



## Host-feeding Preference of *Anopheles* Species under Prolonged Use of Insecticide-treated Bed Nets in Kamuli District, Uganda: Implications for Vector Control

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### Authors' contributions

This work was carried out in collaboration among all authors. Author FGK conceived, designed and carried out the mosquito sampling, analysed the data and drafted the manuscript. Author AY did the blood meal ELISA and provided critical comments on the manuscript. Authors AMA, JBK, EM and AK helped to design the study and provided backstopping during the field work and provided critical comments on the manuscript. Author AWO helped to design the study and provided critical comments on the different versions of the manuscript. All the authors read and approved the final manuscript.

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### ABSTRACT

**Background:** The blood-feeding patterns are crucial in incriminating disease vectors as well as facilitating the design and consolidation of effective vector control interventions in an area.

**Objective:** This study aimed to establish if prolonged use of insecticide-treated bed nets (ITNs) caused a shift in the preferred hosts of the common malaria vectors as the hosts were under the

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bed net. Such a shift would render ITNs less effective and would probably explain the continued morbidity and mortality due to malaria in the highly endemic Kamuli district.

**Methods:** A total of 3,519 indoor and outdoor human biting female *Anopheles gambiae sensu lato* and *An. funestus* mosquitoes were collected from 48 households using human-baited bed net traps. All 187 indoor resting blood-fed anophelines collected were tested by direct enzyme-linked immunosorbent assay (ELISA) for blood meal host identification. Of these, 73 mid guts came from 24 households in villages with a 69% ITNs coverage, while 114 mid guts were from 24 households in non-ITN villages.

**Results:** Blood meal hosts were identified in only 10.96% (n = 8) and 14.91% (n = 17) of the *Anopheles* blood meals from the intervention and non-intervention zones, respectively. Other blood meals could not be clearly identified. Eight (100%) blood meals in the intervention zone were from humans, while in the non-intervention zone, 15 (88.24%), one (5.88%) and one (5.88%) came from humans, cattle and goat, respectively. These findings demonstrated that the malaria vectors in Kamuli district are anthropophilic, with nearly all the mosquitoes collected from both zones feeding on humans during every blood meal (p = 0.82). This indicated high vector-human contacts, and thus implicating these species as important in the transmission of *Plasmodium* species and probably other infections.

**Conclusion:** The use of insecticide-treated bed nets is effective for controlling malaria vectors inside houses, evoking universal coverage of houses in the area.

**Keywords:** *Anopheles* mosquitoes; host preference; ELISA; ITNS.

## 1. INTRODUCTION

Understanding of mosquito behaviour related to host feeding preference is important in understanding of vector-host-pathogen interactions and can facilitate the design and consolidation of effective vector control interventions in an area [1-4]. Identification of the blood meal taken by the vector is the most objective method of identifying its natural blood sources [5,6]. Anophelines exhibit a wide range of host preferences such as humans, livestock, birds, and reptiles, and the prevalence of malaria is influenced by mosquito host selection. Thus, the blood-feeding patterns are crucial in incriminating malaria vectors [7] as well as for control purposes [8]. The degree of anthropophily affects the efficacy of the malaria vector, while climatic, environmental and socio-economic factors also influence vector populations by determining feeding behaviour and vectorial capacity of malaria parasite transmission [7,9].

This study aimed to determine the feeding preferences of *Anopheles gambiae s.l.* and *An. funestus* group in relation to the epidemiology of malaria in Kamuli district, Uganda. Kamuli district is one of the areas in the country where insecticide-treated bed nets have been in use for many years. The study would help in establishing if or not prolonged use of ITNs caused a shift in the preferred hosts of the common malaria vectors from feeding on humans to feeding on other hosts. Such a shift in host preference

would render ITNs less effective, which would probably explain the continued morbidity and mortality due to malaria in Kamuli district and perhaps other areas using ITNs in the country. The study therefore reports the blood feeding patterns and Anthropophilic indices of *Anopheles gambiae s.l.* and *An. funestus* mosquitoes in the ITN and non- ITN zones.

