

Investigating Mosquitoes' Behavior for Malaria Prevention in Webuye, Western Kenya

by

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Thesis submitted in partial fulfillment of
the requirements for the degree of
Master of Science in the Duke Global Health Institute
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ABSTRACT

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Abstract

Insecticide-treated bed nets (ITNs) are widely used in Kenya to prevent the mosquitoes, which can transmit malaria, from biting people at night. However, there is a concern that mosquitoes become resistant to the ITN environment by changing their feeding behavior. The research goal was to observe the current feeding behavior of female *Anopheles* in Webuye, Western Kenya. Prokopack aspirators, CDC light traps, and sticky barrier screens were used for mosquito collection during May – July 2018. 662 collected female *Anopheles*, most of which were *Anopheles gambiae sensu lato*, were included. Descriptive and statistical analyses were applied, and the results show that the feeding proportion was 79.3% in mornings and 13.7% at nights, which was not significantly affected by the frequency of bed net use. 35.9% and 4.3% of the female *Anopheles* were human and *Plasmodium (P.) Falciparum* gDNA positive, respectively. Most of the *P.falciparum* positives were also fed and human gDNA positive, meaning that most of the infected or infectious mosquitoes had human blood meals and may have already attended malaria transmission. The study failed to observe outdoor feeding behavior by sticky barrier screens, and behavioral adaptation may not be responsible for most of the persistence of transmission. The study implicates a full investigation of ITN condition, more research on chemical resistance, and further surveillance of mosquito biting time.

Dedication

This study is dedicated to my hometown, Guangzhou, China, who is inclusive to all kinds of people and all kinds of culture. It is who shapes me into the person that I am, who attracts more than 500 thousand Africans to work and live and makes me interested in the beautiful continent they are coming from, and most importantly who suffered from SARS and aroused my eager to fight against infectious disease.

All start from here.

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1. Introduction

Among over 3,000 mosquito species, a handful of them belong to *Anopheles*, the only genus that transmits human malaria on the earth (Liu et al., 2011; Moutinho, Gil, Cruz, & Ribolla, 2011). They usually bite humans in evening and at night (Kent, Thuma, Mharakurwa, & Norris, 2007; Moutinho et al., 2011), resting indoors or outdoors after blood feeding (Kent et al., 2007). Indoor residual-house spraying (IRS) and insecticide-treated bednets (ITNs), which is more widely used than IRS, are two main approaches of vector control in Kenya (Guyatt, Corlett, Robinson, Ochola, & Snow, 2002). The rationale is —ITNs can prevent mosquito from biting people during their sleeping period and IRS can kill insects and protect people indoors, especially those that rest indoors on walls after feeding. Their effectiveness has been proven – it has been found that sleeping under ITNs and in an area received IRS significantly reduce people’s risk of malaria infection by 63% and 75%, respectively (Guyatt et al., 2002).

However, one challenge for malaria vector control in Africa is the mosquito resistance to ITNs and IRS. These two controlling methods highly rely on pyrethroids, a class of insecticide, for at least 30 years, and very few other classes of insecticides are available for malaria vector controls (Ranson et al., 2011). This has become a defect of the control methods that impairs their effectiveness because the resistance of mosquito emergent, especially the genetic resistance to the toxicant of insecticide (Ranson et al., 2011). In addition to metabolic resistance, malaria vectors can also be selected to avoid

exposure to insecticides by changes in their behavior. (Gatton et al., 2013; Ranson et al., 2011), including but not limited to shortening the period of staying indoors (Akogbeto, Padonou, Bankole, Gazard, & Gbedjissi, 2011), reducing the rest on walls (Akogbeto et al., 2011; Gatton et al., 2013) , earlier indoor blood feeding (Gatton et al., 2013), increasing outdoor bites on human (C. N. M. Mbogo, Baya, Ofulla, Githure, & Snow, 1996), and changing objects when the preferred blood meal source is non-accessible (i.e. human) (Moiroux et al., 2012). The insecticide-resistant behaviors could decrease the efficacy of traditional malarial control methods and even lead to the failure of prevention for malaria transmission (Gatton et al., 2013). Therefore, it is important to study the current behaviors of female *Anopheles* for improving current or finding new preventing methods and understanding the mosquitoes' biting and resting habits can promote better strategies for the disease prevention. Although research findings have demonstrated evidence of behavioral resistance of mosquitoes in some areas, this mechanism of resistance has not been fully studied in Kenya, a country with high malaria prevalence and wide usage of ITNs. Investigating the current feeding behavior of *Anopheles* is thus crucial to malaria surveillance in Kenya.

1.1 Mosquito Trapping Tools

In order to understand the feeding habits of female *Anopheles*, we must carefully consider the trapping methodology. There is a large variety of techniques for mosquito surveillance. Each trapping method depends on specific mosquito behaviors and may be

enriched in subpopulations that exhibit those behaviors. Table 1 shows a brief summary of the trapping tools for *Anopheles* mosquitoes (except sticky traps (Roslan, Ngui, Vythilingam, & Sulaiman, 2017) were for *Aedes*). The tools are classified based on the types of mosquitoes they trap. For instance, window exit traps and indoor hand collections (IHC) are designed for collecting blood-fed mosquitoes indoors while barrier screen is for blood-fed mosquitoes outdoors. Some tools, such as resting box, sticky trap, and clay pot, can be applied to blood-fed mosquito collection both indoors and outdoors. In contrast, CDC light traps and human landing catches (HLC) target for inside and outside mosquitoes which are searching for blood meals. However, there are disadvantages and limitations of some trapping techniques. For example, window exit trap (Dandalo et al., 2017; Gayan Dharmasiri et al., 2017) may trap only a fraction of the exiting mosquitoes since the insects can also fly outside from other doors or gaps. Müller et al. (2017) modified window exit trap into a glue-painted surface version; however, one drawback is the possibility for mosquitoes to escape from the sticky window and return to home. Clay pot (Dandalo et al., 2017; St. Laurent et al., 2018), resting box (St. Laurent et al., 2018), modified plastic bucket (Dandalo et al., 2017), and large tent trap (St. Laurent et al., 2018) are time-consuming and less efficient. CO₂- or other odour-baited traps, such as Odour-baited BG-Malaria (BGM) trap (Batista et al., 2017), BG-Sentinel (BGS) trap (Batista et al., 2017; Mohlmann et al., 2017), and mosquito CO₂-baited trap (Eshun, Gerry, & Hayes, 2016), are not logically suitable for studying natural mosquito

behavior since they are designed to modify behavior to enhance trapping ability. This study tries to discover the natural behavior of the insect on finding and choosing blood source, which is in conflict with the idea of baited trapping. Barrier screen trapping with aspiration is a time-effective choice (Burkot et al., 2013), but mosquitoes may be easy to escape from the screens. Moreover, Mosquito Magnet Liberty Plus (MMLP) trap (Mohlmann et al., 2017), a large trap with a gas cylinder, is efficient but expensive, which may not be affordable and practical to the project.

Prokopack aspirator is one applicable tool of catching indoor blood-fed mosquitoes and can be applied to the study. CDC light traps are also suitable for host-seeking mosquito collection. Nevertheless, there was a lack of suitable tools for capturing blood-fed *Anopheles* exiting after indoor feeding. A new efficient trapping approach on this typical mosquito group is needed for better mosquito surveillance.

Table 1 Summary of Mosquito Trapping Tools

	Type of Trapping Techniques		
	Indoor	Outdoor	Indoor & Outdoor
Blood-Fed	1) Window exit trap (Dandalo et al., 2017; Gayan Dharmasiri et al., 2017) 2) Spray sheet collections (PSC) using pyrethrum (Gayan Dharmasiri et al., 2017) 3) IHC using mouth aspirators (Gayan Dharmasiri et al., 2017)	1) Modified plastic bucket (Dandalo et al., 2017) 2) Barrier screens with mouth aspirators (Burkot et al., 2013) 3) The Furvela tent-trap (Charlwood, Rowland, Protopopoff, & Le Clair, 2017)	1) Sticky traps (Roslan et al., 2017) 2) Clay pot (Dandalo et al., 2017; St.Laurent et al., 2018) 3) Resting box (wooden) (St. Laurent et al., 2018)

	4) Glue painted window exit trap (Müller et al., 2017) 5) CDC Light Trap (Batista et al., 2017; Chen et al., 2017) 6) Prokopack aspiration (Vazquez-Prokopec, Galvin, Kelly, & Kitron, 2009)		
Host-Seeking	1) BGM Trap (Batista et al., 2017)	1) Mosquito CO ₂ -baited Trap (Eshun et al., 2016) 2) Large tent trap (St. Laurent et al., 2018) 3) MMLP Trap (Mohlmann et al., 2017)	1) BGS Trap (Mohlmann et al., 2017) 2) HLC (Batista et al., 2017; Gayan Dharmasiri et al., 2017) 3) LED-baited Trapn(Costa-Neta et al., 2017) 4) CDC Light Trap (Charlwood et al., 2017)

1.2 Study Aims

The research question of the study was: what are the biting and resting habits of *Anopheles* in the rural Kenya where ITN coverage is high? To fill the gaps, the objectives were designed to 1) use innovative techniques to collect *Anopheles* and observe their current feeding and resting habits; 2) explore successful feeding activities of *Anopheles* with the consideration of frequency of ITN use; 3) understand and compare the feeding behavior of *Anopheles* collected by different trapping techniques.

