Biting patterns of malaria vectors of the lower Shire valley, southern Malawi.

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Abstract

Assessing the biting behaviour of malaria vectors plays an integral role in understanding the dynamics of malaria transmission in a region. Biting times and preference for biting indoors or outdoors varies among mosquito species and across regions. These behaviours may also change over time in response to vector control measures such as long-lasting insecticidal nets (LLINs). Data on these parameters can provide the sites and times at which different interventions would be effective for vector control. This study assessed the biting patterns of malaria vectors in Chikwawa district, southern Malawi.

The study was conducted during the dry and wet seasons in 2016 and 2017, respectively. In each season, mosquitoes were collected indoors and outdoors for 24 nights in six houses per night using the human landing catch. Volunteers were organized into six teams of two individuals, whereby three teams collected mosquitoes indoors and the other three collected mosquitoes outdoors each night, and the teams were rotated among twelve houses. All data were analyzed using Poisson log-linear models.

The most abundant species were *Anopheles gambiae* sensu lato (primarily *An. arabiensis*) and *An. funestus* s.l. (exclusively *An. funestus* s.s.). During the dry season, the biting activity of *An. gambiae*s.l. was constant outdoors across the categorized hours (18:00 h to 08:45 h), but highest in the late evening hours (21:00 h to 23:45 h) during the wet season. The biting activity of *An. funestus* s.l. was highest in the late evening hours (21:00 h to 23:45 h) during the dry season and in the late night hours (03:00 h to 05:45 h) during the wet season. Whereas the number of *An. funestus*s.l. biting was constant (P = 0.662) in both seasons, that of *An. gambiae*s.l. was higher during the wet season than in the dry season (P = 0.001). *Anopheles gambiae* s.l. was more likely to bite outdoors than indoors in both seasons. During the wet season, *An. funestus* s.l. was more likely to bite indoors than outdoors but during the dry season, the bites were similar both indoors and outdoors.

The biting activity that occurred in the early and late evening hours, both indoors and outdoors coincides with the times at which individuals may still be awake and physically active, and therefore unprotected by LLINs. Additionally, a substantial number of anopheline bites occurred outdoors. These findings imply that LLINs would only provide partial protection from malaria vectors, which would affect malaria transmission in this area. Therefore, protection against bites by malaria mosquitoes in the early and late evening hours is essential and can be achieved by designing interventions that reduce vector-host contacts during this period.

Keywords: Anophelines;Culicines; HLC; Biting, Indoors; Outdoors; Malawi

Highlights

- *Anopheles arabiensis*was more likely to bite outdoors than indoors in our study
- *Anopheles funestus*biting occurred predominantly indoors
- Humans are at risk of being bitten by malaria mosquitoes before going to bed in the evening
- Outdoor-biting anophelines constitute a considerable risk of malaria transmission

1. Introduction

Vector control remains the most effective measure to prevent malaria transmission (WHO 2006, 2017, 2018). The most common methods of malaria vector control in the last 20 years have been the use of indoor residual spraying (IRS), conventional insecticide-treated nets and long-lasting insecticidal nets (LLINs). These methods provide protection against mosquitoes that bite and rest indoors. The effectiveness of LLINs and IRS in reducing malaria vectors relies on the ability of the vectors coming into contact with the insecticides applied either on the nets or on the inner walls of houses (Killeen and Moore 2012). However, some malaria vector species bite outdoors at least as often as indoors (White et al. 1974, Joshi et al. 1975, Highton et al. 1979, Fornadel et al. 2010, Kenea et al. 2016, Kenea et al. 2017). Additionally, prolonged use of LLINs may lead to changes in the biting preferences of malaria vectors from indoors to outdoors (Reddy et al. 2011, Russell et al.

2011, Padonou et al. 2012, Meyers et al. 2016). In both cases, the vectors biting outdoors are less vulnerable to the insecticides applied indoors (LLINs and IRS), and outdoor biting can sustain or enhance the risk of malaria transmission (Gillies 1964, Antonio-Nkondjio et al. 2006, Killeen et al. 2013, Mwangangi et al. 2013, Killeen 2014).

Besides biting location in relation to indoors or outdoors, knowledge about the peak biting times of malaria vectors is also critical for understanding the impact of LLIN use in a given region. It is evident that the biting behaviour of malaria vectors varies across regions (Pates and Curtis 2005). Thus, there is a need for assessing the biting behaviour of malaria vectors to assess the risk of malaria transmission in a given region. Historically, the highest biting activity of primary malaria vectors in Africa was reported to occur indoors from midnight to late night hours (Fontenille et al. 1990, Githeko et al. 1996, Fontenille et al. 1997), and therefore, the use of bed nets gained interest because people sleeping under LLINs would be protected from most potentially infectious bites. Furthermore, these late-night biting mosquitoes would experience high mortality from the insecticide on the net, reducing vector populations. More recently, shifts in the peak biting times of malaria vectors have been reported following large-scale use of LLINs. For example, in Benin, the peak biting time of *An. funestus* populations shifted from 02:00 h to the early morning hours (05:00 h) (Moiroux et al. 2012), and in Senegal the peak biting time of *An. funestus* was observed in the later morning hours (07:00 h to 11:00 h) (Sougoufara et al. 2014). In Tanzania, the biting activity of *An. arabiensis* and *An. funestus* s.s. was in the early night hours (20:00 h to 23:00 h) (Russell et al. 2011). These regions had high LLIN coverage, suggesting that the malaria vectors sought hosts at times when people were not protected by LLINs.