## 2. MATERIALS AND METHODS

### 2.1 Study Area

The study was conducted in Kamuli district (01°05'N 33°15'E), 68 km North of the source of River Nile (Fig. 1) and divided into intervention zone (five villages using ITNs for at least five years) and non-intervention zone (five villages not using ITNs). The intervention villages were located in Kamuli Town Council and Nabwigulu Sub County, both in Bugabula County [10]. The non-intervention villages were located in Bugaya and Buyende sub counties, both in Budiope County located in the North East of Kamuli Town Council, and well over twenty kilometers away, with households owning no bed nets before the entomological survey [11].

Kamuli district was chosen for the study because the proportion of households that were using bed nets for the past five years in the two sub counties studied (Kamuli Town Council and Nabwigulu) was at least 52%, while at the time of the study coverage stood at 74.8% and 64% for

Kamuli Town Council and Nabwigulu, respectively, with an average of 69% of the households in the two sub counties using at least one net [11]. These villages were privileged with a number of Non Governmental Organizations (NGOs) like Christian Child Fund, CCF and Plan-Uganda that intervened with high quality and durable insecticide-treated bed nets [PermaNet® (Vestergaad Frandsen Laussane [10], Switzerland) and Olyset® (Sumito Chemical Group, Japan)] since the late 1990s to supplement government efforts in the control of malaria targeting pregnant mothers, children under five years and People Living with HIV/AIDS. The NGOs also carried out several community sensitizations in conjunction with the District Health department aimed at promoting ITN use [11].

## 2.2 Climatic and Ecological Characteristics

Kamuli district has two rainy seasons, the heaviest rains in March to June and light rains in August to November, with a dry spell from December to March (Annual average rainfall: 750 mm to 1500mm; average maximum temperature: 27° to 30°C; average minimum temperature 10° to 20°C. Relative humidity: 70 to 80%) [Source: District Agriculture Office, Kamuli].

Both the intervention and non-intervention zones were surrounded by a variety of vegetation types including swamps, crop fields and grazing lands. The predominant vegetation cover in the district was the forest/savannah mosaic which

constituted of a mixture of forest remnants and savannah trees with grass and shrubs [10]. Much of it was secondary vegetation that succeeded the original forest cover as a result of farming, timber and fuel wood harvesting and other forms of land use that took place [11]. Both zones generally had similar climatic and ecological conditions [12], with agriculture (crop and livestock) as the main economic activity. Therefore, by the time of entomological sampling, ITN use was taken to be the only unique factor between the two study zones. This was monitored throughout the sampling period [11].

## 2.3 Mosquito Density

No records of mosquito entomological data were presented; however, high mosquito densities and malaria transmission were reported to occur throughout the year [11].

## 2.4 Sampling Design

The study area was divided into one intervention zone (05 villages where bed nets had been used for more than five years) and one non-intervention zone (05 villages where bed nets had not been used) and households were randomly selected for sampling human biting mosquitoes. Two households from each of the sampling zones were randomly selected for sampling indoor and outdoor biting and indoor resting mosquitoes. Households with the same housing designs (Bricks and iron-roofs) were selected and no household was selected more

