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Ecological and genetic determinants of malaria vectors feeding and resting behaviours

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By

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"True wisdom comes to each of us when we realize how little we understand about life, ourselves and the world around us".

Socrates

"...and what, Socrates, is the food of the soul? Surely, I said, knowledge is the food of the soul".

Plato

"Everybody is a genius. But if you judge a fish by its ability to climb a tree it will live its whole life believing that it is stupid".

Albert Einstein

"The function of education is to teach one to think intensively and to think critically".

Martin Luther King

"...intellectuals have a special contribution to make to the development of our nation, and to Africa. And I am asking that their knowledge, and the greater understanding that they should possess, should be used for the benefit of the society of which we are all members."

Julius Kambarage Nyerere

"Follow up knowledge earnestly, for in the contemporary world, a person without good education is a burden".

Vincent Kilasara Maliti

Abstract

Ecological and genetic factors play a key role in determining the behaviour of mosquito vectors, which in turn influences malaria transmission and epidemiology. Malaria vector control strategies such as long lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) rely on the timing and location of mosquito vector feeding behaviour, and their choice of resting habitat (inside houses versus outside). In some settings where these control measures have been intensively used, the major malaria vectors have been reported to change their behaviour to bite more often outdoors and/or earlier in the evening before people are protected by LLINs, and to increasingly rest outdoors. Such shifts in vector behaviour may jeopardize the effectiveness of LLIN and IRS strategies. The potential for such changes to undermine malaria control can only be understood by: (1) developing better, standardized sampling tools for the surveillance of mosquito behaviours, and (2) identifying the environmental and genetic factors that contribute to these behavioural changes as is required to predict how quickly they can occur and spread.

This study developed and evaluated a range of novel tools to sample host seeking and resting African malaria vectors. These tools were used to characterize a range of epidemiologically relevant malaria vector behaviours within an endemic area of southern Tanzania, and investigate the role of potential ecological and genetic determinants of behavioural variation. Firstly, a novel mosquito electrocuting trap (MET) was developed and evaluated relative to a commercially available insect electrocuting trap (CA-EG) and the gold standard human landing catch (HLC) technique for measuring the abundance and host seeking behaviour of *Anopheles gambiae* s.l. and *An. funestus* s.l. A Latin Square experiment was conducted in a rural setting in the Kilombero Valley of Tanzania where the sampling performance of MET was promising, especially in outdoor sampling where it achieved >58% sampling performance relative to the HLC. In contrast, the CA-EG had poor performance relative to both the MET and HLC and was considered unlikely to be a viable sampling method. This study showed that electrocuting traps can be developed and used as alternative, realistic and exposure-free sampling tools to the HLC technique.

Secondly, a series of new lightweight, portable and standardized sampling traps were developed and compared relative to one another to identify which traps are optimal for measuring African malaria vector resting behaviour. Two existing resting traps, the Resting Bucket (RBU) and Resting Box (RBO) were used along with two modified versions of the RBU designed to test the influence of specific design features to mosquito catchability: a modified entry resting bucket (MERBU) and a sticky resting bucket (SRBU). The performance of all traps for sampling indoor and outdoor resting *An. gambiae* s.l. and *An. funestus* s.l. were evaluated relative to one another, and the back-pack aspirator method (BPA, indoor collections only). Mosquito vector densities in all resting traps were relatively low (<3 per night), but were consistently higher in the RBU and RBO. The SRBU had significantly poorer performance outside than inside, which gave rise to highly biased estimates of exophilic behaviour. The MERBU trap performed consistently poorly inside and outdoors. Based on their relative and consistent sampling performance, the RBO and RBU are recommended as the best choice for wider scale surveillance of vector resting behaviour and its response to control measures.

Thirdly, a candidate gene approach was used to test if variation in the host seeking behaviour of *An. arabiensis* is associated with genetic polymorphisms in their circadian rhythm genes. Single nucleotide polymorphisms (SNPs) from 34 loci across 8 circadian genes in *An. arabiensis* were identified and analyzed for association with the timing ("early=7pm-10pm vs "late" = 4am-7am) and location (indoors vs outdoors) of their host seeking. No associations were found between the host seeking phenotypes and SNP polymorphisms in *An. arabiensis*. However, a strong genetic population structure was detected within *An. arabiensis* from the study area, which was correlated with polymorphisms in the *Timeless* gene (irrespective of the feeding phenotypes or geographical location). The cause of this structuring remains unknown, and further studies to investigate the potential mechanism and epidemiological implications are recommended. Although no association was found in this study, the role of genetics in determining malaria host seeking behaviour cannot be discounted. Other approaches such as transcriptomics and whole genome sequences are recommended in future studies.

In combination, results from this study give insights into the optimality of different sampling tools for reliable, ethically conducive monitoring of malaria vector behaviour. Furthermore, they provide baseline assessment of the contribution of some mosquito genetic and environmental factors to mosquito vector behaviour. It is hoped that the most promising sampling tools developed here can be improved and integrated into malaria vector surveillance programmes to obtain reliable information on vector behaviour and how vectors respond to environmental change and wide-scale use of malaria control measures.

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Acknowledgements

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Lastly, I would like to give very special thanks and honour to my parents and family. More than what can words express, I say thank you very much my beloved father, the late Baba Vincent Kilasara Joseph Maliti, and my beloved Mama Helena, for raising me at a beautiful and industrious home, which inspired my academic excellence since my childhood. My mother was a teacher by profession, and was the very first one to teach me *a, b, c* and 1,2,3, even before I went to school. My mother has been and will remain my very first teacher and my initiator to the informal and formal education. It has been 30 years since my parents sent me to school for the first time, and since then, they have been super supportive by offering me all I needed to make possible my dreams. May Almighty God bless you forever! I am sorry that my beloved father missed to see this work by just a year. I will always remember his usual words of advice to me: "*Soma sana mpaka mwisho*", which means, "follow up knowledge to the end". I also sincerely thank my brothers and sisters; Monica, James, Dolorosa,

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Dedication

To my most beloved father, the late Baba Vincent Kilasara Joseph Maliti (1931-2014), and to my most beloved mom, Mama Helena. I greatly appreciate the resources and the work you invested on me to ensure I went through the best schools throughout my academic journey. I will honour your magnificent work forever!

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May the Almighty God bless you and help you achieve the greatest of the achievements according to His Great Plan in your lives and grant you Eternal Joy in His Kingdom. Amen!

And at this moment and with immense joy and a great sense of accomplishment of the work I started on the 1st of November 2011, and with full of enthusiasm and hope for the work ahead of me, I wind up this work today 23rd October, 2015 the official closure of the British summer and exactly one month since my viva examination, while preparing for my graduation this winter on the 1st of December 2015, I wish that this work be not only for my personal benefit but for the good of all human kind. Now in the words of St. Paul the Apostle I have the courage to say: *For I am already being poured out as a drink offering, and the time of my departure has come. I have fought the good fight, I have finished the race, I have kept the faith. Henceforth there is laid up for me the crown of righteousness, which the Lord, the righteous judge, will award to me on that Day, and not only to me but also to all who have loved his appearing (2 Timothy 4:6-8).* –written immediately after examiners approval for printing as the final version of my PhD thesis on Friday 23rd October 2015 at 18:15pm, in the Graham Kerr Building at the University of Glasgow, Scotland, United Kingdom.

Author's Declaration

I hereby solemnly declare that the work presented in this thesis is entirely my own, except where otherwise stated, and truthfully recognize the contribution of all those collectively remembered, forgotten, recognized or went before. I further declare that no part of this work has been submitted as part of any other degree.

Signed

Deodatus Vincent Maliti

University of Glasgow

October, 2015

Definitions/Abbreviations

AC	Alternating current
bp	Base pair
BPA	Back pack aspirator
CA-EG	Commercially available electrocuting grid
CDC	Centre for Disease Control
CLK	Clock
CRY	Cryptochrome
CYC	Cyclic
DC	Direct current
DNA	Deoxyribonucleic acid
EIR	Entomological inoculation rate
E-Net	Electrocuting net
GLMM	Generalized mixed model
glmmADMB	Generalized mixed model automatic differentiation Model builder
HLC	Human landing catch
HWE	Hardy-Weinberg equilibrium
IHI	Ifakara Health Institute
IHRDC	Ifakara Health Research and Development Centre
IRS	Indoor residual spraying
ITNs	Insecticide treated bed-nets
ITT	Ifakara tent trap
KV	Kilombero valley
Kv	Kilo volt
LEI	Lupiro early indoor
LEO	Lupiro early outdoor
LLI	Lupiro late indoor
LLINs	Long lasting insecticidal nets
LLO	Lupiro late outdoor
mA	millAmpere
MERBU	Modified entry resting bucket
MET	Mosquito electrocuting trap

mtDNA	Mitochondria DNA
NIMR	National Institute for Medical Research
nsSNP	Non-synonymous SNP
OBP	Odor binding proteins
PCA	Principal Component Analysis
PCR	Polymerase chain reaction
Pdp1	Pyruvate dehydrogenase phosphatase
PER	Period
PVC	Polyvinyl chloride
QTL	Quantitative trait locus
RAPD	Random amplified polymorphic DNA
RBO	Resting box
RBU	Resting bucket
SEI	Sagamaganga early indoor
SEO	Sagamaganga early outdoor
SLI	Sagamaganga late indoor
SLO	Sagamaganga late outdoor
SNP	Single nucleotide polymorphism
SRBU	Sticky resting bucket
SSA	Sub-Saharan Africa
sSNP	Synonymous SNP
STIFL	Swiss Tropical Institute Field Laboratory
Thr-Gly	Threonine-glycine
TIM	Timeless
UG	University of Glasgow
UGBU	University of Glasgow bio-electronic unit
V	Volt
WHO	World Health Organization

Chapter 1. General Introduction

1.1 Background

Malaria is one of the leading causes of death and morbidity in tropical regions. This is a vector-borne disease caused by *Plasmodium* parasites, which are transmitted between people by Anopheles mosquitoes. It is estimated that 3.2 billion people worldwide are at risk of being infected every year, with 1.2 billion living in areas with a high risk of being infected (WHO, 2014). An estimated 198 million cases of malaria occurred in 2013 causing 584,000 deaths, with 90% of all mortality occurring in Sub-Saharan Africa (WHO, 2014). Though malaria is still prevalent in many places in Africa, mortality and morbidity due to malaria have decreased by 30% since the year 2000 (WHO, 2014). The decline in malaria cases has been attributed to improved mosquito control measures such as the mass distribution and use of Long Lasting Insecticidal Nets (LLINs) and Indoor Residual Sprays (IRS) (Trape et al., 2014), increased surveillance, improved diagnosis and treatment (O'Meara et al., 2010, Oloifana-Polosovai et al., 2014).

ILLINs and IRS work by targeting female mosquito inside houses during their regular cycle of feeding behaviour. As female mosquitoes need to feed on blood to produce eggs (Gillies, 1953), the time of feeding for anthropophilic female African malaria vectors is usually during the night when people are asleep, with a peak occurring between 10pm and 4am (Gillies, 1957). After blood feeding, mosquitoes find places to rest while digesting blood, which can be either inside or outside houses (Gillies, 1954b, Gillies, 1954a). A few days after blood feeding, the vectors, will find oviposition sites to lay their eggs, with the entire gonotrophic cycle between biting and oviposition taking 2-4 days in *An. gambiae* s.l. (Gillies, 1953). This cycle of feeding, resting and ovipositing is important in controlling malaria vectors and forms the basis for interventions such as ITNs and IRS.

Whilst the use of vector control measures has led to impressive reductions in malaria transmission, as will be discussed in more detail later, there is growing concern that their future effectiveness will be threatened by (1) insecticide resistance and (2) changes in mosquito host seeking and resting behaviour that

reduces their contact with control tools. Both insecticide resistance and mosquito behavioural changes are important, but this study focuses on changes in mosquito behaviours such as a shift in the time of biting towards earlier hours of the night when people are not protected by LLINs (Govella et al., 2010a, Moiroux et al., 2014), an increased tendency to bite outdoors (Kabbale et al., 2013, Reddy et al., 2011, Russell et al., 2011), and a shift to outdoor resting in response to IRS use (Pates and Curtis, 2005). If such changes occur they could pose serious problems to the effectiveness of frontline control measures, and may require introduction of new control strategies including those that offer protection against mosquito bites outdoors (Killeen and Chitnis, 2014). Monitoring if and how rapidly mosquito behaviours change in response to control measures, is crucial for assessment of the long-term effectiveness of LLINs and IRS strategies (Russell et al., 2013, Gatton et al., 2013, Govella and Ferguson, 2012, Briet and Chitnis, 2013).

The biological bases of malaria vector behaviours are not well understood. Variability in their behaviour could be due to simple phenotypic plasticity in response to local environmental conditions, and/or may be due to genetic variation that has arisen through natural selection (Lundsgaard-Hansen et al., 2013, Takken and Verhulst, 2013, Price et al., 2003). Failure to understand the mechanisms driving shifts in mosquito vector behaviour impedes accurate prediction of future changes and consequences for malaria epidemiology.

Accurate study of mosquito vector behaviour will require efficient sampling tools. Among other features, these tools must have the ability to accurately estimate mosquito biting and resting behaviours in both the indoor and outdoor environments. The current gold standard tool for sampling host seeking malaria vectors is the human landing catch (HLC) technique (Le Goff et al., 1997, Hii et al., 2000, Kweka and Mahande, 2009, Loaiza et al., 2008). As will be discussed further, although this method gives the most realistic estimate of human exposure to biting mosquitoes, it has several disadvantages including being labour intensive (Wong et al., 2013, Mboera, 2005) and raising serious ethical concerns due to its requirement that participants expose themselves to potentially infectious mosquitoes (Mathenge et al., 2005, Mboera, 2005, Ndebele

and Musesengwa, 2012). Therefore, there is need to develop alternative sampling tools that can give realistic estimates of the host seeking behaviour of malaria vectors while at the same time not posing any human exposure risk.

The other mosquito behaviour of critical relevance for malaria vector control is their choice of resting habitat. Traditionally, most African vector species were thought to rest primarily inside houses (Gillies, 1954b, Gillies, 1954a, White, 1972), but some species like *An. arabiensis* can also be found resting outdoors in high abundance (Kweka et al., 2009, Odiere et al., 2007). Mosquitoes that rest outdoors are defined as exophilic while those resting indoor are endophilic. In practice, few malaria vector species are entirely exo- or endophilic, and most rest in both indoor and outdoor habitats to varying degrees (Faye et al., 1997, Githeko et al., 1996b). The extent to which malaria vectors rest indoors has a direct impact on the effectiveness of Indoor Residual Spraying (IRS) (Pates and Curtis, 2005). As will be reviewed later on, a variety of methods have been proposed to trap resting mosquitoes. A shortcoming of many previous methods is the lack of standardized approaches for sampling mosquitoes resting in and outside of houses, which makes it difficult to obtain unbiased estimates of exophily. Large-scale surveillance for changes in mosquito vector behaviour will thus require the development of low cost, easy-to-use standardized resting traps that can be used in both indoor and outdoor environments.

The development of appropriate sampling tools will facilitate more detailed investigation of the causes and epidemiological consequences of mosquito vector behavioural variation. For example, investigation of the relative contribution of mosquito genetic factors and environmental variation to malaria vector behaviour will require being able to precisely characterize their behavioural phenotypes within natural populations, and sample individuals with divergent phenotypes for genetic comparison. Estimates of mosquito vector behaviours from different populations could then be tested for association with epidemiological outcomes such as human infection rates. Thus, robust sampling tools will be critical for identifying not only if and why mosquito vector behaviours are changing, but what the consequences of such change could be for malaria transmission.

1.2 Study objectives

In this thesis, I conducted research to address the following 4 objectives which in combination will contribute to addressing the overall aim of assessing the potential for malaria vectors to develop behavioural avoidance in response to control measures:

(1) Develop a new exposure-free method for estimating the human biting behaviour of malaria vectors.

Specifically, I developed a new Mosquito Electrocuting Trap (MET) using optimized voltage that can kill malaria vectors without destroying the specimen.

(2) Evaluate the sampling performance of the MET and CA-EG relative to the current gold standard Human Landing Catch technique (HLC).

The sampling sensitivity of the electrocuting traps and their accuracy in estimating the biting behaviours of the major malaria vectors was evaluated relative to the HLC technique using Latin Square Designs set up in a group of experimental huts.

(3) Develop and evaluate standardized resting traps for sampling indoor and outdoor malaria vectors.

I developed a series of portable resting traps based on simple and readily available materials (buckets and cardboard), and compared them against existing prototypes to test specific hypotheses about how certain physical design features such as trap shape, use of sticky surfaces and size of the entrance into the trap influenced sampling efficiency. The best performing resting trap in this study was used to investigate associations between daily variation in temperature and humidity, and the resting habitat preference (indoor or outdoor) of *An. gambiae* s.l. and *An. funestus* s.l.

(4) Evaluate the genetic basis of *An. arabiensis* feeding behaviour using a candidate gene approach

I adopted a candidate gene approach based on circadian rhythm genes that have known associations with the timing of behaviours in other insects to test for

associations between host-seeking phenotypes and genotypes in a natural *An. arabiensis* population. I focused on testing for associations between the timing of host seeking (early or late in evening) and location (indoors or outdoors) with polymorphisms in circadian rhythm genes.

Before providing a more detailed description of the rationale and approach for the objectives in this study, key aspects of malaria epidemiology and mosquito vector biology are briefly reviewed in the next section.

1.3 Malaria

Malaria is a mosquito-borne disease caused by eukaryotic protists of the genus Plasmodium (Wyborny, 2005) which can infect humans and many other animals (Marcus, 2009, Peterson and Calamandrei, 2011). There are five Plasmodium species that cause malaria in humans: *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae* and *Plasmodium knowlesi* (Chavatte et al., 2007, Perkins and Austin, 2009, Mueller et al., 2007, Singh et al., 2004). Among these species, *P. falciparum* and *P. vivax* are the most common in malaria endemic regions, with *P. falciparum* which occurs in many parts of the Sub-Saharan Africa being the most deadly (Liu et al., 2010, Tanabe et al., 2010, Nozais, 2003, Conway et al., 2000, Molina-Cruz and Barillas-Mury, 2014). Malaria in humans is widespread in many parts of the tropical and subtropical regions including much of Sub-Saharan Africa, Asia and the Americas (Webb, 2009, WHO, 2014), but may have some potential for expansion into temperate areas with climate change (Medlock and Vaux, 2015).

A person contracts malaria through the bite of an infected mosquito during which Plasmodium sporozoites are injected into the bloodstream, and migrate into the liver where they develop into schizonts (Tarun et al., 2006). The time taken for Plasmodium to complete the liver stage is 2-16 days depending on the species (Prudencio et al., 2006). After parasites have developed as schizonts, they are released into the bloodstream as merozoites that replicate asexually through repeated invasion of red blood cells (Abkarian et al., 2011). A small proportion of merozoites differentiate into sexual stage gametocytes which can be transmitted to mosquitoes. Malaria symptoms generally result from

multiplication of Plasmodium parasites within the red blood cells (Bartoloni and Zammarchi, 2012). Additionally, *P. falciparum*-infected red blood cells may also form clumps called rosettes which can block brain capillaries and result in serious brain haemorrhage and death even when parasite numbers are low (Leitgeb et al., 2011).

When a female Anopheles mosquito vector bites an infected person, it ingests these gametocytes with the blood meal. In the mosquito midgut, fertilization of the gametes occurs resulting in the formation of ookinetes which develop into oocysts (Hurd et al., 2006). Transmission stage sporozoites grow within these oocysts and burst out into the mosquito haemocoel approximately 10-12 days after the infectious blood meal, from where they migrate into the mosquito salivary glands (Beier, 1998). Sporozoites are injected again into humans or other vertebrates when an infected mosquito pierces their skin for a blood meal (Cowman and Crabb, 2006).

1.4 African malaria vector ecology and behaviour

The major vectors of malaria in Africa are members of the *An. gambiae s.l.* complex. This group is comprised of 9 morphologically identical species: *An. gambiae sensu stricto, An. colluzzi, An. arabiensis, An. quadriannulatus, An. amharicus, An. melas, An. merus and An. bwambwae* (Coetzee et al., 2013). Of all the sibling species of the *An. gambiae* s.l. complex, *An. gambiae* s.s. is widely considered as the most efficient vector of malaria followed *by An. arabiensis* (Scott et al., 1993, Lemasson et al., 1997). These sister species are sympatric in many localities (Lindsay et al., 1998, Petrarca et al., 1987, Obala et al., 2012) with *An. gambiae* s.s. predominating in more humid environments while *An. arabiensis* is more common in drier areas (Lindsay et al., 1998, Pock Tsy et al., 2003, Caputo et al., 2008). *Anopheles gambiae* s.s. is nocturnal and prefers to feed at night (between 10pm-4am) when humans are asleep (Lemasson et al., 1997, Coluzzi et al., 1979). *Anopheles gambiae* s.s. and *An. arabiensis* exhibit variation in their feeding and resting behaviours. While *An. gambiae* s.s. prefers to feed on humans (anthropophagy) and rest indoors (endophagy), *An. arabiensis* has a more flexible feeding behaviour and is most likely to feed on cattle (zoophagy), and bite and rest outdoors (exophagy and

exophily)(Githeko et al., 1994, Costantini et al., 1999, Killeen et al., 2001, Lyimo and Ferguson, 2009, Obala et al., 2012, Mayagaya et al., 2015, Gillies, 1953, Gillies, 1954a). There are reports of *An. arabiensis* becoming the prominent malaria vector in East African areas where ITN coverage is now high and has had a disproportionate effects in reducing *An. gambiae* s.s. (Russell et al., 2010, Russell et al., 2011, Mutuku et al., 2011, Bayoh et al., 2010, Kitau et al., 2012).

In addition to these primary vectors, there are numerous important vector species such *An. funestus* (Kloke et al., 2011, Mutuku et al., 2011, Lwetoijera et al., 2014), *An. merus* (Cuamba and Mendis, 2009, Collins et al., 1988), *An. coustani* s.l., *An. squamosus* and *An. marshalli* (Fornadel et al., 2011), that also contribute to malaria transmission. These secondary vectors occupy diverse ecological environments across the continent and in most places are found in sympatry with the primary vectors (Kabbale et al., 2013, Oduola et al., 2013, Dadzie et al., 2013, Kiszewski et al., 2014, Munhenga et al., 2014). *Anopheles funestus* is considered a secondary vector in many places in Africa but there are reports of this species becoming more important in vectoring malaria in places where *An. gambiae* s.s. has been shown to diminish (Lwetoijera et al., 2014, Sougoufara et al., 2014, McCann et al., 2014, Moiroux et al., 2014). *Anopheles funestus* s.l. exists as a complex with 9 species; *An. funestus* sensu stricto, *An. parensis* Giles, *An. aruni* Sobti, *An. confusus* Evans and Leesoni, *An. vaneedeni* Giles and Coetzee, *An. rivolorum* Leeson, *An. fuscivenosus* Leeson, *An. leesoni* Evans and *An. brucei* Service (Gillies and Coetzee, 1987a). Some of the members such as *An. funestus* s.s. preferring to bite nocturnally indoors (Moiroux et al., 2012, Seyoum et al., 2012). *Anopheles funestus* s.l. is known to have preference to feed on humans but can also choose to feed on other vertebrates (Githeko et al., 1994).

Tanzania has a diverse range of mosquito species including all major vector species including *An. gambiae* s.s., *An. arabiensis* and *An. funestus* (Drakeley et al., 2003, Ng'habi et al., 2008, Smith et al., 1993b, Lwetoijera et al., 2014). Other anopheline species found in the country include *Anopheles squamosus, An. ziemanni and An. coustani* (Drakeley et al., 2003, Maliti et al., 2014). *Anopheles*

gambiae s.s. and *An. arabiensis* are the two most important malaria vectors in Tanzania with both species being found in many places in the country (Kigadye et al., 2010, Shiff et al., 1995, Mnzava and Kilama, 1986). There is marked seasonal variation in density within the *An. gambiae s.l.* species complex, with both *An. gambiae* s.s. and *An. arabiensis* peaking in the wet season but *An. arabiensis* is able to persist throughout dry season compared to *An. gambiae s.s.*(Charlwood et al., 1995). In Tanzania, *An. gambiae* s.s. has been reported to decline in number, which is thought to be a result of vector control interventions involving ITNs and IRS (Derua et al., 2012), leading to *An. arabiensis* becoming the primary vector of malaria in many places (Russell et al., 2010, Derua et al., 2012). The next section explores some of the prominent control methods against malaria infection.

1.5 Control Methods

1.5.1 Insecticide Treated Nets and Indoor Residual Spraying

From the early 1980s to the present, much effort has been directed to the use of Insecticide Treated Nets (ITNs) to combat malaria in Africa. The entomological impact and efficacy (in terms of morbidity and mortality reduction) of ITNs have been assessed extensively in a number of studies (Lines et al., 1987, Lyimo et al., 1991, Fraser-Hurt et al., 1999, Deribew et al., 2010, White et al., 2011, Smithuis et al., 2013a, Kariuki et al., 2013). Apart from a few challenges to their effectiveness such as insecticide resistance (Ranson et al., 2011) and problems of holes being made during handling and the duration of insecticidal activity (Erlanger et al., 2004, Banek et al., 2010, Ngonghala et al., 2014, Lorenz et al., 2014), ITNs have proven to be an effective strategy for reducing malaria in Africa (Thwing et al., 2011, Bayoh et al., 2010, Pettifor et al., 2009, Noor et al., 2008, Protopopoff et al., 2007, Kariuki et al., 2013).

In the past 5 years, ITN technology has significantly improved through the development and introduction of Long Lasting Insecticidal Nets (LLINs) (WHO, 2014). These nets are similar to earlier generations of ITNs but have been upgraded to have longer lasting insecticidal activity which can remain effective for up to 3 years under field conditions, and therefore avoid the requirement of

regular retreatment with insecticides (Briet et al., 2012, Faulde et al., 2011, Ahmed et al., 2011, Ngufor et al., 2011, Ouattara et al., 2011, Ngufor et al., 2014, Paintain et al., 2013). By 2013 the WHO estimated that at least 44% of people in Sub-Saharan Africa (SSA) were sleeping under an ITN with 67% of households owning at least one LLINs (WHO, 2014). A recent malaria report from the World Health Organization (WHO) has shown a total of 214 million LLINs were expected to be delivered to SSA countries by the end of 2014, bringing the total number of LLINs delivered in this region since 2012 to 427 million (WHO, 2014).

Indoor Residual Spraying (IRS) has also been employed extensively in some parts of SSA as part of the ongoing vector control programs (Al-Arydah and Smith, 2011, West et al., 2014, Simon et al., 2013, Padonou et al., 2012b). In SSA more than half of the malaria endemic countries have adopted ITNs, IRS or both (WHO, 2014). The WHO indicated that about 123 million people in the globe (3.5% of the global population at risk) were protected by IRS in 2013 (WHO, 2014). Unlike ITNs and LLINs, IRS among African countries is not widely used. Ethiopia alone is reported to account for 42% of all IRS use in Africa (WHO, 2014). In Tanzania, IRS is part of the national malaria vector control policy but its use is limited except for Zanzibar where it is being used extensively as part of a local elimination campaign in combination with ITNs, which have shown high success in terms of reduction in malaria cases (Aregawi et al., 2011, Pluess et al., 2010, Beer et al., 2013, Kaufman et al., 2012).

1.5.2 Dependence of vector control measures on mosquito behaviours

Vector-host interactions are the major determinants of malaria transmission intensity (Onori and Grab, 1980, Killeen et al., 2006). The specific features of malaria vector ecology that are most important to malaria transmission are adult emergence rate, gonotrophic cycle length (number of days between bites), adult survival and the frequency with which mosquitoes feed on humans (Garrett-Jones, 1964, Charlwood et al., 1986, Burkot et al., 1989). Of these, adult survival rate and the human feeding index have the greatest impact on

transmission (Smith and McKenzie, 2004), with intensity increasing exponentially in relation to both these factors.

The success of ITNs depends significantly on mosquito feeding behaviours such as the time and place of biting as well as their place of resting (Hamel et al., 2011). The determinants of mosquito host choice and feeding behaviour are not well known, and may include a range of ecological as well as potential genetic factors (Lyimo and Ferguson, 2009). Since the major African malaria vectors are known to largely exhibit a tendency of biting inside houses and at night times when human hosts are available and probably defenceless (Mahande et al., 2007), ITNs are considered a very effective means to reduce the primary source of exposure. Indoor Residual Spraying is also considered to be a very useful intervention against malaria vector species, as its action relies on the tendency of malaria vectors to rest inside houses after feeding, which is a behaviour that many African vectors share (Sharp et al., 2007, Zahar, 1984, Forattini et al., 2000, Kent et al., 2006). Since the efficiency of both ITNs and IRS depends largely on the biting and resting behaviour of malaria vectors, it is anticipated that any change in these behaviours could have a notable impact on malaria transmission and control (Geissbuhler et al., 2007, Killeen et al., 2006, Killeen and Chitnis, 2014), and could necessitate a change in vector control strategies (Killeen, 2014).

1.5.3 Concerns on the efficacy of ITNs and IRS in relation to vector behavioural changes

Universal coverage with LLINs or IRS is advocated as the main malaria prevention strategy by WHO-endorsed malaria control program (WHO, 2014). However, there are growing concerns about the decreasing protective ability of the insecticides applied on the nets (Protopopoff et al., 2013, Okia et al., 2013, Sovi et al., 2014). At present, pyrethroids are the only class of insecticides licensed for use in ITNs and IRS due to their effectiveness in killing insects and lack of toxicity to humans and other animals (Zoulani et al., 1994, Briet et al., 2013). However, all of the major African vectors have capacity to develop resistance against pyrethroids (Ranson et al., 2011), and there are many reports of

increasing development of insecticide resistance, especially where ITN coverage is high, and/or in places with extensive application of agricultural pesticides (Fane et al., 2011, Abilio et al., 2011, Yewhalaw et al., 2011, Sovi et al., 2013, Protopopoff et al., 2013, Haji et al., 2013, Aizoun et al., 2014). It has been argued that, the use of LLINs and/or IRS may prompt other changes in malaria vectors that could reduce the effectiveness of control measures: the emergence of behavioural avoidance (Killeen and Chitnis, 2014) which is a shift in stereotypical patterns of mosquito behaviour in response to introduction of interventions that reduce vector contact with insecticides. Several studies have investigated whether there is evidence of intraspecific shifts in mosquito behaviour in response to interventions. Early work showed mosquitoes become more exophilic following use of IRS and DDT (Gillies and Furlong, 1964, Smith and Gillies, 1960) as well as permethrin (Takken, 2002). Insecticide treated bed-nets use has also been associated with a shift in the host species choice of malaria vectors, with mosquitoes reducing feeding on humans in favour of livestock when net coverage is high (Bogh et al., 1998, Charlwood and Graves, 1987, Sampath et al., 1998b, Sampath et al., 1998a, Iwashita et al., 2014). As the efficacy of ITNs and IRS depend on the feeding and resting behaviours such as indoor/outdoor feeding and indoor/outdoor resting, presence of such changes may reduce the protective ability of the control measures leading to higher malaria transmission.

1.5.4 Changes in the time and place of feeding behaviour in African malaria vectors and possible causes

Over the past few decades, there have been reports of African malaria vectors changing key aspects of their feeding behaviours including the time and place (indoors vs outdoors) of their biting. The quality of evidence supporting claims of long-term shifts in mosquito behaviour is fixed; with the most robust being from high resolution, multi-year longitudinal sampling of vectors from the same place using the same methodology (Bayoh et al., 2010), and other more anecdotal evidence being drawn from limited comparison of mosquito behaviour between just two time points, across which both mosquito sampling methodology and molecular identification methods were different (Russell et al.,

2011, Russell et al., 2010, Kaburi et al., 2009, Ndiath et al., 2014). A range of hypotheses could account for these apparent temporal changes; with the use of vector control measures being the most frequently proposed (Russell et al., 2011, Russell et al., 2010, Kaburi et al., 2009, Wamae et al., 2015, Ndiath et al., 2014). Here I will review the evidence for the occurrence of long-term shifts in either the time or location of biting in African malaria vectors, and its potential causes.

1.5.4.1 Species composition with reference to changes in the feeding behaviour in the *Anopheles gambiae* complex

Changes in malaria vector behaviour have been observed at two scales: changes within a vector species (Reddy et al., 2011), and changes in association with species composition of malaria vectors, whereby species with more exophilic behaviour are favoured over endophilic ones. For example in Western Kenya, it has been shown that *An. gambiae* s.s. predominated in the period 1970-1998 (85%), but has decreased substantially in favour of the more exophilic *An. arabiensis* since 1999, particularly after the mass introduction of ITNs in 2004 (Bayoh et al., 2010). The same study reports that by 2009, *An. gambiae* s.s. comprised only ca. 1% of mosquitoes caught indoors (remainder being *An. arabiensis*). Also in this study, data of species composition collected and analysed prior to the establishment of molecular based techniques relied on polythene chromosome to distinguish between *An. gambiae* s.s. and *An. arabiensis*. A similar pattern of species composition change has been observed in the Kilombero Valley (KV) of Tanzania where it was found that between 1994 and 2008 when ITN coverage increased from 65% to 91.5%, the density of *An. gambiae s.s.* was reduced by 79% whereas that of *An. arabiensis* was reduced by only 38% (Russell et al., 2010). A potential problem with studies based on such historical comparison of mosquito species diversity is that the methodology to distinguish between closely related vector may have changed; with some species being misidentified in early studies. For example, although *An. arabiensis* and *An. gambiae* s.s. have quite distinct biting behaviour (As reviewed in section 1.4) these species are morphologically identical and molecular methods to distinguish them were not developed until 1993 (Scott et al., 1993). From 1960s identification of *An. gambiae* s.s. and *An. arabiensis* was done using banding

patterns on chromosome X of these species (Coluzzi and Sabatini, 1967). Poor species identification could confound results of species composition in studies done before the introduction of molecular assays which are much more reliable. Whilst imprecise methods for vector species identification may be problematic for the interpretation of long-term trends in some data sets, several recent studies where reliable molecular methods have been used have also reported a reduction in the abundance of *An. gambiae* s.s. relative to *An. arabiensis* in areas where ITN coverage is high (Mutuku et al., 2011, Derua et al., 2012, Mwangangi et al., 2013). Consequently I conclude the evidence for long-term changes in malaria vector species composition is relatively robust. As such interspecies changes accompanied by shifts in malaria vector biting behaviour are of strong relevance to vector control.

1.5.4.2 Temporal changes in the feeding behaviour of malaria vectors

In areas of high ITN coverage, there area accounts which suggest malaria mosquitoes are changing their biting behaviour to feed predominantly outdoors or in the early parts of the morning or evening. For example in Benin (West Africa) *An. funestus* mosquitoes have been observed to bite early morning which is linked to massive distribution of ITNs (Moiroux et al., 2014). Although this study did not involve comparison to previous historical data in *An. funestus* biting behaviour, a former study in the same place on *An. funestus* reported a change in its biting peak from 2am to 5am after 3 years of the implementation of LLINs (Moiroux et al., 2012). In Dar es Salaam Tanzania *An. arabiensis* was observed to feed outdoors early before 10pm when most people are still working outdoors and this phenomenon was reported to potentially compromise ITNs effectiveness in the area (Geissbuhler et al., 2007). Other studies have shown an early evening biting in Ethiopia among *An. arabiensis* (Yohannes and Boelee, 2012) as well as in *An. funestus* s.l. and *An. arabiensis* in Kenya (Cooke et al., 2015, Wamae et al., 2015). Although there are reports of the shift in biting time of the major malaria vectors from the classical understanding, a greater body of reports confirming if this phenomenon happens consistently within a species and across a wider geographical area is needed.

1.5.4.3 Spatial changes in the feeding behaviour of malaria vectors

A study conducted on Bioko Island from 2007-2009 revealed a substantially higher rate of outdoor biting by *An. gambiae* s.s. and *An. melas* than had previously been observed before the extension of the IRS programme (Reddy et al., 2011). Between 1997 and 2009 a shift in the biting activity of *An. gambiae* s.l. was observed from mainly indoor biting to outdoor biting, but it is acknowledged that there has been a significant change in species composition from the endophagic *An. gambiae* s.s. to exophagic *An. arabiensis* (Russell et al., 2011). Some observed changes in the feeding behaviour of members of the *An. gambiae* complex involve changes in both the time and place of feeding. Such changes have been reported in Dar es Salaam Tanzania among *An. arabiensis* which was found to feed outside houses and early at dawn before sleeping hours (Govella et al., 2010b). Other reports suggest a possible shift in the biting behaviour of malaria vectors in terms of both the time and the location of feeding. For example *An. arabiensis* in Zambia was reported to be relatively more exophillic and with preference to feed outdoors immediately after sunset and before sunrise potentially avoiding ITNs protective effects (Fornadel et al., 2010a). Though early evening and outdoor transmission occurs in some places in Africa, the majority of transmission still occurs indoor and late at night and there is still no clear evidence for departure from the stereotypical feeding behaviours of malaria vectors (Bayoh et al., 2014, Huho et al., 2013), thus there is need to investigate further on this phenomenon and determine possible epidemiological consequences that may arise out of it.

1.5.4.4 Environmental changes and their contribution to behavioural changes in African malaria vectors

The primary hypothesis put forward to explain the inter- and intra-specific changes in malaria vector feeding behaviors described above is that they have been driven by the use of vector control measures such as ITNs and IRS. Given many of these mosquito-behavioral changes occurred contemporaneously with the scale up of interventions, it is likely that these control measures are at least partially to blame. However, other environmental changes have occurred across Africa in recent decades which may also have contributed. Major changes include changes in land use with increasing urbanization, and increasing

economic development. Although Africa is the least urbanized continent, its rate of urbanization has been the highest in the past decade. With the current 40% of the Africans living in urban areas, the United Nations Habitat predicts that in 2050 about 60% of its population will live in cities (Habitat, 2015).

Changes in economic development and urbanization could impact mosquito vector populations in several ways. First, improvements in socio-economic status may lead to better housing structures that may lead to a lower mosquito biting rate indoors (Dowling et al., 2013, Casas et al., 1994, Lindsay et al., 2003). For example, construction of modern brick houses with window screens and ceiling in Africa have been found to contain few mosquitoes inside (Atieli et al., 2009, Lindsay et al., 2003). Other features of improved housing including use of electric lights indoors have been hypothesized to reduce the number of mosquitoes that come indoors to feed potentially leading to increased outdoor feeding in malaria vectors (Oria et al., 2015). Additionally, differences in the lifestyle of city and rural dwellers may influence their exposure to malaria vectors. For example, it has been reported that people spend longer period of the evening outdoors in rapidly growing urban areas such as in Dar es Salaam Tanzania (Geissbuhler et al., 2007), likely because the availability of electric lighting makes this possible. With the growing urbanization in Africa, it may be hypothesized that better housing condition may reduce the number of biting mosquitoes inside houses or even cause an increase in the outdoor biting behavior of malaria vectors and therefore lead to higher malaria transmission rate in outdoor environments.

Over the past two decades there have been reports of global warming happening in Africa. This climatic change has influenced the spread of malaria to areas which were formerly known to be free of malaria. It is reported for example that some highland areas in Kenya (Chen et al., 2006) and Ethiopia (Woyessa et al., 2012) which did not have record of malaria in the past are now experiencing cases of infection due to rise in temperature following global warming. Climate change models predict invasion of mosquitoes in areas that were mosquito-free in the past (Parham and Michael, 2010b, Parham and Michael, 2010a, Tonnang et al., 2010). As was discussed in section 1.4, drier climate favors *An. arabiensis*

relative to *An. gambiae* s.s. (Lindsay et al., 1998, Pock Tsy et al., 2003, Caputo et al., 2008) it may be hypothesized that with the rise in temperature and increase in drought condition, species composition may change leading to vectors with different feeding and resting behaviors from what used to be known in the past. Deforestation and agriculture are implicated in mosquito population dynamics and behavior. Irrigated areas are more likely to be good breeding sites for mosquitoes compared to drier areas (Ijumba et al., 2002, Ijumba and Lindsay, 2001). The current opening of new irrigation schemes in Africa is expected to influence species composition and consequently malaria transmission. Deforestation leads to drier climate which may favor mosquito species adapted to drier environment such as *An. arabiensis* (Afrane et al., 2012). It is important that inter-species variation in mosquito behavior be delineated from the intra-species to determine the nature of the change in mosquito behavior.

1.6 Metrics of mosquito behaviour and human biting exposure distribution

Malaria epidemiology depends on vector behaviours that in turn determine human exposure to mosquito bites. Estimation of the propensity for vectors to bite inside or outdoors is made using host-seeking collections (Wong et al., 2013, Mathenge et al., 2002), while estimation of mosquito resting behaviour relies on resting traps (Onyango et al., 2013, Govella et al., 2011). Mosquitoes with an inherent tendency to feed indoors and on humans are more capable of spreading malaria especially in the absence of protective measures such as ITNs, while those that prefer to feed outdoors and on other vertebrate hosts are less capable of spreading malaria (Faye et al., 1997, Githeko et al., 1996a). Human behaviours are also known to determine exposure to mosquito bites. For example, humans who go to bed early and use bed-nets are more likely to be protected from indoor feeding mosquito bites than those who stay up to late and spend more time outdoors (Matowo et al., 2013, Killeen et al., 2006).

Three metrics have been proposed to estimate human exposure to infection based on the distribution and location of mosquito biting times. The first one is the proportion of bites occurring indoors (P_i) throughout a nightly host-seeking period (typically defined as 19 to 07 hours)(Seyoum et al., 2012, Govella et al., 2010b). This information is combined with knowledge of the proportion of time when people are indoors to calculate P_{fl}, defined as the proportion of mosquito biting occurring when most people are indoors (Govella et al., 2010b). The time that most people are indoors will differ from place to place and thus should be calculated within a local context. The P_{fl} value can be interpreted as index of what proportion of the total biting is occurring at times and places where it could be prevented by a bed-net (Seyoum et al., 2012, Govella et al., 2010b). The third metric is the proportion of human exposure that occurs indoors (π_i) (Seyoum et al., 2012, Govella et al., 2010b). These metrics provide a useful means to directly associate mosquito behavioural traits with epidemiological consequences for human exposure risk, and are used in this thesis to assess the consistency with which different trapping approaches estimate epidemiologically relevant mosquito vector behaviours. Further details of how these metrics are calculated are provided in chapter 3.

1.7 The need for reliable methods to characterize malaria vectors' behaviours

In order to be able to identify if changes in malaria vectors feeding and resting behaviours are occurring and identify their cause, reliable mosquito sampling methods are required. Desired sampling tools should be accurate in measuring the feeding and resting behaviours of malaria vectors across a wide range of ecological environments while at the same time should be easy to use, cost effective and ethically permissible. The current mosquito sampling tools face a number of bottlenecks for this purpose. These limitations and the new methodologies proposed are discussed in the following sections.

1.7.1 Methods used for sampling host seeking malaria vectors

1.7.1.1 Human landing catches

Historically, the most commonly used means to estimate the density and transmission potential of malaria vectors was the Human Landing Catch (HLC)(Le Goff et al., 1997, Hii et al., 2000, Moore et al., 2001, Mathenge et al., 2002, Mathenge et al., 2005, Mboera, 2005, Kweka and Mahande, 2009, Sikulu et al., 2009). The HLC makes use of human volunteers who act as a natural 'bait' to whom human-host seeking mosquitoes are attracted via kairomones including heat, water vapour, CO_2 emanations and various human odours (Bowen, 1991, Okumu et al., 2010c, Wright and Burgess, 1975). In conducting a human landing catch, a catcher sits on a chair with legs exposed from the foot to the knee and allows host-seeking mosquitoes to land on the skin. Using a mouth aspirator, the catcher sucks in the landing mosquito before it starts feeding. As it provides a highly representative sample of vectors drawn to a human host, the HLC is considered as the gold standard tool for sampling host seeking mosquitoes, and has been used extensively to study malaria vectors density (Govella et al., 2009, Kweka and Mahande, 2009, Mboera, 2005), species composition (Loaiza et al., 2008, Schiemann et al., 2014) and biting activity (Bockarie et al., 1996, Zimmerman et al., 2013).

Although the HLC provides a realistic estimate of malaria vector biting density, this method has numerous drawbacks. Most notably, this method is increasingly considered to be unethical, as it involves exposing human 'bait' to potentially infectious mosquitoes (Mathenge et al., 2005, Mboera, 2005, Gama et al., 2013), and participants are encouraged to be on malaria prophylaxis before doing HLC (Achee et al., 2015, Ndebele and Musesengwa, 2012). Furthermore, this method is logistically demanding and may not be practical or reliable in many settings, because inherent variability in the attractiveness and skill of collectors make it difficult to standardize (Mboera, 2005). Additionally, this method is extremely labour intensive, as it requires vigilance throughout the night with intensive supervision to ensure that the information gathered is reliable.

1.7.1.2 Alternatives to the HLC

The most commonly used alternative to the HLC is the CDC light trap (Main et al., 1979, Faye et al., 1992). The CDC light trap applies a light stimulus to attract mosquitoes into a trap. The trap is composed of a rotating fan placed under a light source. The rotating fan creates suction that draws mosquitoes into a netted collection chamber. CDC traps usually have to be placed indoors close to a person sleeping under a bed-net. Though the CDC trap requires electricity to run, it is easy to use, cheap and it is exposure-free. However, the CDC light trap is not ideal for trapping host-seeking mosquitoes because mosquitoes may be attracted to the host through the light stimulus rather than host body cues. Additionally, CDC light traps have been shown to work well indoors while performing poorly outdoors (Sikaala et al., 2013, Faye et al., 1992).

