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Biting times of *Plasmodium falciparum* infected mosquitoes and transmission intensities following five years of insecticide-Treated bed nets use in Kamuli District, Uganda: Implications for malaria control

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Abstract

This study determined the biting times of malaria vectors and transmission intensities following five years use of Insecticide Treated Nets (ITNs) in Kamuli district, Uganda. A *Plasmodium falciparum* circum-sporozoite protein ELISA was performed on 551 and 1640 *Anopheles* mosquitoes caught at different hours of the night in ITNs intervention and non-intervention zones, respectively. The sporozoite positivity of the vectors was related to the time of biting humans, while the annual entomological inoculation rates (AEIRs) were obtained by multiplying the average annual human biting rate by the sporozoite rate. Infective biting by the vectors occurred throughout the night, while peak infective bites occurred after 22:00 hours in both zones. The annual malaria transmission potential was higher in areas non-intervened with ITNs. ITNs were therefore effective against malaria vectors and should be widely promoted in this area. Other protective interventions when people are not in bed are recommended.

Keywords: *Anopheles,* biting cycle, ELISA, *Plasmodia* circum-sporozoites

1. Introduction

Uganda has one of the world's highest malaria incidences with a rate of 478 cases per 1000 people per year, and 1,502,362 confirmed cases and 7,277 deaths reported in 2014 [1-4]. Malaria is hyper endemic and is the leading cause of morbidity and mortality in the country, especially among young children and pregnant women [5-8]. *Anopheles gambiae sensu lato* and *Anopheles funestus* are the principal malaria vectors in Uganda. These species have for long been known to bite between 22:00 and 05:00 hours [9, 10] as in most parts of Africa. These are hours of the night when most people are in bed and under bed nets if they have them. This was the main entomological justification for the current use of insecticide-treated bed nets (ITNs) for malaria control in Africa [11].

Use of insecticide-treated bed nets is one of the main malaria vector control methods being promoted under the World Health Organisation's Roll Back Malaria (RBM) policy [8, 12-14] and the Uganda National Malaria Control Programme [15]. In Uganda, ITN use has exceeded five years in several areas. Despite this, malaria related deaths continue to be high even in areas under ITN use [16, 17]. Extensive use of impregnated bed nets could result in a greater proportion of the parous and potentially infectious *Anopheles* mosquitoes changing their biting pattern, biting earlier or later in the night when many people are not in bed [11, 18]. This, in addition to insecticide resistance already reported in Uganda [8, 19] and other African countries [8, 15], plus social variables in the different human settings [10], would render bed nets less effective and could explain the continued high rates of morbidity and mortality due to malaria in Uganda.

A study in Southwestern Uganda [19] showed that biting by malaria vectors occurred earlier in the evening following ITN use, which could compromise the effectiveness of vector control interventions in this part of the country. Another recent study in Western Kenya showed an overlap of early biting habit of the malaria vectors and human activity [20]. In Tanzania, a study to test bed net traps for monitoring mosquito populations and time of biting and possible impact of prolonged use of insecticide treated bed nets observed that more of the *Anopheles*

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biting occurred early and late in villages with ITNs, whereas in villages with no history of ITN use, biting was concentrated in the middle of the night. This suggested that behavioural adaptation to avoid contact with ITNs could have begun to evolve in those ITN villages [21].

At the Southeastern coast of Kenya, the malaria transmission intensity of the vectors was reduced following more than five years of 60 to 80% coverage with ITNs [22]. Changes in biting patterns and reduced malaria transmission intensity could have possibly occurred in several areas in Uganda where ITNs/LLINs have been in use for some time. This study therefore aimed to determine the biting times of malaria vectors and transmission intensities following five years use of ITNs/LLINs in Kamuli district. The biting times of the *Plasmodium falciparum* sporozoite-infective mosquitoes and the parasite transmission intensities in the study area are therefore reported. These results may give evidence-based guidance in determining suitable times for deploying the most effective malaria vector control tools, when the vectors are most active and transmission is at its peak. The results would also assess the impact of the ITNs/LLINs intervention on the entomological inoculation rates of the major *Anopheles* mosquito vectors in the study area, and possibly other areas in the country with a modest ITN coverage of 35-65% or at least 80% coverage known to provide equitable community-wide protection [13, 23].

