Mosquito (Diptera: Culicidae) biting behaviour and malaria transmission: interactions between intrinsic host preferences and local host availability

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Abstract

Distribution of mosquito-borne diseases is governed by a complex mix of genetic, environmental and social factors which in turn affect pathogen, vector and host interactions. Different mosquito species show a variety of host biting behaviours with some showing an extreme preference for human blood hosts. However, even the most anthropophilic vectors will source a proportion of their blood meals from non-human hosts, suggesting this preference is not fixed. This thesis investigates mosquito biting behaviour and the interactions between intrinsic host preference and host availability.

Firstly, through investigation of the literature, the HBI was found to be more associated with collection location (R^2= 0.29) than mosquito species (R^2= 0.11). The influence of host availability was then tested in the field using a transect-based collection methodology. *Anopheles* mosquitoes were collected across a range of human host availabilities and significant changes in HBI (OR = 1.50 (95% CIs:1.05 – 2.16)) and BBI (OR = 0.60 (95% CIs:0.49 – 0.73)) were observed over 250 metres. In addition, extrinsic factors (AIC:243) impacted human blood host choice more than intrinsic factors (AIC:359.8). The transect-based collection strategy coupled with a novel molecular measure of blood meal digestion also informed mosquito dispersal. *An. coluzzii* was shown to typically remain within 50m of their host up to seven hours after feeding but disperse up to 250m after sixty hours. This novel molecular method was further optimised for multiple mosquito species of medical importance and compared to the Sella score, a widely used visual measure of blood meal digestion.

This thesis provides compelling evidence of how host availability directly influencing mosquito host preference and describes a novel measure of dispersal utilising bloodmeal digestion. Understanding factors influencing host choice opens the opportunity to synergise current control efforts with alternative methods that exploit this behaviour, ultimately increasing the impact of current and future interventions.

Acknowledgements

The completion of this PhD is really an amalgamation of the help, support and dedication of my colleagues, friends and family. Firstly, I would like to thank both my supervisors Dr Laith Yakob and Dr Thomas Walker for their knowledge, expertise and dedication for making this PhD project a success, allowing me to explore my own ideas, collaborate on other projects but also keeping me on the straight and narrow. I would also like to thank Claire Jeffries and Mojca Kirstan for their knowledge and support in the lab and field; I will miss our lab chats.

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The fieldwork was the biggest challenge of this project. I was so fortunate to work with such a hardworking and knowledgeable team in the shape of Prof Yaw Afrane, Rahim and company. Their commitment was unwavering even when times were tough. I would also like to thank them for welcoming me to their wonderful country of Ghana. I hope to return someday.

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List of abbreviations

Abbreviation	Full description
µl	microlitre
AIC	Akaike information criterion
An.	*Anopheles*
ANOVA	Analysis of Variance
BBI	Bovine blood index
BLAST	Basic local alignment search tool
bp	Base pair
C	Vectorial capacity
CDC	Centre of Disease Control
CI	confidence interval
CO_2	Carbon dioxide
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
EIR	Entomological inoculation rate
ELISA	Enzyme linked immunosorbent assay
GLM	Generalised linear model
HBI	Human blood index
HLC	Human landing catches
IRS	Indoor residual spraying
ITN	Insecticide treated nets
ITS2	Internal transcriber spacer gene 2
IVM	Ivermectin

Kdr	Knockdown resistance
LLINs	Long lasting insecticidal nets
LSHTM	London school of hygiene and tropical medicine
m	metres
min	minutes
ng	nanograms
nM	nanomolar
OR	Odds ratio
p	P-value
PCR	Polymerase chain reaction
PM	Perotrophic matrix
POR	Prevalence odds ratio
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
PSC	Pyrethroid spray catch
qPCR	Qualitative PCR
R0	Basic reproduction number
rDNA	ribosomal Deoxyribonucleic acid
RNA	ribonucleic acid
s	Seconds
s.l.	*sensu lato*
s.s.	*sensu stricto*
spp.	species
TPRI	Tropical Pesticide Research Institute

µg	microgram
UK	United Kingdom
UOG	University of Ghana
USA	United States of America
VBD	Vector-borne disease
WHO	World Health Organisation
WNV	West Nile virus

Declaration of work and contributions

Chapter 1- JO performed the literature search and writing of this chapter. LY and TW provided feedback on drafts before chapter was finalised.

Chapter 2- JO performed the fieldwork with the assistance of YA and ARM. Lab work was supported by TW and CJ. Chapter was written by JO. LY and TW provided feedback on drafts before chapter was finalised.

Chapter 3- JO developed search terms, inclusion/exclusion criteria and performed the systematic literature review as well as extraction of data. LFK performed meta-regression analysis. JO drafted the chapter. LY, TW, YA, ARM, CJ and MK provided feedback on drafts before chapter was finalised.

Chapter 4- JO performed the fieldwork with the assistance of YA and ARM. JO, LY and LFK performed data analysis. JO drafted the chapter. LY, TW, YA, ARM, CJ and MK provided feedback on drafts before chapter was finalised.

Chapter 5- JO and MK reared the mosquito colonies and designed the experiments. JO performed feeding experiment and data collection.TW and CJ advised on molecular analysis with JO performing all laboratory work. JO and LY performed data analysis. JO drafted the chapter. LY, TW, CJ and MK provided feedback on drafts before chapt er was finalised.

Chapter 6- JO performed the fieldwork with the assistance of YA and ARM. JO, LY performed data analysis. JO drafted the chapter. LY, TW, YA, ARM, CJ and MK provided feedback on drafts before chapter was finalised.

Chapter 7- JO performed the literature search and writing of this chapter. LY and TW provided feedback on drafts before chapter was finalised.

Author Abbreviations

JO - James Orsborne (PhD Student)

LY – Dr Laith Yakob (Primary Supervisor)

TW – Dr Thomas Walker (Secondary Supervisor)

LFK – Dr Luis Furuya-Kanamori

CJ – Claire Jeffries

MK – Dr Mojca Kristan

ARM – Abdul Rahim Mohammed

YA – Professor Yaw Afrane

Chapter 1 - Introduction

Background

Malaria is a life-threatening vector-borne disease typically transmitted via the bite of a female *Anopheles* mosquito. Approximately 405,000 people died of malaria in 2018 with a reported 228 million cases globally (1). The vast majority of malaria transmission occurs in sub-Saharan Africa where 93% of malaria cases and 94% of malaria deaths are reported (2). Human malaria is caused by five *Plasmodium* species and is predominantly transmitted from human to human (with the exception of *Plasmodium knowlesi*) via an infectious bite. *Anopheles* species have shown distinct differences in their preferred source of host from which to take a blood meal, with some species showing an extreme preference for human blood over all other hosts whilst others are more indiscriminate (3, 4). This difference in preference is driven by both intrinsic (genetic) and extrinsic (environmental) factors and the interaction between these factors ultimately defines a mosquito's host selection both spatially and temporally (4). One major extrinsic factor is local host abundance within the environment. Although a particular mosquito species shows an inherent preference for a certain host, these species will still switch to feeding on lesser-preferred hosts when the preferred host is scarce (4-6). The relationship between intrinsic preference and local host availability has not been formally investigated in a field setting and the spatial scale on which biting preference can shift has not been identified. Developing a better understanding of this interaction and quantifying the spatial scale on which this behaviour occurs has significant implications. Vector biting behaviour is a key aspect that underpins the current understanding of malaria transmission and control. By investigating the influence of host availability on this behaviour, new and existing control strategies can be better optimised and more effectively implemented through actively targeting this behaviour.

Malaria *(Plasmodium* species)

Malaria is caused by six species of protozoan parasite of the genus *Plasmodium* (Apicomplexa: Haemosporidae). Malaria is endemic throughout the tropics with 95 countries having ongoing transmission (2) and is transmitted via the bite of various female *Anopheles* mosquitoes species. . Human malaria is caused by five main *Plasmodium* species: *falciparum, vivax, malariae, ovale and knowlesi*. *P. falciparum* is the most common and deadly species, contributing to the vast majority of deaths caused by malaria worldwide.

Life cycle

For malaria to be transmitted from one human to another, a mosquito must firstly bite an infectious human ingesting the sexual stage of the parasite (gametocytes) as it feeds (Figure 1.1). The parasite develops in the mosquito and migrates through the mid gut lining, forming oocysts on the exterior surface. These oocysts rupture and sporozoites are released into the body cavity, migrating to the salivary glands. This now infectious mosquito will take another blood meal from a susceptible human and in the process pass the sporozoites into the blood stream of the human.

The human stage of the parasite is divided into two distinct parts; the liver stage and the blood stage (Figure 1.1). When the sporozoites enter the blood stream they migrate to the liver and infect liver cells. Within the liver cell a schizont develops and ruptures the cell sending merozoites into the blood stream. While in the blood stream these merozoites infect red blood cells and develop more schizonts, rupturing the red blood cells and releasing more merozoites. This cycle is repeated indefinitely if the human is left untreated and is the cause of the clinical symptoms of malaria. Some merozoites will differentiate into the sexual stages of malaria gametocytes, of which there are male and female forms. These are ingested by the mosquito as it feeds and the cycle continues.

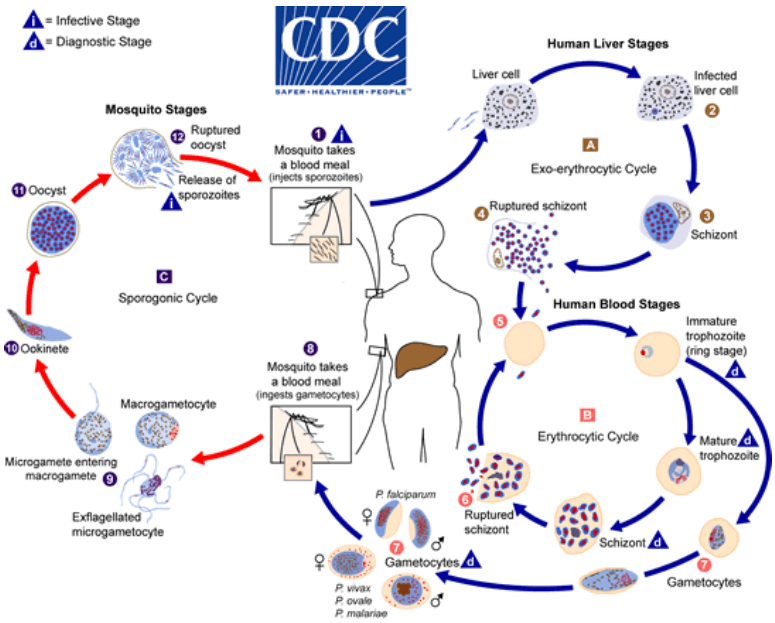


Figure 1.1: Malaria life cycle in both the vertebrate (human) and invertebrate (mosquito) host. Taken from the CDC malaria factsheet (7).

African mosquito vectors of malaria

Mosquito vectors responsible for the transmission of malaria are of the Genus *Anopheles* (Family: *Culicidae*, Subfamily: *Anophelinae*). There are approximately 484 recognised *Anopheles* species, approximately 100 of these have been shown to transmit malaria (8) yet only a few contribute significantly to transmission (9, 10). *Anopheles* species are distributed globally however, human malaria transmission occurs solely in the tropics. In sub-Saharan Africa, the major vector of malaria is *An. gambiae sensu stricto*, a member of the *An. gambiae* species complex comprising of 7 other sibling species namely *An. amharicus*, *An. arabiensis, An. bwambae, An. coluzzii An. melas, An. merus, An. quadriannulatus,* and all of which are capable vectors to varying degrees. *An. funestus* s.s. is seen as the second most important vector although a primary vector in many regions (10-14). Other vector species contribute to malaria transmission in specific areas or circumstances (15-19). These are usually categorised as secondary vectors and can have an important but lesser

impact on malaria transmission in sub-Saharan Africa (20-22). The identification of local mosquito species is critical in understanding local malaria transmission due to different vector species occupying different geographical and ecological niches (23), displaying variable levels of anthropophily (4) and vector competence (24). It is usual for multiple vector species to exist in sympatry, with each species contributing at varying intensities to local malaria transmission (25-27). The presence of sympatric species occupying distinct niches allows malaria to be transmitted across a range of geographies resulting in sustained transmission across sub-Saharan Africa.

Current interventions

Current malaria interventions are centred on both treatment (28) and prevention (29). Treatment has focused on better outcomes for high-risk groups such as children and pregnant women through new drug development and better treatment schedules (28). Preventative measures have included prophylactic drugs, environmental management, improvements to housing and more recently vaccine development (29). However, the largest contribution to the reduction in global malaria to date can be attributed to targeting the mosquito vector itself.

As mosquitoes are obligate blood feeders and for the malaria parasite to be successfully transmitted, two successful bites are required on susceptible human hosts. Vector control strategies utilise this feeding behaviour by targeting where this vector and human host interaction occurs. Many major vectors of malaria exhibit crepuscular and/or nocturnal host seeking behaviours and bite humans predominantly when they are in their homes and/or are sleeping (30). Insecticide treated nets (ITNs) exploit this behaviour by protecting the user when sleeping, whilst indoor residual spraying (IRS) targets mosquito resting on interior walls, a behaviour exhibited by major malaria vectors before and after feeding. By targeting this

fundamental mosquito behaviour these interventions have had a significant role in the reduction of malaria cases across Sub-Saharan Africa (31).

Insecticide treated nets (ITNs) and Long-lasting insecticidal nets (LLINs)

ITN distribution dramatically increased in the 2000s, with the subsequent development of Long-lasting insecticidal nets (LLINs) and realisation of their impact (31-37). ITNs have been successful in the control of a number of mosquito-borne diseases as well as a number of other vector-borne diseases. ITNs protect the individual in two ways. Firstly, by acting as a barrier, reducing the number of infectious bites an individual would receive. Secondly, the insecticidal component (usually a pyrethroid) added or impregnated into the nets actively kills and repels mosquito vectors (38, 39). By protecting the human bed nets, by nature, target the most anthropophilic of disease vectors with these species being most responsible for malaria transmission. Although coverage is rarely 100% in a given setting, the ability to reduce biting rates and vector densities simultaneously has seen a “herd” , or community like, effect with nearby unprotected individuals benefiting from local coverage due to reduced vector survival which stretched the benefits of ITN usage beyond the individual (40, 41).

Indoor residual spraying (IRS)

IRS involves the application of insecticide to the internal walls of houses and other structures. This application targets mosquitoes that rest on interior surfaces and can be used to target endophilic species depending on their resting and feeding behaviours. Like ITNs, pyrethroids are the primary class of insecticide used for IRS however the emergence of resistance to these compounds has resulted in other classes such as Clothianidin being used (42). IRS has a significant effect on reducing

malaria transmission in particular settings however logistical issues and cost has resulted in IRS not being adopted on a wider scale (43, 44) despite evidence of its potential impact (45). Using the same active ingredient as LLINs has also resulted in accelerating insecticide resistance in the mosquito population and therefore it is advised IRS is used focally and performed with a differing class of insecticide to LLINs to slow the emergence of resistance.

Current status of malaria transmission

In 2017, over 3 billion USD was invested in efforts to control or eliminate malaria with approximately three quarters of this investment spent in the WHO Africa region where the highest burden occurs (2) . The WHO reported 624 million ITNs and LLINs were distributed globally in 2017 and 50% of people at risk of malaria in Africa are now sleeping under a bed net (2). Between 2010 and 2015, malaria incidence and mortality fell by 21% and 29% respectively primarily due to the introduction of these control methods (Figure 1.2) (46). Despite the effect of these chemical control measures in recent years, malaria is still a significant burden globally and most notably within sub-Saharan Africa (46). The WHO 2018 malaria report shows progress has stalled and even regressed with bed net coverage only growing marginally from 2015 to 2017 and IRS coverage decreasing over the same period (1). Compounding this stagnation is the lack of bed nets durability and remarkable reduction in bio-efficacy seen once nets are distributed. Although continuous and mass distribution of insecticide-treated bed nets is recommended (47) many distribution campaigns work on a three to five-year cycle based on the expected lifespan of the bed net (48). In reality this lifespan could be overestimated (49). Physical deterioration of nets has been reported after just 6 months (50) and insecticidal activity highly variable due to differences in brands, internal quality control and behaviours related to care after distribution (51-54). As these control tools have

played a crucial role in the success of reducing the burden of malaria it is no surprise to see that as bed net distribution has slowed, no significant progress has been made in the reduction of malaria cases over the same period. This stagnation has also been caused by a multitude of other factors including bureaucratic (funding), political (unrest and conflict) and the ever-changing epidemiology of the disease. The introduction of anti-malaria drugs and insecticide-based control strategies has seen resistance form in both the parasite and the vectors. These developments pose a significant threat to the recent progress made and future progress in reducing malaria burden globally.

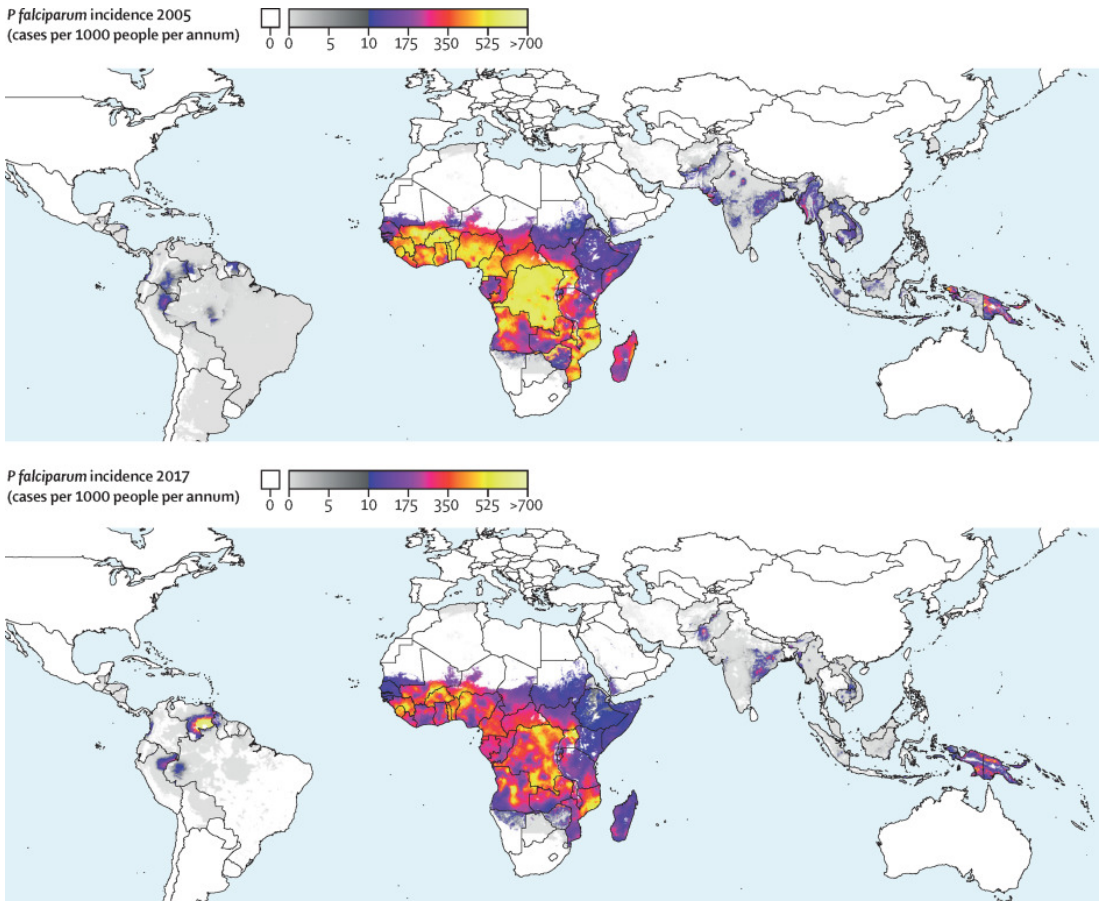


Figure 1.2: *Plasmodium falciparum* incidence maps from 2005 - 2017. Taken from Weiss et al, 2019 (55). These maps demonstrate the reduction in *Plasmodium falciparum* incidence particularly in Africa due largely to the introduction LLINs and IRS.

Insecticide resistance

The development of mosquito resistance to insecticides has been reported for nearly half a century (56). As use of LLINs and IRS has increased, resistance to the insecticides used has developed rapidly and has spread across sub-Saharan Africa with resistance being fixed in some local mosquito populations (46, 57). The development of resistance has occurred through multiple pathways with the mosquito vectors developing physiological adaptations through changes in how the insecticide is metabolised (metabolic resistance) and point mutations to insecticide target sites (target site resistance) resulting in a decrease in insecticide effectiveness (58, 59). The rapid development of these physiological resistance pathways has resulted in resistance being reported across multiple malaria endemic countries (60) with some countries showing resistance to all four classes of insecticides (61). Although evidence that this level of resistance is having a negative effect on control efforts is currently lacking (62, 63) it is widely accepted that resistance must be managed to prolong the effectiveness of these current interventions. The World Health Organisation in response has published the global plan for insecticide resistance management with the aims of better management, reporting and monitoring of insecticide resistance (64). There is also a push for the development of new vector control tools. Novel insecticides such as Chlorfenapyr and Indoxacarb, which target different pathways are currently in development with some showing early stage promise (65, 66). However, pyrethroid based insecticides are still the most commonly used insecticide classes and will remain the cornerstone of vector control through LLINs and IRS for the near future. Research that aids in maximising and extending the efficacy of these control measures is highly necessary. The persistence of malaria transmission where behavioural and physiological resistance has occurred shows the significant threat these rapid adaptations can have on the recent progress made in the reduction of malaria incidence globally (63, 67-70).

Residual and outdoor transmission

Despite the benefits IRS and LLINs have had in reducing malaria incidence globally both these interventions primality target mosquitoes that feed on human hosts when sleeping or resting within households (71). However, the reality is that these interventions target an important but very specific part of the mosquito population (10). Many *Anopheles* mosquito vectors are indiscriminate when it comes to sourcing a blood meal, biting non-human and human hosts readily and mostly outdoors making LLINS and IRS ineffective against these vector species (71). There is therefore a gap in protection, when humans are outside of their households away from the physical protection of bed nets and IRS (65). This gap is evident were sustained malaria transmission has been reported within areas of high coverage of LLINs and IRS (72-74).

This persistent malaria transmission where LLINs and IRS coverage is high has been defined as “residual malaria” transmission and demonstrates that there is a limit to the impact IRS and LLINs can have (71, 72, 75, 76). Residual malaria transmission has been attributed to behaviours that naturally expose the vectors less to these interventions (77). These behaviours include shifting peak biting times, less discriminate host preference and increased outdoor biting since introduction of interventions (71, 76, 78-80). The extent of outdoor biting is estimated to vary between 5% and 40% with a 10% increase since the year 2000, resulting in an estimated 10 million (0.6 – 22.4 million) additional malaria cases a year (81). It is therefore critical that to continue the reduction, and to make malaria elimination a reality, new and improved control strategies that target the mosquito population responsible for residual transmission must be developed and effectively implemented (63, 82-86).

Other interventions

The ever-changing epidemiology of malaria means there is a need for the development of new and novel control tools that can complement current control efforts. These tools aim to increase the effect of current interventions and target mosquito populations maintaining malaria transmission due to insecticide resistance and/or behavioural changes (65). The widespread development of resistance to current classes of insecticide has resulted in the need for development of new insecticides which utilise different pathways to overcome current resistance mechanisms (65, 87) but it is not known how the development of resistance to these new insecticides will occur. The introduction of combinations of insecticides (exploiting distinct pathways), rotation of use and addition of non-chemical components has been timely and hopes to curb the rate of resistance and maintain efficacy in areas of high resistance in the vector population. However, these traditional interventions only target the most anthropophilic vector species and are less effective against residual and outdoor transmission (65, 81, 88). The emergence of residual malaria being supported by *Anopheles* vectors which prefer non-human blood meal sources has seen the use of insecticides that can be applied to cattle to actively target these vector populations which bite outdoors and predominantly on cattle. There is also the use of the systemic endectocide ivermectin (IVM) that kill the mosquito when a blood meal is taken with IVM being shown to have a significant effect on malaria vector population densities (89). Both these strategies allow for the active targeting of outdoor and indiscriminate malaria vector species by suppressing vector population in the same way as LLINs and IRS and these tools can work in tandem to target both indoor and outdoor malaria transmission.

Factors that drive malaria transmission

The reason for the success for both LLINs and IRS to date is the ability for these interventions to reduce vector densities by effectively killing mosquito vectors (increasing the overall mortality rate) and reducing the number of infectious bites an individual may receive. The Ross-Macdonald model of malaria transmission (90) has been used for decades to inform malaria control and demonstrates the success of LLINs and IRS through reducing the human-biting rate (*a*), the mosquito daily survival probability (*p*) and mosquito density (m) (91) (Equation 1). For successful malaria transmission, two successful bites must occur, one on an infectious human and the other on a susceptible individual resulting in this parameter man-biting rate; *a* being squared (Equation 1). The squared dependence of this parameter means targeting the man-biting rate with control strategies will have a squared effect on reducing malaria transmission. With both LLINs and IRS also influencing *m* and *p* by killing mosquitoes, the success of these interventions on the basic reproduction number of malaria can be clearly seen.

Equation 1:

$$R_0 = \frac{\mathrm{m}a^2 bc\, p^n}{\mathrm{r}(-\ln p)}$$

Ross-Macdonald equation describing malaria transmission. R_0 is the basic reproduction number of malaria. *m* represents the density of mosquitoes per person, *a* is the human biting rate per mosquito, *b* is the probability of a human infection from an infective bite, *c* is the probability of a mosquito becoming infected per bite on an infected person. *p* is the daily survival probability, *n* is the incubation period of the vector and *r* is the rate of human recovery from infection.

The mosquito survival probability (*p*) and the human-biting rate (*ma*) are seen as the most important parameters in the Ross-Macdonald model having a large influence on malaria transmission. The human-biting rate is also key in determining the

entomological inoculation rate (EIR), a standard measure of malaria transmission and is used extensively in evaluating control interventions in the field (Equation 2).

Equation 2:

$$EIR = mas$$

The entomological inoculation rate (EIR) gives the number of infective bites a person may receive per unit of time and is calculated by multiplying the human-biting (*ma*) rate by the sporozoite rate (*s*) and unit of time selected (e.g. 365 day or length of season).

The importance of the human-biting rate means identifying this parameter is critical so areas of high risk can be identified and the necessary control strategies implemented (92, 93). Accurately determining the biting rate is highly complex, with the biting rates being highly dependent on climatic variables, spatial distribution of host and aquatic habitats and population density dependant, with EIRs known to vary by more than 10 times across a small spatial scale (94-96). The complexity is further increased when considering the multiple *Anopheles* species that are known to be competent vectors of malaria. Each of these species have different feeding preferences with inter-species biting behaviour known to vary considerably both spatially and temporally (4, 81) which makes attaining an accurately estimate of this key metric problematic (97). However, the importance of the human-biting rate in malaria transmission dynamics means understanding how, why and what influences it is key to developing a better knowledge base of how vector-borne disease can be transmitted within a population and ultimately how they could be controlled.

Mosquito host preference

A key driver of vector-borne disease transmission is the behaviour of the invertebrate vector in its environment (98, 99). As female mosquitoes require blood for egg development, they must find and source this blood meal from a vertebrate host. Transmission of vector-borne disease occurs via the bite of these female mosquito species when blood feeding. Therefore, the blood feeding habit of a vector is critical in the transmission and propagation of vector-borne diseases. The evolution of mosquito host preference has resulted in the development of both specialist and generalist feeders. The evolution of these distinct feeding strategies is linked to ecological specialisation theory where resource management, utilisation and the existence of ecological trade-offs drive the development of specialist or generalist behaviours (100, 101). For mosquito species and other haematophagic insects, these trade-offs have been linked with fitness advantages associated with feeding from a particular blood host (102, 103) although conclusive evidence of the presence and relative strengths of these trade-offs is mixed (104-107). Importantly, the development of these strategies and occupation of specific ecological niches allows many mosquito species to co-exist (108). The vast majority of mosquito species are defined as generalist feeders; however, many vector-borne diseases are species-specific with the pathogen unable to complete its lifecycle unless introduced into the correct host. The success of vector-borne disease and the species-specific nature of many of these pathogens suggests vectors which develop a preference for the correct host is advantageous for disease transmission, with the evolution of these species-specific preferences driven by the constant interaction between the disease vector and pathogen.

Importantly for both pathogen and vector, the environment in which these vector species exist can change overtime with different selection pressures being exerted both spatially and temporally (99). These pressures, particularly effecting foraging

behaviour are driven by intrinsic (genetic) and extrinsic (environmental) factors, with the combination of these ultimately shaping which host is bitten in the environment (4, 109). The natural variability in these factors coupled with the large geographical distribution of mosquito species results in mosquitoes occupying a diverse ecological landscape. As a result, many mosquito species show a diverse set of biting behaviours both across and within species (4, 99, 110, 111). The understanding of the affinity for a particular host species and its critical role in disease transmission has long been known and researched, particularly in human disease. For example, *Anopheles gambiae sensu stricto* and *Aedes aegypti* have shown an extreme preference for humans over other non-human blood-host sources. As a result, their respective influence in the transmission of malaria and arboviruses in human populations is substantial (3, 4, 109, 112-115). Conversely, *An. arabiensis* has been shown to be a more opportunistic vector, indiscriminately feeding on both human and non-human hosts such as cattle (116-119). As a result, their impact on malaria transmission can be highly variable depending on local factors (4, 108). Research into better understanding the host preference and selection behaviour of disease vectors is important as this behaviour has a significant influence on disease transmission.

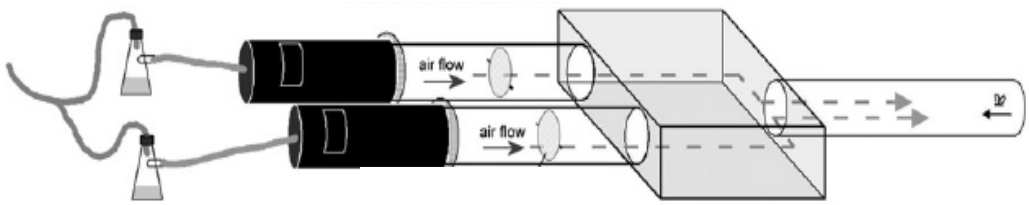
Determining mosquito host preference

Decades of research has produced multiple methods of determining the host preference of mosquitoes, with work to date involving both laboratory and field-based studies. Laboratory-based experiments comprise of assays which give the mosquito a choice between two or more potential hosts. These include the use of experimental wind tunnels, olfactometers and choice chambers (Figure 1.3) (120-123). These assays utilise the mosquito's ability to accurately identify hosts via olfaction. Olfaction is the most dominant sense used by mosquitoes to seek out a host, although other

senses are used. Mosquitoes detect and react to particular olfactory cues produced by the bodies of the vertebrate host (124). The compounds responsible for this reaction, known as kairomones initiate a response in the mosquito, with the level of this response and resulting behaviour indicative of the attractiveness to a particular host.

Similar choice experiments have also been run in semi-field conditions (125-127), again providing two or more hosts in a more open but still controlled setting. The use of a semi-field setting and field-collected mosquitoes (usually F1 generations or reared larvae) provides a more realistic assessment of preference, where a mosquito species can fly more freely than in the laboratory. It also negates the problem of using laboratory-reared insects where host preference and host seeking behavioural traits may diverge from the field due to forced membrane feeding, reduced genetic diversity and prolonged feeding on non-preferential hosts. These experimental designs and resulting research have provided the basis of understanding host preference, however, these only aid in determining the intrinsic host preference of a mosquito species, as many extrinsic factors will be controlled in the experimental design.

A



B

C

Figure 1.3: Examples of methods that are used to assess mosquito host preference. Assessing host preferences in both field and laboratory settings: a) dual-choice olfactometer, b) cattle baited trap (128) and c) human landing catch (HLC) (129).

Field-based studies assess host preference by collecting wild mosquitoes attracted to a certain host using host or odour baited traps human landing catch (HLC) (Figure 1.3) or by collecting and identifying the blood meal source of blood fed mosquitoes after feeding, usually using PCR (130-132), ELISA (133, 134) or precipitin tests (109). Field trapping strategies focus on mosquitoes collected before or after they have obtained a blood meal provides an important distinction in behaviour. Collection of mosquitoes pre-bloodmeal allows host preferences to be assess where availability of hosts can be made equal through using equal number of baited traps or by placing two differing odour baited traps (human vs bovine for example) side by side allowing a mosquito species intrinsic preference to be assessed. Conversely by sampling the blood fed population, the extrinsic effect of availability and effect of control strategies (such as LLINs or IRS) can be assessed as these factors are incorporated once a bloodmeal has been taken.

These collection strategies can be performed both indoors and outdoors. Indoor collections usually take place inside local houses or other man-made structures with pyrethroid spray catches (PSC) or manual/mechanical aspirations the preferred techniques used as they are highly effective in collecting blood fed mosquitoes in large numbers (133, 135-141). Outdoor collections utilise the resting behaviour of mosquitoes after feeding and use specially designed resting traps and artificially created resting sites, which compete with natural resting sites, allowing this population to be sampled (133, 135, 142-145). However, these can be less efficient compared to indoor collections making it difficult to collect a representative sample of the mosquito population. Collecting strategies targeting mosquitoes pre or post bloodmeal have their merits and limitations. The foundations of understanding host

preferences are grounded by choice experiments performed in the laboratory and the field, yet important differences have been shown when sampling pre and post bloodmeal in the same area (6). Therefore, the choice of collection strategy is ultimately driven by the factors, either intrinsic or extrinsic, that are under investigation.

Intrinsic factors

Intrinsic factors (or genetic factors) and their role in host preference have been researched for over 50 years, particularly for mosquito-borne diseases (3, 4). The first recorded experiment to investigate the host preference of a malaria vector was perform by Gillies, where *Anopheles* mosquitoes were released with a choice between a human volunteer or a calf and the numbers of mosquitoes counted in each chamber to infer the preference of this *Anopheles* species (3). Over the 50 years of research and subsequent reviews numerous studies have successfully demonstrated that different mosquito species exhibit a variety of intrinsic host preferences (4).

Due to the critical role of olfaction in identifying hosts, genetic differences in odorant receptors have been explored and differences linked to increased mosquito response to human odour compared with other vertebrates (146). Comparisons between odorant receptors of the major African malaria, *An. coluzzii,* and *An. quadriannulatus,* a secondary vector, demonstrated remarkable transcriptional and sequence differences (147). The influence of genetic variances has also been demonstrated in the field, with the presence of the 3Ra chromosomal inversions in *An. arabiensis* showing an association with an increased preference for humans over cattle and therefore directly influencing host choice (148-150). Presence of this genetic variation also correlates with behavioural connotations inferring differing resting behaviours. In turn influencing parasite exposure, the efficacy of vector control strategies and disease transmission (4, 148, 151-153).

Intrinsic factors remain more rigidly fixed in a mosquito population, however the high reproductive rate of mosquitoes with hundreds of eggs being laid each gonotrophic cycle means genetic drift can occur with highly successful traits selected rapidly. Indeed, Gillies showed host preferences of *An. gambiae* could easily be switched, demonstrating levels of genetic polymorphism which can be expressed as phenotypic behaviour within a few generations (3). Although it is clear host preferences has some genetic basis, the relative role of these genetic traits and how they may influence host preferences is poorly understood. Semi-field and more controlled assays where these genetic differences can be directly compared would be timely and is advocated in this field of research (4, 150). Currently, these studies have been performed in the field where extrinsic factors such as host availability and other ecological factors could not be adequately controlled.

Extrinsic factors

Extrinsic factors can heavily influence which host is ultimately bitten by a mosquito species within the environment and have the ability to shape host preference both spatially and temporally (4). The physical size (154, 155) and more extensively the "smell" of the host itself plays a significant role in mosquito host preference (4, 156). The type of kairomones secreted and the levels of their secretion from the skin of the host play a crucial role in a host's attractiveness (156, 157). *Anopheles gambiae* and more significantly *Ae. aegypti* respond strongly to differing levels of lactic acid, which is a key secretion from human skin (158, 159). There is also compelling evidence that parasite or pathogen infection of the vertebrate and invertebrate hosts can influence host attractiveness and in turn selection. Humans infected with malaria parasites are shown to be more attractive to mosquitoes (160) with particular compounds upregulated in their odour profiles (161). Parasite manipulation of the vectors has also been reported, resulting in increased biting rates and probing of malaria infected mosquitos (162). On a macro scale, climate and seasonal weather changes also

effect host preference with some mosquito species switching host preference between summer and winter months (163). Drought or dehydration effects blood feeding frequency and host choice, with mosquitoes shown to skip sugar feeds for feeding on blood multiple times during a single gonotrophic cycle (164). These seasonal changes in host preference could be attributed to changes in local host availability due to migration and seasonal behavioural changes of the particular host rather than direct effect of the local climate itself (163, 165). Indeed, availability and abundance of the host within the environment often dictates a mosquito's host selection (4). As extrinsic factors can be capricious by nature, their effect on host preference can vary from substantial to negligible and as a result likely contribute to the disparity in biting behaviour seen within the same mosquito species (Figure 1.4). Although host preference does have a genetic grounding, extrinsic factors demonstrate the ability to be highly influential in host selection and therefore their influence should be considered when assessing this behaviour.

