, but this was based on comparison of flight escape responses of fed and unfed insects in a narrow (3.5cm diameter) flight arena. The difference between that finding and the lack of tortuosity in departing tracks in the present study may be accounted for by differences in assay size, or the fact that fewer than half of departing tracks had blood-fed.

Interestingly, the speeds of approaching and departing mosquitoes did not differ significantly. Reports on the influence of host cues on flight speed are ambiguous, with some evidence that host seeking mosquitoes fly slightly slower (Beeuwkes *et al.*, 2008; Chapter 5), and other studies showing flight speed increasing upon host cue detection (Dekker *et al.*, 2005; Dekker & Cardé, 2013; Spitzen *et al.*, 2011). The blood meal can increase a mosquito’s mass by over 60% its original body weight (Reid *et al.*, 2014) and has been observed to reduce flight speed (Roitberg *et al.*, 2003). As fewer than half of departing mosquitoes had blood-fed, and there was no unbaited control, it is not possible to discriminate between the effects of host cues and blood meals on flight speed.

### *7.4.4 Approach and Biting*

The goals of this study were to examine the behaviour of mosquitoes approaching a host to blood-feed, and compare this to the flight of mosquitoes that were not host seeking (i.e. those moving away from the body). In order to isolate approaching track types from other non-host seeking flights, approaching and departing tracks were isolated for further analysis.

By tracking flights and recording bite sites it was possible to examine the relationship between activity prior to landing and location where mosquitoes ultimately chose to bite. The high density of visiting and bouncing flights, and high number of body contacts in region 4 matches biting reports, as most bites were received in this region.

During approach to the host, most flight occurred in the region around the volunteer’s torso and legs. This mirrors attack points on LLINs, which also occur predominantly on the net roof over the torso (Lynd *et al.*, 2013; Sutcliffe & Yin, 2014; Chapter 5). This provides further evidence for the idea that mosquito net attack is focussed on the areas emitting the strongest host cues and attack persistence on a net is driven by host seeking behaviour.

Though relatively few tracks comprised bouncing flight, it is interesting to note this mode of attack was not limited to areas of the body that were covered by clothing. Bouncing flight was observed on the uncovered lower legs and feet, where mosquitoes sometimes made multiple brief landings on the volunteer's skin prior to selecting a bite site. It is unclear what the purpose might have been: one could interpret this as searching or prospecting flight to locate the area of the body emitting strongest attractive cues, but since bites were distributed evenly across the body there is no evidence that mosquitoes preferred any particular area. Alternatively it is speculated that mosquitoes could be testing host responsiveness, making brief landings to assess host defensive behaviour. Volunteers remained still during experiments, hence active disturbance by host defensive action can be excluded (Canyon *et al.*, 1998). Similar behaviour has been observed in *Mansonia* and *Aedes* mosquitoes also, with the suggestion that some species have a longer exploratory period of take-off and resettling than others (Service, 1971).

### *7.4.5 Limitations of Behavioural Mode Definitions*

Tracking in the present study applied track definitions designed for investigating mosquito flight on nets (Chapter 5). These definitions were largely appropriate, but may have limitations in this setting regarding mosquito contact with the volunteer's body. These definitions of contact encompass tracks that make sharp angle turns, or make frequent changes of vector direction, which in the context of an unprotected host may underestimate the number of contact points, as the silhouette of the body may obscure sharp angle changes of direction or brief landings on the body. Methods could be improved by redefining contacts in this context to encompass data on instantaneous velocity of mosquito tracks passing 'behind' the silhouette of the volunteer's body. Where the velocity of 'unseen' track sections is calculated to fall below a certain speed, it can be assumed that mosquitoes contacted the body during the flight section that had been obscured by the body.

In the present study, options were considered for a fifth behavioural category, "feeding", which would be defined as resting tracks that were stationary for longer than a threshold time period. However this idea was not acted on, as little information is available on how long mosquitoes take to initiate feeding after landing on a host, and this threshold would have had to have been chosen arbitrarily without an evidence base to support the definition.

Highest biting occurred in the regions with highest flight density, indicating that after mosquitoes landed on the body they made little movement across the volunteer's body in search of a suitable biting site. The tracking system used was not capable of observing close range flights at the same level as the volunteer's body, due to the nature of the 2 dimensional back-lit arrangement of the cameras: the body of the volunteer blocks the lower part of the field of view, obscuring tracks and making it impossible to track mosquito movements on the sides of the body. Though biting activity broadly corresponded to flight density before landing, there were at least two tests in which volunteers reported bites to body parts, but when tracks were inspected, no corresponding landing trajectories could be found approaching these areas. Assuming volunteers were not over-reporting bites, this suggests that in a low number of instances, mosquitoes made their first approach to a different part of the body and subsequently flew at a height level with the volunteer's body towards the site where they ultimately fed. However it is also possible that volunteers under-reported bites, a problem that is particularly common when individuals are bitten by several mosquitoes in a short space of time (Murihead-Thomson, 1951). The inability to accurately track activity of mosquitoes on the body is a limitation of the back-lit filming set-up: recording the full field of view would not be possible without a change to illumination methods.

### *7.4.6 Preferred Bite Sites*

When a human is standing or sitting, mosquitoes will bite their feet (De Jong & Knols, 1995; Dekker *et al.*, 1998; Braack *et al.*, 2015). This could be related to attractive body odours emanating from the feet as these cues alone can stimulate mosquito host seeking (Spitzen *et al.*, 2013), and attraction to the feet diminishes following foot washing (De Jong & Knols, 1995). However host position plays a major role in choice of bite site, and *An. gambiae s.s.* and *An. arabiensis* mosquitoes show no significant preference for feet when a volunteer lies down, or places their legs in the air (Dekker *et al.*, 1998; Braack *et al.*, 2015). This trend has also been observed in *Cx. quinquefasciatus* (referred to as *Cx. pipiens fatigans* in the publication), which lost the preference for feet when a volunteer was supine (Self *et al.*, 1969).

Results of the present study agreed with this trend, as mosquitoes showed no preferences for any particular body part on the supine volunteer, and bites were distributed evenly across exposed skin, supporting Dekker's (1998) suggestion that mosquitoes bite whichever part of the body is closest to the ground. Analysis of approach tracks reported above shows that this bias was not a simple product of mosquitoes' low flight elevation, as in many cases mosquitoes' approached at heights above the volunteer before descending to bite the body, contradicting the idea that mosquitoes fly towards the host at ground height and bite at the first available area of skin they find.

In the present study, volunteers were partially clothed, wearing a top and short trousers, but in Dekker *et al.* (1998) and Braack *et al.* (2015) volunteers were shirtless, wearing either underwear or short trousers. Though the covered torso is one of the hottest parts of the body (De Jong & Knols, 1995) there is no evidence that mosquitoes in the present study initially approached the torso but were diverted to the other body parts.  If so, then it could be that choice of bite site is guided by attractive cues emanating from exposed skin, and covered skin is less attractive as clothing interferes with or diminishes such emanations by creating a barrier to odour release and blocking outward radiation of heat.

### *7.4.7 Interaction Between Mosquitoes*

The tracking system offers the potential to investigate whether interactions occur between individual mosquitoes during host seeking.  The most frequently reported behaviour in this respect is known as "the invitation effect", the phenomenon where incoming mosquitoes are more likely to bite sites close to where other mosquitoes are already feeding.  To date the effect has only been reported in *Aedes* mosquitoes (Alekseev *et al.*, 1977; Ahmadi & McClelland, 1985; Charlwood *et al.*, 1995), and the evidence derives from small-scale choice tests comparing landings by mosquitoes on hands or legs of the same individual, one of which has actively feeding mosquitoes present, the other a control.

Due to the tracking system's inability to detect accurately the position of mosquito bites on the human body, and the consideration that a single mosquito potentially could have bitten the volunteer more than once in the same area, it was not possible to explore this behaviour reliably. However, with the available data it was possible to investigate the relationship between the number of mosquitoes responding to the host, and the number of bites received (section 7.3.7). These data did not indicate any positive correlation between number of mosquitoes attacking the host and number of bites received. Excluding interactive effects, positive correlation would be expected, as more host seeking mosquitoes would result in a proportional increase in bites. Instead biting propensity appeared to be random, and showed no significant relationship (invitational, or disruptive) to mosquito activity.

Effects of the first mosquitoes' feeding on an incoming mosquito's propensity or likelihood to feed at all has not been well studied but appears to relate to endogenous factors (Lapshin & Vorontsov, 2015). Interference of high densities of mosquitoes on feeding success has been seen in some feeding studies with small mammals, but in these instances the effect was related to defensive behaviours of the host, and did not occur when the host was restrained (Waage & Nondo, 1982; Walker & Edman, 1986). In the present study, hosts were prohibited from making defensive movements, hence active host defence behaviour was unlikely to cause affect results. Repeating this study with different mosquito group sizes could help establish whether anopheline mosquitoes interact during host seeking.

During tests nearly half of mosquitoes blood-fed within the thirty minute testing period. The mosquitoes used were Kisumu strain *An. gambiae* s.s., originally collected in Kenya in 1975, and blood-fed on membrane feeders or small mammals since. Mosquitoes were released at eave height in a 5x5m room: the large number of bites received indicates that the colony mosquitoes, which have not needed to locate a blood meal source in nearly 40 years, have retained the ability to locate and feed on a live human host. This in itself is a useful finding, as concerns have been raised about the adaptation of, or loss of host seeking abilities of mosquitoes in laboratory conditions (Laarman, 1958; Clark *et al.*, 2011).

### *7.4.8 Study Limitations*

An important limitation in this study was the fact that the resolution and observable area of the human body did not allow camera recording of feeding mosquitoes, and hence bite sites had to be reported by the volunteer during and at the end of the test. This increased potential for inaccuracies since mosquitoes (albeit culicines) have been reported to probe in a number of sites prior to choosing a place to bite, and bites may not always be felt by volunteers (Service, 1971; Grossman & Pappas, 1991). Further tests might be better able to identify individual mosquito timelines of approach and blood-feeding using individually released mosquitoes, and camera visible signals from the volunteer as bites occur.

### *7.4.9 Summary*

Analysis of the flight paths of host seeking mosquitoes suggested that mosquitoes follow a plume of attractive cues towards the host. Approaching mosquitoes arrived into the field of view at flight elevations of approximately 1m above ground, and descended to contact the host. Bites were evenly distributed across the whole human body, and the frequency of biting was determined by the availability of uncovered skin rather than a preference for any particular body region. Tracks of mosquitoes approaching the host were more tortuous than departing tracks but flight speed and height were similar. Further studies will be needed to investigate the detailed flight character of blood-fed mosquitoes departing the host.

The results may be useful in the design of new vector control tools, suggesting opportunities for where barriers might be placed to disrupt host seeking flight, though the multiple directions of approach of mosquitoes indicate that interventions that intercept all incoming flight paths, such as bed nets, will be more effective than bed curtains, ceiling screens or other barriers that only block a subset of routes towards the host. Further studies should examine the air currents around the human body to better understand the cues guiding mosquito flight towards the host.

# Chapter 8 General Discussion

## 8.1 Overview

The host seeking behaviour of mosquitoes in and around houses, approaching LLINs and attacking humans is a key point influencing transmission of malaria. To explore this behaviour, the research described in this thesis aimed to develop and evaluate new tools and illumination techniques to ensure that recordings were unobtrusive and did not influence mosquito activity. This work applied small scale 3D, and larger scale 2D recording methods to track nocturnal mosquito flight during house approach, LLIN attack and feeding on a human bait. Initial experiments using a simple small system investigated the close-range impact of insecticide on host seeking behaviour, assessing the spatial repellent properties of two insecticides. Following this, house entry flights were observed using a 3D tracking system, in tests that examined whether mosquitoes moved through window openings in stereotyped routes. Mosquito interactions with LLINs were quantified in a laboratory setting using a new large scale 2D tracking system. This system was then transferred successfully to a semi-field setting in Tanzania and used to track flight of wild *An. arabiensis* mosquitoes at LLINs. A final set of tracking tests investigated the flight paths of mosquitoes blood-feeding on a volunteer who was not protected by a net, following the full and uninterrupted track of mosquitoes as they approached, landed, fed on, and exited the host after feeding.

## 8.2 Mosquito Host Seeking Behaviour

Knowledge of the host seeking flight of mosquitoes is essential in studies seeking to identify the host cues that are important in attraction, and to the design of novel control tools that might be used to intercept the path of unfed mosquitoes. In chapters 5, 6 and 7, host seeking flight of mosquitoes responding to human baits was found to be highly tortuous. Mosquitoes were capable of locating hosts under a number of different experimental conditions, and were able to navigate through windows, around rooms and towards bed nets during approaching flight, an encouraging sign that colonies have retained the ability to host seek after several decades of being fed using membrane feeders.

This thesis is one of the first studies to ‘quantifiably’ observe tortuous flight on host approach that is not within a wind tunnel. Tortuous flight is generally interpreted as relating to casting behaviour of insects flying upwind as they move in and out of a host generated odour plume. However within the still air environment of the test room there were no wind currents to influence flight. Due to random dispersion of odours within plumes, concentration gradients are not thought to be reliable indicators of object position until insects fly within mm distances of a host (Murlis & Jones, 1981; Cardé & Willis, 2008). This would challenge the belief that short-range host location occurs principally via chemotaxis, following attractive chemical cues from the host. As such it is possible that mosquitoes within houses locate hosts by following air currents generated by the host's body, continuing the odour-led upwind flight used in long distance movement (Gibson & Torr, 1999; Cardé & Willis, 2008). Evidence from tests with *Aedes aegypti* supports the importance of convection currents in host location (Khan *et al.*, 1966, 1968; Eiras & Jepsen, 1994). Placing fans around a human can reduce the number of mosquitoes orienting to a host, though the extent to which this is due to disrupted air currents rather than turbulent air disrupting flight is difficult to establish (Hoffman & Miller, 2002). Without full imaging of airspace around a human the importance of convection currents remains hypothetical.