The most direct and favoured method of estimating malaria transmission entomologically is the human landing catch (HLC) (Lines et al. 1991, Service 1993, Davis et al. 1995, Beier 1998, Kline 2006, Govella et al. 2010, Lima et al. 2014). The HLC estimates the peak biting times for vectors, the vectors' indoor/outdoor biting preferences and the number of infectious bites that a single individual can receive per unit time (Charlwood and Graves 1987, Bockarie et al. 1996, Mboera

2005, Pates and Curtis 2005, Oyewole et al. 2007, Bayoh et al. 2014, Sougoufara et al. 2014). Data on these parameters can provide the times at which different interventions would be effective for vector control. In Malawi, the main malaria vectors are *An. gambiae* sensu stricto (s.s.), *An. arabiensis* and *An. funestus* (Spiers et al. 2002, Mzilahowa et al. 2012), but little is known about the biting behaviour of these vectors in the country. This study assessed the vectors' indoor/outdoor biting preferences and the peaks in their biting activities.

2. Methods

2.1. Study site

The study was conducted in two neighbouring villages, Mwalija (-15.96, 34.78) and Njereza (-15.96, 34.77), in Chikwawa District, southern Malawi. The villages are along the low-lying regions that are categorized as hot, wet and humid with high rates of malaria transmission (Kazembe et al. 2006, Kabaghe et al. 2018). Most houses are made of sun-dried or fire-baked bricks with grass-thatched or corrugated iron-sheet roofs. Residents of this region engage mostly in subsistence farming with maize and millet as main crops. The National Malaria Control Programme implemented IRS in Chikwawa District in 2010 and 2012 with alphacypermethrin, and mass distributionsof LLINs wereconducted in 2012 and April 2016.

2.2. Selection of households

The two villages in this study were part of a cluster-randomised control trial assessing the effects of larval source management and house improvement on malaria transmission (McCann et al. 2017). The villages fell under the control arms of the trial (i.e. no larval source management or house improvement were implemented in these two villages).

Inclusion criteria were applied to ensure a degree of uniformity across the houses and these were: houses with grass thatched roofs and open eaves, that were ≥25m apart and ≥ 100m away from any mosquito breeding habitat. Houses that were participating in other mosquito sampling efforts at the time of the current study as part of the cluster-randomised trial referenced above were excluded from the current study. A complete list of households in the two villages was used to randomly select twelve households for the study.

2.3. Mosquito sampling

Mosquito sampling was done during the early months of the dry season (May-June 2016) and following the peak of the rainy season (March-April 2017) using the HLC method (Fig. 1). In each season, the sampling was conducted for 24 nights in 6 of the 12 houses each night. The same houses were used in both seasons. Human volunteers from the study houses were organized into six teams of two individuals. A pair of individuals collected mosquitoes in six houses each night, whereby three teams of HLC volunteers collected mosquitoes indoors, and the other three teams collected mosquitoes outdoors. The collections were from 17:00 h to 09:45 h and were divided into two shifts. The first volunteer in each team sampled mosquitoes from 17:00 h to 01:45 h and the second volunteer sampled from 02:00 h to 09:45 h. Each volunteer was provided with a headlight, wristwatch, pencil, mouth aspirator and mosquito holding containers. Prior to the study, all volunteers were trained in the HLC technique. The volunteers sat on stools exposing the lower part of their legs and collected mosquitoes that landed on their legs. The mosquitoes were placed in holding cups that had been pre-labeled with the house number, hour of collection and location (indoors or outdoors). The volunteers collected mosquitoes for 45 min. and had a 15 min. break within every hour. A research nurse screened the volunteers for malaria on a weekly basisusing a malariarapid diagnostic test (mRDT; SDBioline malaria Ag Pf HRP-2; Standard Diagnostics Inc, Korea). Additionally, all volunteers were provided with doxycycline daily as malaria prophylaxis from one week before the start of the study to one week after the end of the study.

Spot checks were conducted on random days and at random times by the research team and members from a local community watch group. Likewise, sporadic phone calls were made to volunteers' team leaders to check whether there were any challenges.

2.4. Identification of mosquitoes and detection of *Plasmodium falciparum* DNA.

In the laboratory, all mosquitoes were identified morphologically using the protocol by Gillies and Coetzee (1987). All anophelines were classified as *An. gambiae* s.l., *An. funestus* s.l. or *An. tenebrous.* There was no further classification of the culicines beyond the subfamily level. Females from the *An. gambiae* species complex and the *An. funestus* species group were further identified to species level using polymerase chain reaction (PCR) (Scott et al. 1993, Koekemoer et al. 2002, Cohuet et al. 2003), respectively. For the *An. gambiae* species complex, the PCR included species-specific primers for *An. gambiae* s.s., *An. arabiensis*, and *An*. *quadriannulatus*. For the *An. funestus* species group, the PCR included species-specific primers for *An. funestus* s.s., *An. vandeeni*, *An. rivulorum*, *An. rivulorum-like*, *An. parensis*, and *An*. *leesoni*. The heads and thoraces of all female *An. gambiae* s.l. and *An. funestus* s.l. were tested for the presence of *P. falciparum* DNA using real-time polymerase chain reaction (RT-PCR) (Perandin et al. 2004)with a Ct value ≤ 37.0 as the cut-off for *P. falciparum* positive.