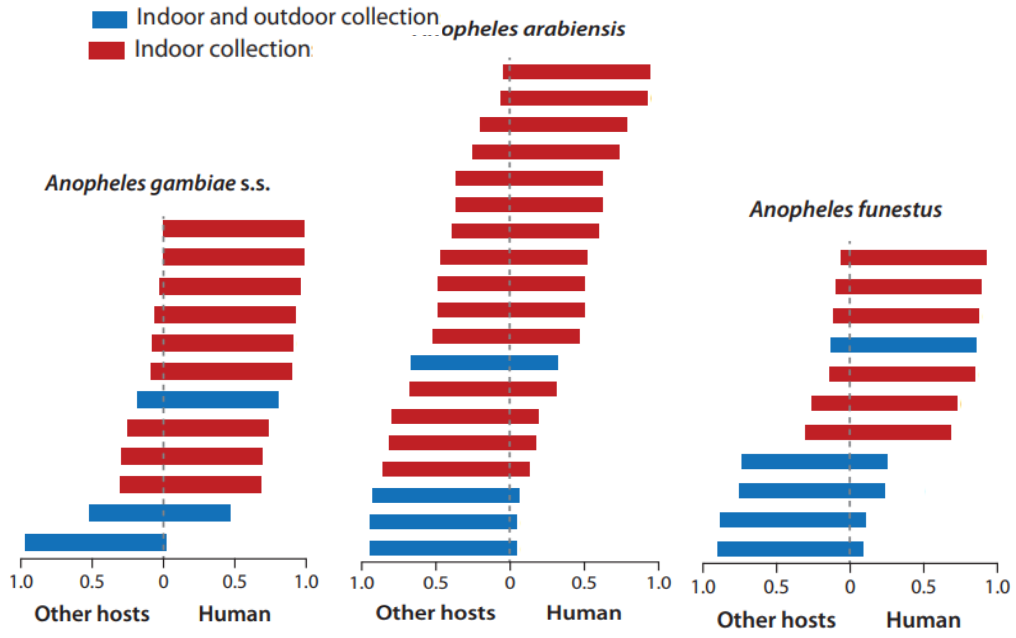


Figure 1.4: Blood meal source variability in *Anopheles gambiae s.s.*, *An. arabiensis* and *An. funestus*. Each bar represents a single study with colour representing collection method demonstrating the variation within species of blood meal source. Taken from Takken (124).

Host availability and blood-host plasticity; their effect on disease transmission and efficacy of control tools

The influence of host abundance on female mosquitoes host selection has significant implications for vector-borne disease transmission (166) . The most well documented example of this influence is in zoonotic disease. West Nile Virus (WNV) transmission in North America is widely supported by *Culex* species that regularly bite the local bird population. However, when this avian population migrate the local availability of these bird species diminishes rapidly. This results in the Culex species switching to biting other more readily available hosts including humans, resulting in a rapid rise in the number of human WNV cases in the US and North America (165). *Trypanosoma cruzi* transmission has also been observed in the USA, where the local *Triatoma species* (a vector of *T. cruzi*) was observed to only start biting humans when local populations of its preferred blood-host , the armadillo, collapsed (167). For zoonotic malaria, *P. knowlesi* has been detected in human populations in South-East Asia where humans have encroached on forested areas for work or to develop new settlements, resulting in a reduced macaques population (the preferred host) while increasing availability of human hosts, consequentially increasing *P. knowlesi* transmission in the area (168). These examples show that when the availability of the preferred host within the environment declines, many mosquito species will switch to the predominantly available host, with this switch having a dramatic effect on disease transmission.

In the context of human malaria, the effect of switching host is more pronounced as successful transmission of the parasite can only occur when two successful bites occur on human hosts. As a result of this the major malaria control tools (namely; LLIN's and IRS) function by killing the vector (by reducing vector population survival) or by reducing the availability of human hosts to host-seeking mosquitoes, thereby reducing the human biting rate. Indeed, due to the success of these intervention, changes in local species composition have been shown to occur with *An. gambiae*

densities decreasing significantly after the introduction of these interventions whilst *An.arabiensis* population numbers remained relatively unchanged (169, 170). However, despite the success of these current interventions and reduction in the availability of human hosts, mosquitoes have shown rapid adaptations to these interventions. These adaptations include both genotypic and phenotypic adaptations such as development of resistance to insecticides (171), increased exophilly (172), shifts in peak biting times (63, 124, 173-176) and changes in preferred blood-host (177). These adaptations have precluded complete control in some regions with malaria transmission still occurring even in areas of high levels of IRS and LLIN usage (87). Indiscriminate feeders such as *An. arabiensis* by nature will be exposed less to these controls (88). This indiscriminate feeding plasticity and perhaps to a degree because of it, has allowed *An. arabiensis to* become the dominant malaria vector in many locations (133, 178-180). This poses a unique problem for IRS and LLINs, as these tools are less effective at targeting vector populations that feed both on human and non-human hosts (181, 182). Therefore, understanding a mosquito's choice to take a blood meal from a specific host in specific scenarios can have an impact on how a vector-borne disease is transmitted and how it can be controlled.

PhD Overview

This thesis investigates the impact local host availability has on the blood-host choice of *Anopheles* malaria vectors in the field. Using current literature, field and laboratory methodologies, the influence host availability has on local mosquito biting behaviour is firstly hypothesised and then investigated in the field using a novel mosquito collection methodology. Blood fed *Anopheles* malaria vectors are collected across a range of host availabilities in southern Ghana across two years of field collections. Mosquito species and host blood meal source are formally identified in the laboratory and correlations between blood meal sources, collection location and local host availability investigated so the relative influence of both intrinsic and extrinsic factors can be explored. Implications of these findings are discussed in the context of better understanding local mosquito biting behaviour and how the consequences of these findings can affect current and future malaria control strategies.

Hypothesis

Host abundance will have a significant effect on the host choice of local *Anopheles* mosquito populations in the field and could even dominate mosquito host choice, overpowering intrinsic preferences.

Thesis Overview

Chapter 2 – Materials and Methodologies

Contains details and development of both field and laboratory methodologies. This chapter demonstrates the design of a unique collection methodology utilising a transect of mosquito traps that were used for all field collections. The optimisation of subsequent molecular techniques used to identify blood meal origin, species identification and molecular techniques for measuring blood meal digestion are also described as well as other methodologies used throughout this thesis.

Chapter 3 – Using the human blood index to investigate host biting plasticity: a systematic review and meta-regression of the three major African malaria vectors (Orsborne *et al*, 2018. *Malaria Journal*)

In this chapter I review the reporting of the human blood index (HBI) systematically for the major malaria vectors *An. arabiensis*, *An. gambiae* and *An. funestus* species complex in sub-Saharan Africa. The effect of key factors such as collection method, collection location and species present are investigated to demonstrate how these factors affect the reporting of the human blood index (HBI). This chapter also informed the methodology used for the field collection of blood fed mosquitoes described in Chapter 2 and provides evidence of host selection plasticity and the role of extrinsic factors in mosquito host selection within these major malaria vectors, complementing the rational of this thesis.

Chapter 4 – Investigating the blood-host plasticity and dispersal of *Anopheles coluzzii* using a novel field-based methodology (Orsborne *et al*, 2019. *Parasites and Vectors*)

This chapter presents the results and findings from a pilot field collection in 2017. Collection methodology designed in Chapter 2 is tested in the field with blood fed mosquitoes collected from areas where human host availability varied from high density to low density. Blood meal origin and species identification of the mosquitoes was performed and correlated with local host abundance allowing the spatial scale on which *Anopheles coluzzii* can vary its host selection to be identified. Molecular analysis is also used to determine post-feeding time of collected mosquitoes with the aim to better inform dispersal and understand post-feeding behaviour of blood fed mosquitoes in the field.

Chapter 5 – Using visual and molecular methodologies to investigate blood meal digestion and estimate post-feeding time for four major vectors of mosquito-borne disease; Anopheles *coluzzii*, *Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefasciatus*.

This chapter describes the development of a time series of blood meal digestion for four major vectors. Four laboratory reared mosquito species are fed on cow blood with sub-samples killed every six hours to generate a panel of mosquitoes at different stages of blood meal digestion. PCR is used to investigate blood meal digestion and findings are compared to a visual scoring system (the Sella score) with the advantages and disadvantages of this technique discussed. Here I investigated and compared these two methodologies of measuring blood meal digestion with the aim to provide a more accurate way of determining time post-feed as well as how this technique can be used to inform dispersal and post-feeding behaviour of mosquito

vectors in the field. The physiological differences in blood meal digestion between species are also discussed.

Chapter 6 – Evidence of extrinsic factors dominating intrinsic blood host preferences of major African malaria vectors (Orsborne *et al*, 2019. *Scientific reports*)

Presents the results of the 2018 field collection using the study design in Chapter 2 with the addition of indoor collections to explore the effect collection location has on HBI (following the findings in Chapter 3). This chapter investigates multiple Anopheles species namely, *An. coluzzii*,*An. gambiae* s.s. *An. rufipes* and *An. pharoensis*. Comparisons are made between indoor and outdoor collection methodologies as well as variation in blood-host selection between these different *Anopheles* species. The hypothesis is that local environmental factors; namely host availability across the transect and collection location would have a greater influence over blood-host selection than the species of the mosquito caught, demonstrating extrinsic factors can dominate a mosquito's host selection despite any intrinsic preference a mosquito species may have. The consequences of these findings for assessing, and perhaps even augmenting, future control strategies are discussed.

Chapter 7 – Discussion and concluding remarks

The findings of this thesis are discussed and summarised in a broader scientific context. Limitations as well as future work and recommendations are also discribed.

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Chapter 2- Materials and Methodologies

Abstract

Introduction: To investigate the scale on which blood host choice can vary with host availability a unique experimental design is required. This chapter describes the study design used in the field, the criteria used for selecting the study sites as well as the description of study sites and trapping schedules. As different mosquito species will exhibit differing blood-host preference and members of species complexes are morphologically identical, molecular assays allow the differentiation of species of malaria vectors present in field samples as well as determine the origin of their blood meal source. Here, the optimisation of these methodologies is described as well as sample storage and DNA extraction methods used.

Methods: The systematic literature search (performed in Chapter 3) was used to develop a collection strategy and identify mosquito trap types best suited for testing the hypothesis of this thesis. Polymerase chain reaction (PCR) assays were optimised to identify mosquito species and blood host.

Results: A collection strategy utilising a 250 m transect with traps placed at 50m intervals was designed starting at an area of low to negligible human density close to cattle resting area and finishing at the centre of a village where human density was at its highest. Optimisation of molecular methods allowed both sensitive and specific assays to be developed which accurately identify mosquito species and blood host sources in the laboratory and previously collected field samples.

Conclusion: The implementation of transect style collection strategy will allow the effect of blood host available on blood host selection to be investigated in the field. The optimised PCR assay will provide high quality data on mosquito species present and blood host source which is critical if this association is to be quantified.

Mosquito collection strategy and study design

Rationale and development of collection design

To investigate the effect host availability had on the host choice of mosquitoes in the field required collection of blood fed mosquitoes from the same population but with deferring access to alternative host species. In Ghana, cattle are usually kept on the periphery of villages. Typically they are not retained inside domestic households (a practice sometimes seen in parts of East Africa (1, 2) but instead are kept in pens close to the village over night to reduce the risk of theft (Professor Yaw Afrane, *personal communication*). This provides a fixed point from which local mosquitoes could access cattle. Malaria vectors in southern Ghana bite between 18.00 and 06.00h with peak biting times between 24.00 – 04.00h (3-5); and based on observations of the local entomological team, most village inhabitants remain within the village from dusk to dawn. This meant there was also a fixed point from which local mosquitoes could access humans during the time they typically blood feed. A transect running from a peripheral cattle pen to the centre of a village would thereby provide a gradient of availability for the alternative hosts.

Final study design

The collection strategy involved clusters of traps placed at approximately 50m intervals for 250m forming a transect comprising six trapping points (Figure 2.1). The transect design allows the availability (defined by distance from the hosts) to be varied within the same collection site and therefore allows the effect of host availability on mosquito host choice to be investigated across a 250m area.

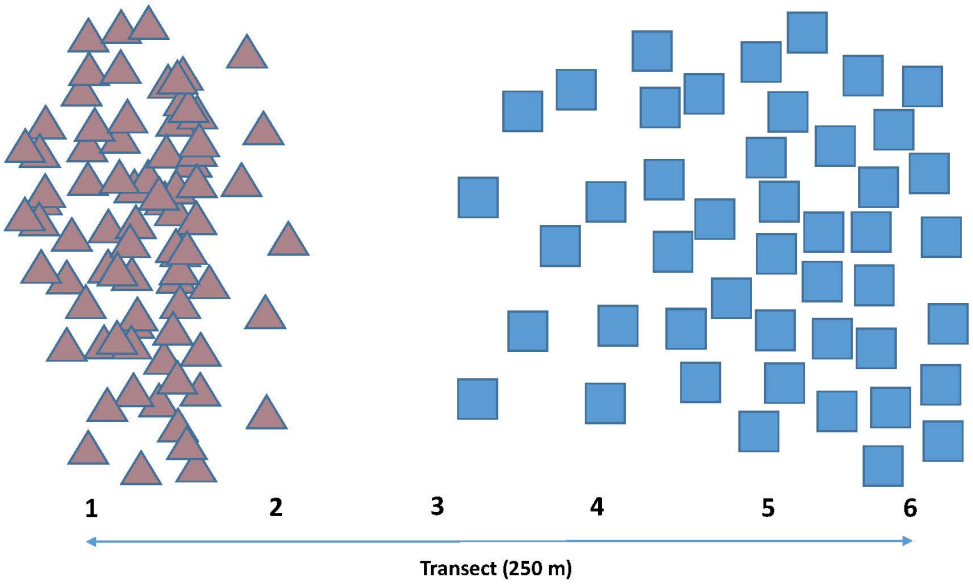


Figure 2.1: Schematic diagram of the study design developed. Houses are represented by blue squares with alternative hosts (e.g. cattle) represented by brown triangles.

Field site criteria

All collections were performed in south-eastern Ghana between May 2017 and July 2018. The specific requirements needed to perform this study meant all potential sites had to meet the following criteria:

Location

Each study site needed to be within the vicinity of an area where a temporary field laboratory could be set up allowing samples to be processed effectively. Sites also needed to be within one day of travel to Accra so supplies could be collected and samples easily transported back to the University of Ghana for long-term storage.

Host species

Each study village was required to have either a holding pen for cattle or an enclosed area in which cattle were kept overnight. Cattle could not be more than 500m from the village periphery to minimise chances that mosquitoes collected from nearby cattle were from a separate population than mosquitoes collected from the village. Five hundred metres was selected from reviewing previous mark-release-recapture studies which describe malaria vectors covering this range routinely (6, 7). No other cattle or other significant animal holdings were to be in the vicinity of the study site (no closer than 2km) though other smaller animals such as chickens, goats, dogs and cats were present in the vicinity of the human population.

Accessibility

Before the sampling began, the village elders were met and study design discussed. The elder in the presence of the local entomology team would also explain the study to the village as a whole, so any questions could be answered. Once the elder granted access to the village, trap locations were selected, recorded and collections would begin the following evening.

Study sites

Twelve sites across southern and northern Ghana (Figure 2.2) were visited in total. Of these, two sites (Obama and Dogo) were identified in 2017 where two small pilot studies were performed. In 2018, based on the pilot study results, Dogo was revisited.

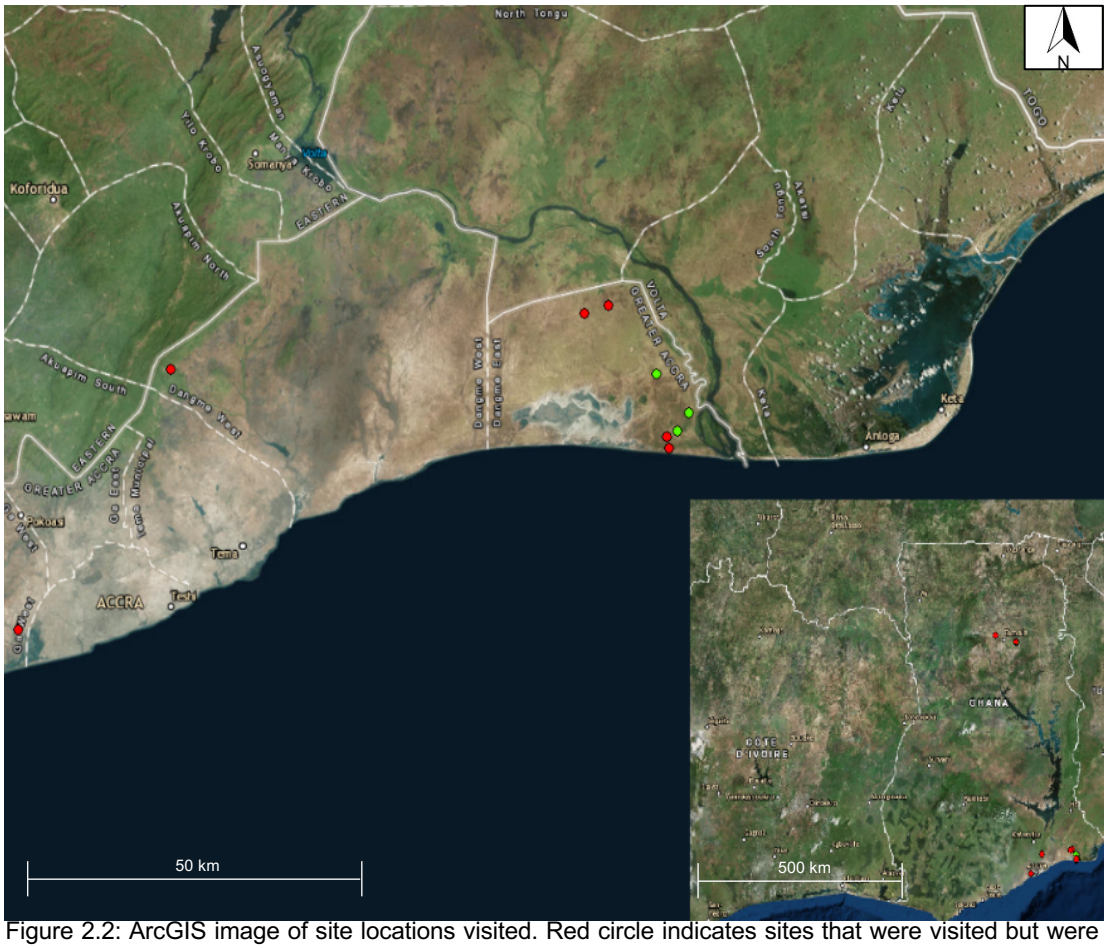


Figure 2.2: ArcGIS image of site locations visited. Red circle indicates sites that were visited but were not suitable for sampling. Green circle indicates sites suitable for sampling.

Obama (5°52'24.9"N 0°33'36.3"E)

A small population of approximately 100 people which were grouped close to the road between Ada and Big Ada. Approximately 150 metres away from the edge of the human inhabitants was a cattle ranch owned by the village chief. The ranch contained approximately 200 cattle kept in a walled, partially covered area from 1700h to 0800h. Between the cattle pen and the first houses there was approximately 50-75m of open ground (Figure 2.3). Six transect points were placed at 50m intervals starting at the cattle pen and ending in a compound of households where the human population density was highest (approximately 40 - 50 human inhabitants).

Figure 2.3: ArcGIS image of Obama field site. Blue line indicates transect route starting at the cattle ranch in the west (circled in red) and finishing within the human settlement in the east.

Dogo (5°52'24.9"N 0°33'36.3"E)

A large farming community (approximately 500 people) located off the same main road as the first site in Obama (Figure 2.4). The community had a large number of cattle (approximately 300) which were kept in adjacent holding pens on the periphery of the village overnight. Cattle were kept approximately 50 m from the edge of the village (Figure S2.2).

Figure 2.4: ArcGIS image of Dogo field site. Blue line indicates transect route from cattle ranch (circled in red) through to the settlement of Dogo.

Adult mosquito collection

To identify the effect of host availability on host choice, blood fed mosquitoes were collected along the transect at the chosen field sites.

Outdoor collections

Four different trap types were used to collect blood fed mosquitoes outdoors (Figure 2.5).

Centre of Disease Control (CDC) miniature light trap

The CDC miniature light traps (John W Hock, USA) (Figure 2.5A) were hung at each transect point a minimum of 1.5 metres from the ground using trees, outdoor housing eaves or fencing, depending on location. The light was used and a carbon dioxide (CO_2) bait was added to increase collection yield and was generated by placing 3g of

yeast in a 500ml plastic bottle with 40g of sugar and 200ml of water, bottles were mixed and lids loosened so any CO_2 produced would be release (8). These bottles were attached to the traps below the rain cover close to the trap entrance and replaced after each night of trapping.

Biogents (BG) Sentinel® 2 Trap

The BG-Sentinel® trap (Biogents, Germany) is usually used to collect *Aedes aegypti*, *Aedes albopictus* and *Culex quinquefasciatus.* However, with the addition of CO_2, the BG-Sentinel can be used as a general surveillance tool for collecting a broader range of mosquito genera and collects *Anopheles* species (9-12). The BG-Sentinel mosquito trap is a collapsible, fabric container with a white lid with holes covering its opening. Air is sucked into the trap through a black catch pipe by an electrical fan, drawing approaching mosquitoes into a catch bag (Figure 2.5B). Traps were placed on the ground at each transect point. CO_2 was added to the trap using the same methodology as the CDC light trap with the bottle placed within the BG trap allowing CO_2 to be emitted from the holes in the lid and into the environment.

CDC resting trap

The CDC resting trap consists of a fibreglass box in which a fan and catchment mechanism sit (Figure 2.5 C). The box is open on one side and dark in appearance. This box acts as a resting area for mosquitoes; once they enter they are caught by the fan and collected in a catchment net. Traps were placed on the ground in darker shaded areas where possible.

Clay pot resting trap

Clay pots have been used successfully to sample resting mosquito populations previously (13) (Yaw Afrane, *personal communication*). The pots used were traditional West African clay pots with a small hole made in the bottom to prevent water gathering inside (Figure 2.5D). Pots were placed on the ground in dark shaded areas and left for the duration of trapping time. Collections involved placing a net over the pot and manually aspirating the mosquitoes out of the pots into paper cups with netting for storage.

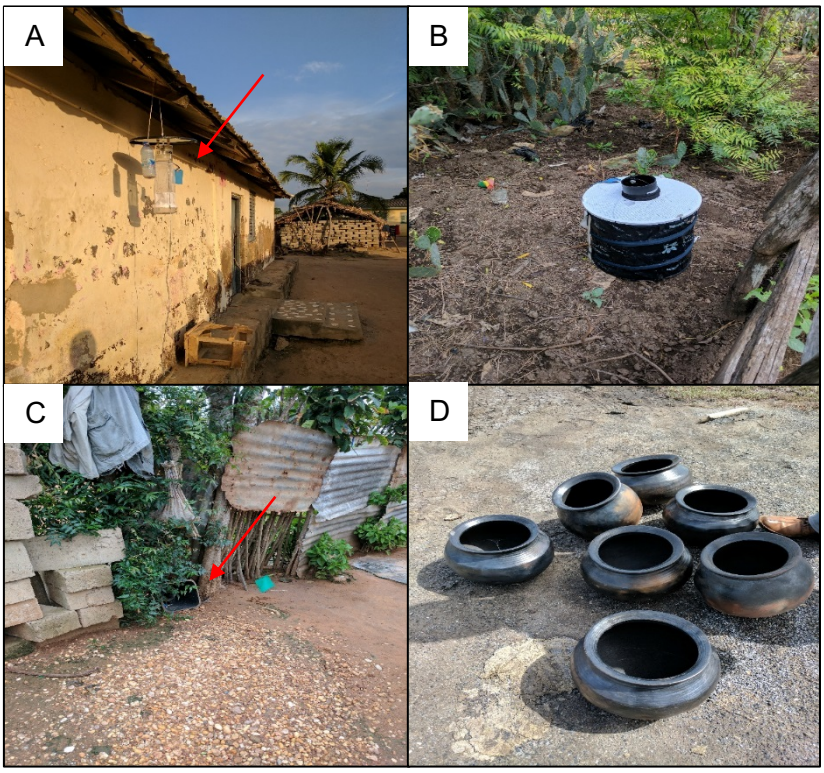


Figure 2.5: Images of the four different trap types used to collect outdoor resting mosquitoes. *A: Centre* of disease control (CDC) miniature light trap. B: Biogents (BG) Sentinel 2 Trap. *C: CDC* Resting Trap. D: Clay pot resting trap.

Indoor mosquito collection

Indoor collections were added to the 2018 field collections to further explore the spatial scale over which host biting plasticity occurs. Due to the housing structure

(Figure S2.2) and frequency of collections required manual collection of mosquitoes using an aspirator was seen as the most effective methodology.

Recruitment of households for indoor collections

Once verbal consent was given by the village elder, households at each transect point were visited to be recruited into the study. The study was explained to the head of the household and informed consent obtained from the head of the household if they agreed to take part in the study. Only once informed consent was obtained from household at each transect point was the study allowed to begin.

Prokopack Aspirator

The Prokopack aspirator (John W Hock, USA) consists of a single fan unit attached to the end of a 50cm pole. Within the unit sits a collection cup. The Prokopack was linked to a 12V battery and hovered over internal walls, eves and the ceiling of participating household (Figure 2.6). Mosquitoes collected during 15 minutes of aspirations were removed and transferred into collection nets labelled by transect number and house ID.

Figure 2.6: Prokopak aspirator used to collect indoor resting mosquitoes. Photo taken at Transect 1.

Trapping schedule

Mosquito traps were set one hour before sunset (approx. 18.00h) and run for 12 hours. At 0600h trap bags were removed and mosquitoes collected. A fully charged battery was used for each trap and night of trapping. Trapping nights varied across the different collection seasons and sites (Table 2.1). Indoor collections performed in 2018 followed the transect design with two houses being selected to represent each transect point. Collections were rotated between these two houses each night of collection, resulting in collections being performed on eight human inhabited households (two for each transect point) for transect points 3, 4, 5 and 6. As the human population did not live close to the cattle holdings, indoor collections for transects 1 and 2 were performed in uninhabited outbuildings/cattle sheds close to each transect point. Indoor resting mosquitoes were collected between 0400h – 0600h each morning.

Table 2.1: Summary of fieldwork performed including details on collection year, site, nights collected, collection location and trap types used.

Year collected	Site name	Location	Nights	Collection locations	Trap types used
2017	Obama	Ada	5	Outdoor	CDC Resting Traps
2017	Dogo	Ada	5	Outdoor	CDC Resting Traps
2018	Dogo	Ada	21	Indoor and Outdoor	CDC Resting Trap, CDC Light Trap, BG Senintel 2 Trap and Prokopack aspirator (Indoor collections)

Transport, handling and storage of specimens

Samples were transported back to the field laboratory where they were placed in a sealed container along with cotton wool soaked in chloroform. Once killed, mosquitoes were sorted based on genus and blood feeding status. Blood fed *Anopheles* were stored individually, abdomens (including blood meal) were either separated from the head/thorax and pressed onto FTA® cards (Sigma-Aldrich, UK) (2017 collection) or whole bodies were stored in RNA later® (Thermo Fisher Scientific, UK) (2018 collection) as both these methods preserve blood meal integrity across a wide range of temperatures. Any non-fed mosquitoes were stored on silica in 5 ml transport tubes (Thermo Fisher Scientific, UK). All samples were labelled and stored based on genus, night of capture, trap type, and transect point.

Morphological identification of adult mosquitoes

Anopheles mosquitoes were identified to species level where possible. As species within the *An. gambiae* species complex are morphologically indistinguishable these specimens were recorded to the complex level with species confirmed using molecular analysis. Based on local expertise, the known species of *Anopheles* present were *An. coluzzii, An. gambiae s.s.* and due to the field sites proximity to the sea and brackish water, *An. melas,* which is also a member of the *An. gambiae* species complex. In addition, secondary malaria vectors species *An. pharoensis* and *An. rufipes* were also known to be present in the area and were morphologically identified using keys developed by Gillies and Coetzee (14).

Laboratory Methodologies

Mosquito samples were transported back to the London School of Hygiene and Tropical Medicine (LSHTM) after each collection period. Before extraction, morphological identification was repeated to confirm species/species complex identification made in the field. Polymerase chain reaction (PCR) was used to identify mosquito species which were morphologically indistinguishable and to identify blood meal source (Flow diagram of sample workflow shown in supplementary material S2.1). DNA was extracted from each collected sample using the methodologies described below.

Extraction of blood-fed mosquito's abdomens and whole bodies

Blood engorged abdomens were separated from the mosquito head and thorax prior to extraction. Abdomens were removed using sterile forceps and dissection pin and placed into 96-well extraction plates provided as part of the Qiagen DNeasy 96 Blood and Tissue Kits (Qiagen, UK). Forceps and dissection pins were sterilised between

each specimen using 100% ethanol and a final wash in sterile water. A 5mm steel bead (Qiagen, UK) was placed onto each specimen followed by 180µl of Buffer ATL and 20µl of proteinase K. Samples were then homogenised using a tissue lyser II machine (Qiagen, UK) for 3 minutes at 30Hz and then incubated at 56^{o}C for 5 minutes. 200 µl of buffer AL was added to each sample followed by 200 µl of 100% ethanol. Racks were shaken manually to ensure mixing. Resulting lysate was transferred individually to DNeasy spin columns (96 well format) (Qiagen, UK). Once transferred, plates were centrifuged for 3 minutes at 14G. DNA was washed twice using recommended wash buffers (Qiagen, UK) and spun for 3 minutes between the two wash steps. DNA was eluted into a new 96 well elution rack (Qiagen, UK) in 100µl of Buffer AE. DNA was stored at -20 ^{o}C until processed.

Extraction from FTA cards

FTA cards containing the mosquito blood meals were punched out using a 3mm hole punch. The resulting 3mm FTA punch was placed into the 96-well extraction plate (Qiagen, UK). The hole punch was sterilised between each specimen using 100% ethanol and a final wash in sterile water. 180µl of Buffer ATL and 20µl of proteinase K was added to each specimen. Samples were then incubated at 56^{o}C for 6 hours; after which supernatant was removed and placed into a fresh extraction plate. DNA was then extracted following the DNeasy 96 Blood and Tissue kit (Quigen, UK) protocol (summarised above). DNA was stored at -20 ^{o}C until processed.

DNA quantification

Total DNA was quantified using the Qubit Fluorometric Quantification system (Invitrogen, UK). Following manufacturer's protocol, a working solution of Qubit high sensitivity (HS) kit (Invitrogen, UK) was made consisting of 1µl of Qubit HS dye and

199µl of buffer solution per reaction. 198µl of the working solution was used per reaction with 2µl of sample DNA added. The sample was vortexed and left for 3 minutes to allow fluorescence develop. The sample was then read using the Qubit Fluorometric Quantification system (Invitrogen, UK) providing a ng/mL reading. This was then converted to ng/ µl for normalisation purposes.

Molecular identification of blood meal source

Polymerase chain reaction (PCR) was used to determined blood meal source. Two PCR assays were used to identify the host blood meal source (Summarised in Table 2.2). As the transect design focused on two dominant species from which blood meals could be sourced (humans and cattle), assays that can accurately detect these species were developed.

Table 2.2: Selected PCR assays optimised and tested for blood meal identification including details on author, host detection capabilities and PCR design

Author name(s)	Design	Host
Gunathalaka *et al*, 2016 (15)	Real-time multiplex PCR using specific primer sets for each target species	Human and Cow
Promega® Plexor HY System	Forensic grade probe based real-time PCR assay. Quantitative PCR capable of determining concentration of total human DNA and male human DNA simultaneously. The assay also contains an internal PCR control (IPC) to test for false-negative and a melt curve analysis. Highly sensitive and specific	Human only (gender identification possible)

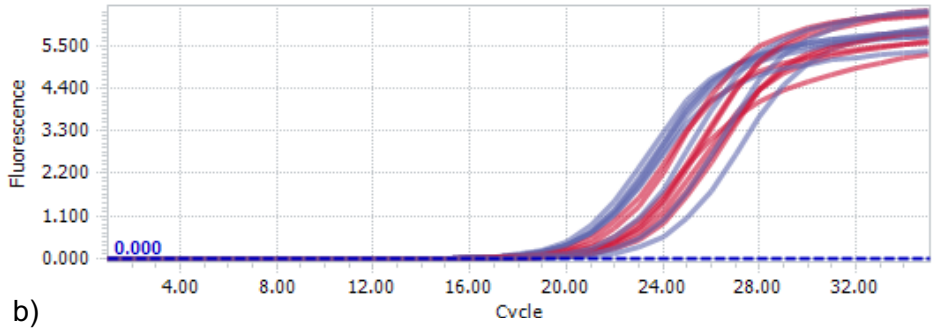
Real-time bovine blood meal detection

The bovine specific PCR primers consisted of a species-specific forward and reverse primer (Table 2.3). PCR reactions were performed using a Roche LightCycler 96 System (Roche, UK). Cycling conditions were as follows; a pre-incubation period of 95 ^{o}C for 5 minutes followed by 40 cycles of 95 ^{o}C for 10 seconds, 62 ^{o}C for 10 seconds, 72 ^{o}C for 30 seconds. A melt curve analysis was then performed to differentiate target amplicons from other non-specific amplification with bovine DNA having a melt temperature of 78^{0}C (Figure 2.7b). All PCR runs contained a positive DNA control and negative control of DNA free water. All analysis was performed using the Roche LightCycler software (Roche, UK).

Table 2.3: Reagents used for bovine DNA detection in mosquito blood meals

Reagent	**Volume (1x)**
Bovine Forward Primer	0.5 µl
- GCCATATACTCTCCTTGGTGACA	
Bovine Reverse Primer	0.5 µl
- GTAGGCTTGGGAATAGTACGA	
PCR Grade H_2O	2 µl
FastStart SYBR Green Master Mix (Roche, UK)	5 µl
Template DNA	2 µl

a)



b)

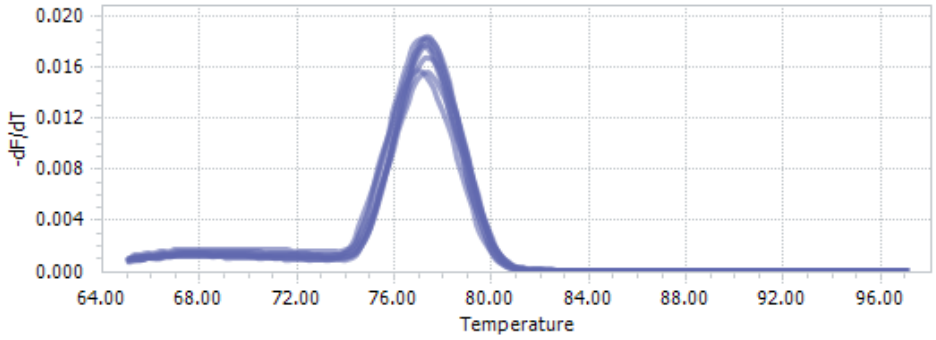


Figure 2.7: Florescence plot (a) and melt curve (b) produced by bovine specific PCR primers designed by Gunathalaka (15)

Real-time human blood meal detection

PCR reactions were performed using a Roche LifeCycler 96 system using the following reaction conditions: 0.5 µl of each human specific forward and reverse primers (each at 10 µM/µl), 2µl of DNA free water and 5µl of FastStart SYBR Green Master Mix (Roche, UK) (Table 2.4). Cycling conditions were identical to the above conditions for the bovine primers with the exception of a higher annealing temperature of 69 ^{o}C with all analysis performed using the Roche LightCycler software (Roche, UK). The human specific primers produced some strong human amplification with Ct values below 28 cycles and species-specific melt curves (Figure 2.8). However, amplification was also present after 30 cycles (Figure 2.8a) with weak indiscriminate melting curves (Figure 2.8b). This was reported in the original publication and as a result, samples with Ct values above 30 were considered potentially beyond the

threshold for detection and reported as negative. Samples with Ct values of 29 and below were confirmed as positive for human blood with an additional real-time PCR assay.

