RESEARCH ARTICLE

Entomological determinants of malaria transmission in Kayin state, Eastern Myanmar: A 24-month longitudinal study in four villages [version 1; referees: 2 approved with reservations]

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Abstract

Background: The Thailand-Myanmar borderland is an area endemic for malaria where transmission is low, seasonal and unstable. The epidemiology has been described but there is relatively few data on the entomological determinants of malaria transmission.
Methods: As part of a pilot study on Targeted Malaria Elimination, entomological investigations were conducted during 24 months in four villages located in Kayin state, Myanmar. *Anopheles* mosquitoes were identified by morphology, and molecular assays were used in order to discriminate between closely related sibling species of malaria vectors. *Plasmodium* infection rate was determined using quantitative real-time PCR.
Results: The biodiversity of *Anopheles* entomo-fauna was very high and multiple species were identified as malaria vectors. The intensity of human-vector contact (mean human-biting rate= 369 bites/person/month) compensates for the low infection rate in naturally infected populations of malaria vectors (mean sporozoite index= 0.4 and 1.7 /1,000 mosquitoes for *P. falciparum* and *P. vivax* respectively), yielding intermediary level of transmission intensity (mean entomological inoculation rate= 0.13 and 0.64 infective bites/person/month for *P. falciparum* and *P. vivax,* respectively). We estimated that 65% of the potential infective bites are not prevented by mosquito bed nets because of outdoor and early biters.
Conclusion: This study provided a unique opportunity to describe the entomology of malaria in low transmission settings of Southeast Asia. Our data

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are important in the context of malaria elimination in the Greater Mekong Subregion.

Keywords
Anopheles, human biting rate, sporozoite index, entomological inoculation rate, parasite load, residual transmission, Plasmodium juxtanucleare, zoophagy index.

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Introduction

For the last two decades, important progress has been made in order to control the global burden of malaria[1]. Unfortunately, artemisinin-resistant strains of *Plasmodium falciparum* have emerged and spread over the entire Greater Mekong Subregion[2]. Multi-drug resistant parasites that are now spreading in Cambodia are a major risk of disease resurgence[3]. Clinical cases have declined in the Greater Mekong Subregion in recent years and it may still be possible to rapidly eliminate malaria, before this trend is reversed by drug resistance[4].

Entomological aspects of malaria transmission are important in the context of elimination as they largely determine intervention design and outcome. For example, the interest of treating asymptomatic infections with mass drug administration or mass screening and treatment obviously depends on the contribution of asymptomatic carriers to the transmission[5,6]. In the field of vector-control, the efficacy of long-lasting insecticide-impregnated mosquito bed nets (LLINs) is greatly influenced by the host seeking behaviour of malaria vectors[7,8].

As part of a pilot study on targeted malaria elimination, entomological investigations were conducted for two years in four villages located on the Myanmar side of the Thailand-Myanmar border. In this area, the transmission of *P. falciparum* is low, seasonal and unstable[9]. Some entomological surveys have been conducted previously, most of them on the Thai side of the border where the transmission of *P. falciparum* is now interrupted[10–14]. Efficient vectors belong to the Minimus Complex (Funestus Group), Maculatus Group and Dirus Complex (Leucosphyrus Group)[11,13]. *Anopheles aconitus* (*s.s.*) (Aconitus Subgroup, Funestus Group), and some members of the Annularis and Barbirostris Groups also play a secondary role in the transmission[15,16]. Numerous aspects of malaria vectors ecology and biology have not been documented and the characteristics of the entomological indices are not known precisely.

The objective of this paper is to describe the entomological determinants of malaria transmission on the Thailand-Myanmar border in order to guide policy making for malaria elimination.

Methods

Study sites

Four villages were included in the study, namely TPN (17° 14' N, 98 ° 29' E), TOT (16° 36' N, 98° 57' E), KNH (17° 18 'N, 98° 24' E) and HKT (16° 85 'N, 98° 47' E) (Figure 1). Study villages were hotspots of malaria transmission (*i.e.* villages with a high prevalence of submicroscopic infection) located on the Myanmar

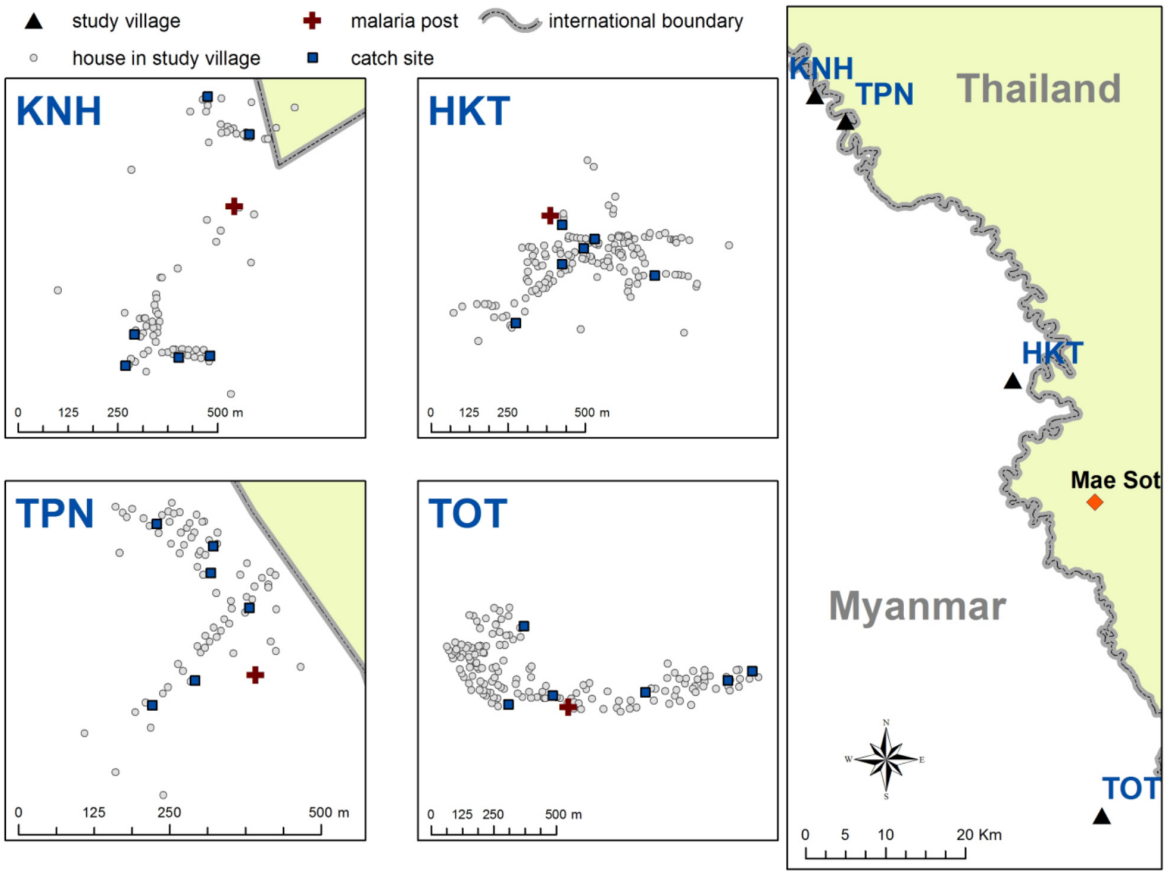

Figure 1. Map of the study sites.

side of the Thailand-Myanmar border. The demographics and malaria epidemiology in the study villages have been described in detail previously[17].

Entomological surveys

Villages were surveyed monthly from 2013 to 2015 for a total of 21 surveys per village. Entomological surveys consisted of five consecutive nights of collection from 06:00 pm to 06:00 am in six sites per villages as described previously[14]. In each village, five traditional houses were selected for mosquito sampling the using human-landing catch (HLC) collection method. In each house, one mosquito collector sat indoors and one mosquito collector sat outdoors, yielding a total of 50 person-nights of collection per survey (25 person-nights indoors and 25 person-nights outdoors). Collectors were asked to collect every mosquito landing on their uncovered legs for 50 min per hour and allowed to rest for 10 min per hour. The sixth collection site was used to set-up the cow bait trap (CBT), yielding an additional five cow-nights of collection per survey. Briefly, one cow was isolated from the herd and a 1m-wide mosquito net was fenced around the animal, 30cm above the ground level. One collector was asked to collect mosquitoes resting on the net for 50 min per hour and allowed to rest for 10 min. Mosquitoes were shipped at the Shoklo Malaria Research Unit (Mae Sot, Thailand) at the end of each night of collection.

