RESEARCH ARTICLE

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

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First published: 31 Aug 2018, 3:109
https://doi.org/10.12688/wellcomeopenres.14761.1

Second version: 05 Feb 2019, 3:109
https://doi.org/10.12688/wellcomeopenres.14761.2

Third version: 09 May 2019, 3:109
https://doi.org/10.12688/wellcomeopenres.14761.3

Latest published: 17 Jun 2019, 3:109
https://doi.org/10.12688/wellcomeopenres.14761.4

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 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.

This article is included in the Mahidol Oxford Tropical Medicine Research Unit (MORU) gateway.
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 \textit{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 \textit{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 \textit{P. falciparum} is now interrupted\(^10-14\). Efficient vectors belong to the Minimus Complex (Funestus Group), Maculatus Group and Dirus Complex (Leucosphyrus Group)\(^11,13\). \textit{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 side of the Thailand-Myanmar border.\(^9\)

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\(^1\).

**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\(^1\). In each village, five traditional houses were selected for mosquito sampling by 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 Rattanarithkul et al.\(^18\). Deoxyribonucleic acid (DNA) was extracted from head/thorax using a cetyltrimethyl ammonium bromide-based method described previously\(^1\). Sibling species to the Funestus, Maculatus and Leucosphyrus Groups were discriminated using allelic-specific polymerase chain reaction (AS-PCR) assays adapted from Garros et al. and Walton et 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\(^2\). DNA extracts were screened for the presence of *Plasmodium* sporozoites using a quantitative real-time PCR (qPCR) 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 qPCR 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 qPCR *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 qPCR *Plasmodium* divided by the corresponding number of person-nights of collection, adjusted over the proportion of collected mosquitoes that were analyzed by qPCR *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, Leucophyry, Tessellatus (*Cellia* Subgenus, Neomyzomyia Series) and Subpictus (*Cellia* Subgenus, Pyretophorus Series). *Anopheles karvarti* (*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, Leucophyry 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 Leucophyry 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*. 

![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.](image-url)

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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 qRT PCR *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
Sporozoites were detected in two An. dirus s.s.). Noteworthy, some species were already infected with a low number of oocysts. Finally, sporozoite-positive samples were screened for Plasmodium vivax sporozoites. Plasmodium vivax sporozoites were detected in two An. minimus (s.s.).

Quantitation of the sporozoite load was possible in 106/108 of the infected specimens carried less than 100 sporozoites (Table 3). The geometric mean was 41 (95%CI= [14; 98]) and 97% (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 werecreened for Plasmodium oocysts (27/108 abdomens were lost or mouldy). Plasmodium oocysts were detected in 57% (46/81) of the sporozoite-positive specimens only. Thirty-two out of the 35 sporozoite-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 sporozoite-positive oocysts-positive specimens (Spearman correlation coef. p= 0.661, p-value= 5.58x10^-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. diras (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).

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**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:** Plasmodium 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.).
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, 0 | 16      |           |       |              |       |
|                    | Geom. mean     | 74, 13, 6, NA | 41      |           |       |              |       |
|                    | 95%CI          | 18-222, 7-23, NA-NA | 41      |           |       |              |       |
|                    | Minimum        | 10, 6, 6, NA | 14-98   |           |       |              |       |
|                    | 1st quartile   | 16, 8.25, 6, NA | 10.75   |           |       |              |       |
|                    | Median         | 26, 13.5, 6 | 21      |           |       |              |       |
|                    | 3rd quartile   | 328, 21, 6 | 89.25   |           |       |              |       |
|                    | Maximum        | 9234, 30, 6 | 9234    |           |       |              |       |
| Pv                 | N              | 78, 7, 3, 2 | 90      |           |       |              |       |
|                    | Geom. mean     | 186, 26, 897 | 162     |           |       |              |       |
|                    | 95%CI          | 101-318, 14-54, 118-22352 | 94-267  |           |       |              |       |
|                    | Minimum        | 6, 4, 36, 20 | 4       |           |       |              |       |
|                    | 1st quartile   | 36, 20, 1485 | 28.5    |           |       |              |       |
|                    | Median         | 57, 26, 2934 | 51.5    |           |       |              |       |
|                    | 3rd quartile   | 472.2, 50, 4884 | 398     |           |       |              |       |
|                    | Maximum        | 517500, 92, 6834 | 517500  |           |       |              |       |
| Total              | N              | 89, 11, 4 | 106     |           |       |              |       |
|                    | Geom. mean     | 166, 20, 257 | 131     |           |       |              |       |
|                    | 95%CI          | 100-270, 12-36, 15-4478 | 79-206  |           |       |              |       |
|                    | Minimum        | 6, 4, 6 | 4       |           |       |              |       |
|                    | 1st quartile   | 27, 13.5, 28.5 | 25.25   |           |       |              |       |
|                    | Median         | 55, 20, 1485 | 45      |           |       |              |       |
|                    | 3rd quartile   | 482, 33, 3909 | 258.2   |           |       |              |       |
|                    | Maximum        | 517500, 92, 6834 | 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. π (indoors, 9pm-5am): proportion of the specimens collected indoors between 09:00 pm and 05:00 am.
Entomological indices of malaria transmission