2.5. Data analysis

Assuming the Poisson distribution for the count of mosquitoes and applying the log link function to the Poisson rate parameter, generalized linear models were fitted to assess differences: a) in the biting times of mosquitoes, b) in vectors' indoor/outdoor biting preference and c) in the abundance of mosquitoes between seasons. Generalized estimating equations were used to account for repeated measures by house. Each of the differences was assessed in a separate model for each taxonomic group and, subsequently, for the pooled counts of all malaria vectors. The cooking locations, number of people that slept in the house during the night of data collection, use of bed-net and kind of livestock that stayed within 20m of the house during the night of data collection were included as covariates in each of the models. Door and roof types were not included in the analysis because all the doors were made of wood and all roofs were grass-thatched. Cooking locations included: inside the house, on the veranda, outside the house but within 2m, and outdoors at more than 2m from the house. Livestock categories were comprised of cattle, goats, and chickens. As the human volunteers worked for 45 min within every hour, the average bites by mosquitoes were divided by 0.75 to obtain the hourly catch rate. The hourly bites were further categorized as early evening (18:00 h to 20:45 h), late evening (21:00 h to 23:45 h), early night (24:00 h to 02:45 h), late night (03:00 h to 05:45 h) and early morning (06:00 h to 08:45 h). Hourly collections at 17:00 h to17:45 h and at 09:00 h to 09:45 h were low and were not considered in the analysis with the categorical hours. All data were analysed using SPSS Version 20.0. Entomological inoculations rates (EIRs) were estimated by pooling all the catches in all the locations (indoors and outdoors) and calculating the average bites. The averages were divided by 0.75 as earlier explained. This was then multiplied by the sporozoite rate that was estimated using RT-PCR.

3. Results

3.1. Abundance of mosquitoes

3.1. 1. Abundance of mosquitoes during the dry season

Combined across all locations, a total of 1,032 mosquitoes was collected during the dry season. Of these, 25 were males (2 anophelines indoors and 4 outdoors; 11 culicines indoors and 8 outdoors) and 1007 were females. Of the 1007 females, 917 (91%) were culicines (400 indoors, 517 outdoors), 43 (4.3%) were *An. tenebrosus (*25 indoors, 18 outdoors) and 47 (4.7%) were malaria vector species. Of the 47 malaria vectors, 22 (46.8%) were *An. gambiae* s.l. (5 indoors and 17 outdoors) and 25 (53.2%) were *An. funestus* s.l. (16 indoors and 9 outdoors; Table 1). Of the 21 malaria vectors caught indoors, 14 were identified by PCR as *An. arabiensis* (n=4) and *An. funestus* s.s. (n=10). DNA of seven of the twenty-one malaria vectors caught indoors failed to amplify (6 *An. funestus* s.l. and 1 *An. gambiae* s.l.). Of the 26 caught outdoors, 23 were identified by PCR as *An. arabiensis* (n=13), *An. gambiae* s.s. (n=1) and *An. funestus* s.s. (n=9). DNA of three of the twenty-six vectors caught outdoors failed to amplify (3 *An. gambiae* s.l.).

Of the 47 malaria vectors tested for the presence of *P. falciparum* DNA, only one was positive for *P. falciparum* (*An. funestus* s.s.). The sporozoite rate was 2.1% and the EIR was 3.4 infectious bites/person /year

3.1.2. Abundance of mosquitoes during the wet season.

Combined across all locations, a total of 1,408 mosquitoes was collected during the wet season. Of these, 18 were males (1 male anopheline outdoors, 10 culicines indoors and 7 outdoors) and 1390 were females. Of the 1,390 females, 1289 (92.7%) were culicines (568 indoors, 721 outdoors), 10 (1%) were *An. tenebrosus (*1 indoors, 9 outdoors) and 91 (6.5%) were malaria vector species. Of the 91 malaria vectors, 69 (75.8%) were *An. gambiae* s.l. (25 indoors and 44 outdoors) and 22 (24.2%) were *An. funestus* s.l. (17 indoors and 5 outdoors; Table 1). Of the 42 caught indoors, 40 were identified by PCR as *An. arabiensis* (n=18), *An. gambiae* s.s. (n=6) and *An. funestus* s.s. (n=16). DNA of two of the forty-two malaria vectors caught indoors failed to amplify (1 *An. funestus* s.l. and 1 *An. gambiae* s.l.). Of the 49 outdoor malaria vectors, 46 were identified by PCR as *An. arabiensis* (n=36), *An. gambiae* s.s. (n=4), *An. funestus* s.s. (n=5) and a hybrid of *An. arabiensis* and *An*. *gambiae* s.s. (n=1). DNA of three of the forty-nine vectors caught outdoors failed to amplify (3 *An. gambiae* s.l.).

Of the 91 malaria vectors tested for the presence of *P. falciparum* DNA, 4 were positive for *P. falciparum* (3 *An. funestus* s.s. and 1 *An. gambiae* s.s.). The sporozoite rate was 4.4% and the EIR was 13.5 infectious bites/person/year

The abundance of female *An. gambiae* s.l. was lower in the dry season than in the wet season (Risk ratio (RR) = 0.32, 95% confidence intervals (CI) = [0.20-0.52], P = 0.001) but that of female *An. funestus* s.l. did not differ between the two seasons (RR = 1.06, CI = [0.56-2.06], P = 0.854).