2. Materials and Methods
2.1. Study sites
The study area was located in Kamuli district (01^0 05'N 33^0 15' E), 68 km North of the source of River Nile (Source: Kamuli District five-year Development Plan, 2005/2006-2009/2010-Unpublished)). The area was 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. 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. The two zones were well over twenty kilometers apart, with households in the non-intervention zone owning no bed nets before the entomological survey (Kamuli District Health Status Reports, 1999/2000-2004/2005; Kamuli District Health Sector Strategic Plan, 2005/06-2009/10-Un published; Personal preliminary house hold survey).

Kamuli district was chosen for the study because the proportion of households that were using bed nets five years before the study 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 [10]. These villages were located in the area of operation of a number of Non Governmental Organizations (NGOs) like Christian Children's Fund (CCF) and Plan-Uganda that intervened with high quality and durable insecticide-treated bed nets [PermaNet® (Vestergaad Frandsen Laussane, 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.

2.2. Climatic and Ecological Characteristics
Kamuli district has a bimodal rainfall pattern, the heaviest rains falling in March to June and light rains in August to November, while the dry spell begins from December to March [10].

The annual average rainfall ranges from 750 mm to 1500 mm. The average maximum temperature is in the range 27° to $30\ ^0$C, while the average minimum temperature is 10^0 to 20 ^{0}C. Relative humidity is 70 to 80 % [10].

Both the intervention and non-intervention zones were surrounded by a variety of vegetation types including swamps, crop fields and grazing lands [10]. 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. 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 (Kamuli District five-year Development Plan, 2005/2006-2009/2010-Unpublished).

Both zones generally had similar climatic and ecological conditions [24], with agriculture (crop and livestock) as the main economic activities (Kamuli District five-year Development Plan, 2005/2006-2009/2010-Unpublished). Therefore, by the time of entomological sampling, ITN use was taken to be the only unique factor between the two study zones, and this was monitored throughout the sampling period.

2.3. Human Population Density
According to the 2002 Population and Housing census, the human population density at the time of the study (December 2009 to November 2010) was higher in the intervention than in the non-intervention zone (Source: Kamuli District Population Reports, 2002/2003-Unpublished).

2.4. Mosquito Density and Malaria Transmission
There was no baseline entomological data; however, high *Anopheles* mosquito densities and malaria transmission were reported to occur throughout the year (Kamuli District Health Sector Strategic Plan 2005/06-2010-Unpublished).

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

2.7. Mosquito Collections and Identification
The catchers were counseled and taught how to trap mosquitoes, and two pairs, one indoor and another outdoor, were positioned at each of the sampling sites. The catchers were replaced in shifts every three hours in each household

and were rotated within and between households. Each month two households from each of the sampling zones were randomly sampled for mosquitoes using human-baited bed net traps [10]. The bed net trap was made by making four to six holes (3 x 3 inches each) on an untreated bed net. The catcher sat under the bed net trap which gave him some protection which is denied when the human-landing catch method is used. The catchers were trained to collect landing mosquitoes prior to blood feeding to minimize the risk of malaria transmission and they were given anti-malarials as this area has high transmission of *P. falciparum* showing resistance to anti-malarial drugs (Dr. Lopita Micah, Pers. Communication*).*

The trap permitted the entrance of mosquitoes and as they rested on the inside of the trap, they were collected using an aspirator and a torch [2]. 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.

From December 2009 to November 2010 indoor and outdoor human biting anopheline mosquitoes were collected every hour from 19:00 to 07:00 hours for four consecutive nights per month by a two-person team of trained catchers positioned indoors and outdoors. Collections were done in the 48 households randomly selected from 10 villages in intervention and non-intervention zones. Mosquitoes were collected for the 12-month period, with 192 collections in total, covering the different periods of high and low rainfall since high and low rainfall intensity influences species density and diversity [25].

People living in a room were protected with a net each, and as hungry mosquitoes persisted in their attempts to look for a blood meal, they got near to the trap and were caught by it. The nets are known to improve the efficiency of the traps [26]. The assumption was that the mosquitoes which entered a trap during any hour were those actively seeking hosts and would, in most cases, bite human hosts in the same hour and room/house if the bed net trap was absent [11].