Only the Funestus, Maculatus and Leucophyclus 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...
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 (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.
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 approximately 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\textsuperscript{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)\textsuperscript{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\textsuperscript{14}. The transmission of P. falciparum is seasonal whereas infective

![Figure 9](image_url)

**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. 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. 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. 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 and contrasts with the high sporozoite loads detected in Africa. 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. The ecology of malaria vectors is a key determinant of intervention efficacy. 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. 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 (zooophagy 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.

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). 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 infection of severe anaemia but not of malaria. 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*. 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. This negative result was explained by the early and outdoor biting pattern of malaria vectors.

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.

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, or the use of
insecticide-treated mosquito nets fenced around cattle 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.

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). However, most mosquito species rest exclusively out of doors in natural settings, and only a relatively few species rest in man-made shelters. 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. 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. 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).

Grant information
This work was supported by the Wellcome Trust [101148]; and the Bill and Melinda Gates Foundation [GH OPP 1081420]. Victor Chaumeau received a PhD scholarship by the Centre Hospitalier Universitaire de Montpellier (CHU Montpellier).

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgments
We thank all study participants and communities from Htoo Pyin Nyar, Tar Au Ta, Ka Nu Hta and Htee Kaw Taw villages for their participation and support to the study. SMRU is part of the Mahidol Oxford University Research Unit, supported by the Wellcome Trust of Great Britain.

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.
Click here to access the data

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Published Abstract | Publisher Full Text
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.

Reviewer Expertise: vector biology, malaria and filariasis transmission dynamics

I confirm that I have read this submission and 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.

Author Response 27 Jan 2019
Victor Chaumeau, Centre hospitalier universitaire de Montpellier, Montpellier, France

We are thankful to the reviewer for her useful feed-back on the manuscript. Part of the introduction was rewritten in order to provide the reader with more background information on malaria vectors, transmission settings and on-going elimination efforts in the area of the study. A full description of the current gaps in our knowledge on vector ecology and biology would be difficult to present in the format of this research article. We instead referred the reader to the review of Sinka et al. that summarize key gaps for each dominant malaria vectors in the Asia-Pacific region. We tried to improve the presentation of the results by keeping only the key tables and figures in the main text and by adding more supplementary figures and tables in the supplementary data. Unfortunately, in was not possible to summarize in one table the statistics of all key indices, together with the raw numbers and confidence intervals. Answer to specific comments are below:

○ 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.

The wording pilot has been removed from the revised manuscript.
Typo under entomological surveys: “mosquito sampling the using human landing catch”

The typo has been corrected.

- It would be more intuitive to presented sporozoite prevalence rather than out of 1000 mosquitoes.

Sporozoite prevalence was expressed as percentages rather than out of 1000 mosquitoes.

- 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?

Yes. Species-specific proportions were used to compute HBR, CBR, EI and CBI estimates at the species level if ≥30 samples in the corresponding *Anopheles* Group were genotyped for each value of the grouping variable. The grouping variables were the collection time (06:00 pm to 06:00 am), the collection method (HCL or CBT) or the location of the mosquito collector (indoors or outdoors). For example, the number of *An. maculatus (s.l.*)* collected by HLC indoors and outdoors was 2240 and 5041 respectively. The proportion of *An. maculatus (s.s.*)* in the indoor and outdoor populations was 0.34 (133/392) and 0.37 (404/1084) respectively. The predicted number of *An. maculatus (s.s.*)* collected by HLC indoors and outdoors was 762 and 1865 respectively. The EI estimate for *An. maculatus (s.s.*)* was therefore 0.71 (1865/2627). More detail were added in the methods section.

- Figure 5 – what are the dashed lines for? It might be more useful to use the line to show a community mean index

Vertical lines have been modified to address reviewer’s suggestion. The solid red line represents the mean index for *Anopheles spp.* mosquitoes, corresponding CIs were too narrow to be shown.

