Blood meal sources of Anopheles vectors of human malaria in Malawi: Implications for malaria transmission and effectiveness of interventions

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Abstract

Background

Selection of blood meal hosts by mosquitoes is a key variable in the vectorial capacity of *Anopheles* mosquitoes for human malaria. Blood feeding on humans is likely to be modulated by use of different types of long-lasting insecticidal nets (LLINs) and the effectiveness of LLINs is impacted by the relative intensity of insecticide resistance. The aim of this study was to test the hypothesis that LLINs containing pyrethroid and the synergist piperonyl butoxide (PBO) would lead to a reduction of human host utilization than LLINs containing only pyrethroid and that blood feeding patterns of *Anopheles* in Malawi compromise malaria interventions.

Methods

Female *Anopheles* mosquitoes were sampled indoors from May 2019 through April 2020 by aspiration, pyrethrum spray catch, and CDC light trap in rural villages of Namanolo (conventional nets) and Ntaja (PBO nets) in Balaka and Machinga districts respectively. *Anopheles* species, blood meal sources, and infection with *Plasmodium falciparum* in the head and thorax of individual mosquitoes were determined with PCR.

Results

Of a total of 6,585 *Anopheles* females sampled indoors in 203 houses, 633 (9.6%) were blood-fed and consisted of *An. arabiensis* (44.1% (n = 279)), *An. gambiae* s.s (16.2% (n = 103)), *An. funestus* s.s (33.5% (n = 212)), *An. parensis* 0.3% (n = 2), and unidentified *Anopheles* spp (5.8% (n = 37)). Of the 541 mosquitoes (85.5%) successfully identified blood meals, 436 (81.0%) were solely human, 28 (5.2%) goat, 11 (2.0%) dog, 60 (11.1%) mixed goat-human, 5 (0.9%) dog-human, and 1 dog-goat. Human blood index and EIR was high in Namanolo than Ntaja (0.96 vs 0.89 (p = 0.001) and 0.11 vs 0.06 infective bites per person per year respectively) despite high net ownership (92%) and nightly use (75%) rates. Relative to host availability, non-human hosts were over selected in the two sites.

Conclusion

The use of PBO nets was associated with lower HBI and EIR, however, the wide availability of LLINs was still associated with extensive successful human blood meals by the main malaria vectors in Malawi. The presence of a small fraction of mixed blood meals indicates constrained plasticity of *Anopheles* vectors to switch to non-human hosts and circumvent malaria control interventions.

Introduction

Malaria is endemic in Malawi with transmission occurring throughout the country, having generally greater transmission in the south than the north [1–3]. The nation-wide prevalence of infection for *Plasmodium falciparum* among 2 to10 year old individuals, sampled from 2010 to 2017,declined from 29.4% in 2010 to 15.2% in 2017 but this change was uneven across the country's 28 jurisdictional districts [4]. Despite these successes, which are largely attributed to reduction in transmission owing to the implementation of long-lasting insecticide insecticidal nets (LLINs) distributed throughout the country, Malawi remains a high malaria burden country with meso-endemic transmission [4]. In 2017, the country-wide incidence of malaria was 247 per 1,000, with an estimated 7,077 deaths [5].

One of the drivers of persistent *Plasmodium* transmission is access to human blood by host-seeking female *Anopheles* mosquitoes. Selection of blood meal hosts by *Anopheles* mosquitoes is a key variable in their vectorial

capacity—a measure of transmission—for human malaria because vectorial capacity increases with the square of the rate of human biting [6]. Further, host selection is likely to be modulated by use of different types of LLINs whose effectiveness is impacted by the extent of insecticide resistance in the *Anopheles* populations, and the relative availability of hosts [7, 8]. These factors will enhance or limit mosquitoes' access to humans relative to non-human hosts. Emergence of pyrethroid resistance could severely compromise malaria control efforts by reducing the effectiveness of LLINs. Newer LLINs which combine pyrethroids with a synergist, piperonyl butoxide (PBO), have shown to be effective at restoring pyrethroid susceptibility [9–11].

Vector incrimination studies in Malawi have identified members of the *Anopheles gambiae* sensu lato (s.l.) and *Anopheles funestus* species assemblage as the most important malaria vectors and pyrethroid insecticide resistance have been reported (Hunt et al., 2010; Mzilahowa et al., 2008, 2016; Spiers et al., 2002). However, only a single study has analyzed mosquito host selection and estimated the human blood index (HBI) of these vectors in the southern region of the country before LLINs were available (Mzilahowa et al., 2012). Accordingly, whether human host selection has changed after LLINs availability is unknown.

The objective of this study was therefore twofold. Firstly, we aimed to expand on knowledge of blood feeding patterns of malaria vectors, a key feature of malaria risk, by conducting host selection analyses and estimation of HBI and EIR. Secondly, we aimed to test the prediction that blood feeding on human hosts is reduced where LLINs containing permethrin and the synergist PBO (i.e., Olyset Net Plus, Sumitomo Corporation, Tokyo, Japan) compared to where standard, pyrethroid-only LLINs (i.e., Olyset Net, Sumitomo) have been distributed.

Methodology

Study area

This study was conducted in two malaria-endemic districts of Malawi (Fig. 1), Balaka (14° 58' 45" S; 34° 57' 20" E) and Machinga (15° 10' 6" S; 35° 18' 0" E). These districts, like the whole of Malawi, have distinct wet and dry seasons where malaria proliferates especially in the rainy wet season [2]. In 2018, residents of Machinga received LLINs with PBO (Olyset Plus net, Sumitono Corporation, Tokyo, Japan), whilst residents of Balaka received standard LLINs only (Olyset net) (Government of Malawi, National Malaria Control Programme, 2017) (*Malawi Malaria Operational Plan FY, 2018*). Households in rural areas located within the catchment areas of Ntaja (Machinga) and Namanolo (Balaka) health centers were enrolled in the study. Household surveys were conducted to determine the extent of LLINs ownership and utilization by householders and to quantify the number of occupants.