The indoor and outdoor human-biting proportions of the mosquito population and time of biting were determined and recorded throughout four repeated nights. Each hourly catch was placed in a disposable polystyrene container pre-labeled with date, location and time of capture, and taken to laboratory for assessment [27]. These were fed on a 10% sugar solution available through a cotton wick [28]. Each hourly catch of the night was identified morphologically using a simplified key [29], while the morphological identifications were confirmed by an Entomologist using a dissecting microscope at the Vector Control Division Laboratory, Ministry of Health, Kampala, Uganda.

2.8. Determination of the Human Biting Rates of the *Anopheles* mosquitoes
The indoor and outdoor human biting rates of the anophelines caught from both zones were calculated as the total number of mosquitoes caught biting humans during the 12-month sampling period divided by the number of people bitten (the catchers) divided by the number of nights of catching (number of mosquitoes per human per night) [10].

2.9. Determination of biting times of *Plasmodium falciparum*-infective mosquitoes and the sporozoite rates
The heads and thoraces of a pool of up to five mosquitoes were tested for *P. falciparum* sporozoites by the Enzyme-Linked Immuno-Sorbent Assay, ELISA, method using monoclonal antibodies [11, 27, 30-33] for *P. falciparum* circum sporozoite protein (CSP) [34].

A maximum of five mosquitoes were used per pool to ensure 99% confidence of detecting at least one infective mosquito per pool [35]. Pooled samples were used as it is a highly efficient and economic method. A total of 511 (112 sample pools) and 1640 (331 sample pools) mosquitoes collected from intervention and non-intervention zones, respectively, were processed for *P. falciparum* sporozoite infection.

The test results were read visually for positivity and scored 30-60 minutes after the substrate was added and then measured spectrophotometrically at 405 nm. Each of the mosquito sample pools was considered positive if the colour changed to green and had absorbance values of twice the average of the optical density values of the negative samples. All the positive pools and 10% of the negatives were repeated to confirm the initial results. All positive pools were regarded as undoubtedly *P. falciparum* sporozoite-infective because only thoraces and heads of the test samples were exclusively used [36, 37]. Sporozoite positivity of the human-biting mosquito proportions were then related to their times of biting.

The sporozoite rate (S) was calculated as the percentage of *Plasmodium* sporozoite positive mosquito sample pools out of the total number of mosquitoes analysed [35], with the assumption that each positive pool had at least one infective mosquito. Crude estimates of sporozoite rates were corrected using Table S1 and associated calculations (Courtesy of Professor Tom Smith of the Swiss Tropical and Public Health Institute) [38].

2.10. Determination of entomological inoculation rates and *Plasmodium* transmission intensity
The average number of sporozoite-positive mosquitoes caught indoor and outdoor at each hour of the night [Daily Entomological inoculation rate, EIR] were obtained from EIR= MaS (where Ma = Human-Biting Rate, HBR, and S = sporozoite rate) for the whole sampling period, while the Annual Entomological Inoculation Rate, AEIR, (i.e. the number of sporozoite-positive bites per person per year in intervention and non-intervention zones) were obtained by multiplying the average annual HBR by the sporozoite rate [35], i.e., AEIR = Ma (HBR) x S.

2.11. Data analysis
The indoor and outdoor human biting rates of the *Anopheles gambiae* complex and *An. funestus* group of mosquitoes for the whole sampling period, the numbers of *P. falciparum* circum-sporozoite protein positive *Anopheles* bites and the numbers of sporozoite-positive sample pools were all compared between intervention and non intervention zones using the Kruskal-Wallis rank sum test of the R-Statistics soft ware, version 2.15.0 (2016.02.02) [39]. The proportions of positive sample pools and the sporozoite rates were compared using Wilcoxon signed rank test with continuity correction [R-Statistics soft ware, version 2.15.0, (2016.02.02)] [39]. 'Adjusted' Wald intervals and 95% confidence intervals were obtained using the confidence interval calculator [38].

2.12. Ethical Considerations
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. Volunteers of the

same sex (male) and approximately equal body weight were recruited from the study area as mosquito catchers. They were selected from the local community to facilitate acceptance from residents. Written informed consent was obtained from each catcher. At least two (02) Bed Nets (LLNS) were donated to each participating household following the study.