Numerous other attempts have been made to evaluate other exposure-free methods that are suitably useful for trapping mosquitoes that bite both humans and animals. In Western Kenya, traps have been developed based on modifying bed-nets to passively trap mosquitoes attracted to sleepers. However, this method was also less effective than the HLC and CDC light trap methods (Mathenge et al., 2004). A further drawback is that this method was only suitable for sampling mosquitoes attracted to humans inside houses (Mathenge et al., 2005). In Tanzania, the 'Ifakara Tent Trap' (ITT)(Govella et al., 2010a, Sikulu et al., 2009, Govella et al., 2009) was developed as a more flexible alternative that could be used outdoors. The ITT is a tent in which a person sleeps. Mosquitoes can enter through 6 funnel entry tunnels which lead into a sealed off chamber (Govella et al., 2010a, Govella et al., 2009). A version of the ITT, the ITT-B, was shown to be ~ 35% as sensitive as the HLC (Sikulu et al., 2009). An upgraded model, the ITT-C was shown to perform well relative to HLC in some settings (Wong et al., 2013, Sikaala et al., 2013, Chaki et al., 2012). While the ITT-C provides advantages over the HLC in that it is 'exposure-free', it is known to preferentially sample mosquitoes with the inherent tendency to feed 'indoors' (endophilic)(Govella et al., 2010a) and thus does not give a good representation of the outdoor biting mosquito population.

Recently, there have been several attempts to develop artificial odour baits mimicking either human or animal cues that could be used to trap mosquitoes in a variety of locations and times (Pombi et al., 2014c, Mweresa et al., 2014, Nyasembe et al., 2014, Mukabana et al., 2012). Early work focused on the use of CO_2 traps (Addison et al., 1979, Kemme et al., 1993, Takken and Kline, 1989), and more recently, blends of chemicals have been created to imitate human odours (Okumu et al., 2010b). One study developed a synthetic human odour that was up to 4 times more attractive than a human in semi-field testing (Okumu et al., 2010c, Okumu et al., 2010b). However such formulations, including those for important non-human hosts, are not yet widely available or calibrated, and it may be unlikely to replace live host baits until they become cheaper and easier to manufacture (Okumu et al., 2010a). At present, the use of live hosts as odour baits provides the most reliable characterization of the host-seeking malaria vector populations.

Videotaping methods have been used to study behaviour of various insects of medical importance. Over the last 3 decades such methodologies have been used for example to observe the swarming behaviour of *Psorophora columbiae* in Texas rice fields (Peloquin and Olson, 1985), to study the activation of *An. gambiae* by carbon dioxide in cages (Healy and Copland, 1995) and to study effects of pyrethroids on the landing and resting behaviour of *Aedes aegypti* mosquitoes (Cooperband and Allan, 2009). Recently video tools have been used to study the modulation of appetite and feeding behaviour of larvae of *Aedes aegypti* (Kinney et al., 2014) and individual male behaviours and coupling in *An. gambiae* s.s. (Manoukis et al., 2014). Though video methods are useful in visualization studies and can generate details of mosquito behaviours in nature, additional tools that can trap the observed specimen for further analysis are required.

1.7.1.3 Potential use of electrocuting traps

Electrocuting surfaces have been used to intercept and kill host seeking insects since the 1970s (Pickens, 1991, Schreck et al., 1975, Torr et al., 2008, Majambere et al., 2013). Traps have been devised in which an electrocuting surface is placed around a host or an attractant odour source which lures

insects, and kills them on contact. One of the first trapping methods that used electrocuting surfaces to sample malaria vectors was the Electrocuting Net trap (E-Net) (Torr et al., 2008, Knols et al., 1998). This trap, based on earlier designs for trapping tsetse and stable flies (Schreck et al., 1975, Vale, 1974), is set up by placing a live host in a sealed tent and piping their odour out to a point source approximately 10m away, which is covered by an electrified net connected to 50kV alternating current (AC). Flies are attracted to the odour source and killed when intercepted by the electric net.

More recently, electrocuting surfaces have been used to test the responses of African malaria vectors to different host odours (Torr et al., 2008) and oviposition cues (Dugassa et al., 2014a). While promising for some mosquito behaviour investigations, the occasional sparking produced by these nets (due to high voltage) and large space required to set them up means they may not be easy or safe to use inside houses. As will be described in more detail later on (Chapter 3), recent work has investigated whether more readily available commercial bug zappers could be modified to kill malaria vectors approaching humans (Majambere et al., 2013). In field trials in Dar es Salaam these bug-zapping devices achieved ~50% sampling efficiency relative to the HLC for the malaria vector *An. gambiae* s.l., but a small proportion of the samples were damaged by the electrocution in a way that made morphological identification difficult. These previous investigations with electrocuting traps show that this approach has good promise as a sampling tool, but likely requires further optimization to generate a practical, safe and efficient tool for sampling malaria vectors in the range of habitats in which vectors seek hosts.

A major aim of this study was to develop a bespoke electrocuting trap that is optimized for killing but not destroying malaria vector specimens (Chapter 2), and demonstrate that it provides an efficient and exposure free alternative to the HLC for sampling malaria vectors in and outside of houses (Chapter 3). This trap will then be used to study the host seeking behaviour of malaria vectors in the Kilombero valley with the aim of identifying if there are shifts in their feeding behaviours.

1.7.1.4 Development of mosquito resting traps and evaluation of designs for optimal sampling efficiency

Previously, different approaches have been used to sample malaria vectors resting in and outside houses. The traditional method for collecting outdoor resting mosquitoes has been the pit shelter trap (Kweka and Mahande, 2009, Mahande et al., 2007). In this method, a pit of approximately 1.5 ft wide x1.5ft deep is dug outside a house and partially covered with grass to let mosquitoes enter. However, as will be discussed in more detail (Chapter 4), this method suffers from a number of shortcomings including only being appropriate for outdoor resting mosquitoes, labour intensive, and difficult to apply at large scale. Methods for sampling resting mosquitoes indoors include backpack aspiration (Clark et al., 1994), prokopack aspiration (Maia et al., 2011) or pyrethrum spray catches (Dia et al., 2002, Okorie et al., 2014, Sabatinelli et al., 1986, Gratz and Carmichael, 1963). Collection of mosquitoes by aspiration depends on visual detection before capture and its efficiency can be highly variable depending on the skill of collectors (Ngo et al., 2014, Turell et al., 2008). Battery-powered aspirators, such as the backpack aspirator (BPA), partially overcome this limitation by mechanising the aspiration process; however, their performance is sensitive to variation in the skill of collectors. The high cost of BPA (~£654 per unit) and their dependence on batteries may limit their use in remote and resource poor areas (Vazquez-Prokopec et al., 2009).

Recently several alternative methods for standardizing resting collections by using small, portable traps that can be used both indoors and outside of houses have been proposed. As will be discussed in Chapter 4, these traps are typically variants of box or bucket type structures that are made from a range of materials (Govella et al., 2011, Sikulu et al., 2009, Williams and Gingrich, 2007, Sandhu et al., 2013, Wong et al., 2013). Examples include the use of cardboard boxes (Sikulu et al., 2009, Mayagaya et al., 2015), clay pots (Odiere et al., 2007, Wong et al., 2013) and plastic buckets (Kreppel et al., 2015). These traps have been used in various settings, but have been evaluated relative to different "gold standards", and rarely evaluated relative to one another. Consequently, it is not yet known which particular trap types are most effective.

Decades of research on tsetse flies have shown that small features of design including colour, size and orientation have a significant impact on trap performance (Colvin and Gibson, 1992). However, relatively little is known about what trap features may be most attractive to resting malaria vectors. Previous research has shown that African malaria vectors prefer to rest on dark surfaces (Scholte et al., 2005, Bidlingmayer, 1994), and sampling methods have incorporated some aspects of this biology by lining resting boxes with dark cloth (Facchinelli et al., 2007, Harris et al., 2011, Marini et al., 2010, Pombi et al., 2014a). Resting mosquitoes are also known to select humid places (Irby and Apperson, 1992). As resting mosquitoes leave their resting sites early in the morning before dawn (Gillies, 1954a), the timing of mosquito collection from resting traps is important.

There has been some investigation of the use of sticky surfaces to fix mosquitoes on traps (Pombi et al., 2014b), thus preventing the problem of having them leave before the trap is checked. Notable advantages of sticky traps (STs) is that they are cheap, do not require power supply and can be used to passively collect resting mosquitoes for a long period of time. On the downside, it can be difficult and time consuming to remove specimens from STs, making them more labour intensive than other resting traps (Service, 1984, Pombi et al., 2014a). Consequently, it is important to know whether the use of sticky surfaces makes a significant improvement to resting trap performance and thus justifies this extra effort. Finally, the influence of trap shape on sampling performance is unknown. Most resting traps that have been recently proposed for use are either bucket or box shaped. It would be useful to know whether this variation in shape has any impact on mosquito catchability before deciding on which kind of trap to use. In this study, I will compare variable features of resting traps such as shape (round vs rectangular), sticky vs non-sticky surface and the size of the entry hole (open vs restricted) and determine their effect on the sampling performance of resting traps. The most effective trap in terms of the abundance of mosquitoes trapped and consistency will be used to study the resting behaviour of the major malaria vectors in the Kilombero valley and determine which environmental factors affect vectors' resting behaviours.

1.7.2 Environmental determinants of mosquito resting behaviours

The distribution and abundance of malaria vectors is known to be significantly related to ecological factors (Simard et al., 2009, Sogoba et al., 2007, Edillo et al., 2002, Minakawa et al., 1999, Tonnang et al., 2014, Soleimani-Ahmadi et al., 2013, Fillinger et al., 2004, Mala and Irungu, 2011, Munhenga et al., 2014). For example, compared to its sibling species *An. gambiae* s.s., *An. arabiensis* has been shown to occupy drier ecological environments (Wondji et al., 2005a) and these species are known to exhibit seasonal variation in their relative abundance (Lenhart et al., 2007, Minakawa et al., 2002). Changes in environmental parameters such as rainfall, temperature, humidity and fresh water availability are also known to influence the species composition and associated malaria infection rates in malaria vectors (Githeko et al., 1996b, Mala et al., 2011, Fillinger et al., 2004). Variation in temperature and humidity has also been linked to malaria vector activities such as flight activity (Lyons et al., 2013, Mordecai et al., 2013, Armstrong and Bransby-Williams, 1961, Ikemoto, 2008, Das et al., 2007).

African malaria vectors are known to have specific thermal and humidity preferences. For example, the optimal relative humidity for survival of *An. gambiae* s.l. is considered to be between 75-85% (Olanga et al., 2010, Das et al., 2007). Correspondingly, *An. gambiae* s.l. are known to prefer to rest in places with high relative humidity (Okal et al., 2013, Yamana and Eltahir, 2013). Warmer temperatures are associated with higher host seeking activity in *An. gambiae* s.l., while lower humidity is correlated with reduced *An. gambiae* s.l. abundance and host seeking activity (Kelly-Hope et al., 2009, Yamana and Eltahir, 2013). A study conducted across different altitudes found correlations between temperature/humidity and the degree of exophily in *An. arabiensis* (Kulkarni et al., 2006). In this study, higher temperatures and humidity were correlated with higher exophilic behaviour. To accurately determine the effect of micro and macro climatic and environmental factors in influencing the resting behaviour of malaria vectors reliable and accurate sampling tools that can sample mosquitoes in and outside houses across different climatic environments are required.

1.7.3 Genetic basis of malaria vector behaviours

1.7.3.1 Methods for studying population genetics of malaria vectors

In recent years, there has been a rapid expansion in the number of techniques and genetic markers available to characterize the genetic structure of mosquito vector populations (Loaiza et al., 2012, Lee et al., 2012, Kemppainen et al., 2015, Lee et al., 2014, Yawson et al., 2007, Slotman et al., 2006, Donnelly et al., 2001, Donnelly and Townson, 2000, O'Loughlin et al., 2014, Ng'habi et al., 2011, Maliti et al., 2014). A variety of genetic markers have been used to identify population structure in mosquitoes including chromosomal inversions, allozymes, random amplified polymorphic DNA (RAPD), mitochondria DNA (mtDNA) sequences, microsatellite loci analysis and single nucleotide polymorphisms (SNPs) (Norris, 2002, Turissini et al., 2014, Sharakhova et al., 2011).

Despite being useful in revealing the genetic structure of malaria vectors in various places, these markers all have some limitations (Fontenille et al., 1993, Collins and Saville, 1990, Collins, 1996). For example, microsatellites and single nucleotide polymorphisms (SNPs) are generally considered to be the most robust approaches due to their higher resolution and thus greater ability to delineate population structure at a fine geographical and evolutionary scale (Hess et al., 2010, Yatsu et al., 2007, Lindgreen et al., 2014), and have thus been used extensively for studying the population genetic structure and gene flow in the African malaria vectors *An. gambiae* s.l.(Lehmann et al., 1996, Donnelly and Townson, 2000, Simard et al., 1999, Kamau et al., 1998a, Kamau et al., 1998b, Ng'habi et al., 2011, Maliti et al., 2014). However, microsatellites have several drawbacks including that they may not be evenly distributed across the genome and thus may underestimate genetic distance (FS_T values) due to homoplasy. In addition, they are often unsuitable for development of high resolution linkage maps needed for Quantitative Trait Loci (QTL) mapping or association studies because only a small number of markers are available in *An. gambiae* s.l. (Wondji et al., 2007b, Putman and Carbone, 2014). Also, most available microsatellite markers were developed for *An. gambiae* s.s. and may not work

efficiently for other related vector species such as *An. arabiensis* (Walton et al., 1998).

Single nucleotide polymorphisms (SNPs) are by far the most common type of molecular variation in all organisms, occurring about 1 in every 1000 base pairs (bp) in humans (Wang et al., 1998) and 1 in every 125 bp in *An. gambiae* s.s.(Morlais and Severson, 2003, Morlais et al., 2004, Lawniczak et al., 2010). A recent study has shown an even higher SNP density in *An. arabiensis* of 1 in every 59 base pairs (Yoosook et al., 2012). While a typical microsatellite based population genetic study utilizes 20-25 markers, a SNP study can easily include several hundred markers thus vastly improving resolution. It has been shown that SNPs located in non-coding regions of the genome, and synonymous SNPs (sSNPs) in coding regions which have no impact on the phenotype, are the most useful markers in population genetic studies, while non-synonymous SNPs (nsSNPs) which alter the structure and function of encoded proteins are useful markers for detecting genetic variations linked with phenotypic traits (Wondji et al., 2007a). SNP based markers require sequencing and advanced equipment which may be expensive, but due to their robustness the use of these markers for population genetic studies in malaria vectors is becoming preferred over other methods.

1.7.3.2 Behavioural phenotypes-genotypes associations in malaria vectors

Several studies have attempted to assess association between malaria vector behaviour and genetics through a variety of different methods (Gibson, 1996, Hii et al., 1991, Rinker et al., 2013, Deng et al., 2013, Dottorini et al., 2013, Xia et al., 2008). Though the use of advanced molecular approaches such as transcriptomics (Das et al., 2010, Rinker et al., 2013), SNP markers (Wondji et al., 2007a, Weetman et al., 2010, Neafsey et al., 2010, Norris et al., 2015, Lee et al., 2014) and whole genome sequencing (Lawniczak et al., 2010, Dottorini et al., 2007) are becoming more common in malaria vector biology, they have not yet been widely applied to assess the genetic basis of behaviours in African malaria vectors. However, various studies have been conducted to test for associations between particular vector behaviours and genotypes based on more coarse scale markers such as chromosomal inversions and karyotypes. One study

showed a significant difference in the frequency of chromosomal inversions within *An. arabiensis* collected either outdoors or indoors (Mnzava et al., 1994). Using cytogenetic markers, Mnzava et al (1995) also showed the presence of chromosomally distinct individuals within *An. arabiensis* that had different preferences for resting sites in Tanzania (Mnzava et al., 1995). In Western Kenya, significant host choice differences among *An. arabiensis* s.s. were observed between mosquitoes carrying different 2Rb inversions in indoor resting sites (Petrarca and Beier, 1992). These studies show the possibility that feeding behaviours in *An. gambiae* s.l. could have a significant genetic component.

1.7.3.3 Use of candidate genes for studying insect behaviour

The candidate gene approach has been used successfully to identify genetic determinants of animal behaviours (Fitzpatrick et al., 2005). Based on quantitative trait loci (QTL) studies, it has been possible to identify candidate genes associated with locomotor activity (Jordan et al., 2006) and aggression in *Drosophila melanogaster* (Osborne et al., 1997), and genes associated with courtship songs in the sand fly *Lutzomya longipalpis* (Oliveira et al., 2001). In mosquito species, a link was recently established between the host species preference of *Aedes aegypti* and the expression of specific odorant binding proteins (McBride et al., 2014). In contrast to approaches based on whole genome sequencing or QTLs which aim to identify genes of importance from a starting point of no information, the candidate gene approach can be a quicker and less expensive means of resolving genotype-phenotype associations, provided the selection of candidates is based on prior knowledge of genes that are likely to have a strong basis for influencing the phenotype of interest.

Although the candidate gene approach can be economic in the sense of requiring less intensive screening than whole genome methods, this approach has drawbacks. One of them is that the relationship between genotype and phenotype may not be fully deterministic in the sense that organisms with the same pair of alleles may behave differently in different environments (Anholt and Mackay, 2004). A second drawback is that candidate genes may have pleitropic effects by being involved in regulating several behaviours (Fitzpatrick, 2004). A third problem with the candidate gene approach is that genes of

interest may require interaction with other genes to influence phenotypic traits (epistasis)(Mackay, 2001), making it impossible to identify their role without consideration of all possible interaction effects. Therefore, the candidate gene approach can be problematic but when backed with good biological intuition can be can a good and useful choice in circumstances where resources are not sufficient to do high-resolution association-mapping studies.

1.7.3.4 Circadian rhythm association studies

Periodicity in physiological activities has been shown to be governed by circadian rhythm genes in a broad range of living organisms ranging from insects to humans (Goldbeter et al., 2010, Rosato et al., 2006, Wager-Smith and Kay, 2000). These endogenous rhythms are close to the 24 hour cycle and are therefore adjusted on a daily basis by external factors such as light and temperature, chemical and social cues which synchronize with the environmental cycles (Ozkaya and Rosato, 2012). In animals rhythmic genes, also known as clock genes, are a network of transcriptional factors which regulate their own production at the level of RNA synthesis. Through negative feedback mechanisms the *Period* (PER) and *Timeless* (TIM) genes have been shown to regulate their own expression in Drosophila by suppressing two activators involved in PER and TIM transcription (known as *Clock* [CLK] and *Cycle* [CYC] (Price et al., 1998)). For example, in light/dark cycles TIM protein levels in Drosophila decreases in late night which is followed by similar but gradual decrease in PER protein level. Degradation of TIM is dependent on PER and its protein and is important in releasing PER from the PER/TIM complex (Zeng et al., 1996). Transcriptional factors such as *Cryptochromes* (CRY) which are photoreceptors are known to regulate circadian activities in living beings (Partch and Sancar, 2005). Other cryptochromes are flaving binding proteins found in mammals, insects and plants which regulate circadian activities by degradation of TIM via ubiquitin in the presence of light (Peschel et al., 2009). Investigation of the role of these genes on organism behaviour is increasing in importance within agricultural and medical fields (Steinmeyer et al., 2012, Johnsen et al., 2007, Lippert et al., 2014, Balmert et al., 2014, Oishi et al., 2009, Weyman et al., 2006).

Host seeking at night, resting during the day and mating at dusk are among the key periodic behavioural activities within malaria vectors like *An. gambiae* s.l. Previous studies indicate that circadian rhythm genes may have some influences on the daily timing of these behaviours. For example under laboratory conditions, the pre-dusk/dusk peak expression of odorant binding proteins (OBPs) in *An. gambiae* s.s. has been shown to be driven by a number of circadian genes, and corresponds to the time of increased blood feeding behaviour (Rund et al., 2013a). Rund et al (2011) applied the DNA microarray technique to study flight activity, swarming, mating, host seeking and egg laying in *An. gambiae* s.s. and found that several genes are under circadian control. Most of the genes studied were specific to heads or bodies of mosquitoes and had peak expression centred on the day/night transition (Rund et al., 2011). When the rhythmic expression of the OBPs collected from heads under light-dark cycles were compared between *An. gambiae* and *Aedes aegypti*, distinct similarities and differences in temporal regulation of the genes in olfaction and vision were observed (Rund et al., 2013b). The host species preference of the major dengue mosquito vector *Aedes aegypti* has also recently been linked to variable expression of OBPs (McBride et al., 2014). This demonstrates the key role played by OBPs in controlling time-related odorant sensitivity therefore enabling coordination of circadian activities in *An. gambiae* s.s.

Natural genetic mutations in the clock genes have also been linked with behaviour in Drosophila. For example a mutation in the circadian clock gene *Timeless* was reported to arise in *Drosophila melanogaster* and was found to affect the incidence of diapause in response to changes in light and temperature (Tauber et al., 2007). An allele of the *Timeless* gene (*ls-tim*) was found to exist at different frequencies across different geographical regions: Italy (0.138), Israel (0.318) and Zimbabwe (0). Another allele of the *Timeles*s gene (*s-tim*) showed geographical gradient with females from the northern population (Netherlands) showing significantly higher levels of diapauses compared to those from southern (Italy) populations (Tauber et al., 2007). In other circadian genes, mutations in various loci of the PER gene have been reported to affect short-term fluctuations in the *D. Melanogaster* male's courtship songs (Kyriacou and Hall, 1980). Mutations on the CLK gene of *Chymomyza costata*

(Drosophilidae) were found to lack photoperiodic diapauses. This mutant did not respond to photoperiods and continued with development irrespective of photoperiods (Riihimaa and Kimura, 1988). Mutations in the clock gene of *Drosophila melanogaster* can lead to differences in eclosion rhythm with the mutant types displaying longer or shorter rhythms from the expected ca. 24 hours periodicity (Konopka and Benzer, 1971b). A homozygous mutation called *Jrk* in the CLK gene of Drosophila was shown to make mutants completely arrhythmic with the flies expressing low levels of PER and TIM proteins (Allada et al., 1998). Given the highly circadian nature of malaria vector feeding behaviour and gene expression, there is good reason to hypothesize that naturally occurring-variation in mosquito biting activity may be associated with polymorphisms in the circadian clock genes.

1.8 Study site: The Kilombero Valley

All field research in this study was conducted within the Kilombero Valley (KV) of southern Tanzania, which has been a site of extensive malaria research since the 1960s (Freyvogel, 1964, Freyvogel and Kihaule, 1968, Maliti et al., 2014, Mayagaya et al., 2015, Ng'habi et al., 2011, Killeen et al., 2006, Smith et al., 1993a, Charlwood et al., 2000, Lwetoijera et al., 2014, Okumu et al., 2010b, Lyimo et al., 2012a), and thus provides strong infrastructure and historical knowledge on local malaria vector ecology, epidemiology and control. This study was done in the two villages of Lupiro (-8.38 S, 36.67 E) and Sagamaganga (-8.07 S, 36.80 E) which are situated about 40km apart within the Kilombero valley.

The Ifakara Health Institute (IHI) has played a remarkable role in conducting basic and applied malaria research in the KV since its first existence under the guise of the Swiss Tropical Institute Field Laboratory (STIFL) in 1957. The Institute was transformed into Ifakara Health Research and Development Centre (IHRDC) in 1991 (Tanner et al., 1994) before being upgraded into an autonomous Tanzanian research institute, the IHI in 2008.

1.8.1 Malaria epidemiology in the Kilombero Valley

The KV lies in the Morogoro region, which historically has been one of the most malaria endemic areas of Tanzania and Africa (Killeen et al., 2006, Charlwood et al., 2000, Drakeley et al., 2003). This has been attributed to the Valley's favourable climatic and ecological characteristics that can support perennial malaria transmission (Alonzo et al., 1995). Malaria remains the leading cause of morbidity and mortality in the KV and in many places in Tanzania, with children under five years old being the most vulnerable (Shabani et al., 2010, Foster and Vilendrer, 2009, Armstrong Schellenberg et al., 2008, Olsen et al., 2002). With the introduction of ITNs in the mid-1980s, malaria transmission in the Kilombero district has been substantially reduced (Rutta et al., 2011, Alba et al., 2011, Gosoniu et al., 2008, Alba et al., 2014).

Before introduction of ITNs in the 1980s, the KV had extremely high malaria transmission as estimated by the entomological inoculation rate (EIR), a measure of the expected number of infected mosquito bites that a resident would be exposed to in a given year. EIR in the KV was estimated to be about 1481 infectious bites per person per year in 2001, but a 14-fold reduction of infectious bites (EIR=108) was documented in 2007 (Killeen and Smith, 2007). Based on data from *An. gambiae* s.l. and *An. funestus*, between 2008 and 2011 there has been a reduction in EIR from 78 infectious bites per year to 31 infectious bites per year (Lwetoijera et al., 2014). However, in 2012 EIR was reported to have increased to 226 infectious bites per person per year, which was attributed to the increasing role of *An. funestus* in malaria transmission in the Valley (Lwetoijera et al., 2014). Studies have shown a high proportion (>90%) of population in the KV own mosquito nets (Russell et al., 2011, Alba et al., 2014).

1.8.2 Malaria vector ecology and behaviour in the Kilombero Valley

The KV has a vast array of mosquito species, including all of the most important African vector species: *An. arabiensis, An. gambiae* s.s. and *An. funestus* (Drakeley et al., 2003, Ng'habi et al., 2008, Smith et al., 1993b, Russell et al., 2011, Killeen et al., 2011, Maliti et al., 2014, Lwetoijera et al., 2014). In

addition to these primary vector species, numerous secondary vector species such as *Anopheles squamosus, An. ziemanni and An. coustani* are present (Drakeley et al., 2003). Historically, *An. gambiae* s.s. was more abundant than *An. arabiensis* within the KV (Killeen et al., 2006). However, in recent years following the widespread distribution of ITNs, there has been a marked reduction in the relative abundance of *An. gambiae* s.s to *An. arabiensis*; with the latter predominating in most areas (Russell et al., 2010, Lwetoijera et al., 2014, Mayagaya et al., 2015), and *An. funestus* now being the second most important malaria vector species after *An. arabiensis* (Lwetoijera et al., 2014).

A study conducted by (Mayagaya et al., 2015) in the KV found no significant differences between numbers of *An. gambiae* s.l. that rested indoor or outdoor in houses without livestock, but in houses with livestock, the number of mosquitoes caught resting outdoors (exophily) was significantly higher than indoors. The Human Biting Index (HBI) showed plasticity with *An. arabiensis*, and even the highly anthropophilic *An. gambiae* s.s. and *An. funestus* showing some evidence of animal feeding at households where cattle were present. Analysis of data collected between 1997 before ITNs were introduced, and in 2009 after their introduction of nets showed an increased proportion of outdoor feeding (exophagy) in *An. gambiae* s.l. and *An. funestus* in the KV (Russell et al., 2011). Knowledge of ecological and genetic factors underlying these changes is important in understanding malaria epidemiology in the Valley as well as in other endemic regions.

1.8.3 Population genetics of malaria vectors in the Kilombero Valley

A limited but valuable number of population genetic studies of malaria vectors have been conducted in the KV. These studies reveal extensive genetic structure in *An. arabiensis* even within this relatively small geographic area (11600 km^2)(Ng'habi et al., 2011). In this study, genetic distances among *An. arabiensis* within the KV were reported to be wider than those between the *An. colluzzi* (formerly M form) and *An. gambiae* s.s. (formerly S form) in West Africa, leading to the hypothesis that, *An. arabiensis* may exist in sub-populations in the

valley. In contrast, a more recent study (Maliti et al., 2014) showed *An. arabiensis* to be a uniform population within the KV, and up to the coast of Dar es Salaam and the Zanzibar islands. However, analysis of *An. gambiae* s.s. within the same study revealed high differentiation unrelated to geographical distance within the KV. These contrasting results between the two studies may be due to differences in sampling strategies and statistical analysis approaches, but at least suggest the possibility of high genetic variability between local vector populations which provides scope for investigation of mosquito vector behaviour phenotype-genotype associations.

1.9 Study hypotheses

Based on the need to develop better sampling tools for malaria vector behaviour and use them to gain a better understanding of the ecological and genetic determinants of their feeding and resting behaviours, the following four hypotheses were developed.

Hypothesis 1: Mosquito electrocuting traps (MET) can be made which have an electrical output sufficient to kill but not destroy malaria vector specimens, and that are safe to use in close range of humans. Application of lower voltages than those applied previously in electrocuting traps (Torr et al., 2008, Majambere et al., 2013) may reduce burning of specimens leaving them suitable for morphological identification and molecular assays.

Hypothesis 2: METs can be used to study the host seeking behaviour of malaria vectors inside and outside households, including estimating the abundance of these vectors by giving similar estimations of the biting behaviour as those obtained from the gold standard human landing catch (HLC) technique.

Hypothesis 3: Mosquito resting trap design influences their sampling performance. Comparing resting traps that have different physical features such as size of entry holes, shape and use of sticky surfaces can help to determine which physical features are important for optimal sampling of resting traps. Microclimatic feature such as indoor and outdoor temperature and humidity influence the resting behaviour of malaria vectors.

Hypothesis 4: Differences in the time (early versus late in the evening) and location of host seeking amongst the malaria vector *An. arabiensis is* associated with polymorphisms in their circadian genes.

These hypotheses were tested as described in the following 5 chapters:

Chapter 1: In this chapter, the general overview of the malaria situation in Africa and specifically in Tanzania is presented. In addition, this chapter provides an overview of different developments in the sampling tools of malaria vectors. Lastly, the ecological and genetic determinants of malaria vectors' behaviour including tools used to study are reviewed, and the key objectives of this thesis presented.

Chapter 2: This chapter presents the methodology used to develop and optimize an electrocuting trap for African malaria vectors. The chapter discusses the experience gained from the field while using the MET, problems encountered while using this trap in the field and suggestions for further improvements of the trap.

Chapter 3: This chapter presents results of a field study that evaluated the MET relative to a commercially available insect electrocuting device (CA-EG) and the Human Landing Catch (HLC). Other criteria such as density dependence and how the MET characterized the feeding behaviour of malaria vectors such as the time and place of feeding relative to the HLC gold standard are discussed.

Chapter 4: This chapter presents results of the evaluation of a series of different traps for collecting indoor and outdoor resting malaria vectors. Trap types were chosen to permit assessment of how different physical features such as size of the trap entrance, application of sticky surfaces and trap shape (round versus rectangular) influenced the sampling sensitivity of four resting traps used for indoor and outdoor sampling. Effect of microclimatic factors such as differences in temperature and humidity between indoor and outdoor environments was investigated.

Chapter 5: This chapter presents results of an association study between variation in the biting behaviour of *An. arabiensis* and polymorphisms in their circadian rhythm genes.

Chapter 6: This chapter presents overview of key findings and general conclusions including specific recommendations to be considered for future work.

Chapter 2: Development of mosquito electric grid trap for exposure-free sampling of host seeking malaria vectors

Contributions from other people

This chapter will not be submitted as a manuscript. However other people who contributed to its writing are listed: Deodatus Maliti[1,2], Dr. Nicodem Govella[1], Dr. Katharina Kreppel[1,2] Nosrat Mizrai[1] & Dr. Heather Ferguson[1].

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The specific contribution of these co-authors to work presented here is as follows: DM NG designed the traps. DM NM constructed trap. DM designed the experiment with guidance from HMF NJG and KK. DM conducted the field experiment. DM conducted all statistical analysis under the guidance of HMF. DM wrote the chapter with guidance from HMF and further comments from HF NM NJG KK who reviewed the chapter.

2.1 Aims and objectives

This chapter describes the early stages of the development and optimization of a mosquito electrocuting trap (MET) designed to sample African malaria vectors inside and outside houses. A key aim of this study was to develop a trap which can be used to characterize the host seeking behaviour of the major African malaria vectors without exposing human participants to mosquito bites. A crucial objective of this work was to develop an electrocuting trap which applies a voltage optimized to be just enough to kill a mosquito whilst leaving the carcass intact and suitable for morphological and molecular identification. A desired feature of this trap was that the output voltage be stable throughout the duration of the nightly trapping experiments to ensure consistent results. In this work, the development of the initial prototypes of the MET are described including the steps taken to improve these prototypes to arrive at advanced working designs, which were suitable for field-testing (Chapter 3). Problems encountered in the development and early-stage testing of these prototypes

including the costs incurred are discussed, and suggestions for improvement of future designs are given.

2.2 Introduction

Insect vector research relies on sampling techniques to identify the species present at a particular location, their abundance and biting behaviour. Malaria vector sampling techniques are a crucial component of vector control because they provide important information for guiding how and where intervention measures should be deployed. For example, by confirming that mosquitoes bite outdoors and at the time people are not yet under bed-nets can lead researchers to devise ways to control outdoor mosquito biting, thus enabling a more successful vector control. The need for more efficient sampling tools has become evident in several current mosquito surveillance programs (James et al., 2014).

As reviewed in Chapter 1, various sampling techniques have been used to study host seeking mosquito vectors of malaria. Of these, the Human Landing Catch (HLC) remains the gold standard and is most often used to estimate the abundance, diversity, infection rate and human-biting behaviour of malaria vectors (Seyoum et al., 2012, Tirados et al., 2006, Dolo et al., 2004, Loaiza et al., 2008, Samarawickrema et al., 1992, Gillies and Coetzee, 1987b). Furthermore, the HLC has often been used as the reference method for estimating the efficiency of numerous intervention methods in terms of their ability to reduce the abundance and infection rates of malaria vector populations (Tirados et al., 2011, Tchicaya et al., 2009, Komalamisra et al., 2009, Bockarie and Dagoro, 2006, Bockarie et al., 2002). However, conducting an HLC involves use of a human bait to attract mosquitoes. As discussed in Chapter 1, the major disadvantage of this technique is its potential to expose the human volunteer to infectious mosquito bites, and so making this technique prone to ethical dilemmas over the course of its application (Ndebele and Musesengwa, 2012, Achee et al., 2015). Despite the risks inherent with this approach, terminating the use of HLC before another suitable alternative method is in place also poses an ethical dilemma, as failure to accurately measure malaria transmission and exposure risks could compromise control

efforts. Thus, there is a clear need for development of alternative 'exposure-free' methods for sampling malaria vectors that can ideally meet similar performance standards as the HLC for representing human exposure risk.

In this thesis, I describe the development, optimization and evaluation (Chapter 3) of a new method for sampling host-seeking malaria vectors based on the use of electrocuting surfaces. The decision to pursue this trapping method was inspired by previous research, which has shown that electrocuting traps can be effective for sampling a variety of insects under some conditions (Vale, 1974, Dugassa et al., 2014b, Dugassa et al., 2012, Majambere et al., 2013, Torr et al., 2008, Knols et al., 1998). I sought to build upon this foundation by creating a specialist mosquito electrocuting trap (MET), that was customized for particular targeting of African malaria vectors, and had a unique advantage in comparison to previous electrocuting traps of being effective and safe for use in both indoor and outdoor settings. Incorporating ability for the trap to be used in both indoor and outdoor environments was an important requirement, given that one of the primary intentions of this trap was for it to estimate key aspects of mosquito vector behaviour including the relative propensity to bite indoors versus outside. However, the most important reason for MET development was the need to provide an exposure-free host-seeking trap for use in malaria research, which is representative of the HLC technique.

2.2.1 Previous development of electrocuting insect traps

The use of electrocuting traps for insect vector surveillance was originally developed for tsetse flies (Vale, 1974). Also called E-nets, these traps use a mesh of positive and negatively charged wires through which high voltage electricity is passed. E-nets use odour from a live host which is placed in a tent, and piped out to a release point several metres away that is covered by an electrocuting surface. Insects are electrocuted when trying to pass through the electrified grids to follow the host odour. Since their initial use for tsetse, electrocuting traps have also been used to sample stable flies (Pickens, 1991), and have been trialled with African malaria vectors in Tanzania (Knols et al., 1998), Zimbabwe (Torr et al., 2008) and recently in Kenya (Dugassa et al., 2014a). Details of the features of previous electrocuting traps mentioned here

are discussed relative to the design developed in this study in section 2.3.2 and are also presented in Table 2.1.

Whilst the e-nets worked with some success in being equally or more efficient in collecting host seeking malaria vectors compared to other techniques such as the CDC light trap, facets of its design may not be optimal for studying malaria vector behaviour. For example the prototype used to sample mosquitoes by (Torr et al., 2008) was based on the design used for tsetse, and had 8mm wide gaps between the electrocuting wires; this spacing would be sufficient to prevent large flies like tsetse passing through, but could be too big to stop mosquitoes. This trap was made up of a series of conductive positive and negative wires through which an insect has to make contact, and the trap uses a high voltage (~50,000V) alternating current (AC) output. This high voltage output is suitable for trapping flies in an open savannah environment, but could pose an electrocution risk when used in close contact to humans, and may not be safe for use inside houses. Additionally, an e-net trap requires a considerable amount of space to set up (a tent for the host followed by several meters of piping), which may be difficult to fit inside houses.

In this work, I developed a mosquito electrocuting trap (MET) which operates on a similar principle to the e-nets developed by (Vale, 1974) and (Torr et al., 2008), in aiming to kill mosquitoes on contact with an electrocuting surface placed around a host odour, but with modification to make it efficient, safe and easy to use for sampling malaria vectors in and outside of houses.

2.2.2 Design concept of Mosquito Electrocuting Trap

The starting principle for MET development was that this trapping method would be set up to mimic the catching approach used in the HLC, with the incorporation of electrocuting surfaces placed around the exposed part of the human catcher's leg to kill mosquitoes attempting to pass through. The HLC technique works through both passive and active engagement of the human catcher in collecting mosquitoes that are attempting to bite. The passive involvement constitutes the attraction of mosquitoes to the human catcher through the emanation of body odours, carbon dioxide and body heat (Mukabana

et al., 2012, Canyon and Hii, 1997, Okumu et al., 2010b, Takken and Knols, 1999, Olanga et al., 2010). Active engagement of the human catchers performing HLC is required to observe mosquitoes landing on exposed regions of their legs, and capture them with an aspirator. Due to people's differential attractiveness and their ability to detect and collect mosquitoes, variability can be introduced in sampling (Mathenge et al., 2002, Kilama et al., 2014). The MET approach developed here sought to eliminate this active collection process by placing electrocuting grids around the host that kill all mosquitoes as they land upon them, and thus shielding collectors from bites.

As a pre-requisite to developing a prototype for field testing, I sought to identify the optimal functional design characteristics of an MET in terms of (1) a structure that could easily be set up to collect mosquitoes both inside and outside homes, (2) an appropriate electrical delivery source and voltage to kill mosquitoes without destroying them (to permit subsequent morphological and molecular identification), (3) use of protective measures to minimize the risk of shock to the human participant, and (4) durability of structure and reliable function under real field conditions (e.g. battery life, consistency of voltage output source). Additionally, I estimated the relative costs of constructing a prototype with these features relative to the HLC to assess whether it could be an affordable sampling tool for mosquito surveillance in low income settings. The above 4 criteria were used to guide the design of a mosquito trap prototype that could be easy-to-use under field conditions.

2.3 Methods

2.3.1 Construction and pilot trials with the first prototype of the mosquito electrocuting trap (MET1)

An initial prototype of the mosquito electrocuting trap (MET1) was developed in Ifakara, Tanzania in 2011. This early version of MET was conceptualized and designed by myself, and co-supervisors Dr Heather Ferguson and University of Glasgow (UG) and Dr Nicodem Govella of the Ifakara Health Institute (IHI). This and all other trap prototypes consisted of two main components: (1) the grid structure that would electrify mosquitoes on contact and (2) the power unit that

delivered electricity to the trap. For MET1, the grid structure was constructed by hand with the help of local technicians in Tanzania, and powered by a high voltage generating power unit which was also locally made by electricians from the Arusha Technical College in Tanzania.

The MET1 was made of four square panels of electrocuting grids measuring $1m^2$ each. Each of these panels was woven by superimposing two steel wire meshes, each with a spacing of approximately 1.6cm, to yield a final grid with wires every 0.8 cm. Following Torr et al (2008), it was assumed that common malaria vectors species in the Ifakara area (*An. gambiae* s.l. and *An. funestus* s.l.) could not pass through a 0.8cm opening without touching one electrocuting wire. A plastic mesh was placed between the two superposed panels in order to prevent contact between the panels (Figure 2.1A). The superimposed wire grids were then mounted onto a wooden frame. The concept was to use four of the wood-mounted grids to form a box-like structure around a human catcher, with the top open and the bottom edge sitting on the ground. During the deployment of this trap, a human volunteer sat surrounded by four panels of the MET1. An insecticide-free bed-net was used to protect the volunteer from mosquito bites during the sampling experiment (Figure 2.1B).

Figure 2.1. An electric grid panel of the first MET prototype (MET1). (A) Showing a single grid panel (B) when set up outdoors. Note a bed-net was used to protect catchers from mosquito bites while the top canvas protected the trap from moisture.

The electric power unit of the MET1 was based on that which was used by (Torr et al., 2008) in that it involved an alternating current (AC) but at a lower voltage (800V) compared to that used in Torr et al (2008). Pulsating electric current (AC) differs from direct current (DC) in that it induces a magnetic field around adjacent positive and negative wires (Herman and Stephen, 2011) which should theoretically be capable of electrocuting a mosquito flying close to the grid, even if it only made contact with one of the wires. It was thought this system would still be sufficient to induce electric shock to a mosquito passing through the grid without having to touch them, while minimizing damage to the specimen as could occur from using voltage as high as 50,000V (as previously by Torr et al. 1998). A voltage amplifier was connected to the superimposed grids through the positive and negative terminals, with each of these terminals being connected to one of the two superimposed grids.

Pilot field trials of the MET1's performance were conducted from Oct 2011-Jan 2012 in Lupiro village, (-8.38 S, 36.67 E) situated within the Kilombero Valley of Tanzania (described in Chapter 1). The aim of these pilot trials was to observe whether the MET1 was reliably operational under field conditions, including if the trap could deliver the desired voltage over the course of the night as expected, and to make preliminary comparison of its performance relative to the HLC technique in outdoor environments.

In Lupiro village, the Ifakara Health Institute (IHI) has constructed a series of experimental huts. Such huts are widely used in medical entomology research for the experimental study of mosquito ecology (Snow, 1987, Smith et al., 1964, Mahande et al., 2007, Seyoum et al., 2002). These huts are constructed to imitate the common design of houses in the local village in terms of size, and having openings between the roof and the wall (eave) which are known to be used by mosquitoes to enter and leave huts. Trials with the MET1 were run in 2 experimental huts, with a 2x2 Latin Square design used to compare the performance of the MET1 to HLC. On each night of experiments, an HLC was conducted both inside and outside at one of the experimental huts, and in the other, an MET was set up outside of the hut. On the following night, the trapping methods were swapped between huts so that one rotation was

completed over 2 days. As this was a pilot study, trials were run only for part of the night, from 7pm to 12pm. The experiment was conducted in 2 rounds each comprised of 2 nights to make a total of 4 experimental nights. As mosquito density was very low in October and November 2011, another round of experiments was conducted after the short rains in the in January of the following over 6 experimental nights.

In each of the experiments described above, volunteers sitting in each of the baited traps (HLC and MET) were rotated after every hour to remove bias resulting from different attractiveness and skill of the volunteers in collecting mosquitoes (for HLC). Volunteers were asked to conduct active trapping for 45 minutes of each hour, and use the remaining 15 minutes to rest and switch to the different trapping method. After the 45 minutes of trapping, the MET1 was switched off and a mouth aspirator was used to collect trapped mosquitoes from the grids. The trap was checked to see if it delivered the correct voltage at the beginning of each sampling hour by using a voltmeter. Trapped mosquitoes were placed in labelled cups with indication of the trapping methodology, hour, experimental hut and place of collection.

2.3.2 Construction and optimization of second prototype of the mosquito electrocuting rap (MET2)

Due to problems arising from the poor function and performance of MET1 under field conditions (as described below), a second prototype was developed with the aim of alleviating shortcomings in the original version. The first step in constructing the second prototype (MET2) involved modification of the design of the electrocuting grid surface by reducing it to cover only the part of the body usually exposed in HLC (the lower legs). In constructing MET2, four square wooden frames each with an inner dimension of 30cm x 30cm were used. Previously larger electrocuting traps measuring 1m x 0.5m were used for trapping ovipositing mosquitoes (Dugassa et al., 2012, Dugassa et al., 2013), host seeking mosquitoes (Torr et al., 2008) and tsetse flies (Vale 1974). Positive and negative wires were placed alternatively and parallel to each other running from one end of the wooden frame to the other. In a change from MET1, the

spacing between wires was reduced from 8mm to 5mm as the former spacing was observed to allow some mosquitoes pass through the grids without being electrocuted. The 8mm spacing was used in previous traps developed for the purpose of trapping larger insects such as Tsetse flies (Vale 1974), and was used also for host seeking traps with *An. arabiensis* (Torr et al., 2008) and for oviposition traps with *An. gambiae* s.l. (Dugassa et al., 2013) and *An. gambiae* s.s. (Dugassa et al., 2012). Other work has used smaller gaps between adjacent wires (2.5mm and 4mm) to trap *An. gambiae* s.l. (Knols et al., 1998). The wooden frames were connected side by side using wires to form a sturdy square frame in which a person's leg could be placed (Figure 2.2A). The bottom of the box was covered with cardboard to provide a platform on which the volunteers placed their legs during the trapping process. The trapping box was designed in such a way that it could be easily assembled or disassembled into separate panels when required by untying the wires.

Figure 2.2. The design of the second MET prototype (MET2) (A), with a close view of mosquitoes being trapped on the grids during field trials (B).

In a second step, optimization was performed on the electrical power unit to identify which voltage level was most effective for killing mosquitoes on contact but keeping their carcass intact, and to improve its safety. As part of this process, a single small grid prototype panel was constructed at the University of Glasgow Bioelectronics Unit (UGBU) and used to experimentally determine the optimum voltage for killing African malaria vectors, while maintaining the specimen intact (e.g. without burning or otherwise destroying it in a way that would prevent identification). This condition was important to ensure that the

electrocuted mosquitoes could be identified using standard morphological keys and PCR identification (Scott et al., 1993).