It has been suggested that visual cues play a role in host location by nocturnal mosquitoes, particularly in long range optomotor control of flight (Bidlingmayer & Hem, 1980; Gibson, 1995; Gibson & Torr, 1999). The experiments of this thesis were conducted in darkness using infrared light, which is invisible to *Anopheles gambiae* (Gibson, 1995). As such, excluding potential light leaks, it is assumed that during the short range host location observed in this work, mosquitoes were capable of navigating without using visual cues.

This thesis characterised host seeking flight of mosquitoes at bed nets by defining four behavioural modes: swooping, visiting, bouncing and resting. These were commonly seen as responses to the presence of the bed net barrier, as mosquitoes persistently attempted to penetrate the net. When no net was used mosquitoes would generally settle rapidly on the human bait after fewer than two contacts with the body, indicating that short 'bouncing' flights across a surface were chiefly a response to the presence of the net barrier. Close range behaviour of mosquitoes around human skin could not always be viewed due to the obstruction of the camera view by the volunteer's body, but in some videos mosquitoes were observed engaging in bouncing flight across exposed skin. It is interesting that mosquitoes did not settle to bite immediately: this resettling behaviour in which mosquitoes do not immediately probe upon landing has been observed in other mosquito species (Khan & Maibach, 1966; Service, 1971; Grossman & Pappas, 1991). As bites were broadly distributed evenly across the volunteer's body it is yet unknown what stimuli evoke settling and biting, and this could be an interesting area for further research.

## 8.3 Effects of Insecticide on Behaviour

The behavioural action of insecticide plays a significant role in determining its function (Killeen *et al.*, 2010, 2011; Killeen & Moore, 2012). As such it is important to properly characterise the repellent and contact irritant action of insecticides used in vector control measures. In chapter 3 small scale tests were designed to identify whether deltamethrin or DDT exhibited spatial repellency against host seeking *An. gambiae s.s.*. Small scale choice tests indicated that deltamethrin had a repellent action, but larger box choice tests failed to sustain this finding. Further tests in chapters 5 and 6 found that at room scale, there was no evidence for a repellent effect of deltamethrin treated LLINs on *An. gambiae s.s.* or *An. arabiensis*. Collectively the results of these chapters highlight the importance of appropriate testing methods in assessing behavioural effects of insecticide. Small scale assay results may not be relevant to practical scenarios of LLIN use, as they constrain flight and artificially increase host attack (Chapter 3; Gerold & Laarman, 1967; Sutcliffe & Colborn, 2015).

The larger scale and less obtrusive test methods used in chapters 5 and 6 make the data more reliable, hence it is considered that under practical conditions the LLIN tested here had no appreciable spatial repellent properties. Instead the drop in activity observed during net attack is thought to represent either contact irritant effects, or sub-lethal insecticide action impairing host seeking abilities of mosquitoes. These factors would be difficult to distinguish without further tests assessing the impact of short term insecticide exposure on flight.

Behavioural effects of insecticide are important in determining personal and community protection offered by bed net use. If insecticide is highly repellent or irritating, causing mosquitoes to disengage with a surface prior to toxic action of the chemical, the burden of bites could be shifted to others in the community not using nets (Lines *et al.*, 1987; Killeen *et al.*, 2007). The work of this thesis shows that mosquitoes disengage from LLINs after a short contact period: whether this is problematic to LLIN action hinges largely on the subsequent fitness of mosquitoes following cessation of attack. Community level reductions in mosquito populations following distribution of permethrin-treated bed nets suggest that mosquitoes unable to feed on a net user suffer fitness consequences (Gimnig *et al.*, 2003; Hawley *et al.*, 2003), but individual novel insecticide formulations should be evaluated for behavioural and physiological effects on mosquitoes to be sure that an intervention will not simply divert the disease burden to vulnerable members of a community without access to nets.

This risk could be averted by improving the speed of insecticide action using synergists, higher concentrations or different insecticides that are faster acting (Hougard *et al.*, 2003b; Darriet & Chandre, 2011). Alternatively short net contact times could favour distribution strategies based on contamination of mosquitoes during one contact instead of requiring a prolonged period of physical contact for action. Biological control with pyriproxyfen larvicides or fungi exploit such methods, as do novel mosquito contamination methods using electrostatic powder coatings that dust mosquitoes' legs with insecticide upon landing (Lwetoijera *et al.*, 2014b; Snetselaar *et al.*, 2014; Wang *et al.*, 2014; Andriessen *et al.*, 2015).

Standard WHO cone tests examine efficacy of insecticide treated nets by giving mosquitoes 3 minutes of forced contact exposure to treated materials (WHO, 2013e). Results of this thesis found that in a free flight scenario, mosquitoes made less than 100 seconds of direct contact with a treated bed net over the course of an hour's test, and that this exposure took the form of several brief 'bounce' or 'visit' type contacts, and short resting periods (Chapters 5 and 6). In a true field scenario in which mosquitoes were allowed to freely move in and out of a room over the course of a night this time could be shorter, as mosquitoes might choose to exit the house. Alternatively, net attack might continue at low levels across the course of the night (though activity decay observed in chapter 5 suggests this is less likely). Nonetheless, results imply that the exposure given in forced contact bioassays may misrepresent actual lethal effects of insecticide treated materials on mosquitoes as cone tests enforce long, uninterrupted periods of insecticide contact, which is not typical of exposure in field situations. WHO cone tests could be improved by modifications to exposure times and methods to better represent contact levels experienced in relevant field settings.

## 8.4 Net Attack Sites

Chapters 5 and 6 identified the areas of the net where mosquitoes spend most time attacking, showing that most net contact occurs on the roof of the net on the surfaces above the volunteer's torso. This finding has been noted in sticky trap tests, but the work of this thesis has verified that attack persists in this area after the first contact point identified by glue trap methods (Lynd & McCall, 2013, Sutcliffe & Yin, 2014). Such information is useful to the design of new nets, which may benefit from placing highest concentrations of insecticides, synergists, or other control agents on this area. This strategy is already employed in the Permanet 3.0, which uses the synergist PBO in combination with deltamethrin on the net roof, but treats the side panels with deltamethrin alone (WHO, 2008b). Other similar designs in earlier stages of development use “smart patches”, smaller treated net patches which cover only the most highly contacted area of the roof (In2Care Holdings B V, 2015). These designs allow targeted distribution of costly or toxic chemicals on a limited surface of the net, reducing costs of net manufacture, and reducing human exposure to certain insecticides.

Results of this thesis discourage the use of bed curtains with no roof (Lines *et al.*, 1988; Curtis *et al.*, 1992), and mosaic net treatments which apply different insecticides to a net’s roof and side panels (Hougard *et al.*, 2003b) as mosquitoes made less contact with net sides, and were observed flying directly to the net roof without first touching the sides. Tracking results are encouraging for the use of Mbita style traps in population sampling: these traps collect mosquitoes attacking the roof of a cotton walled net (Mathenge *et al.*, 2002, 2004). Inconsistent field catches with Mbita traps suggest the existing trap design may need to be modified to improve catch efficiency (Laganier *et al.*, 2003; Braimah *et al.*, 2007). Designs might benefit from adjustments that enable improved airflow through the trap, through changes in the textiles used, and a shorter funnel section, ensuring that strong attractive host cues are more easily located by the searching mosquito.

Analysis of tracks approaching an LLIN did not reveal any stereotyped pathways of incoming flight at the scale investigated by the thesis, with mosquitoes flying towards the host in a variety of direct or tortuous routes. As such it is not possible to recommend a particular position for placement of barrier screens or photonic fences within a house to intercept host locating mosquitoes (Burkot *et al.*, 2013; Foster *et al.*, 2014; Tokitae Llc, 2014): to interrupt attack successfully it is best to block all routes of approach using an LLIN tucked in to a mattress, or alternatively prevent mosquitoes from entering houses using full house screening (Tusting *et al.*, 2015).

It should be noted that in experiments conducted for this thesis, volunteers were positioned to ensure their skin was not in contact with the net surface. It is known that mosquitoes are able to feed through an intact net if the occupant’s skin can be reached through it (Lines *et al.*, 1987; Hossain & Curtis, 1989). The present study suggests that mosquito attack patterns leave the net user relatively safe from this opportunistic biting, as net contact is focussed on the roof, whereas accidental skin contact would be against the side and end panels of the net. However it is plausible that net attack would differ according to the position of the occupant’s body, and that should the body be closer to the net sides contact patterns could change. Similarly, behaviour and persistence could differ according to the number of net occupants, and availability of alternative hosts in the household.

## 8.5 Insect Tracking

Tracking mosquito flight presents a particular technical challenge given the small size of the insect and the requirement to illuminate activity in wavelengths that will not affect behaviour. The camera techniques developed for this thesis represent a significant advance in our ability to record mosquito flight for long periods of time at large scale.

The system developed for this work was never tested to full capacity, but was designed to be theoretically capable of recording tracks of up to 100 mosquitoes, over an 8-10 hour unbroken recording period.

The back-lit and retro-reflective screening systems viewed objects as silhouettes, and as such flights occurring level with the body, or behind a net seam could not be observed. This can be addressed to some extent by careful positioning of the volunteer and net within the field of view to ensure that only a minimal section of the image is lost to tracking. Addition of a third camera to film stereoscopically in 3D could also allow observation of these missing tracks, though this would require a significant adjustment to tracking software to incorporate calculations required for 3D tracking. This thesis has also demonstrated that 3D tracking can be achieved using single camera systems and retro-reflective screening. Such methods offer a computationally less intensive tracking means of obtaining 3 dimensional track information, though at current capabilities the spatial resolution of this method would be lower than stereoscopic filming (D. Towers, *personal communication*).

The present system's field of view could be expanded using additional cameras and computers: each new camera would add a 1.2 x 1.2 x 2m filming area to the existing recording space. Future tracking work may also benefit from the inclusion of tracking methods used in other animal behaviour studies. Attached radio telemetry tags that weigh no more than a mosquito's average blood meal might be used for tracking individual insects over ranges of nearly 1km (Riley & Smith, 2002; Kissling *et al.*, 2014), and could be used for longer range host seeking studies (i.e. around dwellings in villages), or the investigation of post-blood-feeding behaviour, though current tag:insect weight ratios are unfavourable. Acoustic tracking methods may also be useful for 3D tracking of insects (Blumstein *et al.*, 2011), though practical issues of background noise may limit the scope of this technique to soundproofed laboratory settings.

## 8.6 Broader Application of Findings

A large part of this thesis has focussed on the development and testing of two novel camera tracking systems (using Fresnel lenses and retro-reflective screening), produced in collaboration with an engineering team at the University of Warwick. These methods offer new opportunities to investigate mosquito interactions with existing and novel methods of vector control, allowing a state of evaluation between small scale laboratory tests and full experimental hut trials. It is anticipated that the methods developed in this thesis will be applied to test behavioural interactions of mosquitoes with interventions, assisting the development of new tools for mosquito control.

This thesis has not addressed the behaviour of outdoor biting mosquitoes, which is a growing concern for residual malaria transmission (Govella & Ferguson, 2012; Killeen, 2014). However the 2D tracking system has good potential for use outdoors at night time, to observe behaviour of exophagic mosquitoes.

## 8.7 Future Work

This thesis has been able to investigate aspects of mosquito behaviour that could not previously be observed at this level. However interpretation of results is limited to some extent by gaps in our knowledge of the field. Further research on these points would assist our understanding of host seeking behaviour and the impact of LLINs on mosquitoes.

- Though patterns of mosquito approach in large scale experiments are strongly suggestive of attractive cues mosquitoes followed during approach, assertions that mosquitoes follow convection currents to a host, or are stimulated by airborne body odour or $CO_2$ remain speculative. Future work could use methods such as Schlieren imaging to observe influence of body heat on movement of air around the body (Clark & Toy, 1975; Tang *et al.*, 2009), and gas analysis for identification of concentrations of attractive cues in air (Zollner *et al.*, 2004; Cooperband & Cardé, 2006b). Airflow imaging of air around bed nets would be particularly important in verifying the hypothetical suggestion of the chimney effect, in which a volunteer's body heat creates air currents drawing air in to the net from the sides, and channelling it upwards through the roof (Guillet *et al.*, 2001; Sutcliffe & Yin, 2014). With some adjustment to illumination it may be possible to image mosquito flight and air movement simultaneously, to directly observe the interactions of mosquitoes with air currents around the human body (D. Towers, *personal communication*).

- Tests with LLINs in chapters 5 and 6 found that mosquitoes may not contact the net for long enough to be immediately knocked down and killed by insecticide. To assess effects of LLIN distribution on mosquito populations it is important to establish whether these mosquitoes suffer long term behavioural impairment or reduced longevity as a result of their exposure. Chapter 3 provided some evidence that mosquitoes exposed to deltamethrin for 90 seconds show an appreciable reduction in survival time following testing. There is other evidence that sub-lethal doses of insecticide temporarily reduce mosquitoes' fitness by impairing their normal behaviour, though for low insecticide exposure these effects may last for less than 24 hours (Siegert *et al.*, 2009; Glunt *et al.*, 2011). Further work investigating the impact of short insecticide exposure periods on mosquito activity would assist in interpreting the impact of these short attack periods on mosquito longevity and evaluating how problematic contact irritant properties of insecticide are to net function.
- Insecticide resistance is a key concern for vector control, and it has been suggested that genes controlling insecticide susceptibility may also influence host seeking behaviour (Corbel *et al.,* 2004; Spitzen *et al.*, 2014; Diop *et al.*, 2015). Small scale wind tunnels are likely to mask any behavioural impact as host seeking in these scenarios does not require complex navigation, with only short distance flights needed to locate attractant source (see chapter 1, section 1.10.7). The tools developed in this thesis can be applied to precisely quantify impact of insecticide resistance on host seeking behaviour over a more realistic scale, giving more comprehensive data on the fitness effects of the mutations that will affect their spread through mosquito populations.
- Holes in LLINs are expected to present an important problem for future vector control efforts, as insecticide treatment now outlasts net durability (Irish, 2014; Lorenz *et al.*, 2014; Sutcliffe & Colborn, 2015). Tracking tools developed in this thesis can be used to evaluate how rapidly mosquitoes are able to locate holes in nets, and how many holes an LLIN can accrue before its protective value is lost.
- Initial field testing methods in which wild mosquitoes were allowed to enter the experimental hut in Tanzania from the surrounding area had to be

amended when the population was found to consist of a mix of anopheline and culicine mosquitoes. As this thesis focussed solely on the behaviour of malaria vectors, methods were adapted to use *An. arabiensis* that had been caught in the field as larvae and reared in labs. This strategy is less desirable as rearing in the insectaries may select for certain characteristics in a population, and mosquitoes are artificially encouraged to host seek through sugar starvation.