- Typo under “residual malaria transmission”: “35% of infection mosquitoes collected indoors between 9pm and 5pm”

The typo has been corrected.

- Rephrase the final sentence “in order to address specific problematic in the context of malaria elimination”

The final sentence has been changed to “the modalities of vector-control should be retuned to address problematic specific to malaria elimination”.

**Competing Interests:** No competing interests were disclosed.
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.

Reviewer Expertise: molecular entomologist, malaria transmission

I confirm that I have read this submission and 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.

Author Response 27 Jan 2019
Victor Chaumeau, Centre hospitalier universitaire de Montpellier, Montpellier, France

We are thankful to the reviewer for her useful feedback on the manuscript. The manuscript has been edited as per reviewer's suggestions in order to improve the clarity of this report and to provide the reader with more background information on Southeast Asian malaria vectors. We agree with the reviewer that a more detailed explanatory analysis on the heterogeneity of malaria transmission in the four villages would have been valuable. Additional figures were added in the Supplementary File 1 in order to better show the differences in malaria transmission in the four villages. However, in the absence of appropriate datasets, the mechanisms by which local ecological factors and larval habitat interact with malaria transmission cannot be explained. Noteworthy, the primary objective of this paper was not to point out specific differences in particular study villages, but instead to identify key aspects of malaria transmission that are relevant to the Kayin state and other areas in Southeast Asia. Raw numbers used for the calculation of the entomological indices estimates were added in the corresponding tables, additional references on the method used for the calculation of entomological inoculation rate were added in the Methods section. Regarding reviewer's suggestion on the discussion, we wish to keep the outline of the manuscript version 1. Although a comparison of the entomological indices in different geographic areas and across the endemicity spectrum would be of great interest, a review
of the global data seems beyond the scope of the current paper as it would represent a tremendous amount of work and could be the object of a full review article. In addition, the specificities of malaria transmission in the 4 villages (and Southeast Asian malaria transmission settings) were discussed into details in the first version of the manuscript. The point that were discussed included the low infection rates in malaria vectors and high biting rates, the seasonality in *Plasmodium falciparum* transmission, the heterogeneity of the entomological indices, the low parasite loads in infected vectors, the high diversity of malaria mosquitoes, their outdoor and early biting pattern, their opportunistic host type selection and ability to feed on livestock. Finally, clear recommendations for malaria vector-control in this region were made in a specific paragraph using our results as a rational: we suggested to evaluate the use of outdoor spraying of insecticides on daytime resting habitats to tackle exophilic mosquitoes and veterinary approaches to tackle zoophagic malaria vectors. Regarding reviewer’s inquiry about larval source management, it is difficult for us to discuss this intervention because we did not collect data on larval stages. Existing bibliography and our data on adults suggest that larval habitats are diverse, fragmented and poorly accessible, which probably limits the feasibility of larval source management in Kayin state. Regarding the use of “village ecology” as a vector-control tool, it is unclear to us what set of interventions the reviewer was referring to. What may appear to be “dogmatic” for the reviewer (i.e. the specificity of transmission in Asia by mostly exophagic mosquitoes) may be considered by others as one of the main factor explaining the differences in mosquito bed-nets and indoor residual spraying outcomes in Southeast Asia when compared to Africa. In the current context of malaria elimination, we therefore believe that this notion should be at the center of the discussion. Point-to-point answers to specific comments are given below:

- 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.

Reviewer’s suggestion has been addressed by presenting the collection schedule in the Table S1 of the revised manuscript.

- The “Kayin” state is rarely 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”

Reviewer’s concern was addressed by changing “Thailand-Myanmar border” to Kayin state were possible and by mentioning more often “Kayin state” in the main text.

- Lastly, if there is no real discussion on the difference similarity among the 4 villages, why attracting with the “4 villages” in the title?

We agree with the reviewer that a more detailed explanatory analysis on the heterogeneity of malaria transmission in the four villages would have been valuable. However, in the absence of appropriate datasets, this analysis is not possible. Documenting differences is particular villages was not the only objective of including 4 villages in the study design. Extensive longitudinal follow-up in four villages has also allowed us to identify key aspects of malaria entomology that would not have been accurately describe with less sites or time-point collections. Therefore, we think it is important to mention the duration of the follow-up and the number of villages in the title.

- Abstract: Some revision could be done if the authors agree on the following comments:
The abstract was modified as per reviewer's suggestions.

- 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.

Reviewer's comment was addressed by adding more details in the paragraph “Study villages” of the Methods section.

- Was the MDA still active during the whole entomological survey?