Aim 1 Apply novel mosquito trapping techniques for observing populations of malaria vectors: a set of innovative trapping techniques were used with the reference of previous studies (Burkot et al., 2013; Charlwood et al., 2017; Müller et al., 2017; Vazquez-Prokopec et al., 2009) to collect female *Anopheles* indoors and outdoors. The hypothesis was that all the trapping tools applied to the study successfully collect abundant indoor and outdoor mosquitoes.

Aim 2 Examine the prevalence of fed female Anopheles collected at different time and in different locations with the consideration of frequency of use of bednet: the study looked at the prevalence of successful feeding activities among *Anopheles* and the impact of frequency of net use on the prevalence. We hypothesized that the prevalence of fed *Anopheles* was lower in the households that frequently used bednet than in the households that seldomly used bednet.

Aim 3 Compare female Anopheles collected by different trapping tools through key characteristics of feeding activities: the study aimed to compare *Anopheles* collected indoors and outdoors as well as compare fed and host-seeking *Anopheles* by key characteristics of feeding activities, including location, time, abdominal feature, species, blood-meal sources, and infection of malaria parasite (*P. falciparum*). The study also examined the difference between female *Anopheles* collected before and during the sleep time. We hypothesized that indoor and outdoor *Anopheles* and fed and host-seeking

Anopheles were different by their characteristics, and there were more *Anopheles* during than before the sleep time.

2. Methods

This is a cross-sectional study designed for exploring the behavior of *Anopheles* in a rural area where ITNs are widespread. The data collection period was from May to July, 2018.

2.1 Study Area and Population

The study areas were three villages, Kinesamo, Maruti, and Sitabicha, in Webuye, Western Kenya (Figure 1). 12 households were selected in each village by random sampling of an initial household and then enrolling surrounding households in a cluster until 12 households had been enrolled. The study population was the mosquitoes which were active near the households located in Webuye. The enrollment of households did not consider the demographic characteristics of people living in those households.

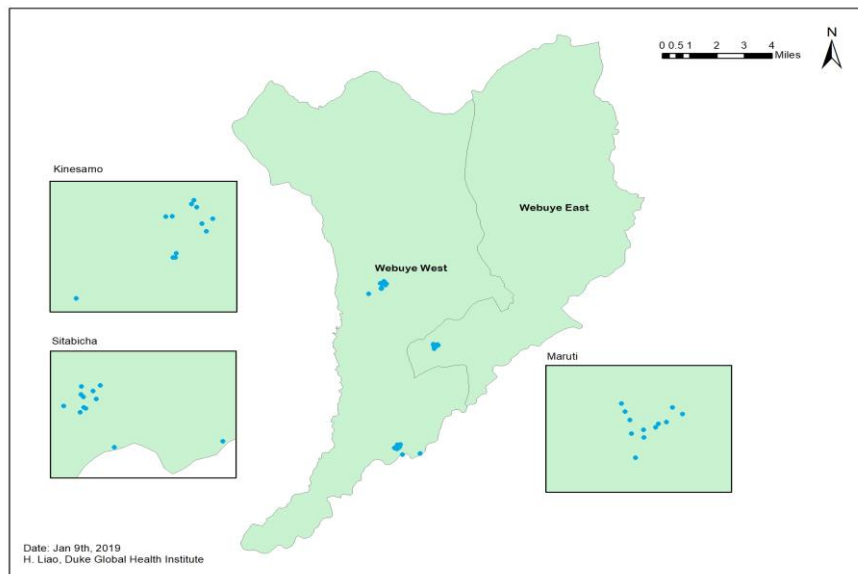


Figure 1 Geographical map of Webuye, Western Kenya

2.2 Setting

All the participating households owned more than one ITN. Therefore, we assumed that all the mosquitoes collected were exposed to ITN environment. Two main outcomes of the study were the feeding habits of female *Anopheles*. Main data variables were location of collection (indoors or outdoors), collection type (Prokopack aspirator collection, sticky barrier screen collection, and CDC light trap collection), abdominal status (unfed, blood-fed, half-gravid, and gravid), human gDNA detection (detected or undetected), *P.falciparum* gDNA detection (detected or undetected), mosquito species (*Anopheles (An.) gambiae sensu lato (s.l.)*, *An. funestus s.l.*, *An. coustani*, and *An. rufipes*), dates, and collection time. Several indicators, including prevalence of successful feeding and parasite infection and mosquito population by abdominal status, by blood source, by time, and by location, were applied to reflect the current feeding habits.

2.3 Data Collection

The study was interested in four categories of female *Anopheles* – the first two were mosquitoes that exhibit traditional anopheles' behaviors – mosquitoes that seek a blood meal indoors during sleeping hours (2.3.3) and mosquitoes that rest indoors after feeding (2.3.1). The second two categories were non-traditional vector behaviors – mosquitoes that seek bloodmeals outdoors while people were active (2.3.4) and mosquitoes that rest outdoors after feeding indoors (2.3.2).

2.3.1 Indoor Blood-fed Mosquitoes Resting Indoors

Prokopack vacuum aspirators were used as indoor collectors to capture blood-fed mosquitoes which were resting on the walls on early mornings (e.g. from 6 a.m.(Reddy et al., 2011)). The collections were done on the mornings of Mondays, Tuesdays, and Wednesdays in Kinesamo, Maruti, and Sitabicha, respectively. The household members were asked to keep the windows and doors closed until the local researchers came to ensure that most mosquitoes could not escape before the collections. These mosquitoes were assumed to feed indoors and rested indoors after feeding.



Figure 2 A local researcher was collecting mosquitoes by Prokopack

2.3.2 Mosquitoes Resting Outdoors after Feeding Indoors

We designed a new mosquito trapping method, a hybrid method of barrier screens(Burkot et al., 2013) and window entry/exit traps(Müller et al., 2017), where barrier screens were set outside with entomology glue (Tanglefoot) on the surface. These

traps were intended to intercept mosquitoes leaving the house after feeding to rest outdoors. Mosquitos which land on the surface would be stuck in the glue. These traps were applied for outdoor mosquito collection in mornings of Mondays to Wednesdays. The barrier screens were first set up with a size of 2mx2m and were then expended to 4m(width)x6m(height). The screens almost touched the ground and were a few meters distant from main exit/entrance windows or doors of the households (Reddy et al., 2011). Due to limited human resources, two households in one of the three villages participated in a collection day. One barrier screen was set at each of two households in the afternoons before the collection days. The tool was used to collect mosquitoes which have fed indoors at nights but leave the house to rest outdoors before laying eggs.



Figure 3 The research team was setting a sticky barrier screen

2.3.3 Indoor Host-Seeking Mosquitoes

CDC light traps were employed to capture host-seeking mosquitoes indoors during times before and after the host go to bed. As a result of a limited number of tools, we randomly selected five households from a village of the cohort to attend the collection on Wednesday and Thursday nights. The nights before Prokopack collections were not selected for light trap collections to avoid the possible impact of light traps on indoor blood-fed mosquito collection. Two traps were set in each house: one was in a living room or kitchen, where the household members spent time in the evening before their sleep; and the other was in a sleeping room near a bed net. On the afternoons of the collection days, the research team went into the houses, set the traps after communication with household heads, and gave the instructions of using them in Swahili (i.e. the local language). The household members were asked to turn on a CDC light trap set in the living/kitchen area after they return to home and then turn it off before sleep; they also turned on a light trap set in the bedroom before they go to bed until they get up in the next morning. The research team picked the trap cups in the next mornings, asked the household members and recorded the collection time.



Figure 4 A local researcher was setting up a CDC light trap

2.3.4 Monthly Household Survey

To achieve the study aim of examining the impact of frequency of net use on prevalence of fed mosquitoes, we derived the information of net use from the household surveys of Dr. O'Meara's spatial R21 project. Each month, the local research team walked into the houses, asked the household heads by Swahili, and filled the surveys by tablets. The survey information was then stored in a digital database for future use.

2.4 Data Management and Pre-Processing

The mosquitoes collected by Prokopack and CDC light traps were first refrigerated in the Webuye office for several hours to ensure that the samples were not alive. Female *Anopheles* were then sorted by morphology in terms of their appeared features. The wings, palps, and the whole bodies were photographed and sent to an entomologist for anopheline species discrimination. To distinguish infected and

infectious mosquitoes, dissection was applied to the study samples collected by Prokopack aspirators. The idea was that mosquitoes infected by *P.falciparum* had the parasites' oocysts in their midguts, and they became infectious once the sporozoites were in their salivary glands (Foley et al., 2012). In other words, we were able to find malaria parasite in the abdomen of infected mosquitoes and in the head of infectious mosquitoes. The research team did the dissection by separating the head and abdomen of each female anopheles and solely stored them in the tubes labeled with unique IDs after photography. CDC light trap collected samples were stored in whole bodies and not dissected with the consideration that most of these mosquitoes were unfed and possibly have nothing in their midguts. On the collection days, the researchers also filled the record forms with date, collection time, and household ID, number of mosquitoes collected by abdominal status, and the unique ID of each sample and its abdominal status. The samples were delivered to and stored at the office in Eldoret every two weeks. When the data collection was completed, all the samples were transferred to Duke Malaria Collaboratory for DNA extraction and detection. The gDNA of samples were derived through a chelex extraction protocol (Appendix C). PCR assay was applied to detect human and *P.falciparum* gDNA and to determine if the sample was fed by human blood and infected by parasites. The DNA extraction and detection were finished by December 2018.