3. Results
3.1. Human Biting Rates of the *Anopheles* mosquitoes
The outdoor biting rates of the anophelines appeared to exceed the indoor biting rates in both zones ((Table 1), however, there was no significant difference shown between the outdoor and indoor human biting rates (Kruskal-Wallis Chi-squared = 0.227, df=1, p = 0.634).

Table 1: Indoor and Outdoor Human Biting Rates by the *Anopheles* mosquitoes in Intervention and Non-Intervention Zones

Intervention Zone (Catch nights, n = 96)			Non- Intervention Zone (Catch nights, n = 96)		
No. of Mosquitoes caught	No. of Persons	HBR*	No. of Mosquitoes caught	No. of Persons	HBR*
Indoor	338	96	0.037	1,306	96	0.142
Outdoor	385	96	0.042	1,490	96	0.162

HBR*(Human Biting Rate) = Mean number of Mosquitoes caught per Person per Night
(Note: Other mosquito species caught but not included in the table were: *An. moucheti* (n > 500), *Culex* species (n > 1,840) and *Aedes aegypti* (n > 150).

### 3.2. Time of malaria-infective biting and peak infection by *Anopheles* mosquitoes
The results showed that the hour-by-hour pooled samples of the-all-night biting *Anopheles* mosquitoes collected from both zones were circum sporozoite protein-positive. Biting by malaria infective mosquitoes occurred during the hours 20:00 to 05:00 and 19:00 to 06:00 hours in the intervention and non-intervention zones, respectively (Table 2). The majority of sporozoite-positive bites occurred in the periods between 22:00 and 04:00 hours in intervention and between 22:00 and 07:00 hours in the non-intervention zone (Figure 1), with more sporozoite-positive mosquitoes observed in the non-intervention than in the intervention zone (Kruskal-Wallis chi-squared = 35.7775, df = 7, p = 7.98 e $^{-0.6}$).

Table 2: Proportions of *Anopheles* mosquitoes positive for *Plasmodium falciparum* Circum- sporozoite protein during the night in Kamuli district

	Intervention Zone					Non-intervention Zone				
Hour beginning	No. caught	No. tested	No. CSP +ve	% CSP +ve pools	MIR*	No. caught	No. tested	No. CSP +ve	% CSP +ve pools	MIR*
19.00	28	5 (1)	0	0.0 (0)	0.00	88	85 (17)	35	41.2 (7)	0.08
20.00	21	21 (5)	4	20.0 (1)	0.05	148	90 (18)	5	5.6 (1)	0.01
21.00	9	20 (4)	20	100.0 (4)	0.20	170	100 (20)	30	30.0 (6)	0.06
22.00	45	25 (5)	5	20.0 (1)	0.04	190	165 (33)	5	3.0 (1)	0.01
23.00	43	43 (9)	43	100.0 (9)	0.21	197	105 (20)	63	60.0 (12)	0.11
00.00	67	60 (12)	49	83.3 (10)	0.17	249	145 (29)	65	44.8 (13)	0.09
01.00	79	70 (14)	10	14.3 (2)	0.03	269	100 (21)	67	66.7(14)	0.14
02.00	82	82 (17)	14	17.6 (3)	0.04	284	130 (27)	72	55.6(15)	0.12
03.00	87	45 (9)	40	88.9 (8)	0.18	329	140 (28)	10	7.1 (2)	0.01
04.00	86	75 (15)	15	20.0 (3)	0.04	334	150 (30)	40	26.7 (8)	0.05
05.00	102	85 (17)	10	11.8 (2)	0.02	272	205 (43)	52	25.6 (11)	0.05
06.00	44	20 (4)	0	0.0 (0)	0.00	266	225 (45)	135	60.0 (27)	0.12
19.00-06.00	723	551 (112)	210	38.4 (43)	0.078	2796	1640 (331)	579	35.4 (117)	0.071