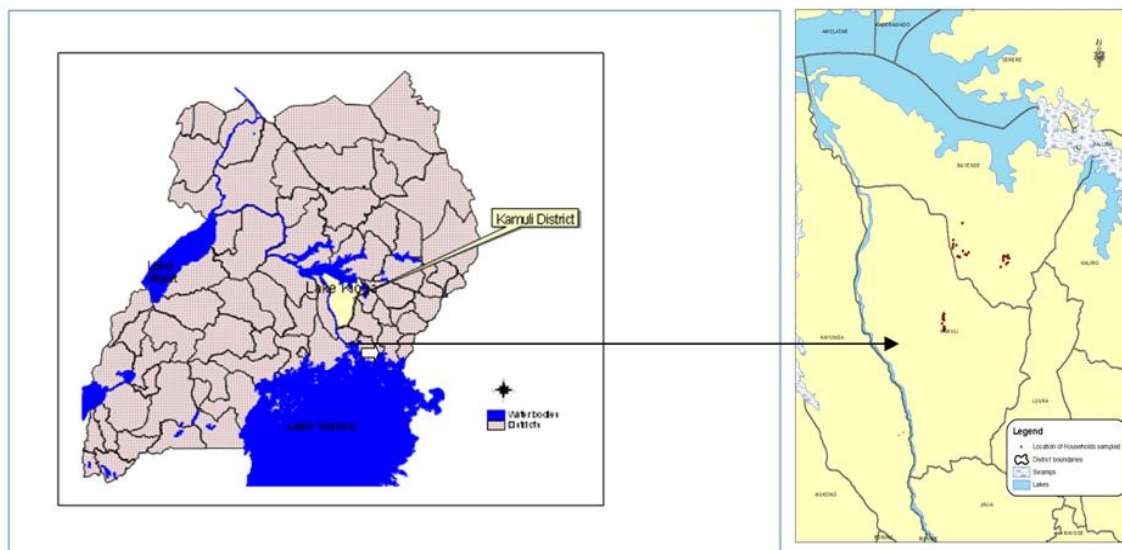


Fig. 1. Map of greater Kamuli District showing study sites

than once for mosquito sampling. A total of four households were randomly selected per month (Two households per zone per month) from the ten villages for a 12-month period. Mosquitoes were sampled for four consecutive nights per household. A total of 48 households were selected and visited for mosquito sampling for the whole sampling period. Volunteers were recruited from the study area, counseled and taught how to trap mosquitoes. Two pairs were positioned at each of the sampling sites. These were replaced in shifts every three hours in each household and were rotated between households.

Latitude and longitude data were recorded for each of the selected households using a hand held Global Positioning System (GPS), and the coordinates were used to map the sampled sites.

## 2.5 Mosquito Collections and Identification

From January to December 2017 indoor and outdoor human biting and indoor resting blood-fed anopheline mosquitoes were collected from 19:00 to 07:00 hours. The indoor and outdoor human biting mosquitoes were caught using human-baited bed net traps [11]. The bed net trap was made by making four to six holes (3 x 3 inches each) on an untreated bed net. This gave some protection to the human-baits sitting under the trap. The trap permitted the entrance of mosquitoes and as they rested on the inside of the trap, they were trapped using an aspirator and a torch. This method was preferred to the CDC light trap (used initially) which was more costly to run overnight, requiring replacement of batteries after a few days.

Collections were done every hour for four consecutive nights per month by a two-person team of trained catchers.

Mosquitoes were collected for the 12-month period covering the different periods of high and low rainfall since high and low rainfall intensity influences species density and diversity [13]. Mosquito collections were made in the 48 households randomly selected from 10 villages in intervention and non-intervention zones using an aspirator and a torch [14].

Each hourly catch of the human biting anophelines was individually placed in a disposable polystyrene container pre-labeled with date, time and location of capture, and taken to laboratory for assessment [15], while feeding

on a 10% sugar solution provided through a cotton wick [16]. The indoor resting mosquitoes were separately placed in a labeled container and were also taken to the laboratory for identification and further analysis. Each catch of the *Anopheles* mosquito population was sorted by sex and identified morphologically using a standard published key [17].

The indoor resting female anophelines were further classified into their respective feeding stages (Unfed, blood-fed, half-gravid and gravid) by examining their abdomens under a dissecting microscope [18]. All the unfed, half-gravid or gravid anophelines were left out of the analysis. The samples were not separated into *An. gambiae s.l.* and *An. funestus* group since both groups were known to have high vectorial capacity [19].