- Preliminary analysis from data from tests with laboratory reared *Cx. quinquefasciatus*, as well as information gained from early tests with mixed species populations indicate that it may be possible to identify mosquitoes to genus using track characteristics and information on insect body size (N. Angarita-Jaimes, *personal communication*). Future work would benefit from the development of algorithms capable of identifying individuals within mixed species groups, as this feature would permit fully field tests to be conducted using wild populations. This would eliminate issues of insectary influences on behaviour. Importantly using an open hut would permit mosquitoes to leave a bait: the current tracking methods, in which mosquitoes are released in to a closed room, may exaggerate attack persistence and LLIN contact as insects do not have the freedom to exit the test room. Species identification by track characteristics is therefore a priority for future research as not only would this improve test design, but it has the potential to reveal interesting differences in host seeking flight of different mosquito genera.

The findings of this thesis contribute to the evidence base necessary for the improvement of existing vector control strategies, and the design of new mosquito control tools. Work has characterised previously unrecognised vector behaviours which may be vulnerable to control using new interventions. These experiments have also observed basic host seeking behaviours, providing a base for further research. Vector control must consider both behavioural and physiological effects of interventions; this thesis has developed tools that can quantify insect interactions with LLINs and other interventions, helping to discern how LLINs influence mosquito behaviour, and offering new methods for evaluation of novel insecticide treatments or net designs.

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# Appendices

## Appendix A: Infrared video tracking of *Anopheles gambiae* at insecticide-treated bed nets reveals rapid decisive impact after brief localised net contact

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# Infrared video tracking of *Anopheles gambiae* at insecticide-treated bed nets reveals rapid decisive impact after brief localised net contact

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**Long-lasting insecticidal bed nets (LLINs) protect humans from malaria transmission and are fundamental to malaria control worldwide, but little is known of how mosquitoes interact with nets. Elucidating LLIN mode of action is essential to maintain or improve efficacy, an urgent need as emerging insecticide resistance threatens their future. Tracking multiple free-flying *Anopheles gambiae* responding to human-occupied bed nets in a novel large-scale system, we characterised key behaviours and events. Four behavioural modes with different levels of net contact were defined: swooping, visiting, bouncing and resting. Approximately 75% of all activity occurred at the bed net roof where multiple brief contacts were focussed above the occupant's torso. Total flight and net contact times were lower at LLINs than untreated nets but the essential character of the response was unaltered. LLINs did not repel mosquitoes but impacted rapidly: LLIN contact of less than 1 minute per mosquito during the first ten minutes reduced subsequent activity; after thirty minutes, activity at LLINs was negligible. Velocity measurements showed that mosquitoes detected nets, including unbaited untreated nets, prior to contact. This is the most complete characterisation of mosquito-LLIN interactions to date, and reveals many aspects of LLIN mode of action, important for developing the next generation of LLINs.**

Many of the important mosquito vectors of malaria feed indoors at night, where and when most human malaria is transmitted in Africa[1,2]. Long-lasting insecticidal bed nets (LLINs) exploit this behaviour and are one of the most effective methods for reducing malaria transmission, fundamental to malaria control (amounting to $2.5 bn in 2013) and to ambitious plans for its elimination[3,4]. However, their future is seriously threatened by emerging resistance in vector populations to pyrethroids, the only insecticide class that can be used with LLINs[5,6] and the need for novel LLIN designs that enable safe use of other insecticides or entirely new control devices or strategies is a global health priority[4].

Delivering the 'next generation' of LLINs or similar tools will require a thorough understanding of how LLINs function, yet remarkably little is known of the mode of action or of precisely how mosquitoes behave at the LLIN interface. Recent studies using 'sticky-nets' reported that host-seeking female *Anopheles spp.* landed preferentially on the top surface of bed nets[7,8] but that lethal capture method recorded only a single landing event and no other behaviours before or after. Although clustering at the net roof is likely to be a response to an attractant 'plume' rising from the human beneath, this too remains

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speculative because knowledge of mosquito flight behaviour prior to blood-feeding and of the identity and location of the key attractants that mediate the host-seeking response is limited[9–12]. Importantly, how insecticide treatments influence that response is unclear. Some studies reported that insecticide residues repelled mosquitoes prior to contact[13,14], which would reduce or eliminate the chance of mosquitoes receiving an effective dose and potentially divert them to unprotected hosts[15]. Others found no evidence for such repellency[16–19] indicating that LLINs attract and impact on mosquitoes by direct contact.

A further complication is the existence of what is termed 'contact-irritancy' or 'excito-repellency', whereby brief exposure to an insecticide can result in mosquitoes exhibiting avoidance behaviour, potentially before a lethal dose has been delivered[13,20]. Remarkably, some basic details are missing: *e.g.* the minimum duration of LLIN contact necessary to deliver an effective dosage is not known. Despite these phenomena being recognised for decades[20–22], when and how they occur and their relative importance in selecting for insecticide resistance have never been fully elucidated.

Consequently, behavioural resistance to insecticides remains poorly understood and rarely reported in mosquitoes, though the risk of vector populations switching blood-feeding times, locations or host preferences in order to avoid LLINs is recognized and closely monitored today[23–25]. However, additional but less apparent or detectable behavioural changes also might exist, potentially conferring partial or complete insecticide resistance (*e.g.* changes in sensitivity to repellents, attractants, or modified flight or resting behaviours). In the absence of definitions or quantifications of the basic behavioural events likely to be affected[26,27], these changes cannot be investigated, let alone monitored.

Ideally, characterisation of mosquito behaviour requires direct observation under conditions that are as 'natural' as possible. Informative studies to date have been limited to wind-tunnel or small-scale laboratory tests, potentially restricting mosquito flight. Frequently, tests use artificial or incomplete attractants such as human breath or limited body parts, carbon dioxide, single attractant chemicals or simple odour blends[9,18,28], rather than an entire human host. Experimental huts[29–31], electrocution grids[32,33], taxis boxes[34] and other methods overcome some of these obstacles but are unsuitable for detailed exploration of behavioural sequences.

Addressing many of the technical challenges that hindered progress to date, we have developed and constructed a novel system that enables tracking, recording and analysis of the flight paths of multiple individual mosquitoes over long periods in the dark at large volumes around the entire human host. In this first application of the system, we describe the flight and landing behaviour of *Anopheles gambiae* responding to human hosts within bed nets in a large experimental laboratory, and we show that human-baited LLINs operate by luring mosquitoes into multiple brief contacts with the net, almost entirely on the net roof, within minutes of commencement of host seeking, virtually eliminating all flight activity by 30 minutes.

## Results

### Classification of mosquito behavioural modes.

In all treatments, mosquito activity was classified into four quantifiably distinct types of behaviours, termed 'modes' (Fig. 1E–H; Supplementary Video), defined as follows:

1. Swooping: Tracks that did not contact the bed net.
2. Visiting: Tracks where relatively long periods of flight were interspersed with infrequent contacts with the bed net. Contacts were characterized by sharp turns of 80° or more in the trajectory and when multiple contacts occurred, the minimum interval between them was 0.4 seconds (*i.e.* an interval of at least 20 frames, at 50 frames per second).
3. Bouncing: Tracks where the mosquito made multiple rapid contacts with the bed net surface, at intervals of less than 0.4 seconds; includes events where the mosquito executed short flights between contacts, or maintained contact with the bed net surface without being static. The latter were brief pauses in movement lasting less than 0.75 seconds and included 'walking' on and 'probing' the bed net.
4. Resting: Mosquito tracks where insects were either completely static for at least 0.75 seconds, or where the velocity of mosquito movement was less than 1.33 mm/s (equivalent to movement of up to one mm in the minimum resting time); assumed constant contact with the bed net surface. Dead mosquitoes were excluded by limiting resting events to a maximum of 300 seconds. Notably, no dead mosquitoes were found on nets at the end of tests.

### Responses at unbaited, baited and insecticide-treated nets.

Figure 1B–D show representative examples of recorded flight tracks at unbaited untreated (henceforth termed 'unbaited'), baited untreated ('untreated') and baited LLIN ('LLIN') nets during 1 hour of recording. Across all treatments, individual flight track durations ranged from 0.22 to 445.1 seconds, with a geometric mean track length of 4.2 seconds (4.0–4.3; n = 7729 tracks).

Activity at an untreated bed net was significantly lower in the absence of human bait, as measured by the mean number of flight tracks (212 [−78–501] at unbaited and 545 [410–679] at untreated nets; generalized linear model, $p < 0.001$) and the total duration of activity recorded for each test (*i.e.* 25 mosquitoes for 1 hour, maximum of 25 hours: geometric means of 19.0 [1.6–223.5] and 124.6 mins [101.5–153.0]

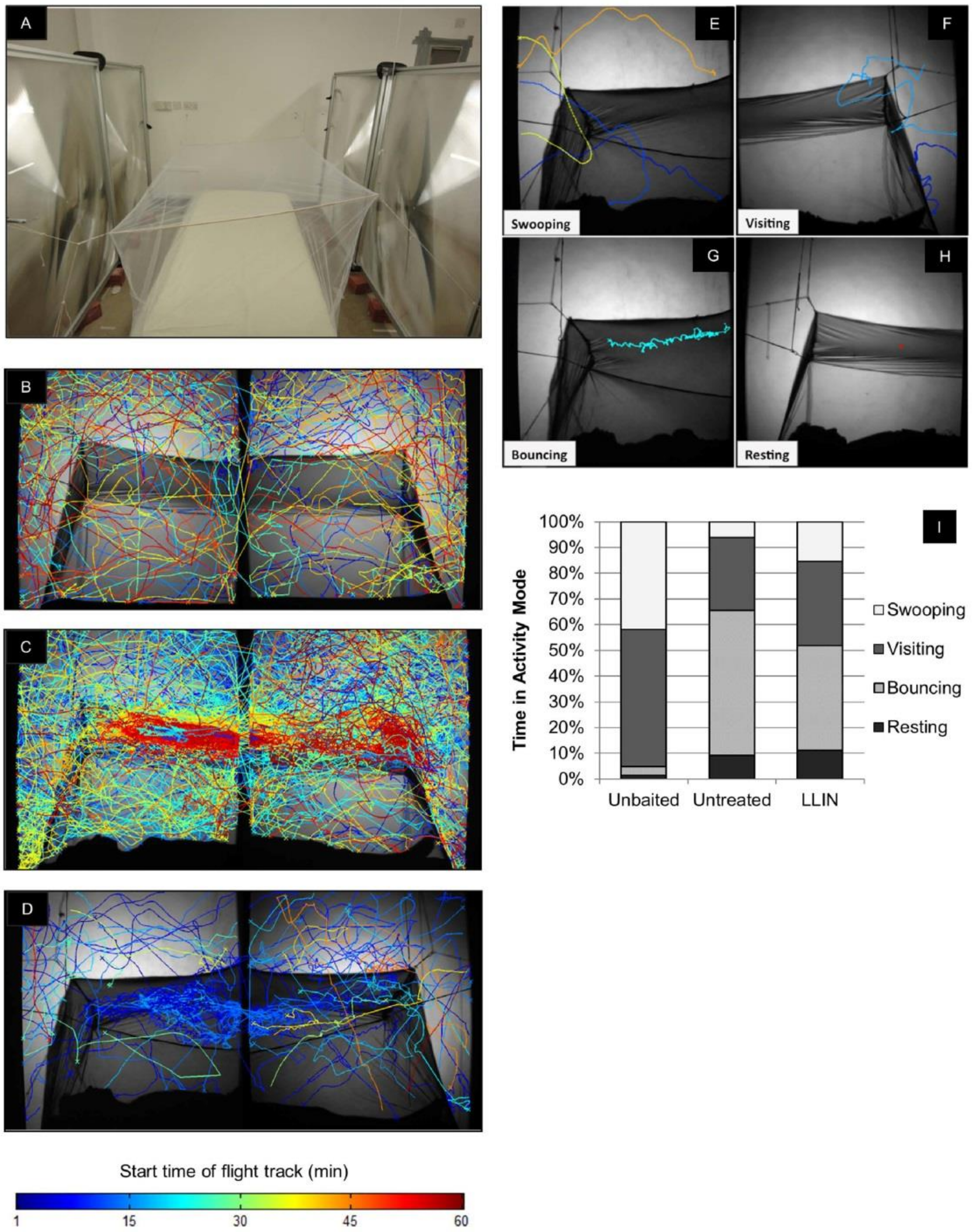

**Figure 1. Flight activity of *Anopheles gambiae* at unbaited, baited and insecticide-treated bed nets.** (**A**) The experimental insectary, showing the bed and fitted bed net, with two pairs of Fresnel lenses visible on the left and right; illumination at 850 nm was from two LEDs (not in image) located behind the right Fresnel lenses with the light from each forming an approximated parallel beam across the bed, through the left Fresnel lenses and focused into the camera beyond (not in photograph). The LEDs and camera were positioned 1.2 m behind the lenses, aligned to an optical axis through the centre of the Fresnel lenses. Mosquitoes were released on the wall behind the photographer at a height of 2 m. (**B–D**) Examples of a full 60-minute record of a test showing flight tracks of *Anopheles gambiae* at: (**B**) an unbaited untreated bed net; (**C**) a human-baited untreated bed net; (**D**) a human-baited insecticide-treated bednet (LLIN; Permanet 2®; Vestergaard-Frandsen, Lausanne, Switzerland). Twenty-five mosquitoes were released in all tests and activity was recorded for 60 minutes. Each coloured track is the path of a single mosquito flight event. Tracks are colour-coded according to time they first appeared in the field of view as shown in the key: blue tracks at the start through to red at the end of the 60-minute test. (**E–H**) Images showing representative tracks for *Anopheles gambiae* flight in each of the four behaviour modes as defined in the text. See also Supplementary Video. (**I**) The proportion of time spent in each behaviour mode for each bed net type: Unbaited = unbaited untreated bed net; Untreated = human-baited untreated bed net; LLIN = human-baited insecticide-treated bed net (LLIN).

| | N | Swooping | Visiting | Bouncing | Resting |
|---|---|---|---|---|---|
| Unbaited | 3 | 7.5 (0.5–116.1) | 10.6 (1.2–96.2) | 0.5 (0–22.1) | 0.1 (0–20.0) |
| | | ab | a | a | a |
| Untreated | 10 | 7.7 (6.1–9.8) | 33.2 (24.0–46.1) | 70.1 (57.7–85.1) | 10.3 (7.0–15.3) |
| | | a | b | b | b |
| LLIN | 10 | 3.4 (1.9–6.2) | 6.9 (3.5–13.6) | 7.7 (3.1–18.7) | 2.0 (0.8–5.0) |
| | | b | a | c | c |

**Table 1. Total activity time of *Anopheles gambiae* recorded in each behaviour mode.** Total duration of all tracks classed in each behaviour mode over 60 minute tests (geometric mean and 95% confidence interval, minutes). Since multiple mosquitoes were often active simultaneously in the field of view, the total activity times could exceed 60 minutes. Values for each mode followed by the same letter are not significantly different at $p < 0.05$ (Generalized Linear Models), between different net types.

at unbaited and untreated respectively; $p < 0.001$). Activity at LLINs (164.7 tracks [91–238]; 21.2 mins [10.6–42.7]) was significantly lower than at untreated nets ($p < 0.001$) but similar to unbaited nets ($p = 0.456$, $p = 0.649$ for track number and duration, respectively).