Table 2.4: Reagents used for real-time Human DNA detection

Reagent	**Volume (1x)**
FastStart SYBR Green Master Mix (Roche, UK)	5 µl
Human Forward Primer - TTCGGCGCATGAGCTGGAGTCC	0.5 µl
Human Reverse Primer -TATGCGGGGAAACGCCATATCG	0.5 µl
PCR Grade H_2O	2 µl
Template DNA	2 µl

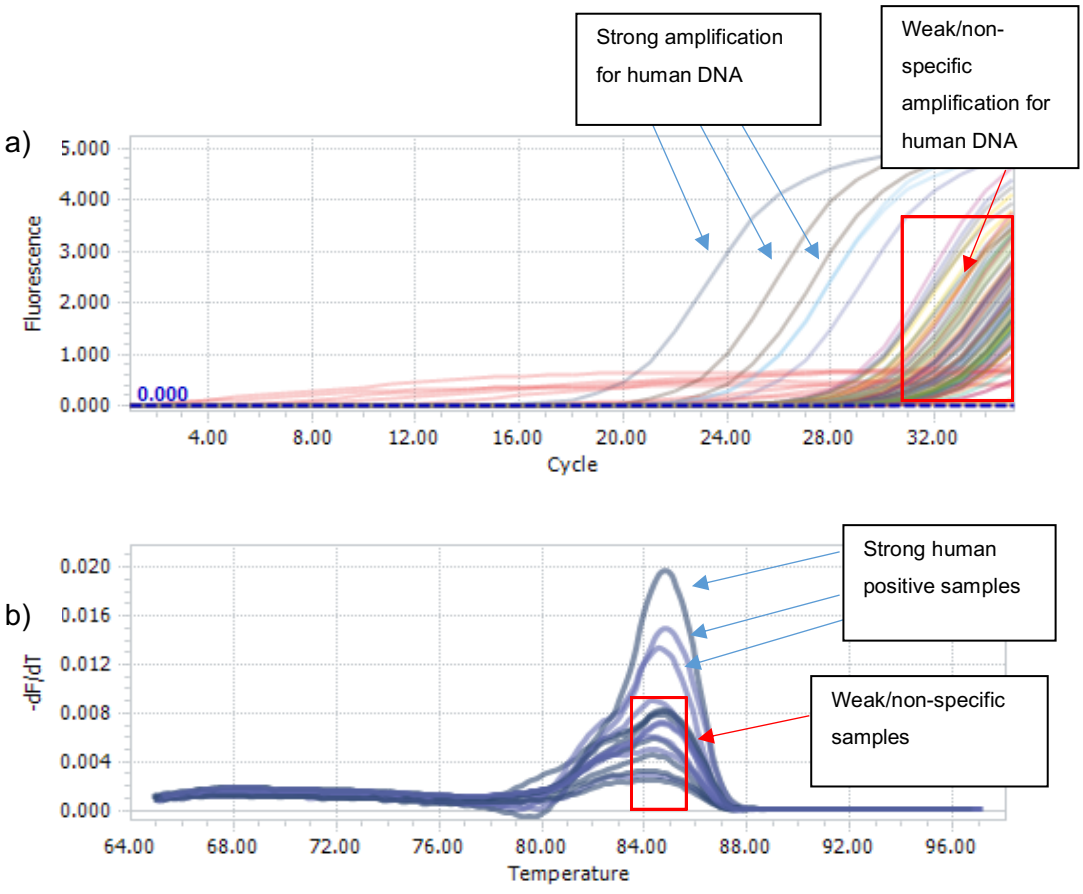


Figure 2.8: Fluorescence plot (a) and melting curve (b) produce by human specific PCR primers designed by Gunathalaka (15)

Human blood meal confirmation

Due to the specificity issues with the primers designed by Gunathalaka (15), an additional confirmation PCR was used to accurately identify human blood meals.

Promega Plexor® HY System

The Promega Plexor® HY system (Promega, USA) is a forensic grade real-time PCR kit capable of detecting human DNA only. The system consisted of two primers, one with a fluorescent reporter and the other with a quencher (Table 2.5). When primers attached to the target DNA sequence during the annealing and extension stage the quencher is in close proximity to the fluorescent reporter resulting in a reduction in fluorescence with this reduction recorded in real-time (Figure 2.9). The Plexor® system was tested on the 20 samples (samples with Ct values >29 and samples with < 30) from the initial human qPCR assay developed by Gunathalaka (15) along with both positive and negative control and a bovine DNA extract to check specificity. Cycling conditions were as follows: A pre-incubation period of 95^{o}C for 2 minutes followed by 38 cycles of 95^{o}C for 5 seconds and 60 ^{o}C for 35 seconds and finally a melting curve. The assay was run on a Stratagene MX3005p qPCR System with analysis performed using the Promega Plexor® analysis software (Promega, USA).

Table 2.5: Reagents used for Plexor® HY system for human blood detection

Reagent	Volume (1x)
Plexor HY® Master mix (Promega, USA)	5 µl
Amplification Grade H_2O	3.5 µl
Plexor HY® Primer/IPC mix (Promega, USA)	0.5 µl
Template DNA	1 µl

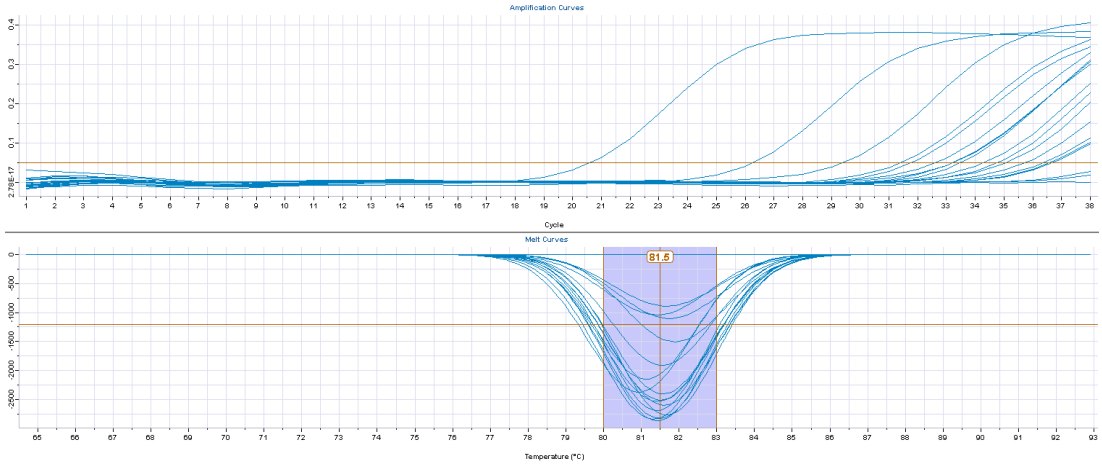


Figure 2.9: Fluorescence plot and melting plot of Plexor® HY system for detection of human blood. The box and red line indicate 81.5°C, the expected melting temperature of the human specific amplicon. The horizontal red line is a threshold of fluorescence required to be a known positive sample with this threshold set by the Plexor® software automatically.

Blood meal identification PCR sensitivity check

After taking a blood meal, female mosquitoes digest it, using the resulting nutrients to develop their eggs. The digestion of the blood meal causes blood host DNA to degrade and fragment. If the DNA is degraded significantly, PCR reactions will be unable to amplify the species-specific sequences they are designed to target (16). The Sella score is used to grade blood meal digestion from freshly fed [2] to full gravid [7] with [1] representing a non-fed mosquito (17). The more digested the blood meal is the less likely the blood meal source can be determined due to DNA degradation

(18). To test the sensitivity of the blood meal assays, the PCRs were tested on *An. gambiae* mosquitoes fed with human blood. Once fed these mosquitoes were periodically removed, killed and DNA extracted to produce a panel of samples representing all stages of the Sella score (Figure 2.10). This panel was then used to test sensitivity (Figure 2.11).

Figure 2.10: Images of *Anopheles gambiae* mosquitoes fed on human blood periodically removed to produce a Sella score panel on which PCR assay sensitivity could be tested. Number represent each Sella score stage based on the original grading developed by Sella (17).

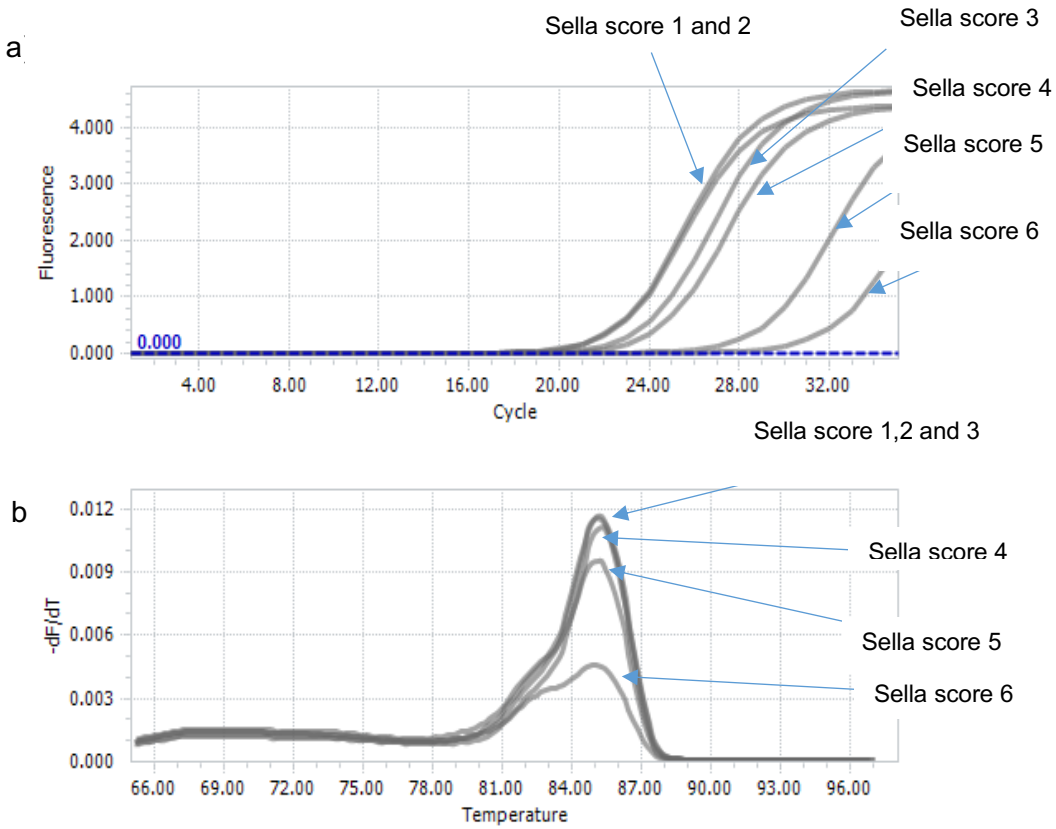


Figure 2.11: PCR Fluorescence (a) and melting curves (b) of Sella score samples. The increase in Ct value is seen to correlate with an increase in Sella score number showing the amount of starting template DNA decreases with time post-feed. An effect that should be considered when analysing field samples.

Mosquito Species identification

Samples were screened with a high throughput assay that can differentiate mosquitoes within the *An. gambiae* species complex. Assays that could detect other members of the complex were also used to confirm findings as well as identify other species within the complex. Morphological ID was used to identify other species present where appropriate.

Identifying Anopheles species within the Anopheles gambiae species complex

An assay developed by Bass *et al* (19) was used to identify species within the morphologically identical *Anopheles gambiae* species complex and required using highly specific TaqMan probes (Table 2.6). The assay was tested on known *An. gambiae* sibling *species.* (including both *An. colluzzii* and *An. gambiae s.s.)* obtained from LSHTM colonies and known *An. arabiensis* samples from the Democratic Republic of Congo confirmed previously. Additional morphologically identified *An. gambiae* species complex caught in Madagascar in 2015 were included to provide additional samples within the *An. gambiae* species complex. Additional mosquitoes that were morphologically identified as species outside of the *An. gambiae* complex namely *Anopheles funestus*, *An. rufipies* and *An. pharoensis*, were also included to check assay specificity. Cycling conditions were as follows: 95^{o}C for 10 minutes followed by 40 cycles of 95^{o}C for 25s and 60s at 66 ^{o}C with fluorescence acquired at the end of each cycle. Results were analysed using the Stratagene MxPro qPCR software.

Table 2.6: Reagents used for real-time detection of species within the *Anopheles gambiae* species complex developed by Bass *et al* (19)

Reagent	**Volume (1x)**
Quantitect Probes Master mix (Qiagen, UK)	6.25 µl
Forward primer - GTGAAGCTTGGTGCGTGCT	1 µl
Reverse primer - GCACGCCGACAAGCTCA	1 µl
Anopheles gambiae TaqMan® MGB probe (Applied biosystems, UK) -TGGAGCGGaACAC	0.1 µl
Anopheles arabiensis Taqman® LNA probe (Sigma-Aldrich, UK) - AC[+A][+T][+A]G[+G]ATGGA[+G][+A][+A]GG	0.25 µl
PCR grade H_20	2.9 µl

Strong amplification was present for all *An. gambiae* species complex samples (Figure 2.12). Fluorescence of the fluorophore FAM indicates *An. gambiae* with *An. arabiensis* represented by the fluorophore Cy5.

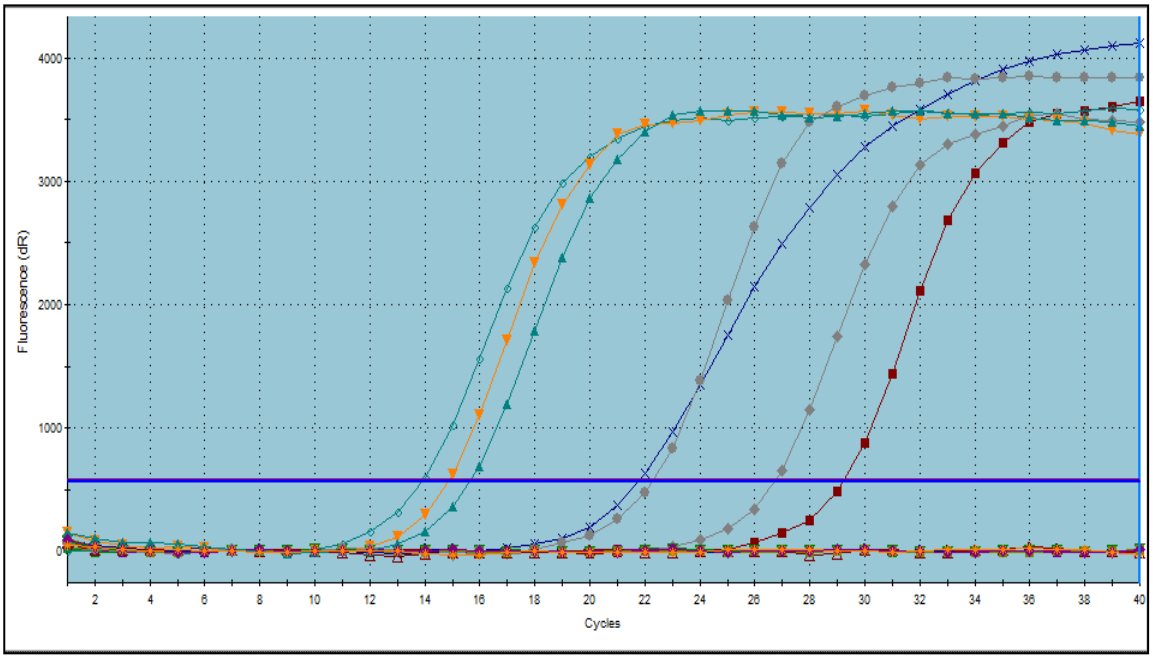


Figure 2.12: Amplification plot of all fluorescence produced by species within *An. gambiae* species complex namely *An. gambiae s.s.* and *An. Arabiensis*. PCR designed by Bass *et al (19)*.

Fluorescence was produced for either FAM or Cy5 for all samples tested, no cross fluorescence with other *Anopheles* mosquitoes was detected, demonstrating good specificity (Figure 2.13).

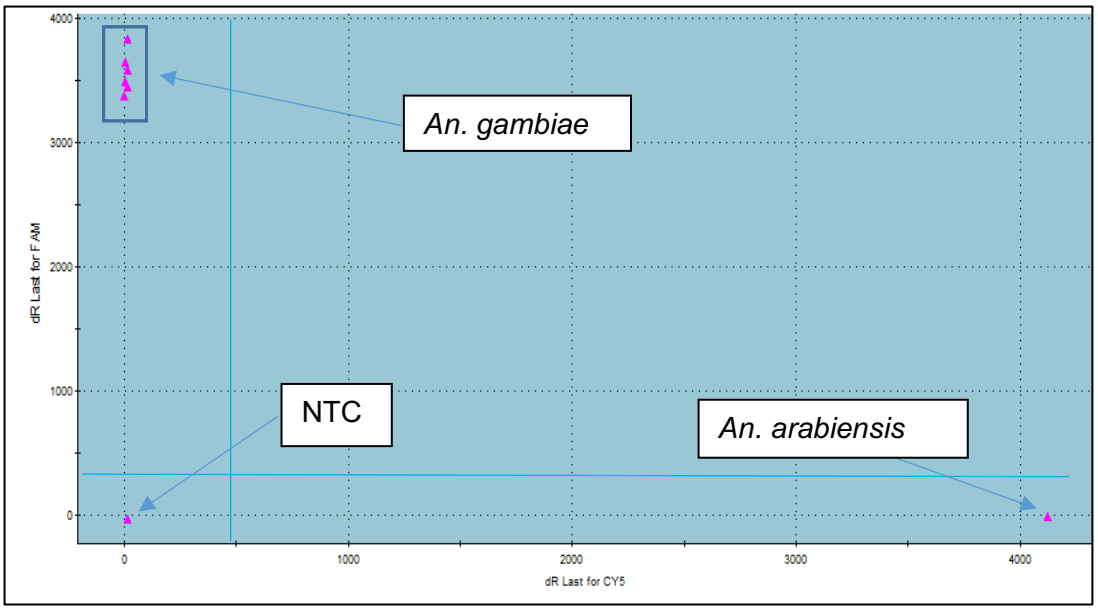


Figure 2.13: Specific fluorescence plot for FAM on the y-axis (*An. gambiae* probe) and CY5 on the x-axis (*An. arabiensis* probe) allowing species ID to be distinguished.

An endpoint PCR assay was also used to distinguish morphologically identical species within the *An. gambiae* species complex including *An. melas* and *An. quadriannulatus* which cannot be distinguished using the assay above. The assay targets the ribosomal rDNA gene and a universal forward primer, with species-specific reverse primers, produces different product sizes (20) (Table 2.7). The product sizes were as follows: 153bp for *An. quadriannulatus*, 315bp for *An. arabiensis*, 390bp for *An. gambiae* and 464bp for *An. melas and An. merus.* As *An. melas* is found along the west coast of Africa and is geographically distinct from *An. merus*, found in coastal regions of eastern and southern Africa, any samples that showed banding for these species could be confirmed as *An. melas*. This assay was used to identify *An. melas* species as well as confirm *An. gambiae* presence prior to *An. coluzzii / An. gambiae s.s.* differentiation where necessary. Reaction volume was 20 µl (Table 2.7) and cycling conditions were as follows: 95 °C for 10 minutes followed by 30 cycles of 95 °C for 30s, 50 °C for 30s and 72 °C for 30s and a final elongation stage of 72 °C for 5

minutes. Assay was performed on a T100 Thermal Cycler (Bio-Rad, UK) with all PCR products visualised on a 2% agarose gel using an E-Gel iBase Power System and E-Gel Safe Imager Real-Time Transilluminator (Invitrogen, UK).

Table 2.7: Reagents used for detection of *An. melas* and confirmation of *An. gambiae* species complex

Reagent	**Volume (1x)**
Hot Start *Taq* 2X Master Mix (New England Biolabs, UK)	10 µl
Universal Forward primer – GTGTGCCCCTTCCTCGATGT	2 µl
An. melas specific reverse primer - TGACCAACCCACTCCCTTGA	2 µl
An. gambiae specific reverse primer - CTGGTTTGGTCGGCACGTTT	1 µl
PCR grade H_2O	3 µl
Template DNA	2 µl

Anopheles coluzzii *and* Anopheles gambiae s.s. *species identification*

An. gambiae s.s is currently in a state of diverging into two different species and these forms were originally described as the S and M form *(21, 22)*. More recently, these have been renamed as *An. gambiae s.s.* and *An. coluzzii*. A PCR targeting the SINE200 retrotransposon and utilising an insertion in this area allows the two species to be distinguished following gel visualisation (23). *An. coluzzii* produces a band at 479 bp and *An. gambiae s.s.* produces a band at 249 base pairs (Figure 2.14). All *An. gambiae* mosquitoes identified from previous assays were run on this assay allowing sibling species to be identified. Reaction volume is described in Table 2.8 and cycling conditions were as follows: 94^{o}C for 10 minutes followed by 35 cycles of

94 °C for 30s, 54 °C for 30s, 72 °C for 60s and a final elongation step of 72 °C for 10 minutes. Assay was performed on a T100 Thermal Cycler (Bio-Rad, UK) with all PCR products visualised on a 2% agarose gel using an E-Gel iBase Power System and E-Gel Safe Imager real-time Transilluminator (Invitrogen, UK).

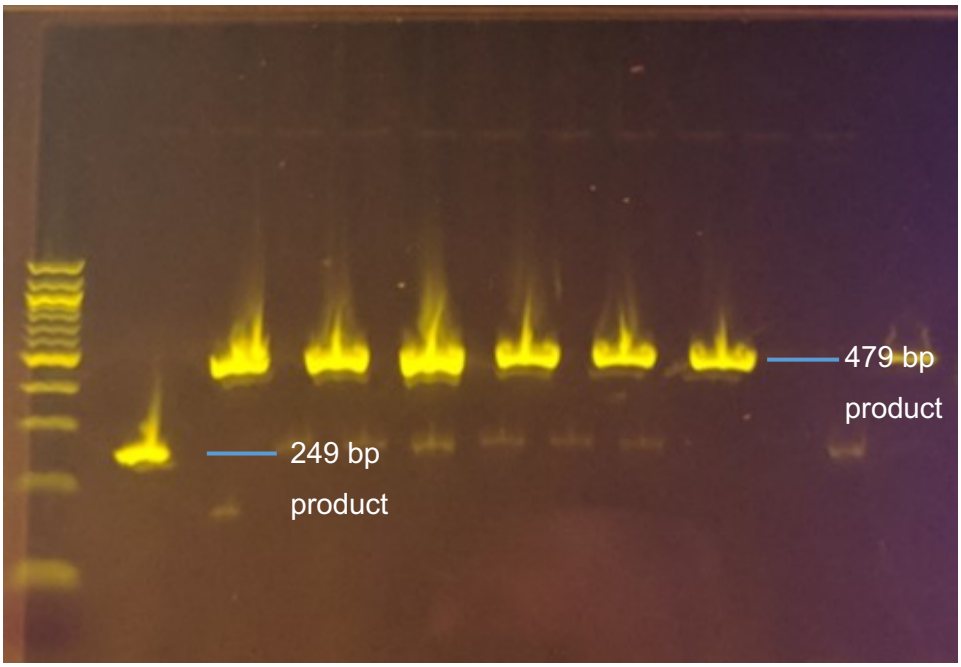


Figure 2.14: Gel visualisation of PCR designed to differentiate between *An. colluzzii* and *An. gambiae s.s.* *An. coluzzii* produces a product size of 479 bp with *An. gambiae s.s.* a product size of 249 bp.

Table 2.8: Reagents used for detection of *An. coluzzii* and *An. gambiae s.s.*

Reagent	**Volume (1x)**
Hot Start *Taq* 2X Master Mix (New England Biolabs, UK)	12.5 µl
Forward primer - TCGCCTTAGACCTTGCGTTA	0.5 µl
Reverse primer - CGCTTCAAGAATTCGAGATAC	0.5 µl
PCR grade H_2O	9.5 µl
Template DNA	2 µl

Species confirmation using Internal transcriber spacer gene 2 (ITS2) sequencing analysis

For further confirmation of PCR results a subset of samples was sequenced by amplifying the ITS2 region using primers designed by Beebe & Saul (Figure 2.15). Sanger sequencing reads were analysed and a consensus was agreed. This consensus was searched against all known sequences in GenBank using the BLAST function. Using the percentage identity and percentage coverage, the *Anopheles* species was determined. This work was performed as part of a larger project and phylogenetic analysis was used for further confirmation of species present, See Jeffries *et al,* 2018 (24).

Reaction volumes are described in Table 2.9 and cycling conditions were as follows: 94 °C for 5 minutes followed by 30 cycles of 94 °C for 60 seconds, 52 °C for 60 seconds and 72 °C for 120 seconds and a final elongation stage of 72 °C for 5 minutes. Assay was performed on a T100 Thermal Cycler (Bio-Rad, UK) with all PCR products visualised on a 2% agarose gel using an E-Gel iBase Power System and E-Gel Safe Imager Real-Time Transilluminator (Invitrogen, UK).

Table 2.9: Reagents used for ITS2 sequencing PCR

Reagent	**Volume(1x)**
Hot Start *Taq* 2X Master Mix (New England Biolabs, UK)	20 µl
ITS2 Forward primer - TGTGAACTGCAGGACA	4 µl
ITS2 Reverse primer - TATGCTTAAATTCAGGGGGT	4 µl
PCR grade H_2O	8 µl
Template DNA	4 µl

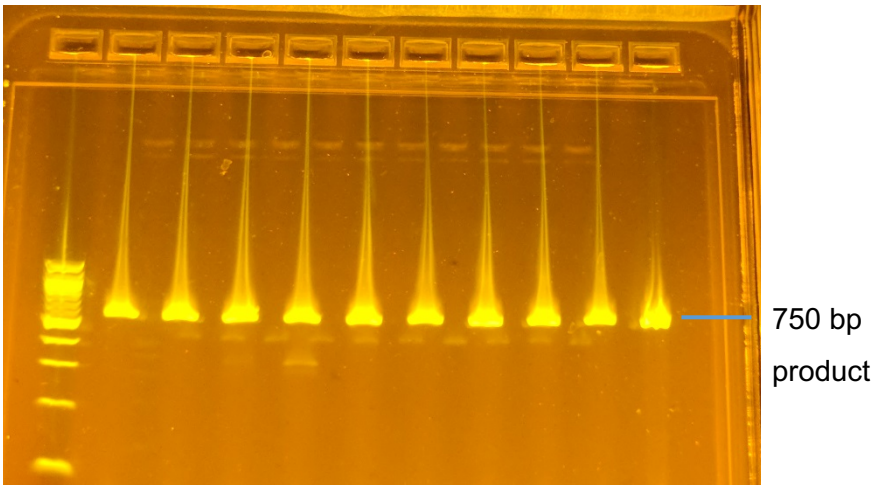


Figure 2.15: Gel image of ITS2 sequence PCR products. Gels were run prior to sequencing analysis to check for successful amplification. All samples that amplified the 750bp ITS2 region were sent for Sanger sequencing.

Plasmodium falciparum screening

Samples were screened for *Plasmodium falciparum* as this is the most dominant malaria species in Ghana. The assay was a real-time PCR assay using specific primers that target the cox1 mitochondrial gene in *Plasmodium falciparum* (Table 2.12). This is a high copy gene increasing assay sensitivity compared to previous *P. falciparum* detection assays (25). The assay was initially tested on a set of field-caught mosquitoes (collected from Madagascar in 2015) with a known *P. falciparum* positive control for assay optimisation (Figure 2.16). Reaction volume is described in Table 2.10 and cycling conditions were as follows: 95 °C for 5 min followed by 35 cycles of 95 °C for 15 s and 58 °C for 30 s. A melt curve was run at the end of the amplification stage to identify target DNA amplification. Samples were run on a Roche light cycler (Roche, UK) and output analysed using Roche LightCycler software (Roche, UK).

Table 2.10: Reagents used for detection of *Plasmodium falciparum* in mosquito samples

Reagent	**Volume(1x)**
FastStart SYBR Green Master Mix (Roche, UK)	5 µl
Pf Forward primer - TTACAATCAGGAATGTTATTGC	4 µl
Pf Reverse primer - ATATTGGATCTCCTGCAAAT	4 µl
PCR grade H_20	8 µl
Template DNA	4 µl

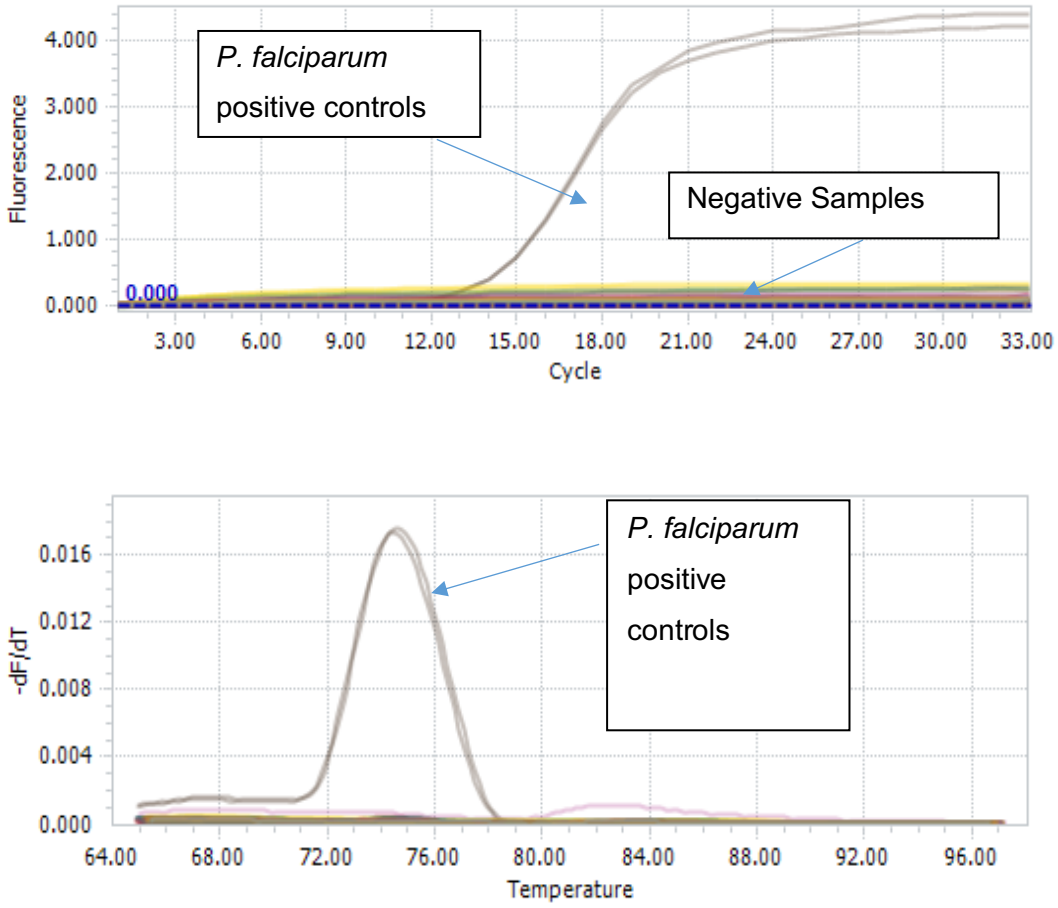


Figure 2.16: Fluorescence and melting curves of *Plasmodium falciparum* specific PCR used to detect the parasite at all life stages targeting the Cox1 gene

Ethical considerations

All ethical clearance (both in the UK and in country) was obtained before any studies commenced. The study design was approved by the London School of Hygiene and Tropical Medicine ethics committee (LSHTM ethics reference:15216). In country clearance was granted by the Noguchi Memorial Institute, University of Ghana (Reference number:DF22).

Conclusion

The use of the transect style collection strategy described in this chapter will allow the collection of blood fed mosquitoes at 50m intervals across a 250m collection area with varying host availability. Through the use of high-throughput screening assays and species-specific end-point PCRs data on mosquito species present in the field will be collected and blood host source (human or bovine, the two dominant hosts in the experimental set up) determined using an initial screening assay for both species and any potential human blood meal confirmed using a second, highly sensitive forensic assay. The combination of transect point (T1 – T6), collection location (indoor or outdoor), mosquito species and bloodmeal source will provide the variables needed to investigate and quantify the relationship between host availability and the host choice of a mosquito species which will be used throughout this thesis.

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Supplementary information

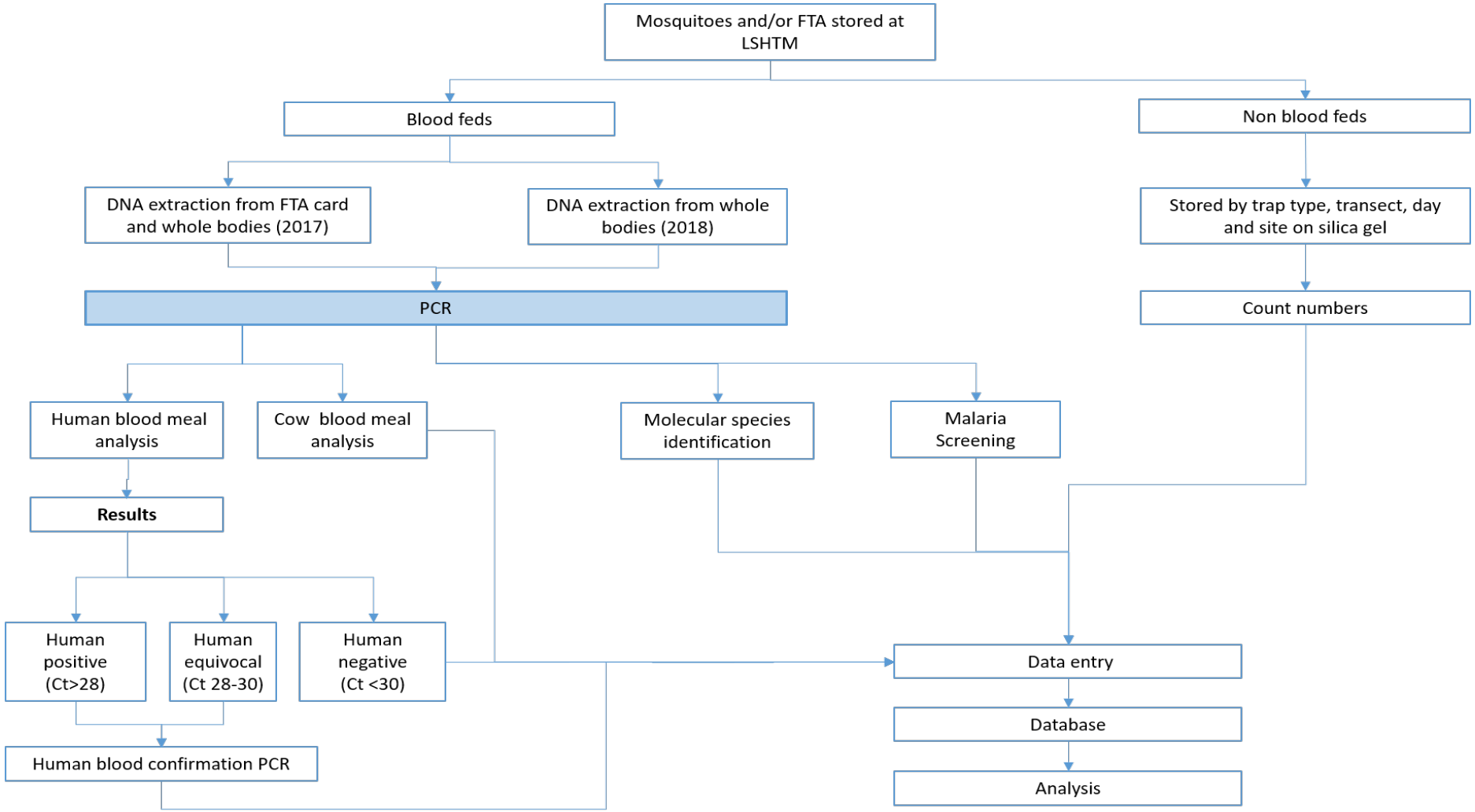

Figure S2.1: Sample analysis and workflow for laboratory work

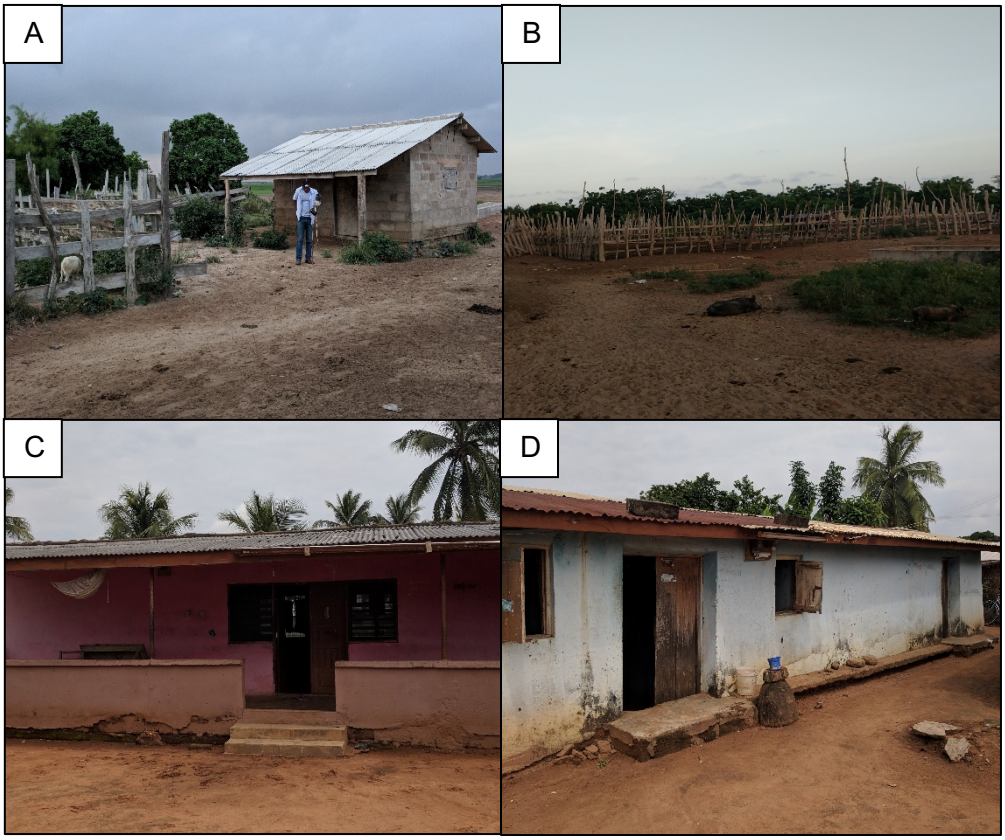

Figure S2.2: Selected images of cattle sheds (A) and cattle enclosure (B) and housing types (C and D) from which mosquitoes were sampled.