Laboratory procedures for the processing of entomological samples

Mosquitoes were immediately identified at the genus level by morphology and *Anopheles* specimens were stored individually at -20°C in 1.5 mL plastic tubes containing silica gel. *Anopheles* mosquitoes were identified at the Group or Complex level using the key developed by Rattanarithikul *et al.*[18]. Deoxyribonucleic acid (DNA) was extracted from head/thorax using a cetyltrimethyl ammonium bromide-based method described previously[19]. Sibling species to the Funestus, Maculatus and Leucosphyrus Groups were discriminated using allele-specific polymerase chain reaction (AS-PCR) assays adapted from Garros *et al.* and Walton *at al.*[20–22]. In case AS-PCR yielded a negative result, identification at the species level was performed by sequencing the internal transcribe spacer 2 (ITS2) mitochondrial marker using universal primers described by Beebe and Saul[23]. DNA extracts were screened for the presence of *Plasmodium* sporozoites using a quantitative real-time PCR (qrtPCR) assay adapted from Mangold *et al.*[24]. Specificity of the signal was confirmed in all positive samples by amplifying a different PCR DNA target with primers described by Cunha *et al.*[25]. In case the confirmation assay yielded a negative result, the PCR product of the screening assay was sequenced (BioSample accessions: SAMN09845988, SAMN09845989, SAMN09845990, SAMN09845991, SAMN09845992). The validation of the qrtPCR assays used for *Plasmodium* detection in this study has been published elsewhere[19]. Detailed laboratory procedures are presented in Supplementary File 1.

Data analysis

HBR and CBR were defined as the number of mosquitoes collected divided by the number of host-nights of collection (person-nights or cow-nights). Results were expressed as a number of bites/host/month. Sporozoite index (SI) was defined as the number of mosquitoes positive in qrtPCR *Plasmodium* divided by the total number of mosquitoes analyzed. Results were expressed as a number of infected mosquitoes /1,000 mosquitoes analyzed. Entomological inoculation rate (EIR) was defined as the number of positive in qrtPCR *Plasmodium* divided by the corresponding number of person-nights of collection, adjusted over the proportion of collected mosquitoes that were analyzed by qrtPCR *Plasmodium*. Results were expressed as a number of infective bites/person/month. The exophagy index (EI) was defined as the number of mosquitoes collected by outdoor HLC over the total number of mosquitoes collected by indoor and outdoor HLC. The zoophagy index (ZI) was defined as the number of mosquitoes collected on CBT divided by the total number of mosquitoes collected by CBT and HLC (the number of mosquitoes collected by HLC was divided by 10 in order to take into account the 1:10 ratio between the number of cow-nights of collection and the number of person night of collection in this study). Confidence Intervals (CIs) for Poisson counts (HBR, CBR and EIR) were estimated using the exact method of the poisson.conf.int() function in the *epitools* package version 05-10 of R software version 3.3. Binomial CIs were estimated for proportions (SI, EI and ZI) using the exact method of the binom.confint() function in the *binom* package version 1.1-1 of R software version 3.3.

Ethics approval

The protocol for mosquito collection and analysis has been approved by the Oxford Tropical Research Ethics Committee (1015–13, dated 29 Apr 2013) and by the Ethics Review Committee for Research Involving Human Research Subjects, Health Science Group, Chulalongkorn University (COA 154/2014). All participants provided their written consent to participate in this study. This consent procedure was approved by the ethics committees.

Results

Biodiversity of the *Anopheles* entomo-fauna

In total, 129,228 *Anopheles* mosquitoes were collected during 4,120 person-nights and 412 cow-nights of collection (63,217 by HLC and 66,011 by CBT). We report the occurrence of 10 Groups of *Anopheles* on the basis of morphological identification: Barbirostris, Hyrcanus (*Anopheles* Subgenus, Myzorynchus Series), Annularis, Jamesii, Maculatus (*Cellia* Subgenus, Neocellia Series), Funestus (*Cellia* Subgenus, Myzomyia Series), Kochi, Leucosphyrus, Tessellatus (*Cellia* Subgenus, Neomyzomyia Series) and Subpictus (*Cellia* Subgenus, Pyretophorus Series). *Anopheles karwari* (*Cellia* Subgenus, Neocellia Series) was also detected at a low frequency. Less than 5% (6,010/129,228) of the specimens could not be identified at the Group level because they were damaged (missing legs or wings).

Potential malaria vectors from the Annularis, Barbirostris, Funestus, Leucosphyrus and Maculatus Groups accounted for >80% and >40% of the *Anopheles* mosquitoes collected by HLC and CBT collection methods respectively (Figure 2). The Funestus Group was the most abundant taxa during both the rainy and dry seasons (June to November and December to May, respectively). Maculatus and Leucosphyrus Groups were mainly collected during the rainy season. The abundance of Annularis and Barbirostris Groups peaked during the transition

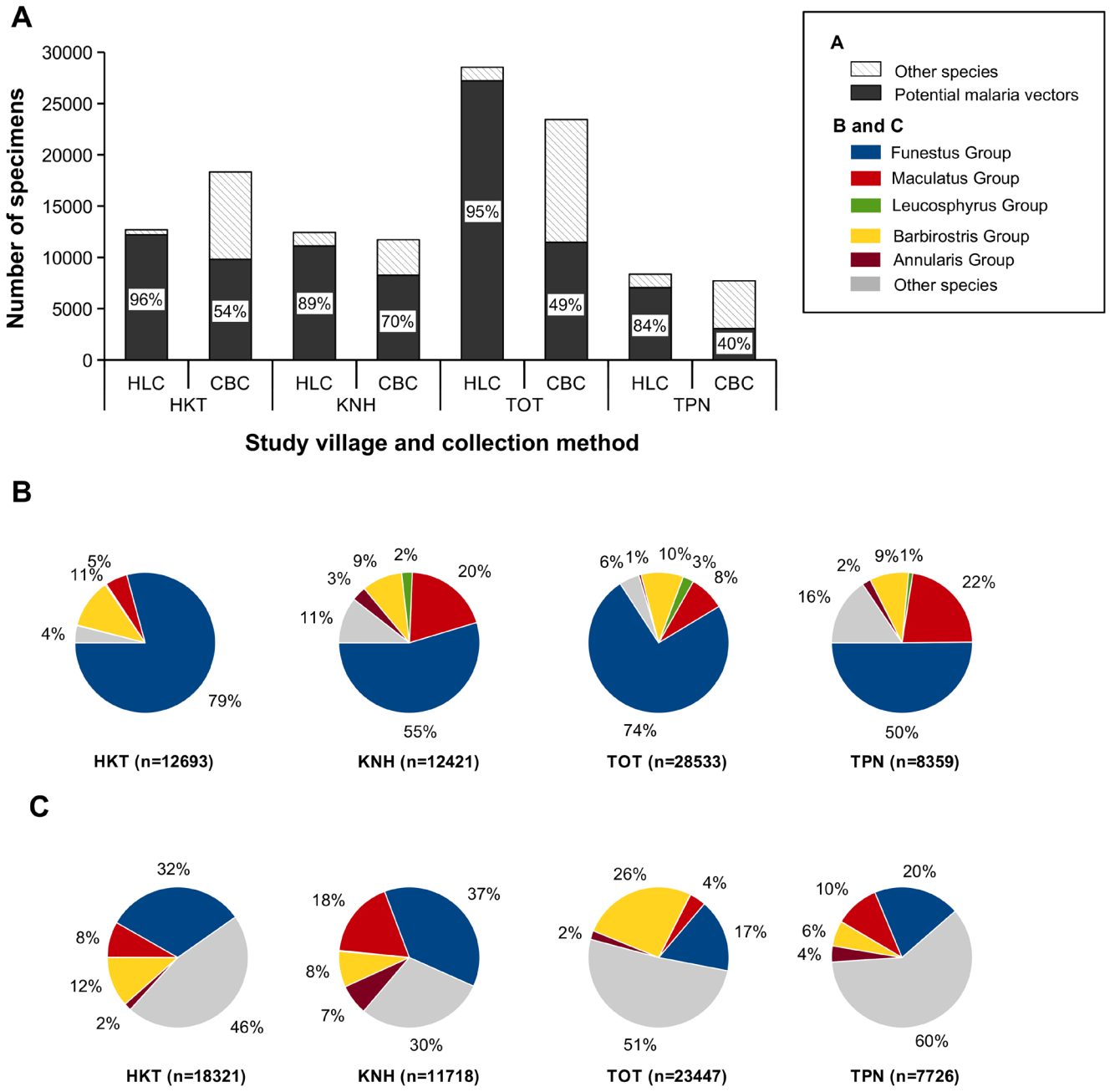

Figure 2. Biodiversity of the *Anopheles* mosquitoes according to the collection method and study village. A) Proportion of malaria vectors in *Anopheles* populations collected by human-landing catch (HLC) and cow bait trap (CBT) collection methods according to the village. **B**) Relative proportions of *sensu lato* malaria vectors collected by HLC according to the village. **C**) Relative proportions *sensu lato* malaria vectors collected by CBC according to the village.

period between the rainy and dry seasons, when the abundance of other groups decreased (Supplementary File 2).

Results of the molecular identification are presented in the Table 1. *Anopheles minimus* (*s.s.*) was by far the most abundant species among the Funestus Group. Its sibling *An. harrisoni* and closely related members from the Aconitus and Culicifacies Subgroups represented <0.5% and 13% of the specimens from the Funestus populations collected by HLC and CBT, respectively. *Anopheles sawadwongporni* was the most frequent species among the Maculatus Group, followed by *An. maculatus* (*s.s.*) and *An. pseudowillmori*. Three species from the Dirus Complex accounted for >99% of the specimens in the Leucosphyrus Group, namely *An. dirus* (*s.s.*) *An baimaii* and *An. cracens*.

Table 1. Molecular identification of sibling species among the Funestus, Maculatus and Leucosphyrus Groups.