3.2. Biting times of mosquitoes

During the dry season, the indoor and outdoor biting by malaria vectors (combined across all species) exhibited bi-modal and uni-modal peaks, respectively. For the indoor biting, the first peak was observed between 21:00 h to 21:45 h and the second peak was at 23:00 h to 23:45 h. For the outdoor biting, the peak was observed between 20:00 h to 20:45 h (Fig.2). Considering each species complex/group separately, the biting activity of *An. gambiae* s.l. was lower indoors than outdoors (RR = 0.29, CI = [0.11-0.80], P= 0.016). The biting activity of *An. gambiae* s.l., outdoors, was constant across all the categorized hours (18:00 h to 08:45 h) ($P \geq 0.05$). Whereas there was no biting activity observed in the early morning hours, indoors, for *An. gambiae* s.l., the biting rates of this species were constant from the late evening hours to the late night hours (21:00 h to 05:45 h) ($P \geq 0.05$) (Fig. 3A). *Anopheles funestus* s.l. biting rates did not differ between indoors and outdoors in the dry season (RR = 1.78, CI = [0.79-4.02], P = 0.167). The biting rate of *An. funestus* s.l. indoors was highest during the late evening hours (21:00 h to 23:45 h) but absent in the early morning hours. The outdoor biting rates of this species were constant from 18:00 h to 05:45 h ($P \geq 0.05$) (Fig. 3B).

During the wet season, the indoor and outdoor biting by malaria vectors (combined across all species) exhibited uni-modal peaks. The highest activity of indoor biting was from 02:00 h to 04:00 h and that of outdoor biting was at 21:00 h (Fig. 2). Similar to the dry season, the biting activity of *An. gambiae* s.l. in the wet season was lower indoors than outdoors (RR = 0.57, CI = [0.35-0.93], P = 0.024). Outdoors, the peak biting time of *An. gambiae* s.l. occurred in the late evening hours (21:00 h to 23:45 h) and this biting activity was higher than that observed in the early evening hours

(P = 0.001), early night hours (P = 0.037) and late night hours (P = 0.001). The indoor biting rates of *An. gambiae* s.l. in the wet season were constant from 18:00 h to 05:45 h (P ≥ 0.05) (Fig. 3A). *Anopheles funestus* s.l. was more likely to bite indoors than outdoors in the wet season (RR= 3.4, CI = [1.25-9.22], P = 0.016). The peak biting time of *An. funestus* indoors in the wet season was in the late night hours (03:00 h to 05:45 h) and was similar to the biting activity that was observed in the early night hours (P = 0.317) but different from the biting activities in the early evening hours (P = 0.021) and in the late evening hours (P = 0.021). The outdoor biting rates of *An. funestus* s.l. were constant from 21:00 h to 05:45 h (P ≥ 0.05) (Fig. 3B).

The biting activity of female culicines was lower in the dry season than in the wet season (RR = 0.65, CI = [0.60-0.71], P = 0.001). Indoor culicine biting rates were lower than the outdoor biting rates in the dry (RR = 0.85, CI = [0.74-0.97], P = 0.014) and wet (RR = 0.8, CI = [0.72-0.89], P = 0.001) seasons (Fig. 4).

The number of people that slept in the house each night, bed net use, cooking locations, presence of cattle, goats and chicken did not influence the biting activity of *An. gambiae* s.l. indoors or outdoors, during both seasons. This was the same for *An. funestus* s.l.with the exception that the presence of chickens was positively associated with the biting activity of this species (Table 2).

4. Discussion

The malaria vectors identified in this study were *An. gambiae* s.l. (primarily *An. arabiensis*) and *An. funestus* s.l. (exclusively *An. funestus* s.s.). Whereas the density of *An. funestus* s.s. was constant in both seasons of this study, the density of *An. gambiae* s.l. was higher in the wet season than in the dry season. In the dry season, the biting activity of *An. gambiae* s.l. was constant across the categorized hours, outdoors, but highest in the late evening hours (21:00 h to 23:45 h) during the wet season. During the dry season, the biting activity of *An. funestus* s.s. was highest in the late evening hours, while in the wet season, the peak biting activity of this species was in the late night hours (03:00 h to 05:45 h). Furthermore, *An. arabiensis* was more likely to bite outdoors than indoors in both seasons, though some biting by this species also occurred indoors.

Previous studies in this region of Malawi conducted in the early 2000s identified three species of malaria vectors: *An. funestus* s.s., *An. gambiae* s.s. and *An. arabiensis* (Spiers et al. 2002, Mzilahowa et al. 2012). The current study identified these same three species, but *An. gambiae* s.s. accounted for only 2% and 10% of the malaria vectors collected in the dry and wet seasons, respectively. This low density of *An. gambiae* s.s. relative to that of *An. arabiensis* and *An. funestus* s.s. agrees with other recent studies in this area (Kabaghe et al. 2018) and warrants further investigation. Generally, similar to the present findings, the densities of malaria vectors in this region have been low with *An. arabiensis* accounting for a sporozoite rate of 5.4% (Kabaghe et al. 2018).

The biting activity by *An. tenebrosus* in both seasons was surprising, as little is known about this species. This species has not been incriminated as a malaria vector (Gillies and De Meillon 1968), though it is closely related to *An. coustani* (Gillies and Coetzee 1987*)*. However, in Tanzania, *An. tenebrosus* was reported with infective larvae of *Dirofilaria immitis* (Gillies and Coetzee 1987), and therefore, it may be a species of medical importance.