Mosquito sampling and analysis

Blood-fed *Anopheles* mosquitoes were sampled in houses, selected by the randomized cluster sampling method. All households were sampled three times each period from May-June 2019, October-November 2019, and December 2019-January 2020. Beginning in February to April of 2020, only a subset of households in each site were sampled once every two weeks. The mosquito samples for each visit were accumulated. The number of humans and goats in the households was also counted, although dogs were too mobile to be counted reliably. Cattle were rare in the study area and scored as absent. Mosquito sampling tools included mouth and battery-powered aspirators, standard miniature CDC light traps (Model 512; John W. Hock Company, Gainesville, Florida, USA) and pyrethrum spray catches (PSCs) [13, 14]. Mosquitoes were morphologically identified [15] into *An. gambiae* sensu lato, *An. funestus* sensu lato, or other *Anopheles* species, stored in tubes with silica gel, and kept at laboratory temperatures.

The abdomen was separated from the head-thorax of each mosquito using sterile, cross-contamination-proof technique. For each mosquito, genomic DNA was extracted from the abdomen and head-thorax separately using the Qiagen extraction kit (DNeasy® Blood & Tissue Kit; Cat. No. 69506) following the manufacturer's standard protocol.

PCR identification of mosquitoes

Mosquitoes of the *An. gambiae* (s.l.) complex were identified to one of two species, *An. gambiae* sensu stricto (s.s.) or *An. arabiensis*, using a published multiplex quantitative PCR (qPCR) method [16]. Mosquitoes of the *An. funestus* (s.l.) species assemblage were identified to one of three species (*An. funestus* (s.s.), *Anopheles parensis* and *Anopheles vaneedeni*) using another multiplex qPCR as follows. A universal primer pair (forward: 5'-AGA ACACTA TGG CGA GCA GC-3', reverse: 5'-TTA CGA CGG ATA CGG TCA ACG-3') that amplifies a region of the internal transcribed spacer region 2 of rRNA gene of members of the *An. funestus* (s.l.) species assemblage was designed along with two oligonucleotide probes specific to *An. funestus* (s.s.) (5'-FAM-CAT GGG GAA ATT CAA TCG AAA ACC TCT-QSY-3') and *An. parensis* (ABY-TGG CGT GCT CGG AAC CTA GC-QSY). The probe specific to *An. vaneedeni* (5'-VIC-CGT TGT GAA AAA TGG AGA TTC ATT TGA AAA CC-QSY-3') was obtained from a published source [17]. After performing optimization tests involving 10-fold dilution series of positive DNA control of the three species, the optimum PCR mixture (10 μL reaction volume) consisted of 1x TaqMan Universal Master Mix (Cat. No. 4304437; Thermo Fisher Scientific, Waltham, MA, USA), 0.6 μM of each primer, 0.4 μM of each probe, and 2 μL of mosquito DNA. The reactions were performed on QuantStudio 7 Flex PCR system (Applied Biosystems, Foster City, CA, USA) using the following cycling conditions: one cycle of 50°C for 2 minutes and 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. PCR sensitivity was one target gene copy/μL sample and efficiency was > 90%. Positive and negative DNA controls were included in each experiment. The PCR results were visualized with QuantStudio software (version 1.3).

Blood meal analysis

Individual mosquito abdominal DNA was first tested for human blood meal using a uniplex qPCR method that involved amplification of a region of intron 1 of the nuclear tyrosine hydroxylase gene with primers and probes (forward: 5'-GGC CTG TTC CTC CCT TAT TT-3', reverse: 5'- TAC ACA GGG CTT CCG AGT-3', probe: FAM-ATG GAG TCT GTG TTC CCT GTG ACC -QSY) as described in Keven et al., (2020). Samples that did not react with the human probe in the qPCR were subjected to a standard PCR to amplify the vertebrate mitochondrial cytochrome B gene using a generic primer pair (forward: 5'-CCC CTC AGA ATG ATA TTT GTC CTC A-3', reverse: 5'-CCA TCC AAC ATC TCA GCA TGA TGA AA-3') [19–23]. The PCR reaction mixture (25 μL volume) consisted of 10 mM Tris at pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 1.0 mM dNTP, 0.5 units of Taq polymerase, 50 pmol of each primer, and approximately 20 ng of DNA template. The reaction condition included one cycle of 95°C for 5 minutes (initial denaturation) followed by 35 cycles of 95°C for 1 minute (denaturization), 57°C for 1 minute (annealing) and 68°C for 1 minute (extension), followed by one cycle of 68°C for 5 minutes (final extension). The PCR products were visualized with 2% agarose gel electrophoresis and amplicons of positive samples were purified using QIAquick PCR purification kit (Cat. No. 2810; Qiagen) following the manufacturer's protocol. The nucleotide sequence of amplicons was determined by direct sequencing and the sequences were subjected to BLAST (Basic Local Alignment Search Tool) search for matches to the available vertebrate host cytochrome B gene sequences in GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi*).* A sequence similarity of 97% or higher was used as the cut-off for an acceptable match, based on literature [20, 25–27].