In the laboratory at UG, two standard insectary cages (30 cm^3) were placed side by side with the open ends facing each other. An electric grid panel (30cm x 30cm) was placed between the open ends of the cages (Figure 2.3), such that mosquitoes attempting to pass from one cage into the other would contact it.

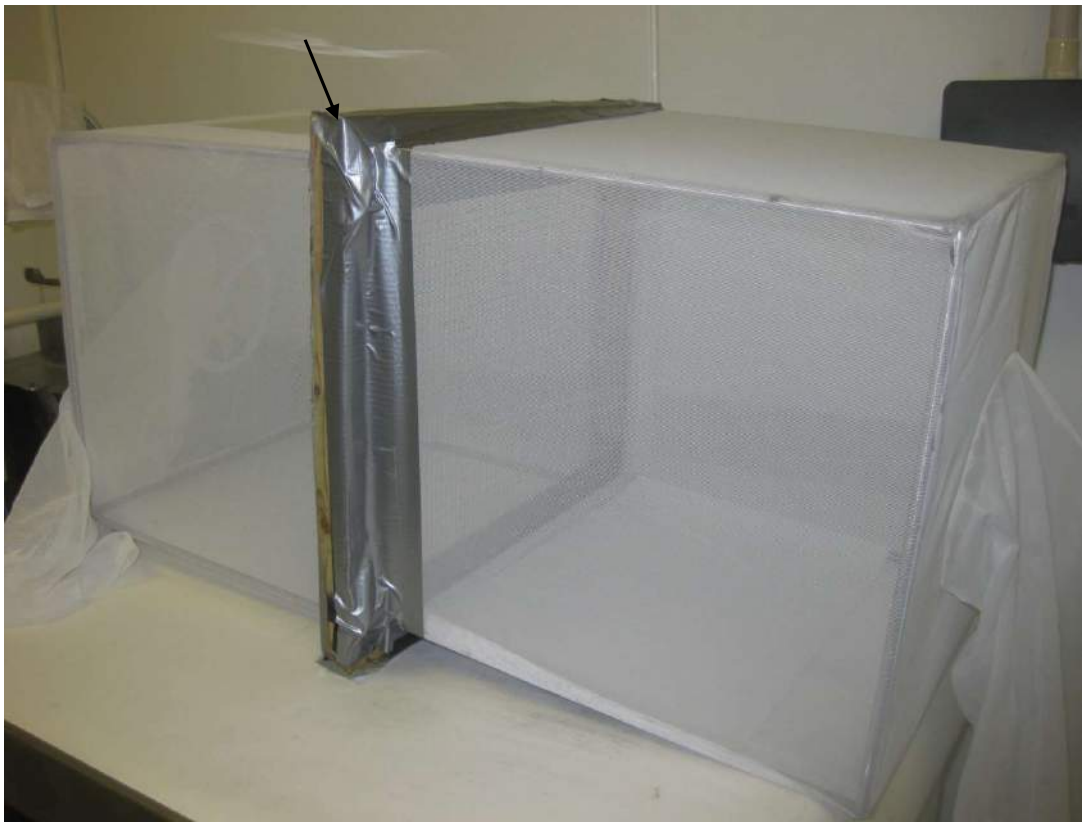

Figure 2.3. Experimental set up used to assess the optimal voltage for killing *An. arabiensis* and *An. gambiae* s.s. mosquitoes by electrocution in the laboratory. Two 30cm^3 mosquito cages were connected with a single panel of MET (pointing arrow) placed between them.

The grid panel was connected to a variable voltage amplifier via the positive and negative terminals. The decision was taken to use a DC rather than AC power source, as advice from the UGBU indicated this could deliver enough voltage to kill insects whilst reducing the sparking often occurring with an AC source which could pose some risk to users. Alternating current has been used in electrocuting traps by (Knols et al., 1998). Other electrocuting traps used in previous studies for sampling *An. gambiae* s.l. and tsetse flies (Dugassa et al., 2013, Dugassa et al., 2014a, Dugassa et al., 2012, Torr et al., 2008) used pulsating DC generated

by spark boxes which is a different kind of electric current from the non-pulsating DC used in this study. The variable voltage amplifier was designed by Nosrat Mizrai of the UGBU and had a voltage output range of 0-1000V DC. A diagrammatic representation of the connection between the variable amplifier, the battery and the grids is shown in Figure 2.4A (as provided by Nosrat Mizrai, UGBU).

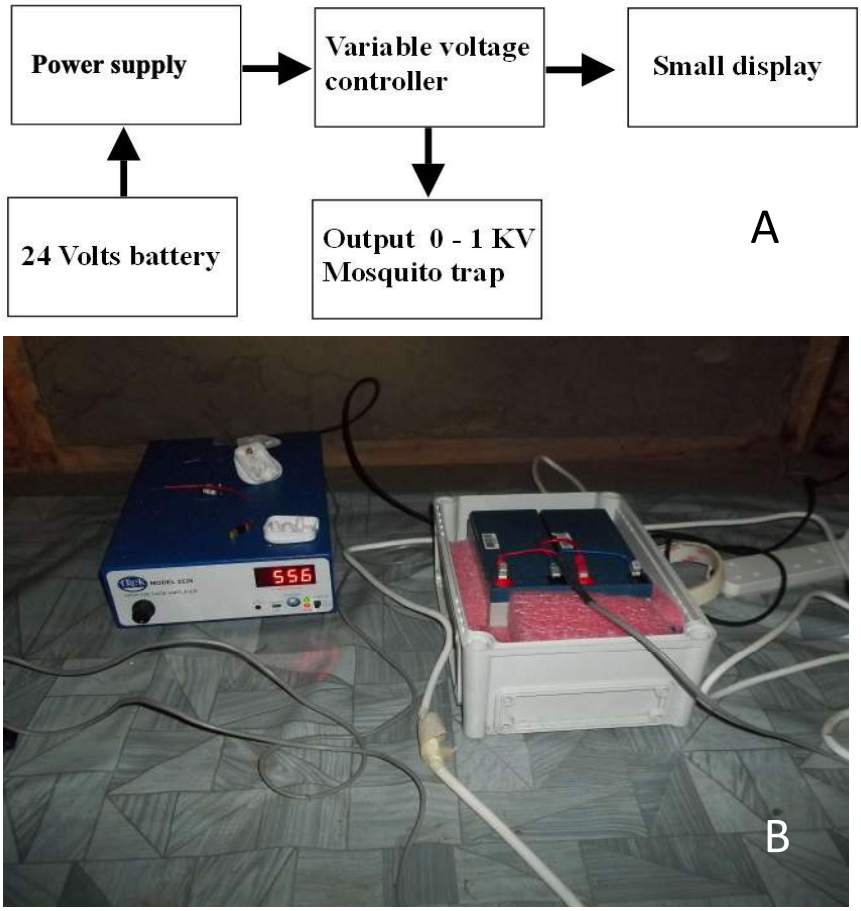

Figure 2.4. (A) Schematic presentation of the circuit used with MET2. (B) Power is supplied from a pack of 2 batteries each with 12V capacity connected in series to give 24V. The variable voltage controller allows for adjustment of the voltage from 0-1kV DC which is supplied into the trap. The circuit diagram was provided by Mr. Nosrat Mizrai from the UGBU.

Experiments were conducted to test ability of these electric surfaces to kill the malaria vector *An. gambiae* s.s. and *An. arabiensis*. *Anopheles gambiae* s.s. were obtained from a laboratory colony at the University of Glasgow, where the Keele line of *An gambiae* s.s. (created from a mixture of 3 Tanzanian populations and one from the Gambia (Hurd et al., 2005) are maintained.

The initial output of the voltage amplifier was set at 20V. Through an opening in one of the cages, 50 female unfed *An. gambiae* s.s. aged between 7-9 days were released into the cage. The cage was shaken slightly for two minutes to force mosquitoes to attempt to cross into the other holding cage via the electrical grid. After two minutes of shaking the cages, the number of mosquitoes that made contact with the grids and were electrocuted was recorded. This procedure was repeated with a 20V increment at each round. Before moving into the next voltage increment, all mosquitoes used in the previous voltage trial were removed and replaced with a fresh batch of mosquitoes. This was necessitated as it was noted that in preliminary trials with low voltages, mosquitoes which received minor, non-lethal electrical shocks in their first attempt to cross the grids were reluctant to approach a second time.

After concluding experiments with *An. gambiae* s.s., a similar lab optimization procedure as described above was repeated using *An. arabiensis,* which is the most abundant vector in the KV (Ng'habi et al., 2010, Lyimo et al., 2012b, Okumu et al., 2013b). The *An. arabiensis* colony used in this experiment was established from a population in Sagamaganga village in the KV (Ng'habi et al., 2010) that has been colonized at the University of Glasgow since 2008. On the basis of results showing that no *An. gambiae* s.s. were killed at voltages lower than 240V (Figure 2.5A), experiments on *An. arabiensis* used a starting voltage of 200V with 100V increases in voltage and used only 20 mosquitoes per round as the number of *An. arabiensis* was limited. Previous electrocuting traps have used high output voltages such as 2.5kV (Dugassa et al., 2012), 2kV-6kV (Knols et al., 1998) and 50kv (Torr et al., 2008) in trapping *An. gambiae* s.l. mosquitoes. This voltage is much higher compared to that used in this experiment (See next section).

Experience from MET1 showed that some loosening of wires within the wooden frame occurred due to its contraction and expansion following temperature fluctuations. This led to adjacent negative and positive wires contacting each other. To solve this problem in MET2, a cylindrical wooden wedge was put close to the place where the grids were tied to the frame to increase tension in the grids (Figure 2.2A). Spring conductors were used in some previous electrocuting traps to avoid loosening of the wires due to contraction and expansion of the

wires (Torr et al., 2008, Dugassa et al., 2012). MET2 was used in a formal field evaluation conducted in Lupiro village in March 2012. Some of the major differences between the MET developed in this study and other electrocuting traps developed previously are described in Table 2.1.

Trap	Author	Target insect	Size of the trap	Space between wires	Electrocuting current type	Power source	Voltage (V) at the grid	Host source
MET	Maliti et al 2015	*Anopheles gambiae* s.l.	30cmx30cm	5mm	DC	24V battery	600	Live human
Oviposition traps	Dugassa et al 2012	*An. gambiae s.s.*	100cm x 50cm	8mm	DC pulse	12V battery	2500	Stagnant water
Electrocuting nets	Torr et al 2008	*An. arabiensis*	100cm x 50cm	8mm	DC pulse	12V battery	50,000	Odors from human and ox via pipe
E-nets	Knols et al 1998	*An. gambiae* s.l.	Small: 15cm x 17cm Large: 28cm x 40cm	Small nets: 2.5mm Large nets: 4mm	AC pulse	12V battery	Small nets: 2000 Large nets: 6000	Human breath (live inhalation through pipe)
E-nets	Vale et al 1974	Tsetse flies	100cm x 50cm	8mm	DC pulse	12V battery	>2000	Odors from animals

Table 2.1: Major features of some of electrocuting traps used in this study and the previous studies. Most of the traps have no specific names and are generally named electrocuting nets (E-nets).

Results of this study are fully presented in Chapter 3 and will not be duplicated here. Results are however presented here for observations made during the development and optimization of this prototype.

2.4 Results

2.4.1 Development of MET1

Pilot field trials of MET1 showed that the performance of MET1 was poor. The total number of mosquitoes captured by these 2 trapping methods in preliminary trials is presented in Table 2.2. Over 2 nights of trials in October and November 2011, only 3 *An. arabiensis* were caught in MET1 compared to 25 in HLC. Furthermore, no *An. funestus* were collected in the MET1 during this period compared to 3 in HLC. Overall mosquito numbers were very low during this period, so further trials were conducted after the short rains in January 2012. The performance of MET1 relative to HLC remained very poor during this period (Table 2.2). Here the MET1 did not collect any malaria vectors while HLC collected 280 (264 *An. gambiae s.*l., 16 *An. funestus*).

Species	October 2011		January 2012	
	MET1	HLC	MET1	HLC
An. gambiae s.l. male	0	0	0	0
An. gambiae s.l. unfed	3	25	0	250
An. gambiae s.l. *fed*	0	0	0	14
Total *An. gambiae* s.l.	3	25	0	264
An. funestus s.l. male	0	0	0	0
An. funestus s.l. unfed	0	3	0	15
An. funestus s.l. fed	0	0	0	1
Total *An. funestus* s.l.	0	3	0	16

Table 2.2. Performance of MET1 relative to HLC, in field trials conducted between October 2011 and January 2012. Fed mosquitoes refer to mosquitoes which were morphologically identified and found to have taken a blood meal, while unfed mosquitoes are those that were not found to have taken a blood meal.

Several problems were observed to arise during the use of MET1 which may have been responsible for its poor performance. Firstly, the voltage amplifier output was not stable. There was frequent, unpredictable shifting of voltage up and down over a range from 0V-800V. When the voltage was around 800V, mosquitoes that attempted to pass through the grids appeared to burn up, and emit a bad smell, which may have deterred other mosquitoes from approaching. These problems were associated with either low or no catch, and when some mosquitoes were captured, they were damaged and therefore difficult to identify by standard morphological procedures. Secondly, in some instances, the opposite terminals of positive and negative wires touched each other which caused the voltage amplifier to short circuit. When this occurred, trials had to be stopped and the voltage amplifier could not be used again until some repair was done. Third, the superimposition of 2 metal grids (16 mm spacing) could not be done with enough precision to yield a final grid with uniform spacing of 8 mm. Thus, the spacing between wires in some places was too large and allowed mosquitoes to pass without being electrocuted. This resulted into low catch, and lowered the amount of protection from biting that the MET1 provided to the

human volunteer. Fourth, it was difficult to observe and extract trapped mosquitoes from the grids as the superimposition of the grids made it difficult to spot trapped mosquitoes. This increased labour and time spent to collect mosquito samples, and was also a potential source of error in the estimated number caught. Finally, the relatively large size of the MET1 panels ($1m^2$) made it bulky and cumbersome to move from place to place. All of these problems were identified as design flaws to be improved upon during the development of the second prototype (MET2).

2.4.2 Development of MET2

In response to the design flaws identified with MET1, several changes were incorporated into MET2. Before assessing the functionality of the new prototype under conditions, laboratory experiments were conducted to identify the optimal voltage for killing but not destroying mosquito samples. No *An. gambiae* s.s. were observed to be killed until the output voltage reached 240V DC. Above this, the proportion of *An. gambiae* s.s. killed on contact continued to increase (Figure 2.5), but at voltages higher than 600V sparking accompanied by a foul, burning smell occurred when mosquitoes touched the grid. Approximately 80% of *An. gambiae* s.s. were observed to be immediately killed at this voltage, with the death rate increasing to just above 90% at the highest voltage of 1000V.

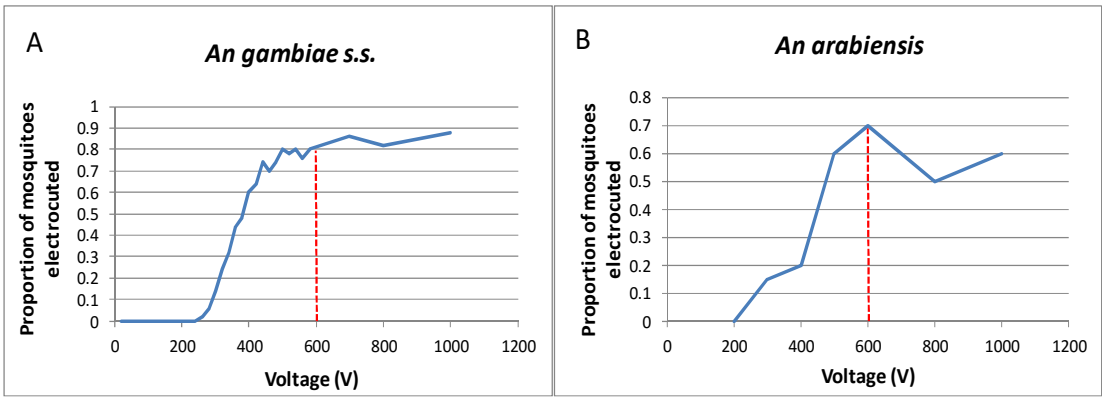

Figure 2.5. The relationship between the voltage (V) of an electrocuting panel and the proportion of (A) *An. gambiae* s.s. and (B) *An. arabiensis* that were killed on contact with the electrocuting grids during laboratory optimizations experiments. The red dotted lines show the optimum voltage which was just enough to kill but not burn a mosquito.

The peak death rate of *An. arabiensis* on contact with grids was ~70%, which was observed to occur at voltages of 600V or higher (Figure 2.5B). As observed in experiments with *An. gambiae* s.s., as voltages were increased above 600V profuse sparking and a foul smell of burning occurred when mosquitoes made contact with the grid surface. Based on these laboratory trials, an output of voltage of 600V was concluded to be the optimal for killing but not destroying malaria vectors on contact.

After an appropriate voltage had been identified from lab experiments, the MET2 was designed for construction in Tanzania using a power supply unit that had been built by the UGBU, and a mesh frame constructed locally. In Tanzania, two MET2 units were constructed in a workshop at IHI with the aid of carpenters who made the outer wooden frame of the trap. This work was relatively easy and straightforward and took the carpenters approximately one week to have the two MET2 box frames ready after spending 2 to 3 hours daily on this work.

Though the 600V used with the MET2 was higher than that supplied in domestic circuits (120V-240V), the current flowing through the grids was reduced by resistors to only 10mA. This resulted in the actual power output of the trap being 6 Watts. Though this level of power output is unlikely to cause electrical harm to humans using the trap (Clifford, 2005), additional protective "safety shields" were built into the inner surface of the grid to prevent direct contact between the user's legs and the electrocuted surface. This shield was built

using a plastic mesh and was placed into the inner surfaces of the grids such that people working with the trap could not get shocked by accidentally moving their legs towards the grids.

The weaving of the grid wires was done by the PhD student with assistance of two technicians trained in mosquito fieldwork. The weaving of the grids was the most challenging part of MET2 construction and took about 5 hours to weave one 30cm x 30cm panel. This was particularly difficult because of the need to ensure a consistent and very small gap of 5mm between wires across a 30cm x 30cm panel. This required taking repeated measurements for each line of wires and tightening the wires to an extent where they would not allow a bigger than required gap which could let mosquitoes pass in without being electrocuted. All tightening of the wires had to be done by hand. Even after tightening the wires, it was observed that some wires became loose as the wooden frame contracted following temperature fluctuation. When this happened, it was necessary to tighten up the wires again to maintain the 5mm gap, which sometimes resulted in breaking of the wires. Compared to MET1, the output voltage from the professionally made variable voltage amplifier was more stable and consistent, though still showed some fluctuations which appeared to be associated with moisture building up on the wooden frames.

The performance of the MET2 relative to the HLC gold standard is described in Chapter 3. Overall, this prototype was judged to perform well with respect to the initial milestones set to assess suitability. However, a number of challenges were noted during field-testing which were recorded to help guide the further development of new prototypes. First, perhaps the most notable challenge was the potential for moisture to become absorbed into wooden frames on which the electric grids were woven. This was problematic as it resulted in short circuiting during some trials, and occasionally caused dramatic reductions in the current and voltage between adjacent positive and negative grids, which could have reduced mosquito catch rate. In addition, occasionally this short-circuiting caused some smoke to emerge from points on the wooden frame. Although this was too minimal to cause safety risks to the mosquito catcher, it necessitated switching off the power supply to fix the problem.

The build up of moisture in the wooden frame may also have lead to an increase in the output load resulting in a higher than expected depletion of battery power. The 24V battery used here was anticipated to last over 2 nights of trapping but stayed charged for only one night under field conditions. These issues were fed back to the UGBU and other colleagues at IHI, and led the production of an improved protocol (MET3)(Figure 2.6) which was tested subsequent to this study by other colleagues at IHI (Meza et al, in preparation).

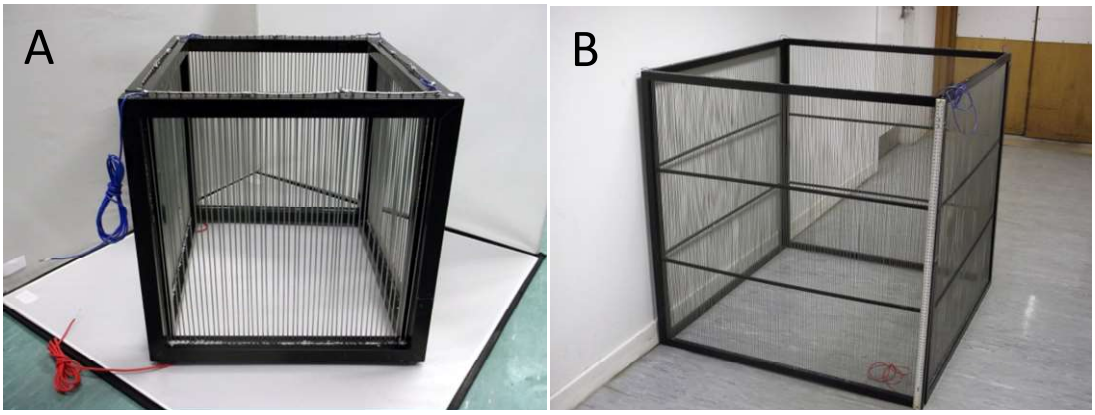

Figure 2.6. The third MET prototype (MET3), whose design was guided by problems encountered with earlier versions. The small version named MET3S (A) is 30cm wide and 30cm wide, and the large version MET3L (B) is 100cm wide and 100cm broad. The small version can be placed around the lower part of two human legs while the large version can enclose an entire host.

2.4.3 Costs of construction

The estimated costs for the construction of one MET2 unit including material and labour were estimated (Table 2.3).

Item	Description	Cost/ item (£)	Quantity	Cost (£)
Voltage amplifier	High voltage amplifier	358.74	1 piece	358.74
Wood	Used to make the MET frame	2.78	4 pieces	11.12
Stainless wire	For electrocuting grids	28	1 roll	28
Insulated cable	Connect the voltage amplifier to the grids	0.48	10 meters	4.48
24V battery	Input source of power	14.94	1 pieces	14.94
Voltmeter	Measures voltage across the grids	11.21	1 pieces	11.21
Charger	For charging batteries	29.89	1 piece	29.89
Labour	Construction of 1 unit of MET	37.37	1 unit	37.37
Total costs	For 1 complete unit of MET			**495.75**

Table 2.3. A breakdown of the estimated costs required for construction of second prototype (MET2). Some of the breakdown estimate costs were provided by Mr. Nosrat Mizrai of the UGBU.

The total cost for constructing one MET2 including the labour charge was approximately £496. The greatest chunk of the cost (£359) was spent on procuring the high voltage generator (Table 2.3). As the cost for procuring the variable high voltage amplifier was high, it was alternatively decided to construct these equipments at the UGBU for future requirements. The wood required for the frame construction represented the smallest cost of the trap components, as this required only a few pieces of ordinary timber which are relatively cheap in Tanzania. Another important cost of MET2 was the wire required to weave the electrocuting grids. This stainless steel wire had to be of high tensile, malleable and stainless to avoid rusting, and was procured from the United Kingdom. At present, MET3 prototypes constructed at the UGBU are estimated to be more expensive (Table 2.4) as they use a much higher quality

PVC frame, and had the wires industrially drilled, inserted and affixed to improve standardization and remove the time consuming process of hand weaving. Future construction of these traps is expected to be much cheaper especially when bulk numbers are ordered for construction.

Item	Cost(£)	
	MET3L	MET3S
Trap frames	244	120
Spacers	204	36
Wire mesh	336	128
Power supply	300	300
Accessories	100	100
Labour	300	210
Total costs	**1484**	**994**

Table 2.4. Cost breakdown including the total costs for construction of MET3L and MET3S. MET3L is the large version of the MET measuring 100cm x 100cm while the small version MET3S is 30cm wide by 30cm broad. The breakdown estimate costs were provided by Mr. Norsrat Mizrai of the UGBU.

2.5 Discussion

Construction of a MET prototype for sampling host seeking malaria vectors has taken an evolutionary process to arrive at a first working prototype. This process began with the first prototype (MET1) which had a number of problems as described in the previous sections. Though MET1 proved to be a failure in the field-testing, the problems observed during field trials provided a useful foundation for the development of the improved prototype MET2. Among problems encountered with MET1, the use of 800V supplied as AC proved to cause much sparking and burning of the specimen. Laboratory optimization of the voltage to obtain a voltage that was just sufficient to kill a mosquito while leaving the specimen morphologically identifiable and suitable for molecular assays. A series of other modifications such as reducing the gap between the grids as well as reducing the size of MET1 from 1m x 1m to 0.3m x 0.3cm resulted in a much better performing MET2. The reduced size of this prototype also made it easier to use indoors as well as outside.

Even with these significant improvements in MET2, a few important problems still remained which had to be addressed in a further, improved version (MET3). Such problems were mainly associated with the use of wooden frames in the MET2. In MET3, the wooden frames have been replaced by PVC frames thereby solving a number of electrical instability problems due to semi-conductive property of the wooden frames. Thus, as far as development of MET is concerned, MET3 is currently the "state of the art" prototype which was developed by improving on earlier prototypes. The MET3 performance is being tested in the field with IHI collaborators. The next section will discuss mostly the experience gained from application of MET2, while a brief description of the development of MET3 is supplied in the Appendix section.

A positive feature of the MET2 trap that was taken forward for evaluation is that the physical structure was relatively simple and could be constructed with basic knowledge of carpentry and electricity. In this study, I was able to construct the electrocuting grids for the MET2 with the help of a carpenter and a technician, requiring about 25 hours of work for us to complete one unit, which could then be powered by the specialist voltage generator. It is further anticipated that this production time could be significantly reduced if parts of the process (e.g. fitting of the wires into the grid) could be done by machine rather than by hand. As construction of the MET was relatively straightforward, its use of high voltage electricity required careful consideration of safety issues.

Based on my laboratory optimization experiments, a voltage of 600V DC was identified as appropriate for killing but not damaging mosquito specimens. Such high voltages could pose serious safety risks to human users if used with high currents and when accidental contact was made with the grid. Electricity in most homes is supplied at 240V and at 20-30 Amperes current flow, which is known to be potentially deadly. In the MET2, only up to 10 miliamperes (mA) current was used which is approximately 3000 times lower than a domestic power supply. As power (P)=VI, where V=voltage and I=current, the relationship between these variables explains how the high voltage (600V) supplied at low amperes (10mA) in the electrocuting grids results in a low power output

(~6Watts) that should be safe to use without causing risk of harm to humans (Clifford, 2005).

Operation of the MET2 was found to be more challenging in outdoor rather than indoor settings because of difficult weather conditions such as rain and wind. Water falling on the wood would make it more conductive to electricity, thus causing short-circuiting. Although tents were used to prevent moisture falling on the trap, windy rains could still cause the traps to get wet. When this happened, it could lead to sudden drops in output voltage, short-circuiting, and occasional spot burning on the wooden frame. This phenomenon necessitated pausing of the experiment for part or all of the trapping night. Solutions to this problem are provided in the new versions (MET3L and MET3S) which use PVC frames. This material is a better insulator of electricity and so is expected to solve operational problems.

The advantage of using the MET in place of HLC lies mainly on preventing the mosquito catcher from the risk of infectious bites and in reducing the labour as well as bias due to varying sampling skills among the catchers. However, as regards to the sampling costs, the use of MET requires more investment in terms of the equipment needed compared to HLC. Additionally, use of HLC is prompt and sampling of mosquitoes could be done with only a mouth aspirator and holding containers, while use of MET requires longer preparation including charging of the batteries, logistics of the traps and other accessories to the sampling site, and setting up of electrical connections. However, these additional logistics and costs would seem to be far outweighed by the advantages the MET provides in terms of in alleviating the risks for infectious bites to users.

2.6 Conclusions

This chapter described the stages through which a prototype of the mosquito-electrocuting trap (MET) for malaria vectors was developed. Although the version used in field-testing (MET2) produced encouraging results for use as a possible replacement of the HLC technique (Chapter 3), some operational and functional challenges remained that required further optimization. Many of

these technical problems have been removed in the latest prototype, MET3, whose development work is presented in the Appendix section 7.1.

In view of the potential application of the MET3 as a replacement of the HLC technique, it is worth investing further development of this trapping method. This is even more evident when weighed against the considerable risks and the hassle involved in using the HLC technique. As ethical concerns over the continued use of HLC are understandably growing, the development of alternative mosquito sampling tools such as the MET is highly warranted even though significant initial investment costs may be required. Based on promising results from ongoing field trials being conducted with the MET3 by colleagues in Tanzania, a joint patent application between the University of Glasgow and the Ifakara Health Institute has recently been submitted (Appendix 7.3 and 7.4). As I have been a major contributor to the development of the MET, I am a named co-inventor on this application.

Chapter 3: Evaluation of mosquito electrocuting traps as an alternative to the human landing catch technique for sampling host-seeking malaria vectors

Contributions from other co-authors

This chapter will form the basis of a paper that is being submitted to Malaria Journal, with the following author list: Deodatus Maliti[1,2], Dr. Nicodem Govella[1], Dr. Gerard Killeen[1,3], Mr. Nosrat Mirzai[1], Dr. Paul Johnson[1,] Dr. Katharina Kreppel[1,2] & Dr. Heather Ferguson[1].

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The specific contribution of these co-authors to work presented here is as follows: DM, NM, NJG and HMF designed the MET trap. DM and NM constructed the MET trap. DM designed the experiment with guidance from HMF NJG and KK. DM conducted the field experiment. DM conducted all statistical analysis under the guidance of HMF, with the exception of the analysis of density dependence which was performed by PJ. DM wrote the manuscript with guidance from HMF, and further comments from GFK HF NJG KK NM and PJ reviewed the manuscript.

3.1 Aims and objectives

The aim of this chapter was to evaluate the efficiency of the mosquito electrocuting trap (MET) whose development was described in Chapter 2 for sampling the major malaria vectors in Africa *Anopheles gambiae* sensu lato and *Anopheles funestus*. A field study was conducted in a rural setting in southern Tanzania to compare performance of this bespoke MET with a commercially available insect-electrocuting device (CA-EG) and the Human Landing Catch (HLC) gold standard. Evaluation of the MET and CA-EG focused on their sampling efficiency as defined by the numbers of malaria vectors sampled relative to the HLC, and its ability to accurately characterize epidemiologically-relevant mosquito behaviours such as the proportion of vectors that were caught

feeding indoors (P_i), the proportion of mosquitoes that were caught feeding when most people were indoors (P_{fl}), and a measure of the proportion of human exposure to mosquito bites that occurs indoors during sleeping hours(π_i). This study also evaluated whether the relative sampling performance of the MET and CA-EG was consistent across the night, and between nights where vector density varied. Based on these results, the viability of replacing the HLC with the MET or CA-EG was discussed.

3.2 Introduction

Efforts to control malaria rely heavily on the application of Long Lasting Insecticidal Nets (LLINs) which are the major strategy to protect against bites from mosquito vectors in African homes (Lengeler, 2004). Rapid increases in the coverage of LLINs over the past decade have been associated with substantial declines in major African vector species (WHO, 2013). A parallel decline in malaria infection rates in people has been reported in several places, as has a decrease in malaria mortality in infants and adults (WHO, 2014). However, the widespread use of these vector control measures may be triggering changes in the ecology and genetics of mosquito populations that could threaten their continued effectiveness, and restrict further progress towards malaria elimination (Wondji et al., 2005b, Chaves et al., 2008, Drake and Beier, 2014).

Insecticide resistance is increasingly being reported in areas where LLINs are widely used (Jones et al., 2012, Temu et al., 2012, Ranson et al., 2009, Kabula et al., 2014). Additionally, there are concerns that LLINs may be selecting for changes in heritable behavioural traits within malaria vector populations that allow them to shift their biting to times and places where people are not protected, which can be defined as "behavioural avoidance" (Pates and Curtis, 2005, Reddy et al., 2011, Govella et al., 2013, Russell et al., 2011, Sougoufara et al., 2014, Killeen and Chitnis, 2014). For example with increases of the coverage of LLINs in Tanzania (Renggli et al., 2013, West et al., 2012, Burgert et al., 2014), *An. arabiensis* has been reported to increasingly feed outdoors and during the earlier hours of the night in Dar es Salaam (Govella et al., 2010b). Further evidence is accruing that mosquito vectors are increasingly biting

outdoors (exophagy) instead of inside houses (endophagy) in response to rising coverage of LLINs and IRS. This shift in feeding behaviours could be due to changes in species composition of the major malaria vectors or due to behavioural changes within species (Kabbale et al., 2013, Reddy et al., 2011, Russell et al., 2011, Ndiath et al., 2014). As a strategy to avoid contact with insecticide treated surfaces, *An. arabiensis* is reported to rapidly exit as soon as it enters in houses treated with IRS or ITNs (Kitau et al., 2012, Okumu et al., 2013b, Okumu et al., 2013a). Other reported behavioural avoidance strategies include a reduction in total feeding time when biting insecticide-treated cattle by minimizing the feeding time (Habtewold et al., 2004). The ability to monitor if and how rapidly these traits are changing in response to control measures is crucial for assessment of the continued effectiveness of LLINs and IRS strategies (Russell et al., 2013, Gatton et al., 2013, Govella and Ferguson, 2012, Briet and Chitnis, 2013).

Exposure of humans to malaria transmitting mosquitoes depends not only on mosquito behaviour, but also on how vectors interact with their human hosts (Killeen et al., 2006). For example, estimation of human exposure risk and their ability to be protected by LLINs will depend on not only the propensity of mosquitoes to bite indoors (endophagy), but also their propensity to bite during times of the night when most humans choose to be indoors and sleeping (nocturnality). Only through combination of these mosquito and human behavioural traits can the proportion of human exposure to malaria that occurs indoors be estimated (Killeen et al., 2007, Govella et al., 2010b, Seyoum et al., 2012) as is required for prediction of the degree of transmission reduction that can be achieved through indoor-based vector control. Given that the effectiveness of current and future vector control strategies, particularly LLINs and IRS are so highly dependent on behavioural interactions between mosquitoes and humans, there is a clear need to establish standardized methods for characterizing these traits.

One of the most important and widely used techniques to sample host-seeking mosquitoes is the human landing catch technique (HLC). This technique is efficient and regarded as the gold standard tool for sampling host-seeking

malaria vectors (Magbity et al., 2002, Lima et al., 2014). Consequently, the HLC is widely used for a range of purposes including estimation of entomological exposure rates (Mboera, 2005, Rohani et al., 2008, Loaiza et al., 2008, Kilama et al., 2014), evaluation of vector control measures (Osse et al., 2013, Malaithong et al., 2010), and for studying mosquito vector behaviour and ecology (Bockarie et al., 1996, Yadouleton et al., 2010, Sougoufara et al., 2014, Mboera, 2005, Rohani et al., 2008, Loaiza et al., 2008). Although it provides a representative estimate of the number of mosquito bites that humans are exposed to, the HLC technique has numerous drawbacks (see Chapter 1). The most notable is the ethical concerns raised by requiring the participating human subjects to expose their legs to attract mosquitoes. The aim is for participants to capture mosquitoes landing on them before they bite, but this is not always possible and thus generates some risk of exposure to infection (Mathenge et al., 2005, Mboera, 2005, Achee et al., 2015). To minimize exposure risk it is recommended that HLC participants use malaria prophylaxis (Gimnig et al., 2013). However even with this precaution, some risk of infection remains when working in areas of high drug resistance, and/or where mosquitoes carry other pathogens (e.g. dengue or filariasis) that pose infection risks (Simonsen et al., 2010). These problems highlight the need for a more efficient, representative and ethical alternative tool for investigation of mosquito biting behaviour.

Previous attempts have been made to develop sampling tools for collecting indoor and outdoor biting mosquitoes. As reviewed in Chapter 1, these techniques include but are not limited to the bed net trap (Mathenge et al., 2004), tent traps (Govella et al., 2009, Govella et al., 2010a, Krajacich et al., 2014), the CDC light traps (Faye et al., 1992), and the mosquito magnet (MM) trap (Vezenegho et al., 2014, Chaves et al., 2014, Sithiprasasna et al., 2004). While these methods have shown promise in some settings, most have limitations that restrict their large-scale application, and/or bias collection towards mosquito species with particular phenotypes that may misrepresent the community of mosquitoes attracted to people. For example, bed net traps are ill-suited for use with animal hosts, and perform poorly compared to HLC in some settings (Mathenge et al., 2005), and tent traps have been shown to preferentially sample mosquitoes with inherent tendency to feed indoors

(Govella et al., 2009, Govella et al., 2010a). While CDC light traps have been shown to work well indoors (Fornadel et al., 2010b), this trap does not give a representative sample of the abundance and diversity of mosquitoes attracted to people when used outdoors (Faye et al., 1992). New approaches are therefore needed that overcome these key limitations.

The approach of killing insect vectors by using electrocuting surfaces was originally developed for trapping tsetse flies outdoors (Vale, 1974). Recently, there has been interest in expanding this approach to sample host-seeking and ovipositing mosquitoes that are lured to a point source (Dugassa et al., 2014b, Dugassa et al., 2012, Majambere et al., 2013). One of the first trapping methods that used electrocuting surfaces to sample malaria vectors was the Electrocuting-Nets (E-Net) (Torr et al., 2008, Knols et al., 1998). This trap is set up by placing a live host in a sealed tent and piping their odour out to a point source approximately 10m away, which is covered by an electrified net. Flies are attracted to the odour source and killed when intercepted by the electric net. Such E-Nets have already shown promise when used to investigate the host species preferences of the African vector species *An. arabiensis* and *An. quadriannulatus* (Torr et al., 2008), and for testing mosquito behaviour in relation to attractant odours (Knols et al., 1998). However, the large amount of space required to set up an E-Net makes it difficult to use for measuring mosquito-biting activity indoors especially if the survey is designed to sample mosquitoes from traditional African houses.

More recently, there has been investigation of the potential use of commercially-available insect-electrocuting devices in close proximity to a live host (Majambere et al., 2013). These devices showed promise in initial field trials conducted in Dar es Salaam Tanzania (Majambere et al., 2013) where they achieved a sampling efficiency of close to 50% relative to the HLC for *An. gambiae* s.l. However, estimates of key metrics of *An. gambiae* s.l. behaviour such as expected human exposure to bites indoors produced by these traps were not consistent with the HLC. An advantage of such devices is that as they are already commercialized, they could provide an easily available and standardized trapping methodology. However, this feature is only advantageous if the

performance of these traps for sampling malaria vectors is sufficient. Given these devices were originally developed to kill large Diptera, their optimality for sampling malaria vector species remains unknown.

Here, I field-tested a custom-made Mosquito Electrocuting Trap (MET) that has an electrical output that was specifically optimized for electrocuting African malaria vector species (as described in Chapter 2). This trap shared the feature of previous electrocuting traps of being an exposure-free method of sampling human biting mosquitoes. I evaluated the performance of this electrocuting trap relative to a previously trialled commercially available electrocuting grid (CA-EG)(Majambere et al., 2013) and the Human Landing Catch (HLC). The primary goal was to assess the potential of these electrocuting methods to provide exposure-free alternatives to the HLC by assessing their relative ability to estimate vector abundance and biting behaviours.

3.3 Methods

3.3.1 Study site

Field experiments were conducted at Lupiro village (-8.38 S, 36.67 E) located in the Kilombero Valley of Southern Tanzania. This village is situated in a malaria endemic region where the most recent estimate of entomological inoculation rate (EIR) was 33.9 infectious bites per person per year (Russell et al., 2010). Further details of the study site are given in Chapter 1.

3.3.2 Trapping methods

Three different trapping methods were used in this study: the human landing catch (HLC), a custom-made Mosquito Electrocuting Trap (MET, Figure 3.2A & B) developed in collaboration between the Ifakara Health Institute (IHI) and the University of Glasgow, and another electrocuting trap made from a commercially available "bug zapper" device (CA-EG), (PlusZap™ model ZE107 PZ40W (http://insect-o-cutor.co.uk/telerikfiles/Insect-O-Cutor%20Catalogue%20300112%20-%20PlusZap.pdf), P + L Systems Ltd,

Knaresborough, UK) which is sold for domestic electrocution of insects. This trap is the same as that used by Majambere et al (2013) in a study in Dar es Salaam, Tanzania. A schematic drawing of one panel of the MET is shown in Figure 3.1.

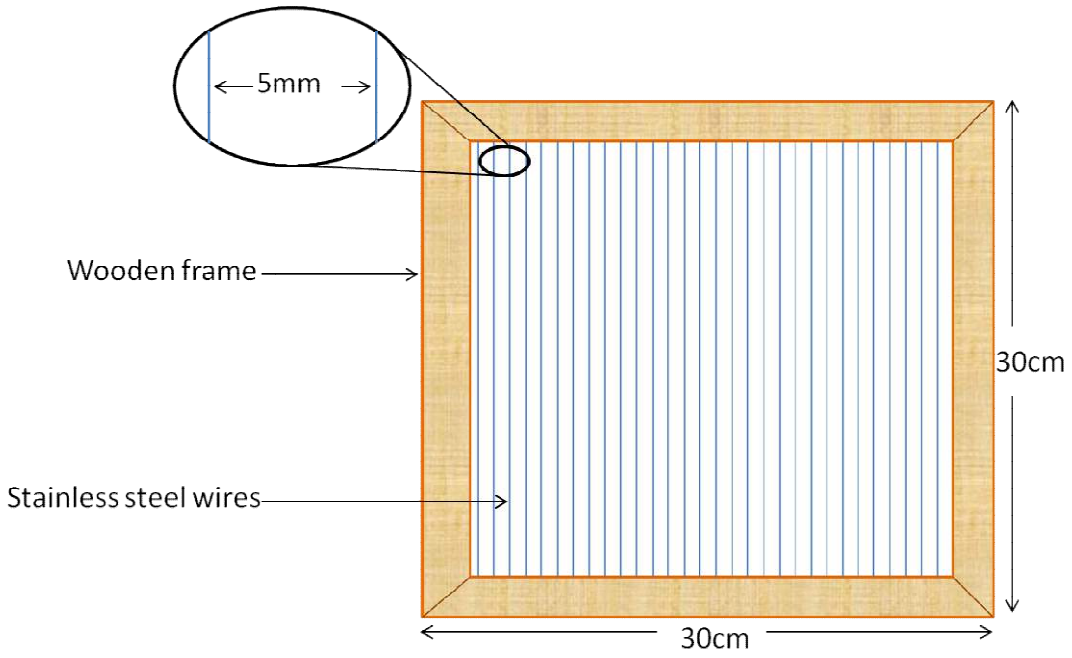

Figure 3.1. Schematic drawing of one panel of the MET. Four units of the same dimensions were connected to form a rectangular sturdy structure as on Figure 3.2A.

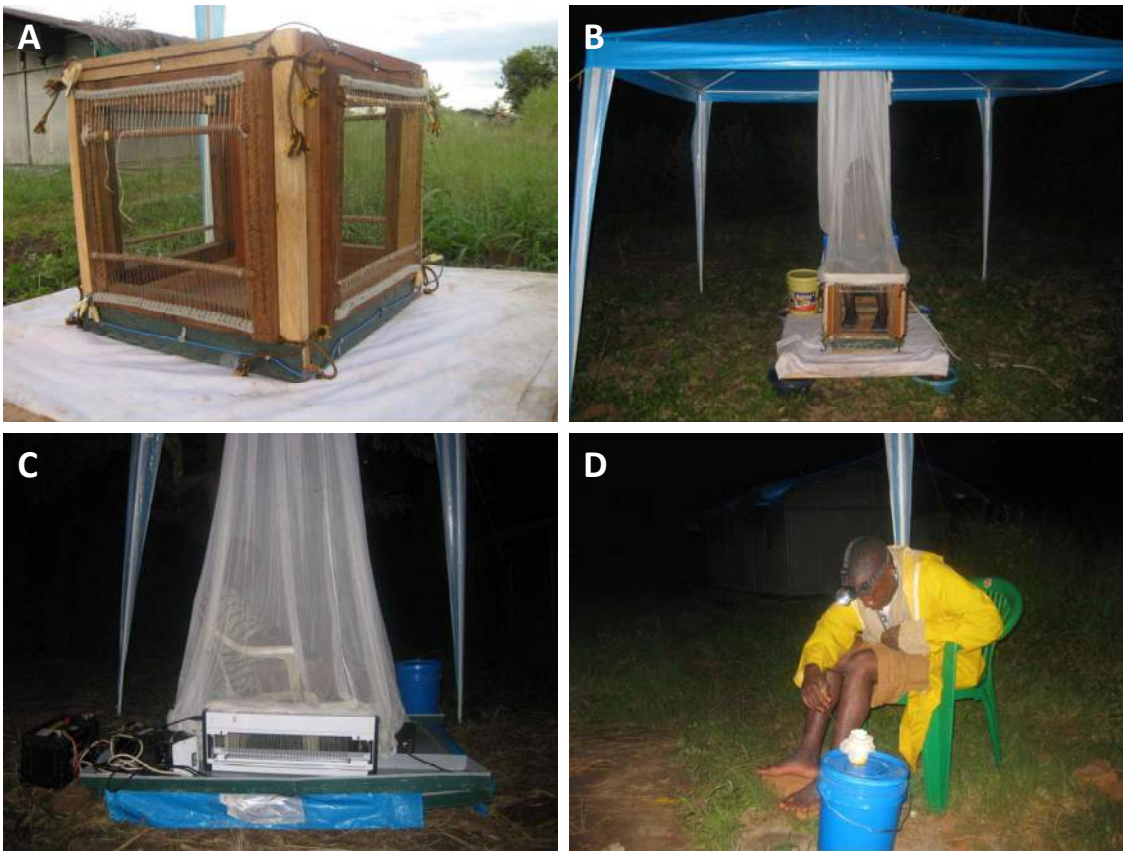

Figure 3.2. Deployment of traps. (A) The MET, (B) the MET with a person sitting with his legs in the trap, (C) a person sitting within the CA-EG trap and (D) a person performing human landing catch.