Exploring this activity by behavioural mode (Fig. 1I) shows that 93.7% of activity on a baited (untreated) net involved net contact (*i.e.* visiting, bouncing or resting modes) compared to 58.1% on an unbaited net. In fact, 65.3% of the activity at a baited net involved frequent (bouncing) or continuous (resting) net contact, in contrast to 4.7% on the unbaited net. Moreover, the mean times spent in each mode involving contact (Table 1) were significantly higher (visiting, $p = 0.009$; bouncing, $p < 0.001$; resting, $p < 0.001$) in the presence of human bait while swooping was not significantly different ($p = 0.953$). At LLINs, activity in all four behaviour modes was significantly lower than at untreated nets, particularly in the three modes with net contact where treated net activity fell to 27% or less than the untreated net values (Table 1, $p < 0.001$ except swooping, $p = 0.002$). However, a response to the host persisted despite the insecticide presence as evidenced by the values for bouncing and resting modes, which were significantly higher than at unbaited nets ($p = 0.001$, and $p = 0.010$).

**Flight speed, tortuosity and height during net approach.** The instantaneous velocity of individual swooping flight tracks ranged from 83.9 to 985.8 mm/s across all tests, with a mean velocity of 346.2 mm/s (341.6–350.8; n = 3234 tracks). Mosquitoes flew slightly faster at baited untreated nets (355.8 mm/s [340.1–371.5]) than at unbaited nets (321.1 mm/s [265.8–376.4]; $p = 0.005$) and LLINs (322.7 mm/s [292.7–352.8]; $p = 0.020$), which were not significantly different from each other ($p = 0.923$).

Track tortuosity was higher in both baited net groups than in the unbaited nets (1.31 [1.16–1.47] unbaited, 1.66 [1.52–1.79] untreated, 1.63 [1.43–1.83] LLIN; $p < 0.001$), but not different between LLINs and baited untreated nets ($p = 0.783$).

Simple analysis of the spatial location of flight path prior to arrival did not indicate any notable bias for low (below top net surface level) or high (above the net) spatial preferences. Adjusting for differences in the visible field of view between high and low areas, equal distribution would result in 36% of tracks starting in the high region. In unbaited and untreated tests, there was no preference (38.0% [34.7–41.2] and 40.3% [32.8–47.9] of tracks starting above the net; $p = 0.134$, $p = 0.237$; one sample t-test), though in baited LLINs marginally more net approaches started above the net than below (41.5% [37.0–46.1]; $p = 0.024$).

**Location of activity at the bed net interface.** The distribution of total activity (seconds/ $m^2$) around the bed net was significantly different at each net type ($p < 0.001$)(Figs 2D and 1B–D). Without human bait, 49.9% of flights occurred in the spatial regions around the net (regions 11–16 in Fig. 2A), compared with 5.5% at untreated nets and 10.5% at LLINs (Fig. 2D). In contrast, activity in baited tests was located primarily on the net roof directly above the human body and to a lesser extent, near the feet: 74.7% and 78.3% of activity occurred on the roof (regions 1–6) and 10.9% and 8.8% at the feet (region 10) in untreated nets and LLINs respectively (Fig. 2D).

Comparing nets by behaviour mode, swooping (Fig. 2E) was distributed unevenly between different net regions in all treatments ($p < 0.001$), with less activity occurring in regions 15 and 16 in front of the vertical net sides. In visiting mode, there was a significant interaction between net type and activity distribution ($p < 0.001$; Fig. 2F): higher visiting rates were recorded in regions 3 and 4 (17% and 16% of activity) on untreated nets, but at LLINs, visiting was higher in regions 3, 7 and 10 (12%, 11% and 10%, respectively). Treatment also affected activity distribution in bouncing mode ($p < 0.001$): bouncing flight was higher in regions 2, 3 and 4 (21%, 35% and 17% respectively) in untreated nets, whereas most bouncing activity at LLINs occurred in regions 1, 2, and 3 (18%, 30%, 24%; Fig. 2G). Finally, net type also significantly affected resting activity ($p < 0.001$; Fig. 2H): on untreated nets, more resting occurred

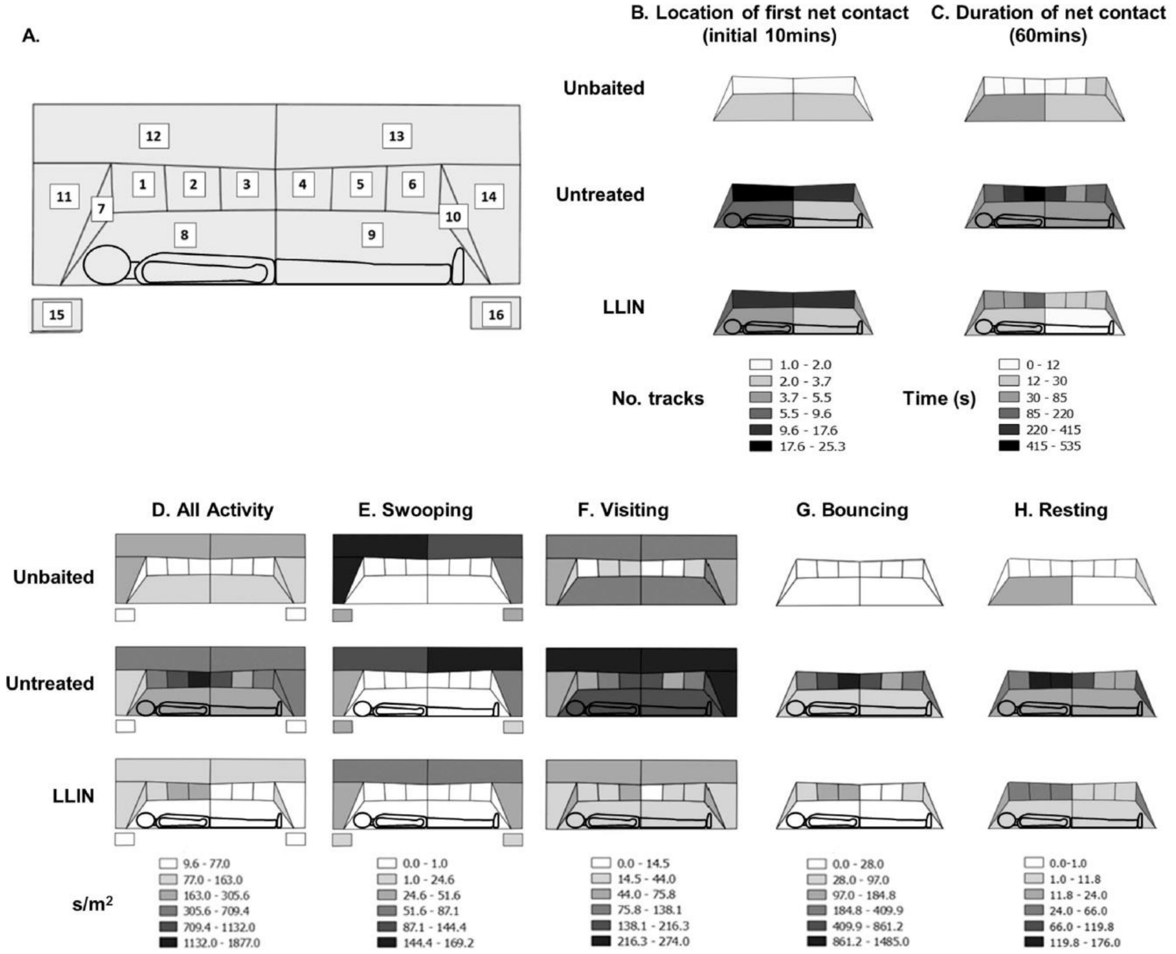

**Figure 2. Distribution of *Anopheles gambiae* flight activity, behaviour modes and net contact at different regions on and around a bed net** (**A**) Distribution map key showing region codes to which each mosquito track was assigned. Bed net surfaces 1–6 were on the horizontal roof, 7 and 10 the vertical head and foot end walls, respectively, 8 and 9 the vertical side walls. Portions of flight tracks visible beyond the net surfaces were assigned to the spatial regions 11–14 as shown. Regions 15 and 16 record flight activity without net contact (*i.e.* swooping) that occurred in front of net, on the left (15) and right (16) portions of the field of view, respectively. (**B**) Distribution of initial net contacts by region, showing the first point of net contact for those tracks occurring in the first ten minutes of testing. (**C**) The total duration (seconds) of all contacts (includes mid-flight brief contacts made during visiting and bouncing, and resting behaviour) by all mosquitoes on each region of the bed net surface over the 60 minute test (means of 3,10 and 10 replicate tests for unbaited, baited and LLIN, respectively). Charts D–H show the density of activity (s/m$^2$) around and on the bed net surface: (**D**) All activity combined; (**E**) Swooping; (**F**) Visiting; (**G**) Bouncing; (**H**) Resting. Although tests controlled for the orientation of the human bait in relation to the mosquito release point, all figures show the volunteer (when present) with the head on the left.

in regions 2, 3 and 10 (26%, 26%, 17%) but on LLINs, resting was higher in regions 1, 2 and 3 (17%, 20%, 33%), with 11% of resting recorded at the feet (region 10).

Hence, although there were marked significant differences between baited and unbaited nets for bouncing ($p = 0.001$) and resting ($p = 0.001$) modes (Fig. 2G,H), there was no evidence that insecticide treatment significantly altered the preference for the roof of the bed net as the focus of activity.

**Velocity of mosquitoes during landing on bed nets.** The mean velocities of mosquitoes during final approach to the net surface, immediately prior to net contact, were determined for each test and compared between bed net types. In total, 896 tracks fitted the conditions for contact analysis. Of this subset, the mean percentages of net contacts classed as 'contacts without deceleration' (*i.e.* tracks that accelerated on their last two points of flight before contact or where deceleration did not start until within 3 mm of

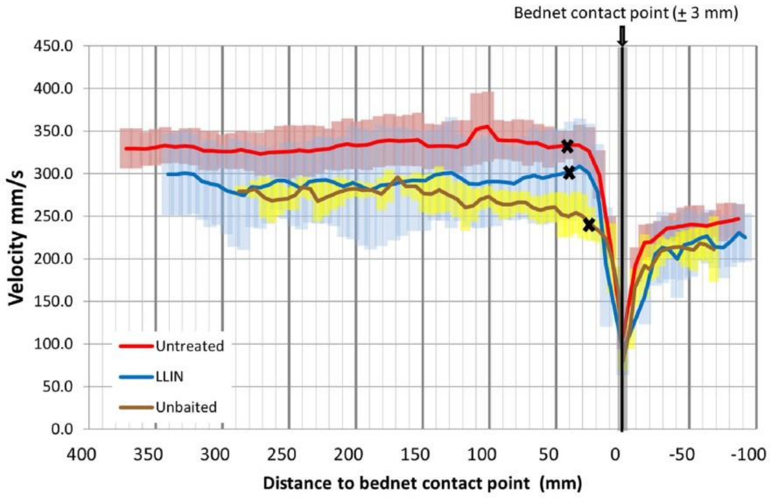

**Figure 3. Velocity of *Anopheles gambiae* during landing at bednets.** Mean velocity of mosquitoes during final approach, contact and departure from the bed net surface. The figure represents a 1.3 s track segment, with the bed net contact point at 0 mm; positive x-axis values indicate position before contact, negative values after contact. The grey region either side of the contact point represents the ± 3 mm region where tarsal contact with the bed net was possible. The average points at which deceleration started for each net type are marked with 'X'. Note that the graph presents the averages of multiple repeat test values and hence the position of the point of deceleration does not correspond perfectly with the average approach track as illustrated. Coloured bars show standard deviation at each track point.

| Duration of physical contact with the bed net surface (60 min test) | | | | |
|---|---|---|---|---|
| | N | Mean total time (all contacts)[1] (min) | Mean time/mosquito (25 mosquitoes)[2] (s) | Mean time/mosquito (observed max number)[3] (s) |
| Unbaited | 3 | 2.4 (−2.1–6.8)[a] | 5.7 (−5.1–16.4)[a] | 41.9 (−19.5–103.4)[a] |
| Untreated | 10 | 33.1 (24.3–41.2)[b] | 79.5 (58.2–100.7)[b] | 334.1 (264.6–403.6)[b] |
| Treated | 10 | 7.3 (3.9–10.7)[c] | 17.5 (9.3–25.7)[c] | 95.6 (57.9–133.2)[c] |

**Table 2. Duration of *Anopheles gambiae* contact with bed nets.** Mean duration of contact with the bed net surface for 60 minutes tests, as calculated for [1]mean total of all contacts observed. [2]mean contact time per mosquito assuming all 25 mosquitoes responded. [3]mean contact time per mosquito based on the maximum number of individual mosquitoes observed simultaneously (mean and 95% confidence interval), values for each mean followed by the same letter are not significantly different between net types at $p < 0.05$).

the net, and leg contact could not be excluded) were calculated as 1.7% [−2.0–5.3] at unbaited nets, 7.5% [5.6–9.4] at untreated nets and 7.6% [−0.1–15.3] at LLINs, and were not significantly different between treatments ($p = 0.392$). Hence, over 90% of mosquitoes decelerated prior to net contact, with deceleration starting at approximately 0.12 seconds prior to landing, at a distance of 26–41 mm from the net.