The different hosts identified in the mosquito blood meals based on the results of BLAST searches aided the development of new qPCR primers and probes specific to those host species; humans, dogs and goats were the only

hosts identified by BLAST searches. Primers and probes (Table 1) for humans and dogs were adopted from literature [18] but those for goats were designed and validated in this study using the same procedure for humans and dogs (Keven et al., 2020). Another set of qPCR procedures was performed on all the blood meal samples. The purpose of this second qPCR was to detect presence of mixed blood meals as well as to confirm the results of the previous uniplex qPCR. The qPCR mixture (10 µL reaction volume) consisted of 1X TaqMan Universal Master Mix (Cat. No. 4304437; Thermo Fisher Scientific), 0.5 µM of each primer, 0.25 µM of each probe, and 2 µL of DNA. PCR cycling condition (QuantStudio 7 Flex PCR System) was the same as described for *An. funestus* (s.l.) above. Positive and negative DNA controls were included in each experiment.

Table 1
Blood meal host primers and probes used in the qPCR reactions

species		Forward	Reverse	probe
human	*Homo sapiens*	5'-GGCCTGTTCCTCCCT TAT TT-3'	5'-TACACAGGGCTTCCGAGT-3'	5'-FAM-ATGGAGTCTGTGTT CCCTGTGACC -QSY- 3'
goat	*Capra hircus*	5'-TAGGCGCCATGCTACTAATTC-3'	5'-GAGTGGATTTGCTGGGATATAG-3'	5'-ABY-ATTCACACCCGAC CTACTCGGAGA-QSY-3'
dog	*Canis lupus familiaris*	5'-TGGACAAAGCAACCCTAACA-3'	5'-CCGGTTTCGTGTAGAAATAGGA-3'	5'-ABY-TCATCCTCCCTTTC ATCATCGCAGC-QSY-3'
*Note: goat primers and probe were developed in this study, human and dog were from Keven et al., (2020)				

Molecular identification of *Plasmodium falciparum*

DNA from both the abdomen and head-thorax of each mosquito was tested for presence of *P. falciparum* using a multiplex qPCR containing two fluorescent-labelled TaqMan probes targeting the 18S rRNA gene of *P. falciparum* (forward primer: 5'-ATT GCT TTT GAG AGG TTT TGT TAC TTT-3' ; reverse primer: 5'-GCT GTA GTA TTC AAA CAC AAT GAA CTC AA-3'; probe: 5'-FAM-CAT AAC AGA CGG GTA GTC AT- MGBNFQ-3'). These primers and probes were developed and tested for specificity to the target organism and gene locus by [28]. The PCR reaction (10 µL volume) consisted of 1X TaqMan Multiplex Master Mix (Cat. No. 4461882; Thermo Fisher Scientific), 0.6 µM of each primer, 0.4 µM of each probe and 2 µL of DNA. PCR amplification (QuantStudio 7 Flex PCR System) was performed using the same cycling condition as with *An. funestus* (s.l.) described above.

Data analysis

Mosquitoes that had fed on one species of host, as determined by blood meal analysis as described above, were classified as single host blood meals. If the blood meal analysis revealed two or more species of vertebrate hosts, then the blood meals were classified as mixed blood meals. The HBI of an *Anopheles* population (i.e., mosquitoes of a particular species from a particular village) was calculated as the proportion of blood-fed mosquitoes that fed on human hosts. Human-fed mosquitoes included both single human blood meals as well as human-nonhuman mixed blood meals. [18, 29–31]. Mosquitoes whose blood meal hosts were not identified were excluded from the analysis.

Differences in host utilization by district with different LLINs were analyzed by contingency table analysis. To test for variation in the propensity of the three main malaria vector species to feed on humans, non-humans and human-nonhuman mix blood meal types, a 3 x 3 contingency table analysis was carried out, and the percentage deviations of observed from expected frequencies were calculated. Host selection of a vector population was quantified using the theta statistic ($\theta = \pi_1/\pi_2$), which tested whether the ratio (θ) of the proportion of a host species in mosquito blood meals (π_1) and proportion of the host in the village (π_2) is different from unity [32]. A host species was considered over-selected by the vector population if theta was significantly greater than 1.0 or under-selected if theta was significantly less than 1.0. A host species was considered to be fed on by the mosquitoes in proportion to its relative availability in the village if theta was not significantly different from 1.0. The theta statistical analysis was performed using the *ci.prat.ak* function of *asbio* package in RStudio Version 1.1.456.

The sporozoite rate (SR) was estimated as the proportion of mosquito heads-thoraces that tested positive for *Plasmodium* sporozoites. The entomological inoculation rate (EIR) can be estimated indirectly from samples obtained by indoor resting mosquitoes, and also directly from samples obtained by the human landing catch (HLC) method [33]. In our study, samples from indoor resting collections were used to indirectly calculate the EIR using the formula: **EIR = (M*SR*HBI)/N**,

where M is the mean number of blood fed mosquitoes per house, SR is Sporozoite rate, HBI is Human blood index and N is the mean number of human occupants per house per night. Annual EIR was estimated by multiplying the EIR by 365 days.

Results

Species composition

Mosquitoes were collected in 203 household yielding 6,585 female *Anopheles* mosquitoes including 633 (9.6%) blood-fed. The blood-fed mosquitoes consisted of the following species: *An. arabiensis* (Overall: 44.1%, n = 280; Namanolo: 59.2%, n = 243; Ntaja: 13.0%, n = 37), *An. funestus* (s.s.) (Overall: 33.5%, n = 213; Namanolo: 26.7%, n = 110; Ntaja: 46.2%, n = 103), *An. gambiae* (s.s.) (Overall: 16.2%, n = 103; Namanolo: 7.8%, n = 32; Ntaja: 30.0%, n = 70), and *Anopheles parensis* (0.3%, n = 2, one each in Namanolo and Ntaja). Thirty-seven (5.8%) were not identified to species by morphological or molecular means.