Group	Collection method (% tested)	Species	N	Percentage
Funestus	HLC (3,294/42,283=8%)	*An. minimus* (*s.s.*)	3,277	99.5
		An. harrisoni	2	0.1
		An. culicifacies B	6	0.2
		An. aconitus	7	0.2
		An. pampanai	0	0
		An. varuna	2	0.1
	CBC (1,543/15,728=10%)	*An. minimus* (*s.s.*)	1,342	87
		An. harrisoni	1	0.1
		An. culicifacies B	90	5.8
		An. aconitus	42	2.7
		An. pampanai	9	0.6
		An. varuna	59	3.8
Maculatus	HLC (1,476/7,281=20%)	*An. maculatus* (*s.s.*)	537	36.4
		An. sawadwongporni	819	55.5
		An. pseudowillmori	114	7.7
		An. dravidicus	6	0.4
	CBC (1,491/5,239=28%)	*An. maculatus* (*s.s.*)	439	29.4
		An. sawadwongporni	975	65.4
		An. pseudowillmori	74	5
		An. dravidicus	3	0.2
Leucosphyrus	HLC (856/1,144=77%)	*An. dirus* (*s.s.*)	205	23.9
		An. baimaii	643	75.1
		An. cracens	5	0.6
		An. balabacensis	3	0.4
	CBC (29/46=57%)	*An. dirus* (*s.s.*)	14	48.3
		An. baimaii	10	34.5
		An. cracens	5	17.2
		An. balabacensis	0	0

Malaria vectors

The contribution of six Groups of *Anopheles* to malaria transmission was determined by processing 56,872 samples collected by HLC in qrtPCR *Plasmodium* (Table 2). Both *P. falciparum* and *P. vivax* were detected in the Funestus, Leucosphyrus and Maculatus Groups, whereas only *P. vivax* was detected in the Barbirostris Group. One specimen of the Funestus Group was co-infected with both *P. falciparum* and *P. vivax*. The Funestus Group was by far the most important taxa contributing to malaria transmission (Pf-EIR= 0.1 and Pv-EIR= 0.6 infective bites/person/month) followed by the Maculatus, Leucosphyrus and Barbirostris Groups. Due to the relatively low sample size analysed in the Annularis and Subpictus Groups (n= 747 and 126 respectively), it was not possible to rule out their contribution to malaria transmission. Positive samples were identified at the species level using molecular assays. Six formally named species were incriminated in malaria transmission: *An. minimus* (*s.s.*), *An. aconitus* (*s.s.*) (Funestus Group), *An. maculatus* (*s.s.*), *An. sawadwongporni* (Maculatus Group), *An. dirus* (*s.s.*) and *An. baimaii* (Leucosphyrus Group). *Plasmodium vivax* sporozoites were detected in all species whereas *P. falciparum* sporozoites were detected only in *An. minimus* (*s.s.*), *An. maculatus* (*s.s.*), *An. sawadwongporni* and *An. dirus* (*s.s.*). Molecular identification at the species level was not possible for 6/104 positive samples because there was no DNA remaining. Interestingly, the avian malaria *P. juxtanucleare* was detected in five specimens of the Funestus, Maculatus and Leucosphyrus Groups collected by HLC (two *An. minimus* (*s.s.*), one *An. baimaii* and two

Table 2. Mean values of the entomological indices of malaria transmission presented at the Group level.

Group	Value of the indicator for the indicated Anopheles Group					
	Proportion analyzed	Mean HBR (95%CI) in b/p/m	Mean Pf-SI (95%CI) in nb. pos. mosq. / 1000 analyzed	Mean Pv-SI (95%CI) in nb. pos. mosq. / 1000 analyzed	Mean Pf-EIR (95%CI) in ib/p/m	Mean Pv-EIR (95%CI) in ib/p/m
Funestus	99% (41797/42283)	307.9 (305-310.8)	0.3 (0.2-0.5)	1.8 (1.5-2.3)	0.01 (0.05-0.16)	0.57 (0.45-0.71)
Maculatus	99% (7178/7281)	53 (51.8-54.2)	0.6 (0.2-1.4)	1 (0.4-2)	0.03 (0.01-0.08)	0.052 (0.02-0.11)
Leucosphyrus	97% (1107/1144)	8.3 (7.9-8.8)	0.9 (0-5)	2.7 (0.6-7.9)	0.008 (0-0.04)	0.023 (0.01-0.07)
Barbirostris	97% (5917/6098)	44.4 (43.3-45.5)	0 (0-0.6)	0.3 (0-1.2)	0 (0-0.03)	0.015 (0.002-0.05)
Annularis	97% (747/772)	5.6 (5.2-6)	0 (0-4.9)	0 (0-4.9)	0 (0-0.03)	0 (0-0.03)
Subpictus	88% (126/144)	1.0 (0.9-1.2)	0 (0-28.9)	0 (0-28.9)	0 (0-0.03)	0 (0-0.03)

b/p/m: bites /person /month; **CI**: confidence interval; **HBR**: human-biting rate; **ib/p/m**: infective bites /person /month**; Pf-EIR**: *P. falciparum* entomological inoculation rate; **Pv-EIR**: *P. vivax* entomological inoculation rate; **Pf-SI**: *P. falciparum* sporozoite index; **Pv-SI**: *P. vivax* sporozoite index.

undetermined species). In addition, 16% (3,308/21,013) of the specimens from the Funestus, Maculatus and Leucosphyrus Groups collected on CBT were screened for *Plasmodium* sporozoites. *Plasmodium vivax* sporozoites were detected in two *An. minimus* (*s.s.*).

Quantitation of the sporozoite load was possible in 106/108 of the *P. falciparum* and *P. vivax* positive samples. Overall, 63% (67/106) of the infected specimens carried less than 100 sporozoites (Figure 3). The geometric mean was 41 (95%CI= [14; 98]) and 162 (95%CI= [94; 167]) sporozoites /infected mosquito for *P. falciparum* and *P. vivax* respectively. *Anopheles maculatus* (*s.l.*) appeared to be infected with lower parasite loads compared to other anopheline species. The range of sporozoite loads was 6 to 9,234 sporozoites for *P. falciparum* and 4 to 517,500 sporozoites for *P. vivax* (Table 3). Moreover, 81/108 abdomens from sporozoite-positive samples were screened for *Plasmodium* oocysts (27/108 abdomens were lost or mouldy). *Plasmodium* oocysts were detected in 57% (46/81) of the sporozoites-positive specimens only. Thirty-two out of the 35 sporozoites-positive oocysts-negative specimens carried less than 500 sporozoites in their salivary glands, suggesting that these specimens were infected with a low number of oocysts. Finally, sporozoite and oocyst loads were found to be correlated in the 46 sporozoites-positive oocysts-positive specimens (Spearman correlation coef. $\rho = 0.661$, p-value= 5.58×10^{-7}).

Host-seeking behaviour of Anopheles mosquitoes

Overall, the mean HBR of *Anopheles* mosquitoes was 460 bites/person/month (95%CI= [457; 463]) and the mean CBR was 4,807 bites/cow/month (95%CI= [4770; 4843]). Mean HBR of primary malaria vectors varied from 2 to 306 bites/person/month in *An. dirus* (*s.s.*) and *An. minimus* (*s.s.*) respectively. Some malaria vectors were also frequently collected on CBT: *Anopheles minimus* (*s.s.*), *An. sawadwongporni* and *An. maculatus* (*s.s.*) had a mean CBR of 996, 249 and 112 bites/cow/month respectively. The data on secondary vectors (*e.g.* Barbirostris and Annularis Groups) are more difficult to interpret because they probably represent a mix of several closely related species (Figure 4).

Leucosphyrus members were the only species to be preferentially anthropophagic and endophagic (mean ZI=0.16 and 0.44 and mean EI= 0.45 and 0.37 in *An. baimaii* and *An. dirus* (*s.s.*) respectively). Other malaria vectors from the Funestus, Maculatus and Barbirostris Groups were preferentially zoophagic and exophagic (mean ZI=0.75-0.95 and mean EI=0.60-0.75). All remaining species were strongly zoophagic and exophagic (mean ZI= 0.83-1.00 and mean EI= 0.63-0.83) (Figure 5). Beyond zoophagy, malaria vectors were found to be opportunistic in the choice of their blood meal source. Indeed, we have shown that *Anopheles* specimens collected by HLC can carry the avian malaria parasite *P. juxtanucleare*, and that *Anopheles* specimens collected on CBT can carry the human malaria parasite *P. vivax* (*i.e. Anopheles* mosquitoes collected on a given host type can carry *Plasmodium* sporozoites acquired from a previous blood meal taken on a different host type). These findings clearly demonstrate that malaria vectors can feed alternatively on different host types during their life span.

Anopheles mosquitoes exhibited an outdoor and early biting pattern (Figure 6 and Figure 7). Noteworthy, some species were already active at 06:00 pm and/or at 06:00 am, suggesting that exposure to malaria vectors stretches out of the standard collection time. The proportion of malaria vectors collected indoors between 09:00 pm and 05:00 am was 29% (range= 15-48% according to the species). Conversely, 65% of the infected specimens were collected out of doors, before 09:00 pm and/or after 05:00 am (Figure 8).