2.5 Data Quality Control

To minimize the errors occurred during data collection, all the data that were collected by incorrect procedures, such as incorrect use of CDC light traps by household members, were recorded and removed from the dataset. To control the recall bias that may occur from the self-reports of CDC light trap collection time, the household members were asked about the information in the next morning of the collection nights. We also carefully checked the dataset to eliminate the errors occurred during data entry. The samples contaminated during DNA extraction were also excluded. To reduce the possibility of misclassification, each mosquito sample was observed under a microscope for the identification of their abdominal status and anopheline species. Moreover, real-time PCR assay was used to ensure the high accuracy of gDNA detection on blood source (i.e. non-human/human) and parasite status (i.e. non-parasite/parasite).

2.6 Data Analysis

All statistical analyses were produced by Stata SE (Release 15; StataCorp, 2017) and RStudio (R Core Team, 2016). Descriptive analysis was generated to compare the characteristics among the collected mosquitoes by species, blood source, abdominal feature, and parasite infection status. Statistical analysis was performed to examine impacts of ITN use on prevalence of successful feeding and to explore the association between collection period and the number of female *Anopheles* collected.

2.6.1 Descriptive Analysis

We applied descriptive analysis to study and summarize the feeding behavior of female *Anopheles* collected in the study area during May – July, 2018. The feeding behavior of mosquitoes was presented by prevalence of successful feeding, prevalence of mosquitoes with positive human gDNA detection, prevalence of mosquitoes with positive *P.falciparum* gDNA detection, and distribution of female *Anopheles* by collection type, village, month, abdominal status, species, human gDNA detection, and *P.falciparum* gDNA detection. Bar charts and pie charts were produced to compare the characteristics of female *Anopheles* in different types of collections.

2.6.2 Statistical Analysis

2.6.2.1 Prevalence of Successful Feeding and ITN Use

The information of ITN use was derived from the monthly household survey, which asked participants the number of nights that they slept under a bed net during last week. The surveys with missing data were excluded from the study. Based on the survey, we defined those households had all surveyed members slept under bed nets for five and over five nights as the households with frequent net use. The households with at least one surveyed member slept under bed net for less than five nights were classified as the households with seldom net use. Two sample z test was applied to determine if the prevalence of successful feeding was significantly different by frequent and seldom net use.

2.6.2.2 Mosquito Population before/during Sleep Time

Poisson regression was used to examine the difference of numbers of female *Anopheles* between the time before and after people go to bed. Duration of collection, month, and village were considered as covariates and included in the regression model.

3. Results

3.1 Demographic Summary

665 female *Anopheles* were collected in the study area from May 7th to July 20th, 2018. Only one sample was trapped outdoor by sticky barrier screen and was excluded since the trapping tool failed to collect adequate amount of outdoor female *Anopheles*. Two samples were trapped through incorrect operating procedures of CDC light traps and removed from the sample population. Therefore, the research includes 662 samples in total, of which 348 were collected by Prokopack aspirators and 314 by CDC light traps (including 32 collected before people sleep and 282 during sleep). The average number of collected female *Anopheles* per household in a single collection by Prokopack aspirators, by CDC light traps before people sleep, and by CDC light traps during people sleep were 0.88, 0.42, and 3.53, respectively, indicating that mosquitoes are more probable to be trapped by CDC light traps during the sleep time. In addition, 314 of the female *Anopheles* were from Kinesamo, 261 from Maruti, 87 from Sitabicha; and 309 of them collected in May, 272 in June, and 81 in July.

3.2 Prevalence of Successful Feeding and Net Use

According to the monthly household survey, 89.3% reported frequent use of mosquito net during sleep, 8.3% reported seldom net use, and 2.4% missing data. The result of two-sample z test demonstrates that there is no significant difference between the fed proportion of frequent-net-use and seldom-net-use groups in Prokopack

aspirator collection sample ($p=0.90$; significance level: 0.05) (Table 2); and the testing result is same for CDC light trap collection sample ($p>0.99$; significance level: 0.05) (Table 3). Therefore, frequency of net use did not moderate the fed proportions. For the CDC light trap collection sample, the prevalence of fed mosquitoes was 13.7% (95%CI [0.103, 0.180]). For the Prokopack collection sample, the prevalence of fed mosquitoes was 79.3% (95%CI [0.747, 0.833]).

Table 2: 2 by 2 table of mosquito type and household net use (Prokopack aspirator collection)

	Seldom Net Use	Frequent Net Use	Total
Unfed	15 (19.5%)	57 (21.0%)	72 (20.7%)
Fed	62 (80.5%)	214 (79.0%)	276 (79.3%)
Total	77 (100.0%)	271 (100.0%)	348 (100.0%)

Table 3: 2 by 2 table of mosquito type and household net use (CDC light trap collection)

	Seldom Net Use	Frequent Net Use	Total
Unfed	38 (86.4%)	233 (86.3%)	271 (86.3%)
Fed	6 (13.6%)	37 (13.7%)	43 (13.7%)
Total	44 (100.0%)	270 (100.0%)	314 (100.0%)

3.3 Comparison of Anopheles in Different Types of Collections by Key Characteristics

3.3.1 Anopheline Species

613 female *Anopheles* were successfully defined by the entomologist while 49 samples were undetermined due to missing fragments, such as wings and palps. We found *An. gambiae s.l.* as the main species in the three villages and all types of collection (Figure 5). 576 of the samples were *An. gambiae s.l.*, which was remarkably larger than *An. funestus s.l.* (19), *An. coustani* (17), and *An. rufipes* (1).

In general, the numbers and types of species were distributed differently in terms of collection type, month, and village. The CDC light traps gathered 22 *An. gambiae s.l.*, and 5 *An. coustani* in the before-sleep collection and 248 *An. gambiae s.l.*, 9 *An. funestus s.l.*, and 12 *An. coustani* in the after-sleep collection. No *An. funestus s.l.* was found in the before-sleep collection and no *An. rufipes* found in the whole CDC light trap collection. The Prokopack aspirator collection gathered 306 *An. gambiae s.l.*, 10 *An. funestus s.l.*, and 1 *An. rufipes*; no *An. coustani* was trapped. More *An. coustani* were collected in June than May and July while more *An. funestus s.l.* and *An. gambiae s.l.* were collected in May than June than July. *An. coustani* was only found in Kinesamo and Maruti in CDC light trap collection, and *An. rufipes* was only found in Kinesamo in Prokopack collection. *An. funestus s.l.* was found in all villages in Prokopack aspirator collection but was only found in Kinesamo and Maruti in the after-sleep period of CDC

light trap collection. Therefore, we found less species diversity in before-sleep collection and in Sitabicha than in the other two collections and villages.

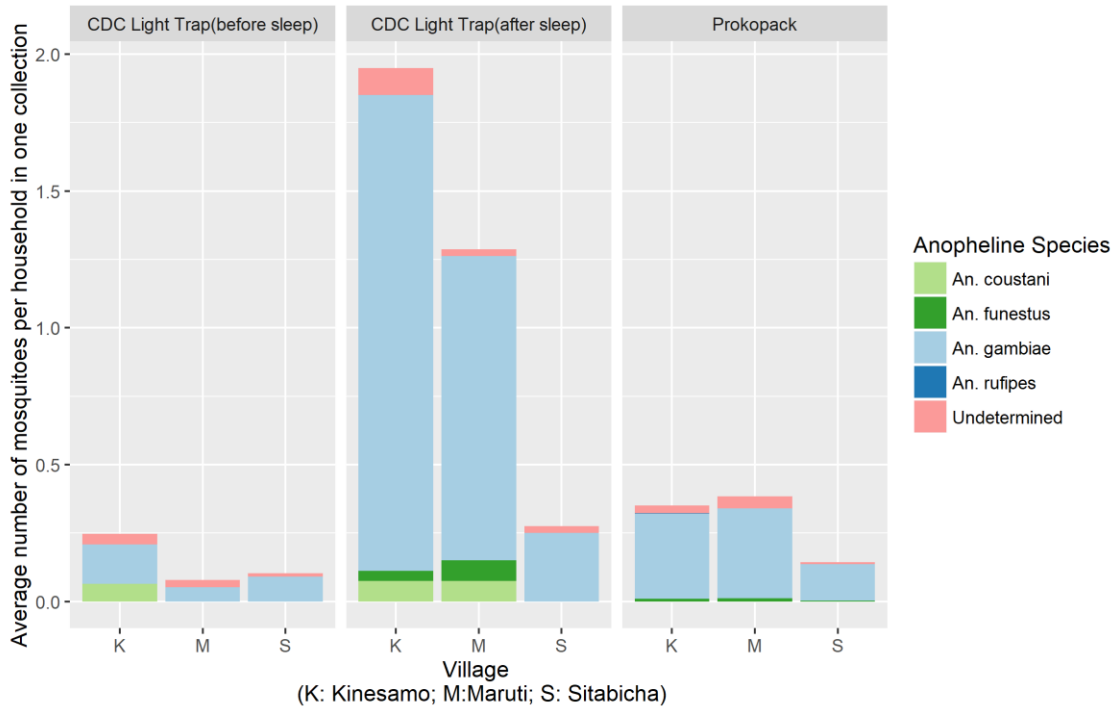


Figure 5 Female anopheline species collected in the three villages by collection type