The reviewer is right to inquire about the timing of mass antimalarial drug administration and to stress that the elimination intervention has been poorly described in the manuscript. As specific paragraph describing the intervention has been added to the Methods section and additional references were provided to the reader.

- Were the houses randomly chosen?

Yes, the houses were randomly selected and this has been stated in the revised manuscript.

- Were the preliminary data (ref14) included in the analysis?

Yes, the preliminary data were included in the analysis.

- Consider simplification: instead “6 sites” use 5 houses plus one cow collection.

The phrasing of this sentence has been simplified as per reviewer’s suggestion.

- Verify the accuracy of the sentence for mosquito collection under the net covering the cow.

The accuracy of the sentence has been verified.

- Expressing SI / 1000 mosquito can be confusing. I would keep with the classical SI expressed as %.

We agree with the reviewer. SI values were expressed as percentages in the revised manuscript.

- 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.

This definition of EIR is based on the work of Drakeley et al. 2003 (An estimation of the entomological inoculation rate for Ifakara: a semi-urban area in a region of intense malaria transmission in Tanzania), also reviewed by Tusting et al. 2014 (Measuring Changes in Plasmodium falciparum Transmission: Precision, Accuracy and Costs of Metrics). When EIR is analysed as the product of SI per HBR (equation 1), computing confidence intervals (CIs) becomes difficult since HBR and SI are not independent of each other; CIs can be computed more easily when analyzing EIR as a biting rate (taking into account only the sporozoite-infected mosquitoes) (equation 2). Adjusting the number of person-nights of collection over the proportion of collected mosquitoes that were analyzed by qrtPCR allow to take into account that not all mosquitoes were screened for Plasmodium infection (equation 3). Of note, this adjustment makes the calculation equivalent to the “classical” definition of EIR (equation 4) and has little impact on the estimate value as 99% (50082/50708) of the collected specimens were analysed by PCR Plasmodium.

\[
(1) \quad \text{EIR}=\text{SI} \times \text{EIR} \\
(2) \quad \text{EIR}=\frac{\text{Nb. sporozoite positive mosquitoes}}{\text{Nb. person.nights of collection}} \\
\]
EIR = \frac{\text{Nb. sporozoite positive mosquitoes}}{\text{Nb. person.nights of collection} \times \frac{\text{Nb. of mosquitoes tested by PCR}}{\text{Nb. of collected specimens}}} \\

EIR = \frac{\text{Nb. sporozoite positive mosquitoes}}{\text{Nb. of mosquitoes tested by PCR}} \times \frac{\text{Nb. of collected specimens}}{\text{Nb. person.nights of collection}}

- The CI calculation might need approval from an external statistician.

The method used for the CI calculation in this paper was also reviewed in Landier et al. WOR, 2017 and Chaumeau et al. JID, 2018.

- From my understanding of the Methods, the total person-nights should be 4200 and total cow-nights 420. Did I miss something?

We are thankful to the reviewer for pointing this ambiguity. Exceptions to the initial study design have been explicitly mentioned in the corresponding paragraph of the Methods section. The number of person-nights and cow-nights for each survey is also presented in the Table S1 of the revised manuscript.

- “Potential malaria vectors”: could you provide your definition of those?

We are thankful to the reviewer for pointing this unclear wording, it has been removed from the revised manuscript.

- A species table as sup data could be useful for non-specialist of Asian mosquito vectors.

Reviewer’s suggestion was addressed by adding a species table in the Introduction.

- In figure 2 “other species” what does this include?

The legend of Figure 2 has been reworded. “Potential malaria vectors” has been changed to “a priori malaria vectors”, which refers to groups of Anopheles species that have been previously found infected with human malaria parasite in other studies (Funestus, Maculatus, Leucosphyrus, Barbirostris and Annularis Groups). “Other species” has been changed to “other groups” and includes all the other groups that have been reported in the paragraph “Anopheles diversity”. More details were also added in the legend note.

- Fig2: why not commenting the comparative results for HLC and CBC and among the villages.

Ranges were cited in the main text to point village differences in the species distribution at the group level. Moreover, differences in species-specific distribution between villages assessed by molecular identification were commented into details in the main text and are reported in the Supplementary File 1, Table S2.

- Homogenize between fig 2 A, Legend, Table 1 and main text : CBC or CBT.

Figure and table captions, and in-text citations were harmonized to CBT in the revised manuscript.

- Table 2: Correct Mean Pf-EIR for Funestus : 0.1, not 0.01.

Mean Pf-EIR for Funestus Group has been corrected.

- 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.