However, the point at which mosquitoes started to decelerate (Fig. 3) was significantly closer to the net at unbaited nets (distance from the net 26.3 mm [18.5–34.1]) than at baited nets, both untreated (41.5 mm [36.8–46.2], $p = 0.010$) and LLINs (40.0 mm [31.0–49.0], $p = 0.019$), which were not significantly different from each other ($p = 0.708$). In addition, unbaited arrival flight velocities (276.6 mm/s [212.6–340.7]) were significantly slower than those at untreated (384.4 mm/s [365.3–403.5], $p < 0.001$) and LLINs (356.9 mm/s [309.5–340.7], $p < 0.007$), which were not significantly different from each other ($p = 0.175$).

**Quantifying duration of net contact.** The mean total time per test where mosquitoes were in physical contact with nets (Table 2) was significantly higher on the untreated baited net (33.1 min [24.3–42.0]) than on both the LLIN (7.3 min [3.9–10.7]; $p < 0.001$) and unbaited nets (2.4 min [−2.1–6.8]; $p < 0.001$; generalized linear model). However, contact time was significantly higher also on LLINs than on unbaited nets ($p = 0.003$). The longest contact time recorded for a single mosquito track was 37.4 s on an unbaited net, 160.4 s on an untreated net and 110.5 s on an LLIN. Since it was not possible to measure the actual

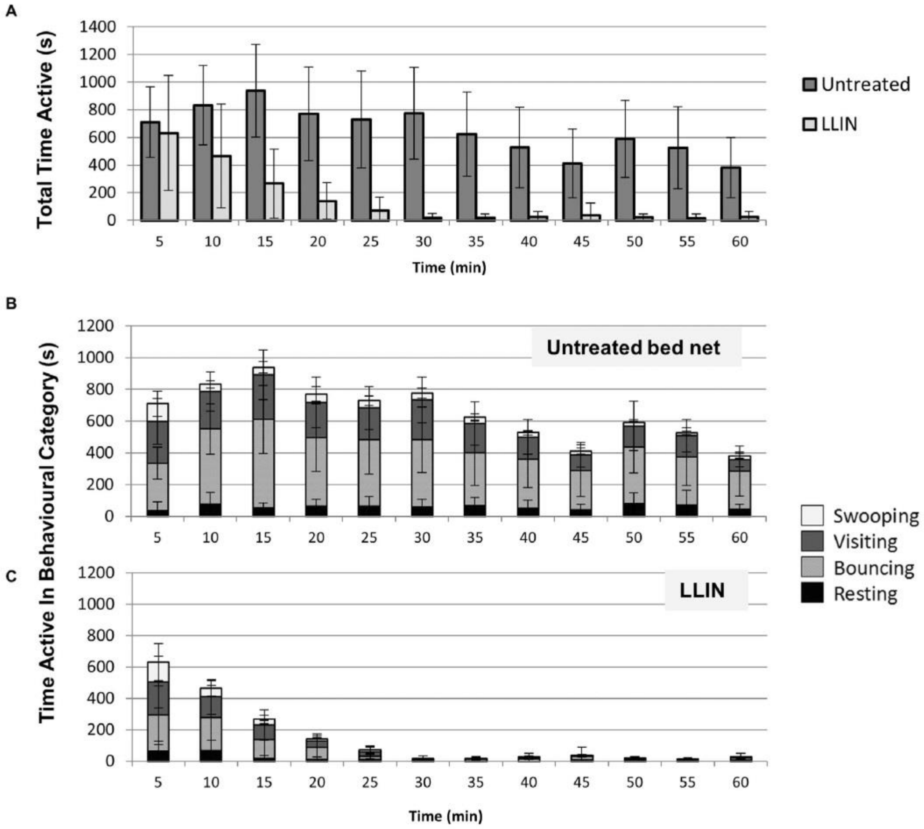

**Figure 4. Rates of *Anopheles gambiae* activity throughout the 60 minute test period.** (**A**) Total activity at untreated baited nets and LLINs.(**B,C**) Mosquito activity as in Fig. 3A separated by behavioural mode, at untreated baited nets (**B**) and LLINs (**C**). X-axis units are mean (±SD) activity per 5-minute inclusive interval, *i.e.* 5 (0–4 min 59 s), 10 (5 min–9 min 59 s), 15 (10–14 min 59 s), etc.

total contact time for individual mosquitoes, we determined plausible mean minimum and maximum contact time values (as defined in Table 2) for a single mosquito of 79.5 to 334.1 seconds at an untreated baited net and 17.5 and 95.6 seconds at an LLIN over 60 minutes of a test.

Total contact time was significantly affected by interactions of net type and region ($p < 0.001$, $p < 0.001$; Fig. 2C). Highest contact times (extracted contact data, of all types, from all tracks) were recorded on the roof in untreated nets (regions 2, 3, 4: 410s, 531s, 306s, respectively), and in the centre of the roof in LLINs (region 3: 126s).

**Interactions with the bed net over time.** Over 60 minutes, total mosquito activity decayed significantly more rapidly at LLINs compared to untreated nets ($p = 0.004$, Generalized Linear Model; Fig. 4A). Reduced activity at the LLIN was indicated after 5–10 minutes, becoming significantly lower from the 10–15 minute period onwards. By 30 minutes, activity at LLINs was negligible and did not recover, while sustained levels of host seeking were recorded at untreated nets for the entire 60 minutes. This rapid fall was apparent in the three behavioural modes involving flight, swooping, visiting, and bouncing ($p = 0.016$, $p = 0.031$, $p = 0.002$, respectively, Generalized Linear Model; Fig. 4B,C).

Mosquito activity during this key initial 10-minute period was explored further. The time lag between appearance of the first mosquito and first net contact was unaffected by the presence of the insecticide or human bait: geometric means unbaited = 18s (0–994); baited = 6s (1–33)($p = 0.438$); LLINs = 17s (6–43) ($p = 0.432$). Comparing untreated baited nets with LLINs, there were no differences in the number (72.8 [53.1–92.5] at untreated nets, 50.3 [28.6–72.0] at LLINs; $p = 0.084$) or the distribution of contacts on different net regions ($p = 0.145$), with a significant majority ($p < 0.001$) of first contacts on the net roof in both (60.6% and 56.5% in untreated and LLINs, respectively)(Fig. 2B). At unbaited nets in contrast, significantly fewer contacts (12.3 [2.3–22.4]; $p < 0.001$) occurred in a significantly different pattern (*i.e.* near uniform distribution) on the net ($p = 0.018$)(Fig. 2B).

These results indicate that LLINs did not repel mosquitoes to any significant level prior to net contact. Yet, while contact with LLINs was significantly lower than untreated nets over 60 minutes (Fig. 1I, Table 1), the majority of LLIN contacts occurred during the first ten minutes: 62.2% on LLINs (4.6 min

[2.2–6.9]), 17.9% on untreated nets (5.9 min [4.0–7.8]). Moreover, this impact was preceded by surprisingly brief time in contact with the LLIN. We calculated that during the initial ten minute period, one mosquito made between 14.3 and 70.3 seconds of contact with an untreated net or 11.0 and 57.1 seconds with an LLIN (minima and maxima calculated as described in the previous section).

## Discussion

These results provide detailed insight into the behaviour of *An. gambiae* at an LLIN. On detection of a human host within a bed net (with or without insecticide), mosquitoes responded immediately in four distinct behaviour modes, with persistent attempts to reach the host resulting in multiple brief net contacts focussed on the net roof above the human torso. Behaviour at an LLIN retained the essential character of the response to untreated nets for the first ten minutes, during which time less than one minute of total contact was made with the LLIN. A rapid decay in all modes of activity resulted and after thirty minutes, mosquito activity was negligible and did not recover. Lag times to response and velocities and deceleration rates prior to net contact were similar in LLINs and untreated nets, demonstrating the absence of LLIN repellency. The results demonstrate that an LLIN is a highly efficient fast-acting baited insecticide trap.

These results were obtained with an optical imaging and flight-tracking system allows remote tracking, recording and quantitative analysis of multiple mosquitoes simultaneously flying without restriction in large fields of view over long periods while they respond to a complete human host in complete darkness. For studies at this scale, the system offers a number of advantages over other approaches. Despite their undoubted value, existing tracking systems, including some three-dimensional (3D) systems, are restricted (in relation to this study's goals) in terms of temporal resolution and test arena size constraints, short recording durations (up to 15 minutes), the low numbers of mosquitoes that can be observed simultaneously (1–4 mosquitoes per experiment) or the need to use isolated host cues such as heat or odour rather than complete human baits[9,28,35,36]. Studies that track multiple mosquitoes have been restricted by short recording periods of less than 3 minutes or the ability to track only initial and final behavioural events[35–38]. An effective stereo video system tracked up to 25 mosquitoes in wild mating swarms[37–39] but required sunlight to generate the images.

The findings are novel and a significant contribution to our understanding of mosquito behaviour generally, and specifically how it is targeted by LLINs. Although the LLIN tested is only one of many types commercially available today, the Permanet® 2.0 is one of the most purchased and widely used LLINs in Sub-Saharan Africa[40]. Clearly further studies must investigate other LLINs.

The immediate and rapid effect of the LLIN and the low level of net contact required to achieve that has never been reported. Less than one minute of contact within the first 10 minutes reduced subsequent foraging such that all flight and host location activity was virtually eliminated by 30 minutes. Whilst acknowledging that a limited number of net contacts may not have been captured by the tracking system (*e.g.* potentially obscured by the host, net seams or wrinkles, or during processing) we consider this to be an accurate measurement of LLIN contact duration.

Activity at baited nets, both untreated and LLINs, was higher than at unbaited nets, particularly in the bouncing and resting behaviour modes when the highest levels of net contact occur. While activity over 60 minutes was lower at LLINs than at untreated nets, there was no difference in the number, distribution or duration of net contacts in the first 10 minutes. Furthermore, velocities measured immediately prior to net contact were virtually identical in both untreated nets and LLINs, with no indication that mosquitoes were repelled or deterred by the insecticide at close range. Finally, there were no significant differences in the time lag prior to the initial mosquito's response, confirming a previous report[18], and indicating there was no distant or spatial insecticidal effect on behaviour.

This finding partially allays fears that LLINs might divert unfed but still hungry mosquitoes to non-users of nets without any LLIN contact[41–44]. However, it remains to be determined whether the observed elimination of activity at the LLIN (Fig. 4) resulted from insecticide-induced knockdown or death, an irreversible sub-lethal flight or sensory impairment, or some other reversible condition. Contact irritancy and impairment of host seeking responses by deltamethrin have been described[45,46] but we were unable to recapture sufficient mosquitoes to determine mortality rates or sub-lethal effects post-exposure. We calculated that an individual mosquito made on average between 17.5–95.6 seconds of contact with the LLIN (Table 2; although the true value depended on the proportion of released mosquitoes responding). Earlier tests with *An. gambiae* and deltamethrin-treated nets reported knockdown and death of some individuals following net contact times of only 0.4 seconds while others survived after 40 seconds of contact[18]. These results suggest that, in reality, the effects of LLINs on individual mosquitoes may be wide ranging in severity. Hence, although our results demonstrate clearly that host seeking ceases rapidly when an LLIN is used, determining the proportion of mosquitoes that survive and remain capable of locating and feeding successfully on a different host following contact with an LLIN, is an important next step.

An important additional point is that these (Table 2 and Spitzen *et al.*[18]) LLIN contact values derive from observed behaviour and are considerably lower than the WHO standard method used for LLIN evaluation[47], where mosquitoes are forced into contact with treated surfaces for 3 minutes. Although further accurate data are essential to confirm this, the duration of exposure used in standard evaluation of LLINs may need to be re-examined to avoid any possibility of overestimating the effectiveness of any material being tested.

Our results emphasise the importance of the bed net roof[7,8] by showing that it is the predominant first point of contact (Fig. 2B), the most commonly visited surface (Fig. 2C), and that most flight activity is also focussed at or around the roof, regardless of the flight path of the arriving mosquito (Fig. 2D–F and 1C,D,G; Supplementary video). Though there was some additional activity near the feet, activity at the net sides was very low (Fig. 2C,G) indicating that mosquitoes oriented primarily to putative olfactory and thermal attractants rising from the prone host[12,48,49]. Within hypothesised models of vector host location[12,28,48,49], the mosquitoes tracked in this study were relatively close to the host throughout, and therefore likely to have been flying in response to 'broad plumes' of host cues that would ultimately lure them to the net. Without knowing the actual location of those 'plumes' or their boundaries, it is not possible at this stage to interpret the observed flight trajectories or assign them to recognised behaviours such as 'casting'[28], where mosquitoes exhibit counterturning on leaving the plume in order to relocate it, or where increased tortuosity and decreased velocity occur as mosquitoes attempt to locate the source of the attractant[50–52].

The four newly described behaviour modes provide a means to measure and compare the effectiveness of different treatments, including repellents or attractants. We hope also that the results will contribute towards the identification of possible new approaches to target anophelines. To maximise LLIN performance, new designs should ensure that novel chemistries or other treatments do not impair the essential attractiveness of a human-baited LLIN; indeed, efforts to enhance or exploit it should be pursued. The results might also lead to improved vector sampling, *e.g.* CDC light traps or other devices placed directly above bed nets might yield better samples[53].