Chapter 3 - A Systematic review and meta-regression analysis of the reported Human blood index (HBI) of the major African malaria vectors: *Anopheles gambiae, Anopheles arabiensis and Anopheles funestus*

Abstract

Background: The proportion of mosquito blood-meals that are of human origin, referred to as the 'human blood index' or HBI, is a key determinant of malaria transmission.

Methods: A systematic review was conducted followed by meta-regression of the HBI for the major African malaria vectors.

Results: Evidence is presented for higher HBI among *Anopheles gambiae* (M/S forms and *Anopheles coluzzii*/*An. gambiae sensu stricto* are not distinguished for most studies and, therefore, combined) as well as *Anopheles funestus* when compared with *Anopheles arabiensis* (prevalence odds ratio adjusted for collection location [i.e. indoor or outdoor]: 1.62; 95% CI 1.09–2.42; 1.84; 95% CI 1.35–2.52, respectively). This finding is in keeping with the entomological literature which describes *An. arabiensis* to be more zoophagic than the other major African vectors. However, analysis also revealed that HBI was more associated with location of mosquito captures ($R^2 = 0.29$) than with mosquito (sibling) species ($R^2 = 0.11$).

Conclusions: These findings call into question the appropriateness of any assumption of fixed host preferences among disease vectors. Explicitly measuring HBI both indoors and outdoors should be included in entomological assessments.

Aim

Using the human blood index (HBI) reported in published literature, show evidence of host biting plasticity for the major malaria vectors in sub-Saharan Africa.

Objectives

1. Provide evidence for biting plasticity using the reported HBI for the major malaria vectors *Anopheles gambiae, Anopheles arabiensis* and *Anopheles funestus s.l.*

2. Investigate the effect collection location (indoor v outdoor) may have on the reported HBI.

3. Identify the major mosquito collection methods used to collect blood fed *Anopheles* mosquitoes in the field and investigate the effect the major collection methods may have on the reporting of the HBI.

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SECTION A – Student Details

Student	James Orsborne
Principal Supervisor	Laith Yakob
Thesis Title	Mosquito (Diptera: Culicidae) biting behaviour and malaria transmission: interactions between intrinsic host preferences and local host availability

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	Malaria Journal		
When was the work published?	18th December 2018		
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion	N/A		
Have you retained the copyright for the work?*	**Yes**	Was the work subject to academic peer review?	**Yes**

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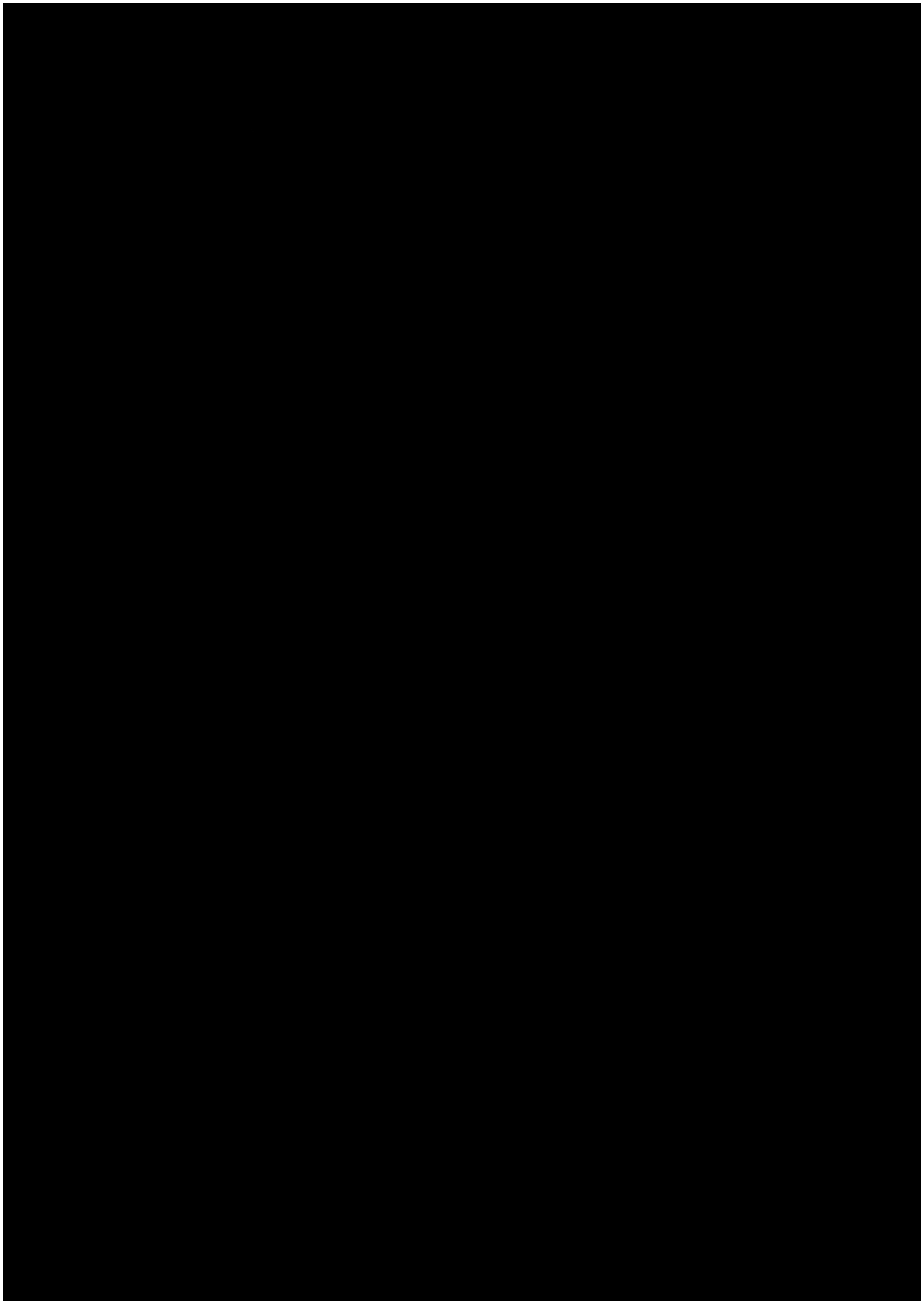
SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	JO created the search terms, inclusion/exclusion criteria and performed the systematic search, extracting and collating of all data used in the manuscript. JO also aided in the drafting and formatting of the final manuscript prior to submission.



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Malaria Journal

RESEARCH Open Access

Using the human blood index to investigate host biting plasticity: a systematic review and meta-regression of the three major African malaria vectors

James Orsborne[1], Luis Furuya-Kanamori[2], Claire L. Jeffries[1], Mojca Kristan[1], Abdul Rahim Mohammed[3], Yaw A. Afrane[3], Kathleen O'Reilly[1], Eduardo Massad[4], Chris Drakeley[5], Thomas Walker[1] and Laith Yakob[1*]

Abstract

Background: The proportion of mosquito blood-meals that are of human origin, referred to as the 'human blood index' or HBI, is a key determinant of malaria transmission.

Methods: A systematic review was conducted followed by meta-regression of the HBI for the major African malaria vectors.

Results: Evidence is presented for higher HBI among *Anopheles gambiae* (M/S forms and *Anopheles coluzzii*/*An. gambiae* sensu stricto are not distinguished for most studies and, therefore, combined) as well as *Anopheles funestus* when compared with *Anopheles arabiensis* (prevalence odds ratio adjusted for collection location [i.e. indoor or outdoor]: 1.62; 95% CI 1.09–2.42; 1.84; 95% CI 1.35–2.52, respectively). This finding is in keeping with the entomological literature which describes *An. arabiensis* to be more zoophagic than the other major African vectors. However, analysis also revealed that HBI was more associated with location of mosquito captures ($R^2 = 0.29$) than with mosquito (sibling) species ($R^2 = 0.11$).

Conclusions: These findings call into question the appropriateness of current methods of assessing host preferences among disease vectors and have important implications for strategizing vector control.

Keywords: Blood meal analysis, Host preference, Mosquito, Biting preference, Blood index

Background

Malaria is transmitted through mosquito bites, making the vectors' choice of which blood-host species to bite a central component of malaria epidemiology and ecology. In Africa, the majority of infections are transmitted by *Anopheles gambiae* sensu stricto (s.s.), *Anopheles coluzzii*, *Anopheles funestus* and *Anopheles arabiensis*. Conventional wisdom indicates that the first three vectors are anthropophagic while the latter sibling species is more zoophagic. Levels of anthropophagy/zoophagy are typically assessed using PCR to identify the host species from blood-meals in field-caught mosquitoes, and are then quantified according to the human blood index (HBI), defined as the proportion of blood-meals that are of human origin [1]. Because two mosquito bites on a human are required to complete the malaria parasite's life-cycle, HBI has an inflated impact on metrics of transmission such as the basic reproduction number, the vectorial capacity and the critical density of mosquitoes for sustained transmission [2].

However, the HBI should not be perceived to have a singular, fixed value; all major African malaria vectors have demonstrable plasticity in the host species that they bite [3–5]. It has long been recognized that the same

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mosquito population will often adjust its biting towards a more locally available host species [1, 6]. This has important implications for malaria control policy. For example, recent studies have observed that increased outdoor biting followed the distribution of insecticide-treated bed nets [7]. In such circumstances, vector control tools that operate effectively outdoors become a critical component for eliminating local malaria transmission. Unfortunately, the huge malaria burden reduction achieved in the years since 2000 has relied disproportionately on control tools operating indoors [8], and there are limited effective malaria-vector control options for outdoor use.

One technology that shows promise for targeting mosquitoes regardless of whether they bite indoors or outdoors involves the use of systemic insecticides—chemicals applied directly to blood-hosts to kill mosquitoes that take a blood meal. This technology arose from the observation that mosquito mortality was increased following the consumption of sugar-meals [9] or blood-meals [10] containing ivermectin—a drug used for onchocerciasis control. Drugs approved for veterinary use, such as fipronil, have subsequently been demonstrated to have similar impact when livestock are dosed orally, or when the chemical is applied topically [11]. More recently, systemic insecticides have had durations of their efficacy extended through dosing with higher concentrations [12], combined dosing with adjuvants [13], and with use of sustained-release devices [14]. The stage is set for progress in development and evaluation of ivermectin for vector control [15]. Therefore, arguably it has never been more important to understand the distribution of malaria-vector bites on alternative host species. Here, the current evidence is systematically reviewed and a meta-regression conducted to identify the factors associated with higher HBI in sub-Saharan Africa.

Methods

Findings from the systematic review were reported following the PRISMA guidelines [16]. The inclusion and exclusion criteria are listed in Table 1 and advanced search terms were developed following initial manual literature searches and a basic PubMed search (Table 2). The purpose of the initial search was to identify keywords and synonyms. The authors agreed on the search terms and inclusion/exclusion criteria before the systematic search was performed. The Ovid database was used to search available MEDLINE and EMBASS literature from inception to February 2018. Books were excluded from all searches as well as articles not written in English. Results were retrieved and collated using Mendeley desktop reference manager.

After eliminating duplications, abstracts for all publications retrieved were reviewed for relevance. Full-text reviews were then conducted on all articles to decide on its inclusion in accordance with the pre-specified inclusion and exclusion criteria. If the inclusion criteria were satisfied the estimated human blood index (HBI) reported was retrieved. Other variables that could have a significant effect on the reported HBI were also retrieved. These variables included (sibling) species (complex),

Table 1 Inclusion and exclusion criteria for systematic review

Inclusion criteria	Exclusion criteria
Studies which used blood meal analysis (PCR, ELISA or precipitin tests) to report the HBI	Semi field studies, studies using baited traps or choice experiments to investigate host preference
Studies performed in sub-Saharan Africa	Entomological studies not specifically reporting the HBI
Studies reporting the HBI for individual mosquito species	Studies not reporting total number of mosquitoes caught
Reporting HBI for *Anopheles gambiae, Anopheles funestus complex* or *Anopheles arabiensis* mosquito species	Data points based on less than 50 blood-fed mosquitoes in total for target species
Studies reporting trapping methodology including location of traps (indoors or outdoors)	

Table 2 Search strategy for systematic review

Ovid MEDLINE® Database
Human blood index OR HBI OR host preference OR trophic preference OR blood meal preference OR blood host preference OR blood meal OR blood meal analysis OR blood-meal analysis OR blood meal source OR host blood OR host blood meal OR blood meal identification
[multiple posting = MeSH subject heading word, abstract, title, original title, text word (title, abstract), key word heading, name of substance, key word heading word, protocol supplementary concept word, synonym]
AND
Anopheles OR Anopheles arabiensis OR Anopheles gambiae OR Anopheles funestus [multiple posting = MeSH subject heading word, abstract, title, original title, text word (title, abstract), key word heading, name of substance, key word heading word, protocol supplementary concept word, synonym]

trapping location (indoors, outdoors or both), trap type(s) used and total number of mosquitoes collected. The primary effect measure of interest was the HBI.

The double arcsine square root transformed HBI (expressed as a proportion of all blood-meals) was used to stabilize the variance across the studies [17] and then back transformed for ease of interpretation. A linear model was performed on all eligible studies to gain additional insight into the effect of trapping location and *Anopheles* species on the proportion of HBI. The linear model was fit using the HBI (proportion) as the response variable weighted by the inverse of each study's variance to allow the observations with the least variance to provide the most information to the model, and using robust error variances. All tests were two-tailed and a p-value < 0.05 was deemed statistically significant. Inverse variance weights were obtained using MetaXL (version 5.3, EpiGear Int Pty Ltd; Sunrise Beach, Australia) and the regression models were run using Stata MP (version 14, Stata Corp, College Station, TX, USA).

Results

The search identified 1243 potentially relevant studies. After collating these results and reviewing all abstracts, 662 studies were deemed relevant. All full text articles were retrieved, reviewed for relevance and reviewed against all inclusion and exclusion criteria. Sixty-one studies resulting in 166 data points fulfilled all criteria and where included in the analysis. Reasons for exclusion at full text stage included inadequate number (fewer than 50) of mosquitoes collected (n = 14) and the use of host-biased trapping methodologies (n = 4) (Fig. 1).

Multiple collection methodologies were identified from the eligible studies. The methodology used was governed by the collection location targeted (indoors or outdoors). Indoor collections were the most widely used (n = 118) with pyrethroid spray catch (PSC) the most commonly used methodology (n = 78). Other collection methods included manual indoor collections (n = 20) and the use of CDC light traps within the household (n = 10). Outdoor collections represented 27 of the total data points extracted with manual collection of mosquitoes being the most common collection method (n = 13). Pit traps (n = 10) and CDC light traps (n = 4) were also an effective collection method. Studies collecting from both indoor and outdoor environments consisted of 21 data points. These studies used a variety of different methods; many used a combination of the most effective indoor and outdoor collection methods. CDC light traps were the most common (n = 12) followed by other combinations of indoor and outdoor methods; CDC light trap plus PSC (n = 2) and pit traps and manual indoor collections (n = 2) (Table 3 and Additional file 1). Collection methods had no significant effect ($p > 0.05$) on the reported HBI when comparing the mean HBI produced by each collection methodology within its respective collection areas (indoor and outdoor) for *An. gambiae, An. arabiensis* and the *An. funestus* species complex (Fig. 2). It should be noted that due to the variety of different methods used and therefore sparsity of data for each methodology within the "both" categories, a meaningful comparison could not be made.

Meta-regression of the data compiled from the 166 data points demonstrated a significantly higher proportion of blood-meals were of human origin (the human blood index, 'HBI') among *An. funestus* (prevalence odds ratios [POR] of 1.84 (95% CI 1.35–2.52, $p < 0.001$) and *An. gambiae* (POR of 1.62, 95% CI 1.09–2.52, $p = 0.02$) compared to *An. arabiensis*. The majority of studies including details of *An. gambiae* did not specify whether they were M or S forms (or, in more modern nomenclature, *An. coluzzii* or *An. gambiae* s.s.), so these were combined. For all three groups, a significantly higher HBI was found from indoor mosquito collections (POR of 2.74, 95% CI 2.00–3.75, $p < 0.001$) as well as combined indoor and outdoor collections (POR of 4.20, 95% CI 3.13–5.62, $p < 0.001$) versus outdoor only collections. *Anopheles* species was not found to be an interaction term for location collection and HBI, indicating that all species follow a similar trend regarding their preferred location for biting humans. The results also revealed that trapping location (R^2 of 0.29) had a larger impact on the blood-meal host species than mosquito species (or species complex) (R^2 of 0.11) and that this difference was statistically significant ($p < 0.01$ resulting from an F-test comparing both univariable models) (Table 4).

Discussion

Control of vector-borne diseases is largely, often entirely, dependent on vector control. For malaria, vector control is achieved primarily through targeting mosquitoes that are host-seeking [8]. The major African malaria vectors, *An. gambiae* s.s., *An. coluzzii* and *An. funestus*, are regularly cited as paragons of anthropophagy, and any non-human biting exhibited by these species has historically been ignored when strategizing control. Here, their biting behaviour was systematically reviewed and clearly demonstrated that the difference in their host choice compared with the zoophilic vector *An. arabiensis* was dwarfed by the difference found when comparing indoor with outdoor collections. In other words, *where* the mosquito was collected was substantially and significantly more influential on host choice than *which* mosquito species was collected.

This raises an important question: where should vectors be collected from in order to provide the most useful HBI

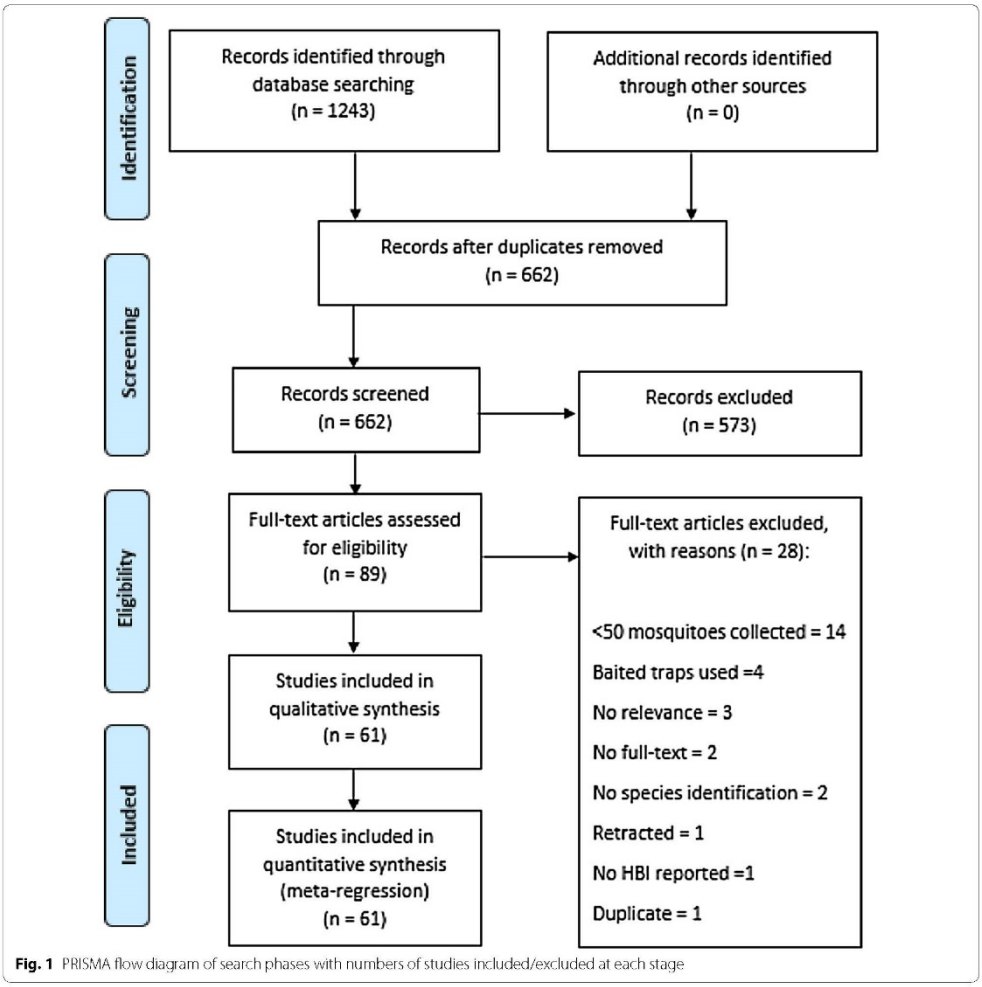

Fig. 1 PRISMA flow diagram of search phases with numbers of studies included/excluded at each stage

estimates? Results indicate that a single HBI for a given location risks presenting quite a biased estimate for local vector biting behaviour. A standardized HBI accounting for both indoor and outdoor behaviours would probably constitute an invalid metric because of the increased difficulty posed by collecting blood-fed mosquitoes outdoors i.e., tools are lacking for the estimation of indoor *versus* outdoor mosquito numbers with any confidence. Therefore, current best practice should be to present both estimates for an indoor HBI and an outdoor HBI. Longitudinal assessments initiated before rolling out control tools, and followed up over the time course of the programme would provide a valuable source of information. For example, these would determine the timeframe across which LLIN-derived exophagy [7], as well as zoophagy [18] occurs, as well as provide unbiased estimates of the magnitude of effect. These entomological data would also be able to inform on whether there is a reversion to behavioural norm after a certain period post-distribution, and the rate at which this occurred.

Table 3 Data points extracted from eligible studies for each collection location and trapping methodology

Species	Collection location			Methodology								
				Indoor			Outdoor			Both (indoor + outdoor)		
	Indoor	Outdoor	Both	Pyrethroid spray catch (PSC)	Manual aspiration	CDC light trap	Manual aspiration	CDC light trap	Pit traps	CDC light trap + PSC	CDC light trap	Others
Anopheles gambiae	37	7	3	22	10	2	7	0	0	1	0	2
Anopheles arabiensis	50	14	10	39	2	7	1	4	9	0	10	0
Anopheles funestus s.l.	31	6	8	17	8	1	5	0	1	1	2	5

The top three methodologies based on number of data points extracted are displayed here

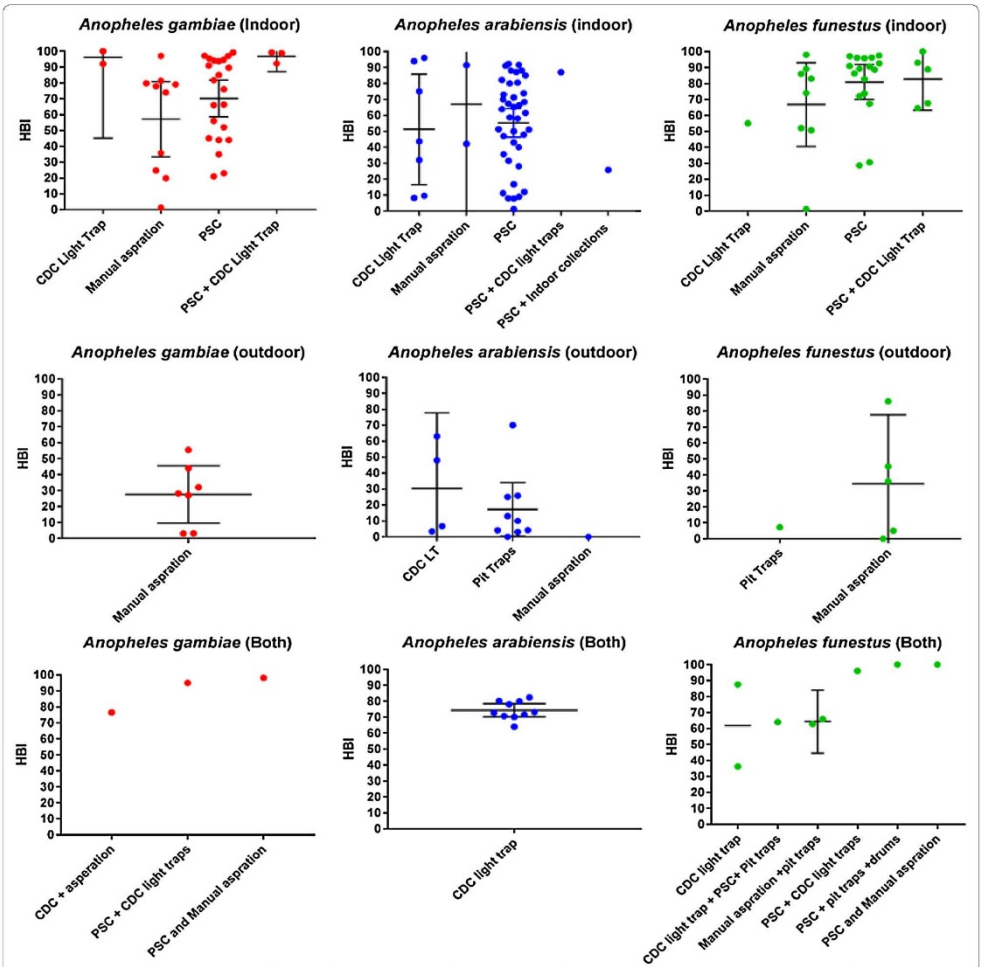

Fig. 2 Reported mean HBI (+ 95% CIs) for individual collection methods when sampling from indoor, outdoor or both (indoor and outdoor) environments for *An. gambiae*, *An. arabiensis* and *An. funestus*

Better data on this behaviour and its temporality will do much more than inform a fundamental aspect of mosquito ecology: it will have considerable ramifications pertaining to malaria control. For example, if significantly reduced HBI is detected immediately following the distribution of LLINs, this may present an excellent opportunity to synergize bed nets with systemic insecticide-treated livestock. Saul [19] described the potential for zooprophylaxis to switch into zoopotentiation if the availability of alternative blood meals increases mosquito survival more than counters the impact of diverting feeds. This risk could be reduced or eliminated with systemic insecticidal dosing that is judiciously timed with LLIN roll-out. Mathematical models already exist for optimal systemic insecticide deployment [20] including its integration with LLINs [21]. These could immediately be capitalized upon once the temporal HBI data became available.

Table 4 Predictors of human blood index: univariable and multivariable regression models

Predictors	Univariable		Multivariable	
	POR (95% CI)	R^2	POR (95% CI)	R^2
Anopheles species		0.11		0.40
An. arabiensis	1.00		1.00	
An. gambiae[a]	1.50 (0.95–2.36)		1.62 (1.09–2.42)	
An. funestus	1.95 (1.34–2.85)		1.84 (1.35–2.52)	
Location		0.29		
Outdoor only	1.00		1.00	
Indoor only	2.83 (2.04–3.93)		2.74 (2.00–3.75)	
Both	3.98 (3.14–5.06)		4.20 (3.13–5.62)	

POR prevalence odds ratio

[a] Most studies did not specify M/S form of *An. gambiae* (and pre-dated the renaming of these forms as *An. coluzzii* and *An. gambiae* s.s., respectively)

One further, important unknown pertaining to HBI is the spatial scale across which within-mosquito population plasticity occurs. Over 50 years ago, Garrett-Jones described differing HBI estimates for mosquitoes collected from proximal locations [1]. Given the current concerns over altered biting behaviour potentially compromising recent gains in malaria burden reduction [22], a fuller comprehension of the scale and magnitude of this variability is timely. A recent study conducted in southern Ghana describes the successful piloting of a novel experimental design to address exactly this phenomenon [23]. It demonstrated that statistically significant alteration in host choice for *An. coluzzii* was detectable over a range of 250 m [23]. Heterogeneity in mosquito biting rates has been demonstrated to be key to malaria transmission, first by theoretical work [24], but more recently with empirical studies using genotyping of blood-meals [25]. Future modelling frameworks will need to account for this additional form of village-level heterogeneity in biting behaviour.

Conclusion

Results demonstrate that where mosquitoes are collected from (indoors *versus* outdoors) is significantly more associated with the HBI than which of the major African malaria-vector mosquito (sibling) species is collected. Some of the more important consequences to disease control of this behaviour are described. Some new theoretical and empirical developments that may improve both HBI assessment and how this metric can inform malaria control optimisation are discussed.

Additional file

Additional file 1. All eligible studies (and corresponding data points) retrieved from systematic search.

Authors' contributions

LY conceived the study. JO, LFK and LY performed the systematic review and meta-regression. All authors contributed to results interpretation and manuscript drafting. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its additional file.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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Addendum

This addendum supplements the publication described in Chapter 3, “Using the human blood index to investigate host biting plasticity: a systematic review and meta-regression of the three major African malaria vectors”. It was noted by the PhD thesis examiners that a number of articles have been missed during the searching and screening phase of our systematic review. Specific reference was made to the exclusion of literature written in French (with potential omission of francophone African region publications) and a list of 16 English articles highlighted by the examiners.

On review, 11 French articles (originating from Senegal, The Ivory Coast and Cameroon) were included in the initial search phase of the review, one of which was included in the original final analysis. These articles had been translated into English. To identify any further missing French articles, we also searched other review articles that stated the inclusion of French literature as part of their inclusion criteria. This combined effort identified 3 potential new hits – one article fulfilled all inclusion criteria and reported an *An. arabiensis* HBI of 22% for indoor collections and an HBI of 4% for outdoor collections. For *An. funestus,* an HBI of 65% and 40% was reported for indoor and outdoor collections respectively. Importantly, these estimates fall well within our current data points for both these species shown in this review. The other articles were excluded in latter rounds of the exclusion criteria due to lack of HBI reporting (n=5), being irrelevant to the study aim (n=3) and the inability to access the full text article (n=1).

The 16 English articles highlighted by the examiners were also checked against the original search database. In total, two of these articles were not identified in the systematic review despite being eligible. The reasons for inclusion or exclusion of all highlighted articles are described below:

Garrett-Jones, C., Boreham, P. F. L. & Pant, C. P. 1980 Feeding habits of anophelines (Diptera: Culicidae) in 1971-78, with reference to the human blood index: a review. *Bull Ent Res.* **70**, 165-185.

This review was missed from the literature search as it was not found by either of the databases used. We are unsure why this has occurred as all other Garrett-Jones reviews and articles on this subject have been included. The review shows HBI measures for *An. funestus, An. arabiensis* and *An. gambiae*. All HBI measures fit the general findings from the systematic review with *An. funestus* showing an average indoor HBI of 98%, *An. arabiensis* HBI of 70% indoors (HBI = 10% outdoors) and *An. gambiae s.s.* 76% indoors. These additional data points are within the range already identified for these vectors and so do not alter the conclusions of the published review.

Aikins, M. K., Pickering, H., Alonso, P. L., D'Alessandro, U., Lindsay, S. W., Todd, J. & Greenwood, B. M. 1993 A malaria control trial using insecticide-treated bed nets and targeted chemoprophylaxis in a rural area of The Gambia, west Africa. 4. Perceptions of the causes of malaria and of its treatment and prevention in the study area. *Trans Roy Soc Trop Med Hyg* 87 Suppl 2, 25-30.

The human blood Index was not reported in this article and therefore it would not be eligible for inclusion in the review.

Boreham, P. F. L. & Port, G. R. 1982 The distribution and movement of engorged females of Giles (Diptera: Culicidae) in a Gambian village. *Bull ent Res* **72**, 489-495.

The species of mosquitoes collected in this study was assumed to be *An. gambiae s.s* based on previous work. However, the inclusion criteria of our review states the species within the *An. gambiae* complex must be identified and therefore this study could not be included in the review.

Boreham, P. F., Lenahan, J. K., Boulzaguet, R., Storey, J., Ashkar, T. S., Nambiar, R. & Matsushima, T. 1979 Studies on multiple feeding by *Anopheles gambiae* s.l. in a Sudan savanna area of north Nigeria. *Trans Roy Soc Trop Med Hyg* **73**, 418-423.

The species of mosquitoes collected in this study was only identified to *An. gambiae s.l.* As the inclusion criteria of our review states the species within the *An. gambiae* complex must be identified, this study could not be included in the review.

Port, G. R. & Boreham, P. F. L. 1982 The effect of bed nets on feeding by Anopheles gambiae Giles (Diptera: Culicidae). *Bull Ent Res.* **72**, 483-488.

The species of mosquitoes collected in this study was assumed to be *An. gambiae s.s* based on previous work. The inclusion criteria of our review states the species within the *An. gambiae* complex must be identified and therefore this study could not be included in the review.

Chandler, J. A., Boreham, P. F., Highton, R. B. & Hill, M. N. 1975 A study of the host selection patterns of the mosquitoes of the Kisumu area of Kenya. *Trans Roy Soc Trop Med Hyg* **69**, 415-425.

***An. funestus* data described in this article showed an HBI of 94% and this should have been included in the review. This data does, however, fit in with the range reported in the literature described in the systematic review. As no attempt was made to identify members of *An. gambiae* species complex despite presence of species A and B reported the *An. gambiae* data could not have been included following our exclusion criteria.**

Lindsay SW, Alonso PL, Armstrong Schellenberg JR, et al. A malaria control trial using insecticide-treated bed nets and targeted chemoprophylaxis in a rural area of The Gambia, west Africa. 7. Impact of permethrin-impregnated bed nets on malaria vectors. *Trans R Soc Trop Med Hyg*. 1993;87 Suppl 2:45–51. doi:10.1016/0035-9203(93)90175-p

Although the HBI is reported in the article, the HBI is not reported for the individual species covered by the review. As our inclusion criteria states the species within the *An. gambiae* complex must be identified, this study cannot be included in the review.

Bøgh, C., Clarke, S. E., Jawara, M., Thomas, C. J. & Lindsay, S. W. 2003 Localized breeding of the Anopheles gambiae complex (Diptera: Culicidae) along the River Gambia, West Africa. *Bull Ent Res* **93**, 279-287.

This manuscript did not report HBI.

Tirados, I., Costantini, C., Gibson, G., Torr, S.J., 2006. Blood-feeding behaviour of the malarial mosquito *Anopheles arabiensis*: implications for vector control. *Med Vet Ent* **20**, 425-437.

Baited traps were used in this study, potentially biasing the sample, and was therefore excluded following our inclusion/exclusion criteria.

Hargrove, J.W., Packer, M.J., 1993. Nutritional states of male tsetse flies (Glossina spp) caught in odourbaite tarps and artificial refuges - models for feeding and disestion.. *Bull Ent Res* **83**, 29-46.

Focused on *Glossina* species and therefore not relevant to the systematic review.

Lindsay, S. W., Wilkins, H. A., Zieler, H. A., Daly, R. J., Petrarca, V., Byass, P., Zieler, R. J., Daly, V., Petrarca, V. & Byass, P. 1991 Ability of *Anopheles gambiae* mosquitoes to transmit malaria during the dry and wet seasons in an area of irrigated rice cultivation in The Gambia. *J Trop Med Hyg* **94**, 313-324.

This article reported *An. gambiae s.l.* data only. Therefore, by following our exclusion criteria, these results could not be included.

Bryan, J. H., Petrarca, V., Di Deco, M. A. & Coluzzi, M. 1987 Adult behaviour of members of the *Anopheles gambiae* complex in The Gambia with special reference to *An. melas* and its chromosomal variants. *Parassit* **29**, 221-249.

This manuscript was only available through contacting colleagues of the author. An HBI of 46% and 73% was reported for *An. gambiae* for indoor collections from two different locations. These data fall in line with the findings from the systematic review and our publication's conclusions would remain unaffected with their inclusion.

Boreham, P. F. L. 1972 Serological identification of arthropod bloodmeals and its Application. *Pans* **18**, 205-209.

The full-text article could not be accessed without payment.

Boreham, P. F. L. 1975 Some applications of bloodmeal identifications in relation to the epidemiology of vector-borne tropical diseases. *Trans Roy Soc Trop Med Hyg* **78**, 83-91.

The full-text article could not be accessed.

Lindsay, S. W., Alonso, P. L., Armstrong Schellenberg, J. R. M., Hemingway, J., Adiamah, J. H., Shenton, F. C., Jawara, M. & Greenwood, B. M. 1993 7. Impact of permethrin-impregnated bednets on malaria vectors. *Trans Roy Soc Trop Med Hyg* **87**, 45-52.

The full-text article could not be accessed without payment.

Lindsay, S. W., Shenton, F. C., Snow, R. W. & Greenwood, B. M. 1989 Responses of *Anopheles gambiae* complex mosquitoes to the use of untreated bednets in The Gambia. *Med Vet Entomol* **3**, 253-262.

The full-text article could not be accessed without payment.

Supplementary information

Code for Meta-analysis

Import dataset

use "D:\Users\lf17118\Desktop\hbi_JO.dta"

Regression analysis

regress arc ib2.collection_area i.spec [aweight = weight_arc], vce(robust)

Generate ORs

eform(exp(Coef.))