The raw number used for the calculation was provided in all tables. Unless specified otherwise, the calculation was made using the entire dataset.

- It would be also informative to provide the raw data as supp file for the identification of the species for the Plasmodium positive samples.
Reviewer’s suggestion was addressed by presenting the raw data of identification of the species for *Plasmodium* positive samples in the Supplementary File 1, Table S4.

- 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.

Reviewer’s suggestion has been added to the revised Supplementary File 2.

- 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.

We are thankful to the reviewer for pointing that the correlation between oocysts and sporozoite densities is not straightforward, especially in wild mosquitoes, and that it may yield to spurious interpretation. The correlation between oocysts and sporozoite densities has been removed from the revised manuscript.

- In addition, please indicate the oocyst detection method, PCR? And how the midgut were preserved?

A statement on oocysts detection method (PCR) and midgut preservation has been added in the Methods section of the revised manuscript in order to address reviewer's comment.

- Table 3 has been moved to supplemental data as per reviewer suggestion.

Table 3 has been moved to supplemental data as per reviewer suggestion.

- First sentence: “overall”, be more precise as taking into account all mosquitoes captured by HLC...

We are thankful to the reviewer for pointing this inaccurate wording. The word “overall” has been changed to “taking into account the whole dataset”.

- Nevertheless, again I cannot obtain the same numbers for both HBR and CBR, though very closed.

We hope that the above explanations will help the reader in understanding the calculation used for the entomological indices.

- 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.

We are thankful to the reviewer for mentioning that estimating zoophagy accurately would require a different approach than that used in our study, although comparing the human- and cow-biting rates as an index can be useful to describe the capacity of some specie to feed on bovines. The wording “zoophagic index” has been changed to “cow-biting index” (CBI) to address reviewer's comment.

- Figure 6 and 7: revised the labeling for Fig 6, no distinction in the grey colours.

Distinction in the grey colours is shown in the Key box in the figure (figure S12 of the revised manuscript).

- Although there is a tendency for early biting it does not stand for all species ie minimus.

“Anopheles mosquitoes exhibited an outdoor and early biting pattern” has been changed to “Anopheles mosquitoes exhibited an outdoor and/or early biting pattern” in order to address reviewer's comment. Although the biting rate of *An. minimus* did not peak during the early
evening/morning, *An. minimus* biting rate between 5-6 pm and 5-6 am was higher than that of any other *Anopheles* species. Moreover, the high biting rates observed at 5-6 pm and 5-6 am suggest that this species was active before and after the standard collection time, which would meet the definition of early biting.

- 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?

Some figure from this section were relocated in the supplementary file as per reviewer’s suggestions.

- 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?

The mean was calculated using the entire dataset for the corresponding grouping variable (village in the table 4 and season in the table 5) using the definition provided in the Method section. Raw data used for the calculation of entomological indices estimates were added to all tables to help the reader understanding the details of the calculation.

- 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.

The figures suggested by the reviewer were added in the Supplementary File 1, Figures S7-S10. We think that presenting mean values of entomological indices in tables 4 and 5 is important to understand malaria transmission in this area and compare with other settings, which would maybe not appear as clearly in the figures presenting the longitudinal data.

- 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.

More details on the elimination intervention were added in the Intervention paragraph, Methods section and more reference were added in the revised version of the manuscript. We think that discussing the the dynamic of gametocyte carriage may be beyond the scope of this article. For example, Pf carriers are also likely to be excellent providers of gametocytes the year around if not treated. A more detailed analysis of the relationship between parasitological and entomological indices was published in the Journal of Infectious Diseases by Chaumeau et al. in 2018.

- 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 typo 05:00pm has been changed to 05:00am. The window 09:00pm to 05:00am indoors is expected to cover the timing when people do sleep under a net. The stronger wording suggested by the reviewer was added to the revised version of the manuscript.

- 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?

The paradigm is that – based on observations made in Sub-Saharan Africa - universal coverage with LLINs would prevent most of the infective bites, and conversely that the part of the transmission that would not be covered by LLINs would be only a residue (i.e. a small part) of transmission at baseline. Some control or elimination programs indeed still rely mainly, and sometimes solely, on mass distribution of LLINs. The data presented in this study (as well as other references cited in the discussion) clearly demonstrate that LLINs have only a marginal impact on disease prevention in the GMS rather than preventing the main part of the transmission. Therefore, we do not think that is would be accurate to refer to the part of the transmission that is not prevented by LLINs as “residual transmission” in the transmission settings described in this study.

**Competing Interests:** No competing interests were disclosed.