3.3.2 Abdominal Status

During the CDC light trap collection, the research team gathered 266 unfed female *Anopheles*, 8 blood-fed, 29 half-gravid, 6 gravid, and 5 undetermined. In the before-sleep collection, 32 female *Anopheles* were collected, including 25 unfed, 3 blood-fed, 2 gravid, and 2 determined; no gravid *Anopheles* was trapped. In the after-sleep collection, 282 female *Anopheles* were collected, including 241 unfed, 5 blood-fed, 24 half-gravid, 9 gravid, and 3 undetermined. In the Prokopack aspirator collection, 71 samples were unfed, 96 were blood fed, 126 were half gravid, 51 were gravid, and 4 were

undetermined. As reflected in Figure 6, unfed and successfully fed *Anopheles* was dominant in CDC light trap collection and Prokopack aspirator collection, respectively, which is consistent with our study design since CDC light traps were used to collect host-seeking mosquitoes and Prokopack aspirators were to collect fed mosquitoes. For Prokopack aspirator collection, all kinds of abdominal status were found in each village, and more half-gravid samples were found than the samples with other abdominal status than in each village. For CDC light trap collection, although the majority was unfed *Anopheles*, the share of successfully fed *Anopheles* was still noticeable in the after-sleep collection. In contrast, the share was very small in the before-sleep collection.

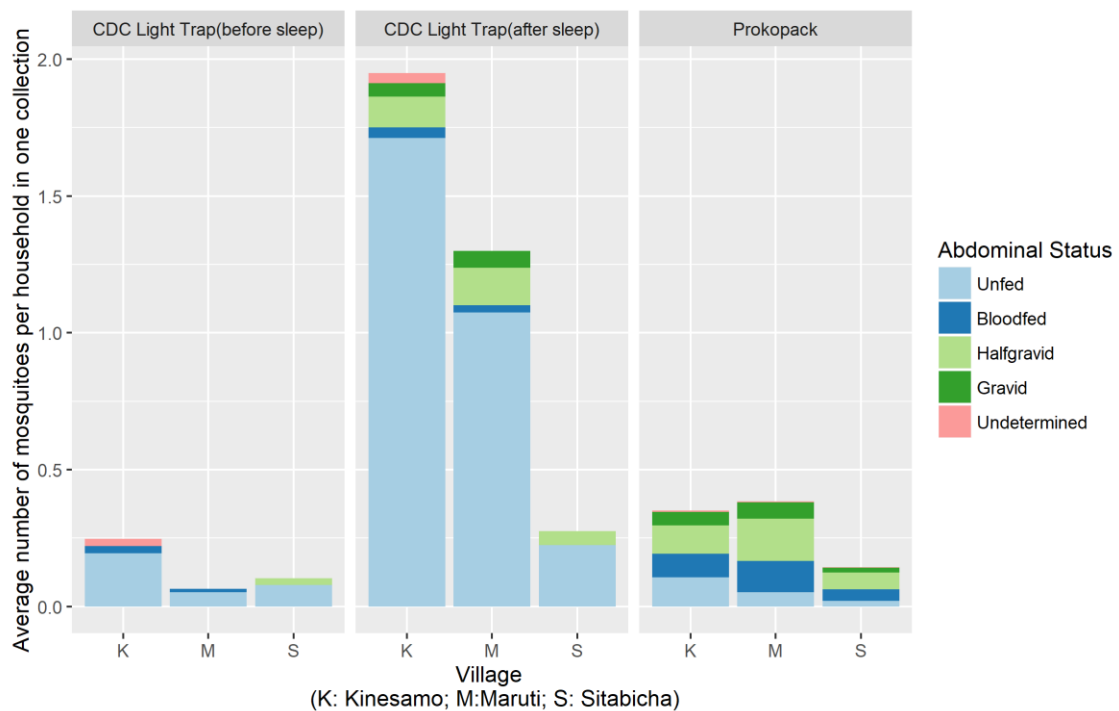


Figure 6 Abdominal status of female *Anopheles* in the villages by collection type

3.3.3 Detection of Human gDNA

647 mosquitoes were tested for human and *P.falciparum* gDNA detection by the real-time polymerase chain reaction (PCR) assay, excluding 10 missing and 5 contaminated samples. The results show 232 positive and 415 negative detections of human gDNA. The proportion of positive detection was 35.9% for total, 35.8% (201/562) for *An. gambiae s.l.*, 22% (4/18) for *An. funestus s.l.*, 29.4% (5/17) for *An. coustani*, 0 (0/1) for *An. rufipes*, and 44.9% (22/49) in undetermined. Figure 7 indicates that the distribution of human gDNA positives and negatives vary by collection type. In the CDC light trap collection, most of the mosquitoes were human gDNA negatives, especially in the after-sleep collection, where there is over 200 difference between the numbers of negatives and positives. *An. gambiae s.l.* contributed to most of the negative results in the whole CDC light trap collection. It also contributed to most of the positive results in the after-sleep collection but was rarely found in the positives of the before-sleep collection. In the Prokopack collection, there were more human gDNA positives than the negatives, but the difference was not as large as the after-sleep CDC light trap collection; and it mainly comes from the positive/negative difference of *An. gambiae s.l.*.

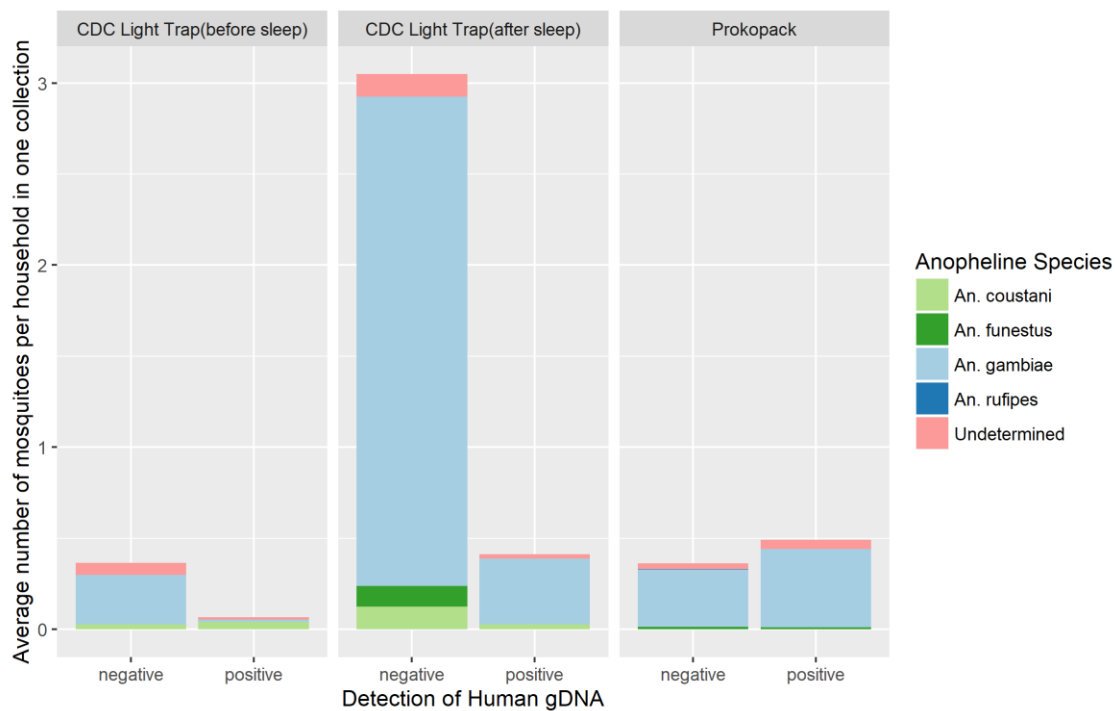


Figure 7 Anopheline species of human-gDNA positives and negatives by collection type

In addition, we found 124 and 192 human gDNA negatives and positives, respectively, were fed mosquitoes; and 291 and 40 human gDNA negatives and positives, respectively, were unfed mosquitoes. In other words, 60.8% fed mosquitoes obtained their blood meal from a human host before they were trapped; and 12.1% of unfed mosquitoes had a history of biting human. Typically, Maruti had the highest proportion of human-blood-fed *Anopheles* (65.1%), followed by Sitabicha (59.6%) and Kinesamo (55.7%). Kinesamo had the highest proportion of unfed *Anopheles* that had human bites before trapped (17.7%), much larger than Maruti (11.0%) and Sitabicha (9.7%). The results indicate human as the main blood source of female *Anopheles* in all villages, but the proportion of human blood source was slightly different by village.