Table S3.1. Study list for systematic review

Lead Author	Year Published	Reference	Location	Species	Collection area (indoor/outdoor/both)	Collection method	Human blood	Total number of blood feds analysed/caught
Das	2017	Beyond the entomological inoculation rate: characterizing multiple blood feeding behavior and *Plasmodium falciparum* multiplicity of infection in *Anopheles* mosquitoes in northern Zambia	Zambia	*Anopheles funestus complex*	Both	PSC + CDC	426	444
Das	2017	Beyond the entomological inoculation rate: characterizing multiple blood feeding behavior and *Plasmodium falciparum* multiplicity of infection in *Anopheles* mosquitoes in northern Zambia	Zambia	*Anopheles gambiae*	Both	PSC + CDC	95	100
Ogola	2017	Composition of Anopheles mosquitoes, their blood-meal hosts, and Plasmodium falciparum infection rates in three islands with disparate bed net coverage in Lake Victoria, Kenya.	Kenya	*Anopheles gambiae*	Both	CDC + outdoor manual collections	236	310

Degefa	2017	Indoor and outdoor malaria vector surveillance in western Kenya: implications for better understanding of residual transmission	Kenya	*Anopheles arabiensis*	Indoors	CDC	10	122
Degefa	2017	Indoor and outdoor malaria vector surveillance in western Kenya: implications for better understanding of residual transmission	Kenya	*Anopheles arabiensis*	Indoors	PSC	1	165
Degefa	2017	Indoor and outdoor malaria vector surveillance in western Kenya: implications for better understanding of residual transmission	Kenya	*Anopheles arabiensis*	Outdoors	CDC	2	59
Degefa	2017	Indoor and outdoor malaria vector surveillance in western Kenya: implications for better understanding of residual transmission	Kenya	*Anopheles arabiensis*	Outdoors	Pit traps	208	298
Kibret	2017	Malaria impact of large dams at different eco-epidemiological settings in Ethiopia	Ethiopia	*Anopheles arabiensis*	Both	CDC	761	924
Kibret	2017	Malaria impact of large dams at different eco-epidemiological settings in Ethiopia	Ethiopia	*Anopheles arabiensis*	Both	CDC	202	278
Kibret	2017	Malaria impact of large dams at different eco-epidemiological settings in Ethiopia	Ethiopia	*Anopheles arabiensis*	Both	CDC	277	392

Kibret	2017	Malaria impact of large dams at different eco-epidemiological settings in Ethiopia	Ethiopia	*Anopheles arabiensis*	Both	CDC	117	168
Kibret	2017	Malaria impact of large dams at different eco-epidemiological settings in Ethiopia	Ethiopia	*Anopheles funestus complex*	Both	CDC	272	311
Kabula	2016	A significant association between deltamethrin resistance, Plasmodium falciparum infection and the Vgsc-1014S resistance mutation in Anopheles gambiae highlights the epidemiological importance of resistance markers	Tanzania	*Anopheles gambiae*	Indoors	PSC	548	575
Kabula	2016	A significant association between deltamethrin resistance, Plasmodium falciparum infection and the Vgsc-1014S resistance mutation in Anopheles gambiae highlights the epidemiological importance of resistance markers	Tanzania	*Anopheles arabiensis*	Indoors	PSC	409	575

Sande	2016	Biting behaviour of Anopheles funestus populations in Mutare and Mutasa districts, Manicaland province, Zimbabwe: Implications for the malaria control programme	Zimbabwe	*Anopheles funestus complex*	Both	CDC,PSC and Pit traps	174	272
Chirebvu	2016	Characterization of an Indoor-Resting Population of Anopheles arabiensis (Diptera: Culicidae) and the Implications on Malaria Transmission in Tubu Village in Okavango Subdistrict, Botswana.	Botswana	*Anopheles arabiensis*	Indoors	PSC + Indoor manual collections	35	139
Lekweiry	2016	Circumsporozoite protein rates, blood-feeding pattern and frequency of knockdown resistance mutations in *Anopheles* spp. in two ecological zones of Mauritania	Mauritania	*Anopheles arabiensis*	Indoors	PSC	46	80
Ndiath	2016	Composition and genetics of malaria vector populations in the Central African Republic	Central African Republic	*Anopheles gambiae*	Indoors	PSC	121	149
Lozano-Fuentes	2016	Evaluation of a topical formulation of eprinomectin against *Anopheles*	Kenya	*Anopheles arabiensis*	Indoors	PSC	10	131

		arabiensis when administered to Zebu cattle (*Bos indicus*) under field conditions						
Lozano-Fuentes	2016	Evaluation of a topical formulation of eprinomectin against *Anopheles arabiensis* when administered to Zebu cattle (*Bos indicus*) under field conditions	Kenya	*Anopheles gambiae*	Indoors	PSC	77	91
Mosqueira	2015	Pilot study on the combination of an organophosphate-based insecticide paint and pyrethroid-treated long lasting nets against pyrethroid resistant malaria vectors in Burkina Faso.	Burkina Faso	*Anopheles gambiae*	Indoors	Indoor manual collection	34	141
Mosqueira	2015	Pilot study on the combination of an organophosphate-based insecticide paint and pyrethroid-treated long lasting nets against pyrethroid resistant malaria vectors in Burkina Faso.	Burkina Faso	*Anopheles gambiae*	Indoors	Indoor manual collection	51	143
Mosqueira	2015	Pilot study on the combination of an organophosphate-based insecticide paint and pyrethroid-treated long lasting nets against	Burkina Faso	*Anopheles gambiae*	Indoors	Indoor manual collection	28	141

		pyrethroid resistant malaria vectors in Burkina Faso.						
Das	2015	Underestimation of foraging behaviour by standard field methods in malaria vector mosquitoes in southern Africa.	Zambia and Zimbabwe	*Anopheles arabiensis*	Indoors	PSC + CDC	559	643
Das	2015	Underestimation of foraging behaviour by standard field methods in malaria vector mosquitoes in southern Africa.	Zambia and Zimbabwe	*Anopheles funestus complex*	Indoors	PSC + CDC	343	343
Das	2015	Underestimation of foraging behaviour by standard field methods in malaria vector mosquitoes in southern Africa.	Zambia and Zimbabwe	*Anopheles funestus complex*	Indoors	PSC + CDC	78	84
Massebo	2015	Zoophagic behaviour of anopheline mosquitoes in southwest Ethiopia: opportunity for malaria vector control	Ethiopia	*Anopheles arabiensis*	Indoors	CDC	93	988
Massebo	2015	Zoophagic behaviour of anopheline mosquitoes in southwest Ethiopia: opportunity for malaria vector control	Ethiopia	*Anopheles arabiensis*	Indoors	PSC	59	352

Massebo	2015	Zoophagic behaviour of anopheline mosquitoes in southwest Ethiopia: opportunity for malaria vector control	Ethiopia	*Anopheles arabiensis*	Outdoors	Pit traps	26	894
Guelbeogo	2014	Behavioural divergence of sympatric Anopheles funestus populations in Burkina Faso.	Burkina Faso	*Anopheles funestus complex*	Indoors	PSC	211	221
Guelbeogo	2014	Behavioural divergence of sympatric Anopheles funestus populations in Burkina Faso.	Burkina Faso	*Anopheles funestus complex*	Indoors	PSC	242	272
Guelbeogo	2014	Behavioural divergence of sympatric Anopheles funestus populations in Burkina Faso.	Burkina Faso	*Anopheles funestus complex*	Outdoors	Pit traps	38	529
Sougoufara	2014	Biting by *Anopheles funestus* in broad daylight after use of long-lasting insecticidal nets: a new challenge to malaria elimination	Senegal	*Anopheles funestus complex*	Indoors	PSC	61	84
Antonio-Nkondjio	2014	High malaria transmission intensity in a village close to Yaounde, the capital city of Cameroon.	Cameroon	*Anopheles funestus complex*	Both	PSC + pit traps +drums	299	299

Kibret	2014	Increased malaria transmission around irrigation schemes in Ethiopia and the potential of canal water management for malaria vector control	Ethiopia	*Anopheles funestus complex*	Both	CDC	20	58
Kibret	2014	Increased malaria transmission around irrigation schemes in Ethiopia and the potential of canal water management for malaria vector control	Ethiopia	*Anopheles arabiensis*	Both	CDC	1680	2101
Kibret	2014	Increased malaria transmission around irrigation schemes in Ethiopia and the potential of canal water management for malaria vector control	Ethiopia	*Anopheles arabiensis*	Both	CDC	171	234
McCann	2014	Reemergence of Anopheles funestus as a Vector of Plasmodium falciparum in Western Kenya after Long-Term Implementation of Insecticide-Treated Bed Nets	Kenya	*Anopheles funestus complex*	Indoors	PSC	697	715
McCann	2014	Reemergence of Anopheles funestus as a Vector of Plasmodium falciparum in Western	Kenya	*Anopheles arabiensis*	Indoors	PSC	58	115

		Kenya after Long-Term Implementation of Insecticide-Treated Bed Nets						
McCann	2014	Reemergence of Anopheles funestus as a Vector of Plasmodium falciparum in Western Kenya after Long-Term Implementation of Insecticide-Treated Bed Nets	Kenya	*Anopheles gambiae*	Indoors	PSC	51	55
McCann	2014	Reemergence of Anopheles funestus as a Vector of Plasmodium falciparum in Western Kenya after Long-Term Implementation of Insecticide-Treated Bed Nets	Kenya	*Anopheles arabiensis*	Indoors	PSC	25	73
Massebo	2013	Blood meal origins and insecticide susceptibility of *Anopheles arabiensis* from Chano in South-West Ethiopia	Ethiopia	*Anopheles arabiensis*	Indoors	CDC	741	988
Massebo	2013	Blood meal origins and insecticide susceptibility of *Anopheles arabiensis* from Chano in South-West Ethiopia	Ethiopia	*Anopheles arabiensis*	Indoors	PSC	204	352
Massebo	2013	Blood meal origins and insecticide susceptibility of *Anopheles arabiensis* from Chano in South-West Ethiopia	Ethiopia	*Anopheles arabiensis*	Outdoors	Pit traps	116	894

Animut	2013	Blood meal sources and entomological inoculation rates of anophelines along a highland altitudinal transect in south-central Ethiopia	Ethiopia	*Anopheles arabiensis*	Indoors	CDC	135	422
Animut	2013	Blood meal sources and entomological inoculation rates of anophelines along a highland altitudinal transect in south-central Ethiopia	Ethiopia	*Anopheles arabiensis*	Outdoors	PSC	227	723
Animut	2013	Blood meal sources and entomological inoculation rates of anophelines along a highland altitudinal transect in south-central Ethiopia	Ethiopia	*Anopheles arabiensis*	Indoors	CDC	27	64
Animut	2013	Blood meal sources and entomological inoculation rates of anophelines along a highland altitudinal transect in south-central Ethiopia	Ethiopia	*Anopheles arabiensis*	Outdoors	PSC	32	114
Dadzie	2013	Role of species composition in malaria transmission by the *Anopheles funestus* group (Diptera: Culicidae) in Ghana	Ghana	*Anopheles funestus complex*	Indoors	PSC	80	89

Dadzie	2013	Role of species composition in malaria transmission by the *Anopheles funestus* group (Diptera: Culicidae) in Ghana	Ghana	*Anopheles funestus complex*	Indoors	PSC	52	64
Dadzie	2013	Role of species composition in malaria transmission by the *Anopheles funestus* group (Diptera: Culicidae) in Ghana	Ghana	*Anopheles funestus complex*	Indoors	PSC	73	76
Obala	2012	Anopheles gambiae and Anopheles arabiensis population densities and infectivity in Kopere village, Western Kenya	Kenya	*Anopheles arabiensis*	Indoors	PSC	59	68
Obala	2012	Anopheles gambiae and Anopheles arabiensis population densities and infectivity in Kopere village, Western Kenya	Kenya	*Anopheles gambiae*	Indoors	PSC	198	205
Mzilahowa	2012	Entomological indices of malaria transmission in Chikhwawa district, Southern Malawi	Malawi	*Anopheles funestus complex*	Indoors	PSC	286	297
Mzilahowa	2012	Entomological indices of malaria transmission in Chikhwawa district, Southern Malawi	Malawi	*Anopheles gambiae*	Indoors	PSC	244	246
Mzilahowa	2012	Entomological indices of malaria transmission in Chikhwawa district, Southern Malawi	Malawi	*Anopheles arabiensis*	Indoors	PSC	289	340

Kibret	2012	How does an Ethiopian dam increase malaria? Entomological determinants around the Koka reservoir.	Ethiopia	*Anopheles arabiensis*	Both	CDC	148	208
Kibret	2012	How does an Ethiopian dam increase malaria? Entomological determinants around the Koka reservoir.	Ethiopia	*Anopheles arabiensis*	Both	CDC	89	111
Kibret	2012	How does an Ethiopian dam increase malaria? Entomological determinants around the Koka reservoir.	Ethiopia	*Anopheles arabiensis*	Both	CDC	56	89
Kawada	2012	Reconsideration of *Anopheles rivulorum* as a vector of *Plasmodium falciparum* in western Kenya: some evidence from biting time, blood preference, sporozoite positive rate, and pyrethroid resistance	Kenya	*Anopheles funestus complex*	Indoors	Indoor manual collection	34	69
Tanga	2011	Daily survival and human blood index of major malaria vectors associated with oil palm cultivation in Cameroon and their role in malaria transmission.	Cameroon	*Anopheles funestus complex*	Indoors	PSC	237	245

Himeidan	2011	Pattern of malaria transmission along the Rahad River basin, Eastern Sudan	Sudan	*Anopheles arabiensis*	Indoors	PSC	176	219
Himeidan	2011	Pattern of malaria transmission along the Rahad River basin, Eastern Sudan	Sudan	*Anopheles arabiensis*	Indoors	PSC	68	102
Himeidan	2011	Pattern of malaria transmission along the Rahad River basin, Eastern Sudan	Sudan	*Anopheles arabiensis*	Indoors	PSC	37	58
Himeidan	2011	Pattern of malaria transmission along the Rahad River basin, Eastern Sudan	Sudan	*Anopheles arabiensis*	Indoors	PSC	361	394
Himeidan	2011	Pattern of malaria transmission along the Rahad River basin, Eastern Sudan	Sudan	*Anopheles arabiensis*	Indoors	PSC	95	119
Himeidan	2011	Pattern of malaria transmission along the Rahad River basin, Eastern Sudan	Sudan	*Anopheles arabiensis*	Indoors	PSC	39	64
Himeidan	2011	Pattern of malaria transmission along the Rahad River basin, Eastern Sudan	Sudan	*Anopheles arabiensis*	Indoors	PSC	272	331
Mala	2011	Plasmodium falciparum transmission and aridity: a Kenyan experience from the dry lands of Baringo and its implications for Anopheles arabiensis control	Kenya	*Anopheles arabiensis*	Outdoors	CDC	55	88

Mala	2011	Plasmodium falciparum transmission and aridity: a Kenyan experience from the dry lands of Baringo and its implications for Anopheles arabiensis control	Kenya	*Anopheles arabiensis*	Indoors	PSC	58	136
Mala	2011	Plasmodium falciparum transmission and aridity: a Kenyan experience from the dry lands of Baringo and its implications for Anopheles arabiensis control	Kenya	*Anopheles arabiensis*	Outdoors	CDC	71	149
Fornadel	2010	Analysis of Anopheles arabiensis Blood Feeding Behavior in Southern Zambia during the Two Years after Introduction of Insecticide-Treated Bed Nets	Zambia	*Anopheles arabiensis*	Indoors	CDC	220	235
Fornadel	2010	Analysis of Anopheles arabiensis Blood Feeding Behavior in Southern Zambia during the Two Years after Introduction of Insecticide-Treated Bed Nets	Zambia	*Anopheles arabiensis*	Indoors	CDC	223	233
Tchuinkam	2010	Bionomics of Anopheline species and malaria transmission dynamics along an altitudinal transect in Western Cameroon.	Cameroon	*Anopheles gambiae*	Indoors	PSC	269	278

Tchuinkam	2010	Bionomics of Anopheline species and malaria transmission dynamics along an altitudinal transect in Western Cameroon.	Cameroon	*Anopheles gambiae*	Indoors	PSC	68	77
Tchuinkam	2010	Bionomics of Anopheline species and malaria transmission dynamics along an altitudinal transect in Western Cameroon.	Cameroon	*Anopheles gambiae*	Indoors	PSC	347	371
Tanga	2010	Climate change and altitudinal structuring of malaria vectors in south-western Cameroon: their relation to malaria transmission	Cameroon	*Anopheles gambiae*	Both	PSC and outdoor manual collections	109	112
Tanga	2010	Climate change and altitudinal structuring of malaria vectors in south-western Cameroon: their relation to malaria transmission	Cameroon	*Anopheles funestus complex*	Both	PSC and outdoor manual collections	63	63
Adeleke	2010	Population dynamics of indoor sampled mosquitoes and their implication in disease transmission in Abeokuta, south-western Nigeria	Nigeria	*Anopheles gambiae*	Indoors	CDC	225	225

Kibret	2010	The impact of a small-scale irrigation scheme on malaria transmission in Ziway area, Central Ethiopia.	Ethiopia	*Anopheles arabiensis*	Both	CDC	93	120
Kasili	2009	Entomological assessment of the potential for malaria transmission in Kibera slum of Nairobi, Kenya	Kenya	*Anopheles gambiae*	Indoors	Indoor manual collection	77	80
Kerah-Hinzoumbé	2009	Malaria vectors and transmission dynamics in Goulmoun, a rural city in south-western Chad	Chad	*Anopheles arabiensis*	Indoors	PSC	92	144
Kerah-Hinzoumbé	2009	Malaria vectors and transmission dynamics in Goulmoun, a rural city in south-western Chad	Chad	*Anopheles funestus complex*	Indoors	PSC	48	53
Caputo	2008	*Anopheles gambiae complex along The Gambia river, with particular reference to the molecular forms of An. gambiae s.s*	Gambia	*Anopheles gambiae*	Indoors	PSC	36	56
Caputo	2008	*Anopheles gambiae complex along The Gambia river, with particular reference to the molecular forms of An. gambiae s.s*	Gambia	*Anopheles gambiae*	Indoors	PSC	71	158

Caputo	2008	*Anopheles gambiae complex along The Gambia river, with particular reference to the molecular forms of An. gambiae s.s*	Gambia	*Anopheles gambiae*	Indoors	PSC	16	73
Caputo	2008	*Anopheles gambiae complex along The Gambia river, with particular reference to the molecular forms of An. gambiae s.s*	Gambia	*Anopheles gambiae*	Indoors	PSC	36	82
Caputo	2008	*Anopheles gambiae complex along The Gambia river, with particular reference to the molecular forms of An. gambiae s.s*	Gambia	*Anopheles gambiae*	Indoors	PSC	29	68
Caputo	2008	*Anopheles gambiae complex along The Gambia river, with particular reference to the molecular forms of An. gambiae s.s*	Gambia	*Anopheles gambiae*	Indoors	PSC	18	89
Caputo	2008	*Anopheles gambiae complex along The Gambia river, with particular reference to the molecular forms of An. gambiae s.s*	Gambia	*Anopheles gambiae*	Indoors	PSC	51	68
Caputo	2008	*Anopheles gambiae complex along The Gambia river, with particular reference to the molecular forms of An. gambiae s.s*	Gambia	*Anopheles gambiae*	Indoors	PSC	62	179

Muturi	2008	Effect of Rice Cultivation on Malaria Transmission in Central Kenya	Kenya	*Anopheles arabiensis*	Indoors	PSC	73	812
Muturi	2008	Effect of Rice Cultivation on Malaria Transmission in Central Kenya	Kenya	*Anopheles arabiensis*	Indoors	PSC	40	334
Muturi	2008	Effect of Rice Cultivation on Malaria Transmission in Central Kenya	Kenya	*Anopheles arabiensis*	Indoors	PSC	65	131
Muturi	2008	Effect of Rice Cultivation on Malaria Transmission in Central Kenya	Kenya	*Anopheles funestus complex*	Indoors	PSC	46	65
Fornadel	2008	Increased Endophily by the Malaria Vector Anopheles arabiensis in Southern Zambia and Identification of Digested Blood Meals	Zambia	*Anopheles arabiensis*	Indoors	PSC	252	289
Abdalla	2008	Insecticide susceptibility and vector status of natural populations of *Anopheles arabiensis* from Sudan	Sudan	*Anopheles arabiensis*	Indoors	PSC	273	310
Kweka	2008	Mosquito abundance, bed net coverage and other factors associated with variations in sporozoite infectivity rates in four villages of rural Tanzania	Tanzania	*Anopheles funestus complex*	Indoors	PSC	719	811

Kweka	2008	Mosquito abundance, bed net coverage and other factors associated with variations in sporozoite infectivity rates in four villages of rural Tanzania	Tanzania	*Anopheles arabiensis*	Indoors	PSC	81	727
Kweka	2008	Vector species composition and malaria infectivity rates in Mkuzi, Muheza District, north-eastern Tanzania	Tanzania	*Anopheles gambiae*	Indoors	PSC + CDC	1129	1224
Kweka	2008	Vector species composition and malaria infectivity rates in Mkuzi, Muheza District, north-eastern Tanzania	Tanzania	*Anopheles funestus complex*	Indoors	PSC + CDC	251	283
Kweka	2008	Vector species composition and malaria infectivity rates in Mkuzi, Muheza District, north-eastern Tanzania	Tanzania	*Anopheles funestus complex*	Indoors	PSC + CDC	51	80
Kweka	2008	Vector species composition and malaria infectivity rates in Mkuzi, Muheza District, north-eastern Tanzania	Tanzania	*Anopheles funestus complex*	Indoors	PSC + CDC	33	50
Mahande	2007	Feeding and resting behaviour of malaria vector, Anopheles arabiensis with reference to zooprophylaxis.	Tanzania	*Anopheles arabiensis*	Indoors	PSC	166	417

Mahande	2007	Feeding and resting behaviour of malaria vector, Anopheles arabiensis with reference to zooprophylaxis.	Tanzania	*Anopheles arabiensis*	Outdoors	Pit traps	0	417
Mahande	2007	Feeding and resting behaviour of malaria vector, Anopheles arabiensis with reference to zooprophylaxis.	Tanzania	*Anopheles arabiensis*	Indoors	PSC	291	417
Mahande	2007	Feeding and resting behaviour of malaria vector, Anopheles arabiensis with reference to zooprophylaxis.	Tanzania	*Anopheles arabiensis*	Outdoors	Pit traps	41	417
Muriu	2007	Host choice and multiple blood feeding behaviour of malaria vectors and other anophelines in Mwea rice scheme, Kenya	Kenya	*Anopheles arabiensis*	Indoors	PSC	194	2467
Muriu	2007	Host choice and multiple blood feeding behaviour of malaria vectors and other anophelines in Mwea rice scheme, Kenya	Kenya	*Anopheles arabiensis*	Outdoors	CDC	5	75
Muriu	2007	Host choice and multiple blood feeding behaviour of malaria vectors and other anophelines in Mwea rice scheme, Kenya	Kenya	*Anopheles funestus complex*	Indoors	PSC	51	181

Temu	2007	Identification of four members of the *Anopheles funestus*(Diptera: Culicidae) group and their role in *Plasmodium falciparum* transmission in Bagamoyo coastal Tanzania	Tanzania	*Anopheles funestus complex*	Indoors	CDC	66	120
Kent	2007	Seasonality, blood feeding behavior, and transmission of Plasmodium falciparum by Anopheles arabiensis after an extended drought in southern Zambia.	Zambia	*Anopheles arabiensis*	Indoors	PSC	415	450
Kulkarni	2006	Entomological Evaluation of Malaria Vectors at Different Altitudes in Hai District, Northeastern Tanzania	Tanzania	*Anopheles arabiensis*	Indoors	PSC	668	905
Kulkarni	2006	Entomological Evaluation of Malaria Vectors at Different Altitudes in Hai District, Northeastern Tanzania	Tanzania	*Anopheles arabiensis*	Outdoors	Pit traps	36	144
Kulkarni	2006	Entomological Evaluation of Malaria Vectors at Different Altitudes in Hai District, Northeastern Tanzania	Tanzania	*Anopheles funestus complex*	Indoors	PSC	57	86

Yohannes	2005	Can source reduction of mosquito larval habitat reduce malaria transmission in Tigray, Ethiopia?	Ethiopia	*Anopheles arabiensis*	Indoors	PSC	141	194
Awolola	2005	Identification of three members of the Anopheles funestus (Diptera: Culicidae) group and their role in malaria transmission in two ecological zones in Nigeria	Nigeria	*Anopheles funestus complex*	Both	Indoor manual collections +pit traps	173	264
Awolola	2005	Identification of three members of the Anopheles funestus (Diptera: Culicidae) group and their role in malaria transmission in two ecological zones in Nigeria	Nigeria	*Anopheles funestus complex*	Both	Indoor manual collections +pit traps	187	299
Kamau	2003	Anopheles parensis: the main member of the Anopheles funestus species group found resting inside human dwellings in Mwea area of central Kenya toward the end of the rainy season.	Kenya	*Anopheles funestus complex*	Indoors	Indoor manual collection	2	139
Wanji	2003	Anopheles species of the mount Cameroon region: biting habits, feeding behaviour and entomological inoculation rates.	Cameroon	*Anopheles gambiae*	Indoors	PSC	156	235

Wanji	2003	Anopheles species of the mount Cameroon region: biting habits, feeding behaviour and entomological inoculation rates.	Cameroon	*Anopheles funestus complex*	Indoors	PSC	72	235
Mwangangi	2003	Blood-meal analysis for anopheline mosquitoes sampled along the Kenyan coast.	Kenya	*Anopheles gambiae*	Indoors	PSC	307	338
Mwangangi	2003	Blood-meal analysis for anopheline mosquitoes sampled along the Kenyan coast.	Kenya	*Anopheles arabiensis*	Indoors	PSC	72	79
Mwangangi	2003	Blood-meal analysis for anopheline mosquitoes sampled along the Kenyan coast.	Kenya	*Anopheles funestus complex*	Indoors	PSC	378	439
Ijumba	2002	Malaria transmission risk variations derived from different agricultural practices in an irrigated area of northern Tanzania.	Tanzania	*Anopheles arabiensis*	Indoors	PSC	380	795
Ijumba	2002	Malaria transmission risk variations derived from different agricultural practices in an irrigated area of northern Tanzania.	Tanzania	*Anopheles arabiensis*	Indoors	PSC	132	193
Ijumba	2002	Malaria transmission risk variations derived from different agricultural practices in an irrigated area of northern Tanzania.	Tanzania	*Anopheles arabiensis*	Indoors	PSC	160	241

Ijumba	2002	Malaria transmission risk variations derived from different agricultural practices in an irrigated area of northern Tanzania.	Tanzania	*Anopheles arabiensis*	Outdoors	Pit traps	21	501
Ijumba	2002	Malaria transmission risk variations derived from different agricultural practices in an irrigated area of northern Tanzania.	Tanzania	*Anopheles arabiensis*	Outdoors	Pit traps	44	174
Ijumba	2002	Malaria transmission risk variations derived from different agricultural practices in an irrigated area of northern Tanzania.	Tanzania	*Anopheles arabiensis*	Outdoors	Pit traps	4	121
Sousa	2001	Dogs as a Favored Host Choice of Anopheles gambiae sensu stricto (Diptera: Culicidae) of Sa˜o Tome´, West Africa	Sao Tome	*Anopheles gambiae*	Indoors	CDC	399	434
Sousa	2001	Dogs as a Favored Host Choice of Anopheles gambiae sensu stricto (Diptera: Culicidae) of Sa˜o Tome´, West Africa	Sao Tome	*Anopheles gambiae*	Indoors	PSC	181	193
Sousa	2001	Dogs as a Favored Host Choice of Anopheles gambiae sensu stricto (Diptera: Culicidae) of Sa˜o Tome´, West Africa	Sao Tome	*Anopheles gambiae*	Outdoors	Indoor manual collection	113	422

Bøgh	2001	Effect of Passive Zooprophylaxis on Malaria Transmission in the Gambia	Gambia	*Anopheles gambiae*	Indoors	PSC	99	177
Bøgh	2001	Effect of Passive Zooprophylaxis on Malaria Transmission in the Gambia	Gambia	*Anopheles gambiae*	Indoors	PSC	96	185
Habtewold	2001	The feeding behaviour and *Plasmodium* infection of *Anopheles* mosquitoes in southern Ethiopia in relation to use of insecticide-treated livestock for malaria control	Ethiopia	*Anopheles arabiensis*	Indoors	Indoor manual collection	27	64
Charlwood	2001	The impact of indoor residual spraying with malathion on malaria in refugee camps in eastern Sudan	Sudan	*Anopheles arabiensis*	Indoors	PSC	123	242
Magbity	1997	Effects of community-wide use of lambdacyhalothrin-impregnated bednets on malaria vectors in rural Sierra Leone.	Sierra Leone	*Anopheles gambiae*	Indoors	PSC + CDC	249	253
Magbity	1997	Effects of community-wide use of lambdacyhalothrin-impregnated bednets on malaria vectors in rural Sierra Leone.	Sierra Leone	*Anopheles gambiae*	Indoors	PSC + CDC	397	401

Hadis	1997	Host choice by indoor-resting Anopheles arabiensis in Ethiopia	Ethiopia	*Anopheles arabiensis*	Indoors	Indoor manual collection	118	130
Githeko	1994	Origin of blood meals in indoor and outdoor resting malaria vectors in western Kenya	Kenya	*Anopheles arabiensis*	Indoors	PSC	108	232
Githeko	1994	Origin of blood meals in indoor and outdoor resting malaria vectors in western Kenya	Kenya	*Anopheles funestus complex*	Indoors	PSC	86	94
Githeko	1994	Origin of blood meals in indoor and outdoor resting malaria vectors in western Kenya	Kenya	*Anopheles arabiensis*	Outdoors	Indoor manual collection	0	186
Mbogo	1993	BLOODFEEDING BEHAVIOR OF ANOPHELES gambiae S.L. AND ANOPHELES FUNESTUS IN KILIFI DISTRICT, KENYA	Kenya	*Anopheles funestus complex*	Indoors	Indoor manual collection	57	64
Garrett-Jones	1964	The human blood index of malaria vectors in relation to epidemiological assessment	Multiple	*Anopheles funestus complex*	Indoors	Indoor manual collection	179	183
Garrett-Jones	1964	The human blood index of malaria vectors in relation to epidemiological assessment	Multiple	*Anopheles funestus complex*	Outdoors	Indoor manual collection	61	72

Garrett-Jones	1964	The human blood index of malaria vectors in relation to epidemiological assessment	Multiple	*Anopheles funestus complex*	Indoors	Indoor manual collection	1768	2056
Garrett-Jones	1964	The human blood index of malaria vectors in relation to epidemiological assessment	Multiple	*Anopheles funestus complex*	Outdoors	Indoor manual collection	110	243
Garrett-Jones	1964	The human blood index of malaria vectors in relation to epidemiological assessment	Multiple	*Anopheles funestus complex*	Indoors	Indoor manual collection	99	120
Garrett-Jones	1964	The human blood index of malaria vectors in relation to epidemiological assessment	Multiple	*Anopheles funestus complex*	Outdoors	Indoor manual collection	152	423
Garrett-Jones	1964	The human blood index of malaria vectors in relation to epidemiological assessment	Multiple	*Anopheles funestus complex*	Indoors	Indoor manual collection	178	344
Garrett-Jones	1964	The human blood index of malaria vectors in relation to epidemiological assessment	Multiple	*Anopheles funestus complex*	Outdoors	Indoor manual collection	9	193

Garrett-Jones	1964	The human blood index of malaria vectors in relation to epidemiological assessment	Cameroon	*Anopheles funestus complex*	Indoors	Indoor manual collection	53	72
Garrett-Jones	1964	The human blood index of malaria vectors in relation to epidemiological assessment	Zimbabwe	*Anopheles funestus complex*	Outdoors	Indoor manual collection	0	161
Garrett-Jones	1964	The human blood index of malaria vectors in relation to epidemiological assessment	Multiple	*Anopheles gambiae*	Indoors	Indoor manual collection	258	327
Garrett-Jones	1964	The human blood index of malaria vectors in relation to epidemiological assessment	Multiple	*Anopheles gambiae*	Outdoors	Indoor manual collection	3	124
Garrett-Jones	1964	The human blood index of malaria vectors in relation to epidemiological assessment	Multiple	*Anopheles gambiae*	Indoors	Indoor manual collection	1844	2311
Garrett-Jones	1964	The human blood index of malaria vectors in relation to epidemiological assessment	Multiple	*Anopheles gambiae*	Outdoors	Indoor manual collection	172	310
Garrett-Jones	1964	The human blood index of malaria vectors in relation to epidemiological assessment	Multiple	*Anopheles gambiae*	Indoors	Indoor manual collection	418	537
Garrett-Jones	1964	The human blood index of malaria vectors in relation to epidemiological assessment	Multiple	*Anopheles gambiae*	Outdoors	Indoor manual collection	147	524

Garrett-Jones	1964	The human blood index of malaria vectors in relation to epidemiological assessment	Multiple	*Anopheles gambiae*	Indoors	Indoor manual collection	566	696
Garrett-Jones	1964	The human blood index of malaria vectors in relation to epidemiological assessment	Multiple	*Anopheles gambiae*	Outdoors	Indoor manual collection	29	964
Garrett-Jones	1964	The human blood index of malaria vectors in relation to epidemiological assessment	Multiple	*Anopheles gambiae*	Indoors	Indoor manual collection	16	1114
Garrett-Jones	1964	The human blood index of malaria vectors in relation to epidemiological assessment	Multiple	*Anopheles gambiae*	Outdoors	Indoor manual collection	300	940
Garrett-Jones	1964	The human blood index of malaria vectors in relation to epidemiological assessment	Multiple	*Anopheles gambiae*	Indoors	Indoor manual collection	196	265
Garrett-Jones	1964	The human blood index of malaria vectors in relation to epidemiological assessment	Multiple	*Anopheles gambiae*	Outdoors	Indoor manual collection	99	226

Chapter 4 - Investigating the blood-host plasticity and dispersal of *Anopheles coluzzii* in the field using a novel field-based methodology

Abstract

Background: The biting behaviour and dispersal of insect vectors in the field underlies the transmission of many diseases. Here, a novel collection methodology coupled with the molecular analysis of blood-meal sources and digestion rates is introduced with the aim of aiding the understanding of two critical and relatively understudied mosquito behaviours: plasticity in blood-host choice and vector dispersal.

Results: A collection strategy utilising a transect of mosquito traps placed at 50 m intervals allowed the collection of blood-fed *Anopheles coluzzii* from a malaria-endemic village of southern Ghana where human host availability ranged from zero (a cattle pen), increasing until humans were the dominant host choice (the middle of the village). Blood-meal analysis using PCR showed statistically significant variation in blood-meal origins for mosquitoes collected across the 250 m transect: with decreasing trend in Bovine Blood Index (OR = 0.60 95% CI: 0.49–0.73, $P < 0.01$) and correspondingly, an increasing trend in Human Blood Index (OR = 1.50 95% CI: 1.05–2.16, $P = 0.028$) as the transect approached the village. Using qPCR, the host DNA remaining in the blood meal was quantified for field-caught mosquitoes and calibrated according to timed blood digestion in colony mosquitoes. Time since blood meal was consumed and the corresponding distance the vector was caught from its blood-host allowed the estimation of *An. coluzzii* dispersal rates. Within 7 hours of feeding, mosquitoes typically remained within 50 m of their blood-host but at 60 hours they had dispersed up to 250 m.

Conclusions: Using this methodology the remarkably small spatial scale at which *An. coluzzii* blood-host choice can change was demonstrated. In addition, conducting qPCR on host blood from field-caught mosquitoes and calibrating with timed experiments with colonised mosquitoes presents a novel methodology for investigating the dispersal behaviour of vectors. Future adaptations to this novel method to make it broadly applicable to other types of setting are also discussed.

Aim

Investigate the spatial range across which host selection varies for major African malaria vectors.

Objectives

1. Implement the field study design described in chapter 2 to collect blood fed *Anopheles* mosquitoes across a range of human host availabilities.

2. Identify the blood meal source and species of *Anopheles* mosquitoes collected from the field using qualitative PCR.

3. Investigate the relationship between blood-host source and host availability for locally captured mosquitoes.

4. Develop a panel of DNA samples to represent a time series of blood meal digestion.

5. Using the time series panel coupled with the field caught data, develop a novel methodology for investigating post-feeding behaviour and dispersal of *An. coluzzii* in the field.

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SECTION A – Student Details

Student	James Orsborne
Principal Supervisor	Laith Yakob
Thesis Title	Mosquito biting behaviour and malaria transmission: interactions between intrinsic host preferences and local host availability

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	Parasites and Vectors		
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SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	JO performed all field and laboratory work shown in the manuscript. JO also aided in the data analysis and statistics performed as well as formatting and drafting of the manuscript prior to submission.

Student Signature: [redacted] 03/03/2019 **Date:**

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Parasites & Vectors

RESEARCH Open Access

Investigating the blood-host plasticity and dispersal of *Anopheles coluzzii* using a novel field-based methodology

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Abstract

Background: The biting behaviour and dispersal of insect vectors in the field underlies the transmission of many diseases. Here, a novel collection methodology coupled with the molecular analysis of blood-meal sources and digestion rates is introduced with the aim of aiding the understanding of two critical and relatively understudied mosquito behaviours: plasticity in blood-host choice and vector dispersal.

Results: A collection strategy utilising a transect of mosquito traps placed at 50 m intervals allowed the collection of blood-fed *Anopheles coluzzii* from a malaria-endemic village of southern Ghana where human host availability ranged from zero (a cattle pen), increasing until humans were the dominant host choice (the middle of the village). Blood-meal analysis using PCR showed statistically significant variation in blood-meal origins for mosquitoes collected across the 250 m transect: with decreasing trend in Bovine Blood Index (OR = 0.60 95% CI: 0.49–0.73, $P < 0.01$) and correspondingly, an increasing trend in Human Blood Index (OR = 1.50 95% CI: 1.05–2.16, $P = 0.028$) as the transect approached the village. Using qPCR, the host DNA remaining in the blood meal was quantified for field-caught mosquitoes and calibrated according to timed blood digestion in colony mosquitoes. Time since blood meal was consumed and the corresponding distance the vector was caught from its blood-host allowed the estimation of *An. coluzzii* dispersal rates. Within 7 hours of feeding, mosquitoes typically remained within 50 m of their blood-host but at 60 hours they had dispersed up to 250 m.