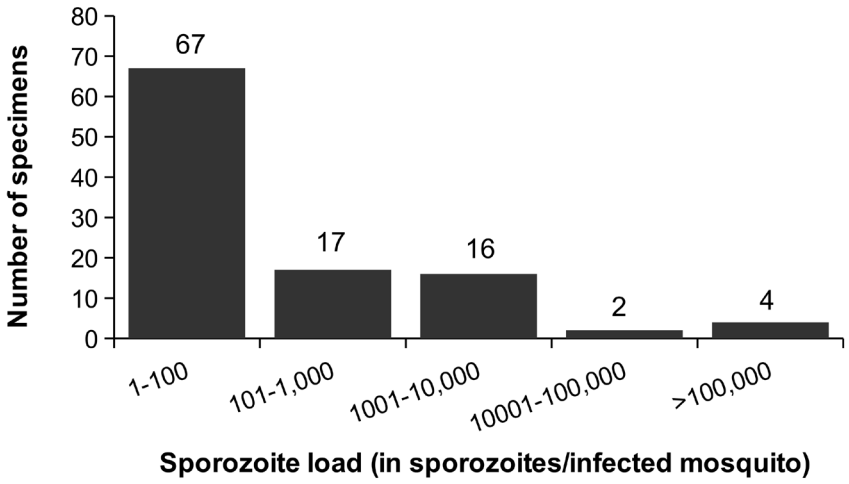

Figure 3. Frequency distribution of the sporozoite load in naturally infected malaria vectors.

Table 3. Descriptive statistics of the sporozoite load in naturally infected malaria vectors from hotspot villages located in the Thailand-Myanmar border area.

Plasmodium species	Parameter	Value of the parameter for the indicated *Anopheles* Group				
		Minimus	Maculatus	Dirus	Barbirostris	Total
Pf	N	11	4	1	0	16
	Geom. mean	74	13	6	NA	41
	95%CI	18-222	7-23	NA-NA	NA	14-98
	Minimum	10	6	6	NA	6
	1st quartile	16	8.25	6	NA	10.75
	Median	26	13.5	6	NA	21
	3rd quartile	328	21	6	NA	89.25
	Maximum	9234	30	6	NA	9234
Pv	N	78	7	3	2	90
	Geom. mean	186	26	897	26	162
	95%CI	101-318	14-54	118-22352	20-34	94-267
	Minimum	6	4	36	20	4
	1st quartile	36	20	1485	23.5	28.5
	Median	57	26	2934	27	51.5
	3rd quartile	472.2	50	4884	30.5	398
	Maximum	517500	92	6834	34	517500
Total	N	89	11	4	2	106
	Geom. mean	166	20	257	26	131
	95%CI	100-270	12-36	15-4478	20-34	79-206
	Minimum	6	4	6	20	4
	1st quartile	27	13.5	28.5	23.5	25.25
	Median	55	20	1485	27	45
	3rd quartile	482	33	3909	30.5	258.2
	Maximum	517500	92	6834	34	517500

NA: not applicable; **N**: number of specimens; **95%CI**: 95% confidence interval; **Geom. mean**: geometric mean.

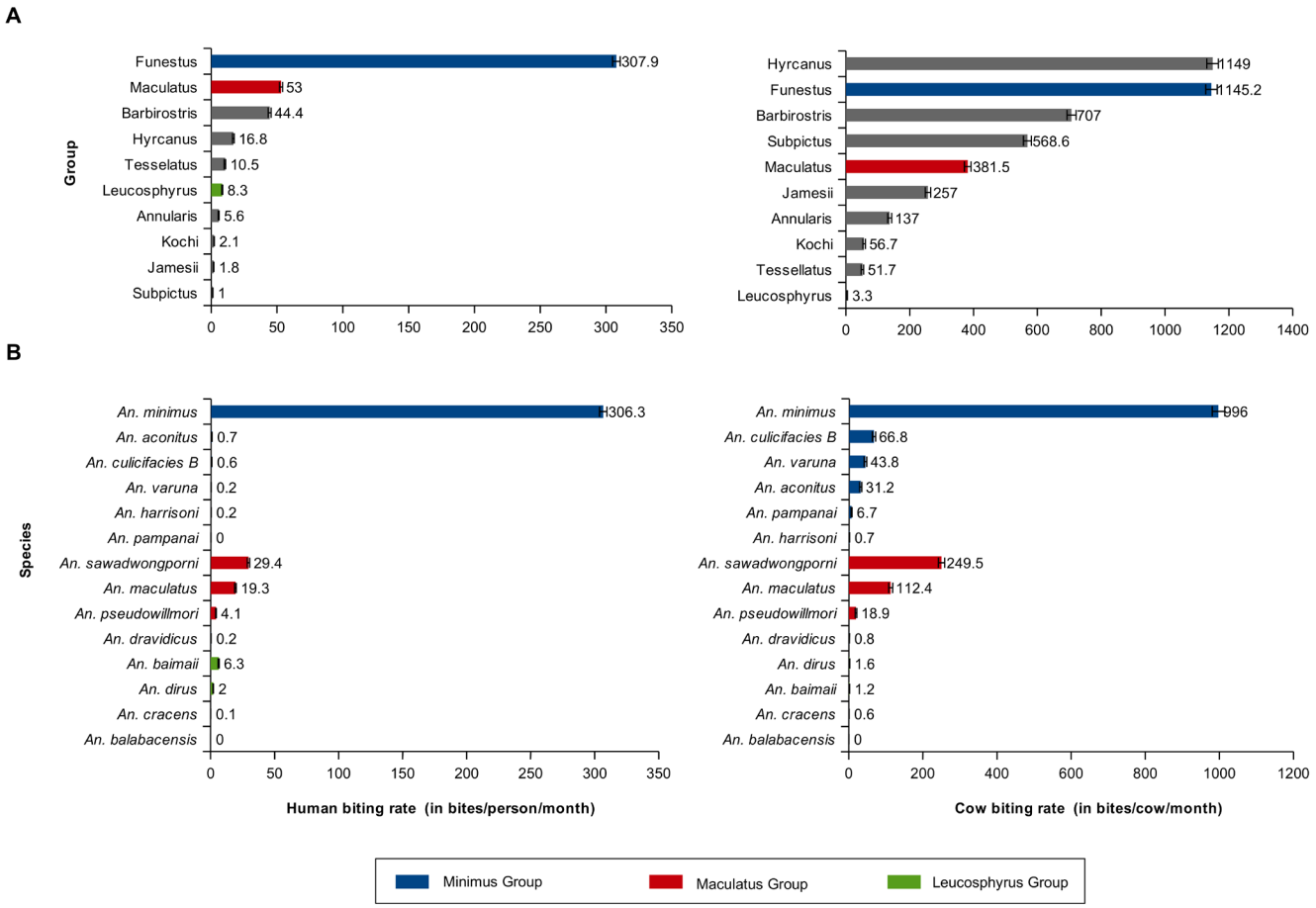

Figure 4. Human-biting rate (HBR) and cow-biting rate (CBR) of *Anopheles* mosquitoes. A) HBR and CBR are presented at the Group level. **B**) HBR and CBR are presented at the species level.

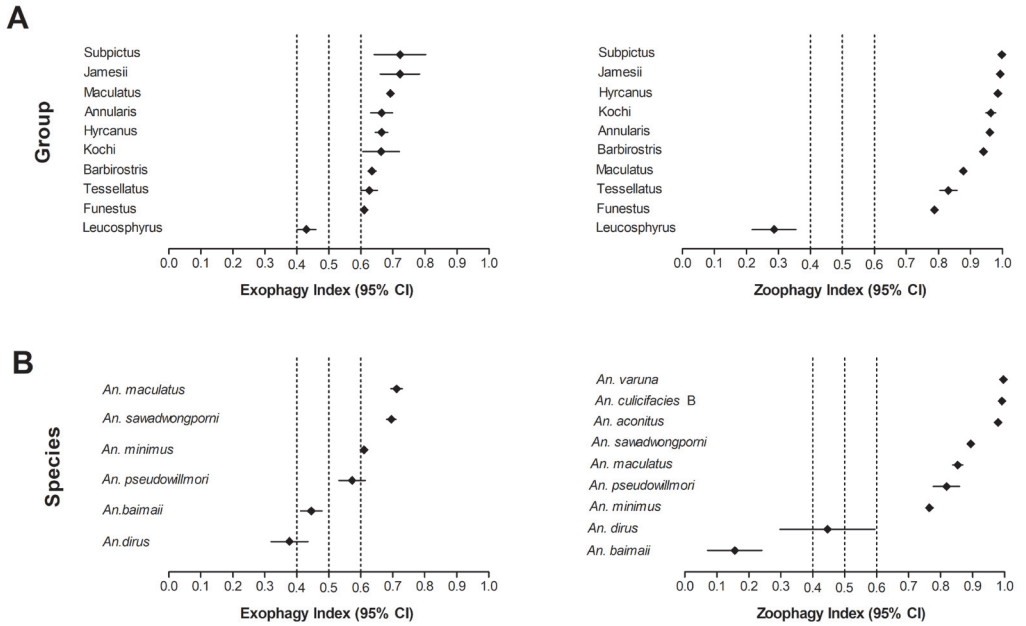

Figure 5. Exophagy index (EI) and zoophagy index (ZI) of *Anopheles* mosquitoes. A) EI and ZI are presented at the Group level. **B**) EI and ZI are presented at the species level.