3.3.4 Detection of *Plasmodium falciparum* gDNA

28 mosquitoes had positive detection of *P.falciparum* gDNA, including one of 310 collected through CDC light trap and 27 of 337 collected through Prokopack aspirator. The Prokopack-collected parasite positives were equally distributed in abdomen-only, in head-only, and in both, regardless of anopheline species. The proportion of *P.falciparum* gDNA positives was 4.3% (28/647) for total, 4.3% (24/563) for *An. gambiae s.l.*, 5.3% (1/19) for *An. funestus s.l.*, 0% for *An. rufipes* and *An. coustani*, and 5.8% (3/52) for undetermined species. *An. gambiae s.l.* contributed to most of the positive detections in abdomen, head, and both. *An. funestus s.l.* only had one positive detection in the abdomen; and no positive detection was found in *An. rufipes* and *An. coustani*.

18 mosquitoes were *P.falciparum*-gDNA positive in head and 18 were positive in abdomen. In other words, we found 18 malaria infectious and infected mosquitoes, respectively. 15 (83.3%) of infectious *Anopheles* were fed from human before they were trapped, which means that they may already infected human through feeding activities. 16 (88.9%) infected *Anopheles* had obtained human blood meals, indicating that these mosquitoes were infected with malaria parasite through human bites.

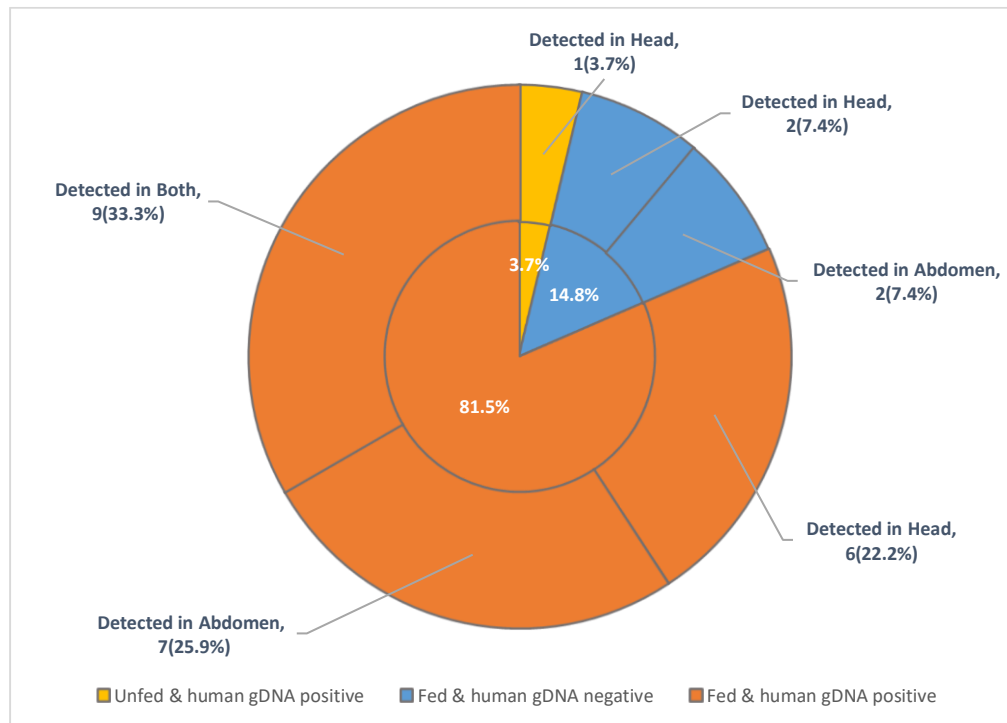


Figure 8 Number of *P.falciparum* gDNA positives in Pokopack collection by detected location, abdominal status, and human gDNA detection

3.3.5 Female Anopheles in Different Time Period

As it is shown in Figure 9, the distribution of female *Anopheles* per hour per household at one night was different between the before-sleep period and the after-sleep period in the CDC light trap collection, and we did not find a correlation of the number of mosquitoes collected before and after people went to bed in a single household at one night. In general, there is higher possibility to collect mosquitoes in an hour in a single household at one night after people go to bed.

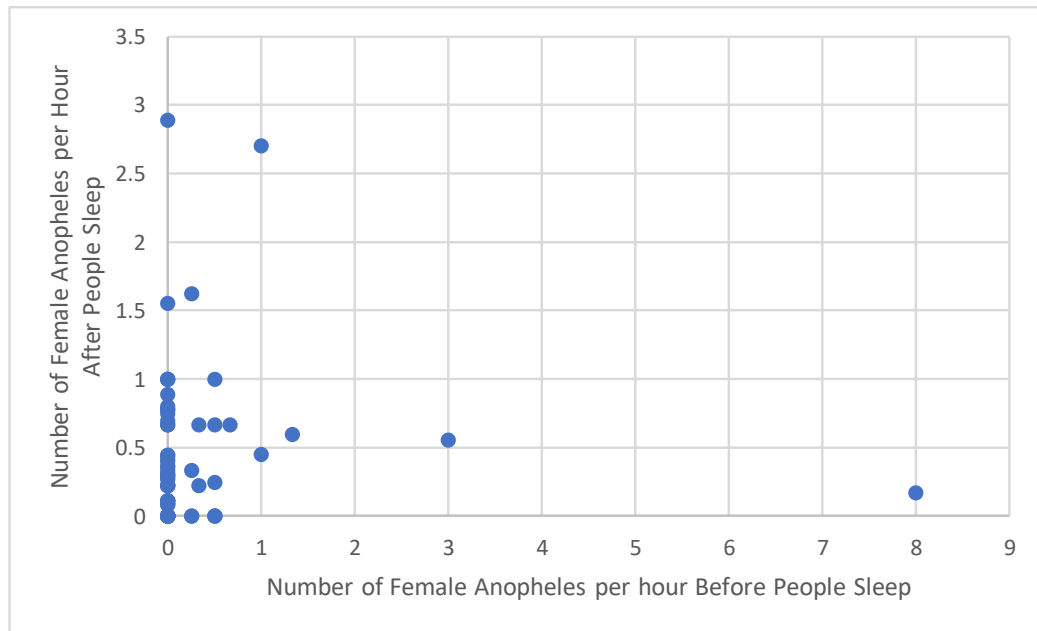


Figure 9 Scatterplot of number of *Anopheles* per hour per household at one night by collection type

Poisson regression was also applied to examine if there was a statistical difference between number of female *Anopheles* in a household before and after people go to bed with the consideration of time exposure. According to the results shown in Table 4, there is a significant difference between number of mosquitoes before and after people go to bed at the 0.05 significance level ($p < 0.05$). We expect about 2.2 (95CI[1.5, 3.1]) more mosquitoes per hour in a household after sleep than before sleep after adjusting month and village.

Table 4 Poisson regression of number of mosquitoes collected in a trap

	Exponentiated Coefficient	P Value	95% CI
Before/After Sleep	2.161	<0.001	[1.499, 3.116]
Month - May	-	-	-
- June	0.990	0.933	[0.777, 1.261]

- July	0.198	<0.001	[0.134, 0.292]
Village - Kinesamo	-	-	-
- Maruti	0.776	0.043	[0.607, 0.992]
- Sitabicha	0.174	<0.001	[0.118, 0.257]
_cons	0.375	<0.001	[0.256, 0.552]

4. Discussion

The study applied a set of mosquito trapping techniques and observed the current feeding behavior of female *Anopheles* in the study area. The influence of frequency of net use on prevalence of successful feeding activities was examined, and the collected mosquitoes were compared by their key characteristics, including species type, abdominal status, human and *P.falciparum* gDNA detection, village, and time.

The study collected 662 female *Anopheles* in total, which were dominated by *Anopheles gambiae* s.l. We also trapped a small amount of *An. funestus* s.l., *An. coustani*, and *An. rufipes*. Prokopack aspirators uniquely trapped one *An. rufipes* in Kinesamo while CDC light traps uniquely collected *An. coustani* in Maruti during the after-sleep collection period and in Kinesamo during the before- and after-sleep period. The results indicate that the anopheline species of the three villages were distinct. It might be a random chance that the only *An. rufipes* was trapped in Kinesamo and by Prokopack aspirator, but the phenomenon implies that *An. rufipes* was rare in the study area and may be exclusive in Kinesamo. Sitabicha had less diversity of anopheline species than Kinesamo and Maruti as we only trapped *An. gambiae* s.l. and very few *An. funestus* s.l. However, since the samples size of Sitabicha is relatively small, it might be possible that other anopheline species were present in the village but were not trapped. The anopheline species was also different by collection type. For example, all the 17 *An. coustani* was trapped by CDC light traps in the study. Similarly, *An. coustani* were mostly

trapped by indoor and outdoor CDC light traps while many *An. gambiae s.l.* and *An. funestus s.l.* were gathered by their aspiration method in four villages of Taita-Taveta county in Kenya (Mwangangi et al., 2013). The authors also demonstrated the major role of *An. coustani* in outdoor transmission, which is consistent with the observation of another study in Western Kenya (Cooke et al., 2015). In Webuye, some *An. coustani* may be active in outdoor feeding activities before they come into houses in evenings as our study had gathered fed *An. coustani*, which may want to digest the blood meals inside the houses, before people went to bed. We also found many host-seeking *An. coustani* after people went to bed and nothing in the early mornings, suggesting that *An. coustani* may not rest indoors at night after obtaining blood meals or may rest indoors but leave the house very early to lay eggs.