Number of sample pools in parentheses; MIR*(Minimum Infection Rate) = Number of CSP positive sample pools/Total number of samples tested
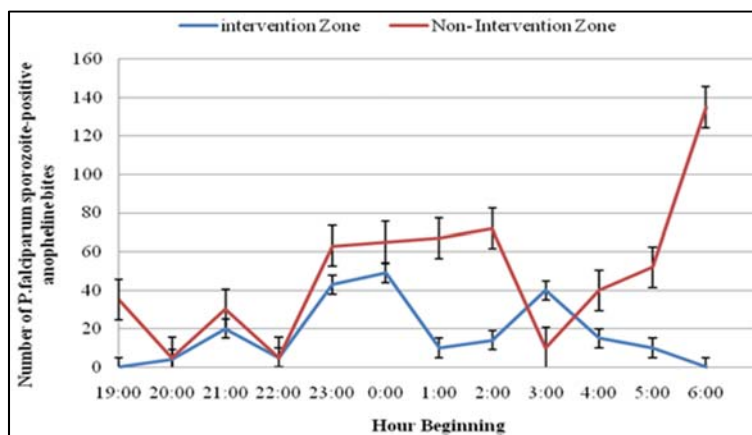
Fig 1: Comparison of peak *P. falciparum* CSP positive-biting (S.E±1) by anopheline mosquitoes between intervention and non-intervention zones in Kamuli district (*p*<0.05)

The distribution of sporozoite positive mosquitoes in three four-hour periods of the night (19:00-22:59, 23:00-02:59 & 03:00-06:00) also showed that infective-biting occurred during all the three periods of the night (Tables 3a and 3b), demonstrating that generally all human bites by the *Anopheles* mosquitoes in the study area were potentially infectious with *P. falciparum* sporozoites and possibly other *Plasmodium* species [40]. However, peaks of infective-biting occurred during the period between 22:00 and 04:00 hours and between 22:00 and 07:00 hours in the intervention and non-intervention zones, respectively. That is, peak exposure occurred at 23:00, 00:00 and 03:00 hours in the intervention and at 23:00, 00:00, 01:00, 02:00 and 06:00 hours in the non-intervention zone (Figure 1). Two unique peaks of infective biting, a minor one at 19:00 hours and a major one at 06:00 hours, were observed in the non-intervention zone.

Table 3 (a): Human-biting *Anopheles* mosquito catches during the first, middle and last thirds of the night and the proportions positive for *P. falciparum* CSP in Kamuli district (Sporozoite positivity rates not corrected)

Period of the night	Intervention Zone (Catch nights, n = 96)					Non-intervention Zone (Catch nights, n = 96)				
	No. caught	No. tested	No. +ve	% CSP +ve pools	MIR*	No. caught	No. tested	No. +ve	% CSP +ve pools	MIR*
19:00- 22:59 Hours	133	71 (15)	29	40.0 (6)	0.08	596	440 (88)	75	17.1 (15)	0.03
23.00- 02:59 Hours	271	255 (52)	116	46.2 (24)	0.09	999	480 (97)	267	55.7 (54)	0.11
03.00-06.00 Hours	319	225 (45)	65	28.9 (13)	0.06	1201	720 (146)	237	32.9 (48)	0.07
19.00- 06.00Hours	723	551 (112)	210	38.4 (43)	0.078	2796	1640 (331)	579	35.4 (117)	0.071
Minimum Infection Rate, MIR*			0.078						0.071	
Number of sample pools in parentheses										
MIR*= Number of CSP positive sample pools/Total number of samples tested										

Table 3 (b): Human-biting *Anopheles* mosquito catches during the first, middle and last thirds of the night and the proportions positive for *P. falciparum* CSP in Kamuli district (Corrected sporozoite positivity rates are based on Table S1 and associated equations, Courtesy of Prof. Tom Smith of the Swiss Tropical and Public Health Institute)

Period of the night	Intervention Zone (Catch nights, n = 96)					Non-intervention Zone (Catch nights, n = 96)				
	No. tested	P_o (%)	P_t (%)	MIR* (Corrected)	95% CI (Adjusted)	No. tested	P_o (%)	P_t (%)	MIR* (Corrected)	95% CI (Wald Adjusted)
19:00- 22:59 Hours	71 (15)	40.0	9.80	0.028	4.58-19.26	440 (88)	17.1	3.68	0.009	2.21-5.87
23.00- 02:59 Hours	255 (52)	46.2	11.70	0.024	8.33-16.34	480 (97)	55.7	15.03	0.031	12.07-18.48
03.00-06.00 Hours	225 (45)	28.9	6.40	0.012	4.00-10.78	720 (146)	32.9	7.67	0.017	5.90- 9.82
19.00- 06.00Hours	551 (112)	38.4	9.24	0.020	7.09-11.98	1640 (331)	35.4	8.37	0.017	7.11-9.80
Minimum Infection Rate, MIR*				0.020					0.017	
Number of sample pools in parentheses										
P_o = Observed proportion of positive pools										
P_t = True sporozoite rate = 1- exp [ln (1- p_o)/n], where n = pool size; pool size was 5										
MIR*= Number of CSP positive sample pools/Total number of samples tested										
95% CI = 95% Confidence Interval, adjusted using Confidence Interval calculator, Courtesy of Sauro (2005)										