The indoor resting blood-fed *Anopheles gambiae s.l.* and *An. funestus* mosquitoes were cut transversely at the thorax between the first and third pairs of legs under a dissecting microscope, 10-20 X. The posterior portion of the mosquito containing the blood meal was squashed on DNA-binding filter paper, dried and kept at -20°C before blood meal analysis [15,20].

## 2.6 Mosquito Blood Meal Sample Preparation and Direct ELISA Analysis

One to two discs (2x2 mm each) were cut from the centre of each of the 187 blood meal sample squashes on the FTA cards using a pre-sterilized hole punch. Discs for each blood meal were placed into a 600 µl eppendorf tube and homogenized in 250 µl of phosphate buffered saline (PBS; PH 7.4). The anophelines (114 and 73 blood meal samples from non-intervention and intervention zones, respectively) were each tested for blood sources of human, bovine, chicken and goat/sheep using direct ELISAs as described by Beier et al. [21] with slight modification. Mosquito triturate (50 µl) was diluted in PBS (1:50) and 50-µl volumes were added to wells of polyvinyl chloride, U-shaped 96-well micro titer plates which were covered and incubated at room temperature for 3 hours. Each well was then washed twice with PBS containing 0.5% Tween 20 (PBS Tw 20). This was followed by addition of 50 µl host-specific conjugate (antihost IgG) diluted 1:2,000 (or 1:250 for bovine) in 0.5% boiled casein containing 0.025% Tween 20. The boiled casein was prepared by dissolving 5 g casein in 100 ml 0.1N NaOH by

boiling, adding 900 ml PBS, adjusting pH to 7.4, adding 0.1 g Thimerosal (Sodium ethylmercurithiosalicylate) and 0.02 g phenol red, and storing at 4°C (all reagents from Sigma Co., St. Louis, Mo). After 1 hour, wells were washed three times with PBS-Tween 20, and 100 ul of ABTS (2,2'-azino-di-[3-ethylbenzthiazoline sulfonate]) peroxidase substrate (Kirkegaard & Perry) was added to each well. Absorbance at 414 nm was determined with an ELISA reader 30 minutes after the addition of substrate. The dark green positive reactions for peroxidase were also determined visually. Blocking buffer (BB) was used as negative control. Samples were recorded positive if absorbance values exceeded the mean plus three times the standard deviation of four negative controls. Positive and negative control samples were tested on each micro titer plate, as interplate variation for absorbance values of controls would be significant if plates were not read at consistent times following substrate addition.

True positive identification was based on only one ELISA. No cross reactions of two or more samples were observed. Preferred hosts and the anthropophilic index (proportion of blood meals obtained from humans multiplied by 100) of the *Anopheles* mosquitoes were determined for the two zones.

## 2.7 Data Analysis

The anthropophilic nature of the *Anopheles* mosquitoes was demonstrated by the proportion of mosquito blood meals obtained from humans, calculated as the anthropophilic index, or human blood index (HBI). Statistical analysis was performed using Graph Pad Prism Version 6.00 [22]. Fisher exact test was used to compare the mosquito anthropophilic indices between intervention and non-intervention zones. The level of significance was set at 5% ( $p < 0.05$ ).

## 3. RESULTS

### 3.1 Species Composition

A total of 3,519 human-biting female *Anopheles* mosquitoes were collected in the 48 households in 1536 man nights. There was lower mosquito abundance in the intervention than the non-intervention zone ( $P < 0.001$ ). Over 70% of the *Anopheles* mosquitoes caught were *Anopheles gambiae s.l.*, with a relatively higher proportion of *An. funestus* group caught in the non-intervention zone as shown in Table 1. Generally, outdoor human-biting catches exceeded the indoor catches, particularly for *An. gambiae s.l.* Other mosquitoes caught included *An. moucheti*, *Culex* and *Aedes* species. A total of 187 indoor resting blood-fed *Anopheles gambiae s.l.* and *An. funestus* mosquitoes (114 in the non-intervention zone and 73 in the intervention zone) were caught during the entire 12-month sampling period (Table 1).