For the distribution of abdominal status, most of the female *Anopheles* collected by Prokopack aspirators were blood-fed, half-gravid, or gravid, and the prevalence of successful feeding was 79.3%. Most of the female *Anopheles* collected by CDC light traps were unfed, and the prevalence of successful feeding was 13.7%. The results were coherent with the study goal of using Prokopack aspirators to catch mosquitoes which had fed and CDC light traps to catch host-seeking mosquitoes. There was high frequency of net use since over 89% of the households had all the members who participated in the monthly survey reported at least five nights of sleep under a bed net during the last week. No statistical significance was found between the prevalence of

successful feeding and use of bed net in each type of collection, which may suggest that mosquitoes are able to feed even when net use is high, although we cannot rule out feeding on alternate sources. Malaria prevalence may thus not be reduced by frequent net use. In contrast, Obala et al., (2015) found frequent ITN use significantly related to low malaria infection among children that had ITNs in Bungoma East sub-county, where Webuye located. The non-association between frequency of the use of bed net and feeding prevalence in this study could be resulting from chemical resistance of *Anopheles* and poor condition or incorrect use of bed nets, which weaken the effectiveness of malaria vector control.

35.9% of the female *Anopheles* were human gDNA positive without the consideration of collection type. The proportion of positives was 35.8% for *An. gambiae s.l.* and slightly lower for *An. funestus s.l.* (22%) and *An. coustani* (29.4%). The only *An. rufipes* was human gDNA negative. In Prokopack aspirator collection, we found more human gDNA positives than negatives. In CDC light trap collection, more human gDNA negatives were detected than positives, and the difference is remarkable in the after-sleep collection. The results were consistent with the finding that the majority were fed with blood meals in Prokopack aspirator collection and unfed in CDC light trap collection.

Twenty-seven female *Anopheles* had positive detection of *P.falciparum* gDNA in Prokopack aspirator collection while only one positive detection was found in CDC light

trap collection. 4.3% of the female Anopheles were *P.falciparum* gDNA positive in total and in *An. gambiae s.l.*, and 5.3% were positives in *An. funestus s.l.*. All the *An. rufipes* and *An. coustani* were *P.falciparum* gDNA negative. For the Prokopack-collected samples, the positive detections were equally found in abdomen, in head, and in both. All the positives were *An. gambiae s.l.* except one *An. funestus s.l.* with positive *P.falciparum* gDNA detection in abdomen. The results indicate the low possibility of an *An. rufipes* or an *An. coustani* to be infectious or infected by *P.falciparum*, but the sample size of the two species was small and made it difficult to conclude if they are not as active as *An. gambiae s.l.* and *An. funestus s.l.* in malaria transmission.

Compared to the prevalence of human gDNA positives, the prevalence of the *P.falciparum* gDNA positives are much rarer in both types of collection and in all anopheline species. Nevertheless, malaria transmission at a low level can still result in remarkable disease prevalence (Charles N. M. Mbogo et al., 1995), and this can be confirmed by one of our findings that most of the infectious and infected *Anopheles* also obtained human blood meals before they were trapped. The finding implies a high possibility of effective malaria transmission through human bites, no matter the direction was vector-to-human or human-to-vector. In addition to the importance of reducing infectious mosquitoes, some researchers also mentioned the vital role of human infectious reservoir and the important idea of controlling malaria by concentrating on the human-to-mosquito pathway (Stone, Gonçalves, Bousema, &

Drakeley, 2015). Since this study found same number of infectious and infected mosquitoes, we believe that taking both the mosquito-to-human and human-to-mosquito transmissions into account are crucial for the effective control of malaria transmission.

In addition, the numbers of female *Anopheles* were different between the period before and after people went to bed. At most of the nights, zero female *Anopheles* was trapped per hour per household during the before-sleep collection. Most of the number of female *Anopheles* per hour per household at one night during the after-sleep collection was above zero with the median slightly larger than zero. Poisson regression also demonstrated a significant statistical difference of female *Anopheles* population between the period before and during people sleep with the adjustment of month and village. As opposed to findings in *An. funestus s.l.* in Benin (Moiroux et al., 2012), my findings do not support a change in behavior in *Anopheles* host seeking in response to control approaches, as the majority of activity was after people went to sleep (Githeko et al., 1996).

Plentiful *An. gambiae* were observed resting outdoors and collected by clay pots and pit shelters in west of Kisumu, Western Kenya (Odiere et al., 2007). However, we did not observe any plausible outdoor resting behavior of female *Anopheles* in the three villages. The sticky barrier screens failed to catch female *Anopheles* though we expanded the size and adjusted the locations and distance. One possible explanation for the failure

of the trapping tool could be the interference of rain, which may prevent *Anopheles*' behavior of resting outside or reduce the stickiness of glue and increase the chances of escape. Another possibility is the absence of outdoor behavior in the study area. A study found the essential oil of *Conyza newii* had mosquito repellency in Webuye and some other areas of Kenya (Mayeku, Omollo, Odalo, & Hassanali, 2014). There could be *Conyza newii* surrounded the households in the three villages, preventing mosquitoes from biting outdoors or resting outside after feeding. However, further research on the environment in the three villages is needed to verify the idea. Moreover, evidence showed the presence of chemical resistance in *An. gambiae s.l.* and *An. funestus s.l.* to insecticide in Suba sub-county, Western Kenya (Kawada et al., 2011). As we observed abundant amount of these two species inside the households, they may have already developed ability to tolerant ITNs and do not need to change their behavior by feeding or resting outdoors.

4.1 Implications for further research

One difficulty of the study was to collect outdoor mosquitoes because of limited time and resources and little knowledge about the outdoor behavior of female *Anopheles* in Webuye. Therefore, larger studies on the outdoor behavior of mosquitoes are needed. Further research with focus on evaluation of ITN use and genetic resistance of female *Anopheles* are also useful to understand effectiveness of malaria prevention. This research only collected the mosquitoes before and during people sleep to study biting

time. Yet one research in Benin found that *An. funestus s.l.* delayed their feeding activities in the area where long-lasting ITN intervention was popular (Moiroux et al., 2012). Therefore, we suggest future research to monitor biting time of female *Anopheles*, especially to compare host-seeking mosquitoes between the late nights and early mornings. In addition, the study found different trapping tools collected different species of mosquitoes during the collection and suggest future mosquito research incorporate multiple trapping techniques to fully understand the characteristics of female *Anopheles*.

4.2 Study strengths and limitations

The study was advanced in using multiple mosquitoes trapping techniques to collect female *Anopheles*. However, there are several limitations in the study. First, this was a cross-sectional study and not able to understand the feeding behavior of mosquitoes in time series. Second, the study area was relatively small and may not be generalizable to Western Kenya. Third, although the study collected more than 650 female *Anopheles* in total, the sample size became small when it was divided by collection type, species, and village. For example, the number of samples collected during the after-sleep collection was small and the power of the study was weakened. Another limitation of the study was not able to identify the subspecies of *An. gambiae s.l.* to study the anopheline feeding behavior in detail. Lastly, as a result of high ITN coverage in the study area, this study was not able to include households that did not

have ITNs into a comparison group to understand how the feeding behavior of female *Anopheles* may change from living in the normal environment to living in ITN environment.

5. Conclusion

The study observed the feeding behavior of female *Anopheles* in the area with high ITN coverage and use in Webuye, Western Kenya during May – July, 2018. The study used a set of mosquitoes trapping tools, including Prokopack aspirators, sticky barrier screens, and CDC light traps, to collect host-seeking and blood-fed mosquitoes indoors and outdoors. Real-time PCR assay was produced for human and *P.falciparum* gDNA detection. The results show the characteristic differences of female *Anopheles* by collection type, village, month, abdominal status, species, human gDNA detection, and *P.falciparum* gDNA detection. The prevalence of successful feeding was not moderated by use of bed net. There was a significant difference of number of mosquitoes between the time before and after people went to bed. This study found that behavioral adaptation may not mainly explain the persistence of malaria transmission. However, since some studies have found mosquitoes resistance in Africa, the feeding behavior of female *Anopheles* should be monitored in the long term. In addition, further research is needed to study the practice of ITN use, genetic resistance of female *Anopheles*, and the biting time of mosquitoes to understand the reasons for the poor effectiveness of malaria prevention in the study area.