Host selection and HBI

Of the blood-fed *Anopheles* mosquitoes (n = 633), the blood meal host of 541 (85.5%) were successfully identified either by qPCR or direct sequencing (Fig. 2 and Table 2). The remaining 92 (15%) were either non-reactors in PCR reactions (n = 42), or amplicons generated by standard PCR failed to match any feasible host (n = 50). Of the 541 mosquitoes whose blood meal host was successfully identified, 436 (81.0%) were solely human blood meals, 28 (5.2%) were solely goat blood meals, 11 (2.0%) were solely dog blood meals, and mixed blood meals were: 1 (0.2%) dog-goat, 5 (0.9%) dog-human, and 60 (11%) goat-human (Fig. 2 and Table 2).

Table 2
Blood meal sources identified from *Anopheles* mosquitoes of Namanolo and Ntaja.

Mosquito Species	Blood meal source							
	No. tested	Human n (%)	Dog n (%)	Goat n (%)	Human/Dog n (%)	Human/Goat n (%)	Dog/Goat n (%)	Other n (%)
An. arabiensis	280	195(69.6)	8(2.9)	11(3.9)	4(1.4)	30(10.7)	1(0.4)	31(11.1)
An. gambiae	103	75(72.8)	0	6(5.8)	0	12(11.7)	0	10(9.7)
An. funestus	212	152(71.8)	3(1.4)	3(1.4)	0	15(7.1)	0	39(18.4)
An. parensis	2	0	0	1(50)	0	1(50)	0	0
An. spp	36	14(38.9)	0	7(19.4)	1(2.8)	2(5.6)	0	10(27.8)
Total	**633**	**436(68.9)**	**11(1.7)**	**28(4.4)**	**5(0.8)**	**60(9.5)**	**1(0.2)**	**90(14.2)**

Humans were the most frequently identified blood-meal host for all three of the most abundant *Anopheles* species (*An. arabiensis*, *An. funestus* s.s. and *An. gambiae* s.s.), at approximately 70% of blood meal samples for each species (Table 2). Mixed blood meals comprising human and goat were present for all three mosquito species and ranged from 7.1 to 11.7% among species. Blood meals identified solely from goats ranged from 1.4 to 5.8% and were also found in all three species. Dog-only, and mixed dog-human or dog-goat blood meals, were present but uncommon. Differences in human or non-human (goat and dog) and mixed (human-nonhuman) host feeding by these three species, and the percentage deviations of observed from expected frequencies are shown in Table 3. Although the chi-square test was not significant ($X^2 = 6.4$, df = 4, p = 0.17), the percentage deviation values were suggestive that *An. arabiensis* tended to feed on nonhuman hosts more so than did *An. gambiae* (s.s.) and *An. funestus* (s.s.), whereas *An. funestus* s.s. tended to underutilize nonhuman hosts compared to *An. gambiae* (s.s.) and *An. arabiensis*. Additionally, *An. funestus* (s.s.) tended to have fewer human-nonhuman mixed blood meals compared to the other species (Table 3).

Table 3
Percent deviation of observed blood meal frequencies from those expected by chi-square analysis, two sites combined.

species	Human	Non-human	Human-nonhuman mix
An. arabiensis	-4.2	+ 27	+ 15.5
An. gambiae	-1.8	+ 7	+ 8.7
An. funestus	+ 7.0	-42.5	-26.9

The results for analysis of host selection by the theta statistic are shown in Fig. 3 In Ntaja, *An. arabiensis* and *An. gambiae* (s.s.) overselected goats and under-selected humans, whilst *An. funestus* (s.s.) selected these two host species in proportion to their relative abundance in the village. In Namanolo, by contrast, all three vector species selected both hosts in proportion to their relative abundance, although there was a nonsignificant tendency for over-selection of goats compared to humans.

The three abundant mosquito species (data from both villages combined) had similar HBI: *An. funestus* (s.s.) (96.5%); *An. gambiae* (s.s.) (93.5%); *An. arabiensis* (92.3%) (X^2 = 0.11, df = 2, p = 0.946). Their combined HBI (i.e., regardless of species) was significantly higher in Namanolo with conventional nets (96.4%) compared to Ntaja with PBO nets (88.9%) (z = 3.32, p = 0.001).

Entomological inoculation rate

Results of PCR analysis for *P. falciparum* sporozoite infection showed that 15.6% (99/633) of the *Anopheles* mosquitoes (regardless of species) were positive in the head-thorax. The infection prevalence according to mosquito species was as follows: *An. arabiensis* 17.7% (50/283), *An. funestus* (s.s.) 12.3% (26/212), *An. gambiae* (s.s.) 22.2% (22/99), *An. parensis* 0% (0/2), and unidentified *Anopheles* spp. 2.6% (1/39).

To estimate EIR, results from 203 houses with 1,106 occupants (97 houses, 488 occupants in Namanolo, 106 houses, 618 occupants in Ntaja) were used. EIR for both sites combined was equal to (633/203 * 16% * 94.0%)/(1106/203) or 0.09 infectious bites per person per night. For Namanolo, the estimated EIR was equal to (410/97 * 14.3% * 96.4%)/(488/97), or 0.11 infectious bites per person per night. For Ntaja, the estimated EIR was equal to (223/106 * 18% * 88.9%)/(618/106) or 0.06 infectious bites per person per night. Annualized EIR was high in Namanolo than Ntaja with 40 and 22 infectious bites/person/year respectively with overall annual EIR of 0.09 translating into ~ 33 infectious bites/person/year.