### 3.2 Blood-meal Host Identification

Blood meal sources were identified in only 10.9% (8 out of 73) and 14.9% (17 out of 114) of the *Anopheles* blood meals in the intervention and non-intervention zones, respectively (Table 2). In total, only 25 blood spots out of the 187 smears were identified. The rest of the blood meals were not identifiable. All the 8 (100%) blood meals in the intervention zone were from human, while in the non-intervention zone, 15 (88.2%), one (5.9%) and one (5.9%) of the identified blood meals were obtained from humans, cattle and goat, respectively. Anophelines in the intervention zone probably exclusively fed on humans, although relatively fewer livestock were also available. In the non-intervention zone, the mosquitoes also fed on other hosts, namely cattle, goats in addition to humans.

**Table 1. Human biting and indoor resting catches of female *Anopheles* mosquitoes in both non-intervention and intervention zones over a 12 month sampling period**

Mosquito group	Non-intervention zone		Intervention zone		Totals	P-Value
	Indoor	Outdoor	Indoor	Outdoor		
<i>Anopheles gambiae s.l.</i>	853	1079	299	346	2,577	
<i>Anopheles funestus</i>	453	411	39	39	942	
<b>Totals</b>	<b>1,306</b>	<b>1,490</b>	<b>338</b>	<b>385</b>	<b>3,519</b>	<b>&lt;0.001</b>
	114*		73*			

\*Number of indoor resting blood-fed anophelines

**Table 2. Summary of *Anopheles* blood-meal host identification by direct ELISA in intervention and non-intervention zones**

Host	Intervention		Non-intervention		P Value
	No. tested	% positive	No. tested	% positive	
Human	73	10.90 (08)	114	13.20 (15)	0.82
Cattle (Bovine)	73	0.0 (00)	114	0.88 (01)	
Chicken	73	0.0 (00)	114	0.0 (00)	
Goat	73	0.0 (00)	114	0.88 (01)	
Total	73	10.9 (08)	114	14.9 (17)	

Number of positive test host samples in parentheses

### 3.3 Anthropophilic Index

The anthropophilic index (or Human Blood Index, HBI), defined as the proportions of *Anopheles* blood meals sourced from humans [5] in intervention and non-intervention zones were 100% and 88.2%, respectively. This showed that anophelines in the intervention zone probably exclusively fed on humans, while in the non-intervention zone, the mosquitoes also fed on other hosts, namely cattle (5.88%), goats (5.88%) in addition to humans (88.24%) (Table 1). Therefore, in this study, *Anopheles gambiae s.l.* and *An. funestus* mosquitoes were highly anthropophilic as humans came out as the probable principal source of blood meals of these mosquito species in both zones ( $p = 0.82$ , Fisher exact test).

## 4. DISCUSSION AND CONCLUSION

### 4.1 Mosquito Species Composition

Over 70% of the *Anopheles* species caught in the study area were *Anopheles gambiae s.l.* and 26.8% were *An. funestus* group. Anopheline catches were higher in the non-intervention zone compared to the intervention zone. The lower mosquito abundance in the intervention zone was probably suggestive of impact of the vector control intervention (ITNs/ LLINs) under use in this zone compared to the non-intervention zone without treated bed nets, although there could be other prevailing factors in the intervention zone such as human behavioural factors [11]. The relatively higher proportion of the *An. funestus* group in the non-intervention zone could be attributed to the presence of more suitable breeding habitats for the *An. funestus* group in the locality [11]. Other mosquitoes caught included *An. moucheti*, *Culex* and *Aedes* species. These may have serious implications as humans are exposed to emerging and re-

emerging parasitic [23-26] and arboviral infections including West Nile virus [27-29] and Chikungunya [26,30] responsible for millions of infections and deaths of humans and animals globally.