Appendix A Adult Mosquito Collection Summary Form

Collection Date [dd/mm/yyyy]: _____ Collection Time: _____

-----This time recording section is for CDC light trap collection only-----

Before-sleep collection: Start Time: _____ End Time: _____

After-sleep collection: Start Time: _____ End Time: _____

Village: _____ Household ID: _____

Collection done by: _____

1) Abdominal status of female *anopheles* mosquitoes caught

<i>Abdominal status of female anopheles mosquitoes (No.)</i>							
Location	Unfed	Blood fed	Half gravid	Gravid	Undetermined	Total	Number archived
Bedroom							
Kitchen							

2) Number of male anopheles collected in bedroom: _____ kitchen: _____

NOTES:

Form checked by-----sign-----date-----

Form entered by-----sign-----date-----

Appendix B Individual Female Anopheles Data Entry

Form

Note: use one form per household per collection day

Collection Date[mm/dd/yyyy]: _____ Collection Time: _____

Village: _____ Household ID: _____

Collection done by: _____ Samples prepared by: _____

Species ID done by: _____

Number	Collected Location Bedroom (sleeping area)/Livingroom (area that people stay before sleep)	Abdominal status (g/hg/fed/unfed/undetermined)	Species (indicate if undetermined and checked by PI)	Sample ID	Comment
1				Head	
				Abdomen	
2				Head	
				Abdomen	
3				Head	
				Abdomen	
4				Head	
				Abdomen	

5				Head	
				Abdomen	
6				Head	
				Abdomen	
7				Head	
				Abdomen	
8				Head	
				Abdomen	
9				Head	
				Abdomen	

Note: head and abdomen sample ID is only applicable to Prokopack aspirator collection. For CDC light trap collection, simply record the sample ID of each mosquito.

Form checked by-----sign-----date-----

Form entered by-----sign-----date-----

Appendix C gDNA Extraction from individual mosquitoes

Authors: Huipeng (Verona) Liao, Daniel Evans, Sam Kim

I. Introduction

This SOP describes the procedure to use Chelex-100 to extract genomic DNA (gDNA) from individual mosquitoes.

II. Background

gDNA extracted from a mosquito – or part of a mosquito – can be used for a variety of applications. This protocol extracts total gDNA, which may include human DNA if the mosquito has recently fed on a human, *Plasmodium spp.* DNA if the human bloodmeal included malaria parasites and the mosquito is capable of transmitting malaria, or other vertebrate DNA if the mosquito is zoophilic.

III. Materials needed

Item	Catalog #
SCIENCEWARE® Wilmad LabGlass Micro Tube	
Homogenizer System (Bel-Art)	VWR 89524-800
Bulk Packaged 1.5 mL Pestle	Fisher 12-141-364
Water bath	
1.5mL microcentrifuge tubes	
2mL deep 96-well plate	Genesee 27-433
1.1mL deep 96-well plate	Genesee 27-431

Saponin	CCF (Sigma) 47036-50G-F
Chelex-100, mol.bio.grade, 200-400 mesh	CCF 1421253
1X PBS	
10% bleach	

IV. Procedure

1. Prepare mosquito

- 1.1. Set water bath to 95-99°C.
- 1.2. Label closed sterile microcentrifuge tubes with full ID numbers for mosquito grinding.
- 1.3. Transfer mosquitoes to the corresponding labeled microcentrifuge tube by inverting and flicking the mosquito tube, or by using a sterile 10uL pipette tip.
- 1.4. Create a plate map of the ground mosquitoes.

2. Homogenize mosquito

- 2.1. Add 100uL of 1% saponin to the microcentrifuge tube containing the mosquito.
- 2.2. Grind mosquito with a sterile pestle attached to the homogenizer.
 - 2.2.1. Position mosquito against the side of the pestle, then pulse the homogenizer 5-10 times to break up the mosquito.
 - 2.2.2. Grind mosquito for ~10s for abdomens, ~20s for heads.
 - 2.2.3. Ensure that the mosquito is not stuck above or below the pestle by occasionally stopping the homogenizer and moving the pestle up and down the tube. Moving the pestle while grinding will create foam.

2.2.4. Typically, the liquid in the tube will turn turbid or brownish with successful grinding.

2.2.5. Place used pestle in 10% bleach.

2.3. Transfer the homogenates to a sterile 2mL deep 96-well plate, drawing up to ~115uL of liquid.

2.3.1. Make sure to transfer all mosquito parts from the tube by resuspending the liquid and scraping the pipette tip along the sides of the tube.

2.4. Incubate at 4°C in refrigerator overnight.

3. Extract gDNA

3.1. Spin plate for 8min at 4,100rpm, with acceleration 9 and deceleration 7, in the Sorvall Legend XTR centrifuge to pellet the ground mosquitoes.

3.2. Aspirate and discard supernatant (up to 95uL) from each well, leaving the pellets undisturbed.

3.3. Wash saponin off pellets.

3.3.1. Add 200uL of 1X PBS to each well and resuspend pellets by vortexing at medium to high speed.

3.3.2. Spin plate for 8min as above.

3.3.3. Aspirate and discard supernatant (up to 195uL) from each well, leaving the pellets undisturbed.

3.4. Add 75uL of sterile water and 25uL of 20% Chelex to each well.

- 3.4.1. Resuspend Chelex beads by vortexing solution for 3-5s at max speed and quickly transfer to reservoir.
- 3.4.2. Use a wide-bore pipette tip to transfer Chelex, and resuspend the beads before each aspiration.
- 3.5. Resuspend pellets by vortexing for 3-5s as above.
- 3.6. Place plate in 95-99°C water bath for 12min.
 - 3.6.1. While in water bath, vortex for 3-5s as above at each 3min interval.
 - 3.6.2. Ensure that the adhesive foil cover is securely attached before and after each vortexing. In order to make the foil cover less likely to detach, leave the water bath lid slightly open.
- 3.7. Place plate on wrinkled paper towels and leave at room temperature for 3min to cool. Using a flat surface may create a partial vacuum between the plate and the surface.
- 3.8. Spin plate for 12min as above.
- 3.9. Aspirate and **save supernatant** (up to 90uL) from each well by transferring it to a sterile 1.1mL deep 96-well plate, leaving the pellets and Chelex beads undisturbed.

4. Clean up

- 4.1. Turn off water bath.
- 4.2. Wipe down work area with 70% ethanol.
- 4.3. Label plate as "Spat ___M-ch.ext.gDNA."

- 4.4. Store plate at 4°C in refrigerator overnight, then move to storage in a -20°C freezer.
- 4.5. Verify plate map data with microcentrifuge tube IDs, then discard the microcentrifuge tubes. Store original mosquito tubes for future verification.
- 4.6. Enter plate name, date of extraction, and plate map data in the electronic database.
- 4.7. Clean and reuse pestles.
 - 4.7.1. Remove pestles from 10% bleach and wash in dilute Alconox.
 - 4.7.2. Rinse pestles with tap water 5 times, then with deionized water 5 times.
 - 4.7.3. Dry overnight and autoclave.

V. References

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Lardeux F, Optimization of a semi-nested multiplex PCR to identify Plasmodium parasites in wild-caught mosquitoes, Trans 2008.

Musapa, M., Kumwenda, T., Mkulama, M., Chishimba, S., Norris, D. E., Thuma, P. E., et al. A Simple Chelex Protocol for DNA Extraction from *Anopheles spp.*. *J. Vis. Exp.* (71), e3281, doi:10.3791/3281 (2013).

Appendix D Mosquito gDNA Testing for Human and *P.*

Falciparum

Authors: Steve Taylor

I. Introduction

This SOP describes the project-specific procedures for testing plates of genomic DNA that was extracted from mosquitos for *Plasmodium falciparum*.

II. Background

In the “Mozzie” study, resting mosquitos were collected weekly from the walls of all enrolled households. These were sorted to identify the female Anopheline mosquitos, these were assigned unique identifiers, and each was split into head and thorax and placed in separate tubes. These mosquito heads and abdomens were deposited into 96-well plates (90 in each plate) and extracted with Chelex-100. These plates of genomic DNA are then tested for P falciparum.

This SOP describes how to implement the duplex real-time PCR assay targeting both P falciparum pfr364 and human β -tubulin that is described in SOP DMC 2017-6. By amplifying both of these targets from the Mozzie samples, we can identify the mosquitos that are infected with P falciparum and that have recently fed on humans.

III. Materials needed

- Reagents for SOP DMC 2017-6

- Two Mozzie mosquito gDNA plates
- 384-well real-time plate
- 25mL reagent reservoir

IV. Procedure

1. Prepare the reagents

1.1. Lab reagents

1.1.1. Defrost two Mozzie mosquito gDNA sample plates that you want to test

1.1.2. Defrost enough primer and probe that you will need in the amounts described below. Protect the probe from exposure to light.

1.1.3. Confirm that you have enough Taqman mastermix.

1.1.4. Confirm that you have positive controls for human and parasite DNA.

1.1.4.1. Human DNA should be 1.0ng/uL.

1.1.4.2. Parasite DNA is extracted 3D7 gDNA at concentrations of 0.7, 0.07, 0.007, 0.0007, 0.00007, 0.000007 ng/uL.

1.1.5. Confirm that nobody else is using the Quantstudio6 machine, and that the 384-well block is currently installed. Its best to have signed up for the use of the instrument.