As described in Chapter 2, the MET was designed for the specific purpose of maximizing the proportion of *An. gambiae* s.l. mosquitoes killed on contact, whilst retaining the specimen intact for further morphological and molecular analyses. In brief, it consists of four panels of 30cm x 30cm electrocuting grid surfaces that are hinged together to make a square trapping box (Figure 3.2A). Electrified wires at a spacing of 5 mm were placed along the length of each panel. This spacing was observed to be sufficiently small to prevent colony-reared *An. gambiae* s.l. from flying through without contacting the wires. Voltage and current values were set at levels that would cause no harm to humans if accidental contact was made, but which were shown to be sufficient to kill >80% of *An. gambiae* s.l. mosquitoes that made contact in preliminary laboratory trials. As described in Chapter 2, when using the MET (Figure 3.2A), a human volunteer sat on a chair and placed the lower part of their legs into the trap while their upper body was protected by an ITN (Figure 3.2B).

The CA-EG was made by placing four commercially-available bug-zapping devices (each unit with an electrocuting surface of 60 cm x 20cm) around a seated human in a square formation (Figure 3.2C). Both the CA-EG and MET were powered by 12V batteries and set up on wooden platforms standing on small water troughs to prevent ants from eating any mosquitoes that dropped to the floor after being shocked by the trap. Human landing catches were performed by seated volunteers who exposed the lower part of their legs and used a torch to search for any mosquitoes landing on them. Volunteers used a mouth aspirator to collect mosquitoes landing on their legs (Figure 3.2D). When trapping methods were used outdoors, human participants sat under a portable canvas roof to protect them and the mosquito traps from rain (Figure 3.2B and C).

3.3.3 Experimental design

This experiment was conducted using a series of experimental huts, which were designed to imitate the typical design of local houses in the study area (Mnyone et al., 2012). These experimental huts had dimensions of 6.5m long, 3.5m wide and 2m high, with a 20cm wide gap between the top of walls and roof to simulate the open eaves found in most local houses. Each of these huts had one big room and not divided into several rooms as the locals' huts. The experimental huts were situated about 30m away from each other. Huts had four windows and one door which were shut during the experiments. The huts had eaves with the opening size of about 20cm x 60cm. Eaves were left unsealed to imitate those of the local people at the site. In each of the huts, a human person slept under a treated bed net just as they would do in a household. Those who slept in the huts are adult researchers who participated in the experiment. Trapping stations were set up inside each hut and at an associated outdoor point approximately 10m away. A 3 x 3 Latin square design was used in which each of the three trapping methods was randomly assigned to 1 of 3 experimental huts on each night of study. On each night, collections were conducted at paired indoor and outdoor trapping stations. Over consecutive nights, all of the 3 trapping methods were trialed at each of the huts to complete a full rotation in

3 days. Seven rounds of trapping were conducted over 21 trapping nights between March and May 2012. The first 2 rounds were conducted in a group of 3 experimental huts defined as A, B and C (at site 1), and the remaining 5 rounds were conducted in a different group of experimental huts (defined D, E and F) which were situated at a second site approximately 200m from the first.

Trapping was conducted from 7pm to 7am. In each hour of experiments, volunteers would spend 45 minutes passively sitting in a trap (MET or CA-EG) or actively collecting mosquitoes (HLC); with the remaining 15 minutes used as a break. Collectors at the six trapping stations were moved to a different trap, position and hut every hour throughout the night following a schedule which ensured that those who were for example working with MET inside hut A in the next hour move to a different trap or different location (e.g. outdoors) in hut B. This was made to ensure volunteers did not stick to the trap they preferred which would have created bias due to variation in human relative attractiveness to mosquitoes. At the end of each hour, MET and CA-EG traps were checked and trapped mosquitoes were removed by mouth aspirators or forceps and placed in labeled cups. On the following morning, mosquitoes from all the three trapping methods were sorted using morphological keys to identify their genera and gender. Female mosquitoes visually identified as belonging to a malaria vector group (*An. gambiae* s.l. or *An. funestus* s.l.) were individually stored in Eppendorf tubes in silica gel. These specimens were later analyzed using the polymerase chain reaction (PCR) technique to confirm their species identity (Scott et al., 1993).

3.3.4 Statistical analysis

All statistical analyses were carried out using the R statistical software version 2.15. Generalized linear mixed models (Crawley, 2007) were used to assess variation in mosquito vector abundance between trap types. Mosquito abundance data was highly over-dispersed and thus modelled as following a negative binomial distribution using the glmmADMB package (Skaug et al., 2011). Here trap type was fit as the primary main effect of interest, and experimental night and hut as random effects. The relative sampling efficiency of the novel

trap types relative to the HLC was estimated by computing the ratio of the predicted nightly abundance of vectors from these statistical models. Analysis was conducted to test if the performance of the novel trap types (relative to the HLC) varied between locations of a trap (in vs out).

Analysis was conducted to test whether the relative performance of the CA-EG and MET traps varied over the course of a night. Here, the proportion of mosquitoes caught by a novel method (CA-EG or MET) out of the total caught from this method and the HLC was modelled as a binomial variable using a logit link function. To test whether there was any systematic increase or decrease in the sampling efficiency of the CA-EG or MET relative to the HLC (e.g. perhaps due to battery decline throughout the night), a model was constructed in which the fraction of the hourly catch occurring either in CA-EG or MET (e.g. "novel trap" / ("novel trap + HLC") was defined as the response variable, and trapping hour (defined as being '1' on the first hour, and increasing through the night to 12 as the last hour) fit as a continuous fixed effect, with experimental night added as a random effect. Likelihood Ratio Tests were performed to assess whether there was a significant, systematic change in the proportional catch over the hours of the sampling night.

Further analysis was conducted to test whether the relative performance of the novel trap types was density dependent. Density dependence was investigated using the Bland-Altman method which assesses the reliability of two measures via regression analysis of the relationship between their difference and their mean (Altman and Bland, 1983). This density dependence analysis was done under the guidance of Dr. Paul Johnson of the University of Glasgow who helped to develop the R code used here. Nonlinearity in this relationship indicates density dependence. Specifically, I modelled density dependence as deviation from linear relationship between y - x and (x + y) / 2, where x and y are mosquito abundances from two different trapping methods recorded on the same night, transformed first by ln(abundance + 1) then standardised to have a mean = 0 and standard deviation = 1. Nonlinearity was modelled as a natural cubic spline with two degrees of freedom. A p-value for density dependence was estimated by comparing the null (linear) model with the density dependent

model (nonlinear) using a likelihood ratio test. Density dependence was quantified by calculating the adjusted R^2 (R^2_{adj}) of the nonlinear model relative to the linear model as R^2 - (1 - R^2) p / (n - p - 1), where p = 2, the number of degrees of freedom of the spline. R^2_{adj} can be interpreted as an estimate of the proportion of deviation from perfect linear correlation that is due to density dependence rather than random error. Thus, a high value of R^2_{adj} indicates density dependence while a low value can result from either density independence, or high error resulting in low power and an imprecise estimate of R^2_{adj}. The precision of the R^2_{adj} estimate was gauged by estimating its 95% confidence interval as the 2.5^{th} and 97.5^{th} centiles from 10,000 bootstrap replicates. Boot strapping analysis was performed by Dr. Paul Johnson.

Finally, analyses were conducted to assess if the three focal trapping methods varied in their prediction of a few key mosquito vector behaviours and their related human exposure outcomes. The predictors of malaria vector behaviours that were analyzed here are the proportion of mosquitoes that were caught feeding indoors (P_i) and the proportion of mosquitoes that were caught feeding when most people were indoors (P_{fl}). A third measure, the proportion of human exposure that occurred indoors (π_i) was derived from these mosquito behavioural traits and local information on human sleeping patterns (Majambere et al., 2013, Huho et al., 2013, Seyoum et al., 2012, Govella et al., 2010b). These measures have been proposed as standardized metrics of assessing epidemiologically relevant mosquito feeding behaviours (Killeen et al., 2006). The proportion of mosquitoes that were caught indoors (P_i) was calculated by dividing the total number of mosquitoes caught indoors by the total number caught outdoors and indoors over 12 hours of the night: $I_{19\rightarrow\ 07\ hrs}/(\ I_{19\rightarrow\ 07\ hrs+}\ O_{19\rightarrow\ 07\ hrs})$ (Govella et al., 2010b); where I and O respectively represent the total number of mosquitoes collected indoors and outdoors while the subscripts represent the start and the end time for each night.

The calculation of P_{fl} and π_i requires definition of the period of the night when most people (>50%) are expected to be indoors and sleeping. This time period was previously estimated for the community living in Lupiro village as running from 9pm-5am (Killeen et al., 2006). Therefore the proportion of mosquitoes

caught when most people were indoors (P_{fl}) was calculated as the proportion of mosquitoes biting either indoors or outdoors during the peak biting as follows: ($I_{21\rightarrow\ 05\ hrs+}\ O_{21\rightarrow\ 05\ hrs}$)/($I_{19\rightarrow\ 06\ hrs+}\ O_{19\rightarrow\ 06\ hrs}$) (Govella et al., 2010b). The proportion of human exposure that occurs indoors (π_i) was calculated by dividing the number of mosquitoes caught indoors during the period that most people are inside (9pm-5am) by itself plus the number of mosquito caught outdoors outside of the sleeping hours ($I_{21\rightarrow\ 05\ hrs}$)/($I_{21\rightarrow\ 05\ hrs+}\ O_{19,20,06\ hrs}$) (Govella et al., 2010b). The binary estimates of P_i, P_{fl} and π_i were estimated using generalized linear mixed models (GLMM) with a binomial distribution and a logit link function (Crawley, 2007). In these models, trap type was fit as a fixed effect, and experimental night fit as a random effect.

3.3.5 Ethical procedures

Ethical approval for this work was granted through research protocols implemented by the Ifakara Health Institute (IHI) that were approved by both the IHI internal institutional review board (Reference IHI/IRB/A.50) and the National Institute for Medical Research (NIMR) certificate number NIMR/HQ/R.8a/Vol. IX/801. Volunteers recruited for the trapping experiments were given informed consent forms with details of the procedures, including the potential risks and mitigation plan associated with and had to read and sign the forms before taking part in the work. All participants were provided with malaria prophylaxis before and during the experiments to prevent infection.

3.4 Results

Over all 21 nights of experiments, 18,497 mosquitoes were collected representing five genera including *Anopheles, Culex, Mansonia, Aedes and Coquillettidia* (Table 3.1). Seven *Anopheles* species were sampled of which *An. gambiae* s.l. was the most abundant. Four hundred of the 5,559 *An. gambiae* s.l. sampled were individually tested using PCR, and all were found to be *An. arabiensis*. This observation matches other recent reports from the study area indicating *An. arabiensis* is now the only remaining member of the *An. gambiae* s.l. species complex in Lupiro (Okumu et al., 2012, Matowo et al., 2013, Mayagaya et al., 2015). As malaria vectors were the prime focus of interest in

this study, all further analyses are restricted to female *An. gambiae* s.l. and female *An. funestus* s.l.

3.4.1 Sampling sensitivity of the traps

Approximately 3.5 times more *An. gambiae* s.l. (N=5,559) were collected than *An. funestus* s.l. (N=1,543, Table 3.1), with more of both species being sampled outdoors than indoors (Figure 3.2).

Species	Total per trapping method			Female	Male	Total	% Composition
	HLC	MET	CA-EG				
An. gambiae s.l.	3443	1786	330	5559	5	5564	30.08
An. funestus s.l.	772	650	121	1543	13	1556	8.41
An. coustani	664	49	26	739	0	739	4.00
An. pharaoensis	10	4	3	17	0	17	0.09
An. squamosus	46	19	14	79	0	79	0.43
An. wellcomei	5	1	1	7	0	7	0.04
An. ziemanni	353	32	6	391	0	391	2.11
Culex	1815	829	383	3027	129	3156	17.06
Mansonia	3829	1429	1603	6861	64	6925	37.44
Aedes	10	1	0	11	0	11	0.06
Coquillettidia	30	12	5	47	2	49	0.26
Grand total	10977	4812	2492	18284	213	18497	100.00

Table 3.1. A summary of the total number of mosquito genera and species caught by different sampling methods in this study (HLC: Human Landing Catch, MET: Mosquito Electrocuting Trap and CA-EG: Commercially Available Electric Grid trap).

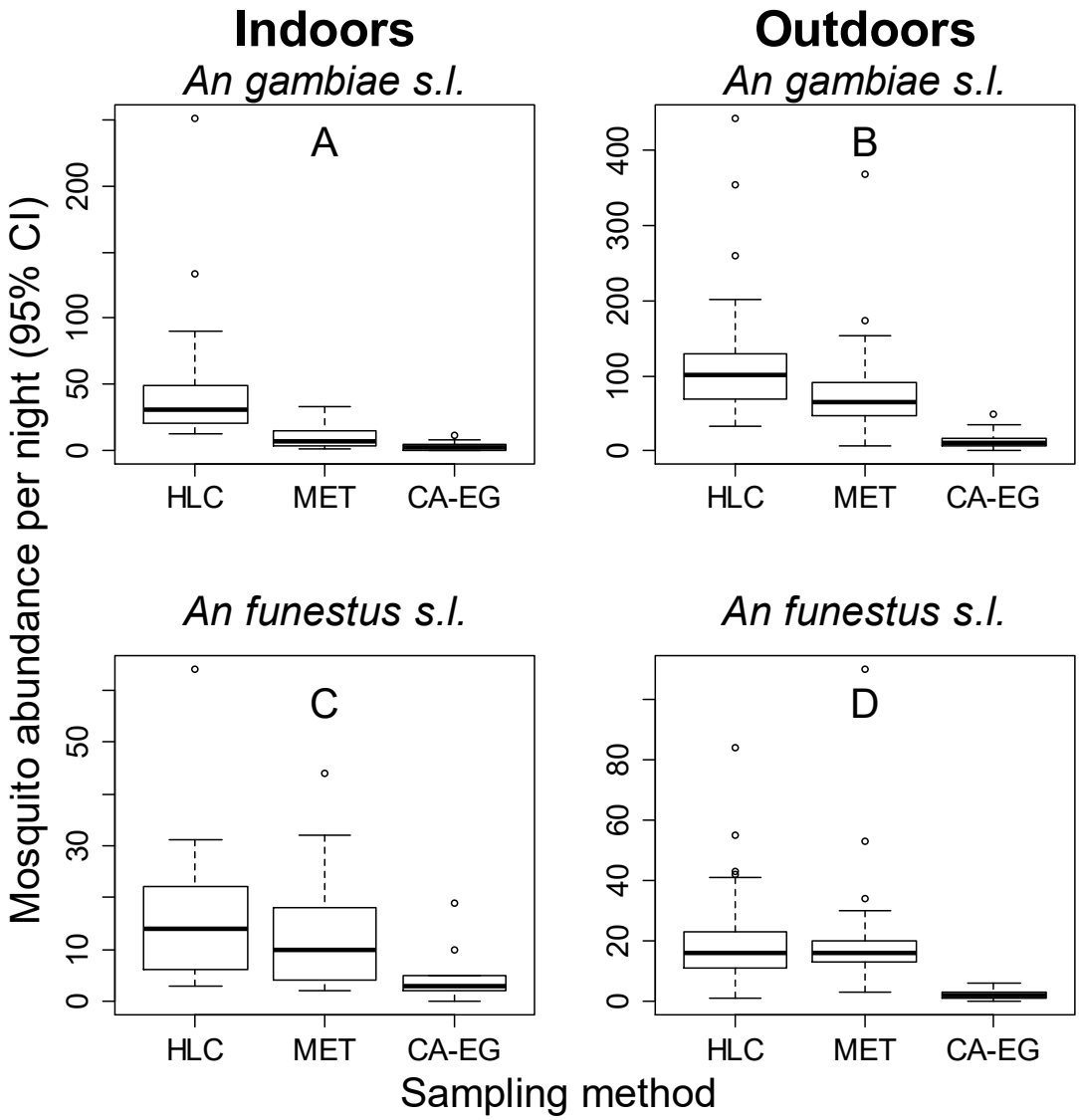

Figure 3.3. A box plot of raw mosquito abundance per sampling night as caught by each of the three sampling methods.

The sampling sensitivity of traps varied between indoor and outdoor environments for both *An. gambiae* s.l. (trap*location: χ^2_2=253.4, p<0.001) and *An. funestus* s.l. (trap*location: χ^2_2=9.0, p=0.003). Regardless of location (indoor vs out), the HLC sampled significantly more *An. gambiae* s.l. than either the MET (outdoors: z=4.10, p<0.001; indoors: z=7.89, p<0.001) or CA-EG (outdoors: z=16.00, p<0.001, indoors: z=11.99, p<0.001, Figure 3.3A &B). The

MET caught significantly more *An. gambiae* s.l. than the CA-EG both indoors (z=4.89, p<0.001, Figure 3.3A) and outdoors (z=12.4, p<0.001, Figure 3.3B).

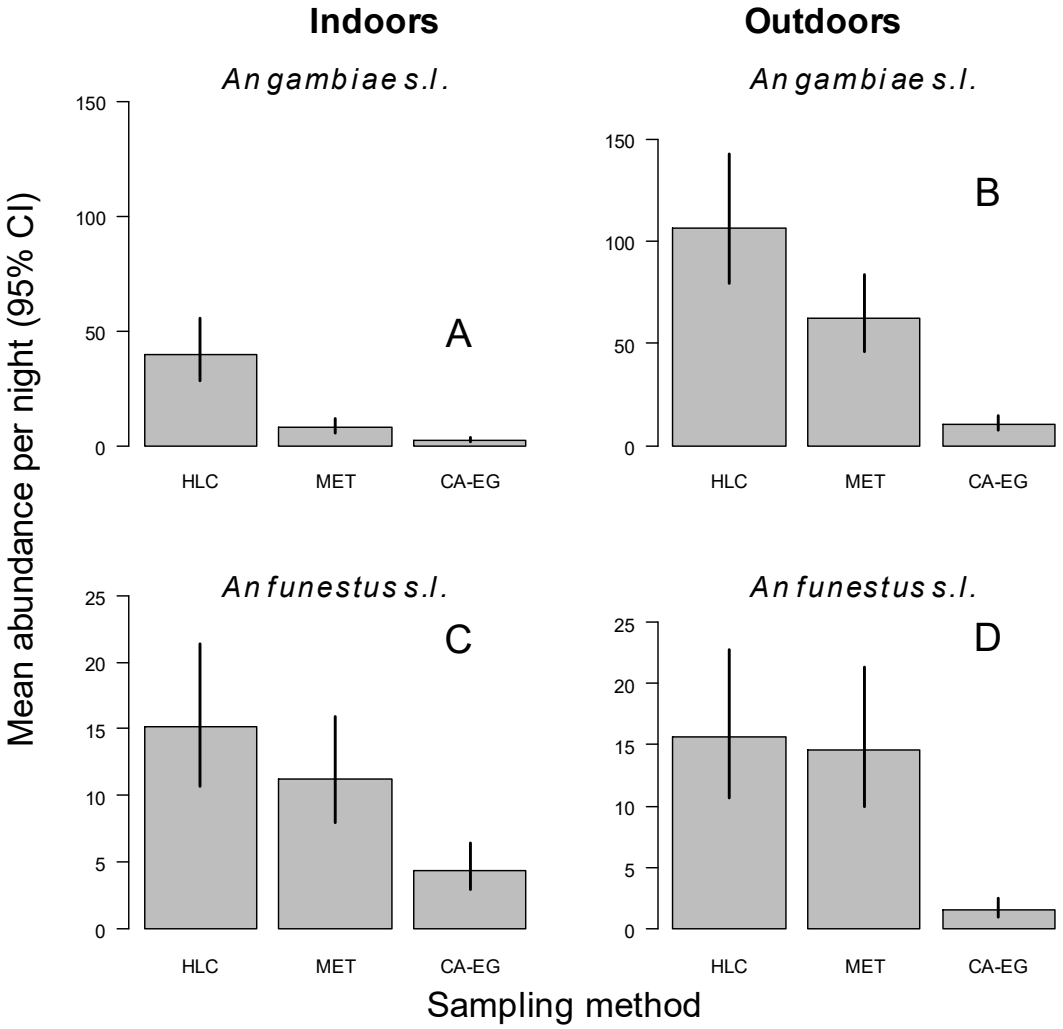

Figure 3.4. The mean abundance of malaria vector species caught per sampling night for each of the three traps evaluated (HLC: Human landing catch, MET: mosquito electricuting trap and CA-EG: commerically available electric grid trap). Bars represent 95% confidence interval.

Based on these results, the sampling efficiency of the MET relative to HLC for *An. gambiae* s.l. was estimated to be 59% outdoors, and 21% indoors (Table 3.2). The sampling efficiency of the CA-EG was considerably lower than the MET and achieved <10% of the HLC indoors and out (Table 3.2).

Species	Location	Trap	Relative sampling efficiency [95% CI]	P value
An. gambiae s.l.	Indoors	MET	20.9[10.3-42.2]	<0.001
		CA-EG	6.1[2.8-13.1]	<0.001
	Outdoors	MET	58.5[32.2-106.2]*	0.55
		CA-EG	9.9[5.3-18.4]	0.023
An. funestus s.l.	Indoors	MET	74.2[37.0-148.9]*	0.12
		CA-EG	28.7[13.9-59.6]	<0.001
	Outdoors	MET	93.5[43.9-199.3]*	0.86
		CA-EG	9.6[4.0-22.7]	0.90

Table 3.2. Predicted sampling efficiency of the novel traps (per night) relative to the HLC gold standard. Asterisks are placed in cases where the upper limit of the 95% confidence interval includes 100%, indicating no significant difference between the performance of a novel trap compared to the HLC.

The number of *An. funestus* s.l. caught per night by the HLC and MET was not significantly different either when used indoors (z=1.71, p=0.09, Figure 3.3C) or outdoors (z=0.58, p=0.56, Figure 3.3D). In contrast, the CA-EG caught significantly fewer *An. funestus* s.l. than either the HLC or MET (p<0.001 for indoors and outdoors, Figure 3.3C & D). The sampling efficiency of the CA-EG for *An. funestus* s.l. was poorer than the MET or HLC (Table 3.2), and had a sampling efficiency of <30% relative the HLC (Table 3.2).

3.4.2 Sampling consistency across the night

The sampling efficiency of the MET relative to the HLC remained constant over all hours of the night when used indoors for *An. gambiae* s.l. (χ^2_1 =0.001, p=0.98), however there was evidence of a moderate decline through time when applied outdoors (χ^2_1=52.11, p<0.001, Figure 3.4A). This trend was reversed for *An. funestus* s.l. where the sampling efficiency of the MET relative to the HLC was predicted to decline over the sampling night when used indoors (χ^2_1=12.42, p<0.001, Figure 3.4B), but appeared to be constant outdoors (χ^2_1=0.76, p=0.38). The relative sampling efficiency of the CA-EG was predicted to increase across the night when used to sample *An. gambiae* s.l. indoors (χ^2_1=10.36, p=0.001,

Figure 3.4C), but declined outdoors (χ^2_1=17.42, p<0.001, Figure 3.4C). The sampling efficiency of the CA-EG relative to the HLC for *An. funestus* s.l. was constant across the night both indoors (χ^2_1=0.39, p=0.54, Figure 3.4D) and outdoors (χ^2_1=2.31, p=0.13, Figure 3.4D).

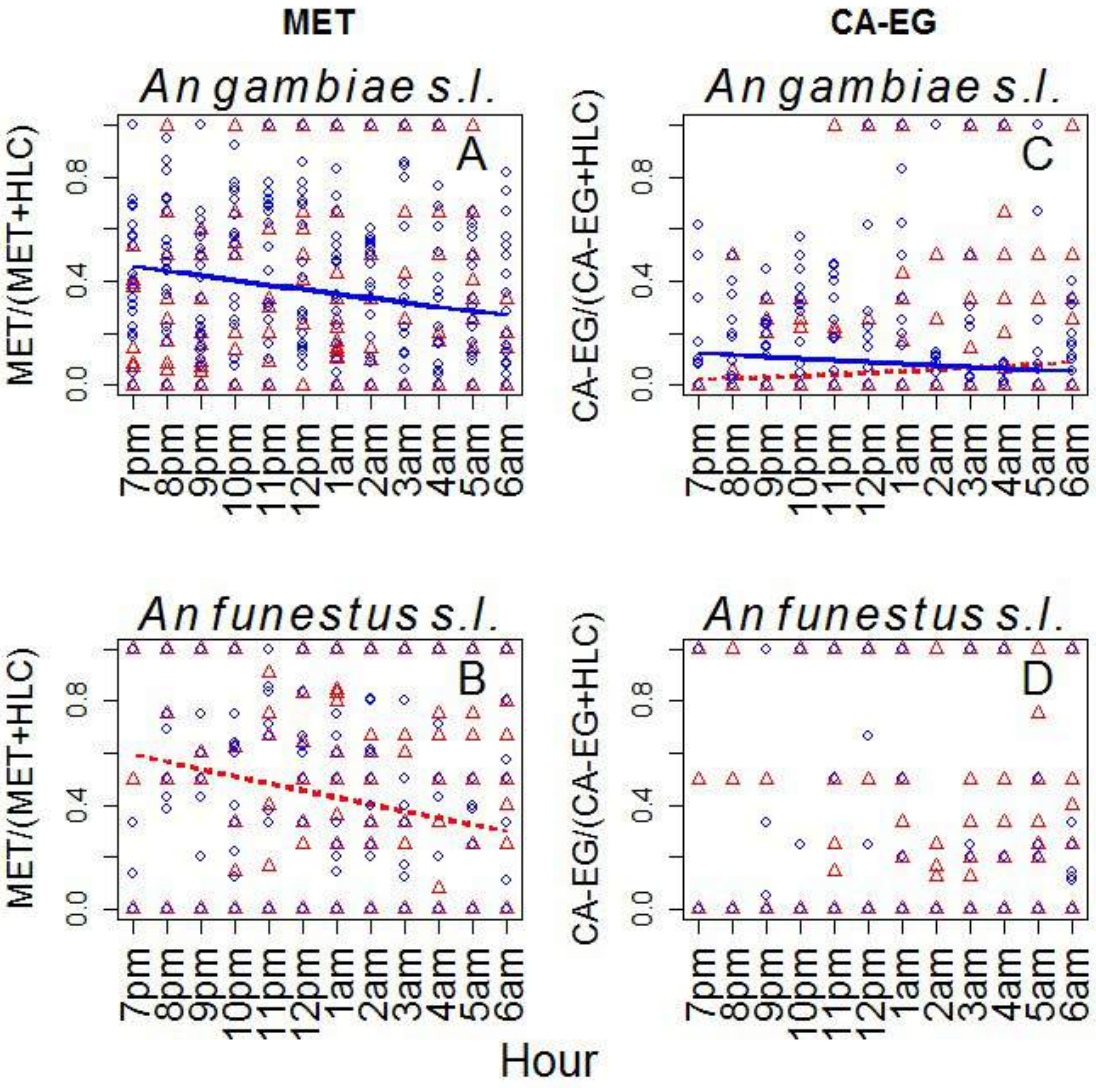

Figure 3.5. The sampling efficiency of the two novel trap types (CA-EG and MET) relative to the HLC gold standard across the hours of a sampling night. Points indicate the proportion of the total catch (new trap + HLC) that was captured by the new trap over each hour of a sampling night (7pm - 7am). Red triangle symbols are for collections made indoors, and blue diamonds for outdoors. Dotted-red and solid-blue lines represent predicted relationship between the relative sampling efficiency across the hours of a sampling night, indoors and outdoors respectively (lines only shown when there was a statistically significant change through time).

3.4.3 Sampling consistency across varying mosquito densities

In general, there was a positive association between the number of malaria vectors caught per night in the MET and the HLC, although the Pearson linear correlation coefficients were not statistically significant in all cases (Table 3.3). A similar pattern of positive but not always statistically significant correlations between nightly captures by CA-EG and the HLC was observed (Table 3.3).

Taxon	location	method	R	P-value
An. gambiae s.l.	Indoors	MET:HLC	0.35	0.12
		CA-EG:HLC	0.28	0.21
	Outdoors	MET:HLC	0.65	0.001
		CA-EG:HLC	0.58	0.005
An. funestus s.l.	Indoors	MET:HLC	0.18	0.43
		CA-EG:HLC	0.12	0.59
	Outdoors	MET:HLC	0.67	<0.001
		CA-EG:HLC	0.22	0.32

Table 3.3. Correlation between the log-transformed [log(1+count)] number of mosquitoes caught by the HLC and those caught by the MET and the CA-EG. R is the Pearson's correlation coefficient. P-values show significance of the correlation between the number of mosquitoes sampled by HLC and that sampled by either MET or CA-EG.

Nightly catches were log(x+1) transformed and plotted for further investigation of potential density dependence as evidenced by deviation from linearity. In all cases, there was much stronger support for a linear relationship between the log-transformed values of nightly catches than a curvilinear association (Figure 3.5).

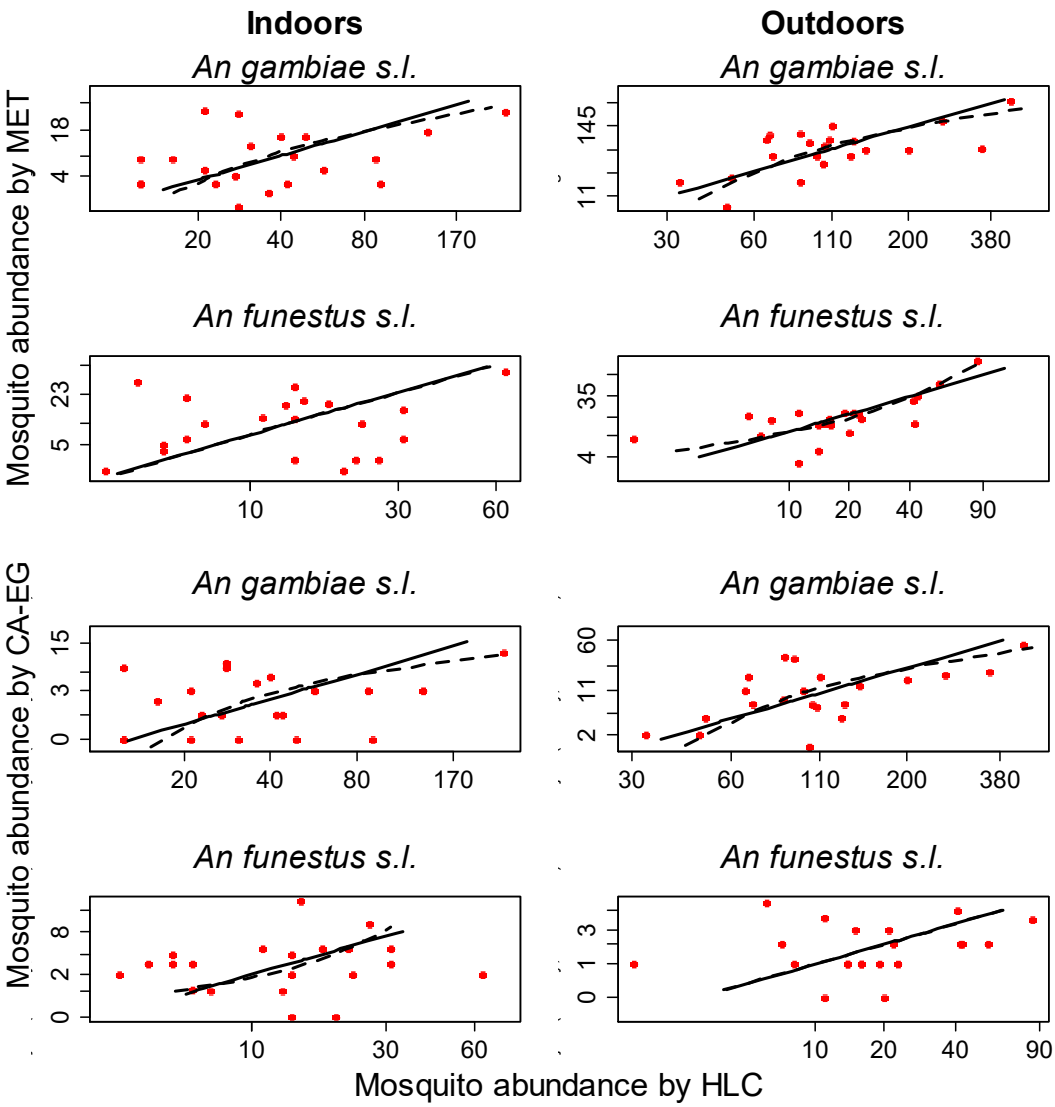

Figure 3.6. Assessment of non-linearity as a measure of density dependence of the electrocuting traps relative to the HLC. Solid lines represent a model of linear relationship between the numbers of mosquitoes collected by a novel trap relative to HLC, while dotted lines were obtained from non-linearity which was modelled as a natural cubic spline with two degrees of freedom. Red dots are the individual data points of mosquito abundance over 21 nights of collection.

All of the estimates of the strength of density dependency (adjusted R^2) were close to zero, but often with a wide confidence interval ranging from below zero to above 40% in some cases (Table 3.4), suggesting that power to detect low-to-moderate levels of density dependence was limited. Thus, based on the range of mosquito densities encountered in this trial, there is no evidence to indicate the relative performance of the CA-EG or MET is density dependent, when used indoors or outside.

Taxon	Location	Method	Adjusted R^2 [95% CI]	P-value
An. gambiae s.l.	Indoors	MET	-9[-11,35]	0.55
		CA-EG	-6[-11,27]	0.36
	Outdoors	MET	-1[-9,44]	0.20
		CA-EG	-3[-9,36]	0.25
An. funestus s.l.	Indoors	MET	-11[-11,16]	0.95
		CA-EG	-9[-11,34]	0.64
	Outdoors	MET	-1[-9,62]	0.20
		CA-EG	-11[-11,40]	0.95

Table 3.4. Quantification of density dependence using the Bland Altman Method. Adjusted R2 values show estimates of the proportion of deviation from perfect linear correlation that is likely to be due to density dependence rather than random error. As adjusted R2 values are penalized for model complexity, negative estimates are possible, but should be interpreted as zero.

3.4.4 Metrics of mosquito behaviour and human biting exposure distribution

Mosquito hourly biting activity was quite variable between nights, and revealed no obvious peaks in biting times for either *An. gambiae* s.l. or *An. funestus* s.l. (Figure 3.6).

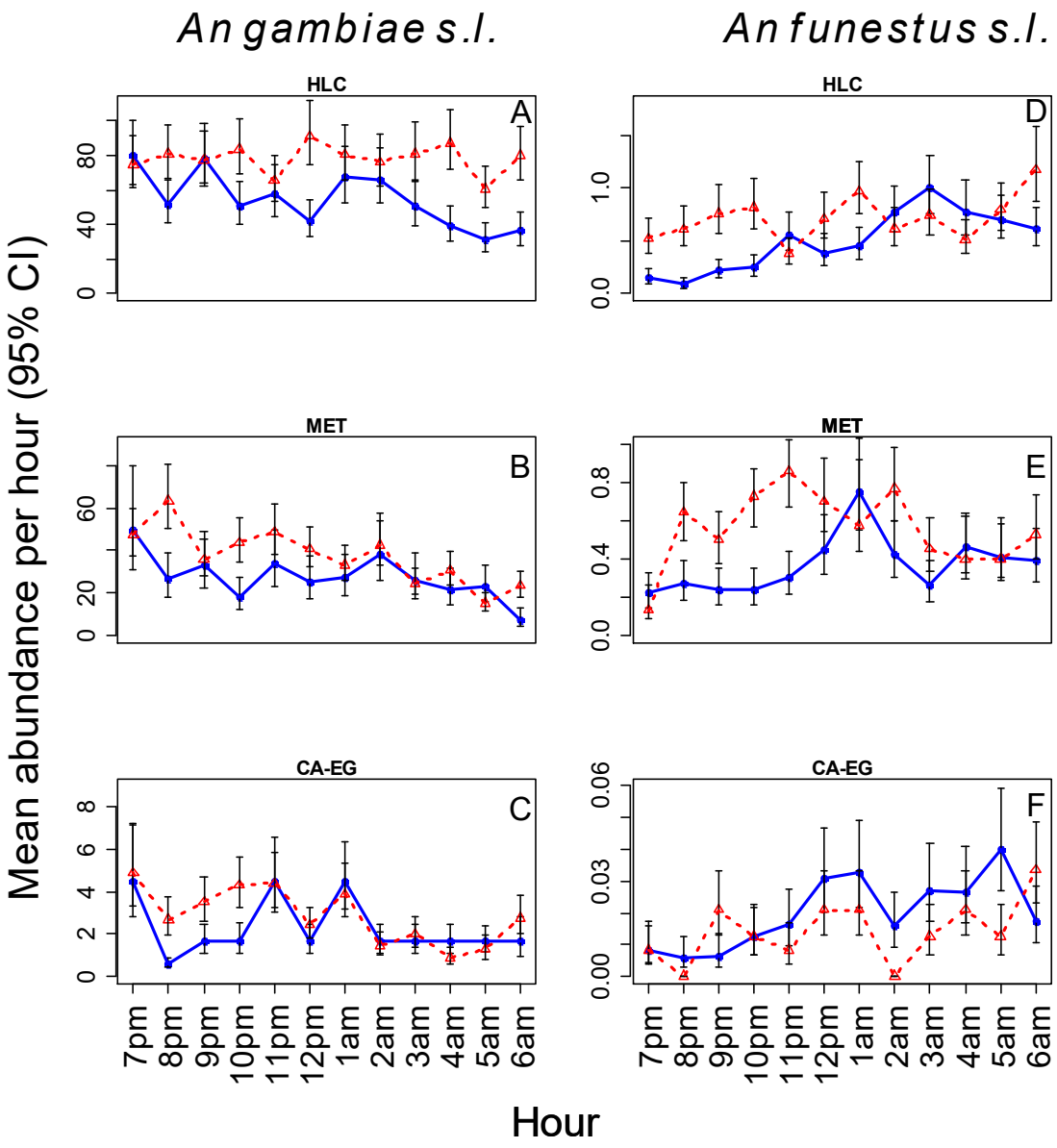

Figure 3.7. Predicted mean abundance of *An. gambiae* s.l. and *An. funestus* s.l. for each hour of the night, in indoor and outdoor environments. Dotted-red and solid-blue lines show predicted mean abundance of mosquito across the night for outdoor and indoor locations respectively. Error bars represent 95% confidence intervals.

All traps indicated that *An. gambiae* s.l. was significantly exophilic (>60% of bites taking outdoors), while *An. funestus* s.l. was estimated to bite indoors and outdoors at similar rates (~50:50 split between indoor and outdoor biting) (Table 3.5). Furthermore, estimates of the proportion of *An. gambiae* s.l. that feed indoors (Pi) obtained from the HLC and MET were similar (z=-1.15, p=0.25, Table 3.5), as were those obtained from the HLC and CA-EG (z=-1.77, p=0.08, Table

3.5). Estimates of the proportion of *An. funestus* s.l. feeding indoors were also similar between the HLC and MET (z=1.65, p=0.10, Table 3.5), and the HLC and CA-EG (z=1.95, p=0.051, Table 3.5).

The novel electrocuting traps were less consistent with the HLC when used to estimate other human exposure risk indicators including the proportion of mosquitoes caught feeding when most people were indoors (P_{fl}), and the proportion of human exposure that occurs indoors (π_i). The HLC predicted that approximately 98% of *An. gambiae* s.l. were attempting to feed during hours when most people would be indoors (Table 3.5). This was underestimated by both the MET (z=9.27, p<0.001, Table 3.5) and CA-EG (z=-12.91, p<0.001, Table 3.5) which estimated only 68% and 40% of *An. gambiae* s.l. biting would occurs during this period respectively. Predictions were less variable for *An. funestus* s.l., in which the proportion of biting occurring during hours when people are indoors was estimated to be 70-75% by the MET and HLC respectively (z=-1.34, p=0.18, Table 3.5), whereas this was underestimated as 65% by the CA-EG (z=-2.62, p=0.009, Table 3.5). It is noted that values of P_{fl} were underestimated in all scenarios where the novel trap type was known to have a lower sampling sensitivity inside and outside. This occurred in all scenarios except when the MET was used for *An. funestus* s.l. (Table 3.5), which notably is also the only scenario in which the estimated value of P_{fl} was similar to that obtained from the HLC.

The MET somewhat underestimated the proportion of human exposure occurring indoors (π_i=36%) in comparison to the HLC for *An. gambiae* s.l. (43%); a difference of borderline statistical significance (z=-2.04, p=0.042). Estimates of the proportion of human exposure occurring indoors as obtained from the CA-EG and HLC were indistinguishable (43-46%, z=0.77, p=0.44, Table 3.5). Both the MET (z=4.21, p<0.001) and CA-EG (z=5.23, p<0.001) overestimated the proportion of human exposure occurring indoors due to *An. funestus* s.l. (73-80% Table 3.5) in comparison to the HLC (55%, Table 3.5).

Taxon	Method	Proportion caught indoors (P_i)		Proportion caught when most people are indoors (P_{fl})		Proportion of human exposure occurring indoors (π_i)	
An. gambiae s.l.		Estimate[95% CI]	p	Estimate[95% CI]	P-value	Estimate[95% CI]	P- value
	HLC[R]	0.37[0.34-0.40]	N/A	0.93[0.89-0.96]	N/A	0.43[0.37-0.50]	N/A
	MET	0.35[0.319-0.38]	0.25	0.681[0.556-0.785]	<0.001	0.36[0.31-0.42]	0.042
	CA-EG	0.34[0.31-0.37]	0.08	0.400[0.278-0.537]	<0.001	0.47[0.40-0.53]	0.44
An. funestus s.l.	HLC[R]	0.51[0.47-0.56]	N/A	0.76[0.68-0.82]	N/A	0.55[0.48-0.63]	N/A
	MET	0.51[0.46-0.55]	0.10	0.70[0.62-0.77]	0.18	0.739[0.675-0.795]	<0.001
	CA-EG	0.55[0.47-0.64]	0.51	0.63[0.53-0.71]	0.009	0.805[0.744-0.855]	<0.001

[R]Reference trap

Table 3.5. Indicators of malaria vector biting behaviour and human exposure metrics (P_i, P_{fl} and π_i) as estimated by each of the three traps for *An. gambiae* s.l. and *An. funestus* s.l. Estimates of the proportion of mosquitoes caught when most people are indoors (P_{fl}) and the proportion of human exposure occurring indoors (π_i) were calculated based on mosquito numbers collected during times when most people are indoors (9pm-5am). The p-values listed are tests of the comparison of the estimates obtained from the electrocuting traps and those from the HLC (as the reference trap).

3.5 Discussion

In this study, I evaluated the potential of two electrocuting traps, the MET and CA-EG, to provide exposure-free alternatives to the HLC technique for sampling African malaria vectors. The HLC generally collected more of the primary vector *An. gambiae* s.l than the MET, but capture rates of *An. funestus* s.l were similar between these methods. The relative efficiency of the MET was reasonably high (~59%) when used for *An. gambiae* s.l. outdoors, but fell to ~20% relative to the HLC when applied indoors. In contrast, the CA-EG performed poorly relative to the HLC in both indoor and outdoor settings, for *An. gambiae* s.l. and *An. funestus*. There was evidence of some decline in the relative sampling efficiency of the novel trapping methods relative to the HLC in sampling *An. gambiae* s.l. outdoors over the course of a sampling night, but this was not detected for *An. funestus* s.l. No evidence of density dependent sampling was observed for the novel traps. Both the MET and CA-EG tended to have higher performance relative to the HLC outdoors compared to indoors. While estimation of the proportion of mosquitoes caught indoors (P_i) by the novel traps were similar to those estimated by HLC, there was tendency of the MET and CA-EG to underestimate (P_{fl}) when sampling *An. gambiae* s.l. while over-estimating πi when sampling *An. funestus* s.l. On balance, the sampling sensitivity of the CA-EG was judged too low to merit further consideration as an alternative to the HLC. However, it is concluded that the MET shows strong promise as an alternative method for exposure-free surveillance of African malaria outdoors outside of houses.

The sampling efficiency of the MET was consistently higher for *An. funestus* s.l. than for *An. gambiae* s.l. Possible explanations for this include differential sensitivity of these species to electrocution. Several biological factors are known to influence the electrical conductivity of insects including their composition of cuticular hydrocarbons (Kenneth, 1976), body size and water content. Although no specific information is available for mosquito species used here, it is possible that *An. funestus* s.l. was more likely to be killed on contact with the grids than *An. arabiensis* (laboratory optimization studies described in Chapter 2 indicate only 70-80% of *An. arabiensis* killed on immediate contact). The voltage and current combination used in the MET were optimized in

laboratory studies using *An. gambiae* s.s. as a model (Chapter 2), but could be even more efficient at killing *An. funestus* s.l. A previous study using the CA-EG also found that sampling efficiency varied between *An. gambiae* s.s. and *An. arabiensis* (Majambere et al., 2013), so vector species-specific sampling may be a common feature of electrocuting traps as has been documented with other methods like CDC light traps (Sikaala et al., 2013). Further investigations in which cohorts of *An. funestus* s.l. and *An. arabiensis* collected in the field are experimentally placed on the electrified grids is necessary to confirm whether species-specific responses to electrocution could explain the variable sampling efficiency reported here.