Household surveys revealed similar and high ownership rates for LLINs in the two sites (Namanolo: 92%, n = 109 households; Ntaja: 90%, n = 158 households) and nightly use rates (Namanolo: 75%, n = 109 households; Ntaja: 74%, n = 158 households).

Discussion

Indoor mosquito sampling of rural houses in Namanolo and Ntaja in Balaka and Machinga Districts, respectively, of southeastern Malawi revealed three major malaria vector species. This finding was consistent with past studies in Malawi and southern Africa [34–36]. The results here provide key malariologic transmission indices (HBI, EIR) that demonstrate the vulnerability of humans to bites of vector *Anopheles* mosquitoes, despite the presence and use of LLINs as the primary anti-malaria intervention. Although no species was numerically dominant, *An. funestus* (s.s.) and *An. gambiae* (s.s.) were relatively more common in Ntaja and *An. arabiensis* was more common in Namanolo. By contrast, indoor collections of mosquitoes at other locations close to both Namanolo and Ntaja, Lindblade et al., (2015) found *An. funestus* (s.s.) to be dominant, while *An. arabiensis* was next in abundance and *An. gambiae* (s.s.) was uncommon. These populations exhibited resistance to the synthetic pyrethroid deltamethrin, with 38% mortality in WHO bioassays for *An. funestus* (s.l.) and 53% mortality for *An. gambiae* s.l. (probably, *An. arabiensis*) (Lindblade et al. 2015). Despite these variations in mosquito species abundances between sites, *An. funestus* (s.s.) and *An. gambiae* (s.s.) are generally considered epidemiologically more important than *An. arabiensis* due to their well-documented anthropophilic and endophilic behaviors [38–41]. This study found high rates of feeding on human blood by all three species, regardless of these variable phenotypes.

These findings are more evident in the fact that human blood comprised most blood meals in unmixed conditions, regardless of species or study site, and that blood meals from other potential sources (goats, dogs) were secondary, with goat blood meal being more frequent than dog. This observation is not surprising because it was commonly observed that people kept goats indoors in special rooms at night, probably for protection against theft, while dogs were left outside as guard dogs. This may explain the higher number of goat blood meals compared to dog blood

meals. Killeen et al., (2001), in modeling mosquito populations of Tanzania and Kenya, demonstrated that there is a relationship between host availability and the amount of time that vectors spend seeking blood meals; by inference, hosts that require less time to locate will be fed upon more frequently. Orsborne et al., (2020), studying mosquito populations in Ghana, reached a similar conclusion, emphasizing that local host availability even for known anthropophilic malaria vectors, is a powerful driver for host selection. In Malawi, there have been no previous studies that consistently quantified relative availability of potential blood meal hosts. The high prevalence of human host blood-feeding by *Anopheles* species observed here is consistent with 2002 findings from southern Malawi, in which blood meals were nearly entirely from humans and secondarily from bovines [13]. In northern and southern Zambia, similar high human host selection (> 90%) and comparatively lower goat selection (< 5%) by *An. gambiae* and *An. funestus* (s.s.) were observed [42, 43]. In contrast, the dominant blood meal of malaria vectors around Lake Victoria in western Kenya was humans for *An. gambiae* (s.s.) and *An. funestus* (s.s.), but for *An. arabiensis* was predominantly bovine [44] or equally bovine and human [45]. Through application of the "ratio of ratios" method of host selection, this study approached the problem of variation in host selection semi-quantitatively in order to assess host selection tendencies of these often behaviorally stereotyped species.

The high frequency of human blood meals detected in this study can be attributed to several factors, in particular bed net use practices. The higher HBI and EIR in Namanolo compared to Ntaja (96.4% vs. 88.9% and 0.11 vs. 0.06, respectively) could be due to widespread use of PBO-containing Olyset Net Plus in Ntaja, which have been shown to be more effective than convention LLINs against pyrethroid-resistant *Anopheles* populations [11, 14]. Lindsay et al., (2021) have suggested that the underlying mechanism of PBO-containing LLINs may simply be that they are more toxic, rather than overcoming insecticide resistance. Regardless, other randomized field trials in Tanzania and Uganda have shown significantly lower human infection prevalence where LLINs with PBO were distributed [9, 10]. Although the entomological mediators of these reductions were not investigated, they are likely due to reduced transmission intensity. In the present study, the lower HBI of *An. arabiensis* and *An. gambiae* (s.s.) in Ntaja than Namanolo could also be explained by the use of LLINs with PBO in Ntaja, which apparently increases their susceptibility to the insecticide as explained above [14, 47].

In the only other study analyzing blood meals of *Anopheles* vectors in Malawi, conducted in Chikwawa district (southern Malawi) during 2002 prior to any mass distribution of insecticide-treated nets, most blood meals were from humans, with relatively few coming from bovine or mixed human-bovine feeding [13]. The 2002 HBI estimates for the three dominant malaria vector species were similar to what was found in the present 2019–2020 study, despite there now being a long history of malaria control and LLIN use. The species-specific HBI estimates for 2002 vs. 2019–2020 were: *An. arabiensis*, 85.0% vs. 92.3%; *Anopheles gambiae* (s.s.), 99.2% vs. 93.5%; and *An. funestus* (s.s.), 99.2% vs. 96.5%. However, the estimated EIR in the present study (33 infectious bites per person per year) was lower than that reported by Mzilahowa et al., (2012) (183 infectious bites per person per year). Both studies used PCR-based detection of sporozoite infection in the head-thorax of individual mosquitoes, although prevalence was lower in the 2002 investigation (4.9%) compared to the present study (16.0%). However, indoor mosquito density was lower at the present study sites, thereby reducing the EIR. Another more recent study in Chikwawa, done during the implementation of a community-based control program, showed that 4 of 91 *Anopheles* (4.4%) tested by PCR were positive for *P. falciparum* infection during the rainy season, with an estimated EIR of 13.5 infectious bites per person per year [48], suggesting a reduced EIR in that region.