Currently, *An. arabiensis* and *An. funestus* s.s. may be considered the primary malaria vectors n southern Malawi. Furthermore, the density of *An. gambiae* s.l. was higher during the wet season than in the dry season, while that of *An. funestus* s.l. was constant in both seasons, similar to previous studies from Mozambique, Malawi and Tanzania (Mendis et al. 2000, Mzilahowa et al. 2012, Finda et al. 2018), and highlighting the different impacts of seasonality on the abundance of different mosquito species. In the case of *An. gambiae* s.l. and *An. funestus* s.s., this difference may reflect differences in the preferred larval habitats of each species. While *An. funestus* s.s. typically inhabits more permanent water bodies during its immature stages, *An. gambiae* s.l. is able to use the more temporary larval habitats that occur more often in the wet season (Gimnig et al. 2001, Mutuku et al. 2009).

Anopheles arabiensis was more likely to bite outdoors than indoors in this study, both in the dry and wet season. This species is considered as a dominant malaria vector in neighbouring southern Zambia (Kent et al. 2007, Fornadel et al. 2010) and has been associated with outdoor biting in other regions (Mendis et al. 2000, Tirados et al. 2006, Geissbühler et al. 2007, Oyewole et al. 2007, Russell et al. 2011). The biting densities of *An. funestus* s.s. were higher indoors than outdoors in the wet season, confirming that this species is predominantly endophagic (Awolola et al. 2003, Antonio-Nkondjio et al. 2006, Mwangangi et al. 2013). However, in the dry season, there was no difference between the indoor and outdoor biting densities of *An. funestus* s.s. In other regions, outdoor biting has been associated with the relative availability of hosts outdoors, when they were sleeping in the courtyards or on the verandas of their houses (Faye et al. 1997). Although the current study did not quantify host availability, some people in the region sleep outdoors during the dry season because of higher temperatures as compared to the wet season. During the rainy season, most people in this region sleep indoors, when many are protected by LLINs. Their exposure to mosquito bites would, therefore, occur mostly at times when they are outdoors in the early evening hours. In this context, outdoor biting activities by both *An. arabiensis* and *An. funestus* s.s. are important factors to consider when selecting and planning malaria control interventions. Because LLINs and IRS target indoor biting vectors, there is a need for additional tools that can provide protection against outdoor biting (Govella and Ferguson 2012, Russell et al. 2013, Killeen et al. 2016).

Studies prior to the large-scale introduction of bed nets in Africa found that the major malaria vectors, *An, gambiae* s.s., *An. arabiensis* and *An. funestus*, are nocturnal with peak biting activity occurring in the late night hours (usually from 23:00 h or 24:00 h to 06:00 h) (Fontenille et al. 1990, Githeko et al. 1996, Fontenille et al. 1997, Pates and Curtis 2005). We refer to this biting as the historic biting time of malaria vectors. These historic biting times coincide with hours that people are usually asleep, which is integral to the effectiveness of LLINs to protect sleepers from infectious bites by malaria vectors. However, some studies have found peak biting activity of malaria vectors outside of these historic biting times. For example, the peak biting activity of *An. arabiensis* in Ethiopia was reported in the early evening hours (19:00 h to 20:00 h), both before and after the implementation of LLINs (Yohannes et al. 2005, Yohannes and Boelee 2012). Such variation in the historic biting times may be explained by regional differences. More recently, in some regions the peak biting times of malaria vectors have been observed outside of the historic biting times, with biting in the early evening (Reddy et al. 2011, Russell et al. 2011) or morning hours (Reddy et al. 2011, Moiroux et al. 2012, Sougoufara et al. 2014). Most of these studies lack data on the biting times of malaria vectors in their specific study sites before the implementation of LLINs (Reddy et al. 2011, Sougoufara et al. 2014) but the high levels of reported LLIN use support the hypothesis that it is possible for malaria vector populations to shift peak biting times to avoid LLINs. In the present study, the biting activities of *An. gambiae* s.l. in the early and late evening hours in the dry and wet season, respectively, and *An. funestus* s.l. in the dry season, also differ from the historic biting times of malaria vectors but are similar to results from studies in Ethiopia (Yohannes and Boelee 2012), Mozambique and Tanzania (Mendis et al. 2000, Geissbühler et al. 2007, Russell et al. 2011). One potential explanation for the observed peak biting time could be that the temperatures are cooler in the late evening hours in this part of Malawi compared to regions closer to the equator, resulting in the activation of the mosquitoes' host-seeking behaviour (Silver 2008). On the other hand, it could be that *An. gambiae* s.l. had limited access to humans at times when people are protected by LLINs as observed in other regions (Charlwood and Graves 1987, Yohannes and Boelee 2012). Regardless of the explanation, our finding of outdoor biting has implications for malaria control in the region because the biting coincides with the times at which many individuals may still be active and therefore unprotected by LLINs. While the observed biting activity of *An*. *funestus* s.l.in the early night hours during the wet season suggests that LLIN use still provides significant protection from malaria transmission, the reported levels of insecticide resistance in *An. funestus* populations in Malawi (Riveron et al. 2015, Mzilahowa et al. 2016) raises further concerns about the long-term effectiveness of LLINs as an intervention.

The biting activity of female culicines was constant from the early evening hours to the late night hours both indoors and outdoors. These mosquitoes are a nuisance and have been implicated as vectors of other diseases. In the present study area, filariasis is prevalent (Nielsen et al. 2002, Ngwira et al. 2007) and culicine species have been reported with infective filarial larvae (Merelo-Lobo et al. 2003) highlighting the need for vector control tools that can also target these mosquitoes.