Both novel electrocuting traps had higher sampling efficiency when used outside than indoors. The reasons underlying this are unknown but could be due to variation in microclimatic conditions (Lorenz et al., 2013) which could differentially impact the function of electrocuting traps, and/or the ability of vectors to find hosts in outdoor vs indoor location. Several other factors such as the direction and concentration of host odours and wind movement vary between indoor and outdoor settings (Lorenz et al., 2013) which could have contributed to the observed trap performance. Regardless of the cause, a simple correction factor could be used to adjust mosquito catch rates for differential sampling efficiency indoors and outdoors if these differences can be confirmed to be consistent. Further investigation of the performance of electrocuting traps in a broader range of ecological settings is required to confirm this.

There were differences in the relative sampling sensitivity of CA-EG as estimated in our study relative to that reported by Majambere et al (2013). Majambere et al (2013) reported that the CA-EG achieved a sampling efficiency of ~50% relative to the HLC, which is considerably higher than the 6-29% recorded this current study. However, in their study human participants were positioned in a lying down rather than a sitting position as used in this current study. In this study, I positioned the participating humans in a sitting position specifically to simulate the way sampling is done by the HLC technique. Enclosing the whole human in the trap (as was the case in Majambere et al) might have intensified

the emanating cues thus leading to a higher performance as opposed to the way the trap was used in the current study. Another major difference was that the study by Majambere et al (2013) was conducted in Dar es Salaam where *An. gambiae* s.s. constitutes a much larger proportion of the *An. gambiae* s.l. species complex. During preliminary laboratory optimization tests conducted in this study (Chapter 2), *An. gambiae* s.s. was shown to be somewhat more sensitive to electrocution than *An. arabiensis*. The lower performance of the CA-EG in the site used for the current study may be due to the increased proportion of the more "resilient" *An. arabiensis*.

In a few occasions there was evidence of decreasing sensitivity of MET and CA-EG over the sampling night, but this did not arise consistently in all settings, or in both vector species. A decreasing sampling efficiency of the CA-EG relative to the HLC over the course of a night was reported in Majambere et al 2013. This was interpreted as a sign of battery drainage over the course of a night, which reduced the electrical output. Given that a decline in the relative sensitivity of electrocuting traps was not consistently detected in this study, it is difficult to interpret what may have been responsible for the somewhat variable performance of traps throughout the night. In addition to battery draining, other factors such as a build up of moisture on traps (especially in outdoor stations) may have contributed. The MET voltage was checked every hour and in some cases it was shown to decrease especially in the late hours of the night. Use of a higher-capacity battery coupled to an alarm system to notify if and when there is any dip in electrical output could resolve any issues of reduced voltage output through time. Additionally, there were a few occasions where traps temporarily short-circuited during experiments because opposing wires came into contact, and/or the wooden frames became moist and mildly conductive (as described in Chapter 2). Experiments were stopped when there was an obvious cessation of current flow, however there could have been more minor dips occurring through the course of a sampling night that went undetected. In considering whether a novel trapping method could replace a gold standard, it is important to assess whether it can sample consistently across mosquito densities. Based on the analysis in this study, no strong evidence of density-dependent sampling was found in the MET or the CA-EG (Figure 3.6).

However, it would be premature to dismiss any possibility of density dependent trapping for a few reasons. First, this study was conducted over 21 consecutive days in the rainy season when mosquito densities were generally high. Thus, the current analysis could not assess density dependence across the full range of high to low mosquito densities that occurs between wet and dry seasons. Additionally, it was noted that the detection of density dependence in trapping studies is sensitive to the analysis method used. Several previous studies have assessed density dependency based on analysis of how the proportional catch rate varies with differing mosquito densities across nights (Govella et al., 2009, Sikulu et al., 2009), whereas others used the Bland-Altman method which is based on comparison of absolute numbers (Altman and Bland, 1983). I chose to use the Bland-Altman method because its use of regression analysis to assess the reliability of two measures based on the relationship between their difference and their mean is not subject to bias inherent in the binomial method. I recommend that future studies which evaluate these trapping methods adopt a similar method so that estimations of density dependence are standardized and comparable.

For any mosquito sampling tool to successfully replace the HLC, it must be able to give similarly representative of key mosquito behaviours and associated human exposure risk factors. Here I investigated three such measures which have been widely used in a number of other studies to assess both human risk and likely degree of protection from Long-Lasting Insecticidal Nets (Russell et al., 2011, Seyoum et al., 2012, Govella et al., 2010b, Huho et al., 2013, Bayoh et al., 2014). One of the most direct measures of indoor exposure is the proportion of mosquitoes that bite indoors (P_i), for which comparable estimates were obtained from both electrocuting traps and the HLC gold standard. However two related exposure metrics, the proportion of mosquitoes caught when most people are indoors (P_{fl}) and the proportion of human exposure to bites that occurs indoors(π_i) were underestimated and overestimated respectively in *An. gambiae* s.l by the CA-EG and MET. This matches results from the previous trial (Majambere et al., 2013) where the CA-EG produced a similar estimate of P_i, but underestimated P_{fl} for *An. arabiensis* relative to the HLC. The likely explanation for this bias is the differential sampling efficiency of the electrocuting traps relative to the HLC when used indoors versus out. This

location-dependent performance would be expected to generate biased estimates of P_{fl} and the proportion of human exposure predicted to occur indoors. If no technical improvements can be made to increase the sampling performance of electrocuting traps indoors, a correction factor could be used to adjust such mosquito behavioural metrics.

The proportion of *An. gambiae* s.l. caught indoors (P_i) estimated by HLC in the Kilombero Valley in 1999 was found to be 0.58 ±0 .01 (Russell et al., 2011), which is higher than the values of 0.37 ±0.03 (HLC) and 0.35 ±0.03 (MET) reported here. In 1999 a much higher proportion of *An. gambiae* s.l. were *An. gambiae* s.s. (an endophilic species) whereas now almost all *An. gambiae* s.l. are *An. arabiensis* which are known to be more exophilic than *An. gambiae* s.s. (White et al., 1974). Therefore, the change in proportion of mosquitoes caught outdoors is likely due to change in vector composition.

The proportion of human exposure occurring indoors (π_i) obtained for *An. gambiae* s.l. and *An. funestus* s.l. using HLC in this study was 0.43 and 0.55 respectively. Assuming that all *An. gambiae* s.l. in this study were *An. arabiensis* (based on PCR results of 400 samples which showed all of them were *An. arabiensis*), the proportion of human exposure occurring indoors (π_i) in this study are low compared to what has been reported for *An. arabiensis* in western Kenya (Bayoh et al., 2014), where π_i for *An. arabiensis* and *An. funestus* s.l. were 0.87 and 0.86 respectively. Also π_i values obtained the current study for *An. gambiae* s.l. are lower than 0.82 estimated for the same species in 2009 (Russell et al., 2011). Another study in Dar es Salaam estimated π_i obtained for *An. arabiensis* to be 53% (Majambere et al., 2013), which is again higher than the 43% estimated here. This indicates that a much lower proportion of human exposure to malaria may be occurring in indoors in the Kilombero Valley relative to other settings, and highlights that interventions based at outdoor biting mosquitoes may be particularly needed to further reduce transmission in this area.

In this work, there were no clear cut peaks in the biting profiles of *An. gambiae* s.l. or *An. funestus* (Figure 3.7). Mosquito biting activity seemed to be spread across the night when using any of the three sampling techniques. Traditional knowledge of *An. gambiae* s.l. biting profile suggests a peak in the biting activity

of this species around midnight (Pates and Curtis, 2005, Gillies and De Meillon, 1968). Therefore, results from this work suggest a departure in the biting activity of *An. gambiae* s.l. from the traditional biting profile. This implies that the protective efficacy of ITNs may be compromised in Lupiro village and therefore appropriate preventive measures should be taken. However, this finding needs to be confirmed with further studies on the same area. Changes in malaria vectors' biting behaviour may be due to a number of other environmental changes such as changes in climate, housing conditions, agriculture, urbanization and change in human behaviour which have been reported to happen in Africa (Dowling et al., 2013, Casas et al., 1994, Lindsay et al., 2003, Atieli et al., 2009, Oria et al., 2015). With further improvements the MET developed here could be used to assess changes in malaria vectors' feeding behaviours in the future.

As the MET applies high voltages to electrocute mosquitoes, human safety in using this trap is a priority. Some measures were taken to ensure no risk of harm to humans using these traps. First, although the MET used relatively high DC voltage (600V), resistors are incorporated to limit the current to no more than 10mA which generates a low power output insufficient to cause harm to a human who momentarily touches them (Clifford et al 2005). Additionally, for future versions an inner protective grid made up of a layer of non-conductor should be placed in the inner side of the grids to make the trap safer for use.

3.6 Conclusions

This study has demonstrated proof-of-principle that the MET can be used with reasonable efficiency to sample malaria vectors outdoors. The CA-EG performance did not merit it further consideration because of its low sampling sensitivity. Whereas the current version of MET may misrepresent some aspects of mosquito behaviour such as the proportion of mosquito bites happening when most people are indoors and human exposure distribution, we hypothesize that the sampling sensitivity of MET can be improved specifically by ensuring generation of stable voltage across the night, and by avoiding short circuiting which can be achieved by replacing the semi-conducting wooden frames with non-conducting Polyvinyl Chloride (PVC). This may improve MET's ability to

accurately represent mosquito biting rates and behaviours relevant to malaria epidemiology. We recommend further testing of the MET in a range of ecological settings to explore its ability to be used as an alternative to the HLC.

Chapter 4: Comparative evaluation of resting traps for surveillance of indoor and outdoor malaria vectors

Contributions from other co-authors

This chapter will form the basis of a paper that will be submitted to Malaria Journal, with the following author list: Deodatus Maliti[1,2], Dr. Nicodem Govella[1], Dr. Katharina Kreppel[1,2] and Dr. Heather Ferguson[1].

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The specific contribution of these co-authors to work presented here is as follows: DM designed the resting traps. DM constructed the traps. DM designed the experiment with guidance from HMF NJG and KK. DM conducted the field experiment. DM conducted all statistical analysis under the guidance of HMF. DM wrote the manuscript with guidance from HMF, and further comments from HF NJG KK reviewed the manuscript.

4.1 Aims and objectives

The aim of this chapter was to evaluate the sampling efficiency of various traps used for sampling resting malaria vectors. Five traps (including two that were developed in this work), were compared in order to assess their relative efficiency as well as their versatility in sampling the major malaria vectors *Anopheles gambiae* sensu lato and *An. funestus* s.l. Traps varied in design to allow evaluation of how such features as the shape of a trap, the size of the entry hole and application of sticky surfaces to resting surfaces had a significant impact on the number of mosquitoes collected. Estimates of exophily (the degree to which malaria vectors prefer to rest outside rather than inside houses) were also compared as predicted from different trapping methods. The influence of daily microclimatic variation in temperature and humidity on the exophilic behaviour of malaria vectors was investigated. These resting traps were evaluated with the intention of identifying simple yet robust and

standardized sampling methods that can be used to study the resting behaviours of malaria vectors both inside and outside of houses.

4.2 Introduction

Female mosquitoes need to rest after consuming a blood meal in order to digest it. After feeding on a human indoors, malaria vectors can rest either on the inside walls of a house (a typical pattern for *An. gambiae* s.s. (Service et al., 1978)) or in the outdoor, peridomestic environment. Resting traps provide unique information on their habitat preference, and are essential for collecting blood fed individuals for subsequent estimation of the Human Blood Index (HBI, which is the proportion of mosquitoes engorged with blood from human hosts), which is a key determinant of malaria transmission (Garrett-Jones and Shidrawi, 1969). Additionally resting collections are essential to estimate the degree of endophily (proportion of indoor resting mosquitoes) within a target vector population as is required to assess the suitability of vector control measures such as Indoor Residual Spraying (IRS) which specifically targets mosquitoes resting in houses (Lengeler, 2004). Compared to sampling methods for host-seeking mosquitoes, resting traps typically catch far fewer mosquitoes (Sikaala et al., 2013, Govella et al., 2011), probably because of competition from natural resting sites (Burkett-Cadena et al., 2013) and that mosquitoes may escape the traps before they are collected. Thus, these traps are generally not suitable for studies of malaria vector population dynamics and infection rates (Sikulu et al., 2009). However, by providing information on mosquito habitat and host choice behaviour that cannot be gathered from other sampling methods, resting traps can unique insights into vector ecology that are of direct relevance to the evaluation and implementation of vector control measures.

Although the need for information on mosquito vector resting behaviour is clear, at present the range of sampling tools is limited and no widely-applicable and standardized method for indoor and outdoor sampling is available. Traditional methods for sampling resting mosquitoes indoors include aspiration (Maia et al., 2011) or pyrethrum spray catches (Dia et al., 2002, Okorie et al., 2014, Sabatinelli et al., 1986, Gratz and Carmichael, 1963). Collection of mosquitoes by aspiration depends on visual detection before capture and its efficiency can

be highly variable depending on the skill of collectors (Ngo et al., 2014, Turell et al., 2008). Battery-powered aspirators, such as the Backpack aspirator (BPA), partially overcome this limitation by mechanising the aspiration process; however, their performance is sensitive to variation in the skill of collectors. Additionally, the high cost of BPA (~£654 per unit) and their dependence on batteries may limit their use in remote and resource poor areas (Vazquez-Prokopec et al., 2009).

Pyrethrum spray catches involve spraying the house ceiling and walls with insecticide to kill all invertebrates resting on it. All furniture has to be removed from a room and a white sheet put on the floor to make it easier to identify dead mosquitoes that fall from walls or ceilings. Limitations of this approach are that its effectiveness may be low in areas of high insecticide resistance (Ranson et al., 2011). This method also involves contamination of walls with insecticides, which may bias succeeding collections on the same house, and can be disruptive to household members by requiring all furniture to be removed before room spraying. Furthermore, both pyrethrum spray catches and aspiration methods only sample mosquitoes that are resting in the house at the time of collection thus may underestimate the total population resting indoors overnight, especially if collection is made after dawn when many endophilic mosquitoes have exited from houses (Rubio-Palis and Curtis, 1992, Jaenson, 1988). Finally, both these approaches are based on active collection by people thereby limiting the wide scale use of these methods in many houses at the same time.

Both aspiration and pyrethrum spray catches are primarily used for collecting mosquitoes resting inside houses, but neither can be easily used outdoors where exhaustive searching with an aspirator and/or wide range, indiscriminate insecticide application is not practical. The current gold standard tool for sampling mosquitoes resting outdoors is the pit shelter trap (PIT) (Pombi et al., 2014b, Kweka and Mahande, 2009, Mahande et al., 2007, WHO, 1975). This trap is made by digging a pit in the ground of about 0.3-0.5 meters deep and similar dimensions wide, usually outside of a house. The pit is covered with materials such as grass or cardboard whilst leaving some gaps for mosquitoes to enter

when looking for resting sites. Although pit traps can yield large numbers of resting mosquitoes (Kweka and Mahande, 2009, WHO, 1975, Fernandez-Salas et al., 1994), this method has some major drawbacks including being time consuming and labor intensive, destructive to property and home gardens, and potentially dangerous as people and animals may fall into the pits. Furthermore, pit trapping can be conducted at only a few fixed locations so may not be suitable for widespread geographical sampling.

A limitation of current approaches is that there is no standardized method for sampling resting mosquitoes that can be used in both indoor and outdoor settings. Consequently, it is difficult to make accurate assessments of the relative propensity of vectors to choose indoor rather than outdoor resting sites, as any comparison between numbers caught in different locations may be confounded by differential efficiency of the sampling methods used. The need for standardized, directly comparable estimates of indoor vs outdoor resting habitat preference is growing due to increasing evidence that the widespread use of vector control measures may be changing vector behaviour (Russell et al., 2011, Fornadel et al., 2010a, Sougoufara et al., 2014, Fornadel and Norris, 2008, Padonou et al., 2012a). There is need for more investigation of this phenomenon across wider geographical areas by monitoring the resting behaviour of malaria vectors in indoor and outdoor locations using standardized sampling tools which can reliably quantify if and where mosquito behavioural shifts are occurring.

A number of alternative trapping approaches for sampling malaria vector resting behaviors have been proposed over the past decade. These methods include but are not limited to the use of clay pots (Odiere et al., 2007, Wong et al., 2013), a variety of resting box designs (RBO)(Govella et al., 2011, Sikulu et al., 2009, Williams and Gingrich, 2007, Sandhu et al., 2013, Wong et al., 2013), sticky traps (Facchinelli et al., 2007, Harris et al., 2011, Marini et al., 2010, Pombi et al., 2014b) and odour baited resting boxes (Kweka et al., 2010, L'Ambert et al., 2012). Some of these methods involve the use of relatively cheap, portable traps that could be used both indoors and outside. However, some degree of logistical and/or technical challenges is involved with the use of these traps, which at present make it difficult to identify a clear best practice. For example,

sticky traps such as the Sticky Resting Box (SRBO) have a sticky surface that binds mosquitoes on contact and thus offers the potential additional advantage of passive collection of mosquitoes over a period of a week or more (Pombi et al., 2014b). However, removing glued specimens from SRBO can be time consuming and cumbersome, and partially destructive such that morphological identification is not always possible.

Previous studies have used Resting Box traps (RBO) made out of cardboard so as to be lightweight, easy to transport and set up (Sikulu et al., 2009, Mayagaya et al., 2015); however the cardboard frame can collapse and disintegrate under conditions of heavy rainfall. Odiere et al (2007) used more robust clay pots (Odiere et al., 2007) which were effective for sampling both male and female *An. gambiae* s.l. resting outdoors. However, a disadvantage of this method is that pots are heavy, fragile and thus may not be suitable for study designs that involve regular shifting of traps. Whilst these alternatives show promise in some settings, few have been directly compared and there is little consensus on what specific approaches, and/or trap design features will be most beneficial for malaria vector collection. Comparative studies of the performance of these trapping methods relative to existing methods and to one another is thus needed to identify which methods are most promising to adopt.

Here a study was conducted to directly compare the sampling efficiency of a variety of recently proposed trapping methods that have potential to be used for standardized sampling of resting malaria vectors. A range of 4 different resting trap types were selected on the basis that all could be used in a standardized manner both inside houses and outdoors, and for which a series of hypotheses relating to the impact of certain trap design features could be tested through specific within-group comparisons. First, by comparing basic resting traps of similar total volume but of different shape (rectangular box versus round bucket), the impact of trap shape on sampling performance was tested. Recent studies have shown that resting traps lined with a sticky surface can be effective for sampling malaria vectors (Pombi et al., 2014a). However, in this study, there was no direct comparison with a trap of similar design without the sticky surface, and thus the additive benefit of the glue has not yet been quantified.

Given that removing mosquito specimens from sticky traps can involve significant handling time, it is important to confirm that the sticky surface leads to a significant improvement in mosquito catch rates. Finally, most recent resting box-based collection methods work by providing a relatively large opening for mosquitoes to enter, with no barrier to their subsequent exit (Govella et al., 2011, Sikulu et al., 2009). Consequently, mosquitoes that are found in these traps may only be a fraction of those that entered, with others having left before the time of collection. Thus in this study, a modified version of a standard resting bucket was developed which had a funnel-entry system which was hypothesized to make it easy for mosquitoes to enter into the trap, but difficult to leave afterwards.

The relative performance of these trapping types with respect to the number of African malaria vectors they collected was compared in outdoor and indoor environments. Additionally, as a secondary aim, these tools were used to investigate associations between naturally-occurring micro-climatic variation occurring during the period of sampling and mosquito resting habitat preference. Microclimate environmental conditions such as temperature and humidity have been shown to influence the preference of mosquitoes for resting indoors or outdoors (Paaijmans and Thomas, 2011), by choosing to rest in the warmer and more humid place. The overall aim of this work was to identify how resting traps can be optimized for collection of indoor and outdoor resting mosquito vectors, and highlight the possibility of using cheap and locally available materials to make efficient resting traps.

4.3 Materials and methods

4.3.1 Study site

The study site used here is the same as that described in section 3.3.1 of chapter 3. Resting collections were conducted in Lupiro village (-8.38 S, 36.67 E) between November 2012 and January 2013. Resting collections were conducted inside and outside of houses. Indoor collections were conducted in the main sleeping room while outdoor collections were conducted about 10m away from the house.

4.3.2 Trapping methods

A total of five different traps were used in this experiment; with all 5 used indoors and a subset of 4 outdoors. In indoor locations, collections were conducted using a CDC backpack aspirator (BPA) (Model 1412, BioQuip Products, Inc.) which was defined as the reference trapping method. This trap uses a 12V battery for its operation which was recharged or changed after every two sampling nights. This trapping method is primarily used to sample mosquitoes resting indoors (Clark et al., 1994). The remaining four trap types were constructed locally at the Ifakara Health Institute using locally available materials. Three of the trap types were constructed using a common basic framework of a standard plastic bucket: (i) a resting bucket (RBU), (ii) a sticky resting bucket (SRBU) and (iii) a modified entry resting bucket (MERBU). These buckets had a diameter of 40cm and measured 55cm high, with a total volume of 69,080cm^3. A schematic drawing of the box and the plastic bucket used to construct the traps is presented in Figure 4.1.

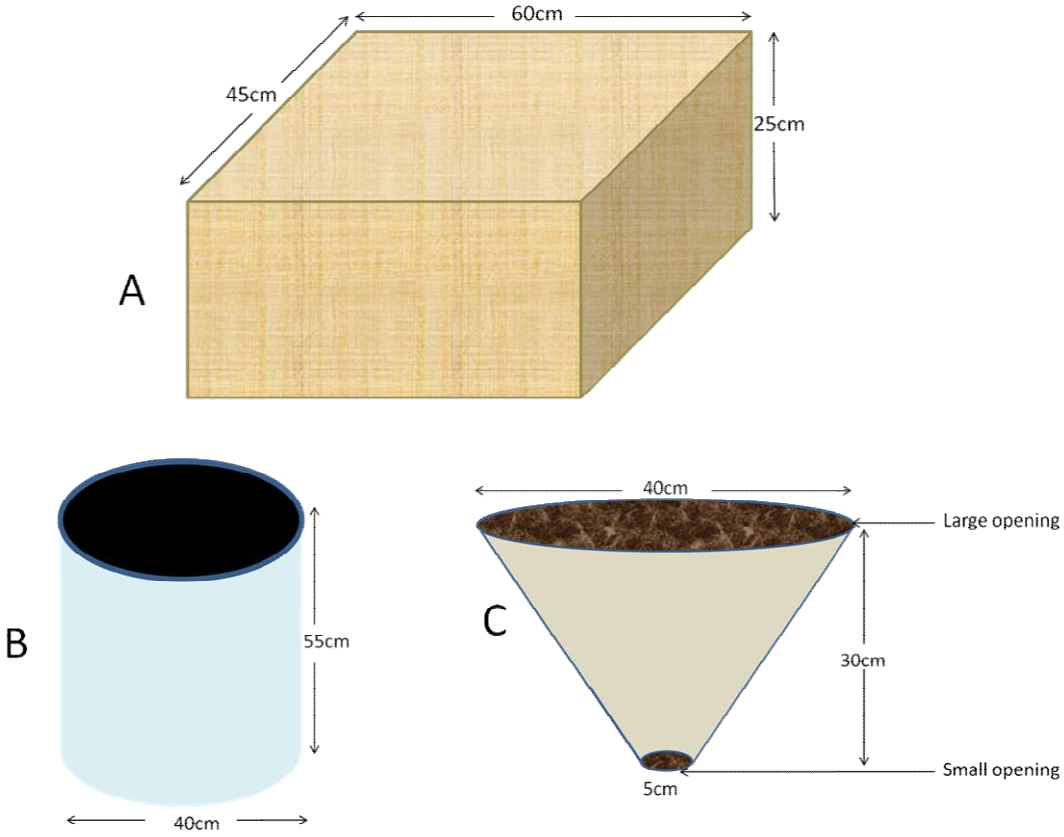

Figure 4.1. Schematic representation of the resting box (A), the basic structure used construction of the resting bucket (RBU) and the modified resting bucket (MERBU) trap (B). The funnel structure (C) was inserted into (B) to make the MERBU.

The RBU has been used by (Kreppel et al., 2015), and consists of bucket fitted with an internal lining of black cloth (Figure 4.2A & B). The SRBU and MERBU are new trap designs developed in this study. The SRBU was made by taking an RBU and lining it with acetate sheets that had been covered with a thin layer of rat glue as described in Pombi et al (2014). Five glued A4 acetate sheets were used to line the inner surface of the trap so that all surfaces on which a mosquito would land (except for the back wall) were sticky (Figure 4.2C & D). The acetate sheets were replaced with new ones after each night of experiments. A MERBU trap was made by creating a cardboard cone that had the same diameter as the RBU at the entrance to the trap, but tapered inwards to an opening of approximately 5cm at the far end (Figure 4.2E & F). Mosquitoes flying into this trap would be funnelled towards this opening for entry into the trap. The resting box trap (RBO) used in this study was similar to that used in previous studies (Govella et al., 2011, Sikulu et al., 2009, Kweka et al., 2009, Mayagaya et al., 2015). This trap was made from a standard cardboard box that

is commonly available from local shops with dimensions of (25cm x 45cm x 60cm) equivalent to a volume of 67,500cm^3 (Figure 4.2 G & H).

Figure 4.2. Resting traps and how they were placed indoors and outdoors. RBU indoors (A) and outdoors (B), SRBU indoors(C) an outdoors (D), MERBU indoors (E) and outdoors (F), RBO indoors (G) and outdoors (H). Traps were either lined with a black cloth in th inner side or were painted with a black chalk to mimic darkness.

4.3.3 Experimental design

Over the course of this experiment, resting mosquitoes were collected from 20 households in Lupiro village (-8.38 S, 36.67 E) situated in the Kilombero valley of Tanzania. Further description of the study site is given in section 3.3.1. Most of these houses were mud houses and grass-thatched. The households were occupied by 3 to 6 inhabitants who slept in during the experiments. Some household members slept under treated bed nets. In some of the households in Lupiro chicken were found staying in the houses at night. Also some of the households used to cook inside houses using traditional wood stoves which generated soot and plated the walls which might have a repellent effect on mosquitoes. Households that were used for experiments were scattered around 15m to 50m from each other. These households had eaves which were about 20cm high and running from one angle of a room to the other. During the experiments household doors and windows were closed and eaves left unsealed as it is the routine of the locals. Sampling took place over 4 discrete rounds in which trapping was conducted at a subset of 5 households. Within a round, a different indoor trapping method was randomly assigned to each of the 5 households on each sampling day: (i) BPA, (ii) RBU, (iii) SRBU, (iv) RBO and (v) MERBU. Four units of the selected resting box or bucket trap (ii-v) were set up inside the main sleeping room on each night as well as outdoors about 10 meters away from the house. In one of the 5 houses, indoor collection of resting mosquitoes was conducted using BPA. Over consecutive nights, all indoor trapping methods were rotated randomly through each of the households to complete a full round in 5 days. Simultaneously with indoor collections, mosquitoes were collected resting outdoors at each household using methods ii-v (no BPA), where 4 units of each of the traps were used. As only 4 sampling methods were used outdoors, a sampling round was concluded in 4 days. Sampling rounds were separated by a few days, and conducted over a total of 20 nights from November 2012 to January 2013. Traps were set in their respective positions by 7pm each night and left to run overnight until 5am the following next morning.

To measure temperature and humidity, tiny tag data loggers (Gemini UK, http://www.geminidataloggers.com/) were deployed to take measurements of temperature and humidity once per hour over the course of the sampling night. In indoor locations, tiny tags were hung in the middle of the main room midway between the roof and the floor, while for outdoor stations tiny tags were placed about 10 meters away from the household, and about 2 meters from the ground and close to where a resting trap was placed. Average nightly temperature and humidity at each trapping point were computed using hourly measurement for each of the 20 nights of experiments.

Each morning, traps were inspected between 5-6am to collect mosquitoes before light emerged. In the household where BPA was allocated for use indoors, collection was conducted between 5-6am by systematically moving the BPA over the roof and wall areas of each sleeping room for approximately 3 minutes. Mosquitoes were collected from RBU, RBO and MERBU by placing the nozzle of a CDC backpack aspirator into the opening of the trap and aspirating for about 10 seconds. For SRBU, acetate sheets were removed and inspected for individual mosquitoes stuck onto their surface. Mosquitoes found on the sheets were removed by placing a drop of acetone onto the specimen to loosen the glue, and the specimen removed using forceps. All sampled mosquitoes were placed in holding cups that were labelled with the date, trap type, household number and location (indoor vs outdoor) of the trap. Mosquitoes were sorted according to genera, gender, feeding status and trap location, using morphological identification keys. After morphological identification, mosquitoes which were visually identified as belonging to the *An. gambiae* s.l. species complex were taken to the laboratory at IHI for further species identification by polymerase chain reaction (PCR) using Scott's method (Scott et al., 1993).

4.3.4 Statistical analysis

All statistical analyses were carried out using the R statistical software version 2.15.3. In all analyses, generalized linear mixed models (GLMM) were used to test the significance of the variables of interest (trap type and/or environmental characteristics) relative to the outcome variables of mosquito vector abundance and exophily (proportion of resting mosquitoes found outdoors), with random

effects for round and household. First, analysis was conducted to test how the abundance of the two major vector groups, *An. gambiae* s.l. and *An. funestus* s.l., varied between resting collection methods. Here, models were constructed to test whether the mean abundance of vectors per collection varied between trap types. Given that mosquito abundance data was highly over dispersed, data were modelled as corresponding to a negative binomial distribution using the package GLMMADMB in R (Skaug et al., 2011) with adjustment for zero inflation. Separate analyses were conducted for indoor and outdoor collections, and for *An. gambiae* s.l. and *An. funestus* s.l.

Secondly, analysis was done to estimate how each of the 4 trap types that were simultaneously used indoors and outside estimated the extent of exophily in each vector species. Here exophily was defined as the proportion of mosquitoes resting outdoors as a fraction of the combined total resting indoors and outdoors. In this analysis, a GLMM model was constructed with a binomial distribution and a logit link (Crawley, 2007). Coefficients derived from this model were used to calculate exophily (proportion resting outdoors) as estimated by each trapping method.

Next, a series of analyses were conducted to test for associations between the abundance of malaria vectors in resting traps and daily microclimatic variation. Here analysis was restricted only to data from the RBO trap because it yielded the highest numbers (catch rates for some trap types were too low for robust analysis), and to facilitate comparison with previous studies where it has been used (Sikaala et al., 2013, Govella et al., 2011). In these analyses mosquito vector abundance per RBO per night was the response variable, and mean nightly temperature and humidity were fit as fixed effects (continuous), with household and experimental round treated as random effects. As described above, variation in mosquito abundance was modelled based on a negative binomial distribution.

A final model was built to test for the effect of the differences between indoor and outdoor temperature and humidity on the resting habitat preference of mosquitoes as defined by exophily. Here the response variable was the

proportion of resting mosquitoes collected outdoors as describe above, , with the differences between average indoor and outdoor temperature and humidity on each night of sampling,(expressed by δTemperature and δHumidity) being fit as explanatory variables. Here δTemperature and δHumidity were calculated by subtracting average indoor values from outdoor values for each night of sampling. As in all other models, household and experimental round were fitted as random effects. In all analyses of environmental variation, likelihood ratio tests using the ANOVA procedure in R were used to compare nested models and test for the significance of each environmental variable (temperature and humidity) on their own and when combined in the same model. This analysis was conducted only for *An. gambiae* s.l. as the total sample size of *An. funestus* s.l. was insufficient for robust analysis.

4.4 Results

A total of 2,875 mosquitoes were caught in resting collections over the 20 nights of this experiment. Four different mosquito genera were sampled: *Anopheles* (1,056), *Mansonia* (12), *Aedes* (14) and *Coquillettidia* (1,793). Amongst the *Anopheles* species, the two most abundant species were *Anopheles gambiae* s.l. (766) and *An. funestus* s.l. (287). Molecular analysis was conducted on a subsample of female *An. gambiae* s.l. (n=200) and all were found to be *An. arabiensis*. Approximately 39% and 47% of the female *An. gambiae* s.l. and *An. funestus* s.l. sampled were observed to be blood fed. The number of female and male *An. gambiae* s.l. and *An. funestus* s.l. sampled in indoor and outdoor locations is given in Appendix 2. A higher proportion of males were caught outdoors than indoors. A summary of female *Anopheles gambiae* s.l. and *An. funestus* s.l. sampled by each of the 5 traps including their location of sampling (indoor vs outdoor) and feeding status is shown in Table 4.1. Only female *An. gambiae* s.l. (366) and *An. funestus* s.l. (153) were analyzed in the next sections.

	Trap											
	BPA	RBO		RBU		SRBU		MERBU		Overall		Total overall
Species	In	In	Out	In	Out	In	Out	In	Out	In	Out	
An. gambiae s.l.												
Fed	14	3	54	3	39	5	0	1	8	26	101	127
Unfed	17	8	71	7	64	45	3	6	14	83	152	235
Partly fed	1	0	0	0	0	2	0	0	0	3	0	3
Gravid	0	0	0	0	0	0	1	0	0	0	1	1
Total *An. gambiae* s.l.	32	11	125	10	103	52	4	7	22	112	254	366
An. funestus s.l.												
Fed	32	7	3	5	2	3	0	1	1	48	6	54
Unfed	15	1	15	12	14	10	0	1	2	39	31	70
Partly fed	6	1	3	1	0	0	0	0	1	8	4	12
Gravid	9	4	2	0	0	1	0	0	1	14	3	17
Total *An. funestus s.l.*	62	13	23	18	16	14	0	2	5	109	44	153
Total mosquitoes	94	24	148	28	119	66	4	9	27	221	298	519

Table 4.1. Total number of resting female *An. gambiae* s.l. and *An. funestus* s.l. caught in resting collections by different methods (BPA= backpack aspiration, RBO=resting box, RBU=resting bucket, SRBU=sticky resting bucket and MERBU= modified entry resting bucket) collected both inside (In) and outside of houses (Out).

4.4.1 Relative performance

The number of female *An. gambiae* s.l. captured in resting collections varied significantly between trapping methods both inside houses (χ^2_4=23.77, p<0.001, Table 4.2) and outdoors (χ^2_3=58.78, p<0.001, Table 4.2). Likewise the number of *An. funestus* s.l. collected was highly dependent on the trap used both indoors (χ^2_4=33.66, p<0.001, Table 4.2) and outside (χ^2_3=20.77, p<0.001, Table 4.2). Generally, the numbers of malaria vectors captured by a trap was highly variable across nights (as indicated by the wide 95% CI in Table 4.2); however, some general trends were observed. When sampling outdoors, the number of *An. gambiae* s.l. and *An. funestus* s.l. tended to be highest in resting buckets (RBU) and resting boxes (RBO), whereas in indoor locations most malaria vectors were caught in SRBU (*An. gambiae* s.l.) or BPA collections (*An. funestus* s.l., Table 4.2). The MERBU trap was consistently the poorest.

Further comparisons were made between three different pairings of the 5 trapping methods to test more specific hypotheses about the impact of trap shape (round vs rectangular), trapping surface (sticky vs non-sticky) and the design of the entrance of the entry hole (funnel entry vs open entry). The impact of trap shape was investigated by comparing trap types made of almost identical materials and of similar volume, but where one was round (RBO) and the other rectangular (RBU). There was no significant difference in the number of *An. gambiae* s.l. caught by round versus rectangular traps either indoors (z=0.44, p=0.66, Table 4.2) or outdoor s (z=1.26, p=0.21, Table 4.2). Similarly, the numbers of *An. funestus* s.l. captured by RBO and RBU were similar indoors (z=-0.81, p=0.42, Table 4.2) and outdoors (z=0.52, p=0.61, Table 4.2). Thus based on these results there is no strong evidence that trap shape (round versus rectangular) influences attractiveness to malaria vectors.

The impact of incorporating a sticky surface into resting traps was investigated by comparing the performance of bucket traps both with standard design (RBU) and modified by lining the interior walls with a sticky surface (SRBU). The addition of the sticky surface significantly increased the catch rate of *An. gambiae* s.l. indoors as evidenced by the mean abundance captured by SRBU being 6.07 times higher than in RBU (z=3.64, p<0.001, Table 4.2). However this

enhancement was not observed when traps were used outdoors, where the SRBU caught significantly fewer *An. gambiae* s.l. than the RBU (z=-5.81, p<0.001, Table 4.2). The mean abundance of *An. funestus* s.l. captured resting indoors by RBU and SRBU was not significantly different (z=-0.72, p=0.47, Table 4.2), while outdoors the number of *An. funestus* s.l. sampled was generally low in all traps, with none being captured in SRBU, thus preventing any robust statistical comparison. Lastly, the sampling efficiency of a trap with large entrance (RBU) was compared to that of a trap with a funnel entry system (MERBU). When used indoors, the mean abundance of *An. gambiae* s.l. captured per trap night was similar in MERBU and RBU (z=-0.61, p=0.54). However, when used outdoors the RBU caught significantly more *An. gambiae* s.l. (3.53 per night) compared to the MERBU (0.78 per night, z=-4.71, p<0.001, Table 4.2). Significantly fewer *An. funestus* s.l. were captured by the MERBU compared to RBU both when used indoor s (~90% reduction, z=-2.80, p=0.01) and outdoors (~67% reduction, z=-1.96, p=0.05, Table 4.2).

Trap	Mean	95% CI	Relative	P-value
An. gambiae s.l. indoors				
RBU*	0.28	0.06-1.24	-	-
MERBU	0.19	0.04-0.88	N.S	0.54
RBO	0.33	0.08-1.42	N.S	0.67
SRBU	1.81	0.48-6.90	6.47	<0.001
BPA	0.89	0.23-3.46	3.17	0.009
An. gambiae s.l. outdoors				
RBU*	3.53	1.79-6.97	-	-
MERBU	0.78	0.36-1.70	0.22	<0.001
RBO	4.85	2.50-9.44	N.S	0.21
SRBU	0.14	0.04-0.46	0.04	<0.001
An. funestus s.l. indoors				
RBU*	0.39	0.01-2.00	-	-
MERBU	0.04	0.01-0.34	0.1	0.005
RBO	0.27	0.05-1.43	N.S	0.42
SRBU	0.28	0.05-1.49	N.S	0.47
BPA	1.24	0.26-6.88	3.17	0.003
An. funestus s.l. outdoors				
RBU*	0.42	0.08-2.33	-	-
MERBU	0.13	0.02-0.79	0.3	0.050
RBO	0.53	0.10-2.74	N.S	0.61
SRBU	0.00	N/A	N/A	N/A

*Reference trap

N.S where there was no significant difference in the performance of a trap

Table 4.2. The mean abundance of malaria vectors captured in different resting traps (per trap per night), and their relative sensitivity compared to the Resting Bucket (RBU) standard method. The mean numbers of mosquitoes collected by each trap were derived from a GLMMadmb model and represents 20 nights of trap comparison. Where N/A is indicated is due to failure of SRBU to collect any mosquitoes outdoors. BPA was used for indoor sampling only and therefore no results for this trap are presented for outdoor locations.

4.4.2 Exophily

For both *An. gambiae* s.l. and *An. funestus* s.l., data from the four sampling methods that were simultaneously used indoors and outdoors (RBU, SRB, RBO &

MERBU) were used to calculate an index of exophily. Generally, all traps estimated *An. gambiae* s.l. to be highly exophilic (e.g. >75% of mosquitoes found resting outdoors) with the exception of the SRBU which indicated only 6% would rest outside (Table 4.3). For *An. gambiae* s.l., the degree of exophily estimated by RBU was higher than in all other traps (Table 4.3), whereas a similar proportion was estimated by the RBO reference method and MERBU (Table 4.3). Estimates of exophily in *An. funestus* s.l. could be obtained from only 3 trapping methods (RBO, MERBU and RBU) because the SRBU did not capture any *An. funestus* s.l. outdoors, and thus no confidence interval for exophily could be calculated for comparison. Of the remaining methods (RBU, RBO & MERBU), all estimated that approximately 50% of *An. funestus* s.l. were found resting outdoors (Table 4.3), indicating that this species (group) has no clear preference for resting indoors or outside (Table 4.3).

Species	Trap	Exophily (95% CI)	P value
An. gambiae s.l.	RBO*	88.49%(79.18-93.96)	
	MERB	76.11%(54.77-89.34)	0.08
	RBU	95.65%(89.23-98.32)	0.041
	SRBU	0.06%(0.02-0.19)	<0.001
An. funestus s.l.	RBO*	58.11%(46.64-68.76)	
	MERB	51.02%(37.30-64.59)	0.44
	RBU	51.35%(40.09-62.48)	0.25
	SRBU	N/A**	

*Reference trap
**No *An. funestus* s.l. were collected outdoors

Table 4.3. Exophily estimates. Estimates of exophily in malaria vectors as estimated by different resting trap methods. P-value refers to statistical comparison between exophily estimates of a resting trap relative to RBO as the reference traps. No *An. funestus* s.l. were caught in SRBU outdoors.

4.4.3 Environmental associations

Further analysis was conducted to investigate associations between microclimatic environmental variation and mosquito resting behaviour during the course of this study. Here, data was used only from *An. gambiae* s.l. captured in

the RBO trap. Generally, nightly values of temperature and humidity were similar inside houses and outdoors over the course of this study, with little difference between their mean values and range (Table 4.4).

		Inside	Outside
Temperature (^{0}C)	Mean	28.22	27.13
	Range	23.34-32.07	23.30-31.01
Relative humidity	Mean	74.41	74.08
	Range	47.14-89.80	46.24-88.50

Table 4.4. Mean and range of nightly temperature and relative humidity values measured inside houses and at outdoor trapping stations during this study. Mean values were calculated from hourly measurements taken from.

These experiments were conducted over a 2-month period just after the short rain season (November-January) that coincides with some of the warmest annual temperatures. Over the sampling period, there was no significant association between mean nightly temperature and the abundance of *An. gambiae* s.l. and *An. funestus* s.l. found resting in either outdoor or indoor locations (Figure 4.3, Table 4.5). Similarly, there was no significant association between mean nightly humidity and the abundance of resting *An. gambiae* s.l. and *An. funestus* s.l. found resting indoors and outdoors respectively (Figure 4.3, Table 4.5).

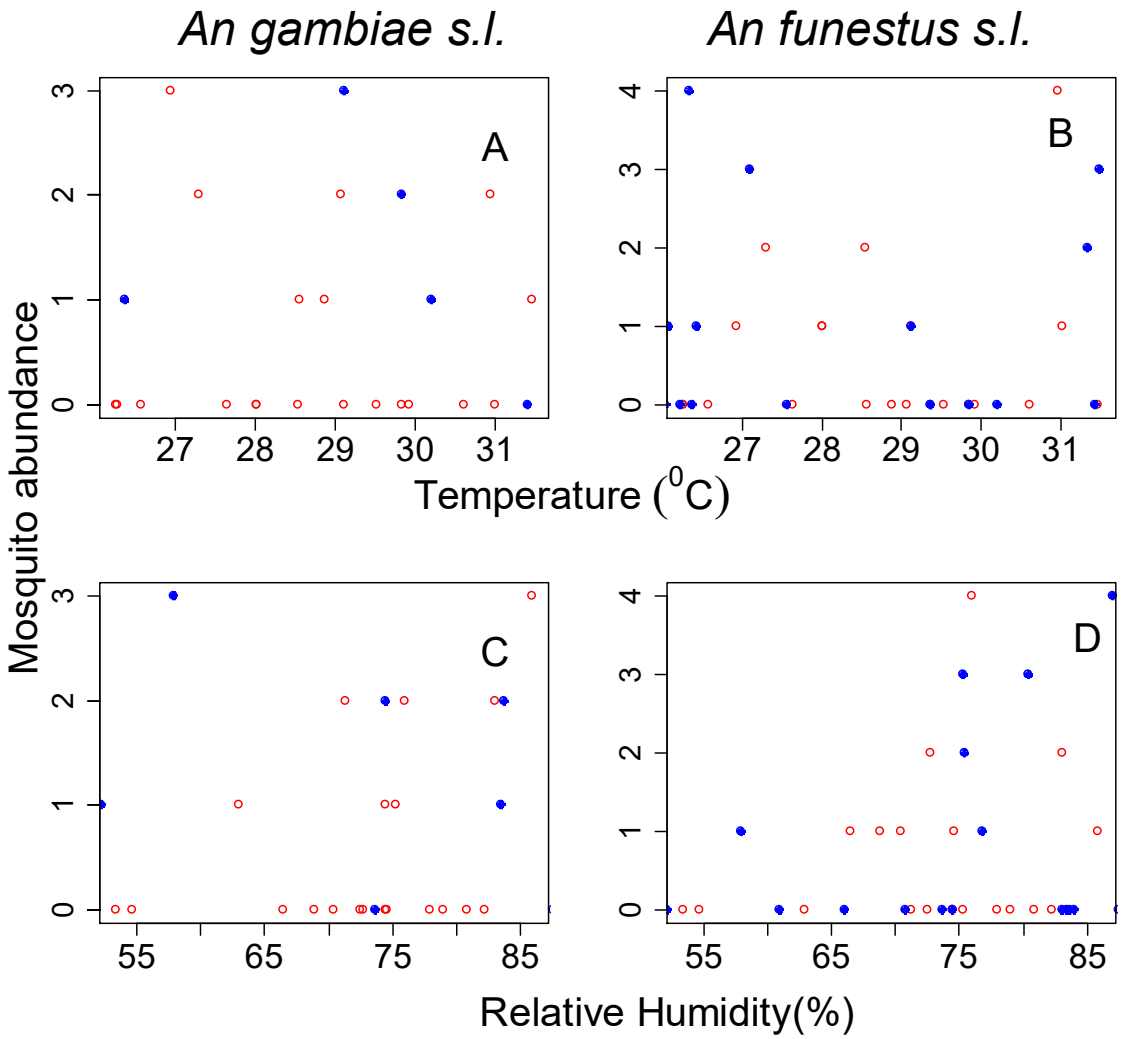

Figure 4.3. A relationship between the mean nightly temperature (A) for *An. gambiae* s.l. and (B) for *An. funestus* s.l.) and humidity (C) for *An. gambiae* s.l. and (D) for A*n. funestus* s.l. and the number of mosquito vectors captured either inside or outside by RBO traps. Blue filled and red-open circles are for number of mosquitoes collected inside and outside houses respectively.