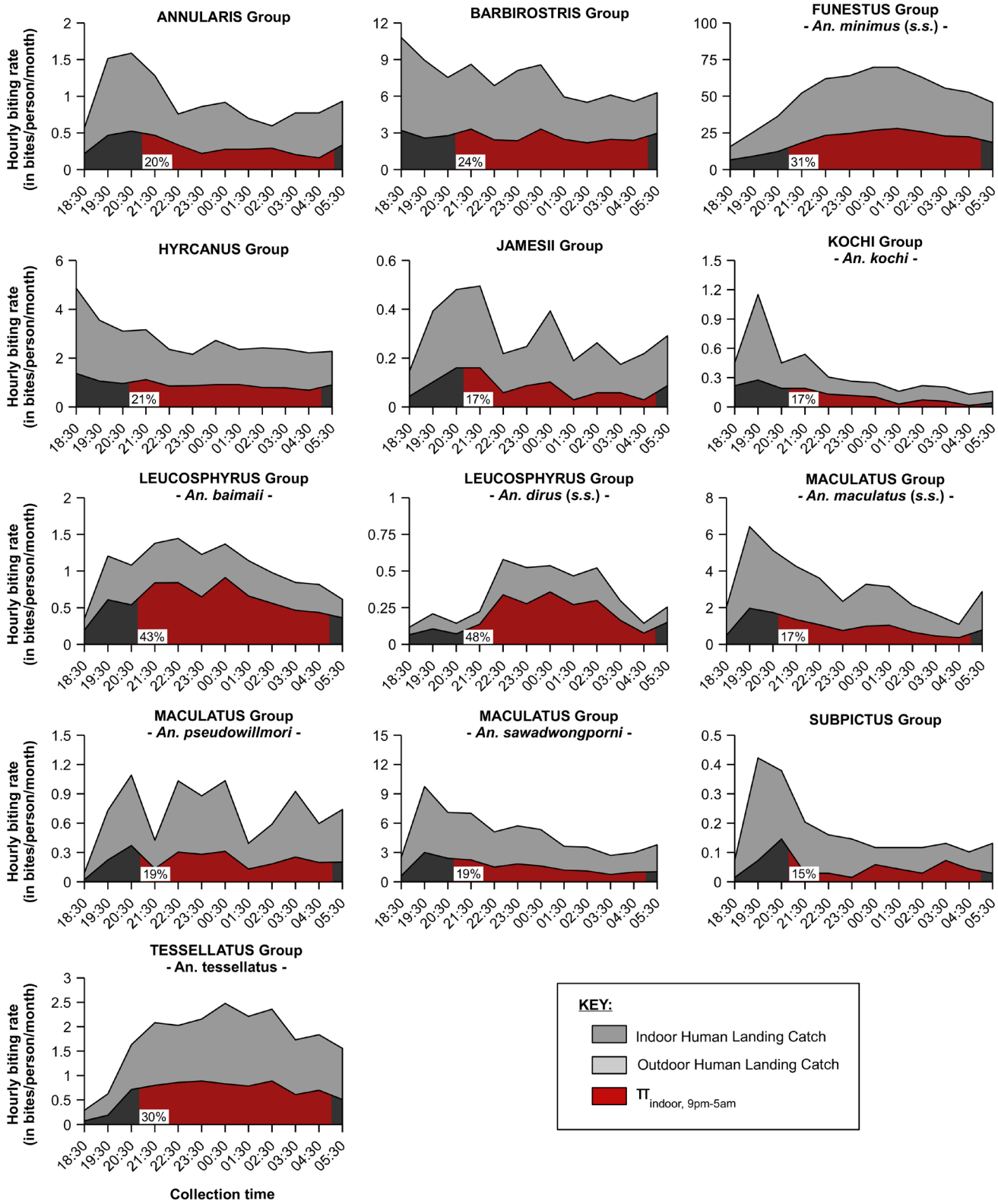

Figure 6. Hourly biting pattern of *Anopheles* mosquitoes collected by human-landing catch. $\pi_{indoors,\ 9pm\text{-}5am}$: proportion of the specimens collected indoors between 09:00 pm and 05:00 am.

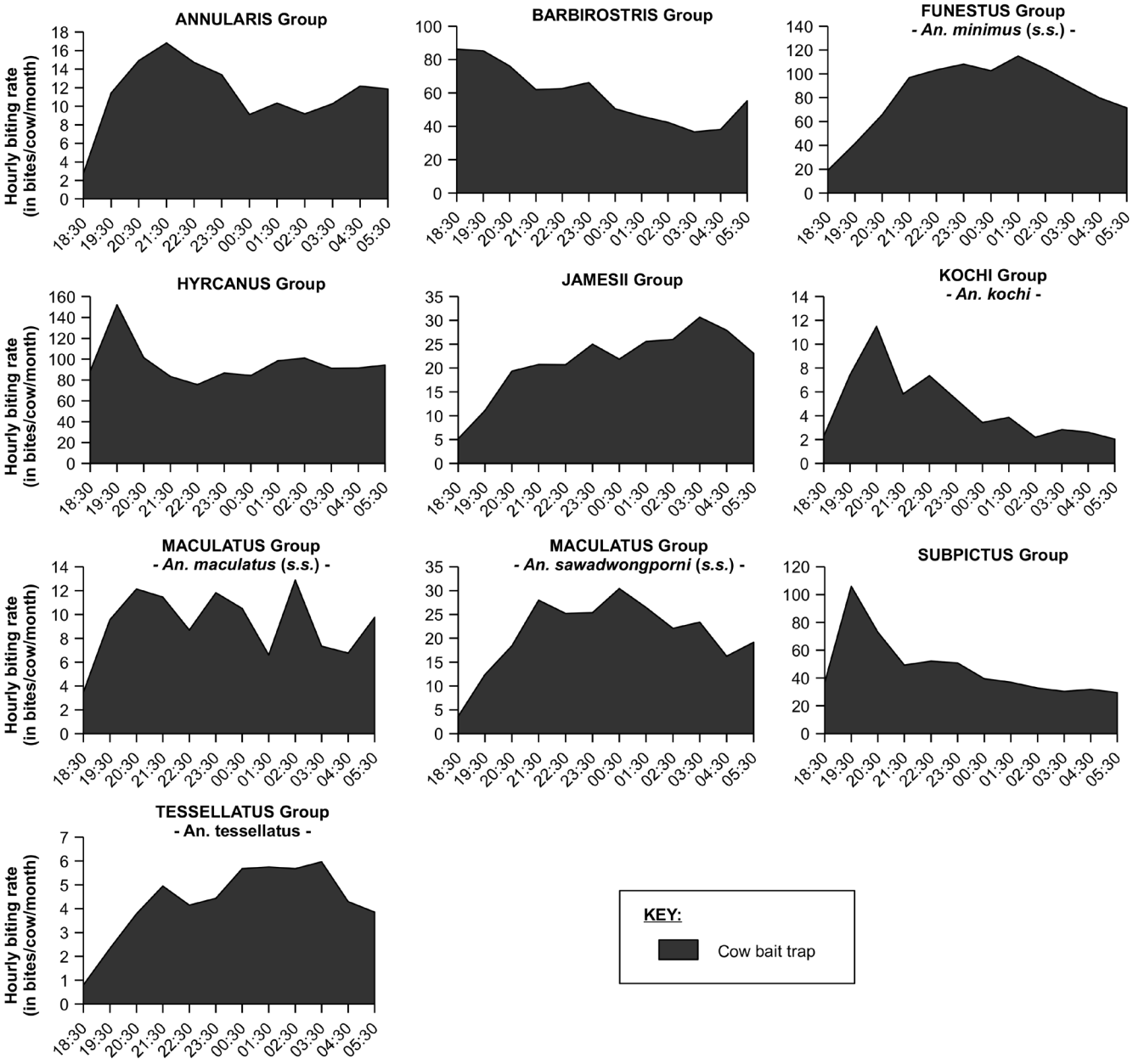

Figure 7. Hourly biting pattern of *Anopheles* mosquitoes collected on cow-bait trap.

Entomological indices of malaria transmission

Only the Funestus, Maculatus and Leucosphyrus Groups were taken into account for the analysis of the entomological indices. Overall, mean HBR was 369 bites/person/month (95%CI= [366; 372]) and compensated for the low infection rate in these naturally infected populations of malaria vectors. Mean Pf-SI was 0.4/1000 (95%CI= [0.2; 0.6]) and mean Pv-SI was 1.7/1000 (95%CI= [1.4; 2.1]), yielding mean Pf-EIR of 0.13 (95%CI= [0.08; 0.21]) and mean Pv-EIR of 0.64 (95%CI= [0.51; 0.79]) infective bites/person/month (Table 4). The transmission of *P. falciparum* was highly seasonal: the rainy season was associated with a 10-fold increase in Pf-EIR. In contrast, mean Pv-EIR was 0.52 (95%CI= [0.34; 0.75]) and 0.73 (95%CI= [0.55; 0.94]) infective bites/person/month during the rainy season and the dry season respectively (Table 5). Only 6% (1/18) of the mosquitoes infected with *P. falciparum* sporozoites were detected during the dry season whereas 32% (28/87) of the mosquitoes infected with *P. vivax* sporozoites were detected during the dry season (two-sided Fisher's exact test, p-value= 0.0218).