Mosquito velocities, measured here in free-flying anophelines responding to complete human hosts, were faster in the presence of the host (previously reported with wind tunnels[9]) but significantly slower (approximately 10%) when the host was protected by an LLIN. However, the velocities measured close to the bed net surface, reported here for the first time, were similar at LLINs and untreated nets. The results are significant, first, because there were no significant differences in the proportions of mosquitoes that decelerated prior to contact and the distance from the net where deceleration occurred, further evidence for the inability of *An. gambiae* to detect the LLIN, and the absence of significant repellent properties, even at close range. Secondly, they indicate that prior to contact, mosquitoes detected the presence of net barriers, including the unbaited untreated net. Landing behaviour in insects is strongly linked to visual interpretation of proximity to a surface[54–56] and the eyes of nocturnally active mosquitoes like *An. gambiae* are sensitive to conditions of low visible light[57]. Tests were carried out using LEDs with a peak wavelength of 850 nm, beyond the visual perception range of *An. gambiae*[58]. Despite our efforts, we cannot guarantee that the test insectary was totally dark and it is possible that a light leak might have allowed *An. gambiae* to navigate visually. Alternatively, mosquitoes may have detected changes in air movement or the odour plume on coming in to proximity with the net surface[59,60], using the Johnston's organ or halteres, which are involved in the detection of mechanosensory cues[61–63]. Notably, *An. gambiae* showed similar responses by avoiding 'invisible' clear plastic obstacles when orienting to host cues in a wind tunnel study[64].

Unlike coordinated landing on the tarsi, uncontrolled collision potentially could influence the quantity of insecticide deposited onto a mosquito, and we questioned whether mosquitoes were responding sufficiently far in advance to avoid 'crashing' into the LLIN. Deceleration started at only 0.12 seconds prior to net contact (26–41 mm from the net). We are unaware of any appropriate studies on mosquitoes for comparison, but *Drosophila melanogaster* flying at 300 mm/s began deceleration when 27 mm away from the landing point[65], values that are remarkably similar to those measured in our study. That study also showed that the deceleration point varied with flight velocity: slower-flying *Drosophila* began deceleration closer to the landing point, also seen in our data. This provides further evidence that mosquitoes detect the presence of net barriers prior to contact.

Ongoing work will explore flight trajectories further and investigate responses in resistant malaria vector populations, other LLINs and other mosquito species. The tracking system has been deployed in rural locations in Africa where preliminary results indicate that these laboratory findings are representative of wild populations (Angarita-Jaimes *et al.* unpublished). Though still at an early stage, already these findings significantly contribute to the evidence base required for improved vector control tools by identifying previously unrecognised vector behaviours that may be vulnerable to targeting via simple interventions, and mechanisms that identify potential routes for reducing quantities of insecticide used or for the use of previously unavailable insecticide classes. They also provide a base for further research on basic behavior and much-needed studies into behavioural mechanisms of insecticide resistance. Not least, the study provides a new platform for elucidation of LLIN function and evaluation of new LLINs[66–68] and other vector control tools such as spatial repellents[69], at a rapid and cost-effective screening stage prior to larger scale testing in the field[70].

## Methods

Three to five day old unfed adult female (25 per experiment) *An. gambiae s. str.* "Kisumu" strain, colonised at Liverpool School of Tropical Medicine (LSTM), were tested in a dedicated insectary at LSTM (5.6 m × 3.6 m in area 2.3 m high; climate controlled at 27 ± 2 °C, 70 ± 10% Relative Humidity).

The LLINs used were Permanet® 2.0 (75 denier polyester net with deltamethrin at 55 mg/m$^2$; Vestergaard, Lausanne, Switzerland), a WHOPES approved product[71]. Untreated nets were assembled from untreated polyester net of similar mesh.

Data were recorded and analysed from 23 laboratory tests (25 mosquitoes/test): 10 with an untreated net and 10 with an LLIN, each with 10 different human bait individuals; 3 tests used an unbaited (*i.e.* no human bait) untreated net. Ten human volunteers were used, a sample size exceeding that used in previous studies investigating similar behaviours[7,8,48] and each volunteer was tested with an LLIN and an untreated bed net.

**Mosquito tracking.** Mosquitoes were tracked using paired identical recording systems (capturing upper or lower body sections, Fig. 1B–D), each comprising a single high power infrared LED and acrylic diffuser, aligned with a pair of Fresnel lenses (mounted either side of the bed net, Fig. 1A) and monochrome camera and lens (as detailed in Supplementary Methods). The complete system captured an area of 1.2 m × 2.4 m, with blind zones of 100 mm × 1200 mm in the centre and 50 mm × 1200 mm on each side (Fig. 1B–D). Cameras were operated from a computer outside the insectary. The set-up was illuminated by two 850 nm (wavelength spectrum from 790–885 nm; invisible to humans and mosquitoes[58]) infrared light emitting diodes (1000 mA minimum; M850L2, Thorlabs, UK), one per Fresnel lens pair, located 1.2 m behind the focusing Fresnel lenses. Minimal barrel type lens distortion was observed, as assessed in multiple planes along the optical axis between the Fresnel lenses. Hence, any image distortions present would have affected the absolute positional accuracy across the entire field of view but have negligible effects on displacements when evaluated during tracking. Mosquito activity was recorded at 50 frames/second, using StreamPix software (www.norpix.com) and data saved as .seq files.

Since multiple mosquitoes were present in all tests and the entire room was not visible, determining the total number of mosquitoes responding or tracking individual mosquitoes throughout the test was not possible. Hence analyses were performed on flight tracks, and as every track theoretically could have been a different mosquito, each track from entry and exit in the field of view was analysed independently.

Segmentation and tracking algorithms were developed using bespoke software written in Matlab (Mathworks), to extract and interpret trajectory duration, time resolved velocity, distance travelled, tortuosity, and the number and duration of contacts with the bed net. Flight track segments were categorised in behavioural modes using existing quantification algorithms (Angarita-Jaimes *et al.* unpublished). A track could comprise up to three different behavioural modes (all except swooping, where no net contact occurred) and where more than one mode occurred, the times spent in each mode were recorded separately.

**Quantifying net activity.** Track duration was analysed using a linear generalised linear model with normal probability distribution. Track numbers were analysed using a generalised linear model with Poisson distribution. The time lag between the first mosquito's first appearance in the field of view (using the natural log to correct for skew) and its first contact with the net, and the effect of net type were assessed with Kaplan Meier Survival Analysis.

**Quantifying net approach.** Analyses were applied only to activity recorded in the first ten minutes. The point where a track first appeared in the field of view was classed as either high (*i.e.* over the net: regions 12 and 13 Fig. 2A) or low (all other positions). Tracks starting on the net were considered likely to be fragments of incomplete tracks (*e.g.* track continuity was lost during movement between lenses or darker net regions or tracks could not be linked with confidence) and discarded; rigorously applying this rule eliminated 24% of tracks from analysis. A one-sample two-tailed *t*-test was used to compare the percentage of tracks making high approaches, with the expected value equal to 36% (the proportion of the total field of view edge, within regions 12 and 13). Location of first net contact was assessed using the definitions of contact stated in relation to activity modes *i.e.* a sharp change in track direction, or frequent semi-periodic change.

**Quantifying velocity and tortuosity.** Flight velocity values were calculated using whole swooping tracks. Tortuosity values were calculated using whole swooping tracks, and track sections prior to first net contact for other flight types. To measure tortuosity, an index of the degree of flight meander, tracks were subdivided into sections comprising 40 sequential positions (average length 280 mm), and tortuosity calculated as the ratio of actual distance travelled to the straight line distance between the two end points on the section; sub-section values were then averaged to provide track value. This method removed bias resulting from extreme meandering tracks that started and ended in close proximity. Although speed and tortuosity data were not normally distributed, results from GLM analysis of transformed data were unchanged, and the untransformed data are shown.

**Determination of velocity/deceleration prior to contact.** To explore mosquito velocity during approach and landing at bed net surfaces, trajectories in which mosquitoes flew for at least one second prior to contacting the net were selected, and a 65-point section of each trajectory, from 1 second before contact to 0.3 seconds after contact was selected. Instantaneous velocities and rates of acceleration/deceleration of these segments were calculated for each track point as described in the Supplementary

Methods, and the point closest to the net where deceleration started was determined. The maximum point therefore that could be classified as the start of deceleration was point 50, net contact occurred at point 51 (Fig. 3), and the track length between the deceleration start point and net contact point was calculated.

With front legs extended during flight[56], mosquitoes potentially could have contacted the bed net with their tarsi before the tracking algorithm that detected the mosquito's body, could detect the change of direction indicating 'collision'. On this basis, the numbers of tracks where deceleration began within 3mm of the net surface (*i.e.* when leg contact could not be excluded) or that accelerated on their last two points of flight towards the net, were quantified for each repeat test. As our interest was in determining whether mosquitoes decelerated prior to contact, these events were classed as "contacts without deceleration" and were excluded from further analysis. Remaining tracks were used to calculate a mean track distance between the point at which deceleration occurred and the net, for each of the 23 test replicates. Average instantaneous velocity at the deceleration was calculated for each test.

**Defining and quantifying contact with a bed net surface.** Bed net contacts were identified as resting tracks, or by sharp changes in mosquito flight direction at the net surface, defined as minimum angle changes of 80° in visiting mode (Fig. 1F). In bouncing mode (Fig. 1G), angle changes during repetitive contacts were often lower, and repetitive oscillations in '$x$' and/or '$y$' co-ordinates were detected from zero crossings of a bandwidth-filtered position vs. time history (Angarita-Jaimes *et al.* unpublished). To avoid spurious connections between unrelated net-arrival and net-departure tracks, resting periods were limited to a maximum of 300 seconds per event.

Time spent in contact with the net was calculated from the sum of all contacts accrued through single visits or rests, and multiple bounces. Since tracking behaviour of individual mosquitoes over the course of any test was not possible, maximum and minimum values of net contact time per mosquito were estimated as follows: the maximum value was total contact divided by the maximum number of mosquitoes observed attacking the net simultaneously in each test; the minimum value assumed that all 25 mosquitoes responded simultaneously, and calculated each mosquito's activity as $\frac{total\ contact}{25}$. If the total number of trajectories recorded was fewer than 25 (only found in estimates for the first 10 minutes) the actual value was used as the total number of recorded trajectories.

**Localisation of activity at the bed net interface.** The field of view was divided into 16 regions, ten on the net surface and six in the surrounding space (Fig. 2A). A mosquito track was assigned to regions 1–10 when contact with that region was detected. Swooping tracks in regions 1–10 were assigned to region 15 or 16 (left or right camera fields, respectively). Mosquito activity showed no bias towards either the right or left camera field (t-test, $p = 0.523$). Total activity, swooping, visiting, bouncing and resting were scaled by region area, giving values of seconds/mm$^2$, to compensate for size differences between regions. Point of first net contact, and duration of net contact were analysed without scaling for area. Larger combined regions were used for analysis of point of first contact (Fig. 2B) as low numbers of data points occurred in the first ten minutes of some tests.

**Rates of mosquito activity throughout the 60 minute test period.** Mosquito activity over the hour's test was grouped in to 12 five-minute intervals. Using Prism 6, these were fitted to an exponential decay equation to find the value of the decay constant ($k$) in the equation $Activity_t = Activity_{t=0} e^{-kt}$ (where $t$ = time in minutes).

**Statistical Analyses.** Statistical analyses used SPSS Statistics 21 (IBM) and Prism 6 (GraphPad). Random effects generalized linear models with normal probability distribution were used for analyses of activity time, behavioural modes, region preferences, velocity, tortuosity, percentage of tracks contacting the net without deceleration, distance from the net and velocity of track at deceleration point, and effects of net type. Where values were not normally distributed according to calculations of skewness and kurtosis, averages were calculated as geometric means. Generalized linear models with Poisson distribution were used to assess differences in numbers of tracks and Kaplan Meier survival tests to assess differences in lag between appearance and net contact. For analyses of rates of activity decay over time, $k$-values were tested for significant differences between untreated nets and LLINs using GLM and 95% confidence intervals used to determine time intervals with significant differences in activity (Fig. 4A). For all tests, the $\alpha$ threshold used was 0.05. In all cases except for 'all treatment' data 95% confidence intervals were calculated using the $t$ distribution to account for small replicate numbers. Unless stated otherwise, data are reported as arithmetic means and 95% confidence intervals.

**Ethical Permission.** Methods were carried out in accordance with the approved guidelines. Informed consent was obtained from all participating human subjects. The study was approved by Liverpool School of Tropical Medicine Research Ethics Committee ('Behaviour of African malaria vectors': Permit no. 12.13, issued 24th May 2012).

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## Acknowledgements

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## Author Contributions

P.J.M. conceived the study; D.T., N.A.J. and C.E.T. designed and built the tracking system; N.A.J. designed and wrote/customized the associated software; P.J.M., J.P. and M.A. designed the experiments; J.P., M.A. and N.A.J. carried out the experiments; J.P. and N.A.J. analysed the data; all authors contributed to and approved the final draft of the manuscript.

## Additional Information

**Supplementary information** accompanies this paper at http://www.nature.com/srep

**Competing financial interests:** The authors declare no competing financial interests.

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## Appendix B: Supplementary Methods: Infrared video tracking of *Anopheles gambiae* at insecticide-treated bed nets reveals rapid decisive impact after brief localised net contact

Supplementary video can be downloaded from: http://www.nature.com/article-assets/npg/srep/2015/150901/srep13392/extref/srep13392-s1.mov

# Infrared video tracking of *Anopheles gambiae* at insecticide-treated bed nets reveals rapid decisive impact after brief localised net contact

Josephine E.A. Parker, Natalia Angarita-Jaimes, Mayumi Abe, Catherine E. Towers, David Towers & Philip J. McCall

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## SUPPLEMENTARY METHODS

### Mosquitoes

Mated unfed adult female *An. gambiae s. str.* Kisumu strain were obtained from a long-established colony at the Liverpool School of Tropical Medicine (LSTM) maintained on human blood, under a L12:D12 hour light: dark cycle. To avoid potential impact of age on responses to insecticide, all insects used were three to five days post-eclosion. Mosquitoes were starved of sugar and water for 4-6 hours, and introduced into the experimental room at least 1 hour before testing. All tests started after the first hour of the scotophase.