Model	AIC	df	Deviance	P-value
An. gambiae s.l. indoors				
Null	49.35			
Temperature	51.27	1	0.08[a]	0.78
Humidity	49.72	1	1.63[a]	0.20
Temperature+Humidity	50.92	1	2.35[b]	0.13
Temperature+Humidity	50.92	1	0.80[c]	0.37
An. gambiae s.l.				
Null	124.80			
Temperature	126.53	1	0.28[a]	0.59
Humidity	126.49	1	0.31[a]	0.58
Temperature+Humidity	128.45	1	0.07[b]	0.79
Temperature+Humidity	128.45	1	0.04[c]	0.84
An. funestu s.l. indoors				
Null	66.70			
Temperature	68.30	1	0.32[a]	0.57
Humidity	68.10	1	0.54[a]	0.46
Temperature+Humidity	68.91	1	1.42[b]	0.23
Temperature+Humidity	68.91	1	1.21[c]	0.27
An. funestus s.l.				
Null	75.95			
Temperature	77.29	1	0.66[a]	0.41
Humidity	76.77	1	1.19[a]	0.28
Temperature+Humidity	78.76	1	0.53[b]	0.47
Temperature+Humidity	78.76	1	0.00[c]	0.95

Table 4.5. Effect of temperature and humidity on the number of mosquitoes captured in resting traps across all nights of experiments. Mosquito abundance was analyzed in association with temperature and humidity in the location where they were found (e.g. indoors or outdoors). Akaike information criterion (AIC), degree of freedom (df), deviance and p-values are given for specific model comparisons as denoted by subscripts as follows: (*a*) Model compared against the null model, (*b*) model was compared against the temperature-only model and (*c*) model was compared against the humidity-only model.

The proportion of *An. gambiae* s.l. and *An. funestus* s.l. resting outdoors was significantly associated with the relative difference in temperature and humidity between indoor and outdoor environments on each night of sampling (Figure 4.4, Table 4.6). The proportion of malaria vectors found resting outdoors was predicted to increase as inside temperatures became warmer than those outdoors; both in *An. gambiae* s.l (z=2.16, p=0.031, Figure 4.4A) and *An. funestus* s.l. (z=2.46, p=0.014, Figure 4.4C). Conversely, the proportion of *An. gambiae* s.l. and *An. funestus* s.l. found resting outdoors decreased as humidity

became higher inside compared to outside (z=-2.86, p=0.004, Figure 4.4B) and (z=-2.31, p=0.020, Figure 4.4D) respectively. When temperature and humidity differences were combined in a single model, both retained a significant impact indicating that they have some independent effects (Table 4.6).

Model	AIC	df	Deviance	P-value
An. gambiae s.l.				
Null	53.88			
Temperature	50.78	1	5.31[a]	0.021
Humidity	44.09	1	11.80[a]	<0.001
Temperature+Humidity	43.45	1	9.13[b]	0.003
Temperature+Humidity	43.45	1	2.64[c]	0.010
An. funestus s.l.				
Null	29.57			
Temperature	25.06	1	6.51[a]	0.011
Humidity	26.25	1	5.32[a]	0.021
Temperature+Humidity	22.24	1	4.82[b]	0.028
Temperature+Humidity	22.24	1	6.01[c]	0.014

Table 4.6. Effect of the difference in temperature and humidity between inside and outside houses on the exophily of *An. gambiae* s.l. and *An. funestus* s.l. Akaike information criterion (AIC), degree of freedom (df), deviance and p-values are presented for specific model comparisons as denoted by subscripts as follows: (*a*) model was compared against the null model, (*b*) model was compared against the temperature-only model and (*c*) model was compared against the humidity-only model.

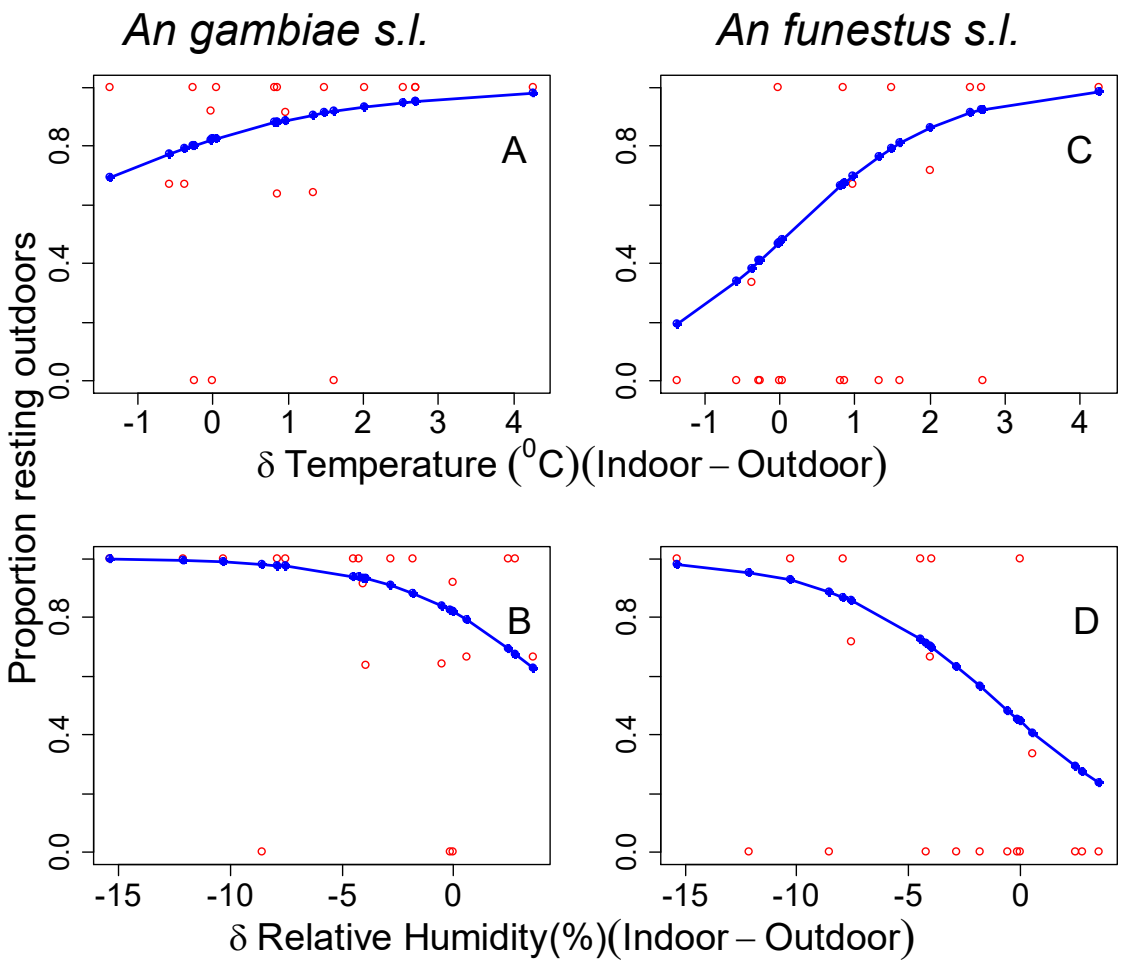

Figure 4.4. Predicted association between the difference in nightly temperature and humidity values between indoor and outdoor settings, and the proportion of resting malaria vectors found outdoors (exophily). Red-open dots represent the proportion of mosquitoes resting outdoor with respect to differences on temperature and humidity between in and outside houses. On the x-axis, negative values indicate occasions where temperatures were warmer or relative humid was higher outdoors than inside houses, while positive values indicate it was warmer and more humid indoors than outdoors.

4.5 Discussion

This study evaluated 5 methods for trapping resting malaria vectors with the aim of assessing their relative sampling performance, and testing hypotheses about the importance of a series of design features (trap shape, use of sticky surfaces, and entry routes). Three of these trapping methods (BPA, RBU, and RBO) have been used before, while two (SRBU and MERBU) were developed in this work. In

terms of sampling efficiency for indoor resting mosquitoes, the SRBU and BPA yielded the highest numbers of *An. gambiae* s.l., whilst the BPA was the best method for *An. funestus* s.l. The sampling performance of MERBU was generally very poor except when used to sample *An. gambiae* s.l. indoors where its efficiency remained low but was statistically indistinguishable from RBO and RBU traps. In outdoor sampling, catches were consistently lower in SRBU than all other methods. In general, catch rates were very low (often <1 mosquito per trap) and quite variable between nights, which limited power to detect anything other than large differences between trap performance. However on the basis of results collected here, the hypotheses that reducing the entry hole in resting traps, and using of sticky surfaces in outdoor trapping improves trapping efficiency can be rejected. Of the options tested, this study hypothesizes that RBU and RBO traps are the best option for investigation of malaria vector resting behaviour (e.g. exophily) due to their relatively high and consistent sampling performance both indoors and outdoors.

In the following paragraphs, the performance of different resting traps relative to each other is discussed in depth. However caution should be taken when comparing the performance of these traps due to the fact that there might not have been enough sample size to give more robust statistical power. Additionally, some traps collected very few mosquitoes while some did not collect any mosquitoes in some nights, resulting into zero inflated data and over-dispersion. Though appropriate statistical models dealing with zero inflation and over-dispersion were used (See section 4.3.4), the raw mean number of mosquitoes collected over 20 nights from the crude data in Table 4.1 may not match well with the mean calculated from the models in Table 4.2. Such discrepancies are characteristic of over-dispersion which when coupled with low sample size could result into a loose model fit as was the case in this chapter.

In this study, RBO had the best performance in sampling outdoor resting *An. gambiae* s.l. and *An. funestus* s.l., but had somewhat lower sampling performance in indoor sampling compared to BPA. A reduced performance of RBO relative to other traps has been shown when sampling *Anopheles quadriannulatus* and *An. funestus* in indoor and outdoor environments (Sikaala et

al., 2013), as well as in sampling *An. gambiae* s.l. outdoors, (Govella et al., 2011, Sikulu et al., 2009). In these studies, RBO had poorer performance compared to window exit trap (WET), and a variety of other host seeking traps including the Ifakara Tent Trap (ITT-C), CDC light trap and Human Landing Catch. Another study reported that RBO captured fewer *An. gambiae* s.l. and *An. funestus* s.l. than other traps including resting pots (Wong et al., 2013). A backpack aspirator was used to suck resting mosquitoes from RBu, MERBu and RBo traps which made it easier and quicker to collect mosquitoes from these traps than using a mouth aspirator. While if mouth aspirators were used a greater number of mosquitoes could fly away and cause a significant underestimation of performance, use of backpack aspirators to collect mosquitoes from RBu, MERBu and RBo reduced the underestimation relative to SRBU due to small fraction of them flying away. It is unsurprising that resting traps have poorer performance than those targeting host seeking or exiting mosquitoes (e.g. WET, ITT, CDC and HLC) as these methods work by attracting mosquitoes towards a host source whereas resting collections are based on active searching for mosquitoes at rest, which may be distributed over a wide area. Clay resting pots were not investigated in this study as it was decided a priori that logistical difficulties involved with these traps (e.g. feasibility of packing and moving large numbers around) would not make it a practical option.

The finding that traps with sticky surfaces performed significantly worse than non-sticky alternatives in outdoor environments matches that of another recent study in the Kilombero valley (Kreppel et al., 2015) in which I am a co-author. This study compared sticky resting boxes and RBU for collecting malaria vectors outside, and found the sticky resting boxes to have significantly poorer performance (~4.6% of RBU). As in the Kreppel et al (2015) study, the sticky and non-sticky traps had different shapes (boxes versus buckets) and were made of different material (wood versus plastic), it was not possible to clearly distinguish the effects of the sticky surface from those of other design features. Results obtained here for traps that were identical except for the use of a sticky surface unambiguously demonstrate that counter to expectation, use of this material significantly reduced the performance of resting traps outdoors. In another study conducted in Burkina Faso, sticky resting boxes had lower but consistent

sampling efficiency in relation to the traditional gold standard methods of backpack aspirators (indoors) and pit shelters (outdoors) (Pombi et al., 2014a). Though resting traps used in other studies may have better performance than some of the traps used in this study (e.g. pit traps or clay pots), the methods used here are likely to be easier to be used for standardized sampling because they are light-weight, cheap and portable.

One of the major aims of this study was to investigate whether physical features such as resting trap shape, use of sticky surface and size of the entry hole had an effect on the sampling efficiency of the traps. The shape of a trap (round or square) had no detectable impact on sampling efficiency, with rectangular (RBO) and round (RBU) traps generally yielding similar numbers of malaria vectors both when used indoors and outside. The resting box trap (RBO) used here was constructed from a cardboard box, and is the same design as used in previous studies (Sikaala et al., 2013, Kweka et al., 2010, Sikulu et al., 2009, Govella et al., 2011, Mayagaya et al., 2015). Whilst the RBU and RBO performed similarly well in this study, a major disadvantage of the latter method is the inability of the cardboard structure to withstand moist environments. This problem did not affect results in this study because sampling was done in dry weather between the short and long rainy season. However, in moist environments, RBU would be expected to be much more robust. A relative advantage of the RBO is that these traps are easily collapsible and can be flat-packed. Whilst RBU are not so compact, their durability and design makes it possible to stack them together for transport with little risk of damage. Furthermore, unlike the RBO, RBU requires no disassembly for transport. Thus on balance the RBU appears a better option given it is more durable and easy to use in the field.

The SRBU showed mixed performance by performing well in collecting *An. gambiae* s.l. in indoor environments while performing poorest in outdoor environments. The reason for this outcome is not well known, but a potential hypothesis is that sticky traps are more quickly covered by dust and/or other insects in outdoor environments, which reduce their stickiness and make them less attractive than non-sticky alternatives. The glue used in the sticky trap has

been tested and found to have no repellent effect to mosquitoes (Pombi et al., 2014a). Another challenge with using sticky traps was the substantial processing time required to remove mosquitoes from the trap compared to other methods. Whilst the relative trapping performance of sticky traps in this and other studies is low, they have a potential advantage over other methods described here in providing the possibility of passive surveillance. As mosquitoes get stuck to the traps, they do not need to be checked daily and be left for periods of a week or longer and still yield specimens that are suitable for morphological and molecular identification (Pombi et al., 2014a). Thus while SRBU do not appear a good option for rapid assessment based on this study, they could be suitable (if used in high numbers) for studies in which it is not possible or desired to check traps daily.

The MERBU trap was designed with the intention of modifying entrance and exit routes into the trap, in order to enhance catch rate by preventing mosquitoes that entered from subsequently leaving before collection. However, the funnel entry system that was incorporated as a strategy to achieve this did not lead to any improvement in sampling efficiency relative to the standard open entry trap (RBU), and in fact generally resulted in significantly poorer performance. Possible reasons for this are that by reducing the size of the entry hole, the funnel entry system also made it harder for mosquitoes to find and enter the trap. Natural objects with large entry holes in the surroundings such as tree cavities have been found to contain more mosquitoes than cavities with small entrances (Burkett-Cadena et al., 2008). The surface of the MERBU funnel might also have contributed to the low catch if there were any repellent effects of the paint used (no known repellent effects but this was not tested). Since results from this work suggest that traps with a wide-open entrance were most effective, other strategies could be explored to limit the chance of mosquitoes to escape from traps. The use of sticky surfaces is one such method, but results obtained here suggest that sticky traps do not lead to consistently better performance. Shuttering mechanisms that automatically close the trap at a specific time before dawn could be incorporated into simple resting traps to ensure mosquitoes cannot exit after a pre-specified time. At present, the best

option is to conduct sampling of the traps early before dawn when mosquitoes have not yet exited.

In this study, resting traps used inside houses were placed in the upper corner of each of the 4 corners of a room. This placement was chosen to try and match the area sampled by BPA (which are conducting by searching roofs and the upper walls of houses). Additionally this placement was most convenient to the householders taking part in the study, as the traps were not in their path when walking in the room. However another recent study of sticky resting boxes inside houses placed them on the floor, and with the open end facing the wall rather than into the room as used here (Pombi et al., 2014a). Kweka et al (2010) compared the performance of RBO placed at different heights from the ground and found this to significantly affect the sampling performance of the RBO inside houses. In this study the optimal height was found to be 105cm from the ground indoors, while outdoors was 15cm (Kweka et al., 2010). In this work, indoor traps were placed about 200cm from the ground, which might have influenced performance of the resting traps. Further study is required to investigate how the placement of resting traps within a house influences their performance.

Estimates of exophily as obtained from most methods indicated that *An. gambiae* s.l. is strongly exophilic (>76% rest outside), while *An. funestus* s.l. were found resting indoors and outdoors in approximately similar proportion. A notable exception to this was results from the SRBU which suggested only 6% of *An. gambiae* s.l. rest outside. These results are consistent with previous studies which have shown high exophily in *An. arabiensis* (Mahande et al., 2007, Oyewole et al., 2007, Tirados et al., 2006) thus suggesting no change in the resting behaviour of *An. arabiensis*. But exophily recorded in this study is higher than what is traditionally known of *An. funestus* (Gillies, 1954b). It is difficult to conclude whether these changes in the resting behaviour of *An. funestus* s.l. reflect a change within species or reflect inter-species differences in the *An. funestus* s.l. which is known to be a complex of many species (Gillies and Coetzee M, 1987, Koekemoer et al., 2002). In this study no molecular analysis was conducted to confirm the identity of species within the *An. funestus* s.l.

group, but more than 90% are expected to be *An. funestus* sensu stricto on the basis of a recent study conducted in the same area (Lwetoijera et al., 2014). Since all inhabitants of the 20 households used in this study were using ITNs at the time this experiment was conducted, this might have played a role in increasing exophily as it is known that mosquitoes that don't find blood meals indoors tend to exit the house thus leading to a higher proportion of mosquitoes resting outdoors (van den Bijllaardt et al., 2009). Estimates of resting habitat preference as obtained from the SRBU were highly biased to the point where a highly exophilic species (*An. arabiensis*) was estimated to be extremely endophilic. This can be explained based on the differential performance of the SRBU indoors (relatively good) versus outdoors (very poor), which will generate a systematic bias towards endophily. For this reason, this study caution against the use of sticky traps in mosquito behaviour surveillance studies.

Microclimatic variation occurring over the course of this study covered a relatively wide range (Mean nightly temperature: 23.3 to 32.1 ^{0}C indoors and 23.3 to 31.0^{0}C outdoors, mean nightly humidity: 47.1 to 89.8% indoors and 46.2 to 88.5% outdoors), but did not vary significantly between indoor and outdoor locations. Mean nightly temperature and humidity values were not significantly associated with the number of malaria vectors found resting either indoors or outside. At the seasonal and macro-geographical level, temperature and humidity have been shown to have strong but opposite associations with the abundance of resting mosquitoes (Paaijmans and Thomas, 2011). Specifically the abundance of indoor and outdoor resting *An. arabiensis* was higher at higher temperatures and negatively correlated with lower humidity values across an altitudinal gradient (Kulkarni et al., 2006), and at seasonal level for resting *An. gambiae* s.l. (Rishikesh et al., 1985). The inability to detect an of association between microclimatic factors and the abundance of resting mosquitoes in this study may be partially due to the fact that this study was conducted over a relatively short period of time (November to January), within which temperature and humidity showed a more limited range of variation than would be expected to occur with altitude or seasons. Larger sample sizes and year-round sampling of vectors will be required to establish whether extreme seasonal variation in

microclimatic conditions can explain variation in the abundance of resting mosquitoes.

Since absolute temperatures and humidity did not correlate with the resting behaviour of mosquitoes, further investigation was conducted to test if the relative difference in microclimatic conditions between indoor and outdoor settings had an association with mosquito resting habitat preference (in terms of the degree of exophily). In this study, exophily in both malaria vector species appeared to increase in association with temperatures becoming relatively warmer indoors compared to outside. Humidity was generally higher outdoors than inside houses, and exophily was predicted to be highest on nights where the relative difference between outdoor and indoor humidity was greatest. Both the relative difference in mean humidity and temperature retained significance when combined in statistical models, indicating these factors both have some independent impact on exophily. Optimal relative humidity in malaria vectors is considered between 75-85% (Olanga et al., 2010, Das et al., 2007). In this experiment, relative humidity ranged from 47.1% to 89.8%, indoors and 46.2% to 88.5%, indicating humidity in both locations could fall within the suboptimal range. However, in general humidity was higher outside than inside which could explain why the majority of malaria chose to rest outdoors. Temperature and humidity depend on house conditions such as ventilation, roofing style and insulation. It is expected that as the house construction style in Africa is improving and changing from the traditional mud and grass thatched houses to block houses, these changes will have impact on the resting behaviour of malaria vectors. Such changes in the behaviour of malaria vectors could include a preference to rest outdoors vs indoors as it may difficult for mosquitoes to enter in screened houses. Other factors such as availability of electricity may improve light conditions indoors and lead to changes in the resting site preference. It could be informative to investigate how housing conditions may influence the resting behaviour of malaria vectors relative to the influence of control measures such as ITNs and IRS. Some of the resting traps such as the RBO and the RBU developed in this study could be used to assess the impact of these environmental changes on the resting behaviour of malaria vectors in endemic areas.

These results demonstrate that, relative differences in temperature and humidity between indoor and outdoor could explain a significant proportion of the daily variation in the exophilic behaviour of malaria vectors. These findings suggest that malaria vectors choose the most humid environments (always outdoors here), and that at least during the hottest times of the year (e.g. when this study was conducted), indoor temperatures may become too high for malaria vectors and prompt them to choose cooler (and more humid) outdoor resting sites. On this basis, it is hypothesized that IRS effectiveness could vary seasonally and be highest at times of year when temperature and humidity are optimal for endophily.

In addition to the relative sampling efficiency of different trapping methods, there are other important logistic factors that should be considered when choosing a trap such as cost and convenience of use. In this study, RBO and RBU were the simplest and cheapest traps to make. The SRBU was made from a similar structure as the RBU, but was more labour intensive and incurred additional cost for glue and acetate sheets. In terms of simplicity, RBO and RBU were the simplest to make and deploy, requiring only either an empty cardboard box or a plastic bucket and a black piece of cloth. Since these materials are easily available in most malaria endemic regions, RBU with additional advantage of being moist tolerant compared to RBO, may be the traps of choice given also that its relative sampling efficiencies surpass those of other novel traps evaluated in this work.

4.6 Conclusions

Lightweight portable resting traps can be useful to collect relatively large number of resting mosquitoes, and estimate key behavioural traits such as exophily. However, small details of design such as the size of the entrance and use of sticky surfaces can have a big impact on trapping success, so specific design details are important. Most traps produced relatively consistent estimates of exophilic behaviour except for the SRBU, which significantly underestimated exophily due to their much poorer performance outdoors than inside. Thus, great care is needed to identify best trap type both for logistic

ease and for unbiased estimation of mosquito behavioural traits. For this purpose, I would recommend the RBU trap.

Chapter 5: Investigating associations of *Anopheles arabiensis* time of feeding behaviour using single nucleotide polymorphisms in the circadian rhythm genes

Contribution from other co-authors

This chapter will be published as a paper in Parasites and Vectors. I mention the names of other people involved with their contribution in the following section:

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DM, GL, CM, HF and YL and conceived the study. DM conducted field collections, laboratory analyses and wrote the manuscript. DM, YL, CM assisted in data analysis. GL, HF, BM, YL, KK, NG and CM reviewed the manuscripts.

5.1 Aims and objectives

The aim of this study was to investigate if polymorphisms in the circadian genes of *An. arabiensis* are associated with heterogeneity in their time of host seeking on people. In field study in southern Tanzania, mosquitoes were collected by Human Landing Catch and divided into phenotype categories of early (7pm-10pm) vs late host seeking (4am-7am), and by their location of capture (indoor vs outdoor). The hypothesis tested was whether variation in single nucleotide polymorphisms within circadian rhythm genes are associated with differences in the time of feeding in *An. arabiensis* (late vs early), in both indoor and outdoor biting mosquitoes. Whether observed differences in the time and location of host seeking in malaria vectors are due to heritable genetic variation or plastic

behavioural adaptations are discussed in the context of epidemiological implications.

5.2 Introduction

In Africa, the prominent malaria vector species include *An. gambiae* s.s., *An. arabiensis* and *An. coluzzi* which are all part of the *An. gambiae* s.l. species complex. Females of these species require vertebrate blood to develop their eggs, and regularly feed upon humans in the wild (Gillies, 1953). The stereotypical pattern of host seeking in these vector species was described in early work by (Gillies, 1957), and is characterized by an onset of limited host seeking just after dusk which increases to a peak around midnight, with 60-80% of bites estimated to occur between 9pm – 3am (Mendis et al., 2000). This host seeking activity coincides with the period when most people are indoors and asleep (Bryan et al., 1987, Gillies, 1953). This pattern of behaviour underlies the success of control measures like LLINs, as by selectively protecting people when they are asleep at night indoors, the majority of mosquito vector bites can be prevented (Smithuis et al., 2013b). However, there is some variation in the stereotypical pattern of host seeking behaviour within the *An. gambiae* s.l. complex. For example, *An gambiae* s.s. are known to be highly endophagic (preference to feed indoors) and anthropophagic (Oyewole et al., 2007), whereas *An. arabiensis* has a more flexible feeding behaviour and can even be relatively exophagic and zoophagic (preferring to feed on animals) (Oyewole et al., 2007, Githeko et al., 1996a, Mahande et al., 2007, White, 1971). Based on this behavioural variability in malaria vectors, malaria vector control programs need to extend their strategies to offer effective measures across diverse species of malaria vectors.

Within the *An. gambiae* s.l. species complex, there have been reports of shifts in their behaviours such as increased tendency to feed outdoors (Russell et al., 2011, Gordicho et al., 2014), to bite earlier or later in the night (Fornadel et al., 2010a), and reduced anthropophagy (Norris and Norris, 2013) in the presence of vector control measures(Bayoh et al., 2010, O'Meara et al., 2010, Derua et al., 2012, Mwangangi et al., 2013, Russell et al., 2010, Lounibos, 2007). These behavioural shifts have been associated with changes in the species composition

towards vector species with more exophilic behaviour (Kitau et al., 2012, Lindblade et al., 2006, Bugoro et al., 2011, Bayoh et al., 2010, Derua et al., 2012), and may also be due to changes in behaviour within species (Govella et al., 2010b).

As a consequence, a substantial amount of malaria exposure (40-50% of bites from *An. gambiae* s.l.) in some parts of sub-Saharan Africa is now thought to occur outdoors (Russell et al., 2011, Seyoum et al., 2012, Majambere et al., 2013), requiring increased efforts to control outdoor biting mosquitoes to move further towards malaria elimination (Govella and Ferguson, 2012). The "behavioural resistance" strategies that allow the major African malaria vectors to avoid exposure to ITNs or IRS (Bayoh et al., 2014) could pose a serious challenge to future control efforts. Given the potential epidemiological importance of these mosquito behavioural changes, there is a need to identify the biological mechanisms responsible for driving them.

Several studies have shown host preference and the location of insect vector biting preference has some genetic basis. For example, when two strains of *Aedes aegypti* and two strains of *Aedes sympsoni* with different host preferences were crossed, the hybrids and their backcrosses were of intermediate host preferences relative to their parental strains (Mukwaya, 1977). Analysis of *An. gambiae* s.s. and *An. arabiensis* caught host seeking and resting inside houses and outdoors in Kano Nigeria showed that certain chromosome inversions occur more significantly in outdoor than indoor collected mosquitoes (Coluzzi et al., 1977). It was hypothesized that mosquitoes with such outdoor-associated chromosome inversions would be more difficult to control using indoor residual spraying (IRS) than the indoor form (Coluzzi, 1984). Further, specific chromosomal inversions in *An. gambiae* s.l. have been associated with higher *Plasmodium* infections in the vectors (Petrarca and Beier, 1992). These chromosome inversion polymorphisms however did not differ within samples collected with human and cow-baited traps (Petrarca and Beier, 1992) and could therefore not explain host preference. These observations indicate that there may be a genetic basis to a broad range of mosquito feeding behaviours such as their time and location of feeding (indoors and outdoors). Furthermore, the use

of more modern, fine-scale genetic analysis approaches (Wondji et al., 2007a, Weetman et al., 2010, Neafsey et al., 2010, Norris et al., 2015, Lee et al., 2014) should enable better detection of phenotype-genotype association than was possible using relatively coarse genetic units such as chromosomal inversions (Mnzava et al., 1994, Petrarca and Beier, 1992).

A wide variety of approaches have been taken to study association between insect genetics and behaviour including single nucleotide polymorphisms (SNPs) (Wondji et al., 2007a, Neafsey et al., 2010, Weetman et al., 2010, Lee et al., 2014) transcriptomic approaches (Das et al., 2010, Rinker et al., 2013) and candidate gene approaches (Fitzpatrick et al., 2005, Ingham et al., 2014). Some of these techniques such as whole genome sequencing require full sequence data and are expensive. The candidate gene approach uses a targeted approach to investigate associations with specific, pre-selected genes that are hypothesized to have a strong impact on the trait of interest through previous work or biological intuition (Fitzpatrick et al., 2005). The candidate gene approach has been used widely in studies of behavioural ecology, and in particular with insect model systems (Fitzpatrick et al., 2005). Notable examples of the successful use of this approach include demonstration that the cGMP-dependent protein kinase (PKG) which is encoded by the *for* gene has a strong influence on the foraging behaviour of *Drosophila* (Osborne et al., 1997), and that its ortholog influences the division of labour in honey bees (Ben-Shahar, 2002). Compared to the other methods, the candidate gene approach seems to be economic as genes linked with behaviour and phenotypic variation are often conserved across insect taxa, and thus may be hypothesized to play the same role in malaria vectors.

There are several ways through which candidate genes may be associated with behaviour. One may be that the genes themselves directly code for a protein that determines behaviour (Robinson, 2004, Osborne et al., 1997), the other is through differential regulation of gene expression (Ben-Shahar, 2002). It is also possible that that candidate genes may not be directly associated with a particular phenotype, but may interact with other genes to result into a phenotype (epistasis) (Mackay, 2001). Candidate genes may also regulate more than one behaviour in an organism (pleiotropy)(Carreira et al., 2013). In cases

where behavioural differences are due to allelic variation, behavioural phenotypes would be heritable and expected to respond to natural selection. In contrast where behavioural variation is due to changes in gene expression which could be influenced by environmental factors (Rittschof and Robinson, 2013), then behavioural changes could occur rapidly even within an individual by phenotypic plasticity, and could be reversed over time. Both types of genetic effects could change mosquito vector behaviour, but distinguishing which mechanism is most prominent for behavioural changes (heritable genetic changes or phenotypic plasticity) will help resolve how quickly vectors can adapt to control measures.

Periodicity has been shown to be governed by circadian rhythm genes across a broad range of living organisms making the study of the influence of these genes on behaviour increasingly important (Steinmeyer et al., 2012, Johnsen et al., 2007, Lippert et al., 2014, Balmert et al., 2014, Oishi et al., 2009, Weyman et al., 2006). Rhythmic genes in animals known as circadian clock genes are a network of transcriptional factors regulating their own production at the level of RNA synthesis. In *Drosophila* for example, the *Period* (PER) and *Timeless* (TIM) genes can regulate their own expression through negative feedback mechanisms by suppressing two activators of PER and TIM transcription (known as Clock [CLK] and Cycle [CYC]; Price et al. 1998). Post translational control of circadian rhythmicity is attributed to *Doubletime* (*DBT*) which is a clock gene found in *Drosophila* which phosphorylates the transcription factor PER (Price et al., 1998). Photoreceptors such as Cryptochromes (CRY) are one of the transcriptional factors regulating circadian activities in living beings (Partch and Sancar, 2005). Cryptochromes which are flavin binding proteins found in mammals, insects and plants (Chaves et al., 2011, Hoang et al., 2008), regulate circadian activities by degradation of TIM via ubiquitin in the presence of light (Peschel et al., 2009).

Circadian genes such as PER which influences diel periodicity were first found in *Drosophila melanogoster* (Konopka and Benzer, 1971a), and later shown to play a similar role in many other organisms including other insects such as flies (Warman et al., 2000, Ikeno et al., 2008, Kostal et al., 2009, Takekata et al.,

2014). In *Drosophila simulans*, the *Thr-Gly* repeat within the PER gene, has been shown to lengthen the circadian period of locomotor activity (Rogers et al., 2004). In other insect vectors, love song patterns in *Lutzomyia cruzi* and *Lutzomyia longipalpis* have also been associated to polymorphisms in the *Period* gene (Vigoder et al., 2010). Further investigation of the role played by polymorphisms in the circadian genes on malaria vector periodicity could shed light in understanding whether the observed intra-specific differences in malaria vector biting behaviours are genetically driven.

So far, only a few studies have investigated circadian rhythm genes in *An. gambiae* s.l. and their potential association with diel activity such as blood feeding. Early studies applied transriptomic approaches to study gene expression patterns (Rund et al., 2013a, Das et al., 2010, Das and Dimopoulos, 2008), and established a genome-wide profiling of circadian gene expression in *An. gambiae* s.s. (Rund et al., 2011). Rhythmically expressed proteins such as the odorant binding proteins (OBPs) that regulate blood feeding behaviour have been shown to have corresponding rhythmic protein levels when measured by quantitative proteomics (Rund et al., 2013a). Specific differences in olfactory sensitivity of antennae during the day have been shown in relation to major host derived odorants. For example, the pre-dusk/dusk peaks in Odorant Binding Proteins (OBPs) coincided with the time of increased olfactory activity and host seeking in a laboratory population of *An. gambiae* s.s. (Rund et al., 2013a). When the rhythmic expression of the OBPs collected from heads under light-dark cycles were compared between *An. gambiae* and *Aedes aegypti*, distinct similarities and differences in temporal regulation of the genes in olfaction and vision were observed (Rund et al., 2013b). This demonstrates the key role played by OBPs in controlling time-related odorant sensitivity therefore enabling coordination of circadian activities in *An. gambiae* s.s. The host species preference of the major dengue mosquito vector *Aedes aegypti* has also recently been linked to variable expression of OBPs (McBride et al., 2014). Natural mutations in some of the genes regulating periodicity in insects have been reported to correlate with specific behaviours or expression levels of the mutant gene. For example male courtship songs in *Drosophila melanogaster* were found to correlate with three allelic mutations in the PER gene (Kyriacou and Hall,

1980). In Drosophila a clock regulated gene known as *to* was implicated in the regulation of the feeding behaviour and the mutants of this gene showed a down-regulated expression of the gene (So et al., 2000). Gene expression studies have been able to find associations between expression levels and behaviour in *An. gambiae* s.l. (Rinker et al., 2013, Rund et al., 2013a, Dottorini et al., 2013), however there has been little investigation of the link between mosquito feeding behaviours and single nucleotide polymorphisms (SNPs) in the circadian genes, which could give rise to consistent variation in diel activity patterns.

In this study, I hypothesized that mutations in circadian rhythm genes may explain variation in the host seeking times of African malaria vectors. I used single nucleotide polymorphisms (SNPs) to test for associations between coding mutations in 8 circadian genes and the times at which *An. arabiensis* were caught host seeking within a natural population in Tanzania.

5.3 Methods

5.3.1 Study site

Host seeking *An. arabiensis* were collected from the two villages of Lupiro and Sagamaganga of the Kilombero Valley using the Human Landing Catch (HLC) technique (Bockarie et al., 1996, Mboera, 2005). In brief, mosquitoes were collected from two villages of the Kilombero Valley between January and March 2012. In each village, mosquitoes were sampled from 3 households using the HLC technique. HLC's were conducted simultaneously indoors and outdoors at all households starting from 7pm-7am. Each of the two villages was sampled for 3 days thus making the whole sampling exercise complete within one week.

5.3.2 Behavioural phenotype selection and mosquito sample collection

To conduct the HLC, a volunteer sat on a chair with his legs exposed from foot to knee. Using a mouth aspirator, the volunteer sucked up mosquitoes that landed on their exposed leg before they started feeding. As described in Chapter 3, mosquitoes collected during each hour were stored in separate holding cups. Those morphologically identified as being *An. gambiae* s.l. were stored in 80%

ethanol to preserve DNA for downstream molecular assays. *An. gambiae* s.l. specimens were classified into one of four categories based on the time and location they were caught host seeking: (1) indoor early feeding, (2) indoor late feeding, (3) outdoor early feeding and (4) e outdoor late feeding. Early feeding was defined as mosquitoes collected whilst host seeking in the early hours of the night (7pm-10pm), while late feeding mosquitoes were collected between 4am-7am. The early and the late biting behaviours were based on research that identified tendency of the major malaria vectors in the Kilombero valley to have changed their nocturnal biting behaviour towards earlier or later night biting (Russell et al., 2011).

The four biting phenotypes from the two villages were abbreviated as: Lupiro early indoor feeders (LEI), Lupiro early outdoor (LEO), Lupiro late indoor (LLI), Lupiro late outdoor (LLO), Sagamaganga early indoor (SEI), Sagamaganga early outdoor (SEO), Sagamaganga late indoor (SLI) and Sagamaganga late outdoor (SLO). *A priori,* it was decided that a target sample size of approximately 100 mosquitoes from each of the four biting phenotypes would be required for robust genetic analysis. This was achieved for all phenotypes in both villages, yielding a total of 800 samples for genetic analysis.

5.3.3 Species diagnosis and SNPs discovery analysis

Genomic DNA extraction was conducted using DNeasy extraction kits (QIAGEN, Valencia, CA). First, PCR analysis was performed on specimens that were morphologically identified as belonging to the *An. gambiae* s.l. complex) according to Scott's method (Scott et al., 1993). Only samples that successfully amplified and were confirmed as *An. arabiensis* were subsequently used in SNP analysis. All successfully amplified samples were *An. arabiensis*. The number of *An. arabiensis* used for SNPs assays from each of the phenotypes from the two villages is given in brackets: LEI (95), LEO (91), LLI (96), LLO (96), SEI (96), SEO (96), SLI (96) and SLO (96).

Three hundred and seventy eight (378) samples from Lupiro and 384 from Sagamaganga were SNP genotyped making a total of 762 samples genotyped for the entire study. Eight circadian candidate genes originally identified from the

An. gambiae s.s. genome were selected for SNP discovery using conventional Sanger sequencing. These genes were Cyclic, *Clock, Period, Timeless, Pdp1, Cryptochrome1, Cryptochrome2* and *Vrille*, which were selected on the basis of their known association with circadian rhythmic behaviours in other insect taxa including *An. gambiae* s.s.(Rund et al., 2013b, Rund et al., 2011, Meireles-Filho et al., 2006). A series of primers were designed for each gene fragment by our project collaborator Yoosook Lee (University of California-Davis) using Primer3 online tools (http://frodo.wi.mit.edu/primer3/). The identity, specific loci sequenced and primer sequences used for all 8 candidate genes are presented in Table 5.1.

Gene	Gene ID	Loci ID	PCR product size (bp)	Forward primer	Reverse primer
Clock	AGAP005711	CLK-E01-267	119	GTAAAATACTCTCCCGGTA	GTAAAATACTCTCCCGGTG
Clock	AGAP005711	CLK-E01-192	113	GCTTCGTTCGAGAGAAAGGAA	GCTTCGTTCGAGAGAAAGGAG
Clock	AGAP005711	CLK-E01-087	106	CTTGCGCACGGTCGACTTGTCCATC	CTTGCGCACGGTCGACTTGTCCATT
Clock	AGAP005711	CLK-E01-240	119	TTCCCGATGATGAACCCGTCC	TTCCCGATGATGAACCCGTCT
cryptochrome1	AGAP001958	CRY1-E04-206	120	TCGACGGCGCAGCACGGA	TCGACGGCGCAGCACGGT
cryptochrome1	AGAP001958	CRY1-E04-097	99	CGCACGTCCATCGTTC	CGCACGTCCATCGTTT
cryptochrome1	AGAP001958	CRY1-E04-240	120	CTACCACCAGCAGCTGTCCA	CTACCACCAGCAGCTGTCCG
cryptochrome1	AGAP001958	CRY1-E04-252	102	CGACCTTGACCGACAGTTC	CGACCTTGACCGACAGTTT
cryptochrome2	AGAP004261	CRY2-E05-378	113	CCACTGCCATTGCCACCA	CCACTGCCATTGCCACCG
cryptochrome2	AGAP004261	CRY2-E05-561	98	GGCGCAGTCGCAGGAAAAC	GGCGCAGTCGCAGGAAAAT
cryptochrome2	AGAP004261	CRY2-E05-501	100	TGAGAATGCTGCAGCTGTGAC	TGAGAATGCTGCAGCTGTGAT
cryptochrome2	AGAP004261	CRY2-E05-407	113	GCCTTGTTTGGTGTCGTCAGGCA	GCCTTGTTTGGTGTCGTCAGGCG
cryptochrome2	AGAP004261	CRY2-E05-045	85	TCCGCTGCCGATGGTC	TCCGCTGCCGATGGTT
cryptochrome2	AGAP004261	CRY2-E05-125	82	CCCCAATACCGCACACCGAA	CCCCAATACCGCACACCGAG
cryptochrome2	AGAP004261	CRY2-E05-351	102	TATCGTGGGTCCGGGCCGCTA	TATCGTGGGTCCGGGCCGCTG
cryptochrome2	AGAP004261	CRY2-E05-051	85	GCGGGAAGCAATCGCA	GCGGGAAGCAATCGCG

Table 5.1 continued

Gene	Gene ID	Loci ID	PCR product size (bp)	Forward primer	Reverse primer
Cyclic	AGAP005655	CYC-E01-177	117	ATTGCTGTTGGAGGGTTTA	ATTGCTGTTGGAGGGTTTG
Cyclic	AGAP005655	CYC-E01-217	118	CCACTCGTTACACCCTGAGGG	CCACTCGTTACACCCTGAGGT
Cyclic	AGAP005655	CYC-E01-093	96	GGCAGCGTCCGATTTAAGCCCA	GGCAGCGTCCGATTTAAGCCCG
Cyclic	AGAP005655	CYC-E01-072	96	GGGTAAAGTGAAGGAGCAACTC	GGGTAAAGTGAAGGAGCAACTG
Cyclic	AGAP005655	CYC-E01-268	118	ACTTTGCACTTCATCCGA	ACTTTGCACTTCATCCGG
Cyclic	AGAP005655	CYC-E01-250	118	TGGAAGAAGGAACGGCGC	TGGAAGAAGGAACGGCGA
Cyclic	AGAP005655	CYC-E01-021	98	TTGATCTTCTTGGGCAGAGC	TTGATCTTCTTGGGCAGAGT

Table 5.1 continued

Gene	Gene ID	Loci ID	PCR product size (bp)	Forward primer	Reverse primer
Pdp1	AGAP006376	PDP1-E02-110	116	ATCGTCGCGGGACCGCTTC	ATCGTCGCGGGACCGCTTT
Period	AGAP001856	PER-PAS-082	101	CGGCTTCCCCAAGGAC	CGGCTTCCCCAAGGAT
Period	AGAP001856	PER-PAS-355	100	AGAAGGCGGAGATCATGAGCGGC	AGAAGGCGGAGATCATGAGCGGT
Period	AGAP001856	PER-PAS-202	114	GGGGAAAGAGCGGCCAGAAGGAC	GGGGAAAGAGCGGCCAGAAGGAT
Period	AGAP001856	PER-PAS-370	100	GGTGGCCGAGATGATC	GGTGGCCGAGATGATG
Timeless	AGAP007801	TIM-E05-075	111	GCCCCGTTGACGCTGTCC	GCCCCGTTGACGCTGTCG
Timeless	AGAP007801	TIM-E05-087	111	GTATCTGCGTTCCGATGTCG	GTATCTGCGTTCCGATGTCT
Timeless	AGAP007801	TIM-E05-189	98	GTCCGCTACGACACAC	GTCCGCTACGACACAT
Timeless	AGAP007801	TIM-E05-495	101	CCTACGCTGATTGCCTGGCTA	CCTACGCTGATTGCCTGGCTG
Vrille	AGAP007801	VRI-E02-427	118	CCCCGATAAGGATGCGGCCACC	CCCCGATAAGGATGCGGCCACT
Vrille	AGAP007801	VRI-E02-355	108	AAGTTGGCGTGCTCGTGA	AAGTTGGCGTGCTCGTGG

Table 5.1. Candidate gene and loci identity with the forward and reverse primer sequences. PCR products sizes are in base pairs (bp).

Eight *An. arabiensis* samples from each of the 4 feeding phenotypes from both Lupiro and Sagamaganga villages were sequenced for SNP discovery. From each of the 8 genes selected, a total of 34 loci were selected for sequencing. These loci were selected from conservative regions of DNA and included synonymous and non-synonymous mutations that have a codon frequency change of 2 or greater, which is a standard approach to identify mutations that are most likely to influence protein function (Kimchi-Sarfaty et al., 2007). Eight circadian gene fragments were amplified and sequenced for each of the 64 samples (e.g. 8 candidate genes x 4 phenotypes x 2 villages). The identity of these genes in *An. gambiae s.s.* (as acquired from the Vectorbase; https://www.vectorbase.org/faqs) and their chromosomal locations are shown in Table 5.2.