1.2. Prepare the electronic file to upload the sample names to the Quantstudio6.

1.2.1. Copy 2 sets of up to 96 sample names from electronic database to designated columns on the sheet "384w – Raw data" in the file "Plate Map

Converter QS6." This file is located in Box in Duke Malaria Collaboratory \ Lab operations \ Protocols \ Active protocols.

1.2.2. Verify that the names were transferred correctly by spot-checking random wells between the database and the simulated plate maps on the first sheet of the file.

1.2.3. Click to the sheet "384w – Output setup."

1.2.4. Save this sheet as a tab-delimited text file with the format Mozzie Plate MX-MX.

1.2.5. Open the txt file and add to the top of this file: "* Instrument Type = QuantStudio 6

* Passive Reference = ROX [Sample Setup]"

1.2.6. Save this altered txt file.

1.2.7. Transfer the setup file to a memory stick in order to import to the Quantstudio6 machine below.

2. Prepare the template-free reaction plate

2.1. The reaction plate is prepared in the dead-air box in Sands 313.

2.1.1. Wipe the p20 multichannel pipet down with ethanol and bring into the box.

2.1.2. Work quickly while preparing the mastermix, in order to prevent prolonged exposure to room temperature and to prevent exposure of the Taqman probes to light.

2.2. Prepare the following amount of mastermix. Because the total volume is nearly 5mL, it is prepared in 4 separate but identical 1.5mL tubes.

	Mastermix		
	ea		Mastermix
	(μ L)		(/4)
TaqMan Environmental MasterMix	6		2484
Pfr364F (20uM) (250nM final)	0.15		62.1
Pfr364_R2 (20uM) (250nM final)	0.15		62.1
Pfr364 probe (20uM) (300nM final)	0.18	x414	74.5
HbtubF (20uM) (250nM final)	0.15	->	62.1
HbtubR (20uM) (250nM final)	0.15		62.1
Hbtub probe (20uM) (300nM final)	0.18		74.5
Template	1		<1>
H ₂ O	4.04		1672.6
Total	12		4968

2.3. Pipet the contents of all 4 tubes of identical mastermix into a 25mL reservoir.

2.4. Distribute 11uL of mastermix to each well of a 384-well reaction plate.

2.4.1. Place the reaction plate on a cold block while doing this.

2.4.2. You can re-use tips at this step but keep an eye on the ability of the individual tips to aspirate and to dispense.

2.5. Using the same water used for the mastermix, add 1uL water to wells H23 and H24.

2.6. When finished, place a real-time cover atop the reaction plate to protect from direct light.

2.7. Put reagents back in the freezer.

2.8. With the reaction plate atop a cold block, transport the reaction plate to Sands 319.

3. Add template to reaction plate

3.1. Wipe down the interior of the PCR hood with ethanol.

3.2. Ensure that you have 4 full boxes of p2 tips, as well as the p10 multichannel, in the hood.

3.3. Add the positive controls.

3.3.1. The positive 3D7 gDNA controls will be added to wells P13 – P24. P13/14 should be 0.7, P15/16 should be 0.07 and so forth, through P23/24.

3.3.2. The positive human controls will be added to wells H21/22.

3.4. Add the Mozzie mosquito gDNA from the sample plate to the reaction plate.

3.4.1. The samples will be tested in duplicate on the reaction plate, in columns next to each other. Therefore, column 1 on the sample plate will become columns

1 and 2 on the reaction plate. Sample plate column 2 ⊗ reaction plate columns 3 and 4, and so forth.

3.4.2. Samples from the first plate (usually the odd-numbered one) will be tested on the top, in rows A-H, and those from the second plate (usu. even-numbered one) on the bottom, in row I – P.

3.4.3. Open the first sample plate.

3.4.4. Using 12 fresh tips, aspirate 1uL from sample plate row A and dispense to the odd-numbered columns of reaction plate row A.

3.4.5. Get fresh tips, and again aspirate 1uL from sample plate row A, but dispense to the even-numbered columns of reaction plate row A.

3.4.6. Get fresh tips, and repeat 3.4.4 – 3.4.5 for rows B – H. Remember that sample plate row H has only 6 samples, so only use 6 tips.

3.4.7. When finished with the first sample plate, re-cover it securely.

3.4.8. Repeat the above steps for the second sample plate, but add to reaction plate rows I – P. Therefore, sample plate row A ⊗ reaction plate row I, sample plate row B ⊗ reaction plate row J, and so forth.

3.4.9. Remember that sample plate row H has only 6 samples so only use 6 tips.

3.4.10. When finished with the second sample plate, re-cover it securely.

3.4.11. Cover securely the reaction plate with a real-time plate cover and spin it in the plate spinner.

3.4.12. While it is spinning:

- Replace sample plates in the fridge/freezer.
- Return controls to fridge/freezer.
- Clean up the PCR hood.

3.4.13. When it has spun 2-3 minutes, remove, place on cool block, and cover to protect from light. Carry to Sands 337.

4. Run the reaction plate on the Quantstudio6

4.1. Open a New Experiment.

4.2. On the Setup – Experiment Properties tab:

4.2.1. Name the file “Mozzie mosquito plateMX-MX Taqman duplex DD-MM-YY”

4.2.2. Choose 384-well block.

4.2.3. Choose Standard Curve as the experiment type

4.2.4. Choose “TaqMan reagents” as the chemistry

4.2.5. Choose Standard as the cycling.

4.3. On the Setup – Define tab:

4.3.1. Choose “Import” at the top of the screen and import your .txt file with the sample names that you created above in Step 1.2.

4.3.1.1. When imported, this should populate the “Sample” pane on the top right.

4.3.1.2. Scan through the Sample pan list and delete any “placeholder” names like “blank” that were also imported.

4.3.2. Add two targets by clicking New twice in the top-left Targets pane.

4.3.2.1. “pfr364”, choose FAM as reporter, “None” as quencher, and red as color.

4.3.2.2. “Hbtub”, choose VIC as reporter, “None” as quencher, and black as color.

4.4. On the Setup – Assign tab:

4.4.1. Select all wells and choose both targets for all in the top left. Task for all is U (for unknown).

4.4.2. Add positive and negative controls.

4.4.2.1. Change these wells to these settings for the positive controls:

Wells	Target	Task	Quantity
H21/22	Hbtub	S	1.0
P13/14	pfr364	S	0.7
P15/16	pfr364	S	0.07
P17/18	pfr364	S	0.007
P19/20	pfr364	S	0.0007
P21/22	pfr364	S	0.00007
P23/24	pfr364	S	0.000007

4.4.2.2. Change these wells to these settings for the negative controls:

Wells	Target	Task
H23/24	pfr364 and Hbtub	N

4.5. On the Setup – Run Method tab:

4.5.1. Change to 12uL the “Reaction volume per well”

4.6. Start the run

4.6.1. Open the plate tray on the instrument and place the reaction plate on it.

4.6.2. Ensure that the reaction plate is in the correct orientation and then close the tray.

4.6.3. Save the file to “Malaria Collaboratory \ Mozzie”

4.6.4. On the Run tab, click the green START RUN button and choose the machine.

4.6.5. You will hear a faint mechanical sound and the screen on the instrument will show the remaining time in large numbers.

5. Get the data

5.1. On the Analysis tab, underneath the amplification plot, un-tick “Auto” for both targets.

5.2. For each target, drag the threshold line above the background fluorescence. Set each threshold at the same y-value for the two targets.

5.3. Click green Analyze button on the top right.

5.4. Export the data

5.4.1. Click to the Export tab

5.4.2. Click Load Export Set. Choose “Mozzie mosquito export”

5.4.3. For Export File Location, click the Browse button, and navigate to “Malaria Collaboratory \ Mozzie \ Exported results.”

5.4.4. For Export File Name, type “Mozzie mosquito Plate Mx-Mx MM-DD-YY”

5.4.5. In the top right, click the Export button.

5.4.6. Close the run file.

5.5. Re-format the export data

5.5.1. Open the file in Excel “Malaria Collaboratory \ Mozzie \ Mozzie export formatter QS6 readonly.xlsx”

5.5.2. Also open the xlsx file that you created in Step 5.4.4.

5.5.2.1. In this second xlsx file, right-click the tab “Results,” and left-click “Move or Copy.”

5.5.2.2. Under the resulting menu, for the field “To book:” select “Mozzie export formatter QS6 readonly.xlsx.” Click OK.

5.5.3. In the file “MESA export formatter....”, click to sheet “Formatted.”

5.5.3.1. Copy everything on this sheet.

5.5.4. Re-open the xlsx file created in Step 5.4.4. (Excel automatically closed this a few steps back)

5.5.5. In this file, create a new sheet and paste the Values that are on the clipboard into it.

5.5.5.1. Save this file and close it.

5.5.6. Click to the file “MESA export formatter...” and delete the “Results” tab.

Close this file without saving.

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