Average values of entomological indices concealed a high heterogeneity. When data are aggregated at the village and survey

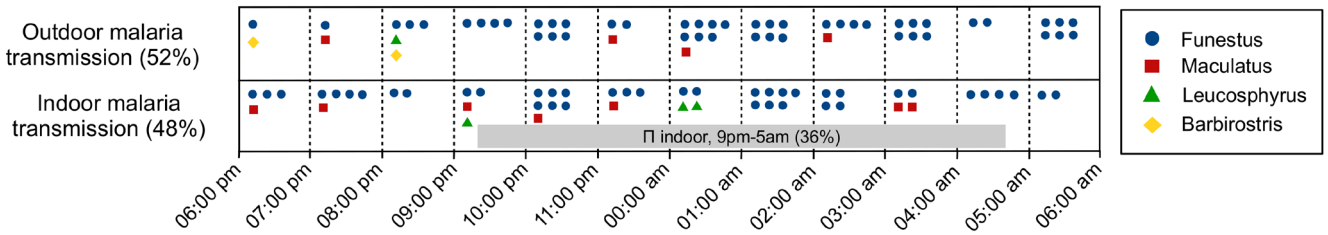

Figure 8. Distribution of infective bites according to the location and the time. $\pi_{\text{indoors, 9pm-5am}}$: proportion of the specimens collected indoors between 09:00 pm and 05:00 am.

Table 4. Mean values of the entomological indices presented per study village.

Village	Value of the parameter for the indicated village				
	Mean HBR (95%CI) *in b/p/m*	**Mean Pf-SI (95%CI) *in nb. pos. mosq. /1000 analyzed***	**Mean Pv-SI (95%CI) *in nb. pos. mosq. /1000 analyzed***	**Mean Pf-EIR *(95%CI) in ib/p/m***	**Mean Pv-EIR *(95%CI) in ib/p/m***
HKT	316 (310-322)	0.5 (0.2-1.1)	3.7 (2.6-5)	0.15 (0.05-0.35)	1.16 (0.82-1.58)
KNH	272 (267-278)	0.4 (0.1-1.1)	2.9 (1.9-4.2)	0.12 (0.03-0.3)	0.78 (0.51-1.14)
TOT	694 (685-703)	0.2 (0.1-0.5)	0.6 (0.3-1)	0.17 (0.06-0.38)	0.43 (0.24-0.71)
TPN	184 (180-189)	0.5 (0.1-1.5)	1 (0.4-2.2)	0.09 (0.02-0.27)	0.19 (0.07-0.4)
Four villages	369 (366-372)	0.4 (0.2-0.6)	1.7 (1.4-2.1)	0.13 (0.08-0.21)	0.64 (0.51-0.79)

b/p/m: bites /person /month; **CI**: confidence interval; **HBR**: human-biting rate; **ib/p/m**: infective bites /person /month; **Pf-EIR**: *P. falciparum* entomological inoculation rate; **Pv-EIR**: *P. vivax* entomological inoculation rate; **Pf-SI**: *P. falciparum* sporozoite index; **Pv-SI**: *P. vivax* sporozoite index.

Table 5. Mean values of entomological indices presented per season.

Season	Value of the parameter for the indicated season				
	Mean HBR (95%CI) *in b/p/m*	**Mean Pf-SI (95%CI) *in nb. pos. mosq. /1000 analyzed***	**Mean Pv-SI (95%CI) *in nb. pos. mosq. /1000 analyzed***	**Mean Pf-EIR *(95%CI) in ib/p/m***	**Mean Pv-EIR *(95%CI) in ib/p/m***
Rainy	477 (472-482)	0.4 (0.3-0.7)	1.5 (1.2-2)	0.21 (0.12-0.33)	0.73 (0.55-0.94)
Dry	208 (204-212)	0.1 (0-0.5)	2.5 (1.6-3.6)	0.02 (0-0.1)	0.52 (0.34-0.75)
Overall	369 (366-372)	0.4 (0.2-0.6)	1.7 (1.4-2.1)	0.13 (0.08-0.21)	0.64 (0.51-0.79)

b/p/m: bites /person /month**; CI**: confidence interval; **HBR**: human-biting rate; **ib/p/m:**infective bites /person /month; **Pf-EIR**: *P. falciparum* entomological inoculation rate; **Pv-EIR**: *P. vivax* entomological inoculation rate; **Pf-SI**: *P. falciparum* sporozoite index; **Pv-SI**: *P. vivax* sporozoite index.

levels, mean HBR ranges from 13 to 2611 bites/person/month, mean Pf-EIR ranges from 0.00 to 3.05 infective bites/person/month and mean Pv-EIR ranges from 0.00 to 9.75 infective bites/person/month. The lowest HBR measured on a single collector and during a single night of collection was 0 bites and the highest HBR was 289 bites. When taking into account the entire follow-up, mean HBR measured on single collectors ranged from 66 to 1253 bites /person /month, mean Pf-EIR ranged from 0 to 0.86 infective

bites/person/month and mean Pv-EIR ranged from 0 to 4.92 infective bites/person/month. The cumulative HBR and EIR measured in the cohort of mosquito collectors followed a logarithmic distribution: 20% of the collectors received approximatively 50% of the bites and of the infective bites. In contrast, 30% of the collectors did not receive any infective bites during the study. Interestingly, the cumulative HBR followed a linear trend when paired to EIR, suggesting that the heterogeneity in the distribution of infective bites is not explained by the mean of exposure to malaria vectors (Figure 9).

Discussion

This study was a unique opportunity to document some entomological aspects of malaria transmission in low transmission settings of Southeast Asia. Our data are important in the context of malaria elimination locally, but also elsewhere in the Greater Mekong Subregion where malaria displays a similar transmission pattern.

The dynamics of entomological indices in an area of low, seasonal and unstable *P. falciparum* transmission setting

Our results confirm previous observations that infection rate is low in naturally infected populations of malaria vectors and compensated by the high biting-rate of malaria vectors[26,27], yielding mean entomological inoculation rate of 1.6 and 7.7 infective bites /person /year for *P. falciparum* and *P. vivax* respectively. These values of EIR were measured in the context of targeted malaria elimination (community wide access to early diagnosis and treatment, and mass drug administration)[17]. Therefore, baseline intensity of malaria transmission in hotspot villages from the Kayin state is likely to be higher than that reported in this study[14]. The transmission of *P. falciparum* is seasonal whereas infective

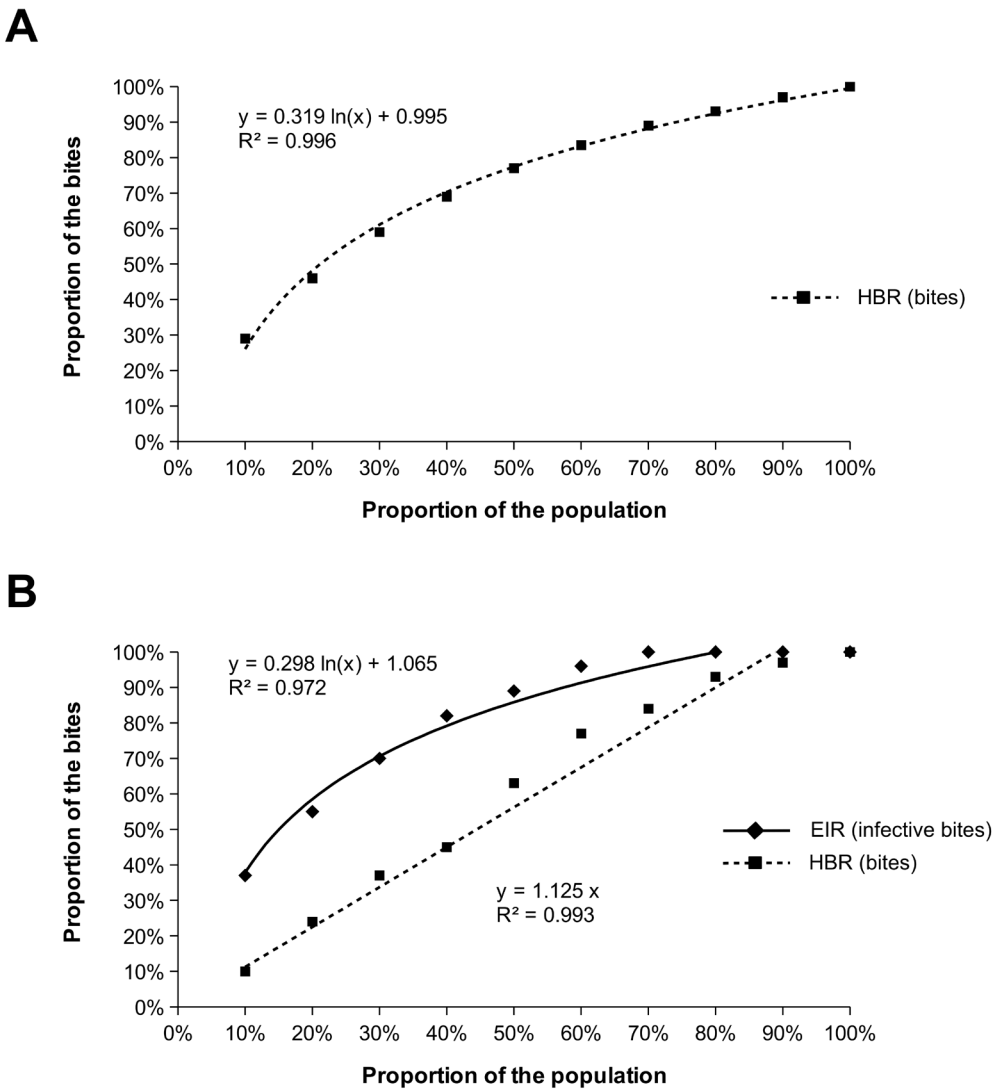

Figure 9. Heterogeneous distribution of bites and infective bites among the cohort of mosquito collectors over the entire period of the study. A) Cumulative distribution of the bites of malaria vectors among the cohort of mosquito collectors. **B**) Paired cumulative distribution of bites of malaria vectors and infective bites among the cohort of mosquito collectors.