SNP ID	Reference Gene ID (*An. gambiae*)	Chromosome	SNP type	Reference *An. gambiae* codon	Variant codon	Reference amino acid	Variant amino acid	Mutation type
PER-PAS-202	AGAP001856	3R	T/C	GAC	GAU	D	D	S
PER-PAS-082	AGAP001856	3R	C/T	GAU	GAC	D	D	S
PER-PAS-301	AGAP001856	3R	G/A	CGG	CGA	R	R	S
PER-PAS-355	AGAP001856	3R	T/C	GGC	GGU	G	G	S
PER-PAS-370	AGAP001856	3R	G/C	CUC	CUG	L	L	S
CRY1_E04-240	AGAP001958	2R	T/C	CGG	UGG	R	W	NS
CRY1_E04-206	AGAP001958	2R	T/A	UCA	UCU	S	S	S
CRY1_E04-097	AGAP001958	2R	A/G	GAA	AAA	E	K	NS
CRY1_E04-252	AGAP001958	2R	T/C	UUU	UUC	F	F	S
CRY2_E05-351	AGAP004261	2R	C/T	CUU	CUC	L	L	S
CRY2_E05-378	AGAP004261	2R	C/T	CUC	CUU	L	L	S
CRY2_E05-407	AGAP004261	2R	G/C	AGG	AGC	R	S	NS
CRY2_E05-501	AGAP004261	2R	A/G	GCA	ACA	A	T	NS
CRY2_E05-561	AGAP004261	2R	T/C	AAC	AAU	N	N	S
CRY2_E05-045	AGAP004261	2R	T/C	GUU	GUC	V	V	S
CRY2_E05-125	AGAP004261	2R	G/A	GCG	ACG	A	T	NS

Table 5.2 continued

SNP ID	Reference Gene ID	Chromosome	SNP type	Reference codon	Variant codon	Reference amino acid	Variant amino acid	Mutation type
CYC_E01-268	AGAP005655	2L	T/C	UGC	UGU	C	C	S
CYC_E01-250	AGAP005655	2L	G/T	UCG	GCG	S	A	NS
CYC_E01-217	AGAP005655	2L	C/A	ACC	CCC	T	P	NS
CYC_E01-093	AGAP005655	2L	G/A	ACG	GCG	T	A	NS
CYC_E01-072	AGAP005655	2L	G/C	CUC	CUG	L	L	S
CYC_E01-021	AGAP005655	2L	T/C	AGU	AGC	S	S	S

Table 5.2 continued

SNP ID	Reference Gene ID	Chromosome	SNP type	Reference codon	Variant codon	Reference amino acid	Variant amino acid	Mutation type
Clk_E01-087	AGAP005711	2L	G/A	GAU	AAU	D	N	NS
Clk_E01-192	AGAP005711	2L	C/T	CUC	UUC	L	F	NS
Clk_E01-240	AGAP005711	2L	G/A	CUG	CUA	L	L	S
Clk_E01-267	AGAP005711	2L	C/T	CAC	UAC	H	Y	NS
PDP1_E02-110	AGAP006376	2L	G/A	GCG	GCA	A	A	S
Vri_E02-355	AGAP007801	3R	C/T	AUC	AUU	I	I	S
Vri_E02-427	AGAP007801	3R	C/T	ACC	ACU	T	T	S
TIM_E05-087	AGAP007801	3R	C/A	CCC	CCA	P	P	S
TIM_E05-189	AGAP007801	3R	A/G	AUG	GUG	M	V	NS
TIM_E05-075	AGAP001856	3R	G/C	GAC	CAC	D	H	NS

Table 5.2. Loci identity showing reference genes, chromosomes of origin, mutated nucleotide and variant codons. The loci were selected from the 8 circadian genes; period (PER), cryptochrome1 (CRY1), cryptochrome2 (CRY2), cyclic (CYC), clock (CLC), Pdp1, vrille (Vri) and Timeless (TIM). Mutation type s means synonymous mutation and ns means non-synonymous mutation.

A priori optimization of PCR was conducted by varying thermocycler conditions for the annealing temperature and initial denaturation while observing which conditions produced the strongest PCR products on a gel. All 8 circadian genes were amplified in a 25µl reaction volume using the resultant optimized circadian gene PCR which included 0.2 X Q solution (Qiagen) (Q solution optimizes the melting behaviour of nucleic acids that have high degree of GC nucleotides), 1X buffer (Sigma-Aldrich), 1 mM MgCl, 0.4mM DNTP, 0.1mM forward primer, 0.1mM reverse primer, 1 Unit of HotstarTaq Plus DNA polymerase (Qiagen), ~8µg/µl DNA and pure water. Negative controls for each of the 8 genes were run along with the samples which was 25µl of TE buffer without any DNA in it. Successful amplification of PCR products was verified using QIAxcel ScreenGel (Qiagen) software version 1.2. The concentration of DNA was measured by spectrophotometry using NanoDrop 1000 V3.7 (Thermo Fisher Scientific Inc.). Ten microliters of the amplified DNA products were purified using DNASap purification kit of which 5µl were sent for conventional post-PCR Sanger sequencing at a DNA sequencing facility at the University of California Davis. Sanger sequencing was used on a subset of the 64 samples used for the purpose of SNP discovery. Each gene fragment was sequenced in both forward and reverse directions. Sequences were then checked for quality control using Geneious software version 6.1(http://desktop-links.geneious.com/download), in which manual alignment of forward and reverse strands was conducted. Poor quality sequences were trimmed, and alignment of reads to reference sequences was made.

5.3.4 SNP genotyping assay and statistical analyses

The Typer® AssayDesigner software (http://www.sequenom.com/home/products—services/genetic-analysis/, San Diego, CA) was used to devise a multiplex SNP genotype assay.

A total of 762 samples were genotyped using Sequenom MassARRAY iPLEX platform for a set of 34 loci from the 8 circadian genes. Negative controls were run for each plate of samples genotyped. A signal to noise ratio of 3 or above the background level was used to call genotypes. The TyperAnalyzer Application (Sequenom Inc.) version 4.0.24.71 was used to score genotypes across all 34 loci. The population genetics software DnaSP version 5 (Librado and Rozas, 2009) was used to identify haplotype sequences from the iPLEX using phase algorithm to score SNP density, calculate Tajima D statistics, the number of nucleotide substitutions per phenotype based on direct sequencing results, and the number of shared mutations between groups. Tajima D statistics were calculated as this method is used to distinguish between a DNA sequence evolving neutrally and one which evolves under non-random process (Tajima, 1989). The test metric, Tajima D, expresses the difference between two estimates of the expected number of single nucleotide polymorphisms relative to their standard error as in the formula:

$$D = \frac{d}{\sqrt{V}}$$

where d is the difference between two estimates of the expected number of single nucleotide polymorphisms and v is the variance of the expected number of SNPs. A negative Tajima D signifies an excess of low frequency polymorphisms relative to expectation, which may indicate a recent selective sweep or population size expansion, while a positive Tajima D signifies low levels of both low and high frequency polymorphisms indicating balancing selection or decrease in population size (Tajima, 1989). Values of D greater than +2 or less than -2 are said to be significant. The number of nucleotide substitutions was also calculated as this can be used to represent the evolutionary substitution of one nucleotide base for another in an exon of a gene coding for a protein. Non-synonymous substitutions result in amino acid sequence changes, while synonymous substitution results in unchanged proteins (Kimchi-Sarfaty et al., 2007).

The Arlequin software version 3.5 (Excoffier and Lischer, 2010) was used to test departure from Hardy-Weinberg equilibrium (HWE) within each loci. The STRUCTURE software (Pritchard et al., 2000) was used to conduct clustering

analysis to assign populations or individuals into their membership groups based on the feeding behavioural phenotypes (i.e. time and location of feeding). An assumption of up to 10 popolations (K=10) was made. STRUCTURE harvester (Earl and vonHouldt, 2012) software was used to assess the ΔK statistic (Evanno et al., 2005) which is used to select the number of distinct genetic clusters(K). IndQsort (http://grassi2.ucdavis.edu/~yoosook/Scripts/indQsort/) was used to reorder individuals according to their membership coefficients. Visualization of population clustering was obtained using the Distruct software (http://www.stanford.edu/group/rosenberglab/distruct.html). A confirmation of the genetic clustering analysis was done using Principal Component Analysis (PCoA) implemented in GenALEx (Peakall and Smouse, 2012) and available as a plug-in in Excel. Variation due to a set of variables is explained by the first three components (principal components) which are picked in the order of how much each of the principal components explains variation in the dataset. The first principal component explains the largest proportion of variation, followed by the second principle component and then the third principal component. In this way PCoA reduces the number of variables explaining variation in multivariate data into just 3 principal variables thus making multivariate analysis simpler. The major output results of this analysis is a scatter plot showing how each data point is scattered between the 3 principal components including the proportion of variation explained by each of the first three principal components.

5.4 Results

In total 1,246 *An. gambiae* s.l. mosquitoes were sampled from the two villages of Lupiro and Sagamaganga. The biting phenotypes, site of collection and the total number collected are given in Table 5.3. For SNP discovery, only 64 samples representing 8 samples from each of the 8 populations were genotyped, while for Iplex genotyping between 91-96 mosquitoes were genotyped from each of the 8 populations making a total of 762 samples that were genotyped using the iPLEX SNP genotyping assay in this study. Species identification of all 762 *An. gambiae*

s.l. from Lupiro and Sagamaganga villages through PCR found all samples to be *An. arabiensis*.

Village	Lupiro				Sagamaganga			
Phenotype	LEI	LEO	LLI	LLO	SEI	SEO	SLI	SLO
Number sampled	110	198	145	133	142	151	167	200
Number analyzed	95	91	96	96	96	96	96	96

Table 5.3. The number of *An. gambiae* s.l. sampled from each of the two villages including their biting phenotypes: LEI (Lupiro early indoors), LEO (Lupiro early outdoors), LLI (Lupiro late indoors), LLO (Lupiro late outdoors), SEI (Sagamaganga early indoors), SEO (Sagamaganga early outdoors), SLI (Sagamaganga late indoors), SLO (Sagamaganga late outdoors).

5.4.1 Sequencing and SNPs discovery

Sequencing of the 8 circadian genes discovered an average of one SNP in every 46.8±34.5 base-pairs, with *Cyc* and *Vri* E027461 having the highest and the lowest densities at one SNP per every 10 bp and 125 bp respectively. The number of haplotype sequences ranged between 16 and 42 per gene across all phenotypes. The number of segregating sites varied from 0 to 32 per gene fragment with a mean number of 9.8±7.6 and a median value of 8. The segregating gene fragments varied in length between 169bp to 593bp. *Pdp1* exon E021381 had the lowest number of segregating sites ranging from 0 to 3, while *Tim* exon E052569 had the highest number of segregating sites varying from 20 to 42.

5.4.2 iPLEX SNP genotyping

There were more synonymous mutations across all genes (n=50) than non-synonymous mutations (n=27) in the coding sequences, indicating most of the SNPs were not involved in altering protein structure. Twenty one (21) out of the 34 loci genotyped had synonymous mutations while the remaining 13 loci had non-synonymous mutations (Table 5.4). SNPs in the *Timeless* gene had the highest number of synonymous mutations (ranging from 10-15, Table 5.4) in all the 4 phenotypes. This was much higher than the range of 0-8 synonymous mutations detected in the remaining circadian genes used in this study (Table 5.4). Non-synonymous mutations in the *Timeless* gene ranged from 1-2, which was notably lower than those reported in the 2 genes with the highest rates of non-synonymous mutations (e.g. *Cryptochrome2* and *Cyclic* genes which ranged from 10-21, Table 5.4). The remaining genes had low to moderate numbers of synonymous and non-synonymous mutations ranging from 0-7 and 0-7 respectively. There were no fixed polymorphisms but there were many shared polymorphisms.

Chr[a]	Gene ID[b]	Gene[c]	Pop[d]	N[e]	n_s[f]	π(%)[g]	D[h]	μ_s[i]	μ_{NS}[j]	μ_{NCS}[k]	SNPd[l]	LE:LL[m]	SE:SL[n]	LE:SE[o]	LL:SL[p]	Cod[q]
2L	AGAP005711	Clk	LE	30	12	0.0057	-1.6620	7	4	7	24.31	6	4	7	4	1
			LL	26	7	0.0034	-1.2588	7	0	7						
			SE	30	8	0.0032	-1.5161	8	0	8						
			SL	24	4	0.0032	-0.1632	4	0	4						
2R	AGAP001958	Cry1	LE	22	8	0.0051	-0.9904	1	7	1	27.36	7	6	7	6	2
			LL	18	10	0.0050	-1.7391	1	9	1						
			SE	28	10	0.0061	-0.9119	1	9	1						
			SL	20	6	0.0054	-0.1223	1	5	1						
2R	AGAP004261	Cry2	LE	28	16	0.0057	-0.5965	0	16	0	31.31	12	13	15	13	1
			LL	20	15	0.0054	-0.9156	0	15	0						
			SE	36	16	0.0050	-0.7496	0	16	0						
			SL	22	15	0.0054	-0.7795	0	15	0						
2L	AGAP005655	Cyc	LE	18	16	0.0138	-0.1541	5	10	5	9.53	13	25	16	23	1
			LL	18	32	0.0301	-0.8217	7	21	7						
			SE	20	26	0.0277	0.5346	6	16	6						
			SL	16	23	0.0198	-0.7512	6	16	6						
2L	AGAP006376	Pdp1	LE	28	3	0.0021	-1.3214	3	0	3	56.33	0	0	0	0	1
			LL	20	0	NA	NA	NA	NA	NA						
			SE	26	0	NA	NA	NA	NA	NA						
			SL	26	2	0.0009	-1.5131	2	0	2						

Table 5.4 continued

Chr[a]	Gene ID[b]	Gene[c]	Pop[d]	N[e]	n_s[f]	Π(%)[g]	D[h]	μ_s[i]	μ_{NS}[j]	μ_{NCS}[k]	SNPd[l]	LE:LL[m]	SE:SL[n]	LE:SE[o]	LL:SL[p]	Cod[q]
3R	AGAP001856	Per PAS	LE	26	5	0.0033	0.0343	0	5	0	68.00	4	3	4	4	1
			LL	18	5	0.0037	0.1080	0	5	0						
			SE	26	4	0.0036	0.1083	0	4	0						
			SL	20	4	0.0028	0.0781	0	4	0						
3R	AGAP001856	Tim	LE	42	17	0.0073	0.4200	15	2	15	31.82	12	11	11	11	1
			LL	32	12	0.0076	1.1916	10	2	10						
			SE	20	11	0.0057	1.6610	10	1	1						
			SL	22	14	0.0071	0.3339	13	1	13						
3R	AGAP007801	Vri	LE	28	4	0.0018	-0.4212	0	4	0	125.75	2	3	2	1	3
			LL	18	2	0.0010	0.9062	0	2	0						
			SE	30	3	0.0014	0.2328	0	3	0						
			SL	18	3	0.0014	-0.2589	0	3	0						

Table 5.4. Tajima's D statistics including synonymous and non-synonymous mutations and nucleotide diversity in 8 circadian genes and among early and late feeding phenotypes of *An. arabiensis*. (a)=Chromosome, (b)=*An. gambiae* reference gene ID, (c)=Gene, (e)=Number of haplotype sequences, (f)=Number of segregating sites, (g)=Nucleotide diversity, (h)=Tajima's D, (i)=Number of synonymous mutations, (j)=Number of non-synonymous mutations, (k)=Number of silent mutations, (l)=SNP density i.e. one SNP found in a given number of nucleotide base-pairs, (m)=shared polymorphisms between early and late in Lupiro, (n)=shared polymorphisms between early and late in Sagamaganga, (o)=shared polymorphisms in early biting between Lupiro and Sagamaganga, (p)=shared polymorphisms in late biting between Lupiro and Sagamaganga, and (q)=Codon at which the SNP occurs.

F_{ST} values were calculated to give an estimate of genetic distance between different feeding phenotypes of *An. arabiensis*. There was no evidence of genetic distance between *An. arabiensis* with different feeding location phenotypes (indoor vs outdoor, (F_{ST} <0.001), feeding times (early vs late, (F_{ST} <0.001), or between geographical locations (Sagamaganga vs Lupiro, F_{ST} <0.001). None of the Tajima's D values were significant (Table 5.4), indicating the SNPs are evolving neutrally with no evidence of selection, demographic expansion or contraction.

All SNPs were in Hardy-Weinberg equilibrium, suggesting an absence of selection on these candidate genes within the populations. Two distinct populations were found across all samples collected in Lupiro and Sagamaganga villages. Delta K statistics showed K=2 as the most probable number of clusters (Figure 5.1).

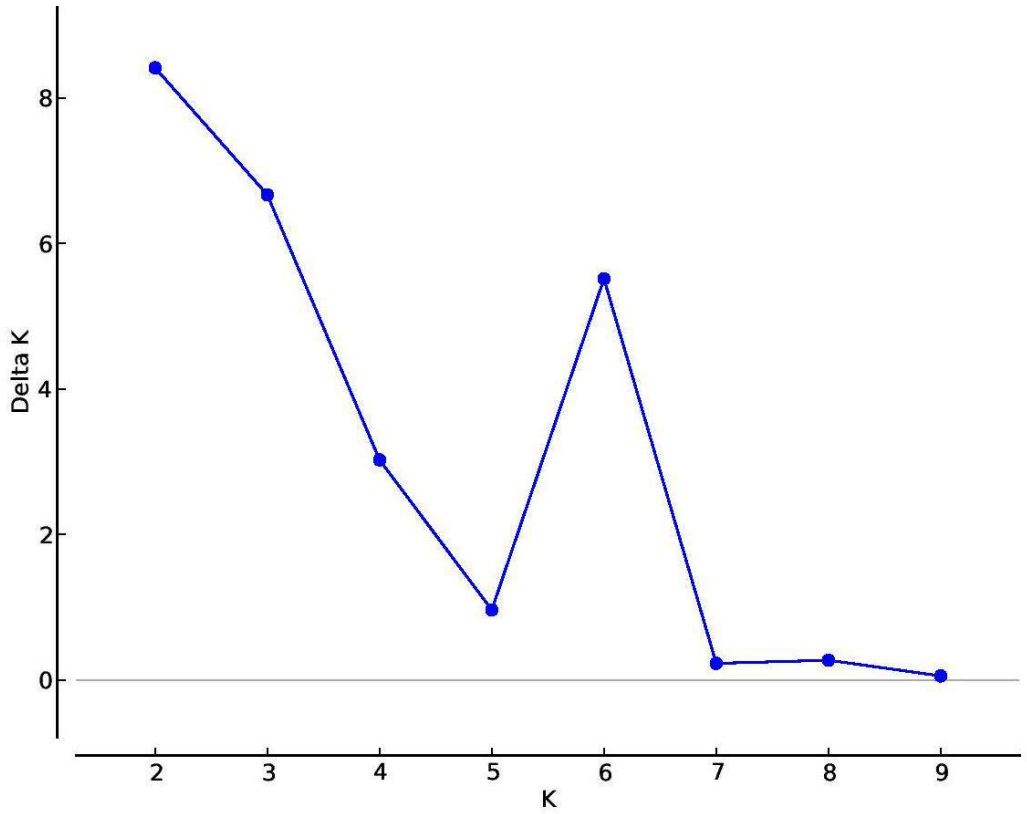

Figure 5.1. Bayesian clustering analysis. The magnitude of ΔK as a function of K showing K=2 as the most probable number of clusters in the samples. Assumption of 10 populations was made *apriori*.

However, based on STRUCTURE analysis these subdivisions were not based on the feeding phenotypes of *An. arabiensis* or geographical location (Figure 5.2).

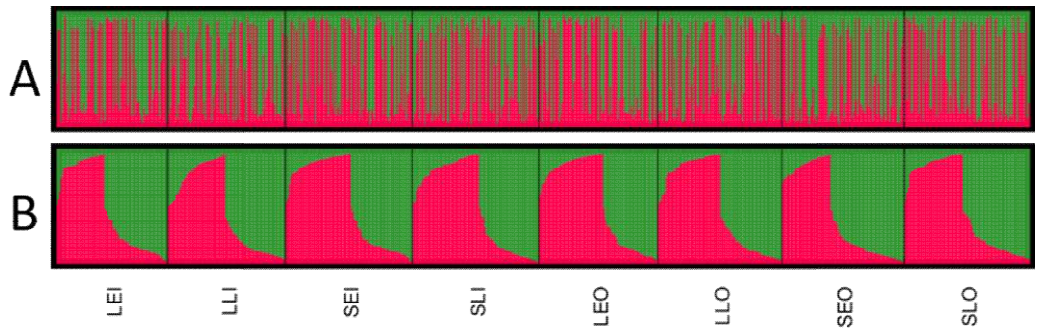

Figure 5.2. STRUCTURE clustering result with parameter K=2. A: individuals unordered. B:individuals sorted based on membership coefficients within each phenotypic group. There appears to be two population sub-divisions (red and green) across samples with different feeding behaviours.

Analysis of the membership coefficients of cluster 1 and 2 revealed that the frequencies of SNPs in the *Timeless* gene were most divergent between two clusters (Table 5.5). Due to the absence of fixed polymorphisms and assignment of all feeding phenotypes from all collection sites into one population by the STRUCTURE analysis, there was no data generated for gene diversity between various feeding phenotypes or sampling sites. Further clustering analysis was done using Principal Component analysis (PcoA). Results from this analysis showed no evidence of clustering based on the 8 feeding phenotypes (Figure 5.3) confirming STRUCTURE results while showing that up to 24.6% of all variation was due to the first 3 components (Figure 5.3).

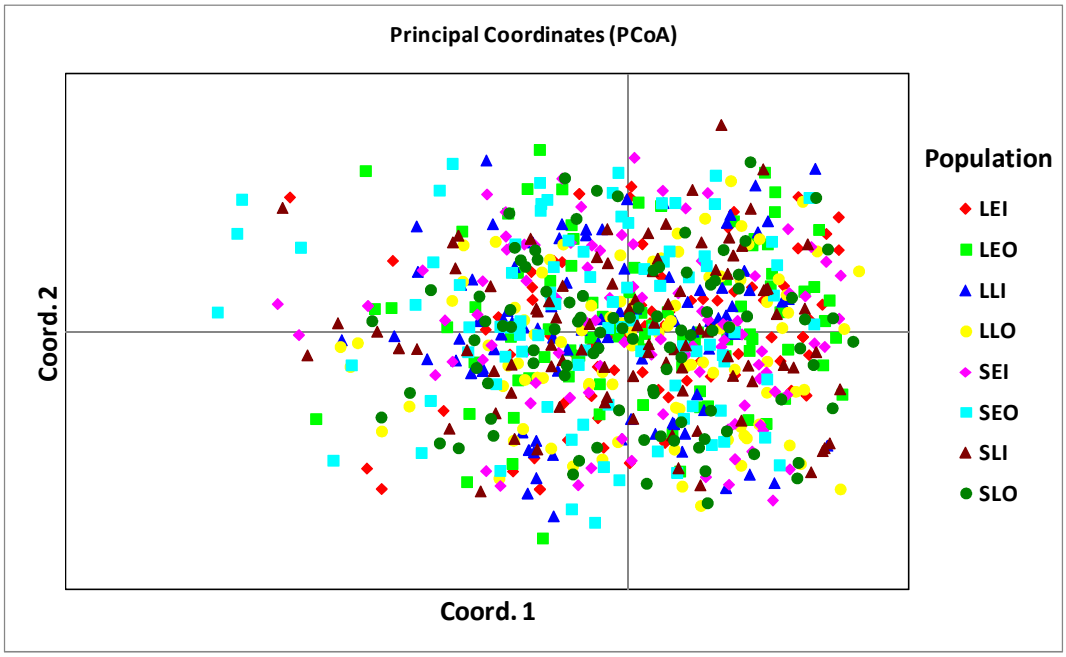

Figure 5.3. Principal Component Analysis based on the genetic distances generated by STRUCTURE at K=2. Coordinate 1 and 2 represent the first and second principal components respectively. PCoA analysis included a total of 730 samples from 8 feeding phenotypes from Lupiro and Sagamaganga: LEI (Lupiro early indoors), LEO (Lupiro early outdoors), LLI (Lupiro late indoors), LLO (Lupiro late outdoors), SEI (Sagamaganga early indoors), SEO (Sagamaganga early outdoors), SLI (Sagamaganga late indoors), SLO (Sagamaganga late outdoors).

Locus	SNP	Cluster 1	Cluster 2	Locus	SNP	Cluster 1	Cluster 2	Locus	SNP	Cluster 1	Cluster 2
clk-e01-087	G	0.957	0.952	cyc-e01-072	C	0.099	0.094	cry2-e05-378	C	0.883	0.889
	A	0.043	0.048		G	0.901	0.906		T	0.117	0.111
clk-e01-192	C	0.865	0.890	cyc-e01-093	G	0.461	0.503	cry2-e05-407	T	0.106	0.082
	T	0.135	0.110		A	0.539	0.497		C	0.894	0.918
clk-e01-267	T	0.847	0.900	cyc-e01-177	T	0.239	0.183	cry2-e05-501	G	0.088	0.088
	C	0.153	0.100		C	0.761	0.817		A	0.912	0.912
clk-e01-240	G	0.933	0.933	cyc-e01-217	C	0.931	0.893	cry2-e05-561	C	0.975	0.971
	A	0.067	0.067		A	0.069	0.107		T	0.025	0.029
cry1-e04-097	C	0.938	0.946	cyc-e01-021	T	0.567	0.630	cry2-e05-051	T	0.913	0.911
	T	0.062	0.054		C	0.433	0.370		C	0.087	0.089
cry1-e04-206	G	0.912	0.917	cyc-e021-250	G	0.119	0.096	cry2-e05-045	C	0.421	0.407
	A	0.088	0.083		T	0.881	0.904		T	0.579	0.593
cry1-e04-240	G	0.969	0.968	cyc-e01-268	T	0.481	0.471	cry2-e05-125	G	0.859	0.888
	A	0.031	0.032	-	C	0.519	0.529		A	0.141	0.112
cry1-e04-252	G	0.771	0.764	-	-	-	-	cry2-e05-351	T	0.533	0.528
	A	0.229	0.236	-	-	-	-		C	0.467	0.472

Table 5.5 continued

Locus	SNP	CLUSTER 1	CLUSTER 2	Locus	SNP	CLUSTER 1	CLUSTER 2	LOCUS	SNP	CLUSTER 1	CLUSTER 2
per-pas-082	C	0.929	0.931	tim-e05-189	G	0.513	0.022	pdp1-e02-110	G	0.967	0.977
	T	0.071	0.069		A	0.487	0.978	-	A	0.033	0.023
per-pas-202	C	0.643	0.624	tim-e05-495	G	0.764	0.991	-	-	-	-
	T	0.357	0.376		A	0.236	0.009	-	-	-	-
per-pas-355	C	0.799	0.829	tim-e05-075	G	0.570	0.994	vri-e02-355	C	0.978	0.987
	T	0.201	0.171		C	0.430	0.006		T	0.022	0.013
per-pas-370	C	0.646	0.569	tim-e05-087	C	0.187	0.489	vri-e02-427	C	0.974	0.963
	G	0.354	0.431		A	0.813	0.511		T	0.026	0.037

Table 5.5. STRUCTURE assignment of allele frequencies of circadian gene SNPs. The *Timeless* gene was associated with the binary clustering into clusters 1 and 2.

5.5 Discussion

The aim of this study was to investigate associations between variations in the time and location of *An. arabiensis* feeding behaviour and polymorphisms in the coding regions of circadian rhythm genes. Though this study did not find any such associations, there was evidence of genetic structuring within the sampled populations which was associated with variation in the *Timeless* gene (Table 5.5). The presence of genetic structuring in *An. arabiensis* irrespective of the feeding behaviour and geographical location is an interesting phenomenon which requires further investigation. Such local population subdivisions may have of relevance to malaria transmission especially if they are linked to epidemiologically significant traits such as insecticide resistance, susceptibility to infection, or other mosquito behavioural traits not investigated here (e.g. host species or habitat preference).

The SNP density reported for *An. arabiensis* in this study was 1 every 47bp. Compared with other recent studies of *An. arabiensis* (Marsden et al., 2014), this study detected a higher level of genetic polymorphism in *An. arabiensis*. One study conducted on *An. arabiensis* populations from Cameroon, Tanzania and Zambia reported one SNP in every 59 bp (Lee et al., 2012), which is comparable to the values reported here. However, another recent study estimated a lower rate of SNP diversity in populations of *An. arabiensis* from the Kilombero valley (Tanzania) and Cameroon based on analysis of SNP markers which were distributed more widely throughout the whole genome (e.g. 1/221bp-1/318bp depending on chromosome region (Marsden et al., 2014). The SNP density reported for *An. arabiensis* here was also several times higher than has been reported for its sibling species *An. gambiae* s.s. For example, data provided by the *An. gambiae* s.s. sequencing project Ensemble (http://www.metazoa.ensembl.org/Anopheles_gambiae/Info/Indexfrom) reports an average of one SNP on every 247 bp of the genome. In another study, a nuclear genome sequence from a laboratory strain of *An. gambiae* s.s. showed one SNP occurred in every 125 coding base-pairs (Morlais et al., 2004), while Lawniczak et al (2010) reported that genome sequences from *An. coluzzi* and *An. gambiae* s.s. contained ~1 SNP in every 130 bp. However in one recent study

(Wilding et al., 2009), a SNP density of 1 per 34 bp was reported, which is the highest reported in *An. gambiae* s.s. Thus while there appears to be a trend of greater nucleotide variation within *An. arabiensis* than in *An. gambiae* s.s, this distinction may not hold for all populations. In contrast to relatively large number of studies on *An. gambiae* s.s, there are relatively few studies characterizing SNP diversity in *An. arabiensis*. The considerable variation in SNP diversity as calculated both within and between malaria vector species may at least partially be due to the particular subset of markers used. This highlights the need for consistency when trying to draw conclusions between populations and the challenges associated with SNP assays in *An. arabiensis* given the highly polymorphic nature of its genome.

This study investigated genetic diversity within *An. arabiensis* based on a set of 8 circadian genes in which 34 loci incorporating 313 polymorphic sites were assayed. Based on this subset of genes, there was no evidence that the feeding behavioural phenotypes (early vs late and indoor vs outdoor feeding) of *An. arabiensis* clustered as distinct genetic subpopulations (Figure 5.1). There may be several reasons for this lack of association. First, it should be recognized that the molecular clock controlled by circadian genes is still poorly understood in haematophagous insects, in contrast to *Drosophila* species (Meireles-Filho et al., 2006). Candidate circadian genes used here were drawn from *Drosophila* and have been shown to have time-dependent expression in *An. gambiae* s.s. (Rund et al., 2013a), but their mechanism of action and daily expression patterns in relation to rhythmic activities in *An. arabiensis* have not yet been confirmed.

Failure to link genetic mutations to feeding behaviour phenotypes in *An. arabiensis* could also be due to other methodological issues including the usage of too few markers (linkage disequilibrium in *An. arabiensis* has been shown to breakdown within 200bp, (Marsden et al., 2014)), use of inappropriate markers, imprecise classification of phenotypes and/or simply due to the fact that extensive phenotypic plasticity in feeding behaviour is possible within one genotype (Whitman and Ananthakrishnan, 2009). Several studies including work in this thesis (e.g. Chapter 3 and 4) indicate that *An. arabiensis* exhibit

substantial variation in their time and location of biting (Kabbale et al., 2013, Reddy et al., 2011, Russell et al., 2011, Ndiath et al., 2014), resting behaviour (Pates and Curtis, 2005) and host species choice (Duchemin et al., 2001). A high plastic adaptability has also been shown in the highly anthropophilic *An. gambiae* s.s. which was reported to extend its host preference to non-human hosts following a reduction in the availability of human hosts (Lefevre et al., 2009). It is therefore possibility that naturally occurring variation in the time and location of *An. arabiensis* biting reflects its ability to plastically adapt to changing environmental conditions (Ancel, 2000, Huey et al., 2003).

Whilst behavioural phenotypes showed no genetic basis here, there was evidence of strong genetic clustering within *An. arabiensis* samples in association with the *Timeles*s gene. The association with *Timeless* gene was so strong that the same pattern of genetic structure was predicted from this gene alone as with all 8 circadian genes combined (Figure 5.1). Studies have shown that the *Timeless* gene is involved in regulating light and cycle rhythms which influence the feeding behaviour of *An. gambiae* s.s. (Das and Dimopoulos, 2008), and light and temperature variations in Drosophila (Tauber et al., 2007). Furthermore, markers based on the *Timeless* gene alone have been used to identify population structure in *An. cruzii* (Rona et al., 2009), and in the *An. triannulatus* complex (Silva-do-Nascimento et al., 2011). Mutations in the *Timeles*s gene have been associated with diapause in *D. melanogaster* (Sandrelli et al., 2007). The *Timeless* gene has also shown variation in expression levels across different times of day in the pitcher-plant mosquito *Wyeomyia smithii* (Mathias et al., 2005). Observation of the natural mutations happening in the *Timeles*s gene across various insect species which were linked with variation in specific behaviours such as diapauses (Tauber et al., 2007) and eclosion (Konopka and Benzer, 1971b) underpins the importance of the *Timeless* gene in controlling periodicity in insects and especially those that are vectors to malaria. In my study, *Timeless* gene seemed to subdivide the samples into two distinct populations with about similar size irrespective of the geographical location of the sample. While it may not be surprising to find that samples from different feeding phenotypes and from the 40km apart villages of Lupiro and Sagamaganga showing no genetic clustering, it is however interesting for samples within the

villages to cluster based on the *Timeless* gene. In a study of frequencies of allele (*ls-tim*) which is one of two alleles of the *Timeless* gene in Drosophila species, variation of frequencies of this allele was shown across geographical location between Italy, Israel and Zimbabwe (Tauber et al., 2007). It may be interesting to investigate how different alleles of the *Timeless* gene cluster across different geographical location in Africa to have a broader insight of the population structure of *An. arabiensis* within and between geographical localities. The link between the *Timeless* gene and population structure in these studies suggests that this gene may be playing a crucial role in population structure, which needs to be investigated further. Furthermore, the existence of such fine-scale genetic structure over such a small geographic area indicates there may be natural barriers to gene-flow within vector populations in the Kilombero Valley. This could have epidemiological relevance by limiting the spread of epidemiologically genotypes such as insecticide resistance.

A few previous studies have investigated the population genetics of *An. gambiae* s.l. in the Kilombero valley where this work was conducted. One study on *An. arabiensis* revealed strong structuring at a village-level in the Kilombero Valley, indicating *An. arabiensis* may exist in genetically distinct populations between villages situated only 40 km apart (Ng'habi et al., 2011). However, a recent study (Maliti et al., 2014) including samples from the Kilombero valley and outside the valley predicted that *An. arabiensis* exists as a single population in the Valley and outside the Valley to the coast of Tanzania and the islands of Zanzibar. Further, a previous continental analysis of *An. arabiensis* population structure predicted there to be relatively high levels of gene flow between populations more than a 1,000 km apart (Donnelly et al., 2001). These contrasting findings on the degree of population structure of *An. arabiensis* highlight may be partially due to limitations and discrepancies due to variation in the methods of analysis and selection of markers used, but alternatively could indicate the existence of population genetic structuring in these vectors which is driven by local or historical causes.

A potential limitation of this study which may have reduced ability to identify clear phenotype-genotype associations was that biting time phenotypes were

quite coarsely and perhaps imprecisely defined. Selection of phenotypes in this study was based on broad categorization into “early” vs “late” feeding groups, with each period spanning 3 hours of collection. Though such categorization was based on evidence from previous studies which show that some mosquitoes prefer to feed early at dawn while some feed late at night (Govella et al., 2010b, Fornadel et al., 2010a, Geissbuhler et al., 2007, Yohannes and Boelee, 2012), it is not evident that the two phenotypes represent two genetically different groups or just the same group feeding at two different periods of the night. Further studies involving finer scale timing of the feeding behaviour for example within an hourly interval could be tested in the future. Another potential imprecision is that the time at which mosquitoes were collected may not necessarily have reflected the time at which they initiated their host seeking. For example, those caught during the late period may actually have begun feeding during the early period of the night, but been unsuccessful in locating a host. It would be difficult to assess this under natural conditions, but more detailed investigation of a small number of mosquitoes under lab or semi-field settings may be viable. Finally, although the circadian genes investigated here were not linked with feeding behaviours, their variation may be associated with other behaviours which influence gene flow. Specifically the clustering of mosquito population into two groups by the *Timeless* gene in this study may mean presence of two coexisting populations which have mating incompatibility possibly through temporal and cytological incompatibility.

Future studies on the population genetics of *An. arabiensis* feeding behaviour may have to involve a broader sampling strategy both with respect to the range and resolution of phenotypes selected, and the number of SNP markers used to increase the possibility of detecting genetic influences on malaria vector feeding time and behaviour. Further, it is possible that circadian candidate genes may not be directly associated with a particular phenotype but may interact with other genes to generate the phenotype (epistasis) (Mackay, 2001). Consequently, other genes associated with the candidate genes used in this study may need to be included in future studies of their potential impact on daily activity phenotypes. A broader range of candidate genes with more loci is also ion into future studies to stronger resolution of potential genotype-phenotype

associations. For this purpose, perhaps whole genome sequencing approach would be preferred as it provides the highest resolution by involving SNPs from the entire genome. However, transcriptomic approaches have also been successfully used to detect associations between host choice behaviours and gene regulation in insect vectors (McBride et al., 2014) therefore such techniques are recommended in future studies.

5.6 Conclusion

This study did not find any association between feeding behavioural phenotypes (early vs late and indoor vs outdoor feeding) in *An. arabiensis* and single nucleotide polymorphisms identified from 8 circadian rhythm candidate genes. However, there was evidence that the population contained two distinct genetic clusters which were associated with the *Timeless* gene, independently of feeding phenotype or geographical location. It is highlighted that investigations of the genetic basis of the feeding behaviour in malaria vectors are still in their infancy, and will y require much further development through use of high-resolution markers distributed across the entire genome, and/ or the application of other methods including transcriptomic approaches to provide a strong test of genotype-phenotype associations. In studies where markers covering the whole genome have been applied, the high density and short LD in *An. arabiensis* observed (Marsden et al., 2014) implies that huge sample sizes could be needed to robustly test for such associations, which at present are not viable due to the high cost and time requirements. These limitations make a candidate gene approach more attractive in the short-term, however in future I would recommend this could be improved through use of a larger set of genes, selected from across the entire genome of malaria vectors.

Chapter 6: General discussions

6.1 Overview

Research conducted for this thesis aimed to develop new sampling tools for characterizing the behaviour of African malaria vectors, and apply these strategies to investigate the role of ecological and genetic factors in shaping mosquito behaviour. To achieve this, four major objectives were pursued: (1) development of mosquito electrocuting trap for sampling host seeking malaria vectors, (2) evaluation of the sampling performance of mosquito electrocuting traps in the field, (3) development and evaluation of resting traps for malaria vectors and (4) determination of the association between heterogeneity in the feeding behaviour of the major African malaria vector *Anopheles arabiensis* and single nucleotide polymorphisms in their circadian rhythm genes. Specifically, mosquito electrocuting traps (MET) were designed with the intention of developing new tools for sampling host seeking malaria vectors that do not expose the participants to potentially infectious mosquitoes. These traps were intended to have comparable sampling efficiency relative to the gold standard human landing technique (HLC); whilst avoiding the exposure risk this method entails.

The development process of the electrocuting traps including experience obtained from using them in the field was discussed in chapters 2 and 3. Chapter 4 describes a study evaluating the sampling efficiency of a variety of standardized resting traps manufactured from simple and low cost materials, with the intention of evaluating how various physical features of resting traps such as their shape, size of entrance and use of sticky surfaces influences their sampling efficiency. Mosquito traps developed and evaluated in this standard were compared with each other and standard methodology to assess the abundance and resting habitat preference of vectors (indoor vs out), and test associations with environmental variation. Lastly, chapter 5 investigated if heterogeneity in the time and location of *An. arabiensis* biting activity can be accounted for by variation in single nucleotide polymorphisms (SNPs) in candidate circadian rhythm genes. Key results from this body of work, their

potential implications for malaria control, and recommendations for future work are discussed in this chapter.

6.2 Summary of principal findings

The second chapter of this study describes the development of a novel mosquito electrocuting trap (MET) which can be used to sample vectors attempting to bite people in indoor and outdoor environments. The key aim was to develop a prototype for an electrocuting trap that delivered an optimal voltage for killing African malaria vector species, without burning or damaging specimens in a way that would prohibit morphological or molecular identification. Another aim was for the trap to be safe to use in close proximity to humans acting as bait sources, and not expose them to mosquito bites. The first prototype of mosquito electrocuting trap (MET1) did not achieve these goals because it had problems of an erratic power supply that was prone to short circuiting, and suboptimal wire spacing which was large enough to allow some mosquitoes to pass through without being killed. An improved prototype (MET2) was developed and evaluated in the field (Chapter 3). This version included reduction of the gap between the wires to prevent mosquitoes from passing through, and a customized and improved power source which delivered an output voltage that was experimentally demonstrated as optimal for high instant kill rates in *An. gambiae* s.s. and *An. arabiensis*. Field-testing of MET2 showed this version to be promising in terms of sampling efficiency (Chapter 3), however minor problems in trap function such as occasional short-circuiting and increased resistance due to semi-conducting effect of the wooden frame remained.

Whilst I set aside further development of the MET at this stage to concentrate on the other objectives of my thesis, my findings and experiences were directly applied to troubleshoot and develop a third, improved prototype (MET3). Following feedback from me and other collaborators, the MET3 was made using non-conducting polyvinyl chloride (PVC) frames (see Appendix 1). Colleagues at IHI are conducting further field-testing of MET3 in the Kilombero Valley. These studies are ongoing but preliminary results (Govella et al, pers comm.) indicate that it has much improved performance relative to the Human Landing Catch gold standard. Due to my contribution to developing this trap, I am a named co-

inventor on a patent application which has been filed jointly between the University of Glasgow and the Ifakara Health Institute for this prototype (see Appendix 2). It is expected that this application will be under review for another few years, and if granted we hope to commercialize this trap on a non-profit basis for use in mosquito vector surveillance throughout Africa and other malaria affected regions.

In the third chapter of this study, the sampling performance of the MET2 and a commercially available bug zapper (CA-EG) were evaluated in the field. The major aim was to find if these traps could replace the HLC by providing similar estimation of mosquito abundance and biting behaviour as estimated by the HLC. A major finding here is that electric traps tended to work better outdoors than indoors, for reasons which are still unknown. MET2 performance was regarded as promising by achieving the predefined threshold of >50% relative to the HLC for sampling malaria vectors in most scenarios (e.g. *An. funestus* s.l. both indoors and outdoors and *An. gambiae* s.l. in outdoor but not indoor locations). The MET2 successfully replicated some characteristics of mosquito behaviour with respect to the HLC gold standard (e.g. degree of exophagy), but gave somewhat biased estimates of more complex human exposure metrics such as the proportion of mosquitoes caught when most people are indoors (P_{fl}) and the proportion of human exposure occurring indoors (π_i). These biases are hypothesized to be due to the variable performance of traps when used indoors and outside; which is identified as an important weakness to address in future prototypes. The relative performance of the CA-EG was <30% in all scenarios (in/out, *An. gambiae s*.l/*An. funestus*), which in my opinion does not merit this trap further consideration as the alternative to the HLC.

In chapter 4, the performance of four different lightweight, portable resting traps were evaluated relative to one another for sampling malaria vectors resting in and outside of houses. Two of these methods were based on recently developed traps, the Resting Box (RBO) and Resting Bucket (RBU) traps. Two further traps were developed based on modification of these designs to test the impact of specific design features: the sticky resting bucket trap (SRBU) which was lined with sticky surfaces to glue mosquitoes on contact, and the modified

entry resting bucket trap (MERBU) which incorporated a funnel-entry system designed to make it easy for mosquitoes to enter but not exit the trap. Inside houses, the performance of these traps was compared relative to the gold standard backpack aspiration approach (BPA). Results suggest that the most efficient designs were traps with open entrances (RBU or RBO). Counter to expectation, the use of sticky surfaces led to much lower trap performance in outdoor environments, and adding a funnel entry system reduced trap performance when used both in and outside of houses. There was no detectable difference between traps of different shapes (round or square), but based on the greater robustness and ease of use of the bucket traps the RBU was recommended as the best approach for the field.

The RBO (which has been used widely in other studies and had the best performance in this study though not statistically different from RBU) was used to investigate the influence of microclimatic environmental variation on mosquito resting behaviour. Specifically, I tested for associations between the exophilic behaviour of malaria vectors (relative number resting outside versus in a house) and nightly variation in temperature and humidity. This analysis found that when temperature was higher inside houses than outside, *An. gambiae* s.l. and *An. funestus* s.l. tended to rest more outdoors, while when humidity was higher outdoors compared to indoors, *An. gambiae* s.l. and *An. funestus* s.l. tended to rest more outdoors.

Chapter five investigated possible links between variation in *An. arabiensis* feeding behaviours and single nucleotide polymorphisms in their circadian activity genes. Although no association was detected, *An. arabiensis* within the study area were observed to be sub-divided into two groups in association with variation in the *Timeless* gene.

The suitability of the tools developed in this work for sampling host seeking and resting malaria vectors, as well as potential epidemiological implications of the ecological and genetic observations are discussed in the next section.

6.3 Potential suitability of sampling tools developed in this study for wide scale surveillance

6.3.1 Suitability of MET2 in large scale surveillance

The novel MET2 developed in this study was a proof of concept that electrocuting traps can be developed that give estimates of the density of host seeking malaria vectors and their biting behaviours close to those obtained from the HLC. One of the weaknesses of MET2 developed in this study however, was its poorer performance when it was used for indoor sampling compared to outdoor use. Further, it was found that MET2 worked better for *An. funestus* s.l. than for *An. gambiae* s.l. It would be important to know if there are consistent collection biases for different mosquito species and different locations (e.g. indoors versus outside) so that simple correction factors could be applied to adjust catches. The relative sampling efficiency of the MET seemed to decline across the night in sampling *An. gambiae* s.l. inside and *An. funestus* s.l. outside houses. This was hypothesized to be due to an unexpected decline in battery power throughout the night, possibly due to the increased electrical resistance building up across the trap as the wooden frame absorbed moisture. These problems need to be corrected before MET prototypes are suitable for use in broad scale vector surveillance. Operation of the MET2 was found to be difficult in outdoor stations when moisture fell on the wooden frame. This problem should now be significantly reduced by using moisture resistant polyvinyl chloride (PVC) frames as have been adopted into the MET3, but even so operation of MET prototypes will likely be improved by having the traps under canvas roofs without walls to protect the trap from moisture in outdoor environments.