3.3. *Anopheles* sporozoite rates
Altogether, 38.4% (43 out of 112) and 35.4% (117 out of 331) of the test sample pools were positive for *P. falciparum* circum-sporozoite protein in the intervention and non-intervention zones, respectively. The percentages were almost similar. Assuming that there was only one infective mosquito in each positive sample pool, the minimum infection rate, MIR, was calculated as the ratio of the number of positive pools to the total number of tested mosquitoes [35, 41, 42]. In table 3(a) with uncorrected sporozoite positivity rates the minimum infection rates peaked during 23:00-02:59 hours in both zones. In table 3(b) with corrected sporozoite positivity rates, MIRs peaked during the period 19:00-22:59 hours in the intervention zone (MIR= 0.028) and during 23:00-02:59 hours in the non-intervention zone (MIR= 0.031). The overall minimum infection rates (MIRs) in the intervention and non-intervention zones were 0.078 and 0.071, respectively (Table 3a). In table 3b (with corrected sporozoite positivity rates), the overall MIRs were 0.020 and 0.017 in the intervention and non-intervention zones, respectively, being apparently greater in the intervention zone. The differences in MIRs between the two zones were, however, very slight and far away from being statistically significant (Wilcoxon signed rank test, p = 0.716).

3.4. Entomological inoculation rates and *Plasmodium* transmission intensity
The minimum infection rates (as calculated in sporozoite rates section above and tables 2, 3a and 3b) were taken as the sporozoite rates for intervention and non-intervention zones, respectively. These results indicated that generally, both indoor and outdoor malaria parasites transmission intensities were at least 3.5 times higher in the non-intervention than in the intervention zone (Table 4), the higher transmission intensities in the non-intervention zone being influenced by the higher human biting rates in this zone (Table 1). In both zones, the indoor AEIRs equaled to the outdoor AEIRs, i.e., the ratio of outdoor AEIR to indoor AEIR in both zones approximated to one. Overall, *P. falciparum* transmission intensity (AEIR) was 3.5 times greater in the non-intervention zone as compared to the intervention zone.

Table 4: Comparison of Annual Entomological Inoculation Rates between Intervention and Non-intervention Zones (*p*<0.05)

	Intervention zone			Non-intervention zone		
	Mean Daily HBR	AHBR*	AEIR**	Mean Daily HBR	AHBR*	AEIR**
Indoor	0.037	13.51	1.04	0.142	51.72	3.67
Outdoor	0.041	15.25	1.19	0.162	59.01	4.19
MEAN			1.12			3.93
AHBR = Mean Daily Human Biting Rate (calculated in table 1as mean bites per person per night) x 365 days; *AEIR = Annual Human Biting Rate (Ma) x Sporozoite Rate (S) as estimated in table 3 (a); S = 0.078 and 0.071 in intervention & non-intervention zones, respectively.						

4. Discussion

4.1. Human Biting Rates of the *Anopheles* mosquitoes

There was no significant difference between outdoor and indoor biting rates (Bites per person per night) of the *Anopheles* species in both zones (Kruskal-Wallis Chi-squared = 0.227, df=1, p = 0.634). This trend implied that probably at one time, the outdoor human biting rates equaled to the indoor biting rates [10]. The outdoor human biting behaviour of the anophelines puts people at risk of receiving malaria infections, and possibly other infections for example lymphatic filariasis [40] before and after bed time and out of the bed net, where people have them.

4.2. Time of malaria-infective biting and peak infection by *Anopheles* mosquitoes

Several studies have been undertaken in Kenya and other countries in East Africa and have shown that mosquitoes are changing their feeding patterns, with vectors feeding early and late following ITN interventions [19, 20, 43, 44]. Some studies, however, reported no change in the biting times of the malaria vectors, but at least reduced the human-vector contact and blood feeding success [45, 46]. In the present study, sporozoite infective biting occurred generally throughout the night, i.e.., from 20:00 to 05:00 hours and 19:00 to 06:00 hours in the intervention and non-intervention zones, respectively.