Molecular-based approaches to blood meal analysis to detect vertebrate host feeding have advanced since the review of this topic by [18, 25, 44, 49]). At the forefront of this advance has been development of qPCR methods using host-specific probes by either TaqMan or SYBR green detection [18, 50]. However, host species-specific probes

in multiplex qPCR targeting *Anopheles* blood-meal hosts were developed only recently [18]. The use of species-specific probes, designed within a qPCR format here, favored the detection of single and multiple (i.e., mixed) blood meals in this study. By screening all blood meals for human blood, and then analyzing by PCR, amplicon sequencing, and BLAST search matching those blood meals not reacting to the human probe, it was possible to reveal the narrow breadth of dominant hosts being utilized by the *Anopheles* community, and then using qPCR to reveal the extent of multiple feeding. This combinatorial approach indicated that ~ 12% of the blood meals were mixed feeding of human and goat (10.8%), human and dog (1%) or goat and dog (0.2%).

The host-selection analysis showed that, in both sites, two of the *Anopheles* species fed more often on goats than humans in proportion to availability of these hosts. *An. arabiensis* and *An. gambiae* (s.s.) over-selected goats and under-selected humans, while *An. funestus* (s.s.) selected the two hosts about equally (i.e., randomly) in proportion to their availability. These results are not surprising. Although *An. arabiensis* is reportedly more zoophilic, while *An. funestus* (s.s.) is more anthropophilic [51]. Plasticity and or opportunistic tendencies have been observed in various *Anopheles* species [7, 38–41]. The explanation to the relatively higher goat feeding in indoor mosquito samples is consistent with goats being kept indoors at night, and malaria control interventions in the area (LLINs) (*Malawi Malaria Operational Plan FY*, 2018). These two activities provide easy accessibility to goat blood meal and makes it more difficult to access human blood meal due increased mosquito-goat contact and reduced mosquito-human contact [38]. The switch in host utilization is indicated by the reduced access to human blood meal and increase in the utilization of the non-human hosts. The comparable increase in mixed blood meal may also suggest disruption in feeding either due to LLINs' activity or otherwise, pushing the mosquito to get a full blood meal from other hosts. Either way this is one of the few studies in Malawi to report blood meal analysis including detection of mixed blood meals. This study will prompt more research in blood meal studies in Malawi to document the range of blood meal hosts, especially those involving goat blood meal which is rarely reported in literature.

The findings of this investigation suggest important implications for *Plasmodium* transmission and malaria control. Multiple host feeding by some *Anopheles* females might allow for increased survival and reproduction [49, 52]. The presence of multiple malaria vector species that successfully obtain human blood meals could lead to an increased *Plasmodium* transmission by increasing the basic reproductive number (R_o) [42, 53]. More widespread use of LLINs, particularly with PBO, could help reduce transmission, but this intervention alone is unlikely to reduce malaria incidence in this meso-endemic setting to acceptable levels where elimination can be contemplated. Residual *Plasmodium* transmission and weakened intervention efforts [54] are likely to persist into the future.

Conclusion

This study has shown that, in southern Malawi, human blood comprises the bulk of the blood meals of the three species of *Anopheles* vectors, yet dog and goat blood meals are also present and commonly mixed with that of humans. Host selection analysis revealed that goats were over-selected compared to humans in proportion to host availability. This could be a direct result of LLIN usage. The presence of mixed-blood meals showed the adaptability of these vectors to switch hosts to obtain a full blood meal, possibly reducing effectiveness of malaria control interventions. The frequent use of humans as a blood meal source elevates the human blood index and consequently the entomological inoculation rate, sustaining malaria incidence. Some evidence suggests that pyrethroid-based LLINs containing PBO reduced mosquito-human contact, as the HBI was significantly lower at the site using these nets. Nonetheless, estimates of EIR remain high, indicating that transmission is well sustained despite the use of PBO-containing LLINs. Other factors, such as net durability and use may be reducing effectiveness of interventions.

Abbreviations

CDC: Centre for Disease Control; DNA: Deoxyribonucleic acid; EIR: Entomological Inoculation Rate; HBI: Human Blood Index; HLC: Human Landing Catches; ICEMR: Center of Excellence in Malaria Research; LLINs: Long lasting insecticidal nets; NIH: National Institutes of Health; PBO: piperonyl butoxide; RNA: Ribonucleic acid; R_o: Basic reproductive number; s.l.: sensu lato; SR: Sporozoite rate; s.s.: sensu stricto; qPCR: quantitative Polymerase Chain Reaction; WHO: World Health Organization.

Declarations

Ethical consideration

The study was approved by the Institutional Research Board (IRB) at Michigan State University and the University of Malawi, College of Medicine, under the International Center of Excellence in Malaria (ICEMR) research program. Written and informed concept was obtained from participants before data collection.

Consent for publication

Not applicable

Availability of data and materials

Data is available upon reasonable request made to the corresponding author.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

RBM, TM, DM, MW, LC, MKL, and EDW conceptualize, designed, and supervised this research, RBM, JBK and EDW analyzed the data, RBM drafted the manuscript, JBK, LC and EDW revised and finalized the paper. All authors read and approved the final manuscript.