bites of *P. vivax* occurred during both the dry and rainy seasons. The seasonality in *P. falciparum* transmission is only partially explained by the increase in malaria vector abundance during the rainy season when compared to the dry season. The longevity of malaria vector is most likely to be the main factor driving the seasonality of *P. falciparum* transmission. During the dry season, the life expectancy of malaria vectors is probably too short for *P. falciparum* to complete its sporogonic cycle in the mosquito. During the rainy season, the longevity of malaria vectors increases and malaria vectors live long enough for *P. falciparum* sporozoites to appear in the salivary glands[26,27]. For *P. vivax*, the duration of the sporogonic cycle is compatible with sporozoite detection throughout the year as this parasite develops faster than any other species in its mosquito vectors[28]. Individuals living in endemic areas receive numerous sporozoites, which aliments the reservoir of hypnozoites in the liver.

Interestingly, the distribution of infective bites among the human population was highly heterogeneous. This pattern was not explained by the mean of exposure to malaria vectors as paired cumulative HBR and EIR did not follow the same trend. The study villages were hotspots of malaria transmission defined by the high prevalence of asymptomatic infection[17]. This implies a substantial degree of premunition in asymptomatic carriers, *i.e.* the development of a protective immunity that maintains parasitaemia at sub-patent levels. The broad spectrum of mean EIR measured in different individuals living in the same village may explain why some people develop such a protective immunity and manage to control the infection while others turn symptomatic once infected.

In this study, the sporozoite loads measured in naturally infected population of malaria vectors were very low (60% of the infected specimens carried less than 100 sporozoites). This is consistent with previous attempts to quantify *P. falciparum and P. vivax* sporozoites in low transmission settings[19,29–31] and contrasts with the high sporozoite loads detected in Africa[32–34]. Importantly, 5% of the positive samples had a high parasite load (>10,000 sporozoites /infected mosquito).

Residual malaria transmission

The two broadly scalable vector-control interventions recommended by the World Health Organization for the control of malaria vectors are mass distribution of LLINs or, where appropriate, indoor residuals spraying (IRS) campaigns[35]. The ecology of malaria vectors is a key determinant of intervention efficacy[7]. By definition, LLINs target malaria vectors seeking a blood meal from a human host, indoors and at a time when people are sleeping under mosquito nets. In order for IRS to be effective, malaria vectors targeted by the intervention must also rest indoors, before or after a blood meal. However, this stereotypical host seeking behavior applies only to a minority of the dominant malaria vectors worldwide[36]. Several behavioral traits drive the refractoriness of malaria vectors to LLINs and IRS including *(i)* their ability to take blood meals from non-human hosts (zoophagy and opportunistic host type selection), *(ii)* their tendency to rest and/or feed outdoors (exophily and exophagy) and *(iii)* their ability to feed before dawn and after dusk, at a time when people are not protected by LLINs or IRS intervention[7].

As previously reported, mosquito bed nets only have a limited efficacy in preventing human-vector contact and disease transmission in the Thailand-Myanmar border area. Somboon *et al*. have evaluated the impact of mosquito bed nets impregnated with lambda-cyhalothrin using entomological endpoints in very similar transmission settings (Karen villages located on the Thai side of the border)[10]. The authors reported that LLINs can prevent 36-78% of the human-vector contact according to the *Anopheles* species. Universal coverage with LLINs failed to reduce the abundance and longevity of malaria vectors, suggesting that this intervention had only a limited impact on vectorial capacity. The impact of permethrin-impregnated mosquito bed nets was also evaluated in pregnant women and children living in refugee camps using epidemiological endpoints. The use of mosquito bed nets during pregnancy was associated with a significant reduction in the incidence of severe anaemia but not of malaria[37]. At a time when EIR was higher, the use of mosquito bed nets in children was associated with a significant decrease of *P. falciparum* malaria incidence but no effect was observed for *P. vivax*[38]. More recently, Smithuis *et al*. failed to observe an impact of LLINs among a cohort of 175 children followed for 10 months in Western Myanmar[39]. This negative result was explained by the early and outdoor biting pattern of malaria vectors[40].

In this study, only 35% of the mosquitoes infected with *Plasmodium* were collected indoors between 09:00 pm and 05:00 pm, because of outdoor and early biters. This proportion might have been underestimated as malaria vectors were already active at 06:00 pm and/or at 06:00 am, suggesting that the exposure stretched out of the collection time. Accurate quantitation of the part of the transmission that LLINs fail to prevent would require collection of additional data on population movements and sleeping habits of people living in this area. Moreover, we have clearly demonstrated an opportunistic host type selection in some vectors, *i.e.* that a given specimen is able to feed on several host types during successive gonotrophic cycles. This opportunism is also an important factor to explain why universal coverage with mosquito bed nets fails to affect the dynamic of anopheline populations and decrease vectorial capacity in the area[10]. Consequently, the paradigm of residual transmission as experienced in high transmission settings of Africa does not apply to the Thailand-Myanmar border area and a drastic shift in vector-control interventions is required.

Shift in vector-control intervention

The design of effective intervention for the control of malaria vectors in Southeast Asia should take into account the dynamics of the transmission, as well as the ecology of malaria vectors present. In this study, we have shown that multiple vectors have different and complementary host-seeking behaviours making their control particularly difficult.

Veterinary approaches such as the injection of livestock with a slow-release formulation of endectocides[41], or the use of

insecticide-treated mosquito nets fenced around cattle[42] may be an interesting strategy to decrease the vectorial capacity of some zoophilic and/or zoophagic malaria vectors (ex: *An. minimus*, *An. maculatus* or *An. sawadwongporni*). We have shown that malaria vectors can readily feed on a wide variety of host type including human, cattle, pigs and birds. However, the diversity of host type and the relative proportion of blood meals taken on a given host type remains to determine. In this regard, targeted sequencing of 16S ribosomal RNA genes detected in DNA extracts from blood-fed specimens is a promising tool for the determination of blood-meal sources in wild populations of malaria vectors[43].

Another important aspect of malaria vector ecology is the nature of their resting habitats, which can be targeted by residual insecticide spraying intervention. Resting habitats have been identified both indoors (ex: roof, wall, ceilings of houses and animal barns) and outdoors (ex: tree holes, rodent holes, dense bushes)[44]. However, most mosquito species rest exclusively out of doors in natural settings, and only a relatively few species rest in man-made shelters[44]. The size and importance of the exophilic population that commonly rest inside houses are typically overlooked because the sampling of outdoor-resting population is more difficult than sampling indoor-resting population. This is especially true in Southeast Asia where most of the life cycle of *Anopheles* mosquitoes is likely to take place out of doors[45]. Therefore, the scope of residual insecticide spraying for the control of malaria vectors may be extended to outdoor applications.

Insecticide resistance may also represent an additional threat to malaria vector control in the target area. We have previously reported that resistance to pyrethroid insecticides is detected at a relatively low level in population of primary malaria vectors from the Funestus and Maculatus Groups[46]. Further investigations are needed in order to document the extent of pyrethroid resistance elsewhere in Kayin state and in order to evaluate the potential effectiveness of alternative class of insecticides such as carbamate (ex. bendiocarb), organophosphate (ex. malathion) or insect growth inhibitors (ex. pyriproxyfen).

Conclusion

This study highlights the importance of entomology in the context of malaria elimination on the Thailand-Myanmar border. A drastic shift in vector-control strategy is required in order to address early and outdoor malaria transmission. Moreover, the place of vector-control should be retuned in order to address specific problematic in the context of malaria elimination.

Data availability

The data is available upon request to the Mahidol Oxford Tropical Medicine Research Unit Data Access Committee (Supplementary File 3; http://www.tropmedres.ac/data-sharing) and following the Mahidol Oxford Tropical Medicine Research Unit data access policy (http://www.tropmedres.ac/_asset/file/data-sharing-policy-v1-0.pdf).

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Supplementary material

Supplementary File 1. Laboratory procedures for the processing of entomological samples.

Click here to access the data

Supplementary File 2. Dynamic of the *Anopheles* entomo-fauna collected by human-landing catch and cow bait trap collection methods in the four villages.