Peaks of sporozoite-infective biting occurred between 22:00 and 04:00 hours in the intervention zone, a period when most people are thought to be in bed. This indicated that use of ITNs in this zone is still protective against malaria infective mosquitoes. Infective biting in the non-intervention zone occurred between 22:00 and 07:00 hours. Therefore, although people can still be protected from the night peak *Plasmodium*-infective bites, they are still at a risk of receiving infective bites at hours before and after bed particularly in the non-intervention zone.

A high exposure risk to outdoor malaria infective bites before 22:00 hours and after 05:00 hours was observed in both zones, with more risk in the non-intervention zone. A person might receive 1.66 and 3.01 infective bites per year outside before 22:00 hours in intervention and non-intervention zones, respectively. A relatively lower risk was observed indoors before 22:00 hours where a person might receive 1.46 and 2.64 infective bites per year in intervention and non-intervention zones, respectively. After 05:00 hours, a high exposure risk to *P. falciparum* infective biting was particularly observed in the non-intervention zone where a person might receive 7.10 and 6.20 infective bites in a year outside and inside, respectively.

In the intervention zone, a low risk to infective biting was observed inside and outside after 05:00 hours. However, many people in this zone wake up as early as 04:00 hours for several economic activities (Personal observation in Kamuli Town Council). These people are at risk as one person is likely to receive 0.27 and 0.31 malaria infective bites per year indoors and outdoors, respectively.

The presence of infective mosquitoes caught at different hours of the night was an indicator that transmission of *P. falciparum* parasites (and possibly other infections, for example lymphatic filariasis) [40] actively occurred in the households of both zones. These results are consistent with earlier reports that *P. falciparum* is the main species of malaria parasites prevalent in Uganda [4, 14, 47, 48] and possibly responsible for most of the morbidity and mortality in this part of the country. However, future studies should include establishing whether or not other *Plasmodium* species, i.e. *P. vivax, P. ovale* and *P. malariae* do exist in this part of the country. It is well known that these species have different life-cycles, hence complicating treatment of disease particularly in cases of mixed infections. For example, *P. malariae* mixed infections with *P. falciparum* have been found in other parts of Uganda, especially among children [48].

4.3. *Anopheles* sporozoite infective rates

Although the method used is most suitable when vector infective rates are low [34, 49, 50], the results may help to cost-effectively estimate the transmission dynamics of *P. falciparum* malaria and the risk averted by using ITNs in Kamuli district. The higher number of sporozoite-positive mosquitoes observed in the non-intervention zone could be due to the higher number of mosquitoes collected and analysed (551 in intervention zone versus 1640 in non-intervention zone). Overall, sporozoite rates appeared to be unaffected by the ITN/LLIN intervention (MIRs: Table 3a: 0.078 and 0.071; Table 3b: 0.020 and 0.017, in intervention and non-intervention zones, respectively, Wilcoxon signed rank test, p = 0.735). However, considering the peak infection period in both zones, i.e., 23:00-02:59 hours, the MIRs were 0.09 and 0.11 (Table 3a), and 0.024 and 0.031 (Table 3b with corrected sporozoite positivity rates) in the intervention and non-intervention zones, respectively. This indicated some impact of the ITNs on the sporozoite infective rates in the intervention zone.

Some studies on effects of ITNs on mosquitoes and malaria transmission potential conducted in The Gambia, Democratic Republic of Congo, Kenya and Ivory Coast that showed sporozoite rates unaffected by ITN use [51]. Similar studies in Ivory Coast, Kenya, Tanzania, Solomon Islands, Senegal, and Burkina Faso showed reduced sporozoite rates [12, 52, 53, 54]. This trend was also observed in Kamuli district in the peak infective period (23:00-02:59 hours) as shown in tables 3a and 3b. A much greater impact may, however, be realized in this area upon intensified ITNs/LLINs ownership and use in the near future.

Since there were no baseline data to compare with, data from this study could be useful in future when monitoring the impacts of ITNs on the malaria vectors following universal/wider coverage of households in the two study zones [10].