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Figures

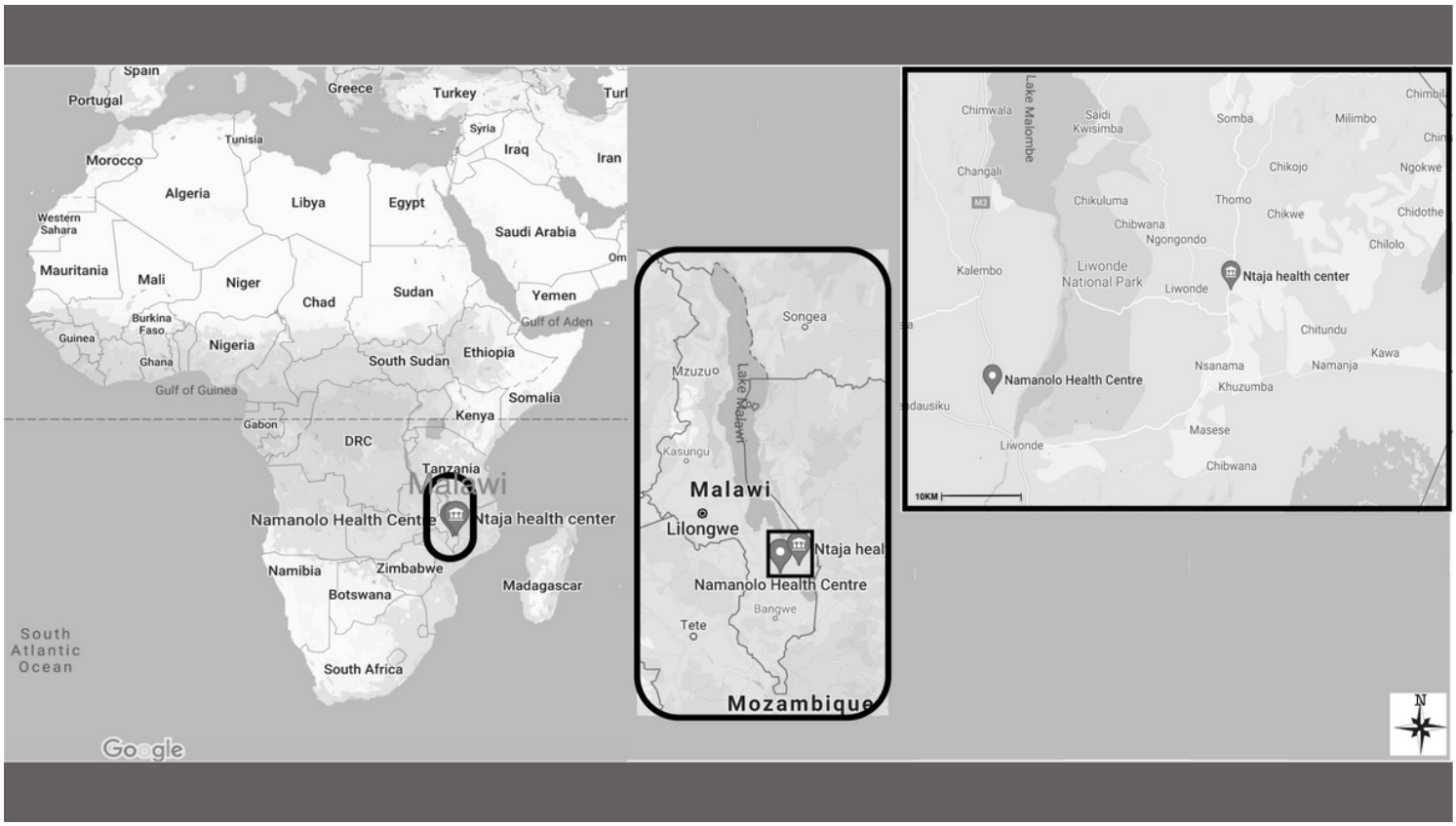

Figure 1

Map of Africa showing the study sites in Namanolo and Ntaja in Malawi.