The use of LLINs is effective against indoor biting in the early and late night hours when many individuals are likely to be asleep. However, the observed biting in the early and the late evening hours before people would be under LLINs, both indoors and outdoors, is a major concern. Future research should incorporate the behaviour of people when assessing the biting patterns of mosquitoes (Monroe et al. 2019). Measuring the behaviour of people alongside mosquito biting behaviour would allow quantification of when and where the two behaviours actually overlap in time and space,and would provide a better understanding of the gap in protection left by current vector control tools. In addition to identifying when and where human-vector contact occurs, it is also important to understand who and why, as the proportion of people spending time indoors or outdoors varies across regions, seasons and economic and social activities (Monroe et al. 2019). Identifying these risk factors is critical for closing any gap in protection against malaria transmission.Potential complementary tools to tackle early biting both indoors and outdoorshave been highlighted by Ferguson et al. (2010) and Williams et al. (2018). For instance, house improvement protects all individuals in a house equally. This is being assessed in a number of regions (Killeen et al. 2017b, McCann et al. 2017) as well as the use of insecticide-impregnated tubes along the eaves, which are the preferred entry points for mosquitoes (Knols et al. 2016, Sternberg et al. 2016, Oumbouke et al. 2018). The development of protective measures that divert malaria vectors from human beings to alternative hosts like cattle is important, especially for species with an opportunistic host-feeding behaviour such as *An. arabiensis*. However, such measures would still sustain the densities of biting malaria vectors and therefore, as suggested by Killeen et al. (2017a), the use of insecticide-treated cattle could be more effective in reducing the density of biting malaria vectors. Other complementary measures that would reduce the densities of biting malaria vectors significantly include the use of insecticide-treated clothes (Kimani et al. 2006, Banks et al. 2014), larval source management and the 'push-pull' approach, which is directed at adult vectors and can be implemented either by the of use of attractive toxic sugar baits (Müller et al. 2010, Beier et al. 2012) or by use of attractants and repellents in traps (Menger et al. (2014), Menger et al. 2016).

5. Conclusion

A considerable proportion of the biting by malaria vectors in this study, both indoors and outdoors, occurred at times in the evening when many people are likely still active and not protected by bed nets. This behaviour is likely to enhance malaria transmission. The development of vector control tools that can tackle the biting activity in the early and late evening hours, both indoors and outdoors, is highly recommended because the current,mostly indoor-based tools provide only partial protection against bites by malaria vectors. (Govella and Ferguson, 2012; Killeen et al., 2016; Russell et al., 2013).

Abbreviations

HLC: Human landing catch; LLINs: Long-lasting insecticidal nets; IRS: Indoor residual spraying; PCR: Polymerase chain reaction; mRDT: malaria rapid diagnostic test.

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Declarations

Ethical approval and consent to participate

This study was approved by the College of Medicine Research and Ethics Committee in Malawi (proposal number P.03/16/1901). Written permission to conduct the study was provided by the District Health Officer of Chikwawa District, southern Malawi. The purpose and procedures of the study were explained in the local language, Chichewa, to local leaders, community watch-team, participating community members, and HLC volunteers. Heads of households and HLC volunteers were only enrolled in the study after providing written consent prior to the start of the study. An impartial witness was present in cases where the head of the household was illiterate.

Consent for publication

Not applicable.

Availability of data and materials

The datasets for this study are available upon a reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

MM, RM, WT conceived the study design. MM, HvdB, TM, RM, and WT were involved in the design and implementation of the study. MM and DC did the molecular work. MM, BA, RM and WT contributed to data analysis. MM wrote the first draft of the manuscript. All authors contributed to the writing of the final manuscript. All authors read and approved the final manuscript.

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Table and Figure legends

Table 1: Mosquito collection during the dry and wet seasons.

Table 2: Effect of covariates on the biting activity of *An. gambiae* s.l. and *An. funestus* s.l.

Fig. 1: Typical house in the present study region (a) and HLC method (b)

Fig 2: Mean number of bites per hour by female anophelines both indoors and outdoors during the dry and wet seasons.

Fig. 3: Mean number of bites (95% CI) per category by female *An. gambiae* s.l. (A) and *An. funestus* s.l. (B) both indoors and outdoors during the dry and wet seasons.

Fig 4: Mean number of bites (95% CI) per category by female culicines both indoors and outdoors during the dry and wet seasons.

Table 1: Mosquito collection during the dry and wet seasons

Mosquito collection	**Indoors**		**Outdoors**		**Totals**	
	Dry season	Wet season	Dry season	Wet season	Dry season	Wet season
No. of nights	72	72	72	72	144	144
An. arabiensis	4	18	13	36	17	54
An. gambiae s.s.	0	6	1	4	1	10
An, arabiensis/An. gambiae s.s *(Hybrid)*	0	0	0	1	0	1
An. gambiae s.l (no amplification)	1	1	3	3	4	4
An. funestus s.s.	10	16	9	5	19	21
An. funestus s.l (no amplification)	6	1	0	0	6	1
An. tenebrosus	25	1	18	9	43	10
Female culicines	400	568	517	721	917	1289
Male Anophelines	2	0	4	1	6	1
Male culicines	11	10	8	7	19	17

Table 2: Effect of covariates on the biting activity of *An. gambiae* s.l. and *An. funestus* s.l.