### 4.2 Blood Feeding Patterns and Anthropophilic Index of the *Anopheles* Mosquitoes

Detailed information on vector frequency by capture time can be seen in Kabbale et al. [11]. *Anopheles gambiae s.l.* and *An. funestus* mosquitoes caught from both the intervention and non-intervention zones were highly anthropophilic (HBIs were 100% and 88.2% in the two zones, respectively) as shown from the identified proportions of the blood meals.

The mosquitoes also exhibited a high degree of endophily as observed from the proportionately similar indoor resting blood-fed catches. These results, although in small numbers, showed that there was a proportionately high incidence of *Anopheles* mosquitoes-human contact in both zones, thus implicating these vector species as important in the transmission of malaria parasites [7] and other infections [18,31] in the study area. Some anophelines in the non-intervention zone, however, fed on non-human hosts, namely cattle (bovines) and goats, as shown in the results above. This could be explained by the fact that during the mosquito sampling period, more livestock, mainly cattle and goats were seen in most households in the non-intervention zone, compared to the intervention zone where livestock were relatively fewer. This phenomenon would be expected as mosquitoes are also known to feed on what is available and accessible in the environment [4,32]. *Anopheles gambiae s.l.* species especially, are known to be less discriminant and more opportunistic in host

selection [33]. Host preference or selection in mosquitoes is determined by extrinsic or intrinsic factors [7] as location, host availability and accessibility, the density and genetic make-up of the mosquito vector population [33-34].

In Kenya, permethrin-impregnated bed nets caused a shift in mosquitoes from human to animal feeding [35,36]. However, earlier studies on mosquito feeding patterns and differences across mosquito species concluded that *Anopheles gambiae* and *An. funestus* feed almost entirely on humans [37-40]. In this study, the lack of a statistically significant difference in the level of anthropophily between the two zones (with ITNs and without ITNs) (p-value =0.82) implied that ITNs could have not had an effect on the blood feeding patterns of *An. gambiae* s.l. and *An. funestus* mosquitoes, and thus possibly still had protective efficacy to humans against mosquito biting inside houses.

A large proportion of the host blood sources could not be identified by the direct ELISA technique. This, however, could not be regarded as a failure of the method, but most probably due to partial digestion of the blood meals between the time of catching in the night and squashing of the blood-fed abdomens on to the filter papers on the next day. This possibly caused considerable deterioration of the ingested blood [41]. It is also possible that the small number of mosquito blood spots identified could be attributed to the inevitably poor field preservation conditions. The dried anopheline blood smears were initially stored under silica gel at room temperature in the field Laboratory before their transfer and storage at -20°C in the Makerere Laboratory prior to the blood meal analysis. This could have caused degradation of the blood DNA [41].

Although the study involved a small sample size, the results of the study give a clue on the prevailing vector-host-pathogen interactions in the area and hence may guide in planning disease outbreak control tools [2]. When resources allow, tests and analyses will be carried out on larger samples in the future.

The observed anthropophilic nature of *An. gambiae* s.l. and *An. funestus* mosquitoes, though with small samples, could confirm the incrimination of these species as the primary human malaria vectors. ITNs/LLINs could

therefore probably still form an effective intervention for controlling malaria vectors inside houses in Kamuli district. This calls for universal coverage of houses in the area with ITNs/LLINs. Considering the endophilic nature exhibited by these mosquito species, indoor residual spraying using ecologically acceptable insecticides should also be employed in this part of Uganda in addition to ITNs/LLINs in the context of integrated vector control strategy. People may also use recommended repellents to protect themselves against the early, later and outdoor anopheline bites when they are not in bed.

## CONSENT AND ETHICAL APPROVAL

Prior to start of the study, approval was sought from the Uganda National Council for Science and Technology and Health Research Ethics Committee (Reference Number: HS 263).

Permission during sensitizations was sought from house hold owners, village and district authorities; and the privacy and psycho-social needs of the individual participants and household members were highly protected. Catchers were selected from the local community to facilitate acceptance from residents. Written informed consent was obtained from each catcher.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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