4.4. Entomological inoculation rates and *Plasmodium* transmission intensity

Minimum infection rates in table 3a were used to calculate the entomological inoculation rates. Therefore, the results under discussion are just at the minimum level and provide an estimate of the transmission dynamics of *P. falciparum* malaria and the risk averted by using ITNs in Kamuli district. The results showed that people in the non-intervention zone received at least 3.5 times more *P. falciparum* sporozoite-positive bites in a year than those in the intervention zone. That is, malaria transmission intensity and potential were 3.5 fold higher in the non-intervention (influenced by the higher human biting rates) than in the intervention zone. For both zones, however, the ratios of outdoor to indoor AEIRs were equal, indicating that the number of sporozoite-positive bites a person received indoor in one year could at one time equal to the number of positive bites received outdoors; in other words, same risk of parasite transmission potential indoors and outdoors in both zones.

The number of positive pools is an approximation of the number of infective mosquitoes in a given sample collection [50], and thus the density of infective mosquitoes being an indicator of the parasite transmission intensity [41]. Therefore, the higher density of infective mosquitoes in the non-intervention zone (tables 2, 3a and 3b, Figure 1) was an indicator of higher transmission intensity in this zone.

Climatic and ecological conditions might have also been influencing mosquito population density. However, these conditions in the two zones were generally similar [24], therefore, the lower EIR in the intervention zone was possibly an effect of the ITNs on the human biting *Anopheles* density in this area.

Similar studies to assess impact of ITNs on malaria transmission and elimination in Tanzania and the Solomon Islands showed reduced annual entomological inoculation rates, although none reduced it to zero [12, 52, 55]. In the present study, both the indoor and outdoor EIRs, influenced by the annual human biting rates in the intervention and non-intervention zones, exceeded one, i.e., each person in the area received more than one *P. falciparum*-positive mosquito bites in a year. Therefore, despite the available malaria control efforts, like effective case management and vector control with ITNS/LLINs [9, 15], a lot is still required to attain a manageable level of the disease, i.e., annual EIRs less than one, to reduce parasite rates to levels that could interrupt *P. falciparum* malaria transmission.

According to the 2012 World Health Organisation report [14], Uganda is one of the African countries that are still in a control or pre-elimination phase as evidenced by the EIRs greater than one sporozoite-infective bite per person per year. Most parts of the country are at levels of EIRs of more than one hundred [17] that must be reduced in order to substantially lower the prevalence of malaria infection as shown by earlier studies [55, 56].

Wider or universal coverage of ITNs/LLINs and the use of insecticide-treated curtains, blankets, and other materials, to benefit from mass killing effect are recommended. Indoor Residual Spraying, integrated with other protective tools when people are not in bed, and environmental management should also be employed. House improvements, including the use of screens in windows, doors, eaves and ventilators may also reduce on the indoor human-biting mosquito densities as this reduces on the entry points of the mosquitoes into the houses [57].

5. Conclusion

The study aimed at determining the biting times of *P. falciparum* sporozoite-infected mosquitoes and transmission intensities and thus the annual transmission potential following five years of ITNs/LLINs use in Kamuli district, Uganda. This further aimed at establishing whether or not ITNs were still protective against malaria-infective biting by the vector mosquitoes. The results indicated that infective biting by *An. gambiae sensu lato* and *An. funestus* mosquitoes in this part of the country occurred throughout all night hours, i.e., in the period 19:00 to 06:00 hours, with peak infective bites occurring in the periods between 22:00 and 04:00 hours and between 22:00 and 07:00 hours in intervention and non-intervention zones, respectively. These results therefore showed that ITNs/LLINs did not cause a shift in the time of biting by sporozoite-infected mosquitoes and so were still protective against the mosquito vectors. This is because most infective biting occurred at hours of the night when people were expected to be under bed nets. Many people were, however, shown to be exposed to infectious bites before and after bed time, depending on human activity and /or behaviour patterns at dusk and dawn.

Results further evidently showed that ITNs/LLINs reduced the indoor and outdoor EIRs more than three- and four-fold, respectively, in the intervention zone. Intensification and proper use of this effective intervention for control of *Plasmodium* vectors as well as its integration with other proven ecologically feasible methods to target the outdoor as well as the earlier and later biting vectors is recommended. The insecticide resistance profile should also be established and monitored regularly.

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