Treatment	**Dry season**		**Wet season**	
	RR	**95% CI**	**RR**	**95% CI**
***An. gambiae* s.l.**				
Indoors	0.29	0.11-0.80	0.57	0.35-0.93
Outdoors	Ref	–		–
People that slept in the house the previous night	0.86	0.61-1.21	1.05	0.83-1.32
Mosquito control-bed-net	1.83	0.4-8.43	0.82	0.43-1.57
Mosquito control-none	Ref	–	Ref	–
Cooking inside the house	-	-	2.27	0.63-8.25
Cooking on the veranda	0.83	0.23-2.97	1.89	0.70-5.08
Cooking outside, within 2m of the house	1.02	0.34-3.08	1.27	0.47-3.40
Cooking outside, more than 2m from the house	Ref	–	Ref	–

Cow	1.27	0.16-9.91	1.68	0.78-3.62
Goat	-	-	1.09	0.56-2.14
Chicken	1.77	0.66-4.8	1.19	0.71-2.01
***An. funestus* s.l.**				
Indoors	1.78	0.79-4.02	3.4	1.25-9.22
Outdoors	Ref	–	Ref	–
People that slept in the house the previous night	0.47	0.30-0.74	0.57	0.32-1.02
Mosquito control-bed-net	1.40	0.31-6.44	1.39	0.33-5.85
Mosquito control-none	Ref	–	Ref	–
Cooking inside the house	2.44	0.56-10.69	0.21	0.02-2.23
Cooking on the veranda	0.63	0.12-3.41	0.48	0.15-1.55
Cooking outside, within 2m of the house	1.39	0.47-4.07	0.17	0.04-0.71
Cooking outside, away from 2m of the house	Ref	–	Ref	–
Cow	1.57	0.34-7.38	2.73	0.70-10.59
Goat	0.90	0.31-2.63	0.61	0.11-3.40
Chicken	7.42	2.61-21.11	3.85	1.43-10.34

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a b

Fig. 1: Typical house in the present study region (a) and HLC method (b)

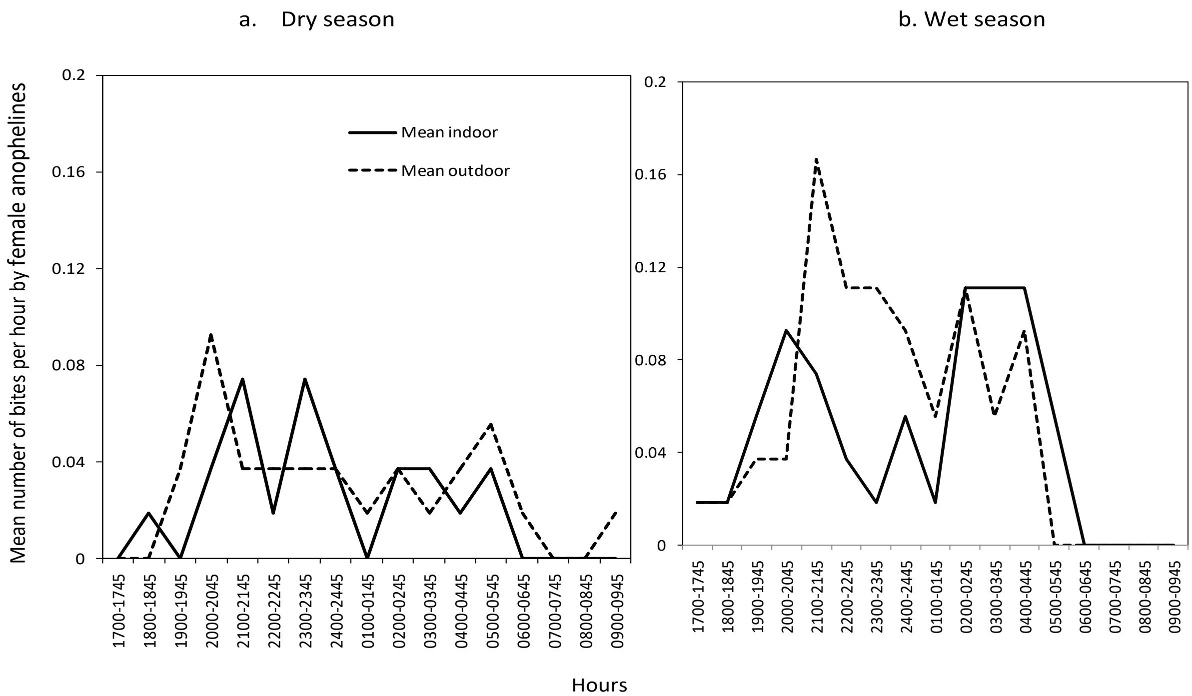

Fig 2: Mean number of bites per hour by female anophelines both indoors and outdoors during the dry and wet seasons.