Click here to access the data

Supplementary File 3. Request form for Mahidol Oxford Tropical Medicine Research Unit Data Access Committee.

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Open Peer Review

Current Referee Status: ? ?

Version 1

Referee Report 10 December 2018

https://doi.org/10.21956/wellcomeopenres.16087.r34267

? **Lisa J. Reimer**
Department of Vector Biology, Liverpool School of Tropical Medicine, Liverpool, UK

This is a very comprehensive study which was completed to a high standard. The knowledge generated is essential to achieve malaria elimination in the sub-region. My two essential recommendations for revision include 1) a deeper description on vector ecology in this region and a summary of which control and prevention activities are currently in place. This background can draw from the work that was described previously in reference 17. The end of the introduction states “Numerous aspects of malaria vectors ecology and biology have not been documented and the characteristics of the entomological indices are not known precisely” but the reader would appreciate a full description of the current gaps in our knowledge. 2) There is a significant amount of data that has been collected and analysed, but this could be organised in a way that is much more accessible. For example a table that summarizes key attributes per vector group (if it is not always available by species) such as mean and range monthly biting rates, perhaps median biting times, EI, ZI, infection prevalence.

Further specific comments are below:

1. This is described as a pilot study on targeted malaria elimination, however this is such a comprehensive entomological survey that it diminishes the significance of the work to call it a pilot.
2. Typo under entomological surveys: “mosquito sampling the using human landing catch”
3. It would be more intuitive to presented sporozoite prevalence rather than out of 1000 mosquitoes
4. Figure 4b and 5b – how is the HBR, EI and ZI calculated at the species level when a smaller proportion of samples were ID’d molecularly? Is it a proportion of those that were morphologically identified?
5. Figure 5 – what are the dashed lines for? It might be more useful to use the line to show a community mean index
6. Typo under “residual malaria transmission”: “35% of infection mosquitoes collected indoors between 9pm and 5pm”
7. Rephrase the final sentence “in order to address specific problematic in the context of malaria elimination”

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Referee Expertise: vector biology, malaria and filariasis transmission dynamics

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Referee Report 17 October 2018

https://doi.org/10.21956/wellcomeopenres.16087.r33968

? **Catherine Bourgouin**
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The objective of the work presented by Chaumeau *et al* was aimed at further describing the entomological determinants of malaria transmission at the Myanmar border with Thailand, in order to guide the policy makers for malaria elimination in this region of low transmission.

The investigation involved a longitudinal survey in four villages, using mosquito collection by Human Landing Catch as well as Cow Landing Catch over 21 months. The manuscript reports on a tremendous amount of work for both collecting mosquitoes, mosquito identification and Plasmodium detection in those mosquitoes by PCR. The data presented provided sound results in line with the objective.

Nevertheless, several parts would benefit from clarification or simplification, while other information, for instance on Asian malaria vectors, would be valuable for readers who are not specialists in malaria transmission in Asia. See specific comments below.
Additionally, I was expecting some deeper analysis or comments on possible differences among the 4 villages investigated and whether the ecology of the villages and the mosquito larvae could be included in malaria control recommendation.

For the global analysis of the entomological indexes for malaria, I have found difficulties in following the calculation of HBR, SI and EIR, when related to the comparison of the 4 villages and the impact of the season (Table 4 and Table 5).

The overall discussion could be better focused 1) on the global data, 2) the specificity or not of the transmission in the 4 villages to finish with stronger arguments for recommendation than the current

discussion that sounds rather dogmatic on the specificity of transmission in Asia by mostly exophagic mosquitoes….

Detailed comments:

Title: "Entomological determinants of malaria transmission in **Kayin** state, Eastern Myanmar: A **24-**month longitudinal study in **four** villages"

The Methods mention a 21 months survey, that indeed might cover 2 years. As the survey in the 4 villages are not fully superimposed, it might be wise to describe the overlap for a better comprehensive value of the data.
The "Kayin" state is barely mentioned in the report, bringing confusion when done ie: appearing only on page 13 beside the title and the abstract with "Therefore, baseline intensity of malaria transmission in hotspot villages from the Kayin state is likely to be higher than that reported in this study"
Lastly, if there is no real discussion on the difference similarity among the 4 villages, why attracting with the "4 villages" in the title?

Abstract: Some revision could be done if the authors agree on the following comments:

Methods

Study sites: Providing the global description of the villages, as done in the J. Vector ecology Paper (ref14), would save time, rather than going to the published document as well as possibly useful for the discussion. Was the MDA still active during the whole entomological survey?
Entomological surveys: Were the house randomly chosen? Were the preliminary data (ref14) included in the analysis? Consider simplification: instead "6 sites" use 5 houses plus one cow collection. Verify the accuracy of the sentence for mosquito collection under the net covering the cow.
Data analysis: Expressing SI / 1000 mosquito can be confusing. I would keep with the classical SI expressed as %. The following sentence "Entomological inoculation rate (EIR) was defined as the number of positive in qrtPCR Plasmodium divided by the corresponding number of person-nights of collection, adjusted over the proportion of collected mosquitoes that were analyzed by qrtPCR Plasmodium" is fully unclear to me. What do you mean by "adjusted over the proportion of collected mosquitoes that were analyzed by qrtPCR Plasmodium"? Again, why not keeping with the classical definition of EIR: HBR * SI%/100.
The CI calculation might need approval from an external statistician.

Results:

Biodiversity of the Anopheles entomo-fauna

From my understanding of the Methods, the total person-nights should be 4200 and total cow-nights 420. Did I miss something?
"Potential malaria vectors": could you provide your definition of those? A species table as sup data could be useful for non-specialist of Asian mosquito vectors. In figure 2 "other species" what does this include?

Fig2: why not commenting the comparative results for HLC and CBC and among the villages.
Homogenize between fig 2 A, Legend, Table 1 and main text : CBC or CBT.

"Results of the molecular identification are presented in the Table 1."

Malaria vectors

Table 2: Correct Mean Pf-EIR for Funestus : 0.1, not 0.01. Also in this table and followings, could not really follow the calculation from HBR and SI to the EIR, from the values of the former indexes, possibly as the mean values were calculated across the different months and/or villages. This might need to be specify at some point.

It would be also informative to provide the raw data as supp file for the identification of the species for the Plasmodium positive samples.

Sporozoite quantification: It is indeed interesting to be able to quantitate the sporozoite load for each positive mosquito. I would suggest to specify in the sup file that the calculated LOD of 6 Pf sporo or 4 Pv sporo are indeed per mosquito, according to your method.

For this load quantification data, I am not sure that any correlation with oocyst detection can be made. There is, to my knowledge, no method to determine in field collected mosquitoes that the oocysts one detects are the one providing the detected sporozoites in head-thoraxes. They might come from a secondary infection. I nevertheless agree that in very low transmission area the probability for a mosquito to feed twice on a gametocyte carrier is rather very low. In addition, please indicate the oocyst detection method, PCR? And how the midgut were preserved?

Table 3 could be placed as supp data.

Host-seeking behaviour of Anopheles mosquitoes

First sentence: “overall”, be more precise as taking into account all mosquitoes captured by HLC… Nevertheless, again I cannot obtain the same numbers for both HBR and CBR, though very closed.

Paragraph on Zoophagy : I am not sure that a zoophagic index can indeed be calculated as the surface of a cow exceed the surface of the human skin for HLC provided by 10 persons , plus volume of air and odors and containment of the cow. This is my opinion on this, but preferentially zoophagic/anthropophagic is OK.

Outdoor and early biting paragraph: Figure 6 and 7: revised the labeling for Fig 6, no distinction in the grey colours. Although there is a tendency for early biting it does not stand for all species ie minimus. Globally there is far too much figures for this section. Relocating some to the sup data will save space for the next section, that is at the center of the question: who is transmitting and when?

Entomological indices of malaria transmission
As said above, this might be the most important analysis. However, I still have some difficulties with the calculation in Table 4 and 5, At what level were the mean calculated?

Because you have all these nice data as monthly collection (see sup file 1) , why not comparing HBR per species (or even group) and EIR for each village, over time. This will clearly show who and when most transmission occurs and easier to visualize as graphs rather than tables as Table 4 and 5.

Discussion:

In my general comment I already mentioned that the discussion could benefit from a better focus.
I would add on the argument Pf and Pv transmission and season (second paragraph) that one needs to keep in mind, that beside having mosquitoes, gametocytes are also needed and Pv carriers who are not totally cured are excellent providers of gametocytes the year around. It is why it is also important that in the Methods section is included whether MDA was on or not and how Pv carriers were treated.

Also this sentence might be strongest :" In this study, only 35% of the mosquitoes infected with Plasmodium were collected indoors between 09:00 pm and 05:00 pm, because of outdoor and early biters", (page 14)., THE TIMING When people do not sleep under a net. The 35% appears as 36% in figure 8 : correct?

Lastly: the last sentence of this paragraph is unclear : "Consequently, the paradigm of residual transmission as experienced in high transmission settings of Africa does not apply to the Thailand-Myanmar border area and a drastic shift in vector-control interventions is required."
Could you explain what is the paradigm?

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Referee Expertise: molecular entomologist, malaria transmission

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.