### Insectary environment

The study was carried out in a dedicated insectary at LSTM, measuring 5.6m x 3.6m in area and 2.3m high, with a controlled climate (27±2˚C, 70±10% Relative Humidity). One hour before tests, the volunteer entered the bed net, the mosquitoes were placed in a paper cup connected to an external release cord, suspended against the wall at a height of 2m (chosen to simulate entry at eave height), and the room was closed. The mosquito release point was located 1.4m from the end of the net.

To avoid any influence of air movements or climate gradients, humidification and air conditioning were switched off. At the end of the 60-minute test period, mosquitoes in the room were collected with aspirators. Between tests with treated and untreated nets, surfaces in the insectary were washed (5% Decon 90) and rinsed and air vented with a fan in the doorway.

### Long-lasting insecticidal bed nets (LLIN)

LLINs were removed from packaging and hung (in a separate room) for four weeks prior to tests. To facilitate image capture, bed nets were altered and sewn to fit the mattress tautly to eliminate wrinkling or folding, and the top surface of the net was tilted on its long axis (measuring 750mm and 450mm high on opposite sides; Figure 1A). Human volunteers lay on a fresh sheet on a 2m x 0.88m mattress (180mm thick, 480mm above the floor at the top) on timber slats mounted on bricks to ensure rigidity and reduce vibration.

### Human baits and test conditions

Volunteers (5 males and 5 females of different ages and a range of ethnicities) were recruited from staff and students at the Liverpool School of Tropical Medicine. Each volunteer was tested once with Permanet 2.0 and once with the untreated net. Volunteers were clothed but barefoot and lay on their backs, as immobile as comfort permitted, to avoid influencing mosquito movement or generation of spurious tracks by the motion detection software. All were asked to refrain from using scented toiletries on the morning prior to testing. To control for any influence of body orientation, half the participants were randomly assigned to one position (*i.e.* 50% with head and 50% with feet towards the mosquito release point), which they retained for both tests. Volunteers were tested with LLIN or untreated net on different days, with an average interval of 13 days between tests.

### Mosquito tracking system

Each of the paired identical recording systems comprised a 12.5mm imaging lens (Kowa LM12HC 1"; Multipix UK) mounted on a monochrome camera (Baumer HXC40NIR, Camera Link, 4Mpix; Lambda Photometrics, UK), a pair of Fresnel lenses (1400 x 1050mm and 3 mm thick; NTKJ Co., Japan) and a single high power infrared LED (850nm, 1000mA minimum; M850L2, Thorlabs, UK) placed behind a 3mm thick acrylic diffuser (Comar Optics, UK). Components were mounted on heavy tripods or aluminium frames to minimise sagging and movement. Fresnel lenses had a focal length of 1.2m and were positioned 1.75m apart to accommodate the width of the bed and mattress, with a gap of approximately 43cm between the lens and mattress on each side (Figure 1A).

In this back-lit set-up, the large aperture Fresnel lens enabled the illumination source to be formed into a large area approximately collimated beam. This allowed optically efficient illumination of the large volume from a single light source (the infrared LED). Moreover, an additional diffuser was placed between the light source and the Fresnel lens to help homogenise the illumination across the entire field of view. This also ensured that the LED source was not directly imaged. The efficiency of the illumination enabled the exposure time of each frame to be reduced to typically 3 milliseconds, thus ensuring that the image was not overexposed.

Both cameras were operated by a single computer (Intel Core i7. 3.4 Ghz.8 Gigabytes RAM, Windows 7 Ultimate; 10 hard drives at 2 Terabytes each, 5 drives per camera).

### Determination of *An. gambiae* velocity and rate of deceleration prior to net contact

To analyse tracks contacting the net a sub-set of trajectories was selected in which mosquitoes flew for at least one second, and then made contact with the net. A 65-point section of the trajectory, starting 1 second prior to contact and extending 0.3 seconds after contact was selected for further analysis. Velocity of these tracks was calculated at each of the points along its length using the equation $v_i = \frac{\|\overrightarrow{r_{i+1}} - \overrightarrow{r_{i-1}}\|}{t_{i+1} - t_{i-1}}$ where $v_i$ is the velocity at point *i*, $r_i$ is the position vector at point *i*, and $t_i$ is time stamp at point *i*. Velocities for each track were filtered with a low pass $3^{rd}$ order Butterworth filter, with a cut-off frequency of 11.25Hz and a sampling frequency of 50Hz.

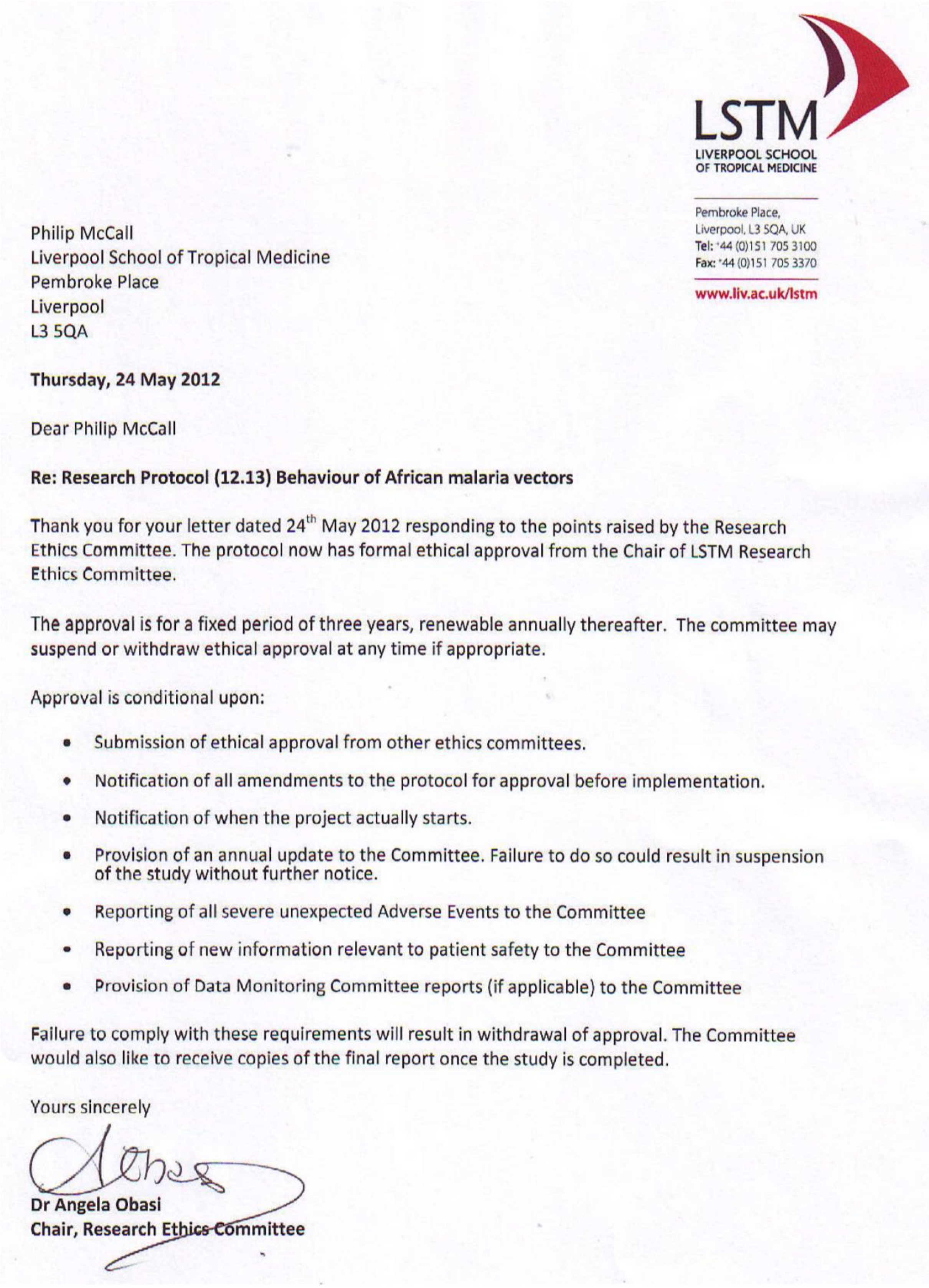
Acceleration at each point was calculated using $a_i = \frac{v_{i+1} - v_{i-1}}{t_{i+1} - t_{i-1}}$ where $a_i$ is acceleration at point *i*. The track point at which pre-contact deceleration started was calculated using methods modified from [51]. An algorithm starting at the point of collision worked backwards along the track to identify the first incidence of two consecutive points with positive acceleration values.

The point following this (i.e. closer to the net) was used as the start of deceleration in the track. Using this system the maximum point that could be classified as the start of deceleration was point 50 (contact occurs at point 51). The remaining track length between deceleration point and contact point was determined for each track.

**Supplementary Video: Mosquito flight at a human-occupied bed net in swooping, visiting, bouncing and resting behavioural modes**

The video demonstrates the characteristic movement patterns within the different behavioural modes: in swooping, mosquitoes fly without contacting the net; visiting flights make infrequent net contacts; bouncing mosquitoes make frequent short persistent attacks on the net surface. In resting the mosquito is stationary, or slow moving. During the resting video, the marker disappears when movement ceases (start of resting) and reappears at the same point when movement restarts (resting mode ends). As markers are attached to moving objects, the mosquito is not highlighted when it stops moving, though the tracking algorithm continues to follow its position.

# Appendix C: Certificates of Ethical Approval



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**Thursday, 24 May 2012**

Dear Philip McCall

**Re: Research Protocol (12.13) Behaviour of African malaria vectors**

Thank you for your letter dated 24th May 2012 responding to the points raised by the Research Ethics Committee. The protocol now has formal ethical approval from the Chair of LSTM Research Ethics Committee.

The approval is for a fixed period of three years, renewable annually thereafter. The committee may suspend or withdraw ethical approval at any time if appropriate.

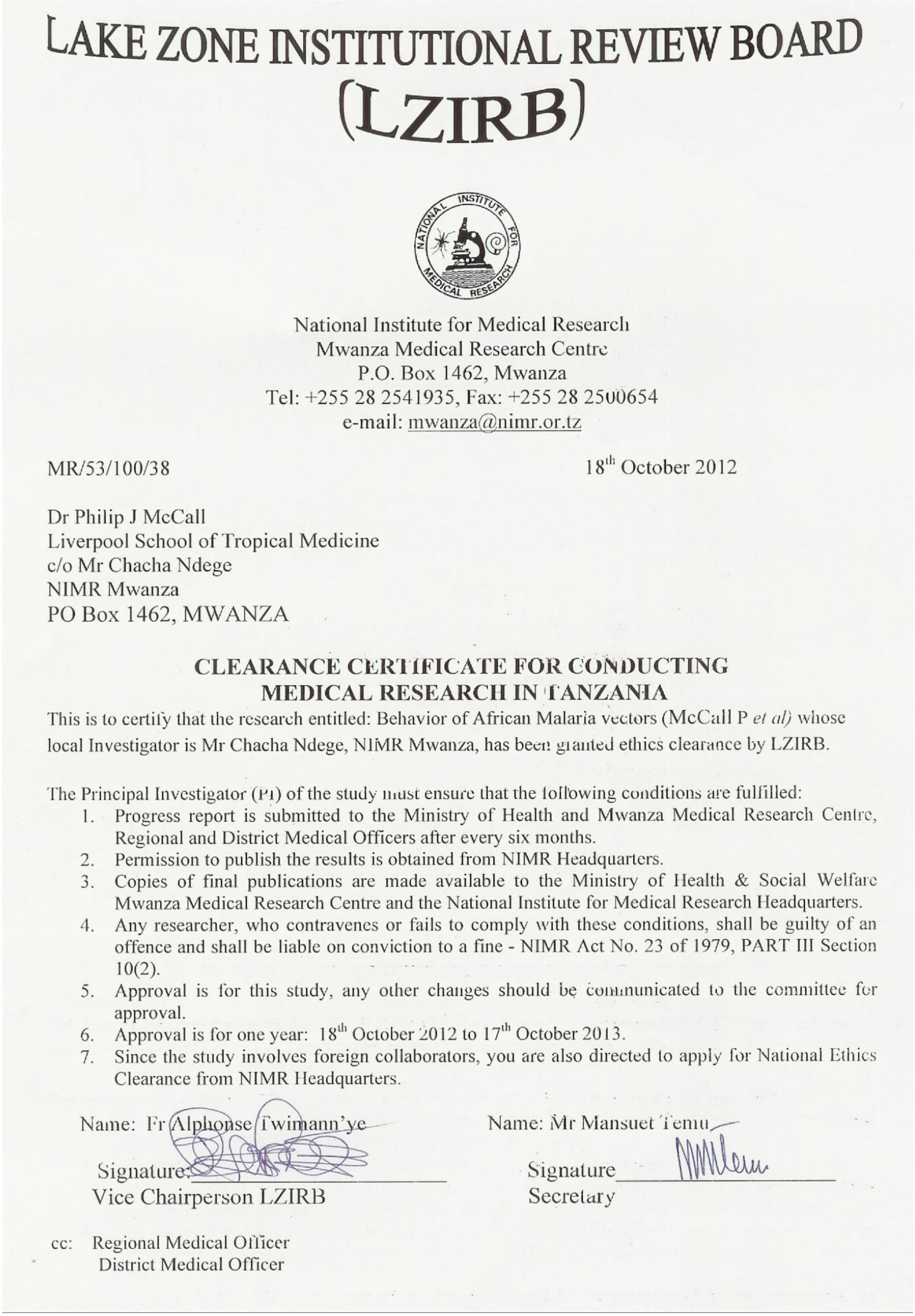
Approval is conditional upon:

- Submission of ethical approval from other ethics committees.
- Notification of all amendments to the protocol for approval before implementation.
- Notification of when the project actually starts.
- Provision of an annual update to the Committee. Failure to do so could result in suspension of the study without further notice.
- Reporting of all severe unexpected Adverse Events to the Committee
- Reporting of new information relevant to patient safety to the Committee
- Provision of Data Monitoring Committee reports (if applicable) to the Committee

Failure to comply with these requirements will result in withdrawal of approval. The Committee would also like to receive copies of the final report once the study is completed.