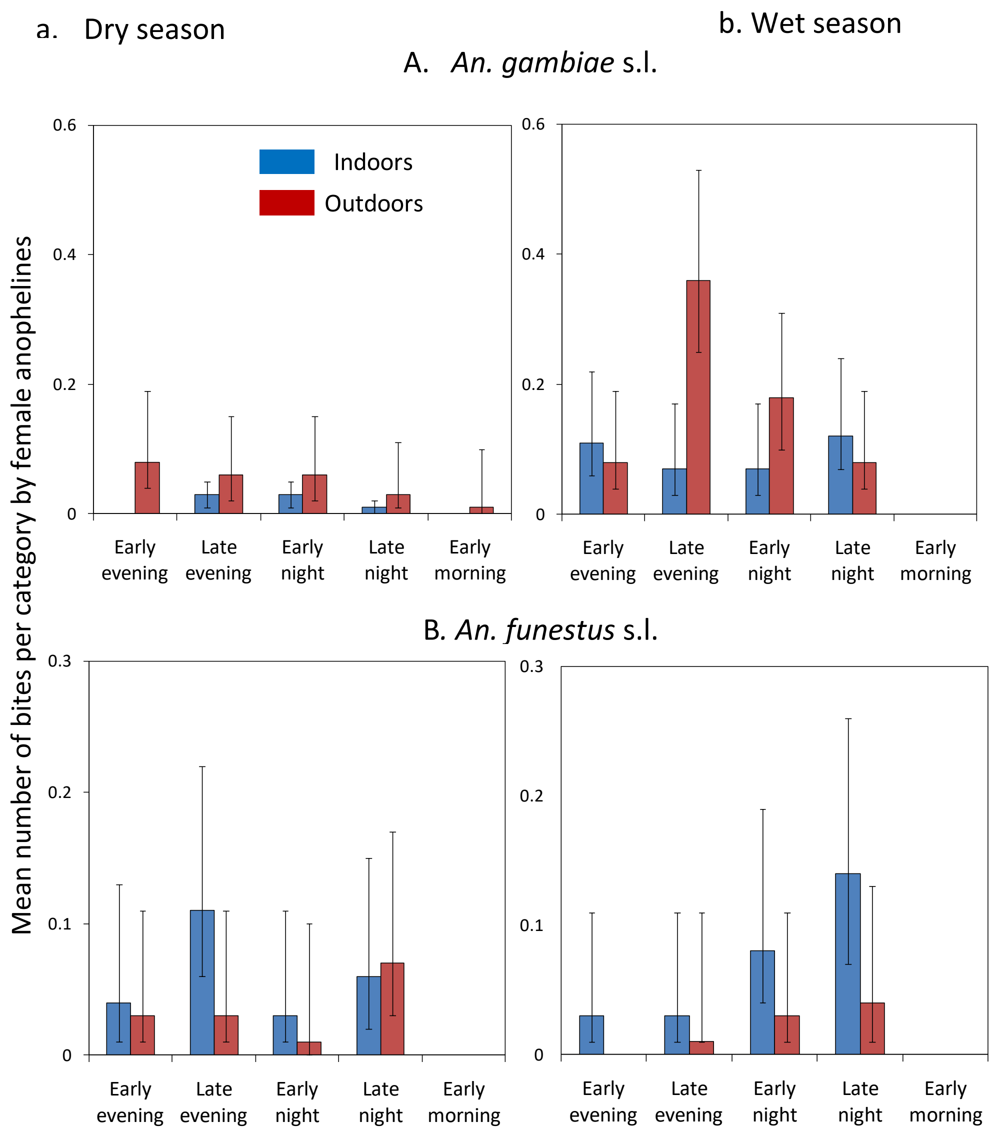

Fig. 3: Mean number of bites (95% CI) per category by female *An. gambiae* s.l. (A) and *An. funestus* s.l. (B) both indoors and outdoors during the dry and wet seasons.

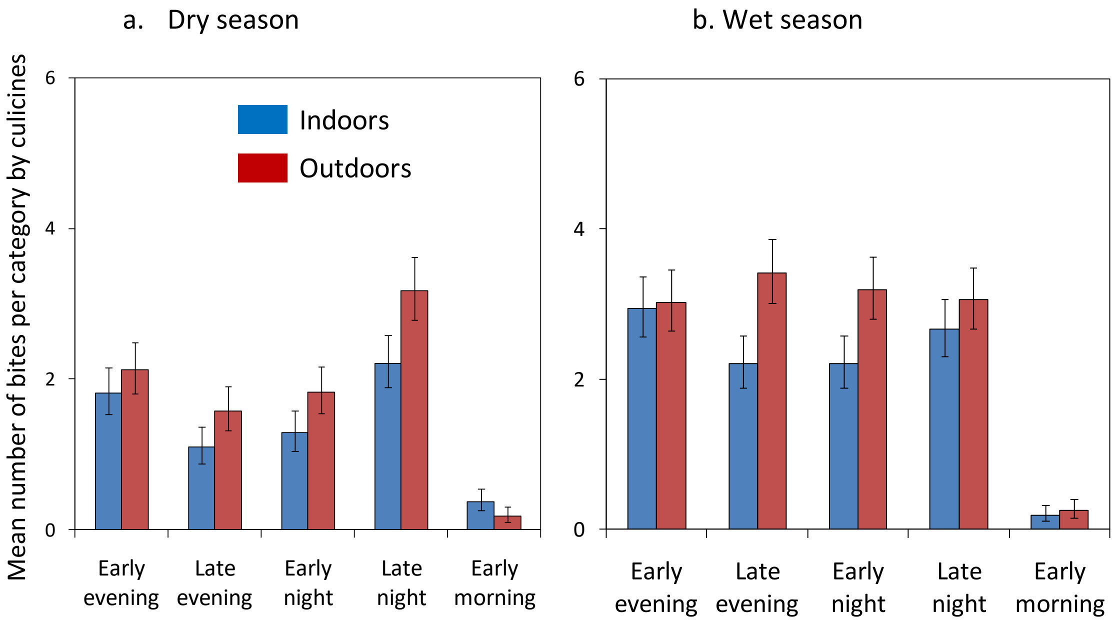

Fig 4: Mean number of bites (95% CI) per category by female culicines both indoors and outdoors during the dry and wet seasons.