The fact that the sampling performance of MET2 was found to be density independent may seem to suggest this trap could give consistent relative performance across varying mosquito densities. However, in this study the density dependence of the MET was tested within the rainy season when mosquito abundance was consistently high, and did not include the large variations in mosquito abundance that occur between rainy and dry seasons. Broad-scale surveillance usually traverses different seasons of the year, and thus

requires sampling methods which show consistence across high and low mosquito abundance seasons. It is recommended that future works investigate how MET2 performs under seasonal fluctuations in mosquito abundance and across different ecological settings where the species composition of vectors differs from those found in these studies in the Kilombero Valley.

Proper operation of MET requires a quick period of training during which important aspects such as safety and user procedures are highlighted. It is expected that use of MET will not require much training (only up 2 hours) for users to become competent, and therefore this tool could be relatively easily used by the same range of volunteers that are capable of working with the traditional HLC technique. It is anticipated that the MET would require much less supervision than the HLC as volunteers working as bait to mosquitoes will not need to conduct active sampling as is the case with the HLC. However, supervisors will need to check the traps regularly to make sure they function consistently.

The current cost for producing a custom-built version of a new version (MET3) is about £995. This is high compared to costs for other commercialized mosquito traps such as the CDC backpack aspirator (~£644) and the CDC light trap (~£106) (http://johnwhock.com/wp-content/uploads/2012/09/jwhorderform.pdf), or the mosquito magnet trap (~ £230)(http://www.amazon.com/Mosquito-Magnet-MM4100-Patriot-Trap/dp/B002RMBDIM). If production costs remained this high for the MET it could hinder its uptake for use in wide scale surveillance. However, it is likely that costs could fall by at least 50% or more if produced at scale, thus cost may not be limiting for future applications. Even with these reductions, it could be argued that the cost of a MET will always be more than that of doing a Human Landing Catch, which requires only staff costs. However, this argument does not take any consideration the substantial non-economic advantages offered by the MET in removing the ethical concerns raised by HLC. Unlike the HLC, people using the MET will be protected from mosquito bites and thus there is no risk of them contracting malaria or any other vector-borne disease while doing the sampling. In my opinion, these substantial ethical benefits outweigh the cost of trap purchase. Even if cost was the only

consideration, there are now many settings where the HLC is no longer allowed and thus where the MET could provide the best alternative to this method.

In trials described here, it was not possible to directly observe all mosquitoes approaching the MET to confirm that none were able to bite the user. However, both based on the physical protection provided to users during trapping and their experiences of not being aware of any bites, I hypothesize this trap is exposure-free. However, there are some critical requirements to ensure the trap is operated in an exposure-free way. First, the MET has to be connected to the power supply set with the recommended output voltage (600V) as lower voltages may not electrocute mosquitoes by allowing them pass through the grids. In addition, the gap between the grids should be no bigger than 5mm to reduce the possibility of a flying adult Anopheles mosquito from passing through it without touching a wire.

6.3.2 Suitability of RBO and RBU in large scale surveillance studies

Of the 4 traps that were trialled inside and outside of houses, RBU and RBO had the most consistent and relatively highest performance with respect to sampling *An. gambiae* s.l. and *An. funestus* s.l. These trapping methods also produced similar estimates of exophily. Estimation of the resting behaviour of malaria vectors requires standard sampling tools that work consistently in indoor and outdoor locations, which was not achieved by the SRBU or MERBU. The SRBU used here produced very biased estimates of exophily in *An. gambiae* s.l. which predicted this species to be highly endophilic (>93%). This prediction is highly misleading both with respect to estimates from other methods, and on the basis of the known exophilic tendency of *An. arabiensis* (Service et al., 1978); the species that constitutes almost 100% of *An. gambiae* s.l. in the study area. The MERBU grossly underestimated abundance of resting mosquitoes especially indoors, so if this trap were applied to evaluate indoor resting spraying (IRS) interventions it would overestimate the efficacy of IRS and mislead vector control policy making. Clearly, the SRBU and MERBU have limitations which at present mean they would not be recommended for wide-scale surveillance.

This study recommends RBO and RBU for wide scale surveillance of resting mosquitoes because they gave consistent results in indoor and outdoor locations. The RBO and RBU offer tools for sampling resting malaria vectors which are easy, cheap and rapid to use in contrast to traditional methods such as pit shelters and pyrethrum sprays. The cost for RBO and RBU are approximately £2 and £3 respectively, which is considerably cheaper than the current cost of a CDC backpack aspirator (£644). RBO and RBU are also relatively easy to transport *en masse* as would be required to conduct large scale surveillance of the resting behaviour of mosquitoes. The simplicity and affordability of RBO and RBU is one of the important factors to consider especially in resource poor areas in Africa. A notable disadvantage of RBO and RBU traps is that they need to be checked up regularly (daily) for mosquito collection. Sticky traps could have overcome this limitation by allowing traps to be put out for up to week for cumulative collection of mosquitoes. Whilst the performance of the sticky traps used here was too low to compensate for their potential use in passive sampling, the development of more effective sticky traps that can be integrated into the RBU and RBO designs used here. Given their consistency in sampling indoor and outdoor resting malaria vectors, RBO and RBU may be used reliably for surveillance of malaria vectors and study if there are trends in their biting behaviour compared to populations across time and location. Their ease of use, simple logistics and affordability make RBO and RBU suitable sampling tools especially in resource poor countries.

6.4 Insights into the ecological and genetic determinants of mosquito vector behaviour

Ecological and genetic factors are proposed to play an important role in determining mosquito vector behaviours (Kelly-Hope et al., 2009, Dana et al., 2005, Lyimo and Ferguson, 2009) . In turn, mosquito behaviours such as the time and location of biting, and resting habitat preference may influence the effectiveness of control strategies such as ITNs and IRS (Sokhna et al., 2013, Russell et al., 2011, Ojuka et al., 2015). Therefore, it is important to understand how mosquito biting and resting behaviours change in relation to genetic and ecological changes to ensure effectiveness of control measures.

This study showed that differences in micro-climatic conditions between indoor and outdoor environments were associated with changes in the resting behaviour of malaria vectors. Specifically, malaria vectors exhibited higher degrees of exophily on nights where temperatures were generally higher inside houses than outside, and when humidity was relatively greater outside than inside. The optimal temperature and humidity range for *An. gambiae* s.l. lies in the range of 25-28^{0}C and 75-85% (Olanga et al., 2010, Das et al., 2007). These thermal optima are narrower than the range of temperatures (23-32^{0}C) and humidity values (43-90%) observed in this study. This means that malaria vectors were exposed to suboptimal and perhaps potentially lethal microclimatic conditions, which would be expected to trigger vectors to vary their resting habitat selection (e.g. indoors or outdoors) on the basis of where microclimatic conditions were most favourable. In the cold season, it may be hypothesized that mosquitoes will tend to rest more indoors than outdoors and therefore this phenomenon could be used to target indoor resting mosquitoes through the IRS strategy.

The changing climatic condition in Africa due to global warming is expected to lead to a change in the resting behaviour of malaria vectors. As the resting behaviour was shown in this study to be determined by the relative temperature and humidity between indoor and outdoor locations, it may be hypothesized that drier climate may lead to vectors seeking humid environments inside houses, therefore causing an increase in the number of mosquitoes resting inside houses. Effect of other ecological changes driven by urbanization and agriculture is also expected to influence the feeding as well as the resting behaviour of malaria vectors. Change in vertebrate populations is one of the major ecological changes that may significantly affect malaria vectors' population as well as behaviour. For example introduction of cattle in places which previously did not have cattle may provide source of blood-meal to malaria vectors especially those inclines to feeding on animals thus raising their numbers relative to species that specialize on humans. Such massive migration of animals has been witnessed in the Kilombero valley of Tanzania, and its effect on vector population and epidemiology needs more investigation. As Africa and many other places in the

world become urbanized and more people afford to build better housing may have impact on the behaviour of malaria vectors. Such behaviours as propensity to feed outdoor may be enhanced when mosquitoes encounter unsuccessful feeding due to things like house screening. As application of repellents inside houses and use of insecticide rise, the feeding and resting behaviour of malaria vector is expected to be shifted towards outdoor feeding.

In addition to investigating ecological determinants of vector behaviour, I also investigated the potential impact of a series of circadian candidate genes on the timing of mosquito behaviours. Here I used a series of candidate genes selected on the basis of their known association with diel activity in mosquitoes (Rund et al., 2013a, Das et al., 2010, Das and Dimopoulos, 2008), to test for association between genetic polymorphisms and the timing of host seeking. Across the 34 loci of the 8 circadian genes I used, no association between candidate gene and host seeking behaviour was found. The lack of association between the feeding phenotypes and the SNPs used in this study was assessed by STRUCTURE cluster analysis and was confirmed by principal component analysis. The finding of clustering based on the *Timeless* gene raises questions about the importance of this gene in the population genetics of *An. arabiensis* in the study area. As has been shown in sections 1.7.3.4 and 5.5 above, the *Timeless* gene has been researched extensively in insects especially Drosophilids where nucleotide polymorphisms in this gene was associated with variation in insect behaviours. Occurrence of this gene and its allelic forms was also found to cause population clustering in some mosquito species. Further, allelic forms of this gene were also shown to exhibit geographical variation between different geographical locations. Further research into the role of this gene in governing periodicity in insects and its ability to cause population structure in insects could shed more light into the population genetics of malaria vectors in Africa and elsewhere. As discussed in depth in chapter 5, several factors could lead to the failure to detect direct associations of phenotypes and genotypes. Insects are known to be capable of extensive behavioural plasticity in response to selection pressures (Whitman and Ananthakrishnan, 2009, Lefevre et al., 2009). It is possible that when confronted with LLINs, malaria vectors can simply vary their time of host

seeking to avoid these measures through phenotypic plasticity without any need for genetic selection.

Alternatively, there may be genetic determinants of feeding behaviour that were not picked here because of the specific markers used in this study may not have been the most relevant ones, or because a much greater number of SNPs would be required to detect associations. Consequently, at present it not possible to dismiss the role of genetic factors in determining malaria vector host seeking behaviour. Further analysis with a broader range of approaches including higher number of markers will be required to provide a conclusive test. For example, transcriptomic approaches have been used in the past to show that onset of host seeking behaviour in An. *gambiae* s.s. is linked to time-dependent expression of olfactory genes (Rund et al., 2013b, Rund et al., 2013a, Das et al., 2010, Das and Dimopoulos, 2008). Thus, it may be that differential expression of genes involved with host seeking, rather than polymorphisms in those genes, is the mechanism through which genes influence malaria vector behaviours. Thus, further work based on transcriptomic approaches combined with analysis of a wider suite of genes (e.g. whole genome sequencing) is recommended for further investigation into the genetics of malaria vector behaviour.

6.5 Possible epidemiological implications of results in this study

This section discusses the potential epidemiological implications of some of the mosquito behaviours recorded within the area of southern Tanzania where these studies were conducted. In chapter 3, estimation of the nightly biting profile of malaria vectors using the reference traps (HLC) and the novel traps (MET2 and CA-EG) revealed no evidence of distinct peaks in the nightly biting trends of either *An. gambiae* s.l. or *An. funestus* s.l. Traditionally, the peak biting activity of *An. gambiae* s.l. is described as being around midnight (Pates and Curtis, 2005, Gillies and De Meillon, 1968). Thus, results obtained from the current study suggest a difference in the biting behaviour of *An. gambiae* s.l. from the traditional expectation. Absence of distinct peaks in the biting activity of *An. arabiensis* indicates their biting activity is spread throughout the night even including the early evening and morning when some people are not under bed-

nets. This more spread out host seeking period may suggest a decreased efficacy of LLINs in contrast to that expected under the traditional assumption that malaria vectors bite primarily during sleeping hours, with a peak around midnight (Pates and Curtis, 2005, Gillies and De Meillon, 1968). However records show that there has been a decrease in malaria prevalence in the area around the Morogoro region in recent years from about 52% in 1990's (Van den Homberg, 1994) to about 13% in 2012 (http://www.nbs.go.tz/takwimu/references/Tanzania_in_figures2012.pdf). The decrease in malaria in this region could also be linked to other interventions such as use of anti-malarial drugs.

The proportion of mosquitoes caught seeking for hosts inside houses during sleeping hours (P_{fl} or nocturnality) was found to be ranging from 34% to 37% as estimated by the 3 traps used in this study. The proportion of human exposure occurring indoors (π_i), estimated that a maximum of 47% of human exposure could be prevented by using bed nets. This means more than half (53%) of human exposure to mosquitoes at Lupiro may not be preventable by ITNs. A previous report from samples collected in 2009 in the Kilombero valley (including samples from Lupiro village) shows higher values of P_{fl} and πi (79%) and 82% respectively)(Russell et al., 2011). Though there is apparently a decrease in *An. gambiae* s.l. nocturnality (P_{fl}) as well as in the protective ability of ITNs (π_i) against *An. gambiae* s.l., this change could be due to shifts in species composition from largely *An. gambiae* s.s. in 2006 (Killeen et al., 2006) to largely *An. arabiensis* currently (Matowo et al., 2013), rather than changes in malaria vectors' biting behaviour. Values for P_{fl} and π_i for *An. arabiensis* obtained in the current study are in the same range to those obtained for this vector elsewhere (e.g. Dar es Salaam, Tanzania (Majambere et al., 2013), showing a consistency in the nocturnality of *An. arabiensis* between both locations.

A clear implication of the moderate degree of protection from exposure (π_i) estimated to be possible from LLINs here is that further progress on control within this area will require the additional use of new vector control measures that target outdoor biting mosquitoes. Measures to control malaria transmission

in outdoor environments should be developed to complement measures used indoors. Of such suggested measures are use of mosquito luring outdoor stations (Okumu et al., 2010c), use of mosquito attractants and repellents (Menger et al., 2015), zooprophylaxis (Iwashita et al., 2014) and larviciding (Tusting et al., 2013). Based on the ecology in the Kilombero valley, methods such as larviciding may be problematic due to the vastness of the breeding sites, while zooprophylaxis would be best for pastoralist communities and may not be suitable in many part s of the valley where inhabitants do not keep cattle. Therefore, personal protective tools such as repellents and outdoor luring strategies could be most suitable alternatives for control of outdoor malaria transmission in the Kilombero valley.

With the exception of the SRBU which did not work well for outdoor sampling, RBO, RBU and MERBU suggested that a greater proportion of *An. gambiae* s.l. (76-96%) and *An. funestus* s.l. (51-58%) prefer to rest outdoors. Such high exophily rates are not uncommon in *An. arabiensis* (Mahande et al., 2007, Oyewole et al., 2007, Tirados et al., 2006) and higher from what is traditionally know of *An. funestus* s.l. (Gillies, 1954b). Exophily results obtained here imply that indoor vector control strategies such as indoor residual spraying (IRS) may be minimally effective to *An. arabiensis* and target only up to 50% of *An. funestus* s.l. A greater effort should therefore be directed to control outdoor resting mosquitoes which may require development of outdoor stations for increasing contact of these vectors to insecticides.

6.6 Questions arising

In the course of conducting this research, several findings were obtained that question conventional wisdom on the ecology of malaria vectors and their susceptibility to control. Here I review a few key areas that raise important and yet unresolved further questions for future study.

6.6.1 More *An. gambiae* s.l. and *An. funestus* s.l. biting outdoor than previously observed. Is it due to mosquito change in biting behaviour or species composition?

Some of the studies which were used to provide reports of shifts in the biting behaviours of malaria vectors in this work date back to 1970s. During this time and up until 1993, there were no diagnostic methodologies that could reliably distinguish different species that are part of species complexes in malaria vectors. It is therefore likely that malaria vectors that were studied during this time and those that were broadly classified under the *sensu lato* species were not identified using reliable methods and so may have mislead researchers to ascribe one species' behaviour in place of another. This study observed lower level of outdoor biting in *An. gambiae* s.l. and *An. funestus* s.l. in comparison to previous studies in the Kilombero valley and elsewhere. As all *An. gambiae* s.l. caught in this study and analyzed by PCR method were found to belong to *An. arabiensis*, I can be sure that we I am ascribing behaviour to a proper species. The following studies and most of those used to argue about shifts in malaria vectors' behaviour in this study should be taken cautiously as they refer to a complex species rather than a specific species. For example in the Kilombero valley, a study using samples collected in 2009 recorded 58% of *An. gambiae* s.l. captured in host-seeking collections were attempting to bite outdoors (Russell et al., 2011) compared to 37% reported in the current study. In western Kenya, proportions of outdoor biting for *An. gambiae* s.l. and *An. funestus* s.l. were estimated to be lower at 13% and 14% respectively (Bayoh et al., 2014) compared to 58% and 51% reported in the current study. Following reports of changes in species composition across many places in Africa, it may be said that changes in malaria vectors' feeding behaviours that were observed across time periods are actually reflecting a change in species composition and not a change within a species. Specifically, whether these reports reflect intraspecific changes in the biting behaviour of *An. arabiensis* from indoor biting to more outdoor biting behaviour, and/or are solely due to a change in the composition of the *An. gambiae* s.l. complex from the dominantly endophilic species like *An. gambiae* s.s. to exophilic sibling *An. arabiensis* is unclear. Recent studies have indicated that large changes in the species composition of the *An. gambiae* s.l.

within the Kilombero Valley have occurred over the last 20 years (Russell et al., 2010) as well as in Western Kenya (Bayoh et al., 2010). Also in these places shifts in *An. gambiae* s.l. biting behaviours from largely indoor/midnight biting to outdoor/late night biting have been reported (Russell et al., 2011, Bayoh et al., 2014). The relative contribution of intra- versus interspecific differences to these changes in malaria vector behaviour is unknown. Further studies should be conducted to repeatedly measure the same vector species and test whether it shows any evidence of an adaptive change within vector species that could be related to the increased coverage of vector control measures.

6.6.2 What are the causes and epidemiological implications of the population sub-division observed in *An. arabiensis* population based on the *Timeless* gene?

Though analysis of the circadian gene SNPs in *An. arabiensis* did not find any population sub-divisions based on the time or location (indoors versus outside) of biting, a population clustering was observed across *An. arabiensis* feeding phenotypes and collection sites. The *Timeless* gene was found to be strongly associated with this clustering. This study is the first to find population structuring based on the *Timeless* gene in *An. arabiensis*. The causes of this association are unclear within *An. arabiensis* though *Timeless* gene is vastly known to cause population structure in other insects especially the Drosophillids and allelic forms of this gene are also known to show frequency variation across geographical locations (Tauber et al., 2007). The *Timeless* gene could be linked to functional traits not measured here like time and place of mating, or it may be associated with other traits that are themselves responsible for the observed sub-division. It is important that possible implications of the *Timeless* gene be investigated further in *An. arabiensis* including how this mutation is inherited within the population and whether this subdivision occurs in other malaria vectors in other geographical localities.

6.7 Future work

While valuable knowledge has been generated in this study, further work needs to be done to seal gaps that this study could not address.

6.7.1 Further development of the Mosquito Electrocuting Trap (MET)

Although the MET2 demonstrated promising results as a proof of principle that electrocuting traps can be developed to be alternative sampling tools to the human landing catch technique, MET2 will need further improvement to achieve this. As will be explained in the appendix 1 section, a further version (MET3) has been developed based on the recommendations in chapter 2. This new version will need to be tested across diverse ecological settings to determine its sampling efficiency across areas with high and low vector densities, while taking into account the effect of environmental factors such as humidity and temperature on its performance. As the performance of MET2 was lower relative to that of the HLC in sampling *An. gambiae* s.l. inside houses, further testing should investigate this phenomenon across a range of localities to establish potential causes of variation of MET performance between inside and outside houses including if MET performance is vector species dependent. As a larger version of the MET has been developed (see appendix 1) which can allow other animals such as calves, goats, etc to be enclosed within the trap, the MET may also prove an effective way to assay the host species preference of malaria vectors. Further investigation of the use of MET for these purposes is required to identify the full range of mosquito behavioural surveillance it could be used for.

6.7.2 Future work on the resting traps

Some of the promising resting traps need to be tested further using a larger sample size and more experimental replicates. These traps need to be tested across diverse sampling sites to be able to confirm the effect of environmental factors such as temperature and humidity on the resting behaviour of the major

malaria vectors. The fact that SRBU worked well in sampling *An. gambiae* s.l. indoors but performed poorly in outdoor location needs further investigation.

The poor performance of the MERBU is hypothesized to be due to the fact that it was more difficult for mosquitoes to enter the trap, but other features of this trap such as a possible repellent effect of a chalk used to paint the funnel entry may also have contributed. Ideally, further testing should be done to confirm that the structural design of the MERU was primarily responsible for its poor performance. Another aspect of resting trap performance that was not thoroughly investigated here was how their placement influenced their performance. Here, all resting traps used indoors were suspended in the corners of a room, with openings positioned towards the centre of the room. This placement was based on biological intuition about where mosquitoes prefer to rest indoors (e.g. usually found on ceilings or the top of walls in backpack aspiration collections) and the logistical practicality of not putting traps on the floor where they could get in people's way and be accidentally disturbed. However, in other studies sticky resting boxes have been placed on the floor of houses (Pombi et al., 2014a) and still caught reasonable numbers of mosquitoes. It is possible that there are optimal placements of traps in terms of height and orientation, both indoors and outside of houses. Future work should contrast resting traps placed at different positions in a house such as on the ceiling, on the wall and on the ground to determine which trap position gives better results.

As the RBO and RBU have been shown to work well both indoors and outdoors, these traps should be tested in a broader range of ecological settings to confirm their suitability for standard sampling of resting malaria vectors including determination of the number of units of these traps that should be regarded as standard in household sampling.

6.7.3 Circadian rhythm genetics and malaria vectors' feeding behaviours

Though in this study polymorphisms in the circadian genes were not linked to variation in the feeding behaviour of *An. arabiensis*, is this not conclusive

evidence that other genetic factors, including circadian genes not included in the subset selected here, are not involved with the regulation of mosquito feeding behaviour. Future studies should to try to find if such links exist in other geographical localities. It is important that such studies be conducted in areas which have proven historical shifts in the feeding behaviours of malaria vectors. As candidate gene approach may still be viable, different sets of genetic markers with stronger function linkage to these phenotypes would need to be used apart from those used here. Whole genome approaches may offer the most powerful approach for analyzing SNPs. Otherwise, transcriptomic approaches have been promising in detecting link between mosquito behaviours and gene regulatory activities and so this technique is recommended in future studies. As the *Timeless* gene was implicated for population sub-division in the Kilombero valley, further studies need to involve other ecological settings to find if mutations in the *Timeless* gene may be linked to epidemiologically important traits such as knock down resistance (kdr) phenotypes and investigate how mutations in this gene are inherited in the population.

6.8 Conclusions

To more effectively control and possibly eliminate malaria within Tanzania and other endemic areas of sub-Saharan Africa, I believe a wider range of tools for sampling and reducing malaria vectors will be needed. These tools should focus on targeting vectors with more diverse behaviours (e.g. outdoor feeding and resting) that are not typically susceptible to the current front line methods based on killing mosquitoes inside houses. It is my belief that some of the sampling tools developed and tested in this study can be useful for this goal, specifically by facilitating safer, more realistic and standardized tools for characterizing mosquito feeding and resting behaviour. Additionally, these tools could be very useful for providing an evidence base of whether the much reported but rarely rigorously tested phenomenon of malaria vectors changing their behaviour in response to control tools is occurring on a wide scale. Future planning and assessment of the sustainability of current control methods will require robust data on mosquito vector ecology as these tools could provide.

In recent years, the Bill and Melinda Gates Foundation have reinvigorated the call for global malaria eradication (Sifferlin, 2014). This aim was abandoned after the failure of the global malaria elimination programme in the 1950-60s (WHO, 1956), with the global health community concluding that control, but not full elimination, was the only realistic prospect. While many in the global health community continue to view malaria elimination as far-fetched (Greenwood, 2009), this may turn into a reality if continued financial and political support is directed on innovation. The Roll Back Malaria Initiative has set a series of short and long term goals (Binka, 2000) for reduction and eventual elimination of malaria. It is my belief that these goals can be achieved in the future even if this will have to happen within a broader timeframe than is currently proposed.

My 3.5 years of PhD work has made me understand that the control of malaria involves a fierce battle which, ultimately, I am optimistic can be won. I am also confident that the knowledge gained and the tools developed in this work will be an important part of the expertise and the arsenal necessary for victory.

Appendices

7.1 Appendix 1. Development and construction costs of MET3

Though performance of MET2 in the field was generally impressive, there were some problems which needed to be solved to come up with a more efficient, user friendly and safer MET prototype. Such problems included the short-circuiting, burning of the wood and widening of the gap between adjacent wires which allowed some mosquitoes to pass in without being electrocuted. To improve performance of MET2, a third version of the trap (MET3) was designed which took into consideration these problems and came up with a more efficient version of the trap. In addition, there was need to construct a version of the MET which apart from being able to be used for host seeking experiments with human hosts, it could also be used in other mosquito behavioural studies such as host choice studies that involve vertebrate hosts such as cattle and goat. The section describes construction of MET3 highlighting what changes were made to improve its performance from MET2.

MET3 was constructed to alleviate shortcomings encountered in MET2 and also to provide a better version of MET2. Based on feedback from myself and collaborators at IHI and UG regarding MET2, the UGBU developed a new improved prototype MET3. Two different models of this prototype were made; small MET3 (MET3S) and a large MET3 (MET3L). Just like MET2, MET3S was made by assembling 4 grid panels each measuring 30cmX30cm (Figure 2.5A), while MET3L were made of 4 panels but each measuring 120cm X 120cm (Figure 2.5B). The frame for MET3 grids were made of polyvinyl chloride (PVC) material in contrast to the wood used in MET2. PVC is non-conductive is more resistant to fire (caused by sparking) than wood. Therefore, use of this material is expected to solve the problems found in MET2 such as the burning of the trap frames, contraction and expansion of the frame, and short-circuiting of the adjacent grid wires.

The MET2 used 0.45mm thick stainless wires to make the electrocuting grids, but in MET3, 1.2mm thick stainless wires were used. Thicker wires have higher tensile strength which is an important feature in preventing adjacent wires from bending which could result in either the adjacent wires touching each other or widening of the gap between them. In making the grid mesh, 1.5mm holes were made on the top and bottom of the PVC frames and at an interval of 5mm. Through these holes, the grid wires were inserted from the top of the frame to the bottom. This was done by industrial drilling rather than hands. As in MET2, adjacent wires had alternating positive and negative charges in which all wires of the same electrical charge were connected together through one bar to an input source of power.

The panels in MET3L were fitted with 2 spacers which were placed at equal distance between the top and bottom frames (Figure 2.5B). The spacers hold the grids in place to prevent the touching of adjacent wires to each other. A variable power supply unit constructed at the UGBU was used to supply power to the grids. The power supplies were constructed to provide an easily adjustable DC voltage range (0V-1000V) with a solid state polarity switching. The power supply units were short-circuit protected, made with high output stability to operate at temperature ranges between 0^0C-50^0C and at high relative humidity of up to 80%. The power supplies were constructed to use with a DC input source of 24V with a capacity of up to 11Amps/hour. A schematic circuit for MET3 is presented in (Figure 2.4A).

The costs for constructing MET3 differ for each of the two versions of MET3. The total cost for construction of MET3L was £1,484 while for MET3S the cost was £994. These costs include labour charge. The greatest part of the costs of constructing one MET3L went on the grids which cost about £336. For the MET3S the largest part of the cost was spent on the variable power supply (£300). A breakdown of the costs for each of the MET3 models including the accessories are presented in Table 2.3.

7.2 Appendix 2. Summary of male and female malaria vectors captured in the resting trap study presented in chapter 4

	Indoors		Outdoors	
	Female	Male	Female	Male
An. gambiae s.l.	112	82	254	316
An. funestus s.l.	109	43	44	91
Total	221	125	298	407

Table 7.1. The number of female and male *An. gambiae* s.l. and *An. funestus* s.l. caught inside and outside houses.

7.3 Appendix 3. Patent letter

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For the attention of Alasdair Street
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G12 8QQ

BY E-MAIL ONLY

31 October 2014

Dear Sirs

We filed the following United Kingdom (GB) patent application for you.

United Kingdom (GB) application number: 1419424.5

Filing date: 31 October 2014

First priority date: None

Applicants: THE UNIVERSITY COURT OF THE UNIVERSITY O F GLASGOW
(and others)

Your ref: Mosquito EGT

Mewburn ref: RAJ/BP7076961

Our full report will follow in due course.

7.4 Mosquito electrocuting trap (MET) patent application details

INSECT TRAP

The invention relates to an electrocution-type insect trap, in particular to a portable, battery-powered electrocution-type trap for catching mosquitoes.

Mosquitoes are responsible for huge losses in human and animal life through their role in transmitting infectious diseases. About half of the world's population live in areas where they are at risk of malaria, which the most important insect-borne disease of humans.

The control of malaria and other mosquito-borne disease is critically dependent on accurate surveillance of the abundance and infection of mosquito vectors. Reliable mosquito sampling methods are fundamental for a number of purposes including to governmental agencies that manage and monitor for vector-borne diseases both in tropical countries and temperate regions where they may emerge (including several areas of western Europe and the UK), to the National Malaria Control Programmes within the 108 countries that have endemic transmission, and to researchers from academia, government and/or industry involved in large-scale mosquito control trials or related research.

Currently, there is only one 'gold standard' method in use for estimating the number of potentially infectious mosquito bites that a person would be exposed to this. This method is called the "Human Landing Catch" (HLC), and involves volunteers sitting over night (when most mosquito biting happens) with one of their legs exposed, with the aim of collecting every mosquitoes that lands on them using a mouth aspirator and storing them. The aim is to collect the mosquitoes from their leg before they bite, but that is not always possible.

Obviously, this method involves considerable risk, as it exposes the human subjects that carry it out to the bite of potentially infected mosquitoes. Additionally the method is quite variable and subject to the particular collection skill of the human participant. Due to these risks, some malaria endemic countries are now restricting or banning the use of the HLC method, which is problematic given the lack of suitable alternative methods. Consequently, there is a clear and urgent need within the global mosquito-borne disease community to develop new trapping methods that provide comparably accurate estimate of mosquito biting rates on

humans, without exposing participants to any infection risk.

Over the last decade, there have been numerous attempts to develop some exposure-free alternatives to the Human landing trap, including such approaches as the Bednet trap, and outdoors tent trap. All of these have shown to have limitations in that their performance compares poorly relative to the HLC, either in terms of overall mosquito abundance and/or giving a skewed representation of the types of mosquitoes that bite people in outdoor environments.

At its most general, the present invention provides a battery-powered electrocution-type insect trap for non-destructive capture of mosquitoes, in which an electric field for mosquito electrocution is optimisable through the provision of a variable voltage power supply.

According to the invention, there is provided an electrocution-type insect trap for non-destructive capture of mosquitoes, the insect trap comprising: a DC energy source for producing an input DC voltage; a variable voltage power supply arranged to controllably multiple the input DC voltage to an output DC voltage; and an electrocution grid surrounding an cavity for receiving a living subject to attract mosquitoes, wherein the electrocution grid comprises a plurality of parallel spaced-apart conductive elements that are electrically connected to the variable voltage power supply so that the output DC voltage creates an electric field between adjacent conductive elements, and wherein the output DC voltage is selectable to optimise non-destructive capture of mosquitoes. The ability to select the output voltage enables the trap to be usable in a wide variety of different environments and to be tunable to optimise the capture of certain species. Herein the term “non-destructive” means that the electric field is capable of supplying an electric shock that is lethal to mosquitoes, but which does not vaporise or cause combustion or other significant damage to the mosquito carcass, whereby it remains suitable for identification by visual or molecular methods.

The DC energy source may be any suitable portable and/or sustainable energy source, e.g. a battery and/or a solar cell. The plurality of parallel spaced-apart conductors may have alternating polarities, i.e. alternate conductive elements may be electrically connected to one another to form two sets of mutually interspersed conductors, and those two sets of mutually interspersed conductors may be connected to opposite polarities of the output DC voltage. This arrangement ensure a uniform electric field presence around the cavity.

The electrocution grid may comprise an insulating frame and the plurality of parallel spaced-apart conductive elements are wires which span across the frame. The wires may extend in a vertical direction, and adjacent wire are preferably separated by 5 mm, which has been found to be optimal for malaria mosquitoes. To ensure uniform separation of the wires, the electrocution grid may include one or more insulating spacers spanning across the frame in a direction offset from (e.g. orthogonal to) the wires, wherein the wires intersect with and are attached to the insulating spacer at their respective intersection points.

The electrocution grid may comprise a plurality of panels which are arranged to surround the cavity. For example, the electrocution grid may comprise four square panels arranged to form a cube-shaped cavity. The panels may be pivotally connected, e.g. hinged, to one another.
The variable voltage power supply may comprise a switched-mode DC-to-DC converter that incorporates a voltage multiplying rectifier. This arrangement may provide a clean, quiet signal with a low output ripple, whilst also enabling an input voltage of 24 V to be multiplied up to 1 kV.
The variable voltage power supply may be controlled using adjustable peripheral circuitry such as a potentiometer, e.g. arranged to vary the input voltage.

As a safety feature and to prolong the battery lifetime, the variable voltage power supply may include an output current limiter arranged to limit the current that flows in the plurality of parallel conductive elements.

The variable voltage power supply may include adjustable peripheral circuitry such as a potential divider connected to the output voltage to provide a reduced voltage output for use as a control parameter, e.g. to compare with the input voltage to ensure that the device is operating as intended
The trap preferably includes a display, e.g. low power LCD panel, arranged to show the selected output voltage.

The variable voltage power supply and electrocution grid may be electrically floating, e.g. to reduce the risk of the high voltages causing unwanted electric shocks. The variable voltage power supply may thus be encased in an insulating, and preferably waterproof, housing.

To prevent accidental physical contact with the electrocution grid, the trap may include an inner shield mounted in front of the electrocution grid inside the cavity and/or an outer shield mounted beyond the electrocution grid outside the cavity.

Embodiment of the invention are discussed below with

reference to the accompanying drawings, in which:
Fig. 1 is a schematic diagram of the power supply and control system for an electrocution-type insect trap that is an embodiment of the invention;
Fig. 2 is a perspective view of an electrocution-type insect trap that is an embodiment of the invention;
Fig. 3 is a perspective view of the electrifiable grids that are used in the electrocution-type insect trap of Fig. 2; and Fig. 4 is a partly assembled view of the electrocution type insect trap of Fig. 2.

The present invention aims to provide an electrocution30 type insect trap that uses electrifiable grids that are optimized to kill but not destroy mosquitoes on contact (to permit subsequent identification), and which can be safely located close enough to a living subject, e.g. human or other animal (e.g. livestock), to enable the living subject to be used as means of attracting the mosquitoes, e.g. in the same manner as an HLC.

Given the desirability to operate such a trap in remote regions, the present invention provides a battery-powered electrocution-type insect trap with a current-limiting capability that ensures both adequate operational lifetime and user safety. The electrocution-type insect trap of the invention can be used either indoors or outside.

The electrocution-type insect trap of the invention includes a controllable power supply capable of setting an optimal voltage for shocking (i.e. killing without destroying) the two main mosquito vector species of malaria in Africa, i.e. *Anopheles arabiensis* and *Anopheles gambiae s.s.* Fig. 1 is a schematic view of a power supply and control system 100 used in an electrocution-type insect trap that is an embodiment of the invention. The energy source for the power supply is a battery 102, e.g. a pair of 12 V batteries connected in series to provide a 24 V DC input voltage. Any suitable energy source may be used, e.g. solar cells, etc.

The input voltage is converted to an output voltage (i.e. an output DC voltage) by variable voltage power supply 104, which may be a self-contained IC unit. The structure and function of the variable voltage power supply 104 is discussed in more detail below. A voltage controller 106 is connected to the variable voltage power supply 104 to provide a means of adjusting the output voltage. For example, the voltage controller 106 may include an external circuit having an potentiometer, e.g. with a user-adjustable dial, to enable the output voltage to be adjustable between 0 V and 1 kV. This enables the device to be set to a specific output voltage, e.g. to a predetermined level that is optimal for a

particular mosquito species and/or ecological setting, but also has the capability of being altered either higher or lower to optimize performance in different settings.

The output voltage is supplied to one or more electrocution grids 108. The structure and configuration of the electrocution grid is discussed in more detail below. To ensure the device can be safely used close to a living subject, the variable voltage power supply 104 includes an output current limiter (e.g. including a resistor having a resistance of 330 Ω or more in series with the output) arranged to limit the output current to 10 mA.

The variable voltage power supply 104 may comprise a switched-mode DC-to-DC converter that incorporates a voltage multiplying rectifier. The switched-mode DC-to-DC converter includes a voltage transformer whose primary coil is driven by a power driver stage that receives the input voltage from the battery 102. The power driver stage is typically a solid-state semiconductor switch, e.g. a power MOSFET, that is arranged to receive a switching control signal from a microcontroller. The secondary coil of the transformed provides an output that is multiplied and rectified by a suitable voltage-multiplying rectifier. This signal is than filtered to give the final output voltage.

The variable voltage power supply 104 may further include an external circuit having a potential divider in the output stage. The potential divider may enable the output voltage to be measured by providing a reduced voltage output suitable for monitoring, etc. In one embodiment, the reduced voltage output may be compared with the input voltage as a means of controlling the control signal for the power driver stage. For example, the difference between the reduced voltage output and the input voltage may generates an error signal used to control the power through the driver stage into the primary of the transformer.

The variable voltage power supply 104 may be a self20 contained integrated circuit component. It may enable a wide range of output voltages to be accurately obtained from a single unit.

The power supply and control system 100 further comprises a display, e.g. a digital LCD screen or the like, which is arranged to shown parameters of interest to the user, e.g. the selected output voltage.

The power supply and control system 100 may be electrically floating, i.e. not connected to ground. It is preferably isolating from the user by being encased in an insulating housing, e.g. plastic box having dimensions

30×20×13 cm. The insulating housing is preferably waterproof, i.e. having an ingress protection rating of IP65 or IP66. The components of the power supply and control system 100 may be chosen to ensure its operational temperature range is 0°C to +50°C, and that it remains operational up to a relative humidity of 80% for temperatures up to 31°C, decreasing linearly to 50% relative humidity at 40°C.

Fig. 2 is a perspective top view of a electrocution-type insect trap that is an embodiment of the invention. Features of the trap that are discussed above with reference to Fig. 1 are given the same reference number. In this embodiment there are four square electrocution grids 108 arranged to enclose a cube-shaped space, but the invention is not limited to this configuration; the trap can work with a variety of frame sizes as dependent on the user needs.

Two different sizes of trap are contemplated:
(i) where each electrocution grid is 30 cm × 30 cm (i.e. 900 cm^2). This trap is designed to be used by a human host who sits in a chair and places their feet in the trap; and (ii) where each electrocution grid is 1.2 m × 1.2 m, i.e. 1.44 m^2. This trap is designed to be used to encircle an entire host, either a seated human or an animal that fits within the interior space.

Each electrocution grid 108 comprises an outer frame, e.g. made of PVC or other suitably robust insulating material. This material ensures that the system remains electrically floating, i.e. not connected to ground. A series of equally spaced holes are fabricated in the top and bottom arms of the each frame. In a preferred embodiment, the holes each have a diameter of 1.5 mm and adjacent holes are separated by a 5 mm pitch. The holes in the top and bottom of the frame are preferably aligned to form vertically spaced pairs. Each of the vertically spaced pairs of holes receives a respective grid wire 116. Each grid wire may be made of stainless steel and have a diameter of 1.2 mm. The area defined by the frame is therefore filled with an array of parallel conductive wires. Sixty grid wires are fit into the each frame of the small trap, and 240 grid wires are used in each of the frames of the large trap.

The grid wires 116 are in electrical communication with the power supply 104. The power supply 104 output comprises two polarities. The grid wires are electrically connected to each other so that adjacent grid wires have opposite polarity (so that the output voltage manifests itself as an electric field between all adjacent grid wires). Thus, adjacent grid wires 116 are not connected to each other, but every

alternative wire is electrically connected. These connections may be made by providing a horizontal conductive connection (e.g. welded rod or solder wiring) at to the top of each frame for the grid wires sharing one polarity, and on the bottom of each frame for the grid wires sharing the opposite polarity. The grid wires 116 may thus be seen as two interlocking sets of fingers having opposite polarity.

To stop adjacent wires short circuiting the power supply, the frames are fitted with horizontal insulating spacers 118, e.g. made from plastic, that hold the grid wires 116 in place. Each small frame is fit with at least one spacer positioned half way down the grid surface. In the large traps, each frame may have at least horizontal three spacers at equidistant points. The large trap may also have at least one vertical insulating column, e.g. positioned in the middle of the frame, that spans from top to bottom to provide additional structural support.

Holes are drilled along the length of horizontal spacers so that wires going from the top to the bottom of the frame are passed through them. This is done by drilling holes for each wire and inserting wires into the spacer. This technique ensures that no adjacent wires will touch each other.

The electrocution grids 108 are surrounded on their inner and outer surfaces by an inner shield 114 and an outer shield 112 respectively. The inner shield and outer shield act as physical barriers to prevent accidental touching of the electrified grid wires by the human participant.

In use, a human sits with their legs inside the trapping box which is surrounded by electrocution grids, and the rest of their body is covered in netting which protects them from mosquito bites.

The electrocution-type insect trap of the invention is preferably portable, and thus it is desirable for it to have a modular construction. Fig. 3 is a perspective view of the electrocution grids 108 in a partly assembled state. Here it can be seen that a pair of electrocution grids 108 can be pivotally connected along one vertical edge, e.g. using a suitable hinge or hinges, to enable them to be folded flat for transport.

Fig. 4 is a perspective view of the electrocution-type insect trap in a partly assembled state. Here it can be seen that the inner shield 114 and the outer shield 112 comprises separate cages that are assembled from four flat face pieces. Once each cage is assembled it can be moved into position relative to the electrocution grids 108. Although this

configuration facilitates transportation of the trap, in other embodiments the shields may be integrally formed with the electrocution grids.

In combination, the current limiting functionality of the variable voltage power supply and the inner and outer shield provide a robust safety precaution against accidental electrical shocks.

It is also desirable to allow the electrocution grids to discharge, e.g. naturally, before they are handled after use. Ideally the user should wait for 30 seconds after turn off before directly touching the grid surfaces to ensure that all charged components have discharged. In a development of the invention, it may be desirable for the variable voltage power supply to remotely and/or wirelessly controlled, e.g. at ranges of up to 1 km. This may assist in efficient optimisation of a plurality of traps in a given location if the environment or other circumstances require the output voltage to change.

CLAIMS

1. An electrocution-type insect trap for non-destructive capture of mosquitoes, the insect trap comprising:
a DC energy source for producing an input DC voltage;
a variable voltage power supply arranged to controllably multiple the input DC voltage to an output DC voltage; and
an electrocution grid surrounding an cavity for receiving a living subject to attract mosquitoes, wherein the electrocution grid comprises a plurality of parallel spaced-apart conductive elements that are
electrically connected to the variable voltage power supply so that the output DC voltage creates an electric field between adjacent conductive elements, and wherein the output DC voltage is selectable to optimise non-destructive capture of mosquitoes.

2. An insect trap according to claim 1, wherein alternate conductive elements are electrically connected to one another to form two sets of mutually interspersed conductors, and wherein the two sets of mutually interspersed conductors are connected to opposite polarities of the output DC voltage.

3. An insect trap according to claim 1 or 2, wherein the electrocution grid comprises an insulating frame and the plurality of parallel spaced-apart conductive elements are wires which span across the frame.

4. An insect trap according to claim 3, wherein the wires extend in a vertical direction.

5. An insect trap according to claim 3 or 4, wherein

adjacent wires are separated by 5 mm.

6. An insect trap according to any one of claims 3 to 5, wherein the electrocution grid includes an insulating spacer spanning across the frame in a direction offset from the wires, wherein the wires intersect with and are attached to the insulating spacer at their respective intersection points.

7. An insect trap according to any preceding claim, wherein the electrocution grid comprises four square panels, and wherein the cavity is cube-shaped.

8. An insect trap according to claim 7, wherein each panel is 30 cm × 30 cm.

9. An insect trap according to claim 7, wherein each panel is 1.2 m × 1.2 m.

10. An insect trap according to any preceding claim, wherein the variable voltage power supply comprises a switched-mode DC-to-DC converter that incorporates a voltage15 multiplying rectifier.

11. An insect trap according to any preceding claim including an adjustable potentiometer connected to the variable voltage power supply to provide a means of adjusting the output voltage.

12. An insect trap according to any preceding claim, wherein the variable voltage power supply includes an output current limiter arranged to limit the current that flows in the plurality of parallel conductive elements.

13. An insect trap according to any preceding claim, wherein the variable voltage power supply includes a potential
divider connected across the output voltage to provide a reduced voltage output for use as a control parameter.

14. An insect trap according to any preceding claim, wherein the variable voltage power supply is a self-contained integrated circuit component.

15. An insect trap according to any preceding claim including a display arranged to show the selected output voltage.

16. An insect trap according to any preceding claim, wherein the variable voltage power supply and electrocution grid are electrically floating.

17. An insect trap according to any preceding claim,

wherein the variable voltage power supply is encased in an insulating housing.

18. An insect trap according to claim 17, wherein the insulating housing is waterproof.

19. An insect trap according to any preceding claim, wherein the DC energy source is a battery and/or solar cell.

20. An insect trap according to claim 19, wherein the DC energy source comprises two 12 V batteries connected in series.

21. An insect trap according to any preceding claim having an inner shield mounted in front of the electrocution grid inside the cavity.

22. An insect trap according to any preceding claim having an outer shield mounted beyond the electrocution grid outside the cavity.

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