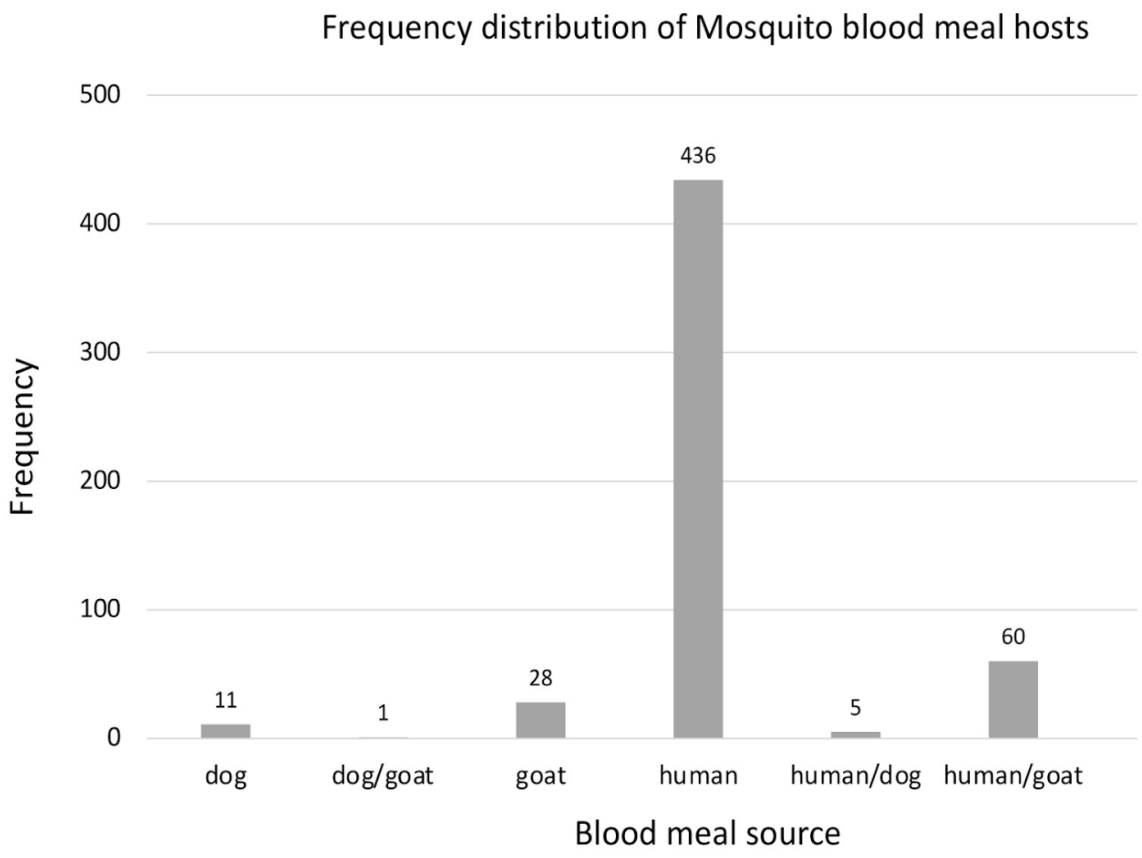

Figure 2

Blood meal identification to vertebrate host, including mixed meals, for Anopheles mosquitoes two sites combined.

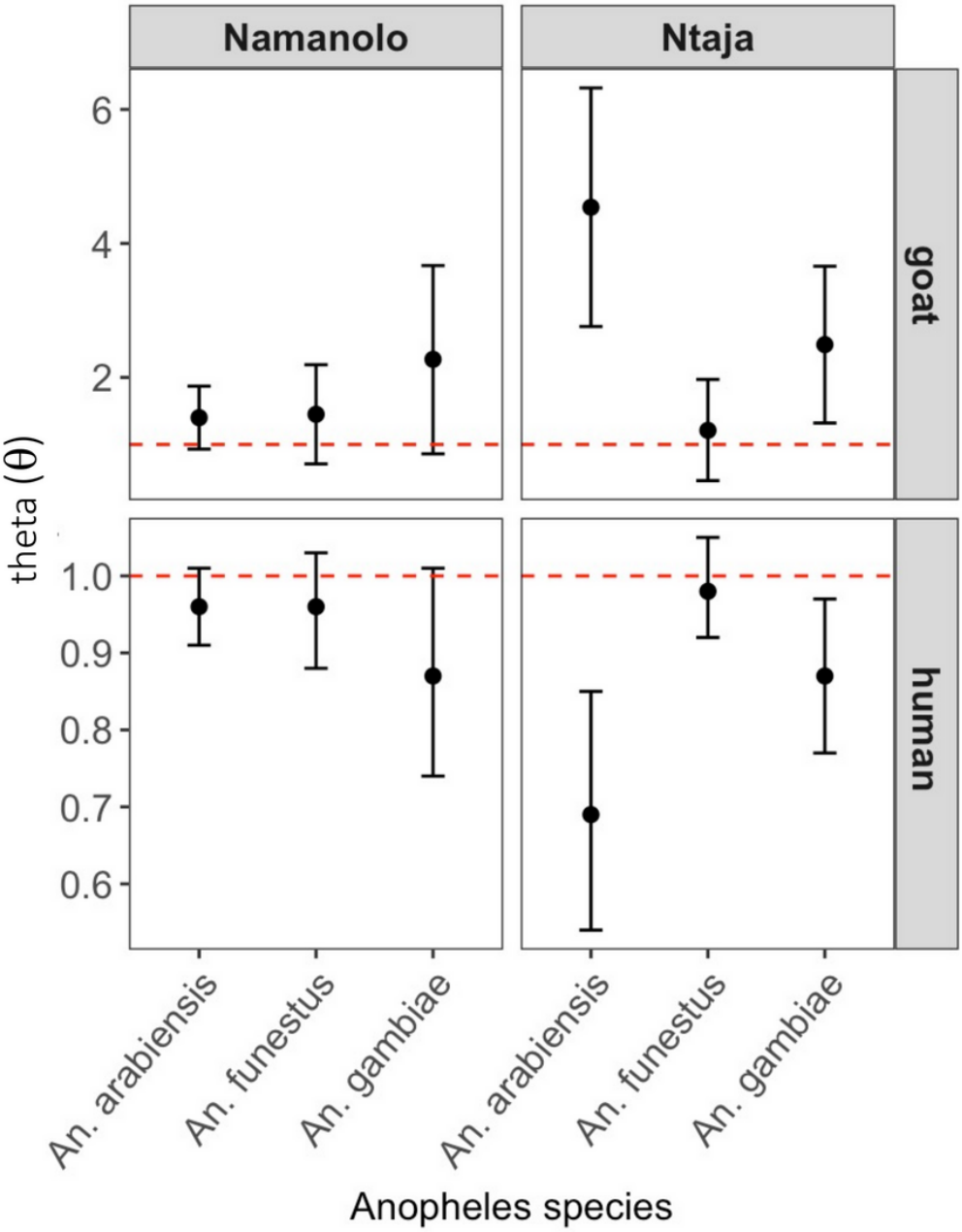

Figure 3

Anopheles species blood meal host selection in relation to number of available hosts. Top panels; goat selection in relation to human, bottom panel; human selection in relation to goats. The black shaded circles are theta values with 95% CI bars. Red dotted line at 1.0 represents random selection in relation to availability of both hosts. The 95% CI bars represent deviation from random selection pointing to over-selection (theta significantly > 1.0) or under-selection (theta significantly < 1.0).