Yours sincerely

**Dr Angela Obasi**
**Chair, Research Ethics Committee**



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MR/53/100/38 18th October 2012

Dr Philip J McCall
Liverpool School of Tropical Medicine
c/o Mr Chacha Ndege
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## CLEARANCE CERTIFICATE FOR CONDUCTING MEDICAL RESEARCH IN TANZANIA

This is to certify that the research entitled: Behavior of African Malaria vectors (McCall P *et al)* whose local Investigator is Mr Chacha Ndege, NIMR Mwanza, has been granted ethics clearance by LZIRB.

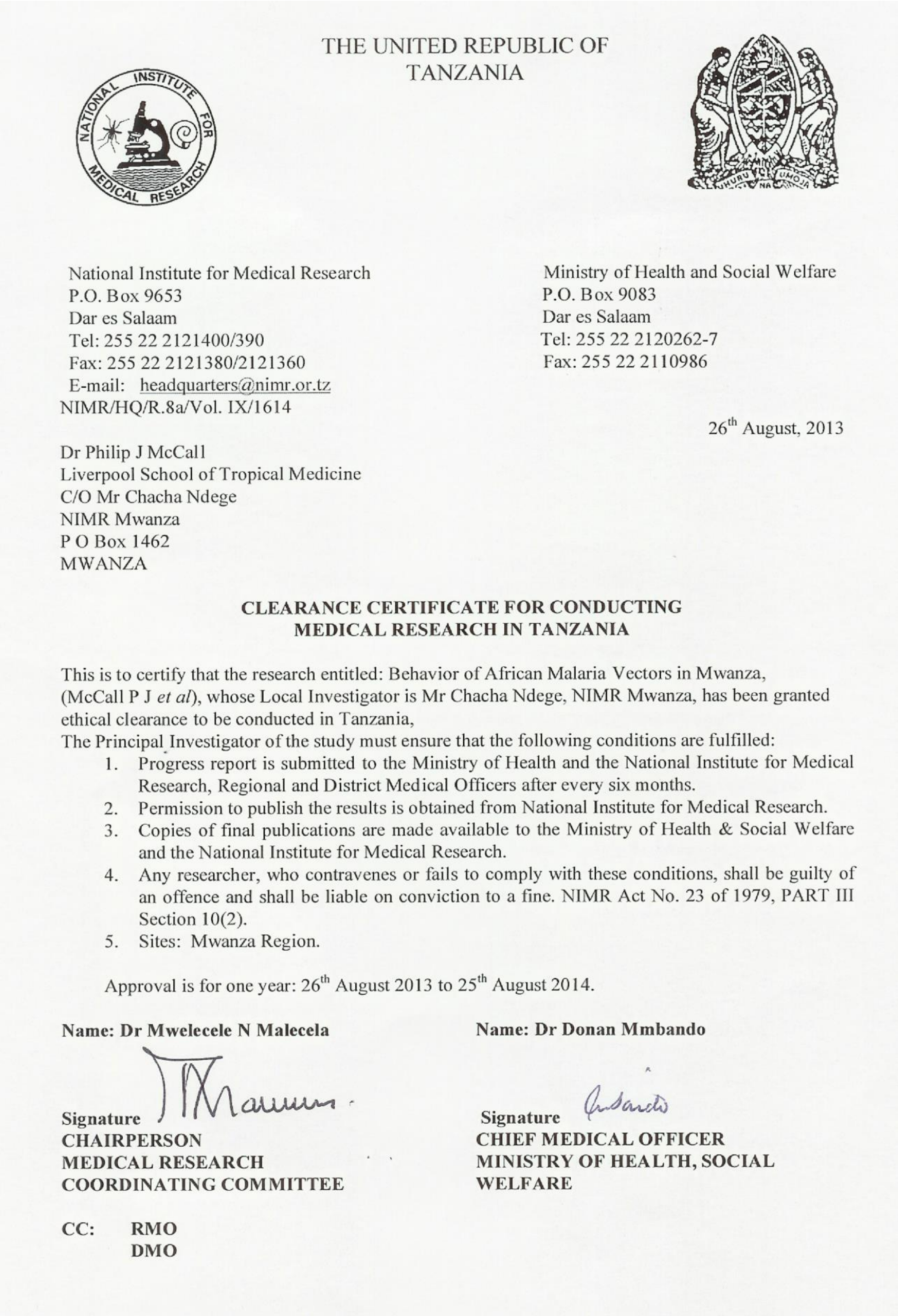
The Principal Investigator (PI) of the study must ensure that the following conditions are fulfilled:

1. Progress report is submitted to the Ministry of Health and Mwanza Medical Research Centre, Regional and District Medical Officers after every six months.
2. Permission to publish the results is obtained from NIMR Headquarters.
3. Copies of final publications are made available to the Ministry of Health & Social Welfare Mwanza Medical Research Centre and the National Institute for Medical Research Headquarters.
4. Any researcher, who contravenes or fails to comply with these conditions, shall be guilty of an offence and shall be liable on conviction to a fine - NIMR Act No. 23 of 1979, PART III Section 10(2).
5. Approval is for this study, any other changes should be communicated to the committee for approval.
6. Approval is for one year: 18th October 2012 to 17th October 2013.
7. Since the study involves foreign collaborators, you are also directed to apply for National Ethics Clearance from NIMR Headquarters.

Name: Fr Alphonse Twimann'ye
Signature:________________
Vice Chairperson LZIRB

Name: Mr Mansuet Temu
Signature________________
Secretary

cc: Regional Medical Officer
District Medical Officer



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NIMR/HQ/R.8a/Vol. IX/1614

26th August, 2013

Dr Philip J McCall
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## CLEARANCE CERTIFICATE FOR CONDUCTING MEDICAL RESEARCH IN TANZANIA

This is to certify that the research entitled: Behavior of African Malaria Vectors in Mwanza, (McCall P J *et al*), whose Local Investigator is Mr Chacha Ndege, NIMR Mwanza, has been granted ethical clearance to be conducted in Tanzania,

The Principal Investigator of the study must ensure that the following conditions are fulfilled:

1. Progress report is submitted to the Ministry of Health and the National Institute for Medical Research, Regional and District Medical Officers after every six months.
2. Permission to publish the results is obtained from National Institute for Medical Research.
3. Copies of final publications are made available to the Ministry of Health & Social Welfare and the National Institute for Medical Research.
4. Any researcher, who contravenes or fails to comply with these conditions, shall be guilty of an offence and shall be liable on conviction to a fine. NIMR Act No. 23 of 1979, PART III Section 10(2).
5. Sites: Mwanza Region.

Approval is for one year: 26th August 2013 to 25th August 2014.

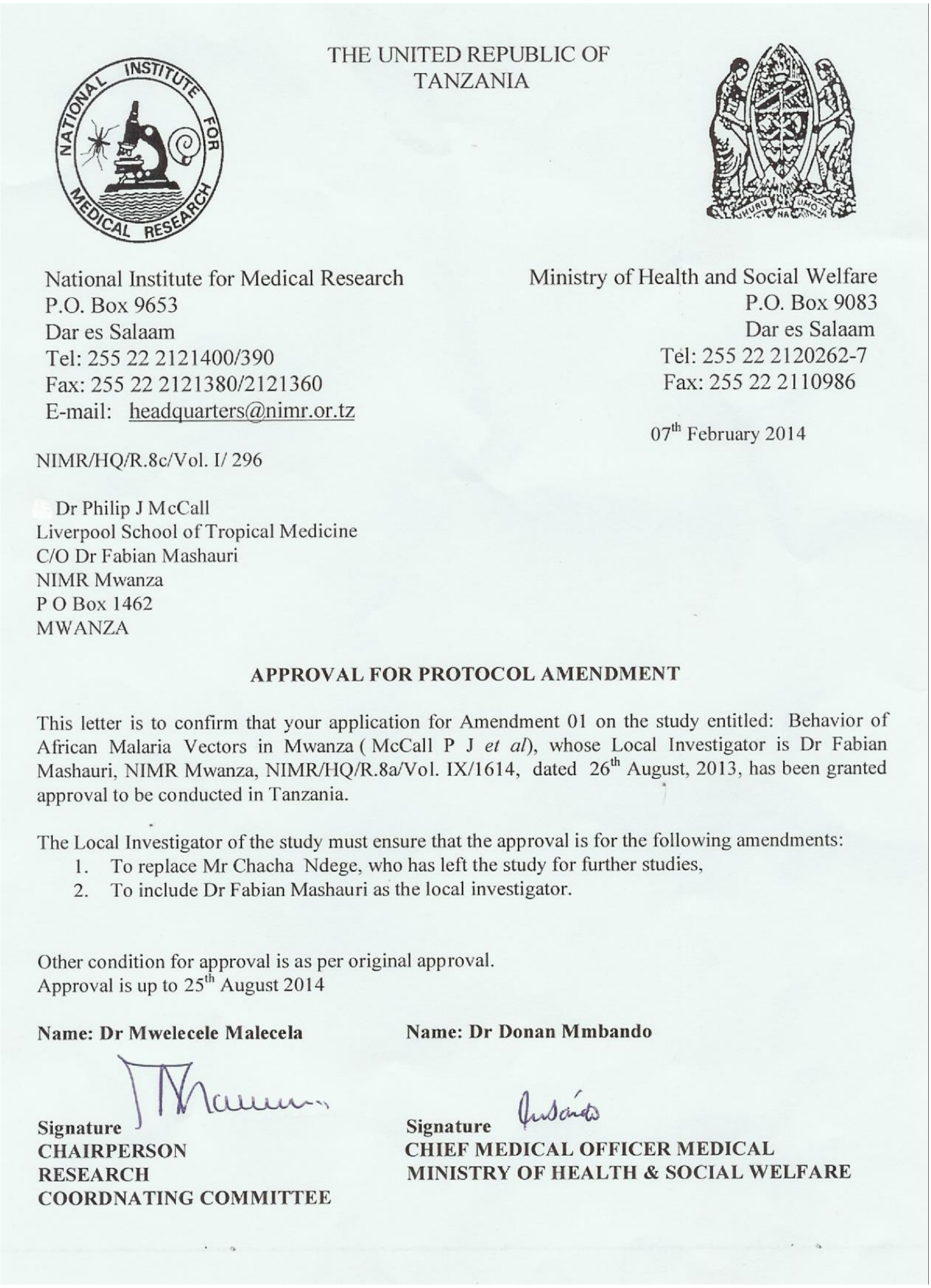
**Name: Dr Mwelecele N Malecela**

**Signature**
**CHAIRPERSON**
**MEDICAL RESEARCH**
**COORDINATING COMMITTEE**

**Name: Dr Donan Mmbando**

**Signature**
**CHIEF MEDICAL OFFICER**
**MINISTRY OF HEALTH, SOCIAL**
**WELFARE**

**CC: RMO**
**DMO**



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07th February 2014

NIMR/HQ/R.8c/Vol. I/ 296

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## APPROVAL FOR PROTOCOL AMENDMENT

This letter is to confirm that your application for Amendment 01 on the study entitled: Behavior of African Malaria Vectors in Mwanza ( McCall P J *et al*), whose Local Investigator is Dr Fabian Mashauri, NIMR Mwanza, NIMR/HQ/R.8a/Vol. IX/1614, dated 26th August, 2013, has been granted approval to be conducted in Tanzania.

The Local Investigator of the study must ensure that the approval is for the following amendments:

1. To replace Mr Chacha Ndege, who has left the study for further studies,
2. To include Dr Fabian Mashauri as the local investigator.

Other condition for approval is as per original approval.
Approval is up to 25th August 2014

**Name: Dr Mwelecele Malecela**

**Signature**
**CHAIRPERSON**
**RESEARCH**
**COORDNATING COMMITTEE**

**Name: Dr Donan Mmbando**

**Signature**
**CHIEF MEDICAL OFFICER MEDICAL**
**MINISTRY OF HEALTH & SOCIAL WELFARE**

# Appendix D: Supplementary CD

CD contains:

**Supplementary Video 5.1: Mosquito flight at a human-occupied bed net in swooping, visiting, bouncing and resting behavioural modes**

The video demonstrates the characteristic movement patterns within the different behavioural modes: in swooping, mosquitoes fly without contacting the net; visiting flights make infrequent net contacts; bouncing mosquitoes make frequent short persistent attacks on the net surface. In resting the mosquito is stationary, or slow moving. During the resting video, the marker disappears when movement ceases (start of resting) and reappears at the same point when movement restarts (resting mode ends). As markers are attached to moving objects, the mosquito is not highlighted when it stops moving, though the tracking algorithm continues to follow its position. Video also accessible online: https://www.dropbox.com/s/h1rbwc0rnkoaxds/SupplementaryVideo_Chapter5_1.wmv?dl=0

**Supplementary Video 6.1: Mosquito flight at a human-occupied LLIN in an experimental hut in Mwanza, Tanzania**

The video is a 45 second clip of mosquito activity at an LLIN in the experimental hut. Tracks on the roof of the net engage in bouncing and visiting behaviour, before one disengages with the net and exits the field of view. Video also accessible at:

https://www.dropbox.com/s/n2l4d8eqd92pcyv/SupplementaryVideo_Chapter6_1.avi?dl=0

**Supplementary Video 7.1: Approach track landing on a volunteer**

The video demonstrates a highly tortuous approach track landing on the volunteer’s feet. Approach tracks were significantly more tortuous than departing tracks ($p$= 0.020). Video also accessible online: https://www.dropbox.com/s/1h8e93g9rcpc62c/SupplemetaryVideo_Chapter7_1.wmv?dl=0