Conclusions: Using this methodology the remarkably small spatial scale at which *An. coluzzii* blood-host choice can change was demonstrated. In addition, conducting qPCR on host blood from field-caught mosquitoes and calibrating with timed experiments with colonised mosquitoes presents a novel methodology for investigating the dispersal behaviour of vectors. Future adaptations to this novel method to make it broadly applicable to other types of setting are also discussed.

Keywords: Blood-meal analysis, Host preference, Mosquito, Biting preference, Blood index

Background

Many disease vectors have demonstrable preference for a particular type of mammalian host to obtain a blood meal, however it is well documented that even the most anthropophilic of disease vectors will still seek a proportion of blood meals from alternative (non-human) host sources [1–4]. Gillies first researched host choice among malaria vectors by releasing *Anopheles* mosquitoes into an enclosed space and comparing the numbers flying into a room holding a human volunteer with those entering a room with a calf [5]. In the subsequent 50 years, the complexity of host preference and biting behaviour has become well documented [2, 6, 7]. While useful, many host-choice experiments have set-ups that can only inform the intrinsic host preference

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of a vector, and may or may not be indicative of what host species is bitten in natural field settings [8].

Many extrinsic as well as intrinsic factors play a part in who or what is ultimately bitten by a disease vector in a field setting, and these have been summarised comprehensively [2]. This balance between intrinsic and extrinsic factors could go some way to explaining the large variability found in the reported human blood index (HBI) of major disease vectors [2, 9]. Although it has been recognised for a long time that the same mosquito population will often adjust its biting towards a more locally available host species [7, 9, 10], the extent of this plasticity and the spatial scale at which it acts remains understudied even for the most important disease vectors. This plasticity is an important factor when it comes to implementing control strategies. The introduction of insecticide treated nets (ITNs) and indoor residual spraying (IRS) has seen the biting behaviour of many major malaria vectors shift [2, 11, 12] with increasing reports of these vectors seeking blood-meals from alternative non-human sources [3]. Outdoor and residual malaria transmission supported by secondary or indiscriminate malaria vectors [13] further highlights the importance of understanding host choice so future control strategies can be better targeted.

Also implicit to the spatial scale across which feeding choice changes is the vector's dispersal ability. For example, if a vector tends not to disperse very far, a reasonable assumption may be that it will be less discerning in its choice of host and therefore be more likely to bite whatever is nearby. However, what is of considerable hindrance to this field's development is the absence of reliable methods for assessing a disease vector's dispersal ability. Conducting experimental studies on mosquito dispersal has been particularly challenging with the majority of such experiments involving the mark-release-recapture of mosquitoes. However, the impact of handling mosquitoes combined with the typically low recapture rates (in the order of < 2% for *An. gambiae* [14–18]) has limited what can be learned.

Here, the blood-meal sources were identified for *An. coluzzii* caught in traps situated across a 250 m transect representing a range of alternative blood-host species availabilities (primarily human or cattle) in a malaria endemic village of southern Ghana. By using this collection methodology coupled with molecular blood-meal identification, we aim to investigate the spatial range across which this principle malaria vector can adjust its targeted blood-host species based on local host availability. In addition, by quantifying host DNA isolated from field-caught vectors and calibrating this with timed laboratory mosquito feeding experiments, an alternative method is presented for measuring dispersal rates for haematophagous disease vectors. Finally, potential future adaptations to these novel methods are discussed in order to make them broadly applicable to investigating host plasticity and dispersal in other settings.

Methods

Study site and mosquito collection

Mosquitoes were collected from the village of Dogo, in the Greater Accra region of Ghana (05°52.418N, 00°33.607E). The village is in the south-eastern coast of Ghana, with the Gulf of Guinea to the south and the Volta River to the east. The average rainfall is approximately 927 mm per year with the main rainy season from April to June and a shorter second season in October. Temperatures range between 23–33 °C. The area is costal savannah with sandy soil, short savannah grass with some small/medium sized trees. The land is used extensively for grazing livestock as well as growing crops for local trade. Housing mostly consisted of concrete structures with concrete/brick walls and flooring. Some traditional mud style houses were also present, more so on the periphery of the village.

Mosquitoes were collected across five consecutive nights in June 2017. The trapping setup consisted of CDC resting traps placed outdoors at 50 m intervals forming a 250 m transect comprising of six trapping points (denoted T1–T6). This transect was set beginning at an area of zero human population density (T1, outside of the village by cattle resting and overnight holding pens) and extending towards a human population in 50 m intervals ending at an area of high human density (see Table 1 for description and Fig. 1 for map of collection site). Mosquitoes were collected overnight from 18:00 to 06:00 h.

Mosquitoes were removed from traps at 06:00 h each morning and immediately killed using chloroform to stop any active blood-meal digestion. Mosquitoes where then sorted with all blood-fed females being processed first. All visually blood-fed and gravid *Anopheles* mosquitoes were processed individually with transect location and night collected being recorded. Abdomens of blood-fed mosquitoes were removed with sterile forceps and pressed onto FTA®Classic cards (Whatman, GE Healthcare, New Jersey, USA) to preserve the blood meal for molecular analysis. Excess blood-fed mosquitoes were preserved in RNA later (Thermo Fisher Scientific Life Technologies, Massachusetts, USA) in a 96-well plate where necessary.

DNA extraction

Mosquito abdomens were extracted individually. Samples were homogenised using a Qiagen TissueLyser II (Qiagen, Manchester, UK) with a 5 mm stainless steel bead (Qiagen) placed in each sample tube in a 96-well plate

Table 1 Description of areas around transects where mosquitoes were collected including number and type of host present

Transect	Description	Approximate no. of hosts
1	Evening holding pen for cattle for the village (used from 18:00 h to 06:00 h), one small uninhabited house next to the pen was used to hold tools and supplies for cattle farmers	Cows ($n=150$)
2	End of cattle pen (as described above), small pig holding and empty cattle shed. Edge of village is approximately 30 m away with empty newly built houses; first house with inhabitants (T3) 50 m away	Cows ($n=150$); pigs ($n=5$)
3	First cluster of 4 small households on periphery of village c.50 m from cattle pen. A small holding of chickens and goats as well as pet dogs which roam the area freely	Humans ($n=$approx. > 20); chickens ($n=7$); dogs ($n=3$); goats ($n=4$)
4	Complex of 5 houses, 3 guinea fowl and 2 cats present, guinea fowl nested in nearby outbuilding, cats roamed freely	Humans ($n=$approx. > 30); guinea fowl ($n=3$); cats ($n=2$)
5	Complex of 8 houses, no fixed animal housing	Humans ($n=$approx. > 45)
6	Dogo village, and the largest density of households; one small chicken coop but no other animal holdings, no dogs or cats seen	Humans ($n=$approx. > 85); chickens ($n=3$)

Fig. 1 Map of collection site and host species present at each transect point (transect 250 m in total) taken from Google Earth Pro

format. Once homogenised, DNA was then extracted using the Qiagen DNeasy 96 kits (Qiagen) following manufacturer's protocol. Blood meals preserved on FTA cards were punched out using a sterile steel 4 mm radius punch. Resulting punches were incubated in ATL buffer and Proteinase K for 6 h before DNA extraction was performed following manufacturer's protocol. Extracted DNA was stored at -20 °C until analysed.

Mosquito species identification

Mosquito species identification was initiated using a real-time multiplex PCR assay targeting the rRNA gene [12]. Standard forward and reverse primers were used in conjunction with two species-specific Taqman probes. The reaction conditions were as follows: a 12.5 µl reaction containing 1 µl of genomic DNA. 6.25 µl of Quantinova (Qiagen) probe master mix, 800 nM of forward and reverse primers (Thermo Fischer Scientific, East Grinstead, UK), 200 nM of *An. arabiensis* probe (Sigma-Aldrich, Gillingham, UK) and 80 nM of *An. gambiae* probe (Applied Biosystems, UK). Samples were run on a Stratagene MX3005P (Agilent Technologies, Santa Clara, USA) using cycling conditions of 10 min at 95 °C, followed by 40 cycles of 95 °C for 25 s and 66 °C for 60 s. The increases in fluorescence were monitored in real time by acquiring at the end of each cycle.

To differentiate between *An. coluzzii* and *An. gambiae* within the *An. gambiae* species complex, a single end-point PCR was performed. This PCR targets the SINE200 retrotransposon and utilising an insertion in this area allows the two species to be distinguished following gel visualisation [13]. *Anopheles coluzzii* produces a band at 479 bp with *An. gambiae* producing a band at 249 bp. Reaction was as follows: a 25 µl reaction containing 0.5 mM of forward (5′-TCG CCT TAG ACC TTG CGT TA-3′) and reverse (5′-CGC TTC AAG AAT TCG AGA TAC-3′) primers, 12.5 µl of Hot start Taq polymerase (New England Biolabs, Ipswich, UK), 9.5 µl of nuclease-free water and 2 µl of template DNA. Cycling conditions were as follows: 10 min at 94 °C followed by 35 cycles of 94 °C for 30 s, 54 °C for 30 s, 72 °C for 60 s, and a final elongation step of 72 °C for 10 min.

PCR products were visualised on a 2% agarose gel using an Egel E-Gel iBase Power System and E-Gel Safe Imager Real-Time Transilluminator (Invitrogen, East Grinstead, UK). The assay was performed on 10% of all samples identified as *An. gambiae* from the first assay with corresponding controls. Samples producing unknown or inconclusive results were sequenced (ITS2 Sanger sequencing) using primers originally developed by Beebe & Saul [19] and sequences were used to perform nucleotide BLAST (NCBI) database queries. PCR reactions were performed on a T100 Thermal Cycler (Bio-Rad Laboratories, Watford, UK) and amplified gene fragments were visualized by electrophoresis on a 2% agarose gel using an E-gel E-Gel iBase Power System and E-Gel Safe Imager Real-Time Transilluminator (Invitrogen).

Blood-meal identification

Samples were initially screened using bovine and human specific primers developed by Gunathilaka et al. [20]. These primers were selected based on the abundance of available host species in the area. The reaction conditions consisted of a 10 µl reaction including 0.5 M of forward and reverse primers (Integrated DNA Technologies, Leuven, Belgium), 5 µl of SYBR green master mix (Roche, Welwyn Garden City, UK), 2 µl of nuclease-free water (Roche) and 2 µl of template DNA. PCR was run on a LightCycler 96 real-time PCR machine (Roche) under the following cycling conditions: pre-incubation of 95 °C for 600 s, 40 cycles of 95 °C for 10 s, 62 °C for 10 s and 72 °C for 30 s followed by a melting analysis.

Human-positive blood meals (including any potentially mixed feeds) from the above assay were confirmed using the Promega Plexor® HY Human DNA forensic detection kit (Promega, Southampton, UK). Assay was performed following manufacturer's protocol using a Stratagene MX3005P (Agilent Technologies, Santa Clara, USA) real-time PCR machine.

Laboratory assessment of blood-meal DNA degradation rate

Approximately 500 female *An. coluzzii* mosquitoes (N'gousso strain originally collected from Yaounde, Cameroon) were placed into an insect cage (Bugdorm, Watkins and Doncaster, UK) and, using a Hemotek, fed for 15 min on bovine blood collected from a UK based abattoir (First Line UK (Ltd), UK). Mosquitoes were reared at the London School of Hygiene & Tropical Medicine under standardized conditions in an incubator (27 °C and 70% humidity with a 12:12 light/dark photocycle) and given access to 10% sugar solution. Female mosquitoes were individually collected and checked for feeding status. Only overtly fully fed mosquitoes were selected for the experiment. Fully-fed females were separated into paper cups covered with netting; each cup contained a maximum of 30 female mosquitoes. Every 6 hours a single cup was removed and placed in a -80 °C freezer to kill the mosquitoes and stop blood-meal digestion. This was repeated until the mosquitoes had completely digested the blood-meal or were visually gravid. DNA was extracted using the above protocol from seven whole bodies for each time point. A 1:10 serial dilution of all time = 0 samples was used to generate a standard curve with dilutions being made down to 1×10^{-7}. The standard curve was used to assess assay sensitivity (limit of detection) with the resulting Ct values from each time point being used to estimate the time post-blood-feed for the field-caught mosquitoes. DNA from the blood meals from the field-caught mosquitoes was quantified using the same protocol. As larger female mosquitoes typically obtain a larger blood meal when feeding [21], we normalised for mosquito body size to account for the possibility that the different quantity of bovine DNA across the transect was due to mosquito size rather than time

post blood-meal. Ct values for bovine DNA were normalised against the Ct values for the corresponding host mosquito ribosomal DNA (rDNA) gene used for species identification, producing a ratio of bovine (*Bos taurus* mtDNA)-to-vector DNA (*An. coluzzii* rDNA). In this way, the quantity of bovine DNA measured for the timed experiments with colonised mosquitoes was used to estimate the time since last blood meal of the mosquitoes caught at the different transect points. In conjunction with the known distances between the hosts and the transect points, this estimated time since last blood meal informed the dispersal rate of the vectors.

Statistical analysis

All statistical analysis was performed using STATA and PRISM. Trends in blood indices across the transect were tested for the field-caught mosquitoes using a generalised linear model (glm) with a binomial function. Odds ratios were calculated for proportion of bovine or human fed mosquitoes across each collection night as a total of *An. coluzzii* collected and *P*-values ($P < 0.05$) were used to interpret any significant trends. Linear regression was performed to investigate the correlation between bovine Ct value and time post-feed recorded in the experiments with colony insects.

Results

A total of 318 blood-fed *Anopheles* mosquitoes were collected over a five-night period. Of these, 307 were identified as part of the *An. gambiae* species complex: 306 were identified as *An. coluzzii* using a combination of species-specific PCRs and Sanger sequencing of a fragment of the ITS2 region. The remaining insect was identified by ITS2 Sanger sequencing as *An. melas* and was excluded from the analysis (Table 2).

The dominant mosquito blood meal was of bovine origin with 73.5% of all meals being sourced from these hosts. Four (1.3%) individual mosquitoes were found to have solely fed on humans with an additional ten (3.3%) having a mixed feed of both bovine and human blood (Table 3). Figure 2 shows how the bovine blood index (BBI) varied significantly across the transect, indicating a decreasing trend with increasing distance from the cattle shed (OR = 0.60, 95% CI: 0.49–0.73, $P < 0.01$). The opposite trend was observed for human blood meals with the HBI increasing significantly towards the village (OR = 1.50, 95% CI: 1.05–2.16, $P = 0.028$).

Focusing on mosquitoes that had fed on cattle ($n = 227$), it was observed that the quantity of blood-host DNA extracted from mosquitoes varied across the transect with the average PCR cycle threshold (Ct) values for bovine blood detection being 20.72 (95% CI: 18.98–22.45) for mosquitoes caught by the cattle pen and 30.15 (23.14–37.16) for mosquitoes caught 250 m away ($P < 0.01$). As detection of rDNA is a proxy for total mosquito DNA extracted and therefore body size, we compared the ratios of bovine-to-vector DNA (*An. coluzzii* rDNA) across the transect to ensure different quantities of bovine DNA detected at different distances from the hosts was not due to mosquito size but rather time post-blood-meal. The correlation between Ct ratio and distance from cattle was retained ($t_{(225)} = -2.18$, $P = 0.03$).

The experimental time series was performed with a laboratory colony of *An. coluzzii* and producing mean Ct values for known time points post-blood-feeding. The time series showed Ct values increased with time

Table 2 Total number of blood-fed mosquitoes caught by species and transect point

	T1	T2	T3	T4	T5	T6	Total
An. coluzzii	17	153	72	26	22	16	306
An. melas	0	1	0	0	0	0	1
Other species	0	9	2	0	0	0	11
Total	17	163	74	26	22	16	318

Table 3 Total number of *An. coluzzii* mosquitoes collected by blood-meal source and transect point

Host source	T1	T2	T3	T4	T5	T6	Total	%
Bovine fed	15	138	39	12	18	5	227	74.18
Confirmed human feds	0	1	0	1	1	1	4	1.31
Mixed human/bovine	0	5	2	1	0	2	10	3.27
Unknown	2	9	31	12	3	8	65	21.24
Total caught	17	153	72	26	22	16	306	100

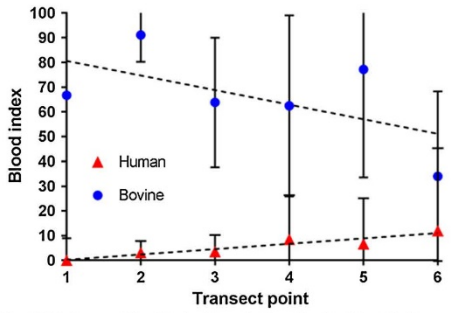

Fig. 2 The human blood index (triangles) and bovine blood index (circles) for each transect point (T1-T6), along with 95% confidence intervals, for all blood-fed *An. coluzzii* mosquitoes collected

post-feed ($P<0.01$, see Fig. 3) with no bovine DNA detected after the 60-hour time point. Regression analysis showed a positive correlation between bovine Ct value and time post-feed in the experimental time series ($R^2=0.92$, slope = 0.183; see Fig. 3). Calibrating the blood-meals of field-caught mosquitoes using the timed experiment with our mosquito colony, the dispersal rate of *An. coluzzii* could then be extrapolated: within 7 hours of feeding, mosquitoes typically remained within 50 m of their blood-host but at 60 hours had dispersed up to 250 m (Fig. 3).

Discussion

Evidence for the influence of local host availability on blood-host selection was demonstrated through analysis of the blood meals of *An. coluzzii* caught from the field using a novel sampling strategy. Previous investigations of HBI in field settings have demonstrated its large variability across and within species [2]. The aim of the present study was to investigate, for the same mosquito population, what level of variability can be expected, and, to determine the spatial scale that this choice can vary.

Here, a relatively low-cost (the chief expense being the PCR for blood-host species identification) and simple experimental setup for investigating host choice in the field is described. It was demonstrated that local host availability plays a crucial role in the host choice of a major malaria vector. Moreover, the remarkably small spatial scale (~250 m) at which this behaviour can be significantly impacted is demonstrated for the first time.

Results could have significant implications for vector control. For example, field studies involving endectocidal applications on livestock have shown encouraging results in terms of long-lasting mosquitocidal effects [22, 23]. However, previously this strategy has only been considered for targeting malaria vector species traditionally viewed as zoophilic (e.g. *An. arabiensis*). Recently, this assumption has been challenged by the demonstration that even the most anthropophagic populations of vectors readily bite non-human hosts, and that the methods for assessing host choice exclusively from mosquitoes

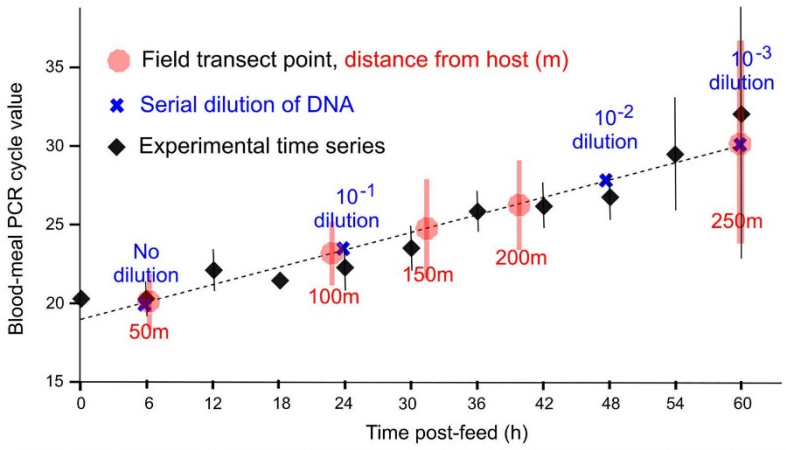

Fig. 3 Effect of time post blood-meal on mean bovine Ct values produced from qPCR. Shown are the means (bars indicate 95% CIs) of experimental time series (black), the serial dilution Ct values to assess assay sensitivity (blue), the mean (and 95% CI) Ct values of each transect point (red) and regression line used to predict time post feeding (dashed black line). Note that 'time post-feed' is from direct observation for colony mosquito blood-meal digestions (black) but is then extrapolated to the estimated time post-feed for field-caught mosquitoes

caught in human habitation may suffer from systematic bias [9]. Therefore, one way by which the current study adds to the discussion of optimal vector control strategy is through the provision of a simple method for assessing the degree to which anthropophagy varies for a given mosquito population. This can then be used to inform strategies for improved targeting of different control methods such as endectocides. Coupled entomological-epidemiological modelling frameworks already exist for using these data to inform projections of this novel vector control [24], including its use as part of an integrated vector management programme [25].

Linking the quantity of host-blood DNA isolated from mosquitoes caught at known distances from the specific host species with timed blood-meal digestion assays conducted on colonised mosquitoes presents a novel method for informing dispersal rates of mosquito populations. Dispersal is recognised to underlie mosquito population structure [17] as well as human exposure to transmission [26] and our ability to control transmission [27]. Yet, knowledge of this critical aspect of behaviour has been hampered by our inability to produce reliable estimates of vector dispersal in the field. To our knowledge, this study provides the first estimates using a non-intrusive and easily repeatable method for measuring malaria vector dispersal that informs the mosquito's dispersal rate across its gonotrophic cycle (approximately 2.5 days). However, it is important to address some of the present study's limitations and to identify some areas of future development of this approach.

First, in this study the numbers of mosquitoes captured nearby humans was low compared to those caught adjacent to cattle. That said, only 5 nights of mosquito captures were needed in order for a statistically significant trend to be identified for host choice across the transect. In the future, increasing the duration of the experiment would improve its ability to inform the likely shape of dispersal (e.g. leptokurtic *versus* Gaussian), something that could not be achieved with the present study.

Secondly, in order to estimate distances from blood-hosts these hosts must remain spatially confined. While this was possible in the present study because cattle were confined to their holding pen, this may require experimental adaptations for other types of environment. It must be made clear that this experiment in this particular field site was not intended to inform *An. coluzzii* dispersal rates everywhere that this vector can be found. Rather, the aim of this study was to present a new method for measuring dispersal that can be adapted to other settings to inform local mosquito behaviour. For example, tethering an animal species not otherwise found in the vicinity of a field site, followed by identifying its DNA from blood-fed mosquitoes caught nearby is one such setup that requires future investigation.

Thirdly, blood-meal digestion levels of field-caught mosquitoes were calibrated with colonised mosquitoes. Here multiple differences can occur: colony fed mosquitoes are reared at controlled densities, temperature and humidity and are able to take a full blood meal without encountering any defensive behaviour from hosts. These are of stark difference to what blood-fed mosquitoes may encounter in the field. A realistic temperature/humidity regimen that better emulates natural diurnal patterns has been shown to significantly impact various aspects of mosquito metabolism [23]; and, artificially controlling larval density can produce mosquitoes of similar size and fitness, something which may not be comparable to the field. Future experiments to ascertain the influence that these factors may have on blood-meal digestion would constitute an important next step.

Conclusions

Results presented in this study provide new insight into fundamental aspects of malaria vectors with important implications for malaria control strategy. Additionally, the novel experimental design presented offers a new methodology in measuring dispersal that with further development could be broadly applicable to other field-caught blood-feeding disease vectors.

Abbreviations

HBI: human blood index; BBI: bovine blood index; Ct: cycle threshold; OR: odds ratio; CDC: centers for disease control and prevention.

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Authors' contributions

LY and JO conceived the study. JO, ARM and YAA performed the field work. JO, CLJ, MK and TW performed laboratory analyses. All authors contributed to experimental designs, results interpretation and manuscript drafting. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Study protocol and all relevant documentation was reviewed by the LSHTM Ethics Committee before the study commenced. In country ethical approval was also obtained by YAA from the University of Ghana's Ethics committee.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Publisher's Note

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Supplementary information

Code used for stats analysis

Import excel “H:\My Documents\PhD work\Lab work\2017\MASTER database Dogo”

identify effect of variables on proportion of human fed *An.coluzzii*

glm pH Transect Night Windspeed, family(binomial mos)

identify effect of variables on proportion of bovine fed *An.coluzzii*

glm pB Transect Night Windspeed, family(binomial mos)

non-significant variables removed from glm

effect of transect on proportion of human fed *An.coluzzii*

glm pH Transect, family(binomial mos)

generate odds ratios

glm pH Transect, family(binomial mos) eform

effect of transect on proportion of boivne fed *An.coluzzii*

glm pB Transect, family(binomial mos)

generate odds ratios

glm pB Transect, family(binomial mos) eform

Field site 1 Data: Obama

The first field site (description in Chapter 2) visited to pilot the transect design. A total of five trap nights were run with one night excluded due to heavy rain and wind. Mosquitoes were caught at both ends of the transect however points in the middle of the transect (T2, 3 and 4) caught low numbers, with transect point 3 failing to catch any blood fed mosquitoes across any collection night. The overall abundance of blood fed *Anopheles* mosquitoes was also low (n= 47), transect point 1 (the cattle ranch) collected over 70% (n= 34) of blood feds (Table S1).

Table S4.1. Total number of blood fed *Anopheles* species caught over 4 nights at site 1 (Obama).

Transect	**Night**				
	1	**2**	**3**	**4**	**Total**
1	13	9	1	11	**34**
2	0	0	0	0	**0**
3	0	0	0	0	**0**
4	8	0	0	2	**10**
5	0	2	0	0	**2**
6	0	1	0	0	**1**
				Total	**47**

The majority of blood fed mosquitoes caught at Obama were of the *Culex* species (Table S2 and S4). However, a similar trend was seen with over 90% of blood fed mosquitoes collected from transect point 1 (Table S2). There were low numbers collected at transect points 2 and 4 with zero collected at transect point 3 over the 4 collection nights.

Table S4.2. Total number of blood fed *Culex* species caught over 4 nights at site 1 (Obama)

Transect	Night				
	1	**2**	**3**	**4**	**Total**
1	201	110	65	208	**584**
2	0	0	10	2	**12**
3	0	0	0	0	**0**
4	0	0	0	2	**2**
5	4	2	1	26	**33**
6	1	1	0	2	**4**
				Total	**635**

Table S4.3. Total number of unfed *Anopheles* mosquitoes caught at site 1 (Obama)

Transect	Night				
	1	**2**	**3**	**4**	**Total**
1	1	0	0	5	**6**
2	0	0	0	2	**2**
3	0	0	1	1	**2**
4	3	0	0	0	**3**
5	1	1	1	0	**3**
6	0	0	1	0	**1**
				Total	**17**

Table S4.4. Total number of unfed *Culex* mosquitoes caught at site 1 (Obama)

Transect	Night				
	1	**2**	**3**	**4**	**Total**
1	5	0	62	8	**75**
2	2	33	33	98	**166**
3	7	3	46	36	**92**
4	42	9	20	40	**111**
5	18	13	21	64	**116**
6	18	12	13	10	**53**
				Total	**613**

Due to the low abundance of both unfed (Table S3) and blood fed (Table S1) *Anopheles* mosquitoes collected at this field site as well as a number of the transect points not collecting any mosquitoes at all it was decided that this site was unsuitable and the samples collected were not analysed in the lab.

Field site 2 Data: Dogo

This location produced significantly higher numbers with blood fed *Anopheles* being collected across the whole transect (Data presented in the manuscript, field site description in Chapter 2). Additional data from the field collection which is not included in the manuscript is shown below. A total of 432 unfed *Anopheles* were collected over the five collection nights with numbers being collected across the whole transect. Blood fed *Culex* mosquitoes were also collected at all transect points at this site with a total of 429 and 2,445 unfed *Culex* species were collected across the 5 nights of capture.

Table S4.5. Total number of unfed *Anopheles* mosquitoes caught at site 2 (Dogo)

Transect	Night					
	1	**2**	**3**	**4**	**5**	**Total**
1	0	8	12	23	3	**46**
2	63	6	27	19	26	**141**
3	14	37	29	18	36	**134**
4	5	16	16	7	9	**53**
5	0	9	15	3	5	**32**
6	0	3	13	3	7	**26**
					Total	**432**

Table S4.6. Total number of blood fed *Culex* mosquitoes caught at site 2 (Dogo)

Transect	Night					
	1	**2**	**3**	**4**	**5**	**Total**
1	17	8	41	22	3	**91**
2	20	26	49	82	11	**188**
3	15	44	16	5	6	**86**
4	4	10	7	6	3	**30**
5	0	0	6	9	2	**17**
6	3	6	1	3	4	**17**
					Total	**429**

Table S4.7. Total number of unfed *Culex* mosquitoes caught at site 2 (Dogo)

Transect	Night					
	1	**2**	**3**	**4**	**5**	**Total**
1	98	13	114	120	27	**372**
2	47	53	68	125	13	**306**
3	164	261	211	85	99	**820**
4	102	81	78	57	54	**372**
5	91	50	72	40	45	**298**
6	21	82	59	70	45	**277**
					Total	**2445**

Chapter 5: Using visual and molecular methodologies to investigate blood meal digestion and estimate post-feeding time for four major vectors of mosquito-borne disease; *Anopheles coluzzii*, *Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefasciatus*.

Abstract

Introduction: The rate of blood meal digestion directly influences the gonotrophic cycle, biting frequency, vectorial capacity as well as mosquito resting and dispersal behaviour. Here, the blood meal digestion of four major vectors of mosquito borne diseases was assessed in the laboratory under controlled conditions using two different approaches.

Methods: *Anopheles coluzzii, Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefasciatus* were fed on bovine blood and, every 6 hours from 0 - 72 hours, a subset was removed, killed and preserved. Mosquito blood meal digestion was first scored using the Sella score (by a microscopist blinded to the times post-meal) and then quantified using qPCR.

Results: Significant differences between species were found when measuring digestion using qPCR ($p < 0.001$). Culicine species demonstrated linear digestion of host blood but the Anopheline species demonstrated a digestion process that diverged significantly from linearity. Both methodologies used to asses digestion estimated time post-feed more-or-less equivalently well. Although, neither method provided reliable estimates for the first 12h post-feed. The molecular methodology generally under estimated post-feeding time after 54h and could not predict post-feeding time after 66 hours for any of the vector species.

Conclusion: These two methodologies have been shown to accurately estimate post-feeding times albeit with some limitations. More refinements are required of the

molecular approach to improve its reliability in estimating time post meal immediately after the blood meal is taken, as well as to the end of the mosquito's gonotrophic cycle and beyond.

Aim

Under laboratory conditions, investigate species specific blood meal digestion and test the ability for PCR and the Sella score to accurately predict time post-feed for four major mosquito vectors.

Objectives

1. Generate a time series of blood meal digestion from 0 – 72 h for each of the four mosquito species (*Anopheles coluzzii*, *Anopheles stephensi*, *Culex quinquefasciatus* and *Aedes aegypti*.), using bovine blood.

2. Sella score each individual mosquito for each time point within the series and extract DNA from each individual to run bovine specific qPCR to generate Ct values.

3. Identify the shape of blood meal digestion (linear or otherwise) for each species using curve fitting statistics.

4. Using this fitted model, predict the time post-feed for each species for both methods.

5. Compare and contrast these methods for predicting the time since a blood meal was taken.

RESEARCH PAPER COVER SHEET

PLEASE NOTE THAT A COVER SHEET MUST BE COMPLETED FOR EACH RESEARCH PAPER INCLUDED IN A THESIS.

SECTION A – Student Details

Student	James Orsborne
Principal Supervisor	Laith Yakob
Thesis Title	Mosquito (Diptera: Culicidae) biting behaviour and malaria transmission: interactions between intrinsic host preferences and local host availability

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?			
When was the work published?			
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion	N/A		
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SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	TBD
Please list the paper's authors in the intended authorship order:	James Orsborne, Mojca Kristan, Claire L. Jeffries, Thomas Walker, Laith Yakob
Stage of publication	**Not yet submitted**

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	JO performed all labwork and analysis. JO drafted the manuscript with LY and TW prior to submission

Student Signature: [redacted] **Date:** 5/12/2019

Supervisor Signature: [redacted] **Date:** 5/12/2019

Using visual and molecular methodologies to investigate blood meal digestion and estimate post-feeding time for four major vectors of mosquito-borne disease; Anopheles *coluzzii*, *Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefasciatus*.

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Abstract

Introduction: The rate of blood meal digestion directly influences the gonotrophic cycle, biting frequency, vectorial capacity as well as mosquito resting and dispersal . Here, the blood meal digestion of four major vectors of mosquito borne diseases was assessed in the laboratory under controlled conditions using two different approaches.

Methods: *Anopheles coluzzii, Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefasciatus* were fed on bovine blood and, every 6 hours from 0 - 72 h, a subset was removed, killed and preserved. Mosquito blood meal digestion was first scored using the Sella score, by a microscopist blinded to the times post-meal and then quantified using qPCR.

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Conclusion: The two methodologies (visual and molecular) have been shown to accurately estimate post-feeding times albeit with some limitations. More refinements are required of the molecular approach to improve its reliability in estimating time post meal immediately after the blood meal is taken, as well as to the end of the mosquito's gonotrophic cycle and beyond.

Introduction

Blood is a vital resource for all female anautogenous mosquitoes (1). To obtain this critical resource, female mosquitoes must go through the process of locating and taking a blood meal from a vertebrate host (1-7). Ingestion of blood by mated females provides key nutrients for supporting egg development. Once an egg batch has developed, the female will locate a suitable oviposition site before seeking another blood meal. This cycle, the gonotrophic cycle, first described by Beklemishev (8), can be described as the time between two consecutive blood meals for an individual mosquito (9). Therefore the rate at which the gonotrophic cycle is completed is a key determinant of the frequency of bites on the host population (10), and directly informs vectorial capacity (11) - a key parameter in mosquito borne disease transmission models (12). Many mosquito species are known to exhibit gonotrophic discordance (7, 13-15), with multiple feeding bouts per egg batch reported in the range 5% - 55% depending on species, location and climate (14, 16-24). Despite this, there is a strong correlation between biting rate and gonotrophy (9).

A key part of the gonotrophic cycle is the time taken to digest the blood meal. Once ingested, the blood meal is digested by various enzymes including trypsin, aminopeptidases and nucleases, during which DNA is fragmented (25, 26) and haemoglobin is broken down into key components for egg development (1, 27). Blood meal digestion is a complex physiological process (1, 28, 29) that is affected by mosquito species (30), initial blood meal size (31), blood meal source (32) and mosquito age (33). Gonotrophy is also known to be highly sensitive to temperature (2, 9, 34). For example, the time to reach maximum proteolytic activity was shown to halve when blood fed *Ae. aegypti* were kept at 32^{o}C compared to 22^{o}C (1). *An. maculipennis* also demonstrates a significantly shorter digestion period (73 hours vs 87 hours) with a temperature increase of 6^{o}C (2); and fluctuations in daily temperature

have shown to significantly affect various mosquito life history traits including gonotrophy under field conditions (35, 36).

Blood meal digestion also has behavioural ramifications (37, 38). As a female mosquito can take up to twice their own bodyweight in a single blood meal (1, 39), flight range is usually highly compromised with energy being diverted to the digestion and subsequent development of eggs (37). As a result, it is assumed female mosquitoes rest close to their host after feeding (40) with flight range increasing with increased digestion and the need to find an oviposition site once gravid (41). This post-feeding behaviour of resting and subsequent dispersal has direct effects on mosquito population dynamics, human exposure to disease (42, 43) and therefore impacts strategies for control (44).

To date, many studies have investigated “who or what” various mosquito species have bitten. These endeavours have made substantial contributions to understanding several aspects of vector borne disease transmission: they have informed the Human Blood Index (HBI) as well as inferred host preference, biting behaviour, and aided in incriminating potential vectors of human disease (45-49). However, investigations of the time elapsed since a blood meal was taken are scant, limiting understanding of post-feeding behaviour of even the vectors of greatest public health significance.

The ability to measuring blood meal digestion and estimate when it was taken has both ecological and epidemiological applications. Post-feeding behaviours of mosquito populations require investigation both spatially and temporally. Mosquito dispersal plays a significant role in mosquito population dynamics and post-feeding dispersal in particular is critical to human exposure to disease (42-44, 50). Yet dispersal research has been hampered through over-reliance on mark-release-recapture which is marred by the unavoidable bias posed by very low recapture rates (51). Recently, a novel method for investigating the post-feeding behaviour of mosquitoes was described (52). This method used qPCR to quantify host DNA in *An.*

coluzzii caught up to 60 h post-feed i.e. long after the mosquito's post-meal resting period (52). Using a transect of traps at varying distances from the hosts, and, correlating this distance with the level of digestion of the blood meal presented a new approach for investigating dispersal (52). We take this research further by investigating the ability of qPCR to accurately estimate time post-feed for four major vectors of human diseases – two anophelines and two culicines. Feasibility is then assessed for simplifying this new method of measuring dispersal by replacing qPCR with a visual, morphological assessment of blood meal digestion (Known as Sella staging (2)) for more rapidly and cheaply estimating time post meal. It is hoped that this new investigation will broaden the applicability of our novel method for measuring dispersal beyond malaria vectors and beyond research teams with access to high-end, molecular biology equipment.

Methods

Blood meal digestion time series

Approximately 500 female *An. coluzzii* (N'gousso strain), *An. stephensi (Sk Strain), Ae. aegypti (LSHTM reference strain,* originally from West Africa*)* and *Cx. quinquefasciatus (TPRI strain)* mosquitoes were placed into separate insect cages (Bugdorm, Watkins and Doncaster, UK) and fed for 15 minutes on bovine blood collected from a UK based abattoir (First Line UK (Ltd), UK) using a Hemotek (Hemotek, UK) membrane feeder. Mosquitoes were reared and kept at the London School of Hygiene & Tropical Medicine under standardized conditions in an incubator (27^{o}C ± 0.2 ^{o}C and 70% ± 3% humidity with a 12:12 light/dark cycle). Female mosquitoes were individually collected and checked for feeding status with only fully fed mosquitoes selected for the time series experiment. Females were separated into paper cups covered with netting, with each cup containing approximately 30 female mosquitoes. Mosquitoes were given access to 10% sugar solution and every 6 h a single cup for each species was removed and placed in a - 80^{o}C freezer to kill the mosquitoes and stop blood-meal digestion. This was repeated until 72 h or the mosquitoes had completely digested the blood meal and appeared gravid or blood meal reabsorption had occurred.

Sella scoring

Each sample was Sella scored morphologically prior to extraction using the original Sella scoring criteria (2) . A single individual who has considerable prior experience with this technique performed the scoring while being blinded to the times post-meal of the mosquito batches. The score ranges from I to VII with I representing a non-fed mosquito, II representing a freshly fed mosquito and VI representing a fully gravid mosquito (Figure 2).

DNA extraction

Mosquito whole bodies were extracted individually. Samples were homogenised using a Qiagen TissueLyser II (Qiagen, UK) with a 5 mm stainless steel bead (Qiagen, UK). After which DNA was extracted using the Qiagen Dneasy individual extraction kits (Qiagen, UK) following manufacturer's protocol. Extracted DNA was stored at -20^{o}C until analysed.

Quantification of DNA

Total DNA was quantified for each mosquito DNA extract using a Qubit 4 fluorometer. 2µl of each DNA extract was added to the Qubit reagents following manufactures protocol to create a 200µl sample. The samples were left at room temperate for three minutes to allow the fluorescence to develop and the Qubit 4 then generated a ng/µl reading.

Normalisation/standardisation of DNA samples

Mosquito body size is known to affect the size of blood meal obtained, with larger female mosquitoes typically obtaining a larger blood-meal when feeding (53). Therefore, all PCR reactions were standardized to a starting DNA concentration of 2 ng/µl. The standardization was performed by using the DNA concentration obtained from the Qubit fluorometer and diluting an aliquot of the sample down to 2 ng/ µl using nuclease free water. The newly standardized samples were stored at -20^{o}C until analysed.

Blood meal Quantification

Normalised samples were run in triplicate and bovine DNA amplified using species specific primers developed by Gunathilaka *et al* (54). The PCR reaction conditions

consisted of a 10 µl reaction including 0.5 M of forward and reverse primers (Integrated DNA Technologies), 5 µl of SYBR green master mix (Roche, UK), 2 µl of nuclease-free water (Roche, UK) and 2 µl of standardized template DNA. PCR reactions were run on a LightCycler 96 real-time PCR machine (Roche, UK) under the following cycling conditions: pre-incubation of 95^{o}C for 5 min followed by 40 cycles of 95^{o}C for 10 s, 62^{o}C for 10 s and 72^{o}C for 30 s followed by a melting analysis. Ct values were generated and averaged across triplicate by the Roche life cycler software.

Statistical Analysis

Curve fitting was initially performed in R studio and visualised using GraphPad PRISM. R^2 was used to compare model fits between linear, quadratic, cubic and quartic models for each species corresponding Ct values over time. An ANOVA was used to select the best-fit model that was then visualised. The analysis of correlation between Sella score and PCR technique was performed in STATA with post-feeding times predicted using the *predict* function. For anophelines, as a non-linear relationship between independent and predictor variables was shown, a non-parametric regression was performed. For the culicines, linear regression was performed. Predicted time for the Sella score for each species was generated using linear regression, the *margins* function was used to obtain predictions. Comparison between predicted time and actual time was performed using t-tests adjusted for multiple comparisons.

Results

Initial regression analysis demonstrated Ct values increase for all species as time post-feed increased ($p < 0.001$). Significant differences between species was also present ($p < 0.001$) and therefore each species was analysed individually. Across all species, replicate had no significant effect on Ct value ($p = 0.92$).

Due to there being a significant difference between species, the linearity of the blood digestion was tested for individual species. Comparisons of the fitted models showed for the two anopheline species, a second order polynomial model fitted the data significantly better than a linear model (*An. coluzzii*: p=0.03, R^2= 0.71 and *An. stephensi*: p< 0.001, R^2= 0.89) whereas none of the alternative model could significantly improve upon a linear model for either *Ae. aegypti* or *Cx. quinquefasciatus* (Table 1).

Table 1: ANOVA comparison table for model fitting of each mosquito species

An. coluzzii			***An. stephensi***			***Ae. aegypti***			***Cx. quinquefasciatus***		
Model	**p-value**	**Sig**	**Model**	**p-value**	**Sig**	**Model**	**p-value**	**Sig**	**Model**	**p-value**	**Sig**
Linear	Ref		**Linear**	Ref		**Linear**	**Ref**		**Linear**	**Ref**	
Quadratic	**0.03**	*	**Quadratic**	**< 0.001**	***	**Quadratic**	0.20		**Quadratic**	0.13	
Cubic	0.85		**Cubic**	0.63		**Cubic**	0.46		**Cubic**	0.25	
Quartic	0.12		**Quartic**	0.82		**Quartic**	0.23		**Quartic**	0.38	

The bloodmeal digestion curves were similar for the two anopheline species ($p = 0.82$) was shown at any time point. Time post-feed did not have a significant association with Ct values until after 12 hours post-feed (Figure 1). For the culicines, a significant association was found between Ct values and species and between Ct values and time post-feed (p<0.001). Analysing these species separately, time had a significant

association after 24 hours for *Ae. aegypti* and after 18 hours for *Cx. quinquefasciatus* (Figure 1).

When analysing the Sella score, time post-feed wasassociated with Sella score (p< 0.001) as did species (p = 0.014) and so species were analysed individually. For *Ae. aegypti,* time post-feed has a significant association with the Sella score after 12 hours. For *An. coluzzii* and *An. stephensi and Cx. quinquefasciatus* differences were undetectable until 18 hours after feeding.

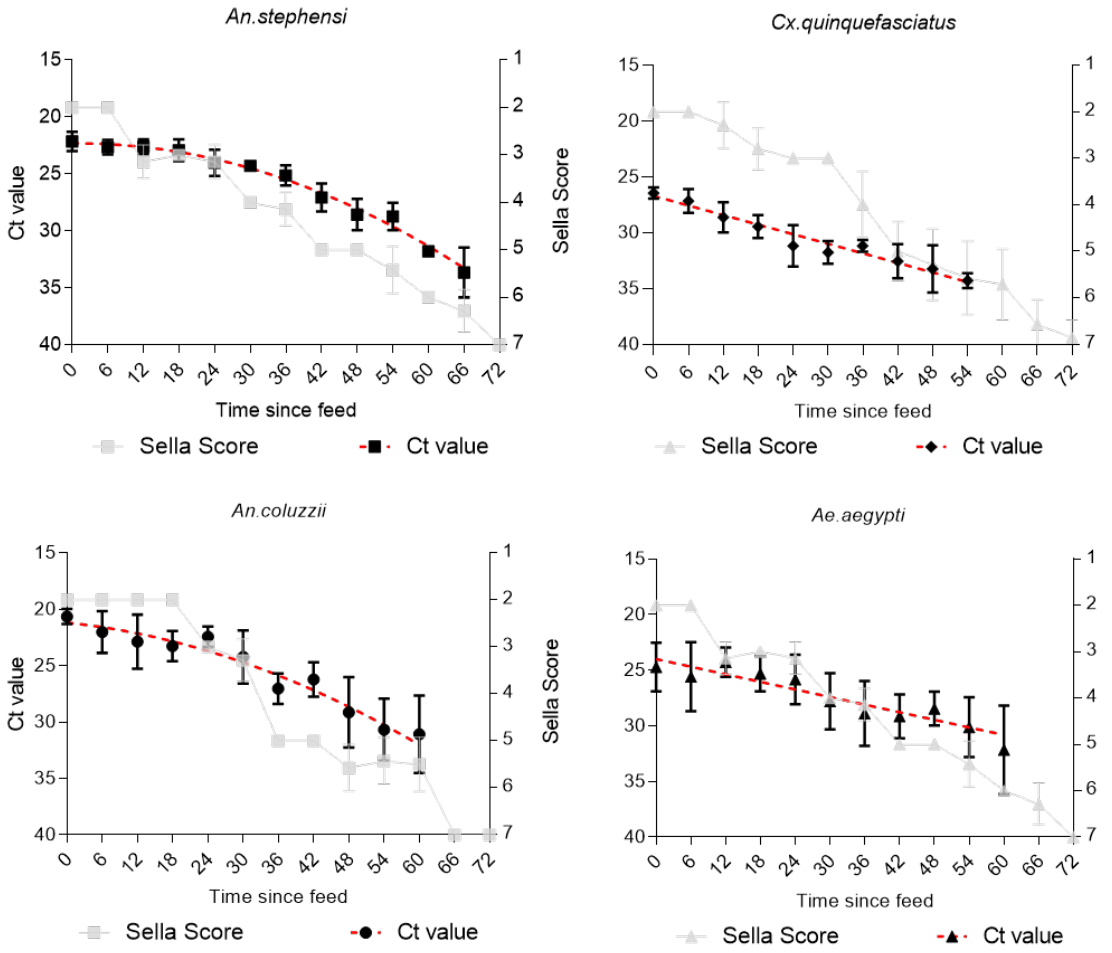

Figure 1: Blood meal digestion over time (in hours) demonstrated by Ct-values for *Anopheles coluzzii, An. stephensi, Ae. aegypti* and *Cx. quinquefasciatus.* Error bars represent 95% confidence intervals.

Predicting time post-feed with visual and molecular methodologies

Using the molecular methodology, actual time post-feed was accurately predicted from 6h to 60h post-feed for *An. coluzzii* with PCR limit of detection reached at 66h (Figure 2). For *An. stephensi,* PCR was unable to predict post-feeding time prior to 12h after feeding. After 12h, time post-feed was accurately predicted up to 60h. After this time, time post-feed was generally underestimated with limit of detection reached at 66h post-feeding (Figure 2). *Ae. aegypti* followed a similar trend to *An. coluzzii* with this methodology unable to accurately predict time post-feed for freshly fed mosquitoes (predicted time of 17h significantly diverged from the actual 0h (p=0.003). After 6h, PCR accurately predicted the time post-feed for *Ae. aegypti* for up to 48h. After 48h, time post-feed was generally under predicted with PCR unable to distinguish post-feeding time after 60 hours (Figure 2). For *Cx. quinquefasciatus*, PCR accurately estimated time post-feed from 0 to 48h (with minor discrepancy at the 36h time point). However, PCR predictions significantly diverged from actual time post-feed at the 54h time point (predicted time= 47h p=0.004) and were unable to predict time post-feed after the 54-hour time point (Figure 2).

For the Sella score, estimated time post-feed was generated for each Sella stage and for each species (Table 2). No significant differences were found between species at any time point (p >0.05) for these estimations. The Sella accurately determined time post-feed across all species with a few exceptions: time point 12h for *Ae. aegypti* was over predicted (p =0.005) and time point 72h was under predicted (Estimated time 65h, p=<0.001).

Table 2: Predicted time post-feed using Sella Score method to the nearest hour with 95% CIs

	Predicted time in hours (95% CIs)					
Species	**2**	**3**	**4**	**5**	**6**	**7**
An. coluzzii	9 (7 - 11)	27 (23 - 30)	30 (21 -39)	46 (43 - 48)	53 (49 - 57)	69 (66 - 72)
An. stephensi	9 (6 - 11)	26 (23 - 29)	37 (35 - 40)	51 (47 - 54)	61 (58 - 65)	69 (65 - 72)
Ae. aegypti	3 (0 - 6)	18 (16 - 20)	31 (28 - 33)	46 (44 -49)	61 (58 - 63)	71 (68 - 74)
Cx. quinquefasciatus	6 (3 - 9)	24 (20 - 27)	38 (31 - 44)	49 (46 - 53)	60 (56 - 64)	66 (62 - 70)

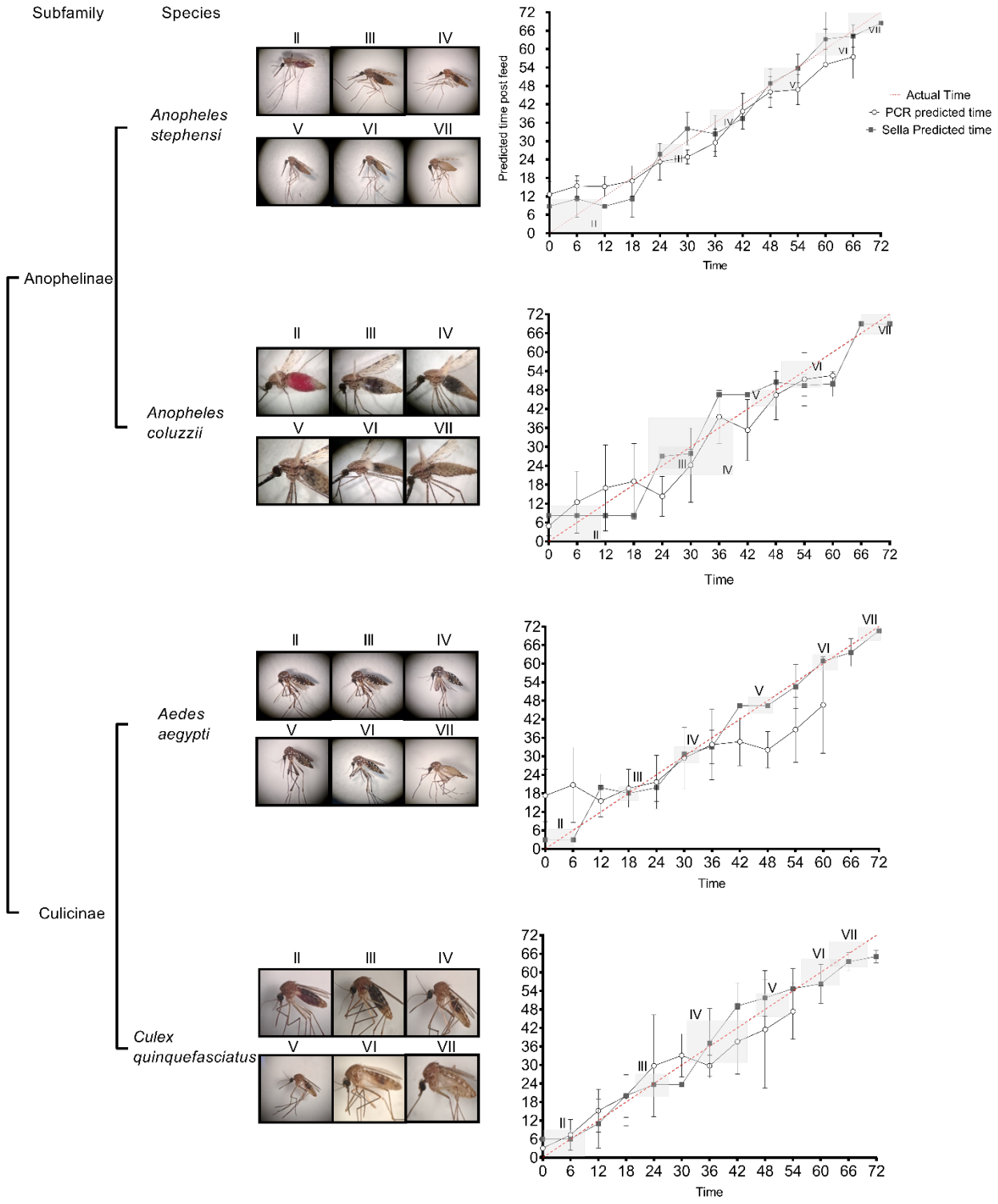

Figure 2: Sella Scores are imaged for each of the species. Predicted time post-feed for the four species for both molecular (PCR, white circles) and visual (Sella score, black squares) methodologies. Grey boxes represent the predicted time range for each Sella score. Red dotted line demonstrates the trend if predicted time post-feed and actual time were equal.

Discussion

Comparison of two different methods of measuring blood meal digestion (molecular and visual) has shown both to have a good fit and predictive performance for the actual time post-feed across four major mosquito vectors.

Differences between the anopheline and culicine subfamilies during the blood meal digestion process were detected: whereas qPCR Ct values for the degradation of host DNA had a linear relationship with time for the culicines, anophelines showed an initially delayed degradation in host DNA resulting in a curvilinear relationship. This difference between subfamilies is likely explained by their physiological differences in blood meal digestion. *Anopheles* mosquitoes undergo diuresis while feeding (expelling excess fluid while actively feeding) resulting in a blood meal with a high density of enterocytes and other blood components (1, 55). This allows them to significantly increase their protein intake while feeding. A consequence of this is that the blood meal contains a higher density of host mDNA - the target for the PCR assay used. This is corroborated by the fact that the Ct values immediately following feeding (at time point 0) were consistently much lower (i.e. higher concentration of host DNA) for the anophelines than the culicines which undergo diuresis during and post blood meal (1).

The development of the peritrophic matrix (PM), a key organ required for the digestion of the blood meal is also a factor in the rate of blood meal digestion. The PM is considered essential for many functions including preventing local tissue damage, compartmentalization of the blood meal, excretion of digestive enzymes, and it acts as a physical barrier which can block pathogens (56, 57). The PM develops around the blood meal shortly after the feed has occurred and the rate at which it forms has been shown to vary across species (1). *Aedes. aegypti* has shown rapid development of the PM after feeding, being detectable after 4 - 8 h and fully developed 12-24 h after feeding (1, 58, 59). For *Cx. quinquefasciatus*, the PM forms in a similar

timeframe, approximately 18 h after feeding (60). Conversely, the PM of *An. gambiae* is only be detected 12 h after feeding and can take up to 48 h to fully develop (1). The differences in rates of PM development may explain why PCR and Sella score were unable to predict blood-feeding times for anophelines up to 24 h after feeding due to the natural lag between taking a blood meal and the physiological changes required for digestion.

The ability to predict when a blood meal was taken from a host allows for a better understanding of biting behaviour, host availability as well as post-feeding behaviour and dispersal. Previously we showed that quantifying host DNA from the blood meals of mosquitoes caught in the field at known distances from their hosts, and calibrating with timed blood-meal digestion of colonised mosquitoes, presents a novel, non-intrusive method for measuring dispersal (52). The work presented here further builds on these findings and shows this methodology is transferable across species and subfamilies of the most influential vectors of mosquito borne diseases. The use of this methodology in a semi-field setting where digestion rates could be assessed for wild mosquitoes exposed to more realistic environmental conditions constitutes an important next step for developing our method. Coupling this work with the recent ability to DNA finger print blood meals and match them to the exact human from which they were obtained would allow when, where and who the blood meal was taken from and this could be a powerful tool for better understanding local malaria transmission (64, 65).

While we must acknowledge the limitation of either proposed method in providing good estimates for blood meal digestion within the first 12h post-feed. Although, this period corresponds with when mosquitoes are most lethargic and least likely to disperse far from their hosts. The inability to distinguish the first 12 hours after feeding is noteworthy and future work to decipher if this is a sensitive issue with the methodologies or a physiological aspect which results in a delay in bloodmeal

digestion is needed. We must also highlight that a key factor which influences digestion was controlled in this study. Changes in temperature directly influences blood meal digestion rates and although the temperatures used in this study were realistic they were also static. In reality temperatures in the field vary considerably both across time, space and can even be affected by household structure (61). Future developmental work will focus on incorporating more realistic temperature regimes into the experimental design allowing the methodology to be more relatable to the field.

By knowing when a bloodmeal was taken and where vectors are dispersing to after they have blood-fed and rested will not just improve the understanding of this key aspect of vector ecology but also has clear public health connotations by better understanding mosquito population dynamics, human exposure to these populations (and the parasites they might be carrying) as well as the necessary measures required for their adequate control a vector population.

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All parties involved in the generation of data and this manuscript were worthy of authorship

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

LY and JO conceived the study. JO and MK reared mosquito colonies. JO, CLJ, MK and TW performed laboratory analyses. All authors contributed to experimental

designs, results interpretation and manuscript drafting. All authors read and approved the final manuscript prior to submission.

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Supplementary information

Code for statistics

Curve fitting for each species

```
rm(list = ls())
install.packages("ggplot2")
library(ggplot2)

#######Data sets#######
Data <-read.csv("Desktop/Master DataBase.csv")
Data
ColuzziiData <-subset(Data,Species=="An. coluzzii")
ColuzziiData
StephensiData <-subset(Data,Species=="An. stephensi")
StephensiData
AegyptiData <-subset(Data,Species=="Ae.aegypti")
AegyptiData

####### Curve fitting repeated for all species, example An. coluzzii #######
ColPlot <- ggplot(ColuzziiData, aes(ColuzziiData$Time, ColuzziiData$Ct.value))+
  geom_point(size=1) +
  geom_smooth(method = 'lm',formula = y ~ x)+
  geom_smooth(method = 'lm',formula = y ~ poly(x, 2))+
  geom_smooth(method = 'lm',formula = y ~ poly(x, 3))+
  geom_smooth(method = 'lm',formula = y ~ poly(x, 4))+
  stat_summary(fun.y = mean, fun.ymin = mean, fun.ymax = mean, geom =
"crossbar", color = "red", size = 0.4)+
  scale_x_discrete(limits=ColuzziiData$Time) + expand_limits(y=18)+
  scale_y_reverse()+
  theme_minimal()

ColPlot
```

```
cfit1<- lm(ColuzziiData$Ct.value~ColuzziiData$Time)
cfit2 <- lm(ColuzziiData$Ct.value~poly(ColuzziiData$Time,2,raw=TRUE))
cfit3 <- lm(ColuzziiData$Ct.value~poly(ColuzziiData$Time,3,raw=TRUE))
cfit4 <- lm(ColuzziiData$Ct.value~poly(ColuzziiData$Time,4,raw=TRUE))

summary(cfit1)
summary(cfit2)
summary(cfit3)
summary(cfit4)

AIC(cfit1)
AIC(cfit2)
AIC(cfit3)
AIC(cfit4)

install.packages("rcompanion")
library(rcompanion)
anova(cfit1,cfit2,cfit3,cfit4)
compareLM(cfit1,cfit2,cfit3,cfit4)
hist(residuals(cfit2))
plot(fitted(cfit2),residuals(cfit2))

ColPlotFinal <- ggplot(ColuzziiData, aes(ColuzziiData$Time,
ColuzziiData$Ct.value))+
  geom_point(size=1) +
  geom_smooth(method = 'lm',formula = y ~ poly(x, 2))+
  stat_summary(fun.y = mean, fun.ymin = mean, fun.ymax = mean, geom =
"crossbar", color = "red", size = 0.4)+
  scale_x_discrete(limits=ColuzziiData$Time) + expand_limits(y=18)+
  scale_y_reverse()+
  theme_minimal()+
  labs(title = "BLood meal digestion Time series",
                    subtitle = "An. coluzzii",
```

```
                x = "Time (h)",
                y = "Ct Value",
                colour = "Gears")
ColPlotFinal
```

####### Curve fitting repeated for all species using same code above #######

####### Generate plots #######

```
library(gridExtra)
grid.arrange(ColPlotFinal,StephPlotFinal,AegyptiPlotFinal)
```

####### STATA to predict times for PCR and Sella methods from models #######

*Anopheles* npregression (repeat for both *Anopheles* species) to predict time

```
import excel "H:\My Documents\PhD Work\Lab work\2019\Molecular Sella Score\Data\Master DataBase.xlsx", sheet("Anophs") firstrow clear
generate Ct2 = Ctvalue*Ctvalue
encode Species, generate(S)
```

compare regress to npregress using R-squared

```
regress Ctvalue Time S Replicate
regress Ctvalue Time
```

R-squared better for npregresss- as suggested by model fit in R

```
npregress kernel Ctvalue Time, vce(bootstrap, reps(100) seed(123))
```

predict time post feed for PCR

```
npregress kernel Time Ctvalue,vce(bootstrap, reps(100) seed(123))
.npgraph
```

predict pcrTime

Culex and Aedes regression (repeated for each species) to predict time post feed for PCR. Sella Score predicted time performed for all species

Test and explore

import excel "H:\bloodfeed.xlsx", sheet("Ae.aeg") firstrow

recode ctvaluepcr 0 = 40

scatter ctvaluepcr visualscore

scatter ctvaluepcr actualtime

scatter visualscore actualtime

corr ctvaluepcr visualscore

regress ctvaluepcr visualscore

* mixed ctvaluepcr visualscore || actualtime: , mle

predict pred_pcr1 , xb

predict res_pcr1, rstandard

qnorm res_pcr1

twoway scatter res_pcr1 pred_pcr1

estat hettest visualscore

######modelling time ######

Predict PCR time

regress actualtime ctvaluepcr

predict time_pcr1, xb

twoway (scatter actualtime ctvaluepcr) (line actualtime time_pcr1)

Predict Sella time

regress actualtime i.visualscore

predict time_visual1, xb

margins visualscore

twoway (scatter actualtime visualscore) (line time_visual1 visualscore)

Chapter 6 - Evidence of extrinsic factors dominating intrinsic blood host preferences of major African malaria vectors

Abstract

Introduction: One of the key determinants of a haematophagous vector's capacity to transmit pathogens is its selection of which host to secure a blood meal from. This choice is influenced by both intrinsic (genetic) and extrinsic (environmental) factors, but little is known of their relative contributions.

Methods: Blood fed *Anopheles* mosquitoes were collected from a malaria endemic village in Ghana. Collections were conducted across a range of different host availabilities and from both indoor and outdoor locations.

Results: These environmental factors were shown to impact dramatically the host choice of caught malaria vectors: mosquitoes caught indoors were ten-fold more likely to have sourced their blood meal from humans; and a halving in odds of being human-fed was found for mosquitoes caught only 25 m from the centre of the village. For the first time, we demonstrate that anthropophagy was better explained by extrinsic factors (namely, local host availability and indoor/outdoor trapping location) than intrinsic factors (namely, the (sibling) species of the mosquito caught) (respective Akaike information criterion estimates: 243.0 versus 359.8).

Conclusions: Instead of characterizing biting behaviour on a taxonomic level, we illustrate the importance of assessing local entomology. Accounting for this behavioural plasticity is important, both in terms of measuring effectiveness of control programmes and in informing optimal disease control strategies.

Aim

Developing on the findings from the systematic review and pilot study, investigate if local extrinsic factors have the potential to have a greater influence over blood host selection than mosquito taxa.

Objectives

1. Using the methodology piloted in Chapter 3, repeat this fieldwork over an extended period of time with the inclusion of indoor collections across the transect

2. Identify mosquito species of *Anopheles* mosquitoes collected from the field using morphological identification and molecular methods

3. Identify blood meal source of all blood fed *Anopheles* collected

4. Statistically analyse the effect of intrinsic (species) vs extrinsic (host availability and indoor or outdoor location) on the HBI and BBI.

5. Identify which factors (intrinsic or extrinsic) are driving host selection for major *Anopheles* species in this vector population

RESEARCH PAPER COVER SHEET

PLEASE NOTE THAT A COVER SHEET MUST BE COMPLETED FOR EACH RESEARCH PAPER INCLUDED IN A THESIS.

SECTION A – Student Details

Student	James Orsborne
Principal Supervisor	Dr Laith Yakob
Thesis Title	Mosquito (Diptera: Culicidae) biting behaviour and malaria transmission: interactions between intrinsic host preferences and local host availability

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?			
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If the work was published prior to registration for your research degree, give a brief rationale for its inclusion			
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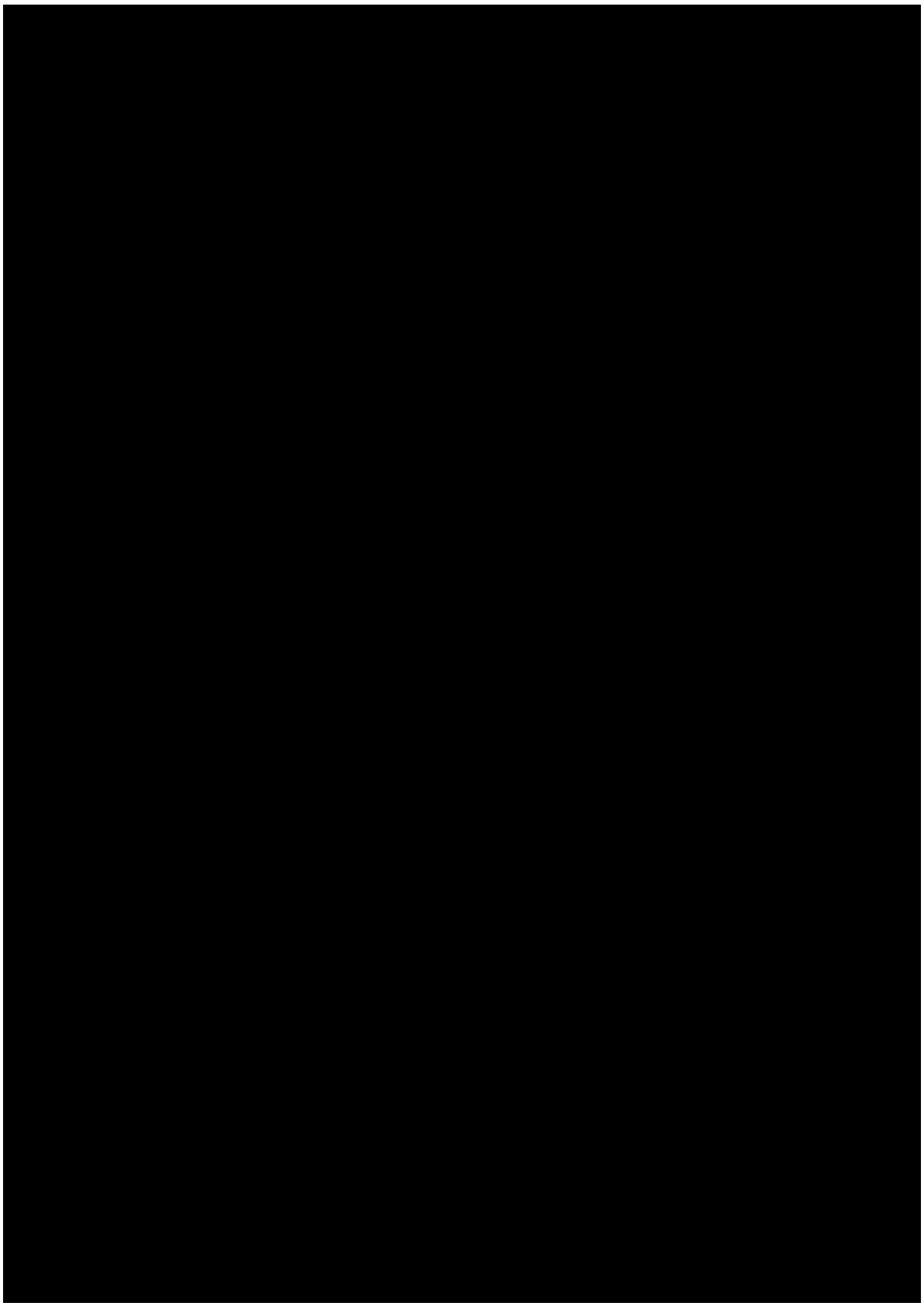
SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	Scientific Reports
Please list the paper's authors in the intended authorship order:	James Orsborne, Abdul Rahim Mohammed, Claire L. Jeffries, Mojca Kristan, Yaw A. Afrane, Thomas Walker and Laith Yakob
Stage of publication	**In press**

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	JO performed all fieldwork, lab work and data generation. JO also aided in drafting the manuscript prior to submission.

Student Signature: _____ **Date:** 5/12/2019



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Evidence of extrinsic factors dominating intrinsic blood host preferences of major African malaria vectors

James Orsborne[1], Abdul Rahim Mohammed[2], Claire L. Jeffries[1], Mojca Kristan[1], Yaw A. Afrane[2], Thomas Walker[1] & Laith Yakob[1*]

One of the key determinants of a haematophagous vector's capacity to transmit pathogens is its selection of which host to secure a blood meal from. This choice is influenced by both intrinsic (genetic) and extrinsic (environmental) factors, but little is known of their relative contributions. Blood fed *Anopheles* mosquitoes were collected from a malaria endemic village in Ghana. Collections were conducted across a range of different host availabilities and from both indoor and outdoor locations. These environmental factors were shown to impact dramatically the host choice of caught malaria vectors: mosquitoes caught indoors were ten-fold more likely to have sourced their blood meal from humans; and a halving in odds of being human-fed was found for mosquitoes caught only 25 m from the centre of the village. For the first time, we demonstrate that anthropophagy was better explained by extrinsic factors (namely, local host availability and indoor/outdoor trapping location) than intrinsic factors (namely, the (sibling) species of the mosquito caught) (respective Akaike information criterion estimates: 243.0 versus 359.8). Instead of characterizing biting behaviour on a taxonomic level, we illustrate the importance of assessing local entomology. Accounting for this behavioural plasticity is important, both in terms of measuring effectiveness of control programmes and in informing optimal disease control strategies.

Mosquitoes are responsible for the transmission of multiple human pathogens with malaria the most prominent of the resulting diseases. Malaria is transmitted by the bite of several *Anopheles* mosquito species when infected with *Plasmodium* parasites. As the successful transmission of the parasite requires two successful bites (one for the mosquito to become infected and one for the parasite to be transmitted to the human host), the mosquito's choice of which host species to bite has an exaggerated, non-linear impact on malaria transmission intensity[1]. The way in which host availability impacts the choice of host is largely unknown despite the dramatic impact it has on transmission and control of vector-borne diseases of diverse aetiology[2]. Similarly little is known of the short- and long-term impacts of different control methods on host choice.

The introduction of indoor residual spraying and insecticidal nets has resulted in a change in the biting behaviour of major malaria vectors with many mosquito species seeking a higher proportion of blood meals outdoors[3–6] and/or from non-human hosts[7–10]. Coupled with increasing rates of insecticide resistance[11], these behavioural changes indicate potential inadequacies of current leading vector-control tools to target outdoor biting and, correspondingly, sustained residual malaria transmission[12–19]. Therefore, understanding this behaviour is becoming increasingly important when implementing vector control strategies.

In the field, which host is ultimately bitten is a complex balance between intrinsic (genetic) and extrinsic (environmental) factors including behavioural conditioning driven by exposure to insecticides or previous successful feeds from a particular host[20,21]. The amalgamation and balance of these factors results in a significant level of host biting plasticty which can have considerable consequences for disease control[2]. One of the most popular methods of investigating host biting behaviour in the field is to collect blood fed mosquitoes and identify the blood meal source. The proportion (or percentage) of blood meals that are of human origin is referred to as

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Figure 1. Map of collection site and transect points (shown in red). Location of buildings and houses used for indoor sampling for each transect point are indicated by the blue squares.

the Human Blood Index (HBI)[22]. Malaria-transmitting mosquitoes are often described as primary or secondary vectors with this categorisation informed, in part, by their level of anthropophagy as indicated by their HBI. Owing to the influences of different environmental settings, however, studies reporting HBI for the same vector species demonstrate considerable variability[21,23–25]. We recently showed that the HBI of *Anopheles coluzzii* varied significantly over an extraordinarily small spatial scale, thus demonstrating how localised host biting behaviour can be[26]. Further, a recent systematic review indicated that the location a mosquito was caught (indoors versus outdoors) may have just as much influence, if not more influence, on host selection than the taxa collected[23]. However, these studies were performed by multiple researchers using a variety of methods on different mosquito populations, and spanned several decades. To the best of our knowledge, the relative influence of intrinsic and extrinsic factors on host selection has not been reported for any major disease vector.

In this study, multiple species of blood fed *Anopheles* mosquitoes were collected across a range of host availabilities, both from indoors and outdoors. Our hypothesis was that local environmental factors had a greater influence over blood host selection than the (sibling) species of the caught mosquitoes. We discuss the consequences of our findings to assessing, and perhaps even augmenting, future control strategies.

Methods

Study site and mosquito collection. Mosquitoes were collected from the village of Dogo, in the Greater Accra region of Ghana (05°52.418N, 00°33.607E) over 22 nights across June and July 2018 (Fig. 1). The village is on the south-eastern coast of Ghana, with a main rainy season from April to June and a shorter second rainy season in October. Temperatures were measured using a datalogger (EasyLog USB, UK) with windspeed recorded using an anemometer (Holdpeak, UK). Housing mostly consisted of concrete structures with concrete/brick walls and flooring, with predominantly iron roofs although some traditional mud style houses were also present. Several criteria were used to select the site: it had a defined area where cattle were kept and rested during the collection period on the outskirts of the village; the village had no other nearby cattle holdings; human housing density gradually increased towards the village centre and away from the cattle pen. Critical for this site being selected was that it offered both indoor and outdoor mosquito collection points within a full range of availabilities of alternative host species. Mosquito species known to be present in the area were primarily *An. gambiae* and *An. coluzzii* as well as *An.pharoensis* and *An. rufipes*. Due to the proximity to the sea, the potenital of *An. melas* being present was also noted and considered during molecular analysis.

Outdoor mosquito collections consisted of three outdoor trapping types: Centers for Disease Control and Prevention (CDC) resting traps (Bioquip products, CA, USA), BG Sentinel 2 traps (Biogents AG, Regensburg, Germany) and CDC light traps (Bioquip products, CA, USA). These traps were placed in clusters (<10 m apart) at 50 m intervals to form a 250 m transect comprising of six trapping points (Fig. 1). The transect began at an area of zero human population density (termed 'T1') outside of Dogo village and nearby the cattle resting and

Transect	Approximate number of hosts in area	Approximate number of indoor hosts
1	cows n = 150	n = 0, empty house used for sorting farming and cattle rearing equipment, open window frames and eves
2	cows n = 150 pigs n = 5	n = 0, empty cattle shed with open window frames but door closed to stop animals resting within
3	humans n = approx.' 20 chickens n = 7 dogs n = 3 goats n = 4	n = 6 permanent human inhabitants, dogs sometimes found in porch at night
4	humans n = approx.' 30 guinea fowl n = 3 cats n = 2	n = 7 permanent human inhabitants
5	humans n = approx.' 45	n = 6 permanent human inhabitants
6	humans n = approx.' 85 chickens n = 3	n = 9 permanent human inhabitants

Table 1. Approximate number of hosts across the transect.

overnight holding pen. The transect extended towards a human population ending at an area of high human density, the middle of Dogo village (denoted 'T6'). Details of hosts located at each transect point are described in Table 1. Mosquitoes were collected overnight from 6 pm to 6 am. Indoor collections were performed using a Prokopack aspirator (John.W.Hock, FL, USA) at two houses per transect point within the village (T3–T6) with each house being alternated for each collection night. Indoor collections at T1 and T2 were performed in out-buildings, which had no residents. Indoor collections were performed for 15 minutes in each building between 4 am and 6 am of each collection night. Bednets were absent from most households and only seen to be used sporadically in remaining households during sampling.

Ethical clearance. The study was reviewed and cleared by the London School of Hygiene & Tropical Medicine (LSHTM) ethics committee (LSHTM ethics reference: 15216). Ethical clearance was also obtained in country and cleared by the University of Ghana (UOG) ethics committee (University of Ghana, Noguchi Memorial Institute reference: DF22). Ethical clearance was obtained prior to any part of the study being performed. All methods in this study were performed in accordance with all relevant guidelines and regulations.

Recruitment of households for indoor collection. Households within 10 metres of each outdoor trap cluster were approached to be recruited into the study. Households were provided with a participant information sheet explaining the study and for those who could not read or speak English, a local translator was present. If the head of the household agreed to take part in the study, informed consent was provided via the signature of a consent form and the household was recruited into the study. All households were allowed to withdraw at any time during the study.

Morphological identification of mosquito species. *Anopheles pharoensis* and *An. rufipes* are morphologically distinguishable from the *An. gambiae* complex as well as from each other. Morphological Identification of these species was performed in the field prior to sample processing using a key developed by Gillies and Coetzee[27]. *An. gambiae* were identified only to the species complex until molecular analysis.

Sella staging of blood-fed mosquitoes. The Sella score is a visual measure of blood meal digestion and is comprised of seven stages. Stage two (II) is a freshly blood fed mosquito and stage seven (VII) is a fully gravid mosquito, stages between are defined by set criteria. Each blood fed mosquito in this study was assessed under light microscope and scored using the Sella score originally described by Detinova[28].

Sample storage. *Anopheles* mosquitoes collected from each transect point were immediately killed using chloroform to stop any active blood meal digestion. Mosquitoes where then sorted with all blood-fed females being processed first. All overtly blood-fed *Anopheles* mosquitoes were processed individually, noting their Sella score[28] (a visual measure of blood meal digestion), transect location, trap type, night collected and collection location (indoor or outdoor). All blood-fed mosquitoes were preserved in RNAlater® (Thermo Fisher Scientific, Life Technologies,UK) in a 96 well plate and stored at 4 °C and transported in this format back to the UK. All samples were stored at −70 °C once transported back to laboratories at the London School of Hygiene & Tropical Medicine.

DNA extraction. DNA was extracted from each *Anopheles* mosquito individually. Each sample was firstly homogenised using a Qiagen TissueLyser II (Qiagen, UK) with a 5 mm stainless steel bead (Qiagen, UK) and placed in each sample tube in a 96 well plate format (Qiagen UK). Once homogenised, DNA was extracted using the Qiagen DNeasy 96 kits (Qiagen, UK) following the manufacturer's protocol. Extracted DNA was stored at −20 °C until analysis was performed.

***An. gambiae* species complex identification.** Mosquito species identification first involved a real-time multiplex PCR assay targeting the rRNA gene developed by Bass *et al.*[29]. Standard forward and reverse primers

were used in conjunction with two species-specific Taqman© probes. The reaction conditions were as follows: a 12.5 µl reaction containing 1 µl of genomic DNA. 6.25 µl of QuantiNova (Qiagen, UK) probe master mix and 2.9 µl of DNA free H_2O. 800 nM of forward and reverse primers (Thermo Fischer Scientific, UK), 200 nM of *An. arabiensis* probe (Sigma-Aldrich, UK) and 80 nM of *An. gambiae* probe (Applied Biosystems, UK). Samples were run on a Stratagene MX3005P (Agilent Technologies, USA) using cycling conditions of 10 min at 95 °C, followed by 40 cycles of 95 °C for 25 s and 66 °C for 60 s. The increases in fluorescence were monitored in real time by acquiring at the end of each cycle. Analysis was carried out using the Stratagene MxPro software.

***An. coluzzii* and *An. gambiae* sensu stricto identification.** To differentiate between *An. coluzzii* and *An. gambiae* s.s. within the *An. gambiae* species complex, a single end-point PCR was performed. This PCR targets the SINE200 retrotransposon and utilising an insertion in this area allows the two species to be distinguished following gel visualisation[30]. *An. coluzzii* produces a band at 479 bp with *An. gambiae* producing a band at 249 bp. Reaction was as follows: a 25 µl reaction containing 0.5 mM of forward and reverse primers, 12.5 µl of Hot start Taq polymerase (New England Biolabs NEB, UK), 9.5 µl of nuclease-free water and 2 µl of template DNA. Cycling conditions were as follows: 10 min at 94 °C followed by 35 cycles of 94 °C for 30 s, 54 °C for 30 s, 72 °C for 60 s, a final elongation step of 72 °C for 10 minutes finished the cycling program.

***An. melas* identification.** *An. melas* has been found in coastal areas of Ghana. With the study site close to the sea and <5 km from saltwater lagoons, an endpoint assay developed by Scott *et al.*[31] was used to identify this *An. gambiae* sibling species. The PCR targets the ribosomal rDNA gene and used a universal forward primer with species-specific reverse primers to produce different product sizes allowing species to be identified. The product size was 464 bp for *An. melas*. Reaction volume consisted of 10 µl of Hot start Taq polymerase (New England Biolabs NEB, UK), 2 µl of universal forward primer (10 µM) 2 µl of *An. melas* specific reverse primer (10 µM), 4 µl of DNA/RNA free H_2O and 2 µl of template DNA. Cycling conditions were as follows: 10 mins at 95 °C followed by 30 cycles of 30 s at 95 °C, 30 s at 50 °C and 30 s at 72 °C and a final elongation stage of 5 mins at 72 °C.

End-point PCR gel visulisation. All endpoint PCR products were visualised on a 2% agarose gel cartridge using an Egel E-Gel iBase Power System and E-Gel Safe Imager Real-Time Transilluminator (Invitrogen, UK).

Blood meal identification. Due to the nature of the experimental set up with humans and bovines dominating all blood meal sources, samples were initially screened using bovine and human specific primers developed by Gunathilaka *et al.*[32]. The reaction conditions consisted of a 10 µl reaction including 0.5 M of forward and reverse primers (Integrated DNA Technologies), 5 µl of SYBR green master mix (Roche, UK), 2 µl of nuclease-free water (Roche, UK) and 2 µl of template DNA. PCR was run on a LightCycler 96 real-time PCR machine (Roche, UK) under the following cycling conditions: pre-incubation of 95 °C for 600 s, 40 cycles of 95 °C for 10 s, 62 °C for 10 s and 72 °C for 30 s followed by a melting analysis to determine specific amplification. Any potential human positive samples were confirmed using the Promega Plexor® HY Human DNA forensic detection kit (Promega, UK). This assay was performed following manufacturer's protocol using a Stratagene MX3005P (Agilent Technologies, USA) real-time PCR machine and analysed using the Promega®Plexor Analysis software.

Statistical analysis. For each blood-fed mosquito, several variables were recorded: its species, the night and location (transect point and indoors/outdoors) of its capture, Sella stage and source host species of its blood meal. Wind speed and rainfall were also recorded for each capture night. Logistic regression was used to test the association between source species of blood meal (bovine vs non-bovine, and, human vs non-human) and i) species of mosquito, ii) transect point and iii) indoors/outdoors capture. Potential interactions between the variables and wind speed and rainfall were also sought. The Akaike Information Criterion (AIC) was used to estimate the relative quality of statistical models generated using intrinsic versus extrinsic factors for describing the data on blood meal sources. Our previous work identified that the level of blood meal digestion increased significantly the further away from the blood source that mosquitoes were caught[26]. Therefore, we conducted an ordered logistic regression to ascertain whether the Sella stage (for either bovine-fed or human-fed mosquitoes) varied significantly across the transect. All data analyses were performed in STATA. P values < 0.05 were assumed statistically significant.

Results

Summary of collection. A total of 904 blood fed *Anopheles* mosquitoes were collected across the 22 trapping nights. Of these, 665 (73.6%) were identified as members of the *Anopheles gambiae* species complex with 519 identified as *An. coluzzii* (57.4%), 70 were *An. gambiae ss* (7.7%) and 32 were *An. melas* (3.5%). 44 (4.9%) were *An. gambiae sensu lato* but could not be identified past the species complex level (Table 1). In addition, 108 (11.9%) were *An. pharoensis*, 54 (6%) were *An. rufipes* and 77 (8.5%) samples could not be morphologically or genetically identified (Table 2).

A similar number of blood-fed mosquitoes were obtained from indoor (n = 493, 55%) and outdoor (n = 411, 45%) collections. Of the 493 collected indoors, 413 were shown to have blood meal sources of bovine origin (84%), 20 human (4%), nine (2%) of mixed human/bovine origin and 51 (10%) unknown. For outdoor collections, 364 (89%) had bovine blood meal sources, 6 (1%) had human sources, 10 (2%) human/bovine mix and 31 (8%) unknown.

The HBI and Bovine Blood Index (BBI) for each species and collection location is shown in Table 3. *An. coluzzii* had an overall HBI of 6%, with indoor HBI of 8.5% significantly higher than outdoor HBI of 0.6% (Pearson $\chi^2 = 12.4068$, $p < 0.001$). This general trend was maintained across the different species: overall, mosquitoes caught indoors had a higher HBI (Pearson $\chi^2 = 8.7848$, $p = 0.003$) and lower BBI (Pearson $\chi^2 = 9.0013$, $p = 0.003$).

Species	Collection Location*	T1	T2	T3	T4	T5	T6	Total
An. coluzzii	Indoors	100	202	15	12	4	21	354
	Outdoors	110	21	25	5	3	1	165
	Total	210	223	40	17	7	22	519
An. gambiae s.s.	Indoors	12	8	0	2	1	2	25
	Outdoors	35	5	3	0	1	1	45
	Total	47	13	3	2	2	3	70
An. pharoensis	Indoors	1	4	1	1	1	2	10
	Outdoors	65	20	8	1	4	0	98
	Total	66	24	9	2	5	2	108
An. rufipes	Indoors	0	17	0	0	0	0	17
	Outdoors	20	9	6	1	0	1	37
	Total	20	26	6	1	0	1	54
An. melas	Indoors	0	17	1	1	1	0	20
	Outdoors	3	2	4	1	2	0	12
	Total	3	19	5	2	3	0	32
An. gambiae s.l.	Indoors	1	24	1	0	0	0	26
	Outdoors	2	3	12	1	0	0	18
	Total	3	27	13	1	0	0	44
Unknown	Indoors	7	26	1	3	0	0	37
	Outdoors	22	9	3	2	3	1	40
	Total	29	35	4	5	3	1	77

Table 2. Summary of blood-fed mosquito species collected by transect point and collection location. *Indoor collections were performed in uninhabited outbuildings for T1 and T2 and inhabited houses for T3-T6.

Species	Collection location	Human Fed (HBI)*	Bovine Fed (BBI)*	Unknown (%)
An. coluzzii	Indoors (n = 354)	30 (8.47)	308 (87.01)	23 (6.50)
	Outdoors (n = 165)	1 (0.61)	154 (93.33)	10 (6.06)
	Both (n = 519)	31 (5.97)	462 (89.02)	33 (6.36)
An. gambiae s.s.	Indoors(n = 25)	2 (8)	22 (88)	3 (12)
	Outdoors (n = 45)	3 (6.66)	40 (88.89)	4 (8.89)
	Both (n = 70)	5 (7.14)	62 (88.57)	7 (10)
An. pharoensis	Indoors (n = 10)	2 (20)	8 (80)	1 (10)
	Outdoors (n = 98)	6 (6.12)	95 (96.94)	3 (3.06)
	Both (n = 108)	8 (7.41)	103 (95.37)	4 (3.70)
An. rufipes	Indoors (n = 17)	0 (0)	11 (64.71)	6 (35.29)
	Outdoors (n = 37)	1 (2.70)	32 (86.49)	5 (13.51)
	Both (n = 54)	1 (1.85)	43 (79.63)	11 (10.37)

Table 3. Summary of HBI and BBI calculated for each mosquito species and collection location. *Includes mixed feeds.

Impact of local host availability on mosquito host choice. Categorising all blood-fed mosquitoes as human-fed (n = 45) or non-human-fed (n = 859), logistic regression demonstrated a significant impact of the distance (in metres) from the village centre where a mosquito was caught (OR: 0.9790 (95% CI: 0.9749–0.9830), $p < 0.001$). This equates to approximately halved odds of being human-fed for mosquitoes caught 25 m from the village centre. Keeping human-fed mosquitoes as the response variable, logistic regression was repeated – first using mosquito (sibling) species as the (intrinsic) explanatory variable, then using transect point and trapping location (extrinsic) explanatory variables. Estimates of the Akaike information criterion identified the superiority of the model including extrinsic factors (model with extrinsic factors AIC: 243.0, versus, model with intrinsic factors AIC: 359.8).

Rainfall (yes/no) was not found to have a significant impact on whether or not a blood-fed mosquito sourced its meal from humans ($p = 0.484$) nor did it significantly modify the impact of indoors versus outdoors captures ($p = 0.115$). Windy nights (defined as nights with wind speeds recorded over 3 m/s) were also shown not to have a significant impact ($p = 0.432$) on whether or not a blood-fed mosquito sourced the blood meal from humans. However, windy nights were a significant modifier for whether human-fed mosquitoes were caught indoors versus outdoors ($p = 0.041$).

For mosquitoes which were bovine-fed (n = 796) or not bovine-fed (n = 108), a significant impact of the distance (in metres) from the cattle holding pen was found (OR: 0.9894 (95% CI: 0.9866–0.9922), $p < 0.001$). This equates to approximately halved odds of being bovine-fed for mosquitoes caught 50 m from the cattle pen which is an equivalent order of magnitude in difference found when comparing indoor- versus outdoor-caught mosquitoes (OR: 0.5875 (95% CI: 0.3644–0.9472), $p = 0.029$). Keeping bovine-fed mosquitoes as the response variable, logistic regression was repeated – first using mosquito (sibling) species as the (intrinsic) explanatory variable, then using transect point and trapping location (extrinsic) explanatory variables. Estimates of the Akaike information criterion identified the superiority of the model including extrinsic factors (model with extrinsic factors AIC: 611.3, versus, model with intrinsic factor AIC: 660.6).

Both rainfall and windy nights were negatively associated with mosquitoes having sourced their meals from cattle (respectively, OR: 0.2357 (95% CI: 0.1194–0.4654), $p < 0.001$; OR: 0.4830 (95% CI: 0.2902–0.8037), $p = 0.005$). Neither rainfall nor windy nights were a significant modifier for whether bovine-fed mosquitoes were caught indoors versus outdoors (respectively, $p = 0.999$ and $p = 0.292$). Ordered logistic regression did not find any significant association between the Sella stage of bovine blood meal digestion and distance from the cattle pen, nor between the Sella stage of human blood meal digestion and distance from the village centre (respectively, $p = 0.081$ and $p = 0.464$).

Discussion

Mosquito vectors of human diseases differ markedly in their preference for human hosts[21,25,33]. This preference is driven by both intrinsic (genetic) and extrinsic (environmental) factors, meaning that the same species of vector may exhibit different host-biting behaviours when confronted with a different setting. Malaria vector species are often described as primary or secondary vectors and this is largely contingent on how much they typically bite humans. However, just how much flexibility a vector exhibits according to its local environment is poorly understood. A recent systematic review indicated that where a mosquito is caught (indoors or outdoors) may be as influential on host selection as which vector taxa is collected[23]. In a malaria endemic setting of southern Ghana, we sought to quantify the relative influence of extrinsic and intrinsic factors in determining which host species is bitten by local vectors.

Over several weeks during the rainy season, mosquitoes were collected across a range of alternative host (bovine or human) availabilities, both from aspirating indoors and from traps placed outdoors. A general preference for bovine blood hosts was demonstrated by local malaria vectors including sibling species that are considered anthropophagic (*An. gambiae* s.s. and *An. coluzzii*). Although atypical, the very low HBI for these vectors (respectively 7% and 6%) is not without precedent; a recent systematic review presented a very wide range of HBI values (~0–100%) found among *An. gambiae* caught across Africa[23]. More remarkable was the significant associations found between feeding indices and the extrinsic factors under investigation.

Across the different species found locally, blood-fed mosquitoes caught indoors had a significantly higher HBI and significantly lower BBI than those caught outdoors. Despite being an intuitive result, the number of studies describing both indoor and outdoor blood indices are quite limited; and most of these have sought to document the widely recognised generalist ('catholic') biting behaviour of *An. arabiensis*[34,35]. Perhaps, therefore, even the paragons of anthropophagy such as *An. gambiae* s.s. are just as unfussy in their host choice; but, we acknowledge that the result we have found in our field site would need replication elsewhere to develop the evidence base to confirm this speculation.

Regression analysis showed that whether or not a blood-fed mosquito obtained its meal from humans was better predicted by its proximity to humans and whether it was caught indoors than by the species of the mosquito. Similarly, whether or not a blood-fed mosquito obtained its meal from cattle was better predicted by its proximity to cattle and whether it was caught indoors than by the species of the mosquito. Sella staging of the blood-fed mosquitoes showed no significant difference in blood meal digestion across the transect. Hence, mosquitoes were moving freely over the 250 m transect indicating that there was no unobserved barrier between village- and cattle pen-adjacent mosquitoes. In other words, individuals from the same sibling species across the transect likely comprised the same mosquito population and observed differences in behaviour were indeed driven through extrinsic factors. We believe this to be the first evidence from the field demonstrating the high degree of potency that the relative influence of extrinsic factors has on host selection by a major disease vector. Our findings suggest categorizing biting behaviour solely on taxonomy or on a broadly spatial level (by region or country) risks missing the highly localized impact extrinsic factors such as host availability can have on mosquito host choice; and this could have significant public health consequences.

Malaria control is chiefly centered on mosquito control, and mosquito control is largely dependent on exploiting human-biting mosquito behaviour. The two most prominent malaria-control mainstays over the past 20 years in Africa have been insecticidal bednets, which target human-seeking mosquitoes, and indoor residual spray of insecticides, which targets mosquitoes that have recently fed. Historically, effectiveness of new formulations and combinations was assessed solely through measuring epidemiological endpoints (usually malaria incidence). More recently, clustered randomised trials have also incorporated the measurement of entomological endpoints[36–38], following recognition that this is critical in linking insecticidal deployments with the mechanism(s) of disease control. However, monitoring of biting behaviour which fails to account for extrinsically driven plasticity risks misattribution of the mechanism(s) of disease control which would lead to misleading effectiveness measures and consequently jeopardise the rigour of any extrapolations or projections.

In addition to elucidating the mechanisms of effectiveness of current control trials and programmes, a better understanding of plasticity in host choice could qualitatively change disease control strategy. One burgeoning approach to malaria control is the treatment of blood-hosts with systemic insecticides. These insecticides are delivered topically, orally or parenterally and operate through the uptake of toxic compounds during a bite. They have been demonstrated in trials to significantly reduce malaria incidence[39]. These drugs are not restricted to

human use and several studies have documented their efficacy in controlling mosquito vectors when applied to local livestock[40,41]. Models have demonstrated when and where this technology could be most successful either as a standalone tool[42,43] or as part of an integrated vector management programme[44,45]. For these projection efforts to be built upon to inform operational control, reliable data are required for describing: how bites are distributed among different host species; how host choice is impacted by local host availability; and, how this behaviour is impacted in the presence of different control measures (e.g. bednets). In the setting of the present study, bednets were seen and used sporadically but also completely absent from some households during sampling. Therefore the host selection plasticity identified among the local vectors here could be exploited by complementing mass bednet deployments (i.e. a time that is arguably when local vectors will try to exploit alternative blood host species) with systemic insecticidal applications in livestock.

Conducting experiments such as those described in the current study but in different epidemiological/entomological settings – including communities before/after mass distribution of LLINs – is an important focus of future work. There is no reason to believe that host choice is always dominated by extrinsic factors – it is important to know how much this behaviour varies in different settings and what are the key drivers. Hopefully the ease with which the experiment is set up; its relatively modest costs; and, the broadly recognised criticality of human biting rate in malaria epidemiology will encourage others to capitalise on our methods to investigate mosquito biting behaviour in other settings. To that end, findings from this first study could be exploited by others in conducting power analyses to determine appropriate sample sizes for their mosquito collections.

Conclusions

Local host availability is a powerful driver for host selection of even the most discerningly anthropophilic malaria vectors. The importance of not characterizing biting behaviour on a taxonomic level but through explicit study of local mosquito populations is demonstrated. A better understanding of plasticity in host choice is critical for attributing disease reductions to the correct control mechanisms and is key to implementing the most effective malaria control strategy.

Data availability

All data generated or analysed during this study are included in this published article.

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Author contributions

L.Y. and J.O. conceived the study. J.O., A.R.M. and Y.A.A. performed the fieldwork. J.O., C.L.J., M.K. and T.W. performed laboratory analyses. All authors contributed to experimental designs, results interpretation and manuscript drafting. All authors read and approved the final manuscript prior to submission.

Competing interests

The authors declare no competing interests.

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Chapter 7 – General Discussion

The 2018 world malaria report states that progress in the control and reduction of the global malaria burden has stalled (1). The reasons for this stagnation include political, economic, bureaucratic and epidemiological aspects. From the perspective of vector control, research efforts have been focused on curbing the spread of insecticide resistance (1, 2), however, this must not distract from the commitment to increase bed net coverage, whilst continuing to development their durability and bio-efficacy. What is most apparent from this most recent report is that simply maintaining current control efforts will be insufficient to eradicate malaria in many areas (3-8). New technologies are needed, and, in the interim period, optimal use of currently available technologies is paramount if we are to avoid reversion back to ~1 million malaria deaths every year (9).

Control of vector-borne diseases, particularly malaria, is and will remain largely reliant on mosquito management, therefore it is imperative that we identify ways of improving the targeting of mosquito vectors. The research I have conducted over the past three years was driven by this need.

This thesis aimed to investigate the interaction between the intrinsic host preference of major malaria vectors and local host availability to improve understanding of how this interaction drives mosquito host choice - who or what is bitten by the vector has clear impact on malaria transmission as well as implications for its control.

First, initial evidence for plasticity in host choice was demonstrated in a systematic review of the literature (10). Next, a novel, field-based methodology for investigating this behaviour was developed and tested (11). Further refinement of the transect method to incorporate indoor as well as outdoor collections allowed for the first indication of the dominant role of extrinsic factors in host choice for the major African

malaria vectors (Chapter 6). This methodology, along with developmental laboratory work also demonstrated a novel means of both measuring mosquito dispersal (Chapter 4) and estimating time post-feeding (Chapter 5).

HBI estimates are correlated more to "where" a mosquito is collected, not "which" species is collected

Across the literature reviewed, the HBI for each major malaria vector species ranged 0-100%, and this included species that are widely referred to as paragons of anthropophagy. With the exception of a small number of studies demonstrating plasticity (12-14), the majority of studies reported singular HBI values meaning the comparison was drawn across numerous African countries and across decades. This motivated the systematic collection of vectors from the same time and place but across a transect of alternative host availabilities. We found that the HBI was better informed by the location of capture than the mosquito (sibling) species caught.

These results question the categorisation of mosquito species based on their intrinsic preference alone. They also highlight the difficulty of gaining an unbiased and accurate estimate of the HBI. The methodology designed and implemented here further highlighted this challenge, demonstrating the highly localised and small spatial scale on which the HBI can vary with host availability (15). The fact that the HBI can significantly vary over a matter of a few hundred metres suggests single point estimates of the HBI from one type of location or using a single technique is likely to be inadequate (10, 16-18).

Producing a robust measure (or range) for the HBI is important. It is a key metric used for several aspects of medical entomology, from behavioural ecology to vector incrimination (19, 20). It also forms a key parameter in estimating the vectorial

capacity and entomological inoculation rate (EIR) for disease transmission (21-25). Garrett-Jones acknowledged the risk of bias in the HBI due to location in which the mosquitoes were collected in the 1960s (17, 18); and while this has been acknowledged in subsequent studies (25-28), suggestions for resolving this issue are scant. The method described in this thesis hopefully offers a step towards a more robust measure of the HBI. It is relatively easy to perform, inexpensive and easily transferable to alternative settings and mosquito species.

Quantifying blood meal digestion offers a novel measure of post-feeding behaviour and dispersal

An unexpected but significant outcome from this research was the demonstrated potential to use blood meal digestion as a tool to measure a mosquito's dispersal distance. It was observed that the quantity of host DNA extracted and amplified during qPCR decreased with distance from the hosts (15). Previous work investigating the effect of digestion on PCR amplification showed that as digestion progressed DNA degradation increased, resulting in less template DNA from which amplification could occur with this process being indicated by higher Ct values (29-32). Linking the quantity of host-blood DNA to each of the transect points (which were at known distances from the cattle population) with a timed blood meal digestion assay (described in Chapter 5) resulted in a novel methodology for informing mosquito dispersal post blood meal.

Dispersal of mosquitoes underlies population structure (33), species density (34, 35), the potential of human exposure to disease (36) and directly affects the ability/effort required to control transmission (37-39). Despite this, dispersal is frequently the poorest understood among all life-history traits. For many arthropod vectors of disease, including the most important species globally, knowledge of dispersal

behaviour has hardly advanced over the past 50 years (40). Of considerable hindrance to this field's development is the absence of reliable methods. These experiments have involved the mark-release-recapture of insects. A major disadvantage of this method is that the numbers of marked mosquitoes recaptured is typically very low; translating the dispersal of 1% of the insects to the remaining 99% is fraught with problems such as bias. The negative impact of marking mosquitoes combined with the incredibly low recapture rates (41-44) particularly for *An. gambiae* (33, 41, 45-47) has limited progress in this field. Using the blood meal as a "natural" biological marker to track dispersing mosquitoes has been used previously by radioactively labelling the blood of an animal host (48); however, the use of host blood DNA is less intrusive and more broadly applicable.

Chapter 5 developed these findings further, showing that the Sella score and qPCR can follow the digestion process with a remarkable level of correlation across multiple species of medical importance. Understanding how mosquito species disperse within the environment is important if localised transmission is to be better understood (39). Recently *An. coluzzii* females in Mali were shown to disperse over huge distances – in the order of 100s of kilometres (49) with this movement being key to re-establishment of mosquito populations (50). New methods described in the aforementioned Mali study coupled with the new methods in this thesis offer the beginnings of a completely novel toolbox to revitalise the field of vector dispersal at the macro and micro scale.

How can our new findings inform malaria control?

Plasticity in biting behaviour must be considered when strategising malaria control. This plasticity is likely to be an important contributing factor in preventing elimination interruption in elimination settings (51-55). On the surface it may seem like the ability

of a local vector population to shift its biting onto non-human hosts should only serve to benefit malaria control; however, it is possible that this ability enables transient zoophagy to act as a short term respite from contact with insecticidal control tools (e.g. LLINs and IRS) while they are at their most potent (56-58).

That said, this behaviour also opens opportunities to synergise current control strategies with other complementary interventions (52, 59). Mathematical models have demonstrated very good returns from combining LLINs with endectocides applied either to humans (60) or to cattle to offset host choice plasticity (61). Zooprophylaxis, the process of diverting blood seeking mosquitoes away from humans and onto domestic animals has been shown to effective in particular setting where vector biting behaviour has been effectively characterised (62) and supplementing LLINs with human or cattle odour-baited traps has shown both theoretical (63) and real-world promise on reducing malaria transmission in particular settings (64). Importantly, the data produced within this thesis is unique in its ability to inform the spatial scale across which these alternative interventions, amongst others, could be optimised.

Study limitations

The primary limitations of this study were found in the fieldwork. Due to the strict criteria required for a field site to be eligible for sampling, a significant amount of time was taken by visiting and assessing potential sites. Although this was critical to the success of this study, it only allowed one field site to be tested for a relatively short period of time; 27 nights of capture across a two year period. Although enough data was collected to inform statistical significance, this work should be viewed as a pilot study with the aim to performed extended field collections across multiple sites and collection seasons to further strengthen these findings. This is important as mosquito biting behaviour can be highly localised, varying both spatially and temporally (12, 15,

27)and the identification of a second or multiple other sites would have provided a valuable comparison.

For many malaria-endemic areas, peak mosquito density and malaria cases follow the rainy season as transmission and distribution of malaria is highly associated with climactic factors (65-70). Different *Anopheles* species peak density also varies over space and time (71-74). Although this work was timed with the rainy season and targeted the peak mosquito density and malaria transmissions period, a longer duration in the field would have allowed collection across a wider window of both vector density and transmission period. This could have allowed for an increased collection of other vector species. The fact that species diversity was markedly different between the two collection years could be evidence for this seasonal variation in species composition.

The critical aspect of any collection is providing a representative sample of the mosquito population and in this case its behaviour. Trapping methods will always inherit some bias into mosquito collections due to placement of traps and the type of trap used (75, 76). Many studies investigating the HBI have used PSC and indoor aspiration of human dwellings for example, and in doing so, inflate HBI estimates and over-represent anthropophilic mosquito species. Conversely, collection from outdoor trapping could underestimate the HBI and miss blood fed endophagic mosquitoes resting indoors (12, 27, 28). Whilst it is our recommendation that both indoors and outdoors collections are used to inform local HBI, we have not managed to resolve a precise strategy for informing best practice in summarising this behaviour.

The research output from the field was also limited by man-power. Additional traps and transect points (perhaps at differing orientations) would have increased the yield of blood fed mosquitoes. This would have provided more granularity in how biting behaviour changes with host availability. However, the balance between sample numbers and sample quality was key as processing of samples as soon as they were

collected was required to accurately measure the Sella score and halt blood meal digestion (29, 31, 77, 78). Indoor collections also needed to be performed early morning before sun rise to maximise yield (79-81) and this time constraint limited the number of traps and households that could be used by a small team in the field.

The experimental design also targeted the two predominant host species. Although aiding in the simplification of the blood meal analysis there were a number of unknown blood meals from both years of field collection. If resources had allowed, identification of these blood meals would have provided more clarity as to how blood host selection occurs where chicken coops and other domestic animals were present and living in close proximity to the human population.

The sensitivity of the molecular assays could also have resulted in blood meals and species being unidentified. More sensitive methodologies have allowed detection of blood meals from visually “unfed” mosquitoes (82, 83), however, these methods are usually costlier to perform. Sequencing would have allowed potentially more blood meal sources to be identified and in an unbiased manner as species specific primers do not have to be selected. Studies using this technique have identified blood meals from hosts which would not have otherwise been identified using other techniques (82, 84). The ability to identify more blood meal sources and in larger quantities may have informed the shape of dispersal in the field (e.g. Gaussian versus leptokurtic) as well as better informing the relationship between host availability and host choice (85).

Current malaria control strategies are known to influence mosquito biting behaviour through increasing exophilly as well as shifting peak biting times and host preference (86-94). However, LLIN and IRS usage was not directly measured during this study. Bed nets were present in the community, although not used in some of the houses sampled. The age, condition and insecticidal potency of these nets could not be tested, and bed net usage is known to vary from ownership (95-99) and is notoriously

difficult to measure (100). The presence of insecticide-treated bed nets in the area may have impacted local mosquito biting behaviour, shifting feeding preference towards cattle, resulting in the high levels of zoophagy and outdoor biting identified at this site. Genetic resistance to these interventions was also not formally measured. The kdr West mutation was screened for in a subset of 2017 samples and was found to be fixed in all *An. coluzzii* samples tested, falling in line with findings from the region (101) and neighbouring areas (102, 103) but additional screening for other resistance mechanisms did not take place. Genotypic resistance to insecticides allows for phenotypic behavioural changes in the presence of insecticide-based interventions (104-107) and the addition of screening for genetic biomarkers of resistance could provide a deeper insight into the biting behaviour.

The addition of experiments focused on identifying the intrinsic preference of the mosquito population would provide valuable information. Here, the HBI was the only metric measured and although a key metric in investigating malaria transmission and host preference, it is ultimately the final host choice of that individual mosquito once the various extrinsic factors have been accounted for (14). The addition of experiments to formally investigate the intrinsic preference of this population would perhaps show contrasting results to the findings using the HBI alone (14).

Measuring mosquito dispersal has been hampered by the limitations of previous methodologies (36, 39). Here, the methodology for measuring dispersal shows promise in improving understanding of this behaviour but with some caveats. First, due to the limit of detections of the molecular techniques, mosquitoes that had blood fed more than 60 hours ago could not have host DNA detected. This is insufficiently long to capture mosquitoes where they only take a single blood meal across their entire gonotrophic period (which, for many parts of Africa is closer to 72 hours). That said, Anopheline mosquitoes are routinely shown to exhibit gonotrophic discordance (108-111) and our preliminary calculations estimate that the average time since last blood meal for captured mosquitoes was 39 hours which corroborates this

discordance (Orsborne *et al*, *unpublished work*). This would mean that the proportion of the mosquito population we are missing because of molecular detection limits is considerably reduced. Neither methodology (Sella stage or qPCR) was able to distinguish time since feed for the first hours following the blood meal. Although much of this time is likely spent stationary and resting for the processing of the blood meal, further work is required if this aspect of mosquito behaviour is to be better understood.

Future work

Malaria transmission is highly heterogeneous in its distribution with each foci of transmission being driven by a variety of factors including climate, proximity of breeding sites but also biting behaviour of the vector species present (112-115). Although the findings here are suggestive of extrinsic factors having the ability to dominate host preferences, there may well be areas where the intrinsic preferences for human blood is more pronounced and dominates environmental settings. Repetition of this work is therefore crucial. Much of the methodology described in this thesis can easily be replicated in other areas, for other malaria vectors and other mosquito-borne diseases. Of particular interest would be to perform this experimental set up in areas with varying histories of indoor control tools to ascertain the influence that interventions have had on mosquito behaviour. In a similar vein, longitudinal data from the same intervention site would also be useful. This strategy would allow for a more adaptive and reactionary approach to control implementation.

Only experimentally controlled assays can provide an accurate representation of a mosquito populations intrinsic preferences by providing equal opportunity of available hosts and where the influence of extrinsic factors are controlled (12, 14, 116, 117). It was assumed, that *An. coluzzii* collected in an endemic malaria area would be strongly anthropophilic – perhaps except where cattle populations dominated local availability. Yet this was not the case in the study area selected here and the intrinsic

aspect of host preference may be contributing to this behaviour and therefore should be further investigated. Laboratory or semi-field experiments involving the collection of live adults or reared larvae from the field site followed by choice experiments would identify the intrinsic preference of this mosquito population. As selection of particular traits for host preferences can occur rapidly (118, 119), performing this fundamental work alongside more complex field-based entomological research in the future could aid in explaining perhaps the unusual host biting behaviour demonstrated here.

A promising finding from this research was the use of blood meal digestion as a proxy for time post-feed and to inform dispersal. This methodology warrants further investigation. Next generation sequencing is currently prohibitively expensive but as it becomes more routine, it might offer a way of significantly increasing the sensitivity of host DNA detection beyond the current limits. Additionally, dispersal in the field was calibrated to blood meal digestion under laboratory conditions. Blood digestion is heavily influenced by temperature, species and initial blood meal size in the field (120-123). For the purposes of mass rearing of these insects for research purposes many of these variables are controlled. Optimisation of these calibration curves would require a more realistic temperature and humidity regime and perhaps larval densities which are more representative of the field. Performing this work in the field by collecting larvae and using the emerging adults would also be beneficial as it would incorporate field conditions and avoid the potentially significant amount of genetic divergence shown to occur in lab insect strains when mass rearing over many years (12).

Conclusion

Understanding mosquito biting behaviour is critical if local transmission dynamics are to be better understood and control strategies more effectively implemented. Through the development of a novel field methodology, this thesis presents for the first time

the remarkably small spatial scale on which host biting plasticity can occur and demonstrates extrinsic factors have the ability to dominate mosquito host choice in the field. This methodology coupled with timed laboratory experiments also provides a new methodology that can inform mosquito flight and dispersal post-feeding, contributing to an important but poorly understood area of mosquito ecology.

In the era where malaria elimination is seen as an achievable goal with vector control at the forefront of these efforts, a better understanding of mosquito biting behaviour is critical so the effectiveness of current control strategies can be maintained whilst new interventions are developed. As vector control will likely remain a key facet in tackling malaria transmission, it is essential that research in this area continues to progress, as the success of current and future vector control strategies likely hangs on comprehending and ultimately exploiting this behaviour.

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